AMELIORATIVE EFFECT OF BIOMOLECULES IN TUMOR CONTROL

Thesis Submitted to

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For the degree of
DOCTOR OF PHILOSOPHY IN BOTANY
(Faculty of Science)

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MAY 2022

DECLARATION

I hereby declare that the thesis entitled **"Ameliorative effect of biomolecules in tumor control"** is based on the original research carried out by me at Sree Narayana College, Nattika, Thrissur under the guidance of **Dr. C.T ANITHA**, Asso. Professor, PG Department of Botany & Research, Sree Narayana College, Nattika, Thrissur, and no part thereof has been presented for the award of any other degree, diploma or other similar titles.

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My Amma and Sister

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Abstract

Cancer has increasingly become a global health issue, addressing the rising cancer incidence and death rates it has become a challenge at multiple levels. The continuous discharge of reactive oxygen species (ROS) from various sources resulted in oxidative damages to cells and changes many cellular functions. Among the various biological targets, DNA, enzymes, and lipid membranes are most vulnerable to oxidative stress. The various treatment modalities available for cancer include radiotherapy, chemotherapy and surgery. During radiotherapy, the radiation produce many biological changes in cells. It mainly aims at enhancing DNA damage of tumor cells, as double strand breaks, leads to cell death. But, due to various doses of radiation, cell death occurs to normal cells also. Ionizing radiation creates oxidative damages to cells through the generation of ROS and the main sub cellular target as the DNA.

Many xenobiotics supplied to living organisms are metabolized within the body by uniting with the cellular antioxidant enzymes like GSH, causing their depletion and lead to oxidative damages. Most of the common chemotherapeutic agents used for cancer treatment are immunosuppressant, cytotoxic and exert several side effects such as nephrotoxicity, cardiotoxicity, hepatotoxicity, hematopoietic depression, gastrointestinal-toxicity, hemorrhagic cystitis, hemopoetic suppression and urotoxicity. Thus, the role of radioprotective and chemo protective compounds are very important in such clinical cancer treatment strategies. Such compounds can decrease the side effects of chemotherapeutics, and can stimulate immunity, will be of great help in improving cancer treatments.

In the present study, the biological activities of the nanoparticle bound DAO complex, ferulic acid, Ascorbic acid mono glucoside and phytoceuticals like Galangin and Kaempferide were evaluated for their active role in tumor control.

Application of nanoparticles for targeting drugs to tumor cells for tumor treatment was explored using magnetic Fe_2O_3 nanoparticles and complexing this with the enzyme D amino acid oxidase (DAO). DAO was isolated from fresh porcine kidney; its cytotoxic potential was studied under *in vitro* and *in vivo* conditions. The isolated DAO was complexed with Fe_2O_3 nanoparticles and its potential as an oxidative therapeutic agent was analysed. The ability of the complex in eliciting H_2O_2 mediated cytotoxicity was studied on Dalton's lymphoma ascites cells (DLA). The induction of apoptosis in DLA cells by Fe_2O_3-DAO complex was studied by morphological examination and alkaline single cell gel electrophoresis (comet assay). The antitumor activity of the complex was investigated by oral administration of the complex and the substrate D-alanine to tumor bearing Swiss albino mice and by targeting the complex to the tumor site, using an externally applied magnetic field. Fe_2O_3-DAO along with D-alanine showed remarkable cytotoxicity in a substrate concentration-dependent manner. Both morphological examination and comet assay revealed that Fe_2O_3-DAO/D-alanine induced apoptosis. Oral administration of Fe_2O_3-DAO and D-alanine along with magnetic targeting significantly suppressed tumor growth in mice. The study provides the evidence for the promising application of enzyme bound nanoparticles for targeted oxidation therapy.

The anti inflammatory activities of ferulic acid and Ascorbic acid mono glucoside were examined and found to possess significant anti-inflammatory activity and the oxidative stress induced by selected xenobiotics, acetaminophen induced hepatotoxicity, doxorubicin induced cardiotoxicity and cisplatin induced nephrotoxicity were also studied. The combination of nanomaterials with biology led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications, and drug delivery vehicles. The thesis encompass the application of a magnetic iron oxide nanoparticle as an anticancer agent when complexed with the enzyme D aminoacid oxidase (DAO) for targeted tumor therapy in a preclinical situation using an animal solid tumor model.

Preface

Living systems are regularly exposed to a variety of reactive oxygen species (ROS) and reactive nitrogen species (RNS) from both exogenous and endogenous sources. The exogenous sources consist of different drugs, range of physical and chemical agents, which causes cellular and genetic changes leading to mutations and cell death. Cancer is said to be one of the leading causes of morbidity and mortality in a number of populations of the world. Radiotherapy and chemotherapy are commonly used treatment modalities for cancer treatment.

Radiation affects the biological system in several ways. On one side, it helped the life forms to evolve and on the other it creates deleterious effects on life forms. At present, ionizing radiation is used for a large number of therapeutic, diagnostic, and industrial purpose. Other applications include the generation of nuclear power, developing new varieties of high yielding crops, sterilization of surgical and medical materials and increasing the storage period of food materials. In many instances where radiation is used, organisms may be subjected to low level exposures. High exposures to radiation may occur due to nuclear accidents or during nuclear war. In radiotherapy, radiation injury to normal tissues surrounding the tumor is one of the major problems limiting the success. So there is need to protect normal tissues from deleterious effects of radiation during radiotherapy.

A number of drugs of both synthetic and biological origin have been tested as radioprotectant in the past few decades (Weiss and Landauer, 2003). But, their toxicity at optimum protective dose prohibited their human use. Hence there is still an urgent need to identify novel, nontoxic, effective, and convenient compounds to protect humans from the damaging effects of ionizing radiation.

A number of drugs used for various treatments such as chemotherapy can increase the production of free radicals and can act as a major source of ROS (Rav *et al*., 2001). These drugs include antibiotics containing quinoid groups or bound metals, antineoplastic agents like bleomycin, anthracyclines like adriamycin. The adriamycin or doxorubicin induced cardiotoxicity has been associated with diverse mechanisms as ROS generation, increased membrane lipid peroxidation, mitochondrial damage and iron-dependent oxidative damage to various macromolecules (Xu *et al*., 2001; Kalyanaraman, 2020). Cisplatin is another important chemotherapeutic drug causing oxidative stress. The major dose limiting adverse reaction associated with cisplatin is nephrotoxicity associated with oxidative damage of tissues leading to renal cell death.

Human body is exposed to different environmental pollutants and xenobiotics. Liver is the prime organ, that metabolize all foreign agents. Due to severe toxicity, it is vulnerable to different diseases such as hepatitis, cirrhosis, liver fibrosis, alcohol-related disorders and liver cancer. The reactive oxygen species (ROS) play an important role in pathological changes in the liver causing liver cell death (Mahmood *et al*., 2014). The most commonly used analgesic, acetaminophen (paracetamol) cause free radical flux, causing severe damage to the hepatic tissues (Ratheea *et al*., 2018).

In order to protect the cells and organ systems of the body against ROS, humans have developed a complex antioxidant protection system. The main antioxidant enzymes directly involved in the neutralization of ROS and RNS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx). (Halliwell, 2007; Valko *et al*., 2007). Exogenous antioxidants are agents supplied exogenously as supplements, that cannot be produced by human body but protect against pro-oxidant activities of free radicals. These exogenous antioxidants are available from various dietary sources. Among them Vitamin C, Vitamin E, flavonoids and β-carotene are the most commonly used dietary antioxidants.

Phenolics are commonly distributed in the plant kingdom and are integral part of human diet. As an antioxidant, Ferulic acid (FA) play a key role in the body's defense against carcinogenesis and inhibit the formation of N-nitroso compounds (Kuenzig *et al*., 1984).

Moreover, FA is a strong scavenger of free radicals and it has been approved in certain countries as food additive to prevent lipid peroxidation. FA possesses radioprotective abilities and reduces ionizing radiation induced damages to DNA and membranes in biological systems (Sreenivasan *et al*., 2007). The present study also aims to develop a screening method to analyse ferulic acid from various plant sources like wheat bran, rice bran and bamboo shoot.

Nanotechnology is a multidisciplinary branch involving the design and engineering of nano materials smaller than 100 nm in diameter level to get unique properties. So they can be correctly manipulated for the desired applications (Salvioni *et al*., 2019).

Nanosized particles employed as a tool to explore certain avenues of medicine such as imaging, sensing, targeted drug or gene delivery systems, artificial implants etc. Due to enhanced effectiveness, the new age drugs developed are nanoparticles of polymers, metals or ceramics, which can be used in treatment for cancer (Brigger *et al*., 2002; Stoimenov *et al*., 2002).

The combination of nanomaterials with biology led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications, and drug delivery vehicles. The thesis encompass the application of a magnetic iron oxide nanoparticle as an anticancer agent when complexed with the enzyme D aminoacid oxidse (DAO) for targeted tumor therapy in a preclinical situation using an animal solid tumor model.

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•OH	-hydroxyl radical
$1O_2$	-singlet oxygen
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8-OH-dG	-8-hydroxy-2' -deoxyguanosine
ABTS	-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))
AGENT	- Antibody- guided enzyme nitrile therapy
AP-1	-Activator Protein-1
APAP	- *N*-acetyl-*p*-aminophenol
AsAG	- Ascorbic Acid-2 Glucoside
bcl-2	-B-cell lymphoma 2
Ca^{2+}	- Calcium ion
CASP	-Comet assay software project
Cis	-Cisplatin
CK	- Creatine kinase
CK-BB	-Creatine kinase (Brain)
CK-MB	-Creatine kinase (Heart)
CK-MM	-Creatine kinase (Skeletal muscle)
$CO_3 \bullet^-$	- Carbonate radical anion
Cu^+	-Cupric ion
Cys-	-Cysteine
DAO	- D-amino acid oxidase
DEAE	-Diethylaminoethyl
DILI	- Drug-induced liver injury
DLA	-Dalton's Lymphoma Ascites

DMEM	-Dulbecco's Modified Eagle Medium
DMSO	-Dimethyl sulfoxide
DNA	- Deoxy ribo nucleic acid
DOX	-Doxorubicin
DPPH	- 2,2-diphenyl-1-picrylhydrazyl
DTNB	- 5,5'-dithiobis-(2-nitrobenzoic acid)
e_{aq}^-	-Hydrated electron
EC_{50}	-Half maximal effective concentration
EDTA	-Ethylenediamine tetraacetic acid
ESRD	- End-stage renal disease
ETC	- Electron transport chain
FA	-Ferulic acid
FAD	-Flavin adenine dinucleotide
Fe^{2+}	-Ferrous ion
Fe_2O_3	-Ferric oxide
Fe_3O_4	- ferrouso ferric oxide
FMN	-Flavin mononucleotide
FTIR	-Fourier Transform Infrared Spectroscopy
GFR	- Glomerular filtration rate
GM-CSF	-Granulocyte-macrophage colony stimulating factor
GPx	-Glutathione peroxidase
GR	-Glutathione reductase
Grx	-Glutaredoxins
GSH	- Reduced Glutathione
GSNO	-*S*-nitrosoglutathione adduct
GSSG	- Oxidized glutathione
GST	-Glutathione S-transferase

H_2O_2	-Hydrogen peroxide
H_3O^+	-Hydronium
H_3PO_4	- Phosphoric acid
HDL	-High-density lipoprotein
HIV	- Human immuno deficiency virus
HNO_2	-Nitrous acid
HOCl	-Hypochlorous acid
ICAM-1	- Intercellular Adhesion molecule-1
IFN-	- Interferons
IL-	- Interleukin
KCl	- Potassium chloride
KH_2PO_4	-Potassium dihydrogen phosphate
KIO_4	- Potassium periodate
KOH	- Potassium hydroxide
LDH	-lactate dehydrogenase
LDL	-low density lipoproteins
MDA	-Malondialdehyde
MRI	- Magnetic resonance imaging
N_2O_3	- Dinitrogen trioxide
Na_2HPO_4	-Disodium hydrogen phosphate
NaCl	- Sodium Chloride
NaCN	-Sodium cyanide
NADP	-Nicotinamide adenine dinucleotide phosphate
NADPH	-Nicotinamide adenine dinucleotide phosphate (reduced)
NaN_3	-Sodium Azide
NAPQI	- *N*-acetyl-*p*benzoquinoneimine
NBT	- Nitro blue tetrazolium

NF-*k*B	-Nuclear Factor-*k*B
NH_3	-Ammonia
NO	-Nitric oxide
NO_3	-nitrate
NOS	- Nitric oxide synthase
$O_2^{\bullet-}$	- Superoxide radical
OH-	-Hydroxide
-OH	- hydroxyl radical
ONOO-	- peroxynitrite
$ONOOCO_2^-$	-nitroso peroxocarboxylate
ONOOH	-peroxonitrous acid
PBS	-Phosphate-buffered saline
PGHS-2	- Prostaglandin-endoperoxide synthase- 2
Prx	-Peroxiredoxins / thioredoxin peroxidises
PVP	-Poly Vinyl Pyrolidone
RNA	-Ribo nucleic acid
RNS	-Reactive nitrogen species
RO•	-alkoxy radicals
ROO•	-peroxyl radicals
ROS	- Reactive oxygen species
SGOT	-Serum glutamate oxaloacetate transaminase
SGPT	-Serum glutamate pyruvate transaminase
SPIOs	- Superparamagnetic iron oxides
SOD	- Superoxide Dismutase
SOR	- Superoxide reductases
TBA	-Thiobarbituric acid
TBARS	-Thiobarbituric acid reacting substance

TCA	-Trichloroacetic acid
TNF-α	-Tumor necrosis factor alpha
Trx	-thioredoxins
TrxR	-thioredoxin reductase
XRD	-X-ray diffraction
XO	- Xanthine oxidase

Chapter-1

INTRODUCTION AND REVIEW OF LITERATURE

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1.1 CANCER

Cancer is the abnormal development of cells caused by various changes in gene expression leads to deregulated balance of cell proliferation and cell death and finally evolving into a population of cells. Later those cells invade other tissues and metastasize to far sites, causing significant morbidity. If the disease is untreated, leads to the death of the host. Being a genetic disease, it can be traced to alterations within specific genes. Due to these genetic changes, cancer cells multiply uncontrollably, producing malignant tumors that can attack surrounding healthy tissue. If the growth of the tumor remains localized, it can be treated and cured by surgical removal of the tumor. However malignant tumors metastasize, and the spawn cells breaks away from the parent mass, enter into the lymphatic or vascular circulation, and establish as lethal secondary tumors (metastases) that are no longer treated by surgical removal.

Cancer is a complex multistep process, that turn a normal cell in the body into an abnormal cancer cell (carcinogenesis). The diseases involve molecular defects in cell function resulting from common types of alterations to a cell's genes. Thus cancer is a disease of abnormal gene expression which occurs by a number of mechanisms such as gene mutation, translocation, amplification, deletion, loss of heterozygosity, or abnormal gene transcription or translation. The disease is characterized by imbalance of cell replication and cell death in a tumor cell population that later leads to an expansion of tumor tissue. But in normal tissues, cell proliferation and cell loss are in a state of balance.

Tumors are of two different types- benign and malignant. Major features are following:

1. Malignant tumors usually invade adjacent normal tissues and destroy them; on the other hand, benign tumors expands, generally encapsulated, and never invade surrounding tissue. Benign tumors can become life threatening if they press on nerves or blood vessels or sometimes they can secrete biologically active substances, like hormones, that later alter normal homeostasis of cells.

2. Malignant tumors metastasize, and enter into lymphatic channels or blood vessels to lymph nodes and other tissues in the body. Benign tumors always remain localized and do not metastasize.

3. Malignant tumor cells are ‘‘anaplastic,’’ tend to be well differentiated than normal cells of the tissue in which they arise. Benign tumors generally show resemblance to normal tissue than malignant tumors.

4. Malignant tumors usually grow more speedily than benign tumors. Whereas benign tumors grow slowly over several years.

1.1.1 Radiotherapy and chemotherapy for cancer treatment

Cancer is a cause of death in developing as well as in developed countries. The treatment modalities for cancer includes radiotherapy, chemotherapy and surgery. The conventional treatments have various modalities, all focussed at killing tumor cells and preventing their multiplication.

Radiotherapy is consider as the common modality for treating human cancers. It has been used in cancer treatment for decades, and used to destroy cancer cells and as a palliative to reduce pain associated with metastases. More than eighty percent of cancer patients require radiotherapy for curative or palliative purpose. During the course of treatment, radiation produce many biological changes in cells. Various

approaches are developed to make a balance between killing cancer cells and protecting normal tissues (Mohan *et al*., 2019). The main focus in radiotherapy is to enhance DNA damage of tumor cells, as double strand breaks, leads to cell death. Another option is to change cellular homeostasis, modifying signal transduction pathways, redox state, and disposition to apoptosis. The cellular changes would increase the tumor cells killing, while it should reduce normal cell death (Borek, 2004; Niyazi *et al*., 2011; Lu *et al*., 2016). To obtain optimal results, a sensible balance between the total dose of radiotherapy delivered and the threshold limit of the surrounding normal critical tissues is required. In order to attain better tumor control with a high dose, the normal tissues should be protected against radiation injury. Thus, the role of radioprotective compounds is very important in such clinical radiotherapy situations (Nair *et al.,* 2001; Mann *et al*., 2017).

Among the common therapeutic modalities of cancer, chemotherapy plays another important role. Most of the available synthetic chemotherapeutic agents are immunosuppressant, cytotoxic and exert several side effects (Diwanay *et al.,* 2004; Nurgali *et al*., 2018).

Immunosuppression is one of the major drawback of chemotherapy (Hersh and Freiriech, 1968). The cytotoxic drugs create major toxic side effects like nephrotoxicity, cardiotoxicity, hepatic toxicity, hematopoietic depression, gastrointestinal toxicity, hemorrhagic cystitis, hemopoetic suppression and urotoxicity etc (Morandi *et al.,* 2005; Papaldo *et al.,* 2005; Schwartz *et al.,* 2005; Amudha *et al.,* 2007; Niyazi *et al*., 2011; Chan and Ismail, 2014; Lu *et al*., 2016). Cytotoxicity to normal tissue is the major dose-limiting factor in chemotherapy. Thus reduces the quality and restricts treatment protocol. Therefore there is a constant search and need for the discovery and development of effective and non-toxic chemo protective

compound, that can decrease the side effects of chemotherapeutics. Such drugs can stimulate immunity, and help in improving cancer treatment strategies.

Any agent or drug, that offers protection to normal cells during therapy of cancer is essential for modifying the therapeutic index and to improve the life quality during radiation and chemotherapy (Baliga *et al*., 2013; Bendale *et al*., 2015).

1.1.2 Side effects of chemotherapy

For the treatment of cancer, chemotherapy has opened new possibilities for development of the quality of life in cancer patients and for the cure of disease. Although the success rate is very high, treatment with the most effective anticancer drugs shows symptoms of direct toxicity. Studies have documented the ability of chemotherapeutic agents in causing chromosomal damage and apoptosis (D'Agostini *et al.*, 2005; Rao *et al.*, 2005; Aslam *et al*., 2014; Zhou *et al*., 2018). Doxorubicin (DOX) and Cisplatin (Cis) are two of the most commonly used chemotherapeutic agents used in the treatment of a number of human neoplasms such as testicular, head, neck and lung carcinomas.

1.1.2.1 Doxorubicin

Doxorubicin (DOX) has been used in cancer treatment since the late 1960s. Its a potent drug in the fight against cancer. The various side effects of doxorubicin treatment, include myelosuppression, nausea, vomiting, and arrhythmia etc (Lefrak *et al.*, 1973; Injac and Strukelj, 2008). But, reports on fatal cardiotoxic effects of doxorubicin have restrained the interest for this drug. The various mechanism by which anthracyclines cause cardiotoxicity includes development of free radicals and superoxides (Rajagopalan *et al.*, 1988; Alderton *et al.*, 1992; Rossi *et al.*, 1994; Vasquez-Vivar *et al.*, 1997; Mobaraki *et al*., 2017; Varricchi *et al*., 2018).

Doxorubicin induces free radical production (Sinha *et al.*, 1987), and causing oxidative stress along with cellular injury and liver damage (Rosen and Halpern, 1990). Cardiac cells may be more vulnerable to oxidative stress due to low levels of antioxidant enzymes in the heart tissues (Doroshow *et al.*, 1980; McGowan *et al*.,2017). Various works in rat hearts suggest that doxorubicin is able to cause more depletion of antioxidant enzymes (Li and Singal, 2000). More than that DOX causes different metabolic errors within cardiac cells with major undesirable side effects leads to cardiotoxicity and bone marrow suppression (Chatham *et al.*, 1990; Beanlands *et al.*, 1994). The toxicity can be marked by elevated levels of serum enzymes such as SGOT, LDH, Creatinine phosphokinase etc (al-Harbi *et al.*, 1992). The dominant cellular target of DOX is DNA, causing two major types of DNA damages such as DNA adducts and protein associated single and double strand DNA breaks (Cullinane *et al.*, 2000; Sandamali *et al*., 2020). The scission of DNA by DOX toxicity is believed to be either by the action of topoisomerase II (Tewey *et al.*, 1984) or by production of free radicals by DOX (Gutteridge and Toeg, 1982; Albert *et al*., 2018). DOX causes sister chromatid exchanges, chromosome aberrations and DNA double strand breaks both in *in vivo* and *in vitro* system (Noviello *et al.*, 1994;Gulkac *et al.*, 2004; Elblehi *et al*., 2021).

1.1.2.2 Cisplatin

Cisplatin (Cis or CP) contain a central platinum atom, a chlorine atom and an ammonia molecule in the cis-position, and is effective in the treatment of a wide variety of neoplastic diseases (Lebwohl and Canetta, 1998; dos Santos *et al*., 2012). The cytotoxicity by Cis is often associated with its ability to combine DNA to form cisplatin–DNA adducts (Goldstein and Mayor, 1983; Prasaja *et al*., 2015). Even if Cis is effective, it is associated with various undesirable drug reactions, like renal

damage, gastrointestinal dysfunction, auditory toxicity, and peripheral nerve toxicity etc (Cooley *et al.*, 1994; Zicca *et al.*, 2002; Miller *et al*., 2010). Nephrotoxicity in particular is a major complication and a dose-limiting factor for Cis therapy (Safirstein *et al.*, 1986). Cisplatin induced nephrotoxicity is closely associated with an increase in lipid peroxidation in the kidney tissues (Krakoff, 1979; Matsushima *et al.*, 1998) and elevation in serum markers such as urea and creatinine (Mizushima *et al.*, 1987). The drug can generate ROS, such as superoxide anion and hydroxyl radicals (Greene, 1992; Cross *et al.*, 1996; Overbeck *et al.*, 1996; Khynriam and Prasad, 2001; Alhoshani *et al*., 2017). It has been shown that cisplatin-based chemotherapy induces a fall in patient plasma concentrations by depleting various antioxidants (Borch and Markman, 1989; Volarevic *et al*., 2019). Beside with its toxic effects, Cis can induce DNA damages and chromosomal abberations (Crul *et al.*, 1997; McSweeney *et al*., 2021). Studies have shown that Cis can cause genotoxic effects in cultured mammalian cells (Zwelling *et al.*, 1979) and bone marrow cells (Giri *et al.*, 1998; Fang *et al*., 2021).

1.1.3 Inflammation

Inflammation is a physiological process occurs in response to tissue damage from microbial pathogen infection, chemical irritation, wounding and/or toxicity. The relation between inflammation and various diseases such as cancer, diabetes, alzheimer's, atherosclerosis, arthritis has been well studied (Ohshima and Bartsch, 1994; Stevens *et al.*, 2005; Maiti and Agrawal, 2007; Virani *et al.*, 2008). It is a complex action mediated by few factors like prostaglandins, cytokines, and free radicals etc. They have direct or indirect effect on the pathophysiology of diseases. Inflammatory responses are classified based on the duration of the reaction to injury, that is acute and chronic inflammation. Chronic inflammation formed from

unresolved symptomatic acute inflammation with and/or without any clinical manifestations. This may trigger macrophages and lymphocytes, they release inflammatory mediators and result in excess formation of reactive oxygen and nitrogen species that damage DNA and cell membranes (Sprung *et al*., 2015). The inflammatory cells liberate prostaglandins along with increased expression of key enzyme cyclooxygenase that later can activate several transcription factors including NF-κB (Hofseth and Ying, 2006).

Carrageenan and dextran induced acute inflammation in animals are the most appropriate test procedures used to study anti-inflammatory agents. Carrageenan induce edema and releases histamine, 5-hydroxy tryptamine, kinins and prostaglandins etc. It directs protein rich exudates and neutrophils at the site of inflammation (Lo *et al.*, 1982; Larsen and Henson, 1983; Vane and Botting, 1987; Brooks and Day, 1991). Dextran directs histamine and serotonin to the site of inflammation and fluid accumulation by mast cell degradation (Ghosh *et al.*, 1963). Formalin induced inflammation is the test procedure commonly used to screen chronic anti-inflammatory agents, because it resembles human arthritis (Greenwald, 1991). The nociceptive effect of formalin is biphasic, an early neurogenic component which is followed by tissue mediated response (Panthong *et al.*, 2004).

Irradiation and radio-chemotherapy in cancer treatment raise the level of endogenous cytokines and other growth factors and exert their regulatory functions by autocrine, paracrine or endocrine modes of action resulting in inflammatory responses (Michalowski, 1994). Inflammation also facilitate the instigation of normal cells to malignancy through the production of inflammatory oxidants. Proper treatment of inflammation with various anti-inflammatory agents, inhibitors of oxidant generating

enzymes, and scavengers of oxidants are to be explored to prevent development of human cancers associated with chronic inflammation (Ohshima *et al.*, 2003).

1.1.3.1 Inflammation and cancer

The association between the development of cancer and inflammation has long been reportd (Balkwill and Mantovani, 2001; Coussens and Werb, 2002 ;Reuter *et al*., 2010). Earlier studies focused on different aspects of the relationship between cancer and inflammation, like the role of inflammatory cells (Balkwill and Mantovani, 2001; Egeblad *et al.*, 2005; Condeelis and Pollard, 2006), mediators (Egeblad *et al.*, 2005; Wang and Dubois, 2006; Zlotnik, 2006), or signaling pathways (Karin and Greten, 2005) in cancer. Tumor promotion require not only the survival of initiated cells, but also their development. Various inflammatory mediators as cytokines, chemokines, and eicanosoids are able to stimulate the proliferation of both untransformed and transformed tumor cells (Balkwill and Mantovani, 2001).

The connection between cancer and inflammation can be studied by two pathways: an intrinsic and an extrinsic pathway (Mantovani *et al.*, 2008; Khandrika *et al*., 2009). At the level of the tumor cell initiation, both pathways join and bring the activation of several transcription factors leads to the formation of numerous pro-inflammatory molecules that direct and activate leucocyte populations into the tumor microenvironment (Multhoff *et al.*, 2011). Tumor cell-derived pro-inflammatory molecules, then activates the same transcription factors within the cells of microenvironment, so tumor cells themselves resulted in a more pronounced generation of inflammatory mediators leading to tumor-promoting amplification loop. This amplification mechanism enhances the action of inflammatory stimuli within the tumor environment and creates a cancer-related inflammatory milieu contributing to tumor growth and invasiveness (Elinav *et al*., 2013). Some lines of evidence shows

that inflammation also plays a key role in modulating radiation responsiveness of tumors. Radiation treatment is evidently a two-edged sword. On one hand, the sub lethal doses of ionizing radiation induces a DNA damage response. On the other hand, they activate a cellular damage response in tumors by inducing pro-inflammatory pathways predominantly mediated via activation of NF-κB, the central linker between inflammation, carcinogenesis, and radioresistance. Apart from NF-κB activation, radiation also activates/ up-regulates the expression of early genes for c-Fos, c- Myc, c-Jun (Hong *et al.*, 1997) as well as TNF (Zhou *et al.*, 2001), GM-CSF (Akashi *et al.*, 1992), PGHS-2 (Steinauer *et al.*, 2000), and ICAM-1(Son *et al.*, 2006).

1.1.4 Nanomedicine

Nanotechnology is a multidisciplinary branch focused on the design and manufacturing of materials smaller than 100 nm in diameter to attain unique properties, so that they can be suitably manipulated for various applications (Gleiter, 2000). As most of the natural processes occur in the nanometre scale regime, the union of nanotechnology and biology can tackle several biomedical problems, and can revolutionize the field of health and medicine (Curtis and Wilkinson, 2001; Salvioni *et al*., 2019). Nanosized particles are amenable to various biological functionalization and are presently employed as a tool in medicine like imaging (Chan and Nie, 1998), sensing (Vaseashta and Dimova-Malinovska, 2005), targeted drug delivery (Langer, 2001), gene delivery systems (Roy *et al.,* 1999), artificial implants (Sachlos *et al.,* 2006) *etc*. Based on improved effectiveness, the new generation drugs are nanoparticles of polymers, metals or ceramics, which can be used in situations like cancer (Zhang *et al.,* 1996; Brigger *et al.,* 2002; Farokhzad *et al.,* 2006) and fight human pathogens (Stoimenov *et al.,* 2002; Sondi and Sondi, 2004; Baker *et al.,* 2005; Elechiguerra *et al.,* 2005;Morones *et al.,* 2005).

Nanomedicine is the medical application of nanotechnology in experimental and clinical challenges. Among its numerous nonmedical applications, the use of nanoparticles as an alternative therapeutics against various diseases is under research.

1.1.4.1 Nanoparticles for drug delivery and cancer therapy

Nanoparticles are widely used in cancer diagnosis, treatment, and as delivery vectors for biologic or pharmacologic agents (Brigger *et al.,* 2002; ONeal *et al.,* 2004; Paciotti *et al.,* 2004; Bhattacharya *et al.,* 2005; Jain, 2005; Mukherjee *et al.,* 2005; Cuenca *et al.,* 2006; Kam *et al.,* 2006; Zhang *et al.,* 2006) because of the unique size and large surface area-to-volume ratio of particles (Youn and Bae, 2018).

The use of nanoscale devices helped cancer biology in various ways: for early detection, tumor imaging purpose by using radiocontrast nanoparticles or quantum dots, and for targeted drug delivery using nanovectors and nanoparticles (Wilhelm *et al*., 2016). Nanotechnology also employed in management of various diseases, in drug resistance in leukemia by blocking drug efflux from cancer, in inducing efficient delivery of siRNA into lymphocytes to block apoptosis in sepsis and in targeting tumors. The immune cells with nanocrystal labeling considered as nanoimmunotherapy (Prabhu *et al.,* 2011). Cancer nanotherapeutics are rapidly progressing and are used to solve limitations of conventional drug delivery systems such as nonspecific biodistribution and targeting, lack of water solubility, poor oral bioavailability, and low therapeutic indices. In order to use nanoparticles as cancer drugs, they have to be designed for optimal size and surface characteristics, so that the drugs can be maintain in bloodstream for a long time. They can able to carry loaded active drugs to cancer cells by selectively using the unique pathophysiology of tumors, such as their enhanced permeability and retention effect and the tumor microenvironment. Nowadays active targeting strategies using ligands or antibodies

directed against selected tumor targets magnify the specificity of these therapeutic nanoparticles. Drug resistance, is another obstacle that reduces the efficacy of both molecularly targeted and conventional chemotherapeutic agents, which might also be overcome or be reduced using nanoparticles. Nanoparticles have the ability to accumulate in cells without being recognized by P-glycoprotein, one of the main mediators of multidrug resistance, resulting in the increased intracellular drug accumulation (Cho *et al.,* 2008; Nakamura *et al*.,2016).

Gold nanoparticles conjugated with molecules such as antibodies, carbohydrates, and pharmacologic agents, can be used to target cancer cells (Hirsch *et al.,* 2005; Huang, 2006; Niidome *et al.,* 2006; Ding *et al.,* 2007;Mukherjee *et al.,* 2007). Studies have shown that the gold nanoparticles showed anti-proliferative activity in multiple myeloma cells (Bhattacharya *et al.,* 2007) and anti-angiogenic properties (Mukherjee *et al.,* 2005). Studies have shown that gold-silica nanoshells or gold nanoparticles release significant heat when exposed to near-infrared (NIR) light (650–950 nm) or radiofrequency radiation and can be used to produce thermal cytotoxicity *in vitro* (Gobin *et al.,* 2007; Ding *et al*., 2018).

Magnetic drug targeting is a different promising novel field of research. Gilchrist injected maghemite particles (20–100 nm) and inducted selective heating of lymph nodes near surgically removed cancer (Gilchrist *et al.,* 1957; He *et al*., 2019). Meyers *et al*. (1963) studied the accumulation of small iron particles by intravenous injection into the leg veins of dogs, through an externally applied magnetic field. The use of magnetic particles for the therapy of liver cancer were also found successful (Wu *et al.,* 1995; Jones and Winter, 2001). The use of more defined spherical magnetic microspheres were made at the end of the 1970s (Widder *et al.,* 1979). Magnetic drug delivery can be used to attain very high concentrations of chemotherapeutic or

radiological agents close to the target site, such as a tumor, without causing any toxic effects to the normal surrounding tissues. A drug or therapeutic radioisotope is bound to a magnetic nanoparticles, then injected into a patient's blood stream, and a powerful magnetic field is applied in the target area. Based on the type of drug, it is then slowly release from the magnetic carriers or conferred to a local area (e.g. irradiation from radioactive microspheres; hyperthermia with magnetic nanoparticles). It is thus possible to replace large amounts of freely circulating drug with much lower amounts of drug restricted in disease sites ensuring effective and several-fold increased localized drug levels (Widder *et al.,* 1979; Gupta and Hung, 1989; Hafeli *et al.,* 1997).

1.1.4.2 Applications of magnetic nanoparticle systems in diagnosis and treatment

For molecular imaging purpose, four different classes of nanoparticles are of prime interest: (a) magnetic nanoparticles, (b) magnetofluorescent nanoparticles, (c) fluorescent nanoparticles (e.g., quantum dots, fluorochrome doped nanoparticles), and (d) isotope tagged nanoparticles.

The main diagnostic application of magnetic nanospheres as contrast agents for magnetic resonance imaging (MRI). Superparamagnetic iron oxides (SPIOs) are used for the imaging of liver metastases or to distinguish bowel loops from other abdominal structures since 1994.

1.1.4.3 Magnetic delivery of chemotherapeutic drugs to tumors

Molecular targeted therapeutics is better than nonspecific cytotoxins due to the higher therapeutic index (Kim, 2003). Nanoparticles of diverse compositions and purposes can be used (Brigger *et al.,* 2002; El-Readi and Althubiti, 2019). Size, charge, hydrophilicity, and composition of nanoparticles can be altered to attain optimal

uptake within a tumor and specific targeting ligands (*i.e*., peptides, monoclonal antibodies, small molecules) (Merisko-Liversidge *et al.,* 1996).

Prolonged exposure of tumor to high drug concentration is a prerequisite for therapeutic efficacy (Gutman *et al.,* 2000). But, passive biodistribution of a systemically administered drug is governed by the physicochemical properties like molecular weight, lipophilicity, *etc*. of the compound and thus results in subtherapeutic drug levels at the tumor site (Motl *et al.,* 2006) which fail to irradicate the lesion and can stimulate overgrowth of resistant malignant cells (Heimberger *et al.,* 2000). The capacity to selectively target antineoplastic agents to tumor can be carried out by encapsulation carriers such as liposomes (Gregoriadis and Ryman, 1971) and albumin microspheres (Kramer, 1974). But, the circulation of liposomes and carriers result in the rapid clearance by reticuloendothelial system. Magnetic nanoparticles are excellent candidate for developing drug delivery systems, as it is feasible to make and characterize (Tiefenauer *et al.,* 1993; Alexiou *et al.,* 2000; Zhang *et al.,* 2002;Gupta *et al.,* 2003; Berry *et al.,* 2004;Gupta and Wells, 2004; Bi *et al*., 2016) and an external localized magnetic field gradient can be applied to target it (Alexiou *et al.,* 2002; Rani *et al*., 2012).

1.2 OXIDATIVE STRESS

In recent years there's an increasing awareness among people in prevention of disease especially the role of free radicals in health and disease. Oxygen is an element indispensable for life. When cell uses oxygen to get energy, free radicals are produced by the mitochondria (Tiwari, 2004). These by-products are generally reactive oxygen species (ROS) also as reactive nitrogen species (RNS) that result from the cellular redox process. The free radicals have a special affinity for lipids, proteins and nucleic acid (DNA) (Velavan, 2011). In a normal cell there's balance between formation and

removal of free radicals. However this balance are often shifted towards more formation of free radicals or when levels of antioxidants are diminished. This state is called 'oxidative stress' and can result in serious cell damage (Butnariu and Samfira, 2012). Oxidative stress plays a major role in the development of chronic and degenerative diseases such as cancer, arthritis, ageing, autoimmune disorders, cardiovascular and neurodegenerative diseases (Lian, 2008).

In biological system oxidative stress may result from (i) decrease in endogenous antioxidant levels like SOD, catalase or GPx, depletion of dietary antioxidants and other essential dietary constituents (ii) Generation of oxidative compounds, physiologically important as a neighborhood of a defense reaction against invading microorganisms or malignant cells, also as of tissue repair, healing and remodelling (Locatelli *et al*., 2003; Ahmadinejad *et al*., 2017).

The human body has some mechanisms to counter oxidative stress by producing antioxidants which are either naturally formed in body, or externally supplied through foods and or supplements. Endogenous and exogenous antioxidants act as radical scavengers and thus can enhance the immune defence and lower the danger of cancer and degenerative diseases (Valko *et al*., 2006; Chandra *et al*., 2015). Recently it has been claimed that the imbalances within the amount of free radicals and antioxidants in saliva may play an important role within the onset of periodontal diseases, therefore measurement of oxidative stress in saliva represents major intraoral condition and this is often ready to provide a more accurate account of the oral environment (Cully *et al*., 2002).

1.2.1Generation of free radicals

Most of the oxygen haunted by the cells is converted to water by the action of cell enzymes. However a number of these enzymes leak electrons into the oxygen

molecules and cause the formation of free radicals and are formed during normal biochemical reaction involving oxygen. There are two important sources of radical formation, one among the interior factors i.e. normal cellular metabolism like mitochondrial ETC, endoplasmic reticulum oxidation and lots of enzymatic activities (Bandyopadhyay *et al*., 1999; Tandon *et al*., 2005). Other external factors includes radiation, oxidation of engine exhaust, carbon tetrachloride , cigarette smoke and oxygen itself (Slater, 1984; Mustacich and Powis,2000; Nordberg and Arner, 2001; Imlay, 2003) (**Figure 1.1**).

Intracellular generation of ROS mainly comprises superoxide (O_2•−) radicals and nitric oxide (NO•) radicals. Under normal physiological conditions, nearly 2% of the oxygen consumed by the body is converted into O_2•− through mitochondrial respiration, phagocytosis, etc (Winterbourn, 2008). ROS percentage increases during infections, exercise, exposure to pollutants, UV light, radiation, etc. NO•, is an endothelial relaxing factor and neurotransmitter, produced through gas synthase enzymes. The NO• and O_2•− radicals, are converted to powerful oxidizing radicals like hydroxyl radical (•OH), alkoxy radicals (RO•), peroxyl radicals (ROO•), singlet oxygen ($^1O_2$) by complex transformation reactions (Pickrell *et al*., 2009).

Some of the novel species are converted to molecular oxidants like hydrogen peroxide (H_2O_2), peroxynitrite (ONOO-), hypochlorous acid (HOCl). Sometimes these molecular species act as source of ROS for instance, H_2O_2 is converted to •OH radicals by Fenton reaction and HOCl through its reaction with H_2O_2 are often converted to $^1O_2$. At physiological concentrations of carbon dioxide, peroxynitrite (ONOO-) becomes a source of carbonate radical anion (CO_3 •−). The varied pathways involved within the generation of ROS are given in **figure 1.2.**

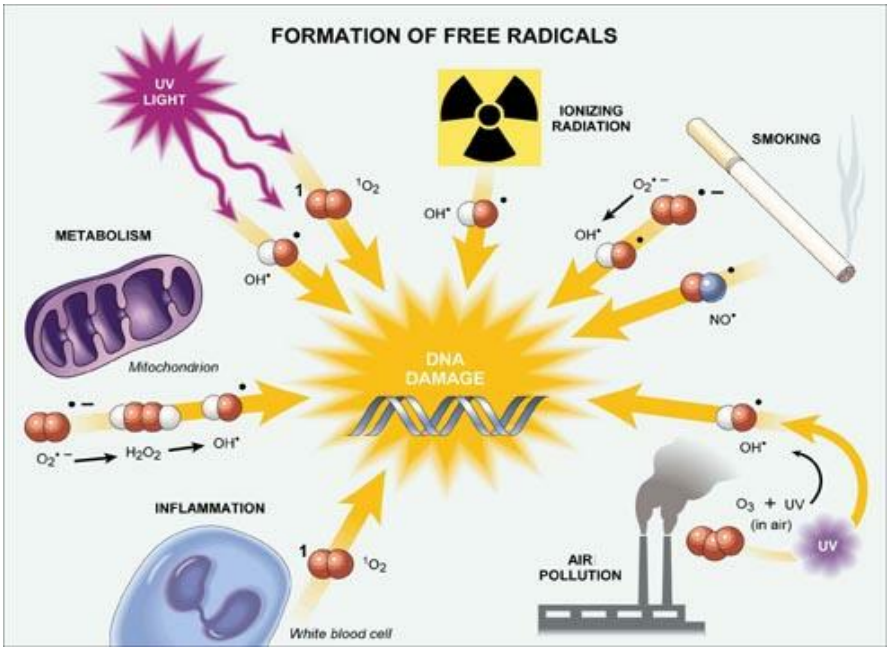

Figure 1.1 Sources of free radical formation (Vasudevan and Sreekumari, 2004;Shinde *et al*.,2012**)**

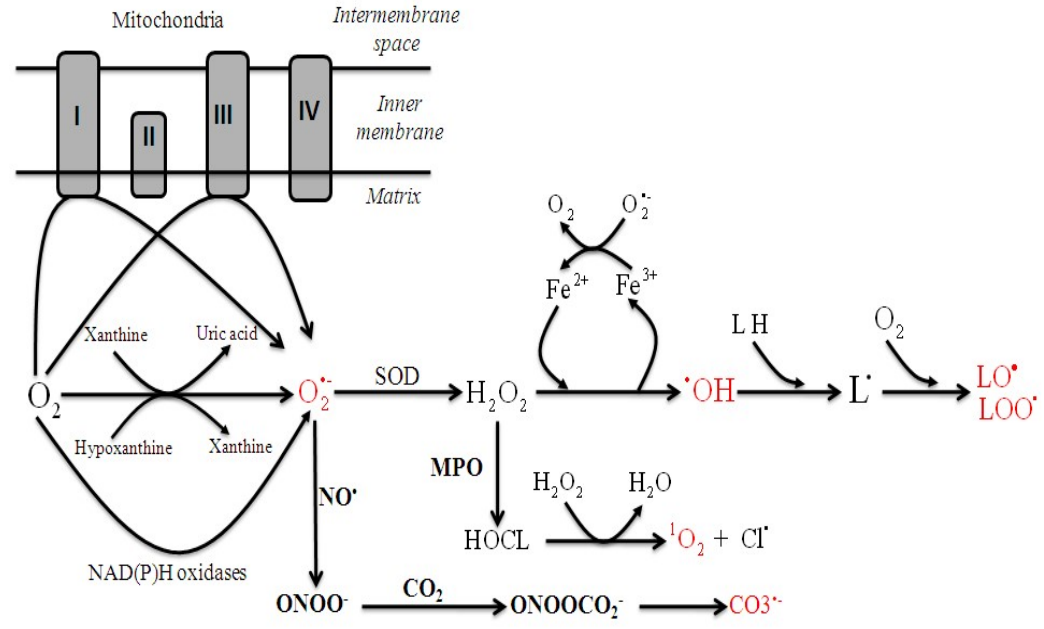

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Figure 1.2 Production of free radicals via different routes (Kunwar and Priyadarsini,2011**)**

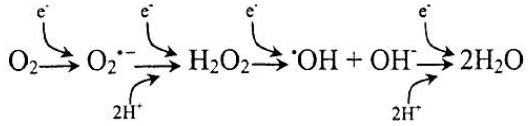
1.2.2 Reactive oxygen species (ROS)

ROS include a number of chemically reactive molecule derived from oxygen (Halliwell, 1996; Fridovich, 1999; Halliwell, 1999; Betteridge, 2000; Halliwell, 2007). Some of those molecules are extremely reactive (the hydroxyl) , while some are less reactive (superoxide and hydrogen peroxide). Free radicals and ROS can readily react with most biomolecules, starting a sequence reaction of radical formation (Rizzo *et al*., 2010). In order to prevent this chain reaction, a newly formed radical must either react with another radical , eliminating the unpaired electrons, or react with a radical scavenger— a chain-breaking or primary antioxidant. **Table 1.1** shows the common intracellular sorts of ROS, key cellular sources of production and the relevant enzymatic antioxidant systems scavenging these ROS molecules.

Table 1. 1 The Major ROS Molecules and Their Metabolism

ROS molecule	Main sources	Enzymatic defence systems	Product (s)
Superoxide (O_2•−)	Leakage of electrons from electron transport chain, Activated phagocytes, xantine oxidase, flavoenzymes	Supeoxide dismutase(SOD), Superoxide reductase (in some bacteria,	$H_2O_2 + O_2$ H_2O_2
Hydrogen peroxide (H_2O_2)	From O_2•− via SOD, NADPH oxidase, Glucose oxidase, Xanthine oxidase	Glutathione peroxidise, Catalases, Peroxiredoxins (Prx)	H_2O_2 + GSSG $H_2O + O_2$ H_2O
Hydroxyl radical (•OH)	From O_2•− and H_2O_2 via transition metal(Fe or Cu)	Glutathione/TrxR	GSNO
Nitric oxide (NO)	Nitric oxide synthases	Glutathione/TrxR	GSNO

The step-wise reduction of molecular oxygen producing and as well connecting the ROS molecules shown in Table 1.1, can be summarized as follows:



(Reaction 1)

1.2.2.1 Superoxid*e* (O_2•–)

The superoxide created from molecular oxygen by the addition of an electron is, in spite of being a radical, not highly reactive. It lacks the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it was produced (Cynshi *et al*., 2010). The formation of superoxide takes place spontaneously, especially in the electron-rich aerobic environment in vicinity of the inner mitochondrial membrane with the respiratory chain (**Figure 1.3**). Superoxide (as well as hydrogen peroxide) can be produced endogenously by flavoenzymes, e.g., xanthine oxidase (Kuppusamy and Zweier, 1989) activated in ischemia-reperfusion (Zimmerman and Granger, 1994). Other superoxide-producing enzymes are lipoxygenase and cyclo oxygenase (Kontos *et al*., 1985; McIntyre *et al*., 1999).

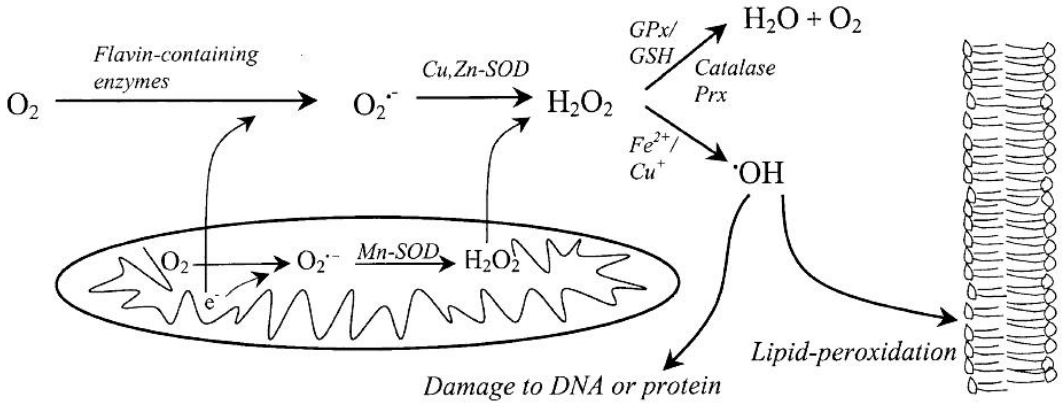


Figure 1.3 Schematic representation of oxidative and antioxidative systems in cells (Nordberg and Arner, 2001)

1.2.2.2 Hydrogen peroxide (H_2O_2)

H_2O_2 is not a free radical but is nonetheless highly important much because of its ability to penetrate biological membranes. It plays a radical forming role as an intermediate in the production of more reactive ROS molecules including HOCl (hypochlorous acid) (Reaction 2) (Winterbourn *et al*., 2000; Wolin, 2009).

$$H^+ + Cl^- + H_2O_2 \rightarrow 3\ HOCl + H_2O$$

(Reaction 2)

Another important function of H_2O_2 is carried out in its role as an intracellular signaling molecule (Sundaresan *et al*., 1995;Rhee, 1999). H_2O_2 once produced by the above mentioned mechanisms is removed by at least three antioxidant enzyme systems, namely catalases, glutathione peroxidases, and peroxiredoxins. (Chae *et al*., 1999[a] ; Chae *et al*., 1999[b] ; Mates *et al*., 1999).

1.2.2.3 Hydroxyl radical (•OH)

Due to its strong reactivity with biomolecules, •OH is probably capable of doing more damage to biological systems than any other ROS (Halliwell, 1987; Betteridge, 2000). The radical is formed from hydrogen peroxide in a reaction catalyzed by metal ions (Fe^{2+}or Cu^+), often bound in complex with different proteins or other molecules. This is known as the Fenton reaction:

$$H_2O_2 + Cu^+/Fe^{2+} \rightarrow \bullet OH + OH^- + Cu2^+/Fe^{3+}$$

(Reaction 3)

Superoxide also plays an important role in connection with Reaction 3 by recycling the metal ions:

$$Cu^{2+}/Fe^{3+} + O_2 \bullet^- \rightarrow Cu^+/Fe^{2+} + O_2$$

(Reaction 4)

The sum of Reactions 3 and 4 is the Haber-Weiss reaction; transition metals thus play an important role in the formation of hydroxyl radicals (Halliwell, 1987; Halliwell, 1999). Transition metals may be released from proteins such as ferritin (Harris *et al*., 1994) and the [4Fe-4S] center of different dehydrases by reactions with O_2 •–. This mechanism is specific for living cells has been called the *in vivo* Haber-Weiss reaction (Fridovich, 1997).

1.2.2.4 Nitric oxide (NO)

Nitric oxide represents an odd member of the radical family and is analogous to O_2•_ in several aspects therein it doesn't readily react with most biomolecules despite its unpaired electron. On the opposite hand it simply reacts with other free radicals (e.g., peroxyl and alkyl radicals), releasing less reactive molecules, so in reality functioning as a radical scavenger; NO has, for instance , been shown to prevent lipid peroxidation in cell membranes (Hogg and Kalyanaraman, 1998; Rubbo *et al*., 2000). Though, if O_2 •_ is produced in large amounts in parallel with NO, both react with one another to offer OONO_ (peroxynitrite), which is very cytotoxic (Beckman and Koppenol ,1996). Peroxynitrite can directly react with diverse biomolecules in one- or two-electron reactions, and readily react with CO_2 to make highly reactive nitroso peroxocarboxylate ($ONOOCO_2$_), or protonated as peroxonitrous acid (ONOOH) undergo homolysis to make either •OH and •NO_2 or rearrange to nitrate (NO_3). The individual rates of those different reactions of peroxynitrite will depend on the pH, temperature, and sort of compounds present within the surrounding milieu (Radi *et al*., 2001). Peroxynitrite, directly or via its reaction products, may oxidize low density lipoproteins (LDL), release copper ions by destroying ceruloplasmin, and usually attack tyrosine residues in several proteins, as observed in many inflammatory diseases (Halliwell, 1997).

NO is synthesized enzymatically from L-arginine by NO synthase (NOS) (Andrew and Mayer, 1999; Beck *et al*., 1999; Bredt, 1999) (Reaction 5).

L-arginine + O_2 +NADPH → L-citrulline + NO + $NADP^+$

(Reaction 5)

In physiologic concentrations NO functions mainly as an intracellular messenger stimulating guanylate cyclase and protein kinases, thereby relaxing smooth muscle in blood vessels, among other effects.

NO has the ability to cross cell membranes and can thereby also transmit signals to other cells (Ignarro, 1990). When produced in larger amounts, as is the case when iNOS is induced by endotoxin and IFN-_, NO becomes an important factor in redox control of cellular function (Wink and Mitchell, 1998).

1.2.3 PHYSIOLOGICAL FUNCTIONS OF ROS

1.2.3.1 Defense against infection

When phagocytes are activated, they produce ROS in amounts enough to kill intruding bacteria (Thomas *et al*., 1988). ROS are in this system produced by the NADPH oxidase complex that converts O_2 to $O_2^{\bullet-}$ (Babior, 1999; Nauseef, 1999). Superoxide is then reduced in the phagosome by SOD to H_2O_2 that can be further converted to HOCl by myeloperoxidase (Rossi *et al*.,1985) (Reaction 2). Hypochlorous acid can suddenly form •OH by Reactions 6 or 7. Here reaction 7 is analogous to the Fenton reaction (Reaction 3) but HOCl taking the place of H_2O_2.

HOCl + O2 $^{\bullet-}$ → •OH + O2+ Cl^- (Reaction 6)

HOCl + Cu^+/Fe^{2+}→ •OH + Cl^- + Cu^{2+}/Fe^{3+} (Reaction 7)

The two heavily reactive ROS molecules thus formed in phagosomes (HOCl and •OH) are extremely toxic to bacteria ingested by the phagocyte and bring the direct antimicrobial effects of ROS. The hypochlorous acid produced in the

myeloperoxidase reaction is also an important part of the antimicrobial defense by destruction of the DNA anchoring at the bacterial membrane, resulting in cessation of DNA replication (Rosen *et al*., 1990; Elsayed, 2019).

1.2.3.2 Redox regulation of transcription factor activity

ROS can directly affect the conformation activities of all sulfhydryl-containing molecules, such as proteins or GSH, by oxidation of their thiol moiety. This type of redox regulation affects many proteins important in signal transduction and carcinogenesis such as protein kinase C, Ca^{2+}-ATPase, collagenase, and tyrosine kinases (Dalton *et al*., 1999; Fisher, 2011), among many other enzymes and membrane receptors. For several transcription factors, ROS function as physiological mediators of transcription control (Sen and Packer, 1996; Arrigo, 1999; Dalton *et al*., 1999; Morel and Barouki, 1999; Allen and Tresini, 2000). Well-known examples of redox-sensitive transcription factors are Nuclear Factor-*k*B (NF-*k*B) and Activator Protein-1 (AP-1) (Schreck *et al*., 1991; Schreck *et al*., 1992; Baeuerle and Henkel, 1994; Foletta *et al*., 1998; Sen, 1998; Dalton *et al.,* 1999; Kamata and Hirata, 1999; Kyriakis, 1999; Morel and Barouki, 1999; Marshall *et al*., 2000).

1.2.3.3 ROS as Cause of Oxidative Damage

Due to the high reactivity, ROS prone to cause damage, and are thereby also potentially toxic, mutagenic, or carcinogenic. The targets for ROS damage include all major groups of biomolecules, summarized as follows.

1.2.3.3.1 DNA

ROS have shown to be mutagenic (Mates *et al*., 1999; Marnett, 2000), that chemically modifies DNA molecules. A number of alterations such as cleavage of DNA, DNA-protein cross links, oxidation of purines, etc are due to reactions with ROS, like •OH. If the DNA repair systems doesn't work, mutation will occurs results

in erroneous base pairing during replication. This mechanism may somewhat explains the high prevalence of cancer in individuals with oxidative stress (Mates *et al*., 1999; Marnett, 2000). The reality is that apoptosis in some cases is mediated by ROS (Kamata and Hirata, 1999) or may due to ROS-derived damage to DNA, but is also related to increased mitochondrial permeability, released cytochrome C, increased intracellular Ca^{2+}, and other effects (Kroemer *et al*., 1998).

The ROS plays an important factor in cellular and whole organism ageing due to damage to mitochondrial DNA (Ames *et al*., 1993; Beckman and Ames, 1997; Butterfield *et al*., 1998; Cortopassi and Wong, 1999). This concept was newly challenged by a study based on extensive gene array analysis signifying errors in the mitotic machinery and maybe in arachidonic acid metabolism as important determinants for the ageing process (Ly *et al*., 2000). However, studies indicate that ROS contribute to ageing (Finkel and Holbrook, 2000) and, on the other hand, superoxide dismutase and catalase increases the life span in *C. elegans* (Melov *et al*., 2000).

1.2.3.3.2 Lipids

Lipid peroxidation is possibly the most explored area of research in ROS production (Steinberg, 1997; Yla-Herttuala, 1999). The excellent targets for free radical includes the polyunsaturated fatty acids because of their multiple double bonds. Such type of oxidation is necessary for the production of atherosclerotic plaques (Halliwell, 1993; Frei, 1999). The oxidation of low density lipoproteins (LDL) and uptake of these particles by phagocytes in the sub endothelial space by way of their scavenger receptor, stimulate the formation of atherosclerotic plaques. Cardiovascular disease with plaque formation is a common disese reported in western countries. Therefore, minimize the amount of lipid peroxidation is of significant medical importance.

1.2.3.3.3 Proteins

In vitro studies have shown that ROS have been directly react with several amino acid residues, generating anything from modified and less active enzymes to denatured, non-functioning proteins (Butterfield *et al*., 1998; Stadtman and Berlett, 1998). The sulfur- (or selenium)-containing residues are the most susceptible amino acids. General antioxidant systems (Lowther *et al*., 2000), offers protection to such proteins from modification.

1.2.4 CELLULAR ANTIOXIDANT ENZYMES

There are two major groups of cellular anti oxidant systems- enzymatic and nonenzymatic.

1.2.4.1 Superoxide dismutases (SOD)

The first genuine ROS-metabolizing enzymes discovered was Superoxide dismutases (SOD) (McCord and Fridovich, 1969). In eukaryotic cells, O_2 •– can be metabolized to H_2O_2 by two metal containing SOD isoenzymes, one present in mitochondria, 80-kDa tetrameric Mn-SOD, and the second one include cytosolic 32-kDa dimeric Cu/Zn-SOD. Bacteria possess both Mn-SOD and Fe-SOD type of SOD (Yost and Fridovich, 1973) and in some cases Cu, Zn-SOD also present in them (Fridovich, 1997). In the reaction catalyzed by SOD, two molecules of superoxide form H_2O_2 and molecular O_2 and it constitute a source of cellular hydrogen peroxide (Reaction 8). The reaction catalyzed by SOD is particularly efficient.

$2O_2 \bullet^- + 2H^+ \rightarrow H_2O_2 + O_2$ (Reaction 8)

In mitochondria, superoxide is formed in high concentrations due to the outflow of electrons from the respiratory chain. The mitochondrial Mn-SOD (Weisiger and Fridovich, 1973) is essential since no inherited diseases have been found in which Mn-SOD is deficient, and knockout mice lacking Mn-SOD die soon after birth or

suffer severe neurodegeneration (Melov *et al*., 1998). Expression of Mn-SOD is, in contrast to Cu, Zn-SOD, induced by oxidative stress and also by thioredoxins (Trx) (Das *et al*., 1997).

1.2.4.2 Superoxide reductases (SOR)

For catalyzing the direct reduction of superoxide a novel type of superoxide scavenging enzyme was discovered recently.

$O_2^{\bullet -} + e^- + 2H^+ \rightarrow H_2O_2$ (Reaction 9)

These enzymes, the superoxide reductases (SOR), contain iron and have found in anaerobic sulfate-reducing bacteria (Lombard *et al*., 2000[a]) such as *Pyrococcus furiosus*, (Jenney *et al*., 1999) or the microaerophilic bacterium, *Treponema pallidum* causing venereal syphilis (Lombard *et al*., 2000[b]). The *Treponema pallidum* organism lacks SOD and seems to rely on SOR for its elimination of superoxide. No direct evidence for a mammalian SOR till now.

1.2.4.3 Catalases

Catalases are heme-containing enzymes found in many organisms (Aebi, 1974). It is found mainly in peroxisomes of mammalian cells, where catalase catalyze the dismutation of hydrogen peroxide to water and molecular oxygen:

$2H_2O_2 \rightarrow O_2 + 2H_2O$ (Reaction 10)

Catalase also has functions as detoxifying agent of different substrates, e.g., phenols and alcohols, by coupled reduction of hydrogen peroxide:

$H_2O_2 + R'H_2 \rightarrow R' + 2H_2O$ (Reaction 11)

1.2.4.4 Peroxiredoxins (Prx)

Peroxiredoxins (Prx; thioredoxin peroxidases) e.g., hydrogen peroxide and different alkyl hydroperoxides, are enzymes capable of directly reducing peroxides, (Kim *et al*., 1988). Thioredoxin (in mammalian cells), trypanothione (in trypanosomatids) or

AhpF, a component of the alkyl hydroperoxide reductase system in *Salmonella typhimurium* (Poole *et al*., 2000). In the mitochondria of mammalian cells the mitochondrial thioredoxin system is most likely a specific reductant of Prx (Miranda-Vizuete and Spyrou, 2000). Peroxiredoxins shown to inhibit apoptosis induced by p^{53} (Zhou *et al*., 2000) and by hydrogen peroxide on a level upstream of bcl-2 (Zhang *et al*., 1997). Till date, at least 13 mammalian peroxiredoxins are known (Butterfield *et al*., 1999).

1.2.4.5 Glutathione peroxidases (GPx)

There are four different types of GPx reported in mammals (GPx1–4), all of them containing selenocysteine (Ursini *et al*., 1995). GPx1 and GPx4 (or phospholipid hydroperoxide GPx) are both cytosolic enzymes rich in most tissues (de Haan *et al*., 1998). GPx4 has been found to have dual functions in sperm cells by being enzymatically active in spermatids but insoluble and working as a structural protein in mature spermatozoa (Ursini *et al*., 1999; Sharma *et al*., 2004).

1.2.4.6 Other glutathione-related systems

Reduced Glutathione (GSH) is the abundant intracellular thiol-based antioxidant, common in millimolar concentrations in all living aerobic cells. Its functions as a sulfhydryl buffer, but GSH detoxify compounds either by conjugation reactions catalyzed by glutathione S-transferases (Armstrong, 1997; van Bladeren, 2000) or directly, as is the case with hydrogen peroxide in the GPx catalyzed reaction (Reaction 12).

GSH glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathione transferase and others and help to scavenges hydroxyl radical and singlet oxygen and detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase. In general, the

antioxidant ability of thiol compounds is due to the sulphur atom which can easily accommodate the loss of a single electron (Karoui *et al*., 1996). In addition the lifetime of sulphur radical species thus generated, i.e. a thiyl radical (GS•), may be longer than many other radicals generated during the stress. The reaction of glutathione with the radical R• can be described:

GSH + R• → GS• + RH

Thiyl radicals generated may dimerise to form the nonradical product, oxidised glutathione (GSSG):

GS• + GS• → GSSG

Oxidised glutathione (GSSG) is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Hwang *et al*., 1992).

GSH is an important factor in the protection against apoptosis, the efficiency of anticancer drug-induced apoptosis requires depletion of GSH, thus facilitate tumor treatment. Studies have shown that an association between cancer incidence and various disorders of GSH-related enzyme functions, in which alterations of glutathione *S*-transferases (GSTs) being most commonly reported. GSTs utilize glutathione in reactions contributing to the transformation of a wide range of compounds, including carcinogens, therapeutic drugs, and products of oxidative stress (Mitchell and Russo, 1987; Valko *et al*., 2006).

Glutathione is able to stimulate the most important antioxidants, vitamins C and E back to their active forms; glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, via reduction of semidehydro ascorbate to ascorbate. The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide–glutathione couple (GSSG/2GSH) (Jones

et al., 2000). This, in turn, has a high impact on the overall redox environment of the cell.

Another class of proteins intimately related to GSH is the glutaredoxins (Grx), with functions overlapping those of thioredoxins (Trx). A major qualitative difference between Grx and Trx is that Grx can be reduced by GSH and is capable of reducing GSH mixed protein disulfides formed at oxidative stress, which should play an important role in the total cellular antioxidant defense (Holmgren, 2000). **Figure 1.4** summarizes the different GSH-related antioxidant systems.

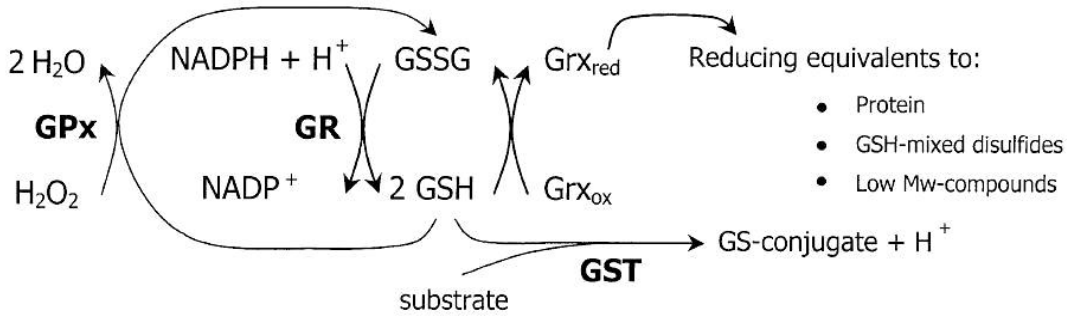


Figure 1.4 Schematic summary of the major glutathione-associated antioxidant systems (Nordberg and Arner 2001).

1.3 OXIDATIVE STRESS AND DISEASES

The evidence for a role of free radicals in disease is of several types. A large number of human diseases are associated with more production of activated species or with increased level of radical forming substances (Sowers, 2002; Mahmoudian-sani *et al*., 2017). Free radicals have also been implicated in the etiology of diseases which include cancer, cataract, coronary heart disease, stroke, arthritis, Alzheimer's disease, and aging process (Finkel and Holbrook, 2000; Kaynar *et al*., 2005; Ojo *et al*., 2006).

1.3.1 Damage to DNA by ROS : role in inflammatory disease and progression to cancer

It is proposed that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a key role in human cancer development (Totter, 1980; Ames, 1989; Cerutti, 1994; Oshima and Bartsch,1994). ROS and RNS have been shown to possess many characteristics of carcinogens (Cerutti, 1994; Mukhtar and Ahmad, 2000; Ahmadinejad *et al*., 2016). Mutagenesis by ROS/RNS could add to the beginning of cancer, as being important in the promotion and progression phases. ROS/RNS can have the effects.

(1) Cause structural alterations in DNA, e.g. base pair mutations, rearrangements, deletions, insertions and sequence amplification. ROS be able to make gross chromosomal alterations in addition to point mutations and thus involved in the inactivation or loss of the second wild-type allele of mutated proto-oncogene or tumor-suppressor gene that can occur during tumor promotion and progression, making the expression of the mutated phenotype to happen (Cerutti, 1994; Jami *et al*., 2014).

(2) Affect cytoplasmic and nuclear signal transduction pathways (Schreck *et al*., 1992; Burdon *et al*., 1995). For example, H_2O_2 (which crosses cell and organelle membranes easily) can lead to displacement of the inhibitory subunit from the cytoplasmic transcription factor, nuclear factor *k*B, allowing the activated factor to migrate to the nucleus (Schreck *et al*., 1992; Valko *et al*., 2006). Nitration of tyrosine residues by $ONOO^-$ can block phosphorylation.

(3) Modulate the activity of the proteins and genes that respond to stress and which act to regulate the genes that are related to cell proliferation, differentiation and apoptosis (Schreck *et al*., 1992; Cerutti, 1994; Jackson,

1994). For example, H_2O_2 can stimulate transcription of c-*jun* (Rao *et al*., 1993) and can activate mitogen-activated protein kinase in NIH 313 cells (Stevenson *et al*., 1994; Zhang *et al*., 2010).

1.3.2 Chemistry of DNA Damage

The endogenous reactions that are likely to contribute to ongoing DNA damage are oxidation, methylation, depurination and deamination (Totter, 1980; Ames, 1989; Chalansonnet *et al*., 2013). Nitric oxide or, and the reactive products derived from it, such as NO_2 •, $ONOO^-$, N_2O_3 and HNO_2, are mutagens, with the potential to produce nitration, nitrosation and deamination reactions on DNA bases (Cerutti, 1994;Oshima and Bartsch,1994). Methylation of cytosines in DNA is significant for the regulation of gene expression, and normal methylation patterns can be altered during carcinogenesis (Weitzman *et al*., 1994; Cairns *et al*., 2011). Conversion of guanine to 8- hydroxyguanine is a mutagenic result of ROS attack (Halliwell and Aruoma, 1991; Dizdaroglu, 1993; Box *et al*., 1995) has been found to alter the enzyme-catalysed methylation of adjacent cytosines (Box *et al*., 1995), thus providing a link between oxidative DNA damage and altered methylation patterns.

Damage to DNA by ROS/RNS appears to occur naturally, in that low steady-state levels of base damage products have been detected in nuclear DNA from human cells and tissues (Floyd, 1986; Ames, 1989). The pattern of damage to the purine and pyrimidine bases suggests that at least some of the damage occurs by OH• attack, telling that OH• is formed in the nucleus *in vivo* (Halliwell and Dizdaroglu, 1992).

1.3. 3 Mitochondrial DNA damage

ROS/RNS can also damage mitochondrial DNA, and such damage has been suggested to play an important role in several human diseases and in aging process (Shigenaga *et al*., 1994). The mitochondrial electron transport chain generates ROS *in vivo*

(Ambrosio *et al*., 1993; Guidot *et al*., 1993; Sena and Chandel, 2012) and results in mitochondrial DNA damage. Oxidative DNA base damage has been detected in mitochondrial DNA at steady-state levels several-fold higher than in nuclear DNA (Shigenaga *et al*., 1994;Sohal *et al*., 1994). This increased net oxidative damage in mitochondrial DNA compared with nuclear DNA could be of the proximity of mitochondrial DNA to ROS formed during electron transport, the lack of histone proteins to guard the DNA against attack, or inefficient repair, so as to base damage accumulates to higher levels. Oxidative damage could contribute to the deletions and mutations in mitochondrial DNA that accumulate with age at a higher rate than in nuclear DNA (Arnheim and Cortopassi, 1992).

Oxidative mechanisms have been demonstrated to possess a potential role in the initiation, promotion, and malignant conversion (progression) stages of carcinogenesis. Active oxygen may be involved in carcinogenesis through two possible mechanisms: (i) the induction of gene mutations that result from cell injury and (ii) the effects on signal transduction and transcription factors (Mates *et al*., 1999). Numerous studies have attempted to establish a relationship between levels of oxidative DNA damage and cancer. Elevated levels of damage are purported to arise as a consequence of an environment in the tumor tissues, because of low antioxidant enzymes and high ROS generation.

Although many studies have furthered the hypothesis that oxidative DNA damage may be an important risk factor for carcinogenesis, it has been argued that the mere presence of 8-OH-dG in DNA is unlikely to be necessary or sufficient to cause tumor formation. Lesions such as 8-OH-dG are established biomarkers of oxidative stress; coupled with their potential mutagenicity in mammalian cells, this has led to their proposed potential as intermediate markers of a disease endpoint. Supportive of this

proposal are the findings that GC→TA transversions potentially derived from 8-OH-dG have been observed *in vivo* in the *ras* oncogene and the *p53* tumor suppressor gene in lung and liver cancer. The GC→TA transversions are not unique to 8-OH-dG, whereas CC→TT substitutions in the absence of UV in internal tumors have been identified as signature mutations for ROS (Cooke *et al*., 2003). But all cases of the increased incidence of carcinogenesis are not always associated with oxidative DNA damage. The mere presence of elevated levels of damage in tumors does not indicate it as oxidative damage that led to the tumorigenic changes. Elevation in levels may have occurred as a result of well-established characteristics of tumors, e.g., increased metabolism or cell turnover.

More over for DNA mutations to arise from oxidative damage, the nuclei of undifferentiated, proliferating stem cells must be affected. Hence not only the DNA of target cells can be affected; to result in a mutation the damage must be within a coding region of the DNA. It is possible that the antioxidants themselves, may allow clonal expansion and tumor promotion by protecting initiated cells from excessive oxidant toxicity and apoptosis that would otherwise kill them. Finally, in linking oxidative stress with promotion, it must not be forgotten that biomolecules other than DNA may be oxidatively modified and that these may have a significant effect (Cooke *et al*., 2003). Therefore, although the role of oxidative stress in carcinogenesis appears well established, the extent to which oxidative DNA damage contributes has not been well defined.

1.3.4 Inflammation

The relationship between inflammation and oxidative stress is well accepted (Srivastava, 2007). Inflammation may ultimately result from damage to membrane lipids by reactive oxygen species (ROS) such as peroxide, superoxide anion, hydroxyl

radical and singlet oxygen (Symons and King, 2003). Bactericidal species ($O_2^{\bullet-}$ and H_2O_2), generated from the respiratory burst of invading neutrophils, macrophages, and eosinophils damage surrounding tissue, initiating further radical reactions and potentially oxidative stress. Chronic inflammation and hence oxidative stress have been closely linked to the pathogenesis of such autoimmune diseases as rheumatoid arthritis (Bashir *et al*., 1993) and systemic lupus erythematosus (Lunec *et al*., 1994) with radical production resulting not only in connective tissue damage, but also modified biomolecules being exposed to the systemic circulation, postulated to be the antigen driving autoantibody production. Mechanistically, chronic inflammation can be closely linked to carcinogenesis, although there is little evidence to suggest that patients with chronic inflammatory diseases such a systemic lupus erythrematosis have an increased rate of cancer development (Sultan *et al*., 2000).

1.3. 5 Cardiovascular disease and renal dysfunction

Under physiological conditions, ROS are produced in low concentrations can act as a signaling molecule that regulate vascular smooth muscle cells contraction and relaxation, and participate in their growth (Rao and Berk, 1992; Touyz and Schiffrin, 1999). But under pathophysiological conditions, these free radicals play an important role in various conditions, including atherosclerosis, arrhythmias, cardio mayopathy, congestive heart failure cancer and diabetes (Harrison, 1997; Zalba *et al*., 2001). Various other potential sources of cardiovascular diseases includes xanthine oxidase, cyclooxygenase, lipooxygenase, mitochondrial respiration, cytochrome P450, uncoupled nitric oxide synthase and NAD(P)H oxidase. They have been identified as the sources of ROS generation in all type of vasculature (Vijayalakshmi *et al*., 2009).

ROS are involved in intracellular signaling. When ROS production is increased, deregulation of physiological processes occurs. $O_2^{\bullet-}$ and other radicals may react with

NO (nitric oxide) causing endothelial dysfunction. The reaction of $O_2^{\bullet-}$ with NO led to the production of peroxynitrite. Peroxynitrite itself is a potent oxidant which can induce oxidation of proteins, lipids and DNA. In addition, ROS can stimulate vascular smooth muscle cell hypertrophy and hyperplasia. Furthermore, elevations in levels of ROS may, via a variety of mechanisms, initiate development of a vascular pro-inflammatory state. This pro-inflammatory state may be promoted via activation of redox-sensitive transcription factors, such as nuclear factor κB and the leukocyte adhesion molecule by reduction in levels of NO or by Ang-II-dependent pathways (Wolin, 2000;Landmesser and Harrison, 2001). Endothelial dysfunctions (ED) is an early event in artherosclerotic disease, preceding clinical manifestations and complications. Evidences have shown that ED is a strong predictor of future cardiovascular events in patients with cardiovascular risk factor (Fichtlscherer *et al*., 2004).

ROS has been implicated as important mechanism that contributes ED and may function as intracellular messenger that modulate signaling pathways. Increased ROS production is a major cause of ED in experimental and clinical atherosclerosis (Cai and Harrison, 2000). Atherosclerosis occurs as a result of ED and inflammation. The free radicals are involved throughout the atherogenic process, beginning from ED in an otherwise intact vessel wall up to the rupture of a lipid rich atherosclerosis plaque, leading to acute myocardial infarction or sudden death (Kunsch and Medford, 1999). The development of atherosclerosis is a multifactorial process in which both elevated plasma cholesterol levels and proliferation of smooth muscle cells play a central role. Its still a topic of debate whether this increased oxidative stress has any causative role in cardiovascular disease pathogenesis or is a vascular consequence of disease progression. There is, however, emerging evidence for genetic components both from

careful functional genomic studies (McBride *et al*., 2003) and from systemic evaluation of the candidate genes within the oxidative stress pathway (Guzik *et al*., 2000). In both cases it appears that re-establishment of vascular ROS to normal is a significant and commonly neglected therapeutic target.

Studies have shown a significant imbalance in pro-oxidant and antioxidant activities in patients with renal dysfunction. Oxidative stress has been found in the early stages of chronic kidney disease (CKD), as an increased level of plasma 8- isoprostane. Furthermore, oxidative stress seems to increase as CKD progresses and correlates significantly as inversely with the level of glomerular filtration rate (Dounousi *et al*., 2006). Indirect confirmation that oxidative stress is especially important in renal dysfunction comes from several main studies (Boaz *et al*., 2000; Yusuf *et al*., 2000). Inflammation is another important cause for the increased oxidative stress observed in patients with advanced renal disease, with factors such as malnutrition, chronic volume overload and autonomic dysfunction being among some of the factors implicated in the increased inflammatory state seen in renal impairment. Thus oxidative stress has been increasingly linked to the high incidence of cardiovascular events in patients with chronic kidney disease (CKD); especially as traditional cardiovascular risk factors seem to not be able to account for the huge cardiovascular morbidity and mortality in this population group.

1.4 DRUG INDUCED OXIDATIVE STRESS

Many xenobiotics supplied to living organisms are metabolized inside the body by conjugation with the cellular antioxidant enzymes mainly GSH, catalysed by glutathione S-transferase (GST) enzymes and cause the depletion of GSH that lead to oxidative stress (Valko *et al*., 2007).

RX + GSH →RSG + HX

1.4.1 Hepatotoxicity

The liver may be considered as the most important organ in drug toxicity for two reasons: on the one hand it is functionally interposed between the site of absorption and the circulation and may be a major site of metabolism and elimination of foreign substances; but on the opposite hand these features also render it a preferred target for drug toxicity. Therefore being an important organ, its protection features a special status in therapeutics. Despite of the superb regeneration capacity of this organ, a small injury or toxicity may cause fatal complications. Drug-induced liver injury (DILI) therefore possess a major clinical problem. DILI has become the leading cause of acute liver failure and transplantation in western countries (Ostapowic *et al*., 2002; Lee, 2003). Drug such as paracetamol, diclofenac etc., alcoholism, exposure to certain xenobiotic, pollutants and certain disease state are found to affect liver functioning.

Drug metabolism is considered into two phases. Phase I reaction introduce a polar functional group within the molecule, often by oxidation involving cytochrome P450. Concentration of total P450 is higher in liver. Phase II reactions are conjugation reactions where an endogenous molecule is added to the phase I reaction product. The cytochrome P450 monooxygenase system of the hepatic endoplasmic reticulum (microsomes) also generates a substantial amount of ROS in the process of metabolizing a chemically diverse group of compounds that includes most of the drugs that we administer as well as environmental substances (Spahn-Langguth and Benet, 1992; Boelsterli *et al*., 2002; Almroth *et al*., 2005). Drug metabolism and formation of reactive toxic metabolites by the hepatic microsomal enzyme system plays a role in the hepatotoxic mechanism**.** Xenobiotics are metabolized to inert

metabolites that are excreted by the kidney, but some are metabolized to more reactive compounds that are more toxic than the parent compound.

1.4.1.1 Acetaminophen-induced Hepatotoxicity

Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP), also referred to as paracetamol, is widely used as an analgesic and antipyretic drug throughout the world. In the United Kingdom, about 3.2×10^{9} tablets of APAP are consumed every year, which is equivalent to an average of 55 tablets/person (Jones, 1998). As an over-the-counter drug, it can be readily obtained without prescription. Although APAP is generally harmless at therapeutic doses, accidental or intentional intake of high doses often induced liver failure with acute hepatocellular necrosis with high morbidity and mortality (Lee *et al*., 2008). APAP is biotransformed and eliminated as nontoxic glucuronic acid and sulphate conjugates. Glucuronide is provided by UDP-glucuronic acid, and sulfation is dependent on phosphoadenylsulfate (Clements *et al*., 1984). Furthermore, the mixed function oxidase system cytochrome P450 (CYP) participates in metabolizing a small proportion of APAP at therapeutic doses (Azab and Albasha, 2018).

The metabolism of APAP by CYP leads to form *N*-acetyl-*p*benzoquinoneimine (NAPQI), a highly reactive intermediate metabolite (Dahlin *et al*., 1984), which in general detoxified by conjugation with reduced glutathione (GSH). After heavy doses of APAP, the ability for its elimination by hepatic conjugation with glucuronide and sulfate is exceeded, and more of the reactive metabolite NAPQI is created (**Figure 1.5**). Consequently, more NAPQI is conjugated with GSH, and when hepatic GSH is depleted, more NAPQI will bind covalently to cellular macromolecules (Jollow *et al*.,

1973; Chen *et al*., 2009). This is thought to lead to a loss of protein thiol groups and ultimately to cell death (Moore *et al*., 1985; Fox, 2017).

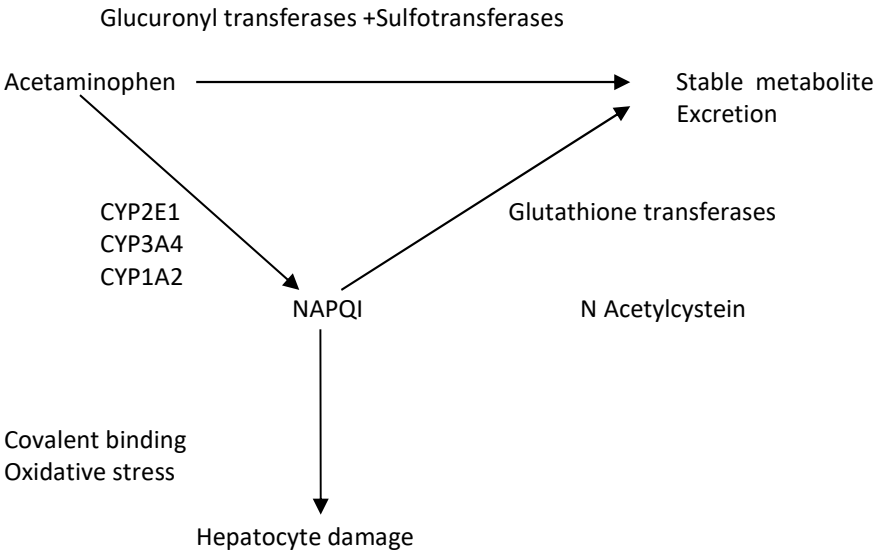


Figure 1.5 Acetaminophen-induced Hepatotoxicity

1.4. 2 Cardiotoxicity

Chemotherapy is a known therapeutic approach for several malignancies, but major drawback of such therapy is that leads to cardiomyopathy, possibly evolving into heart failure. Doxorubicin (DOX) (also known as Adriamycin) is an anthracycline antibiotic that has been used for more than 30 years for the treatment of a number of cancers. It can be made from *Streptomyces peucetius* or totally chemical synthesis may also possible. Breast and oesophageal carcinomas, Hodgkin's and non-Hodgkin's lymphomas, osteosarcoma, Kaposi's sarcoma and soft tissue sarcomas have shown good responses to DOX. In spite of extensive and long-standing clinical utilization, the mechanisms responsible for the antiproliferative and cytotoxic effects of DOX are still uncertain and have been subject to considerable controversy (Buschini *et al*., 2003).

Doxorubicin is an antineoplastic drug which is targeted for cell cycle specific for the S phase of cell division (Kusyk and Hsu, 1976). Several mechanisms seem to account for the effects of this anthracycline, both in terms of anticancer action and cardiac and other organ toxicities. Antineoplastic activity of DOX may be due to binding with DNA by intercalating between base pairs resulting in inhibition of synthesis of DNA and RNA by template disordering and steric obstruction (Chachoua *et al*., 1988). In addition, reduction of DOX by membrane bound reductase enzymes generates various free radicals such as quinones (Ramos *et al*., 2001). One of the primary targets for the activity of anthracycline antibiotics include Topoisomerase II. The induction of strand breaks in the DNA of L1210 leukemic cells by DOX was described more than 20 years ago (Quiles *et al*., 2002).

Tewey *et al*. (1984) have shown that topoisomerase II to be a target enzyme for DOX. The capacity of DOX to inhibit DNA synthesis has been assumed to be a mechanism of action of DOX. This mechanism may be related to DNA intercalation or inhibition of DNA polymerase activity. It is possible that this effect is due to growth arrest signalling events and p^{53} function. Another mode of action is through alterations in DNA is induction of enzymatically or chemically activated DNA adducts and DNA cross-linking by DOX. Interference with DNA strand separation and helicase activity has also been postulated as mechanism of action for DOX (Quiles *et al*., 2002). Two different routes of free radical formation by DOX have been described. The first implicates the formation of a semiquinone free radical by the action of several NADPH dependent reductases that produces a one-electron reduction of the DOX to the corresponding DOX semiquinone.

In the presence of oxygen, this free radical rapidly donates its electron to oxygen to generate superoxide anion ($O_2^{\bullet -}$) (Bachur *et al*., 1978). The dismutation of

superoxide yields hydrogen peroxide (H_2O_2). Under biological situation, the anthracycline semiquinone or reduced metal ions such as iron reductively cleave hydrogen peroxide to make the hydroxyl radical, the most reactive and destructive chemical species ever identified. This ultimately leads to lipid peroxidation, causing irreversible damage of membrane structure and function.

In the second route, DOX free radicals come from a non-enzymatic mechanism that involves reactions with iron (Quiles *et al.*, 2002). DOX can undergo futile redox cycling that result in the production of oxygen free radicals. This free redox cycling iron species have been found to play important roles in the development of DOX-induced cardiomyopathy. These reactive oxygen species may oxidize proteins, lipids and nucleic acids and potentially cause DNA strand scission (Ramos *et al*., 2001). The involvement of oxygen radical-induced injury of membrane lipids has been reported as the main causative factor for DOX -induced cardiotoxicity (Myers *et al*., 1977). As free radicals play an important role in DOX -induced cardiotoxicity, it is logical to consider antioxidants as primary potent therapeutic agent to prevent such toxic effect.

1.4.3 Nephrotoxicity

Nephrotoxicity is a toxic result of some substances, both chemicals and medicines, on the kidneys. Nephrotoxicity is frequently seen with many common medications in use today. Kidneys are the dynamic organs and represent the major control system maintaining body homeostasis, affected by many chemicals and drugs (Okada *et al*., 2001;Lakshmi *et al*., 2012). Kidneys are most exposed organ to administered medication, as it receives 25% of the resting cardiac output over a large endothelial surface area. With a high metabolic rate involving numerous enzymes and transtubular cell transport processes, renal glomerular, tubular, and interstitial cells

may come in contact with drugs and their metabolites at greater concentrations than expected. Some medications can affect the vasoregulatory mechanisms of the kidney to cause a functional limitation (Azab *et al*., 2017).

Nephropathy is often recognized only when filtration is affected, as seen by a rise in the blood urea nitrogen (BUN) and serum creatinine, because there are as yet no good serologic markers to determine the subtle insults to the kidneys. Drugs exposed to root nephrotoxicity use their poisonous effects by one or more general pathogenic mechanisms (Cyril *et al*., 2016). Drug-induced nephrotoxicity tend to be more frequent among some patients and for certain precise clinical situations. Oxygen free radicals largely contribute to complications observed in the end-stage renal disease (ESRD) and also in patients treated with haemodialysis (Canaud *et al*., 1999; Handelman, 2000).

Antineoplastic drugs in the treatment of cancers shows variable renal tolerance profile. Drugs with potential renal toxicity, platinum salts, particularly cisplatin is a familiar agent that may make acute and chronic renal failure. Cisplatin (*cis*-diamine-dichloroplatinum) is a well-known member of the efficient broad-spectrum antitumor drug. However, its clinical usage is restricted due to some adverse side effects, such as nephrotoxicity and ototoxicity (Fouladi *et al*., 2008; Afifi, 2010). The mechanism by which cisplatin kill tumor cells is distinct from the mechanism by which it selectively kills the proximal tubule cells (Townsend *et al*., 2003).

Investigators suggested diverse mechanisms by which cisplatin kill the proximal tubule cells selectively. It was also said that cisplatin is activated in the kidney to toxic metabolite through a platinum-glutathione conjugate, then to a cysteinyl-glycine-platinum-conjugate, later formed to a cysteine conjugate, is a metabolically

reactive thiol (Salahudeen *et al*., 1998; Elena *et al*., 2009). More than that, two distinct pathophysiological mechanisms have also been documented as promoters of cellular damage, i.e. inhibition of protein synthesis and glutathione depletion (Moayeri *et al*., 2006). It induce ROS in renal epithelial cells chiefly by lessening the activity of antioxidant enzymes and by depleting the amount of intracellular GSH (Huang *et al*., 2001; Jariyawat *et al*., 2009).

Evidences have shown that the side effect is closely related to reactive oxygen species (ROS) that cause mitochondrial damage, inhibition of membraneous transport proteins and lipid peroxidation etc (Rebillard *et al*., 2007). Studies demonstrated that treatment with cisplatin induced biochemical signs of nephrotoxicity manifested by increased serum creatinine and urea levels with increased lipid peroxides (MDA) and depletion of antioxidants as GSH, Vitamin E and Vitamin C and inhibition of SOD, CAT, GPx and gene expression (Afifi, 2010; Sundararajan *et al*., 2014).

In addition to these Gur *et al*. (2001), demonstrated that cisplatin induce renal cell death through the activation of initiator caspases-8, -9 and -2, and executioner caspase-3 and cause a marked induction of Akt/PKB phosphorylation pathway in a time and dose-dependent manner during the course of cisplatin injury (**Figure 1.6**). Since Oxidative stress has been reported as the major cause of cisplatin-induced nephrotoxicity, administration of antioxidants has been shown to ameliorate cisplatin-induced nephrotoxicity in various species of animals (Afifi, 2010; Ibrahim *et al*., 2010).

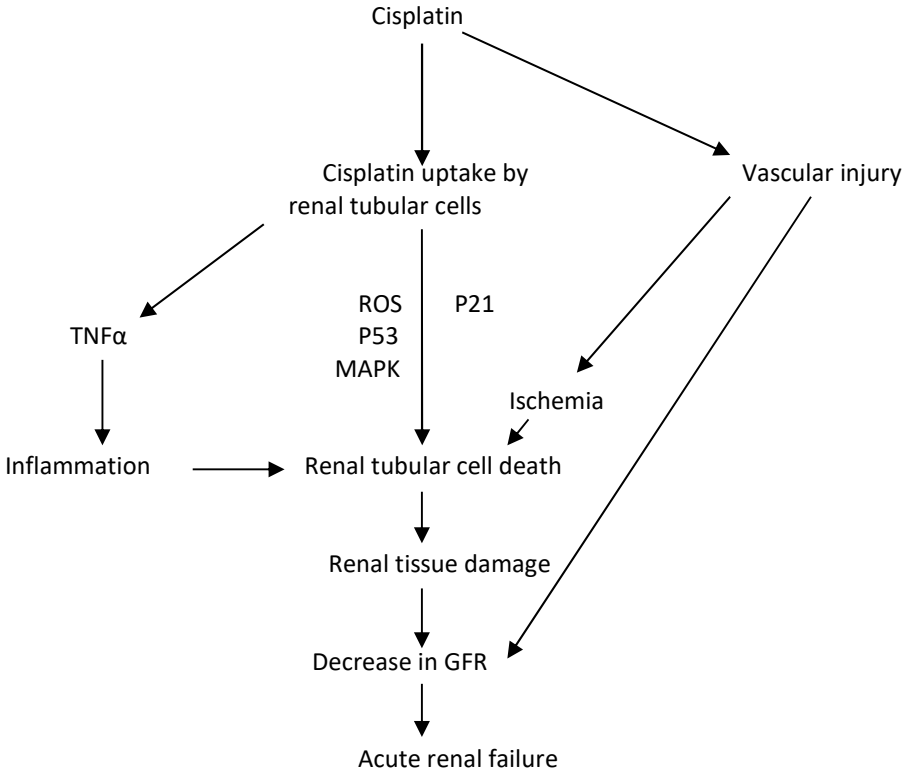


Figure 1.6 Cisplatin induced nephrotoxicity

1.5 RADIATION INDUCED OXIDATIVE STRESS

Living systems are constantly exposed to a variety of physical and chemical agents which cause cellular damages including genetic alterations leading to mutations and cell death. Among the physical agents, ionizing and non-ionizing radiations are the major ones which cause damage to living organisms. Radiation is a type of energy in the form of waves or moving subatomic particles emitted by an atom or other body as it changes from a higher energy state to a lower energy state. Radiation can propagate through space or medium, usually with transfer of energy. Ionizing radiation can be defined as any type of electromagnetic (such as X- or gamma rays) or particle radiation (such as neutron or alpha particles) with sufficient energy to ionize atoms or molecules; that is, to eject electrons from their outer orbitals.

The ionizing event involves the ejection of an orbital electron from a molecule, producing a positively charged or "ionized" molecule. These molecules are highly unstable and rapidly undergo chemical change. This change results in the production of free radicals; these are atoms or molecules containing unpaired electrons. These free radicals are extremely reactive and may lead to permanent damage of critical macromolecules, such as DNA, proteins or membranes, and can induce cell damage and, potentially, cell dysfunction and death. Exposure to ionizing radiations occurs from natural as well as from man-made sources including, cosmic rays, nuclear weapons, nuclear reactors, aircrafts, medical diagnostics and cancer treatment. The use of ionizing radiation has become an integral part of modern medicine. Hence is used for both diagnostic and therapeutic purposes.

In some cases, radiation may be the single best treatment of cancer. The radiation therapy of cancer depends on achieving a therapeutic differentiation between the cancer cell cytotoxicity and normal tissue toxicity. The therapeutic differentiation may be achieved with chemical radiation sensitizers or protectors. Ionizing radiation inflicts deleterious effects to living cells through the generation of reactive oxygen species that damage vital cellular target such as DNA and membrane. The toxic effect of radiation results mostly from oxidative damages through the generation of several reactive oxygen species such as superoxide, hydrogen peroxide, hydroxyl radicals etc, and the most important sub cellular target is the DNA.

When cells are exposed to ionizing radiation, radiochemical damage can occur either by direct action or indirect action (Nair *et al*., 2001). Direct action occurs when alpha particles, beta particles or x-rays create ions which physically break one or both of the sugar phosphate backbones or break the base pairs of the DNA. The bonding of the base pairs can also be affected by the direct action of ionizing radiation. Ionizing

radiation creates two types of breaks in the sugar phosphate backbone. A single strand break occur only when one of the sugar phosphate backbones is broken. Single strand breaks are readily repaired using the opposite strand as a template (Hall and Giaccia, 2006). However, base pair substitutions and frame shift mutations can still occur. Double-strand breaks are believed to be the most detrimental lesions produced in chromosomes by ionizing radiation. Because such breaks are difficult to repair, they can cause mutations and cell death (Jackson, 2002). Both direct and indirect effects of ionizing radiation damage cellular DNA.

In the indirect effect, DNA damage is mainly induced by abstraction of the H atom from the C- 4 position of the deoxyribose or by attack of the bases via the hydroxyl radicals produced by the radiolysis of water (Spotheim-Maurizot *et al*., 1992). Indirect action of ionizing radiation occur when it interacts with water molecules in the cell resulting in the production of highly reactive free radicals such as $OH^{\bullet}$, $H^{\bullet}$, $^{1}O_{2}$, e_{aq}^{-}, $HO_2^{\bullet}$, H_3O^{+}, etc. Apart from DNA damage membrane protein and lipids can be damaged by radiation. In protein it can lead to formation of protein carbonyls and loss of protein thiols besides loss of activity of membrane bound enzymes (Devasagayam and Tilak, 2006). Lipid peroxidation also considered to be critical event of ionizing radiation effect (Agrawal and Kale, 2001).

1.5.1 Radioprotectors

The use of ionizing radiation has become an integral part of modern medicine. In some cases, radiation may be the single best treatment of cancer. As the tumor cells proliferate very rapidly, they usually overgrow their vascular supply, resulting in centrally necrotic and hypoxic regions, rendering radiation ineffective in these areas. To overcome this problem, higher doses of radiation must be delivered to control the tumor. This is clinically not feasible, since the normal tissues surrounding the tumor

are well perfused, vascularised and remain oxygenated, and are therefore more prone to radiation damage. This necessitates the protection of normal cells surrounding the tumor from radiation injury (Bendale *et al*., 2015). The identification of radiation-protecting agents is an important goal for radiation oncologists and basic radiation biologists. A large number of chemical substances have been reported to possess radioprotective activity (Baliga *et al*., 2013).

The attention over the past 15 years has shifted towards the evaluation of plant products as radioprotectors, due to their efficacy and low toxicity. The radioprotective efficiency of plant extracts is due to the presence of rich active constituents, such as antioxidants, immuno-stimulants showing antimicrobial activity. Most efficacy studies on plants have been on total extracts for their ability to protect against radiation-induced chromosomal aberrations and micronuclei formation; they were assessed by genotoxic tests, such as micronucleus and metaphase analysis. The radioprotective effect seems to be largely due to the high levels of phenolic and flavonoid compounds with strong antioxidant activities.

1.6 ENZYME THERAPY AND DRUG TARGETING IN CANCER TREATMENT

Enzymes can be normally used for therapeutic purposes. Enzymes are used as an external source for impaired wound healing (e.g. in the presence of varicose ulcers) and it becomes part of the medical practitioners for centuries . Parental lytic therapy with streptolinase or urokinase for cardiac infarct or for vascular occlusions is standard throughout the world. Enzyme therapy or enzyme replacement therapy is such a medical treatment replacing an enzyme in patients in whom that particular enzyme is deficient or lacking. Usually this is done by giving the patient an intravenous infusion containing an enzyme. Replacement in intestinal enzyme deficit

conditions is a classical treatment modality. Enzyme therapy is now available for all kinds of lysosomal diseases: Gaucher disease, Fabry disease (X-linked recessive glycosphingolipid storage disorder caused by deficiency of the lysosomal enzyme α - galactosidase A), Glycogen storage disease type II, etc. Taking enzyme supplements would be beneficial for treatment of some acute and chronic diseases such as hypoglycemia, endocrine gland deficiencies, obesity, anorexia nervosa, and stress-related problems, etc (Desnick, 1976). Individual enzymes have also been used to alleviate skin ailments (psoriasis, acne, eruptions, stretch marks, etc.), chronic inflammation, cardiovascular disease, circulatory problems, high blood pressure, arteriosclerosis, high cholesterol, fatigue, Epstein-Barr Syndrome, asthma, allergies, arthritis, swelling, bee stings, joint mobility, diabetes, respiratory ailments, fever, all types of infections (including cold, AIDS, flu, sore throat, yeast, parasites, Candida albicans, etc.) multiple sclerosis, fibromyalgia, tumors, depression, anxiety, ADD/ADHD and insomnia (Dale *et al*., 2001). In traditional medicine, enzyme supplements are given for patients suffering from disorders that affect the digestive process, such as cystic fibrosis, Gaucher's disease, diabetes, and celiac disease (Desnick *et al*., 1976).

Use of enzymes for treatment of cancer started about 100 years ago when a British embryologist, John Beard used pancreatic enzymes to treat cancer in 1902 (Ernst, 2001). German researchers used enzyme therapy for treating patients with multiple sclerosis, viral infections and cancer. Some enzyme mixtures are still commonly used in several European countries. Dr. John Beard proposed in 1906 that pancreatic proteolytic digestive enzymes represent the body's main defense against cancer, and that enzyme therapy would be useful as a treatment for all types of cancer (Beard, 1906). There were several reports about proteolytic enzymes therapy resulting in

tumor regression and remission in terminal cancer patients (Wiggin, 1906; Campbell, 1907; Cutfield, 1907; Goeth, 1907). In 1911, Dr. Beard published a monograph entitled "The Enzyme Therapy of Cancer", which summarized his therapy and the supporting evidence of use of pancreatic juices to reduce tumors in humans and animals. The work on enzyme therapy did not have much follow up in later years. Periodically, alternative therapists have rediscovered Dr.Beard's work, and used pancreatic proteolytic enzymes as a treatment for cancer (Gonzalez, 2000). However some scientific evidence exists that pancreatic enzymes derived from animal sources are helpful in cancer treatment (Gonzalez and Isaacs, 1999). The enzymes may be able to dissolve the coating on cancer cells and may make it easier for the immune system to attack the cancer (Fonorow, 2004; Kelley, 2005).

A recent study conducted on cancer cells by antibody – guided enzyme therapy shows enzyme catalysis of the substrate that generate cyanide, a metabolic poison that asphyxiates cells and leads them to a necrotic-like cell death . This system has been called antibody- guided enzyme nitrile therapy (AGENT). Antibodies have been widely developed as biopharmaceuticals for cancer therapy because they can effectively target tumor related antigens. They have been used against malignant cells as delivery vehicles for cytotoxic therapy. Antibody-enzyme-mediated prodrug activation strategies are powerful approaches that exploit the selectivity of the antibody-antigen reaction to generate cytotoxic molecules specifically in tumors (Kousparou, 2002).

Another promising area of cancer research is nanotechnology. Here enzymes like D-amino acid oxidase, Xanthine oxidase, etc, which generate oxidative radicals as H_2O_2 when reacts with the substrate. Targeting these enzymes specifically to tumor sites

can be of greater advantage. Complexes of these enzymes could be designed to release H_2O_2 at specific target site so that the complete deterioration of tumor cells occurs (Fang *et al*., 2002). Xanthine oxidase (XO) mediates anticancer activity because of its ability to generate cytotoxic reactive oxygen species (ROS), including superoxide anion radical and hydrogen peroxide (Haddow *et al*., 1958; Wada *et al*., 1990;Yoshikawa *et al*., 1995). However, the high binding affinity of XO to blood vessels would cause systemic vascular damage and hence limits the use of native XO in clinical settings and critical re- evaluation of the antitumor effect of native XO showed that the results were variable and the efficacy was insignificant (Bray and Swann, 1972).

Various reports says that Glucose oxidase (GO), which generates H_2O_2 during oxidation of glucose, showed antitumor activity in solid tumor models (Nathan and Cohn, 1981; Ben-Yoseph and Ross, 1994). However, regulation of H_2O_2 production by exogenously administered enter tumor-bearing hosts is problematic because the supply of its substrates, oxygen and glucose, can't be significantly modulated, with the possible induction of severe systemic side effects due to systemic generation of H_2O_2. In fact, GO administration to supply H_2O_2 required injection of antioxidants to attenuate systemic toxicity (Nathan and Cohn, 1981; Ben-Yoseph and Ross, 1994).

Sawa *et al*. (2000) developed a conjugate of PEG and XO (PEG-XO) for effective delivery of XO to tumor. Infusion of the substrate of XO to generate superoxide thus caused tumor regression (Sawa *et al*., 2000). Xanthine oxidase (XO) is an iron-containing flavoprotein that catalyzes the oxidation of purines, such as hypoxanthine, through xanthine, to uric acid. In this oxidation, molecular oxygen (O_2) is used as an electron acceptor; and hence, ROS including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are generated (Davies, 1995). It has been reported that XO showed

antitumor activity (Sundaresan *et al*., 1995; Beckman and Ames, 1997), probably via the generation of ROS. ROS formed from antitumor drugs exhibit antitumor effects on the basis of high cytotoxicity. However, systemic distribution of these drugs causes undesirable side effects (Halliwell and Gutteridge, 1984). To enhance the therapeutic efficacy of anticancer agents while reducing systemic side effects, it is necessary to deliver the drug (*e.g.,* XO) selectively to tumor tissue.

It was found that all biocompatible macromolecular drugs and lipids accumulate selectively in tumor tissue compared with other normal tissues and organs (Sugiura, 1958; Mealey, 1965; Kaibara *et al*., 1971). In addition, they are retained in tumor tissue for a long time, *e.g.,* .100 h (Makino and Tanaka, 1953;Sugiura, 1958). This phenomenon is known as the Enhanced permeability retension (EPR) effect of macromolecules and lipids in solid tumor. This EPR effect was validated in many experimental solid tumors, *e.g.,* Walker 256 carcinoma of the rat (Nathan and Cohn, 1981); VX-2 carcinoma of the rabbit (Ben-Yoseph and Ross, 1994; Sawa *et al*., 2000); sarcoma 180 (S-180) (Sugiura, 1958; Yagi,1971; Matsumura and Maeda, 1986; Konno and Yasumura,1992) and B16 melanoma (Yagi,1971) of the mouse; and human hepatocellular carcinoma (Maeda *et al*., 1992; Kojima , 1993). Very recently, a clinical trial of hydroxypropylmethacrylamide-copolymer conjugate with doxorubicin was also demonstrated (Yagi, 1971; Seymour *et al*., 1995; Noguchi *et al*., 1998).

Fang *et al*. (2002) reported the antitumor activity of PEG- D- amino acid oxidase (PEG-DAO) complex. This flavoprotein catalyzes the stereoselective oxidative deamination of D-amino acids to the corresponding α-keto acids. Here molecular oxygen (O_2) is used as an electron acceptor, and hence H_2O_2 is formed (Yagi, 1971). D-Amino acids do not usually exist in mammalian organisms to a significant level

(Matsumura and Maeda, 1986). Hence generation of H_2O_2 could be regulated by exogenous administration of D-amino acids. DAO does have a pharmacological drawback, however: a short *in vivo* half-life. The molecular size of DAO (Mr 39,000) is somewhat smaller than the renal excretion threshold (Mr ~50,000), so it would be excreted gradually for other small proteins or polymer drugs smaller than Mr 40,000 (Matsumura and Maeda, 1986; Maeda *et al*., 1992; Kojima , 1993; Tsutsumi *et al*., 1994; Seymour *et al*., 1995; Noguchi *et al*., 1998).

To overcome this drawback, DAO was chemically modified by conjugation with a biocompatible polymer, PEG. This modification results in an increased *in vivo* half-life, a reduced antigenicity of the native protein, and an inhibition of proteolytic degradation, as reported previously (Pyatak *et al*., 1980; Tsutsumi *et al*., 1994;Sawa *et al*., 2000). The biocompatible macromolecules accumulate and remain in solid tumor because of the unique features of the tumor vasculature and the impaired lymphatic clearance system due to the the EPR effect of macromolecules and lipids in solid tumor (Matsumura and Maeda, 1986; Maeda *et al*., 1992; Kojima , 1993; Tsutsumi *et al*., 1994; Seymour *et al*., 1995; Marecos *et al*., 1998; Noguchi *et al*., 1998; Duncan, 1999; Muggia, 1999). Thus, on the basis of the EPR effect, DAO conjugated to PEG (PEG-DAO), a high blood level retension of DAO for a long period and better accumulation of DAO in tumor occur. Presence of D- proline is essential for effective antitumor activity by PEG-DAO via extensive generation of H_2O_2 These novel modes of enzyme therapy for effective tumor control which depends on targeting the H_2O_2-generating enzymes to the tumor site where the enzyme converts a pharmacologically inert product to a highly cytotoxic metabolite, H_2O_2. Thus "Systemic enzyme therapy", is a promising area in the treatment of various diseases and cancer in particular.

1.7 AMELIORATION OF DRUG AND RADIATION INDUCED OXIDATIVE STRESS BY PHYTOCEUTICALS

Several natural agents are widely used to ameliorate toxic and carcinogenic xenobiotics and drugs toxicity, which include Vitamin E and C, carotenoids and extracts of medicinal plants. Among this more interest is focused on the use of herbal and its phytoconstituents for therapeutic uses because of their antioxidant property and are less harmful when compared to the synthetic chemicals. The extract of *Ginkgo biloba* has been used in Chinese medicine for thousands of years. The extract has antioxidant properties, mainly due to the presence of flavonoids in it. In Indian system of medicines and ayurveda several plants have been used due to their beneficial use as antioxidants or source of antioxidants (Scartezzini and Speroni 2000;Al-Mamary *et al*., 2002;Govindarajan *et al*., 2006; Fetouh and Azab, 2014).

Herbal-based therapeutics for liver disorders has been used worldwide for a long time and been popularized by leading pharmaceuticals. A large number of plants and their formulations showed hepatoprotective activity (Handa *et al*., 1986). Several plants were reported to possess hepatoprotecting property include, *Tridax procumbens, Glycyrrhiza glabra, Phyllanthus niruri, Momordica dioica, Asparagus racemosus, Tephrosia purpurea, Glycyrrhiza glabra, Amaranthus spinosus, Boerhaavia diffusa, Kalanchoe pinnata, Phyllanthus amarus, Equisetum arvense, Vitis vinifera, Rubia cordifolia, Aloe barbadensis, Aegle marmelos, Phyllanthus emblica, Flacourtia indica , Orthosiphon stamineus, ,Cassia fistula, Azadirachta indica, Cassia fistula, Fumaria indica, Phyllanthus polyphyllus, Launaea intybacea, Hygrophila difformis, Cajanus acutifolius , Phyllanthus niruri, Eclipta alba, and Solanum indicum etc.*(Sandeep and Nair, 2010; Kumar *et al*., 2011).

Heart is an organ easily vulnerable to injury by anthracyclin induced reactive oxygen species (ROS) because of less developed antioxidant defence mechanism. It is suggested that endogenous antioxidant deficits play a major role in doxorubicin induced cardiomayopathy and heart failure. As free radical generation plays an important role in DOX-induced cardiotoxicity, it is logical to consider antioxidants as primary potential therapeutic agent without reducing the therapeutic effect of doxorubicin in cancer patients. A series of antioxidants have been studied to destroy ROS. However, many of them have a limited cardioprotective effect or have other side-effects (Wouters *et al*., 2005; Elbl *et al*., 2006). From our experimental studies it is shown that ferulic acid and ascorbic acid mono glucoside were capable in reducing the oxidative stress induced by doxorubicin in mice. Various plants like *Curcuma longa, Murraya, koenigii, Terminalia arjuna, Schisandra Chinensis, Lycium barbarum, Kohautia grandiflora* etc also shown to possess cardio protective activity against doxorubicin induced cardiotoxicity.

There is a continuous search for agents that provide nephroprotection against the renal impairment caused by drugs like cisplatin. It is thus imperative that we turn toward alternative systems of medicine for solutions. Several medicinal plants such as *Bauhinia variegata, Aerva lanata, Carica papaya, Cucurbita pepo, Ficus religiosa, Vernonia cinerea, Pongamia pinnata, Alviae radix, Cassia auriculata, Portulaca oleracea etc* were found to be effective against cisplatin induced nephrotoxicity (Joy and Nair, 2008; Gaikwad *et al*., 2012).

Radiation also forms an important cause of oxidative stress. Several natural and synthetic compounds were found to protect against radiation induced damage in biological systems (Nair *et al*., 2001). However most of them showed inherent toxicity and side effects at the radioprotective concentrations. So an interest in search

of efficient and non-toxic compounds with radioprotective capacity leads to investigations on naturally occurring antioxidants and phytoceuticals. Several plant extracts, herbal preparations and phytochemicals have been reported to have radioprotective action in *in vitro* and *in vivo* studies (Divakaran *et al*., 2013).Some examples for plant showing radioprotecting property include *Eugenia jambolana, Curcuma longa, Alpinia galanga, Tinospora cordifolia, Ginkgo biloba, Centella asiatica, Ocimum sanctum, Podophyllum hexandum, Amaranthus paniculatus, Biophytum sensitivum , Allium sativum, Adhathoda vasica, Mentha piperita, Myristica fragrans, Aegle marmelose, Aloe vera, Boerhaavia diffusa, Zingiber officinale, Piper betle, Moringa oliefera, Azadirachta indica, Syzigium cumini, Terminalia arjuna, Emblica officinalis, Lycium barbarum, Citrus aurantium, Panax ginseng, Glycyrrhiza glabra, Tephrosia purpurea etc.* (Sandeep and Nair, 2010; Bhandari, 2013).

In fact, many of the radioprotectors are antioxidants. Many compounds showing antioxidant activities are proved to be effective radio protectors (Weiss and Landauer, 2003). Even though, there are a number of enzyme systems found within the body that scavenge free radicals, the principle antioxidants are α-tocopherol (Vitamin E), beta-carotene, and ascorbic acid (Vitamin C). In addition, selenium, a trace metal that is also required for proper function of the body's antioxidant enzyme systems, is sometimes included in this category. The body cannot make these micronutrients so they have to be supplied in the diet. The use of natural antioxidants present in food and other biological materials has involved considerable interest due to their presumed safety, nutritional and therapeutic value (Ajila *et al*., 2007). Antioxidants obtained from fruits, vegetables, spices and cereals are very efficient and have reduced interference with the body's ability to use free radicals constructively (Kahkonen *et al*., 1999; Wolfe *et al*., 2003). The hunt for natural antioxidants for

dietary, cosmetic and pharmaceutical use has become a major industrial and scientific research challenge over the last two decades.

1.7.1 Antioxidant vitamins

The antioxidant property of vitamins has been well studied and are important candidates for the development of effective and nontoxic radioprotectors and efforts are directed (i) to improve their chemical, oxidative or heat stability and (ii) to alter their hydrophile-lipophile balance (HLB), yielding a series of "semi synthetic" antioxidants (e.g. tocopherol monoglucoside, tocopheryl acetate, L-ascorbyl palmitate).

1.7.1.1 Ascorbic acid (VITAMIN C)

Ascorbic acid (AsA) or vitamin C is a water-soluble and potent dietary antioxidant in biological fluids that has been confirmed to be an effective free radical scavenger and radioprotector (Frei, 1989; Duarte and Lunec,2005). It is a necessary micronutrient required for normal metabolic activities and it plays numerous functions in the body.

The various physiological activities of AsA include antioxidant activity, inhibition of melanin synthesis, antimutagenic activity, antiviral activity and also support the immune system by stimulating the activity of antibodies and immune system cells. It supports cardiovascular system by facilitating fat metabolism and protecting tissues from free radical damage and it assists the nervous system by converting certain amino acids into neurotransmitters. Moreover, it also improved the inducible hepatocyte growth factor (HGF) production (Wu *et al*., 2000). Acute lack of vitamin C leads to scurvy, manifested by blood vessel fragility, connective tissue damage, fatigue, and, finally, death. The intestinal absorption and renal excretion decide the serum level of vitamin C and thus its bioavailability. Vitamin C, consumed either with diet or dietary supplements, is absorbed by the epithelial cells of the small intestine by

the SVCT1 transporter or, then, diffuses into the nearby capillaries and then the circulatory system (Hornig *et al*., 1973; Malo and Wilson 2000;Takanaga *et al*., 2004). But, at high concentrations, SVCT1 becomes saturated, so that limits the amount of ascorbic acid absorbed from the intestine and reabsorbed from the kidney (Wilson, 2005). This imposes a physiological restriction on the maximal effective serum vitamin C concentration (or its bioavailability) which is attainable by oral consumption (Padayatty *et al*., 2004; Linster and Schaftingen,2007).

1.7.1.2 Ascorbic acid derivatives

AsA is easily oxidized by the oxygen in aqueous solution, followed by its rapid degradation. This limits its use under physiological conditions and requires a large dose for medical use. AsA is not synthesized in human body and is easily oxidized due to unstable nature. Due to pro-oxidant nature and well known susceptibility of vitamin C to heat, UV light, metal ions and oxidants etc, led to the development of stable derivatives of ascorbic acid with improved chemical, oxidative and heat stability. The chemical modification of hydroxyl group at the C-2 position of vitamin C and chemical modification by introducing substituting groups to improve the stability.

The enzymatic alteration of AsA by the addition of a glucoside unit at the 2- carbon position is the most important among these as it offers more stability and water solubility than AsA while retaining its vitamin C activities. Various glucoside derivatives of AsA include Ascorbic acid 2-glucoside (AsAG) and a series of 6-acyl ascorbic acid 2-glucosides with branched and unbranched acyl units attached at the 6-carbon position of AsAG (6-Acyl-AsAG). The other stable AsA derivatives include ascorbic acid 2- phosphate (AsAP), ascorbic acid 2-sulfate (AsAS), 2- O-octadecylascorbic acid (CV-3611), and ascorbyl 2,6-dipalmitate (2,6-Palm-AsA) etc,

and it is found that they themselves have no two-electron reductivity (Yamamoto *et al*., 1990; Aga *et al*., 1991).

The stable derivatives of ascorbic acid made with the aim of increasing stability and bioavailability of AsA. So they are found to be useful in industry, nutrition and medicine. Most of the derivatives, particularly glucoside derivatives have inherent vitamin C activity. They retains all the physiological activities of AsA and found to be more stable and can act as supplement to AsA. Some of the derivatives of AsA include 6-Palmitoyl Ascorbic Acid-2-Glucoside and AsAG are found to be as potent radioprotectors in cancer studies (Mathew *et al*., 2007; Chandrashekharan *et al*., 2009).

1.7.1.3 Ascorbic acid-2-glucoside (AsAG)

Ascorbic acid-2-glucoside (AsAG) is a stable derivative of ascorbic acid, here at the second carbon position of Vitamin C, a glucoside unit is added. The stabilization of AsA is focused on the chemical modification of a 2, 3-enediol moiety present at the five-membered lactone ring. The 2, 3-enediol moiety is the main reason causing the instability of AsA (**Figure.1.7**)

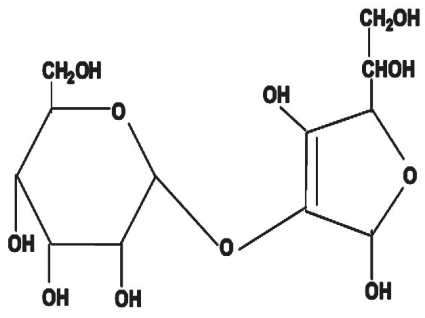


Figure1.7. Structure of Ascorbic acid-2 glucoside

Ascorbic acid-2-glucoside (AsAG) is synthesized from AsA and maltose or other α -glucans by regioselective transglucosylation with α-glucosidase or cyclomaltodextrin

glucanotransferase (Mandai *et al.*, 1992). Here the C-2 hydroxyl group of AsA is masked with glucose, so AsAG is non reducible and very stable to oxidative conditions. This stable hydrophilic vitamin C derivative showed vitamin C activity *in vitro* and *in vivo* after enzymatic hydrolysis to ascorbic acid (AsA) by α -glucosidase (Yamamoto *et al.*, 1990; Wakamiya *et al.*, 1992; Kumano *et al*., 1998). AsAG has two basic properties; it has high stability against thermal and oxidative degradation, and it can be easily converted to AsA by α- glucosidase in the blood and liver cells (Yamamoto *et al*., 1990). The bioavailability studies, showed that AsAG administration resulted in a gradual increase in the plasma AsA concentration in rats and reached a maximum at 1 hour (Tai *et al*., 2002). AsAG showed antioxidant and free radical scavenging activity under *in vitro* and *in vivo* conditions and inhibited DPPH radical thus indicating its antioxidant ability (Fujinami *et al*., 2001;Takebayashi *et al*., 2002)

In the present study, we have investigated the anti inflammatory, hepatoprotective cardioprotective , and nephroprotective activity of AsAG in Swiss albino mice and shows its utility to offer protection against deleterious effects of chemotherapeuticals for cancer therapy.

1.8 SECONDARY METABOLITES AND DISEASES

Secondary metabolites (SM) are a range of chemical compounds formed by the plant cell through metabolic pathway, derived from the primary metabolic pathway. The concept of secondary metabolites was first described by Albercht Kossel, Nobel Prize winner for physiology and medicine. Secondary metabolites have shown to possess various biological effects when provided the scientific bases for the use of herbs in the traditional medicine in many ancient communities. They have been described as

antibiotic, antifungal and antiviral and therefore are able to protect plants from various pathogens (Frei and Higdon, 2003; Genova *et al*., 2012). Secondary plant metabolites are classified based on their chemical structure to several classes. The classes of secondary plant metabolites include:

- Phenolics
- Alkaloids
- Saponins
- Terpenes
- Lipids
- Carbohydrate

The Secondary metabolites are not that much essential to the life of the plant. They can be used as pigments, hormones and chemical agents that can attack other plants. The secondary plant metabolites are not directly involved in the normal growth, development or reproduction of plants (Samanta *et al*., 2011). Unlike primary metabolites, their absence does not result in immediate death. The study of plant metabolites is thought to have started in the early 1800s when Fredrich Willhelm Serturner isolated morphine from opium poppy and after that new discoveries were made rapidly. In early 1900s, the research around secondary plant metabolites was mainly on formation of secondary metabolites in use of tracer techniques that made metabolic pathways easier (Dixon and Pasinetti, 2010). Earlier in 1970s, secondary plant metabolites were considered as simply waste products, but new research showed that secondary plant metabolites played a vital role in the survival of plants in its environment (Zhao and Moghadasian, 2008).

Secondary metabolites are low molecular weight compounds. These compounds regularly differ between individuals from the same population of plants in their amount and types. They guard plants against stress, both biotic (bacteria, fungi, nematodes, insects or grazing via animals) and abiotic (higher temperature and moisture, shade, injury or occurrence of heavy metals) types. SMs are used mainly as chemical such as drugs, flavour, fragrance, insecticides, and dyes by human for the reason that of a great economic value (Kumar and Pandey, 2013). The place of biosynthesis are compartmentalised at cellular or sub-cellular level. SMs can be transported to long distances and accumulate from their site of production. SMs can be of water soluble (hydrophilic) or lipophilic compounds (organic solvents), therefore have diverse cellular mechanism for their transport, storage and turnover. Generally hydrophilic SMs are stored in the vacuole once they are formed in cytoplasm, while lipophilic substances are transported in resin ducts, trichomes, thylakoid membranes, glandular hairs, laticifers, or on the cuticle (Zheng and Wang, 2001).

Plant secondary metabolites are currently the subject of research interest, but their extraction as division of phytochemical or biological investigations represent specific challenges that have to be addressed all over the solvent extraction process. Successful extraction starts with careful selection and preparation of plant samples. During the extraction of plant material, it is important to minimize interference of certain compounds that may co- extract with the target compounds, also to avoid contamination of the extract, and to avoid decomposition of important metabolites or artifact formation due to extraction conditions or solvent impurities (Croteau *et al*., 2000). Researchers from a diversity of scientific disciplines are confront with the challenge of extracting plant material with solvents, regularly as a first step toward

isolating and identifying the specific compounds responsible for biological activities related with a plant or a plant extract. Presence of volatile monoterpenes or essential oils in the plants give an essential defense strategy to the plants, mainly against herbivorous insect pests and pathogenic fungi (Krolicka *et al*., 2006). The volatile terpenoids play a crucial role in plant-plant interactions and serve as attractants for pollinators (Tholl, 2006). They work as signalling molecules and represent evolutionary relationship with their functional roles. Soluble secondary compounds like cyanogenic glycosides isoflavoids and alkaloids be able to be toxic to animals (Pagare *et al*.,2015).

1.8.1 FLAVONOIDS

Flavonoids are an important class of natural products; particularly, belong to a class of plant secondary metabolites having a polyphenolic structure, generally found in fruits, vegetables and certain beverages (Pietta, 2000). They have favourable biochemical and antioxidant effects related with various diseases such as cancer, Alzheimer's disease (AD), atherosclerosis, etc (Burak and Imen, 1999; Lee *et al*., 2009; Castaneda- Ovando *et al*., 2009). Flavonoids possess broad spectrum of health promoting effects and are an essential part in a range of nutraceutical, pharmaceutical, medicinal and cosmetic applications. These may be due to the antioxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties along with their ability to change key cellular enzyme functions. They are also known to be strong inhibitors for several enzymes, such as xanthine oxidase (XO), cyclo-oxygenase (COX), lipoxygenase and phosphoinositide 3-kinase (Hayashi *et al*.,1988; Metodiewa *et al*.,1997; Walker *et al*., 2000).

In nature, flavonoid compounds are products extracted from plants and they are seen in several parts of the plants. Flavonoids in plants help in their growth and also to

protect them from plaques. They comprise one of the most characteristic classes of compounds in higher plants. Many flavonoids are simply recognised as flower pigments generally in angiosperm families (Iwashina, 2013). Though, their occurrence is not limited to flowers but are found in all parts of plants. Flavonoids are also rich in fruits, vegetables, tea, cocoa and wine, hence they are termed as dietary flavonoids. Flavonoids have a number of subgroups, which include chalcones, flavones, flavonols and isoflavones. These subgroups have sole major sources. For example, onions and tea are key dietary sources of flavonols and flavones (Cerezo *et al*., 2010).

Flavonoids participate in various biological activities in plants, animals and bacteria. In plants, flavonoids have long been known to be are accountable for the colour and aroma of flowers, and fruits. That helps to attract pollinators, helping in fruit and seed dispersal. Jorgensen (1995) has reported that the early advances in floral genetics were mainly due to mutation techniques creating an impact on flavonoid derived flower colours, and established that functional gene silencing in plants was connected with flavonoid biosynthesis. Flavonoids are reported to have major role in human and animal health and the current interest is for disease therapy and chemoprevention. At present there are about 6000 flavonoids, that contribute the colourful pigments of fruits, herbs, vegetables and medicinal plants(Ross and Kasum, 2002).

Flavonoids are produced in plants from the aromatic amino acids phenylalanine and tyrosine and malonate. Flavonoids includes many classes such as flavones, flavanones, isoflavonones, flavonols, flavanonols and anthocyanidins *etc.* Flavonoids usually occur in plants as glycosylated derivatives and they add to the brilliant shades of blue, scarlet and orange in leaves, flowers and fruits. Distant from various vegetables and fruits, flavonoids are found in seeds, nuts and grains. Flavan oligomers

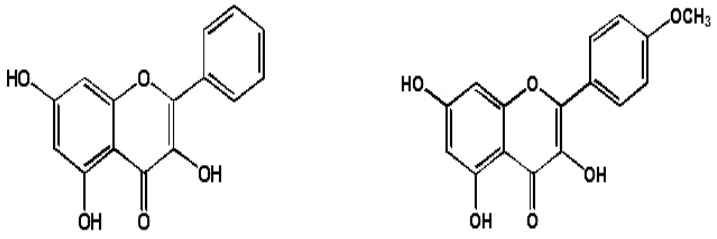
are found in apples, grapes, berries, persimmon, black currant and sorghum and barley grains. Flavonoids perform as catalysts in the light phase of photosynthesis or as regulators of iron channels involved in phosphorylation. They can also work as stress protectants in plant cells by scavenging (Basli *et al*., 2012). Flavonoids keeps plants from UV radiation of sun and scavenge UV generated reactive oxygen free radicals. The level of intake of flavonoids from diet is very much high as compared to those of vitamin C, vitamin E and carotenoids. High clients of fruits and vegetables have a healthy lifestyle, which may be an important factor for their resistance against various chronic diseases. Studies has shown that consumption of flavonoids is inversely correlated with lung cancer (Lee *et al*., 2009).

Flavonoids inhibit the enzymes accountable for superoxide ion production, such as xanthine oxidase and protein kinase C. A number of flavonoids powerfully elevate trace metals which play an important role in oxygen metabolism. Dietary flavonoids stand for an important source of antioxidants, as their intake may reach 800 mg /day. Small portion of the ingested dietary flavonoids is absorbed in their glycoside form, while the major part is widely degraded to different phenolic acids (Staniszewska *et al*., 2003). Both the absorbed flavonoids and their metabolites may show as *in vivo* anti oxidant activity, which seems to involve differently the physiological antioxidants, resulting in aspiring effect on α-tocopherol and β- carotene.

Flavonoids are related with a broad spectrum of health promoting effects and are indispensable pharmaceutical, medicinal and cosmetic applications. All these are because of their anti oxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties (Lopez-Lazaro, 2009). Many flavonoids have evolved as bioactive compounds that interfere with nucleicacid or proteins and show antimicrobial and pharmacological properties. Anti oxidants interfere with free radical

producing systems and protect the cells from destruction. Flavonoids such as quercetin, harvingin, hesperetin possess a variable degree of anti-viral activity. Some flavonoids show hormone-like activities and they bear resemblance to steroid hormones, particularly with oestrogen and are present in fruits and vegetables, tea ,red wine and cereals (Schmeda-Hirschmann *et al*., 2004). A number of flavonoids of dietary significance have been shown to import beneficial impact on parameters associated with atherosclerosis, including lipoprotein oxidation, blood platelet aggregation and cardiovascular activity.

1.8.1.1 GALANGIN AND KAEMPFERIDE



Galangin (1) Kaempferide (2)

Figure 1.8 Structure of galangin (1) and kaempferide (2).

Flavonoids are a class of plant secondary metabolites, found abundantly in fruits, vegetables, and beverages. They have been referred to as "nature's biological response modifiers" because of strong experimental proof of natural ability to change the body's reaction to allergens, viruses, and carcinogens. The antioxidative property as well as the effectiveness of the ethanol extract of *Alpinia galanga* inhibited lipid peroxidation in cooked ground pork (Dai *et al*., 2006; Divakaran *et al*., 2013). An in

depth study of *A. galanga* appear to be significant as dietary constituent and is widely used in traditional medicines.

The present study include free radical scavenging, antioxidant and radioprotective activities of two important flavonoids, viz., galangin (3,5,7-Trihydroxyflavone) and kaempferide (4'-Methylkaempferol) **(Figure1.8)** isolated from *Alpinia galanga L.* rhizome.

1.8.2 FERULIC ACID

Ferulic acid is derivative of cinnamic acid with molecular formula $C_{10}H_{10}O_4$. Ferulic acid (FA) or 4-Hydroxy-3-Methoxycinnamic Acid (**Figure. 1.9**), an omnipresent natural phenolic phyto chemical seen in seeds, leaves, its free form and covalently conjugated to the plant cell wall polysaccharides, glycoprotein, polyamines, lignin and hydroxyl fatty acids.

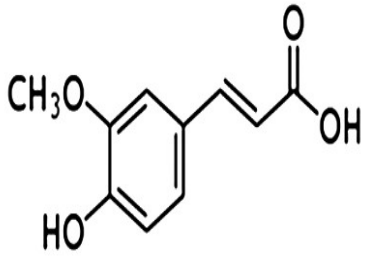


Figure. 1.9 Ferulic acid (FA)

It is found in a number of vegetable sources and seen in high concentration in popcorn and bamboo shoots. Due to antioxidant property, FA display a major role in the body's defense against carcinogenesis and inhibits the formation of N-nitroso compounds (Kuenzig *et al*., 1984). Moreover, it is a strong scavenger of free radicals and used in many countries as food additives. It was reported that FA shows

radioprotective abilities and protects normal cells from harmful effects of ionizing radiation (Roginsky and Lissi, 2005).

Earlier ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) was isolated from *Ferula foetida* for determining the structure, and its name was given based on the botanical name of plant. Later in 1925, FA was chemically synthesized and structurally confirmed by spectroscopic techniques, depicted the presence of an unsaturated side chain in FA, and also exist in both cis and trans isomeric forms (Dutt, 1925; Nethaji *et al*., 1988).

FA is commonly seen in commelinid plants (rice, wheat, oats,and pineapple), grasses, grains, vegetables, flowers, fruits, leaves, beans, seeds of coffee, artichoke, peanut and nuts Cell walls of cereal grains and a variety of food plants (spinach, pineapple, banana, and beetroot) contains 0.5–2% extractable amount of FA, mostly in the trans-isomeric form, and esterified with the specific polysaccharides (Kim *et al*., 2006; Buranov and Mazza, 2009).

The extraction of FA offers business fortuity, and gives supplementary environmental and economic encouragement for industries as it is used in ingredients of many drugs, functional foods and nutraceuticals. Numerous alkaline, acidic and enzymatic methods for the extraction of FA from different sources have been proposed in literature (Mathew and Abraham, 2005; Xu *et al*., 2005;Mathew and Abraham, 2006; Soto *et al*.,2011).

Even though, FA is found ubiquitously in the cell wall of woods, grasses, and corn hulls, but it is not readily available from these natural sources as it is covalently linked with a variety of carbohydrates as a glycosidic conjugate, or an ester or amide. Therefore, it can released from these natural products by alkaline hydrolysis (Tilay *et al*., 2008).

Generally, FA obtained from the chemical process cannot be considered as natural, hence various attempts have been made enzymatically for the release of FA from natural sources. Isolation of FA for commercial production by enzymatic method is a difficult one because most of the FA contents in plants are covalently linked with lignin and other biopolymers. Uraji *et al*. (2013) studied the enzymatic production of FA from defatted rice bran, and suggested that the enzymes (a-L-arabinofuranosidase, multiple xylanases, and an acetyl xylan esterase) from Streptomyces can also be used for the extraction of FA from other sources viz., raw rice bran, wheat bran and corncob.

The TLC separation of crude extracts and visualization by a range of spraying reagents and UV-light offers an easy way for the regular high-throughput detection of FA. About >45% (>2.0%/g dry weight) of total FA content was released during enzymatic treatment of sweet potato stem that had been achieved through the incubation period of 12 hr with 1.0% Ultraflo L (Min *et al*., 2006).

Various biotransformation studies for the production of FA from eugenol carried out by using the recombinant strain of Ralstonia eutropha H16 (Overhage *et al*., 2002).

In the present study, we have made an attempt to develop a simple, cost effective VIS Spectrophotometric method for the determination and quantification of ferulic acid from various plant materials and also investigated the anti inflammatory, hepatoprotective and nephroprotective activity of FA in Swiss albino mice.

1.9 OBJECTIVES OF THE STUDY

The various objectives of the study includes

1. To study *in vitro* cytotoxicity of DAO in murine tumor cells.
2. To study nanoparticle complex mediated antitumor activity .
3. To study the ameliorative and anti - tumor effect of biochemicals .

4. To study the antioxidant capacity and radioprotective properties of phytochemicals .
5. To estimate the presence of ferulic acid (FA) from selected plant materials.

1.10 SCOPE OF THE THESIS

The present work is aimed to evaluate the biological activities of ferulic acid , Ascorbic acid 2-glucoside, iron oxide nanoparticle complex of DAO and flavonoids like galangin and kaempferide from *Alpinia galanga* L. Oxidative stress was induced in mammalian system by using pharmaceuticals like paracetamol, doxorubicin, and cisplatin.

The radioprotecting ability of flavonoids, galangin and kaempferide against whole body γ-radiation was studied by exposing mammalian system to ionizing radiation.

Investigations were carried out to analyze the efficacy of targeting enzyme DAO, bound to magnetic Fe_2O_3 nanoparticles to tumor site thereby minimizing toxic side effects and enhancing therapeutic efficacy.

Ferulic acid and Ascorbic acid 2-glucoside, was examined for their protecting ability against formalin and carrageenan induced inflammation, paracetamol induced hepatotoxicity, doxorubicin induced cardiotoxicity and cisplatin induced nephrotoxicity.

Chapter - 2

MATERIALS AND METHODS

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2.1. MATERIALS

2.1. 1. Animals

Swiss albino mice, 6-8 weeks old (body weight 23-28 g) were obtained from the Small Animal Breeding Station (SABS), Mannuthy, Thrissur, Kerala. They were maintained under standard conditions of temperature and humidity in the Centre's Animal House Facility. The animals were given standard mouse chow and water *ad libitum.* All animal experiments in study be carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted firmly adhering to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

2.1.2. Cell lines

Ehrlich's ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) cell lines were obtained from Cancer Institute, Adayar, Chennai. The cells were maintained in mice by intraperitoneal inoculation of 1x 10^6 viable cells.

2.1.3 Instruments used

Upright research microscope - Meiji, Japan, Labex, Labovision

Centrifuge - Remi, India

Micro centrifuge - Eppendorf India Ltd.

Incubator - Beston

Water bath - Remi, India

Hemoanalyser - BC 2800 VET

Biochemical analyzer - Mispa Plus

High speed cooling centrifuge - Remi, India

Deep freezer, -70^{0}C, -20^0 C - Remi, India

UV-Vis Spectrophotometer - Elico Ltd., Hyderabad

pH meter - Eutech, Merck, Singapore

Fluorescent microscope - Olympus, Japan

Electrophoresis unit - Bangalore Genei, Bangalore

Gel documentation system - Biotech, Yercaud

Elisa Reader - Thermo Scientific

Laminar air flow - Labline, Kochin

Tissue homogenizer - Remi, India

Ultrasonicator - Rotek

VIS spectrophotometer - Systronics

2.1.4 Chemicals

Sodium pyrophosphate	-Sigma-Aldrich, St Louis, USA.
Catalase	-do
Flavin adenine dinucleotide (FAD)	-do
D-alanine	-do
4-amino-3-hydrazino-5-mercapto -1, 2, 4-triazole (purpald reagent)	-do
Potassium periodate (KIO_4)	-do
DEAE cellulose	-do

Poly Vinyl Pyrolidone (PVP)	-do
Thiobarbituric acid (TBA)	-do
Polyoxy ethylene-25-propylene glycol stearate (POES)	-do
Normal melting point agarose	-do
Carrageenan	-do
Dextran	-do
Low melting point agarose	-do
Dimethyl sulphoxide (DMSO)	-do
Ascorbic acid	-do
Hydrochloric acid (HCl)	-do
Triton X	-do
Ethidium Bromide	-do
Bovine serum albumin	-do
Nitroblue tetrazolium (NBT)	-do
Riboflavin	-do
Reduced glutathione (GSH)	-do
5-5' dithiobis-2- nitrobenzoic acid (DTNB)	-do
Acridine orange	-do
Sodium benzoate	Himedia Laboratories Pvt. Ltd. Mumbai
Ammonium sulphate ($(NH_4)_2SO_4$)	-do
RPMI medium	-do
Methanol	-Merck India Ltd., Mumbai
H_2O_2	-do
Silver nitrate	-do
Ammonium nitrate	-do

Zinc sulphate hepta hydrate	-do
Tungstosilicic acid	-do
Sodium dodecyl sulfate	-do
Formaldehyde	-do
pBR322	-Bangalore Genei, Bangalore, India.
Bromophenol blue	-do
Cisplatin	- Samasth Pharma, Mumbai. India.
Doxorubicin Hydrochloride	-Cadila pharmaceuticals Ltd., Ahmedabad, India
Paracetamol	-Variety Pharmaceuticals, Kerala
Trichloro acetic acid	-Sisco research laboratories Ltd., Mumbai, India
Ethylenediamine tetra acetic acid (EDTA)	- Sisco Research Ltd (SRL), Mumbai.
Tris Buffer	-do
Tris-HCl	-do
Folin's reagent	-do
Sodium azide	-do
Ethyl acetate	- Nice Chemicals
Sodium carbonate	-do
Ferulic acid	-do-

2.1.5 Plant Materials

Plant materials selected includes

- Maize
- Rice Bran,

- Wheat bran
- Bamboo shoot

Materials were collected from various reliable sources. They were dried, powdered and kept in an air tight container for future use.

2.1.6 Diagnostic reagent kits

Serum Urea and Creatinine analysis kit : Agappe Diagnostics, Thane

Glutamate pyruvate transaminase (GPT) : Agappe Diagnostics, Thane

Glutamate oxaloacetate transaminase : Agappe Diagnostics, Thane

Alkaline phosphatase (ALP) : Agappe Diagnostics, Thane

Creatine phospho kinase : Agappe Diagnostics, Thane

Lactate dehydrogenase : Agappe Diagnostics, Thane

2.1.7 Reagents and stains

a. Phospahate Buffered saline (PBS)

NaCl	: 8.00 g
KCl	: 0.20 g
KH_2PO_4	: 0.20 g
$Na_2HPO_4.2H_2$	: 1.44 g

These are dissolved in distilled water, made up to 1000 mL. pH was adjusted to 7.4 and was sterilized by autoclaving at 15 lbs for 15 min.

b. Trypan blue

Trypan blue	: 0.4 g
Normal saline (0.9% NaCl)	: 100 mL

Trypan blue stain was dissolved in saline followed by filtration using whatmann No. 1 filter paper.

c. May-Grunwald stain

May-Grunwald powder	: 0.25 g
Methanol	: 100mL

The stain was dissolved in methanol by stirring and filtered through whatmann No.1 filter paper and stored at room temperature.

d. Giemsa stain

Giemsa powder	: 0.8 g
Glycerol	: 50 mL
Methanol	: 50 mL

Giemsa powder was dissolved in glycerol at 60^{o} C with shaking. The mixture was cooled to room temperature and methanol was added, mixed well for 5 minutes and allowed to stand 4 minutes. The solution was filtered through whatmann No.1 filter paper and stored at room temperature.

e. Silver stain - Silver stain - 66 ml solution A + 34 ml solution B

Silver stain a: 5 g Na_2CO_3 was dissolved in 100 mL distilled water.

Silver stain b:

NH_4NO_3	: 1 g
$AgNO_3$	: 0.1 g
Tungstosilicic acid	: 0.25 g
Formaldehyde	: 0.15 mL

These are dissolved in 100 mL distilled water and 0.15 mL.

f. Comet neutralization solution

0.0 32 g Tris HCl was added and dissolve in 500 mL distilled water and pH was 7.4.

g. Comet lysis Buffer, pH- 10.0

NaCl	: 2.9 g
Tris	: 0.12 g

EDTA	: 2.9 g
Sodium sarcosinate	: 1 g
Triton X 100	: 1 mL

These are dissolved in 100 mL distilled water and 1 mL Triton X 100 was added and pH was adjusted to 10.0.

h. Comet stop solution : 1% Acetic acid in distilled water.

i. Fix solution

TCA	: 15 g
$ZnSO_4 .7H_2 O$	: 5 g
Glycerol	: 5 mL

These are dissolved in distilled water and 5 mL Glycerol was added and made up to100 mL.

j. Comet electophoresis Buffer - pH≥ 13.0

NaOH	: 1.2 g
EDTA	: 0.292 g
DMSO	: 0.2 mL

These are dissolved in distilled water and 0.2 mL DMSO was added and made up to100 mL.

k. Bouin's fixative

Saturated picric acid (1.2%)	: 15 mL
Formalin (30-40%)	: 5 mL
Glacial acetic acid	: 1 mL

Saturated picric acid was prepared and formalin and glacial acetic acid was mixed with it.

l. TE Buffer - pH 8

Tris buffer	: 0.12 g
EDTA	: 0.029 g

These are dissolved in distilled water and made up to100 mL and pH was adjusted to 8.0

m. TBE Buffer - pH 8

Tris buffer	: 1.076 g
Boric acid	: 0.55 g
EDTA	: 0.058 g

These are dissolved in distilled water and made up to100 mL and pH was adjusted to 8.0

n. Lysis Buffer for DNA isolation - pH 8

Tris	: 0.61 g
EDTA	: 0.584 g
SDS	: 2 g

These are dissolved in distilled water and made up to100 mL and pH was adjusted to 8.0

2.2 METHODS

2.2.1 Preparation of standard stock solution of ferulic acid (FA)

The stock solution of FA (1 mg/ml) was prepared by dissolving 10 mg FA in ethyl acetate, final volume was made up to 10 ml with ethyl acetate in volumetric flask. From this stock, 1 ml was taken out and added to 10 ml volumetric flask, and the volume was adjusted to 10 ml by adding double distilled water to get 100 µg/ml concentration. This solution was used for further analysis for making calibration curve.

2.2.2 Preparation of calibration curve of ferulic acid

From the stock solution (100 μg/ml) of FA, 0.1 ml to 0.8 ml aliquots were added to a volumetric flask (10ml). To this flask, 2ml of sodium carbonate solution (15 %) and Folin–Ciocalteu reagent (0.5 ml) diluted with double distilled water (1:2 ratio) were added. The final volume was added with double distilled water to get a solution ranging in concentration from 1μg/ml - 8μg/ml of FA. The mixture showed maximum

absorption at 718 nm when calculated against the blank solution. The absorbance of all solutions can be measured, and a calibration curve was plotted.

2.2.2. 1 Reaction Mechanism of Folin–Ciocalteu reagent

Folin–Ciocalteu assay is based on of oxidation-reduction reaction, containing molybdates, tungstates as the main components of the reagent. This assay is a commonly used method for the quantification of phenolic acids in samples. The phenolic compounds reduces the heteropolyphosphotungstates–molybdates into a blue coloured chromogen. The reaction is carried out only under basic conditions in the presence of washing soda solution. The Phenolate anion formed from phe-nolic compound reduces Folin–Ciocalteu reagent to form the blue coloured substance. Spectrophotometer can be used to measure the colour intensity of blue chromogen (Jadhav *et al*., 2012).

2.2.3 Preparation of Ethyl acetate extract of Plant materials

Accurately weighed 10 g of maize, wheat bran, rice bran, and bamboo shoot was extracted separately with 100ml of ethyl acetate with the help of the Soxhlet apparatus. The resulting crude extract was used for further analysis.

2.2.4 Preparation of Sample solution

The extract solution (1ml) was added to the volumetric flask (10ml). To the flask, Folin – Ciocalteu reagent (0.5 ml) diluted with double distilled water (1:2 ratio) and 2 ml sodium carbonate solution (15 %) was added, the final volume was made up to 10ml with double distilled water. Absorbance was measured at 718 nm with a Spectrophotometer.

2.2.5 Isolation of D- amino acid oxidase (DAO)

DAO was isolated from fresh porcine kidney using ammonium sulphate ((NH4)2SO4) precipitation technique (Choi, 1978). The synthesis is described in brief; fresh porcine

kidneys were obtained from Veterinary College, Mannuthy, Thrissur, Kerala, India. It was homogenized in 0.016 M pyrophosphate buffer (pH 8.3). To the solution, Sodium benzoate (1 g/L) was added and heated at 40°C for 30 minutes and then was cooled at 25°C. After cooling process, pH was adjusted to 5.2 with 2N acetic acid, centrifuged at 10,000 rpm for 10 minutes. After centrifugation the supernatant was taken and 250 g/L ammonium sulphate was added. Later centrifugation was carried out at 10,000 rpm to get the precipitate which was dissolved in 0.016 M pyrophosphate buffer (pH 8.3). Heating was repeated at 52°C, and centrifugation and reprecipitation with ammonium sulphate was performed thrice. The obtained final precipitate dissolved in pyrophosphate buffer and dialyzed next to the same buffer. The final enzyme was stored at –20°C.

2.2.6 Enzyme Activity of DAO

Enzyme activity was measured colorimetrically using D-alanine as a substrate (Watanabe *et al.,* 1978). The standard assay mixture consists of 0.133 M pyrophosphate buffer (pH 8.3) containing catalase (700 IU/ml), 0.1 M D-alanine, 0.1 mM FAD, and 70% (V/V) methanol. The reaction was started by the addition of 0.1 ml of the diluted enzyme solution. The reaction was carried out at 37^0C for 15-30 minutes and terminated by the addition of 1 ml of 10% trichloroacetic acid. The precipitate was removed by centrifugation (700 x g, 20 min). To 0.5 ml of the supernatant solution were added 0.5 ml of 5 N KOH and 0.5 ml of 0.5% purpald reagent in 0.5 N HCl. The mixture was kept for 15 min at room temperature. 0.5 ml of 0.75% KIO_4 in 0.2 N KOH were added to the mixture with vigorous shaking, then absorbance at 550 nm was measured. One unit of activity is defined as the rate of formation of 1 µmol of H_2O_2 per min. Enzyme activity was calculated from absorbance obtained for the corresponding concentration by substituting to the

equation, *Activity* = (µmol min^{-1}) =2.584 *A/t,* where, 'A' is a differential absorbance at 550 nm between the sample and the blank, and 't' *is* the reaction time in minutes.

2.2.7 Preparation of Fe_2O_3 Nanoparticles Coated with PVP

Fe_2O_3 nanoparticles were prepared by co-precipitation technique in an inert atmosphere. A typical synthesis process is discussed below. In brief, stoichiometric quantities of metal chlorides (ferric chloride and ferrous chloride 2:1 molar ratio) were dissolved in milli-Q water. Ammonia solution (28%) was added drop wise into the metal chloride solution, followed by vigorous stirring so the final pH became 11 - 12, and subsequently heating the slurry at 80°C for 1 h. PVP (K-15, *M.W* = 15,000) was added to the above slurry and further heated for 1 h and cooled to room temperature and the salt solution was decanted and the precipitate was repeatedly washed to remove any impurity ions. The resulting Fe_3O_4 nanoparticles were heated at 180°C - 210°C to yield Fe_2O_3 nanoparticles (Grabis *et al*.,2008; Jayakumar *et al*., 2009).

2.2.8 Preparation of Fe_2O_3-DAO Complex

Fe_2O_3 (300 mg) was mixed with 0.3 ml of isolated DAO (Enzyme activity-12.6 U/ml, protein concentration -1 mg/ml) in 0.133 M pyrophosphate buffer (pH 8.3), incubated overnight at 4°C in refrigerator and was sonicated using an ultrasonicator for ten minutes. The enzyme nanoparticle complex was centrifuged at 15,000 rpm for 15 minutes. The supernatant was collected and the pellet was suspended in 0.5 ml of 0.133 M pyrophosphate buffer (pH 8.3) and kept in refrigerator. Enzyme activity of both the pellet and supernatant were measured. As the enzyme activity was detected only in pellets, it is further used for the experimental studies.

2.2.9 Characterization of Fe_2O_3 and Fe_2O_3-DAO Complexes

The Fe_2O_3-DAO Complexes were characterized by X-ray diffraction (XRD), FTIR spectroscopy and Scanning electron microscopy (SEM).

2.2.10 Maintenance of cells in animals and tumor measurement

Dalton's Lymphoma Ascites (DLA) cells were maintained in the peritoneal cavity of Swiss albino mice. The cells were transplanted in every 2 week from animal to animal. For this tumor cells were aspirated from the peritoneal cavity, washed with PBS and $1.5X10^6$cells were injected intraperitoneally to a fresh animal to transplant Ascites tumor.

For solid tumor transplantation, DLA cells ($1x10^6$cells/animal) were injected in to the right hind legs of Swiss albino mice (6-8 weeks old, body weight 20-25 g). The experiments were started when the tumor reached a size of 1.0 cm^3. The hind leg thicknesses were measured using a vernier caliper once in two/three days.

The tumor volume was calculated as follows:

Tumor radius

$$= \text{Radius of tumor induced leg} - \text{Radius of normal leg}$$

Tumor volume = $4/3\pi r^3$ where r is the tumor radius.

2.2.11 Irradiation of animals

Irradiation was carry out using a ^{60}Co-Theratron Phoenix Teletherapy Unit (Atomic Energy Ltd., Ottawa, Canada) at dose rate of 1.88 Gy per minute. Swiss albino mice were restrained in specially designed well ventilated cages and exposed to whole body gamma irradiation in a field size of 25x25 $cm^2$and at a distance of 80 cm from the source.

2.2.12 Administration of Drugs

a. Fe_2O_3 nanoparticle–DAO complex

The animals were administered orally with DAO (0.1 mg protein/ml) or Fe_2O_3-DAO (30 mg Fe2O3/ml, 0.1 mg protein/ml) with or without D-alanine (0.5 mmol/mouse). One group of the animals those received Fe_2O_3-DAO and D-alanine were given a magnetic treatment for 15 minutes with a horse shoe magnet, 20 minutes after the drug administration for targeting of the drug nanoparticle complex to the solid tumor site. The drug administration continued for 5 days continuously. The route of administration was oral using an oral needle.

b. Ascorbic acid mono glucoside (AsAG) and ferulic acid (FA)

Ferulic acid (100mg/kg & 200 mg/kg) or AsAG (250 mg/kg & 500 mg/kg) were dissolved in distilled water was given to animals using oral gavage. Both FA or AsAG were administered to animals, one hour after Doxorubicin or cisplatin or paracetamol administration. For anti inflammatory studies, AsAG or FA and diclofenac were administered one hour prior to the sub plantar injection of carrageenan or formalin.

2.2.13 Anti-inflammatory activity

Anti-inflammatory activity of the drug/nanoparticle complex was determined by using two different inflammatory agents

a) Carrageenan induced acute paw edema

   Female Swiss albino mice were subjected to acute inflammation by subplanar injection of 0.02 ml of freshly prepared 0.1% suspension of carrageenan in phosphate buffered saline on the right hind paw (Winter *et al.,*1962). The paw thicknesses were measured using vernier calipers and the thickness was measured up to 6 hours.

b) Formalin induced chronic paw edema

Female Swiss albino mice were subjected to chronic inflammation by sub-planar injection of freshly prepared 0.02 ml of 2% formalin on the right hind paw (Chaw, 1989). The paw thickness was measured using vernier calipers before and after injection of formalin. The readings were measured for 6 days continuously.

2.2. 14 Biochemical parameters

a) Preparation of tissue homogenate

Animals were sacrificed by cervical dislocation after experiments and liver, brain, kidney, heart, tumor tissue or a portion of intestine were quickly excised, and the surface was washed with ice-cold phosphate buffered saline and kept at -20^{o}C. The intestinal mucosa was scrapped off using a clean glass slide and kept at -20^{o}C until analysis. The tissues (liver, brain, kidney, heart, tumor tissue or intestinal mucosa) were homogenized in ice cold phosphate buffered saline (pH 7.4) and 10 % of homogenate was prepared. The homogenate was centrifuged at 10,000 rpm at 4^{o}C and the clear supernatant was taken for the assay. It was stored at -70^{o}C till analysis.

b) Determination of superoxide dismutase (SOD) activity in the tissue homogenate

SOD activity was carried out by following the method of Mc Cord and Fridovich. (1969) and Sandeep and Nair. (2010). The photo-illumination of riboflavin solution in the presence of EDTA causes a reduction of the flavin. It then re-oxidizes and simultaneously reduces oxygen to O_2^-, which is allowed

to react with the detector molecule, NBT. Upon reaction NBT is reduced to a formazan blue. The SOD in the sample inhibits the formazan production.

100 μL of supernatant was mixed with 200 μL of 0.1 M EDTA (containing 0.0015% NaCN), 100 μL of 1.5 mM NBT and phosphate buffer (67 mM, pH 7.8) in a total volume of 2.95 mL. After adding 0.05 mL of riboflavin, the absorbance of the solution was measured against distilled water at 560 nm. The tubes were then uniformly illuminated with an incandescent lamp for 15 min and absorbance was taken again at 560 nm. The difference in absorbance at two time interval (before and after illumniation) was calculated for each sample. Percent of inhibition was calculated by comparing absorbance of sample with the absorbance of control (the tube containing no enzyme activity). The volume of the sample required to scavenge 50% of the generated superoxide anion was considered as 1 unit of enzyme activity and was expressed as U/mg protein for tissue.

c) Determination of reduced glutathione (GSH) content in the tissue homogenate

Reduced glutathione in tissue homogenate was determined according to the method of Moron *et al*.,(1979) with minor modifications. Reduced glutathione forms a yellow colored complex with DTNB with an absorbance at 412 nm. 100 μL of the tissue homogenate was mixed with 63 μL of 25% TCA and cooled on ice for 5 minutes followed by further dilution of the mixture with 300 μL of 5% TCA and these were then subjected to centrifugation at 3000 g for 5 minutes to settle down the precipitate. 150 μL of the supernatant was mixed with 350 μL of sodium phosphate buffer (0.2M, pH 8.0) and 1.0 mL of DTNB (0.6 mM in 0.2M, pH 8.0 phosphate buffer). The yellow color obtained was measured at 412 nm. A standard graph was prepared using different

concentrations (10-50 nmoles) of GSH. The GSH content of the sample was calculated from the standard graph and expressed as n mol/mg protein.

d) Determination of glutathione peroxidase (GPx) activity in the tissue homogenate

Glutathione peroxidase activity was determined according to the method of Hafeman *et al*.,(1974). The GPx enzyme degrades the H_2O_2 in presence of GSH by the following reaction

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O$$

The remaining GSH was measured by its reaction with DTNB

25 μL of tissue homogenate was treated with 50 μL of 5mM GSH, 250 μL of 1.2 mM H_2O_2, 50 μL of 25 mM NaN_3 and phosphate buffer (1M, pH7.0) in a total volume of 1.25 mL at 37ºC for 6 min. The reaction was stopped by adding 1.0 mL of 1.67 % H_3PO_4 and the reaction mixture was centrifuged at 3000 rpm for 10 min to settle down the precipitate. 1.0mL of the supernatant was mixed with 1.0 mL 0.4 M Na_2HPO_4 and 1mL of 1mM DTNB (in buffer). The absorbance of the yellow colored complex was measured at 412 nm after incubation for 10 min at 37º C against distilled water. A sample without the tissue homogenate was processed in the same way and was kept as the blank. The activity is expressed as U/mg protein.

The GPx activity = O.D of blank – O.D of sample / 0.001 X mg protein

One unit of enzyme activity was defined as decrease in log GSH by 0.001/min after subtraction of the decrease in log GSH per minute for the non-enzymatic reaction.

e) Determination of lipid peroxidation in the tissue homogenate

The level of lipid peroxidation was measured as malondialdehyde (MDA) according to the method of Buege and Aust (1978). The malondialdehyde (MDA) is formed

mainly from the peroxidation of PUFAs. MDA is a TBA reacting substance (TBARs) and the product formed between the reaction of MDA and TBA is estimated at 532 nm.

Briefly, the reaction mixture contained 1 mL of tissue homogenate(100 mL of 10 % tissue homogenate + 900 mL distilled water) and 1mL of TBA reagent (which contains 0.375% thiobarbituric acid, 0.025 N HCl, 15% trichloroacetic acid and 6.0 mM EDTA). The reaction mixture was heated at 90^{o}C for 30 minutes, cooled and centrifuged at 10,000xg for 10 minutes. The amount of TBARS in the supernatant was estimated by measuring the absorption at 532 nm. The lipid peroxidation values are expressed as n moles of MDA per mg protein.1, 1, 3, 3-tetraethoxypropane was used as the standard.

f) Determination of the total protein in the tissue homogenate

Protein content in the tissue was determined according to the method of Lowry *et al*. (1951) and Mathew *et al*. (2007). The tyrosine and tryptophan residues of proteins cause reduction of the phosphomolybdate and phosphotungstate components of Folin-Ciocalteau reagent in an alkaline medium to give a bluish purple colour with absorbance at 660 nm.

10 μL of the homogenate was mixed with 990 μL of distilled water, 5 mL of alkaline $CuSO_4$ (0.5 % CuSO4 in 1 % sodium potassium tartarate and 2% Na_2CO_3in 0.1 N NaOH mixed in the ratio 1:50) was kept for 10 min at room temperature. 0.5 mL of 1 N Folin-Ciocalteau reagent was added and absorbance was measured after 30 min at 660 nm against the reagent blank. Protein content was calculated from the standard graph plotted using different concentrations (0-500 μg/mL) of bovine serum albumin (BSA).

2.2. 15 Serum parameters

a) Serum glutamate oxaloacetate transaminase (SGOT)

Serum SGOT activity was determined according to the method of Thefeld *et al*., (1974).

SGOT (AST) catalyzes the transfer of amino group between L-Aspartate and α-Ketoglutarate to form Oxaloacetate and Glutamate. The formed Oxaloacetate reacts with NADH in the presence of Malate Dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is calculated as a decrease in absorbance which is proportional to the SGOT (AST) activity in the sample

$$\text{L-Aspartate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+$$

Reagents used were from Agappe diagnostic kit. The working reagent was prepared by mixing 4 volume of Reagent 1 [Tris buffer (88 mmol/L, pH 7.8), L-Aspartate (260 mmol/L), LDH (1500 U/L), MDH (900U/L)] with 1 volume of Reagent 2 [α-Ketoglutarate (12mmol/L, NADH (0.24mmol/L)] provided in the kit. 0.1 ml of serum was mixed with 1.00 ml of the working reagent. Mixed well and incubated for 1 minute at 37º C. The change in absorbance was measured per minute for 3 minutes at 340 nm.

SGOT activity (U/L) = (Δ OD / min) x 1745

b) Serum glutamate pyruvate transaminase (SGPT)

Serum SGPT activity was determined according to the method of Thefeld *et al*., (1974).

SGPT (ALT) catalyzes the transfer of amino group between L-Alanine and α-Ketoglutarate to form Pyruvate and Glutamate. The formed Pyruvate react with NADH in the presence of Lactate dehydrogenase forming NAD. The rate of oxidation of NADH to NAD is calculated as a decrease in absorbance which is proportional to the SGPT (ALT) activity in the sample.

$$\text{L-Alanine } \alpha\text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{L-Glutamate+ Pyruvate LDH}$$

$$\text{Pyruvate + NADH + H}^{+} \xrightarrow{\text{MDH}} \text{Lactate + NAD}^{+}$$

Reagents used were from Agappe diagnostic kit. The working reagent was prepared by mixing 4 volume of Reagent1 [Tris buffer (110 mmol/L, pH 7.5), L-Alanine (660 mmol/L), LDH (1500 U/L)] with 1 volume of Reagent2 [α-Ketoglutarate (16mmol/L, NADH (0.24mmol/L)] provided in the kit. 0.1 ml of serum was mixed with 1.00 ml of the working reagent. Mixed well and incubated for 1 minute at 37^0 C. The change in absorbance was measured per minute for 3 minutes at 340 nm.

$$\text{SGPT activity (U/L)} = (\Delta \text{ OD / min}) \times 1745$$

c. Serum bilurubin

Serum bilurubin activity was determined according to the method of Walter and Gerarde (1970).

Sulfanilic acid react with sodium nitrite to form diazotized sulfanilic acid. Serum bilurubin react with diazotized sulfanilic acid to form colored azobilurubin which measured photometrically.

Reagents used were from Agappe diagnostic kit. 0.05 ml of serum was mixed with 0.02 ml of activator and I ml of bilurubin reagent as test and mixed 0.02

ml of activator alone with I ml of bilurubin reagent as blank. Incubate for 5 minutes and measured the absorbance of test against reagent blank at 546 nm.

Total Bilurubin = OD of test – OD of reagent blank X 29.

d) Serum urea

Serum urea was determined following the method of Chaney and Marbach (1962).

Urea is hydrolyzed in presence of water and urease to produce ammonia and carbon dioxide. Under alkaline conditions, thus formed ammonia, reacts with hypochlorite and phenolic chromogen forming coloured indophenol, which is measured at 578 nm. Sodium nitroprusside acts as a catalyst. The intensity of the colour is proportional to the concentration of urea in the sample.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2\,NH_3 + CO_2$$

$$NH_3 + \text{Salicylate} \xrightarrow[\text{Hypochlorite}]{\text{Nitroprusside}} \text{2-2-Dicarboxy Indophenol}$$

Reagents used were from Agappe diagnostic kit. Working reagent was prepared by mixing one bottle of Reagent 3 [Enzyme concentrate Ureases (> 500K U/L)] with one bottle of reagent1 [Buffer (pH 7, 120 mmol/L), Sodium Salicylate (60 mmol/L), Sodium nitroprusdide (5 mmol/L)]. 0.01 ml of serum was mixed with 1 ml of working reagent and 0.01 ml of Urea-B Standard Concentration (40mg/dl) provided in the kit was added to the working reagent as standard. Mixed well and incubated it for 5 minutes at 37^0C. Then Reagent 2 (Phosphate buffer (pH-<13, 120 mmol/L), Sodium hypochlorite (10 mmol/L)] provided was added to it andagain incubated it for 5 minutes at 37^0

C. The absorbance of the sample and standard was read against the reagent blank at 578 nm.

Serum urea (mg/dl) = Absorbance of Sample / Absorbance of Standard X 40

e) Serum creatinine

Serum creatinine was determined according to the method of Bonsnes and Taussky (1945).

Creatinine forms a yellow-orange compound in alkaline medium with picric acid. The intensity of the color is measured at 520 nm. The change in absorbance is proportional to the creatinine concentration. Reagents used were from Agappe diagnostic kit. Working reagent was prepare by mixing 1 volume of Reagent 1 [picric acid (8.73 mmol/L), Surfactant] with 1 volume of Reagent 2 [Sodium hydroxide (300 mmol/L), Sodium phosphate (25 mmol/L)]. 0.1 ml of serum was mixed with 1 ml of working reagent and 0.1ml of creatinine standard was also mixed with the working reagent. The optical density was read at 60 second after (T_1) the sample or standard addition. Exactly after 60 seconds after the first reading the second reading (T_2) was taken .

Creatinine conc. (mg/dl) = (T1- T2 of sample)/ (T1- T2 of sample) X 2

f) Serum lactate dehydrogenase (LDH)

Serum LDH was determined according to the method of McQueen (1975). The reduction of pyruvate with NADH forming NAD was catalyzed by Lactate dehydrogenase. The rate of oxidation of NADH to NAD is calculated

as a decrease in absorbance, which is proportional to the LDH activity in the sample.

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{L- Lactate} + NAD^{+}$$

Reagents used were from Agappe diagnostic kit. 0.01ml of serum was added to 1 ml of working reagent prepared by mixing 4 volume of Reagent 1, [Tris buffer(pH 7.4, 80mmpl/L), Pyruvate (1.6 mmol/L), Sodium Chloride (200 mmol/L)] with 1 volume of reagent 2, [NADH (240 μ mol/L)] provided in the kit. Mixed well and incubated for 1 minute at 37° C.

LDH activity (U/L) = (Δ OD / min) x 16030

g) Creatinine kinase (CK)

Serum CK was determined according to the method of Witt and Trendelenburg (1982).

The procedure involve analysis of CK-activity in the presence of an antibody to CK-M monomer. This antibody completely inhibits the activity of CK-MM and half of the activity of CK-MB, while not affecting the B subunit activity of the CK-MB and CK-BB. CK method was used to quantitatively determine CK-B activity. The CK-MB activity is obtain by multiplying the CK-B activity.

Reagents used were from Agappe diagnostic kit. 0.04 ml of serum was added to 1 ml of working reagent prepared by mixing 4 volume of Reagent 1 [Imidazole (pH6.7, 125mmol/L), D-Glucose (25mmol/L), N-Acetyl-L-cysteine (25mmol/L), magnesium acetate (12.5 mmol/L), NADP (2.55 mmol/L), EDTA (2.025 mmol/L), hexokinase (> 6800 U/L)] with 1 volume of reagent 2 [Creatinine phosphate (250.5 mmol/L), ADP (15.25 mmol/L), AMP

(25 mmol/L), Diadenosine pentaphosphte (103 mmol/L), G-6-PDH (> 8800 U/L)] provided in the kit. Mixed well and incubated for 5 minute at 37°C.

CK-MB activity (U/L) = (Δ OD / min) x 8254

2.2. 16 Histopathological analysis

A portion of the intestine, kidney, liver or heart was fixed in 10% formalin solution immediately after sacrifice, passed through ascending grade of alcohol, cleared in xylene impregnated and embedded in paraffin. Sections were made using a microtome, stained with Hematoxylene and Eosin and mounted in DPX. The slides were observed in light microscope under oil immersion microscope (100X) and photographed.

2.2.17 Determination of cell viability and apoptosis

a) The cell viability was determined by Trypan blue dye exclusion method (Talwar, 1974). As the cells selective in the compounds that passes through the membrane, in a viable cell, trypan blue is not absorbed while it traverses the membrane in a dead cell. Hence, dead cells have blue colour under a microscope. The method is an index of the dead cells in a cell population.

   0.1 mL of cell suspension was mixed with 0.01 mL of 0.4% Trypan blue, kept for 2-3 minutes and loaded on a haemocytometer. The no. of stained and unstained cells was counted separately and more than 100 cells were counted. The percentage survival of cells were calculated and plotted against concentration of drug.

   % dead cells = Number of dead cells / Number of viable cells + No. of dead cells X 100

b) May Grunwalde-Giemsa staining (Baskic *et al.,*2006). To analyze the morphological changes, thick smears of cells were prepared on clean glass slides, stained with May Grunwald stain for 10 minutes followed by Giemsa stain for 30 minutes, washed, dried and visualized for apoptotic cells using a bright field microscope.

The cells having condensed and fragmented nuclei, blebbing of plasma membrane and decreased size and apoptotic bodies were identified as apoptotic cells (Rajagopalan *et al*., 2003). Necrotic cells will be having nuclear and cytoplasmic swelling, chromatin flocculation, cytoplasmic and nuclear membrane dissolution or lysis.

Apoptotic index was calculated using the formula,

Apoptotic index = Number of apoptotic cells/ Number of total cells X 100

2.2.18 Determination of DNA damage

a) Plasmid relaxation assay

The plasmid pBR322 DNA (100 mg), in potassium phosphate buffer (0.1M, pH 7.4) was exposed to various doses of gamma radiation in the presence or absence of drugs or nanoparticle complexes. After irradiation, the DNA samples were analyzed in 1% agarose gel prepared in TBE buffer (89mM Tris, 89mM Boric acid, 0.2mM EDTA) by electrophoresis at 50 V for 3 hours. The DNA was stained with EB (0.5 mg/ml) and visualized using a gel documentation system, DNA bands were photographed and analyzed using Digital Gel Documentation and Analysis Software, Biotech R&d Laboratories, Yercaud.

b) Alkaline single cell gel electrophoresis or comet assay

The comet assay, also called single cell gel electrophoresis (SCGE), is an electrophoretic technique for direct visualization of DNA damage in individual cells.

In this technique, cells are fixed in agarose gel on microscope slides. The cells are lysed by detergents and high salt and electrophoresed under alkaline conditions. Cells with increased DNA damage display increased migration of their DNA from the nucleus towards the anode, giving the appearance of the tail of a comet (the head being the residue of the nucleus). The comets are observed by fluorescence microscopy after staining with suitable dyes such as ethidium bromide, acridine orange or propidium iodide or by light microscopy following silver staining.

Alkaline single cell gel electrophoresis was performed by the method given by Singh (2000), with minor modifications. In brief, microscopic slides were coated with normal melting agarose (1% in PBS), immediately cover sliped and kept at 4^{o}C for 10 minutes to allow the agarose to solidify. After removal of the cover slip, 200 μL of 0.8% low melting point agarose containing 50μL of treated cells were added onto the slide, cover slips were placed immediately and the slides were kept at 4^{o}C. After solidification, the cover slips were removed and the slides were immersed in pre-chilled lysing solution containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, pH-10, 1% DMSO, 1% Triton X and 1% sodium sarcosinate and kept for 1 hour at 4^{o}C. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank filled with freshly prepared alkaline buffer containing 300

mM NaOH, 1 mM EDTA, and 0.2% DMSO (pH≥13). The slides were equilibrated in the same buffer for 20 minutes and electrophoresis was carried out for 30 minutes at 20 V. After electrophoresis the slides were washed gently with 0.4 mM Tris-HCl buffer, pH-7.4 to neutralize alkali. The slides were again washed with distilled water, kept at 37^{o}C for 2 hours. Alkaline single cell gel electrophoresis was performed by the method given by Singh (2000), with minor modifications. In brief, microscopic slides were coated with normal melting agarose (1% in PBS), immediately cover sliped and kept at 4^{o}C for 10 minutes to allow the agarose to solidify. After removal of the cover slip, 200 μL of 0.8% low melting point agarose containing 50μL of treated cells were added onto the slide, cover slips were placed immediately and the slides were kept at 4^{o}C. After solidification, the cover slips were removed and the slides were immersed in pre-chilled lysing solution containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, pH-10, 1% DMSO, 1% Triton X and 1% sodium sarcosinate and kept for 1 hour at 4^{o}C. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank filled with freshly prepared alkaline buffer containing 300 mM NaOH, 1 mM EDTA, and 0.2% DMSO (pH≥13). The slides were equilibrated in the same buffer for 20 minutes and electrophoresis was carried out for 30 minutes at 20 V. After electrophoresis the slides were washed gently with 0.4 mM Tris-HCl buffer, pH-7.4 to neutralize alkali. The slides were again washed with distilled water, kept at 37^{o}C for 2 hours.

c) Micronucleus assay in peripheral blood reticulocytes

Acridine orange (AO) - coated glass slides were prepared according to the method of Hayashi *et al.,* (1990). AO was dissolved in distilled water at a concentration of 1 mg/mL. 10 μL of this solution was placed on a pre-heated clean glass slide. The solution was spread by moving a glass rod back and forth over it and then air-dried. The AO-coated glass slides were stored at room temperature under dry conditions.

Peripheral blood was collected from Swiss albino mice under study by tail vein puncture without any anticoagulant at 24 h and 48 h after 1 0r 2 Gy-whole body gamma-irradiation. 5 μL of blood were kept on AO-coated slides and covered with cover slip. The reticulocytes (RETs) and micro nucleated reticulocytes (MNRETs) were monitored using a fluorescent microscope. The slides are observed under a blue excitation (488 nm) and a yellow to orange barrier filter (515 nm). The slides be observed for 2000 reticulocytes of peripheral blood (identified by their reticulum structure with red fluorescence) and % of micronucleated (round in shape with a strong yellow-green fluorescence) reticulocytes were scored.

2.3 Statistical Analysis

The statistical data were expressed as mean ± standard deviation (SD). Statistical analyses of the results were performed using ANOVA with Tukey-Kramer multiple comparisons test, using the software (GraphPad inStat 3.1) where $p<0.05$, $p<0.01$ and $p<0.001$ are considered to be significant.

Chapter- 3

ISOLATION OF PIG KIDNEY D-AMINO ACID OXIDASE (DAO) AND *IN VITRO* CYTOTOXICITY OF DAO IN MURINE TUMOR CELLS

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3.3 RESULTS

3.3.1 Enzyme purification

3.3.2 *In Vitro* Cytotoxicity of DAO

3.3.3 Determination of Cellular DNA Damage by Comet Assay (Alkaline single cell gel electrophoresis)

3.4 DISCUSSION

3.1 INTRODUCTION

D-Amino-acid oxidase (DAO) (D-amino-acid: oxygen oxidoreductase (deaminating); EC 1.4.3.3) catalyzes the oxidative deamination of D-amino acids, producing the corresponding α-keto acid and ammonia with concomitant reduction of molecular oxygen to hydrogen peroxide (H_2O_2). All DAO s characterized so far contain noncovalently bound FAD as their prosthetic group (Warburg and Christian, 1938). Recent researches on D-amino acid oxidases (DAO) and amino acid metabolism have shown new, interesting properties of the flavoenzyme and enlighted new biotechnological uses of this catalyst. The presence of DAO has been reported in many organisms and it is used as a marker of peroxisomes in higher organisms and microbodies in yeast (De Duve and Baudhuin, 1966). The only D- amino acid oxidase species available in a homogeneous form and in large quantities so far is the one obtained from pig kidney (Bright and Porter, 1975).

Reactive oxygen species (ROS) *e.g.,* superoxide anion (O_2^{-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (**.**OH), are potentially harmful byproducts of normal cellular metabolism that directly affect cellular functions (*eg.* development, growth, and aging) and survival as well as causing mutation (Davies, 1995; Beuth, 2008). ROS are generated by all aerobic organisms and seem to be indispensable for signal transduction pathways that regulate cell growth (Beckman and Ames, 1997) and reduction-oxidation (redox) status. However, overproduction of these highly reactive metabolites can initiate lethal chain reactions that involve oxidation and that damage cellular integrity and survival. Because of its highly cytotoxic nature, ROS may exert remarkable antitumor potential if they can be selectively delivered into tumors (Baig

et al., 2019). This strategy of cancer treatment by generation of ROS selectively in tumor site has been named 'oxidation therapy' (Ben-Yoseph and Ross, 1994; Stegman *et al*., 1998). In oxidation therapy, the main aim lies in generating excess ROS in tumor tissue directly and causing maximum tumor killing without affecting the normal tissues. Nowadays, use of H_2O_2-generating enzymes has been proposed as an alternative approach to developing an H_2O_2 dependent antitumor system.

The enzyme possess certain common properties such as it is stabilized toward heating and the loss of its FAD prosthetic group by the inhibitor benzoate, with which it forms a complex, at a low molarity of ammonium sulphate it is precipitated (Kubo *et al*., 1960;Yagi *et al*., 1971) possess a low affinity for calcium phosphate that is in the form of either a gel or hydroxylapatite (Massey *et al*.,1961). These properties have been exploited for its purification.

The present chapter reveals the isolation procedure of D-amino acid oxidase (DAO), and its use as an effective anti tumor agent by preferential production of H_2O_2 in murine tumor cells, by administration of corresponding D-aminoacid.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Fresh porcine kidneys were obtained from Veterinary college, Mannuthy, Thrissur, Kerala, India.

3.2.2 Chemicals

Sodium pyrophosphate, catalase, flavin adenine dinucleotide (FAD), D-alanine, 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole (purpald reagent), potassium periodate

(KIO_4) and DEAE cellulose were from Sigma chemical company Inc., St.louis, MO, USA. Sodium benzoate and ammonium sulphate ($(NH_4)_2SO_4$) were from Himedia Laboratories Pvt. Ltd. Mumbai. Methanol was from Merck India Ltd., Mumbai. Trichloro acetic acid was from Sisco research laboratories Ltd., Mumbai, India. All other reagents were all of analytical grade and purchased from reputed Indian manufacturers.

3.2.3 Assay of enzyme activity

Detailed methodology is explained in Chapter 2, section, 2.2.6

3.2.4 Protein determination

The protein concentration of the mixture was determined according to the method of Lowry *et al*. (1951) using bovine serum albumin as a standard. D-Amino-acid oxidase activity is finally expressed as the amount of D-alanine oxidized per min per milligram of protein (Lowry *et al*., 1951).

3.2.5 Isolation procedure

The fresh kidneys were sliced, trimmed of fat and the ureters, cut into pieces of less than 3-cm dimension, and the deep red medulla removed and discarded. The cleaned cortices were used immediately or stored overnight at -20^{o}C. Unless otherwise specified, all operations were performed at 0-5^{0}C.

Fresh porcine kidney (1000g) was homogenized in 3.3 liters of cold 0.016 MM pyrophosphate-HCl buffer, pH 8.3 in a blender. To the homogenate, sodium benzoate (1g/L) was added, heated at 40^{o} C for 30 min in a water bath and cooled at 25°C. After cooling, pH was adjusted to 5.2 with 2N acetic acid followed by centrifugation at 10,000 rpm for 10 min in a cooling centrifuge. After centrifugation, supernatant were taken, and 250 g/L ammonium sulphate was added, kept for 15

minutes at 5^0C. Again centrifugation was carried out at 10,000 rpm to obtain the precipitate.

The precipitate was dissolved in 0.016 M pyrophosphate-HCl buffer (pH 8.3) and added sodium benzoate (1g/L). The pH was again adjusted to 5.2 with 2N acetic acid. After heating at 52ºC for 7 min followed by sudden cooling at 10^0C, the solution was centrifuged and reprecipitated with $(NH_4)_2SO_4$ (113g/L). Again centrifugation (10,000 rpm, 10 min) was carried out to collect the precipitate. It was dissolved in 35ml of 0.016 M pyrophosphate-HCl buffer (pH 8.3) followed by the addition of $(NH_4)_2SO_4$ (54g/L). The solution was kept for 15 minutes, sodium benzoate (1g/L) was again dissolved, pH was adjusted to 5.2 with 2N acetic acid. After centrifugation, 56g/L $(NH_4)_2SO_4$ was added to the supernatant, followed by heating at 52ºC. Suddently, cooled the supernatant to 10^0C . Centrifugation (10,000 rpm, 10 min) was carried out.

Precipitate was dissolved in a small volume of 0.016 M pyrophosphate-HCl buffer (pH 8.3). To the supernatant, obtained after centrifugation, $(NH_4)_2SO_4$ (205 g/ L) was added and kept for 20 minutes. Final centrifugation was carried out at 10,000 rpm for 10 min and precipitate was dissolved in 5 ml of 0.025 M sodium phosphate buffer, pH 7.5, containing 2 x 10^{-4} M benzoate. The buffer and $(NH_4)_2SO_4$ from enzyme preparation were then removed by dialysis against the same buffer, kept overnight in the cold room.

3.2.5 Final purification

After dialysis, enzyme solution was added to 60×3 cm-DEAE cellulose column previously equilibrated with 0.02 M phosphate buffer (pH 7.5), which had been freshly prepared, in the cold room. The enzyme moves down the column as a yellow band leaving a white impurity at the top. The enzyme was washed into the column

with of 0.01 M sodium phosphate buffer, pH 7.5, and eluted with the 0.5 M sodium phosphate buffer, pH 7.5 at a rate of 3-5 ml/min. The DAO is clearly visible, as an intensely yellow band, as it passes down the column and fractions containing the enzymes are can be pooled after by performing enzyme assay. After pooling the active enzyme fractions, FAD (0.2ml/ml) and sodium benzoate (1g/L) were added and dissolved by vigorous stirring to keep the enzyme in benzoate complex to maintain its stability. Final dialysis of purified enzyme was carried out against 0.025 M sodium phosphate buffer, pH 7.5, containing 2 x 10^{-4} M benzoate and dialyzed twice for 4 hours against 250-ml portion of the medium above. $(NH_4)_2SO_4$ was slowly added to 25% saturation to the final dialysed enzyme. After standing for 30 min, the solution was centrifuged at 10,000 rpm for 20 min and the precipitate dissolved in a small volume of 0.0125 M phosphate buffer (pH 7.5) containing 0.2mM sodium benzoate. The final pure enzyme may be stored at -20^0C.

3.2.6 Electrophoretic methods and molecular weight determination

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE) was carried out to check the purity of final pure enzyme (Laemmli, 1970) on a 12% polyacrylamide slab gel containing 0.1% SDS at a constant current (20 mA) for about 1 hr. Molecular weight standards (Genei, Bangalore, India) used were myosin rabbit muscle (205,000 Da) phosphorylase b (97,400 Da), bovine serum albumin (66,000 Da), ovalbumin (43,000 Da), and bovine carbonic anhydrase (29,000 Da). The gel was stained by using silver staining proposed by Oakley *et al*., 1980.

3.2.7 *In Vitro* Cytotoxicity Assay

In vitro cytotoxicity of DAO was determined by the use of different concentrations of substrate with Dalton's lymphoma ascites (DLA), to analyze their ability to produce H_2O_2 and to induce cytotoxicity on cancer cells. The tumor cells, DLA

($1x10^6$cells/ml) were incubated in phosphate buffered saline (PBS) with 10% FCS for 2 hours with various concentrations of D-alanine (0.01-0.2 M) for different time intervals in presence of the DAO (0.27 U/ml). At specific time intervals (0, 30, 60 and 120 min), the cell viability was estimated by means of trypan blue dye exclusion method using 0.2% trypan blue. Toxicity was quantified as the number of cells surviving relative to untreated controls.

3.2.8 Determination of Cellular DNA Damage by Comet Assay (Alkaline single cell gel electrophoresis)

DLA cells from a stock of 1×10^6 cells/ml were incubated with DAO (0.27 U/ml) and D-alanine (0.2M) to study the generation of H_2O_2 and its effect in causing DNA strand breaks in DLA cells. The DNA strand breaks in Daltons Lymphoma ascites (DLA) were measured using alkaline single cell gel electrophoresis (Chandrasekharan *et al*., 2009). Microscopic slides were coated with 0.8% low melting point agarose containing 50 μl of the treated cells and the slides were kept at 4°C. After solidification, the slides are immersed in pre-chilled lysing solution containing 2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris-HCl, pH 10, 1% DMSO, 1% Triton X and kept for 1 hour at 4°C. After the process of lysis, slides were drained well and placed in a horizontal electrophoretic apparatus filled with freshly prepared electrophoretic buffer containing 300 mM NaOH, 1 mM EDTA, 0.2% DMSO, pH ≥ 13. Then slides are equilibrated in buffer for 20 minutes and electrophoresis was carried out for 30 minutes at 20 V, 300 mA. After electrophoresis the slides are washed properly with 0.4 mM Tris-HCl buffer, pH-7.4 to remove alkali. The slides were again washed with distilled water, kept at 37°C for 2 hours to dry the gel and silver staining was carried out (Cerda *et al*., 1997). The comets were observed using a microscope and the images captured and analyzed using the software 'CASP' which gives % DNA in tail,

tail length, tail moment and olive tail moment directly. The parameter tail moment (TM) is the product of tail length and % DNA in tail and olive tail moment (OTM) is the product of the distance between the centre of the head and the centre of the tail and % DNA in tail (Konca *et al*., 2003). Results are given as mean ± standard deviation.

3.3 RESULTS

3.3.1 Enzyme purification

The purification of the enzyme according to our method is summarized in **Table 3.1**. The enzyme activity and specific activity were determined at every stages of isolation. The recovery of the enzyme was 8.4 % and a yield of 1.5 mg can be obtained from a typical preparation (1000 g of kidney). The final specific activity of the DAO at 37ºC is also reported in Table 3.1. The DEAE cellulose chromatographic step purified the enzyme preparation so that the specific activity was increased to about 6 U/mg protein. The purified preparation was homogenous, since a single band (Mr = 38,000 Da) was detected after SDS- PAGE (**Figure 3.1**) .

Table 3. 1 Characterization of DAO at various steps of isolation

Samples	Volume (ml)	Enzyme Activity (µmol/ml/ min)	Enzyme unit (U)	Concentration of Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield
I Homogenate - Crude extract	3300	0.0164	108.24	31.26	103158	0.0010	1	100
I Homogenate after adding sodium benzoate	3300	0.0598	394.68	29.75	98175	0.0040	4	364
Homogenate after heating	3300	0.0524	345.84	10	33000	0.0105	10.5	319.5
II step supernatant after adding ammonium sulphate	200	0.0423	16.92	3.75	750	0.0226	22.6	15.63
Final enzyme after precipitation with ammonium sulphate	50	0.1269	12.69	1	50	0.2538	254	11.72
After DEAE cellulose column chromatography	3	1.523	9.1	0.5	1.5	6	6000	8.4

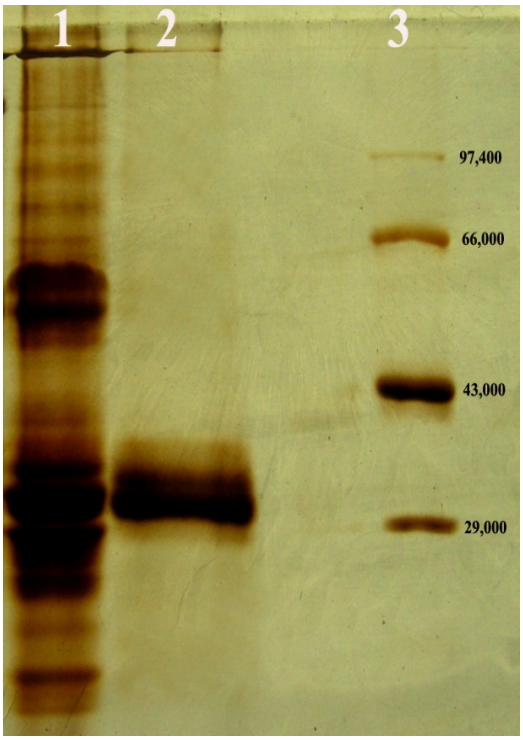

Figure 3.1 SDS- polyacrylamide gel electrophoresis of D-amino acid oxidase a) lane 1: DAO before purification, lane 2: Purified DAO showing single band, lane 3: Marker proteins: phosphorylase b (97,400 Da), bovine serum albumin (66,000 Da), ovalbumin (43,000 Da), and bovine carbonic anhydrase (29,000 Da)

3.3.2 *In Vitro* Cytotoxicity of DAO

Cytotoxicity of DAO was first examined via an *in vitro* system using Dalton's lymphoma ascites (DLA) cells. D-alanine was used as the substrate because of its high turnover rate. DAO alone and D-alanine alone showed no cytotoxicity against DLA cells. In contrast, DAO along with D-alanine showed remarkable cytotoxicity in a concentration-dependent manner (**Figure 3.2**). This is attributed to the H_2O_2 generation by the enzyme-substrate reaction system. Maximum percentage mortality (96 %) was obtained when 0.2 M D-alanine was used.

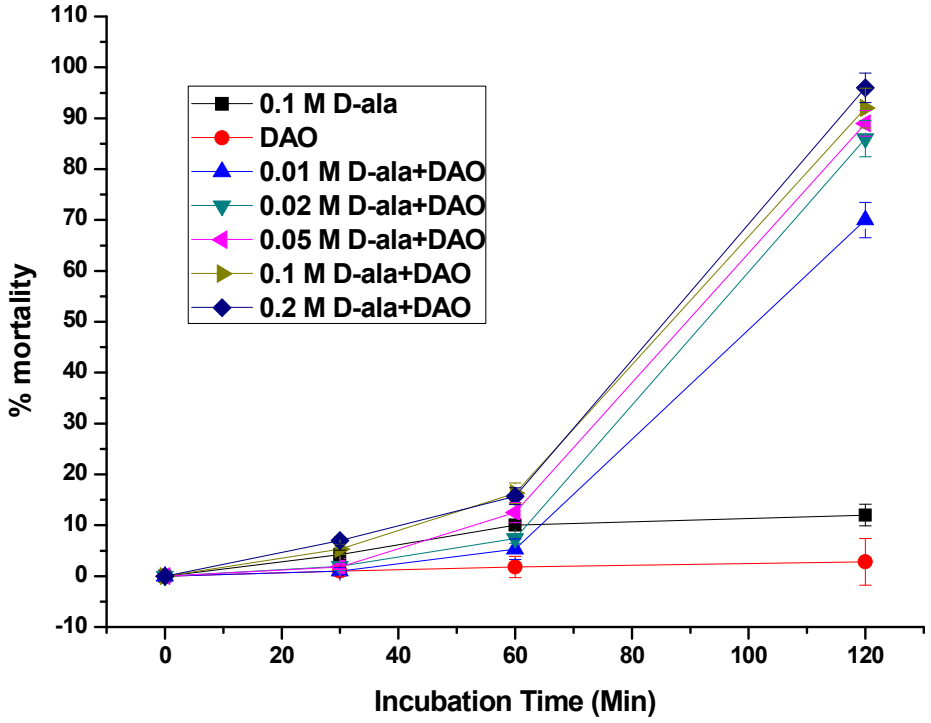

Figure 3. 2 Cytotoxic effect of different concentrations of D-alanine on DLA cells

3.3.3 Determination of Cellular DNA Damage by Comet Assay (Alkaline single cell gel electrophoresis)

To understand the extent of cellular DNA damage, DLA cells were incubated with DAO and D-alanine and subjected to comet assay. The result showed that the DLA cells on treatment with different concentrations of enzyme and D-alanine, resulted in DNA damage indicating the *in vitro* generation of H_2O_2 in the enzyme- substrate reaction system (**Figure 3.4**). The cells treated with either substrate or enzyme alone did not show any damage to DNA (**Figure 3. 3a**). Thus the result provided evidence for the induction of cellular DNA damage through the oxidative stress generated by H_2O_2, in *situ* through enzyme action (**Figure 3. 3b**).

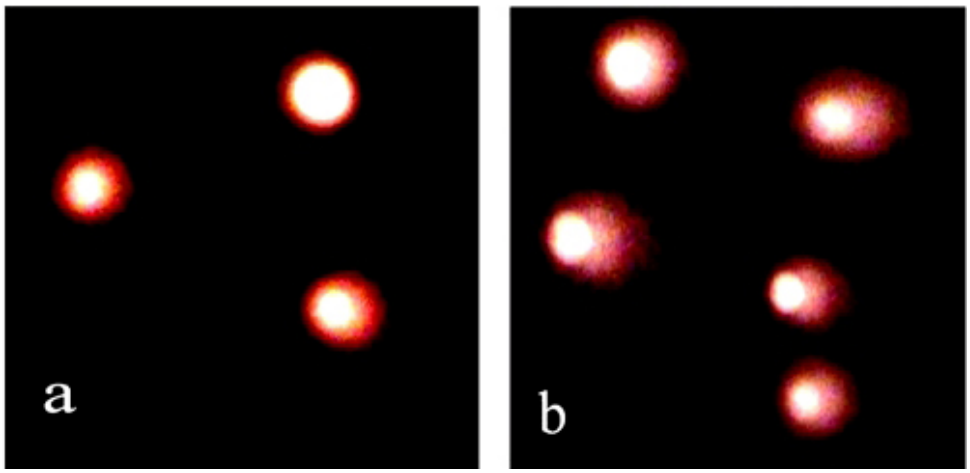

Figure 3.3 Comet assay a) Untreated DLA cells b) DLA cells treated with isolated enzyme and D- alanine showing DNA damage.

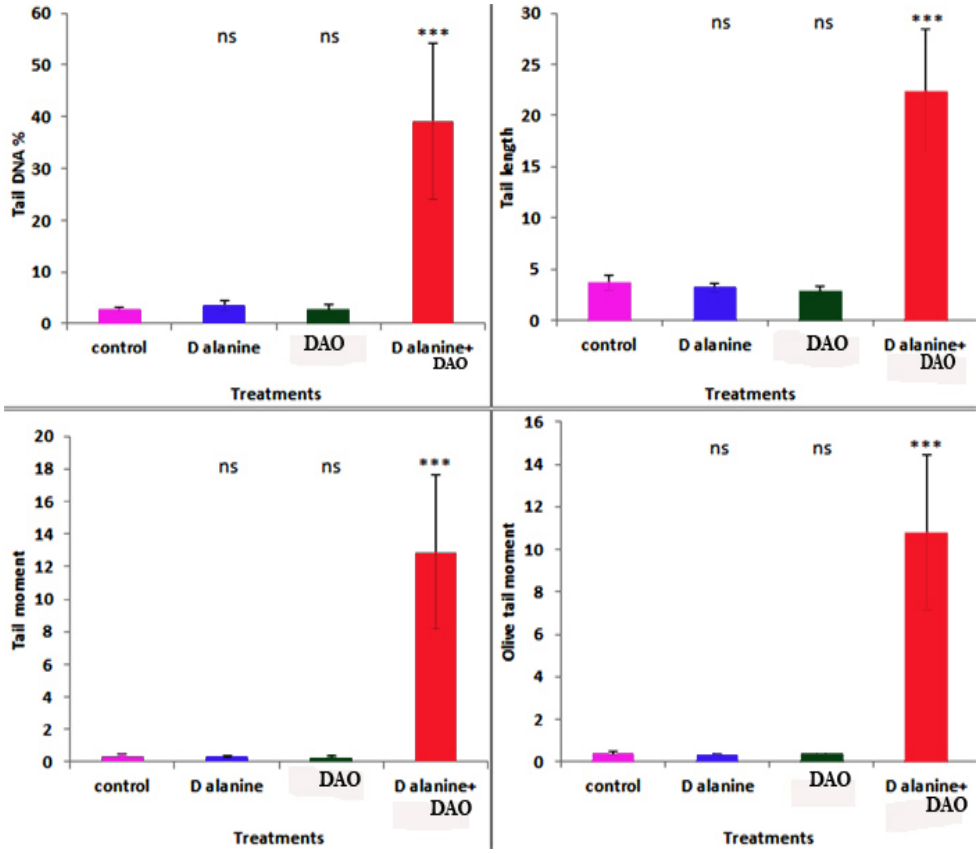

Figure 3.4 Effect of D–aminoacid oxidase (DAO) and D-alanine on inducing DNA damage in Daltons lymphoma ascites(DLA cells) assessed by comet assay. Percentage DNA in tail, tail length, tail moment and olive tail moment are presented as mean ± SD. The result shows that DAO and its substrate D-alanine causes DNA damage on tumor cells. 'ns' indicates not significant and * indicates p< 0.001 when compared to control.**

3.4 DISCUSSION

The attempts carried out in different laboratories in order to improve the already published purification procedures (Miyake *et al*.,1965;Henn and Ackers,1969; Mizon *et al*.,1969; Yagi, K *et al*.,1970) of DAO seems to indicate some difficulties in obtaining a homogenous protein. The main advantage of our method consists in obtaining a homogenous protein from porcine kidney as judged by SDS- PAGE, that showed a unique single band of DAO. The higher specific activity of our preparation as reported in Table 3.1 may be due to higher FAD content per mg of protein. Considerable discrepancies exist in the literature considering the molecular weight of DAO. Values ranging from 35,000 to 55, 000 have been reported for the monomer of this flavoprotein while the published amino acid analyses differ widely on the basis of the FAD content (Brumby and Massey, 1968).

The enzyme activity of the crude homogenates was found to be lower than the ammonium sulphate precipitated homogenates in the later steps, which may be due to the degradation of enzyme by protease or the presence of protein inhibiting substances. The final enzyme yields a 6000- fold purification factor with respect to crude extract. The recovery of the enzyme was 8.4 %. The final specific activity of the DAO at 37°C is 6 U/mg protein.

In organisms, redox status in cells is of crucial importance, and its imbalance, for instance overproduction of reactive oxygen species (ROS), leads to severe cellular damage and cell death (Beckman and Ames, 1997; Berlett and Stadtman, 1997). Thus the anticancer therapy by modulating the redox status of tumor cells, can be regarded as a mode of ‘‘oxidation therapy’’. One way to achieve this antitumor therapy is to deliver excess ROS into tumor tissue directly. Among the ROS, H_2O_2 is perhaps the

best candidate for this therapeutic challenge because of its cytotoxicity. H_2O_2 can cause oxidative damage to DNA, proteins and lipids, most probably via formation of hydroxyl radical (**.**OH), which is one of the most reactive type of ROS. More important, H_2O_2 induces apoptosis to a wide range of tumor cells via activation of the caspase cascade (Suhara *et al*., 1998; Matsura *et al*., 1999; Yamakawa *et al*., 2000; Ren *et al*., 2001; Chu *et al*., 2020). The present chapter aims to exploit the potential of isolated D- amino acid oxidase to generate H_2O_2, when its substrate D-amino acids (D- alanine) is supplied exogenously as D-amino acids do not usually exist in mammalian organisms in significant levels (Konno and Yasumura,1992; Sawa *et al*., 2000; Fang *et al*., 2002; Fang *et al*., 2008). The anticancer effect was primarily analyzed *in vitro* by using the murine tumor cells, Dalton's lymphoma ascites (DLA). The results clearly showed that DAO in combination with D-alanine had significant antitumor activity against DLA, suggesting the rationale of development of a new anticancer strategy.

Under *in vitro* conditions, the killing of DLA cells were enhanced in presence of the enzyme DAO and its substrate D- alanine. The result suggests that the cytotoxic mechanism of DAO and its substrate is through inducing cellular DNA damage, which was assessed by alkaline single cell gel electrophoresis or comet assay. As shown in *in vitro* experiments, DAO alone or D-alanine alone did not have any cytotoxicity; it became apparent only when DAO and D-alanine were accessible simultaneously (**Figure 3.3**). The cellular DNA damage induced by the enzyme-substrate complex on DLA cells, might possibly be due to apoptosis via DNA fragmentation in cells. The result suggests that this enzyme could be used for the production of H_2O_2 in the tumor site and thereby inducing apoptosis at those sites. This strategy will ensure that the enzyme will convert the nontoxic substrate D-

alanine to a potent cytotoxic metabolite, H_2O_2. This finding strongly suggests that the antitumor activity of DAO / D-alanine is mostly due to the generation of H_2O_2, kills the tumor cells by oxidative injury to cellular vital molecules. (Beckman and Ames, 1997; Berlett and Stadtman, 1997).

Recent study demonstrated that intravenous administration of DAO conjugated with poly ethylene glycol (PEG) along with intra peritoneal administration D-proline exhibited marked antitumor activity under *in vivo* condition by increasing ROS formation at the tumor site (Fang *et al*., 2002). These novel modes of enzyme therapy exploit the mechanism of targeting the H_2O_2-generating enzymes to the tumor site where the enzyme converts a pharmacologically inert product to a highly cytotoxic metabolite, H_2O_2 for effective tumor control. Antitumor therapeutics using PEG-conjugated D-amino acid oxidase exhibited potent antitumor activity by generating toxic reactive oxygen species, namely Oxidation therapy, showed remarkable antitumor effect on murine Sarcoma 180 solid tumor, by taking advantage of the enhanced permeability and retention effect (EPR effect) (Fang *et al*., 2008). Another enzyme, Xanthine oxidase (XO) generate cytotoxic reactive oxygen species (ROS), such as superoxide anion radical and hydrogen peroxide, have found to shown anticancer activity when its substrate hypoxanthine is used. The pegylated form of XO (PEG-XO) accumulates preferentially in tumor tissue resulted in significant suppression of tumor growth (Sawa *et al*., 2000).

From the study, generation of H_2O_2 mediated cellular damage by the combination of enzyme, DAO and its substrate D-alanine was examined with the objective of exploiting its potential in therapeutic situations. Results of the *in vitro* studies with Daltons Lymphoma Ascites (DLA) cells, suggest that enzyme generated H_2O_2 can have cytotoxic effect for effective tumor control.

Chapter- 4

PREPARATION OF Fe_2O_3, Fe_2O_3-DAO NANOPARTICLE COMPLEX, CHARACTERIZATION AND *IN VIVO* ANTI TUMOR ACTIVITY IN SWISS ALBINO MICE

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4.4 DISCUSSION

4.1 INTRODUCTION

Cancer remains one among the leading causes of death within the world. Despite advances in our understanding of molecular and cancer biology, discovery of cancer biomarkers and conventional surgical procedures, radiotherapy, and chemotherapy, the overall survival rate from cancer has not significantly improved within the past 20 years (Jemal *et al*., 2008). The development of new approach for timely detection and cancer marker-specific and personalized treatment of cancers is urgently required to increase patient survival. In cancer chemo therapy, the chemotherapeutic agent exerts anticancer action through cytotoxic mechanisms, where prolonged exposure of tumor to high drug concentrations is a prerequisite for therapeutic efficacy (Gutman *et al*., 2000). The biodistribution of a systemically administered drug depends on the physical- chemical properties like molecular weight, lipophilicity, etc and often results in sub-therapeutic drug levels at the tumor site (Motl *et al*., 2006). This sub-therapeutic exposure may not only fail to eradicate the tumor, but can even stimulate overgrowth of resistant malignant cells (Heimberger *et al*., 2000).

Recent advances in nanoscience and nanotechnology have led to the preparation of nanomaterials for molecular and cellular imaging, cancer therapy, and usage of integrated nanodevices for cancer detection and screening (Jain, 2005; Nie *et al.,* 2007; Sengupta and Sasisekharan, 2007; Wang *et al.,* 2007; Li *et al*., 2017). The nanoparticles cannot only provide sensitive and specific imaging information in cancer patients but also deliver anticancer drugs to tumor sites selectively (Shi *et al*., 2011; Shanthi, 2014). Most chemotherapeutic agents possess poor selectivity toward the target tissue and can harm normal cells as well as cancer cells. During chemotherapy, pharmacologically active cancer drugs arrive at the tumor tissue with poor specificity and make dose-limiting toxicities. Nanoparticle drug delivery may

provide a more efficient, less harmful solution to overcome these problems (Kamaly *et al*., 2012).

To improve the anticancer potential of chemotherapeutic agents, specific targeting to tumor tissue is crucial (Langer, 2001; Irvine, 2011). Specific targeting of nanoparticles to tumor has been accomplished in various experimental systems (Denardo *et al*., 2005; Cai *et al*., 2006). A wide range of targeting molecules like humanized antibodies, peptides (Weiner and Adams, 2000), nucleic acid ligands like aptamers (Ellington and Szostak, 1990; Tuerk and Gold, 1990), colloidal drug delivery modalities such as liposomes, micelles or nanoparticles are intensively studied for use in cancer therapy (Davis *et al*., 2010; Lee and Lee, 2012; Liu *et al*., 2014). However, the unrestricted circulation of most of these carriers results in the rapid clearance by reticuloendothelial system and also in off target site specificity.

Magnetic nanoparticles can be injected intravenously and transported through blood stream to the desired area of treatment (Berry *et al*., 2004). Super paramagnetic particles do not retain any magnetism after removal of magnetic field and they are physiologically well tolerated *in vivo*. Magnetic nanoparticles can be deposited on tumor tissues and heated by an alternating magnetic field to destroy the tumor (Forbes *et al*., 2003; Muller *et al*., 2005). It was reported that magnetic nanoparticles can be retained at tumor sites, after local administration along with a locally applied external magnetic field, due to the magnetic responsiveness of the iron oxide core, thereby enabling magnetic targeting (Widder *et al*., 1983; Pulfer *et al*., 1999; Alexiou *et al*., 2003; Leakakos *et al*., 2003). Magnetic nanoparticles can be injected intravenously and transported through blood stream to the desired area of treatment (Berry and Curtis, 2003).

Magnetic nanoparticles that are specifically and differentially taken up by the targeted cells and release their payload over an extended period to achieve a clinical response is important (Langer, 2001; Ferrari, 2005). The unique pathophysiology of tumors such as their enhanced permeability and retention effect and the tumor microenvironment further improves the efficacy of nanoparticle carriers. In addition to passive targeting mechanism, active targeting strategies using ligands or antibodies focussed against selected tumor targets amplify the specificity of the therapeutic nanoparticles. Drug resistance, was said to be an obstacle that hinder the efficacy of both molecularly targeted and traditional chemotherapeutic agents, may also be overcome or be reduced using nanoparticles. Nanoparticles have the power to concentrate in cells without being recognized by P-glycoprotein, acts as one among the most mediators of multidrug resistance, that leads to the increased intracellular accumulation of medicine (Cho *et al*., 2008). Targeting by magnetic particles offers a unique opportunity to treat tumors due to magnetic responsiveness and are excellent candidates for developing drug delivery systems, as it is feasible to produce and characterize (Tiefenauer *et al*., 1993; Alexiou *et al*., 2000; Zhang *et al*., 2002; Gupta *et al*., 2003; Berry *et al*., 2004; Gupta and Wells, 2004) and an external localized magnetic field gradient may be used to target it (Alexiou *et al*., 2002). Some anticancer chemotherapeutics like paclitaxel (Fonseca *et al*., 2002; Koziara *et al*., 2006), doxorubicin (Yoo *et al*., 2000), 5-fluorouracil (Bhadra *et al*., 2003) and dexamethasone (Panyam and Labhasetwar, 2004) have been effectively formulated using nanomaterials.

Reactive oxygen species (ROS) e.g., superoxide anion (O_2 -), hydrogen peroxide (H_2O_2), and hydroxyl radical (.OH) are generated in all aerobic organisms and are indispensable for signal transduction pathways that regulate cell growth (Beckman

and Ames, 1997) and reduction-oxidation (redox) status. On the other hand, overproduction of highly reactive metabolites can start lethal chain reactions that involve oxidation of varied bio molecules causing damage to cellular integrity and survival. If they will be selectively delivered into tumors, ROS may exert remarkable antitumor potential due to their reaction with vital cellular targets. This strategy of cancer treatment by generation of ROS selectively in tumor cells has been named "oxidation therapy" (Ben-Yoseph and Ross,1994; Stegman *et al*., 1998). In oxidation therapy, the principle is to get excess ROS selectively in tumor tissue causing maximum tumor killing without affecting the normal tissues.

Among the ROS, H_2O_2 readily crosses cellular membranes and causes oxidative damage to DNA (Beckman and Ames, 1997) proteins (Berlett and Stadtman, 1997) and lipids by direct oxidation (Halliwell and Gutteridge, 1984). It was also reported that H_2O_2 induces apoptosis in many tumor cells *in vitro* (Bladier *et al*., 1997; Matsura *et al*.,1999) *via* activation of the caspase cascade. Various antitumor agents, like doxorubicin, vinblastine, inostamycin and camptothecin shows antitumor activity via H_2O_2 -dependent activation of apoptotic cell death, that explains potential use of H_2O_2 as an antitumor principle (Simizu *et al*.,1998). H_2O_2 is comparatively unstable and is a small water-soluble molecule. Such characteristics impede the utility of H_2O_2 as an antitumor agent *in vivo* for selective delivery to tumor site. H_2O_2 used alone was ineffective when injected into tumor or into the circulation (Green and Westrop, 1958; Sugiura, 1958) because of its rapid clearance and decomposition by catalase in erythrocytes. Therefore the use of H_2O_2 -generating enzymes has been proposed as an another approach to an H_2O_2 dependent oxidation therapy.

Nathan and Cohn (1981) and Ben-Yoseph and Ross (1994) reported that Glucose oxidase (GO), which generates H_2O_2 during oxidation of glucose, showed antitumor

activity in solid tumor models. The regulation of H_2O_2 production by exogenously administered GO in tumor-bearing hosts is challenging because the availability of its substrates, oxygen and glucose, cannot be efficiently modulated, with the possible induction of severe systemic side effects because of systemic generation of H_2O_2. In fact, GO administration to supply H_2O_2 required injection of antioxidants to attenuate systemic toxicity.

In this study we isolated, D-aminoacid oxidase (DAO) from porcine kidney and exploited its potential as an H_2O_2-generating enzyme for cancer treatment. D-Aminoacid oxidase (DAO) (D-aminoacid: oxygen oxidoreductase (deaminating); EC 1.4.3.3) catalyzes the oxidative deamination of D-amino acids, producing the corresponding α-keto acid and ammonia with concomitant reduction of molecular oxygen to hydrogen peroxide (H_2O_2). The generation of H_2O_2 are often easily modulated by varied infusion of its substrates (D-aminoacids) as shown by the *in vitro* data. Since the D-amino acids are scarce in mammalian organisms, it is to be introduced as the substrate for effective oxidation therapy (Fang *et al*., 2002).

DAO have a short *in vivo* half-life. The estimated molecular size of DAO (Mr 39,000) is smaller than the renal excretion threshold (Mr 50,000), so it would be excreted gradually as observed earlier for other small proteins or polymer drugs smaller than Mr 40,000 (Maeda *et al*., 1992). To overcome the drawback, isolated DAO was conjugated with a Fe_2O_3 (coated with Poly Vinyl Pyrolidone (PVP)) nanoparticles. Earlier reports showed that chemical modification results in an improved *in vivo* half-life, a reduced antigenicity of the native protein, inhibition of proteolytic degradation and also helps in the effective targeting (Fang *et al*., 2002; Fang *et al*., 2008). When the enzyme complexed with nanoparticles, th stability of the enzyme increases.

Earlier reports showed that Fe_3O_4- doxorubicin complexes can be targeted directly to the site of the tumor by using external magnetic field (Jayakumar *et al*., 2009).

Studies have revealed that biocompatible macromolecules accumulate and remain in solid tumor because of the special characteristics of the tumor vasculature and the weakened lymphatic clearance system. This phenomenon was known as 'EPR effect' of macromolecules and lipids in solid tumor. This EPR effect was validated in many experimental solid tumors (Seymour *et al*., 1995; Seymour *et al*., 1998). In the present work we try to take advantage of the potential of magnetic nanopartcle-enzyme complex to be targeted using an external magnetic flux and therefore due to this EPR effect, the nanocomplex once entered the tumor may get trapped within the tumor vasculature. This chapter, describes the synthesis of Fe_2O_3 nanoparticles and preparation of Fe_2O_3 nanoparticles-DAO complex. At first, the prepared complex was analysed for its anti tumor potential under *in vitro* conditions. The *in vivo* antitumor activity was analysed in tumor bearing swiss albino mice by targeting the nanoparticle- DAO complexes to the tumor site using an externally applied magnetic flux, after oral administration of the complexes and D-alanine (substrate).

4.2 MATERIALS AND METHODS

4.2.1 Animals

Female Swiss albino mice (8 - 10 weeks old), weighing 22 - 25 g were obtained from Small Animal Breeding Section (SABS), Mannuthy, Thrissur, Kerala.

4.2.2 Chemicals

Sodium pyrophosphate, Poly Vinyl Pyrolidone (PVP), D-alanine, 4-amino-3-hydrazino - 5-mercapto-1,2,4-triazole (purpald reagent) were from Sigma chemical company Inc., St.louis, MO, USA. Sodium benzoate and ammonium sulphate ($(NH_4)_2SO_4$) were from

Himedia Labo- ratories Pvt. Ltd. Mumbai. All other reagents were all of analytical grade and purchased from reputed Indian manufacturers.

4.2.3 Preparation of Fe_2O_3 Nanoparticles Coated with PVP

Detailed methodology is given in chapter 2, section 2.2.7

4.2.4 Enzyme Activity of DAO

Enzyme activity was determined colorimetrically using, 4-amino-3-hydrazino-5-mercapto-1, 2,4-triazole (purpald reagent) as a colouring reagent (Watanabe *et al*., 1978) and color developed was measured at 550 nm. D-Alanine was used as the substrate with an initial concentration of 0.1 M. The enzyme reaction was carried out at 37°C in 0.133 M pyrophosphate buffer (pH 8.3), where 1 unit of DAO activity is defined as the rate of formation of 1 μmol of H_2O_2 per min.

4.2.5 Preparation of Fe_2O_3-DAO Complex

Detailed methodology is given in chapter 2, section 2.2.8

4.2.6 Stability studies of DAO and Fe_2O_3-DAO Complex

The effect of pH on the activities of the free DAO and Fe_2O_3 bound DAO was investigated in the pH range of 3-10 in pyro phosphate buffer at 25°C. The thermal stability of DAO and Fe_2O_3-DAO were determined by measuring the enzyme activity of the enzyme, after being exposed to different temperatures (25–70°C) in pyro phosphate buffer (0.133 M, pH 8.3) for 30 min. Aliquots of the reacting solution were taken and assayed for enzymatic activity as described above. The storage stability was examined by measuring the change in the enzyme activity by placing the DAO and Fe_2O_3 bound DAO at room temperature 25°C for 30 days. The storage stability of the free and Fe_2O_3-DAO Complex was determined by assaying for their enzyme activity.

4.2.7 Cytotoxic effect of Fe_2O_3-DAO in Presence of Different Concentrations of D-Alanine, *in Vitro*

The capacity of Fe_2O_3-DAO complex to make H_2O_2 in presence of different concentrations of D-alanine was determined. *In vitro* cytotoxicity of the complex to DLA (Dalton's lymphoma ascites) cells was also studied. The DLA cells (1×10^6 cells/ml) were treated with Fe_2O_3-DAO complex in presence of different concentrations of D-alanine. After the incubation, the cell viability was estimated by means of Trypan blue dye exclusion method. The percentage mortality of cells was calculated and a graph was plotted with percentage mortality of cells against time of incubation (in min).

4.2.8 Induction of Apoptosis *in Vitro*

DLA cells were aspirated from ascitic mice. The cells were washed three times using phosphate buffered saline (PBS). The cells (1×10^6 cells/ml) are incubated in DMEM medium contains 10% foetal calf serum (FCS) in presence or absence of the Fe_2O_3-DAO complex with 0.2 M D-alanine.

4.2.9 Morphological Analysis

To detect the morphological changes during apoptosis, cell suspension was smeared on a clean glass slide. The smear was stained with May-Grunwald-Giemsa staining. The slides were observed under bright field microscope for apoptotic cells (having condensed or fragmented nuclei and cytoplasmic blebbing). Apoptotic index was calculated using the equation.

Apoptotic index = (Number of apoptotic cells / Total number of cells) × 100

4.2.10 Comet Assay

In order to detect apoptotic induction by Fe_2O_3-DAO complex, on DLA cells, comet assay was performed (Chandrasekharan *et al*., 2009) with minor modifications.

4.2. 11 *In Vivo* Antitumor Activity of Fe_2O_3-DAO

Solid tumor was grown up in hind limbs of animals by transferring DLA cells (1×10^6 cells/animal) simultaneously in to the right hind legs of thirty female Swiss albino mice of about 6 - 8 weeks old, body weight 20 - 25 g. When the tumor was grown to a diameter 0.8 - 10 mm (10^{th} day after tumor transplantation), the animals were divided into six groups of five animals each. Fe_2O_3 nanoparticles complexed with the enzyme DAO were orally administered to the animals, as well as nanoparticles and enzyme alone or water. Thirty minutes later, the animals were orally administered with D- alanine (0.5 mmoles in 0.2 ml/mouse) and treated as detailed below:

Group I : 0.1 ml Distilled water *p. o*

Group II : 0.1 ml Fe_2O_3 (30 mg/ml) *p.o*

Group III : 0.1 ml Fe_2O_3 -DAO (30 mg Fe_2O_3 /ml, 0.1 mg protein/ml) *p.o*

Group IV : 0.1 ml DAO (0.1 mg protein/ml) + D-alanine (0.5 mmol/mouse) *p.o*

Group V: 0.1 ml Fe_2O_3-DAO (30 mg Fe_2O_3/ml, 0.1 mg protein/ml) + D-alanine (0.5 mmol/mouse) *p.o*

Group VI: 0.1 ml Fe_2O_3 -DAO (30 mg Fe_2O_3/ml, 0.1 mg protein/ml) + D-alanine (0.5 mmol/mouse) *p.o* + magnetic treatment for 15 minutes.

The animals in the groups II, III and VI were given a magnetic treatment for 15 minutes on the tumor bearing limb with a horse shoe magnet (1 G field) each day, 20 minutes after the D-alanine administration. The treatments were continued for five consecutive days. The hind leg thicknesses were measured using a vernier calliper once in two days from 10^{th} day of tumor induction. The tumor volume was calculated as follows:

Tumor radius = Radius of tumor induced leg − Radius of normal leg

Tumor volume = $4/3\pi r^3$ where r is the tumor radius.

4.3 RESULTS

4.3.1 Characterization of Fe_2O_3 and Fe_2O_3-DAO Complexes

The FTIR spectra of magnetic Fe_2O_3 and Fe_2O_3-DAO are shown in the **Figures 4.1** and **4.2**. A weak peak shown at 2061.88 cm^{-1} in the spectra of Fe_2O_3-DAO might be assigned to the C-O bonds in the enzyme molecule. This peak was totally absent in the spectra of Fe_2O_3 signifying enzyme attachment onto the particles. The results of the XRD analysis of the nanoparticle and enzyme complexes are presented in **Figures 4.3** and **4.4**. The results of the Scanning electron microscopy (SEM) profile is shown in **Figure 4.5**.

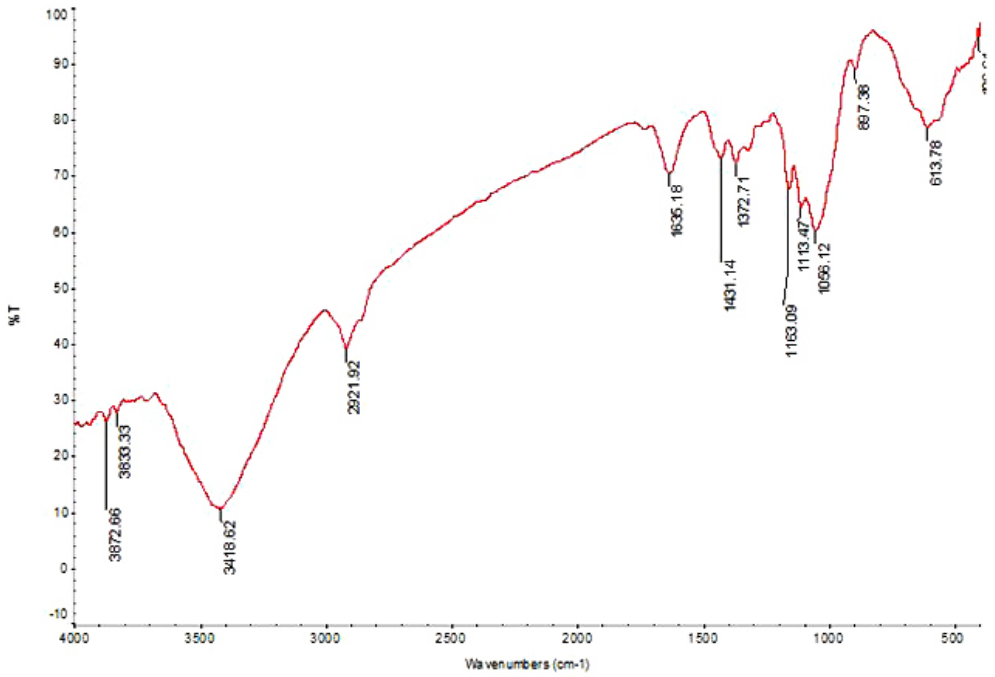

Figure 4.1. FTIR spectra of Fe_2O_3 magnetic nanoparticles.

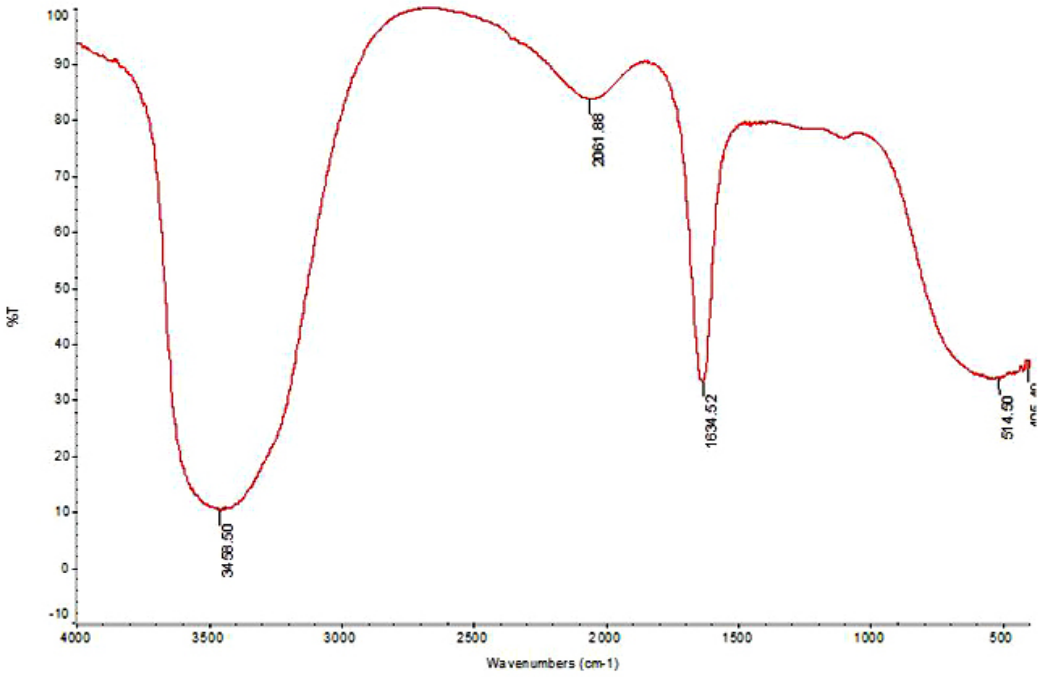

Figure 4.2 . FTIR spectra of Fe_2O_3-DAO complex.

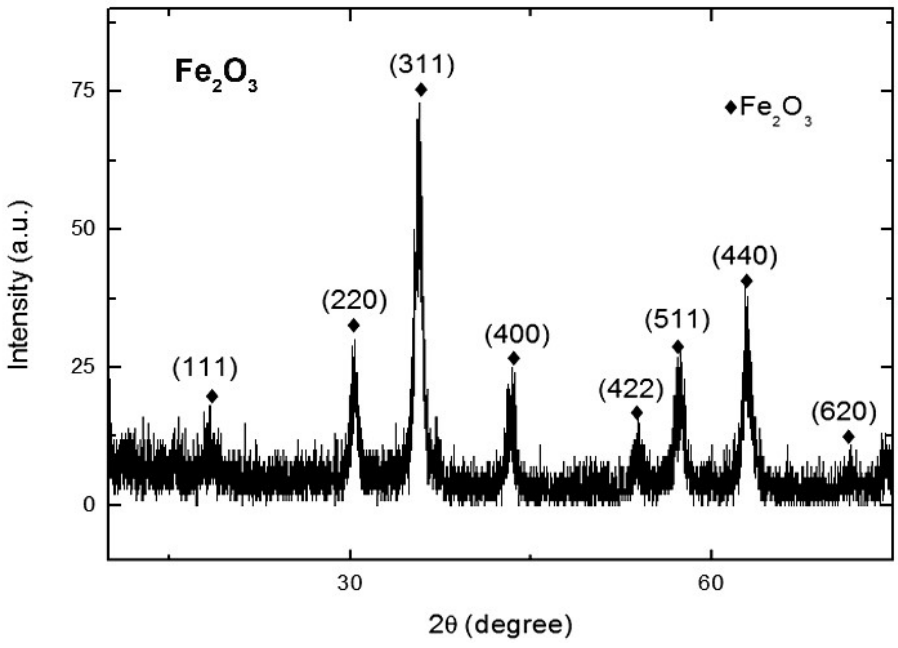

Figure 4.3. XRD pattern of Fe_2O_3 nanoparticles coated with PVP.

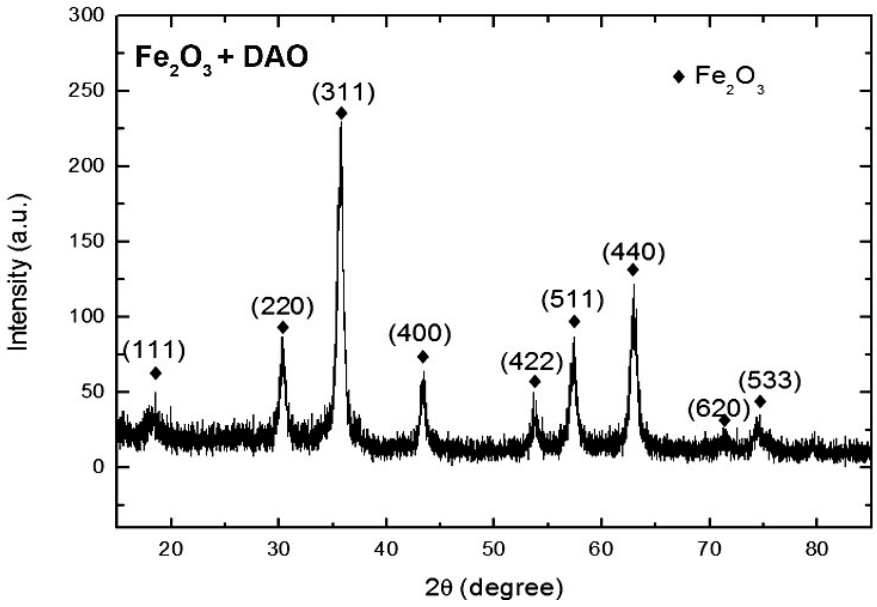

Figure 4.4 . XRD pattern of Fe_2O_3-DAO complex.

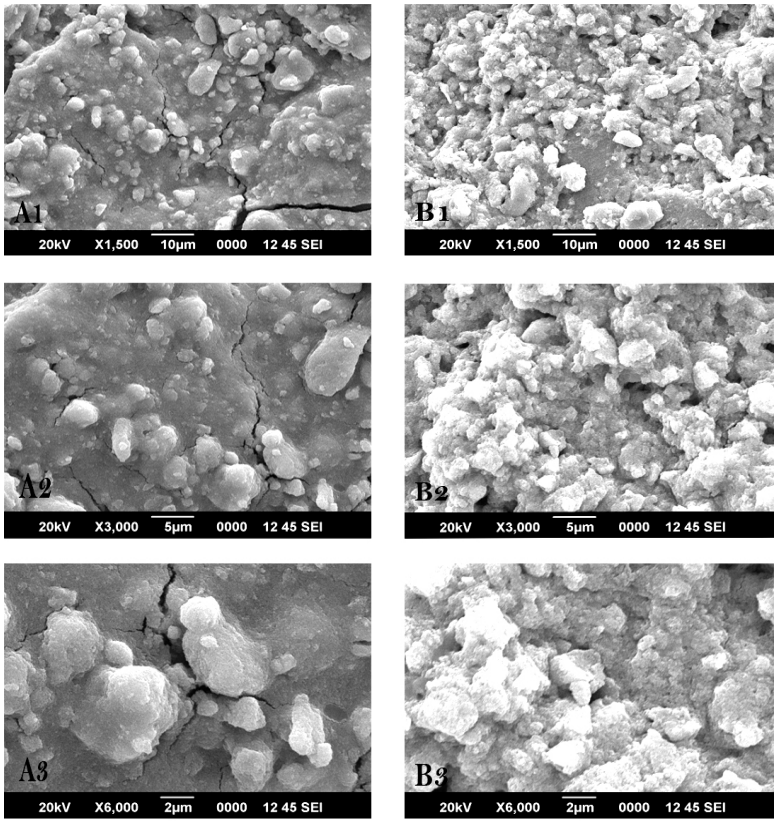

Figure 4.5. Characterization of Fe_2O_3 and Fe_2O_3-DAO complex. Scanning electron microscopic image of Fe_2O_3 (A1, A2, A3) and Fe_2O_3-DAO nanoparticles complex (B1, B2, B3)

4.3.2 Effect of pH

The effect of pH on the activities of the free and bound DAO was investigated in the pH range of 3-10 at 25°C and presented in **Figure 4.6**. In the pH range between 6 and 7.3 the activities of the free and bound DAO were found increased, stable and reached a maximum at pH 8. The activity then decreased from pH 8 to 9.5.

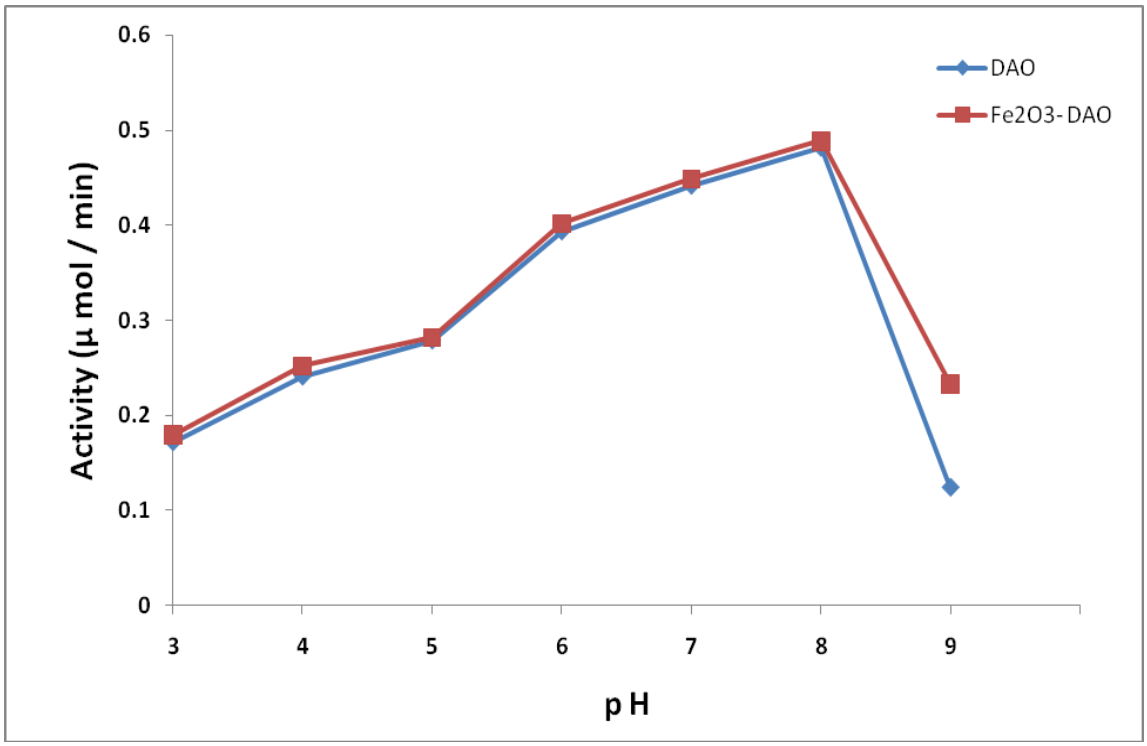

Figure 4.6. Effect of pH on the activities of DAO and Fe_2O_3 bound DAO

4.3.3 Thermal stability

The thermal stability of free and bound DAO was investigated after 40 min of storage in the temperature range of 25–70°C (**Figure 4.7**). There was no apparent change in activity in the free DAO as well as in the Fe_2O_3 bound DAO, in the temperature range of 25–37°C. Above this temperature range, the enzyme activity decreased in both systems. However, the bound DAO showed higher retained activity than the free DAO. The remaining activity at 60°C was about 2 fold that of the free DAO.

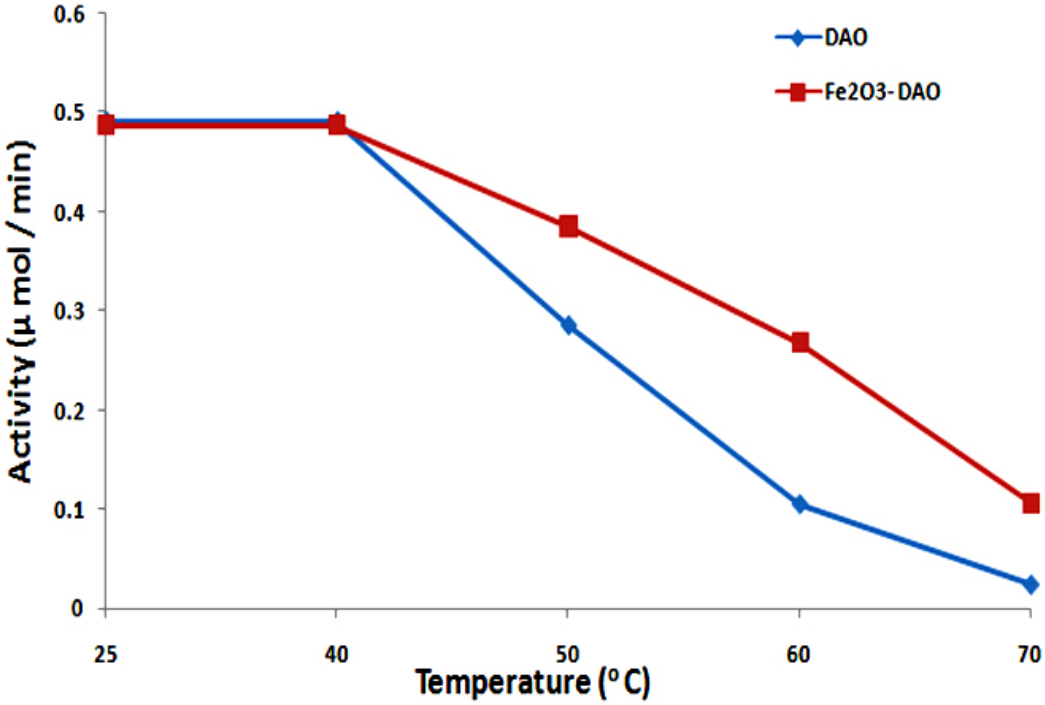

Figure 4.7. Thermal stability of free DAO and Fe_2O_3-DAO at pH 8.3. The samples were stored at 50, 60, or 70°C for 40 min and the activities were then measured at 25°C.

4.3.4 Storage stabilities

Figure 4.8 shows the storage stabilities of free and bound DAO at 25°C at pH 8.3. After 15 days, no activity was observed in free DAO. However, the activity of bound DAO remains greater during the same time period, and continues after 30 days indicating a considerable enhancement on its stability.

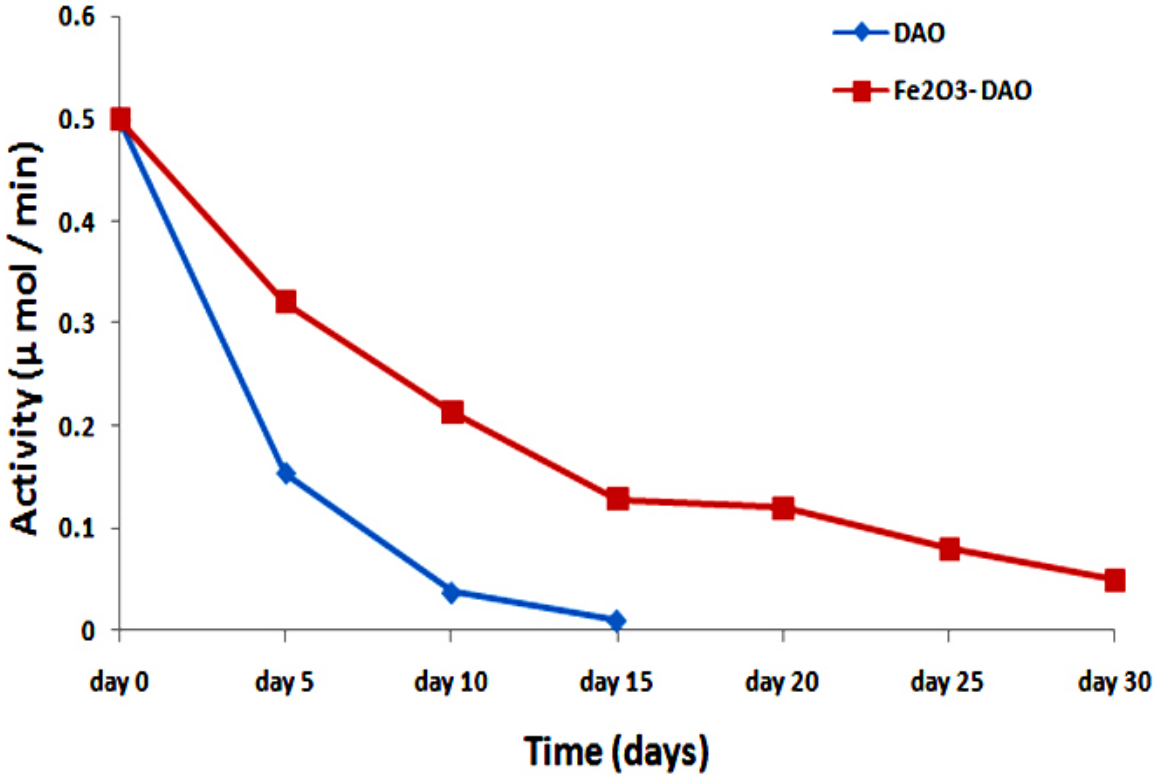

Figure 4.8. Storage stability of free DAO and Fe_2O_3-DAO. The activities measurements were performed at pH 8.3, at 25°C

4.3.5 *In Vitro* Cytotoxicity of Fe_2O_3-DAO

Cytotoxicity of Fe_2O_3-DAO was examined by an *in vitro* system with Dalton's lymphoma ascites (DLA) cells. Different concentrations of D-alanine was used as the substrate because of its high turnover rate. In the study, DAO alone and D-alanine alone groups show no clear cytotoxicity against DLA cells. In contrast, Fe_2O_3-DAO along with D-alanine showed remarkable cytotoxicity in a substrate concentration-dependent manner (**Figure 4.9**). This is attributed to the H_2O_2 generation by the enzyme-substrate reaction system. Here maximum percentage mortality (99%) was noticed when 50 mM D-alanine was used.

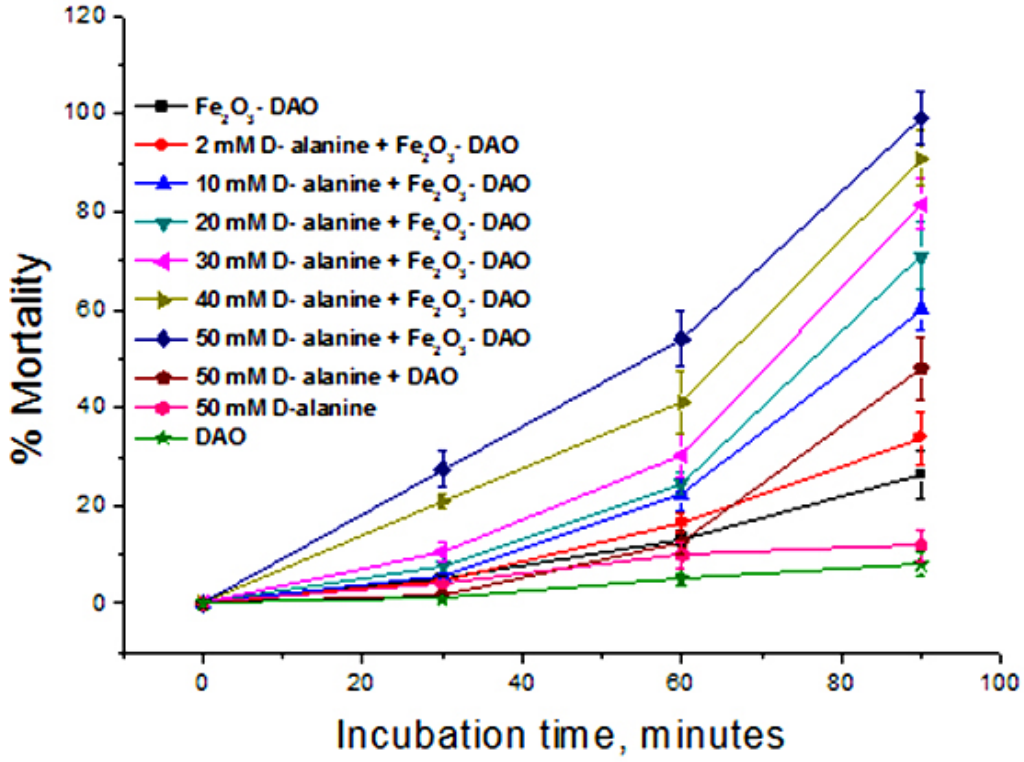

Figure 4.9. Cytotoxic effect of Fe_2O_3-DAO in presence of different concentrations of D-alanine on DLA cells *in vitro.*

4.3.6 Induction of Apoptosis in DLA Cells, *in Vitro*

Induction of apoptosis on DLA cells was analyzed by studying the morphological pattern of DLA cells. The apoptotic cells were visualized as having cytoplasmic blebbing and nuclear shrinkage (**Figure 4.10**). The results shows that D-alanine along with Fe_2O_3-DAO gives maximum apoptotic index (95.5%). The DAO (0.36 U) along with substrate (0.2 M) gave an apoptotic index of about 85.2 ± 5.55. The cells treated with D-alanine or Fe_2O_3-DAO alone showed less apoptosis. The results are shown in **Table 4.1**.

Table 4.1 . Apoptoic index in DLA cells treated with Fe2O3- DAO in presence of D-alanine. ("a" represents, p < 0.001 compared to respective control, "b" represents, p < 0.05 compared to respective control).

Treatments	Apoptoic index (%)
Control	0
0.2 M D-Alanine + DAO (0.36 U)	85.2 ± 5.55^a
0.2 M D-Alanine + Fe_2O_3-DAO (0.36 U)	95.5 ± 3.53^a
0.2 M D-Alanine	6.5 ± 2.12^b
Fe_2O_3-DAO (0.36U)	11.0 ± 1.41^a

To further study the induction of apoptosis, DLA cells incubated with Fe_2O_3-DAO and D-alanine were subjected to comet assay (**Figure 4.11**). Cellular DNA damage was seen when DLA cells treated with Fe_2O_3- DAO with substrate D-alanine. The comets posses a fan like tail indicative of apoptosis (Figure 4.10 (b)). The extent of DNA damage produced was nearly equal when the cells treated with DAO with its substrate D-alanine (Figure 4.10 (c)). The cells treated with Fe_2O_3-DAO alone (Figure 4.10 (d)) and D-alanine alone (Figure 4.10 (e)) did not exhibit any DNA damage which may be due to the lack of formation of H_2O_2 at the reaction site. Thus the result indicated the extent of cellular DNA damage obtained through an induced oxidative stress by H_2O_2 formed *in situ* through enzyme action.

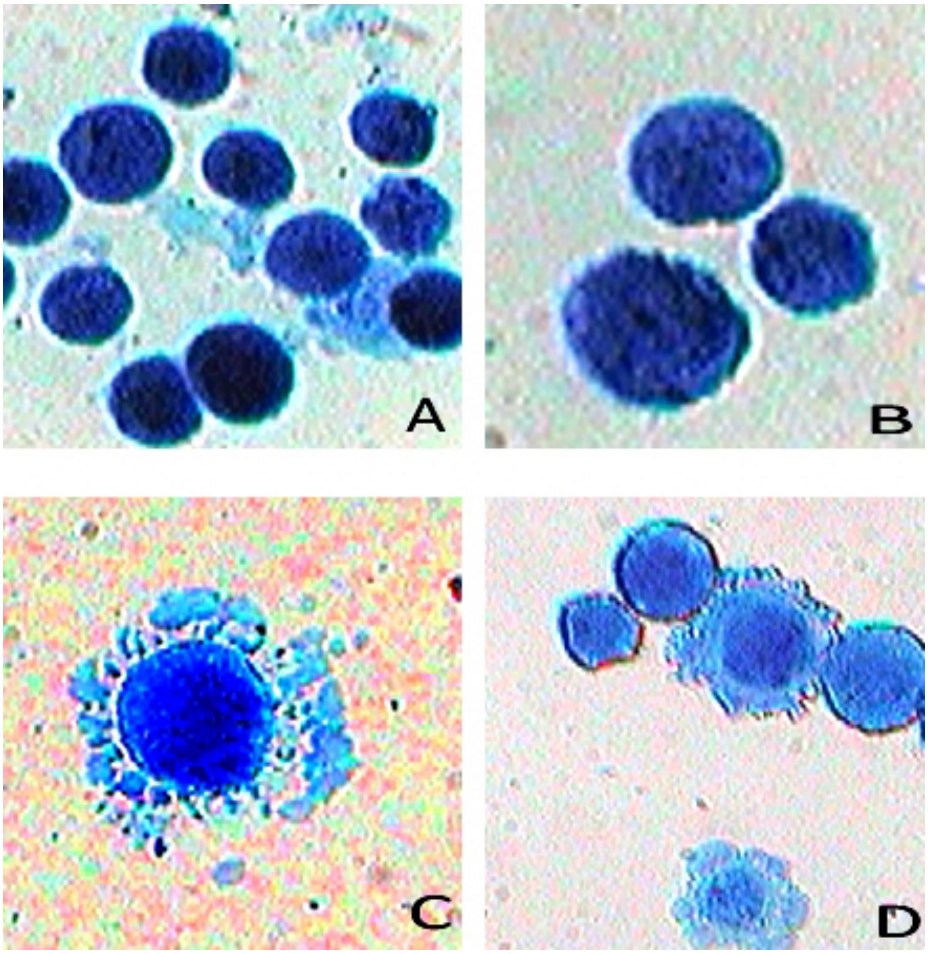

Figure 4.10. Representative figures of DLA cells showing apoptosis after May-Grunwald-Giemsa staining. (a) Untreated cells; (b) Cells treated with Fe_2O_3-DAO; (c) Cells treated with Fe_2O_3-DAO and D-alanine showing cytoplasmic blebbing; (d) Cells treated with DAO and D-alanine showing apoptotic and non-apoptotic cells.

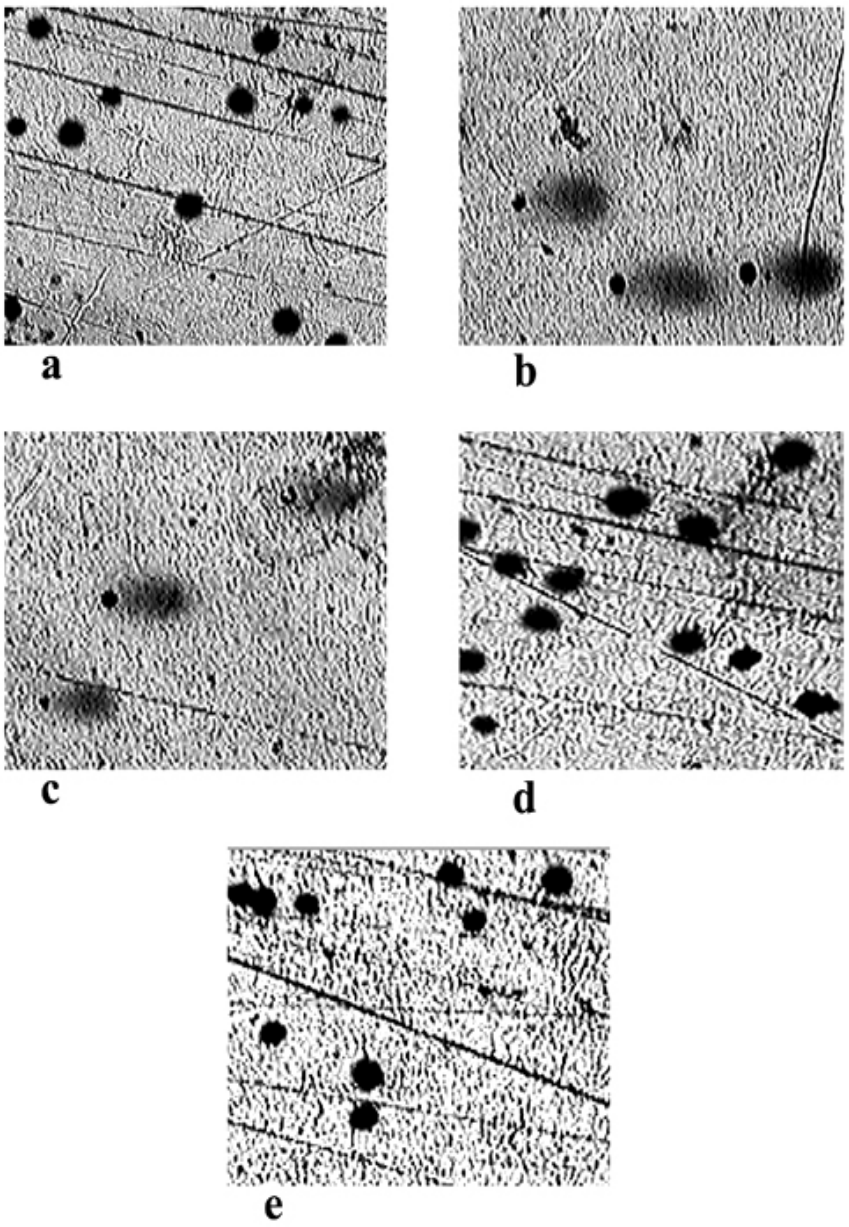

Figure 4.11. Representative images of DLA cells after comet assay. (a) The untreated DLA cells; (b) DLA cells treated with Fe_2O_3-DAO and D-alanine showing Fan-like comets indicative of apoptosis; (c) DLA cells treated with enzyme (DAO) and D-alanine showing DNA damaged and apoptotic comets; and (d) DLA cells treated with Fe_2O_3-DAO; (e) DLA cells treated with D-alanine.

4.3.7 *In Vivo* Antitumor Activity of Fe_2O_3-DAO

Fe_2O_3-DAO was administrated orally and after an adequate lag time to allow the accumulation of Fe_2O_3-DAO in the tumor by the application of an external magnetic field, the substrate D-alanine was administered. This treatment procedure allows formation of cytotoxic H_2O_2, mainly at the tumor site.

After oral administration of DAO-Fe_2O_3 nanoparticles and D-alanine, the tumor sites of tumor bearing animals were allowed to place under the influence of an external magnetic field "*m*" by keeping a magnet at the tumor site for 15 minutes every day for 5 consecutive days, 20 minutes after the drug administration (**Figure 4.12**). The results are shown in Figures 4.13 and 4.14. **Figure 4.13** presents the data on the tumor volume, following the treatments and **Figure 4.14** gives representative photographs of the animals and their tumor bearing limbs

Figure 4.12. Magnetic therapy, after oral administration of DAO-Fe_2O_3 nanoparticles and D- alanine to tumor bearing animals by keeping the magnet for 15 min everyday, 20 minutes after the drug administration

As shown in Figure 4.13 (5a), tumor growth was significantly suppressed in mice administered Fe_2O_3-DAO and D-alanine along with magnetic treatment in the fourth day. In contrast, no significant antitumor effect was observed in mice treated with native Fe_2O_3-DAO plus D- alanine without magnetic treatment (Figures 4.13 (4 a) and (4 b). Fe_2O_3-DAO and D-alanine alone treatment did not show any antitumor activity (Figures 4.13 (2 a), (2b), (3a), and (3b)).

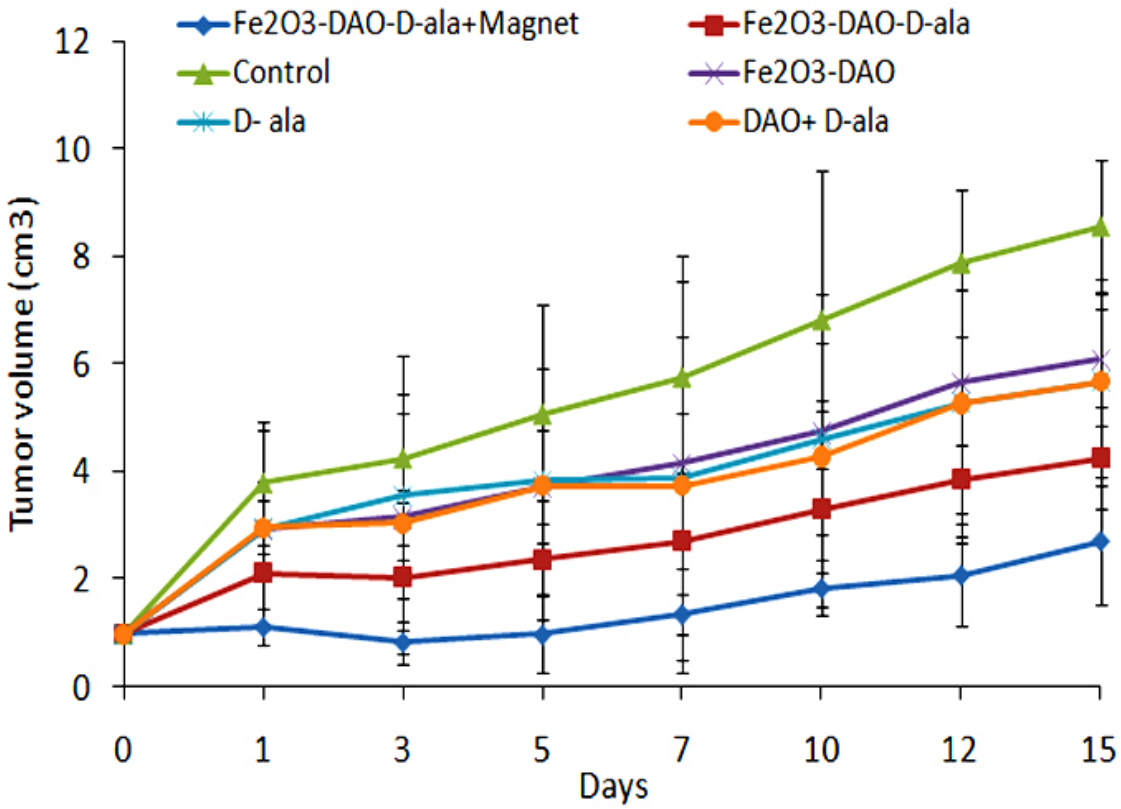

Figure 4. 13. Effect of administration of Fe_2O_3 nanoparticles coated with PVP and complexed with DAO and magnetic targeting on growth of DLA solid tumor on hind limb of mice. After transplanting the DLA cells (1×10^6 cells), on 10th day the tumor grows to a size of 1 cm^3 and different treatments are started as detailed in the text.

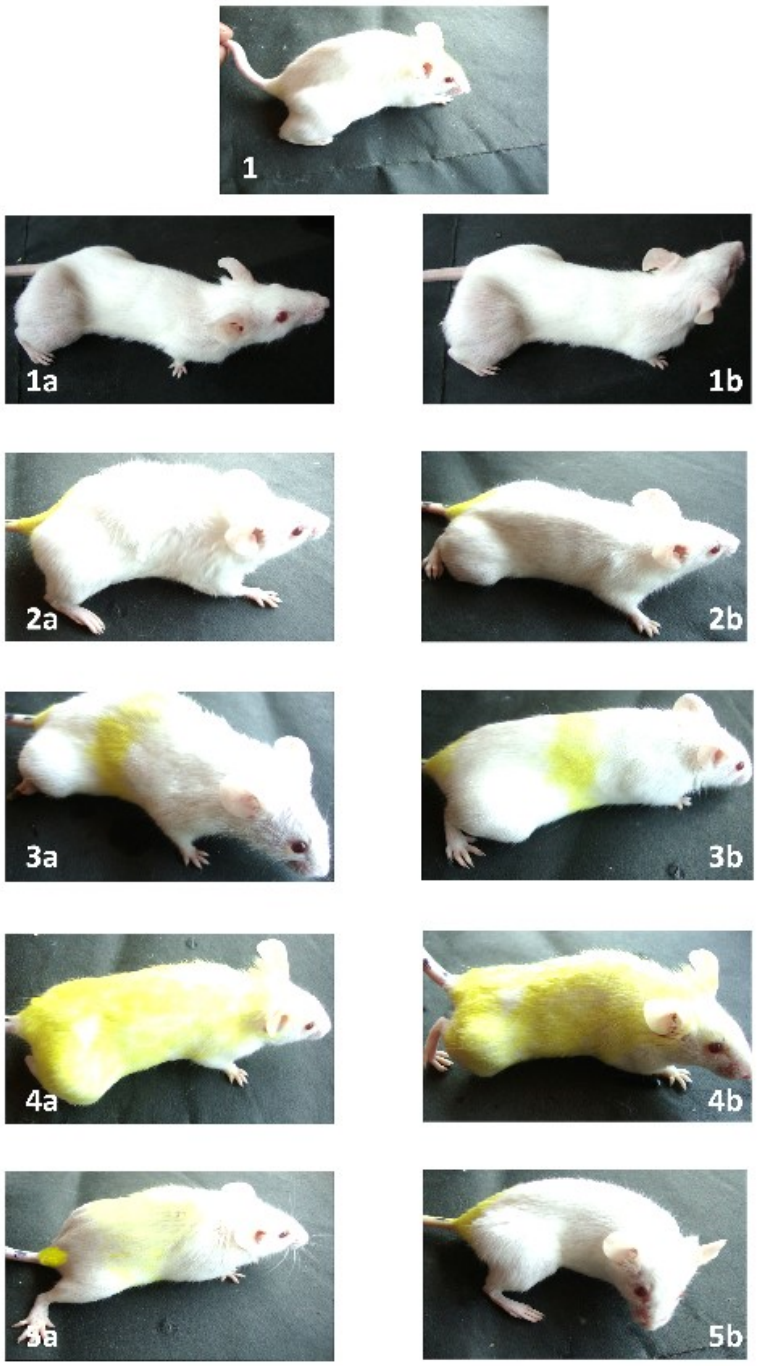

Figure 4.14. Effect of administration of Fe_2O_3 nanoparticles coated with PVP and complexed with DAO and magnetic targeting on DLA solid tumor growth on hind limb of mice. 1 represent animals on the initial day of commencement of the experiment, 1a & 1b represents untreated control animals, 2a & 2b represents animals treated with Fe_2O_3- DAO, 3a& 3b represents animals treated with D- alanine. 4a & 4b represents animals treated with Fe_2O_3- DAO and D- alanine without magnetic treatment, 5a&5b represents animals treated with Fe_2O_3- DAO and D- alanine with magnetic treatment. The suffix 'a' indicates 4th day of treatment and 'b' indicates15th day of treatment.

4.3.8 Effect of Fe_2O_3-DAO nanoparticle complexes on apoptosis in solid tumor, *in vivo*

The extent of apoptosis was analyzed in the tumor and spleen tissue of solid tumor bearing Swiss albino mice using Maygrunwald-Giemsa staining on 7th day of treatment. The animals were treated with Fe_2O_3-DAO complexes, DAO and Fe_2O_3 alone with or without magnetic treatment for seven days, 20 minutes after the drug administration. The results are given in figure 4.15 and 4.16.

In the tumor tissues, it can be seen that the apoptotic index was increased in animals treated with Fe_2O_3-DAO complexes along with D-alanine compared to respective control (**Figure 4.16**). The complexing of Fe_2O_3 along with DAO showed a much significant increase in the apoptotic index in the tumor tissue of animals. When the animals were treated with Fe_2O_3-DAO complexes along with D-alanine and a magnetic treatment for 15 minutes with a horse shoe magnet (1G field) each day for seven days, 20 minutes after the drug administration, the apoptotic index was found to be significantly high in the tumor tissue.

It was found that the spleen tissue of untreated animals and animals treated with Fe_2O_3-DAO and D- alanine along with or without magnetic treatment did not show any apoptosis (**Figure 4.15**).

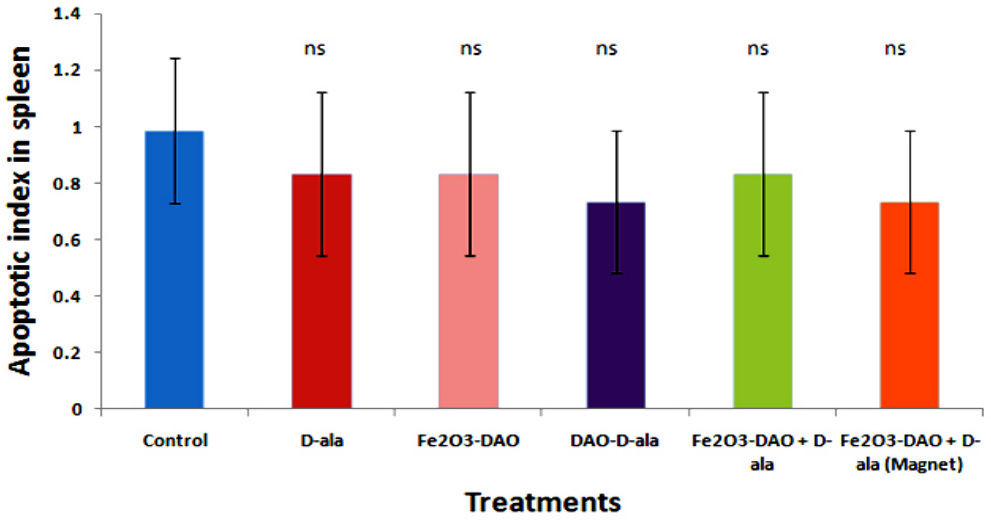

Figure 4.15. Effect of Fe_2O_3 - DAO complex and magnetic treatment on induction of apoptosis in spleen cells of tumor bearing mice. The animals were treated with D-alanine, Fe_2O_3–DAO, Fe_2O_3-DAO and D-alanine with or without magnetic treatment for 7 days. (ns indicates not significant $p>0.05$ when compared to untreated control)

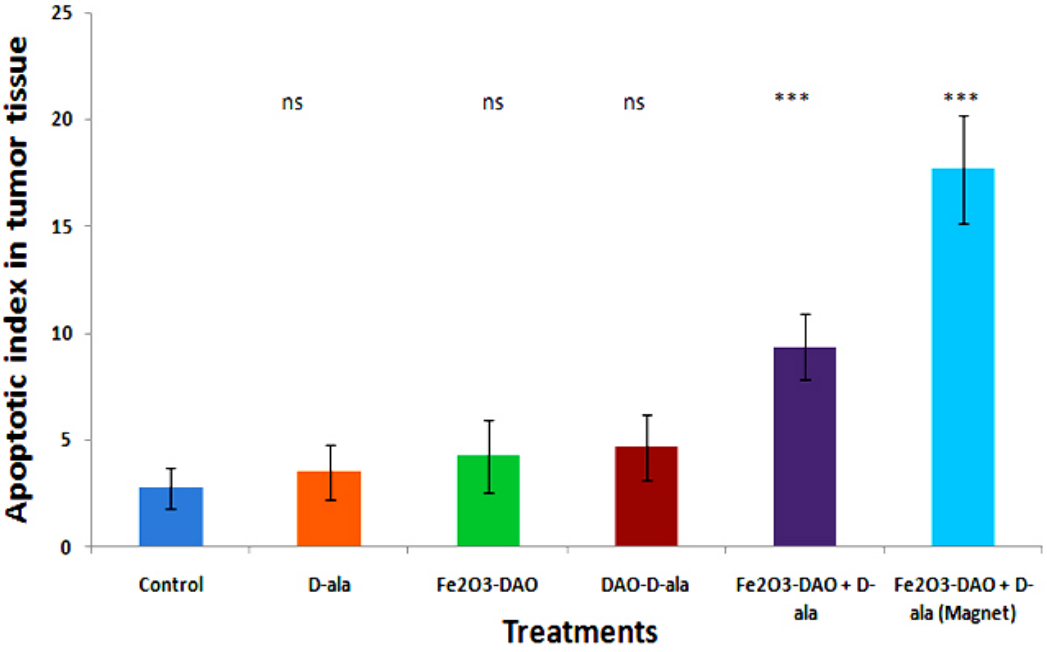

Figure 4.16. Effect of Fe_2O_3 - DAO complex and magnetic treatment on induction of apoptosis in solid tumor on hind limb of mice. The animals were treated with D-alanine, Fe_2O_3–DAO, Fe_2O_3-DAO and D-alanine with or without magnetic treatment for 7 days. (ns indicates not significant when $p>0.05$ and * indicates $p<0.001$ when compared to untreated control)**

4.3.9 Magnet mediated targeting of Fe_2O_3-DAO nanoparticle complexes to the tumor for decreased systemic toxicity

The levels of tissue antioxidant molecule, reduced glutathione (GSH) was measured in various tissues of tumor bearing Swiss albino mice such as Liver, Kidney, Heart, Intestine and Tumor. The level of GSH in all tissues except tumor tissue was near to normal in animals treated with Fe_2O_3-DAO nanoparticle complexes with magnetic treatment (**Table 4.2**). In the tumor tissue of animals treated with Fe_2O_3-DAO nanoparticle complexes and D- alanine with magnetic treatment, the level of GSH was significantly reduced (**Figure 4.17**).

The level of lipid peroxidation was also near to normal levels in all tissues except tumor tissue in animals treated with Fe_2O_3-DAO nanoparticle complexes with magnetic treatment (**Figure 4.18 &Table 4.3**).

Table 4.2. Levels of GSH in various tissues of tumor bearing animals treated with D-alanine, Fe_2O_3–DAO, DAO- D-alnine, Fe_2O_3-DAO and D-alanine with or without magnetic treatment for 7 days. (a- $p>0.05$, b- $p<0.05$, c- $p<0.01$ and d- $p<0.001$ when compared to untreated control group)

GSH (nmol/mg protein	Control	D-alanine	Fe_2O_3-DAO	DAO-D-alanine	Fe_2O_3-DAO+ D-ala	Fe_2O_3-DAO+D-ala (Magnet)
Liver	15.82±3.18	13.6±2.4[a]	14.78±2.0[a]	14.63±1.65[a]	13.1±1.20[b]	12.63±1.76[b]
Kidney	57.71±6.7	47.0±2.0[c]	46.6±4.20[c]	45.3±3.0[d]	48.3±3.50[b]	49.6±5.50[b]
Heart	47.3±6.80	41.3±1.15[a]	44.0±5.65[a]	37.6±5.50[b]	40.6±3.0[a]	39.6±6.80[a]
Intestine	41.6±2.5	40.0±2.0[a]	35.0±3.0[b]	34.3±4.04[c]	33.3±5.0[c]	39.0±2.64[a]

Table 4.3. Levels of MDA in various tissues of tumor bearing animals treated with D-alanine, Fe_2O_3–DAO, DAO- D-alnine, Fe_2O_3-DAO and D-alanine with or without magnetic treatment for 7 days. (a- $p>0.05$, b- $p<0.05$, c- $p<0.01$ and d- $p<0.001$ when compared to untreated control group)

MDA (nmol/mg protein	Control	D-alanine	Fe_2O_3-DAO	DAO-D-alanine	Fe_2O_3-DAO+ D-ala	Fe_2O_3-DAO+D-ala (Magnet)
Liver	2.34±0.23	1.83±0.20[a]	1.9±0.42[a]	1.6±0.56[b]	2.15±0.21[a]	1.8±0.30[a]
Kidney	3.36±0.5	2.6±0.61[a]	2.8±0.2[a]	3.0±0.34[a]	2.13±0.42[d]	2.0±0.4[d]
Heart	2.32±0.25	2.0±0.18[a]	1.83±0.25[c]	2.2±0.2[a]	1.9±0.10[c]	1.83±0.12[c]
Intestine	2.9±0.12	2.54±0.24[a]	2.13±0.26[c]	2.24±0.35[c]	2.35±0.27[b]	2.16±0.42[c]

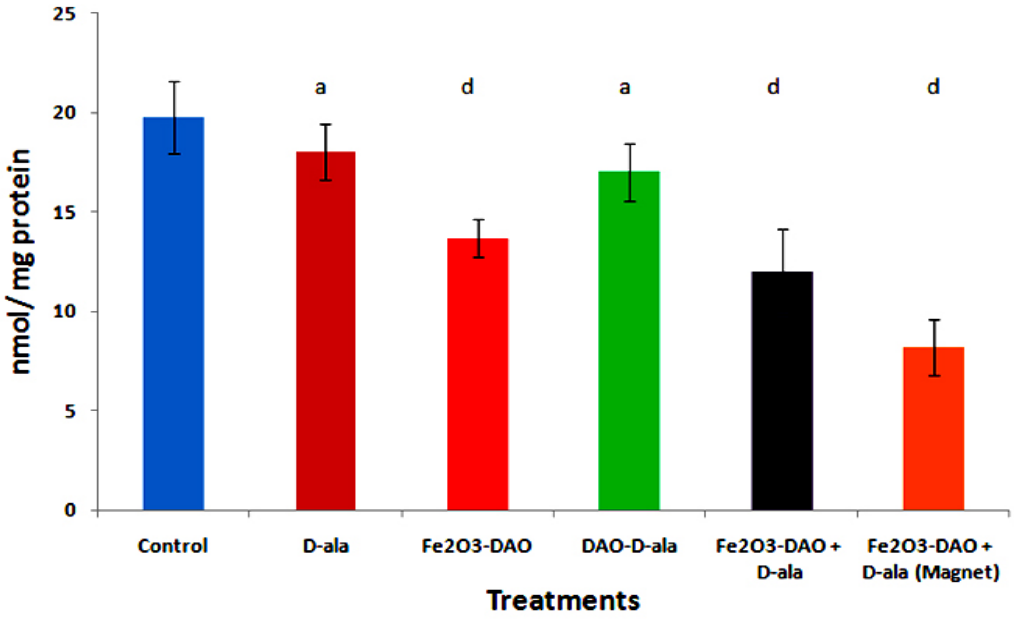

Figure 4.17. Levels of GSH in tumor tissues of tumor bearing animals treated with D-alanine, Fe_2O_3–DAO, DAO- D-alnine, Fe_2O_3-DAO and D-alanine with or without magnetic treatment for 7 days. (a- $p>0.05$, d- $p<0.001$ when compared to untreated control group)

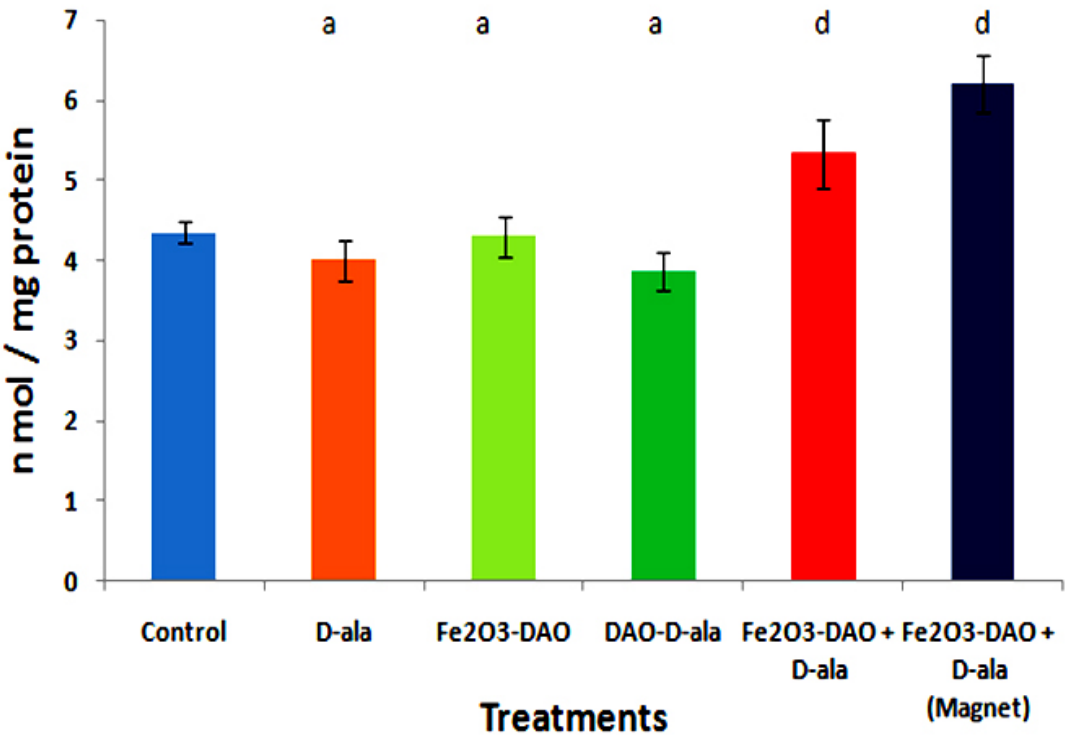

Figure 4.18. Levels of MDA in tumor tissues of tumor bearing animals treated with D-alanine, Fe_2O_3–DAO, DAO- D-alnine, Fe_2O_3-DAO and D-alanine with or without magnetic treatment for 7 days. (a- $p>0.05$, d- $p<0.001$ when compared to untreated control group)

5.4 DISCUSSION

Reactive oxygen species (ROS), including hydroxyl radical (OH .), superoxide anion (O_2 -), peroxynitrite (ONOO–) and hydrogen peroxide (H_2O_2), are highly reactive and potentially hazardous by-products of cellular metabolism. Overproduction of ROS, e.g. H_2O_2, cause oxidative stress to the DNA, proteins and lipids leading to cell death. ROS are highly cytotoxic and may exert remarkable antitumor potential if they can be selectively produced in cells of tumors for "oxidation therapy" (Fang *et al*., 2002). Nanoparticles can be used to target certain drugs selectively to produce ROS at the tumor site (Hu *et al*., 2013; El-Readi and Althubiti, 2019). Present study aims to exploit the potential of ferric nano complex of D-amino acid oxidase (Fe_2O_3-DAO) to

produce H_2O_2 at tumor site when its substrate D-amino acids (D-alanine) was supplied exogenously. Since D-amino acids are not generally present in mammalian organisms in significant amount, it is to be administered exogenously.

Usually it is seen that the stability and activity of the enzymes are naturally reduced during storage. However the activity of Fe_2O_3–DAO remained unchanged after 30 days indicating the protective function of nanoparticles. It has been suggested that this higher stability of the bound enzyme was due to its fixation over the surface of magnetic nanoparticles, preventing its auto-digestion and thermal inactivity (Liao and Chen, 2001; Janib *et al*., 2010). Another plausible explanation is that the binding of DAO on Fe_2O_3 nanoparticles might allow a better spatial orientation of the FAD prosphetic groups and the side chains of DAO providing a better stability to the enzyme. The activity of the enzyme was maximum at pH of 8. After this range the activity was found to be decreased . In this range, the activity of the Fe_2O_3 bound DAO was much higher than its free counterpart. This shows that the bound enzyme showed improved tolerance to the change of solution pH. The similarities in these activities in the pH range of 6 to 7.3 indicate that in these conditions, DAO did not suffer from any major activity constraint. Rather, this pH range appears to be suitable for DAO activity. It is well known that the ability of the amino acids at the active sites of the enzyme to interact with the substrate depends on their electrostatic state (Kang *et al*., 1994). The decrease in activity observed after pH 8 and 9.5 shows that DAO faces some limitations as the pH increased toward more alkaline conditions. The thermal stability was significantly improved upon binding of DAO to magnetic nanoparticles. The binding to nanoparticles suggests a far better resistance of the enzyme to temperature. We hypothesize that the bound enzyme could possibly

undergo a conformational change and a spatial rearrangement that could slow down the folding process and denaturation of the enzyme.

Under *in vitro* conditions, the killing of DLA cells were enhanced in presence of the Fe_2O_3-DAO and its substrate D-alanine. The result suggests that the cytotoxic mechanism of Fe_2O_3-DAO and its substrate is through inducing apoptosis, which was assessed by alkaline single cell gel electrophoresis or comet assay. As shown in *in vitro* studies, DAO alone, D-alanine alone or Fe_2O_3- DAO alone didn't have any cytotoxicity. The cytotoxicity became apparent only when DAO and D-alanine were accessible simultaneously. The cellular DNA damage produced by the ferric-enzyme-substrate complex on DLA cells is due to the stimulation of apoptosis in cells. The generation of apoptosis by ferric enzyme nanocomplex with D-alanine causes extensive damage to cellular DNA as shown in comet assay. In this study, a greater portion of DNA has fragmented and migrated sufficiently to make the long tail. The intense damage to cellular DNA may need caused the cells to travel for apoptosis as revealed by morphological pattern of the DLA cells with cytoplasmic blebbing and nuclear shrinkage. Treatment of the cells with D-alanine along with Fe_2O_3-DAO showed an apoptotic index of 95.5% (Wilkins *et al*., 2002;Wada *et al*.,2003).

The extent of apoptosis was higher in tumor tissue of animals treated with Fe_2O_3-DAO complex and D-alanine with magnetic treatment compared to the unbound drug or nanoparticle controls and Fe_2O_3- DAO group without the magnetic treatment. Thus it was shown that the complex can be targeted by means of an external magnetic field. This targeting enabled the drug to be concentrated at the tumor site where an enhanced apoptosis was obtained and decreasing the systemic concentration of the drug as evidenced by the decreased level of apoptosis in spleen tissue. The administration of DAO alone or complexing of DAO to the nanoparticle alone,

without the magnetic treatment did not result in an increased concentration at the tumor site, but maintained a circulatory level, as can be realized from the *in vivo* apoptosis results.

This targeting enhanced the efficacy of the treatment by decreasing the circulatory concentrations of the drug and helps to minimize its toxic side effects which was also shown by the levels of tissue GSH and lipid peroxidation. Decrease in GSH and increase in the TBARS level (MDA) on tumor tissue of animals treated with Fe_2O_3-DAO complex and D-alanine with or without magnetic field application may be due to the oxidative stress induced by the drug. Magnet mediated targeting of the drug nanoparticle complex to the tumor site decreased the circulating levels of the drug complex and thus helped to maintain normal levels of GSH and TBARS in various tissues except tumor tissue.

The new type of cancer therapy, described here, depends on targeting the H_2O_2-generating enzyme (DAO) to the tumor site using magnetic nanoparticles and the enzyme converting a pharmacologically inert substance (D-alanine) to a highly cytotoxic metabolite, H_2O_2. Re- cent studies shows that intravenous administration of DAO in conjugation with poly ethylene glycol (PEG) with intra peritoneal administration of D- proline exhibited noticeable antitumor activity under *in vivo* condition by rising ROS formation at the tumor site (Maeda *et al*., 1992;Fang *et al*., 2002; Saggar *et al*., 2013; Bi *et al*., 2016). Tumor targeting by PEG-xanthine oxidase (PEG- XO) complex along with the substrate hypoxanthine also has been reported to enhance the tumor targeting efficacy and the antitumor activity of xanthine oxidase (XO) (Sawa *et al*., 2000). The present study, demonstrated the remarkable antitumor activity of two nontoxic components, Fe_2O_3-DAO and D-alanine on mouse solid tumor model. Tumor targeted delivery of Fe_2O_3-DAO by applying an external

magnetic field was possible and subsequent administration of D-alanine selectively generated potent cytotoxic agent, H_2O_2 at the tumor site. Consequently, effective antitumor activity by H_2O_2 could be accomplished, minimising toxicity to normal tissues and organs. Key findings of the present study are: 1) tumor-targeted delivery of an H_2O_2-generating enzyme (DAO) can be accomplished; and 2) tumor regression due to generation of H_2O_2 by exogenous administration of the substrate, D-alanine.

The present work strongly recommended the possibility of effectively controlling tumor growth by targeting orally administered nanoparticle bound enzyme with the help of an external magnetic field to enhance the efficacy of the treatment, suggesting the rationale of advancement of a new anticancer strategy.

Chapter- 5

ANTI INFLAMMATORY ACTIVITY OF ASCORBIC ACID MONO GLUCOSIDE (AsAG) AND FERULIC ACID (FA) AGAINST CARRAGEENAN AND FORMALIN INDUCED PAW EDEMA

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5.3.1 Anti-Inflammatory Activity

5.3.1.1 Carrageenan induced paw edema

5.3.1.2 Formalin induced paw edema

5.4 DISCUSSION

5.1 INTRODUCTION

Inflammation is a reaction of living tissues towards injury and it comprises systemic and local responses (Joel *et al*.,1998). The formation of free radicals, particularly reactive oxygen species and their high activity plays an important role in the progression of a great number of pathological disturbances like atherosclerosis, inflammation, heart disease, stroke, multiple sclerosis, diabetes mellitus, Parkinson's disease, cancer, Alzheimer's disease, etc (Nickavar *et al*., 2007; Hafeez *et al*., 2013). Many present day diseases are reported to be due to the shift in balance of pro-oxidant and antioxidant homeostasis in the body (Schulz *et al*., 2000). Reactive oxygen species (ROS), which include superoxide radical, hydrogen peroxide (H_2O_2) and the hydroxyl radical (-OH) are well documented as cytotoxic intermediates. These ROS differ significantly in their interactions and can cause extensive cellular damage such as nucleic acid strand scission (Adelman *et al.*,1988; Mohanty *et al.,* 2015), modification of polypeptides, lipid peroxidation etc. (Pryor and Porter, 1990) leading to cell membrane disintegration, membrane protein damage and DNA mutation.

Inflammation activates a range of inflammatory cells, that induce and trigger several oxidant generating enzymes such as NADPH oxidase, nitric oxide synthase, myeloperoxidase and eosinophil peroxidase etc. These enzymes generate high concentrations of different free radicals and oxidants such as superoxide anion, nitric oxide, nitroxyl, nitrogen dioxide, hydrogen peroxide etc, which react with each other to produce more potent reactive oxygen and nitrogen species that can damage DNA, RNA, lipids and proteins and also leads to multistage carcinogenesis (Ohshima *et al*., 2003). Therefore much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit peroxidation and to protect DNA and other macromolecules from damage due to free radicals (Wu *et al*., 2017).

In spite of our dependence on modern medicine and the tremendous advances in synthetic drugs, a large number of world populations cannot afford the products of the western pharmaceutical industry and have to rely upon the use of traditional medicines, which are mainly derived from plant materials. A large number of aromatic, spicy, medicinal and other plants have chemical compounds, exhibiting antioxidant properties. There are various sources of natural antioxidants mainly, plant phenolics that may seen in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Pratt and Hudson, 1990; Naik and Krishnamurthy, 2018). Such antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent (Sala *et al*., 2002). Many anti-inflammatory, digestive, anti-necrotic, neuroprotective, and hepatoprotective drugs have recently known to have an antioxidant and/or radical scavenging mechanism as part of their activity (Lin *et al*., 2000; Repetto and Lesuy, 2002). Crude extracts of herbs, fruits, cereals, vegetables and other plant materials rich in phenolic are increasingly of interest in the food industry, because they hinder oxidative degradation of lipids and thereby improve the quality and nutritive value of food (Sevgi *et al*., 2015). Hence interest in natural antioxidants, especially phytochemicals has greatly increased in recent years. Many phytochemicals including phenolics, flavonoids, tannins, proanthocyanidins, vitamins and various herbal extracts have been reported as antioxidants (Kumar *et al*., 2010;Sandeep and Nair 2010).

The present chapter reports the investigation on the anti-inflammatory properties of AsAG and FA.

5.2 MATERIALS AND METHODS

5.2.1Animals

Female Swiss albino mice weighing 22- 25 g were obtained from the Small Animal Breeding Section (SABS), Mannuthy, Thrissur, Kerala.

5.2.2 Studies on anti-inflammatory activity

5.2.2.1 Carrageenan induced acute paw edema

Animals were divided into 6 groups of 5 animals in each group. For all groups acute inflammation was induced by sub plantar injection of 0.02 ml freshly prepared 1% of carrageenan in normal saline in the right hind paw of mice (Winter *et al*., 1962). The administration of AsAG, FA and diclofenac and induction of paw edema is described below.

Group I- Distilled water (0.1ml) + 0.02 ml of 1% carrageenan injection

Group II- Ferulic acid 100 mg/kg (in 0.1 ml distilled water *p.o*) + 0.02 ml of 1% carrageenan injection

Group III - Ferulic acid 200 mg/kg (in 0.1 ml distilled water *p.o*) + 0.02 ml of 1% carrageenan injection

Group IV- AsAG 250 mg/kg (in 0.1 ml distilled water *p.o*) + 0.02 ml of 1% carrageenan injection

Group V - AsAG 500 mg/kg (in 0.1 ml distilled water *p.o*) + 0.02 ml of 1% carrageenan injection

Group VI- Diclofenac 10 mg/kg body weight (in 0.1 ml distilled water *p.o*) + 0.02 ml of 1% carrageenan injection

Animals which received carrageenan injection alone were kept as control. Diclofenac (10 mg/kg body weight) served as standard reference drug. AsAG, FA and diclofenac were administered one hour prior to the sub plantar injection of carrageenan. The paw

thickness was measured using vernier callipers at one hour intervals for 6 hours following carrageenan injection.

5.2.2.2 Formalin induced chronic paw edema

The animals were treated in the same way as in the above models; except formalin (20 µl of freshly prepared 2% formalin) was used as the edematogenic agent instead of carrageenan. The treatment was continued for 6 consecutive days. Diclofenac (10 mg/kg body weight) was used as the reference drug.

In all the above models, the degree of edema formation was determined as increase in paw thickness. The increase in paw thickness and percent inhibition were calculated as follows.

Increase in paw thickness in control (P_C) or treatment (P_T) = Pt-Po,

Percent inhibition = $\frac{P_C - P_T}{P_C} \times 100$

Where P_t indicates paw thickness at time t, P_0 is initial paw thickness, P_C represents increase in paw thickness of the control group and P_T is the increase in paw thickness of the treatment groups.

5.3 RESULTS

5.3.1 Anti-Inflammatory Activity

5.3.1.1 Carrageenan induced paw edema

The sub plantar injection of carrageenan in Swiss albino mice produced a local inflammatory response. The paw edema found to reach the peak at 2nd hour and after

that it was found to reduce. Administration of the ferulic acid produced 28 % inhibition in the paw thickness at a dose of 100 mg/kg body weight at 6th hour and 37.5% inhibition in paw thickness at a dose of 200 mg/kg at 6th hour (**Figure 5.1 & Table 5.1**).

Table 5.1 Percent inhibition in the paw thickness on carrageenan induced paw edema in mice

Treatments	% inhibition		
	2 hour	4hour	6 hour
Diclofenac	28.57	39.5	44.5
FA100	19	26.3	28
FA200	23.8	34.2	37.5
AsAG250	21.42	38	40.6
AsAG500	28.6	45	50

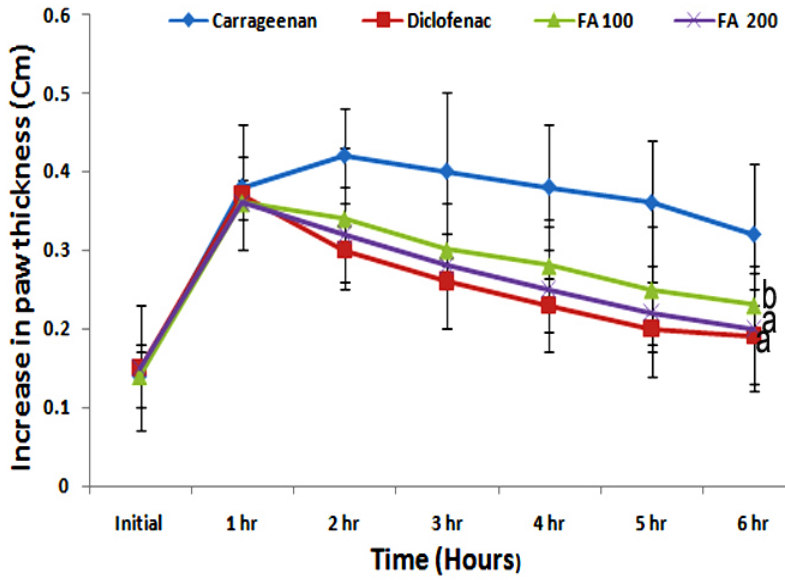

Figure 5.1 Effect of Ferulic acid (100mg/kg &200 mg/kg) on carrageenan induced paw edema in mice. All values expressed as Mean ± S.D, (n=5). 'a' denotes p<0.001; 'b' denotes p<0.01

Similarly administration of AsAG also exhibited decrease in paw thickness and produced 40.6 % inhibition in the paw edema at a dose of 250 mg/kg body weight at 6th hour as evident from Figure 5.2

Administration of AsAG produced reduction in paw thickness and produced 50% inhibition at a dose of 500 mg/kg at 6th hour when compared with that of the carrageenan induced control animals (**Figure 5.2 & Table 5.1**).

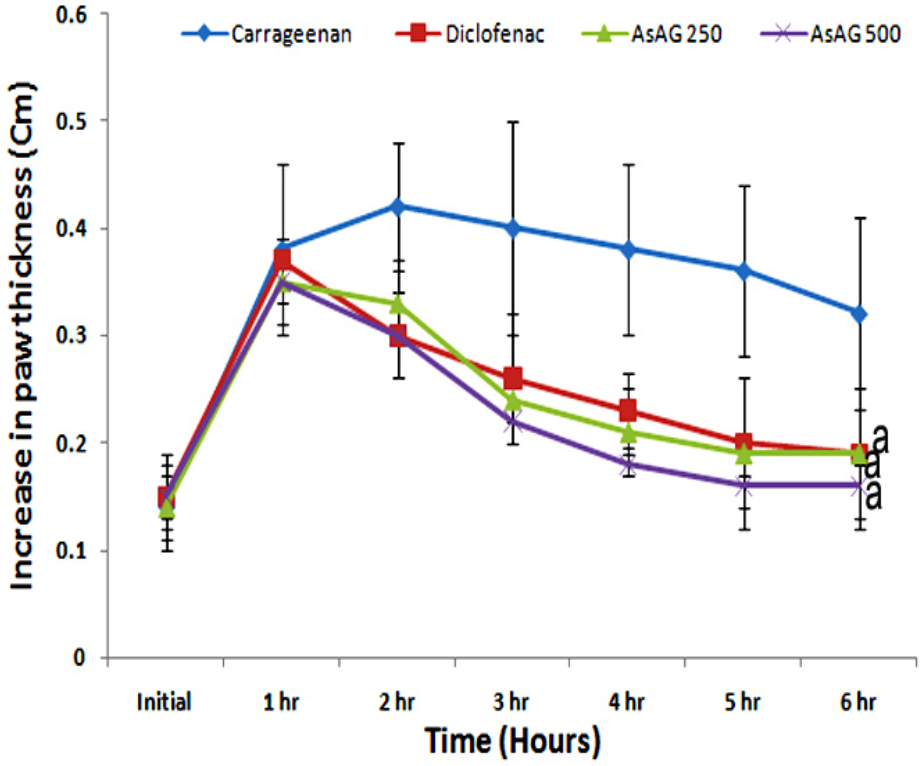

Figure 5.2 Effect of Ascorbic acid monoglucoside (AsAG) (250mg/kg & 500 mg/kg) on carrageenan induced paw edema in mice. All values expressed as Mean ± S.D, (n=5). 'a' denotes p<0.001

5.3.1.2 Formalin induced paw edema

In the formalin-induced paw edema test for chronic inflammation, the sub plantar injection of formalin in Swiss Albino mice produced a local inflammatory response which reached a maximum intensity of edema at the 3rd day. Administration of ferulic acid at doses of 100 mg/kg body weight showed 31.4 % inhibition of edema on 6th

day, where as ferulic acid exhibited 46.87 % inhibition at a dose of 200 mg/kg on 6th day (**Figure 5.3 &Table 5.2**).

Table 5.2 Percent inhibition in the paw thickness on Formalin induced paw edema in mice.

Treatments	% inhibition		
	Day 2	Day 4	Day 6
Diclofenac	25	40	42.8
FA100	20.5	27.5	31.42
FA200	25	35	46.87
AsAG250	27.3	39	42.8
AsAG500	31.8	50	54.3

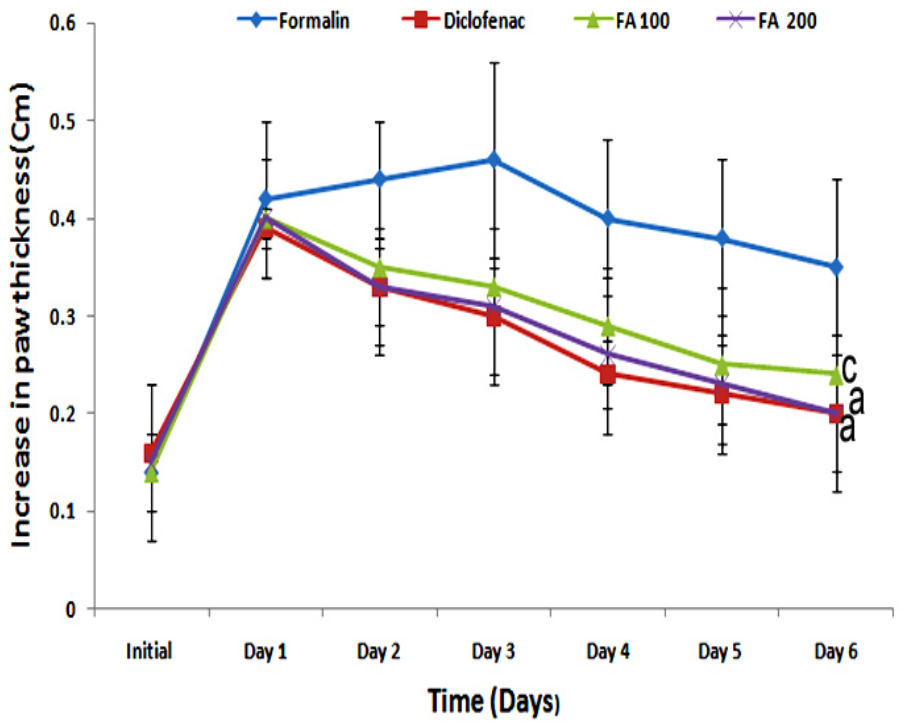

Figure 5.3 Effect of Ferulic acid (100mg/kg &200 mg/kg) on formalin induced paw edema in mice. All values expressed as Mean ± S.D, (n=5). 'a' denotes p<0.001; 'c' denotes p<0.05

AsAG produced 42.8% inhibition at a concentration of 250 mg/kg (Figure 5.4) and 54.3 % inhibition of edema at a concentration of 500 mg/kg on 6th day (**Figure 5.4 &Table 5.2**).

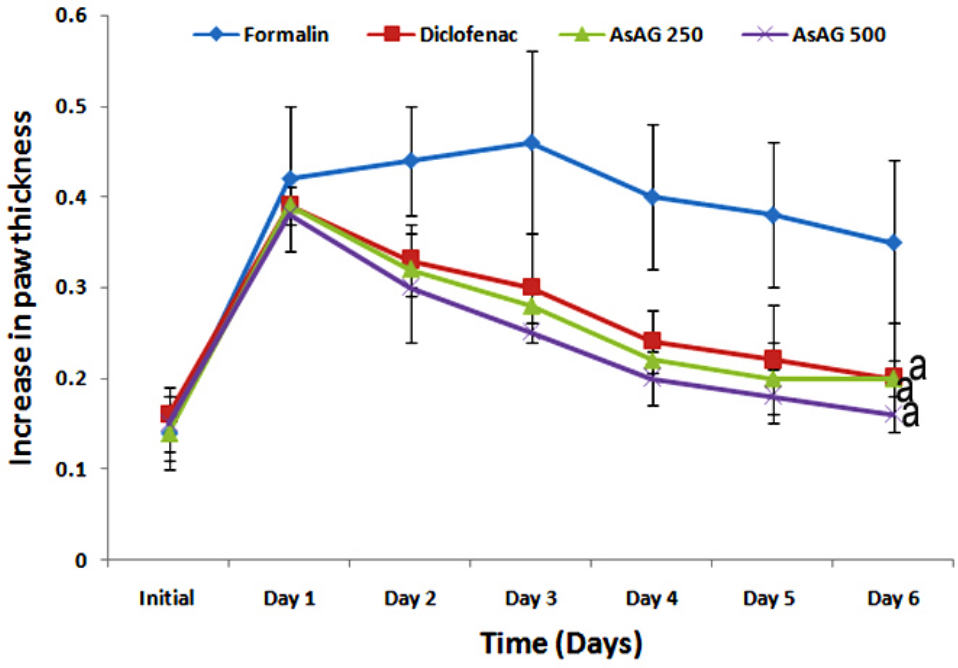

Figure 5.4 Effect of Ascorbic acid monoglucoside (AsAG) (250mg/kg & 500 mg/kg) on formalin induced paw edema in mice. All values expressed as Mean ± S.D, (n=5). 'a' denotes p<0.001

5.4 DISCUSSION

Various studies suggest that the inflammatory tissue damages are due to the liberation of reactive oxygen species from phagocytes invading the inflammation sites (Cross *et al*., 1987; Winrow *et al*., 1993). Studies have shown that nitric oxide is implicated in inflammation, cancer, and other pathological conditions (Hemnani and Parihar, 1998). Interactions between superoxide and nitric oxide regulate the vascular tone or inflammation (Conner and Grisham, 1996).

Inflammation is a complex process and ROS plays an important role in the pathogenesis of inflammatory reactions (Halliwell and Gutteridge, 1985). As the inflammation is mainly produced by the oxidative burst of macrophages, antioxidants which can scavenge ROS may be effective to reduce inflammatory disorders. Inflammation, which is a pattern of response to injury, involves the accumulation of cells and exudates in irritated tissues that allows protection from further damage (Azab *et al*., 2016). Significant ameliorative activity against carrageenan and formalin induced anti-inflammation was shown by AsAG and FA.

Ascorbic acid has been reported to have protective effect against radiation-induced mortality and cytogenetic damage (El-Nahas, *et al*., 1993). The radioprotective effect of ascorbic acid has been ascribed to its interactions with radiation-induced free radicals (Nair *et al*., 2001). AsAG also possess free radical scavenging activity as evidenced from its ability to scavenge DPPH, ABTS and OH- radicals. The anti-inflammatory activity of AsAG may also be due to its ability scavenge ROS. The ability of AsAG to scavenge OH radicals and *in vivo* anti-inflammatory effect indicated that addition of a glucose moiety to ascorbic acid does not alter its free radical scavenging ability.

Carrageenan induced acute inflammation in animals is said to be the most suitable test procedures to monitor anti-inflammatory agents. The carrageenan induced edema is mediated by activation of platelet activating factor (PAF), prostaglandins and other inflammatory mediators (Hwang *et al*., 1986; Mansouri *et al*., 2015). Carrageenan-induced edema is a biphasic response in which the involvement of the cyclo-oxygenase products of arachidonic acid metabolism and the production of reactive oxygen species are well established (Chen, 1993; Madhuri *et al*., 2016). The first phase is mediated through the release of histamine, serotonin, and kinins, whereas the

second phase is related to the release of prostaglandin oxygen-derived free radicals and production of inducible cyclo-oxygenase which peak at 2-3 hours (Panthong *et al*., 2004). Carrageenan induces the formation of protein rich exudates containing large number of neutrophills (Lo *et al*., 1982; Naik and Krishnamurthy, 2018). The ferulic acid produced considerable inhibition of carrageenan-induced paw edema comparable in magnitude with the inhibitory action of the standard drug diclofenac. But the effect is more with the AsAG than the standard drug.

Formalin induced paw edema is also an appropriate test procedure to screen chronic anti-inflammatory agents as it strongly resembled human arthritis (Greenwald, 1991). The nociceptive effect of formalin is biphasic, an early neurogenic component followed by tissue mediated response (Panthong *et al*., 2004; Zhao *et al*., 2018). Ferulic acid obtained from wheat bran by a new strain of *A. niger* showed good anti-inflammatory activity and better antioxidant ability (Yin *et al*., 2019). The ferulic acid and AsAG showed significant anti-inflammatory activity against formalin induced paw edema and thus found to be effective in chronic inflammatory conditions. Among these maximum inhibition of edema was observed in AsAG treated animals.

There is a strong relationship between antioxidants and inflammation (Ma and Huang, 2014). Chronic inflammation is always followed by increased production of tissue reactive oxygen and nitrogen intermediates. Oxygen free radicals and non radical reactive oxygen intermediates released by neutrophils and other phagocytes have been increasingly implicated in inflammation/ immune disorders. Inflammation also facilitates the initiation of normal cells and their progression to malignancy through the production of inflammatory oxidants (Dhingra *et al*., 2018). Appropriate treatment of inflammation with anti-inflammatory agents, inhibitors, inhibitors of oxidant

generating enzymes, and scavengers of oxidants should be explored to prevent development of human cancers associated with chronic inflammation (Ohshima *et al*, 2003).

To conclude, the present study reveals the profound anti-inflammatory activity of both the ferulic acid and Ascorbic acid monoglucoside and the effect showed by them might probably due to their significant antioxidant power.

Chapter- 6

AMELIORATION OF DOXORUBICIN INDUCED CARDIOTOXICITY IN TUMOR BEARING MICE BY ASCORBIC ACID MONOGLUCOSIDE (AsAG) AND FERULIC ACID (FA)

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6.4 DISCUSSION

6.1 INTRODUCTION

Anthracycline antibiotics are a class of potent anticancer drugs against many types of hematological and solid malignancies. However, its use has been limited primarily due to cardiotoxicity after an acute dose or cumulative doses (Singal and Iliskovic, 1998). Therefore, reducing this side effect while maintaining their efficacy is critical for the success of chemotherapy. Doxorubicin (DOX), an anthracyclin antibiotic, used in the treatment of a variety of solid tumors and hemopoietic malignancies (Abdel-Raheem *et al*., 2013). The chronic cardiotoxicity is dose-dependent and results in irreversible myocardial damage, following dilated cardiomyopathy with fatal congestive heart failure (Von Hoff *et al.*, 1979). The mechanisms proposed for cardiotoxic effects of DOX include free radical induced myocardial injury, membrane lipid peroxidation, (Myers *et al*., 1977) mitochondrial damage, activity of Na^+ K^+ ATPase, (Geetha and Devi, 1992) vasoactive amine release, impairment in myocardial adrenergic signalling regulation, increase in serum parameters such as total cholesterol, triglycerides, and low density lipoproteins and also iron-dependent oxidative damage to macromolecules (Xu *et al.*, 2001). It also causes the elevation of serum enzymes like lactate dehydrogenase (LDH), and creatine phosphokinase (CPK) (Aniss *et al*., 2014).

Several studies have demonstrated that DOX induces the generation of a cascade of reactive oxygen species (ROS) such as $O_2{-}{\cdot}$, ·OH and H_2O_2, which are implicated in the DOX- induced cardiotoxicity (Kalivendi *et al.*, 2001; Wang *et al.*, 2004). The semiquinone form, formed by the reduction of DOX by some endogenous enzymes, creates $O_2{-}$ radical by transferring electrons to molecular oxygen. The superoxide radicals are rapidly transformed, either spontaneously or enzymatically, into other forms of ROS such as ·OH and H_2O_2 (Lee *et al.*, 1991).

Endogenous antioxidant deficits have been suggested to play a major role in doxorubicin induced cardiomyopathy and heart failure (Naidu *et al.*, 2002; Abou-El-Hassan *et al.*, 2003). Various polyherbal formulations or their secondary metabolites serve as antioxidant have shown protective effects in doxorubicin induced cardiotoxicity without affecting their therapeutic efficacy. Antioxidants protect cells and tissues against free radicals which caused oxidative damage and injury and they have been reported to have beneficial effects against DOX induced cardiotoxicity in mice and rats (Liu *et al.*, 2002). Antioxidants such as vitamin E provide protection from cardiac cell damage with a simultaneous decrease in lipid peroxidation (Speyer *et al.*, 1985; Al-Harthi *et al*., 2014).

The most widely used of all vitamin supplements, Ascorbic acid (AsA) is a powerful antioxidant and free radical scavenger that has a myriad of functions and also helps to strengthen the immune system. Both under *in vitro* and *in vivo* conditions, AsA reduces oxidative free radical induced damages to DNA and membranes in biological systems (Wilson, 1983). As an antioxidant, AsA helps in cardiovascular disease by protecting the linings of arteries from oxidative damage. AsA neutralizes reactive oxygen metabolites (ROM) and reduces oxidative DNA damage and genetic mutations (Wilson, 1983). Though AsA has been proved as an effective antioxidant, its pro-oxidant activity and easily oxidizable nature limit its use in many of the applications. The glucoside derivative of AsA is more stable, and in many plant sources AsA exists as glucoside. Moreover, ascorbic acid monoglucoside (AsAG) is found to exhibit *in vitro* free radical scavenging activity (Fujinami *et al*., 2001) and antioxidant and radioprotective abilities (Mathew *et al*., 2007). AsAG exhibits vitamin C activity *in vivo* after enzymatic hydrolysis by α-glucosidase (Fujinami *et al*., 2001).

Phenolics are widely distributed in the plant kingdom and are integral part of human diet (Graf,1992). As an antioxidant, FA play a major role in the body's defense against carcinogenesis by inhibiting the formation of N-nitroso compounds (Kuenzig *et al*., 1984; Batista, 2014).

The present study envisages exploring the effect of glucoside derivatives of ascorbic acid (AsAG) / ferulic acid (FA) or 4-Hydroxy-3-Methoxycinnamic Acid on DOX-induced cardio toxicity in tumor bearing Swiss albino mice.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Female Swiss albino mice, 6–8-weeks old (body weight 25–30 g), were kept under standard conditions of temperature (25 ± 5℃) and humidity in the Centre's Animal House Facility.

6.2.2 Chemicals

Ascorbic acid monoglucoside (AsAG) were obtained from Dr. V. T. Kagiya, Health Research Foundation, Kyoto, Japan. Ferulic acid (FA), Nitroblue tetrazolium (NBT), riboflavin, reduced glutathione (GSH), 5-5' dithiobis-2- nitro benzoic acid (DTNB) were purchased from Sigma Chemical Company Inc., St. Louis, MO, USA. Doxorubicin (DOX) was purchased from Cadila pharmaceuticals Ltd., Oncocare Di-vision, Ahmedabad, India. EDTA was from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. H_2O_2 was from Merck India Ltd., Mumbai, India. Thiobarbituric acid (TBA) was purchased from Hi-media Laboratories, Mumbai, India. All other chemicals and reagents used in this study were of analytical grade procured from reputed Indian manufacturers.

6.2.3 Administration of Drugs

Doxorubicin was given intraperitoneally as a single dose of 25mg/kg b.w. Ferulic acid/ AsAG dissolved in distilled water was given to animals using oral gavage.

Solid Tumor in Animals- Solid Tumor was formed in hind limbs of mice by transferring Dalton's Lymphoma Ascites (DLA) cells (1×10^6 cells/animal) via subcutaneous injection. The animals were given standard mouse chow and water *ad libitum*. When the tumor size reached approximately 1.0 cm^3, the treatments started on the 13^{th} day of transplantation of DLA cells. The animals were randomly divided into 6 groups of five each and treated as follows.

Group – I were kept untreated control group, 0.1 ml saline *i.p* and 0.1 ml distilled water *p.o* . Group – II DOX control group, doxorubicin 25mg/kg b.w **(**in 0.1 ml saline *i.p* as single dose).

Group – III Doxorubicin 25mg/kg b.w (in 0.1 ml saline *i.p* as single dose) + AsAG 250 mg/kg b.w **(**in 0.1 ml distilled water *p.o*)

Group – IV Doxorubicin 25mg/kg b.w (in 0.1 ml saline *i.p* as single dose) + AsAG 500 mg/kg b.w **(** in 0.1 ml distilled water *p.o*)

Group V- Doxorubicin 25mg/kg b.w (in 0.1 ml saline *i.p* as single dose) + Ferulic acid 100 mg/kg b.w **(**in 0.1 ml distilled water *p.o*).

Group VI- Doxorubicin 25mg/kg b.w **(**in 0.1 ml saline *i.p* as single dose) + Ferulic acid 200 mg/kg b.w **(**in 0.1 ml distilled water *p.o*).

AsAG was administered to group III and IV, Ferulic acid was administered to Group V and VI animals, one hour after DOX (25 mg/kg b.w) administration.

6.2.4 Assessment of Toxicity on Heart and Tumor Tissues

The toxicity to cardiac and tumor tissues were studied by histopathological examinations, change in biochemical parameters and cellular DNA damage in animals administered with the drugs either alone or in combination. From the animals of different groups, the blood was collected by direct cardiac puncture under light ether anesthesia following 24 hours of various treatment as described above and serum was separated for the biochemical estimations. The heart and tumor tissues were dissected, from each group and were taken for histopathological examination. Single cell suspension of tissues prepared in 0.1 M phosphate buffer (pH.7) (10^6 cells/ml) were used to carry out comet assay (Alkaline single cell gel electrophoresis).

Biochemical parameters such as creatine kinase (CK) lactate dehydrogenase (LDH), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and high density lipoprotein (HDL) were analyzed as cardiac biomarkers using diagnostic kits (Agappe Diagnostic Pvt. Ltd., Thane, India). The oxidative damage in cardiac and tumor tissues following various treatments were estimated by monitoring superoxide dismutase (SOD) (Mc Cord and Fridovich, 1969), glutathione peroxidase (GPx) (Hafemann *et al*., 1974), reduced glutathione (GSH) (Moron *et al*., 1979) and for lipid peroxidation (LPO) (Buege and Aust, 1978) in 10% homogenates of the tissues in PBS.

For histopathological studies, tissues were fixed in 10% formalin and embedded in paraffin wax. Sections were made with 5 micron thickness using a microtome and stained with haematoxylin-eosin. The histopathological examinations were carried out at Sudharma Metropolis Pathological Laboratory, Thrissur, Kerala, India.

6.2.5 Estimation of Cellular DNA Damage in Heart and Tumor Tissues by Comet Assay (Alkaline Single Cell Gel Electrophoresis)

Administration of doxorubicin to animals cause oxidative stress in the tissues, which in turn can cause damage to cellular DNA. The damage in cellular DNA subsequent to various treatments were analysed by alkaline comet assay or single cell gel electrophoresis as DNA single strand breaks in heart and tumor tissues.

6.3 RESULTS

6.3.1 Serum Enzyme Levels

Animals treated with DOX produced significant increase in the levels of serum enzyme levels (**Table 6.1**). A single *i.p* injection of DOX significantly elevated serum SGOT, SGPT, CK and LDH levels from 227.48±53.61 U/L, 49.41± 6.45 U/L, 65±12.98 U/L and 297.63 ±39.58 U/dL to 4510±842.93 U/L, 156±11.52 U/L, 425.66±102.76 U/L and 3598.86 ±618.15 U/dL respectively, showing cardio toxicity. The post treatment with AsAG (250mg/Kg b.w and 500 mg/Kg b. w) / FA (100 mg/kg b.w and 200 mg/kg b.w) in tumor bearing animals significantly ameliorated SGOT, SGPT, LDH and CK enzyme activities in a dose dependent manner. Treatments with FA/ AsAG to DOX-challenged animals significantly decreased ($p<0.001$) the activities of these enzymes elevated by doxorubicin, though not to normal. Animals treated with DOX produced slight difference in HDL levels compared to untreated control. The administration of DOX and AsAG / FA caused significant increase in the level of HDL as compared to DOX control (**Figure 6.1& 6.2**).

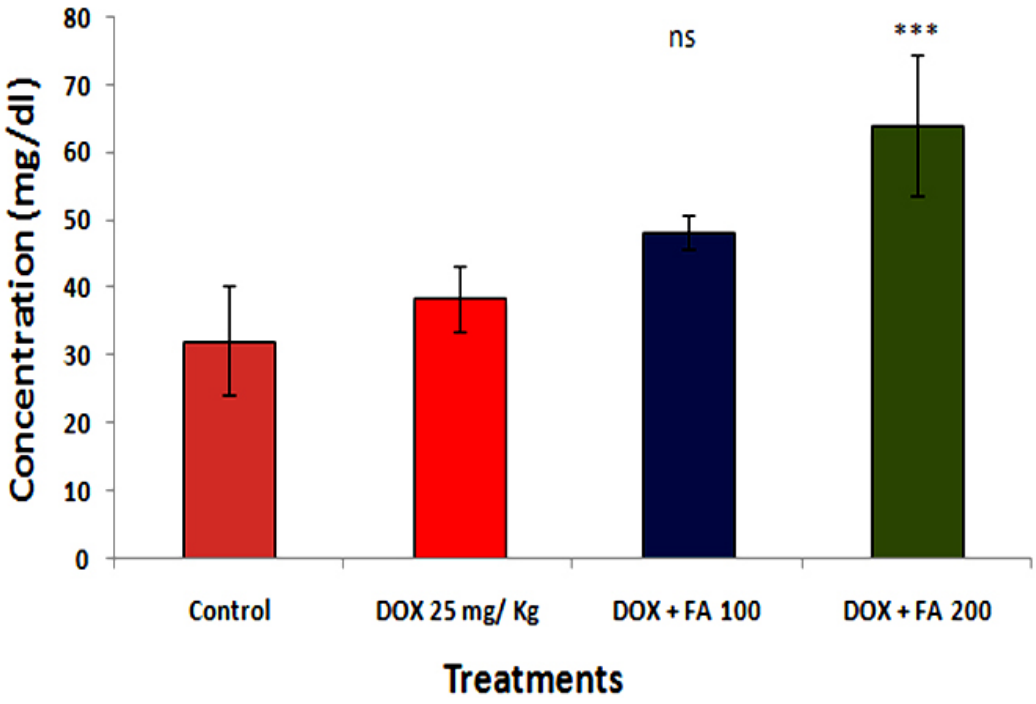

Figure 6.1 Effect of administration of ferulic acid (100mg/kg and 200mg/kg) on HDL level in doxorubicin induced cardiotoxicity. (ns indicate p>0.05 and * indicate p < 0.001 when compared with DOX alone treated group)**

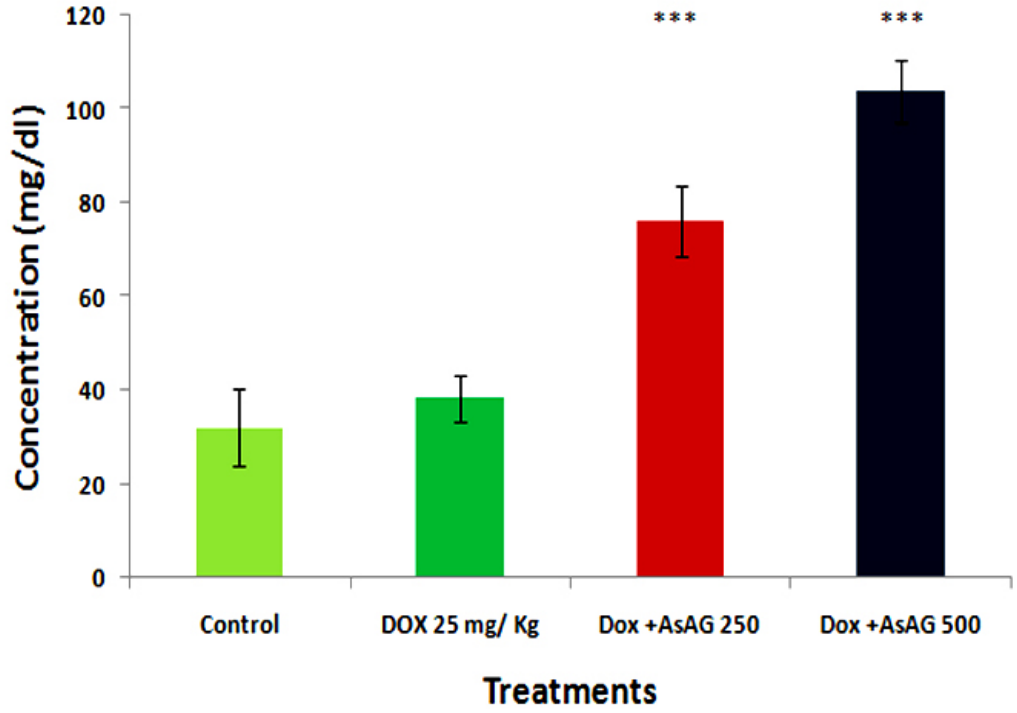

Figure 6.2 Effect of administration of AsAG (250 mg/kg and 500 mg/kg) on HDL level in doxorubicin induced cardiotoxicity. (* indicate p < 0.001 when compared with DOX alone treated group)**

Table 6.1 Effect of ferulic acid and AsAG on serum marker enzymes in doxorubicin induced cardio toxicity in mice. values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. 'a' represents p< 0.001 when compared with DOX control.

Treatment	SGOT (U/L)	CK (U/L)	LDH(U/dl)	SGPT(U/L)
Control	227.48±53.61	65±12.98	297.63±39.58	49.41±6.45
Dox 25 mg/ kg	4510±842.93	425.66±102.76	3598.86±618.15	156±11.52
Dox +FA 100 mg/kg	1650±588.64[a]	263.77±15.6[a]	2609.28±206.7[a]	72±6.22[a]
Dox +FA 200 mg/kg	1272.44±337.04[a]	153.3±11.23[a]	1725.64±205.27[a]	62.62±12.12[a]
Dox +AsAG 250 mg/kg	1141.3±449.58[a]	140.6±7.5[a]	1665.12±178.4[a]	63.0±7.0[a]
Dox +AsAG 500 mg/kg	823.98±181.16[a]	107.3±11.0[a]	1237.53±152.95[a]	52.75±6.70[a]

6.3.2 Biochemical Measurements and Antioxidant Status

MDA (Malondialdehyde) was measured as a marker of lipid peroxidation and an indicator of oxidative injury. The MDA levels in heart tissue were increased significantly in the DOX treated group compared with the untreated control group. The increase in MDA by DOX was significantly attenuated by the administration of AsAG / FA (**Figure 6.3**).

Different antioxidant enzymes were examined in the heart tissue from all the groups. The DOX-treated mice showed a significant decrease in SOD, GPx and GSH activities compared with the untreated control (**Figure 6.4, 6.5 & 6.6**). The decrease in activities of SOD, GPx and GSH due to doxorubicin was attenuated by both AsAG or FA.

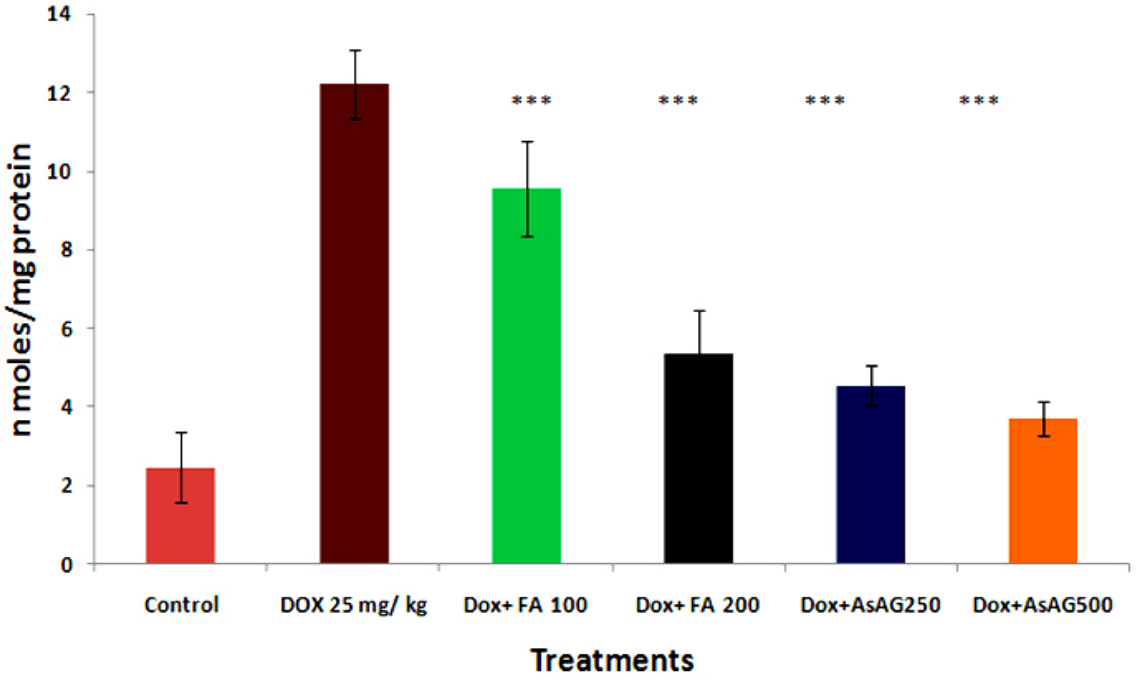

Figure 6.3 Effect of administration of AsAG or ferulic acid on doxorubicin-induced lipid peroxidation (MDA formation) in heart tissues of mice. (* indicate p<0.001 when compared with the DOX alone treated group)**

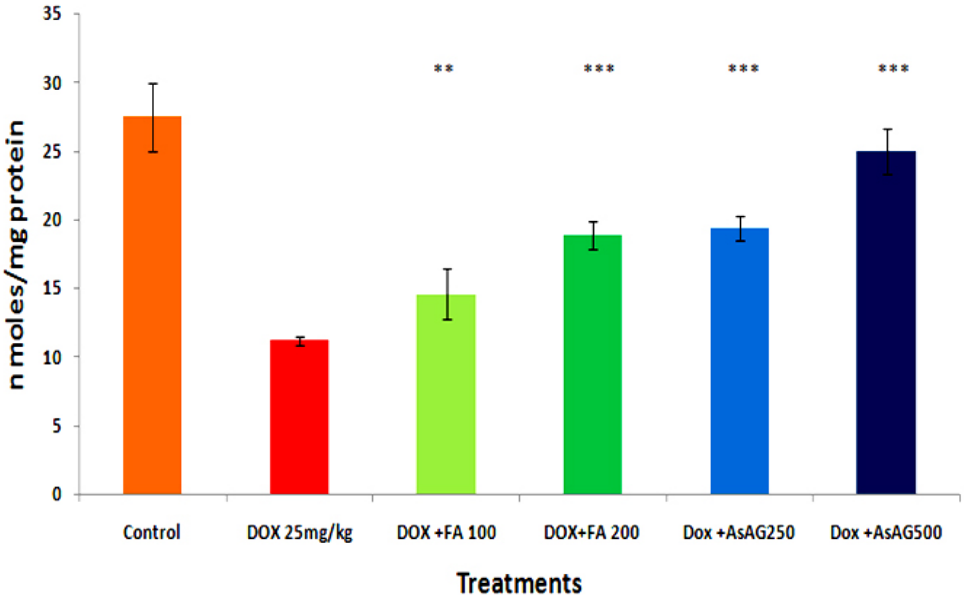

Figure 6.4 Effect of post administration of AsAG or FA on doxorubicin induced depletion of GSH levels in heart tissue of tumor bearing mice. Values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. ** represent p< 0.01 vs DOX control; * represent p< 0.001 vs DOX control**

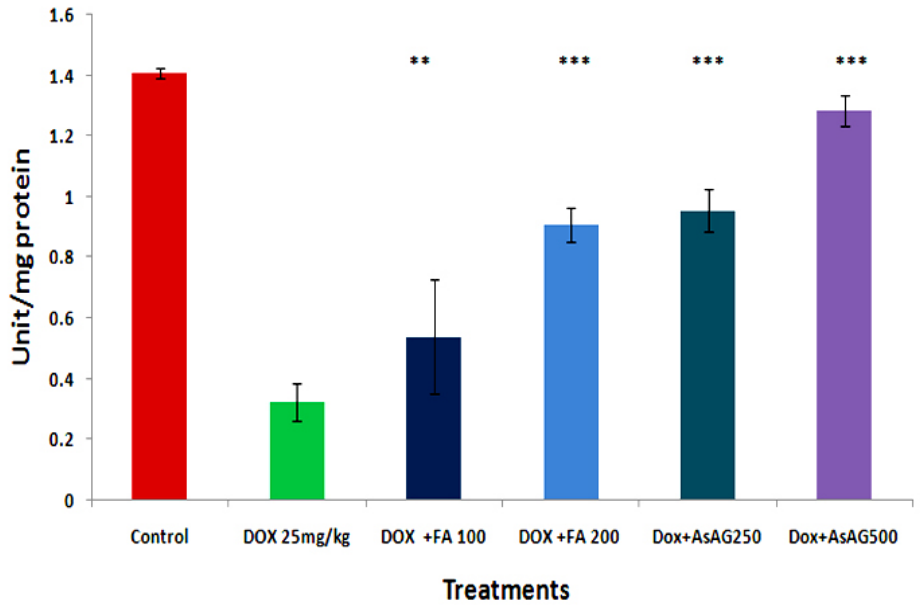

Figure 6.5 Effect of post administration of AsAG or FA on doxorubicin induced depletion of SOD levels in heart tissue of tumor bearing mice. Values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. ** represent p< 0.01 vs DOX control; * represent p< 0.001 vs DOX control**

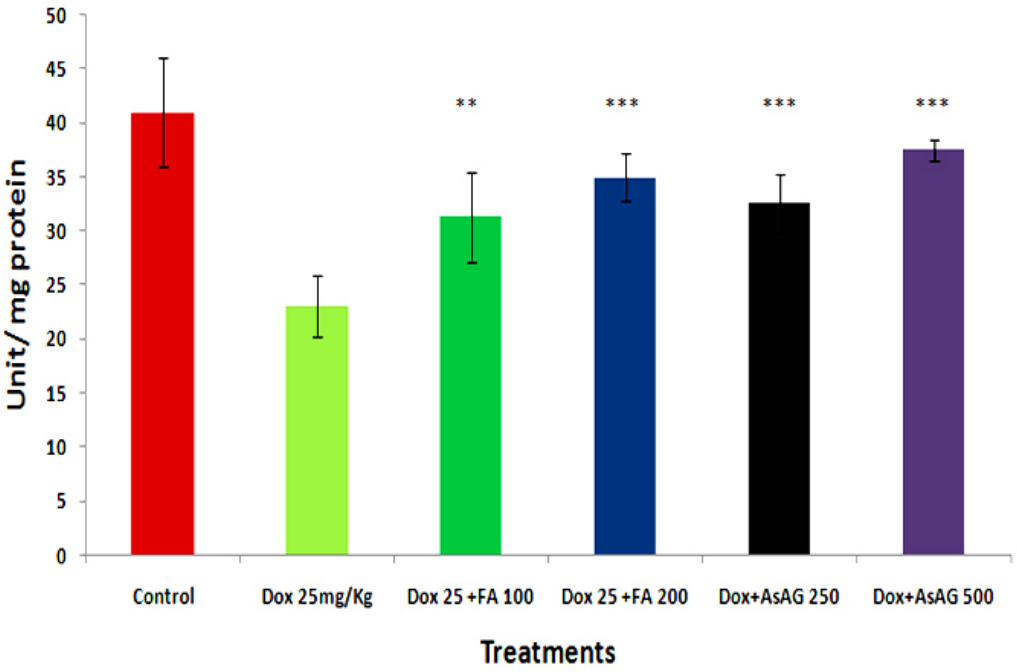

Figure 6.6 Effect of post administration of AsAG or FA on doxorubicin induced depletion of GPx levels in heart tissue of tumor bearing mice. Values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. ** represent p< 0.01 vs DOX control; * represent p< 0.001 vs DOX control**

In tumor tissues the MDA levels were increased significantly in the DOX treated group compared with the untreated control group. The post administration of both AsAG / FA along with DOX effectively potentiated the formation of MDA in a significant manner (**Figure 6.7**). The antioxidant enzymes such as GPx, SOD and GSH activities in the tumor tissues were significantly reduced in the DOX and AsAG or ferulic acid treated group when compared to the respective control (**Figure 6.8, 6.9 & 6.10**).

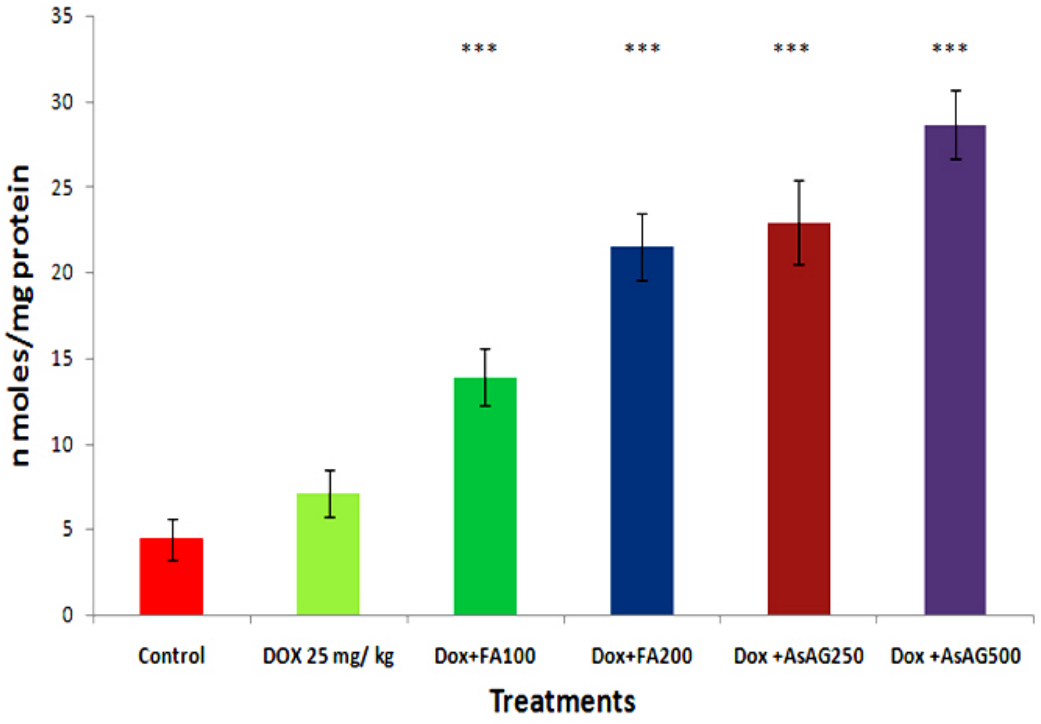

Figure 6.7 Effect of administration of AsAG or Ferulic acid on doxorubicin-induced lipid peroxidation (MDA formation) in tumor tissues of mice. (* indicate p<0.001 when compared with the DOX alone treated group).**

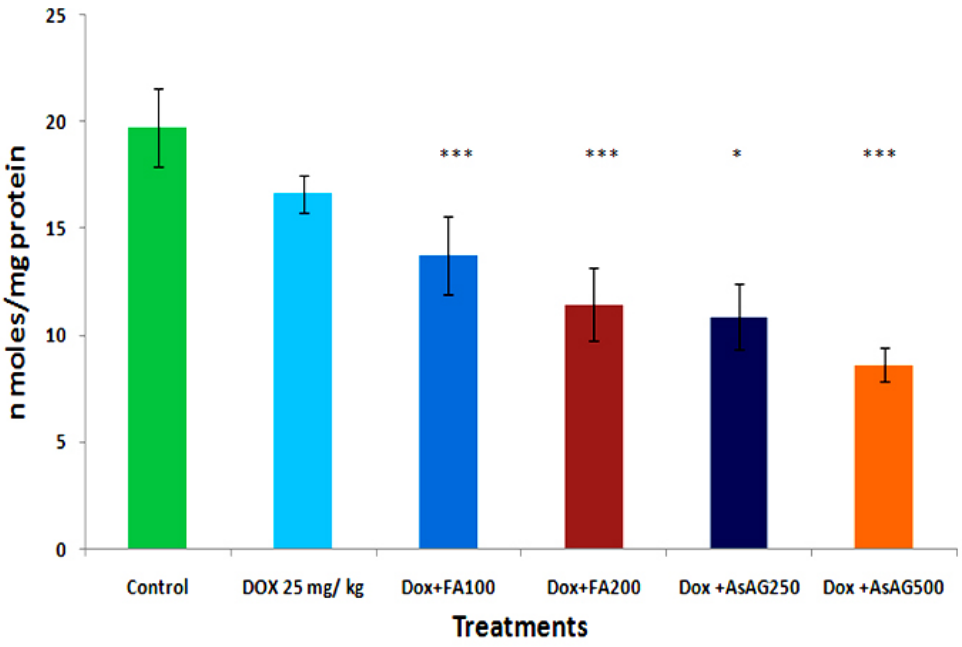

Figure 6.8 Effect of *p.o* administration of AsAG or ferulic acid on doxorubicin induced depletion of GSH levels in tumor tissues of mice. Values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. *represents p<0.05 vs DOX control; ***represent p< 0.001 vs DOX control

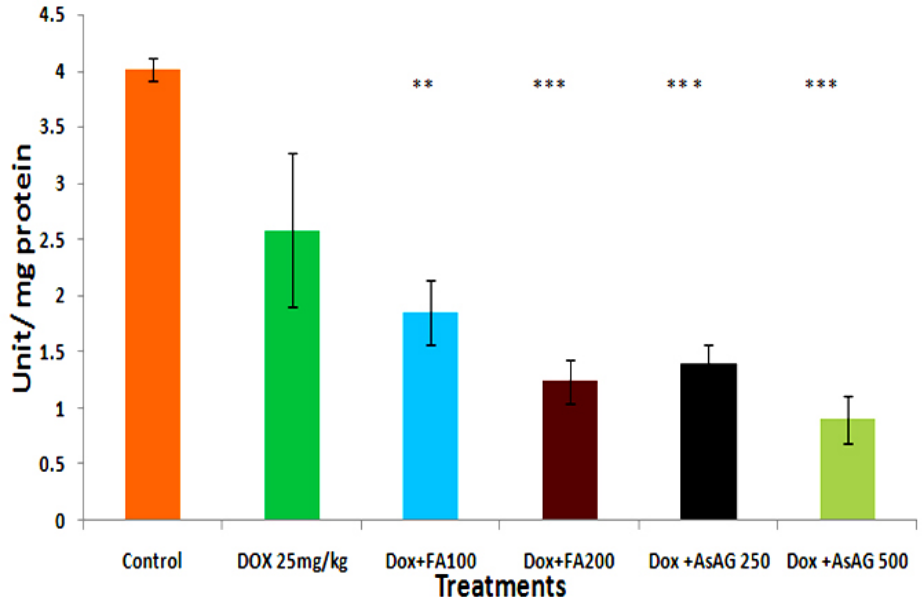

Figure 6.9 Effect of *p.o* administration of AsAG or ferulic acid on doxorubicin induced depletion of SOD levels in tumor tissues of mice. Values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. ** represent p< 0.01 vs DOX control ; ***represent p< 0.001 vs DOX control

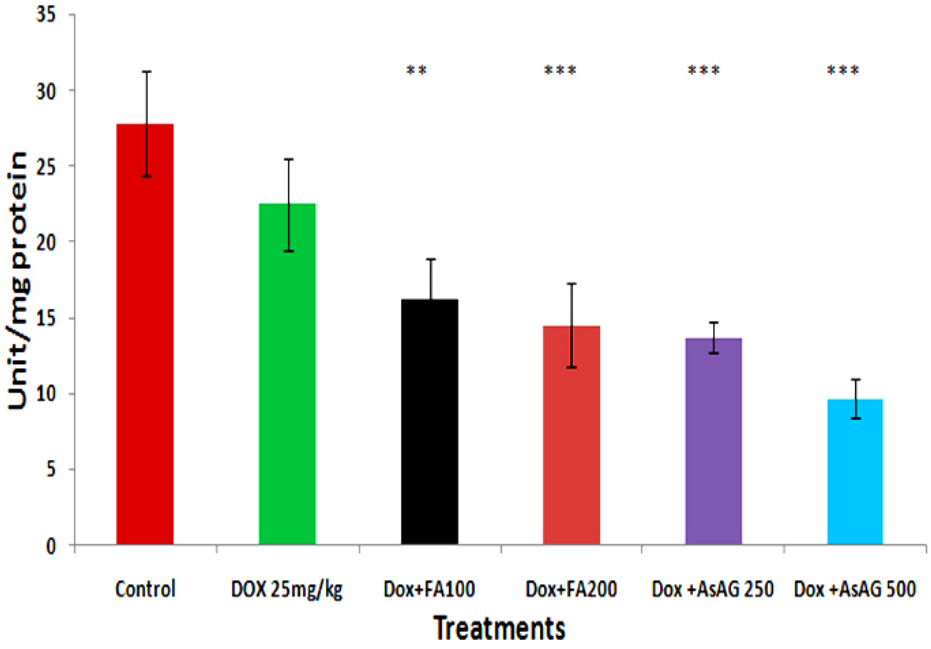

Figure 6.10 Effect of *p.o* administration of AsAG or ferulic acid on doxorubicin induced depletion of GPx levels in tumor tissues of mice. Values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. ** represent p< 0.01 vs DOX control ; ***represent p< 0.001 vs DOX control

6.3.3 Morphological Study

Histopathological examination of the heart tissues of normal animals showed clear integrity of myocardial cell membrane (**Figure 6.11 A**), and no inflammatory cell infiltration was seen. Light microscopic examination of hearts stained with hematoxylin and eosin of the control and FA and AsAG treated animals displayed a normal morphological appearance. Whereas the hearts of the DOX-treated animals showed myocardial degeneration including the loss of myofibrils, focal cytoplasmic vacuolization and endocardium appear disrupted in some places (**Figure 6.11 B**). In Tumor bearing mice treated with AsAG / FA and DOX, a significant reduction in the severity of myocardial degeneration was observed (**Figure 6.11 C & D**).

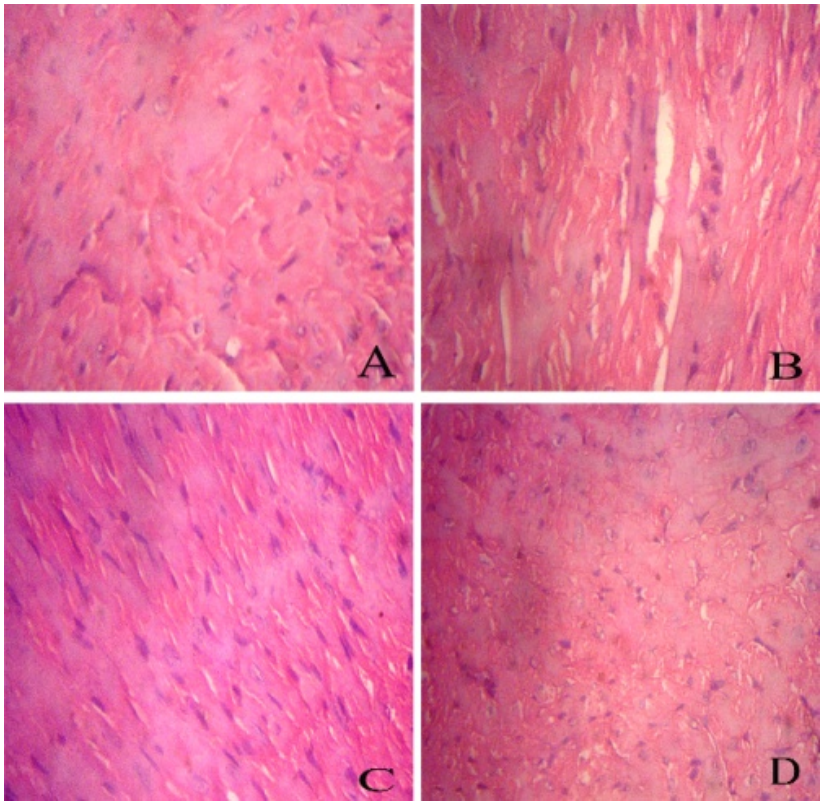

Figure 6. 11 Histopathology. Representative sections of heart tissue of tumor bearing mice (A) untreated (B) DOX 25 mg/kg (C) DOX+FA 200 mg/kg and (D) DOX+AsAG 500 mg/kg

The histopathological examination of tumor tissues is detailed in the **Figure 6.12**. Tumor tissues of control animals showed compact arrangement of carcinomatous cells having clear cytoplasm and hyperchromatic nuclei (**Figure 6.12 A**). While the DOX treated animals showed many degenerating cells, stroma displayed extensive areas of necrosis and haemorrhage (**Figure 6.12 B**). Tumor bearing animals administered with AsAG or FA and DOX, showed a significant increase in the number of degenerating cells. Stroma displayed extensive areas of necrosis and cellular integrity was found lost in many regions. The results showed that AsAG or FA potentiates the cytotoxic action of doxorubicin in tumor cells (**Figure 6.12 C & D**).

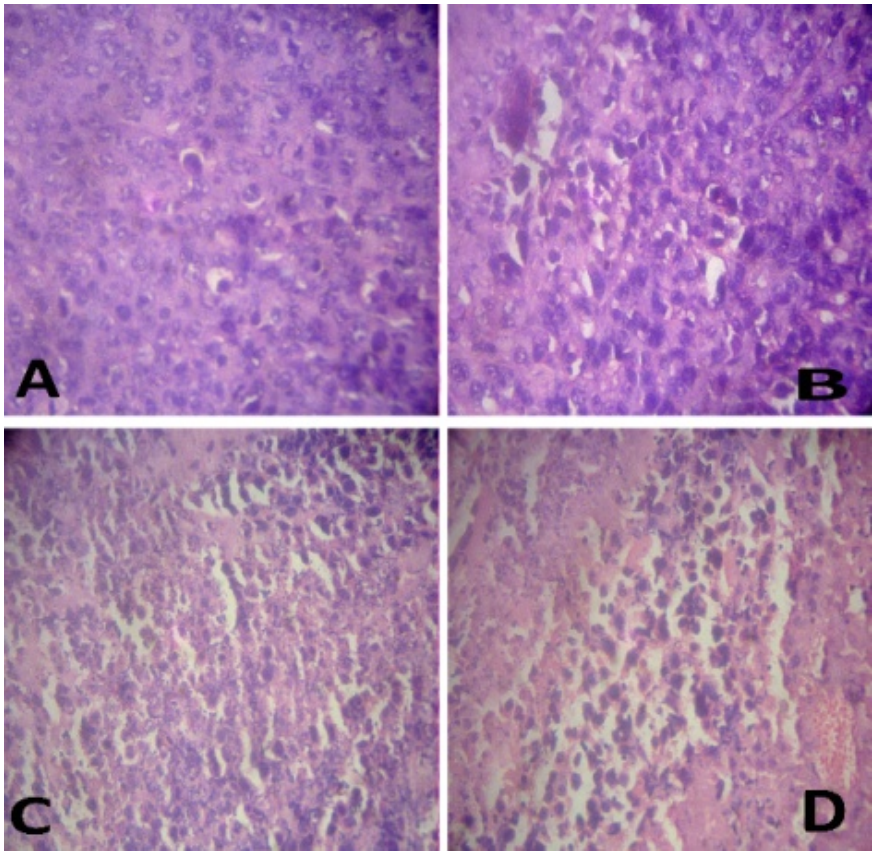

Figure 6.12 Histopathology. Representative sections of tumor tissue of tumor bearing mice (A) untreated (B) DOX 25 mg/kg (C) DOX+FA 200 mg/kg and (D) DOX+AsAG 500 mg/kg

6.3.4 Estimation of Cellular DNA Damage in Heart and Tumor Tissues of Tumor Bearing Mice

The administration of DOX caused damage to the cellular DNA in both tissues such as heart (**Figure 6.13 & 6.14**) and tumor (**Figure 6.15 &6.16**) as evident from the increase in the comet parameters. In the case of heart tissues, tail DNA %, tail length, tail moment and Olive tail moment were increased from 3.51±0.67, 3.30±0.79, 0.20±0.06 and 0.23±0.09 to 8.15±1.52, 8.61±1.83, 1.19±0.19, 0.95±0.11 in the control DOX- treated group. When 100 mg/kg body weight of FA was given orally one hr after DOX- administration, it was found that there is a significant decrease in the comet parameters in heart tissue, as can be seen in **Figure 6.13**. The parameters such as % DNA in tail, tail length, tail moment and olive tail moment were brought down

to levels of 6.13±0.62, 6.37±1.11, 0.90±0.25 and 0.76±0.15 respectively (P<0.001). While, 200mg/kg body weight of FA significantly decreased the comet parameters to 4.58±0.34, 3.51±0.89, 0.45±0.16 and 0.46±0.12 showing cardio protection (P<0.001).

When 250 mg/ Kg body weight of AsAG was administered the parameters were brought down to 5.87±.58, 5.86±0.79, 0.56±0.09 and 0.55±0.07 respectively. While 500 mg/ Kg body weight of AsAG significantly decreased the comet parameters in a dose dependent manner (**Figure 6.14**). The results clearly indicated the ability AsAG or FA to offer protection to cellular DNA against DOX- induced cardiomyopathy.

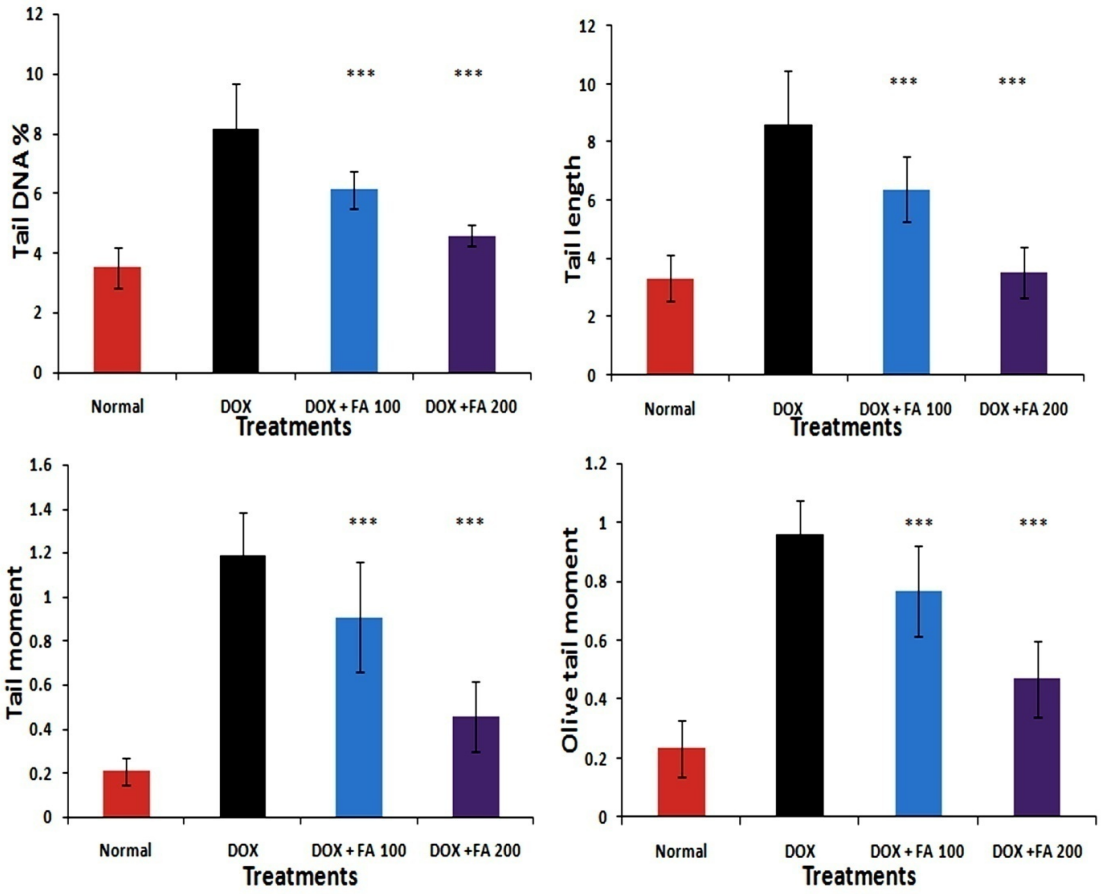

Figure 6.13 Effect of Ferulic acid (100 mg/kg and 200 mg/kg) on DNA damage in mice cardiomyocytes induced by administration of doxorubicin (25mg/kg) assessed by comet assay. Percentage DNA in tail, tail length, tail moment and olive tail moment is presented as mean ± sd (* indicate p < 0.001 when compared with DOX alone treated group)**

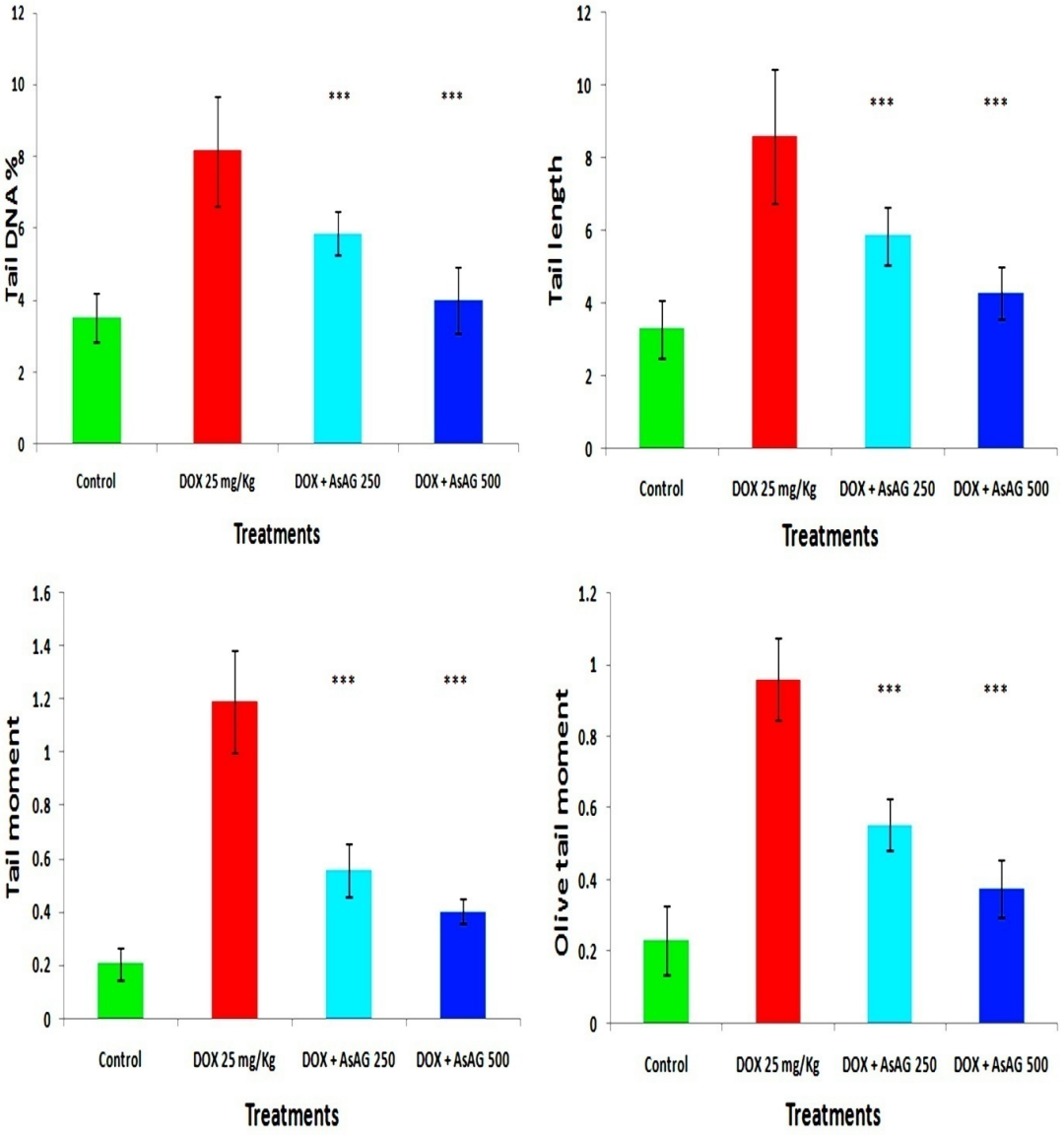

Figure 6.14 Effect of AsAG (250 mg/kg and 500 mg/kg) on DNA damage in mice cardiomyocytes induced by administration of doxorubicin (25mg/kg) assessed by comet assay. Percentage DNA in tail, tail length, tail moment and olive tail moment is presented as mean ± sd (* indicate $p < 0.001$ when compared with DOX alone treated group**

In tumor tissues, administration of DOX caused DNA damage and percentage DNA in tail, tail length, tail moment and Olive tail moment were increased from 4.47± 0.90, 3.50 ± 1.02, 0.16 ± 0.02 and 0.26 ± 0.06 to 5.39 ± 0.79, 5.94 ± 0.52, 1.37 ± 0.12 and 0.96 ± 0.25 in the DOX treated group (**Figure 6.15 & 6.16**). The comet parameters such as tail DNA %, tail length, tail moment and Olive tail moment were increased to

6.13 ± 0.35, 6.8 ± 0.24, 1.68 ± 0.09 and 1.93± 0.15 in the DOX with 100 mg/kg body weight of FA treated group. When 200mg/kg body weight of FA along with DOX significantly increased the comet parameters to 7.55 ± 0.11, 7.66± 0.05, 1.92 ±0.08 and 1.93 ± 0.15 showing the antitumor potential of FA (**Figure 6.15**). While 500 mg/ Kg body weight of AsAG significantly increased the parameters to 8.4±0.52, 8.1±0.73, 3.04±0.36 and 3.2±0.57 showing enhanced antitumor efficacy (**Figure 6.16**).

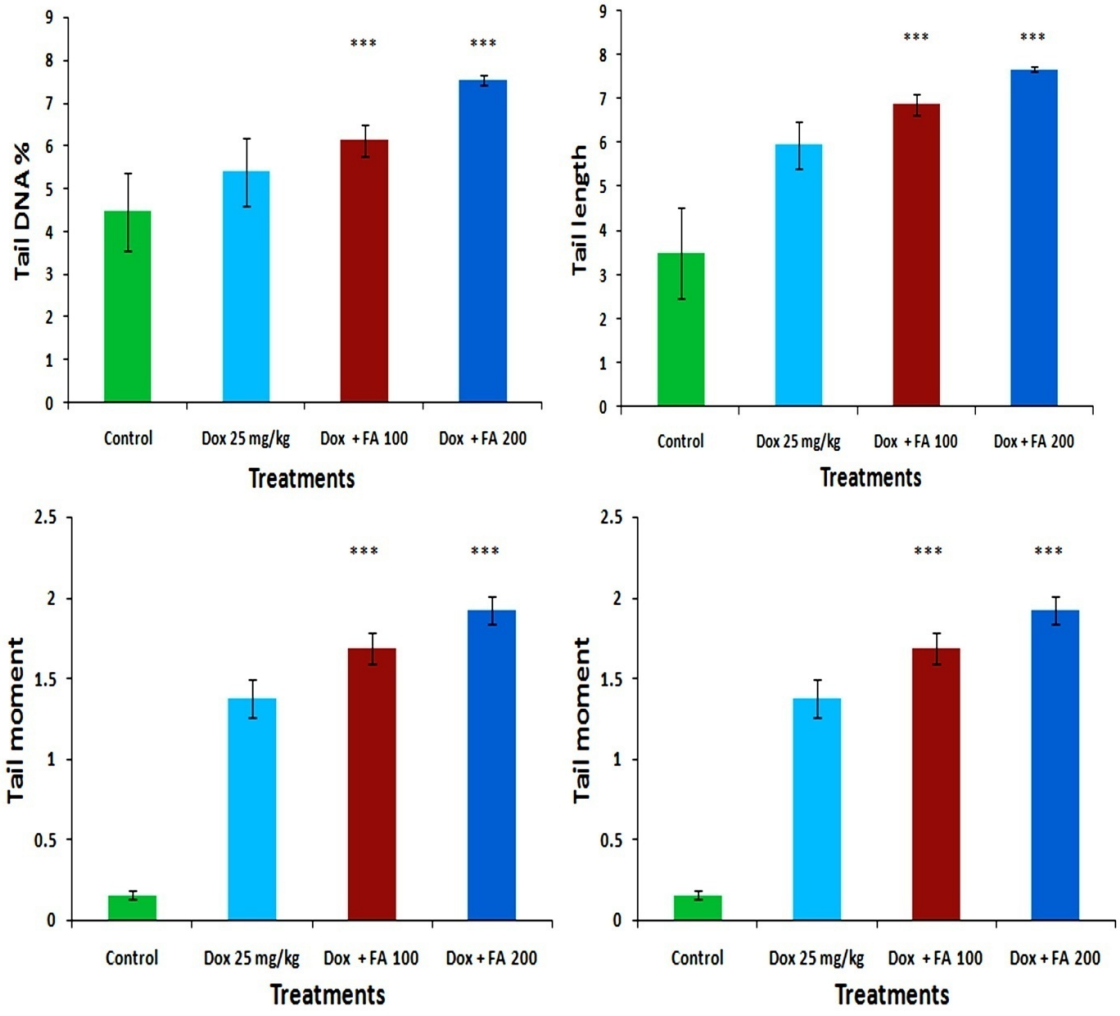

Figure 6.15 Effect of Ferulic acid (100 mg/kg and 200 mg/kg) on DNA damage in tumor tissues of tumor bearing mice induced by administration of doxorubicin (25mg/kg) assessed by comet assay. Percentage DNA in tail, tail length, tail moment and olive tail moment is presented as mean ± sd (* indicate $p < 0.001$ when compared with DOX alone treated group)**

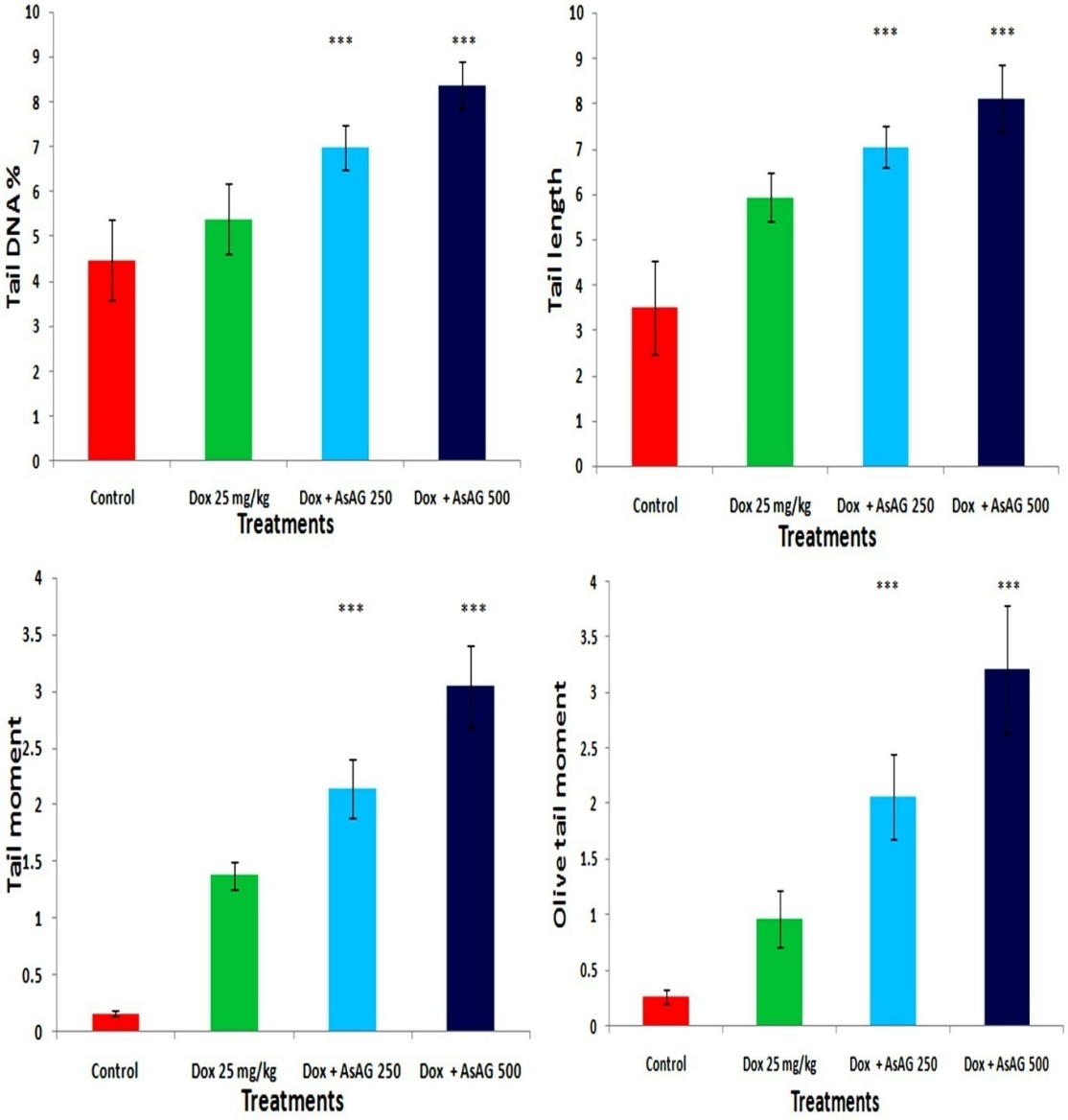

Figure 6.16 Effect of AsAG (250 mg/kg and 500 mg/kg) on DNA damage in tumor tissues of tumor bearing mice induced by administration of doxorubicin (25mg/kg) assessed by comet assay. Percentage DNA in tail, tail length, tail moment and olive tail moment is presented as mean ± sd (* indicate $p < 0.001$ when compared with DOX alone treated group)**

6.4 DISCUSSION

Various studies have demonstrated the generation of a cascade of reactive oxygen species (ROS) such as O_2-, ·OH and H_2O_2, which are implicated in the DOX-induced car-diotoxicity (Kalivendi *et al*., 2001; Wang *et al*., 2004). The semiquinone form, produced by the reduction of DOX by several endogenous enzymes, generates O_2- radical by transferring electrons to molecular oxygen. The superoxide radicals are rapidly transformed, either spontaneously or enzymatically, into other forms of ROS such as ·OH and H_2O_2 (Lee *et al*., 1991). Among the postulated mechanisms of doxorubicin cardiomyopathy, like radical formation with lipid peroxidation, depletion of cardiac reduced glutathione level, inhibition of cardiac coenzyme Q10 and a decrease in cardiac adenylate charge. A number of measures to protect against cardiomyopathy have been investigated. The most encouraging ones among them are concurrent administration of vitamin E, coenzyme Q10, N-acetylcysteine, cysteamine, razoxane (ICRF 159) and its isomer, ICRF 187, and administration of doxorubicin by continuous intravenous infusion or within liposomal carriers (Saltiel and McGuire, 1983; Afsar *et al*., 2017).

The results of the study specify that *i.p* injection of DOX at a dose of 25 mg /kg b.w, induced oxidative stress in cardiac tissues as shown by elevated serum LDH and CK levels. These enzymes are mostly considered as important markers of early and late cardiac injury particularly during clinical follow-up of doxorubicin therapy (Alkuraishy *et al*., 2017; Kalyanaraman, 2020). Earlier reports have demonstrated similar elevations in cardiac enzymes activities in rats following challenge with a single cumulative dose of doxorubicin (Nagi and Mansour 2000; Yagmurca *et al*., 2003; Vijay *et al*., 2011; Ragavendran *et al*., 2012). Administration of AsAG or FA, significantly protected mice from DOX-induced elevated levels of LDH and CK. The

levels of serum SGOT and SGPT were also elevated in DOX treated animals and post administration of AsAG or FA significantly reversed the levels of these enzymes when compared to DOX alone treated control animals. The increased elevation of SGOT and SGPT levels in serum could result from tumor cell production. The elevated SGOT and SGPT levels indicate abnormal liver function secondary to Tumor growth (Popp *et al*., 1984). Increase in the level of HDL indicates that doxorubicin may be interfering with metabolism or biosynthesis of lipids (Koti *et al*., 2009; Wu *et al*., 2019). Post-treatment with AsAG or FA following DOX administration showed significant increase in HDL cholesterol.

Administration of Doxorubicin induced oxidative stress in cardiac tissues as determined by the alterations observed in cardiac antioxidant defense systems both enzymatic and non-enzymatic. The present study, showed that DOX significantly reduced the cardiac GSH content, in addition it notably lowered the cardiac enzymatic activities of SOD and GPx associated with a notable increase in cardiac lipid peroxidation as indicated by increased MDA level. Administration of both the doses of 100 mg/kg and 200 mg/kg of FA or 250 mg/ kg and 500 mg/kg of AsAG inhibited the DOX induced consequences and significantly increased the antioxidant enzymes SOD and GPx. In addition, the enhancement in cardiac GSH status and decrease in tissue MDA levels, indicated a significant reduction in the extent of cellular oxidative damage by ASAG or FA. Moreover the histopathological reports suggest that post-treatment of AsAG or FA greatly inhibited the DOX induced changes in cardiac tissue supporting the protective action of both AsAG or FA against DOX induced cardiotoxicity (Othman *et al*., 2020).

The stimulation of oxidative damage by DOX marks in extensive damage to cellular DNA as seen in comet assay. A greater portion of DNA has fragmented and migrated

sufficiently to make the long tail. Due to high myocardial infarction the cellular DNA undergo damage, as revealed in the increase in comet parameters (tail length, % DNA in tail, tail moment and olive tail moment) in DOX treated groups. Post administration of AsAG or FA decreased the comet parameters indicative of its protecting ability.

A main prerequisite for any compound to be used as a cardioprotective agent during the treatment of cancer is that it should not interfere with the antitumor activity of the chemotherapy. Reports indicate that AsAG exhibits vitamin C activity *in vivo* following enzymatic hydrolysis by α-glucosidase (Fujinami *et al*., 2001). Several studies in animals and humans confirmed that FA demonstrates anticancer properties (Lesca, 1983; Asanoma *et al*., 1994). The MDA levels of tumor tissues in FA and DOX treated animals showed an increase in the level when compared to the untreated control animals. The antioxidant levels in tumor tissues were also found to be significantly reduced by the administration of doxorubicin and ferulic acid or AsAG. The comet assay analysis showed that DNA damage was significantly enhanced in the tumor tissues revealing the anti-tumor potential of the combination treatment.

Administration of Ascorbic acid mono glucoside or Ferulic acid after DOX treatment in tumor bearing Swiss albino mice restored all the biochemical parameters altered by this cytotoxic anticancer drug to near normal levels in the cardiac tissue. AsAG or FA prevented the DOX induced myocardial toxicity by boosting the endogenous antioxidant activity, lowering the cardiac biomarker enzymes (CK and LDH) and preventing the degeneration of cardiac tissue. Further, the combined modality showed better antitumor activity as seen from the antioxidant enzyme levels, extent of membrane lipid peroxidation, DNA damage (comet assay) and histopathological analysis. The combined treatment of DOX and AsAG /FA holds promise as a safe and effective chemotherapeutic strategy.

Chapter- 7

AMELIORATION OF CISPLATIN INDUCED NEPHROTOXICITY IN TUMOR BEARING ANIMALS BY ASCORBIC ACID MONOGLUCOSIDE (AsAG) AND FERULIC ACID (FA)

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7.4 DISCUSSION

7.1 INTRODUCTION

Nephrotoxicity arises as a result of direct exposure to drugs and environmental chemicals to renal tissue (Sales and Foresto, 2020). Many therapeutic agents such as oxaliplatin, cisplatin, paclitaxel, carboplatin, doclitaxel, vinorelbine, topotecan, etc. are responsible for the induction of nephrotoxicity. These agents are used as chemotherapeutic drugs against different forms of human cancers (Jagieła *et al*., 2021). To control neoplastic disorders and distant metastases, these agents are administered systemically, which adversely affects normal cells, resulting in cytotoxicity (Janakiraman and Jayaprakash, 2015).

Cis-Diamminedi chloroplatinum II (Cisplatin or CP or Cis) is one of the foremost effective cancer therapeutic agents, used in the treatment of several solid tumors and refractory non-Hodgkin's lymphomas (Giaccone, 2000). The therapeutic efficacy of cisplatin is usually related to nephrotoxicity (Arany and Safirstein, 2003;George and Anushree, 2014). The nephrotoxicity by cisplatin is associated with cellular necrosis, alterations within the number and size of the lysosomes, morphological damage of intracellular organelles, mitochondrial vacuolization, loss of microvilli, and functional changes as inhibition of protein synthesis, lipid peroxidation, mitochondrial damage, and GSH depletion (Awdishu and Mehta, 2017). Several natural agents like vitamins and/or dietary supplements have the potential to diminish the physiological side effects of those drugs (Ali and AlMoundhri, 2006; Rad *et al*., 2017). Such antioxidants protect normal tissues against the deleterious effects of the drug and may be safely manipulated without toxic manifestations (Ojha *et al*., 2016).

Several distinct mechanisms have been proposed for cisplatin cytotoxicity in renal tubule cells, including direct DNA damage (Leibbrandt *et al*., 1995), caspase

activation (Kaushal *et al*., 2001), mitochondrial dysfunction (Sugiyama *et al*., 1989), formation of reactive oxygen species (Matsushima *et al*., 1998), effects on the endoplasmic reticulum (Baliga and Liu, 2004) and activation of TNF-α apoptotic pathways (Ramesh and Reeves, 2002). It has also been reported that cisplatin induced nephrotoxicity is closely associated with an increase in lipid peroxidation in the kidney. Besides, cisplatin has been found to lower the activities of antioxidant enzymes and to induce depletion of GSH.

This antitumor drug also causes generation of reactive oxygen species (ROS), such as superoxide anion and hydroxyl radical that deplete the GSH levels and inhibit the activity of antioxidant enzymes in renal tissue. The ROS can produce cellular injury and necrosis via some mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage (Kim *et al*., 1997; Mora *et al*., 2003). Glutathione, an endogenous free thiol, has been reported to decrease cisplatin nephrotoxicity (Somani *et al*., 1995). The depletion of GSH seems to be a prime factor that permits lipid peroxidation (Younes and Siegers, 1981). Administration of superoxide dismutase (SOD) and antioxidants (GSH) ameliorates cisplatin nephrotoxicity in experimental animals (Anderson *et al*., 1990). SOD plays an important role in the dismutation of superoxide anions by catalyzing their conversion to hydrogen peroxide and singlet oxygen. Glutathione peroxidise (GPx) activity was found decreased in the rat renal mitochondria incubated with cisplatin and has been linked to disturbances in GSH metabolism (Sugiyama *et al*., 1989).

The importance of reactive oxygen metabolites (ROM) in cisplatin-induced renal cell apoptosis has been documented in several studies (Ueda *et al*., 2000). It is well known that mitochondria continuously produce ROM such as superoxide. Also mitochondria continuously scavenge ROM via the action of antioxidant enzymes such

as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase and glutathione- S-transferrase (GST) (Richter *et al*., 1995). Cisplatin is known to accumulate in mitochondria of renal epithelial cells (Gemba and Fukuishi, 1991). Several investigators have demonstrated that cisplatin induces ROM in renal epithelial cells primarily by decreasing the activity of antioxidant enzymes and depleting the intracellular concentration of GSH (Sadzuka *et al*., 1992).

A large number of studies have reported the beneficial effects of a variety of antioxidants in cisplatin induced nephrotoxicity. Various agents like SOD, dimethyl thiourea and GSH have been shown to reduce the degree of renal failure and tubular cell damage when administered along with cisplatin in rats (Sadzuka *et al*., 1992; Matsushima *et al*., 1998). Much consideration has been given to the possible role of dietary antioxidants in protecting the kidney against cisplatin induced nephrotoxicity. There are various reports available on the chemoprotecting activities of vitamin C, E, curcumin, selenium, bixin and other dietary components that scavenge free radicals induced by exposure to cisplatin (Antunes *et al*., 2001; Silva *et al*., 2001).

FA is a strong scavenger of free radicals and it has been approved in certain countries as food additive to prevent lipid peroxidation (Sreenivasan *et al*., 2007; Panche *et al*., 2016). FA possesses radioprotective abilities and reduces ionizing radiation induced damages to DNA and membranes in biological systems (Maurya *et al*., 2005; Kumar and Goel, 2019).

The present study envisages exploring the effect of ferulic acid (FA) and ascorbic acid mono glucoside (AsAG) on Cis- induced nephrotoxicity in tumor bearing Swiss albino mice.

7.2 MATERIALS AND METHODS

7.2.1 Animals

Female Swiss albino mice (8 - 10 weeks old) weighing 22 - 25 g, obtained from the Small Animal Breeding Section (SABS), Mannuthy, Thrissur, Kerala were used for the study.

7.2.2 Chemicals

Ascorbic acid monoglucoside (AsAG) were from Dr. V. T. Kagiya, Health Research Foundation, Kyoto, Japan. Ferulic acid (FA), Nitroblue tetrazolium (NBT), riboflavin, reduced glutathione (GSH), 5-5' dithiobis-2- nitro benzoic acid (DTNB) were purchased from Sigma Chemical Company Inc., St. Louis, MO, USA. H_2O_2 was from Merck India Ltd., Mumbai, India. Thiobarbituric acid (TBA) was purchased from Hi-media Laboratories, Mumbai, India. All other chemicals and reagents used in this study were of analytical grade procured from reputed Indian manufacturers.

7.2.3 Administration of Drugs

Cisplatin was administered intraperitoneally as a single dose of 12mg/kg b.w.

*Solid tumor in Animals***-** Solid tumor was induced in hind limbs of mice by transferring Dalton's Lymphoma Ascites (DLA) cells (1×10^6 cells/animal) through subcutaneous injection. The animals were given standard mouse chow and water *ad libitum*. The treatments were started when the tumor size reach upto 1.0 cm^3 on the 13^{th} day of transplantation of DLA cells. The animals were randomly divided into 6 groups of five each and treated as follows.

Group – I were kept untreated control group, 0.1 ml saline *i.p* and 0.1 ml distilled water *p.o* . Group – II served as Cis control, cisplatin 12 mg/kg b.w **(**in 0.1 ml saline *i.p* as single dose).

Group – III Cisplatin (12 mg/kg b.w in 0.1 ml saline) *i.p* as single dose + AsAG 250 mg/kg b.w **(**in 0.1 ml distilled water *p.o*)

Group – IV Cisplatin (12 mg/kg b.w in 0.1 ml saline) *i.p* as single dose +AsAG 500 mg/kg b.w **(** in 0.1 ml distilled water *p.o*)

Group V- Cisplatin (12 mg/kg b.w in 0.1 ml saline) *i.p* as single dose + Ferulic acid 100 mg/kg b.w **(**in 0.1 ml distilled water *p.o*).

Group VI- Cisplatin (12 mg/kg b.w in 0.1 ml saline) i.p as single dose + Ferulic acid 200 mg/kg b.w **(**in 0.1 ml distilled water *p.o*).

AsAG was administered to group III and IV, Ferulic acid was administered to Group V and VI th group animals, one hour after Cis (12 mg/kg b.w) administration.

7.2.4 Assessment of nephrotoxicity

Serum creatinine was determined by the alkaline picric acid method (Allen *et al*., 1982) using a diagnostic kit (Agappe Diagnostic Pvt. Ltd; Ernakulam, Kerala, India). Serum urea was determined by diacetylmonoxime (DAM) reagent using the Agappe diagnostic kit (Kassirer, 1971). In the case of cisplatin-treated animals, seventy-two hours after cisplatin treatment (12 mg/kg as an intraperitoneal injection) animals were sacrificed using ether anesthesia, blood was collected directly from the heart, serum separated for urea and creatinine analysis. Kidneys and tumors were excised for analyzing the antioxidant status and for histopathological examinations (Divakaran and Nair, 2012). Reduced glutathione (GSH) level was measured colorimetrically using DTNB as the substrate. The concentrations of malondialdehyde (MDA) as indices of lipid peroxidation were assessed according to the method of Buege and Aust (1978). Superoxide dismutase activity was determined by the nitroblue tetrazolium reduction method of Mc Cord and Fridovich (1969). GPx activity was determined by the method of Hafemann *et al*. (1974), based on the degradation of

H_2O_2 in the presence of GSH. Tissue protein was studied according to the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

From the animals, peripheral blood was collected by tail veining method at 24th hour of CP treatment.

An aliquot of blood (5µL) was placed directly onto the acridine orange coated glass slide and covered with a cover slip (Hayashi *et al.*, 1990) for micronuclei analysis.

7.2.5 Histopathological studies

Histopathological examinations of kidney and tumors from all the treated groups were evaluated using light microscopy. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin-eosin. The histopathological studies were done at Sudharma Metrapolis Pathological Laboratory, Thrissur, Kerala, India.

7.3 RESULTS

7.3.1 Effect on serum creatinine and urea levels

Administration of cisplatin to mice was induced a marked renal failure, characterized by a significant increase in serum urea and blood creatinine levels. As shown in **Figure 7.1** and **Figure 7.2** serum urea and serum creatinine concentrations were significantly increased in the cisplatin (151.66±7.63 and 2.5±0.46) treated group compared to the untreated control group (44.33±4.0 and 0.53±0.11).

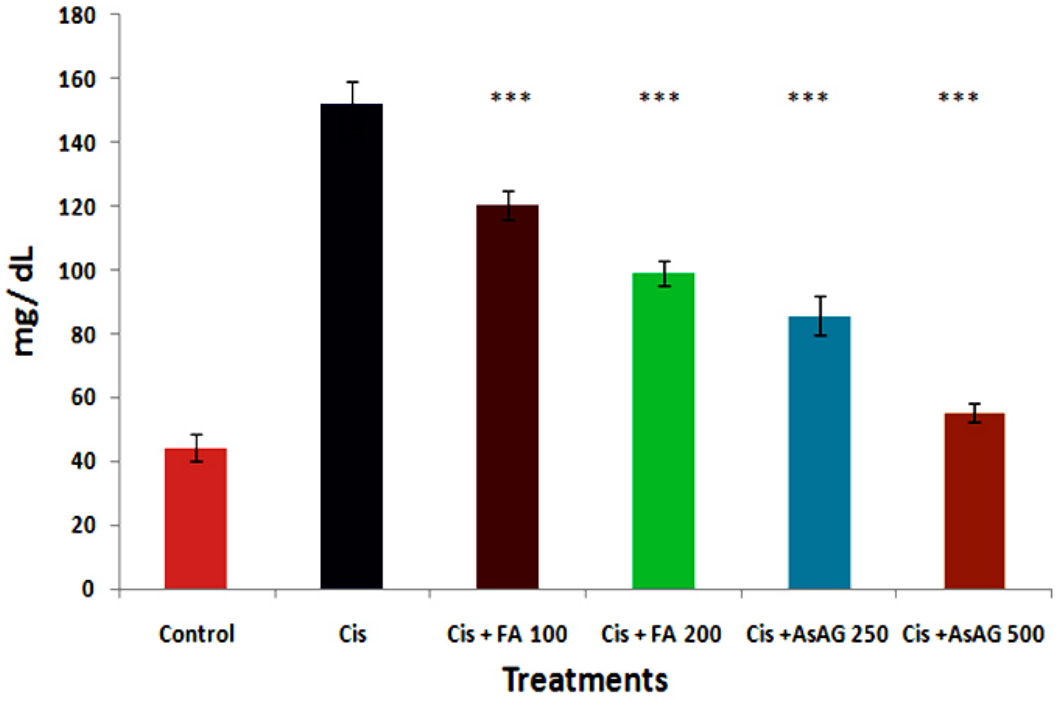


Figure 7.1 Effect of AsAG and FA on serum urea levels in tumor bearing mice treated with cisplatin (*represent p <0.001 when compared with cisplatin alone treated group).**

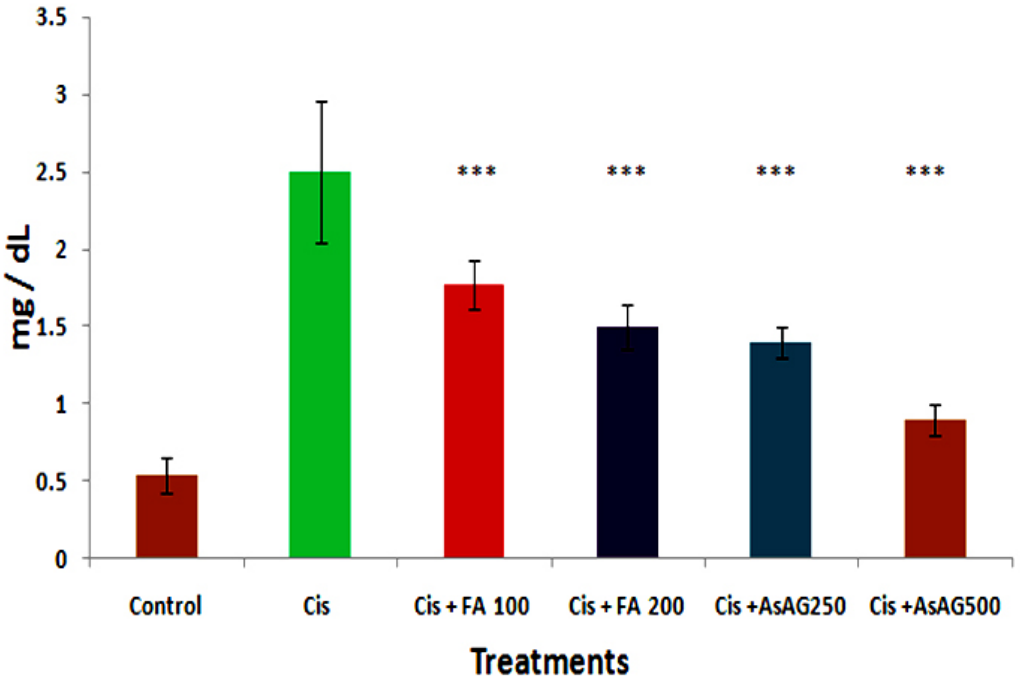


Figure 7.2 Effect of AsAG and FA on blood creatinine levels in tumor bearing mice treated with cisplatin (*represent p <0.001 when compared with cisplatin alone treated group).**

Treatment with the both AsAG and FA to tumor bearing animals showed marked decrease in concentrations of serum urea and creatinine compared to control group ($p<0.001$). Treatment with 500 mg/kg body weight of the AsAG restored the urea level to 55.33 ± 3.05 mg/dL and creatinine level to 0.9 ± 0.1 mg/dL in cisplatin administered animals. The post treatment with FA (100 mg/kg b.w and 200 mg/kg b.w) in tumor bearing animals significantly ameliorated urea and creatinine level in a dose dependent manner. Treatments with FA to Cis-challenged animals significantly decreased ($p<0.001$) the activities of serum urea and creatinine elevated by cisplatin, though not to normal.

7.3.2 Biochemical Measurements and Antioxidant Status

MDA (Malondialdehyde) was measured as a marker of lipid peroxidation and an indicator of oxidative injury. The MDA levels in kidney tissue were increased significantly in the Cis treated group compared with the untreated control group. The increase in MDA by Cis was significantly attenuated by the administration of AsAG and FA (**Figure 7.3**). Different antioxidant enzymes were examined in the kidney tissues from all the groups. The Cis-treated mice showed a significant decrease in SOD, GSH and GPX activities compared with the untreated control (**Figure 7.4, 7.5 & 7.6**). The decrease in activities of SOD, GPx and GSH due to cisplatin was attenuated by AsAG and FA.

Cisplatin treatment caused micronuclei formation in peripheral blood lymphocytes of tumor bearing mice. Administration of FA or AsAG to cisplatin treated animals significantly decreased the micronuclei frequency when compared to respective control groups (**Figure 7.7 &7.8**).

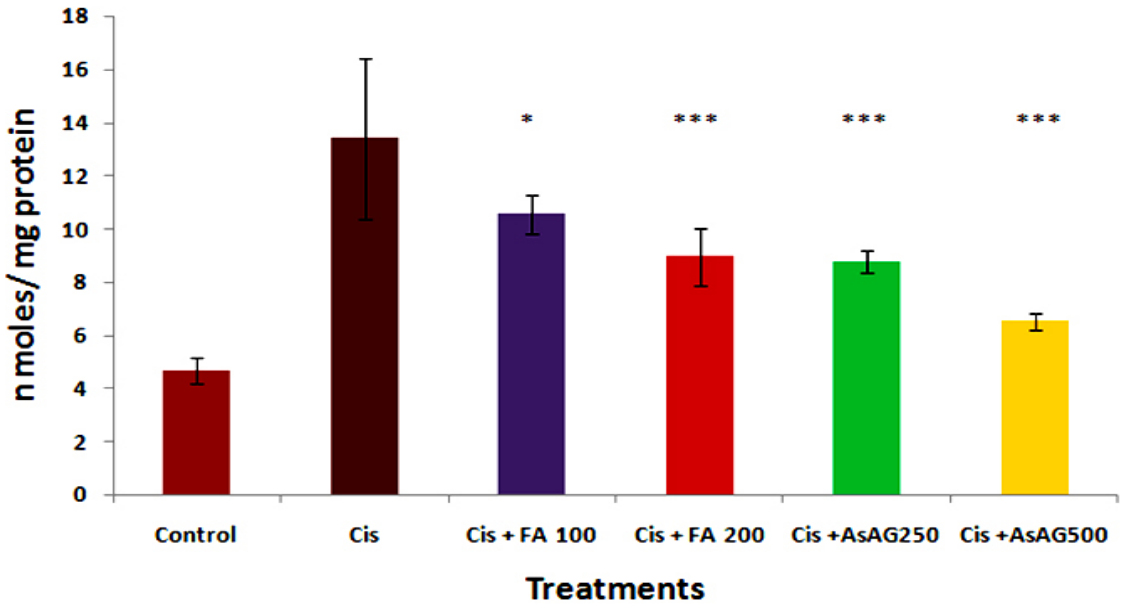


Figure 7.3 Effect of administration of AsAG and FA on cisplatin-induced lipid peroxidation. The lipid peroxides formed are expressed as nanomoles of MDA per mg potein ± SD.(* represent p <0.001 when compared with cisplatin alone treated group).**

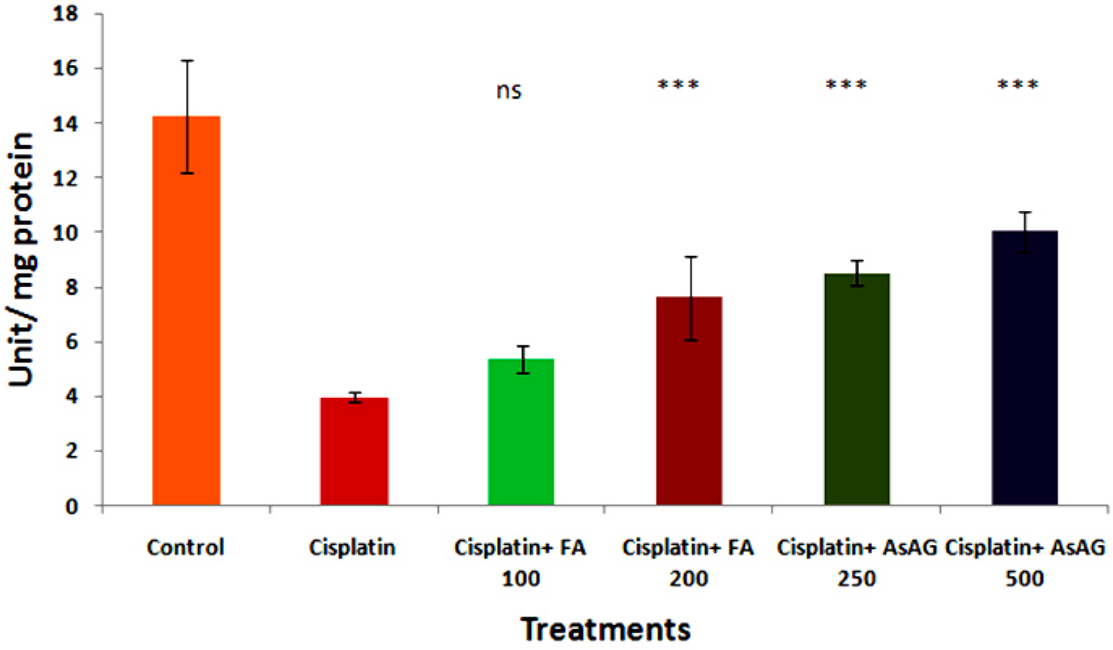


Figure 7.4 Effect of *p.o.* administration AsAG and FA on cisplatin-induced decrease in renal antioxidant, SOD enzymes (ns, not significant; ***p <0.001 when compared with cisplatin alone treated group).

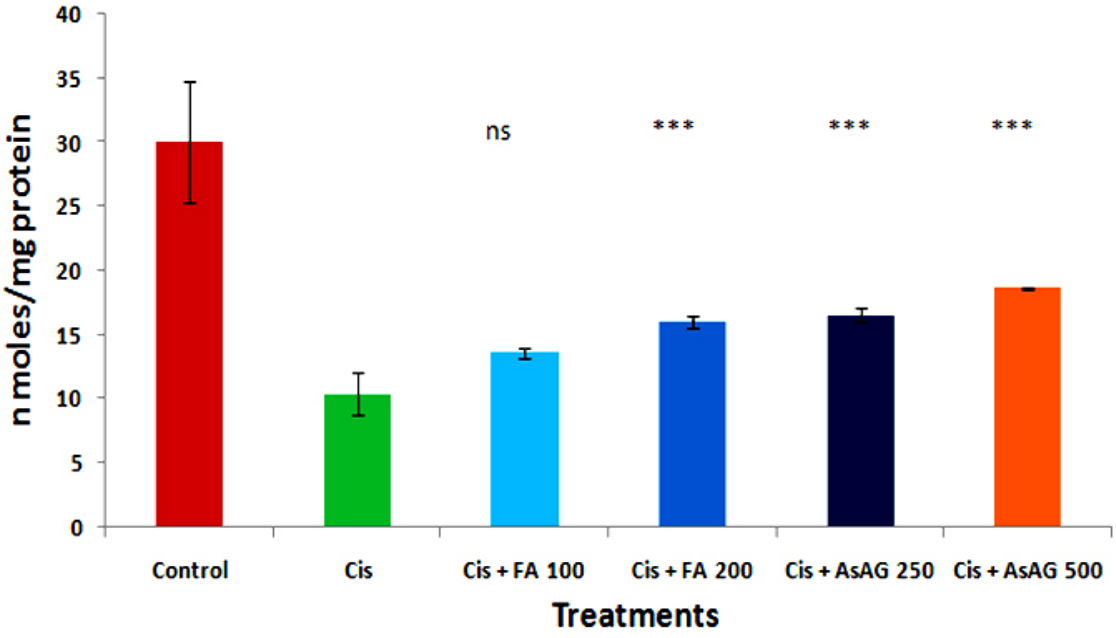


Figure 7. 5 Effect of *p.o.* administration AsAG and FA on cisplatin-induced decrease in renal antioxidant, GSH enzymes (ns, not significant; ***p <0.001 when compared with cisplatin alone treated group).

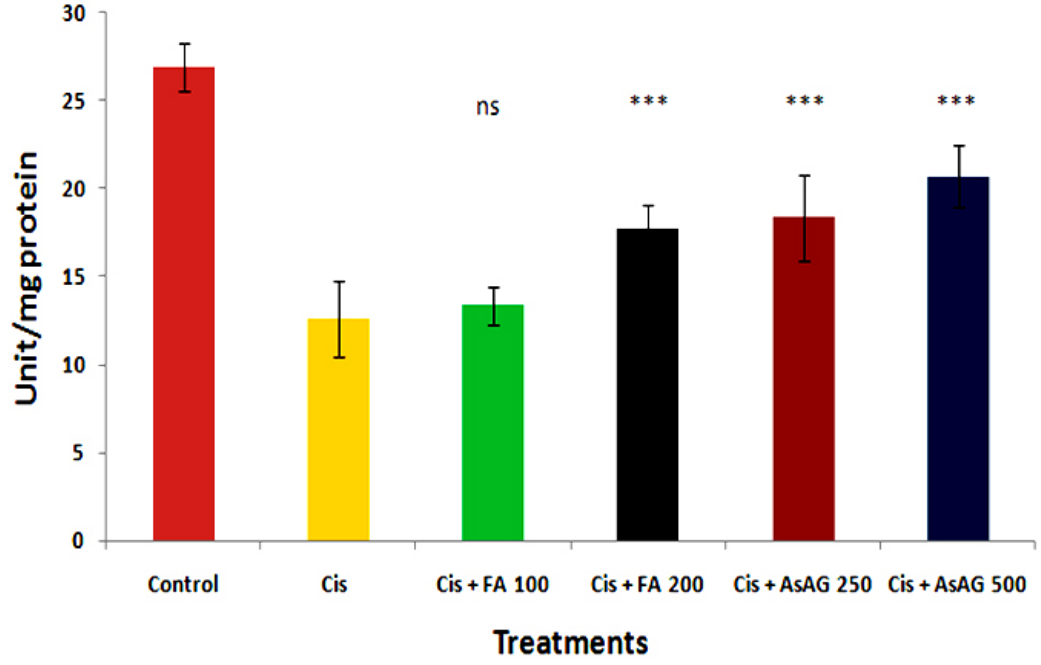


Figure 7.6 Effect of *p.o.* administration AsAG and FA on cisplatin-induced decrease in renal antioxidant, GPx enzymes (ns, not significant; ***p <0.001 when compared with cisplatin alone treated group).

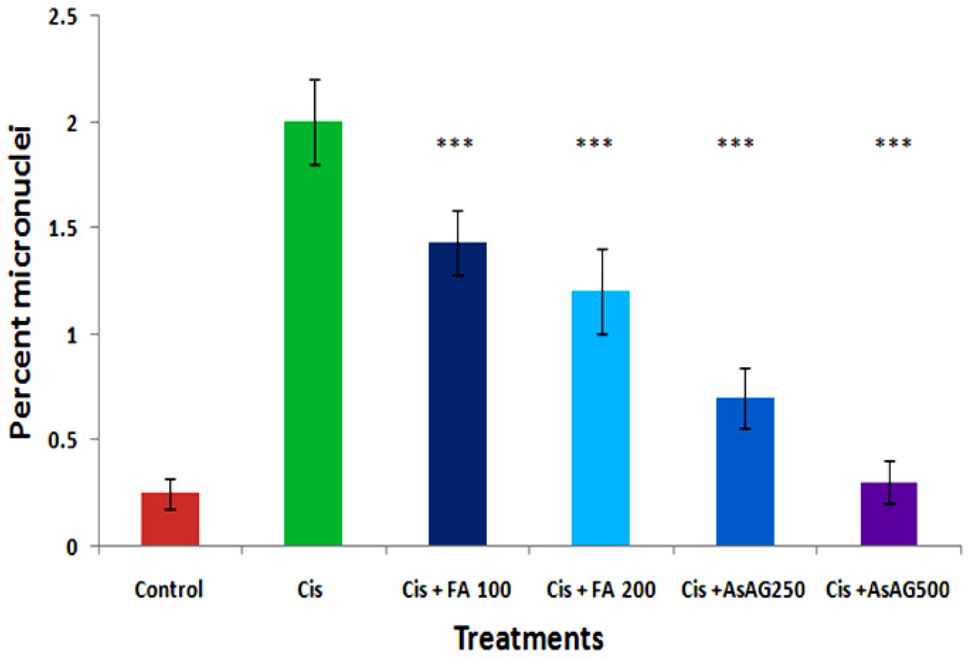
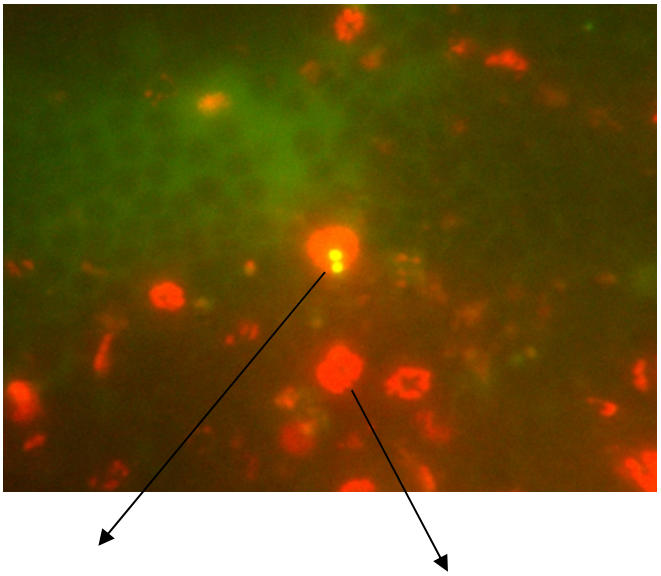


Figure 7.7 Effect of FA and AsAG administration on micronuclei frequency in tumor bearing mice. Values are expressed as mean ±SD. (* indicates p<0.001 when compared to Cisplatin treated group)**



Micronucleated reticulocyte **Normal reticulocyte**

Figure 7.8 Representative images of micronucleated and normal reticulocyte.

In tumor tissues the MDA levels were increased significantly in the Cis treated group compared with the untreated control group. The post administration of AsAG and FA along with Cis effectively potentiated the formation of MDA in a significant

manner (**Figure 7.9**). The antioxidant enzymes such as SOD, GPx and GSH activities in the tumor tissues were significantly reduced in the Cis and AsAG/FA treated group when compared to the respective control (**Figure 7.10, 7.11 &7.12**).

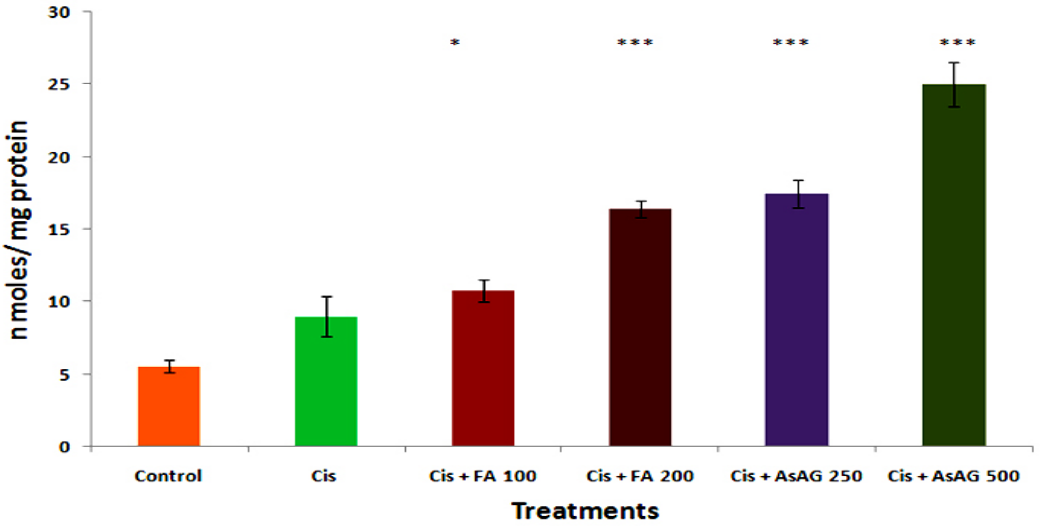


Figure 7.9 Effect of administration of AsAG and Ferulic acid on cisplatin induced lipid peroxidation (MDA formation) in tumor tissues of mice. (* indicate p<0.05, * indicate p<0.001 when compared with the Cis alone treated group).**

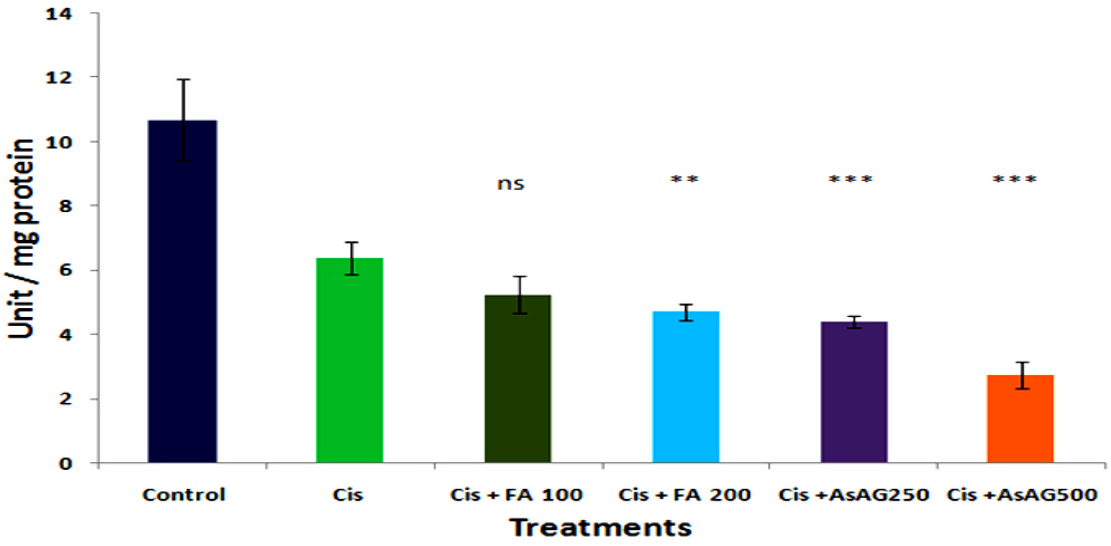


Figure 7.10 Effect of *p.o* administration of AsAG and Ferulic acid on cisplatin induced depletion of SOD levels in tumor tissues of mice. values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. (ns – not significant represent p>0.05 vs Cis control ; * represent p< 0.001 vs Cis control ; ** represent p< 0.01 vs Cis control)**

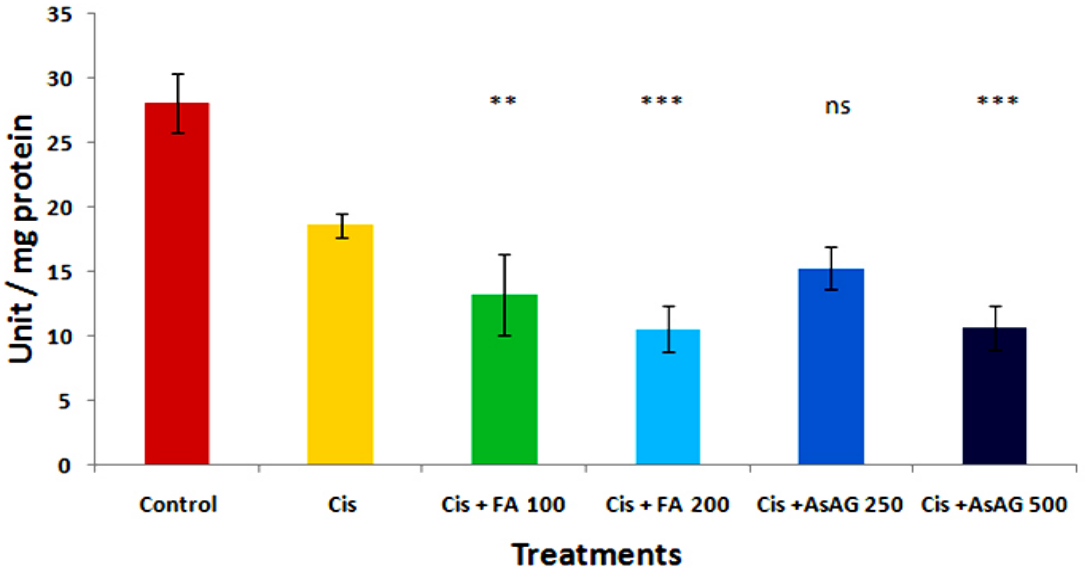


Figure 7.11 Effect of *p.o* administration of AsAG and Ferulic acid on cisplatin induced depletion of GPx levels in tumor tissues of mice. values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. ns – not significant represent p>0.05 vs DOX control ; * represent p< 0.001 vs DOX control ; ** represent p< 0.01 vs Cis control**

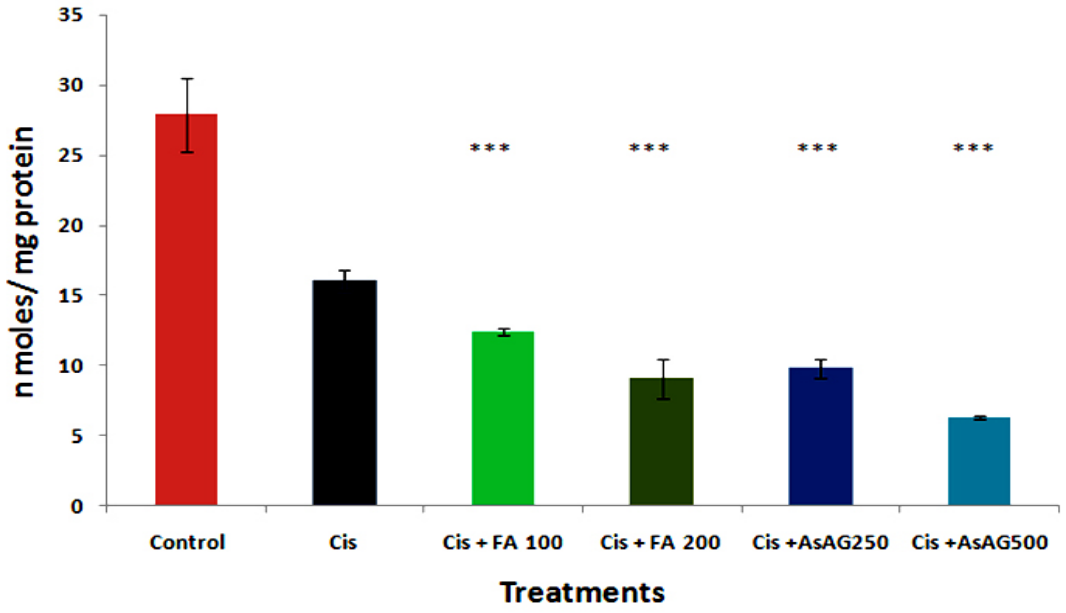


Figure 7.12 Effect of *p.o* administration of AsAG and Ferulic acid on cisplatin induced depletion of GSH levels in tumor tissues of mice. values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. (* represent p< 0.001 vs Cis control)**

7.3.3 Morphological Study

Histopathological investigation showed that, in cisplatin treated mice kidney there is a decreased cellularity of the glomeruli and edema of the lining of epithelial cells in the renal tubules. More over the nuclei of the lining cells show vacuolation. The interstitial tissue also showed edema as can be evident from **figure 7.13 (B).** The renal tissue architecture of the untreated mice kidney (**Figure 7.13 (A)**) was unaffected with normal glomeruli. The renal tissues of cisplatin treated mice when administered with AsAG or FA after the cisplatin treatment, showed near normal architecture with normal glomerular, renal tubules and interstitial tissue appearance (**Figure.7.13 (C)** and **(D)**).

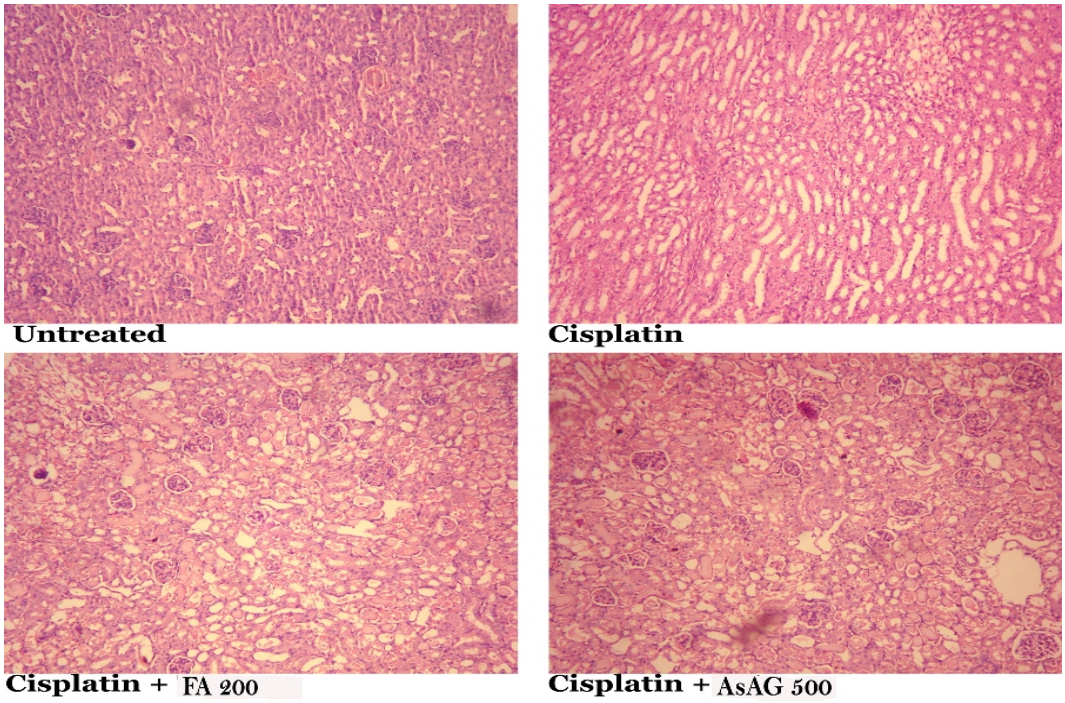


Figure 7.13 Micrograph of kidney of mice (A) control, (B) 72 hr of cisplatin injection, (C) cisplatin and FA 200 mg/kg, (D) cisplatin and AsAG 500 mg/kg. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin-eosin. The histopathological examinations were carried out using light microscopy.

The histopathological examination of tumor tissues is detailed in the **figure 7.14**. Tumor tissues from control animals showed compact arrangement of carcinomatous cells having clear cytoplasm and hyperchromatic nuclei. Whereas the CP treated animals showed many degenerating cells, stroma showed extensive areas of necrosis and haemorrhage. Tumor bearing animals administered with AsAG / FA and CP, showed a significant increase in the number of degenerating cells. Stroma showed more necrotic cells, cellular integrity was misplaced in many regions. The results showed that both AsAG / FA potentiates the cytotoxic action of cisplatin in tumor cells.

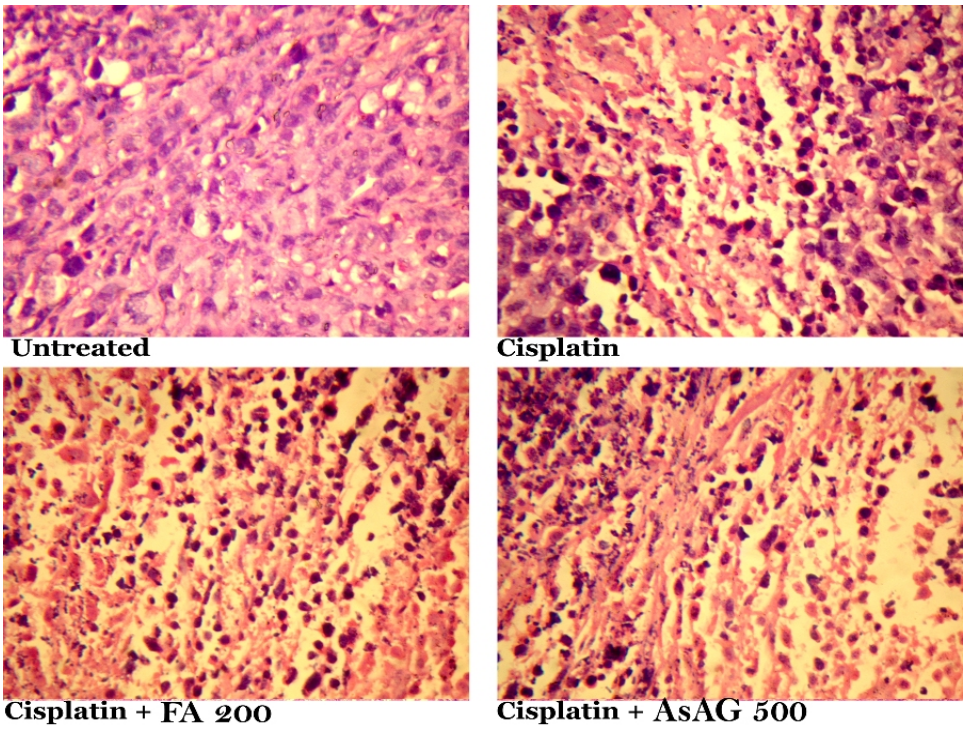


Figure 7. 14 Histopathology. Representative sections of tumor tissue of tumor bearing mice (A) control, (B) 72 hr of cisplatin injection, (C) cisplatin and FA 200 mg/kg, (D) cisplatin and AsAG 500 mg/kg. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin-eosin. The histopathological examinations were carried out using light microscopy.

7.4 DISCUSSION

Cisplatin is the most widely used anti-cancer agent, best against many solid organ tumors. The major side-effect of cisplatin is nephrotoxicity and its dose is the main limiting factor for its clinical use (O'Dwyer, 2006;Rana *et al*., 2016). Results of previous studies have shown that a minimum dose of cisplatin (5 mg/kg body weight, *i.p*) was sufficient to induce nephrotoxicity in rats (Boogaard *et al*., 1991). The highly reactive platinum complexes of Cisplatin binds with nucleophilic DNA *via* inter and intra-strand crosslinking with guanine nucleotide (Holditch *et al*., 2019). These events end up in denaturation of DNA and cell cycle arrest. Cisplatin *via* cytochrome P450 (CYP) in microsome and mitochondrial dysfunctioning creates reactive oxygen species (ROS) and damages the renal tissue (Humanes *et al*., 2017;Nematbakhsh *et al*., 2017).

One of the foremost important mechanisms observed in cisplatin toxicity is oxidative stress (Mohamed and Shenouda, 2021). Under normal physiological conditions, production and removal of reactive oxygen species (ROS) in cells are controlled by the endogenous scavenging system including catalase, superoxide dismutase, and reduced glutathione (Pabla and Dong, 2008). Under oxidative stress conditions, ROS levels are elevated, and many cellular structures (proteins, lipids, DNA) can be damaged. Increased oxidative stress can make cellular injury and necrosis in the kidney and other tissues. Previous studies (Kim *et al*., 2012; Saisruthi and Sreedevi, 2017) suggested the key role of lipid peroxidation and antioxidant enzymes like SOD and CAT in free radical metabolisms. These antioxidants enzymes scavenge free radicals or convert them into non-toxic end products and are measured as a first-line cellular defense mechanism against oxidative damage. The second line of antioxidant defense consists of the non-enzymatic scavengers such as GSH, which scavenge

remaining free radicals escaping from the primary line of antioxidant enzymes defense (Humanes *et al*., 2012; Mody *et al*., 2020).

Vitamin C and Vitamin E are reported to prevent this nephrotoxicity at very high doses (1.0 g/kg body weight) administered *i.p*. (Appenroth *et al*., 1997). The glucose derivatives of AsA viz. AsAG, has higher stability and better bioavailability. Studies have reported that it has *in vitro* free radical scavenging activity as well as antioxidant and radioprotective abilities (Mathew *et al*., 2007). Reports indicate that AsAG exhibits vitamin C activity in vivo following enzymatic hydrolysis by α-glucosidase (Fujinami *et al*., 2001). Several studies in animals and humans confirmed that FA demonstrates anticancer properties (Lesca, 1983;Asanoma *et al*.,1994). Earlier studies have reported the nephro protective activities of pieperazine ferulate on rats (Shaojun *et al*.,2002). Recent studies have demonstrated that the administration of ferulic acid ameliorated the doxorubin induced cadiotoxicity in tumor bearing animals (Divakaran and Nair, 2012). Here FA provides protection to the cardiac tissues, on the other hand, it potentiated the anticancer efficacy of DOX in tumor tissues as evident from different antioxidant enzyme levels and the extent of lipid peroxidation.

Administration of cisplatin shows a significant increase in blood creatinine and serum urea concentrations compared to normal, which indicates the intrinsic acute renal failure (Joy and Nair, 2008; Wu and Huang, 2018). The results of the current study showed that cisplatin administration to tumor bearing mice significantly raised the levels of lipid peroxidation and caused depletion of GSH, SOD, and GPx in renal tissues. The declined antioxidant status partially explains the mechanism of nephrotoxicity induced by cisplatin. The renal accumulation of platinum, covalent binding of renal protein also play a crucial role in nephrotoxicity (Hanigan and

Devarajan, 2003). The decreased concentration of GSH increases the sensitivity of organs to oxidative and chemical injury.

Treatment of AsAG or FA together with cisplatin could significantly prevent the depletion of these renal antioxidant systems. Treatment with AsAG or FA rendered protection because of the increase in GSH concentration could keep the renal cells from oxidants attack. Moreover, the protection of GSH is additionally by forming the substrate for the GPx activity that may react directly with various aldehydes produced from the peroxidation of membrane lipids. Treatment with AsAG or FA and cisplatin enhances the activity of Se-GPx (selenium-dependent GPx) compared to the cisplatin alone-treated animals. Thus the improved GPx activity partially explained the role in nephro protection. The decreased activity of SOD and GPx in renal tissues enhanced the lipid peroxidation in cisplatin treated group (Jia *et al*., 2011; Moreno-Gordaliza *et al*., 2018). Treatment with AsAG or FA prevented lipid peroxidation by enhancing the renal SOD and GPx activities.

Micronuclei are derived from chromosomal fragments or chromosomes which are not included in the daughter nuclei at the time of cell division. Frequency of micronuclei formation depends on the rate of chromosomal breakage or of chromosomal loss. Treatment with the FA or AsAG significantly reduced the micronuclei frequency in both experimental sets, suggesting their salubrious activity against chemotherapy induced clastogenesis.

The main prerequisite for any compound to be used as a nephroprotective agent during the treatment of cancer is that it should not interfere with the antitumor activity of the chemotherapy (Fang *et al*., 2021). Various studies in animals and humans confirmed that FA FA and AsA could demonstrate anticancer properties (Lesca,1983; Asanoma *et al*., 1994; Gorton and Javis,1999; Ohno *et al*., 2009; Divakaran and Nair,

2012). Vitamin C and Ferulic acid is effective in protecting against oxidative damage in tissues, and also suppresses the formation of carcinogens like nitrosamines. The MDA levels of tumor tissues in FA or AsAG and Cis-treated animals showed an increase in the level when compared to the untreated control animals. The antioxidant levels in tumor tissues were also found to be significantly reduced by the administration of cisplatin and ferulic acid. The results significantly showed the antitumor potential of the combination treatment (Humanes *et al*., 2012). Moreover, the histopathological reports suggest that post-treatment of FA or AsAG greatly inhibited the Cis-induced changes in kidney tissue supporting the protective action of FA or AsAG against Cis-induced nephrotoxicity and offer maximum destruction to tumor tissues (Zhao and Dai, 2020).

The combined strategy showed high antitumor activity as evident from MDA formation, antioxidant enzyme levels, and histopathological examination. The strategy ensures a secure and efficient anticancer treatment modality. Thus, the combination treatment improves the chemotherapeutic index of cytotoxic anticancer drug, Cisplatin.

Chapter- 8

AMELIORATION OF PARACETAMOL INDUCED HEPATOTOXICITY IN MICE BY ASCORBIC ACID MONOGLUCOSIDE (AsAG) AND FERULIC ACID (FA)

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8.4 DISCUSSION

8.1 INTRODUCTION

Liver is a very important organ in maintaining homeostasis of the body. It involved in almost every biochemical pathways connected to growth, to fight against disease, nutrient supply, energy provision and reproduction. It plays a pivotal role in regulating metabolism of endogenous as well as xenobiotic substances, secretion and storage. Liver has got the capacity to detoxicate toxic substances and manufacture useful chemical principles. Liver disease is a worldwide health problem. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders (Ganguly *et al.,* 2009). Most of the hepatotoxic chemicals harm liver cells mainly by producing reactive species which form covalent bond with the lipids of the tissue. The analgesic acetaminophen or paracetamol causes a potentially fatal, hepatic centrilobular necrosis when taken in overdose (James *et al.,* 2003). Hence, damage to the liver by hepatotoxic agents is of great concern (Shahani, 1999). On the other hand, natural protective mechanisms combat the hazardous reactions associated with the free radicals. Due to excessive exposure to hazardous chemicals, sometimes the free radicals generated are so high that they overpower the natural defencive system leading to hepatic damage and cause jaundice, cirrhosis and fatty liver (Jyoti *et al*., 2008). Production of the reactive species manifest in tissue thiol depletion, lipid peroxidation, plasma membrane damage etc., leading into severe hepatic injury (Kanter *et al*., 2005).

Acetaminophen or paracetamol (PAR) is one of the most widely used analgesics with few side effects when taken in therapeutic doses (Black,1980;Larson *et al*., 2005) and hepatotoxicity is a common consequence of acetaminophen overdose (Makin and Williams, 1997; Ratheea *et al*., 2018). Acetaminophen (Paracetamol) is a well known antipyretic and analgesic agent. A number of reports indicate that overdose of

paracetamol can produce centrizonal hemorrhagic hepatic necrosis in humans and experimental animals (Ganguly *et al*., 2009). Acetaminophen toxicity is caused by the reaction metabolite N-acetyl-p-benzoquinoneimine (NAPQI), which is partly metabolized by cytochrome P-450 (Thummel *et al*., 1993). This species causes severe oxidative damage and glutathione depletion leading to liver necrosis. Subsequent to an overdose of acetaminophen, elevated levels of the toxic NAPQI metabolite are formed, which can deplete hepatocellular GSH and covalently modify cellular proteins resulting in hepatocyte death. It is generally recognized that at higher doses the drug induces lipid peroxidation and oxidative stress, each contributing to hepatocellular damage and also produces hepatic necrosis. Introduction of cytochrome or depletion of hepatic glutathione is a prerequisite for acetaminophen -induced hepatotoxicity (James *et al*., 2003; Elkomy *et al*., 2016). Acetaminophen is considered as a predictable hepatotoxin, where biochemical signs of liver damage appears within 24 to 48 hours after the time of overdose and create a dose-related centrilobular necrosis in the liver (Black, 1980; Lee, 1995). Mechanisms of acetaminophen hepatotoxicity include generation of a toxic metabolite, mitochondrial dysfunction, and alteration of innate immunity.

Acetaminophen overdose can cause mitochondrial dysfunction by covalent binding to mitochondrial proteins or by some other mechanisms. The modified mitochondrial proteins and high levels of cytosolic calcium can lower mitochondrial respiration and adenosine triphosphate (ATP) synthesis and induce mitochondrial oxidant stress with increased production of peroxynitrite, a potent oxidant and nitrating agent. Peroxynitrite can able to produce extra covalent bonds with cellular proteins, causing more mitochondrial dysfunction. Finally there is change of membrane permeability leading to collapse of mitochondrial membrane potential, distraction of ATP

synthesis, release of mitochondrial proteins into the cell cytoplasm, and oncotic necrosis of hepatocytes (Jaeschke and Bajt, 2006). Oxidative stress is one more mechanism that is said to be important in the development of acetaminophen toxicity. Therefore, increased formation of superoxide leads to hydrogen peroxide and peroxidation reactions by Fenton-type reactions.

Liver is one of the principal organs in human body and the main site for intense metabolism and excretion. So it possess a leading role in the maintenance, performance and regulating homeostasis of the body. It is concerned with all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction. Liver diseases are treated as some of the fatal disease in the world today. They create a serious confront to international public health. Modern medicines have offer little for alleviation of hepatic diseases. So plant based preparations are used for the treatment of liver disorders. But presently, there are not much drugs available for the treatment of liver disorders (Karan *et al.*, 1999). Liver dysfunction is a clinically significant problem, for people of all the reported cases of acute liver failure (Yamazaki *et al*., 2005). Therefore very often hepatoprotective drugs are recommended to ameliorate liver problems. Herbal drugs are often used widely because of their effectiveness, fewer side effects and relatively low cost (Valiathan, 1998). Reports says that about 160 phyto-constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotinoids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthenes from 101 plants have hepatoprotective activity (Chaterjee, 2000).

Thus the present chapter concerns, prevention of paracetamol induced hepatotoxicity in mice by AsAG and FA.

8.2 MATERIALS AND METHODS

8.2.1 Animals

Female Swiss albino mice of 8-10 week old weighing 26-28g, selected from inbred group was used for hepatototoxicity studies.

8.2.2 Administration of Drugs

The animals were randomly divided into7 groups of five each and treated as follows.

Group I - Untreated Control

Group II - Paracetamol control, administered with paracetamol (150 mg/kg) as single dose after 18 h starvation.

Group III - Silymarin (75 mg/kg body weight) one hour after paracetamol administration

Group IV- AsAG 250 mg/kg b.w **(**in 0.1 ml distilled water *p.o*) one hour after paracetamol administration

Group V- AsAG 500 mg/kg b.w **(**in 0.1 ml distilled water *p.o*) one hour after paracetamol administration

Group VI- Ferulic acid 100 mg/kg b.w **(**in 0.1 ml distilled water *p.o*) one hour after paracetamol administration

Group VII- Ferulic acid 200 mg/kg b.w **(**in 0.1 ml distilled water *p.o*) one hour after paracetamol administration

Group I administered with distilled water alone was kept as untreated control. Group II was given acetaminophen (150 mg/kg body weight) as single dose orally after 18 h starvation and kept as control. Group III served as reference control, received Silymarin (75mg/kg body weight), a clinically used hepatoprotective drug, one hour after paracetamol administration.

AsAG was administered to group IV and V, Ferulic acid was administered to Group VI and VII animals, one hour after paracetamol (150 mg/kg) administration. The test drugs and paracetamol were administered orally to all animals.

After 24 hours of paracetamol feeding, the animals were sacrifised and blood was collected by direct cardiac puncture under light ether anesthesia and serum was separated for the biochemical estimations. The liver was removed, washed with ice-cold PBS, weighed and morphological changes were observed. Then liver homogenates (10% w/v) was analyzed for antioxidant status.

8.2.3 Evaluation of liver function enzymes in serum

Serum was used for the determination of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), Serum albumin and serum Bilirubin.

8.2.4 Determination of antioxidant status in the liver

Liver was excised after sacrificing the animals and washed with ice-cold PBS and 10% homogenate was prepared in PBS (pH 7). A part of the homogenate was used for the determination of reduced glutathione (GSH). Rest of the homogenate was centrifuged at 1,000 rpm for 10 min for removing the cell debris, unbroken cells, nuclei, erythrocytes and mitochondria. The supernatant was used for the estimation of superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA). The protein was estimated by Lowry's method.

8.2.5 Histopathological studies

Small sections taken from each lobe of the liver were fixed immediately in 10% neutral formalin for a period of at least 24 hr, and embedded in paraffin wax. Thin sections of 5 micron thickness were made using a microtome and later stained with

haematoxylin-eosin. The histopathological examinations were carried out at Sudharma Metropolis Pathological Laboratory, Thrissur, Kerala, India.

8.3 RESULTS

8.3.1 Evaluation of liver function enzymes in serum

Administration of acetaminophen (150 mg/kg body weight) results in manifestation of hepatotoxicity as can be revealed from the elevated levels of serum marker enzymes. The levels of serum GOT (46.06±9.4), GPT (45.5± 10.35), ALP (110.26 ± 22.16), bilirubin (0.72±0.04) and albumin (3.27±0.27) were elevated in acetaminophen treated animals [GOT (250.75±11.78), GPT (258.5±12.6), ALP (213.6 ± 22.35), bilirubin (1.67±0.88) and albumin (4.91±0.12)] when compared to control, indicating liver damage. Silymarin is a known hepatoprotective agent and administration of this compound at a dose rate of 75 mg/kg body weight prevented the onset of hepatotoxicity induced by acetaminophen. Administration of AsAG and FA after paracetamol treatment prevented elevated levels of serum marker enzymes in a dose dependent manner. Post treatment with AsAG and FA significantly reversed the levels of GOT, GPT, ALP, bilirubin and albumin when compared to acetaminophen alone treated mice (**Figure 8.1, 8.2, 8.3, 8.4 & 8.5**).

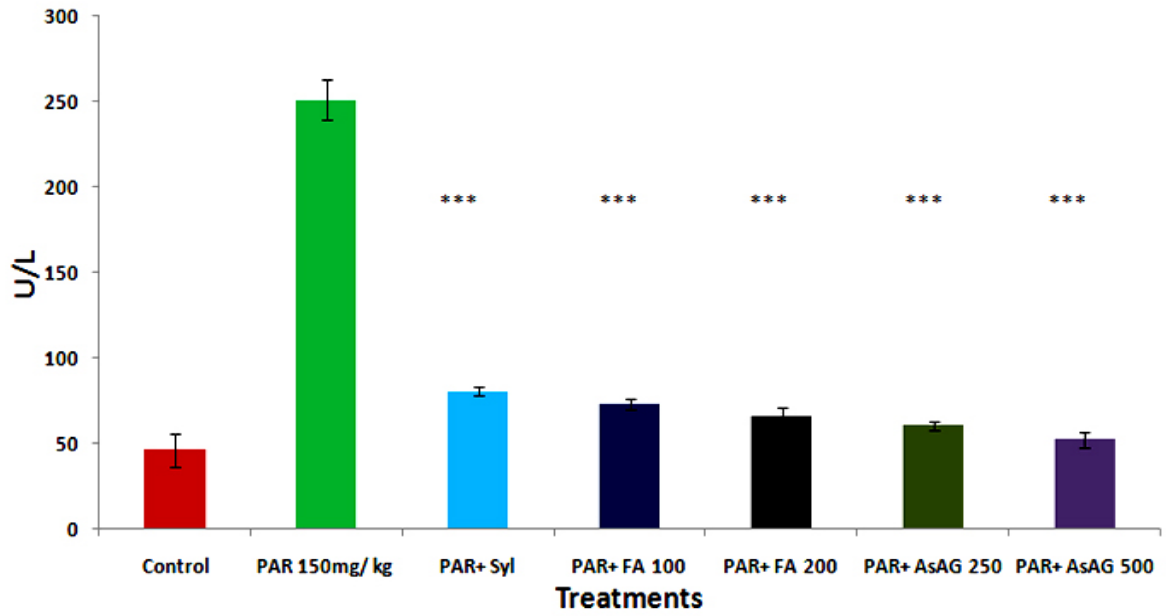

Figure 8.1 Effect of post treatment of FA and AsAG on serum SGOT in paracetamol induced hepatotoxicity in mice. Values are expressed as mean ± SD (n = 5). * indicate P< 0.001 Vs paracetamol.**

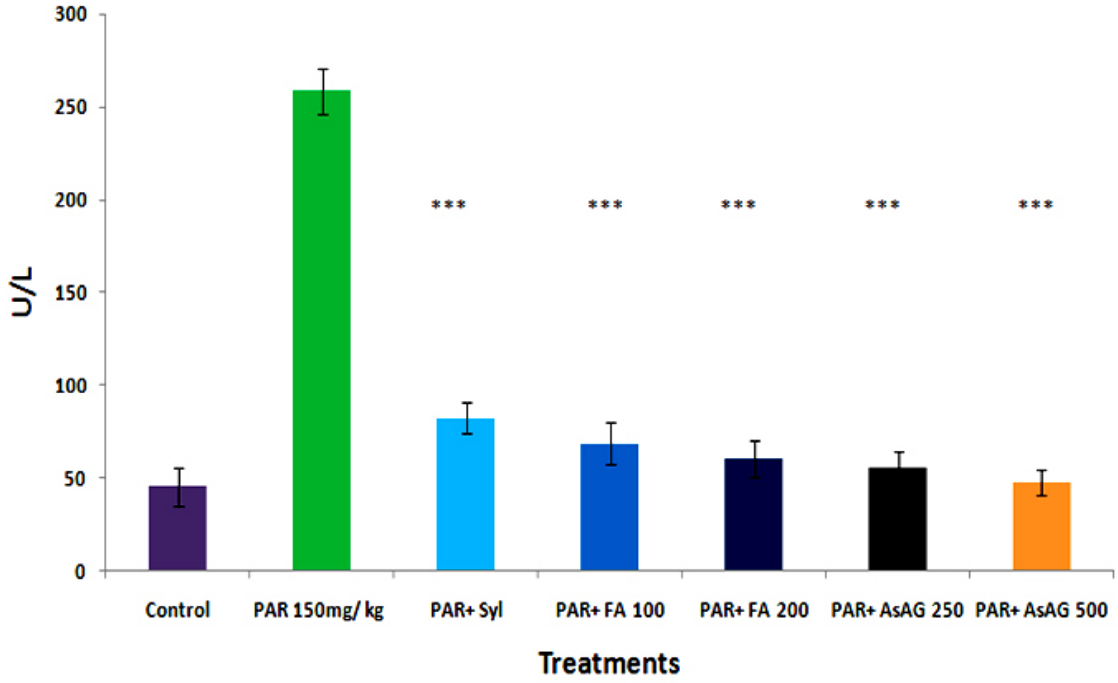

Figure 8.2 Effect of post treatment of FA and AsAG on serum SGPT in paracetamol induced hepatotoxicity in mice. Values are expressed as mean ± SD (n = 5). * indicate P< 0.001 Vs paracetamol.**

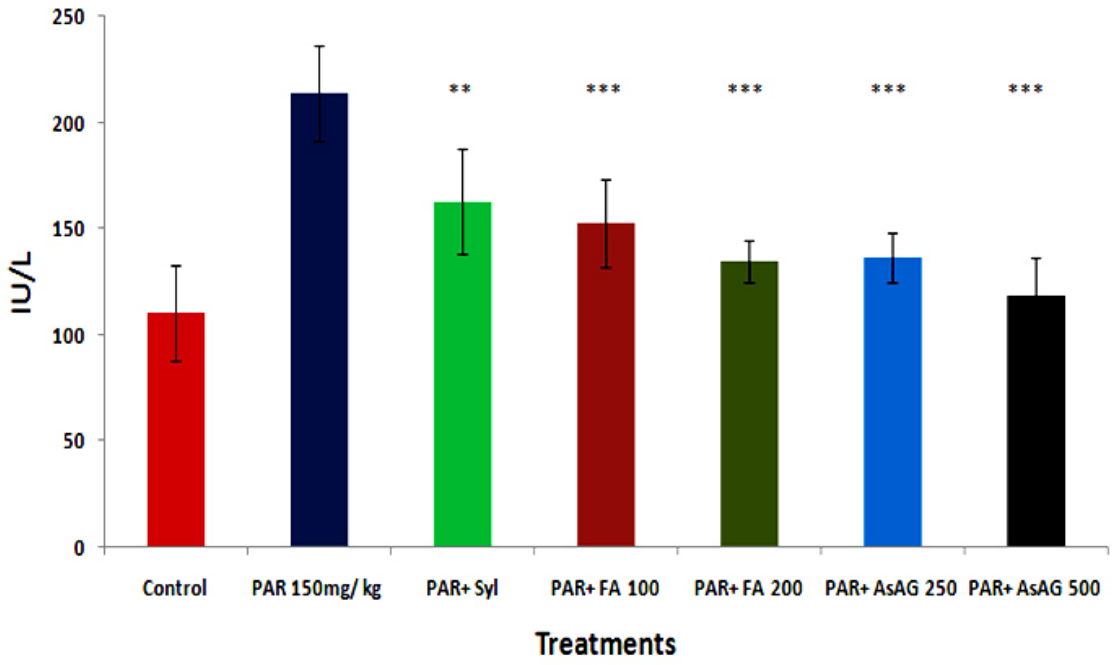

Figure 8.3 Effect of post treatment of FA and AsAG on serum ALP in paracetamol induced hepatotoxicity in mice. Values are expressed as mean ± SD (n = 5). ** indicate p <0.01 Vs paracetamol * indicate P< 0.001 Vs paracetamol.**

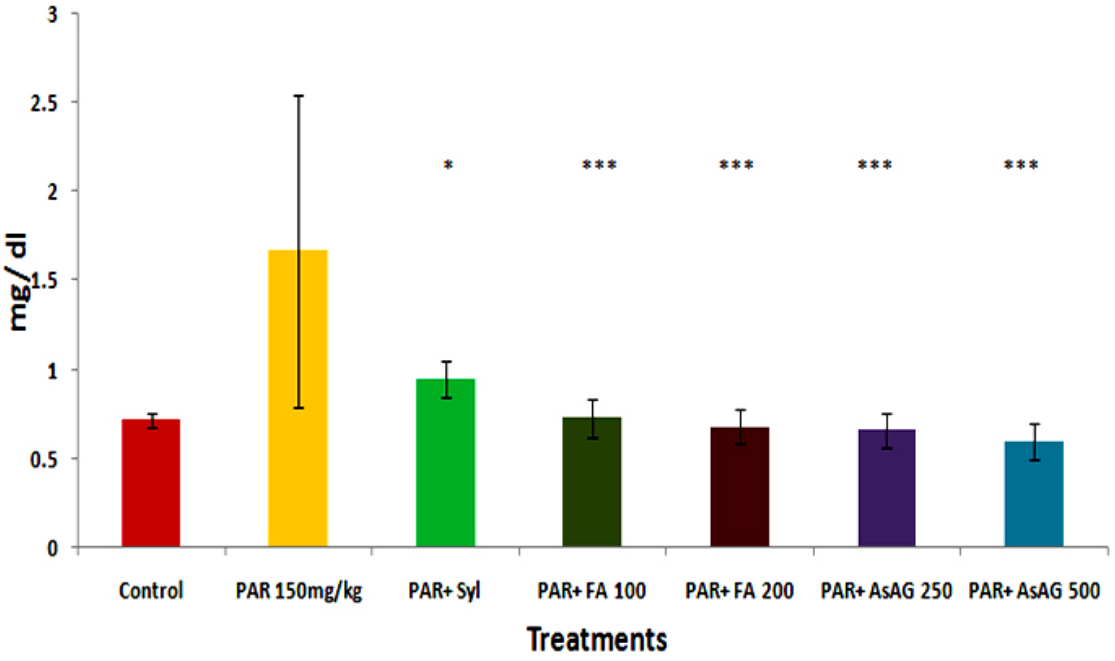

Figure 8.4 Effect of post treatment of FA and AsAG on serum bilirubin in paracetamol induced hepatotoxicity in mice. Values are expressed as mean ± SD (n = 5). * indicate p <0.05 Vs paracetamol * indicate P< 0.001 Vs paracetamol.**

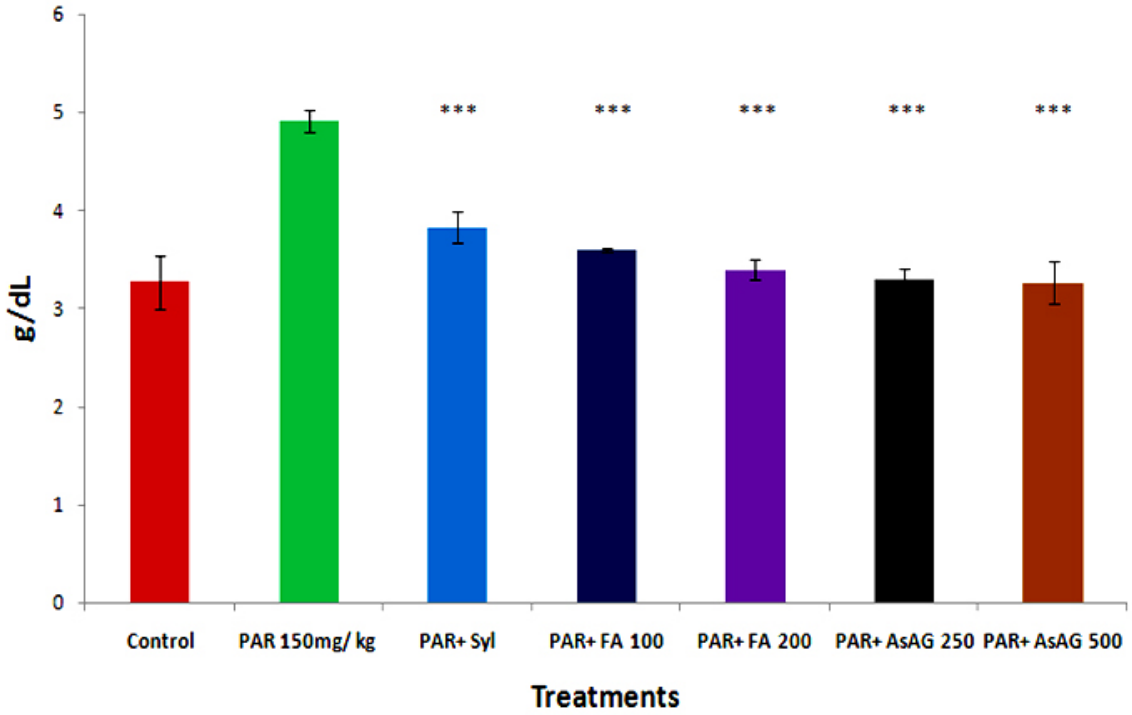

Figure 8.5 Effect of post treatment of FA and AsAG on serum albumin in paracetamol induced hepatotoxicity in mice. Values are expressed as mean ± SD (n = 5). * indicate p <0.05 Vs paracetamol * indicate P< 0.001 Vs paracetamol.**

Thus it can be clearly seen that AsAG and FA prevented the manifestation of acetaminophen induced hepatotoxicity by acting in dose dependent manner.

8.3.2 Antioxidant status in liver

The total antioxidant activity, as a measure of antioxidant status, was significantly decreased in the liver tissue of the acetaminophen treated group. Acetaminophen treatment caused a significant decrease in the level of SOD, GPx and GSH in liver tissue when compared with control group. The activities of all the antioxidant enzymes were significantly enhanced in the animals treated with AsAG and FA. The post-treatment with AsAG (250 and 500 mg/kg) or FA (100 and 200 mg/kg) resulted in significant increase of SOD, GPx and GSH in a dose dependent manner (**Figure 8.6, 8.7 & 8.8).** The silymarin treated animals also showed a significant increase in antioxidant enzymes levels compared to acetaminophen treated animals.

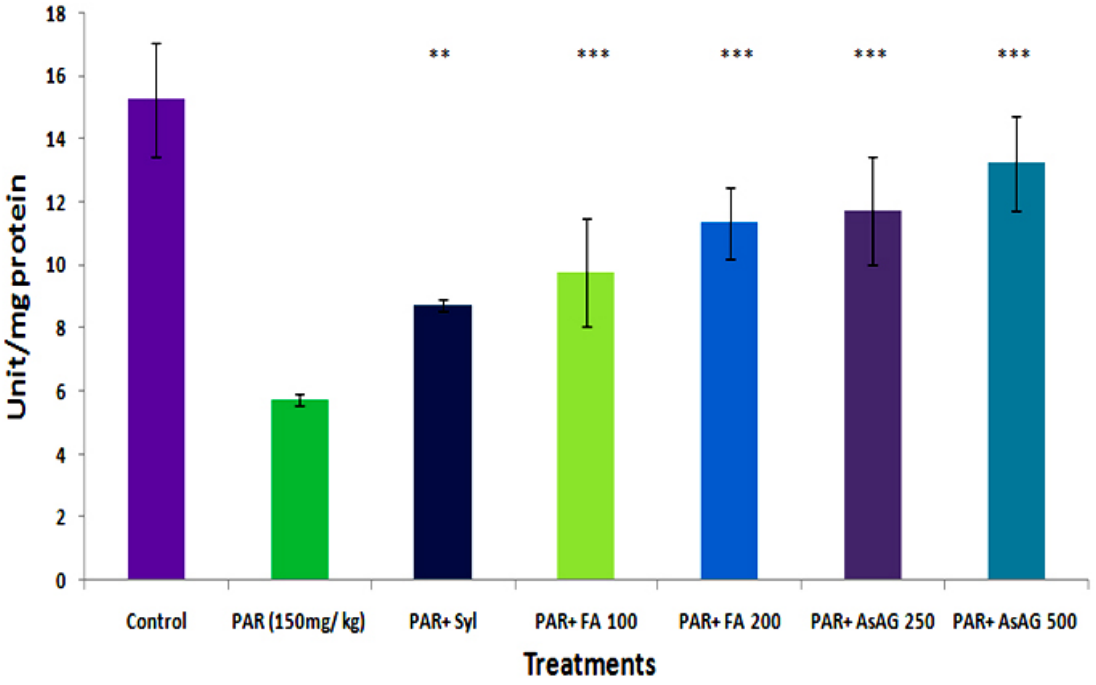

Figure 8.6 Effect of post administration of FA and AsAG on SOD level in paracetamol induced hepatotoxicity in mice. Values are expressed as mean ± SD (n = 5). Data were analyzed by using one-way ANOVA followed by Tukey multiple comparison test. ** indicate p<0.01 Vs paracetamol * indicate p<0.001 Vs paracetamol.**

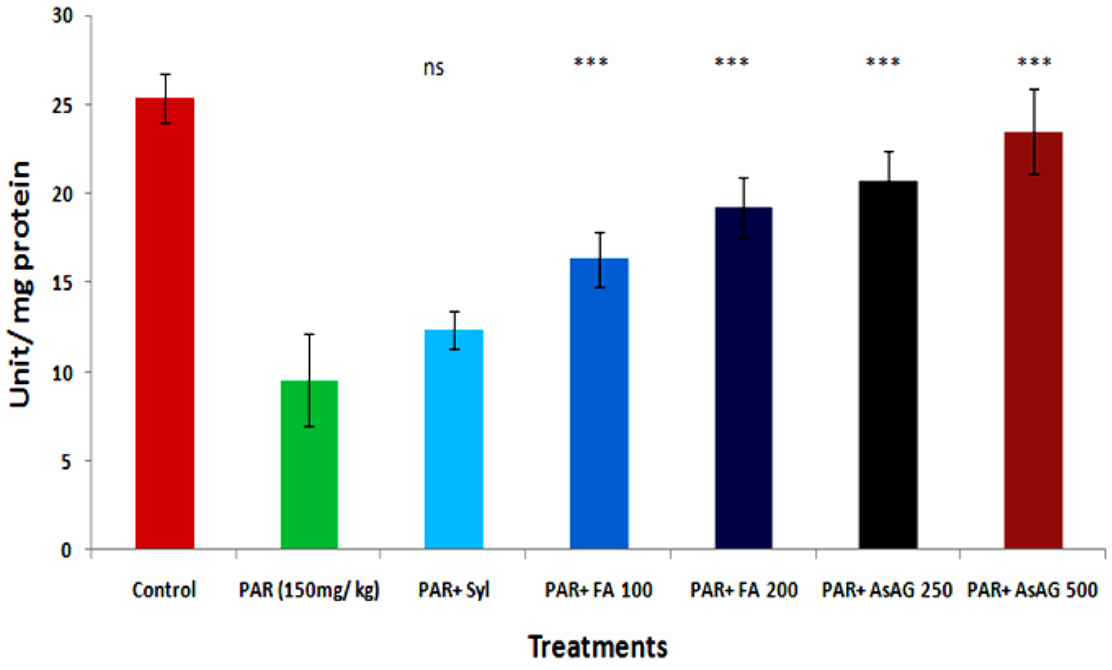

Figure 8.7 Effect of post administration of FA and AsAG on GPx level in paracetamol induced hepatotoxicity in mice. Values are expressed as mean ± SD (n = 5). Data were analyzed by using one-way ANOVA followed by Tukey multiple comparison test. ns indicate p>0.05 Vs paracetamol * indicate p<0.001 Vs paracetamol.**

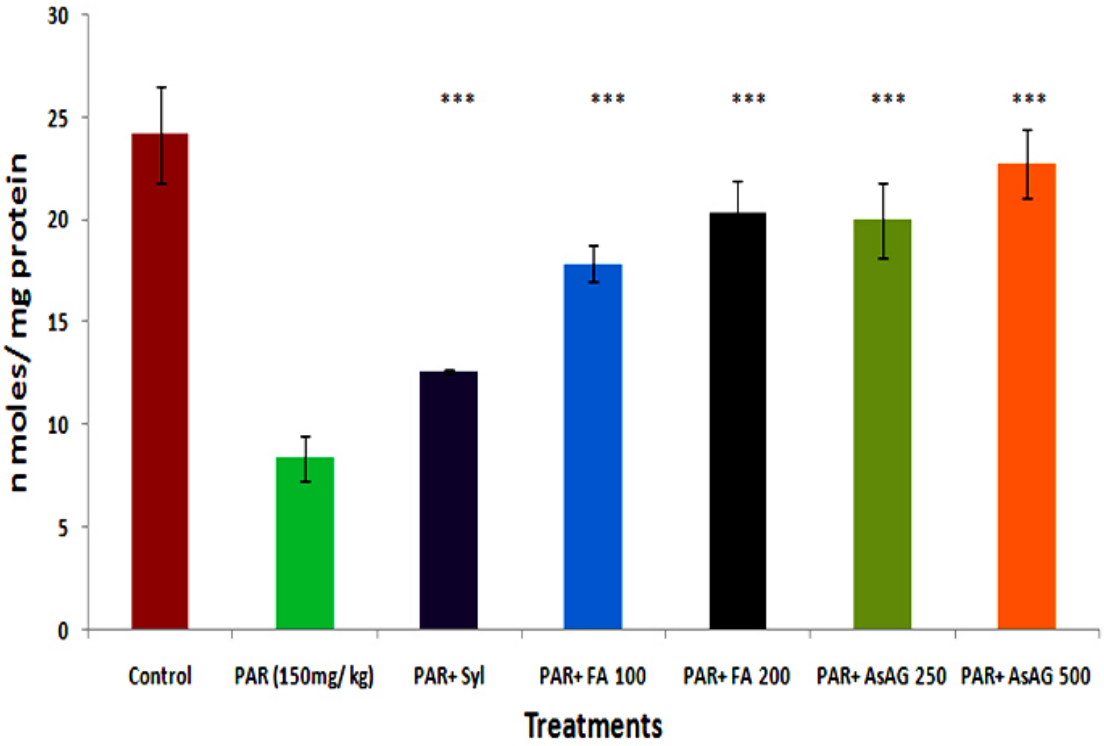

Figure 8.8 Effect of post administration of FA and AsAG on GSH level in paracetamol induced hepatotoxicity in mice. Values are expressed as mean ± SD (n = 5). Data were analyzed by using one-way ANOVA followed by Tukey multiple comparison test. * indicate p<0.001 Vs paracetamol.**

Generation of malondialehyde (MDA) was measured as a marker of lipid peroxidation and an indicator of oxidative injury. Analysis of lipid peroxidation (LPO) levels by thiobarbituric acid reaction showed a significant increase in the acetaminophen treated mice. The post treatment with AsAG (250 and 500 mg/kg) or FA (100 and 200 mg/kg) significantly prevented the increase in LPO level which was brought to near normal level. The effect of AsAG or FA was comparable with that of standard drug silymarin (**Figure 8.9**).

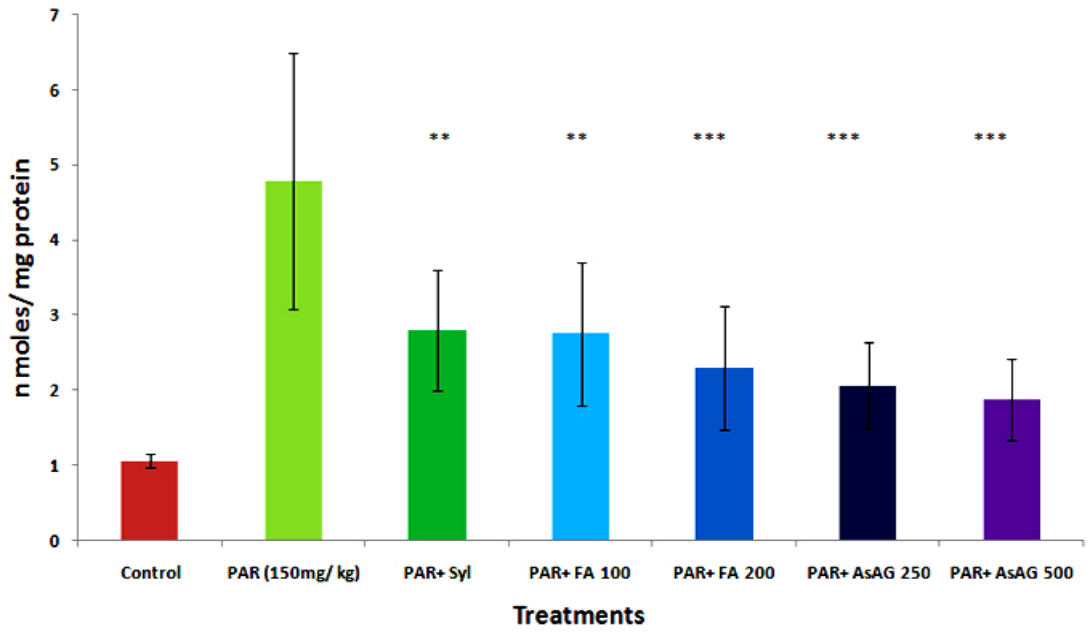

Figure 8.9 Effect of post administration of FA and AsAG on Lipid peroxidation level in paracetamol induced hepatotoxicity in mice. Values are expressed as mean ± SD (n = 5). Data were analyzed by using one-way ANOVA followed by Tukey multiple comparison test. ** indicate p<0.01 Vs paracetamol, * indicate p<0.001 Vs paracetamol.**

8.3.3 Histopathological observations

Histological analysis was performed to confirm AsAG or FA mediated hepatoprotection. In the untreated control animals the morphological observations of liver tissues showed normal architecture of hepatic cells with clear cytoplasm and slightly dilated central veins. Here the kupffer cells are appeared normal and all cells having normal large nuclei (**Figure 8.10 [A]**). In acetaminophen treated animals the liver tissue shows distorted architecture. Central veins are markedly congested. There are extensive area of necrosis and haemorrhage. Nuclei are distorted and some of the hepatocytes contain vacuolated cytoplasm (**Figure 8.10 [B]**). The silymarin treated animals also showed similar results as that of AsAG or FA but here the sinusoidal spaces are compressed. And some areas showed minimal nuclear pleomorphism (**Figure.8.10 [C]**). In the post-treated animals with FA (100 and 200 mg/kg) , the normal architecture of the liver tissue can be seen (**Figure 8.10 [D&E]**). Here central

veins are slightly dilated and hepatocytes are slightly pleomorphic of them have larger nuclei. Similarly in the post AsAG (250 and 500 mg/kg) treated group a similar type of cellular architecture was seen with a small area of area hemorrhage. Sinusoidal space appears normal, and also the kupffer cells (**Figure 8.10 [F&G]**). Thus the histological observations supported the results obtained from liver enzyme assays.

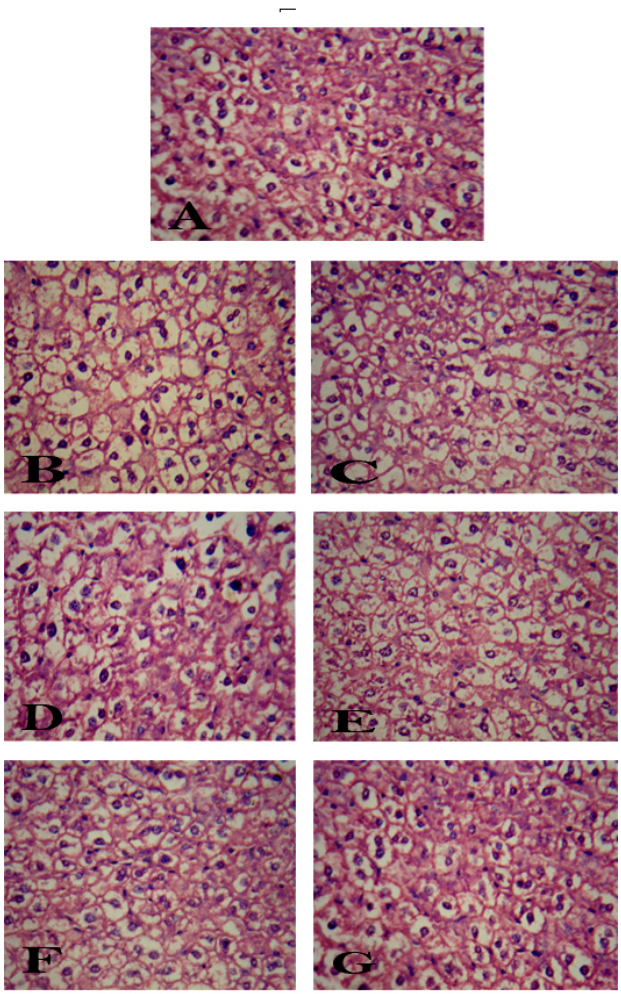

Figure 8.10 Effect of FA and AsAG on paracetamol induced liver damage in mice (X 40). [A] liver from untreated control mice shows normal cellular architecture with distinct hepatic cells, sinusoidal space and a central vein; [B] Liver from mice treated with acetaminophen (150mg/kg after 18hr starvation) exhibited severe hepatocyte degeneration and necrosis; [C] Liver from mice treated with acetaminophen (150mg/kg , after 18hr starvation) and silymarin (75mg/kg); [D & E] Liver treated with FA(100 mg/kg and 200 mg/kg after acetaminophen treatment (150mg/kg after 18hr starvation) with mild hepatocyte degeneration; [F & G] Liver treated with AsAG (250 mg/kg and 500 mg/kg after acetaminophen treatment (150mg/kg after 18hr starvation) with mild hepatocyte degeneration and near normal architecture

8.4 DISCUSSION

Subsequent to an overdose of paracetamol, toxic NAPQI metabolite are elevated, which can widely deplete hepatocellular GSH and covalently change cellular proteins resulting in hepatocyte death. Assessment of liver function is made by estimating the activities of serum GPT, GOT and bilirubin which are present higher concentration in cytoplasm. When there is hepatopathy, these molecules leak into the blood stream in compliance with the extent of liver damage (Nkosi *et al.,* 2005; Hamza and Al-Harbi, 2015). Bilirubin is one of the most useful clinical clues to the level of damage of necrosis and its increase is a measure of binding, conjugation and excretory capacity of hepatocyte.

Paracetamol overdose result in toxic liver damage leading to potentially fatal, hepatic centrilobular necrosis and liver failure (James *et al.,* 2003) associated with metabolic activation by the P-450 system to form a quinine imine metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), which covalently binds to proteins and other macromolecules to cause cellular damage. At low doses, NAPQI is well detoxified, mainly by conjugation with glutathione. At higher doses glutathione becomes depleted and the excess of NAPQI arylates and oxidizes hepatic proteins (Fountoulakis *et al.,* 2000).

Oxidative stress is also a mechanism that has been postulated to be important in the development of paracetamol toxicity. Toxicity starts with the change in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures (Recknagel, 1983). Assessment of liver function can be made by estimating the activities of serum ALT, AST and Bilirubin which are originally present higher concentration in cytoplasm. When there is heaptopathy, these leaks into the blood stream in conformity with the extent of liver damage (Nkosi *et al.,*

2005; Mahmood *et al*., 2014). The elevated level of these marker enzymes seen in the group II, paracetamol treated mice shows the extensive liver damage induced by toxin.

The experimental results indicate that AsAG or FA were able to delimit the liver damage induced by acetaminophen. The protective effect was found to be significant and can be compaired with the standard hepatoprotective drug silymarin (Hamza Hamza and Al-Harbi, 2015). Accidental or incidental acetaminophen overdose may be associated with toxic liver damage leading to potentially fatal, hepatic centrilobular necrosis and liver failure (James *et al*., 2003). Oxidative stress is another mechanism that is said to be important in the progress of acetaminophen toxicity.

It was seen that the levels of cellular antioxidant enzymes and molecules are lowered significantly in paracetamol treated animals. This further results in the peroxidative damage to membranes results in the leakage of enzymes, and metabolites to circulation. In the present study, it was observed that, the animals treated with paracetamol showed elevated levels of serum markers such as SGPT, SGOT, ALP and bilirubin. Normally, a higher concentrations of SGOT and ALP are present in liver. Due to hepatocyte necrosis or abnormal membrane permeability, these enzymes are released from the cells and their levels in the blood increases. SGPT is a sensitive indicator of severe liver damage and increased level of this enzyme in non hepatic diseases is unusual (Mahmood *et al*., 2014). SGPT is more selectively a liver parenchymal enzyme than SGOT (Shah *et al.,* 2002).

Intake of alcohol results in excessive generation of free radicals (Rouach,1997) which alter the biomembrane and cause severe damage. FA can work well in herbal antioxidant formula, vitamin and herbal health supplement, and body's immune

system can be benefited from FA. The reports greatly support the idea that regular ingestion of FA may provide substantial protection against alcohol and polyunsaturated fatty acid (PUFA) induced toxicity and may provide the body with the ability to triumph over the deleterious effects of alcohol and PUFA (Rukkumani *et al*., 2004). Treatment with FA significantly lower the activities of these enzymes in plasma. FA is shown to protect the physiological integrity of the cells exposed to various stress. These can be attributed to the effective antioxidant property of FA. Usually phenolic compounds acts by scavenging free radicals and quenching the lipid peroxidative side chain. Phenolic compounds can act as free radical scavengers by virtue of their hydrogen donating ability and forming aryloxyl radicals (Francisco *et al*., 2000). Srinivasan *et al.* (2005) have also been reported FA protects against carbon tetrachloride (CCl_4) induced toxicity in an experimental animal model, which ascribed to antioxidant potential.

Oral administration of AsAG or FA exhibited a significant reduction in acetaminophen induced levels of serum GOT, GPT, ALP, and bilirubin which is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage. This could be an evidence for the protective effect of AsAG or FA which helps to maintain the functional integrity of hepatic cells. More over the decrease in serum bilirubin after treatment with the AsAG or FA in liver damaged mice induced by acetaminophen indicated their effectiveness in normal functional status of the liver.

Thus above alterations can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells. The silymarin with a dose of 75 mg/kg, body weight has also provided a better inhibition of the elevated level of SGOT, SGPT, ALP, and serum bilirubin content. Thus the data obtained from the serum parameters of the present

study clearly indicates that the antihepatotoxic activity of the AsAG or FA probably through the correction of cellular integrity of hepatic cell and its regeneration.

Oxidative stress is considered to be associated with many diseases, including cell damage. Glutathione is one of the most abundant tripeptide, a non-enzymatic biological antioxidant present in the liver. Its functions include, processes like removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals and maintenance of membrane protein thiols and as a substrate for glutathione peroxidase (GPx) and GST (Prakash *et al*., 2001). We observed that the level of GSH was decreased in association with acetaminophen treatment and administration of AsAG or FA significantly increased ($P< 0.001$) the level of glutathione in a dose dependent manner.

The increase in MDA level in liver of acetaminophen treated mice suggests provoked lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Lipid peroxidation has been postulated as being a destructive process in liver injury caused by acetaminophen administration (Muriel *et al*., 1992; Elkomy *et al*., 2016). The post treatment with AsAG or FA on reversed the increase in MDA levels more significantly ($P< 0.001$). The coincidence of antioxidant activity and protective effect on liver tissues after acetaminophen administration suggest that both free radical generation and lipid peroxidation may be involved in this type of drug injury process.

SOD and GPx enzymes are important scavengers of superoxide ion and hydrogen peroxide. These enzymes prevent generation of hydroxyl radical and protect the cellular constituents from oxidative damage (Scott *et al*., 1991;Subramanya *et al*.,

2018). In the present study, it was observed that both AsAG or FA significantly increased the hepatic SOD activity in acetaminophen treated mice. This shows that both can reduce reactive free radicals and cause lesser oxidative damage to the tissues and improve the activities of the hepatic antioxidant enzyme. Hence it may be possible that the mechanism of hepatoprotection of AsAG or FA is due to its antioxidant effect.

Chapter- 9

ANTIOXIDANT CAPACITY AND RADIOPROTECTIVE PROPERTIES OF THE FLAVONOIDS GALANGIN AND KAEMPFERIDE ISOLATED FROM *ALPINIA GALANGA* L. AGAINST RADIATION INDUCED CELLULAR DNA DAMAGE

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9.4 DISCUSSION

9.1 INTRODUCTION

The deleterious effects of ionizing radiation in biological systems arise directly (interaction between radiation and target macromolecules) or indirectly (mediated by the generation of free radicals) (Mathew *et al*., 2007;Chandrasekharan *et al*., 2009). The generated free radicals and related reactive oxygen species (ROS) damage vital cellular targets. The most important target in a living cell affected by ionizing radiation is genomic DNA. The radiation induced damages to DNA may lead to cell death and increase the risk for numerous genetically determined diseases like cancer. The radiation induced damages to DNA may be of single and double strand breaks, oxidative damage to sugar and base residues, chromosomal aberration and mutation that may lead to cell death and increase the risk for numerous genetically determined diseases such as cancer, atherosclerosis, diabetes, immune system impairment, Parkinson's and Alzheimer's diseases, arthritis as well as premature body aging. Efforts to reduce toxicity to normal tissues and organs led to the search for radiation-protecting drugs and compounds with potential application during planned radiation exposures such as radiotherapy, diagnostic scanning, undertaking clean-up operations in nuclear accidents, space expeditions, etc and unplanned radiation exposures such as accidents in nuclear industry, nuclear terrorism and natural background radiation (Nair *et al*., 2001).

Many compounds with antioxidant activities are proved to be effective radio protectors (Weiss and Landauer, 2000; Weiss and Landauer, 2003) and since flavonoids and other polyphenolic compounds possess antioxidant activity the interest in these molecules as radioprotector is increased (Nair *et al*., 2001; Weiss and Landauer, 2003; Maurya *et al*., 2006;Sankaranarayanan, 2006). Flavonoids are a class of plant secondary metabolites, present ubiquitously in fruits, vegetables, and

beverages. They have been referred to as "nature's biological response modifiers" because of strong experimental proof of natural ability to change the body's reaction to allergens, viruses, and carcinogens.

Alpinia galanga L. (also known as 'greater galangal', family: *Zingiberaceae*), is a rhizomatous herb, which grows widely in many parts of India, China and south-east Asia. *Alpinia galanga L*. rhizome is an important ingredient in Thai curry paste and is commonly used as a flavouring agent (Uhl, 1996). The rhizome is used in Indian traditional medicines ("Ayurveda" and "Sidha") to treat diabetes, bronchitis, rheumatism and diseases of the heart (Sukhdev, 2006). Lack of published reports on the presence of flavonoids in *Alpinia galanga* L. rhizomes appeared significant that led us to investigate the flavonoids present in the rhizomes, as the biological activitiy of most of the natural products arise from the phenolic compounds/flavonoids present in them. Also only a few investigations have been carried out on the antioxidant properties of the plant. The antioxidative property as well as the effectiveness of the ethanol extract of *Alpinia galanga* L. as inhibitor of lipid peroxidation in cooked ground pork has been reported (Juntachote *et al*., 2006). Thus an in depth study of *A. galanga* L. appeared significant as it is an important dietary constituent in many parts of the world and also is extensively used in traditional medicines of India and China for chronic diseases like rheumatism where inflammation is perhaps initiated through Reactive Oxygen Species (ROS).

The present report summarizes free radical scavenging, antioxidant and radioprotective activities of two important flavonoids, viz., galangin (3,5,7-Trihydroxyflavone) and kaempferide (4'-Methylkaempferol) from *Alpinia galanga L*. rhizome.

9.2 MATERIALS AND METHODS

9.2.1 Chemicals

pBR 322 DNA was obtained from Bangalore Genei, India. Folin-Ciocalteu phenol reagent, Nitro blue tetrazolium (NBT), Ethylene diamine tetra acetic acid disodium salt (EDTA) were purchased from Sisco Research Lab, Mumbai, India. DPPH˙(2,2-diphenyl 1- picryl hydrazyl) was obtained from Sigma Aldrich. All other chemicals were of analytical grade procured from reputed Indian manufacturers.

9.2.2 Irradiation

^{60}Co- Theratron Phoenix teletherapy unit (Atomic energy Ltd., Ottawa, Canada) was used to carry out irradiation at a dose rate of 1.88 Gy per minute.

9.2.3 Plant material and extraction

Alpinia galanga L. belonging to the family Zingiberaceae is obtained from the Ayurvedic Research Institute (Poojappura, Trivandrum, Kerala, India) and was identified by their resident botanist. For the isolation of major constituents from the rhizomes of *A. galanga* L., a total of 250 g of the crudely powdered dried rhizomes were extracted with acetone (3 x 1000 mL) at room temperature (27 °C) (we observed that crude extract obtained by extraction with acetone carried out using Soxhlet apparatus tended to polymerize during concentration procedure). The solvent was removed under reduced pressure at 40°C to get 6.8 g of the crude acetone extract. 0.5 g of this extract was kept for antioxidant assays. 6.3 g of the acetone extract was subjected to silica gel column chromatography (100-200 mesh) using increasing polarites of hexane–ethyl acetate (EtOAc-Hexane) mixture.

In order to obtain the ethanol extract, 100 g of dried and powdered rhizomes were extracted with ethanol (2000 mL, 24 h) using a Soxhlet apparatus. This extract was found to be stable and was used as such for antioxidant assays.

9.2.4 Phytochemical Screening

Total phenolic constituents in the ethanolic and the acetone extract of *Alpinia galanga* L. rhizomes was analyzed by employing the method of Slinkard and Singleton (1977). By using gallic acid as a standard, the total phenolic content of the ethanol and acetone extracts of *Alpinia galanga* L. rhizomes has been calculated and expressed as gallic acid equivalents (mg GAE/100 g dry rhizomes). Total flavonoid content were determined according to a colorimetric method (Woisky and Salatino, 1998) employing quercetin as the standard.

9.2.5 Assay for total antioxidant capacity

The total antioxidant capacity of the extracts was evaluated by the phosphomolybdenum method (Prieto *et al*., 1999) using ascorbic acid was used as the standard.

9.2.6 Free radical scavenging activity of ethanolic and acetone extract of *Alpinia galanga*

In order to determine the free radical scavenging activity of ethanolic and acetone extract of *Alpinia galanga,* the following parameters were assayed. Superoxide radical scavenging activity was done by the NBT (nitroblue tetrazolium) reduction method (Oktay *et al*., 2003). The deoxyribose method was used to study the hydroxyl radical scavenging property of the samples (Halliwell *et al*., 1987). Hydroxyl radical scavenging activity was calculated by studying the competition between deoxyribose and extract for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2

system. DPPH radical scavenging was determined by the method of Sanchez-Moreno *et al*. (1998) with minor modifications.

9.2.7 Isolation of compounds

The acetone extract was subjected to silica gel column chromatography and eluted with EtOAc-Hexane mixtures of increasing polarity starting from (9:1) to (1:9). The fractions were pooled according to the similarities in their thin layer chromatography plates (TLC's). The fractions from 51-77 were combined and evaporated to get 1.5 g of a solid which crystallizes to yield galangin (compound **1**) as yellow flakes (1.398 g). Fractions 78–94 were combined and evaporated to yield a solid (0.150 g) which on crystallization from chloroform–methanol yielded kaempferide (compound **2**) as pure yellow crystals (0.108 g) (**Figure 9.1**).

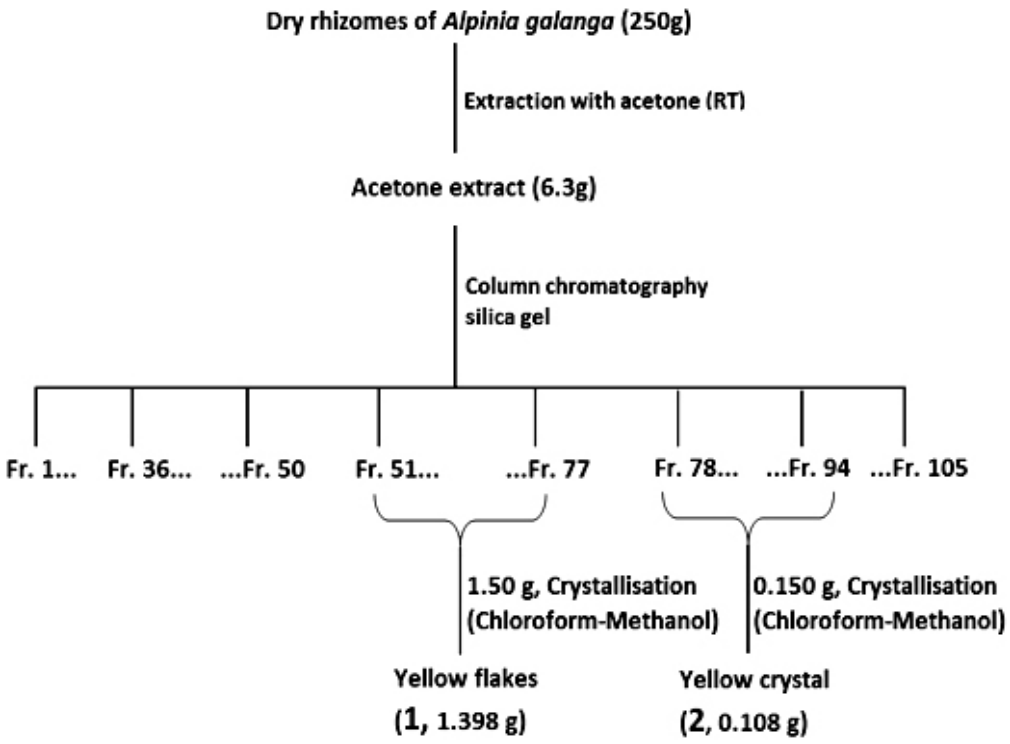

Figure 9.1 Pictorial representation of isolation of Compound 1 (Galangin) and Compound 2 (Kaempferide).

9.2.8 Effect of Galangin and Kaempferide on γ- radiation induced DNA damage

Plasmid DNA in phosphate buffer (0.1 M, pH 7.4) (100 ng) was exposed to 25 Gy γ-radiation in presence or absence of 10 mM flavanoids (*galangin or kaempferide*), on ice. After irradiation plasmid DNA was electrophoresed on 0.8% agarose in 0.08 M Tris borate/0.2 mM EDTA buffer (pH 8.3) at 55 V, 75 mA for 2 hours and the amount of DNA damaged was studied by Digital Gel Documentation and Analysis Software, Biotech R&D Laboratories, Yercaud, Tamil Nadu, India.

Human blood samples were collected from 3 healthy non-smoking volunteers. The blood samples were subjected to 4 Gy gamma-radiation at ambient temperature in presence or absence of 10 mM of galangin or kaempferide. The blood samples were subjected to alkaline single cell gel electrophoresis. The DNA strand breaks in human peripheral blood leucocytes were measured using alkaline single cell gel electrophoresis performed using method given by Chandrasekharan *et al*. (2009). The comets were visualized after silver staining (Cerda *et al*., 1997) and the images captured were analyzed using the software 'CASP' which gives % DNA in tail, tail length, tail moment and olive tail moment directly. The parameter tail moment (TM) is the product of tail length and % DNA in tail and olive tail moment (OTM) is the product of the distance between the centre of the head and the centre of the tail and % DNA in tail (Konca *et al*., 2003). The results are presented as mean±SD of the studied groups. Statistical analyses were performed using ANOVA with Tukey–Kramer multiple comparisons test.

9.3 RESULTS

9.3.1 Phytochemical Screening

The total amount of phenolics in *A. galanga* show 1.93 ± 0.7 g GAE /100 g of dry rhizomes in the ethanol extract and 2.17 ± 0.3 g GAE /100 g of dry rhizomes in the acetone extract. The total flavonoids content expressed in grams as the number of equivalents of quercetin for the ethanolic extract of the rhizomes of the *A. galanga* was 3.3 ± 0.9 and the acetone extract contained 0.01 ± 0.004.

9.3.2 Total antioxidant capacity

The ethanol extract of *Alpinia galanga* rhizomes showed antioxidant capacity of 9.6 ± 1.6 g ascorbic acid equivalents/100 g dry weight of the rhizomes whereas the acetone extract showed a higher antioxidant capacity of 11.1 ± 4.8 g ascorbic acid equivalents/100g dry weight of the rhizomes.

9.3.3 Antioxidant activity of *A. galanga* ethanol extract (EE), acetones extract (AE), galangin and kaempferide

The antioxidant capacity of the extracts and pure compounds against three reactive species, *viz*., the superoxide ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$) and DPPH free radicals ($DPPH^{\bullet}$) were studied by comparing with the standard antioxidants.

The compounds demonstrated a concentration dependent scavenging capacity by neutralizing the superoxide radicals. A considerably lower concentration (400-1000 ppm) of the pure flavonoids showed good antioxidant capacity and their activity was compared with the standard flavonoid, quercetin. Kaempferide showed the highest superoxide scavenging property with an EC_{50} value of 868 ppm compared to galangin (EC_{50} value, 903 ppm) and Quercetin (EC_{50} value, 1560 ppm). At this concentration, the extracts did not show any superoxide radical scavenging capacity. Therefore the activity of AE and EE were evaluated at higher concentrations (2000-8000 ppm)

along with Tocopherol as the standard. Of these, the standard tocopherol showed maximum superoxide scavenging capacity with an EC_{50} value of 3663 ppm whereas EE and AE showed EC_{50} value of 7033 ppm and 10509 ppm respectively (**Table 9.1**).

The extracts and pure compounds scavenged hydroxyl radicals in a concentration dependent manner. The activity was compared with that of the standards, butylated hydroxy anisole (BHA) and quercetin. The high hydroxyl radical scavenging activity of the flavonoids and the extracts could be attributed to the active hydrogen donating ability. All the tested samples showed more or less the same EC_{50} values ranging from 1.39-1.51 ppm (**Table 9.1**).

The stable free radical DPPH with characteristic absorption at 515 nm was reduced by the flavonoids, galangin and kaempferide resulting in decrease in the absorption, which is directly related to the electron scavenging capacity of the flavonids (**Table 9.1**).

Table 9.1 The antioxidant capacity expressed as EC_{50} values of the AE, EE, galangin, Kaempferide and the standards

	Radical scavenging capacity, EC_{50} (ppm)		
Sample	**Superoxide**	**Hydroxyl**	**DPPH$^{\cdot}$**
Alpinia galanga ethanol extract (EE)	7033	1.51	895
Alpinia galanga acetone extract (AE)	10509	1.70	851
Galangin	903	1.40	442
Kaempferide	868	1.41	541
BHA	-	1.39	139
Tocopherol	3663	-	-
Quercetin	1560	2.00	1.67

9.3.4 Isolation of galangin (1) and kaempferide (2)

Based upon the observation that the acetone extract of *A. galanga* showed higher phenolic content, higher flavonoid content as well as high total antioxidant capacity, the flavonoids galangin (**1**, 1.398 g) and kaempferide (**2**, 0.108 g) were isolated and characterized. The compounds isolated corresponded to different spectral values with that reported in the literature (Markham and Mabry, 1975) and found to possess the same melting points as galangin (214-215 °C) and kaempferide (198-199°C) (Bleier and Chirikdjian, 1972). The concentration of galangin and kaempferide in the rhizomes of *A. galanga* were 5.592 g/kg and 0.432 g/kg of the plant material respectively. The structures of the isolated compounds are given in **Figure 1.8.**

9.3.5 Effect of Galangin and Kaempferide on γ- radiation induced DNA damage

The agarose gel electrophoresis pattern of pBR 322 DNA exposed to 25 Gy γ-radiation in the presence or absence of 10 mM galangin, and kaempferide is given in **Figure 9.2(a)**. Exposure of plasmid pBR 322 DNA to gamma radiation resulted in strand breaks by which the super coiled/ covalently closed circular form (ccc) of plasmid DNA was converted to open circular (oc) form or linear form. The disappearance of ccc form of plasmid DNA following exposure to gamma radiation can be taken as an index of DNA damage induced by radiation. Both galangin, and kaempferide gave protection to the plasmid DNA against radiation induced strand breaks as shown in **Figure 9.2(b)**.

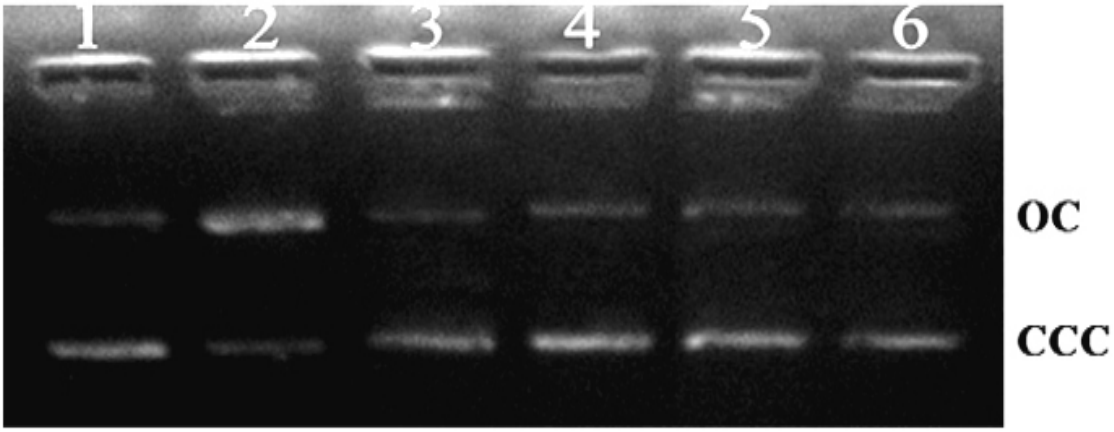

Figure 9.2 (a). Effect of Galangin and Kaempferide (10 mM) on Plasmid DNA (pBR 322) damage induced by Gamma irradiation. Lane1: 0 Gy, Lane 2: 25 Gy, Lane 3: Galangin +0 Gy, Lane 4: Kaempferide +0 Gy, Lane 5: Galangin +25 Gy, Lane 6: Kaempferide +25 Gy. 'OC' represent open circular form of pBR, 'CCC' represent covalently closed circular form of pBR.

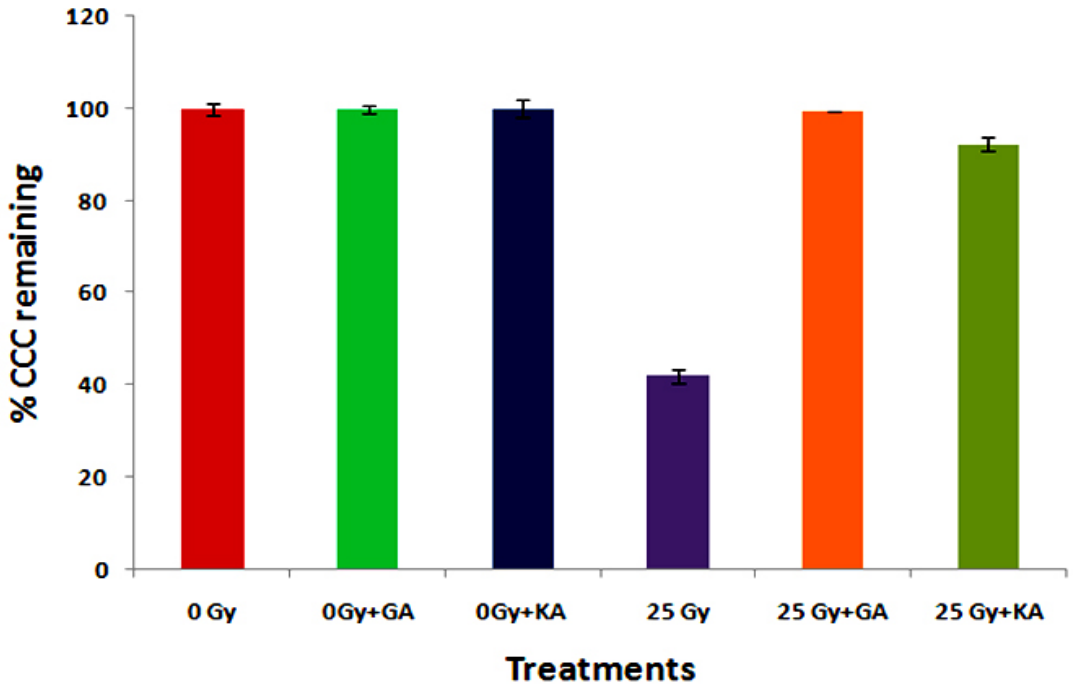

Figure 9. 2(b). Protection of plasmid pBR 322 DNA by Galangin and kaempferide (10 mM) against different doses of gamma radiation (25 Gy).

The results of comet assay performed on human blood leukocytes irradiated *ex vivo* in the presence or absence of 10 mM kaempferide or galangin is shown in **Figure 9.3**. Exposure of human peripheral blood leucocytes to 4 Gy gamma radiation caused

damage to the cellular DNA as evident from the increase in the comet parameters. Percentage DNA in tail, tail length, tail moment and Olivetail moment were increased from 3.56±0.90, 3.15±0.53, 0.12±0.04 and 0.51±0.24 to 11.10±2.77, 9.31±0.73, 4.26±0.40, 2.65±0.80 in the control irradiated group. The presence of 10 mM kaempferide helped in reducing the extent of DNA damage as can be seen from the comet parameters which were brought down to 4.87 ± 0.58, 4.41±1.65, 0.45±0.16, and 0.47 ±0.04 respectively ($P < 0.001$). Galangin (10 mM) caused reduction of comet parameters to 6.07 ±0.52, 7.28 ±1.55, 0.55 ±0.14 and 0.53 ±0.17 respectively.

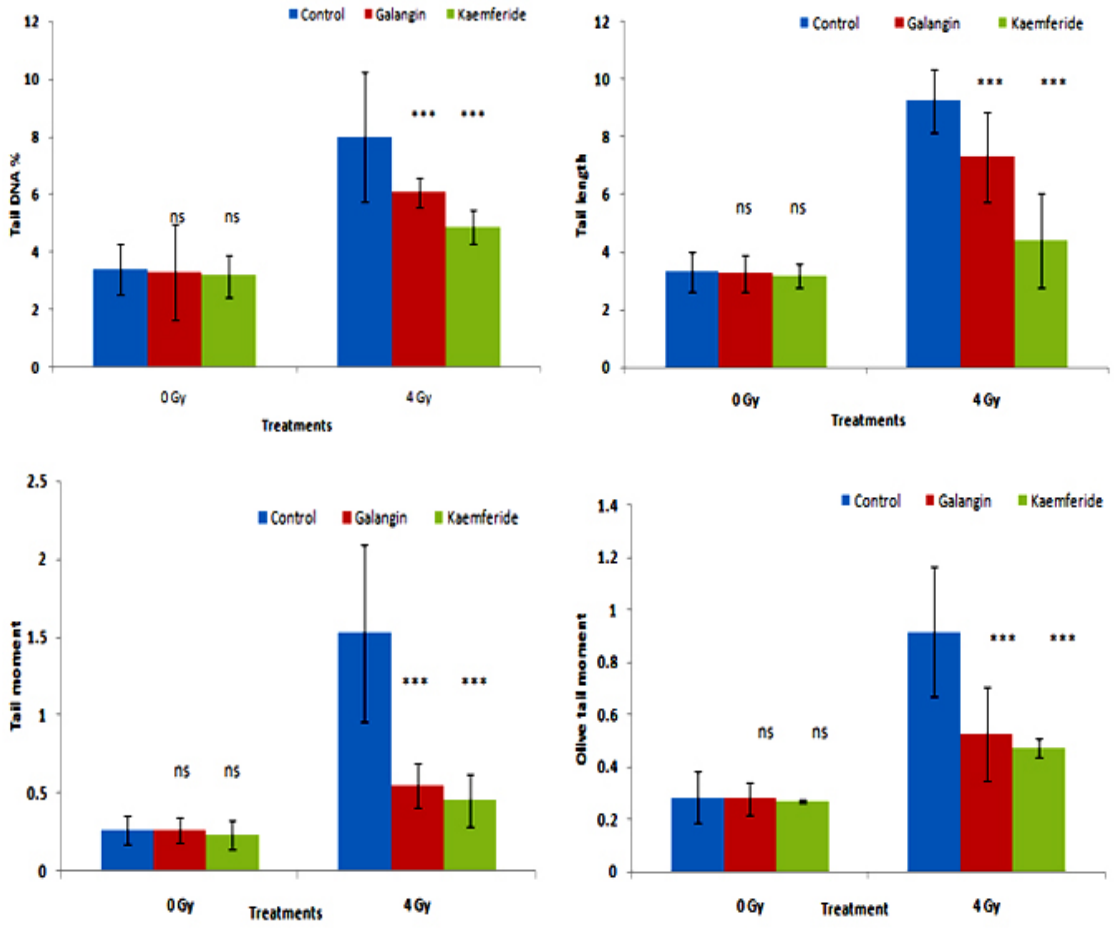

Figure 9. 3. Effect of Galangin and Kaempferide (10 mM) on DNA damage in human peripheral blood leukocytes induced by exposure (*ex vivo*) to gamma radiation (4 Gy) assessed by comet assay. Percentage DNA in tail, tail length, tail moment and olive tail moment are presented as mean ± sd. 'ns' indicate not significant and * indicate p < 0.001 when compared with respective control.**

9.4 DISCUSSION

Flavonoid's potential health benefits are due to their antioxidant effects which can be attributed to the phenolic hydroxyl groups attached to the flavonoid structure (Kanner *et al*., 1994). Flavonoids due to their free radical scavenging properties has been suggested to have radioprotective activities (Shimoi *et al*., 1996;Umadevi *et al*., 1998). The present study focuses on free radical scavenging and radioprotecting properties of *Alpinia galanga* L. and the two bio-flavonoids isolated *viz*. galangin, and kaempferide.

A. Galanga L. extract has been reported to possess several pharmacological activities. Earlier reports have shown that it exhibits antioxidant properties (Tachakittirungrod and Chowwanapoonpohn, 2007). Moreover *A. Galanga L*. also found to have anti inflammatory (Min *et al*., 2009), immune-stimulating (Bendjeddou *et al*., 2003), and anticancer (Lee and Houghton, 2005) activities.

The *Alpinia galanga* L. rhizome extract prepared in acetone had higher content of phenolics, flavonoids and total antioxidant capacity than the extract prepared in ethanol. The current study indicates that rhizome extracts and the isolated flavonoids effectively scavenged superoxide and hydroxyl radicals in a dose dependent manner. These radicals are generated inside the body during the normal metabolism or in presence of xenobiotics. The stable free radical DPPH was also scavenged by the extracts and flavonoids.

The search for non-toxic, selective and effective cytoprotective compounds that preferentially protect normal tissues, without protecting malignant tissue, is of prime concern in radiation biology. Such compounds could protect against the genetic damage, mutation, immune system alterations, as well as the teratogenic effects of

radiation. Several phytochemicals have been shown to be effective radioprotectors (Weiss and Landauer, 2000; Weiss and Landauer, 2003).

In living systems DNA constitutes the primary vital target for cellular inactivation by ionizing radiation. The damages to cellular DNA produced by exposure to ionizing radiation are mainly lesions in DNA like double and single – strand breaks, base damages, elimination of bases, sugar damage, DNA- DNA and DNA- protein cross-links. The evaluation of these lesions is an essential step in the examination of the sequence of events leading to mutagenic, carcinogenic and other lethal effects of radiation. In the present study it is clear that the presence of the galangin or kaempferide protected plasmid DNA from the radiation induced damages. Thus the flavonoids effectively protect DNA against ionizing radiation in a system devoid of repair and replication machinery.

Alkaline comet assay is a sensitive technique to monitor strand breaks and alkali labile DNA lesions and is widely used to study genotoxicity, cellular DNA lesions such as single strand breaks or double strand breaks, apoptosis and DNA repair (Tice *et al*., 2000; Maurya *et al*., 2005). When human peripheral blood leukocytes are exposed to γ-radiation *ex vivo*, the cellular DNA undergo damage, as seen in the increase in comet parameters (tail length, % DNA in tail, tail moment and olive tail moment). Presence of galangin or kaempferide during irradiation of the cells decreased the comet parameters indicative of its radioprotecting ability.

The protection given to DNA by the flavonoids is maybe due to the scavenging of radiation-induced primary as well as secondary reactive oxygen species. More over the study also revealed that these flavonoids does not induce any DNA damage by itself. Various studies reported that *in vitro* or *in vivo* treatment of lymphocytes with galangin suppressed the stimulation of chromosome aberrations induced by

bleomycin in a dose-dependent manner (Heo *et al*., 1994). Murray *et al.* (2006) have reported that galangin inhibited transition of cells from the G0/G1 to the S phases of cell growth, through total elimination of cyclins D3,A and E and inhibits Hs578T cell proliferation and the activity of the AhR, (a transcription factor implicated in the initiation and growth of mammary tumors).

Thus the present work showed the ability of isolated bioflavonoids, galangin and kaempferide in protecting DNA against ionizing radiation induced damages under *in vitro* and *ex vivo* conditions. The mechanism of radioprotection by these compounds could be ascribed to its antioxidant and free radical scavenging activities. The present study suggests the possibility of using the extract of *Alpinia galanga L.* or the isolated bioflavonoids for the prevention of deleterious effects of ionizing radiation in situations of radiation exposure.

Chapter- 10

ESTIMATION OF FERULIC ACID (FA) FROM SELECTED PLANT MATERIALS

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10.3RESULTS AND DISCUSSION

10.1 INTRODUCTION

Antioxidants are compounds needed by most organisms, where they prevent oxidative damage caused by free radicals. The formation of free radicals causes the development of various diseases like cancer, cardio-vascular diseases and cataracts in humans (Zhang *et al*., 2007). The antioxidant activities of plants have been mainly due to their phenolic content, which is one class of natural antioxidants (Castaneda-Ovando *et al*., 2009). Thus, plants containing a high-level of phenolic acids considered a source of potent natural antioxidants (Ranusova *et al*., 2021). Ferulic acid (FA), together with dihydroferulic acid, could be a component of lignocelluloses, confers plasma membrane rigidity by cross linking lignin and polysaccharides. It is commonly found in seeds of plants like rice, wheat and oats (Buranov and Mazza, 2009). Ferulic acid will be easily absorbed by the body and stays within the blood longer than the other antioxidant, even longer than vitamin C. Thus FA can be considered as an important antioxidant and commonly used in nutrition purposes and food supplements (de Oliveira and Batista, 2017).

Ferulic acid is found in many vegetable sources and occurs in particularly high concentration in popcorn and bamboo shoots. As an antioxidant, FA plays a major role in the body's defence against carcinogenesis by inhibiting the formation of N-nitroso compounds (Kuenzig *et al*., 1984; Lee *et al*., 2009; Aarabi *et al*., 2016). Ferulic acid possesses anticancer, antioxidant, and anti-aging potentials and can decrease blood glucose levels. Like other phenolic compounds, FA showed radioprotective abilities and reduced ionizing radiation-induced damages to DNA and membranes in biological systems (Roginsky and Lissi, 2005; Divakaran *et al*., 2013; Kumar and Goel, 2019).

Therefore, we exploited a simple, repeatable, sensitive and cost-effective VIS Spectrophotometric method for the quantification and determination of FA from various plant materials. Folin–Ciocalteu assay is used for the quantification of phenolic acid in the presence of alkali (15 % sodium carbonate). High-Performance Liquid Chromatography (HPLC) method is considered to be as an appropriate method for estimation of chemical constituents from plant materials. Therefore, HPLC analysis has also been used for the quantitative determination of ferulic acid.

10.2 MATERIALS AND METHODS

10.2.1 Instrument

Soxhlet apparatus, VIS spectrophotometer (Systronics), HPLC (Agilent Technologies 1200 Infinity Series) were used.

10.2.2 Chemicals

AR grade chemicals such as ethyl acetate, Folin – Cio-calteu reagent and Sodium carbonate were obtained from Nice chemicals. Double distilled water was obtained after purification. Ferulic acid (FA) of 98% purity was purchased from NICE.

10.2.3 Plant Materials

Plant materials selected such as rice bran (*Oryza sativa*), wheat bran (*Triticum aestivum*) and Bamboo shoot (*Bambusa vulgaris*) were collected from homestead region. Rice bran and wheat bran as whole and young bamboo shoot tip were dried, powdered with the help of blender and kept in sealed containers for future use.

10.2.4 Folin–Ciocalteu assay

Detailed methodology is explained in Chapter 2, section **2.2.2.**

10.2.5 Standard solution of FA preparation (Stock)

Detailed methodology is explained in Chapter 2, section **2.2.1.**

10.2.6 Calibration curve of FA

Detailed methodology is explained in Chapter 2, section **2.2.2.**

10.2.7 Preparation of Ethyl acetate extract of Plant materials

Detailed methodology is explained in Chapter 2, section **2.2.3.**

10.2.8 Preparation of Sample solution

Detailed methodology is explained in Chapter 2, section **2.2.4.**

10.2.9 Validation of the proposed method

10.2.9.1 Linearity

The linearity was determined by constructing the calibration curve and evaluating it by linear least square regression analysis.

10.2.10 Chromatography

Column : C18 4.6×250mm×5µm

Flow rate : 1.0mL/Minute

Inj.Volume : 20µL

Wave length : 319nm

Run time : 10 minute

Column temperature: 30^0C

10.2.10.1 Method

Mobile phase : 5% Glacial Acetic acid in HPLC water : Acetonitrile(80:20)

10.2.10.2 Standard preparation

Ferulic acid stock solution of 1000ppm was prepared in methanol. From this, working standard of 2.5,5,10,15,20 ppm were prepared by serial dilution of the stock solution with methanol.

10.2.10.3 Sample preparation

The ethyl acetate extract of plant samples were concentrated, filtered with 0.45µm disposable filter, 20µL samples were injected to the HPLC system for the analysis..

10.3 RESULTS AND DISCUSSION

Ferulic acid is a strong scavenger of free radicals and it has been accepted as a food additive to prevent lipid peroxidation. The selected plant materials such as rice bran, wheat bran, bamboo shoot are recognized in different systems of traditional medicine for the treatment of various diseases (Kumar and Pruthi, 2014). The mixture of ferulic acid along with Folin Ciocalteu reagent in an alkaline medium yielded a maximum absorbance at 718 nm. A linear relationship was obtained when a graph was plotted for concentration v/s absorbance within the concentration range of 1µg/ml - 8 µg/ml with a correlation coefficient value r2=0.988 and there-fore, the rectilinear regression equation was y= 0.094x – 0.001(**Table 10.1**).

Table 10.1 Regression Analysis Data

Regression equation	y= 0.094x – 0.001
Range	1µg/ml - 8 µg/ml
Co-relation coefficient r2	0.988
Slope m	0.094
y-intercept	0.001

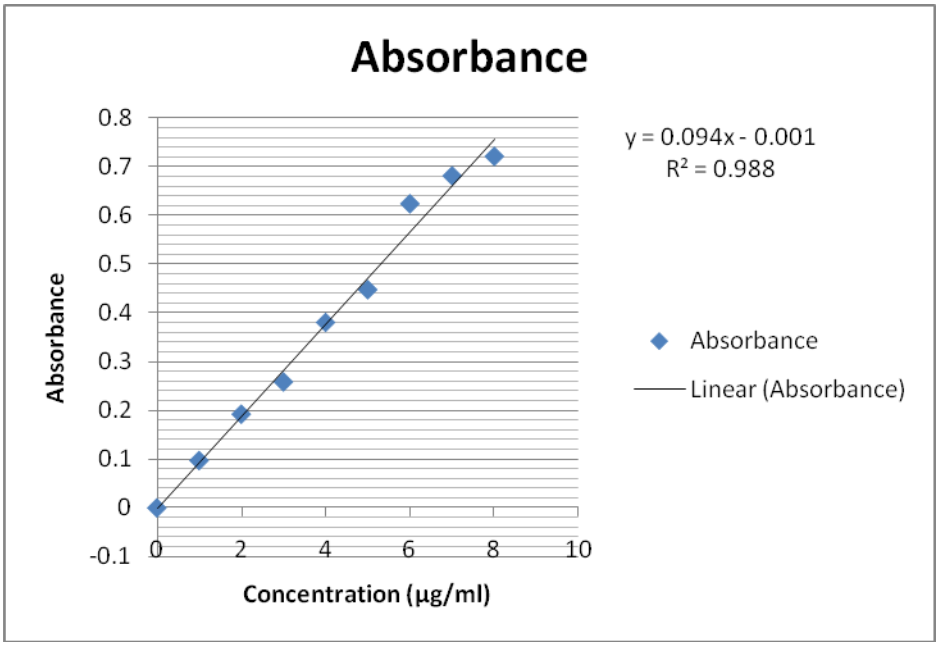


Figure 10 .1 Calibration curve of ferulic acid

Table 10. 2 Concentration of FA from various plant samples calculated from standard Graph

Sample	Unknown (µg/ml)
Bamboo shoot	1.7
Wheat bran	7.3
Rice bran	8.0

The various samples used for the studies yielded different concentrations of FA, calculated by using the standard graph (**Figure 10 .1**). The bamboo shoot sample used for the study contained 1.7 µg/ml of FA, the wheat bran sample contains 7.3 µg/ml, and the rice bran sample yielded a maximum amount as 8 µg/ml (**Table 10. 2**).

Chromatographic studies

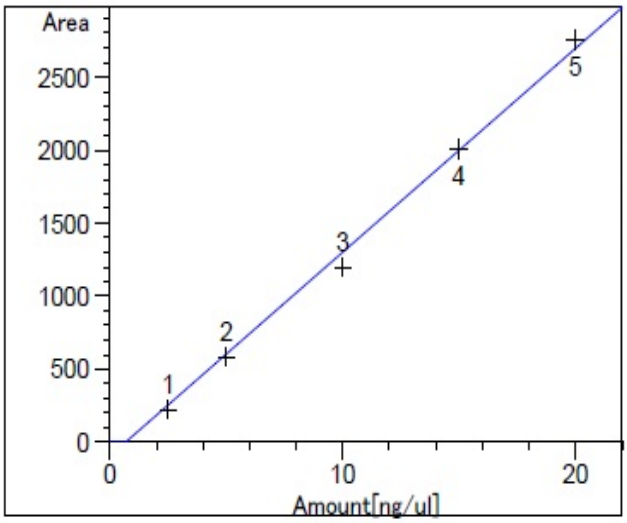
In the HPLC analysis, Ferulic acid got eluted at 6.82 min (**Figure 10. 3**) and the peak for the same was found to be at 6.794 in the bamboo shoot sample (**Figure 10. 4**), 6.804 for the wheat bran sample (**Figure 10. 5**) and 6.791 for rice bran sample (**Figure 10. 6**) under the conditions of detection at 319 nm and temperature 30°C. The amount of ferulic acid in bamboo shoot, wheat bran and rice bran was found to be 1.92mg/kg, 11.03mg/kg and 14.03mg/kg, respectively, as calculated from the calibration curve of FA (**Figure 10. 2**). The present study showed that rice bran yielded a higher amount of FA than bamboo shoot and wheat bran. Recent studies also unveiled the fact that rice bran is a rich source of antioxidant molecules such as γ-oryzanol and ferulic acid (Arumsari *et al*., 2019; Tam *et al*., 2021).

The extraction of Ferulic acid has been found much attention nowadays because it exhibits a wide variety of biological activities, including antimicrobial, anti-inflammatory, anti-thrombosis, anticancer, and antioxidant activities. However, the extraction procedure of phenolic acids from biomass is very complicated and proper methodology is yet to be developed (Zavala-Lopez and Garcia-Lara, 2017; Zhong *et al*., 2019).

The extraction and purification of phenolic acid viz. ferulic acid from rice bran and orange peels by solvent extraction method was studied by Gogoi *et al*. (2017). One of the major drawbacks of this procedure is its requirement for large quantities of different solvents and chemicals, which generates a significant quantity of toxic solvent waste (Acosta-Estrada *et al*., 2014). Ideia *et al*. (2020) reported the use of autoclave to perform alkaline hydrolysis and partial purification by adsorption on a synthetic resin to obtain ferulic acid from brewer's spent grain. The procedure is

additionally very time consuming, making the handling of several samples without delay a challenging task.

In the present study, the estimation of phenolic acid viz. ferulic acid from rice bran, wheat bran and bamboo shoot by spectrophotometric method and the HPLC technique seems to be simple, sensitive, reproducible with minimum sample quantity, solvent and extraction time compared to methods proposed by Gogoi *et al*. (2017) and Ideia *et al*. (2020). The result obtained from the quantitative estimation of FA by Spectrophotometry and HPLC shows a parallel relationship. The chromatogram developed from HPLC also explains the isolation of ferulic acid with minimum impurities; hence can be recommended as a precise technique for the estimation of ferulic acid from plant samples.



at exp. RT: 6.833
VWD1 A, Wavelength=319 nm
Correlation: 0.99786
Residual Std. Dev.: 78.96265
Formula: y = mx + b
m: 139.83489
b: -95.38780
x: Amount
y: Area

Figure 10 2. Calibration curve of Ferulic acid

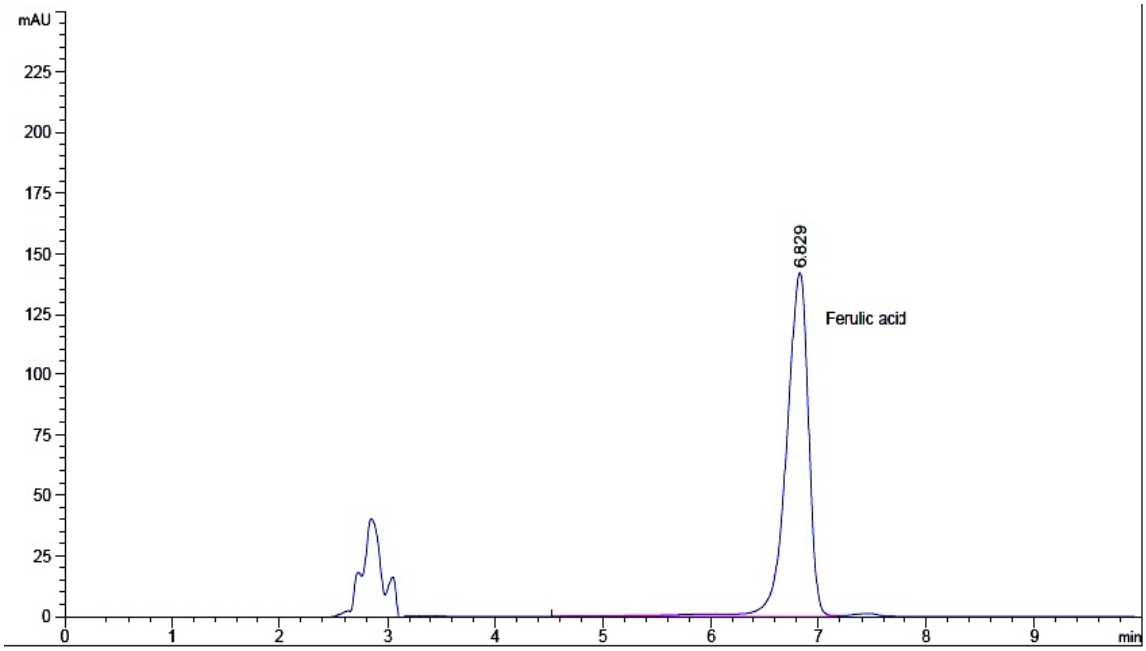


Figure 10. 3. HPLC chromatogram of Standard Ferulic acid

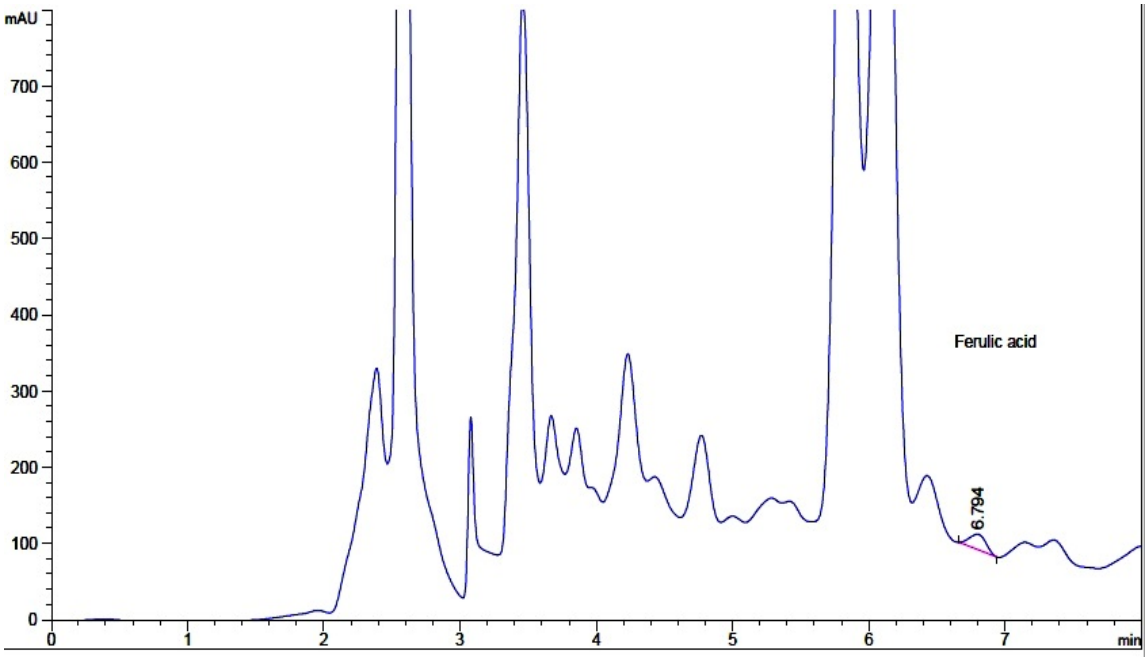


Figure 10. 4. HPLC chromatogram of Ethyl acetate extract of bamboo shoot

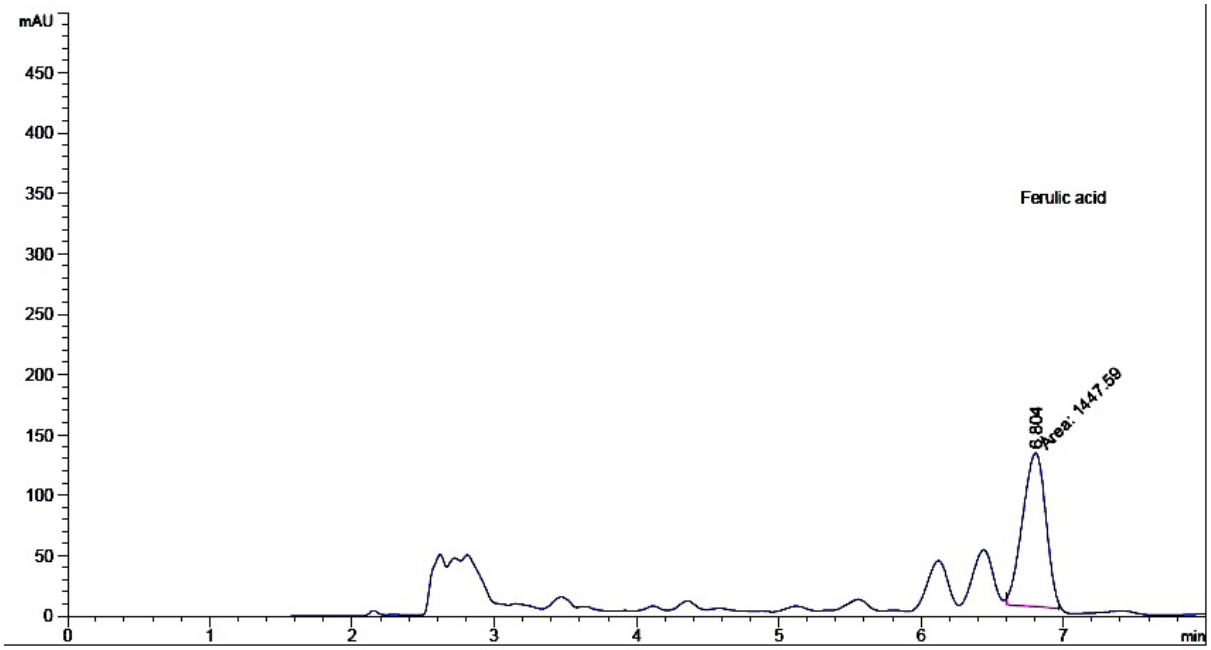


Figure 10. 5.HPLC chromatogram of Ethyl acetate extract of Wheat bran

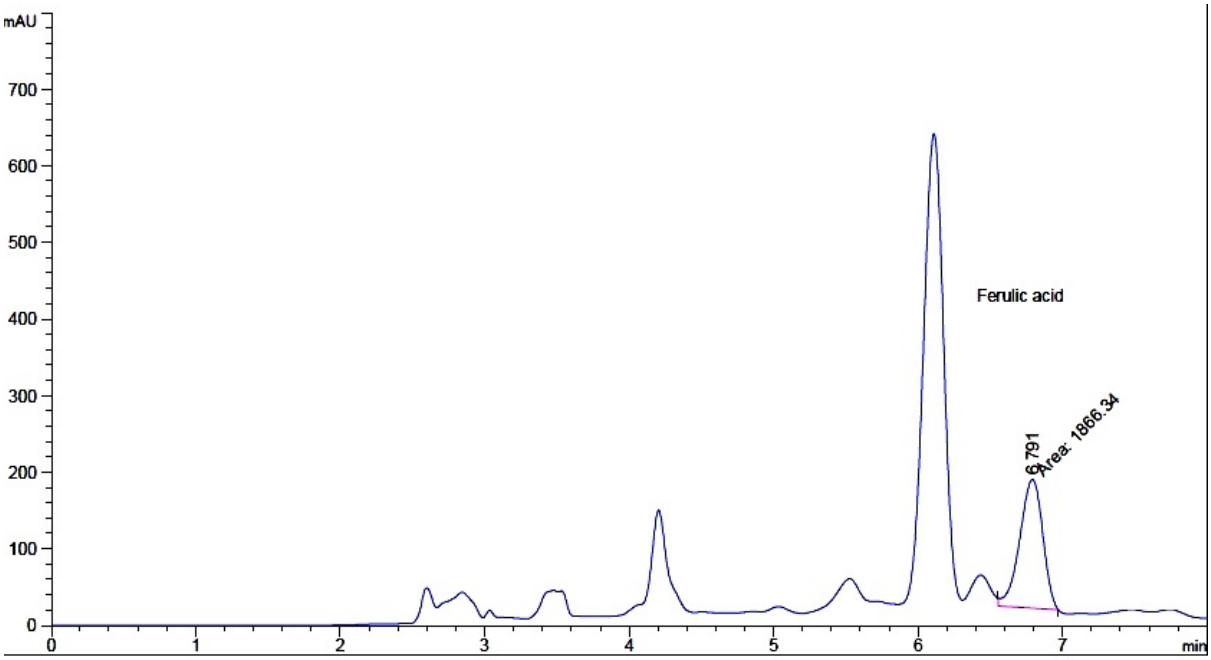


Figure 10. 6. HPLC chromatogram of Ethyl acetate extract of Rice bran

A simple, sensitive and reproducible VIS-Spectrophotometric method has been exploited to estimate and quantify ferulic acid in various plant materials like bamboo shoots, rice bran and wheat bran *etc*. using Folin Ciocalteu reagent in the presence of an alkaline medium. The method can be employed for the routine analysis of ferulic acid from the various plant species. Moreover, they can prove to be helpful for nutritional and clinical investigations of ferulic acid levels in a variety of samples. The method for estimating ferulic acid in bamboo shoots, rice bran and wheat bran by HPLC is accurate, precise and reproducible.

Chapter- 11

SUMMARY AND CONCLUSION

Cancer remains one of the leading causes of death in the world. Recent advances in nanoscience and nanotechnology have led to the development of nanomaterials for molecular and cellular imaging, cancer therapy, and integrated nanodevices for cancer detection and screening. It is highly desirable that nanoparticles cannot only provide sensitive and specific imaging information in cancer patients but also selectively deliver anticancer drugs to tumor sites. Currently, there is limited knowledge of suitable biomarkers for imaging, selection of the imaging target and contrast enhance materials, and the chemistry required to assemble the bioactive imaging probe. In addition, numerous obstacles are faced in developing cancer-specific imaging agents, such as 1) delivery of the probe to the targeted tissue/tumor; 2) biocompatibility and toxicity; 3) stability of the probe and effective signal enhancement *in vivo*; 4) adequate imaging methods and strategies. During chemotherapy, pharmacologically active cancer drugs reach the tumor tissue with poor specificity and induce dose-limiting toxicities. Nanoparticle drug delivery may provide a more efficient, less harmful solution to overcome these problems.

In the present study we have isolated and purified DAO, synthesized Fe_2O_3 nanoparticles and prepared Fe_2O_3 nanoparticles-DAO complex. The prepared complex was analysed for its anti tumor potential under *in vitro* and *in vivo* conditions.

The isolated DAO from porcine kidney yields a 6000- fold purification factor with respect to crude extract. The *in vitro* cytotoxic studies with DAO and D- alanine proved that cellular DNA damage of DLA cells occurred as a result of apoptosis. DAO alone or D-alanine alone did not showed any cytotoxicity. The result suggests that DAO and D-alanine could be used for the production of H_2O_2 in the tumor site and thereby inducing apoptosis at those sites.

The stability studies with ferric nano complex of D-amino acid oxidase (Fe_2O_3-DAO) showed that activity of Fe_2O_3–DAO remained unchanged after 30 days indicating the protective function of nanoparticles. The Fe_2O_3–DAO to generate H_2O_2 at tumor site when its substrate D-amino acids (D-alanine) are supplied exogenously. The induction of apoptosis to cancer cells by ferric enzyme nanocomplex with D-alanine results in extensive damage to cellular DNA as revealed in comet assay. Under *in vitro* conditions, the killing of DLA cells were enhanced in presence of the Fe_2O_3-DAO and its substrate D-alanine. Treatment of the cells with D-alanine along with Fe_2O_3-DAO showed an apoptotic index of 95.5%. Under *in vivo* antitumor studies tumor growth was significantly suppressed in mice administered Fe_2O_3-DAO and D-alanine along with magnetic treatment in the fourth day. Decrease in GSH and increase in the TBARS level on tumor tissues of animals treated with Fe_2O_3-DAO complex and D-alanine with or without magnetic field application may be due to the oxidative stress induced by the drug, and also helped to maintain normal levels of GSH and TBARS in normal tissues. The results suggest, this method can used to control tumor growth by targeting orally administered nanoparticle bound enzyme with the help of an external magnetic field to enhance the efficacy of the treatment.

AsAG and FA showed significant ameliorative activity against carrageenan and formalin induced anti-inflammation which may be due to its radical scavenging activity. Administration of ferulic acid (100 mg/kg body weight) showed 31.4 % inhibition of edema on 6th day, where as ferulic acid exhibited 46.87 % inhibition at a dose of 200 mg/kg on 6th day. AsAG produced 42.8% inhibition at a concentration of 250 mg/kg (Figure 8.4) and 54.3 % inhibition of edema at a concentration of 500 mg/kg on 6th day.

Various xenobiotics are metabolized within the body of living organisms, by conjugation with the cellular antioxidant, GSH, reactions are catalysed by glutathione S-transferase (GST) enzyme thus causing the depletion of GSH , which lead to oxidative stress. Several drugs like acetaminophen, doxorubicin and cisplatin can increase the formation of free radicals in presence of increased oxygen tensions and can act as a major source of ROS. Therefore attempts have been made to alleviate the side effects of these drugs in mammalian system by the administration of AsAG and FA.

Our studies shown that the ferulic acid and ascorbic acid mono glucoside protected liver from paracetamol induced hepatotoxicity in mice and also showed inhibition of lipid peroxidation, free radicals scavenging and enhancement of antioxidant enzyme levels and protected serum marker enzymes.

Paracetamol (Acetaminophen) is a renowned antipyretic and analgesic agent. Hepatotoxicity is the major side effect due to the over dosage of paracetamol. Administration of paracetamol (150 mg/kg b.wt) resulted in hepatotoxicity as revealed from the elevated levels of serum marker enzymes. Post administration of AsAG or FA exhibited a significant reduction in acetaminophen induced levels of serum SGOT, SGPT, ALP, albumin and bilirubin which is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage. The results clearly indicates that the antihepatotoxic activity of the AsAG or FA probably through the correction of cellular integrity of hepatic cell and its regeneration.

Doxorubicin (adriamycin), an anthracycline antibiotic is well established and highly efficacious drug in the fight against many kinds of cancer, but the clinical usefulness is still restricted due to its specific toxicities to cardiac tissue. From the present study

it is clear that administration of DOX (25 mg /kg b.wt, *i.p*.) induced oxidative stress in cardiac tissues of tumor bearing mice as manifested from the elevated serum levels of SGOT, SGPT, LDH and CK. The increased levels LDH in serum suggested an increased leakage of these enzymes from mitochondria as a result of toxicity induced by DOX treatment. Administration of ASAG and FA after DOX treatment in tumor bearing Swiss albino mice restored all the biochemical parameters altered by this cytotoxic anticancer drug to near normal levels in the cardiac tissue. Both ASAG and FA prevented the DOX induced myocardial toxicity by boosting the endogenous antioxidant activity, lowering the cardiac biomarker enzymes (CK and LDH) and preventing the degeneration of cardiac tissue.

Cisplatin is another important antineoplastic agent widely used for the treatment of metastatic tumors. Nephrotoxicity and oxidative stress are the major side effects associated with the over dosage of cisplatin. Administration of cisplatin (12 mg/kg b. wt, *i.p*.) shows significant increase in blood creatinine and serum urea concentrations in tumor bearing mice compared to normal, which is an indication of acute renal failure. Treatment with AsAG or FA prevented the lipid peroxidation by enhancing the renal GSH, SOD and GPx activities. It also significantly reduced the micronuclei frequency in both experimental sets, suggesting their nephroprotection in tumor bearing Swiss albino mice. Moreover they also helped to prevent the decrease in antioxidant levels induced by cisplatin injection and restored the cellular integrity of the renal tissues.

Radiation protecting drugs and compounds are of great importance owing to their potential application during planned radiation exposures such as radiotherapy, diagnostic scanning, and undertaking clean up operations in nuclear accidents, space expeditions etc. and unplanned radiation exposures such as accidents in nuclear

industry, nuclear terrorism and natural background radiation. An ideal radioprotective agent should fulfill several criteria such as a general protective effect on the majority of organs and tissues, suitable for oral administration, rapid absorption and distribution in mammalian systems, should be devoid of any toxicity and adverse side effects, chemical stability, ready availability, low cost etc.

The medicinal effects of plants are often attributed to the antioxidant activity of the phytochemical constituents. Plants having significant medicinal values have often been found to be rich in phenolics and to have high antioxidant potentials. The extract of *Alpinia galangal L.* rhizome prepared in acetone yield higher amount of phenolics, flavonoids and showed higher total antioxidant capacity than the extract prepared in ethanol. The rhizome extracts and the isolated flavonoids effectively scavenged superoxide and hydroxyl radicals in a dose dependent manner. The stable free radical DPPH was also scavenged by the extracts and flavanoids. The presence of the galangin or kaempferide protected plasmid DNA from the radiation induced damages. Thus the result showed that flavonoids effectively protect DNA against ionizing radiation in a system . Alkaline comet assay analysis with human peripheral blood leukocytes exposed to γ-radiation, showed a decrease in the comet parameters indicative of radioprotecting ability of galangin or kaempferide. The mechanism of radioprotection by these compounds could be ascribed to its antioxidant and free radical scavenging activities.

A simple, sensitive and reproducible spectrophotometric method has been exploited by means of Folin-ciocalteu's reagent in 15 % sodium carbonate for quantitative estimation of ferulic acid from selected plant materials such as Rice Bran, Wheat bran and Bamboo shoot etc. The blue coloured chromogen formed after the reaction was measured at wavelength of maximum absorption 718 nm for ferulic acid against the

blank reagent. The developed method can be employed for the routine analysis of ferulic acid from the various plant species.

In summary the present study revealed the biological potential of nanoparticle bound DAO complex, ferulic acid, Ascorbic acid mono glucoside and bioflavonoids like Galangin and kaempferide. The present study clearly showed that administration of the phytoceuticals and nutraceuticals resulted in amelioration of radiation induced damages and drug induced oxidative stress like hepatotoxicity, cardiotoxicity and nephrotoxicity in mammalian system. The protective effect of these compounds is mainly associated with their antioxidant properties, as they showed significant biological activities. Even though the potential applications is evident in situations like radiation exposures or oxidative stress conditions. Further studies are needed for the use of phytoceuticals, nutraceuticals and nanoparticle complexes as promising therapeutic supplements in man.

Chapter- 12

RECOMMENDATIONS

The medicinal effects of plants are often ascribed to the antioxidant activity of their phytochemical contents. Plants with high medicinal values are found to be rich in phenolics and to own high antioxidant potentials (Akinmoladun *et al.,* 2007). The antioxidant activity of phenolics attribute to their redox properties, allow them to act as reducing agents, metal chelators, chain breakers or radical scavengers depending on their chemical structures (Rice-Evans *et al*., 1996; Shahidi, 2000). Naturally occurring phytophenols (eg. Ferulic acid (FA), Gallic acid (GA)) comprise a good form of compounds divided into several classes that occur in fruits and vegetables, wine and tea, and chocolate and other cocoa products.

A number of natural agents have been used to ameliorate toxicity induced by carcinogenic xenobiotics and drugs. These include Vitamin C, E, carotenoids and extract of some medicinal plants. Plant products have a range of pharmacological properties and are used for the treatment of varied diseases long years ago.

Therefore, screening of herbal drugs is a vital area for new drug discovery. In this way, attention has shifted towards the evaluation of plant products as radioprotectors, hepato protectors, nephro protectors and cardioprotectors for their efficacy and low toxicity. Compounds with antioxidant activities can neutralize various free radicals and can be used to alleviate the side effects of xenobiotics like chemotherapeutic drugs and radiotherapy (Divakaran *et al*., 2013). The present study developed a screening method to analyse ferulic acid from various plant sources like wheat bran, rice bran and bamboo shoot *etc.*

The use of flavonoids galangin and kaempferide from *Alpinia galanga L*. as a radioprotector can be used under situations of radiation exposure. Anti-inflammatory activity of Ferulic acid and Ascorbic acid 2-glucoside is demonstrated. Ferulic acid and Ascorbic acid 2-glucoside also offered protection against

paracetamol induced hepatotoxicity, doxorubicin induced cardiotoxicity and cisplatin induced nephrotoxicity. The results of our present study gave evidences for the utilization of phytoceuticals, nutraceuticals and nanoparticle complexes as promising therapeutic supplements.

Nanoparticle drug delivery may provide a more efficient, solution to beat problems like poor specificity and dose-limiting toxicities induced by pharmacologically active cancer drugs. Nanotechnology deals with the planning and engineering of nano materials smaller than 100 nm in diameter. So they can be correctly manipulated for the specified applications like imaging, sensing, targeted drug or gene delivery systems, artificial implants etc. (Salvioni *et al*., 2019). Due to enhanced effectiveness, the developing of new age nanoparticle drugs can be employed in treatment for cancer (Brigger *et al*., 2002; Stoimenov *et al*., 2002).

The development of radioprotective agents has been the topic of intense research in view of their potential to be used within a radiation environment, like space exploration, radiotherapy and even nuclear war. Nowadays, many man made as well as natural compounds are investigated for their efficacy to shield against radiation damage. They include sulfhydryl compounds, antioxidants, plant extracts, immunomodulators, and other agents (Upadhyay *et al.*, 2005; Weiss & Landauer, 2000). However, no ideal, safe synthetic radioprotectors are available to this point, that the seek for alternative sources, including plants, has been ongoing for several decades. A scientific screening approach can provide results in identifying potential new candidate drugs from plant sources, for mitigation of radiation injury. Hence the work opens a window for research and developing of more phytoceutically based safe drug against cancer treatment.

BIBLIOGRAPHY

Aarabi, A., Honarvar, M., Mizani, M., Faghihian, H., &Gerami, A.(2016). Extraction and purification of ferulic acid as an antioxidant from sugar beet pulp by alkaline hydrolysis. *Italian Journal of Food Science*, *28*(3), 362–375. https://doi.org/10.14674/1120-1770/ijfs.v143

Abate, C., Patel, L., Rauscher, F. J., &Curran, T.(1990). Redox regulation of fos and jun DNA-binding activity in vitro. *Science*, *249*(4973), 1157–1161. https://doi.org/10.1126/science.2118682

Abdel-Raheem, I.T., Taye, A.&Abouzied, M.M.(2013). Cardioprotective effects of nicorandil, a mitochondrialpotassiumchannelopener against doxorubicin-inducedCardiotoxicity in rats. *Basic and Clinical Pharmacology and Toxicology*, *113*(3), 158–166. https://doi.org/10.1111/bcpt.12078

Abou-El-Hassan, M. A., Rabelink, M. J., van der Vijgh, W. J., Bast, A., &Hoeben, R. C.(2003). A comparative study between catalase gene therapy and the cardioprotector monohydroxyethylrutoside (monoher) in protecting against doxorubicin-induced cardiotoxicity in vitro. *British Journal of Cancer*, *89*(11), 2140–2146. https://doi.org/10.1038/sj.bjc.6601430

Acosta-Estrada,B.A., Gutiérrez-Uribe, J.A., &Serna-Saldívar, S.O.(2014). Bound phenolics in foods, a review *Food Chemistry*, *152*, 46–55. https://doi.org/10.1016/j.foodc hem.2013.11.093

Adelman, R., Saul, R. L., &Ames, B. N.(1988). Oxidative damage to DNA: Relation to species metabolic rate and life span. *Proceedings of the National Academy of Sciences of the UnitedStates of America*, *85*(8), 2706–2708. https://doi.org/10.1073/pnas.85.8.2706

Aebi, H.(1974). Catalase. InH. U.Bergmeyer(Ed.), *Methods of enzymatic analyses* (pp. 673–683). Academic Press.

Afifi, M.E.M.(2010). Effect of Camel's milk on cisplatin-inducednephrotoxicity in Swiss albinomice. *AmericanJournal of Biochemistry andBiotechnology*, *6*(2), 141–147. https://doi.org/10.3844/ajbbsp.2010.141.147

Afsar, T.,Razak, S.,Batoo, K.M., &Khan, M.R.(2017). Acacia hydaspica R. Parker prevents doxorubicin-induced cardiac injury by attenuation of oxidative stress and structural cardiomyocyte alterations in rats. *BMC Complementary and Alternative Medicine*, *17*(1), 554. https://doi.org/10.1186/s12906-017-2061-0

Aga, H., Yoneyama, M., Sakai, S., &Yamamoto, I.(1991). Synthesis of 2-O-α-D-glucopyranosyl L-ascorbic acid by cyclomaltodextrin glucanotransferase from Bacillus stearothermophilus. *Agricultural and BiologicalChemistry*, *55*(7), 1751–1756. https://doi.org/10.1080/00021369.1991.10870856

Agrawal, A., &Kale, R.K.(2001). Radiation induced peroxidative damage: Mechanism and significance. *Indian Journal of Experimental Biology*, *39*(4), 291–309.

Ahmadinejad, F., Geir Møller, S.G.,Hashemzadeh-Chaleshtori, M., Bidkhori, G., &Jami, M.S.(2017). Molecular mechanisms behind free radicalscavengersfunction against oxidativestress. *Antioxidants*, *6*(3), 51. https://doi.org/10.3390/antiox6030051

Ahmadinejad, F., Honardoost, M.A., Mowla, S.J., Teimori, H., &Ghaedi, K.(2016). miR-218 as a multifunctionalregulator of oncogenicprocesses in differentsolidtumors. *Genetics. 3rd Millenn*, *14*, 4128–4145.

Ajila, C.M., Naidu, K.A., Bhat, S., &Rao, U.(2007). Bioactive compounds and antioxidant potential of mango peel extract. *Food Chemistry*, *105*(3), 982–988. https://doi.org/10.1016/j.foodchem.2007.04.052

Akashi, M., Hachiya, M., Koeffler, H.P., &Suzuki, G.(1992). Irradiation increases levels of GM-CSF through RNA stabilization which requires an AU-rich region in cancer cells. *Biochemical and Biophysical Research Communications*, *189*(2), 986–993. https://doi.org/10.1016/0006-291x(92)92301-d

Akinmoladun, A.C., Ibukun, E.O., Afor, E., Akinrinlola, B.L., Onibon, T.R., Akinboboye, A.O., Obuotor, E.M., &Farombi, E.O.(2007). Chemical constituents and antioxidant activity of *Alstoniaboonei*. *African Journal of Biotechnology*, *6*,1197–1201.

Albert, Z., Luu, B.S.C., Chowdhury, B., Al-Omran, M.,Teoh, H., Hess, D.A., &Verma,S.(2018). Role of endothelium in doxorubicin-inducedcardiomyopathy. *ACC: Basic to TranslationalScience*, *3*(6), 861–870.

Alderton, P.M., Gross, J., &Green, M.D.(1992). Comparative study of doxorubicin, mitoxantrone, and epirubicin in combination with ICRF-187 (ADR-529) in a chronic cardiotoxicity animal model. *Cancer Research*, *52*(1), 194–201.

Alexiou, C., Arnold, W., Klein, R.J., Parak, F.G., Hulin, P., Bergemann, C., Erhardt, W., Wagenpfeil, S., &Lübbe, A.S.(2000). Locoregional cancer treatment with magnetic drug targeting. *Cancer Research*, *60*(23), 6641–6648.

Alexiou, C., Jurgons, R., Schmid, R.J., Bergemann, C., Henke, J., Erhardt, W., Huenges, E., &Parak, F.(2003). Magnetic drug targeting- biodistribution of the magnetic carrier and the chemotherapeutic agent mitoxantrone after locoregional cancer treatment. *Journal of Drug Targeting*, *11*(3), 139–149. https://doi.org/10.1080/1061186031000150791

Alexiou, C., Schmidt, A., Klein, R., Hulin, P., Bergemann, C., &Arnold, W.(2002). Magnetic drug targeting: Biodistribution and dependency on magnetic field strength. *Journal of Magnetism and Magnetic Materials*, *252*, 363–366. https://doi.org/10.1016/S0304-8853(02)00605-4

al-Harbi, M.M., al-Gharably, N.M., al-Shabanah, O.A., al-Bekairi, A.M., Osman, A.M., &Tawfik, H.N.(1992). Prevention of doxorubicin-induced myocardial and haematological toxicities in rats by the iron chelator desferrioxamine. *Cancer Chemotherapy and Pharmacology*, *31*(3), 200–204. https://doi.org/10.1007/BF00685548

Al-Harthi, S.E.,Alarabi, O.M., Ramadan, W.S., Alaama, M.N., Al-Kreathy, H.M.,Damanhouri, Z.A.,Khan, L.M., &Osman, A.M.(2014). Amelioration of doxorubicin-induced cardiotoxicity by resveratrol. *Molecular Medicine Reports*, *10*(3), 1455–1460. https://doi.org/10.3892/mmr.2014.2384

Alhoshani, A.R.,Hafez, M.M., &Husain, S., Al-sheikh, AM., Alotaibi, MR., Al Rejaie, SS., Alshammari, MA., Almutairi, MM & Al-Shabanah, OA.(2017). Protective effect of

rutin supplementation against cisplatin-induced Nephrotoxicity in rats. *BMC Nephrology*.18:194.

Ali, B.H., &Al Moundhri, M.S.(2006). Agents ameliorating or augmenting the nephrotoxicity of cisplatin and other platinum compounds: A review of some recent research. *Food and Chemical Toxicology*, *44*(8), 1173–1183. https://doi.org/10.1016/j.fct.2006.01.013

Ali, H., &Dixit, S.(2012). Identification of chemicalconstituent of combinesample of somemedicinalplants for antioxidantactivity. *Asian Journal of Plant Science and Research*, *2*(3), 269–273.

Alkuraishy, H.M., Al-Gareeb, AI & al-hussaniy, HA.(2017). Doxorubicin-inducedcardiotoxicity: Molecular mechanism and protection by conventionaldrugs and naturalproducts. *International Journal of Clinical Oncology and Cancer Research*, *2*(2), 31–44. https://doi.org/10.11648/j.ijcocr.20170202.12

Allen, L.C., Michalko, K., &Coons, C.(1982). More on cephalosporin interference with creatinine determinations. *Clinical Chemistry*, *28*(3), 555–556. https://doi.org/10.1093/clinchem/28.3.555

Allen, R. G., &Tresini, M.(2000). Oxidative stress and gene regulation. *Free Radical Biology and Medicine*, *28*(3), 463–499. https://doi.org/10.1016/s0891-5849(99)00242-7

Al-Mamary, M., Al-Meeri, A., &Al-Habori, M.(2002). Antioxidant activities and total phenolics of different types of honey. *Nutrition Research*, *22*(9), 1041–1047. https://doi.org/10.1016/S0271-5317(02)00406-2

Almroth, B.C., Sturve, J., Berglund, A., &Förlin, L.(2005). Oxidative damage in eelpout (*Zoarcesviviparus*), measured as protein carbonyls and TBARS, as biomarkers. *Aquatic Toxicology*, *73*(2), 171–180. https://doi.org/10.1016/j.aquatox.2005.03.007.

Ambrosio, G., Zweier, J.L., Duilio, C., Kuppusamy, P., Santoro, G., Elia, P.P., Tritto, I., Cirillo, P., Condorelli, M., &Chiariello, M.(1993). Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabit heart subjected to ischemia and reflow. *Journal of Biological Chemistry*, *268*(25), 18532–18541. https://doi.org/10.1016/S0021-9258(17)46660-9

Ames, B. N., Shigenaga, M. K., &Hagen, T. M.(1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(17), 7915–7922. https://doi.org/10.1073/pnas.90.17.7915

Ames, B.N.(1989). Endogenous oxidative DNA damage, aging and cancer. *Free Radical Research Communications*, *7*(3–6), 121–128. https://doi.org/10.3109/10715768909087933

Amudha, G., Josephine, A., Mythili, Y., Sundarapandiyan, R., &Varalakshmi, P.(2007). Therapeutic efficacy of dl-lipoic acid on cyclosporine A induced renal alterations.*European Journal of Pharmacology*, *571*(2–3), 209–214. https://doi.org/10.1016/j.ejphar.2007.05.047

Anderson, M.E., Naganuma, A., &Meister, A.(1990). Protection against cisplatin toxicity by administration of glutathione ester. *FASEB Journal*, *4*(14), 3251–3255. https://doi.org/10.1096/fasebj.4.14.2227215

Andrew, P. J., &Mayer, B.(1999). Enzymatic function of nitric oxide synthases. *Cardiovascular Research*, *43*(3), 521–531. https://doi.org/10.1016/s0008-6363(99)00115-7

Aniss,H.A., Said, Ael M., El Sayed, I.H.E., &Adly, C.(2014). Amelioration of adriamycin-induced cardiotoxicity by Salsola kali aqueous extract is mediated by lowering oxidative stress. *Redox Report: Communications in Free Radical Research*, *19*(4), 170–178. https://doi.org/10.1179/1351000214Y.0000000088

Antunes, L.M.G., Darin, J.D.C., &Bianchi, Nde L.(2001). Effects of the antioxidants curcumin or selenium on cisplatin induced nephrotoxicity and lipid peroxidation in rats. *Pharmacological Research*, *43*(2), 145–150. https://doi.org/10.1006/phrs.2000.0724

Appenroth, D., Fröb, S., Kersten, L., Splinter, F.K., &Winnefeld, K.(1997). Protective effects of vitamin E and C on cisplatin nephrotoxicity in developing rats. *Archives of Toxicology*, *71*(11), 677–683. https://doi.org/10.1007/s002040050444

Arany, I., &Safirstein, R.L.(2003). Cisplatin nephrotoxicity. *Seminars in Nephrology*, *23*(5), 460–464. https://doi.org/10.1016/s0270-9295(03)00089-5

Armstrong, R. N.(1997). Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chemical Research in Toxicology*, *10*(1), 2–18. https://doi.org/10.1021/tx960072x

Arnelle, D.R., &Stamler, J.S.(1995). NO_, NO, and NO_ donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Archives of Biochemistry and Biophysics*, *318*, 179–185.

Arnheim, N., &Cortopassi, G.(1992). Deleterious mitochondrial DNA mutations accumulate in ageing human tissue. *Mutation Research*, *275*(3–6), 157–167. https://doi.org/10.1016/0921-8734(92)90020-p

Arrigo, A. P.(1999). Gene expression and the thiol redox state. *Free Radical Biology and Medicine*, *27*(9–10), 936–944. https://doi.org/10.1016/s0891-5849(99)00175-6

Arumsari, A.G., Nugrahani, R. A., &Hendrawati, T. Y.(2019). The extraction of ferulic acid from the phenolic fraction from rice bran oil using ultrasonic methods and analysis of antioxidant effectiveness. *Advances in Health Sciences Research*, *15*, 37–39.

Asanoma, M., Takahashi, K., Miyabe, M., Yamamoto, K.,Yoshimi, N., Mori, H., &Kawazoe, Y.(1994). Inhibitory effect of topical application of polymerized ferulic acid, a synthetic lignin, on tumor promotion in mouse skin two stage tumori-genesis. *Carcinogenesis*, *15*(9), 2069–2071. https://doi.org/10.1093/carcin/15.9.2069

Aslam, M.S., Naveed, S., Ahmed, A., Abbas, Z., Gull, I., &Athar, M.A.(2014). Side effects of chemotherapy in cancerpatients and evaluation of patientsopinion about

starvationbaseddifferentialchemotherapy. *Journal of Cancer Therapy*, *05*(8), 817–822. https://doi.org/10.4236/jct.2014.58089

Ausprunk, D. H., &Folkman, J.(1977). Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvascular Research*, *14*(1), 53–65. https://doi.org/10.1016/0026-2862(77)90141-8

Awdishu, L., &Mehta, R.L.(2017). The 6R's of drug induced nephrotoxicity. *BMC Nephrology*, *18*(1), 124. https://doi.org/10.1186/s12882-017-0536-3

Azab, A.,Nassar, A., &Azab, A.N.(2016). Anti-inflammatoryactivity of naturalproducts. *Molecules*, *21*(10), 1321. https://doi.org/10.3390/molecules21101321

Azab, A.E., &Albasha, M.O.(2018). Hepatoprotective effect of some medicinal plants and herbs against hepatic disorders induced by hepatotoxic agents. *Biotechnology and Bioengineering*, *2*(1), 8–23.

Azab, A.E., Albasha, M.O., &Elsayed, A.S.I.(2017). Prevention of nephropathy by some natural sources of antioxidants. *Yangtze Medicine*, *01*(4), 235–266. https://doi.org/10.4236/ym.2017.14023

Babior, B.M.(1999). NADPH oxidase: An update. *Blood*, *93*(5), 1464–1476

Bachur, N.R., Gordon, S. L., &Gee, M.V.(1978). A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Research*, *38*(6), 1745–1750.

Baeuerle, P.A., &Henkel, T.(1994). Function and activation of NF-kappa B in the immune system. *Annual Review ofImmunology*, *12*, 141–179. https://doi.org/10.1146/annurev.iy.12.040194.001041

Baguley, B.C., &Ching, L.M.(2002). DMXAA: An antivascular agent with multiple host responses. *International Journal of Radiation Oncology, Biology, Physics*, *54*(5), 1503–1511. https://doi.org/10.1016/s0360-3016(02)03920-2

Baig, M.H., Adil, M., Khan, R., Dhadi, S., Ahmad, K., Rabbani, G., Bashir, T., Imran, M. A., Husain, F. M., Lee, E. J., Kamal, M. A., &Choi, I.(2019). Enzyme targeting strategies for prevention and treatment of cancer: Implications for cancer therapy. *Seminars in Cancer Biology*, 56, 1–11. https://doi.org/10.1016/j.semcancer.2017.12.003

Baker, C., Pradhan, A., Pakstis, L., Pochan, D.J., &Shah, S.I.(2005). Synthesis and antibacterial properties of silver nanoparticles. *Journal of Nanoscience and Nanotechnology*, *5*(2), 244–249. https://doi.org/10.1166/jnn.2005.034

Balasubashini, M.S., Rukkumani, R., Viswanathan, P., &Menon, V.P.(2004). Ferulic acid alleviates lipid peroxidation in diabetic rats. *Phytotherapy Research*, *18*(4), 310–314. https://doi.org/10.1002/ptr.1440

Baliga, M.S., Meera, S., Vaishnav, L.K., Rao, S., &Palatty, P.L.(2013). Rasayana drugs from the Ayurvedic system of medicine as possible radioprotective agents in cancer treatment.

Integrative Cancer Therapies, *12*(6), 455–463. https://doi.org/10.1177/1534735413490233

Baliga, R., &Liu, H.(2004). *Activation of caspase 12 by cisplatin (CP) induced endoplasmic reticulum (ER) stress mediates apoptosis in LLC-PK1 cells*, *15* p. 39. American Society ofNephrology.

Balkwill, F., &Mantovani, A.(2001). Inflammation and cancer: Back to Virchow?*Lancet*, *357*(9255), 539–545. https://doi.org/10.1016/S0140-6736(00)04046-0

Bandyopadhyay, U., Das, D., &Banerjee, R.K.(1999). Reactive oxygen species: Oxidative damage and pathogenesis. *Current Science*, *77*, 658–665.

Bashir, S., Harris, G., Denman, M.A., Blake, D.R., &Winyard, P.G.(1993). Oxidative DNA damage and cellular sensitivity to oxidative stress in human autoimmune diseases. *Annals of the Rheumatic Diseases*, *52*(9), 659–666. https://doi.org/10.1136/ard.52.9.659

Baskić, D., Popović, S., Ristić, P., &Arsenijević, N. N.(2006). Analysis of cycloheximide-induced apoptosis in human leukocytes: Fluorescence microscopy using annexin V/propidium iodide versus acridin orange/ethidium bromide.*Cell Biology International*, *30*(11), 924–932. https://doi.org/10.1016/j.cellbi.2006.06.016

Basli, A., Soulet, S., Chaher, N., Mérillon, J. M., Chibane, M., Monti, J. P., &Richard, T.(2012). Wine polyphenols: Potential agents in neuroprotection. *Oxidative Medicine andCellular Longevity*, 2012, 805762. https://doi.org/10.1155/2012/805762

Batista,R.(2014). Uses and potential applications of ferulic acid. InB.Warren(Ed.), *Ferulic acid: Antioxidant properties, uses and potential health benefits. 1ed edn* (pp. 39–70). Nova Science Publishers, Inc.

Beanlands, R.S., Shaikh, N.A., Wen, W.H., Dawood, F., Ugnat, A.M., McLaughlin, P.R., Carere, R., &Liu, P.P.(1994). Alterations in fatty acid metabolism in adriamycin cardiomyopathy. *Journal of Molecular and Cellular Cardiology*, *26*(1), 109–119. https://doi.org/10.1006/jmcc.1994.1012

Beard, J.(1906). The action of trypsin upon the living cells of Jensen's mouse tumor. *British Medical Journal*, 1(2351), 140–141.

Beck, K.F., Eberhardt, W., Frank, S., Huwiler, A., Messmer, U.K., Mühl, H., &Pfeilschifter, J.(1999). Inducible NO synthase: Role in cellular signalling. *Journal of ExperimentalBiology*, *202*(6), 645–653. https://doi.org/10.1242/jeb.202.6.645

Beckman, J.S., &Koppenol, W.H.(1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *AmericanJournal of Physiology*, *271*(5 Pt 1), C1424–C1437. https://doi.org/10.1152/ajpcell.1996.271.5.C1424

Beckman, K. B., &Ames, B.N.(1997). Oxidative decay of DNA.*Journal of Biological Chemistry*, *272*(32),19633–19636. https://doi.org/10.1074/jbc.272.32.19633

Bendale, Y., Bendale, V., Birari-Gawande, P., Kadam, A., &Gund, P.(2015). Tumor regression with ayurvedic rasayana therapy in squamous cell carcinoma of lungs. *Rasamruta*, *7*, 1–5.

Bendjeddou,D., Lalaoui, K., &Satta, D.(2003). Immunostimulating activity of the hot water soluble polysaccharide extracts of *Anacyclus pyrethrum*, *Alpinia galanga*, and *Citrulluscolocythis*.*Journal of Ethnopharmacology*, *88*(2–3), 155–160. https://doi.org/10.1016/s0378-8741(03)00226-5

Ben-Yoseph, O., &Ross, B.D.(1994). Oxidation therapy: The use of a reactive oxygen species-generating enzyme system for tumour treatment. *British Journal of Cancer*, *70*(6), 1131–1135. https://doi.org/10.1038/bjc.1994.460

Berlett, B.S., &Stadtman, E.R.(1997). Protein oxidation in aging, disease, and oxidative stress. *Journal ofBiological Chemistry*, *272*(33), 20313–20316. https://doi.org/10.1074/jbc.272.33.20313

Berry, C. C., &Curtis, A.S.G.(2003). Functionalisation of magnetic nanoparticles for applications in biomedicine. *Journal of Physics. Part D*, *36*(13), R198–R206. https://doi.org/10.1088/0022-3727/36/13/203

Berry, C.C., Charles, S., Wells, S., Dalby, M.J., &Curtis, A.S.(2004). The influence of transferrin stabilised magnetic nanoparticles on human dermal fibroblasts in culture.*International Journal of Pharmaceutics*, *269*(1), 211–225. https://doi.org/10.1016/j.ijpharm.2003.09.042

Betteridge, D.J.(2000). What is oxidative stress?*Metabolism: Clinical and Experimental*, *49*(2) (Suppl. 1), 3–8. https://doi.org/10.1016/s0026-0495(00)80077-3

Beuth, J.(2008). Proteolytic enzymetherapy in evidence-basedcomplementaryoncology: Fact or fiction?*Integrative Cancer Therapies*, *7*(4), 311–316. https://doi.org/10.1177/1534735408327251

Bhadra, D., Bhadra, S., Jain, S., &Jain, N.K.(2003). A pegylated dendritic nanoparticulate carrier of fluorouracil. *International Journal of Pharmaceutics*, *257*(1–2), 111–124. https://doi.org/10.1016/s0378-5173(03)00132-7

Bhandari, P.R.(2013). A review of radioprotective plants. *International Journal of Green Pharmacy*, *7*(2), 90–101. https://doi.org/10.4103/0973-8258.116379

Bhattacharya, R., Patra, C.R., Verma, R., Kumar, S., Greipp, P.R., &Mukherjee, P.(2007). Gold nanoparticlesinhibit the proliferation of multiplemyelomacells.*Advanced Materials*, *19*(5), 711–716. https://doi.org/10.1002/adma.200602098

Bhattacharya, R., Senbanerjee, S., Lin, Z., Mir, S., Hamik, A., Wang, P., Mukherjee, P., Mukhopadhyay, D., &Jain, M.K.(2005). Inhibition of vascular permeability factor/vascular endothelial growth factor mediated angiogenesis by the Krüppel-like factor KLF2. *Journal of Biological Chemistry*, *280*(32), 28848–28851. https://doi.org/10.1074/jbc.C500200200

Bi, Y., Hao, F., Yan, G., Teng, L., Lee, R. J., &Xie, J.(2016). Actively targeted nanoparticles for drug delivery to tumor.*Current Drug Metabolism*, *17*(8), 763–782. https://doi.org/10.2174/1389200217666160619191853

Black, M.(1980). Acetaminophen hepatotoxicity. *Gastroenterology*, *78*(2), 382–392. https://doi.org/10.1016/0016-5085(80)90593-4

Bladier, C., Wolvetang, E.J., Hutchinson, P., de Haan, J.B., &Kola, I.(1997). Response of a primary human fibroblast cell line to H2O2: Senescence-like growth arrest or apoptosis?*Cell Growth and Differentiation*, *8*(5), 589–598.

Blakey, D.C., Westwood, F.R., Walker, M., Hughes, G.D., Davis, P.D., Ashton, S.E., &Ryan, A.J.(2002). Antitumor activity of the novel vascular targeting agent ZD6126 in a panel of tumor models. *Clinical Cancer Research*, *8*(6), 1974–1983.

Bleier, W., &Chirikdjian, J.J.(1972). Flavonoids from galanga rhizome (*Alpiniaofficinarum* Hance).*Planta Medica*, *22*(2), 145–151. https://doi.org/10.1055/s-0028-1099597

Bloemendal, H.J., Logtenberg, T., &Voest, E.E.(1999). New strategies in anti-vascular cancer therapy. *European Journal of Clinical Investigation*, *29*(9), 802–809. https://doi.org/10.1046/j.1365-2362.1999.00534.x

Boaz, M., Smetana, S., Weinstein, T., Matas, Z., Gafter, U., Iaina, A., Knecht, A., Weissgarten, Y., Brunner, D., Fainaru, M., &Green, M. S.(2000). Secondary prevention with antioxidants of cardiovascular disease in end stage renal disease (SPACE): Randomised placebo-controlled trial. *Lancet*, *356*(9237), 1213–1218. https://doi.org/10.1016/s0140-6736(00)02783-5

Boelsterli, U.A.(2002). Mechanisms of NSAID-induced hepatotoxicity: Focus on nimesulide. *Drug Safety*, *25*(9), 633–648. https://doi.org/10.2165/00002018-200225090-00003

Bonsnes, R.W., &Taussky, H.H.(1945). On the colorimetric determination of creatinine by the Jaffe reaction. *Journal of Biological Chemistry*, *158*(3), 581–591. https://doi.org/10.1016/S0021-9258(19)51334-5

Boogaard, P.J., Lempers, E.L., Mulders, G.J., &Meerman, J.H.N.(1991). 4-methylthiobenzoic acid reduces cisplatin nephrotoxicity in rats without compromising anti-tumor activity. *Biochemical Pharmacology*, *41*(12), 1997–2003. https://doi.org/10.1016/0006-2952(91)90141-q

Borch, R.F., &Markman, M.(1989). Biochemical modulation of cisplatin toxicity. *Pharmacology and Therapeutics*, *41*(1–2), 371–380. https://doi.org/10.1016/0163-7258(89)90114-9

Borek, C.(2004). Dietary antioxidants and humancancer.*Integrative Cancer Therapies*, *3*(4), 333–341. https://doi.org/10.1177/1534735404270578

Box, H.C., Freund, H.G., Budzinski, E. E., Wallace, J.C., &Maccubbin, A.E.(1995). Free radical-induced double base lesions. *Radiation Research*, *141*(1), 91–94. https://doi.org/10.2307/3579095

Bradley, M.O., &Erickson, L.C.(1981). Comparison of the effects of hydrogen peroxide and X-ray irradiation on toxicity, mutation, and DNA damage/repair in mammalian cells (V-79). *Biochimica et Biophysica Acta*, *654*(1), 135–141. https://doi.org/10.1016/0005-2787(81)90146-5

Bray, R.C., &Swann, J.C.(1972). Moribudenum- containing enzymes. *Structure and Bonding*, *11*, 107–144.

Bredt, D.S.(1999). Endogenous nitric oxide synthesis: Biological functions and pathophysiology. *Free Radical Research*, *31*(6), 577–596. https://doi.org/10.1080/10715769900301161

Brigger, I., Dubernet, C., &Couvreur, P.(2002). Nanoparticles in cancer therapy and diagnosis.*Advanced Drug Delivery Reviews*, *54*(5), 631–651. https://doi.org/10.1016/s0169-409x(02)00044-3

Bright,H.J., &Porter,D.J.T.(1975). *The enzymes*, *12*, part B (pp. 445–446).Academic Press.

Brooks, P.M., &Day, R.O.(1991). Nonsteroidal antiinflammatory drugs—Differences and similarities. *New England Journal of Medicine*, *324*(24), 1716–1725. https://doi.org/10.1056/NEJM199106133242407

Brumby,P.E., &Massey, V.(1968). InW. E. M.Lands(Ed.). *Biochemical preparations*, *12*(pp. 29–41). Wiley.

Buege, J. A., &Aust, S. D.(1978). Microsomal lipidperoxidation. *Methods in Enzymology*, *52*, 302–310. https://doi.org/10.1016/s0076-6879(78)52032-6

Burak, M., &Imen. (1999).Flavonoids and their antioxidant properties. *Türkiye Klinikleri Journal of Medical Sciences*, *19*, 296–304.

Buranov, A. U., &Mazza, G.(2009). Extraction and purification of ferulic acid from flax shives, wheat and corn bran by alkaline hydrolysis and pressurised solvents. *Food Chemistry*, *115*(4), 1542–1548. https://doi.org/10.1016/j.foodchem.2009.01.059

Buranov, A.U., &Mazza, G. (2009). Extraction and purification of ferulic acid from flax shives, wheat and corn bran by alkaline hydrolysis and pressurised solvents. *Food Chemistry*, *115*(4), 1542–1548. https://doi.org/10.1016/j.foodchem.2009.01.059

Burdon, R.H., Alliangana, D., &Gill, V.(1995). Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radical Biology and Medicine*, *18*(4), 775–794. https://doi.org/10.1016/0891-5849(94)00198-s

Buschini, A., Poli, P., &Rossi, C.(2003). Saccharomyces cerevisiae as an eukaryotic cell model to assess cytotoxicity and genotoxicity of three anticancer anthraquinones. *Mutagenesis*, *18*(1), 25–36. https://doi.org/10.1093/mutage/18.1.25

Butnariu, M., &Samfira, I.(2012).Free radicals and oxidativestress. *Journal of Bioequivalence and Bioavailability*, *04*(3), 3. https://doi.org/10.4172/jbb.10000e13

Butterfield, D.A., Koppal, T., Howard, B., Subramaniam, R., Hall, N., Hensley, K., Yatin, S., Allen, K., Aksenov, M., Aksenova, M., &Carney, J.(1998). Structural and functional changes in proteins induced by free radical-mediated oxidative stress and protective action of the antioxidants N-tert-butyl-alpha-phenylnitrone and vitamin E. *Annals of the New York Academy ofSciences*, *854*, 448–462. https://doi.org/10.1111/j.1749-6632.1998.tb09924.x

Butterfield, L.H., Merino, A., Golub, S.H., &Shau, H.(1999). From cytoprotection to tumor suppression: The multifactorial role of peroxiredoxins. *Antioxidants and Redox Signaling*, *1*(4), 385–402. https://doi.org/10.1089/ars.1999.1.4-385

Cai, H., &Harrison, D.G.(2000). Endothelial dysfunction in cardio vascular diseases:The role of oxidative stress. *Circulation Research*, *87*(10), 840–844. https://doi.org/10.1161/01.res.87.10.840

Cai, W., Shin, D.W., Chen, K., Gheysens, O., Cao, Q., Wang, S.X., Gambhir, S.S., &Chen, X.(2006). Peptide-labeled near-infrared quantum dots for imaging tumor vasculature in living subjects. *Nano Letters*, *6*(4), 669–676. https://doi.org/10.1021/nl052405t

Cairns, R.A., Harris, I.S., &Mak, T.W.(2011). Regulation of cancer cell metabolism. *Nature Reviews. Cancer*, *11*(2), 85–95. https://doi.org/10.1038/nrc2981

Campbell, J. T.(1907).Trypsin treatment of a case of malignant disease. *JAMA*, *48*, 225–226.

Canaud, B., Cristol, J., Morena, M., Leray-Moragues, H., Bosc, J., &Vaussenat, F.(1999). Imbalance of oxidants and antioxidants in haemodialysis patients. *Blood Purification*, *17*(2–3), 99–106. https://doi.org/10.1159/000014381

Castañeda-Ovando, A., Pacheco-Hernández, MdL., Páez-Hernández, M. E., Rodríguez, J. A., &Galán-Vidal, C. A.(2009). Chemical studies of anthocyanins: A review. *Food Chemistry*, *113*(4), 859–871. https://doi.org/10.1016/j.foodchem.2008.09.001

Cerda, H., Delincée, H.,Haine,H., &Rupp, H.(1997). The DNA “cometassay” as a rapidscreeningtechnique to controlirradiatedfood. *Mutation Research*, *375*(2), 167–181. https://doi.org/10.1016/s0027-5107(97)00012-2

Cerezo, A.B., Tesfaye, W., Soria-Díaz, M.E., Torija, M. J., Mateo, E., Garcia-Parrilla, M. C., &Troncoso, A. M.(2010). Effect of wood on the phenolic profile and sensory properties of wine vinegars during ageing. *Journal of Food Composition andAnalysis*, *23*(2), 175–184. https://doi.org/10.1016/j.jfca.2009.08.008

Cerutti, P. A.(1994). Oxy-radicals and cancer. *Lancet*, *344*(8926), 862–863. https://doi.org/10.1016/s0140-6736(94)92832-0

Chachoua, A., Hoschster, H., &Muggia, F.M.(1988). Doxorubicin. In J. P.Droz, E.Cvitkovic, J. P.Armand&S.Khoury(Eds.), *Handbook of chemotherapy in clinicaloncology. Rhöne Poulenc, Houston, TX*, *125–127*.

Chae, H.Z., Kang, S.W., &Rhee, S.G.(1999[a]). Isoforms of mammalian peroxiredoxin that reduce peroxides in presence of thioredoxin. *Methods in Enzymology*, 300, 219–226. https://doi.org/10.1016/s0076-6879(99)00128-7

Chae, H.Z., Kim, H.J., Kang, S.W., &Rhee, S.G.(1999[b]). Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. *Diabetes Research and ClinicalPractice*, 45(2–3), 101–112. https://doi.org/10.1016/s0168-8227(99)00037-6

Chalansonnet, M., Carabin, N., Boucard, S., Cosnier, F., Nunge, H., &Gagnaire, F.(2013). Study of the potential oxidative stress induced by six solvents in the rat brain. *Neurotoxicology*, *35*, 71–83. https://doi.org/10.1016/j.neuro.2012.12.002

Chan, H. K., &Ismail, S.(2014).Side effects of chemotherapy among cancerpatients in a Malaysian generalhospital: Experiences, perceptions and informationalneeds from clinicalpharmacists.*Asian Pacific Journal of Cancer Prevention: APJCP*, *15*(13), 5305–5309. https://doi.org/10.7314/apjcp.2014.15.13.5305

Chan, W. C., &Nie, S.(1998). Quantum dot bioconjugates for ultra sensitive nonisotopic detection.*Science*, *281*(5385), 2016–2018. https://doi.org/10.1126/science.281.5385.2016

Chandra, K., Salman A., Sweety, R & Ali, KN. (2015). Protection against FCA induced oxidative stress induced DNA damage as a model of arthritis and in vitro anti-arthritic potential of Costus speciosus rhizome extract. *International Journal of Pharmacognosy and Phytochemical Research*. Mohd, *7*, 383–389.

Chandrasekharan, D. K., Kagiya, T. V., &Nair, C.K.K.(2009). Radiation protection by 6-palmitoyl ascorbic acid- 2-glucoside: Studies on DNA damage *in vitro, ex vivo, in vivo* and oxidative stress *in vivo*. *Journal ofRadiation Research*, *50*(3), 203–212. https://doi.org/10.1269/jrr.08090

Chaney, A.L., &Marbach, E.P.(1962). Modified reagents for determination of urea and ammonia. *Clinical Chemistry*, *8*, 130–132. https://doi.org/10.1093/clinchem/8.2.130

Chaplin, D.J., &Dougherty, G.J.(1999). Tumour vasculature as a target for cancer therapy. *BritishJournal of Cancer*, *80* (Suppl. 1), 57–64.

Chaplin, D.J., Pettit, G.R., &Hill, S.A.(1999). Anti-vascular approaches to solid tumour therapy: Evaluation of combretastatin A4 phosphate. *Anticancer Research*, *19*(1A), 189–195.

Chatham, J.C., Cousins, J.P., &Glickson, J.D.(1990). The relationship between cardiac function and metabolism in acute adriamycin-treated perfused rat hearts studied by 31P and 13C NMR spectroscopy. *Journal of Molecular and Cellular Cardiology*, *22*(10), 1187–1197. https://doi.org/10.1016/0022-2828(90)90082-d

Chatterjee, T.K.(2000). Medicinal plants with hepatoprotective properties. In*Herbal opinions*(3rd ed). Books & Allied (P) Ltd p. 135.

Chaw, T.T.(1989). Analgesic testing in animal models. InAlan R Liss, Inc.(Ed.), *Pharmacological methods in the control of inflammations* (pp. 448–452).

Cheeseman, K.(1993). InB.Halliwell&O. I.Aruoma(Eds.). *DNA and free radicals* (pp. 109–144).Ellis Horwood.

Chen, Q.(1993). *Methodology in pharmacological study on Chinese materia medica* p. 360.People's Medical Publishing House.

Chen, X., Sun, C.K., Han, G.Z., Peng, J.Y., Li,Y., Liu, Y.X., Lv, Y. Y., Liu, K. X., Zhou, Q., &Sun, H. J.(2009).Protective effect of tea polyphenols against paracetamol-induced hepatotoxicity in mice is significantly correlated with cytochrome P450 suppression. *World Journal of Gastroenterology*, *15*(15), 1829–1835. https://doi.org/10.3748/wjg.15.1829

Cho, K., Wang, X., Nie, S., Chen, Z.G., &Shin, D.M.(2008). Therapeutic nanoparticles for drugdelivery in cancer.*Clinical Cancer Research*, *14*(5), 1310–1316. https://doi.org/10.1158/1078-0432.CCR-07-1441

Choi,J.D.(1978). The nature of N-5 reactivity in flavins and inhibitorcomplexes. http://etd.lib.ttu.edu/theses/available/etd-04102009-1295015070716/unrestricted/31295015070716.pdf

Choo, W.S., &Yong, W.K.(2011). Antioxidant properties of two species of. Hylocereus fruits.*Advances in Applied Science Research*, *2*(3), 418–425.

Chu, D. T., Nguyen, T.T., Tien, N.L.B., Tran, D. K., Jeong, J. H., Anh, P. G., Thanh, V. V., Truong, D. T., &Dinh, T. C.(2020). Recent progress of stem celltherapy in cancertreatment: Molecular mechanisms and potentialapplications. *Cells*, *9*(3), 563.https://doi.org/10.3390/cells9030563

Clements, J.A., Critchley, J.A., &Prescott, L.F.(1984). The role of sulphate conjugation in the metabolism and disposition of oral and intravenous paracetamol in man. *BritishJournal of Clinical Pharmacology*, *18*(4), 481–485. https://doi.org/10.1111/j.1365-2125.1984.tb02495.x

Condeelis, J., &Pollard, J.W.(2006). Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis. *Cell*, *124*(2), 263–266. https://doi.org/10.1016/j.cell.2006.01.007

Conner, E.M., &Grisham, M.B.(1996). Inflammation, free radicals and antioxidants. *Nutrition*, *12*(4), 274–277. https://doi.org/10.1016/s0899-9007(96)00000-8

Cooke, M. S., Evans, M.D., Dizdaroglu, M., &Lunec, J.(2003). Oxidative DNA damage: Mechanisms, mutation, and disease. *FASEB Journal*, *17*(10), 1195–1214. https://doi.org/10.1096/fj.02-0752rev

Cooley, M.E., Davis, L., &Abrahm, J.(1994). Cisplatin: A clinical review. Part II—Nursing assessment and management of side effects of cisplatin. *Cancer Nursing*, *17*(4), 283–293. https://doi.org/10.1097/00002820-199408000-00001

Cortopassi, G.A., &Wong, A.(1999). Mitochondria in organismal aging and degeneration. *Biochimica et Biophysica Acta*, *1410*(2), 183–193. https://doi.org/10.1016/s0005-2728(98)00166-2

Coussens, L.M., &Werb, Z.(2002). Inflammation and cancer. *Nature*, *420*(6917), 860–867. https://doi.org/10.1038/nature01322

Cross, C.E., Halliwell, B., Borish, E.T., Pryor, W.A., Ames, B.N., Saul, R.L., McCord, J. M., &Harman, D.(1987). Oxygen radicals and human diseases. *Annals of Internal Medicine*, *107*(4), 526–545. https://doi.org/10.7326/0003-4819-107-4-526

Cross, H.J., Tilby, M., Chipman, J.K., Ferry, D.R., &Gescher, A.(1996). Effect of quercetin on the genotoxic potential of cisplatin. *International Journal of Cancer*, *66*(3), 404–408. https://doi.org/10.1002/(SICI)1097-0215(19960503)66:3<404::AID-IJC23>3.0.CO;2-9

Croteau, R., Kutchan, T.M., &Lewis, N.G.(2000). Natural Products (Secondary metabolites). InB. B.Buchanan, w.Gruissem&R. L.Jones(Eds.), *USA: Courier companies, Inc. Biochemistry & molecular biology of plants*., 1250–1318.

Crul, M., Schellens, J.H., Beijnen, J.H., &Maliepaard, M.(1997). Cisplatin resistance and DNA repair. *Cancer Treatment Reviews*, *23*(5–6), 341–366. https://doi.org/10.1016/s0305-7372(97)90032-3

Cuenca, A.G., Jiang, H., Hochwald, S.N., Delano, M., Cance, W.G., &Grobmyer, S.R.(2006).Emerging implications of nanotechnology on cancer diagnostics and therapeutics.*Cancer*, *107*(3), 459–466. https://doi.org/10.1002/cncr.22035

Cullen, B.R.(1991). Regulation of HIV-1 gene expression. *FASEB Journal*, *5*(10), 2361–2368. https://doi.org/10.1096/fasebj.5.10.1712325

Cullinane, C., Cutts, S.M., Panousis, C., &Phillips, D.R.(2000). Interstrand cross-linking by adriamycin in nuclear and mitochondrial DNA of MCF-7 cells. *Nucleic Acids Research*, *28*(4), 1019–1025. https://doi.org/10.1093/nar/28.4.1019

Cully, D., Simon, C., &Langely, G.(2002). Salivary antioxidants and periodontal disease status. *Preceding of the Nutrition Society*, *16*(1), 137–143.

Curtis, A., &Wilkinson, C.(2001). Nanotechniques and approaches in biotechnology. *Trends in Biotechnology*, *19*(3), 97–101. https://doi.org/10.1016/s0167-7799(00)01536-5

Cutfield, A.(1907). Trypsin treatment in malignant disease. *British Medical Journal*, *5*, 525.

Cynshi, O., Tamura, K., &Niki, E.(2010). Design, synthesis, and action of antiatherogenic antioxidants. *Methods in Molecular Biology*, *610*, 91–107. https://doi.org/10.1007/978-1-60327-029-8_6

Cyril, D.G., Landry, K.S., François, K.Y.K., Abou, B., Felix, Y., &Timothée, O.(2016). Evaluation of nephroprotectiveactivity of aqueous and Hydroethanolic extracts of Trema guineensis Leaves (*Ulmaceae*) against gentamicin-inducednephrotoxicity in rats. *International Journal of Biochemistry Research and Review*, *15*(2), 1–10. https://doi.org/10.9734/IJBCRR/2016/30539

D'Agostini, F., Izzotti, A., Balansky, R.M., Bennicelli, C., &De Flora, S.(2005). Modulation of apoptosis by cancer chemopreventive agents. *Mutation Research*, *591*(1–2), 173–186. https://doi.org/10.1016/j.mrfmmm.2005.03.034

Dahlin, D.C., Miwa, G.T., Lu, A.Y.H., &Nelson, S.D.(1984). *N*-Acetyl*p*- benzoquinone imine: A cytochrome P450-mediated oxidation product of acetaminophen. *Proceedings*

of theNational Academy ofSciences of the UnitedStates of America, *81*(5), 1327–1331. https://doi.org/10.1073/pnas.81.5.1327

Dai, F., Miao, Q., Zhou, B., Yang, L., &Liu, Z. L.(2006). Protective effects of flavonols and their glycosides against free radical-induced oxidative hemolysis of red blood cells. *Life Sciences*, *78*(21), 2488–2493. https://doi.org/10.1016/j.lfs.2005.10.009

Dai, Y., &Cederbaum, A.I.(1995). Cytotoxicity of acetaminophen in human cytochrome P4502E1- transfected Hep G2 cells. *Journal of Pharmacology and Experimental Therapeutics*, *273*(3), 1497–1505.

Dale,P.S., Tamhankar,C.P., George,D., &Daftary,G.V.(2001). Co-medication with hydrolytic enzymes in radiation therapy of uterine cervix: Evidence of the reduction of acute side effects. *Cancer Chemotherapy and Pharmacology*, *47* (Suppl.), S29–S34. https://doi.org/10.1007/s002800170006

Dalton, T.P., Shertzer, H.G., &Puga, A.(1999). Regulation of gene expression by reactive oxygen. *Annual Review of Pharmacology and Toxicology*, *39*, 67–101. https://doi.org/10.1146/annurev.pharmtox.39.1.67

Das, K.C., Lewis-Molock, Y., &White, C.W.(1997). Elevation of manganese superoxide dismutase gene expression by thioredoxin. *American Journal of Respiratory Cell and MolecularBiology*, *17*(6), 713–726. https://doi.org/10.1165/ajrcmb.17.6.2809

Davies, K.J.(1995). Oxidative stress: The paradox of aerobic life.*Biochemical Society Symposium*, *61*, 1–31. https://doi.org/10.1042/bss0610001

Davis, M.E., Zuckerman, J.E., Choi, C.H.J., Seligson, D., Tolcher, A., Alabi, C.A., Yen, Y., Heidel, J.D., &Ribas, A.(2010). Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*, *464*(7291), 1067–1070. https://doi.org/10.1038/nature08956

De Duve, C., &Baudhuin, P.(1966). Peroxisomes (microbodies and related particles).*Physiological Reviews*, *46*(2), 323–357. https://doi.org/10.1152/physrev.1966.46.2.323

de Haan, J.B., Bladier, C., Griffiths, P., Kelner, M., O'Shea, R.D., Cheung, N.S.,Bronson, R.T., Silvestro, M.J., Wild, S., Zheng, S.S., Beart, P.M., Hertzog, P.J., &Kola, I.(1998). Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. *Journal of Biological Chemistry*, *273*(35), 22528–22536. https://doi.org/10.1074/jbc.273.35.22528

de Oliveira Silva, E., &Batista, R.(2017). Ferulic acid and naturally occurring compounds bearing a feruloyl moiety: A review on their structures, occurrence, and potential health ben-efits. *Comprehensive Reviews in Food Science and Food Safety*, *16*(4), 580–616. https://doi.org/10.1111/1541-4337.12266

De Vries, J.(1981). Hepatotoxic metabolic activation of paracetamol and its derivatives phenacetin and Benorilate: Oxygenation or electron transfer?*Biochemical Pharmacology*, *30*(5), 399–402. https://doi.org/10.1016/0006-2952(81)90622-5

Denardo, S.J., Denardo, G.L., Miers, L.A., Natarajan, A., Foreman, A.R., Gruettner, C., Adamson, G.N., &Ivkov, R.(2005). Development of tumor targeting bioprobes (^{111}In-Chimeric L6 monoclonal antibody nanoparticles) for alternating magnetic field cancer therapy. *Clinical Cancer Research*, *11*(19 Pt 2), 7087s–7092s. https://doi.org/10.1158/1078-0432.CCR-1004-0022

Desnick, R. J., Thorpe, S. R., &Fiddler, M. B.(1976). Toward enzyme therapy for lysosomal storage diseases. *Physiological Reviews*, *56*(1), 57–99. https://doi.org/10.1152/physrev.1976.56.1.57

Devasagayam, T.P.A., &Tilak, J.C.(2006).Oxidative damage to mitochondria. InK. K.Sing(Ed.), *Oxidative stress, disease and cancer* (pp. 85–150).Imperial College Press. *Oxidative Stress, Disease and Cancer*.

Dhingra, A.K., Chopra, B., &Bonthagarala, B.(2018). Natural anti-inflammatoryagents: Recent Progress and futureperspectives. *Annals of Pharmacology and Pharmaceutics, 3*(5), 1158.

Ding, L., Yao, C., Yin, X., Li, C., Huang, Y., Wu, M., Wang, B., Guo, X., Wang, Y., &Wu, M.(2018). Size, shape, and proteincoronadeterminecellularuptake and removalmechanisms of goldnanoparticles. *Small*, *14*(42), e1801451. https://doi.org/10.1002/smll.201801451, PubMed: 30239120

Ding, Y., Liu, J., Wang, H., Shen, G., &Yu, R.(2007). A piezoelectric immunosensor for the detection of alpha-fetoprotein using an interface of gold/hydroxyapatite hybrid nanomaterial. *Biomaterials*, *28*(12), 2147–2154. https://doi.org/10.1016/j.biomaterials.2006.12.025

Divakaran, S. A, & Nair, C.K.K(2012). Amelioration of doxorubicin induced cardiotoxicity in tumor bearing mice by ferulic acid: A mechanistic study at cellular and biochemical level. *International Journal of Tumor Therapy*, *1*(2), 6–13. https://doi.org/10.5923/j.ijtt.20120102.01

Divakaran, S.A., Hema, P.S., Nair, M.S., &Nair, C.K.K.(2013). Antioxidant capacity and radioprotective properties of the flavonoids galangin and kaempferide isolated from *Alpinia galanga L*. (Zingiberaceae) against radiation induced cellular DNA damage. *International Journal of Radiation Research*, *11*(2), 81–89.

Diwanay, S., Chitre, D., &Patwardhan, B.(2004). Immunoprotection by botanical drugs in cancer chemotherapy. *Journal of Ethnopharmacology*, *90*(1), 49–55. https://doi.org/10.1016/j.jep.2003.09.023

Dixon, R. A., &Pasinetti, G. M.(2010). Flavonoids and isoflavonoids: From plant biology to agriculture and neuroscience. *Plant Physiology*, *154*(2), 453–457. https://doi.org/10.1104/pp.110.161430

Dizdaroglu, M.(1993). DNA and freeradicals. InB.Halliwell&O. I.Aruoma(Eds.)p. 19±39.Ellis Horwood.

Doroshow, J.H., Locker, G.Y., &Myers, C.E.(1980). Enzymatic defenses of the mouse heart against reactive oxygen metabolites: Alterations produced by doxorubicin. *Journal of Clinical Investigation*, *65*(1), 128–135. https://doi.org/10.1172/JCI109642

dos Santos, N. A., Carvalho Rodrigues, M. A., Martins, N. M., &dos Santos, A. C.(2012). Cisplatin-induced nephrotoxicity and targets of nephroprotection: An update. *Archives of Toxicology*, *86*(8), 1233–1250. https://doi.org/10.1007/s00204-012-0821-7

Dounousi, E., Papavasiliou, E., Makedou, A., Ioannou, K., Katopodis, K.P., Tselepis, A., Siamopoulos, K. C., &Tsakiris, D.(2006). Oxidative stress is progressively enhanced with advancing stages of CKD. *American Journal ofKidney Diseases*, *48*(5), 752–760. https://doi.org/10.1053/j.ajkd.2006.08.015

Dreher, I., Schmutzler, C., Jakob, F., &Köhrle, J.(1997). Expression of selenoproteins in various rat and human tissues and cell lines. *Journal of Trace Elements in Medicine and Biology: Organ of the Society for Minerals and Trace Elements*, *11*(2), 83–91. https://doi.org/10.1016/S0946-672X(97)80031-4

Duarte, T.L., &Lunec, J.(2005). Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. *Free Radical Research*, *39*(7), 671–686. https://doi.org/10.1080/10715760500104025

Duncan, R.(1999). Polymer conjugates for tumour targeting and intracytoplasmic delivery. The EPR effect as a common gateway?*Pharmaceutical Science and Technology Today*, *2*(11), 441–449. https://doi.org/10.1016/s1461-5347(99)00211-4

Dutt, S.(1925). General synthesis of a-unsaturated acids from malonic acid.*Quarterly Journal of the ChemicalSociety*, 297–301.

Egeblad, M., Littlepage, L.E., &Werb, Z.(2005). The fibroblastic coconspirator in cancer progression. *Cold Spring Harbor Symposia on Quantitative Biology*, *70*, 383–388. https://doi.org/10.1101/sqb.2005.70.007

Elbl, L., Hrstkova, H., Tomaskova, I., &Michalek, J.(2006). Late anthracycline cardiotoxicity protection by dexrazoxane (ICRF-187) in pediatric patients: Echocardiographic follow-up. *Supportive Care in Cancer*, *14*(2), 128–136. https://doi.org/10.1007/s00520-005-0858-8

Elblehi, S.S., El-Sayed, Y.S., Soliman, M.M., &Shukry, M.(2021). Date palmpollenextractavertdoxorubicin- inducedcardiomyopathyfibrosis and associatedoxidative/nitrosativestress, inflammatorycascade, and apoptosis-targeting Bax/Bcl-2 and caspase-3signalingpathways. *Animals: An Open Access Journal from MDPI*, *11*(3), 886. https://doi.org/10.3390/ani11030886

Elechiguerra, J.L., Burt, J.L., Morones, J.R., Camacho-Bragado, A., Gao, X., Lara, H.H., &Yacaman, M.J.(2005). Interaction of silver nanoparticles with HIV-1.*Journal of Nanobiotechnology*, *3*, 6. https://doi.org/10.1186/1477-3155-3-6

Elena, M., Quiroga, A. G., Castro, J., Ortiz, A., Aller, P., &Mata, F.(2009). Inhibition of p38-MAPK potentiatescisplatin-inducedapoptosis via GSH depletion and increasesintracellulardrugaccumulation in growth-arrestedkidneytubularepithelialcells. *Toxicological Sciences*, *111*(2), 413–423. https://doi.org/10.1093/toxsci/kfp145

Elinav, E., Nowarski, R., Thaiss, C.A., Hu, B., Jin, C., &Flavell, R.A.(2013). Inflammation-induced cancer: Crosstalk between tumours, immune cells and microorganisms. *Nature Reviews. Cancer*, *13*(11), 759–771. https://doi.org/10.1038/nrc3611

Elkomy, A.,Aboubakr, M., Soliman, A.,Abdeen, A., Abdelkader, A., &Hekal, H.(2016). Paracetamol induced hepatic toxicity and amelioration by cinnamon in rats. *International Journal of Pharmacology and Toxicology*, *4*(2),187–190. https://doi.org/10.14419/ijpt.v4i2.6529

Ellington, A.D., &Szostak, J.W.(1990). *In vitro* selection of RNA molecules that bind specific ligands.*Nature*, *346*(6287), 818–822. https://doi.org/10.1038/346818a0

Ellis, L.M., Liu, W., Ahmad, S.A., Fan, F., Jung, Y.D., Shaheen, R.M., &Reinmuth, N.(2001). Overview of angiogenesis: Biologic implications for antiangiogenic therapy. *Seminars in Oncology*, *28*(5) (Suppl. 16), 94–104. https://doi.org/10.1016/s0093-7754(01)90287-8

El-Nahas, S.M., Mattar, F.E., &Mohamed, A.A.(1993). Radioprotective effect of vitamins C and E. *Mutation Research*, *301*(2), 143–147. https://doi.org/10.1016/0165-7992(93)90037-v

El-Readi, M.Z., &Althubiti, M.A.(2019). Cancer nanomedicine: A New Era of successfultargetedtherapy. *Journal of Nanomaterials*, 2019, 1–13. https://doi.org/10.1155/2019/4927312

Elsayed Azab, A., A Adwas, Ibrahim Elsayed, A. S., A Adwas, A., Ibrahim Elsayed, S., &Quwaydir, F. A.(2019).Oxidative stress and antioxidant mechanisms in human body.*Journal of Applied Biotechnology and Bioengineering*, *6*(1),43–47. https://doi.org/10.15406/jabb.2019.06.00173

Ernst, E.(2001). Complementary therapies in palliative cancer care. *Cancer*, *91*(11), 2181–2185. https://doi.org/10.1002/1097-0142(20010601)91:11<2181::aid-cncr1247>3.0.co;2-l

Fang, C.Y.,Lou, D., Zhou, L.,Wang, J., Yang, B., He, Q.,Wang, J., &Weng, Q.(2021). Natural products: Potential treatments for cisplatin-induced nephrotoxicity. *Acta Pharmacologica Sinica*, *42(12),0* 1951–1969. https://doi.org/10.1038/s41401-021-00620-9’

Fang, J., Deng, D., Nakamura, H., Akuta, T., Qin, H., Iyer, A. K., Greish, K., &Maeda, H.(2008). Oxystress inducing antitumor therapeutics via tumor-targeted delivery of PEG-conjugated D-amino acid oxidase.*International Journal of Cancer*, *122*(5), 1135–1144. https://doi.org/10.1002/ijc.22982

Fang, J., Sawa, T., Akaike, T., &Maeda, H.(2002). Tumor-targeted delivery of polyethylene glycol-conjugated D-aminoacidoxidase for antitumortherapy via enzymaticgeneration of hydrogenperoxide.*Cancer Research*, *62*(11), 3138–3143.

Farokhzad, O.C., Cheng, J., Teply, B.A., Sherifi, I., Jon, S., Kantoff, P.W., Richie, J.P., &Langer, R.(2006). Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(16), 6315–6320. https://doi.org/10.1073/pnas.0601755103

Ferrari, M.(2005). Cancer nanotechnology: Opportunities and challenges. *Nature Reviews. Cancer*, *5*(3), 161–171. https://doi.org/10.1038/nrc1566

Fetouh, F.A., &Azab, A.E.(2014). Ameliorating effects of curcumin and propolis against the reproductive toxicity of gentamicin in adult male Guinea pigs: Quantitative analysis and morphological study. *American Journal of Life Sciences*, *2*(3), 138–149. https://doi.org/10.11648/j.ajls.20140203.13

Fichtlscherer, S., Breuer, S., Schächinger, V., Dimmeler, S., &Zeiher, A.M.(2004). C-reactive protein levels determine systemic nitric oxide bioavailability in patients with coronary artery disease. *European Heart Journal*, *25*(16), 1412–1418. https://doi.org/10.1016/j.ehj.2004.06.026

Filho, A.C.M., &Meneghini, R.(1984). In vivo formation of singlestrand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss reaction. *Biochimica et Biophysica Acta*, *781*(1–2), 56–63. https://doi.org/10.1016/0167-4781(84)90123-4

Finkel, T., &Holbrook, N.J.(2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, *408*(6809), 239–247. https://doi.org/10.1038/35041687

Fisher, A.B.(2011). Peroxiredoxin 6: A bifunctional enzyme with glutathione peroxidase and phospholipase A_2 activities. *Antioxidants andRedox Signaling*, *15*(3), 831–844. https://doi.org/10.1089/ars.2010.3412

Floyd, R.A., Watson, J.J., Wong, P.K., Altmiller, D.H., &Rickard, R.C.(1986). Hydroxyl free radical adduct of deoxyguanosine: Sensitive detection and mechanisms of formation. *Free Radical Research Communications*, *1*(3), 163–172. https://doi.org/10.3109/10715768609083148

Foletta, V.C., Segal, D.H., &Cohen, D.R.(1998). Transcriptional regulation in the immune system: All roads lead to AP-1. *Journal ofLeukocyte Biology*, *63*(2), 139–152. https://doi.org/10.1002/jlb.63.2.139

Folkman, J.(1986). How is blood vessel growth regulated in normal and neoplastic tissue? GHA Clowes memorial Award lecture. *Cancer Research*, *46*(2), 467–473.

Fonorow, O.(2004). The cure for cancer: Theory, history and treatment.*Townsend Letter for Doctors and Patients*.

Fonseca, C., Simões, S., &Gaspar, R.(2002). Paclitaxel-loaded PLGA nanoparticles: Preparation, physicochemical characterization and *in vitro* anti-tumoral activity. *Journal of Controlled Release*, *83*(2), 273–286. https://doi.org/10.1016/s0168-3659(02)00212-2

Forbes, Z.G., Yellen, B.B., Barbee, K.A., &Friedman, G.(2003). An approach to targeted drug delivery based on uniform magnetic fields.*IEEE Transactions on Magnetics*, *39*(5), 3372–3377. https://doi.org/10.1109/TMAG.2003.816260

Fouladi, M., Chintagumpala, M., Ashley, D., Kellie, S., Gururangan, S., Hassall, T., Gronewold, L., Stewart, C. F., Wallace, D., Broniscer, A., Hale, G. A., Kasow, K. A., Merchant, T. E., Morris, B., Krasin, M., Kun, L. E., Boyett, J. M., &Gajjar, A.(2008). Amifostine protects against cisplatin-induced ototoxicity in children with average-risk medulloblastoma. *Journal of Clinical Oncology*, *26*(22), 3749–3755. https://doi.org/10.1200/JCO.2007.14.3974

Fountoulakis, M., Berndt, P., Boelsterli, U.A., Winter, M., &Suter, L.A.(2000). Two diamensional database of mouse liver proteins: Changes in hepatic protein levels following treatment with acetaminophen or its nontoxic regioisimer 3-acetamidophenol.*Electrophoresis*, *21*, 2148–2161

Fox, S.I.(2017). *Human physiology*(16th ed) (pp. 666–670). McGraw-Hill Education.

Francisco, A., Tomás-Barberán, F. A., Hess-Pierce, B., Holcroft, D. M., &Kader, A. A.(2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal ofAgricultural and Food Chemistry*, *48*(10), 4581–4589. https://doi.org/10.1021/jf000404a

Frei, B.(1999). On the role of vitamin C and other antioxidants in atherogenesis and vascular dysfunction. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine*, *222*(3), 196–204. https://doi.org/10.1046/j.1525-1373.1999.d01-136.x

Frei, B., &Higdon, J. V.(2003). Antioxidant activity of tea polyphenols in vivo: Evidence from animal studies. *Journal ofNutrition*, *133*(10), 3275S–3284S. https://doi.org/10.1093/jn/133.10.3275S

Frei, B., England, L., &Ames, B.N.(1989). Ascorbate is an outstanding antioxidant in human blood plasma. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(16), 6377–6381. https://doi.org/10.1073/pnas.86.16.6377

Fridovich, I.(1997). Superoxide anion radical (O_2–•), super oxide dismutases, and related matters. *Journal of Biological Chemistry*, *272*(30), 18515–18517. https://doi.org/10.1074/jbc.272.30.18515

Fridovich, I.(1999). Fundamental aspects of reactive oxygen species, or what's the matter with oxygen?*Annals of the New York Academy ofSciences*, *893*, 13–18. https://doi.org/10.1111/j.1749-6632.1999.tb07814.x

Fujinami, Y., Tai, A., &Yamamoto, I.(2001). Radical scavenging activity against 1,1-diphenyl-picrylhydrazyl of ascorbic acid 2-glucoside (AA-2G) and 6-acyl-AA-2G. *Chemical and PharmaceuticalBulletin*, *49*(5), 642–644. https://doi.org/10.1248/cpb.49.642

Gaikwad, K.,Dagle, P., Choughule, P., Joshi, Y.M., &Kadam, V.A.(2012). Review onsomenephroprotectivemedicinalplants. *International Journal of Pharmaceutical Sciences and Research*, *3*(8), 2451–2454.

Ganguly, P., Gupta, A.K., Majumder, U.K., &Ghosa, S.(2009). Hepatoprotective and antioxidant effects of naturally occurring with a steroid metal ion conjugates of Withania somnifera in paracetamol induced hepatotoxicity in rats. *Pharmacology Online*, *1*, 1044–1056.

Geetha, A., &Devi,C. S.(1992). Effect of doxorubicin on heart mitochondrial enzymes in rats: A protective role for alphatocopherol.*Indian Journal of Experimental Biology*, *30*(7), 615–618.

Gemba, M., &Fukuishi, N.(1991). Amelioration by ascorbic acid of cisplatin induced injury in cultured renal epithelial cells. *Contributions to Nephrology*, *95*, 138–142. https://doi.org/10.1159/000420651

Genova, G., Iacopini, P., Baldi, M., Ranieri, A., Storchi, P., &Sebastiani, L.(2012). Temperature and storage effects on antioxidant activity of juice from red and white grapes. *International Journal of Food Science and Technology*, *47*(1), 13–23. https://doi.org/10.1111/j.1365-2621.2011.02801.x

George, SP and Anushree CS..(2014). Drug-induced impairment of renal function.*InternationalJournal of Nephrology and Renovascular Disease*, *7*, 457–468. https://doi.org/10.2147/IJNRD.S39747

Ghosh, M.N., Banerjie, R.M., &Mukherji, S.K.(1963). Capillary permeability-increasing property of hyaluronidase in rat. *Indian Journal of Physiology and Pharmacology*, *7*, 17–21.

Giaccone, G.(2000). Clinical perspectives on platinum resistance. *Drugs*, *59* (Suppl. 4), 9–17; discussion 37. https://doi.org/10.2165/00003495-200059004-00002

Gilchrist, R.K., Medal, R., Shorey, W.D., Hanselman, R.C., Parrott, J.C., &Taylor, C.B.(1957). Selective inductive heating of lymph nodes. *Annals of Surgery*, *146*(4), 596–606. https://doi.org/10.1097/00000658-195710000-00007

Gill, C. I., Boyd, A., McDermott, E., McCann, M., Servili, M., Selvaggini, R., Taticchi, A., Esposto, S., Montedoro, G., McGlynn, H., &Rowland, I.(2005). Potential anti_cancer effects of virgin olive oil phenols on colorectal carcinogenesis models in vitro. *International Journal of Cancer*, *117*(1), 1–7. https://doi.org/10.1002/ijc.21083

Giri, A., Khynriam, D., &Prasad, S.B.(1998). Vitamin C mediated protection on cisplatin induced mutagenicity in mice. *Mutation Research*, *421*(2), 139–148. https://doi.org/10.1016/s0027-5107(98)00158-4

Gleiter, H.(2000). Nanostructured materials, basic concepts and microstructure.*Acta Materialia*, *48*(1), 1–29. https://doi.org/10.1016/S1359-6454(99)00285-2

Gobin, A.M., Lee, M.H., Halas, N.J., James, W.D., Drezek, R.A., &West, J.L.(2007). Near-infrared resonant nanoshells for combined optical imaging and photothermal cancer therapy.*Nano Letters*, *7*(7), 1929–1934. https://doi.org/10.1021/nl070610y

Goeth, R. A.(1907). Pancreatic treatment of cancer, with report of a cure. *JAMA*, *XLVIII*(12), 1030. https://doi.org/10.1001/jama.1907.25220380046002d

Gogoi, P., Gogoi, A., Rajbangshi, C., Bhuyan, P., &Bhuyan, B.K.(2017). Extraction, purification and spectroscopic characterization of ferulic acid by alkaline hydrolysis from brans of Assam, India. *Imperial Journal of Inter-Disciplinary Research*, *3*(1), 358–361.

Goldstein, R.S., &Mayor, G.H.(1983). Minireview. The nephrotoxicity of cisplatin. *Life Sciences*, *32*(7), 685–690. https://doi.org/10.1016/0024-3205(83)90299-0

Gonzalez, G. S.(2000). Treatment for cancer: Gland extracts, coffee enemas, vitamin megadoses, and diets. *Scientific Review of Alternative Medicine*, *2*(2), 25–30.

Gonzalez, N. J., &Isaacs, L. L.(1999). Evaluation of pancreatic proteolytic enzyme treatment of adenocarcinoma of the pancreas, with nutrition and detoxification support. *Nutrition and Cancer*, *33*(2), 117–124. https://doi.org/10.1207/S15327914NC330201

Gorton, H.C., &Jarvis,K.(1999). The effectiveness ofvitamin C in preventing and relieving the symptoms of virus-induced respiratoryinfections. *Journal of Manipulative and PhysiologicalTherapeutics*, *22*(8), 530–533. https://doi.org/10.1016/s0161-4754(99)70005-9

Govindarajan, R., Vijayakumar, M., Shirwaikar, A., Rawat, A.K.S., Mehrotra, S., &Pushpangadan, P.(2006). Antioxidant activity of *Desmodiumgangeticum* and its phenolics in arthritic rats. *Acta Pharmaceutica*, *56*(4), 489–496.

Grabis, J., Heidemane, G., &Rasmane, D.(2008). Preparation of Fe3O4 and *γ*-Fe2O3 nanoparticles by liquid and gasphaseprocesses.*Materials Science* (Medziagotyra), *14*,292–295.

Graf, E.(1992). Antioxidant potential of ferulic acid. *Free Radical Biology and Medicine*, *13*(4), 435–448. https://doi.org/10.1016/0891-5849(92)90184-i

Green, H. N., &Westrop, J. W.(1958). Hydrogen peroxide and tumour therapy. *Nature*, *181*(4602), 128–129. https://doi.org/10.1038/181128a0

Greene, M.H.(1992). Is cisplatin a human carcinogen?*Journal of the National Cancer Institute*, *84*(5), 306–312. https://doi.org/10.1093/jnci/84.5.306

Greenwald, R.A.(1991). Animal models for evaluation of arthritis drugs. *Methods and Findings in Experimental and Clinical Pharmacology*, *13*(2), 75–83.

Gregoriadis, G., &Ryman, B.E.(1971). Liposomes as carriers of enzymes or drugs: A new approach to the treatment of storage diseases.*Biochemical Journal*, *124*(5), 58P. https://doi.org/10.1042/bj1240058p

Guidot, D.M., McCord, J.M., Wright, R.M., &Repine, J.E.(1993). Absence of electron transport (rho0 state) restores growth of a manganese-superoxide dismutase-deficient Saccharomyces cerevisiae in hyperoxia: Evidence for electron transport as a major source of superoxide generation *in vivo*. *Journal of Biological Chemistry*, *268*(35), 26699–26703. https://doi.org/10.1016/S0021-9258(19)74369-5

Gulkac, MD., Akpinar, G., Ustün, H., &Ozön Kanli, A.(2004). Effects of vitamin A on doxorubicin-induced chromosomal aberrations in bone marrow cells of rats. *Mutagenesis*, *19*(3), 231–236. https://doi.org/10.1093/mutage/geh021

Gupta, A.K., &Wells, S.(2004). Surface-modified superparamagnetic nanoparticles for drug delivery: Preparation, characterization, and cytotoxicity studies. *IEEE Transactions on Nanobioscience*, *3*(1), 66–73. https://doi.org/10.1109/tnb.2003.820277

Gupta, A.K., Berry, C., Gupta, M., &Curtis, A.(2003). Receptor-mediated targeting of magnetic nanoparticles using insulin as a surface ligand to prevent endocytosis. *IEEE Transactions on Nanobioscience*, *2*(4), 255–261. https://doi.org/10.1109/tnb.2003.820279

Gupta, P.K., &Hung, C.T.(1989). Magnetically controlled targeted micro-carrier systems.*Life Sciences*, *44*(3), 175–186. https://doi.org/10.1016/0024-3205(89)90593-6

Gur, KP., Kaushal, V., Hong, X., &Shah, S.V.(2001). Role and regulation of activation of caspases in cisplatin-induced injury to renal tubular epithelial cells. *Kidney International*, *60*(5), 1726–1736. https://doi.org/10.1046/j.1523-1755.2001.00026.x

Gutman, R.L., Peacock, G., &Lu, D.R.(2000). Targeted drug delivery for brain cancer treatment.*Journal of Controlled Release*, *65*(1–2), 31–41. https://doi.org/10.1016/s0168-3659(99)00229-1

Gutteridge, J.M.C., &Toeg, D.(1982). Adriamycin-dependent damage to deoxyribose: A reaction involving iron, hydroxyl and semiquinone free radicals. *FEBS Letters*, *149*(2), 228–232. https://doi.org/10.1016/0014-5793(82)81106-X

Guzik, T.J., West, N.E.J., Black, E., McDonald, D., Ratnatunga, C., Pillai, R., &Channon, K. M.(2000). Vascular superoxide production by NAD(P)H oxidase:association with endothelial dysfunction and clinical risk factor. *Circulation Research*, *86*(9), E85–E90. https://doi.org/10.1161/01.res.86.9.e85

Haddow,A.,DeLamirande,G.,Bergel,F.,Bray,R.C., &Gilbert,D. A.(1958).A Anti-tumor and biochemical effects of purified bovine xanthine oxidase in C3H and C mice. *Nature*, *182*(4643), 1144–1146. https://doi.org/10.1038/1821144a0

Hafeez, A., Jain, U., Sajwan, P.,Srivastava, S., &Thakur, A.(2013).Evaluation of carrageenan induced anti-inflammatory activity of ethanolic extract of bark of *Ficusvirens* Linn. in swiss albino mice. *Journal of Phytopharmacology*, *2*(1–3), 39–43. https://doi.org/10.31254/phyto.2013.21307

Hafeli, U.O., Schutt, W., Teller, J., &Zborowski, M.(1997). *Scientific and clinicalapplications of magneticcarriers*.Plenum Press.

Hafeman, D.G., Sunde, R.A., &Hoekstra, W.G.(1974). Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat.*Journal of Nutrition*, *104*(5), 580–587. https://doi.org/10.1093/jn/104.5.580

Hahnfeldt, P., Panigrahy, D., Folkman, J., &Hlatky, L.(1999). Tumor development under angiogenic signaling: A dynamical theory of tumor growth, treatment response, and postvascular dormancy. *Cancer Research*, *59*(19), 4770–4775.

Hall, E.J., &Giaccia, A.J.(2006). *Radiobiology for radiobiologist. Lippincott Williams and Walkins. 6th*eds (pp. 16–28).

Halliwell, B.(1987). Oxidants and human disease: Some new concepts. *FASEB Journal*, *1*(5), 358–364. https://doi.org/10.1096/fasebj.1.5.2824268

Halliwell, B.(1993). The role of oxygen radicals in human disease, with particular reference to the vascular system. *Haemostasis*, *23*(Suppl. 1), 118–126. https://doi.org/10.1159/000216921

Halliwell, B.(1996). Antioxidants in human health and disease. *Annual Review of Nutrition*, *16*, 33–50. https://doi.org/10.1146/annurev.nu.16.070196.000341

Halliwell, B.(1997). Antioxidants and human disease: A general introduction. *Nutrition Reviews*, *55*(1 Pt 2), S44–S459; discussion S49–S52. https://doi.org/10.1111/j.1753-4887.1997.tb06100.x

Halliwell, B.(1999). Antioxidant defence mechanisms: From the beginning to the end (of the beginning). *Free Radical Research*, *31*(4), 261–272. https://doi.org/10.1080/10715769900300841

Halliwell, B.(2007). Biochemistry of oxidative stress. *Biochemical Society Transactions*, *35*(5), 1147–1150. https://doi.org/10.1042/BST0351147

Halliwell, B., &Aruoma, O.I.(1991). DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. *FEBS Letters*, *281*(1–2), 9–19. https://doi.org/10.1016/0014-5793(91)80347-6

Halliwell, B., &Dizdaroglu, M.(1992). The measurement of oxidative damage to DNA by HPLC and GC/MS techniques.*Free Radical Research Communications*, *16*(2), 75–87. https://doi.org/10.3109/10715769209049161

Halliwell, B., &Gutteridge, J.M. (1984). Free radicals, lipid peroxidation, and cell damage. *Lancet*, *2*(8411), 1095. https://doi.org/10.1016/s0140-6736(84)91530-7

Halliwell, B., &Gutteridge, J.M.(1984). Free radicals, lipidperoxidation, and celldamage.*Lancet*, *2*(8411), 1095. https://doi.org/10.1016/s0140-6736(84)91530-7

Halliwell, B., &Gutteridge, J.M.(1985). The importance of free radicals and catalytic metal ions in human diseases. *Molecular Aspects of Medicine*, *8*(2), 89–193. https://doi.org/10.1016/0098-2997(85)90001-9

Halliwell, B., Gutteridge, J.M.C., &Aruoma, O.I.(1987). The deoxyribose method: A simple "test tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Analytical Biochemistry*, *165*(1), 215–219. https://doi.org/10.1016/0003-2697(87)90222-3

Hamza, R.Z., &Al-Harbi, M.S.(2015). Amelioration of paracetamol hepatotoxicity and oxidative stress on mice liver with silymarin and Nigella sativa extract supplements. *Asian PacificJournal of Tropical Biomedicine*, *5*(7), 521–531. https://doi.org/10.1016/j.apjtb.2015.03.011

Handa, S.S., Sharma, A., &Chakraborti, K.K.(1986). Natural products and plants as liver protecting drugs. *Fitoterapia*, *57*, 307–352.

Handelman, G.J.(2000). Evaluation of oxidant stress in dialysis patients. *Blood Purification*, *18*(4), 343–349. https://doi.org/10.1159/000014460

Hanigan, M.H., &Devarajan, P.(2003).Cisplatin nephrotoxicity: Molecular mechanisms. *Cancer Therapy*, 1, 47–61.

Harris, L.R., Cake, M.H., &Macey, D.J.(1994). Iron release from ferritin and its sensitivity to superoxide ions differs among vertebrates. *Biochemistry Journal*. *Biochemical Journal*, *301*(2), 385–389. https://doi.org/10.1042/bj3010385

Harrison, D. G.(1997). Cellular and molecular mechanisms of endothelial cell dysfunction. *Journal of Clinical Investigation*, *100*(9), 2153–2157. https://doi.org/10.1172/JCI119751

Hayashi, M., Morita, T., Kodama, Y., Sofuni, T., &Ishidate, M.(1990). The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. *Mutation Research*, *245*(4),245–249. https://doi.org/10.1016/0165-7992(90)90153-b

Hayashi, T., Sawa, K., Kawasaki, M., Arisawa, M., Shimizu, M., &Morita, N.(1988). Inhibition of cow's milk xanthine oxidase by flavonoids. *Journal of Natural Products*, *51*(2), 345–348. https://doi.org/10.1021/np50056a030

Hayes, J. D., Flanagan, J. U., &Jowsey, I. R.(2005). Glutathione transferases. *Annual Review of Pharmacology and Toxicology*, *45*, 51–88. https://doi.org/10.1146/annurev.pharmtox.45.120403.095857

He, H., Liu, L., Morin, E.E., Liu, M., &Schwendeman, A.(2019). Survey of clinicaltranslation of cancernanomedicines-lessonslearned from successes and failures. *Accounts of Chemical Research*, *52*(9), 2445–2461. https://doi.org/10.1021/acs.accounts.9b00228

Heimberger, A.B., Archer, G.E., McLendon, R.E., Hulette, C., Friedman, A.H., Friedman, H.S., Bigner, D.D., &Sampson, J.H.(2000). Temozolomide delivered by intracerebral microinfusion is safe and efficacious against malignant gliomas in rats.*Clinical Cancer Research*, *6*(10), 4148–4153.

Hemnani, T., &Parihar, M.S.(1998). Reactive oxygen species and oxidative DNA damage. *Indian Journal of Physiology andPharmacology*, *42*(4), 440–452.

Henn, S. W., &Ackers, G. K.(1969). Molecular Sieve Studies of Interacting Protein Systems. *Journal of Biological Chemistry*, *244*(2), 465–470. https://doi.org/10.1016/S0021-9258(18)94452-2

Heo, M.Y.,Lee, S.J.,Kwon, C.H.,Kim, S.W., Sohn, D.H., &Au, W.W.(1994). Anticlastogenic effects of galangin against bleomycin-induced chromosomal aberrations in mouse spleen lymphocytes.*Mutation Research*, *311*(2), 225–229. https://doi.org/10.1016/0027-5107(94)90180-5

Herrmann, K.(1989). Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *Critical Reviews in Food Science and Nutrition*, *28*(4), 315–347. https://doi.org/10.1080/10408398909527504

Herrmann, K.(1990). Significance of hydroxycinnamic compounds in food. I. Antioxidant activity-effects on the use, digestibility, and microbial spoilage in food. *Chemie, Mikrobiologie, Technologie der Lebensmittel*, *12*, 137–144.

Hersh, E.M., &Freiriech, E.J.(1968). InH.Busch(Ed.). *Methods in cancerresearch* p. 355A.Academic Press.

Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., &Yodoi, J.(1997). AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(8), 3633–3638. https://doi.org/10.1073/pnas.94.8.3633

Hirsch, L.R., Halas, N.J., &West, J.L.(2005). Whole-blood immunoassay facilitated by gold nanoshell-conjugate antibodies.*Methods in Molecular Biology*, *303*, 101–111. https://doi.org/10.1385/1-59259-901-X:101

Hoffmann, M. E., &Meneghini, R.(1979). Action of hydrogen peroxide on human fibroblast in culture. *Photochemistry and Photobiology*, *30*(1), 151–155. https://doi.org/10.1111/j.1751-1097.1979.tb07128.x

Hofseth, L.J., &Ying, L.(2006).Identifying and defusing weapons of mass inflammation in carcinogenesis. *Biochimica et Biophysica Acta*, *1765*(1), 74–84. https://doi.org/10.1016/j.bbcan.2005.08.005

Hogg, N., &Kalyanaraman, B.(1998). Nitric oxide and low-density lipoprotein oxidation. *Free Radical Research*, *28*(6), 593–600. https://doi.org/10.3109/10715769809065815

Holditch, S.J., Brown, C.N., Lombardi, A.M., Nguyen, K.N., &Edelstein, C.L.(2019). Recent advances in models, mechanisms, biomarkers, and interventions in cisplatin-inducedacutekidneyinjury. *International Journal of Molecular Sciences*, *20*(12), 3011. https://doi.org/10.3390/ijms20123011

Hollcroft, J.W., Lorenz, E., &Matthews, M.(1952). Factors modifying the effect of X-irradiation on regression of a transplanted lymphosarcoma. *Journal of the National Cancer Institute*, *12*(4), 751–763.

Holmgren, A.(2000). Antioxidant function of thioredoxin and glutaredoxin systems. *Antioxidants and Redox Signaling*, *2*(4), 811–820. https://doi.org/10.1089/ars.2000.2.4-811

Hong, J.H., Chiang, C.S., Sun, J.R., Withers, H.R., &McBride, W.H.(1997). Induction of c-fos and junB mRNA following in vivo brain irradiation. *Brain Research. Molecular Brain Research*, *48*(2), 223–228. https://doi.org/10.1016/s0169-328x(97)00095-8

Hornig, D., Weber, F., &Wiss, O.(1973). Site of intestinal absorption of ascorbic acid in guinea pigs and rats. *Biochemical and Biophysical Research Communications*, *52*(1), 168–172. https://doi.org/10.1016/0006-291x(73)90969-8

Hu, C.M.J., Fang, R.H., Copp, J., Luk, B. T., &Zhang, L.(2013).LA biomimetic nanosponge that absorbs pore-forming toxins. *Nature Nanotechnology*, *8*(5), 336–340. https://doi.org/10.1038/nnano.2013.54

Huang, D., Ou, B., &Prior, R. L.(2005). The chemistry behind antioxidant capacity assays. *Journal ofAgricultural and Food Chemistry*, *53*(6), 1841–1856. https://doi.org/10.1021/jf030723c

Huang, Q., Dunn, R.T., Jayadev, S., DiSorbo, O., Pack, F.D., Farr, S. B., Stoll, R. E., &Blanchard, K. T.(2001). Assessment of cisplatin induced nephrotoxocity by microarray technology. *Toxicological Sciences*, *63*(2), 196–207. https://doi.org/10.1093/toxsci/63.2.196

Huang, S.H.(2006). Gold nanoparticle-based immunochromatographic test for identification of *Staphylococcus aureus* from clinical specimens. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *373*(1–2),139–143. https://doi.org/10.1016/j.cca.2006.05.026

Huber, H.E., Tabor, S., &Richardson, C.C.(1987). Escherichia coli thioredoxin stabilizes complexes of bacteriophage T7 DNA polymerase and primed templates. *Journal of BiologicalChemistry*, *262*(33), 16224–16232. https://doi.org/10.1016/S0021-9258(18)47719-8

Humanes, B., Lazaro, A., Camano, S., Moreno-Gordaliza, E., Lazaro, J. A., Blanco-Codesido, M., Lara, J. M., Ortiz, A., Gomez-Gomez, M. M., Martín-Vasallo, P., &Tejedor, A.(2012).Cilastatin protects against cisplatin-induced nephrotoxicity without compromising its anticancer efficiency in rats. *Kidney International*, *82*(6), 652–663. https://doi.org/10.1038/ki.2012.199

Humanes,B., Camaño, S.,Lara,J.M.,Sabbisetti,V., González-Nicolás,M. Á,Bonventre,J.V., Tejedor, A., &Lázaro, A.(2017).Cisplatin-induced renal inflammation is ameliorated by cilastatin nephroprotection.*Nephrology, Dialysis, Transplantation*, *32*(10), 1645–1655. https://doi.org/10.1093/ndt/gfx005

Hwang, C., Sinskey, A.J., &Lodish, H.F.(1992). Oxidized redox state of glutathione in the endoplasmic-reticulum. *Science*, *257*(5076), 1496–1502. https://doi.org/10.1126/science.1523409

Hwang, S., Lam, M.L., &Shen, T.(1986). Release of platelet activating factor and its involvement in the first phase of carrageenan rat foot oedema. *European Journal of Pharmacology*, *120*, 33–41

Ibrahim, M.Y., Abdul, A.B.H., Ibrahim, T.A.T., Wahab, S.I.A., Elhassan, M.M., &Mohan, S.(2010). Attenuation of cisplatin-induced nephrotoxicity in rats using zerumbone. *African Journal of Biotechnology*, *9*(28), 4434–4444.

Ideia, P., Sousa-Ferreira, I., &Castilho, P.C.(2020). A novel and simpler alkaline hydrolysis methodology for extraction of ferulic acid from brewer's spent grain and its (partial) purification through adsorption in a synthetic resin. *Foods*, *9*(5), 600. https://doi.org/10.3390/foods9050600

Ignarro, L.J.(1990). Haem-dependent activation of guanylate cyclise and cyclic GMP formation by endogenous nitric oxide: A unique transduction mechanism for transcellular signaling. *Pharmacology and Toxicology*, *67*(1), 1–7. https://doi.org/10.1111/j.1600-0773.1990.tb00772.x

Imlay, J.A.(2003). Pathways of oxidative damage. *Annual Review ofMicrobiology*, *57*, 395–418. https://doi.org/10.1146/annurev.micro.57.030502.090938

Imlay, J.A., Chin, S.M., &Linn, S.(1988). Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science*, *240*(4852), 640–642. https://doi.org/10.1126/science.2834821

Injac, R., &Strukelj, B.(2008). Recent advances in protection against doxorubicin-induced toxicity. *Technology in Cancer Research and Treatment*, *7*(6), 497–516. https://doi.org/10.1177/153303460800700611

Irvine,D.J.(2011). Drug delivery: One nanoparticle, one kill. *Nature Materials*, *10*(5), 342–343. https://doi.org/10.1038/nmat3014

Iwashina, T.(2013). Flavonoid properties of five families newly incorporated into the order Caryophyllales[Review]. *Bulletin of Natural Museum of Nature and Sciences*, *39*, 25–51.

Jackson, J.H.(1994). Potential molecular mechanisms of oxidant-induced carcinogenesis.*Environmental Health Perspectives*, *102* (Suppl. 10), 155–157. https://doi.org/10.1289/ehp.94102s10155

Jackson, S.P.(2002). Sensing and repairing DNA double-strand breaks. *Carcinogenesis*, *23*(5), 687–696. https://doi.org/10.1093/carcin/23.5.687

Jadhav, A.P., Kareparamban, J.A., Nikam, P.H., &Kadam, V.J.(2012). Spectrophotometric estimation of ferulic acid from *Ferula asafoetida* by Folin—Ciocalteu's Reagent. *Pharmacia Sinica*, *3*(6), 680–684.

Jaeschke, H., &Bajt, M.L.(2006). Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicological Sciences*, *89*(1), 31–41. https://doi.org/10.1093/toxsci/kfi336

Jagieła, J., Bartnicki, P., &Rysz, J.(2021). Nephrotoxicity as a complication of chemotherapy and immunotherapy in the treatment of colorectalcancer, melanoma and non-smallcelllungcancer. *International Journal of Molecular Sciences*, *22*(9), 4618. https://doi.org/10.3390/ijms22094618

Jain, K.K.(2005). Nanotechnology-based drug delivery for cancer. *Technology in Cancer Research and Treatment*, *4*(4), 407–416. https://doi.org/10.1177/153303460500400408

Jain, K.K.(2005). Role of nanobiotechnology in developing personalized medicine for cancer. *Technology in Cancer Research and Treatment*, *4*(6), 645–650. https://doi.org/10.1177/153303460500400608

James, L.P., Mayeux, P. R., &Hinson, J. A.(2003). Acetaminophen-inducedhepatotoxicity. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *31*(12), 1499–1506. https://doi.org/10.1124/dmd.31.12.1499

Jami, M.S., Hou, J., Liu, M., Varney, M.L., Hassan, H., Dong, J., Geng, L., Wang, J., Yu, F., Huang, X., Peng, H., Fu, K., Li, Y., Singh, R. K., &Ding, S. J.(2014). Functional proteomic analysis reveals the involvement of KIAA1199 in breast cancer growth, motility and invasiveness. *BMC Cancer*, *14*, 194. https://doi.org/10.1186/1471-2407-14-194

Janakiraman, M., &Jayaprakash, K.(2015). Nephroprotective potential of medicinal plants: *A review*. *International Journal ofScientific and EngineeringResearch*, *4*(9), 543–547.

Janib, S.M., Moses, A.S., &MacKay, J.A.(2010).Imaging and drug delivery using theranostic nanoparticles. *AdvancedDrug Delivery Reviews*, *62*(11), 1052–1063. https://doi.org/10.1016/j.addr.2010.08.004

Jariyawat, S., Kigpituck, P., Suksen, K., Chuncharunee, A., Chaovanalikit, A., &Piyachaturawat, P.(2009). Protection against cisplatin-induced nephrotoxicity in mice by *Curcumacomosa* Roxb. ethanol extract. *Journal of Natural Medicines*, *63*(4), 430–436. https://doi.org/10.1007/s11418-009-0345-5

Jayakumar, O.D., Ganguly, R., Tyagi, A.K., Chandrasekharan, D.K., &Nair, C.K.K.(2009). Water dispersible Fe3O4 nanoparticlescarryingdoxorubicin for cancertherapy.*Journal of Nanoscience and Nanotechnology*, *9*(11), 6344–6348. https://doi.org/10.1166/jnn.2009.1369

Jemal, A., Siegel, R., Ward, E.*et al*.(2008). Cancer statistics, CA.*Cancer Journal of Clinicians*, *58*, 71–96.

Jenney, F.E.J., Verhagen, M.F., Cui, X., &Adams, M.W.(1999). Anaerobic microbes: Oxygen detoxification without superoxide dismutase. *Science*, *286*(5438), 306–309. https://doi.org/10.1126/science.286.5438.306

Jia, Z., Wang,N., Aoyagi, T.,Wang, H., Liu, H., &Yang, T.(2011). Amelioration of cisplatin nephrotoxicity by genetic or pharmacologic blockade of prostaglandin synthesis. *Kidney International*, *79*(1), 77–88. https://doi.org/10.1038/ki.2010.331

Joel, A. H., Lee, E. L., &Alfred, G.(1998). *Pharmacological basis of therapeutics*, *1465*.

Jollow, D.J., Mitchell, J.R., Potter, W.Z., Davis, D.C., Gillette, J.R., &Brodie, B.B.(1973). Acetaminophen-induced hepatic necrosis. II. Role of covalent binding *in vivo*. *Journal of Pharmacology and Experimental Therapeutics*, *187*(1), 195–202.

Jones, A.L.(1998). Mechanism of action and value of Nacetylcysteine in the treatment of early and late acetaminophen poisoning: A critical review. *Journal of Toxicology. Clinical Toxicology*, *36*(4), 277–285. https://doi.org/10.3109/15563659809028022

Jones, D.P., Carlson, J.L., Mody, V.C., Cai, J., Lynn, M.J., &Sternberg, P.(2000). Redox state of glutathione in human plasma. *Free Radical Biology and Medicine*, *28*(4), 625–635. https://doi.org/10.1016/s0891-5849(99)00275-0

Jones, S.K., &Winter, J.G.(2001). Experimental examination of a targeted hyperthermia system using inductively heated ferromagnetic microspheres in rabbit kidney.*Physics in Medicine and Biology*, *46*(2), 385–398. https://doi.org/10.1088/0031-9155/46/2/308

Jorgensen, R. A.(1995). Co-suppression, flower color patterns, and metastable gene expression states. *Science*, *268*(5211), 686–691. https://doi.org/10.1126/science.268.5211.686

Joy, J., &Nair, C.K.K.(2008).Amelioration of cisplatin induced nephrotoxicity in Swiss albino mice by *Rubiacordifolia* extract. *Journal of Cancer Research and Therapeutics*, *4*(3), 111–115. https://doi.org/10.4103/0973-1482.43139

Juntachote, T., Berghofer, E., Siebenhandl, S., &Bauer, F.(2006). The antioxidative properties of Holy basil and Galangal in cooked ground pork. *Meat Science*, *72*(3),446–456. https://doi.org/10.1016/j.meatsci.2005.08.009

Jyoti, T.M., Prabhu, K., Lakshminarasu, S., &Ramachandra, S.S.(2008). Hepatoprotective and antioxidant activity of *Euphorbiaantiquorum*. *Pharmacognosy Magazine*, *4*, 127–133.

Kahkonen, MP., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S., &Heinonen, M.(1999). Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry*, *47*(10), 3954–3962. https://doi.org/10.1021/jf9901461

Kaibara, N., Ikeda, T., Hattori, T., &Inokuchi, K.(1971). Experimental studies on enhancing the therapeutic effect of mitomycin-C with hydrogen peroxide. *Japanese Journal of Experimental Medicine*, *41*(4), 323–329.

Kalivendi, S. V., Kotamraju, S., Zhao, H., Joseph, J., &Kalyanaraman, B.(2001). Doxorubicin-induced apoptosis is associated with increased transcription of endothelial nitric-oxide synthase. Effect of antiapoptotic antioxidants and calcium. *JournalofBiologicalChemistry*, *276*(50), 47266–47276. https://doi.org/10.1074/jbc.M106829200

Kalyanaraman, B.(2020). Teaching the basics of the mechanism of doxorubicin-induced cardiotoxicity: Have we been barking up the wrong tree?*Redox Biology*, *29*, 101394. https://doi.org/10.1016/j.redox.2019.101394

Kam, N.W., Liu, Z., &Dai, H.(2006). Carbon nanotubes as intracellular transporters for proteins and DNA: An investigation of the uptake mechanism and pathway.*Angewandte Chemie*, *45*(4), 577–581. https://doi.org/10.1002/anie.200503389

Kamaly, N., Xiao, Z., Valencia, P.M., Radovic-Moreno, A.F., &Farokhzad, O.C.(2012). Targeted polymeric therapeutic nanoparticles: Design, development and clinical translation. *Chemical Society Reviews*, *41*(7), 2971–3010. https://doi.org/10.1039/c2cs15344k

Kamata, H., &Hirata, H.(1999). Redox regulation of cellular signalling. *Cellular Signalling*, *11*(1), 1–14. https://doi.org/10.1016/s0898-6568(98)00037-0

Kang, Y., Marangoni, A.G., &Yada, R.Y.(1994). Effect of two polar organic_aqueous solvents systems on the structure-functions relationships of proteases III, *papain, and trypsin*. *Journal of Food Biochemistry*, *17*, 389–405.

Kanner, J., Frankel, E., Granit, R., German, B., &Kinsella, J.E.(1994). Natural antioxidants in Grapes and Wines. *Journal of Agricultural and Food Chemistry*, *42*(1), 64–69. https://doi.org/10.1021/jf00037a010

Kanter, M., Coskun, O., &Budancamanak, M.(2005). Hepatoprotective effect of *Nigellasativa*L. and *Utricadioica*L. on lipid peroxidation, antioxidant enzyme systems and liver enzymes in carbon tetrachloride treated rats. *World Journal of Gastroenterology*, *11*(42), 6684–6688. https://doi.org/10.3748/wjg.v11.i42.6684

Kanzok, S.M., Fechner, A., Bauer, H., Ulschmid, J.K., Müller, H.M., Botella-Munoz, J., Schneuwly, S., Schirmer, R., &Becker, K.(2001). Substitution of the thioredoxin system for glutathione reductase in Drosophila melanogaster. *Science*, *291*(5504), 643–646. https://doi.org/10.1126/science.291.5504.643

Kanzok, S.M., Schirmer, R.H., Turbachova, I., Iozef, R., &Becker, K.(2000). The thioredoxin system of the malaria parasite Plasmodium falciparum. Glutathione reduction revisited. *Journal of Biological Chemistry*, *275*(51), 40180–40186. https://doi.org/10.1074/jbc.M007633200

Karan, M., Vasisht, K., &Handa, S.S.(1999). Antihepatotoxic activity of Swertia chirata on carbon tetrachloride induced hepatotoxicity in rats. *Phytotherapy Research*, *13*(1), 24–30. https://doi.org/10.1002/(SICI)1099-1573(199902)13:1<24::AID-PTR378>3.0.CO;2-L

Karin, M., &Greten, F.R.(2005).NF-kappaB: Linking inflammation and immunity to cancer development and progression. *Nature Reviews. Immunology*, *5*(10), 749–759. https://doi.org/10.1038/nri1703

Karoui, H., Hogg, N., Fréjaville, C., Tordo, P., &Kalyanaraman, B.(1996). Characterization of sulfur-centered radical intermediates formed during the oxidation of thiols and sulfite by peroxynitrite—ESR-SPIN trapping and oxygen uptake studies. *Journal of Biological Chemistry*, *271*(11), 6000–6009. https://doi.org/10.1074/jbc.271.11.6000

Kassirer, J.P.(1971). Clinical evaluation of kidney function- glomerular function. *New England Journal of Medicine*, *285*(7), 385–389. https://doi.org/10.1056/NEJM197108122850706

Kaushal, G.P., Kaushal, V., Hong, X., &Shah, S.V. (2001). Role and regulation of activation of caspases in cisplatin induced injury to renal tubular epithelial cells. *Kidney International*, *60*(5), 1726–1736. https://doi.org/10.1046/j.1523-1755.2001.00026.x

Kawabata, K., Yamamoto, T., Hara, A., Shimizu, M., Yamada, Y., Matsunaga, K., Tanaka, T., &Mori, H.(2000). Modifying effects of ferulic acid on azoxymethane-induced colon carcinogenesis in F344 rats. *Cancer Letters*, *157*(1), 15–21. https://doi.org/10.1016/s0304-3835(00)00461-4

Kaynar, H., Meral, M., Turhan, H., Keles, M., Celik, G., &Akcay, F.(2005). Glutathione peroxidase, glutathione-S-transferase, catalase, xanthine oxidase, Cu− Zn superoxide dismutase activities, total glutathione, nitric oxide, and malondialdehyde levels in erythrocytes of patients with small cell and non-small cell lung cancer. *Cancer Letters*, *227*(2), 133–139. https://doi.org/10.1016/j.canlet.2004.12.005

Kelley, W.(2005). *Cancer: Curing the incurable without surgery, chemotherapy, or radiation* (pp. 3–13). New Century Promotions.

Kerbel, R.S.(2000). Tumor angiogenesis: Past, present and the near future. *Carcinogenesis*, *21*(3), 505–515. https://doi.org/10.1093/carcin/21.3.505

Khandrika, L., Kumar, B., Koul, S., Maroni, P., &Koul, H.K.(2009). Oxidative stress in prostate cancer. *Cancer Letters*, *282*(2), 125–136. https://doi.org/10.1016/j.canlet.2008.12.011

Khynriam, D., &Prasad, S.B.(2001). Hematotoxicity and blood glutathione levels after cisplatin treatment of tumor-bearing mice. *Cell Biology and Toxicology*, *17*(6), 357–370. https://doi.org/10.1023/a:1013784115683

Kim, H.K., Jeong, T.S., Lee, M.K., Park, Y.B., &Choi, M.S.(2003). Lipid-lowering efficacy of hesperetin metabolites in high-cholesterol fed rats. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *327*(1–2), 129–137. https://doi.org/10.1016/s0009-8981(02)00344-3

Kim, J.,Long, K.E.,Tang, K., &Padanilam, B.J.(2012). Poly(ADP-ribose) polymerase 1 activation is required for cisplatin nephrotoxicity.*Kidney International*, *82*(2), 193–203. https://doi.org/10.1038/ki.2012.64

Kim, J.A.(2003). Targeted therapies for the treatment of cancer. *American Journal of Surgery*, *186*(3), 264–268. https://doi.org/10.1016/s0002-9610(03)00212-5

Kim, K., Kim, I.H., Lee, K.Y., Rhee, S.G., &Stadtman, E.R.(1988). The isolation and purification of a specific "protector" protein which inhibits enzyme inactivation by a thiol/Fe(III)/O2 mixed function oxidation system. *Journal of Biological Chemistry*, *263*(10), 4704–4711. https://doi.org/10.1016/S0021-9258(18)68840-4

Kim, K.H., Tsao, R., Yang, R., &Cui, S.W.(2006). Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. *Food Chemistry*, *95*(3), 466–473. https://doi.org/10.1016/j.foodchem.2005.01.032

Kim, Y.K., Jung, J.S., Lee, S.H., &Kim, Y.W.(1997). Effects of antioxidants and Ca^{2+}in cisplatin-induced cell injury in rabbit renal cortical slices. *Toxicology and Applied Pharmacology*, *146*(2), 261–269. https://doi.org/10.1006/taap.1997.8252

Kirkman, H.N., &Gaetani, G.F.(1984). Catalase: A tetrameric enzyme with four tightly bound molecules of NADPH. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(14), 4343–4347. https://doi.org/10.1073/pnas.81.14.4343

Kirkman, H.N., Rolfo, M., Ferraris, A.M., &Gaetani, G.F.(1999). Mechanisms of protection of catalase by NADPH. Kinetics and stoichiometry. *Journal of Biological Chemistry*, *274*(20), 13908–13914. https://doi.org/10.1074/jbc.274.20.13908

Kojima, Y., Haruta, A., Imai, T., Otagiri, M., &Maeda, H.(1993). Conjugation of Cu,Zn-superoxide dismutase with succinylated gelatin: Pharmacological activity and cell-lubricating function. *Bioconjugate Chemistry*, *4*(6), 490–498. https://doi.org/10.1021/bc00024a011

Końca, K.,Lankoff, A., Banasik, A.,Lisowska, H.,Kuszewski, T.,Góźdź, S.,Koza, Z., &Wojcik, A.(2003). Across platformpublic domain PC Image Analysis Programme for the Comet Assay. *Mutation Research*, *534*(1–2), 15–20. https://doi.org/10.1016/s1383-5718(02)00251-6

Konno, R., & Yasumura, Y.(1992). D-amino-acid oxidase and its physiological functions. *International Journal of Biochemistry*, *24*(4),519–524. https://doi.org/10.1016/0020-711X(92)90322-R

Kontos, H.A.,Wei, E.P., Ellis, E.F., Jenkins, L.W., Povlishock, J.T., Rowe, G.T., &Hess, M.L.(1985). Appearance of superoxide anion radical in cerebral extracellular space during increased prostaglandin synthesis in cats. *Circulation Research*, *57*(1), 142–151. https://doi.org/10.1161/01.res.57.1.142

Koti,B. C., Vishwanathswamy, A. H. M., Wagawade, J., &Thippeswamy, A. H. M.(2009). Cardioprotective effect of lipistat against doxorubicin induced myocardial toxicity in albino rats. *Indian JournalofExperimentalBiology*, *47*(1), 41–46.

Kousparou, C. A., Epenetos, A. A., &Deonarain, M. P. (2002).Antibody-guidedenzymetherapy of cancerproducingcyanideresults in necrosisoftargetedcells. *International Journal of Cancer*, *99*(1), 138–148. https://doi.org/10.1002/ijc.10266

Koziara, J.M., Whisman, T.R., Tseng, M.T., &Mumper, R.J.(2006). In-vivo efficacy of novel paclitaxel nanoparticles in paclitaxel-resistant human colorectal tumors. *Journal of Controlled Release*, *112*(3), 312–319. https://doi.org/10.1016/j.jconrel.2006.03.001

Krakoff, I.H.(1979). Nephrotoxicity of cis-dichlorodiammineplatinum(II). *Cancer Treatment Reports*, *63*(9–10), 1523–1525.

Kramer, P.A. (1974).Letter: Albumin microspheres as vehicles for achieving specificity in drug delivery.*Journal of Pharmaceutical Sciences*, *63*(10), 1646–1647. https://doi.org/10.1002/jps.2600631044

Kroemer, G., Dallaporta, B., &Resche-Rigon, M.(1998). The mitochondrial death/life regulator in apoptosis and necrosis. *Annual Review of Physiology*, *60*, 619–642. https://doi.org/10.1146/annurev.physiol.60.1.619

Krolicka, A., Kartanowicz, R.,Wosiński, S. A., Szpitter, A., Kamiński, M., &Łojkowska, E.(2006). Induction of secondary metabolite production in transformed callus of Ammi majus L. grown after electromagnetic treatment of the culture medium. *Enzyme and Microbial Technology*, *39*(7), 1386–1391. https://doi.org/10.1016/j.enzmictec.2006.03.042

Kroncke, K.D., Suschek, C.V., &Kolb-Bachofen, V.(2000). Implications of inducible nitric oxide synthase expression and enzyme activity. *Antioxidants and Redox Signaling*, *2*(3), 585–605. https://doi.org/10.1089/15230860050192341

Kubo,H., Yamano, T.,Iwatsubo,M.,Watari, H., Soyama, T.,Shiga, T., &Isomoto, A.(1960).*Bulletin de la Société Chimique de France*, *42*, 569–582.

Kuenzig, W., Chau, J., Norkus, E., Holowaschenko, H., Newmark, H., Mergens, W., &Conney, A. H.(1984).Caffeic and ferulic acid as blockers of nitrosamine formation. *Carcinogenesis*, *5*(3), 309–313. https://doi.org/10.1093/carcin/5.3.309

Kuhlmann, M.K., Horsch, E., Burkhardt, G., Wagner, M., &Köhler, H.(1998). Reduction of cisplatin toxicity in cultured renal tubularcells by bioflavonoid quercetin. *Archives of Toxicology*, *72*(8), 536–540. https://doi.org/10.1007/s002040050539

Kumano, Y., Sakamoto, T., Egawa, M., Iwai, I., Tanaka, M., &Yamamoto, I.(1998). In vitro and in vivo prolonged biological activities of novel vitamin C derivative, 2-O-alpha-D-glucopyranosyl-L-ascorbic acid (AA-2G), in cosmetic fields. *Journal of Nutritional Science and Vitaminology*, *44*(3), 345–359. https://doi.org/10.3177/jnsv.44.345

Kumar, C.H., Ramesh, A., Kumar, J.N.S., &Ishaq, B.M.(2011). A reviewonhepatoprotectiveactivityofmedicinalplants. *International Journal of Pharmaceutical Sciences and Research*, *2*(3), 501–515.

Kumar, D., Kumar, S., Singh, J., Narender, R., Vashistha, B.D.*et al*.(2010). Free radical scavenging and analgesic activities of *Cucumis sativus* L. fruit extract. *Pharmacognosy*, *4*, 365–368

Kumar, N., &Goel, N.(2019). Phenolic acids: Natural versatile molecules with promisingtherapeutic applications. *Biotechnology Reports*, *24*, e00370. https://doi.org/10.1016/j.btre.2019.e00370

Kumar, N., &Pruthi, V.(2014). Potential applications of ferulic acid from natural sources. *Biotechnology Reports*, *4*, 86–93. https://doi.org/10.1016/j.btre.2014.09.002

Kumar, S., &Pandey, A.K.(2013). Chemistry and biological activities of flavonoids: An overview. *The Scientific World Journal*, 2013, 162750. https://doi.org/10.1155/2013/162750

Kunsch, C., &Medford, R.M.(1999). Oxidative stress as a regulator of gene expression in the vasculature. *Circulation Research*, *85*(8), 753–766. https://doi.org/10.1161/01.res.85.8.753

Kunwar, A., &Priyadarsini,K.I.(2011). Free radicals, oxidative stress, and importance of antioxidants in human health. *Journal of Medical and AlliedSciences*, *1*(2), 53–60.

Kuppusamy, P., &Zweier, J.L.(1989). Characterization of free radical generation by xanthine oxidase. Evidence for hydroxyl radical generation. *Journal ofBiological Chemistry*, *264*(17), 9880–9884. https://doi.org/10.1016/S0021-9258(18)81740-9

Kusyk, C.J., &Hsu, T.C.(1976). Adriamycin-induced chromosome damage: Elevated frequencies of isochromatid aberrations in G2 and S phases. *Experientia*, *32*(12), 1513–1514. https://doi.org/10.1007/BF01924424

Kyriakis, J.M.(1999). Activation of the AP-1 transcription factor by inflammatory cytokines of the TNF family. *Gene Expression*, *7*(4–6), 217–231.

Laemmli, U. K.(1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685. https://doi.org/10.1038/227680a0

Lakshmi, M.S., Reddy, U.K., &Rani, S.R.K.S.(2012). A review on medicinalplants for nephroprotectiveactivity. *Asian Journal of Pharmaceutical and Clinical Research*, *5*, 8–14.

Lambert, F.,Zucca, J.,Ness, F., &Aigle, M.(2013). Production of ferulic acid and coniferyl alcohol by conversion of eugenol using a recombinant strain of Saccharomyces cerevisiae. *Flavour and Fragrance Journal*, *29*, 14–21.

Landmesser, U., &Harrison, D.G.(2001). Oxidative stress and vascular damage in hypertension. *Coronary Artery Disease*, *12*(6), 455–461. https://doi.org/10.1097/00019501-200109000-00004

Langer, R.(2001). Drug delivery: Drugs on target. *Science*, *293*(5527), 58–59. https://doi.org/10.1126/science.1063273

Larsen, G.L., &Henson, P.M.(1983). Mediators of inflammation. *Annual Review of Immunology*, *1*, 335–359. https://doi.org/10.1146/annurev.iy.01.040183.002003

Larson, A.M., Polson, J., Fontana, R.J., Davern, T. J., Lalani, E., Hynan, L. S., Reisch, J. S., Schiødt, F. V., Ostapowicz, G., Shakil, A. O., Lee, W. M., &Acute Liver Failure Study Group. (2005). Acetaminophen induced acute liver failure: Results of a United States multicenter, prospective study. *Hepatology*, *42*(6), 1364–1372. https://doi.org/10.1002/hep.20948

Leakakos, T., Ji, C., Lawson, G., Peterson, C., &Goodwin, S.(2003). Intravesical administration of doxorubicin to swine bladder using magnetically targeted carriers. *Cancer Chemotherapy and Pharmacology*, *51*(6), 445–450. https://doi.org/10.1007/s00280-003-0597-9

Lebwohl, D., &Canetta, R.(1998). Clinical development of platinum complexes in cancer therapy: An historical perspective and an update. *European Journal of Cancer*, *34*(10), 1522–1534. https://doi.org/10.1016/s0959-8049(98)00224-x

Lee, C.C., &Houghton, P.(2005). Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer.*Journal ofEthnopharmacology*, *100*(3), 237–243. https://doi.org/10.1016/j.jep.2005.01.064

Lee, J.H., &Lee, M.J.(2012).Liposome mediated cancer gene therapy: Clinical trials and their lessons to stem cell therapy. *Bulletin of the Korean Chemical Society*, *33*(2), 433–442. https://doi.org/10.5012/bkcs.2012.33.2.433

Lee, V., Randhawa, A. K., &Singal, P. K.(1991). Adriamycin-induced myocardial dysfunction *in vitro* is mediated by free radicals. *American Journal of Physiology*, *261*(4 Pt 2), H989–H995. https://doi.org/10.1152/ajpheart.1991.261.4.H989

Lee, W.M.(1995). Drug-induced hepatotoxicity. *New England Journal of Medicine*, *333*(17), 1118–1127. https://doi.org/10.1056/NEJM199510263331706

Lee, W.M.(2003). Acute liver failure in the United States. *Seminars in Liver Disease*, *23*(3), 217–226. https://doi.org/10.1055/s-2003-42641

Lee, W.M., Squires, R.H., Jr., Nyberg, S.L., Doo, E., &Hoofnagle, J.H.(2008). Acute liver failure: Summary of a workshop. *Hepatology*, *47*(4), 1401–1415. https://doi.org/10.1002/hep.22177

Lee, Y. K., Yuk, D. Y., Lee, J. W., Lee, S. Y., Ha, T. Y., Oh, K. W., Yun, Y. P., &Hong, J. T.(2009). Epigallocatechin-3-gallate prevents lipopolysaccharide-induced elevation of β-amyloid generation and memory deficiency. *Brain Research*, *1250*, 164–174. https://doi.org/10.1016/j.brainres.2008.10.012

Lefrak, E.A., Pitha, J., Rosenheim, S., &Gottlieb, J. A.(1973).Rosenheim, S and Gottlieb, JAA clinicopathologic analysisof adriamycin cardiotoxicity. *Cancer*, *32*(2), 302–314. https://doi.org/10.1002/1097-0142(197308)32:2<302::aid-cncr2820320205>3.0.co;2-2

Leibbrandt, M.E., Wolfgang, G.H., Metz, A.L., Ozobia, A.A., &Haskins, J.R.(1995). Critical subcellular targets of cisplatin and related platinum analogs in rat renal proximal tubule cells. *Kidney International*, *48*(3), 761–770. https://doi.org/10.1038/ki.1995.348

Lesca, P.(1983). Protective effects of ellagic acid and other plant phenols on benzo[a] pyrene-induced neoplasia in mice. *Carcinogenesis*, *4*(12), 1651–1653. https://doi.org/10.1093/carcin/4.12.1651

Li, T., &Singal, P.K.(2000). Adriamycin-induced early changes in myocardial antioxidant enzymes and their modulation by Probucol. *Circulation*, *102*(17), 2105–2110. https://doi.org/10.1161/01.cir.102.17.2105

Li, Z.,Tan, S., Li, S., Shen, Q., &Wang, K.(2017). Cancer drug delivery in the Nano era: An overview and perspectives [Review]. *Oncology Reports*, *38*(2), 611–624. https://doi.org/10.3892/or.2017.5718

Lian Ai Pham-Huy., He, H., &Pham-Huy, C.(2008).Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science*, *4*(2), 89–96.

Liao, M.H., &Chen, D.H.(2001). Immobilization of yeast alcohol deshydrogenase on magnetic nanoparticles. *Biotechnology Letters*, *23*(20), 1723–1727. https://doi.org/10.1023/A:1012485221802

Lin, J.K., Chen, P.C., Ho, C.T., &Lin-Shiau, S.Y.(2000). Inhibition of xanthine oxidase and suppression of intracellular reactive oxygen species in HL-60 cells by theaflavin-3,3′-digallate, (−)-epigallo- catechin-3-gallate, and propyl gallate. *Journal of Agricultural and Food Chemistry*, *48*(7), 2736–2743. https://doi.org/10.1021/jf000066d

Linster, C.L., &Van Schaftingen, E.(2007). Vitamin C: Biosynthesis, recycling and degradation in mammals. *FEBS Journal*, *274*(1), 1–22. https://doi.org/10.1111/j.1742-4658.2006.05607.x

Liu, X., Chen, Z., Chua, C. C., Ma, Y. S., Youngberg, G. A., Hamdy, R., &Chua, B. H.(2002). Melatonin as an effective protector against doxorubicin-induced cardiotoxicity. *American Journal of Physiology. Heart and CirculatoryPhysiology*, *283*(1), H254–H263. https://doi.org/10.1152/ajpheart.01023.2001

Liu, Z., Xiong, M., Gong, J., Zhang, Y., Bai, N., Luo, Y., Li, L., Wei, Y., Liu, Y., Tan, X., &Xiang, R.(2014). Legumain protease-activated TAT-liposome cargo for targeting tumours and their microenvironment. *Nature Communications*, *5*, 4280. https://doi.org/10.1038/ncomms5280

Lo, T.N., Almeida, A.P., &Beaven, M.A.(1982). Dextran and carrageenan evoke different inflammatory responses in rat with respect to composition of infiltrates and effect of indomethacin. *Journal of Pharmacology and Experimental Therapeutics*, *221*(1), 261–267.

Locatelli, F., Canaud, B., Eckardt, K.U., Stenvinkel, P., Wanner, C., &Zoccali, C.(2003). Oxidative stress in end-stage renal disease: An emerging threat to patient outcome. *Nephrology, Dialysis, Transplantation*, *18*(7), 1272–1280. https://doi.org/10.1093/ndt/gfg074

Lombard, M., Fontecave, M., Touati, D., &Nivière, V.(2000a). Reaction of the desulfoferrodoxin from Desulfoarculus baarsii with superoxide anion. Evidence for a superoxide reductase activity. *Journal of Biological Chemistry*, *275*(1), 115–121. https://doi.org/10.1074/jbc.275.1.115

Lombard, M., Touati, D., Fontecave, M., &Nivière, V.(2000b). Superoxide reductase as a unique defense system against superoxide stress in the microaerophile Treponema pallidum. *Journal of Biological Chemistry*, *275*(35), 27021–27026. https://doi.org/10.1074/jbc.M004201200

Lopez-Lazaro, M (2009). Distribution and biological activities of the flavonoid luteolin. *Mini Reviews in Medicinal Chemistry*, *9*(1), 31–59. https://doi.org/10.2174/138955709787001712

Lowry,O. H.,Rosebrough,N. J.,Farr, A. L., &Randall,R. J.(1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*(1), 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6

Lowther, W.T., Brot, N., Weissbach, H., Honek, J.F., &Matthews, B.W.(2000). Thiol-disulfide exchange is involved in the catalytic mechanism of peptide methionine sulfoxide reductase. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(12), 6463–6468. https://doi.org/10.1073/pnas.97.12.6463

Lu, L., Shan, F., Li, W., &Lu, H.(2016). Short-term side effects after radioiodine treatment in patients with differentiated thyroid cancer. *BioMed Research International*, *2016*, 4376720, article ID4376720. https://doi.org/10.1155/2016/4376720

Lunec, J., Herbert, K., Blount, S., Griffiths, H.R., &Emery, P.(1994). 8-hydroxydeoxyguanosine. A marker of oxidative DNA damage in systemic lupus erythematosus. *FEBS Letters*, *348*(2), 131–138. https://doi.org/10.1016/0014-5793(94)00583-4

Ly, D.H., Lockhart, D.J., Lerner, R.A., &Schultz, P.G.(2000). Mitotic misregulation and human aging. *Science*, *287*(5462), 2486–2492. https://doi.org/10.1126/science.287.5462.2486

Ma, Y., &Huang, H.(2014). Characterisation and comparison of phenols, flavonoids and isoflavones of soymilk and their correlations with antioxidant activity. *International Journal ofFood Science andTechnology*, *49*(10), 2290–2298. https://doi.org/10.1111/ijfs.12545

Madhuri, A.S., Mohanvelu, R., &Ramabhimaiah, S.(2016). Evaluation of anti-inflammatory activity of aqueous extract of Mangifera indica leaves in albino rats. *International Journal ofBasic and Clinical Pharmacology*, *5*, 635–638. https://doi.org/10.18203/2319-2003.ijbcp20161451

Maeda, H., Seymour, L.W., &Miyamoto, Y.(1992). Conjugates of anticancer agents and polymers: Advantages of macromolecular therapeutics *in vivo*. *Bioconjugate Chemistry*, *3*(5), 351–362. https://doi.org/10.1021/bc00017a001

Mahmood, N.D., Mamat, S.S., Kamisan, F.H., Yahya, F., Kamarolzaman, M.F.F., Nasir, N.,Mohtarrudin, N.,Tohid, Md., S. F., &Zakaria, Z.A.(2014). Amelioration of paracetamol-inducedhepatotoxicity in rat by the administration of methanolextract of *Muntingiacalabura* L. Leaves.*BioMed Research International*, 2014, article ID695678 https://doi.org/10.1155/2014/695678

Mahmoudian-Sani, M.R., Mehri-Ghahfarrokhi, A., Ahmadinejad, F., Hashemzadeh-Chaleshtori, M., Saidijam, M., &Jami, M.S.(2017). MicroRNAs: Effective elements in ear-related diseases and hearing loss. *European Archives ofOto-Rhino-Laryngology*, *274*(6), 2373–2380. https://doi.org/10.1007/s00405-017-4470-6

Maiti, R., &Agrawal, N.K.(2007). Atherosclerosis in diabetes mellitus: Role of inflammation. *Indian Journal of Medical Sciences*, *61*(5), 292–306. https://doi.org/10.4103/0019-5359.32098

Makin, A.J., &Williams, R.(1997). Acetaminophen-induced hepatotoxicity: Predisposing factors and treatments. *Advances inInternal Medicine*, *42*, 453–483.

Makino, S., &Tanaka, T.(1953). The cytological effects of chemicals on ascites sarcomas.II. Selective damage to tumor cells by CaCl3 and H2O2. *Gan*, *44*(1), 39–46.

Malo, C., &Wilson, J.X.(2000). Glucose modulates vitamin C transport in adult human small intestinal brush border membrane vesicles. *Journal of Nutrition*, *130*(1), 63–69. https://doi.org/10.1093/jn/130.1.63

Mandai, T., Yoneyama, M., Sakai, S., Muto, N., &Yamamoto, I.(1992). The crystal structure and physicochemical properties of L-ascorbic acid 2-glucoside. *Carbohydrate Research*, *232*(2), 197–205. https://doi.org/10.1016/0008-6215(92)80054-5

Mann, A. K., Indira, A.P., &David, M.P.(2017). Recent advances in radiotherapy for head and neck cancer: A comprehensive review. *International Journal of Contemporary Medical Research*, *4*(4), 927–932.

Mansouri, M.T., Hemmati, A.A., Naghizadeh, B., Mard, S.A., Rezaie, A., &Ghorbanzadeh, B.(2015). A study of the mechanisms underlying the anti-inflammatory effect of ellagic acid in carrageenan-induced paw edema in rats. *Indian Journalof Pharmacology*, *47*(3), 292–298. https://doi.org/10.4103/0253-7613.157127

Mantovani, A., Allavena, P., Sica, A., &Balkwill, F.(2008).Cancer-related inflammation. *Nature*, *454*(7203), 436–444. https://doi.org/10.1038/nature07205

Marecos, E., Weissleder, R., &Bogdanov, A., Jr. (1998). Antibody-mediated versus nontargeted delivery in a human small cell lung carcinoma model. *Bioconjugate Chemistry*, *9*(2), 184–191. https://doi.org/10.1021/bc970146w

Markham, K.R., &Mabry, T.J.(1975). Ultraviolet-visible and proton magnetic resonance spectroscopy of flavanoids. InJ. B.Harborne, T. J.Mabry&H.Mabry(Eds.), *The flavonoids*.Chapman & Hall, 45-77.

Marklund, S.L.(1982). Human copper-containing superoxide dismutase of high molecular weight. *Proceedings of the National Academy of Sciences of the United States of America*, *79*(24), 7634–7638. https://doi.org/10.1073/pnas.79.24.7634

Marnett, L.J.(2000). Oxyradicals and DNA damage. *Carcinogenesis*, *21*(3), 361–370. https://doi.org/10.1093/carcin/21.3.361

Marshall, H.E., Merchant, K., &Stamler, J.S.(2000). Nitrosation and oxidation in the regulation of gene expression. *FASEB Journal*, *14*(13), 1889–1900. https://doi.org/10.1096/fj.00.011rev

Massey, V., Palmer, G., &Bennett, R.(1961). The purification and some properties of d-amino acid oxidase. *Biochimica et Biophysica Acta*, *48*, 1–9. https://doi.org/10.1016/0006-3002(61)90508-x

Mates, J.M., Perez-Gómez, C., &Núñez de Castro, I.(1999). Antioxidant enzymes and human diseases. *ClinicalBiochemistry*, *32*(8), 595–603. https://doi.org/10.1016/s0009-9120(99)00075-2

Mathew, D., Nair, C.K.K., Jacob, J. A., Biswas, N., Mukherjee, T., Kapoor, S., &Kagiya, T. V. (2007). Ascorbic acid monoglucoside as antioxidant and radioprotector. *Journal of Radiation Research*, *48*(5), 369–376. https://doi.org/10.1269/jrr.07007

Mathew, S., &Abraham, T.E.(2005). Studies on the production of feruloyl esterase from cereal brans and sugar cane bagasse by microbial fermentation.*Enzyme and Microbial Technology*, *36*(4), 565–570. https://doi.org/10.1016/j.enzmictec.2004.12.003

Mathew, S., &Abraham, T.E.(2006). Bioconversions of ferulic acid, an hydroxycinnamic acid. *CriticalReviews in Microbiology*, *32*(3), 115–125. https://doi.org/10.1080/10408410600709628

Matsumura, Y., &Maeda, H.(1986). A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANCS. *Cancer Research*, *46*(12 Pt 1), 6387–6392.

Matsura, T.,Kai, M.,Fujii, Y., Ito, H., &Yamada, K.(1999). Hydrogen peroxide-induced apoptosis in HL-60 cells requires caspase-3 activation. *Free Radical Research*, *30*(1),73–83. https://doi.org/10.1080/10715769900300081

Matsushima, H., Yonemura, K., Ohishi, K., &Hishida, A.(1998). The role of oxygen free radicals in cisplatin-induced acute renal failure in rats. *Journal ofLaboratory and Clinical Medicine*, *131*(6), 518–526. https://doi.org/10.1016/s0022-2143(98)90060-9

Maurya, D.K., Balakrishnan, S., Salvi, V.P., &Nair, C.K.K.(2005). Protection of cellular DNA from γ- radiation- induced damages and enhancement in DNA repair by troxerutin.*Molecular andCellular Biochemistry*, *280*(1–2), 57–68. https://doi.org/10.1007/s11010-005-8052-3

Maurya, D.K., Devasagayam, T.P.A., &Nair, C.K.K.(2006). Some novel approaches for radioprotection and the beneficial effect of natural products. *Indian Journal ofExperimental Biology*, *44*(2), 93–114.

Maurya, D.K., Salvi, V.P., &Nair, C.K.K.(2005).Radiation protection of DNA by ferulic acid under *in vitro* and *in vivo* conditions. *Molecular andCellular Biochemistry*, *280*(1–2), 209–217. https://doi.org/10.1007/s11010-005-0170-4

McBride, M.W., Carr, F. J., Graham, D., Anderson, N. H., Clark, J. S., Lee, W. K., Charchar, F. J., Brosnan, M. J., &Dominiczak, A. F.(2003). Microarray analysis of rat chromosome 2 congenic strains. *Hypertension*, *41*(3 Pt 2), 847–853. https://doi.org/10.1161/01.HYP.0000047103.07205.03

McCord, J. M., &Fridovich, I.(1969). Superoxide dismutase. *Journal of Biological Chemistry*, *244*(22), 6049–6055. https://doi.org/10.1016/S0021-9258(18)63504-5

McGowan, J.V., Chung, R., Maulik, A., Piotrowska, I., Walker, J. M., &Yellon, D. M.(2017). Anthracycline chemotherapy and cardiotoxicity. *Cardiovascular Drugs and Therapy*, *31*(1), 63–75. https://doi.org/10.1007/s10557-016-6711-0

McIntyre, M., Bohr, D.F., &Dominiczak, A.F.(1999). Endothelial function in hypertension: The role of superoxide anion. *Hypertension*, *34*(4 Pt 1), 539–545. https://doi.org/10.1161/01.hyp.34.4.539

McQueen, M.J.(1975). True Arrhenius relationships of human lactate dehydrogenase. *Zeitschrift für Klinische Chemie und Klinische Biochemie*, *13*(1), 17–19. https://doi.org/10.1515/cclm.1975.13.1.17

McSweeney, K.R., Gadanec, L.K., Qaradakhi, T., Ali, B.A., Zulli, A., &Apostolopoulos, V.(2021). Mechanisms of cisplatin-inducedacutekidneyinjury: Pathological mechanisms, pharmacologicalinterventions, and geneticmitigations. *Cancers*, *13*(7), 1572. https://doi.org/10.3390/cancers13071572

Mealey, J., Jr. (1965). Regional infusion of vinblastine and hydrogen peroxide in tumor bearing rats. *Cancer Research*, *25*(11), 1839–1843.

Melov, S., Ravenscroft, J., Malik, S., Gill, M. S., Walker, D.W., Clayton, P.E., Wallace, D.C., Malfroy, B., Doctrow, S.R., &Lithgow, G.J.(2000). Extension of life-span with superoxide dismutase/catalase mimetics. *Science*, *289*(5484), 1567–1569. https://doi.org/10.1126/science.289.5484.1567

Melov, S.,Schneider, J.A., Day, B.J., Hinerfeld, D., Coskun, P.,Mirra, S.S., Crapo, J.D., &Wallace, D.C.(1998). A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase. *Nature Genetics*, *18*(2), 159–163. https://doi.org/10.1038/ng0298-159

Merisko-Liversidge, E., Sarpotdar, P., Bruno, J., Hajj, S., Wei, L., Peltier, N., Rake, J., Shaw, J.M., Pugh, S., Polin, L., Jones, J., Corbett, T., Cooper, E., &Liversidge, G.G.(1996). Formulation and antitumor activity evaluation of nanocrystalline suspensions of poorly soluble anticancer drugs.*Pharmaceutical Research*, *13*(2), 272–278. https://doi.org/10.1023/a:1016051316815

Metodiewa, D., Kochman, A., &Karolczak, S.(1997). Evidence for antiradical and antioxidant properties of four biologically active N, N- diethylaminoethyl ethers of flavanone oximes: A comparison with natural polyphenolic flavonoid (rutin) action. *Biochemistry and Molecular Biology International*, *41*(5), 1067–1075. https://doi.org/10.1080/15216549700202141

Meyers, P.H., Cronic, F., &Nice, C.M.(1963). Experimental approach in the use and magnetic control of metallic iron particles in the lymphatic and vascular system of dogs as a contrast and isotopic agent. *American Journal of Roentgenology, Radium Therapy, and Nuclear Medicine*, *90*, 1068–1077.

Michalowski, A.S.(1994). On radiation damage to normal tissues and its treatment. II. Anti-inflammatory drugs. *Acta Oncologica*, *33*(2), 139–157. https://doi.org/10.3109/02841869409098397

Miller, R.P., Tadagavadi, R.K., Ramesh, G., &Reeves, W.B.(2010). Mechanisms of cisplatinnephrotoxicity. *Toxins*, *2*(11), 2490–2518. https://doi.org/10.3390/toxins2112490

Min, H.J., Nam, J. W., Yu, E. S., Hong, J. H., Seo, E. K., &Hwang, E. S.(2009).EK and Hwang, ES. Effect of naturally occurring hydroxy chavicol acetate on the cytokine production in T helper cells.*International Immunopharmacology*. Hong. Seo, *9*(4), 448–454. https://doi.org/10.1016/j.intimp.2009.01.008

Min, J.Y., Kang, S.M., Park, D.J., Kim, Y.D., Jung, H.N., Yang, J.K., Seo, W.T., Kim, S.W., Karigar, C.S., &Choi, M.S. (2006). Enzymatic release of ferulic acid from Ipomoea batatas L. (Sweet Potato) stem. *Biotechnology and Bioprocess Engineering*, *11*(4), 372–376. https://doi.org/10.1007/BF03026256.

Miranda-Vilela, A.L., Portilho, F.A., de Araujo, V. G. B., Estevanato, L. L. C., Mezzomo, B. P., de Almeida Santos, MdF. M., &Lacava, Z. G. M.(2011). The protective effects of nutritional antioxidant therapy on Ehrlich solid tumorbearing mice depend on the type of antioxidant therapy chosen: Histology, genotoxicity and hematology evaluations. *Journal of Nutritional Biochemistry*, *22*(11), 1091–1098. https://doi.org/10.1016/j.jnutbio.2010.09.009

Miranda-Vizuete, A.D.A., &Spyrou, G.(2000). The mitochondrial thioredoxin system. *Antioxidants and Redox Signaling*, *4*, 801–810

Mitchell, J.B., &Russo, A.(1987). The role of glutathione in radiation and drug induced cytotoxicity. *British Journal of Cancer. Supplement*, *8*, 96–104.

Miura, Y., Chiba, T., Tomita, I., Koizumi, H., Miura, S., Umegaki, K., Hara, Y., Ikeda, M., &Tomita, T.(2001). Tea catechins prevent the development of atherosclerosis in apoprotein E-deficient mice. *Journal of Nutrition*, *131*(1), 27–32. https://doi.org/10.1093/jn/131.1.27

Miyake, Y., Aki, K., Hashimoto, S., &Yamano, T.(1965). Crystallization and some properties of D-amino acid oxidase apoenzyme.*Biochimica et Biophysica Acta*, *105*(1), 86–99. https://doi.org/10.1016/s0926-6593(65)80177-1

Mizon,J., Biserte,G., &Boulanger, P.. *Bulletin Societie Chimie Biol*ogique.(1969), *51*, 419–423.

Mizushima, Y., Nagahama, H., Yokoyama, A., Morikage, T., &Yano, S.(1987). Studies on nephrotoxicity following a single and repeated administration of cis-diamminedichloroplatinum (CDDP) in rats. *Tohoku Journal of Experimental Medicine*, *151*(2), 129–135. https://doi.org/10.1620/tjem.151.129

Moayeri, M., Wiggins, J.F., Lindeman, R.E., &Leppla, S.H.(2006). Cisplatin inhibition of anthraxlethaltoxin. *Antimicrobial Agents and Chemotherapy*, *50*(8), 2658–2665. https://doi.org/10.1128/AAC.01412-05

Mobaraki,M., Faraji,A., Zare, M.,Dolati,P., Ataei,M., &Manshadi, H.R.(2017).Molecular mechanisms of cardiotoxicity: A review on the majorside-effect of doxorubicin.*Indian Journal of Pharmaceutical Sciences*, *79*(3), 335–344

Mody, H., Ramakrishnan, V., Chaar, M., Lezeau, J., Rump, A., Taha, K., Lesko, L., &Ait-Oudhia, S.(2020). A review on drug-inducednephrotoxicity: Pathophysiological mechanisms, drugclasses, clinicalmanagement, and recentadvances in mathematicalmodeling and simulationapproaches. *Clinical Pharmacology in Drug Development*, *9*(8), 896–909. https://doi.org/10.1002/cpdd.879

Mohamed, H.Z., &Shenouda, M.B.(2021). Amelioration of renal cortex histological alterations by aqueous garlic extract in gentamicin induced renal toxicity in albino rats: A histological and immunohistochemical study. *Alexandria Journal of Medicine*, *57*(1), 28–37. https://doi.org/10.1080/20905068.2020.1871179

Mohan, G., T P, A. H., A J, J., K M, S. D., Narayanasamy, A., &Vellingiri, B.(2019). Recent advances in radiotherapy and its associated side effects in cancer—A review. *Journal of Basic and Applied Zoology*, *80*(1), 14. https://doi.org/10.1186/s41936-019-0083-5

Mohanty, S.K., Swamy, M.K., Middha, S.K., Prakash, L., Subbanarashiman, B., &Maniyam, A.(2015). Analgesic, anti- inflammatory, Anti- lipoxygenase Activity and Characterization of three Bioactive compounds in the Most Active Fraction of *Leptadenia reticulata* (Retz.)Wight and Arn. – A Valuable Medicinal Plant. *Iranian Journal of Pharmaceutical Research*, *14*(3), 933–942.

Moore, M., Thor, H., Moore, G., Nelson, S., Moldéus, P., &Orrenius, S.(1985). The Toxicity of acetaminophen and N-acetyl-p-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased cytosolic Ca2+. *Journal of Biological Chemistry*, *260*(24), 13035–13040. https://doi.org/10.1016/S0021-9258(17)38834-8

Mora, Lde O., Antunes, L.M., Francescato, H.D., &Bianchi, Mde L.(2003). The effects of oral glutamine on cisplatin-induced nephrotoxicity in rats. *Pharmacological Research*, *47*(6), 517–522. https://doi.org/10.1016/s1043-6618(03)00040-9

Morandi, P., Ruffini, P.A., Benvenuto, G.M., Raimondi, R., &Fosser, V.(2005). Cardiactoxicity of high-dose chemotherapy. *Bone Marrow Transplantation*, *35*(4), 323–334. https://doi.org/10.1038/sj.bmt.1704763

Morel, Y., &Barouki, R.(1999). Repression of gene expression by oxidative stress. *Biochemical Journal*, *342*(3), 481–496. https://doi.org/10.1042/bj3420481

Moreno-Gordaliza, E.M., Esteban-Fernández, D., Lázaro, A., Aboulmagd, S., Humanes, B., Tejedor, A., Linscheid, M.W., &Gómez-Gómez, M.M.(2018). Lipid imaging for visualizing cilastatin amelioration of cisplatin-induced nephrotoxicity. *Journal of Lipid Research*, *59*(9), 1561–1574. https://doi.org/10.1194/jlr.M080465

Moron, M. S., Depierre, J. W., &Mannervik, B.(1979). Levels of glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochimica et BiophysicaActa*, *582*(1), 67–78. https://doi.org/10.1016/0304-4165(79)90289-7

Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramírez, J.T., &Yacaman, M.J.(2005). The bactericidal effect of silver nanoparticles. *Nanotechnology*, *16*(10), 2346–2353. https://doi.org/10.1088/0957-4484/16/10/059

Motl, S., Zhuang, Y., Waters, C.M., &Stewart, C.F.(2006).Pharmacokinetic considerations in the treatment of CNS tumours.*Clinical Pharmacokinetics*, *45*(9), 871–903. https://doi.org/10.2165/00003088-200645090-00002

Muggia, F.M.(1999). Doxorubicin-polymer conjugates: Further demonstration of the concept of enhanced permeability and retention. *Clinical Cancer Research*, *5*(1), 7–8.

Mukherjee, P., Bhattacharya, R., Bone, N., Lee, Y.K., Patra, C.R., Wang, S., Lu, L., Secreto, C., Banerjee, P.C., Yaszemski, M.J., Kay, N.E., &Mukhopadhyay, D.(2007). Potential therapeutic application of gold nanoparticles in B-chronic lymphocytic leukemia (BCLL): Enhancing apoptosis. *Journal of Nanobiotechnology*, *5*, 4. https://doi.org/10.1186/1477-3155-5-4

Mukherjee, P., Bhattacharya, R., Wang, P., Wang, L., Basu, S., Nagy, J.A., Atala, A., Mukhopadhyay, D., &Soker, S.(2005). Antiangiogenic properties of gold nanoparticles. *Clinical Cancer Research*, *11*(9), 3530–3534. https://doi.org/10.1158/1078-0432.CCR-04-2482

Mukhtar, H., &Ahmad, N.(2000). Tea polyphenols: Prevention of cancer and optimizing health. *AmericanJournal of Clinical Nutrition*, *71*(6) (Suppl.), 1698S–702S; discussion 1703S. https://doi.org/10.1093/ajcn/71.6.1698S

Muller,A. J.,DuHadaway,J. B.,Donover, P. S., Sutanto-Ward, E., &Prendergast, G. C.(2005). Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. *Nature Medicine*, *11*(3), 312–319. https://doi.org/10.1038/nm1196

Multhoff, G., Molls, M., &Radons, J.(2011). Chronic inflammation in cancer development. *Frontiers in Immunology*, *2*, 98. https://doi.org/10.3389/fimmu.2011.00098

Muriel, P., Garciapiña, T., Perez-Alvarez, V., &Mourelle, M.(1992). Silymarin protects against paracetamol-induced lipid peroxidation and liver damage. *Journal of Applied Toxicology*, *12*(6), 439–442. https://doi.org/10.1002/jat.2550120613

Murray, T. J.,Yang, X., &Sherr, D.H.(2006). Growth of a human mammary tumor cell line is blocked bygalangin, a naturally occurring bioflavonoid, and is accompaniedby down-regulation of cyclins D3, E andA. *Breast Cancer Research*, *8*(2), R17. https://doi.org/10.1186/bcr1391

Mustacich, D., &Powis, G.(2000). Thioredoxin reductase. *Biochemical Journal*, *346*(1), 1–8. https://doi.org/10.1042/bj3460001

Myers, C.E., McGuire, W.P., Liss, R.H., Ifrim, I., Grotzinger, K., &Young, R.C.(1977). Adriamycin: The role of lipid peroxidation in cardiac toxicity and tumor response. *Science*, *197*(4299), 165–167. https://doi.org/10.1126/science.877547

Nagi, M. N., &Mansour, M. A.(2000).Protective effect of thymoquinone against doxorubicin-induced cardiotoxicity in rats: A possible Mechanism of protection. *PharmacologicalResearch*, *41*(3), 283–289. https://doi.org/10.1006/phrs.1999.0585

Naidu, M. U., Kumar, K. V., Mohan, I. K., Sundaram, C., &Singh, S.(2002). Protective effect of *Gingkobiloba* extract against doxorubicin-induced cardiotoxicity in mice. *Indian Journal of ExperimentalBiology*, *40*(8), 894–900.

Naik, K., &Krishnamurthy, R.(2018). Anti-inflammatory activity of methanolic extracts of *Pseudarthriaviscida* and *Urariapicta* against carrageenan induced paw edema in albino rat. *Open Access Journal of Medicinal and Aromatic Plants*, *9*(1), 1–11.

Nair, C.K.K., Parida, D.K., &Nomura, T.(2001). Radioprotectors in radiotherapy. *Journal of Radiation Research*, 42(1), 21-37.

Nair, G.G., &Nair, C.K.K.(2011). Amelioration of *γ*-Radiation Induced Genomic Insult and Oxidative Stress in Whole Body Irradiated Swiss albino Mice by sesamol.*International Journal of Low Radiation*, *8*(1), 20–34. https://doi.org/10.1504/IJLR.2011.040646

Nakamura, H., Nakamura, K., &Yodoi, J.(1997). Redox regulation of cellular activation. *Annual Review ofImmunology*, *15*, 351–369. https://doi.org/10.1146/annurev.immunol.15.1.351

Nakamura, Y., Mochida, A., Choyke, P.L., &Kobayashi, H.(2016). Nanodrug delivery: Is the enhancedpermeability and retention Effect Sufficient for curingcancer?*Bioconjugate Chemistry*, *27*(10), 2225–2238. https://doi.org/10.1021/acs.bioconjchem.6b00437

Nathan, C.F., &Cohn, Z.A.(1981). Antitumor effects of hydrogen peroxide *in vivo*. *Journal ofExperimentalMedicine*, *154*(5), 1539–1553. https://doi.org/10.1084/jem.154.5.1539

Nauseef, W.M.(1999). The NADPH-dependent oxidase of phagocytes. *Proceedings of the Association ofAmerican Physicians*, *111*(5), 373–382. https://doi.org/10.1111/paa.1999.111.5.373

Nematbakhsh, M., Pezeshki,Z., Eshraghi Jazi, F.E., Mazaheri, B., Moeini, M., Safari, T.,Azarkish, F.,Moslemi, F., Maleki, M., Rezaei, A., Saberi, Sh,Dehghani, A.,Malek, M.,Mansouri, A., Ghasemi, M., Zeinali, F.,Zamani, Z., Navidi, M., Jilanchi, S., … Ashrafi,F.(2017). Cisplatin-inducednephrotoxicity; protectivesupplements and genderdifferences.*Asian Pacific Journal ofCancer Prevention: APJCP*, *18*(2), 295–314. https://doi.org/10.22034/APJCP.2017.18.2.295

Nethaji, M., Pattabhi, V., &Desiraju, G.R.(1988). Structure of 3-(4-hydroxy-3-methoxyphenyl)- 2-propenoic acid (ferulic acid).*Acta Crystallographica Section C Crystal Structure Communications*, *44*(2),275–277. https://doi.org/10.1107/S0108270187009211

Nickavar, B., Kamalinejad, M., &Izadpanah, H.(2007). *In vitro* free radical scavenging activity of five Salvia species. *Pakistan Journal of Pharmaceutical Sciences*, *20*(4), 291–294.

Nie, S., Xing, Y., Kim, G.J., &Simons, J. W.(2007). Nanotechnology applications in cancer. *Annual Review of BiomedicalEngineering*, *9*, 257–288. https://doi.org/10.1146/annurev.bioeng.9.060906.152025

Niidome, T., Yamagata, M., Okamoto, Y., Akiyama, Y., Takahashi, H., Kawano, T., Katayama, Y., &Niidome, Y.(2006).PEG-modified gold nanorods with a stealth character for *in vivo* applications.*Journal of Controlled Release*, *114*(3), 343–347. https://doi.org/10.1016/j.jconrel.2006.06.017

Nikitovic, D., &Holmgren, A.(1996). S-nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *Journal of Biological Chemistry*, *271*(32), 19180–19185. https://doi.org/10.1074/jbc.271.32.19180

Niyazi, M., Maihoefer, C., Krause, M., Rödel, C., Budach, W., &Belka, C.(2011). Radiotherapy and "new" drugs-new side effects?*Radiation Oncology*, *6*(1), 177. https://doi.org/10.1186/1748-717X-6-177

Nkosi, C.Z., Opoku, A.R., &Terblanche, S.E.(2005). Effect of pumpkin seed (*Cucurbita pepo*) protein isolate on the activity levels of certain plasma enzymes in CCl4-induced liver injury in low protein fed rats. *Phytotherapy Research*, *19*(4), 341–345. https://doi.org/10.1002/ptr.1685

Noda, N., &Wakasugi,H.(2001). Cancer and oxidativestress. *JMAJournal*, *44*(12), 535–539.

Noguchi, Y., Wu, J., Duncan, R., Strohalm, J., Ulbrich, K., Akaike, T., &Maeda, H.(1998). Early phase tumor accumulation of macromolecules: A great difference in clearance rate between tumor and normal tissues. *Japanese Journal ofCancer Research: Gann*, *89*(3), 307–314. https://doi.org/10.1111/j.1349-7006.1998.tb00563.x

Nordberg, J., &Arnér, E.S.(2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radical Biology and Medicine*, *31*(11), 1287–1312. https://doi.org/10.1016/s0891-5849(01)00724-9

Noviello, E., Aluigi, M.G., Cimoli, G., Rovini, E., Mazzoni, A., Parodi, S., De Sessa, F., &Russo, P.(1994). Sister-chromatid exchanges, chromosomal aberrations and cytotoxicity produced by topoisomerase II-targeted drugs in sensitive (A2780) and resistant (A2780-DX3) human ovarian cancer cells: Correlations with the formation of DNA double-strand breaks. *Mutation Research*, *311*(1), 21–29. https://doi.org/10.1016/0027-5107(94)90069-8

Nurgali, K., Jagoe, R.T., &Abalo, R.[Editorial]. (2018).Editorial: Adverse effects of cancerchemotherapy: Anything new to improvetolerance and reducesequelae?*Frontiers in Pharmacology*, *9*, 245. https://doi.org/10.3389/fphar.2018.00245

O'Dwyer, P.J.*University of Pennsylvania Cancer Center NtSMPPAUSA, Stevenson JP, University of Pennsylvania Cancer Center NtSMPPAUSA, Johnson SW, University of Pennsylvania Cancer Center NtSMPPAUSA. Clinical status of cisplatin, carboplatin, and otherplatinum-basedantitumordrugs*. (2006); 29–69.

O'Reilly, M.S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W.S., Flynn, E., Birkhead, J.R., Olsen, B.R., &Folkman, J.(1997). Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. *Cell*, *88*(2), 277–285. https://doi.org/10.1016/s0092-8674(00)81848-6

Ohno, S., Ohno, Y., Suzuki, N., Soma, G., &Inoue, M.(2009). High-dose vitamin C (ascorbic acid) therapy in the treatment of patients with advancedcancer. *Anticancer Research*, *29*(3), 809–815.

Ohshima, H., &Bartsch, H.(1994). Chronic infections and inflammatory processes as cancer risk factors: Possible role of nitric oxide in carcinogenesis. *Mutation Research*, *305*(2), 253–264. https://doi.org/10.1016/0027-5107(94)90245-3

Ohshima, H., Tatemichi, M., &Sawa, T.(2003). Chemical basis of inflammation-induced carcinogenesis. *Archives of Biochemistry and Biophysics*, *417*(1), 3–11. https://doi.org/10.1016/s0003-9861(03)00283-2

Ojha, S., Venkataraman, B., Kurdi, A., Mahgoub, E., Sadek, B., &Rajesh, M.(2016). Plant-derivedagents for counteractingcisplatin-inducednephrotoxicity. *Oxidative Medicine and Cellular Longevity*, 2016, article ID4320374. https://doi.org/10.1155/2016/4320374

Ojo, O.O., Kabutu, F.R., Bello, M., &Babayo, U.(2006). Inhibition of paracetamol-induced oxidative stress in rats by extracts of lemongrass (*Cymbropogoncitratus*) and green tea (*Camelliasinensis*) in rats. *African Journal of Biotechnology*, *5*, 1227–1232.

Okada, K., Wangpoengtrakul, C., Tanaka, T., Toyokuni, S., Uchida, K., &Osawa, T.(2001). Curcumin and especially tetrahydrocurcumin ameliorate stress-induced renal injury in mice. *Journal ofNutrition*, *131*(8), 2090–2095. https://doi.org/10.1093/jn/131.8.2090

Oktay, M., Gülçin, İ, &Küfrevioğlu, Öİ. (2003). Determination of in vitro antioxidant activity of fennel (*Foeniculumvulgare*) seed extracts. *LWT – Food Science and Technology*, *36*(2), 263–271. https://doi.org/10.1016/S0023-6438(02)00226-8

ONeal, D.P., Hirsch, L.R., Halas, N.J., Payne, J.D., &West, J.L.(2004). Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles. *Cancer Letters*, *209*(2), 171–176. https://doi.org/10.1016/j.canlet.2004.02.004

Orrell, R.W.(2000). Amyotrophic lateral sclerosis: Copper/zinc superoxide dismutase (SOD1) gene mutations. *Neuromuscular Disorders*, *10*(1), 63–68. https://doi.org/10.1016/s0960-8966(99)00071-1

Ostapowicz, G., Fontana, R.J., Schiødt, F.V., Larson, A., Davern, T.J., Han, S.H., McCashland, T. M., Shakil, A. O., Hay, J. E., Hynan, L., Crippin, J. S., Blei, A. T., Samuel, G., Reisch, J., Lee, W. M., &U.S. Acute Liver Failure Study Group. (2002). Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Annals of Internal Medicine*, *137*(12), 947–954. https://doi.org/10.7326/0003-4819-137-12-200212170-00007

Othman, S.N.N., Lum, P.T.,Gan, S.H., Mani, S., &Sekar, M.(2020). Protective effect of naturalproducts against chemotherapy- inducedcardiotoxicity: A review. *Pharmacognosy Journal*, *12*(5), 1180–1189. https://doi.org/10.5530/pj.2020.12.166

Overbeck, T.L., Knight, J.M., &Beck, D.J.(1996). A comparison of the genotoxic effects of carboplatin and cisplatin in Escherichia coli. *Mutation Research*, *362*(3), 249–259. https://doi.org/10.1016/0921-8777(95)00056-9

Overhage, J., Steinbüchel, A., &Priefert, H.(2002). Biotransformation of eugenol to ferulic acid by a recombinant strain of Ralstonia eutropha H16.*Applied and Environmental Microbiology*, *68*(9), 4315–4321. https://doi.org/10.1128/AEM.68.9.4315-4321.2002

Pabla, N., &Dong, Z.(2008). Cisplatin nephrotoxicity: Mechanisms and renoprotective strategies. *Kidney International*, *73*(9), 994–1007. https://doi.org/10.1038/sj.ki.5002786

Paciotti, G.F., Myer, L., Weinreich, D., Goia, D., Pavel, N., McLaughlin, R.E., &Tamarkin, L.(2004). Colloidal gold: A novel nanoparticle vector for tumor directed drug delivery.*Drug Delivery*, *11*(3), 169–183. https://doi.org/10.1080/10717540490433895

Padayatty, S.J., Sun, H., Wang, Y.H., Riordan, H.D., Hewitt, S.M., Katz, A., Wesley, R.A., &Levine, M.(2004). Vitamin C pharmacokinetics: Implications for oral and intravenous use. *Annals of Internal Medicine*, *140*(7), 533–537. https://doi.org/10.7326/0003-4819-140-7-200404060-00010

Pagare, S., Bhatia, M.,Tripathi,N., Pagare, S., &Bansa, Y.K.(2015). Secondary metabolites of plants and their role: Overview. *Current Trends in Biotechnology and Pharmacy*, *9*(3), 293–304.

Panche, A.N.,Diwan, A.D., &Chandra, S.R.(2016). Flavonoids: An overview. *Journal of Nutritional Science*, *5*(47), e47. https://doi.org/10.1017/jns.2016.41

Panthong, A., Kanjanapothi, D., Taesotikul, T., Phankummoon, A., Panthong, K., &Reutrakul, V.(2004). Anti-inflammatory activity of methanolic extracts from Ventilago Harmandiana Pierre. *Journal of Ethnopharmacology*, *91*(2–3), 237–242. https://doi.org/10.1016/j.jep.2003.12.025

Panyam, J., &Labhasetwar, V.(2004). Sustained cytoplasmic delivery of drugs with intracellular receptors using biodegradable nanoparticles. *Molecular Pharmaceutics*, *1*(1), 77–84. https://doi.org/10.1021/mp034002c

Papaldo, P., Lopez, M., Marolla, P., Cortesi, E., Antimi, M., Terzoli, E., Vici, P., Barone, C., Ferretti, G., Di Cosimo, S., Carlini, P., Nisticò, C., Conti, F., Di Lauro, L., Botti, C., Di Filippo, F., Fabi, A., Giannarelli, D., &Calabresi, F.(2005). Impact of five prophylactic filgrastim schedules on hematologic toxicity in early breast cancer patients treated with epirubicin and cyclophosphamide.*Journal of Clinical Oncology*, *23*(28), 6908–6918. https://doi.org/10.1200/JCO.2005.03.099

Patel, J.M., Zhang, J., &Block, E.R.(1996). Nitric oxide-induced inhibition of lung endothelial cell nitric oxide synthase via interaction with allosteric thiols: Role of thioredoxin in regulation of catalytic activity. *AmericanJournal of Respiratory Cell and Molecular Biology*, *15*(3), 410–419. https://doi.org/10.1165/ajrcmb.15.3.8810647

Pfeifer, H., Conrad, M.,Roethlein, D.,Kyriakopoulos, A., Brielmeier, M., Bornkamm, G. W., &Behne, D.(2001). Express Article 10.1096. Identification of a specific sperm nuclei selenoenzyme necessary for protamine thiol cross-linking during sperm maturation. *FASEB Journal*, 15(7), 1236–1238. https://doi.org/10.1096/fj.00-0655fje

Philp, H.A.(2003). Hot flashes-A review of the literature on alternative and complementary treatment approaches. *Alternative Medicine Review*, *8*(3), 284–302.

Pickrell, A.M., Fukui, H., &Moraes, C.T.(2009). The role of cytochrome c oxidase deficiency in ROS and amyloid plaque formation. *Journal ofBioenergetics and Biomembranes*, *41*(5), 453–456. https://doi.org/10.1007/s10863-009-9245-3

Pietta, P.G.(2000). Flavonoids as antioxidants. *Journal of Natural Products*, *63*(7), 1035–1042–10422. https://doi.org/10.1021/np9904509

Poole, L. B., Godzik, A., Nayeem, A., Schmitt, J. D., &Ahp, D.(2000). AhpF can be dissected into two functional units: Tandem repeats of two thioredoxin-like folds in the N-terminus mediate electron transfer from the thioredoxin reductase-like C-terminus to ahpC. *Biochemistry*, *39*(22), 6602–6615. https://doi.org/10.1021/bi000405w

Popp, M. B., Kirkemo, A. K., Morrison, S. D., &Brennan, M. F.(1984). Tumor and host carcass changes during total parenteral nutrition in an anorectic rat-tumor system. *Annals ofSurgery*, *199*(2), 205–210. https://doi.org/10.1097/00000658-198402000-00013

Prabhu, V., Siddik, U., Berlin Grace, V.M., &Chandrasekharan, G.(2011). Nanoparticles in drugdelivery and cancertherapy. The giantratstail.*Journal of Cancer Therapy*, *2*, 325–334

Prakash, J., Gupta, S. K., Kochupillai, V., Singh, N., Gupta, Y.K., &Joshi, S.(2001). Chemopreventive activity of *Withania somnifera* in experimentally induced fibro sarcoma tumors in Swiss albino mice. *Phytotherapy Research*, *15*(3), 240–244. https://doi.org/10.1002/ptr.779

Prasaja, Y., Sutandyo, N., &Andrajati, R.(2015).Incidence of cisplatin-inducednephrotoxicity and associatedfactors among cancerpatients in Indonesia. *Asian Pacific Journal of Cancer Prevention: APJCP*, *16*(3), 1117–1122. https://doi.org/10.7314/apjcp.2015.16.3.1117

Pratt, D.E., &Hudson, B.J.F.(1990)B. J. F.Hudson(Ed.). *Natural antioxidants not exploited commercially. Food Antioxidants*. PubMed: 171192.Elsevier.

Prieto, P., Pineda, M., &Aguilar, M.(1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E.*Analytical Biochemistry*, *269*(2), 337–341. https://doi.org/10.1006/abio.1999.4019

Pryor, W.A., &Porter, N.A.(1990). Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids. *Free Radical Biology and Medicine*, *8*(6), 541–543. https://doi.org/10.1016/0891-5849(90)90153-a

Pulfer, S. K., Ciccotto, S. L., &Gallo, J. M.(1999). Distribution of small magnetic particles in brain tumor-bearing rats.*Journal of Neuro-Oncology*, *41*(2), 99–105. https://doi.org/10.1023/a:1006137523591

Pyatak, P.S., Abuchowski, A., &Davis, F.F.(1980). Preparation of a polyethylene glycol: Superoxide dismutase adduct, and an examination of its blood circulating life and anti-inflammatory activity. *Research Communications in Chemical Pathology and Pharmacology*, *29*(1), 113–127.

Quiles, J.L., Huertas, J.R., Battino, M., Mataix, J., &Ramírez-Tortosa, M.C.(2002). Antioxidant nutrients and adriamycin toxicity. *Toxicology*, *180*(1), 79–95. https://doi.org/10.1016/s0300-483x(02)00383-9

Rad, A.K., Mohebbati, R., &Hosseinian, S.(2017). Drug-induced nephrotoxicity and medicinalplants. *Iranian Journal of Kidney Diseases*, *11*, 169–179.

Radi, R., Peluffo, G., Alvarez, M. N., Naviliat, M., &Cayota, A.(2001). Unraveling peroxynitrite formation in biological systems. *Free Radical Biology and Medicine*, *30*(5), 463–488. https://doi.org/10.1016/s0891-5849(00)00373-7

Ragavendran, P., Sophia, D., Arulraj, C., &Gopalakrishnan, V. K.(2012). Cardioprotective effect of aqueous, ethanol and aqueous ethanol extract of Aerva lanata (Linn.) against dox-orubicin induced cardiomyopathy in rats. *Asian Pacific Journal ofTropicalBiomedicine*, 1–5

Rajagopalan, R., Kagiya, T. V., &Nair, C. K. K.(2003). Radiosensitizer sanazole (AK2123) enhances up radiation-induced apoptosis in murine fibrosarcoma. *Journal of Radiation Research*, *44*(4), 359–365. https://doi.org/10.1269/jrr.44.359

Rajagopalan, S., Politi, P.M., Sinha, B.K., &Myers, C.E.(1988). Adriamycin-induced free radical formation in the perfused rat heart: Implications for cardiotoxicity. *Cancer Research*, *48*(17), 4766–4769.

Ramesh, G., &Reeves, W. B.(2002). TNF alpha mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. *Journal ofClinical Investigation*, *110*(6), 835–842. https://doi.org/10.1172/JCI15606

Ramos, K.S., Melchert, R.B., Chacon, E., &Acosta, D., Jr.(2001). Toxic response of the heart and vascular systems. InC. D.Klaassen(Ed.), *Casarett and Doull'stoxicology: Basic science of poisons* (pp. 597–651).McGraw-Hill.

Rana, M.A.,Khan, R.A., Nasiruddin, M., &Khan,A.A.(2016).Amelioration of cisplatin-inducednephrotoxicity by ethanolicextract of *Bauhiniapurpurea*: An *in vivo*study in rats. *Saudi Journal of Kidney Diseases and Transplantation*, *27*(1), 41–48. https://doi.org/10.4103/1319-2442.174068

Rani, D., Somasundaram, V.H., Nair, S., &Koyakutty, M.(2012). Advances in cancernanomedicine. *Journal of the Indian Institute of Science*, *92*, 2.

Ranusova, P., Matušíková, I., &Nemeček, P.(2021). Optimization of plant extract purification procedure for rapid screening analysis of sixteen phenolics by liquid chromatography. *Separations*, *8*(2), 13. https://doi.org/10.3390/separations8020013 arations8020013

Rao, G.N., &Berk, B.C.(1992). Active oxygen species stimulates vascular smooth muscle cell growth and protooncogene expression. *Circulation Research*, *79*, 593–599.

Rao, G.N., Lassègue, B., Griendling, K.K., &Alexander, R.W.(1993). Hydrogen peroxide stimulates transcription of c-jun in vascular smooth muscle cells: Role of arachidonic acid. *Oncogene*, *8*(10), 2759–2764.

Rao, V.K., Knutsen, T., Ried, T., Wangsa, D., Flynn, B.M., Langham, G., Egorin, M.J., Cole, D., Balis, F., Steinberg, S.M., Bates, S., &Fojo, T.(2005). The extent of chromosomal aberrations induced by chemotherapy in non-human primates depends on the schedule of administration. *Mutation Research*, *583*(2), 105–119. https://doi.org/10.1016/j.mrgentox.2005.01.013

Ratheea, D., Kamboj, A., Sachdev, R.K., &Sidhu, S.(2018). Hepatoprotective effect of Aegle marmelos augmented with piperine co-administration in paracetamol model. *Revista Brasileira de Farmacognosia*, *28*(1), 65–72. https://doi.org/10.1016/j.bjp.2017.11.003

Rav, R.S., Mehrotra, S., Shankar, U., Babu, G.S., Joshi, P.C., &Hans, R.K.(2001). Evaluation of UV-induced superoxide radical generation potential of some common antibiotics. *Drug and Chemical Toxicology*, *24*(2), 191–200. https://doi.org/10.1081/dct-100102610

Rebillard, A., Tekpli, X., Meurette, O., Sergent, O., LeMoigne-Muller, G., Vernhet, L., Gorria, M., Chevanne, M., Christmann, M., Kaina, B., Counillon, L., Gulbins, E., Lagadic-Gossmann, D., &Dimanche-Boitrel, M. T.(2007). Cisplatin-induced apoptosis involves membrane fluidification via inhibition of NHE1 in human colon cancer cells. *Cancer Research*, *67*(16), 7865–7874. https://doi.org/10.1158/0008-5472.CAN-07-0353

Recknagel, R.O.(1983). A new direction in the study of carbon tetrachloride hepatoprotective. *Life Sciences*, *33*(5), 401–408. https://doi.org/10.1016/0024-3205(83)90787-7

Ren, J. G.,Xia, H. L., Just, T., &Dai, Y. R.(2001). Hydroxyl radical-induced apoptosis in human tumor cells is associated with telomere shortening but not telomerase inhibition and caspase activation.*FEBS Letters*, *488*(3), 123–132. https://doi.org/10.1016/s0014-5793(00)02377-2

Repetto, M. G., &Llesuy, S. F.(2002). Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. *Brazilian Journal of Medical and Biological Research*, *35*(5), 523–534. https://doi.org/10.1590/s0100-879x2002000500003

Reuter, S., Gupta, S.C., Chaturvedi, M.M., &Aggarwal, B.B.(2010). Oxidative stress, inflammation, and cancer: How are they linked?*Free Radical Biology and Medicine*, *49*(11), 1603–1616. https://doi.org/10.1016/j.freeradbiomed.2010.09.006

Rhee, S.G.(1999). Redox signaling: Hydrogen peroxide as intracellular messenger. *Experimental and Molecular Medicine*, *31*(2), 53–59. https://doi.org/10.1038/emm.1999.9

Rice-Evans, C.A., Miller, N.J., &Paganga, G.(1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, *20*(7), 933–956. https://doi.org/10.1016/0891-5849(95)02227-9

Richter,C., Gogvadze, V., Laffranchi, R., Schlapbach, R., Schweizer, M., Suter, M., Walter, P., &Yaffee, M.(1995). Oxidants in mitochondria: From physiology to diseases. *Biochimica et Biophysica Acta*, *1271*(1), 67–74. https://doi.org/10.1016/0925-4439(95)00012-s

Rizzo, A.M., Berselli, P., Zava, S., Montorfano, G., Negroni, M., Corsetto, P., &Berra, B.(2010). Endogenous antioxidants and radical scavengers. *Advances in Experimental Medicine and Biology*, *698*, 52–67. https://doi.org/10.1007/978-1-4419-7347-4_5

Roginsky, V., &Lissi, E.A.(2005). Review of methods to determine chain-breaking antioxidant activity in food. *Food Chemistry*, *92*(2), 235–254. https://doi.org/10.1016/j.foodchem.2004.08.004

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., &Deng, H. X.(1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, *362*(6415), 59–62. https://doi.org/10.1038/362059a0

Rosen, G.M., &Halpern, H.J.(1990). Spin trapping biologically generated free radicals: Correlating formation with cellular injury. *Methods in Enzymology*, *186*, 611–621. https://doi.org/10.1016/0076-6879(90)86156-p

Rosen, H., Orman, J., Rakita, R.M., Michel, B.R., &VanDevanter, D.R.(1990). Loss of DNA-membrane interactions and cessation of DNA synthesis in myeloperoxidase-treated Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(24), 10048–10052. https://doi.org/10.1073/pnas.87.24.10048

Ross, J.A., &Kasum, C.M.(2002). Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annual Review ofNutrition*, *22*, 19–34. https://doi.org/10.1146/annurev.nutr.22.111401.144957

Rossi, F., Bellavite, P., Berton, G., Grzeskowiak, M., &Papini, E.(1985). Mechanism of production of toxic oxygen radicals by granulocytes and macrophages and their function in the inflammatory process. *Pathology, Research and Practice*, *180*(2), 136–142. https://doi.org/10.1016/S0344-0338(85)80161-8

Rossi, F., Filippelli, W., Russo, S., Filippelli, A., &Berrino, L.(1994). Cardiotoxicity of doxorubicin: Effects of drugs inhibiting the release of vasoactive substances. *Pharmacology and Toxicology*, *75*(2), 99–107. https://doi.org/10.1111/j.1600-0773.1994.tb00330.x

Rouach, H., Fataccioli, V., Gentil, M., French, S.W., Morimoto, M., &Nordmann, R.(1997). Effect of chronic ethanol feeding on lipid peroxidation and protein oxidation in relation to liver pathology. *Hepatology*, *25*(2), 351–355. https://doi.org/10.1002/hep.510250216

Roy, K., Mao, H.Q., Huang, S.K., &Leong, K.W.(1999). Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy.*Nature Medicine*, *5*(4), 387–391. https://doi.org/10.1038/7385

Rubbo, H., Radi, R., Anselmi, D., Kirk, M., Barnes, S., Butler, J., Eiserich, J. P., &Freeman, B. A.(2000). Nitric oxide reaction with lipid peroxyl radicals spares alpha-tocopherol during lipid peroxidation. Greater oxidant protection from the pair nitric oxide/alphatocopherol than alpha-tocopherol/ascorbate. *Journal ofBiological Chemistry*, *275*(15), 10812–10818. https://doi.org/10.1074/jbc.275.15.10812

Rukkumani, R., Aruna, K., Varma, P. S., &Menon, V. P.(2004). Influence of ferulic acid on circulatory prooxidant antioxidant status during alcohol and PUFA induced toxicity. *Journal of Physiology and Pharmacology*, *55*(3), 551–561.

Sachlos, E., Gotora, D., &Czernuszka, J.T.(2006). Collagen scaffolds reinforced with biomimetic composite nano-sized carbonate-substituted hydroxyapatite crystals and shaped by rapid prototyping to contain internal microchannels.*Tissue Engineering*, *12*(9), 2479–2487. https://doi.org/10.1089/ten.2006.12.2479

Sadzuka, Y., Shoji, T., &Takino, Y.(1992). Effect of cisplatin on the activities of enzymes which protect against lipid peroxidation. *Biochemical Pharmacology*, *43*(8), 1872–1875. https://doi.org/10.1016/0006-2952(92)90725-x

Safirstein, R., Winston, J., Goldstein, M., Moel, D., Dikman, S., &Guttenplan, J.(1986). Cisplatin nephrotoxicity. *American Journal of Kidney Diseases*, *8*(5), 356–367. https://doi.org/10.1016/s0272-6386(86)80111-1

Saggar, JK., Yu, M.,Tan Q &Tannock IF. (2013). *The tumor microenvironment and strategies to improve drug distribution Frontiers in Oncology*, *3*, 154.

Saisruthi, K., &Sreedevi, A.(2017).Amelioration of cisplatin- induced Nephrotoxicityby roots of *Anthocephaluscadamba*. *Biomedical and Pharmacology Journal*, *10*(3), 1433–1439. https://doi.org/10.13005/bpj/1250

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., &Ichijo, H.(1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO Journal*, *17*(9), 2596–2606. https://doi.org/10.1093/emboj/17.9.2596

Sala, A., Recio, M.D., Giner, R.M., Máñez, S., Tournier, H., Schinella, G., &Ríos, J.L.(2002). Antiinflammatory and antioxidant properties of *Helichrysumitalicum*. *Journal of Pharmacy and Pharmacology*, *54*(3), 365–371. https://doi.org/10.1211/0022357021778600, PubMed: 11902802

Salahudeen, A., Poovala, V., Parry, W., Pande, R., Kanji, V., Ansari, N., Morrow, J., &Roberts, J.(1998). Cisplatin induced N-acetylcysteine suppressible F2-isoprostane production and injury in renal tubular epithelial cells. *Journal of the AmericanSociety ofNephrology*, *9*(8), 1448–1455. https://doi.org/10.1681/ASN.V981448

Sales, G.T., &Foresto, R.D.(2020). Drug-induced nephrotoxicity. *Revista da AssociaçãoMédica Brasileira*, *66*(1), 82–90

Saltiel, E., &McGuire, W.V.(1983). Doxorubicin (Adriamycin) cardiomyopathy (Medical Progress). *Western Journalof Medicine*, *139*, 332–341.

Salvioni, L., Rizzuto, M. A., Bertolini, J. A., Pandolfi, L., Colombo, M., &Prosperi, D.(2019). Thirty years of cancernanomedicine: Success, frustration, and hope. *Cancers*, *11*(12), 1855. https://doi.org/10.3390/cancers11121855

Samanta, A., Das, G., &Das, S.(2011);Tech 6. Roles of flavonoids in plants. *International Journal of Pharmaceutical Sciences*, 12–35.

Sanchez-Moreno, C., Larrauri, J.A., &Saura-Calixto, F.A.(1998). A Procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture*, *76*(2), 270–276. https://doi.org/10.1002/(SICI)1097-0010(199802)76:2<270::AID-JSFA945>3.0.CO;2-9

Sandamali, J.A.N., Hewawasam, R.P., Jayatilaka, K. A. P. W., &Mudduwa, L.K.B.(2020). Cardioprotective potential of *Murraya koenigii (L.)* Spreng. Leaf Extract against Doxorubicin-Induced Cardiotoxicity in Rats. *Evidence-Based Complementary and Alternative Medicine: eCAM*, 2020, 6023737. https://doi.org/10.1155/2020/6023737

Sandeep, D., &Nair, C.K.K.(2010). Protection of DNA and Membrane from γ-radiation induced damage by the extract of *Acoruscalamus* Linn.: An *in vitro* study. *Environmental Toxicology and Pharmacology*, *29*(3), 302–307. https://doi.org/10.1016/j.etap.2010.02.006

Sankaranarayanan, K.(2006). Estimation of the genetic risks of exposure to ionizing radiation in humans: Current status and emerging perspectives. *Journal of Radiation Research*, *47* (Suppl. B), B57–B66. https://doi.org/10.1269/jrr.47.b57

Sawa, T., Wu, J., Akaike, T., &Maeda, H.(2000). Tumor-Targeting Chemotherapy by a xanthine oxidase-Polymer Con- jugate That Generates Oxygen-free Radicals in Tumor Tissue.*Cancer Research*, *60*(3), 666–671.

Scartezzini, P., &Speroni, E.(2000). Review on some plants of Indian traditional medicine with antioxidant activity. *Journal of Ethnopharmacology*, *71*(1–2), 23–43. https://doi.org/10.1016/s0378-8741(00)00213-0

Schenk, H., Klein, M., Erdbrügger, W., Dröge, W., &Schulze-Osthoff, K.(1994). Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-B and AP-1. *Proceedings of the National Academy of Sciences*, *91*(5), 1672–1676. https://doi.org/10.1073/pnas.91.5.1672

Schmeda-Hirschmann, G., Jordan, M., Gertn, A., Wilken, D., Hormazabal, E., &Tapia, A.A.(2004). Secondary metabolite content in Fabiana imbricate plants and in vitro cultures. *Z. Naturforsch.*, *5*, 48–54f.

Schreck, R., Albermann, K., &Baeuerle, P. A.(1992). Nuclear factor kappa B: An oxidative stress-responsive transcription factor of eukaryotic cells (a review)[A review]. *Free*

Radical Research Communications, *17*(4), 221–237. https://doi.org/10.3109/10715769209079515

Schreck, R., Rieber, P., &Baeuerle, P. A.(1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO Journal*, *10*(8), 2247–2258. https://doi.org/10.1002/j.1460-2075.1991.tb07761.x

Schulz, J.B., Lindenau, J., Seyfried, J., &Dichgans, J.(2000). Glutathine oxidative stress and neurodegeneration. *European Journal of Biochemistry*, *267*(16), 4904–4911. https://doi.org/10.1046/j.1432-1327.2000.01595.x

Schwartz, J., Domchek, S.M., Hwang, W.T., &Fox, K.(2005). Evaluation of anemia, neutropenia and skin toxicities in standard or dose-dense doxorubicin/cyclophosphamide (AC)-paclitaxel or docetaxel adjuvant chemotherapy in breast cancer.*Annals of Oncology*, *16*(2), 247–252. https://doi.org/10.1093/annonc/mdi058

Schweigerer, L.(1995). Antiangiogenesis as a novel therapeutic concept in pediatric oncology. *Journal of Molecular Medicine*, *73*(10), 497–508. https://doi.org/10.1007/BF00198901

Scott, M.D., Lubin, B.H., Zuo, L., &Kuypers, F.A.(1991). Erythrocyte defense against hydrogen peroxide: Preeminent importance of catalase. *Journal of Laboratory and Clinical Medicine*, *118*(1), 7–16.

Scott, P. A., &Harris, A.L.(1994). Current approaches to targeting cancer using antiangiogenesis therapies. *Cancer Treatment Reviews*, *20*(4), 393–412. https://doi.org/10.1016/0305-7372(94)90020-5

Sen, C.K.(1998). Redox signaling and the emerging therapeutic potential of thiol antioxidants. *Biochemical Pharmacology*, *55*(11), 1747–1758. https://doi.org/10.1016/s0006-2952(97)00672-2

Sen, C.K., &Packer, L.(1996). Antioxidant and redox regulation of gene transcription. *FASEB Journal*, *10*(7), 709–720. https://doi.org/10.1096/fasebj.10.7.8635688

Sena, L.A., &Chandel, N.S.(2012). Physiological roles of mitochondrial reactive oxygen species. *Molecular Cell*, *48*(2), 158–167. https://doi.org/10.1016/j.molcel.2012.09.025

Sengupta, S., &Sasisekharan, R.(2007). Exploiting nanotechnology to target cancer. *British Journal of Cancer*, *96*(9), 1315–1319. https://doi.org/10.1038/sj.bjc.6603707

Sevgi, K., Tepe, B., &Sarikurkcu, C.(2015). Antioxidant and DNA damage protection potentials of selected phenolic acids. *Food and Chemical Toxicology*, *77*, 12–21. https://doi.org/10.1016/j.fct.2014.12.006

Seymour, L.W., Miyamoto, Y., Maeda, H., Brereton, M., Strohalm, J., Ulbrich, K., &Duncan, R.(1995). Influence of molecular weight on passive tumour accumulation of a soluble macromolecular drug carrier. *European Journal of Cancer*, *31A*(5), 766–770. https://doi.org/10.1016/0959-8049(94)00514-6

Seymour, L.W., Olliff,S.P., Poole, C.J., De Takats, P.G., R., Orme, F., D. R., Maeda, H., Konno, T., &Kerr, D.J.(1998). A noveldosageapproach for evaluation of SMANCS [poly(strene-co-maleyl-half-*n*-butylate)-neocarzinostatin] in the treatment of primaryhepatocellularcarcinoma.*International Journal of Oncology*, *12*(6), 1217–1223

Shah, M., Patel, P., Phadke, M., Menon, S., Francis, M., &Sane, R.T.(2002). Evaluation of the effect of aqueous extract from powders of root, stem, leaves and whole plant of *Phyllanthusdebilis* against CCL4 induced rat liver dysfunction. *Indian Drugs*, *39*, 333–337.

Shah, R.M., Aarsh, S.S., &Mamta, B.S.(2012). Estimation offerulic acidin*Syzygiumcumini Seeds extract by HPLC. International Journal Of Pharmaceutical And Chemical Sciences*, *1*(1).

Shahani, S.(1999). Evaluation of hepatoprotective efficacy of APCL-A poly herbal formulation *in vivo* in rats. *Indian Drugs*, *36*, 628–631.

Shahidi, F.(2000). Antioxidant factors in plant foods and selected oilseeds. *BioFactors*, *13*(1–4), 179–185. https://doi.org/10.1002/biof.5520130129

Shanthi, M.(2014). *Global statusreport on noncommunicable diseases*.WHO Press, World Health Organization.

Shaojun,L.,Yong, GU & Sujuan,LI. (2002). Nephroprotective effects of piperazine ferulate on rat remnant kidney. *Chinese Journal of IntegratedTraditional and WesternNephrology*, 2002–2005.

Sharma, R., Yang, Y., Sharma, A., Awasthi, S., &Awasthi, Y. C.(2004). Antioxidant role of glutathione S-transferases: Protection against oxidant toxicity and regulation of stress-mediated apoptosis. *Antioxidants and Redox Signaling*, *6*(2), 289–300. https://doi.org/10.1089/152308604322899350

Sharma, R.P.(1985). Interactions of cisplatin with cellular zinc and copper in liver and kidney tissues. *Pharmacological Research Communications*, *17*, 1097–1020

Shi, J., Xiao, Z., Kamaly, N., &Farokhzad, O.C.(2011). Self-assembled targeted nanoparticles: Evolution of technologies and bench to bedside translation. *Accounts of Chemical Research*, *44*(10), 1123–1134. https://doi.org/10.1021/ar200054n

Shigenaga, M.K., Hagen, T.M., &Ames, B.N.(1994). Oxidative damage and mitochondrial decay in aging.*Proceedings of the National Academy of Sciences of the UnitedStates of America*, *91*(23), 10771–10778. https://doi.org/10.1073/pnas.91.23.10771

Shimoi, K., Masuda, S., Shen, B., Furugori, M., &Kinae, N.(1996). Radioprotective effects of antioxidative plant flavonoids in mice. *Mutation Research*, *350*(1), 153–161. https://doi.org/10.1016/0027-5107(95)00116-6

Shinde, A., Ganu, J., &Naik, P.(2012). Effect of free radicals and antioxidants on oxidativestress. *Journal of Dental and AlliedSciences*, *1*(2), 63–66. https://doi.org/10.4103/2277-4696.159144

Siemann, D.W., Bibby, M.C., Dark, G.G., Dicker, A.P., Eskens, F.A.L.M., Horsman, M.R., Marmé, D., &LoRusso, P.M.(2005). Differentiation and definition of vascular-targeted therapies. *Clinical Cancer Research*, *11*(2 Pt 1), 416–420.

Siemann, D.W., Chaplin, D.J., &Horsman, M.R.(2004). Vascular-targeting therapies for treatment of malignant disease. *Cancer*, *100*(12), 2491–2499. https://doi.org/10.1002/cncr.20299

Silva, C., Antunes, L.M.G., &Bianchi, M.L.P.(2001). Antioxidant action of bixin against cisplatin induced chromosome aberrations and lipid peroxidation in rats. *Pharmacological Research*, *86*, 271–278

Simizu, S., Takada, M., Umezawa, K., &Imoto, M.(1998). Requirement of caspase-3 (-like) protease-mediated hydrogen peroxide production for apoptosis induced by various anticancer drugs. *Journal of Biological Chemistry*, *273*(41), 26900–26907. https://doi.org/10.1074/jbc.273.41.26900

Singal, P.K., &Iliskovic, N.(1998). Doxorubicin-induced cardiomyopathy. *New England Journal of Medicine*, *339*(13), 900–905. https://doi.org/10.1056/NEJM199809243391307

Singh, N.P.(2000). Microgels for estimation of DNA strand breaks, DNA- protein cross links and apoptosis.*Mutation Research*, *455*, 119–135.

Sinha, B.K., Katki, A.G., Batist, G., Cowan, K.H., &Myers, C.E.(1987). Adriamycin stimulated hydroxyl radical formation in human breast tumor cells. *Biochemical Pharmacology*, *36*(6), 793–796. https://doi.org/10.1016/0006-2952(87)90164-x

Slater, T.F.(1984). Free radical mechanism in tissue injury. *Biochemical Journal*, *222*(1), 1–15. https://doi.org/10.1042/bj2220001

Slinkard, J., &Singleton, V.L.(1977). Total phenol analyses: Automation and comparison with manual methods. *American Journal ofEnology and Viticulture*, *28*, 49–55.

Sohal, R.S., Ku, H.H., Agarwal, S., Forster, M.J., &Lal, H.(1994). Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. *Mechanisms of Ageing and Development*, *74*(1–2), 121–133. https://doi.org/10.1016/0047-6374(94)90104-x

Somani, S.M., Ravi, R., &Rybak, L.P.(1995).Diethyldithiocarbamate protection against cisplatin nephrotoxicity: Antioxidant system. *Drug and Chemical Toxicology*, *18*(2–3), 151–170. https://doi.org/10.3109/01480549509014318

Son, E.W., Rhee, D.K., &Pyo, S.(2006). Gamma-irradiation-induced intercellular adhesion molecule-1 (ICAM-1) expression is associated with catalase: Activation of AP-1 andJNK. *Journal of Toxicology and Environmental Health. Part A*, *69*(24), 2137–2155. https://doi.org/10.1080/15287390600747759

Sondi, I., &Salopek-Sondi, B.(2004). Silver nanoparticles as antimicrobial agent: A case study of *E. coli* as a model for gram-negative bacteria.*Journal of Colloid and Interface Science*, *275*(1), 177–182. https://doi.org/10.1016/j.jcis.2004.02.012

Soto, M.L., Moure, A., Domínguez, H., &Parajó, J.C.(2011). Recovery, concentration and purification of phenolic compounds by adsorption: A review.*Journal ofFood Engineering*, *105*(1), 1–27. https://doi.org/10.1016/j.jfoodeng.2011.02.010

Sowers, J.R.(2002). Hypertension, angiotensin II and oxidative stress. *New England Journal of Medicine*, *346*(25), 1999–2001. https://doi.org/10.1056/NEJMe020054

Spahn-Langguth, H., &Benet, L.Z.(1992). Acyl glucuronides revisited: Is the glucuronidation process a toxification as well as a detoxification mechanism?*Drug Metabolism Reviews*, *24*(1), 5–47. https://doi.org/10.3109/03602539208996289

Speyer, J. L., Green, M. D., Dubin, N., Blum, R. H., Wernz, J. C., Roses, D., Sanger, J., &Muggia, F. M.(1985). Prospective evaluation of cardiotoxicity during a six-hour doxorubicin infusion regimen in women with adenocarcinoma of the breast.*American Journal ofMedicine*, *78*(4), 555–563. https://doi.org/10.1016/0002-9343(85)90395-x

Spotheim-Maurizot, M., Garnier, F., Sabattier, R., &Charlier, M.(1992). Metal ions protect DNA against strand breakage induced by fast neutrons. *International Journal of RadiationBiology*, *62*(6), 659–666. https://doi.org/10.1080/09553009214552601

Sprung, C. N., Ivashkevich, A., Forrester, H. B., Redon, C. E., Georgakilas, A., &Martin, O. A.(2015). Oxidative DNA damage caused by inflammation may link to stress-induced non-targeted effects. *Cancer Letters*, *356*(1), 72–81. https://doi.org/10.1016/j.canlet.2013.09.008

Sreenivasan, M., Sudheer, A.R., &Menon, V.P.(2007). Ferulic acid: Therapeutic Potential Through its antioxidant property. Recent Advances In Indian Herbal Drug Research. *Journal ofClinical Biochemistry and Nutrition*, *40*, 92–100

Srinivasan, M., Rukkumani, R., Ram Sudheer, A., &Menon, V.P.(2005). Ferulic acid, a natural protector against carbon tetrachloride-induced toxicity.*Fundamental and Clinical Pharmacology*, *19*(4), 491–496. https://doi.org/10.1111/j.1472-8206.2005.00332.x

Srivastava, K.S.(2007). A novelpathwaylinkingoxidativestress and inflammation: Prevention of cancer, sepsis, asthma, diabetes and uveitis by aldosereductaseinhibition. *Indian Journal ofClinical Biochemistry*; 22: 11.

Stadtman, E.R., &Berlett, B.S.(1998). Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metabolism Reviews*, *30*(2), 225–243. https://doi.org/10.3109/03602539808996310

Stamler, J.S.(1994). Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell*, *78*(6), 931–936. https://doi.org/10.1016/0092-8674(94)90269-0

Staniszewska, I., Królicka, A., Maliński, E., Łojkowska, E., &Szafranek, J.(2003). Elicitation of secondary metabolites in in vitro cultures of Ammi majus L.*Enzyme and Microbial Technology*, *33*(5), 565–568. https://doi.org/10.1016/S0141-0229(03)00180-7

Stegman, L.D., Zheng, H., Neal, E.R., Ben-Yoseph,O., Pollegioni, L., Pilone, M. S., &Ross, B.D.(1998). Induction of cyto- toxic Oxidative Stress by D-alanine in Brain Tumor Cells

Expressing *Rhodotorulagracilis*: A Cancer Gene Therapy Strategy.*Human Gene Therapy*, *9*(2), 185–193. https://doi.org/10.1089/hum.1998.9.2-185

Steinauer, K.K., Gibbs, I., Ning, S., French, J.N., Armstrong, J., &Knox, S.J.(2000). Radiation induces upregulation of cyclooxygenase-2 (COX-2) protein in PC-3 cells. *International Journal of Radiation Oncology, Biology, Physics*, *48*(2), 325–328. https://doi.org/10.1016/s0360-3016(00)00671-4

Steinberg, D.(1997). Low density lipoprotein oxidation and its pathobiological significance. *Journal of Biological Chemistry*, *272*(34), 20963–20966. https://doi.org/10.1074/jbc.272.34.20963

Stevens, R.J., Douglas, K. M. J., Saratzis, A.N., &Kitas, G.D.(2005). Inflammation and atherosclerosis in rheumatoid arthritis. *Expert Reviews in Molecular Medicine*, *7*(7), 1–24. https://doi.org/10.1017/S1462399405009154

Stevenson, M.A., Pollock, S.S., Coleman, C.N., &Calderwood, S.K.(1994). X-irradiation, phorbol esters, and H2O2 stimulate mitogen-activated protein kinase activity in NIH-3T3 cells through the formation of reactive oxygen intermediates. *Cancer Research*, *54*(1), 12–15.

Stoimenov, P.K., Klinger, R.L., Marchin, G.L., &Klabunde, K.J.(2002). Metal oxide nanoparticles as bactericidal agents.*Langmuir*, *18*(17), 6679–6686. https://doi.org/10.1021/la0202374

Strack, D. Metabolism of hydroxycinnamic acid conjugates, Bull. Liaison *Groupe Polyphen* .1990;15 ,55–64.

Strohmeyer, D.(1999). Pathophysiology of tumor angiogenesis and its relevance in renal cell cancer. *Anticancer Research*, *19*(2C), 1557–1561.

Subramanya, S. B., Venkataraman, B., Meeran, M. F. N., Goyal, S. N., Patil, C. R., &Ojha, S.(2018). Therapeutic potential of plants and plantderivedphytochemicals against acetaminophen-inducedliverinjury. *International Journal of MolecularSciences*, *19*(12), 3776. https://doi.org/10.3390/ijms19123776

Sugiura, K.(1958). Effect of hydrogen peroxide on transplanted rat and mouse tumours. *Nature*, *182*(4645), 1310–1311. https://doi.org/10.1038/1821310a0

Sugiyama, S., Hayakawa, M., Kato, T., Hanaki, Y., Shimizu, K., &Ozawa, T.(1989). Adverse effect of antitumor drug cisplatin on rat kidney mitochondria: Disturbences in glutathione peroxidase activity. *Biochemical and Biophysical Research Communications*, *159*(3), 1121–1127. https://doi.org/10.1016/0006-291x(89)92225-0

Suhara, T., Fukuo, K., Sugimoto, T., Morimoto, S., Nakahashi, T., Hata, S., Shimizu,M., &Ogihara, T.(1998). Hydrogen peroxide induces up-regulation of Fas in human endothelial cells. *Journal of Immunology*, *160*(8), 4042–4047.

Sukhdev, A.(2006). *Selection of primeayurvedicplantdrugs—Ancient modernconcordance*. Anshan Publishing.

Sultan, S.M., Ioannou, Y., &Isenberg, D.A.(2000). Is there an association of malignancy with systemic lupus erythematosus? An analysis of 276 patients under long-term review. *Rheumatology*, *39*(10), 1147–1152. https://doi.org/10.1093/rheumatology/39.10.1147

Sundararajan, R., Bharampuram, A., &Koduru, R.A.(2014). Review on phyto- constituents for nephroprotectiveactivity. *Pharmaceuticals*, *5*, 160–182.

Sundaresan, M., Yu, Z.X., Ferrans, V.J., Irani, K., &Finkel, T.(1995). Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. *Science*, *270*(5234), 296–299. https://doi.org/10.1126/science.270.5234.296

Symons, A.M., &King, L.J.(2003). Inflammation, reactive oxygen species and cytochrome P450.*Inflammopharmacology*, *11*(1), 75–86. https://doi.org/10.1163/156856003321547121

Tachakittirungrod, S., &Chowwanapoonpohn, S.(2007). Comparison of antioxidant and antimicrobial activities of essential oils from *Hyptissuaveolens* and *Alpinia galanga* growing in Northern Thailand.*Journal of Natural Sciences*, *6*, 31–42.

Tai, A., Fujinami, Y., Matsumoto, K., Kawasaki, D., &Yamamoto, I.(2002). Bioavailability of a series of novel acylated ascorbic acid derivatives, 6-O-acyl-2-O-alpha-Dglucopyranosyl-L-ascorbic acids, as an ascorbic acid supplement in rats and guinea pigs. *Bioscience, Biotechnology, and Biochemistry*, *66*(8), 1628–1634. https://doi.org/10.1271/bbb.66.1628

Takanaga, H., Mackenzie, B., &Hediger, M.A.(2004). Sodium-dependent ascorbic acid transporter family SLC23. *Pflügers Archiv*, *447*(5), 677–682. https://doi.org/10.1007/s00424-003-1104-1

Takebayashi, J., Tai, A., &Yamamoto, I.(2002). Long-termradicalscavengingactivity of AA-2G and 6-acyl-AA-2G against 1,1-diphenyl-2-picrylhydrazyl. *Biological and Pharmaceutical Bulletin*, *25*(11), 1503–1505. https://doi.org/10.1248/bpb.25.1503

Talwar, G.P.(1974). Hand book of practicalimmunology. InG. P.Talwar(Ed.), *National booktrust* (pp. 339–336).New Delhi.

Tam, L.T., Ha, N.C., &Thom, L.T.(2021). Ferulic acid extracted from rice bran as a growth promoter for the mi-croalga*Nannochloropsisoculata*. *Journal of Applied Phycology*, *33*, 37–45. https://doi.org/10.1007/s10811-020-02 166-5

Tandon, V., Gupta, B.M., &Tandon, R.(2005). *Free radicals/reactive oxygen species*, *12* (pp. 143–148). Practioner.

Teengam,P., Nunant, N.,Rattanarat,P.,Siangproh, W., &Chailapakul,O.(2013). Simple and rapiddetermination of ferulic acidlevels in food and cosmeticsamplesusingpaper-basedplatforms. *Sensors*, *13*(10), 13039–13053. https://doi.org/10.3390/s131013039

Tewey, K.M., Rowe, T.C., Yang, L., Halligan, B.D., &Liu, L.F.(1984). Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science*, *226*(4673), 466–468. https://doi.org/10.1126/science.6093249

Thannickal, V.J., &Fanburg, B.L.(2000). Reactive oxygen species in cell signaling. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *279*(6), L1005–L1028. https://doi.org/10.1152/ajplung.2000.279.6.L1005

Thefeld, W., Hoffmeister, H., Busch, E.W., Koller, P.U., &Vollmar, J.(1974). Reference values for the determination of GOT, GPT, and alkaline phosphatase in serum with optimal standard methods (author's transl). *Deutsche Medizinische Wochenschrift*, *99*(8), 343–4 passim. https://doi.org/10.1055/s-0028-1107760

Tholl, D.(2006). Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Current Opinion in Plant Biology*, *9*(3),297–304. https://doi.org/10.1016/j.pbi.2006.03.014

Thomas, E.L., Lehrer, R.I., &Rest, R.F.(1988). Human neutrophil antimicrobial activity. *Reviews of Infectious Diseases*, *10* (Suppl. 2), S450–S456. https://doi.org/10.1093/cid/10.supplement_2.s450

Thorpe, P.E.(2004). Vascular targeting agents as cancer therapeutics. *Clinical Cancer Research*, *10*(2), 415–427. https://doi.org/10.1158/1078-0432.ccr-0642-03

Thummel, K.E., Lee, C.A., Kunze, K.L., Nelson, S.D., &Slattery, J.T.(1993). Oxidation of acetaminophen to N-acetyl-p-aminobenzoquinone imine by human CYP3A4. *Biochemical Pharmacology*, *45*(8), 1563–1569. https://doi.org/10.1016/0006-2952(93)90295-8

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A.,Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., &Sasaki, Y.F.(2000). Single cell gel/comet assay: guidelines for the *in vitro* and *in vivo* genetic toxicology testing.*Environmental and Molecular Mutagenesis*, *35*(3), 206–221. https://doi.org/10.1002/(sici)1098-2280(2000)35:3<206::aid-em8>3.0.co;2-j

Tiefenauer, L.X., Kühne, G., &Andres, R.Y.(1993). Antibody-magnetite nanoparticles: *In vitro* characterization of a potential tumorspecific contrast agent for magnetic resonance imaging.*Bioconjugate Chemistry*, *4*(5), 347–352. https://doi.org/10.1021/bc00023a007

Tilay, A., Bule, M., Kishenkumar, J., &Annapure, U.(2008). Preparation of ferulic acid from agricultural wastes: Its improved extraction and purification.*Journal of Agricultural and Food Chemistry*, *56*(17), 7644–7648. https://doi.org/10.1021/jf801536t

Tiwari, A.K.(2004). Antioxidants: New- generation therapeutic base for treatment of polygenic disorders. *Current Science*, *86*(8), 1092–1102.

Totter, J.R.(1980). Spontaneous cancer and the possible relationship to oxygen metabolism.*Proceedings of the National Academy of Sciences of the UnitedStates of America*, *77*(4), 1763–1767. https://doi.org/10.1073/pnas.77.4.1763

Touyz, R.M., &Schiffrin, E.L.(1999). AngII-stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells. *Hypertension*, *34*(4 Pt 2), 976–982. https://doi.org/10.1161/01.hyp.34.4.976

Townsend, D.M., Deng, M., Zhang, L., Lapus, M.G., &Hanigan, M.H.(2003). Metabolism of cisplatin to a nephrotoxin in proximal tubule cells. *Journal of theAmerican Society of Nephrology*, *14*(1), 1–10. https://doi.org/10.1097/01.asn.0000042803.28024.92

Toyokuni, S., Okamoto, K., Yodoi, J., &Hiai, H.(1995). Persistent oxidative stress in cancer. *FEBS Letters*, *358*(1), 1–3. https://doi.org/10.1016/0014-5793(94)01368-b

Tsutsumi, Y., Kihira, T., Yamamoto, S., Kubo, K., Nakagawa, S., Miyake, M., Horisawa, Y., Kanamori, T., Ikegami, H., &Mayumi, T.(1994). Chemical modification of natural human tumor necrosis factor-alpha with polyethylene glycol increases its anti-tumor potency. *Japanese Journal of Cancer Research: Gann*, *85*(1), 9–12. https://doi.org/10.1111/j.1349-7006.1994.tb02879.x

Tuerk, C., &Gold, L.(1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science*, *249*(4968), 505–510. https://doi.org/10.1126/science.2200121

Turner, F.C.(1953). Studies with various experimental tumours. *Cancer Research*, *13*(Suppl 1) (Suppl. 1), 81–89.

Twardowski, P., &Gradishar, W.J.(1997). Clinical trials of antiangiogenic agents. *Current Opinion in Oncology*, *9*(6), 584–589. https://doi.org/10.1097/00001622-199711000-00015

Ueda, N., Kaushal, G.P., &Shah, S.V.(2000). Apoptotic mechanisms in acute renal failure. *American Journal of Medicine*, *108*(5), 403–415. https://doi.org/10.1016/s0002-9343(00)00311-9

Uhl, S.(1996). Ingredients: The building blocks for developing “new” ethnic foods. *Food Technology*, *50*, 79–84.

Umadevi,P., Bisht, KS & Vinitha, MA.(1998). comparative study ofradioprotection by Ocimum flavanoids and synthetic aminothiol protectors in the mouse.*British Journal of Radiology*, *71*, 782–784.

Upadhyay, S.N., Dwarakanath, B.S., Ravindranath, T., &Mathew, T. L.(2005). Chemical radioprotectors.*Defence Science Journal*, *55*(4), 403–425. https://doi.org/10.14429/dsj.55.2003

Uraji, M., Kimura, M., Inoue, Y., Kawakami, K., Kumagai, Y., Harazono, K., &Hatanaka, T.(2013). Enzymatic production of ferulic acid from defatted rice bran by using a combination of bacterial enzymes. *Applied Biochemistry and Biotechnology*, *171*(5), 1085–1093. https://doi.org/10.1007/s12010-013-0190-6

Ursini, F., Heim, S., Kiess, M., Maiorino, M., Roveri, A., Wissing, J., &Flohé, L.(1999). Dual function of the selenoprotein PHGPx during sperm maturation. *Science*, *285*(5432), 1393–1396. https://doi.org/10.1126/science.285.5432.1393

Ursini, F., Maiorino, M., Brigelius-Flohé, R., Aumann, K. D., Roveri, A., Schomburg, D., &Flohé, L.(1995). Diversity of glutathione peroxidases. *Methods in Enzymology*, *252*, 38–53. https://doi.org/10.1016/0076-6879(95)52007-4

Valiathan, M.S.(1998). Healing plants. *Current Science*, *75*, 1122–1127.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., &Telser, J.(2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*, *39*(1), 44–84. https://doi.org/10.1016/j.biocel.2006.07.001.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., &Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions*, *160*(1), 1–40. https://doi.org/10.1016/j.cbi.2005.12.009.

van Bladeren, P.J.(2000). Glutathione conjugation as a bioactivation reaction. *Chemico-Biological Interactions*, *129*(1–2), 61–76. https://doi.org/10.1016/s0009-2797(00)00214-3

Van Boven, M.V.,Toppet, S., Cokelaere, M.M., &Daenens, P.(1994). Isolation and structural identification of a new simmondsin ferulate from jojoba meal. *Journal of Agricultural and Food Chemistry*, *42*(5), 1118–1121. https://doi.org/10.1021/jf00041a014

Vane, J., &Botting, R.(1987). Inflammation and the mechanism of action of anti-inflammatory drugs. *FASEB Journal*, *1*(2), 89–96. https://doi.org/10.1096/fasebj.1.2.3111928

Varricchi, G., Ameri, P., Cadeddu, C., Ghigo, A., Madonna, R., Marone, G., Mercurio, V., Monte, I., Novo, G., Parrella, P., Pirozzi, F., Pecoraro, A., Spallarossa, P., Zito, C., Mercuro, G., Pagliaro, P., &Tocchetti, C. G.(2018). Antineoplastic drug-induced cardiotoxicity: A redox perspective. *Frontiers in Physiology*, *9*, 167. https://doi.org/10.3389/fphys.2018.00167

Vaseashta, A., &Dimova-Malinovska, D.(2005). Nanostructured and nanoscale devices, sensors and detectors. *Science and Technology of Advanced Materials*, *6*(3–4), 312–318. https://doi.org/10.1016/j.stam.2005.02.018

Vasquez-Vivar, J., Martasek, P., Hogg, N., Masters, B.S., Pritchard, K.A., Jr., &Kalyanaraman, B.(1997). Endothelial nitric oxide synthase-dependent superoxide generation from adriamycin. *Biochemistry*, *36*(38), 11293–11297. https://doi.org/10.1021/bi971475e

Vasudevan, D.M., &Sreekumari, S.*Textbook of Biochemistry for Medical Students* (4th ed).Jaypee 2004(pp. 338–340).

Velavan, S.(2011). *Free radicals in health and diseases- Pharmacology online*(pp. 1062–1077).

Vijay, T., Rajan, M. S. D., Sarumathy, K., Palani, S., &Sakthivel, K.(2011). Cardioprotective, antioxidant activities and phytochemical analysis by GC-MS of *Gmelinaarborea* (GA) in doxorubicin-induced myocardial necrosis in *Albino* rats. *Journal ofAppliedPharmaceuticalSciences*, *01*(05), 198–204.

Vijayalakshmi, S.V., Padmaja, G., Kuppusamy, P., &Kutala, V.K.(2009). Oxidative Stress in cardiovascular disease. *Indian Journal of Biochemistry and Biophysics*, *46*(6), 421–440.

Virani, S.S., Polsani, V.R., &Nambi, V.(2008). Novel markers of inflammation in atherosclerosis. *Current Atherosclerosis Reports*, *10*(2), 164–170. https://doi.org/10.1007/s11883-008-0024-0

Volarevic, V., Djokovic, B.,Jankovic, M.G.,Harrell,C.R., Fellabaum, C., Djonov, V., &Arsenijevic, N.(2019). Molecular mechanisms of cisplatin-induced nephrotoxicity: A balance on the knife edge between renoprotection and tumor toxicity. *Journal of Biomedical Science*, *26*(1), 25. https://doi.org/10.1186/s12929-019-0518-9

Von Hoff,D.D., Layard, M.W., Basa, P., Davis, H. L., Von Hoff, A. L., Rozencweig, M., &Muggia, F. M.(1979).Risk factors for doxorubicin-induced congestive heart failure.*Annals ofInternal Medicine*, *91*(5),710–717. https://doi.org/10.7326/0003-4819-91-5-710

Wada, S., Khoa, T.V., Kobayashi, Y., Funayama, T., Yamamoto, K., Natsuhori, M., &Ito, N.(2003). Detection of Radiation-induced apoptosis using the cometassay.*Journal of Veterinary Medical Science*, *65*(11), 1161–1166. https://doi.org/10.1292/jvms.65.1161

Wada,H., Imamura,I., Sako,M., Katagiri,S.,Tarui, S., Nishimura,H., &Inada,Y.(1990). *Antitumor enzyme: Polyethylene glycol –mediated asparaginase*, *613* (pp. 95–108).Annals Publishing of the New York Academy.

Wagh, S., Gangadhar, S., &Shinde, B.(2010). Antioxidant and hepatoprotective activity of *Tridaxprocumbens* Linn, against paracetamol induced hepatotoxicity in male albino rats. *Advanced Studies inBiology*, *2*(3), 105–112.

Wakamiya, H., Suzuki, E., Yamamoto, I., Akiba, M., Otsuka, M., &Arakawa, N.(1992). Vitamin C activity of 2-O-α- glucopyranosyl-L-ascoric acid in guinea pigs. *Journal of Nutritional Science and Vitaminology*, *38*(3), 235–245. https://doi.org/10.3177/jnsv.38.235

Walker, E. H., Pacold, M. E., Perisic, O., Stephens, L., Hawkins, P. T., Wymann, M. P., &Williams, R. L.(2000). Structural determinations of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Molecular Cell*, *6*(4), 909–919. https://doi.org/10.1016/s1097-2765(05)00089-4

Walker, R., &Edwards, C.(Eds.). (1999) (pp. 195–212).Churchill Livingstone.

Walter, M. I., &Gerarde, H. W.(1970). An Ultramicromethod for the determination of conjugated and total bilirubin in serum or plasma. *Microchemical Journal*, *15*(2), 231–243. https://doi.org/10.1016/0026-265X(70)90045-7

Wang, D., &Dubois, R.N.(2006). Prostaglandins and cancer. *Gut*, *55*(1), 115–122. https://doi.org/10.1136/gut.2004.047100

Wang, M.D., Shin, D.M., Simons, J.W., &Nie, S.(2007). Nanotechnology for targeted cancer therapy. *Expert Review of Anticancer Therapy*, *7*(6), 833–837. https://doi.org/10.1586/14737140.7.6.833

Wang, S., Konorev, E.A., Kotamraju, S., Joseph, J., Kalivendi, S., &Kalyanaraman, B.(2004). Doxorubicin induces apoptosis in normal and tumor cells via distinctly different

mechanisms. intermediary of H(2)O(2). *Journal of Biological Chemistry*, *279*(24), 25535–25543. https://doi.org/10.1074/jbc.M400944200

Warburg, O., &Christian, W.(1938). Isolation of the prosthetic group of the amino acid oxydase.*Biochemische Zeitschrift*, *298*, 150–168.

Watanabe, T., Motomura, Y., &Suga, T.(1978). A newcolorimetricdetermination of and urate oxidaseactivity.*Analytical Biochemistry*, *86*(1), 310–315. https://doi.org/10.1016/0003-2697(78)90347-0

Weiner,L.M., &Adams, G.P.(2000).New approaches to antibody therapy. *Oncogene*, *19*(53), 6144–6151. https://doi.org/10.1038/sj.onc.1204000

Weisiger, R.A., &Fridovich, I.(1973). Mitochondrial superoxide dismutase: Site of synthesis and intramitochondrial localization. *Journal of Biological Chemistry*, *248*(13), 4793–4796. https://doi.org/10.1016/S0021-9258(19)43735-6

Weiss, J.F., &Landauer, M.R.(2000). Radioprotection by antioxidants. *Annals of the New York Academy of Sciences*, *899*, 44–60.

Weiss, J.F., &Landauer, M.R.(2003). Protection against ionizing radiation by antioxidant nutrients and phytochemicals. *Toxicology*, *189*(1–2), 1–20. https://doi.org/10.1016/s0300-483x(03)00149-5

Weitzman, S.A., Turk, P.W., Howard-Milkowski, D., &Kozlowski, K.(1994). *Proceedings of the National Academy of Sciences of the UnitedStates of America*, *91*, 1261–1264

Widder, K.J., Morris, R.M., Poore, G.A., Howard, D.P., &Senyei, A.E.(1983). Selective targeting of magnetic albumin microspheres containing low-dose doxorubicin: Total remission in Yoshida sarcoma-bearing rats.*European Journal of Cancer and Clinical Oncology*, *19*(1), 135–139. https://doi.org/10.1016/0277-5379(83)90408-x

Widder, K.J., Senyei, A.E., &Ranney, D.F.(1979). Magnetically responsive microspheres and other carriers for the biophysical targeting of antitumor agents.*Advances in Pharmacology and Chemotherapy*, *16*, 213–271. https://doi.org/10.1016/s1054-3589(08)60246-x

Wiedau-Pazos, M., Goto, J.J., Rabizadeh, S., Gralla, E.B., Roe, J.A., Lee, M.K., Valentine, J.S., &Bredesen, D.E.(1996). Altered reactivity of superoxide dismutase in familial amyotrophic lateral sclerosis. *Science*, *271*(5248), 515–518. https://doi.org/10.1126/science.271.5248.515

Wiggin, F. H.(1906). Case of multiple fibrosarcoma of the tongue, with remarks on the use of trypsin and amylopsin in the treatment of malignant disease. *JAMA*, *XLVII*(24), 2003–2008. https://doi.org/10.1001/jama.1906.25210240037001j

Wilhelm, S., Tavares, A. J., Dai, Q., Ohta, S., Audet, J., Dvorak, H. F., &Chan, W. C. W.(2016). Analysis of nanoparticle delivery to tumours. *Nature Reviews Materials*, *1*(5), 16014. https://doi.org/10.1038/natrevmats.2016.14

Wilkins, R.C., Wilkinson, D., Maharaj, H.P., Bellier, P.V., Cybulski, M.B., &McLean, J.R.(2002). Differential Apoptotic Response to Ionizing Radiation in subpopulations of Human whitebloodcells.*Mutation Research*, *513*(1–2), 27–36. https://doi.org/10.1016/s1383-5718(01)00290-x

Williams, C.H., Jr. (1992). In*Mu¨ller, F*., ed. Chemistry and biochemistry of flavoenzymes (pp. 121–211). CRC Press, Inc.

Wilson, J.X.(2005). Regulation of vitamin C transport. *Annual Review of Nutrition*, *25*, 105–125. https://doi.org/10.1146/annurev.nutr.25.050304.092647

Wilson, R. L.(1983). Free radical repair mechanisms and the interaction of glutathion and vitamin C and E. In. InO. F.Nyga&M. G.Simic(Eds.), *Radioprotectors and anticarcinogens* p. 23.Academic Press.

Wink, D.A., &Mitchell, J.B.(1998). Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radical Biology and Medicine*, *25*(4–5), 434–456. https://doi.org/10.1016/s0891-5849(98)00092-6

Winrow, V.R., Winyard, P.G., Morris, C.J., &Blake, D.R.(1993). Free radicals in inflammation: Secondmessengers and mediators of tissue destruction. *British Medical Bulletin*, *49*(3), 506–522. https://doi.org/10.1093/oxfordjournals.bmb.a072627

Winter, C.A., Risley, E.A., &Nuss, G. W.(1962). Carrageenan induced paw oedema in hind paw of rats—An assay for its anti-inflammatory drugs.*Experimental Biology and Medicine*, *111*(3), 544–547. https://doi.org/10.3181/00379727-111-27849

Winterbourn, C.C.(2008). Reconciling the chemistry and biology of reactive oxygen species. *Nature Chemical Biology*, *4*(5), 278–286. https://doi.org/10.1038/nchembio.85

Winterbourn, C.C., Vissers, M.C., &Kettle, A.J.(2000). Myeloperoxidase. *Current Opinion in Hematology*, *7*(1), 53–58. https://doi.org/10.1097/00062752-200001000-00010

Witt, I., &Trendelenburg, C.(1982). Joint study to establish reference values for clinical chemical parameters in childhood (author's transl).*Journal of Clinical Chemistry and Clinical Biochemistry. Zeitschrift Fur Klinische Chemie und Klinische Biochemie*, *20*(4), 235–242.

Woisky, R. G., &Salatino, A.(1998). Analysis of propolis: Some parameters and procedures for chemical quality control. *Journal of Apicultural Research*, *37*(2), 99–105. https://doi.org/10.1080/00218839.1998.11100961

Wolfe, K., Wu, X., &Liu, R.H.(2003). Antioxidant activity of apple peels. *Journal of Agricultural and Food Chemistry*, *51*(3), 609–614. https://doi.org/10.1021/jf020782a

Wolin, M.S.(2000). Interactions of oxidants with vascular signaling systems. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *20*(6), 1430–1442. https://doi.org/10.1161/01.atv.20.6.1430

Wolin, M.S.(2009). Reactive oxygen species and the control of vascular function. *American Journal of Physiology. Heart and Circulatory Physiology*, *296*(3), H539–H549. https://doi.org/10.1152/ajpheart.01167.2008

Wouters, K.A., Kremer, L.C., Miller, T.L., Herman, E.H., &Lipshultz, S.E.(2005). Protecting against anthracycline-induced myocardial damage: A review of the most promising strategies. *British Journal of Haematology*, *131*(5), 561–578. https://doi.org/10.1111/j.1365-2141.2005.05759.x

Wu, C.B., Wei, S.L., &He, S.M.(1995).Studies on adriamycin magnetic gelatin microspheres. *Journal of Clinical Pharmacology and Science*, *4*, 1–6.

Wu, H., &Huang, J.(2018). Drug induced nephrotoxicity: Pathogenic mechanisms, Biomarkers and Prevention strategies. *Current Drug Metabolism*, *19*(7), 559–567. https://doi.org/10.2174/1389200218666171108154419

Wu, X., Ranganathan, V., Weisman, D. S., Heine, W. F., Ciccone, D. N., O'Neill, T. B., Crick, K. E., Pierce, K. A., Lane, W. S., Rathbun, G., Livingston, D. M., &Weaver, D. T.(2000). ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response. *Nature*, *405*(6785), 477–482. https://doi.org/10.1038/35013089

Wu, X.W., Wei, W., Yang, X.W., Zhang, Y. B., Xu, W., Yang, Y. F., Zhong, G. Y., Liu, H. N., &Yang, S. L.(2017). Anti-inflammatoryphenolic Acid Esters from the Roots and Rhizomes of Notopterygium incisium and Their Permeability in the Human Caco-2 Monolayer Cell Model.*Molecules*, *22*(6), 935. https://doi.org/10.3390/molecules22060935

Wu, Y. Z., Zhang, L., Wu, Z. X.,Shan, T. T., &Xiong, C.(2019). Berberine amelioratesdoxorubicin-inducedcardiotoxicity via a SIRT1/p66Shc-mediatedpathway. *Oxidative Medicine and Cellular Longevity*, 2019, 2150394. https://doi.org/10.1155/2019/2150394

Xu, F., Sun, R.C., Sun, J.X., Liu, C.F., He, B.H., &Fan, J.S.(2005). Determination of cell wall ferulic and p-coumaric acids in sugarcane bagasse.*Analytica ChimicaActa*, *552*(1–2), 207–217. https://doi.org/10.1016/j.aca.2005.07.037

Xu, M. F., Tang, P. L., Qian, Z. M., &Ashraf, M.(2001). Effects by doxorubicin on the myocardium are mediated by oxygen free radicals. *Life Sciences*, *68*(8), 889–901. https://doi.org/10.1016/s0024-3205(00)00990-5

Yagi, K.(1971). Reaction mechanism of D-amino-acid oxidase. *Advances in Enzymology and Related Areas of Molecular Biology*, *34*, 41–78. https://doi.org/10.1002/9780470122792.ch2

Yagi,K., Sugiura,N., Ōhama,H., &Kotaki, A.(1970). A Simple Purification Method of D-Amino Acid Oxidase.*Journal of Biochemistry*, *68*(4), 573–575. https://doi.org/10.1093/oxfordjournals.jbchem.a129387

Yagmurca, M., Fadillioglu, E., Erdogan, H., Ucar, M., Sogut, S., &Irmak, M. K.(2003). Erdosteine prevents doxorubicin induced cardiotoxicity in rats. *PharmacologicalResearch*, *48*(4), 377–382. https://doi.org/10.1016/s1043-6618(03)00185-3

Yamakawa, H., Ito, Y., Naganawa, T., Banno, Y., Nakashima, S., Yoshimura, S., Sawada, M., Nishimura, Y., Nozawa, Y., &Sakai, N.(2000). Activation of caspase 9 and 3 during H2O2-induced apoptosis of PC12 cells independent of ceramide formation. *Neurological Research*, *22*(6), 556–564. https://doi.org/10.1080/01616412.2000.11740718

Yamamoto, I., Muto, N., Nagata, E., Nakamura, T., &Suzuki, Y.(1990).Formation of a stale L-ascorbic acid α-glucoside by mammalian α-glucosidase-catalysed transglucosylation. *Biochimica et Biophysica acta*, *1035*(1), 44–50. https://doi.org/10.1016/0304-4165(90)90171-r

Yamazaki, Y., Kakizaki, S., Horiguchi, N., Takagi, H., Mori, M., &Negishi, M.(2005). Role of nuclear receptor CAR in carbon tetrachloride induced hepatotoxicity. *World Journal of Gastroenterology*, *11*(38), 5966–5972. https://doi.org/10.3748/wjg.v11.i38.5966

Yin, X., Zhou, J., Jie, C., Xing, D., &Zhang, Y.(2004). Anticancer activity and mechanism of *Scutellariabarbata* extract on human lung cancer cell line A549. *Life Sciences*, *75*(18), 2233–2244. https://doi.org/10.1016/j.lfs.2004.05.015

Yin, Z. N., Wu, W. J., Sun, C. Z., Liu, H. F., Chen, W. B., Zhan, Q. P., Lei, Z. G., Xin, X., Ma, J. J., Yao, K., Min, T., Zhang, M. M., &Wu, H.(2019). Antioxidant and anti-inflammatorycapacity of ferulic acidreleased from wheatbran by solid-state fermentation of Aspergillus niger.*Biomedical andEnvironmental Sciences*, *32*(1), 11–21. https://doi.org/10.3967/bes2019.002

Yla-Herttuala, S.(1999). Oxidized LDL and atherogenesisa. *Annals of the New York Academy of Sciences*, *874*(1 HEART IN STRE), 134–137. https://doi.org/10.1111/j.1749-6632.1999.tb09231.x

Yoo, H.S., Lee, K.H., Oh, J.E., &Park, T.G.(2000).*In vitro* and *in vivo* anti-tumor activities of nanoparticles based on doxorubicin-PLGA conjugates. *Journal of Controlled Release*, *68*(3), 419–431. https://doi.org/10.1016/s0168-3659(00)00280-7

Yoshikawa,T., Kokura,S., Tainaka,K., Naito, Y., &Kondo,M.(1995).A novelcancer therapy based on oxygenradicals. *Cancer Research*, *55*(8), 1617–1620.

Yost, F.J.J., &Fridovich, I.(1973). An iron-containing superoxide dismutase from Escherichia coli. *Journal of Biological Chemistry*, *248*(14), 4905–4908. https://doi.org/10.1016/S0021-9258(19)43649-1

Youn, Y.S., &Bae, Y.H.(2018). Perspectives on the past, present, and future of cancer nanomedicine. *Advanced Drug Delivery Reviews*, *130*, 3–11. https://doi.org/10.1016/j.addr.2018.05.008

Younes, M., &Siegers, C.P.(1981). Mechanistic aspects of enhanced lipid peroxidation following glutathione depletion *in vivo*. *Chemico-Biological Interactions*, *34*(3), 257–266. https://doi.org/10.1016/0009-2797(81)90098-3

Yousuf, M., Aslam, K., Wani, B.A., Aslam, N., Dar, N.A., &Nawchoo, I.A.(2012). In vitro antibacterial activity and phytochemical studies of methanolic extract of leaves of Hypericum perforatum L. growing wild in Kashmir Himalaya.*Asian Journal ofPlant Science and Research*, *2*(4), 414–420.

Yusuf, S., Dagenais, G., Pogue, J., Bosch, J., &Sleight, P.(2000). Vitamin E supplementation and cardiovascular events in high-risk patients. *New England Journal ofMedicine*, *342*(3), 154–160. https://doi.org/10.1056/NEJM200001203420302

Zalba, G., San José, G., Moreno, M.U., Fortuño, M.A., Fortuño, A., Beaumont, F.J., &Díez, J.(2001). Oxidative stress in arterial hypertension: Role of NADPH oxidase. *Hypertension*, *38*(6), 1395–1399. https://doi.org/10.1161/hy1201.099611

Zavala-López, M., &García-Lara, S.(2017). An improved microscale method for extraction of phenolic acids from maize. *Plant Methods*, *13*, 81. https://doi.org/10.1186/s13007-017-0235-x

Zhang, C., Liu, S., Logan, J., Mazumder, R., &Phelps, T.J.(1996). Enhancement of Fe(III), Co(III), and Cr(VI) reduction at elevated temperatures and by a thermophilic bacterium. *Applied Biochemistry and Biotechnology*, *57–58*(1), 923–932. https://doi.org/10.1007/BF02941773

Zhang, J., Li, Y. D., Patel, J. M., &Block, E.R.(1998). Thioredoxin overexpression prevents NO-induced reduction of NO synthase activity in lung endothelial cells. *American Journal of Physiology*, *275*(2), L288–L293. https://doi.org/10.1152/ajplung.1998.275.2.L288

Zhang, P., Liu, B., Kang, S.W., Seo, M.S., Rhee, S.G., &Obeid, L.M.(1997). Thioredoxin peroxidase is a novel inhibitor of apoptosis with a mechanism distinct from that of Bcl-2. *Journal of Biological Chemistry*, *272*(49), 30615–30618. https://doi.org/10.1074/jbc.272.49.30615

Zhang, Y., Kohler, N., &Zhang, M.(2002). Surface modification of superparamagnetic magnetite nanoparticles and their intracellular uptake.*Biomaterials*, *23*(7), 1553–1561. https://doi.org/10.1016/s0142-9612(01)00267-8

Zhang, Y.T., Xu, M.T., Du, M., &Zhou, F.M.(2007). Comparative studies of the interaction between ferulic acid and bovine serum albumin by ACE and surface plasmon resonance. *Electrophoresis*, *28*(11), 1839–1845. https://doi.org/10.1002/elps.200700025

Zhang, Z., Huang, L., Zhao, W., &Rigas, B.(2010). Annexin 1 induced by anti-inflammatory drugs binds to NF−_B and inhibits its activation: Anticancer effects in vitro and in vivo. *Cancer Research*, *70*(6), 2379–2388. https://doi.org/10.1158/0008-5472.CAN-09-4204

Zhang, Z., Yang, X., Zhang, Y., Zeng, B., Wang, S., Zhu, T., Roden, R.B., Chen, Y., &Yang, R.(2006). Delivery of telomerase reverse transcriptase small interfering RNA in complex with positively charged singlewalled carbon nanotubes suppresses tumor growth.*Clinical Cancer Research*, *12*(16), 4933–4939. https://doi.org/10.1158/1078-0432.CCR-05-2831

Zhao, Y., &Dai, W.(2020). Effect of phloretintreatmentameliorated the cisplatin-inducednephrotoxicity and oxidativestress in experimentalrats. *Pharmacognosy Magazine*, *16*(69).

Zhao, Z., &Moghadasian, M.H.(2008). Chemistry, natural sources, dietary intake and pharmacokinetic properties of ferulic acid: A review. *Food Chemistry*, *109*(4), 691–702. https://doi.org/10.1016/j.foodchem.2008.02.039

Zhao,J., Maitituersun, A.,Li, C., Li,Q., Xu, F., &Liu, T.(2018). Evaluation on analgesic and anti-inflammatoryactivities of totalflavonoids from Juniperus sabina. *Evidence-Based Complementary and Alternative Medicine: eCAM*, 2018, 7965306. https://doi.org/10.1155/2018/7965306

Zheng, W., &Wang, S.Y.(2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, *49*(11), 5165–5170. https://doi.org/10.1021/jf010697n

Zhong, L., Yuan, Z., Rong, L., Zhang, Y., Xiong, G., Liu, Y., &Li, C.(2019). Extraction and Characterization of phenoliccompounds in Dendranthema indicum var. aro-maticum Flower. *Scientific Reports*, *9*(1),7745. https://doi.org/10.1038/s41598-019-44102-9

Zhou, D., Yu, T., Chen, G., Brown, S.A., Yu, Z., Mattson, M.P., &Thompson, J.S.(2001). Effects of NF-kappaB1 (p50) targeted gene disruption on ionizing radiation-induced NF-kappaB activation and TNFalpha, IL-1alpha, IL-1beta and IL-6 mRNA expression in vivo. *International Journal of Radiation Biology*, *77*(7), 763–772. https://doi.org/10.1080/09553000110050047

Zhou, Y., Kok, K.H., Chun, A.C., Wong, C.M., Wu, H.W., Lin, M.C., Fung, P.C., Kung, H., &Jin, D.Y.(2000). Mouse peroxiredoxin V is a thioredoxin peroxidase that inhibits p53-induced apoptosis. *Biochemical and Biophysical ResearchCommunications*, *268*(3), 921–927. https://doi.org/10.1006/bbrc.2000.2231

Zhou, Y.Q., Liu, D.Q., Chen, S.P., Sun, J., Zhou, X. R., Rittner, H., Mei, W., Tian, Y. K., Zhang, H. X., Chen, F., &Ye, D. W.(2018). Reactive oxygen species scavengers ameliorate mechanical allodynia in a rat model of cancer-induced bone pain. *Redox Biology*, *14*, 391–397. https://doi.org/10.1016/j.redox.2017.10.011

Zicca, A., Cafaggi, S., Mariggiò, M.A., Vannozzi, M.O., Ottone, M., Bocchini, V., Caviglioli, G., &Viale, M.(2002).Bocchin,i V,[Reduction of cisplatin hepatotoxicity by procainamide hydrochloride in rats]. *European Journal of Pharmacology*, *442*(3), 265–272. https://doi.org/10.1016/s0014-2999(02)01537-6

Zimmerman, B.J., &Granger, D.N.(1994). Mechanisms of reperfusion injury. *American Journal of the Medical Sciences*, *307*(4), 284–292. https://doi.org/10.1097/00000441-199404000-00009

Zlotnik,A.(2006). Chemokines and cancer. *International Journal of Cancer*, *119*(9), 2026–2029. https://doi.org/10.1002/ijc.22024

Zwelling, L.A., Bradley, M.O., Sharkey, N.A., Anderson, T., &Kohn, K.W.(1979). Mutagenicity, cytotoxicity and DNA crosslinking in V79 Chinese hamster cells treated with cis- and trans-Pt(II) diamminedichloride. *Mutation Research*, *67*(3), 271–280. https://doi.org/10.1016/0165-1218(79)90021-1

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4. Divakaran, SA., CT, Anitha. Anti Inflammatory Activity of Ferulic Acid against Carrageenan and Formalin Induced Paw Edema in Swiss Albino Mice. *International Journal of Science and Research*. 2020; 9 (11), 788-792.
5. Divakaran, S A., Sreekanth, KM., Rao, KV and Nair, CKK. D-Aminoacid Oxidase-Fe2O3 Nanoparticle Complex Mediated Antitumor Activity in Swiss Albino Mice. *Journal of Cancer Therapy*. 2011; 2, 666-674.
6. Divakaran, S A., Nair, CKK. Amelioration of Doxorubicin Induced Cardiotoxicity in Tumor Bearing Mice by Ferulic Acid: a Mechanistic Study at Cellular and Biochemical Level. *International Journal of Tumor Therapy*. 2012; 1(2): 6-13.
7. Divakaran, SA., Hema, PS., Nair, MS., Nair, CKK. Antioxidant capacity and radioprotective properties of the flavonoids galangin and kaempferide isolated from *Alpinia galanga L.* (Zingiberaceae) against radiation induced cellular DNA damage. *International Journal of Radiation Research.* 2013; 11(2): 81-89.

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Research Article

Estimation of ferulic acid from selected plant materials by Spectrophotometry and High performance liquid chromatography

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Abstract
Ferulic acid (FA) is an abundant phytophenolic compound present in plant cell wall. Ferulic acid possesses anticancer, antioxidant, and anti-aging properties. A simple, sensitive and reproducible spectrophotometric method has been exploited for quantitative estimation of ferulic acid from selected plant materials such as rice bran (*Oryza sativa*), wheat bran (*Triticum aestivum*) and bamboo shoot (*Bambusa vulgaris*). The blue coloured chromogen obtained after the Folin–Ciocalteu assay was measured at a wavelength of 718 nm for ferulic acid against the blank reagent. The chromogen obeyed linearity over the range of 1µg/ml - 8µg/ml. High-Performance Liquid Chromatography (HPLC) method was also developed for the estimation of ferulic acid from selected plant materials. In HPLC analysis, ferulic acid got eluted and the amount of FA was found to be higher in rice bran (14.03mg/kg) compared to bamboo shoot (1.92mg/kg) and wheat bran (11.03mg/kg). The method can be used for the routine analysis of ferulic acid from various plant species and can be applied for nutritional and clinical investigations in a variety of samples.

Keywords: Bamboo shoot, Ferulic acid, Rice bran, Spectrophotometry, Wheat bran

INTRODUCTION

Antioxidants are compounds needed by most organisms, where they prevent oxidative damage caused by free radicals. The formation of free radicals causes the development of various diseases like cancer, cardiovascular diseases and cataracts in humans (Zhang *et al*., 2007). The antioxidant activities of plants have been mainly due to their phenolic content, which is one class of natural antioxidants (Castaneda *et al*., 2009). Thus, plants containing a high-level of phenolic acids considered a source of potent natural antioxidants (Ranusova *et al*., 2021). Ferulic acid (FA), together with dihydroferulic acid, could be a component of lignocelluloses, confers plasma membrane rigidity by cross linking lignin and polysaccharides. It is commonly found in seeds of plants like rice, wheat and oats (Buranov and Mazza, 2009). Ferulic acid will be easily absorbed by the body and stays within the blood longer than the other antioxidant, even longer than vitamin C. Thus FA can be considered as an important antioxidant and commonly used in nutrition purposes and food supplements (Silva and Batista, 2017).

Ferulic acid is found in many vegetable sources and occurs in particularly high concentration in popcorn and bamboo shoots. As an antioxidant, FA plays a major role in the body's defence against carcinogenesis by inhibiting the formation of N-nitroso compounds (Kuenzig *et al*., 1984; Lee *et al*., 2009; Aarabi *et al*., 2016). Ferulic acid possesses anticancer, antioxidant, and anti-aging potentials and can decrease blood glucose levels. Like other phenolic compounds, FA showed radioprotective abilities and reduced ionizing radiation-induced damages to DNA and membranes in biological systems (Roginsky & Lissi, 2005; Divakaran *et al*., 2013; Kumar and Goel, 2019).

Therefore, we exploited a simple, repeatable, sensitive and cost-effective VIS Spectrophotometric method for the quantification and determination of FA from various plant materials. Folin–Ciocalteu assay is used for the quantification of phenolic acid in the presence of alkali (15 % sodium carbonate). High-Performance Liquid

Chromatography (HPLC) method is considered to be as an appropriate method for estimation of chemical constituents from plant materials. Therefore, HPLC analysis has also been used for the quantitative determination of ferulic acid.

MATERIALS AND METHODS

Instruments

Soxhlet apparatus, VIS spectrophotometer (Systronics), HPLC (Agilent Technologies 1200 Infinity Series) were used.

Chemicals

AR grade chemicals such as ethyl acetate, Folin – Ciocalteu reagent and Sodium carbonate were obtained from Nice chemicals. Double distilled water was obtained after purification. Ferulic acid (FA) (Fig. 1) of 98% purity was purchased from NICE.

Plant materials

Plant materials selected such as rice bran (*Oryza sativa*), wheat bran (*Triticum aestivum*) and Bamboo shoot (*Bambusa vulgaris*) were collected from homestead region. Rice bran and wheat bran as whole and young bamboo shoot tip were dried, powdered with the help of blender and kept in sealed containers for future use.

Folin–Ciocalteu assay

Folin–Ciocalteu assay is based on of oxidation-reduction reaction, containing molybdates, tungstates as the main components of the reagent. This assay is a commonly used method for the quantification of phenolic acids in samples. The phenolic compounds reduces the heteropolyphosphotungstates–molybdates into a blue coloured chromogen. The reaction is carried out only under basic conditions in the presence of washing soda solution. The Phenolate anion formed from phenolic compound reduces Folin–Ciocalteu reagent to form the blue coloured substance. Spectrophotometer can be used to measure the colour intensity of blue chromogen (Jadhav *et al.*, 2012).

Standard solution of FA preparation (Stock)

The stock solution of FA (1 mg/ml) was prepared by dissolving 10 mg FA in ethyl acetate, final volume was made up to 10 ml with ethyl acetate in volumetric flask. From this stock, 1 ml was taken out and added to 10 ml volumetric flask, and the volume was adjusted to 10 ml by adding double distilled water to get 100 µg/ml concentration. This solution was used for further analysis for making calibration curve.

Calibration curve of FA

From the stock solution (100 µg/ml) of FA, 0.1 ml to 0.8 ml aliquots were added to a volumetric flask (10ml). To this flask, 2ml of sodium carbonate solution (15 %) and Folin–Ciocalteu reagent (0.5 ml) diluted with double distilled water (1:2 ratio) were added. The final volume was added with double distilled water to get a solution ranging in concentration from 1µg/ml - 8µg/ml of FA. The mixture showed maximum absorption at 718 nm when calculated against the blank solution. The absorbance of all solutions can be measured, and a calibration curve was plotted.

Preparation of ethyl acetate extract of plant materials

Accurately weighed 10 g of maize, wheat bran, rice bran, and bamboo shoot was extracted separately with 100ml of ethyl acetate with the help of the Soxhlet apparatus. The resulting crude extract was used for further analysis.

Preparation of sample solution

The extract solution (1ml) was added to the volumetric flask (10ml). To the flask, Folin – Ciocalteu reagent (0.5 ml) diluted with double distilled water (1:2 ratio) and 2 ml sodium carbonate solution (15 %) was added, the final volume was made up to 10ml with double distilled water. Absorbance was measured at 718 nm with a Spectrophotometer.

Validation of the proposed method:

Linearity

The linearity was determined by constructing the calibration curve and evaluating it by linear least square regression analysis.

Chromatography

Column : C18 4.6×250mm×5µm
Flow rate : 1.0mL/Minute
Inj.Volume : 20µL
Wave length : 319nm
Run time : 10 minute
Column temperature: 30^0C
Mobile phase: 5% Glacial Acetic acid in HPLC water : Acetonitrile (80:20)

Standard preparation

Ferulic acid Stock solution of 1000ppm was prepared in Methanol. From this, working standard of 2.5, 5, 10, 15, 20 ppm were prepared by serial dilution of the stock solution with methanol.

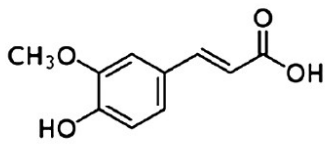

Fig. 1. *Structure of ferulic acid.*

Sample preparation

The ethyl acetate extract of plant samples were concentrated, filtered with 0.45µm disposable filter, 20µL samples were injected to the HPLC system for the analysis.

RESULTS AND DISCUSSION

Ferulic acid is a strong scavenger of free radicals and it has been accepted as a food additive to prevent lipid peroxidation. The selected plant materials such as rice bran, wheat bran, bamboo shoot are recognized in different systems of traditional medicine for the treatment of various diseases (Kumar and Pruthi, 2014). The mixture of ferulic acid along with Folin Ciocalteu reagent in an alkaline medium yielded a maximum absorbance at 718 nm. A linear relationship was obtained when a graph was plotted for concentration v/s absorbance within the concentration range of 1µg/ml - 8 µg/ml with a correlation coefficient value r2=0.988 and therefore, the rectilinear regression equation was y= 0.094x – 0.001 (Table.1).

The various samples used for the studies yielded different concentrations of FA, calculated by using the standard graph (Fig. 2). The bamboo shoot sample used for the study contained 1.7 µg/ml of FA, the wheat bran sample contains 7.3 µg/ml, and the rice bran sample yielded a maximum amount as 8 µg/ml (Table 2).

Chromatographic studies

In the HPLC analysis, Ferulic acid got eluted at 6.82 min (Fig. 4) and the peak for the same was found to be at 6.794 in the bamboo shoot sample (Fig. 5), 6.804 for the wheat bran sample (Fig. 6) and 6.791 for rice bran sample (Fig. 7) under the conditions of detection at 319 nm and temperature 30°C. The amount of ferulic acid in bamboo shoot, wheat bran and rice bran was found to be 1.92mg/kg, 11.03mg/kg and 14.03mg/kg, respectively, as calculated from the calibration curve of FA (Fig.3). The present study showed that rice bran yielded a higher amount of FA than bamboo shoot and wheat bran. Recent studies also unveiled the fact that rice bran is a rich source of antioxidant molecules such as γ-oryzanol and ferulic acid (Arumsari *et al*., 2019; Tam *et al*., 2021).

The extraction of Ferulic acid has been found much attention nowadays because it exhibits a wide variety of biological activities, including antimicrobial, anti-inflammatory, anti-thrombosis, anticancer, and antioxidant activities. However, the extraction procedure of phenolic acids from biomass is very complicated and proper methodology is yet to be developed (Zavala-Lopez and Garcia-Lara, 2017; Zhong *et al*., 2019).

The extraction and purification of phenolic acid viz. ferulic acid from rice bran and orange peels by solvent extraction method was studied by Gogoi *et al*. (2017). One of the major drawbacks of this procedure is its requirement for large quantities of different solvents and chemicals, which generates a significant quantity of toxic solvent waste (Acosta-Estrada *et al*., 2014). Ideia *et al*. (2020) reported the use of autoclave to perform alkaline hydrolysis and partial purification by adsorption on a synthetic resin to obtain ferulic acid from brewer's spent grain. The procedure is additionally very time consuming, making the handling of several samples without delay a challenging task.

In the present study, the estimation of phenolic acid viz. ferulic acid from rice bran, wheat bran and bamboo shoot by spectrophotometric method and the HPLC technique seems to be simple, sensitive, reproducible

Table 1. Regression analysis.

Regression equation	y= 0.094x – 0.001
Range	1µg/ml - 8 µg/ml
Co-relation coefficient r2	0.988
Slope m	0.094
y-intercept	0.001

Table 2. Concentration of FA from various plant samples.

Sample	Unknown yield (µg/ml)
Bamboo shoot	1.7
Wheat bran	7.3
Rice bran	8.0

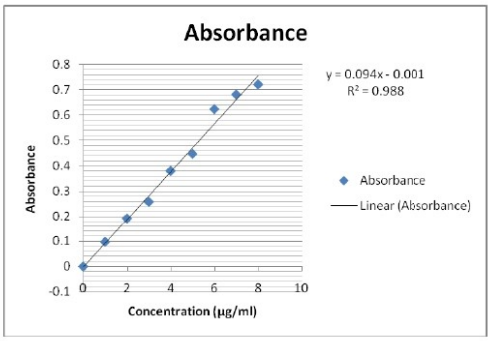

Fig. 2. *Calibration curve of ferulic acid.*

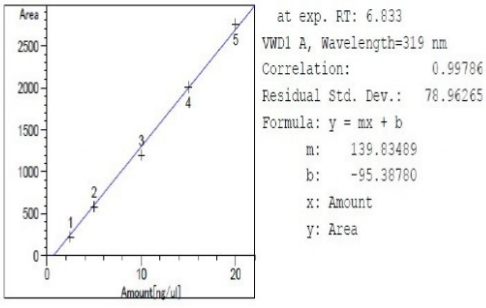

Fig. 3. *Calibration curve of ferulic acid.*

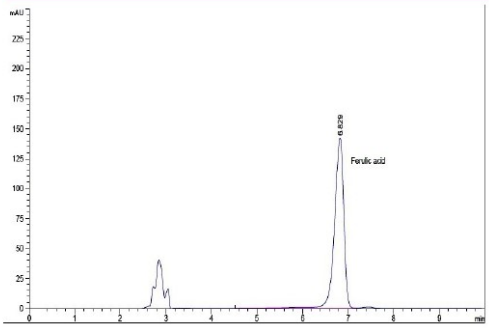

Fig. 4. *HPLC chromatogram of standard ferulic acid.*

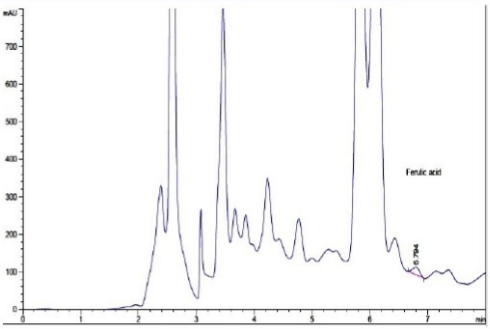

Fig. 5. *HPLC chromatogram of ethyl acetate extract of bamboo shoot.*

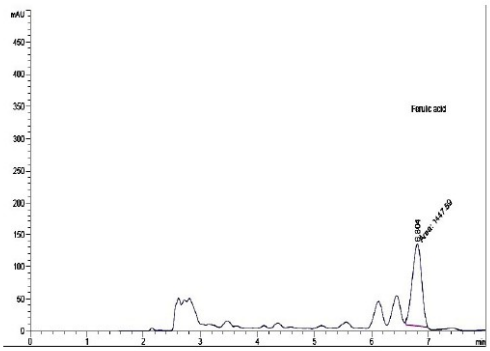

Fig. 6. *HPLC chromatogram of ethyl acetate extract of wheat bran.*

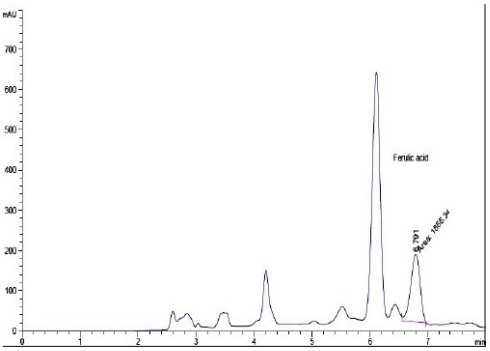

Fig.7. *HPLC chromatogram of ethyl acetate extract of rice bran.*

with minimum sample quantity, solvent and extraction time compared to methods proposed by Gogoi *et al*. (2017) and Ideia *et al*. (2020). The result obtained from the quantitative estimation of FA by Spectrophotometry and HPLC shows a parallel relationship. The chromatogram developed from HPLC also explains the isolation of ferulic acid with minimum impurities; hence can be recommended as a precise technique for the estimation of ferulic acid from plant samples.

Conclusion

A simple, sensitive and reproducible VIS-Spectrophotometric method has been exploited to estimate and quantify ferulic acid in various plant materials like bamboo shoots, rice bran and wheat bran etc., using Folin Ciocalteu reagent in the presence of an alkaline medium. The method can be employed for the routine analysis of ferulic acid from the various plant species. Moreover, they can prove to be helpful for nutritional and clinical investigations of ferulic acid levels in a variety of samples. The method for estimating ferulic acid in bamboo shoots, rice bran and wheat bran by HPLC is accurate, precise and reproducible.

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Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

1. Aarabi, A., Honarvar, M., Mizani, M., Faghihian, H. & Gerami, A. (2016). Extraction and purification of ferulic acid as an antioxidant from sugar beet pulp by alkaline hydrolysis. *Italian Journal of Food Science*, 28(3), 362-375. doi https://doi.org/10.14674/1120-1770/ijfs.v143
2. Acosta-Estrada , B.A., Gutiérrez-Uribe, J.A. & Serna-Saldívar, S.O. (2014). Bound phenolics in foods, a review. *Food Chemistry*, 152, 46–55. doi: 10.1016/j.foodchem.2013.11.093
3. Arumsari, A.G., Nugrahani, R. A. & Hendrawati, T. Y. (2019). The extraction of ferulic acid from the phenolic

fraction from rice bran oil using ultrasonic methods and analysis of antioxidant effectiveness. *Advances in Health Sciences Research*, 15, 37-39.

4. Buranov, A.U & Mazza, G. (2009). Extraction and purification of ferulic acid from flax shives, wheat and corn bran by alkaline hydrolysis and pressurised solvents. *Food Chemistry*,115(4), 1542–1548. doi: 10.1016/j.foodchem.2009.01.059
5. Castaneda, A., Hernandez, D., Hernandez, E., Rodríguez, J.A. & Galan, C.A. (2009). Chemical studies of anthocyanins: a review. *Food Chemistry*, 113, 859–871. doi:10.1016/j.foodchem.2008.09.001
6. Divakaran, S.A., Hema, P.S., Nair, M.S. & Nair, C.K.K. (2013). Antioxidant capacity and radioprotective properties of the flavonoids galangin and kaempferide isolated from *Alpinia galanga L.* (Zingiberaceae) against radiation induced cellular DNA damage. *International Journal of Radiation Research,* 11(2), 81-89.
7. Gogoi, P., Gogoi, A., Rajbangshi, C., Bhuyan, P. & Bhuyan, B.K. (2017). Extraction, purification and spectroscopic characterization of ferulic acid by alkaline hydrolysis from brans of Assam, India. *Imperial Journal of Interdisciplinary Research,* 3(1), 358-361.
8. Ideia, P., Sousa-Ferreira, I & Castilho, P.C. (2020). A novel and simpler alkaline hydrolysis methodology for extraction of ferulic acid from brewer's spent grain and its (partial) purification through adsorption in a synthetic resin. *Foods*, 9 (5), 600. doi:10.3390/foods9050600.
9. Jadhav, A.P., Kareparamban, J.A., Nikam, P.H. & Kadam, V.J. (2012). Spectrophotometric estimation of ferulic acid from *Ferula asafoetida* by Folin - Ciocalteu's Reagent. *Der Pharmacia Sinica*, 3 (6), 680-684.
10. Kuenzig, W., Chau, J., Norkus, E., Holowaschenko, H., Newmark, H., Mergens, W. & Conney, A.H. (1984). Caffeic and ferulic acid as blockers of nitrosamine formation. *Carcinogenesis*, 5(3), 309-313. doi: 10.1093/carcin/5.3.309
11. Kumar, N. & Goel, N. (2019). Phenolic acids: Natural versatile molecules with promisingtherapeutic applications. *Biotechnology Reports*, 24, e00370. doi: 10.1016/j.btre.2019.e00370
12. Kumar, N. & Pruthi, V. (2014). Potential applications of ferulic acid from natural sources. *Biotechnology Reports*, 4, 86–93. doi: 10.1016/j.btre.2014.09.002.
13. Lee, Y., Yuk, D., Lee, J., Lee, S., Ha, T., Oh, K., Yun, Y. & Hong, J. (2009). Epigallocatechin-3-gallate prevents lipopolysaccharide-induced elevation of β-amyloid generation and memory deficiency. *Brain Research,* 1250, 164–174. doi: 10.1016/j.brainres.2008.10.012.
14. Ranusova, P., Matušíková, I & Nemecek, P. (2021). Optimization of plant extract purification procedure for rapid screening analysis of sixteen phenolics by liquid chromatography. *Separations*, 8, 13. https://doi.org/10.3390/separations8020013.
15. Roginsky, V. & Lissi, E.A. (2005). Review of methods to determine chain-breaking antioxidant activity in food. *Food Chemistry*, 92(2), 235-254. doi:10.1016/j.foodchem.2004.08.004
16. Silva, E. & Batista. R. (2017). Ferulic acid and naturally occurring compounds bearing a feruloyl moiety: A review on their structures, occurrence, and potential health benefits. *Comprehensive Reviews in Food Science and Food Safety*, 16, 580-616. doi: 10.1111/1541-4337.12266.
17. Tam, L.T., Ha, N.C. & Thom, L.T. (2021). Ferulic acid extracted from rice bran as a growth promoter for the microalga *Nannochloropsis oculata*. *Journal of Applied Phycology,* 33, 37–45. https://doi.org/10.1007/s10811-020-02166-5
18. Zavala-Lopez, M. & Garcia□Lara, S. (2017). An improved microscale method for extraction of phenolic acids from maize. *Plant Methods,* 13, 81. doi 10.1186/s13007-017-0235-x
19. Zhang, Y.T., Xu, M.T., Du, M. & Zhou, F.M. (2007). Comparative studies of the interaction between ferulic acid and bovine serum albumin by ACE and surface plasmon resonance. *Electrophoresis*, 28(11), 1839–1845. doi: 10.1002/elps.200700025
20. Zhong, L., Yuan, Z., Rong, L., Zhang, Y., Xiong, G., Liu, Y. & Li, C. (2019). Extraction and Characterization of Phenolic Compounds in Dendranthema indicum var. aromaticum Flower. *Scientific Reports*, 9,7745. doi:10.1038/s41598-019-44102-9

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TREATMENT WITH FERULIC ACID AMELIORATED CISPLATIN-INDUCED NEPHROTOXICITY AND OXIDATIVE STRESS IN TUMOR BEARING MICE

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Nephrotoxicity

Oxidative stress

Antioxidant

Tumor

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ABSTRACT

Cisplatin (Cis) is one of the most widely used cytotoxic therapeutic agents for the treatment of cancer. Overdose of the drug resulted in various side effects of genotoxicity and nephrotoxicity. The toxicity of the drug has been attributed to the generation of oxidative free radicals. The current study aims to explore the effect of Ferulic acid (FA) in ameliorating Cis-induced renal toxicity in tumor bearing Swiss albino mice. Nephrotoxicity was induced in tumor bearing mice by a single dose of Cis (12mg/kg, *i.p*). Post administration of FA was carried out (100 mg/kg *p.o* and 200 mg/kg *p.o*) one hour after Cis administration. Toxicity was measured by analyzing the amount of serum urea, creatinine, and antioxidant status of renal and tumor tissues. Treatment of cisplatin-administered tumor animals with the FA could prevent the drug-induced oxidative damage as evidenced by the decreased levels of lipid peroxidation and enhanced activities of the antioxidants in the renal tissues. The treatment also protected the renal tissues from the toxic effects of Cis by reducing the levels of serum urea and creatinine. FA protected the renal tissues, whereas it enhanced the anticancer efficacy of Cis in tumor tissues. The histopathological observations support that ferulic acid has a protective effect against Cisplatin-induced nephrotoxicity and can be used to improve the chemotherapeutic index of Cisplatin for cancer treatment.

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1 Introduction

Nephrotoxicity arises as a result of direct exposure to drugs and environmental chemicals to renal tissue (Sales & Foresto, 2020). Many therapeutic agents such as oxaliplatin, cisplatin, paclitaxel, carboplatin, doclitaxel, vinorelbine, topotecan, etc. are responsible for the induction of nephrotoxicity. These agents are used as chemotherapeutic drugs against different forms of human cancers (Jagiela et al., 2021). To control neoplastic disorders and distant metastases, these agents are administered systemically, which adversely affects normal cells, resulting in cytotoxicity (Janakiraman & Jayaprakash, 2015).

Cis-Diamminedi chloroplatinum II (Cisplatin or CP or Cis) is one of the foremost effective cancer therapeutic agents, used in the treatment of several solid tumors and refractory non-Hodgkin's lymphomas (Giaccone, 2000). The therapeutic efficacy of cisplatin is usually related to nephrotoxicity (George & Anushree, 2014). The nephrotoxicity by cisplatin is associated with cellular necrosis, alterations within the number and size of the lysosomes, morphological damage of intracellular organelles, mitochondrial vacuolization, loss of microvilli, and functional changes as inhibition of protein synthesis, lipid peroxidation, mitochondrial damage, and GSH depletion (Awdishu & Mehta, 2017). Several natural agents like vitamins and/or dietary supplements have the potential to diminish the physiological side effects of those drugs (Ali & AlMoundhri, 2006; Rad et al., 2017). Such antioxidants protect normal tissues against the deleterious effects of the drug and may be safely manipulated without toxic manifestations (Ojha et al., 2016).

The present study envisages the effect of ferulic acid (FA) on CP-induced nephrotoxicity in tumor bearing Swiss albino mice. Phenolics are widely distributed in the plant kingdom and are an integral part of the human diet as an antioxidant, ferulic acid (FA) or 4-Hydroxy-3-Methoxycinnamic Acid (Figure 1) play a key role within the body's defense against carcinogenesis by inhibiting the formation of N-nitroso compounds (Batista, 2014). Moreover, FA could be a strong scavenger of free radicals and approved in certain countries as an additive to stop lipid peroxidation (Sreenivasan et al., 2007; Panche et al., 2016). FA possesses radioprotective abilities and reduces ionizing radiation-induced damages to DNA and membranes in biological systems (Kumar & Goel, 2019).

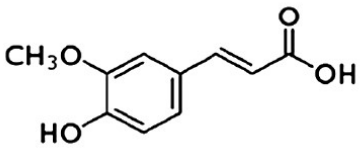

Figure 1 Ferulic acid (FA)

2 Materials and Methods

2.1 Animals

Female Swiss albino mice (8 - 10 weeks old) weighing 22 - 25 g, obtained from the Small Animal Breeding Section (SABS), Mannuthy, Thrissur, Kerala were used for the study. These experimental animals were kept under standard conditions of temperature and humidity within the Centre's Animal House Facility. The animals got standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad-libitum*. All animal experiments during this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) by strictly adhering to the rules of the Committee for the aim of Control and Supervision of Experiments on Animals (CPCSEA) governed by the Animal Welfare Division of Government of India.

2.2 Chemicals

Ferulic acid (FA), riboflavin, reduced glutathione (GSH), Nitroblue tetrazolium (NBT), 5-5' dithiobis-2- nitro benzoic acid (DTNB) were bought from Sigma Chemical Company Inc., St. Louis, MO, USA. Hydrogen peroxide (H_2O_2) was purchased from Merck India Ltd., Mumbai, India. Thiobarbituric acid (TBA) was from Hi-media Laboratories, Mumbai, India. All chemicals and reagents utilized in this study were of AR grade from reputed Indian manufacturers.

2.3 Drug administration to tumor induced mice

Cisplatin (12mg/kg *i.p*) was administered as a single dose to tumor animals.

2.4 Solid tumor in Animals

A solid tumor was induced in the hind limbs of mice by transferring Dalton's Lymphoma Ascites (DLA) cells (1×10^6 cells/animal) through subcutaneous injection. The treatments are started when the tumor size reached about 1.0 cm^3 on the 13^{th} day of transplantation of DLA cells. The animals were randomly divided into 4 groups of five animals each and treated as Group – I animals were kept as the untreated control group, provided with 0.1 ml saline *i.p* and 0.1 ml distilled water *p.o*; Group – II served as Cisplatin control group administered with cisplatin 12 mg/kg b.w (in 0.1 ml saline *i.p* as a single dose); Group – III animals were given Cisplatin (12 mg/kg b.w in 0.1 ml saline) *i.p* as a single dose with Ferulic acid 100 mg/kg b.w (in 0.1 ml distilled water *p.o*); and Group – IV animals were provided with Cisplatin (12 mg/kg b.w in 0.1 ml saline) *i.p* as a single dose with Ferulic acid 200 mg/kg b.w (in 0.1 ml distilled water *p.o*). FA was administered to groups III and IV, one hour after Cisplatin (12 mg/kg b.w) administration

2.5 Assessment of nephrotoxicity

Serum creatinine was determined by the alkaline picric acid method (Allen et al., 1982) using a diagnostic kit (Agappe Diagnostic Pvt. Ltd; Ernakulam, Kerala, India). Serum urea was determined by diacetylmonoxime (DAM) reagent using the Agappe diagnostic kit (Kassirer, 1971). In the case of cisplatin-treated animals, seventy-two hours after cisplatin treatment (12 mg/kg as an intraperitoneal injection) animals were sacrificed using ether anesthesia, blood was collected directly from the heart, serum separated for urea and creatinine analysis. Kidneys and tumors were excised for analyzing the antioxidant status and for histopathological examinations (Divakaran & Nair, 2012).

Reduced glutathione (GSH) level was measured colorimetrically using DTNB as the substrate. The concentrations of malondialdehyde (MDA) as indices of lipid peroxidation were assessed according to the method of Buege & Aust (1978). Superoxide dismutase activity was determined by the nitroblue tetrazolium reduction method of Mc Cord & Fridovich (1969). GPx activity was determined by the method of Hafemann et al. (1974), based on the degradation of H_2O_2 in the presence of GSH. Tissue protein was studied according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.6 Histopathological studies

Histopathological examinations of kidney and tumors from all the treated groups were evaluated using light microscopy. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin-eosin. The histopathological studies were done at Sudharma Metrapolis Pathological Laboratory, Thrissur, Kerala, India.

2.7 Statistical Analysis

The statistical results are represented as the Mean ± SD of the studied group. All the analyses were subjected to ANOVA with Tukey- Kramer multiple comparisons test.

3 Results

3.1 Serum Enzyme Levels

Administration of cisplatin to mice is found to induce a marked renal failure, characterized by a significant increase in serum urea and creatinine levels. As shown in table 1, the levels of serum creatinine and urea concentrations were significantly increased within the cisplatin-treated group when compared with the normal untreated group. Further, the concentrations of serum creatinine and urea in the mice treated by FA (200 mg/kg body weight) shows drastic reduction up to 60 % and 65.27 %, respectively, concerning the cisplatin-treated group.

Table 1 Effect of the FA treatment on the serum urea and creatinine levels in mice treated with cisplatin

Treatments	Urea (mg/dL)	Creatinine (mg/dL)
Normal (Untreated)	44.33±4.04	0.53±0.11
Cisplatin	151.66±7.63	2.5±0.45
Cis+FA 100 mg/Kg	120.33±4.50[a]	1.76±0.15[a]
Cis+FA 200 mg/Kg	99±3.60[a]	1.5±0.14[a]

Values given in the table are the mean of five replicates and are expressed as mean ±SD, 'a' represents $p< 0.001$ vs Cis control

3.2 Biochemical Measurements and Antioxidant Status

MDA (Malondialdehyde) was measured as a marker of lipid peroxidation and an indicator of oxidative injury. The MDA levels in nephro tissue were increased significantly within the cisplatin-treated group compared with the untreated control group. The increase in MDA by cispaltin was significantly attenuated by the administration of FA (Figure 2). Different antioxidant enzymes were examined in the kidney tissue from all the groups. The Cis-treated mice showed a significant decrease in SOD, GPX, and GSH activities compared with the untreated control (Table 2). The decrease in activities of SOD, GPX, and GSH due to cisplatin was suppressed by the treatment of FA.

In tumor tissues, the MDA levels were increased significantly within the Cis treated group compared with the untreated control group. The post administration of FA together with Cis effectively potentiated the formation of MDA in an exceedingly significant manner (Figure 3). The antioxidant enzymes such as GPx, SOD, and GSH activities in the tumor tissues were significantly reduced within the Cis and ferulic acid treated group compared to the respective control (Table 3).

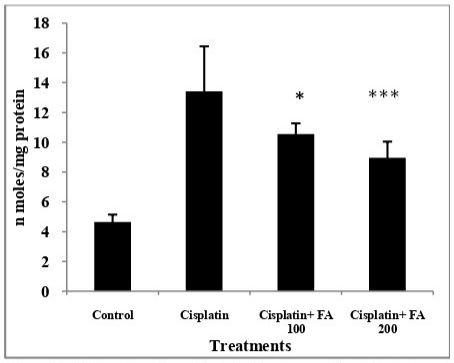

Figure 2 Effect of ferulic acid administration on Cisplatin-induced lipid peroxidation (MDA formation) in kidney tissues of mice. (* indicate $p<0.05$ and *** indicate $p<0.001$ when compared with the Cis alone treated group)

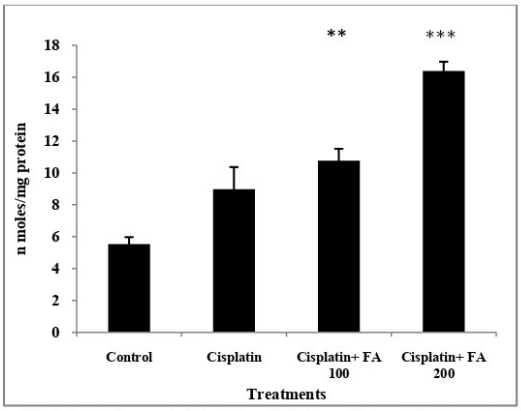

Figure 3 Effect of Ferulic acid administration on cisplatin- induced lipid peroxidation (MDA formation) in tumor tissues of mice. (*** indicate p<0.001 vs Cis control, ** indicate p< 0.01 vs Cis control)

Table 2 Effect of *p.o.* administration of FA on cisplatin-induced damages to renal antioxidant enzymes

Treatments	GPx (U/mg protein)	GSH (n moles/ mg protein)	SOD (unit/mg protein)
Normal (Untreated)	26.9±1.34	30.08±4.77	14.22±2.07
Cisplatin	12.61±2.14	10.41±1.67	3.96±0.19
Cis+FA 100 mg/Kg	13.39±1.08^{ns}	13.6±0.33^{ns}	5.36±0.52^{ns}
Cis+FA 200 mg/Kg	17.75±1.35^{a}	16.08±0.49^{b}	7.6±1.54^{a}

Values given in the table are the mean of five replicates and are expressed as mean ±SD, 'a' represent p< 0.001 vs Cis control; 'b' represent p< 0.01 vs Cis control, 'ns' represent p>0.05 vs Cis control

Table 3 Effect of ferulic acid (*p.o*) administration on the Cisplatin induced depletion of antioxidant levels in tumor tissues of mice

Treatments	GPx (U/mg protein)	GSH (n moles/ mg protein)	SOD (unit/mg protein)
Normal (Untreated)	28.06±2.32	27.89±2.59	10.68±1.25
Cisplatin	18.60±0.88	16.08±0.77	6.36±0.51
Cis+FA 100 mg/Kg	15.26±2.66^{b}	12.4±0.28^{b}	5.25±0.57^{c}
Cis+FA 200 mg/Kg	10.71±1.70^{a}	9.06±1.40^{a}	4.7±0.26^{b}

Values are expressed as mean ± SD (n = 5). 'a' represent p< 0.001 vs Cis control ; 'b' represent p< 0.01 vs Cis control ; 'c'represent p< 0.05 vs Cis control

3.3 Morphological Study

Histopathological analysis of cisplatin-treated mice kidney showed a decreased cellularity of the glomeruli, and edema of the epithelial cells lining in the renal tubules as compared to control (Figure 4A), and also the interstitial tissue as evident from figure 4B. The renal tissues of cisplatin treated mice administered with FA showed normal glomerular, renal tubules, and interstitial tissue appearance. In tumor bearing mice treated with FA and Cis, shows a significant reduction in the severity of renal degeneration was observed (Figure 4 C and D).

The histopathological examination of tumor tissues is detailed in figure 5. Tumor tissues of control animals showed a compact arrangement of carcinomatous cells having clear cytoplasm and hyperchromatic nuclei. Whereas the Cis-treated animals showed many degenerating cells, stroma showed extensive areas of necrosis and hemorrhage. Tumor bearing animals administered with FA and Cis showed a significant increase in the number of degenerating cells. Stroma showed extensive areas of necrosis, cellular integrity was lost in many regions. The results showed that FA potentiates the cytotoxic action of cisplatin in tumor cells (Figure 5 C and D).

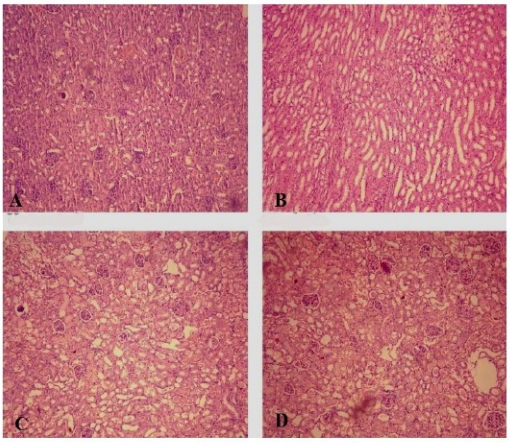

Figure 4 Histopathology study of the representative sections of kidney tissue of tumor bearing mice (A) untreated (B) Cisplatin 12 mg/kg (C) Cis+FA 100 mg/kg and (D) Cis+FA 200 mg/kg

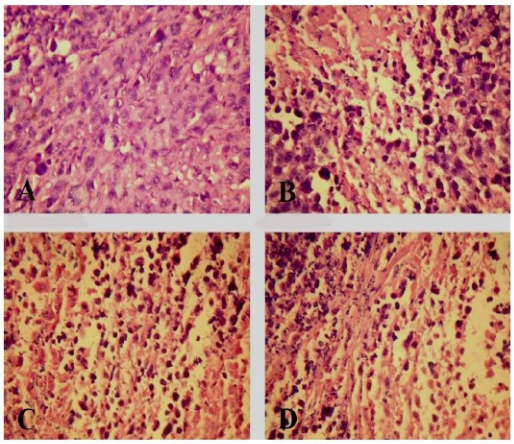

Figure 5 Histopathology study of the representative sections of tumor tissue of tumor bearing mice (A) untreated (B) Cisplatin 12 mg/kg (C) Cis+FA 100 mg/kg and (D) Cis+FA 200 mg/kg

4 Discussion

Cisplatin is the most widely used anti-cancer agent, best against many solid organ tumors. The major side-effect of cisplatin is nephrotoxicity and its dose is the main limiting factor for its clinical use (Rana et al., 2016). Results of previous studies have shown that a minimum dose of cisplatin (5 mg/kg body weight, *i.p*) was sufficient to induce nephrotoxicity in rats (Boogaard et al., 1991). The highly reactive platinum complexes of Cisplatin binds with nucleophilic DNA *via* inter and intra-strand crosslinking with guanine nucleotide (Holditch et al., 2019). These events end up in denaturation of DNA and cell cycle arrest. Cisplatin *via* cytochrome P450 (CYP) in microsome and mitochondrial dysfunctioning creates reactive oxygen species (ROS) and damages the renal tissue (Nematbakhsh et al., 2017).

One of the foremost important mechanisms observed in cisplatin toxicity is oxidative stress (Mohamed & Shenouda, 2021). Under

normal physiological conditions, production and removal of reactive oxygen species (ROS) in cells are controlled by the endogenous scavenging system including catalase, superoxide dismutase, and reduced glutathione (Pabla, & Dong, 2008). Under oxidative stress conditions, ROS levels are elevated, and many cellular structures (proteins, lipids, DNA) can be damaged. Increased oxidative stress can make cellular injury and necrosis in the kidney and other tissues. Previous studies (Kim et al., 2012; Saisruthi & Sreedevi, 2017) suggested the key role of lipid peroxidation and antioxidant enzymes like SOD and CAT in free radical metabolisms. These antioxidants enzymes scavenge free radicals or convert them into non-toxic end products and are measured as a first-line cellular defense mechanism against oxidative damage. The second line of antioxidant defense consists of the non-enzymatic scavengers such as GSH, which scavenge remaining free radicals escaping from the primary line of antioxidant enzymes defense (Mody et al., 2020).

Administration of cisplatin shows a significant increase in blood creatinine and serum urea concentrations compared to normal, which indicates the intrinsic acute renal failure (Joy & Nair, 2008; Wu & Huang, 2018). The results of the current study showed that cisplatin administration to tumor bearing mice significantly raised the levels of lipid peroxidation and caused depletion of GSH, SOD, and GPx in renal tissues. The declined antioxidant status partially explains the mechanism of nephrotoxicity induced by cisplatin. The renal accumulation of platinum, covalent binding of renal protein also play a crucial role in nephrotoxicity (Hanigan & Devarajan, 2003). The decreased concentration of GSH increases the sensitivity of organs to oxidative and chemical injury. Treatment of FA (100 & 200 mg/kg body weight, *p.o*) together with cisplatin could significantly prevent the depletion of these renal antioxidant systems. Treatment with FA rendered protection because of the increase in GSH concentration could keep the renal cells from oxidants attack. Moreover, the protection of GSH is additionally by forming the substrate for the GPx activity that may react directly with various aldehydes produced from the peroxidation of membrane lipids. Treatment with FA and cisplatin enhances the activity of Se-GPx (selenium-dependent GPx) compared to the cisplatin alone-treated animals. Thus the improved GPx activity partially explained the role in nephro protection. The decreased activity of SOD and GPx in renal tissues enhanced the lipid peroxidation in cisplatin treated group (Jia et al., 2011; Moreno-Gordaliza et al., 2018). Treatment with FA prevented lipid peroxidation by enhancing the renal SOD and GPx activities.

The main prerequisite for any compound to be used as a nephroprotective agent during the treatment of cancer is that it should not interfere with the antitumor activity of the chemotherapy (Fang et al., 2021). Various studies in animals and humans confirmed that FA could demonstrate anticancer properties (Divakaran & Nair, 2012). Ferulic acid is effective in protecting against oxidative damage in tissues, and also suppresses the formation of carcinogens like nitrosamines. The MDA levels of tumor tissues in FA and Cis-treated animals showed an increase in the level when compared to the untreated control animals. The antioxidant levels in tumor tissues were also found to be significantly reduced by the administration of cisplatin and ferulic acid. The results significantly showed the antitumor potential of the combination treatment (Humanes et al., 2012). Moreover, the histopathological reports suggest that post-treatment of FA greatly inhibited the Cis-induced changes in kidney tissue supporting the protective action of FA against Cis-induced nephrotoxicity and offer maximum destruction to tumor tissues (Zhao & Dai, 2020).

Conclusion

The results of this study can be concluded that ferulic acid (100 &200 mg/kg body weight) rendered significant protection against cisplatin-induced oxidative renal damage. FA offers renal protection by preventing the cisplatin-induced decline of renal antioxidant status. The protective effect may result to direct scavenging of the electrophilic free radicals by antioxidant FA. Thus, the present study indicates the potential antitumor use of FA in preventing cisplatin-induced renal toxicity in clinical practice. The combination treatment of ferulic acid and cisplatin in tumor bearing mice restored all the biochemical parameters altered by the cytotoxic drug nearer to normal levels in renal tissues. FA protected the kidney tissues from Cis-induced renal toxicity by enhancing the endogenous antioxidant levels, lowering the serum urea and creatinine levels. Thus prevents the damages of renal tissues. The combined strategy showed high antitumor activity as evident from MDA formation, antioxidant enzyme levels, and histopathological examination. The strategy ensures a secure and efficient anticancer treatment modality. Thus, the combination treatment improves the chemotherapeutic index of cytotoxic anticancer drug, Cisplatin.

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Conflict of interest

The authors report no conflicts of interest. The authors only are answerable for the content and inscription of the paper

References

Ali BH, AlMoundhri MS (2006) Agents ameliorating or augmenting the nephrotoxicity of cisplatin and other platinum

compounds: a review of some recent research. Food and Chemical Toxicology 44: 1173–1183.

Allen LC, Michalko K, Coons C (1982) More on cephalosporin interference with creatinine determinations. Clinical chemistry 28 (3): 555-556.

Awdishu L, Mehta RL (2017) The 6R's of drug induced nephrotoxicity. BMC Nephrology 18:124.

Batista R (2014) Uses and potential applications of ferulic acid. In: Warren B (ed). Ferulic acid: antioxidant properties, uses and potential health benefits, 1 Ed. Nova Science Publishers, Inc., New York, USA 39–70.

Boogaard PJ, Lempers EL, Mulder GJ, Meerman JHN (1991) 4-methylthiobenzoic acid reduces cisplatin nephrotoxicity in rats without compromising anti-tumor activity. Biochemical Pharmacology 41: 1997–2003.

Buege AJ, Aust SD (1978) Microsomal Lipid Peroxidation. Methods in Enzymology 52: 302-310.

Divakaran SA, Nair CKK (2012) Amelioration of Doxorubicin Induced Cardiotoxicity in Tumor Bearing Mice by Ferulic Acid: a Mechanistic Study at Cellular and Biochemical Level. International Journal of Tumor Therapy 1(2): 6-13.

Fang C, Lou D, Zhou L., et al. (2021) Natural products: potential treatments for cisplatin-induced nephrotoxicity. Acta Pharmacologica Sinica. https://doi.org/10.1038/s41401-021-00620-9.

George SP, Anushree CS (2014) Drug-induced impairment of renal function. International Journal of Nephrology and Renovascular Disease 7: 457–68.

Giaccone G (2000) Clinical perspectives on platinum resistance. Drugs 59:9–17.

Hafemann DG, Sunde RA, Hoekstra WG (1974) Effect of dietary selenium on erythrocyte and live glutathione peroxides in the rat. Journal of Nutrition 104: 580-87.

Hanigan MH, Devarajan P (2003) Cisplatin nephrotoxicity: molecular mechanisms. Cancer Therapy 1-47.

Holditch SJ, Brown CN, Lombardi AM, Nguyen KN, Edelstein CL (2019) Recent Advances in Models, Mechanisms, Biomarkers, and Interventions in Cisplatin-Induced Acute Kidney Injury. International Journal of Molecular Sciences 20: 3011.

Humanes B, Lazaro A, Camano S, Gordaliza EM, Lazaro JA, Codesido MB, Lara JM, Ortiz A, Gomez MG, Vasallo PM, Tejedor A (2012) Cilastatin protects against cisplatin-induced nephrotoxicity without compromising its anticancer efficiency in rats. Kidney International 82: 652–663; doi:10.1038/ki.2012.199

Jagieła J, Bartnicki P, Rysz J (2021) Nephrotoxicity as a Complication of Chemotherapy and Immunotherapy in the Treatment of Colorectal Cancer, Melanoma and Non-Small Cell Lung Cancer. International Journal of Molecular Sciences 22: 4618. https:// doi.org/10.3390/ijms22094618

Janakiraman M, Jayaprakash K (2015) Nephroprotective potential of medicinal plants: A Review. International Journal of Science and Research 4(9): 543-547.

Jia Z, Wang N, Aoyagi T, Wang H, Liu H, Yang T (2011) Amelioration of cisplatin nephrotoxicity by genetic or pharmacologic blockade of prostaglandin synthesis. Kidney International 79:77–88; doi:10.1038/ki.2010.331

Joy J, Nair CKK (2008) Amelioration of cisplatin induced nephrotoxicity in Swiss albino mice by Rubia cordifolia extract. Journal of Cancer Research and Therapeutics 4(3): 111-115.

Kassirer JP (1971) Clinical evaluation of kidney function-glomerular function. The New England Journal of Medicine 285 (7):385-389.

Kim J, Long KE, Tang K, Padanilam BJ (2012) Poly(ADP-ribose) polymerase 1 activation is required for cisplatin nephrotoxicity. Kidney International 82: 193–203; doi:10.1038/ki.2012.64

Kumar N, Goel N (2019) Phenolic acids: Natural versatile molecules with promisingtherapeutic applications. Biotechnology Reports 24: e00370.

Lowry OH, Rosenblum NJ, Farr AL, Randall J (1951) Protein measurement with folin fenol reagent. Journal of Biological Chemistry193:265–75.

McCord JM, Fridovich I (1969) Superoxide dismutase enzyme function for erythrocuprein (hemocuprein). Journal of Biological Chemistry 244: 6049-6056.

Mody H, Ramakrishnan V, Chaar M, Lezeau J, Rump A, Taha K, Lesko L, Ait- Oudhia S (2020) A Review on Drug-Induced Nephrotoxicity: Pathophysiological Mechanisms, Drug Classes, Clinical Management, and Recent Advances in Mathematical Modeling and Simulation Approaches. Clinical Pharmacology in Drug Development 9(8): 896-909.

Mohamed HZ, Shenouda MB (2021) Amelioration of renal cortex histological alterations by aqueous garlic extract in gentamicin induced renal toxicity in albino rats: a histological and immunohistochemical study. Alexandria Journal of Medicine 57 (1): 28-37.

Moreno-Gordaliza EM, Esteban-Fernández D, Alberto Lázaro A, Aboulmagd S, Humanes B, Tejedor A, Linscheid MW, Gómez-Gómez MM (2018) Lipid imaging for visualizing cilastatin amelioration of cisplatin-induced nephrotoxicity. Journal of Lipid Research 59: 1561-1574.

Nematbakhsh M, Pezeshki Z, Jazi FE, Mazaheri B, Moeini M, Safari T, Azarkish F, Moslemi F, Maleki M, Rezaei A, Saberi S, Dehghani A, Malek M, Mansouri A, Ghasemi M, Zeinali F, Zamani Z, Navidi M, Jilanchi S, Shirdavani S, Ashrafi F (2017) Cisplatin-Induced Nephrotoxicity; Protective Supplements and Gender Differences. Asian Pacific Journal of Cancer Prevention 18 (2): 295-314.

Ojha S, Venkataraman, B, Kurdi A, Mahgoub E Sadek B, Rajesh M (2016) Plant-Derived Agents for Counteracting Cisplatin-Induced Nephrotoxicity. Oxidative Medicine and Cellular Longevity Article ID 4320374. doi: 10.1155/2016/4320374.

Pabla N, Dong Z (2008) Cisplatin nephrotoxicity: Mechanisms and renoprotective strategies. Kidney International 73: 994–1007. doi:10.1038/sj.ki.5002786

Panche AN, Diwan AD, Chandra SR (2016) Flavonoids: an overview. Journal of Nutritional Science 5:e47. doi: 10.1017/jns.2016.41.

Rad AK, Mohebbati R, Hosseinian S (2017) Drug-induced Nephrotoxicity and Medicinal Plants. Iranian journal of Kidney Disease 11:169-79.

Rana MA, Khan RA, Nasiruddin M, Khan AA (2016) Amelioration of Cisplatin-Induced Nephrotoxicity by Ethanolic Extract of Bauhinia purpurea: An in vivo Study in Rats. Saudi Journal of Kidney Disease and Transplantation 27(1):41-48.

Saisruthi K, Sreedevi A (2017) Amelioration of Cisplatin- Induced Nephrotoxicityby Roots of Anthocephalus cadamba. Biomedical & Pharmacology Journal 10(3): 1433-1439.

Sales GT, Foresto RD (2020) Drug-induced nephrotoxicity. Revista da Associacao Medica Brasileira 66(1): 82-90.

Sreenivasan M, Sudheer AR, Menon VP (2007) Ferulic acid : Therapeutic Potential Through its antioxidant property. Recent Advances In Indian Herbal Drug Research. Journal of Clinical Biochemistry and Nutrition 40: 92-100.

Wu H, Huang J (2018) Drug induced nephrotoxicity: Pathogenic mechanisms, Biomarkers and Prevention strategies. Current Drug Metabolism 19 (7): 559-567.

Zhao Y, Dai W (2020) Effect of Phloretin Treatment Ameliorated the Cisplatin-Induced Nephrotoxicity and Oxidative Stress in Experimental Rats. Pharmacognosy Magazine 16(69): 207-2013.

Amelioration of Paracetamol-induced Hepatotoxicity in Mice with Ferulic acid

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Abstract— Paracetamol (PAR) is a widely used analgesic used in therapeutic purposes but its use is limited by hepatotoxicity. The present work concerns on the ability of ferulic acic (FA) to offer protection against acute hepatotoxicity induced by paracetamol (150 mg/kg) in Swiss albino mice. Oral administration of FA (100 mg/kg *p.o* and 200 mg/kg *p.o*) offered a significant dose dependent protection against paracetamol induced hepatotoxicity as assessed in terms of biochemical and histopathological parameters. The paracetamol induced elevated levels of serum marker enzymes such as serum glutamate pyruvate transaminase (GPT), serum glutamate oxaloacetate transaminase (GOT), alkaline phosphatase (ALP), and bilirubin in peripheral blood serum and deformed hepatic tissue architecture along with increased levels of lipid peroxides (LPO) and reduction of superoxide dismutase (SOD), catalase, reduced glutathione (GSH) and glutathione peroxidase (GPx) in liver tissue. Post administration of the FA after paracetamol insult restored the levels of these parameters to control (untreated) levels. Thus the present study revealed that FA offered protection against hepatotoxicity induced by paracetamol.

Keywords- Hepatotoxicity, Paracetamol, antioxidant, lipid peroxidation

1 INTRODUCTION

Liver is a very important organ in maintaining homeostasis of the body. It involved in almost every biochemical pathways connected to growth, to fight against disease, nutrient supply, energy provision and reproduction. It plays a pivotal role in regulating metabolism of endogenous as well as xenobiotic substances, secretion and storage. Liver has got the capacity to detoxicate toxic substances and manufacture useful chemical principles. Liver disease is a worldwide health problem. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders [1]. Most of the hepatotoxic chemicals harm liver cells mainly by producing reactive species which form covalent bond with the lipids of the tissue. Due to excessive exposure to hazardous chemicals, sometimes the free radicals generated are so high that they overpower the natural defencive system leading to hepatic damage and cause jaundice, cirrhosis and fatty liver [2]. Production of the reactive species manifest in tissue thiol depletion, lipid peroxidation, plasma membrane damage etc., leading into severe hepatic injury [3].

Acetaminophen or paracetamol (PAR) is one of the most widely used analgesics with few side effects when taken in therapeutic doses [4] and hepatotoxicity is a common consequence of acetaminophen overdose [5],[6]. A number of reports indicate that overdose of paracetamol can produce centrizonal hemorrhagic hepatic necrosis in humans and experimental animals [1]. Acetaminophen toxicity is caused by the reaction metabolite N-acetyl-p-benzoquinoneimine (NAPQI), which is partly metabolized by cytochrome P-450 [7]. This species causes severe oxidative damage and glutathione depletion leading to liver necrosis. An overdose of acetaminophen, causes elevated levels of toxic NAPQI metabolite, which can extensively deplete hepatocellular GSH and covalently modify cellular proteins resulting in hepatocyte death. It is generally recognized that at higher doses the drug induces lipid peroxidation and oxidative stress, each contributing to hepatocellular damage and also produces hepatic necrosis. Introduction of

cytochrome or depletion of hepatic glutathione is a prerequisite for acetaminophen -induced hepatotoxicity [8], [9].

Liver dysfunction is a clinically significant problem, for people of all the reported cases of acute liver failure [10]. Therefore very often hepatoprotective drugs are recommended to ameliorate liver problems. Herbal drugs are often used widely because of their effectiveness, fewer side effects and relatively low cost [11]. It has been reported that about 160 phyto constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotenoids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthenes from 101 plants have hepatoprotective activity [12], [13].

Phenolics are widely distributed in the plant kingdom and are integral part of human diet [14]. As a phenolic compound, Ferulic acid (FA or 4-Hydroxy-3-Methoxycinnamic Acid) (**Figure. 1**) play a major role in the body's defense against carcinogenesis by inhibiting the formation of N-nitroso compounds [15]. Moreover, FA is a strong scavenger of free radicals and it has been approved in certain countries as food additive to prevent lipid peroxidation [16]. FA is shown to preserve physiological integrity of the cells exposed to various stress. This can be attributed to the effective antioxidant property of FA. Usually phenolic compounds acts by scavenging free radicals and quenching the lipid peroxidative side chain. Phenolic compounds can act as free radical scavengers by virtue of their hydrogen donating ability and forming aryloxyl radicals [17]. Thus the present chapter concerns, prevention of paracetamol induced hepatotoxicity in mice by Ferulic acid.

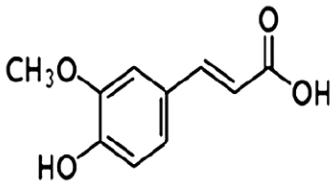

Figure. 1 Ferulic acid (FA)

2 MATERIALS AND METHODS

2.1 Animals

Female Swiss albino mice of 8-10 week old weighing 26-28g, selected from inbred group was used for hepatototoxicity studies and were kept under standard conditions of tempera-ture (25 ± 5ºC) and humidity in the Centre's Animal House Facility. The animals were given standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. All experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) consti-tuted by the Animal Welfare Division of Government of India.

2.2 Chemicals

Ferulic acid (FA), Nitroblue tetrazolium (NBT), riboflavin, reduced glutathione (GSH), 5-5' dithiobis-2- nitro benzoic acid (DTNB) were purchased from Sigma Chemical Company Inc., St. Louis, MO, USA. Paracetamol was obtained from Variety Pharmaceuticals (P) Ltd., Shornur, Kerala, India. EDTA was from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. H_2O_2 was from Merck India Ltd., Mumbai, India. Thiobarbituric acid (TBA) was purchased from Hi-media Laboratories, Mumbai, India. All other chemicals and reagents used in this study were of analytical grade procured from reputed Indian manufacturers.

2.3 Administration of Drugs

The animals were randomly divided into 5 groups of five each and treated as follows.

Group I - Untreated Control

Group II - Paracetamol control, administered with paracetamol (150 mg/kg) as single dose after 18 h starvation.

Group III - Silymarin (75 mg/kg body weight) one hour after paracetamol administration

Group IV- Ferulic acid 100 mg/kg b.w (in 0.1 ml distilled water *p.o*) one hour after paracetamol administration

Group V- Ferulic acid 200 mg/kg b.w (in 0.1 ml distilled water

p.o) one hour after paracetamol administration

Group I administered with distilled water alone was kept as untreated control. Group II was given acetaminophen (150 mg/kg body weight) as single dose orally after 18 h starvation and kept as control. Group III served as reference control, received Silymarin (75mg/kg body weight), a clinically used hepatoprotective drug, one hour after paracetamol administration. Ferulic acid was administered to Group IV and V animals, one hour after paracetamol (150 mg/kg) administration. The test drugs and paracetamol were administered orally to all animals. After 24 hours of paracetamol feeding, the animals were sacrificed and blood was collected by direct cardiac puncture under light ether anesthesia and serum was separated for the biochemical estimations. The liver was removed, washed with ice-cold PBS, weighed and morphological changes were observed. Then liver homogenates (10% w/v) was analyzed for antioxidant status.

2.4 Evaluation of liver function enzymes in serum

Serum was used for the determination of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), Serum albumin and serum Bilirubin.

2.5 Determination of antioxidant status in the liver

Liver was excised after sacrificing the animals and washed with ice-cold PBS and 10% homogenate was prepared in PBS (pH 7). A part of this homogenate was used for the determination of reduced glutathione (GSH). Rest of the homogenate was centrifuged at 1,000 rpm for 10 min for removing the cell debris, unbroken cells, nuclei, erythrocytes and mitochondria. The supernatant was used for the estimation of superoxide dismutase (SOD), glutathione peroxidise (GPx) and malondialdehyde (MDA). The protein was estimated by Lowry's method.

2.6 Histopathological studies

Small sections taken from each lobe of the liver were fixed immediately in 10% neutral formalin for a period of at least 24 hr, and embedded in paraffin wax. Thin sections of 5 micron thickness were made using a microtome and later stained with haematoxylin-eosin. The histopathological examinations were carried out at Sudharma Metropolis Pathological Laboratory, Thrissur, Kerala, India.

2.7 Statistical Analysis

The results are presented as Mean ± SD of the studied group. Statistical analyses of the results were performed using ANOVA with Tukey-Kramer multiple comparisons test.

3 RESULTS

3.1 Evaluation of liver function enzymes in serum

Administration of acetaminophen (150 mg/kg body weight) results in manifestation of hepatotoxicity as can be revealed from the elevated levels of serum marker enzymes (**Table.1**). The levels of serum GOT (46.06±9.4), GPT (45.5± 10.35), ALP (110.26 ± 22.16), bilirubin (0.72±0.04) and albumin (3.27±0.27) were elevated in acetaminophen treated animals GOT (250.75±11.78), GPT (258.5±12.60), ALP (213.6 ± 22.35), bilirubin (1.67±0.88) and albumin (4.91±0.12)] when compared to control, indicating liver damage. Silymarin is a known hepatoprotective agent and administration of this compound at a dose rate of 75 mg/kg body weight prevented the onset of hepatotoxicity induced by acetaminophen. Administration of FA after paracetamol treatment prevented elevated levels of serum marker enzymes in a dose dependent manner. Post treatment of FA significantly reversed the levels of GOT, GPT, ALP, bilirubin and albumin when compared to acetaminophen alone treated mice.

Table 1. Effect of ferulic acid on serum marker enzymes in paracetamol induced hepato toxicity in mice. values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. 'a' represents p< 0.001 vs PAR control; 'b' represents p< 0.01 vs PAR control

Treatments	SGOT (U/L)	SGPT(U/L)	ALP (IU/L)	BILIRUBIN (mg/dL)	ALBUMIN(g/dL)
Control	46.06±9.4	45.5±10.35	110.26±22.16	0.72±0.04	3.27±0.27
PAR(150mg/Kg)	250.75±11.78	258.5±12.60	213.6±22.35	1.67±0.88	4.91±0.12
PAR +Syl	80.5±2.64[a]	82.25±8.30[a]	162.6±24.60[a]	0.95±0.1[b]	3.82±0.16[a]
PAR+FA(100 mg/kg)	73.25±2.98[a]	68.5±11.4[a]	152.6±20.40[a]	0.73±0.11[b]	3.6±0.02[a]
PAR+FA(200 mg/kg)	66.25±4.34[a]	60.5±10.20[a]	134.3±10.06[a]	0.68±0.1[a]	3.3±0.1[a]

3.2 Biochemical Measurements and Antioxidant Status

The total antioxidant activity, as a measure of antioxidant status, was significantly decreased in the liver tissue of the acetaminophen treated group (**Table.2**). Acetaminophen treatment caused a significant decrease in the level of SOD, GPx and GSH in liver tissue when compared with control group. The activities of all the antioxidant enzymes were significantly enhanced in the animals treated with FA. The post-treatment with FA (100 and 200 mg/kg) resulted in significant increase of SOD, GPx and GSH in a dose dependent manner. The silymarin treated animals also showed a significant increase in antioxidant enzymes levels compared to acetaminophen treated animals.

Generation of malondialdehyde (MDA) was measured as a marker of lipid peroxidation (LPO) and an indicator of oxidative injury. Analysis of LPO levels by thiobarbituric acid reaction showed a significant increase in the acetaminophen treated mice. The post treatment with FA (100 and 200 mg/kg) significantly prevented the increase in LPO level which was brought to near normal level. The effect of FA was comparable with that of standard drug silymarin (**Figure.2**).

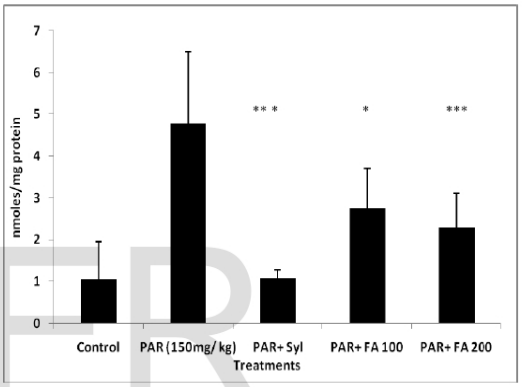

Figure 2. Effect of administration of ferulic acid on paracetamaol-induced lipid peroxidation (MDA formation) in liver tissues of mice. (* indicate p<0.05 and *** indicate p<0.001 when compared with the PAR alone treated group)

3.3 Morphological Study

Histological analysis was performed to confirm FA mediated hepatoprotection. In the untreated control animals the morphological observations of liver tissues showed normal architecture of hepatic cells with clear cytoplasm and slightly dilated central veins. Here the kupffer cells are appeared normal and all cells having normal large nuclei (**Figure.3[A]**). In acetaminophen treated animals the liver tissue shows distorted architecture. Central veins are markedly congested. There are extensive area of necrosis and haemorrhage. Nuclei are distorted and some of the hepatocytes contain vacuolated cytoplasm (**Figure.3[B]**). The silymarin treated animals also showed similar results as that of FA but here the sinusoidal spaces are compressed. And some areas

showed minimal nuclear pleomorphism (**Figure 3.[C]**). In the extract post-treated animals with FA (100 and 200 mg/kg), the normal architecture of the liver tissue can be seen (**Figure3. [D&E]**). Here central veins are slightly dilated and hepatocytes are slightly pleomorphic of them have larger nuclei. Sinusoidal space appears normal, and also the kupffer cells. Thus the histological observations supported the results obtained from liver enzyme assays.

Table 2. Effect of post administration of FA on paracetamol induced depletion of GPx, GSH, and SOD levels in liver tissue of mice. Values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. 'a' represents p< 0.001 vs PAR control; 'b' represents p< 0.01 vs PAR control

Treatments	GPx(Unit/mg protein))	GSH(n moles/mg protein)	SOD(Unit/mg protein)
Control	25.4±1.39	24.16±2.33	15.26±1.8
PAR(150mg/Kg)	9.56±2.63	8.39±1.1	5.75±0.2
PAR +Syl	12.38±1.04ª	12.6±0.08ª	8.73±0.17ᵇ
PAR+FA(100 mg/kg)	16.3±1.52ª	17.87±0.85ª	9.75±1.70ª
PAR+FA(200 mg/kg)	19.25±1.70ª	20.33±1.52ª	11.3±1.15ª

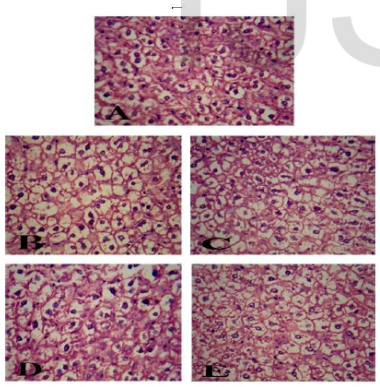

Figure.3 Histopathology. Representative sections of liver tissue of mice **(A)** untreated **(B)** PAR(150mg/kg) **(C)** PAR+Syl and **(D)** PAR +FA 100 mg/kg **(E)** PAR +FA 200 mg/kg

4 DISCUSSION

After an overdose of paracetamol, elevated levels of the toxic NAPQI metabolite are generated, which can extensively deplete hepatocellular GSH and covalently modify cellular proteins resulting in hepatocyte death. Assessment of liver function is made by estimating the activities of serum GPT, GOT and bilirubin which are present higher concentration in cytoplasm. When there is hepatopathy, these molecules leak into the blood stream in compliance with the extent of liver damage [18], [19]. Bilirubin is one of the most useful clinical clues to the level of damage of necrosis and its increase is a measure of binding, conjugation and excretory capacity of hepatocyte.

Paracetamol overdose result in toxic liver damage leading to potentially fatal, hepatic centrilobular necrosis and liver failure [8] associated with metabolic activation by the P-450 system to form a quinine imine metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), which covalently binds to proteins and other macromolecules to cause cellular damage. At low doses, NAPQI is well detoxified, mainly by conjugation with glutathione. At higher doses glutathione becomes depleted and the excess of NAPQI arylates and oxidizes hepatic proteins [20]. Oxidative stress is also a mechanism that has been postulated to be important in the development of paracetamol toxicity. Toxicity begins with the change in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures [21]. Assessment of liver function can be made by estimating the activities of serum Alanine amino transferase (ALT), Aspartate Amino transferase (AST) and Bilirubin which are originally present higher concentration in cytoplasm. When there is heaptopathy, these leaks into the blood stream in conformity with the extent of liver damage [18], [22]. The elevated level of marker enzymes seen in the group II, paracetamol treated mice shows the extensive liver damage induced by toxin.

The experimental results indicate that FA were able to delimit the liver damage induced by acetaminophen. The protective effect was found to be significant and can be compared with the standard hepatoprotective drug silymarin [23]. Accidental or incidental acetaminophen overdose may be associated with toxic liver damage leading to potentially fatal, hepatic centrilobular necrosis and liver failure. Oxidative stress is another mechanism that is said to

be important in the progress of acetaminophen toxicity. It was observed that the levels of cellular antioxidant enzymes and molecules are decreased significantly in paracetamol treated animals. This further results in the peroxidative damage to membranes results in the leakage of enzymes, and metabolites to circulation. In the present study, it was observed that, the animals treated with paracetamol showed elevated levels of serum markers such as SGPT, SGOT, ALP and bilirubin. Normally, a higher concentrations of SGOT and ALP are present in liver. Due to hepatocyte necrosis or abnormal membrane permeability, these enzymes are released from the cells and their levels in the blood increases. SGPT is a sensitive indicator of severe liver damage and increased level of this enzyme in non hepatic diseases is unusual [22].

Oral administration of FA exhibited a significant reduction in acetaminophen induced levels of serum GOT, GPT, ALP, and bilirubin which is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage. This could be an evidence for the protective effect of FA which helps to maintain the functional integrity of hepatic cells. More over the decrease in serum bilirubin after treatment with the FA in liver damaged mice induced by acetaminophen indicated their effectiveness in normal functional status of the liver. Thus above alterations can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells. The silymarin with a dose of 75 mg/kg, body weight has also provided a better inhibition of the elevated level of SGOT, SGPT, ALP, and serum bilirubin content. Thus the data obtained from the serum parameters of the present study clearly indicates that the antihepatotoxic activity of the FA probably through the correction of cellular integrity of hepatic cell and its regeneration.

Oxidative stress is considered to be associated with many diseases, including cell damage. Glutathione is one of the most abundant tripeptide, a non-enzymatic biological antioxidant present in the liver. Its functions are concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals and maintenance of membrane protein thiols and as a substrate for glutathione peroxidase (GPx) and GST [24]. We observed that the level of GSH was decreased in association with acetaminophen treatment and administration of FA significantly increased ($P< 0.001$) the level of glutathione in a dose dependent manner.

The increase in MDA level in liver of acetaminophen treated mice suggests provoked lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Lipid peroxidation has been postulated as being a destructive process in liver injury caused by acetaminophen administration [25], [26], [9]. The post treatment with FA reversed the increase in MDA levels more significantly ($P< 0.001$). The coincidence of antioxidant activity and protective effect on liver tissues after acetaminophen administration suggest that both free radical generation and lipid peroxidation may be involved in this type of drug injury process.

SOD and GPx enzymes are important scavengers of superoxide ion and hydrogen peroxide. These enzymes prevent generation of hydroxyl radical and protect the cellular constituents from oxidative damage [27], [13]. In the present study, it was observed that FA significantly increased the hepatic SOD activity in acetaminophen treated mice.

In conclusion, the results of this study suggest that in mice, post administration of FA possesses ameliorating effects on paracetamol induced hepatotoxicity. This shows that administration of FA can reduce reactive free radicals there for cause lesser oxidative damage to the tissues and improve the activities of the hepatic antioxidant enzyme. Hence it may be possible that the mechanism of hepatoprotection of FA is due to its antioxidant effect.

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REFERENCES

[1]. Ganguly P., Gupta AK., MajumderUK., Ghosa S, Hepatopro-

tective and antioxidant effects of naturally occurring with a steroid metal ion conjugates of Withania somnifera in paracetamol induced hepatotoxicity in rats, *Pharmacol online*., **2009**, 1, 1044-1056

[2]. Jyoti TM., Prabhu K., Lakshminarasu S., Ramachandra SS, Hepatoprotective and antioxidant activity of *Euphorbia antiquorum*, *Pharmacog magazine*., **2008**, 4:127-133.

[3]. Kanter M., Coskun O., Budancamanak M, Hepatoprotective effect of *Nigella sativa* L and *Utrica dioica* L on lipid peroxidation, antioxidant enzyme systems and liver enzymes in carbon tetrachloride treated rats, *World J Gastroenterol.*, **2005**, 11: 6684-6688.

[4]. Larson AM., Polson J., Fontana RJ, Acetaminopheninduced acute liver failure: results of a United States multicenter, prospective study, *Hepatology*, **2005**, 42:1364–1372.

[5]. Makin AJ., Williams R, Acetaminophen-induced hepatotoxicity: predisposing factors and treatments, *Adv Intern Med.*, **1997**,42:453–483.

[6]. Ratheea, D., Kambojb, A., Sachdevc, RK., Sidhuc, S, Hepatoprotective effect of Aegle marmelos augmented with piperine co-administration in paracetamol model. Brazilian journal of pharmacology 28 (**2018**) 65–72.

[7]. Thummel KE., Lee CA., Kunze KL., Nelson SD., Slattery JT, Oxidation of acetaminophen to N-acetyl-p-aminobenzoquinone imine by human CYP3A4, *Biochem Pharmacol.*,**1993**, 45: 1563-1569.

[8]. James LP., Mayeux RP., Hinson AJ, , Acetaminophen-Induced Hepatotoxicity, *Drug Metabolism and Disposition.*, **2003**, 31, 1499–1506.

[9]. Elkomy, A., Aboubakr, M., Soliman, A., Abdeen, A., Abdelkader, A., Hekal, H, Paracetamol induced hepatic toxicity and amelioration by cinnamon in rats. *International Journal of Pharmacology and Toxicology, 4 (2) (**2016**) 187-190 .*

[10]. Yamazaki Y., Kakizaki S., Horiguchi N., Takagi H., Mori M., Negishi M., Role of nuclear receptor CAR in carbon tetrachloride induced hepatotoxicity, *World J Gastroenterol.*, **2005**, 11: 5966-5972.

[11]. Valiathan MS, Healing plants, *Curr Sci.*, **1998**, 75: 1122-1127

[12]. Chatterjee TK. Medicinal plants with hepatoprotective properties, In Herbal Opinions, third ed. Books & Allied (P) Ltd., Calcutta, **2000**, 135.

[13]. Subramanya, SB., Venkataraman, B., Meeran, MFN., Goyal, SN., Chandragouda R. Patil, CR., Ojha, S, Therapeutic Potential of Plants and Plant Derived Phytochemicals against Acetaminophen-Induced Liver Injury. International journal of molecular sciences **2018**, 19, 3776; doi:10.3390/ijms19123776.

[14]. Graf, E, Antioxidant potential of ferulic acid, *Free Radical Biol Med.*, **1992**, 13, 435-48.

[15]. Kuenzig W., Chau J., Norkus E., Holowaschenko H., Newmark H., Mergens W., Conney A H, , Caffeic and ferulic acid as blockers of nitrosamine formation, *Carcinogenesis.*, **1984**,5, 309-313.

[16]. Srinivasan M., Rukkumani R., Ram Sudheer A., Menon VP, Ferulic acid, a natural protector against carbon tetrachloride-induced toxicity, *Fundam Clin Pharmacol.*, **2005**, 19(4):491-6.

[17]. Francisco A., Barberan T., Pierce B.H., Holcroft D.M., Kader A.A, Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing, *J. Agric. Food Chem.*, **2000**, 48(10): 4581-4589.

[18]. Nkosi CZ., Opoku AR., Terblanche SE, Effect of pumpkin seed (*Cucurbita pepo*) protein isolate on the activity levels of certain plasma enzymes in CCl4-induced liver injury in lowprotein fed rats, *Phytotherapy Research.*, **2005**,19, 341–345.

[19]. Hamza,RZ., Al-Harbi, MS, Amelioration of paracetamol hepatotoxicity and oxidative stress on mice liver with silymarin and Nigella sativa extract supplements. Asian Pac J Trop Biomed 2015; 5(7): 521–531.

[20]. Fountoulakis M., Berndt P., Boelsterli UA., Winter M., Suter LA, Two diamensional database of mouse liver proteins: changes in hepatic protein levels following treatment with acetaminophen or its nontoxic regioisimer 3-acetamidophenol, *Electrophoresis.*, **2000**,21, 2148-2161.

[21]. Recknagel RO, A new direction in the study of carbon tetrachloride hepatoprotective, *Life Science.*, **1983**,33, 401-408.

[22]. Mahmood, ND., Mamat, SS., Kamisan, FH., Yahya, F., Kamarolzaman, MFF., Nasir, N., Mohtarrudin, N., Md. Tohid, SF.,

Zakaria, ZA, Amelioration of Paracetamol-Induced Hepatotoxicity in Rat by the Administration of Methanol Extract of *Muntingia calabura* L. Leaves. BioMed Research International Volume 2014, Article ID 695678, 10 pages http://dx.doi.org/10.1155/2014/695678.

[23]. Hamza,RZ., Al-Harbi, MS, Amelioration of paracetamol hepatotoxicity and oxidative stress on mice liver with silymarin and Nigella sativa extract supplements. Asian Pac J Trop Biomed 2015; 5(7): 521–531.

[24]. Prakash J., Gupta S., Kochupillai V., Singh N., Gupta YK., Joshi S, Chemo preventive activity of *Withania somnifera* in experimentally induced fibro sarcoma tumors in Swiss albino mice, *Phytother Res*., **2001**, 15: 240-244.

[25]. Muriel P., Garciapina T., Pierz-Alvarez V., Murelle M, Silymarin protects against acetaminophen-induced lipid peroxidation and liver damage, *J Appl Toxicol*., **1992**, 12: 439-442.

[26]. Adeneye AA., Benebo AS, Ameliorating the effects of acetaminophen-induced hepatotoxicity in rats with African red palm oil extract. *Asian Journal of Traditional Medicines*., **2007**, 2 (6), 244-249.

[27]. Scott MD., Lubin BH., Zuo L., Kuypers FA, Erythrocyte defense against hydrogen peroxide: Preeminent importance of Catalase, *J Lab Clin Med*., **1991**, 118: 7-16.

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Anti Inflammatory Activity of Ferulic Acid against Carrageenan and Formalin Induced Paw Edema in Swiss Albino Mice

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Abstract: ***The anti-inflammatory activity of ferulic acid (FA) was evaluated using carrageenan and formalin-induced paw edema models in Swiss albino mice. The anti-inflammatory activity was found to be dose dependent in both paw edema model. Administration of the FA showed significant ($p<0.001$) inhibition of paw edema, 28% and 37.5% on 6th hour at the doses of 100 and 200 mg/kg body weight respectively in Carrageenan induced paw edema model. Similar pattern of paw edema inhibition was seen in formalin-induced paw edema model. The maximum percentage inhibition in paw edema was 31.4 % and 46.87 % on 6th day at the doses of 100 and 200 mg/kg, respectively. The results of present study demonstrate that FA possess significant anti-inflammatory potential.***

Keywords: anti-inflammatory activity, paw edema, carrageenan, formalin

1. Introduction

Inflammation is a reaction of living tissues towards injury and it comprises systemic and local responses (Joel *et al*.,1998). The generation of free radicals, particularly reactive oxygen species and their high activity plays an important role in the progression of a great number of pathological disturbances like inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, cancer, Parkinson's disease, Alzheimer's disease, etc (Nickavar *et al*., 2007; Hafeez *et al*., 2013). Many present day diseases are reported to be due to the shift in balance of pro-oxidant and antioxidant homeostasis in the body (Schulz *et al*., 2000). Reactive oxygen species (ROS), which include superoxide radical, hydrogen peroxide (H_2O_2) and the hydroxyl radical (-OH) are well documented as cytotoxic intermediates. These ROS differ significantly in their interactions and can cause extensive cellular damage such as nucleic acid strand scission (Adelman *et al*.,1988; Mohanty*a et al.,* 2015), modification of polypeptides, lipid peroxidation etc leading to cell membrane disintegration, membrane protein damage and DNA mutation.

Inflammation activates a range of inflammatory cells that induce and trigger several oxidant generating enzymes such as NADPH oxidase, nitric oxide synthase, myeloperoxidase and eosinophil peroxidise etc. These enzymes generate high concentrations of different free radicals and oxidants such as superoxide anion, nitric oxide, nitroxyl, nitrogen dioxide, hydrogen peroxide etc, which react with each other to produce more potent reactive oxygen and nitrogen species that can damage DNA, RNA, lipids and proteins and also leads to multistage carcinogenesis (Ohshima *et al*., 2003). Therefore much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit peroxidation and to protect DNA and other macromolecules from damage due to free radicals (Wu *et al*., 2017).

In spite of our dependence on modern medicine and the tremendous advances in synthetic drugs, a large number of world populations cannot afford the products of the western pharmaceutical industry and have to rely upon the use of traditional medicines, which are mainly derived from plant materials. A large number of aromatic, spicy, medicinal and other plants have chemical compounds, exhibiting antioxidant properties. Well known source of natural antioxidants are mainly, plant phenolics that may seen in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Naik and Krishnamurthy, 2018). These antioxidant compounds hold anti-inflammatory, antiatherosclerotic, antitumor, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent (Sala *et al*., 2002). Many anti-inflammatory, digestive, anti-necrotic, neuroprotective, and hepatoprotective drugs have recently known to have an antioxidant and/or radical scavenging mechanism as part of their activity (Lin *et al*., 2000; Repetto and Lesuy, 2002). Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolic are increasingly of interest in the food industry, because they retard oxidative degradation of lipids and thereby improve the quality and nutritive value of food (Sevgi *et al*., 2015). Hence interest in natural antioxidants, especially phytochemicals has greatly increased in recent years (Kumar *et al*., 2010).

Ferulic acid (FA) or 4-Hydroxy-3-Methoxycinnamic Acid, derivative of cinnamic acid with molecular formula $C_{10}H_{10}O_4$, a ubiquitous natural phenolic phyto chemical present in seeds, leaves, its free form and covalently conjugated to the plant cell wall polysaccharides, glycoprotein, polyamines, lignin and hydroxyl fatty acids (**Figure. 1**). As an antioxidant, FA play a major role in the body's defense against carcinogenesis by inhibiting the formation of N-nitroso compounds (Dai and Mumper, 2010). Moreover, FA is a strong scavenger of free radicals and it has been approved in certain countries as food

additive to prevent lipid peroxidation (Roginsky & Lissi, 2005; Balasubashini *et al*., 2004). The present paper reports the investigation on the anti-inflammatory properties of FA.

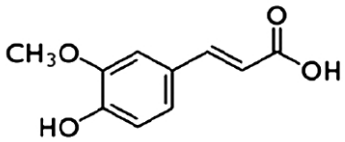

Figure 1: Ferulic acid (FA)

2. Materials and Methods

Animals

Female Swiss albino mice weighing 22- 25 g were obtained from the Small Animal Breeding Section (SABS), Mannuthy, Thrissur, Kerala. They were maintained under standard conditions of temperature and humidity in the Centre's Animal House Facility. The animals were given standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. All animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

Studies on anti-inflammatory activity

Carrageenan induced acute paw edema

Animals were divided into 4 groups comprising of 5 animals in each group. For all groups acute inflammation was induced by sub plantar injection of 0.02 ml freshly prepared 1% of carrageenan in normal saline in the right hind paw of mice (Winter *et al.*, 1962). The administration of FA and diclofenac and induction of paw edema is described below.

Group I- Distilled water (0.1ml) + 0.02 ml of 1% carrageenan injection

Group II- Ferulic acid 100 mg/kg (in 0.1 ml distilled water *p.o*) + 0.02 ml of 1% carrageenan injection

Group III - Ferulic acid 200 mg/kg (in 0.1 ml distilled water *p.o*) + 0.02 ml of 1% carrageenan injection

Group IV- Diclofenac 10 mg/kg body weight (in 0.1 ml distilled water *p.o*) + 0.02 ml of 1% carrageenan injection

Animals which received carrageenan injection alone were kept as control. Diclofenac (10 mg/kg body weight) served as standard reference drug. FA and diclofenac were administered one hour prior to the sub plantar injection of carrageenan. The paw thickness was measured using vernier callipers at one hour intervals for 6 hours following carrageenan injection.

Formalin induced chronic paw edema

The animals were treated in the same way as in the above models; except formalin (20 µl of freshly prepared 2% formalin) was used as the edematogenic agent instead of carrageenan. The drug treatment was continued for 6 consecutive days. Diclofenac (10 mg/kg body weight) was used as the reference drug.

In all the above models, the degree of edema formation was determined as increase in paw thickness. The increase in paw thickness and percent inhibition were calculated as follows.

Increase in paw thickness in control (P_C) or treatment (P_T) = P_t-P_0,

$$\text{Percent inhibition} = \frac{P_C - P_T \times 100}{P_C}$$

Where P_t indicates paw thickness at time t, P_0 is initial paw thickness, P_C represents increase in paw thickness of the control group and P_T is the increase in paw thickness of the treatment groups.

3. Results

Carrageenan induced paw edema

The sub plantar injection of carrageenan in Swiss albino mice produced a local inflammatory response. The paw edema found to reach the peak at 2nd hour and after that it was found to reduce. Administration of the ferulic acid produced 28 % inhibition in the paw thickness at a dose of 100 mg/kg body weight at 6th hour and 37.5% inhibition in paw thickness at a dose of 200 mg/kg at 6th hour ($p<0.001$) (**Figure .2**).

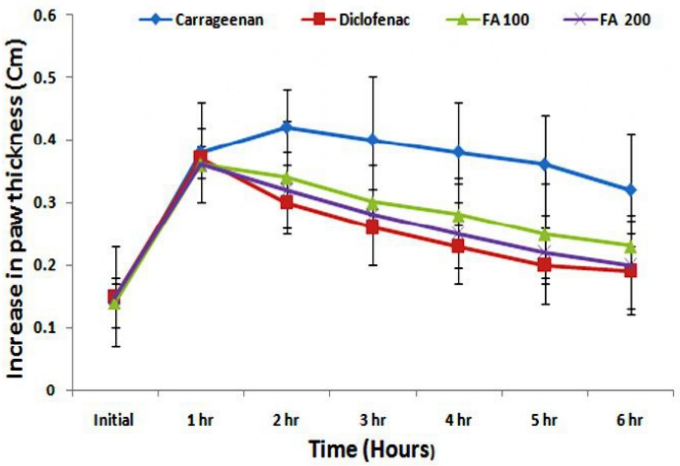

Figure 2: Effect of Ferulic acid (100mg/kg &200 mg/kg) on carrageenan induced paw edema in mice. All values expressed as Mean ± S.D, (n=5).

Formalin induced paw edema

In the formalin-induced paw edema test for chronic inflammation, the sub plantar injection of formalin in Swiss Albino mice produced a local inflammatory response which reached a maximum intensity of edema at the 3rd day. Administration of ferulic acid at doses of 100 mg/kg body weight showed 31.4 % inhibition of edema on 6th day, where as ferulic acid exhibited 46.87 % inhibition at a dose of 200 mg/kg on 6th day ($p<0.001$) (**Figure. 3**).

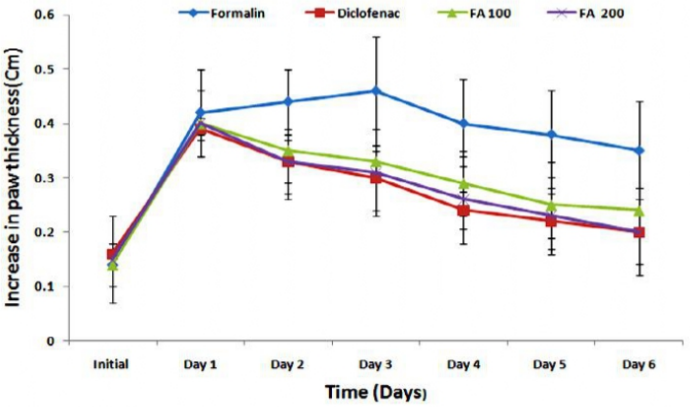

Figure 3: Effect of Ferulic acid (100mg/kg &200 mg/kg) on formalin induced paw edema in mice. All values expressed as Mean ± S.D, (n=5).

4. Discussion

Various studies suggest that the inflammatory tissue damages are due to the liberation of reactive oxygen species from phagocytes invading the inflammation sites (Cross *et al*., 1987; Winrow *et al*., 1993). In addition to this, nitric oxide is also implicated in inflammation, cancer, and other pathological conditions (Hemnani and Parihar, 1998). Interactions between superoxide and nitric oxide regulate the vascular tone or inflammation (Conner and Grisham, 1996).

Inflammation is a complex process and ROS plays an important role in the pathogenesis of inflammatory reactions (Halliwell and Gutteridge, 1985). As the inflammation is mainly produced by the oxidative burst of macrophages, antioxidants which can scavenge ROS may be effective to reduce inflammatory disorders. Inflammation, which is a pattern of response to injury, involves the accumulation of cells and exudates in irritated tissues that allows protection from further damage (Azab *et al*., 2016). Significant ameliorative activity against carrageenan and formalin induced anti-inflammation was shown by FA.

Carrageenan induced acute inflammation in animals is said to be the most suitable test procedures to monitor anti-inflammatory agents. The carrageenan induced edema is mediated by activation of platelet activating factor (PAF), prostaglandins and other inflammatory mediators (Hwang *et al*., 1986; Mansouri *et al*., 2015). Carrageenan-induced edema is a biphasic response in which the involvement of the cyclo-oxygenase products of arachidonic acid metabolism and the production of reactive oxygen species are well established (Madhuri *et al*., 2016). The first phase is mediated through the release of histamine, serotonin, and kinins, whereas the second phase is related to the release of prostaglandin oxygen-derived free radicals and production of inducible cyclo-oxygenase which peak at 2-3 hours (Panthong *et al*., 2004). Carrageenan also induces a protein rich exudates containing large number of neutrophills (Lo *et al*., 1982; Naik and Krishnamurthy, 2018). The ferulic acid produced considerable inhibition of carrageenan-induced paw edema comparable in magnitude with the inhibitory action of the standard drug diclofenac.

Formalin induced paw edema is also one of the most suitable test procedure to screen chronic anti-inflammatory agents as it closely resembled human arthritis (Greenwald, 1991). The nociceptive effect of formalin is also biphasic, an early neurogenic component followed by tissue mediated response (Zhao *et al*., 2018). The ferulic acid showed significant anti-inflammatory activity against formalin induced paw edema and thus found to be effective in chronic inflammatory conditions. Ferulic acid released from wheat bran by a new strain of *A. niger* showed good anti-inflammatory activity and better antioxidant ability (Yin *et al*., 2019)

There is a strong relationship between antioxidants and inflammation (Ma and Huang, 2014). Chronic inflammation is accompanied by increased production of tissue reactive oxygen and nitrogen intermediates. Oxygen free radicals and non radical reactive oxygen intermediates released by neutrophils and other phagocytes have been increasingly implicated in inflammation/ immune disorders. Inflammation also facilitates the initiation of normal cells and their progression to malignancy through the production of inflammatory oxidants (Dhingra *et al*., 2018). Appropriate treatment of inflammation with anti-inflammatory agents, inhibitors, inhibitors of oxidant generating enzymes, and scavengers of oxidants should be explored to prevent development of human cancers associated with chronic inflammation (Ohshima *et al*, 2003).

In conclusion, the present study reveals the profound anti-inflammatory activity of ferulic acid and the effect showed by them might probably due to its significant antioxidant power.

References

[1] Joel A Hardmann, Lee E Limbard, Alfred Goodmann. Pharmacological basis of therapeutics, 1998, p.1465.
[2] Nickavar B Kamalinejad M and Izadpanah H. *In vitro* free radical scavenging activity of five *Salvia* species. *Pakistan J Pharm Sci*. 2007; 20: 291-294.
[3] Hafeez, A., Jain, U., Sajwan, P., Srivastava, S., Thakur,A. Evaluation of Carrageenan induced anti-inflammatory activity of ethanolic extract of bark of *Ficus virens* Linn. in swiss albino mice. The Journal of phyto pharmacology, 2013; 2(3): 39-43
[4] Schulz JB, Lindnau J, Seyfriend J and Dichgans J. Glutathine oxidative stress and neurodegeneration. European Journal Biochemistry, 2000;267: 4904-4911.
[5] Adelman R, Saul R and Ames B. Oxidative damage to DNA: Relation to species metabolic rate and life span. Proceedings of the National Academy of Sciences, USA, 1988;85:2706–8.
[6] Mohantya,SK., Swamy, MK., Middha, SK., Prakash, L., Subbanarashiman, B and Maniyama, A. Analgesic, Anti- inflammatory, Anti- lipoxygenase Activity and Characterization of Three Bioactive Compounds in the Most Active Fraction of *Leptadenia reticulata* (Retz.)Wight & Arn. – A Valuable Medicinal Plant. Iranian Journal of Pharmaceutical Research, 2015; 14 (3): 933-942
[7] Ohshima H, Tatemichi M, Sawa T. Chemical basis of inflammation-induced carcinogenesis, Arch Biochem Biophys, 2003; 417, 3-11.
[8] Wu XW, Wei W, Yang XW. Anti-Inflammatory Phenolic Acid Esters from the Roots and Rhizomes of Notopterygium incisium and Their Permeability in the Human Caco-2 Monolayer Cell Model. Molecules, 2017; 22, 935.
[9] Naik, K., Krishnamurthy, R. Anti-inflammatory activity of methanolic extracts of *Pseudarthria viscida* and *Uraria picta* against carrageenan induced paw edema in albino rat. Open Access Journal of Medicinal and Aromatic Plants, 2018; Vol. 9(1): 1-11
[10] Sala A, Recio MD, Ginner RM, Manez S, Tournier H, Schinella G, Rios JL. Antiinflammatory and antioxidant properties of *Helichrysum italicum. J Pharm Pharmacol*. 2002; 54: 365371.
[11] Lin JK, Chen PC, Ho CT, Lin-Shiau SY. Inhibition of xanthine oxidase and suppression of intracellular reactive oxygen species in HL-60 cells by theaflavin-3,3'-digallate, (−)-epigallo- catechin-3-gallate, and propyl gallate. *J Agric Food Chem*. 2000; 48: 2736-2743.
[12] Repetto M, Lesuy S. Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. *Braz J Med Biol Res*. 2002; 35: 523–534.
[13] Sevgi K, B Tepe, C Sarikurkcu. Antioxidant and DNA damage protection potentials of selected phenolic acids. Food Chem Toxicol, 2015;77, 12-21.
[14] Kumar D, Kumar S, Singh J, Narender, Rashmi, Vashistha BD et al. Free radical scavenging and analgesic activities of *Cucumis sativus* L. fruit extract. *Pharmacog*. 2010; 4: 365-368.
[15] Dai J, RJ Mumper. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules, 2010; 15, 7313-52.
[16] Roginsky, V., Lissi, E.A. Review of methods to determine chain-breaking antioxidant activity in food. *Food Chem*, 2005;*92*, 235-254.
[17] Balasubashini MS, Rukkumani R, Viswanathan P. Ferulic acid alleviates lipid peroxidation in diabetic rats. Phytotherapy Research, 2004; 18, 310-4.
[18] Winter CA, Risley EA and Nuss GW. Carrageenan-induced oedema in the hind paw of rat as an assay for anti-inflammatory activity. Proceedings of the Society

for Experimental Biology and Medicine, 1962; 111: 544-547.
[19] Cross CE, Halliwell B, Borish ET, Pryor WA, Ames BN, Saul RL. Oxygen radicals and human diseases. *Annal Int Med*. 1987; 107: 526-45.
[20] Winrow VR, Winyard PG, Morris CJ, Blake DR. Free radicals in inflammation: second messangers and mediators of tissue destruction. *Br Med Bull*. 1993; 49: 506-522.
[21] Hemnani T, Parihar MS. Reactive oxygen species and oxidative DNA damage. *Indian J Physiol Pharmacol*. 1998;42: 440-52.
[22] Conner EM, Grisham MB, Inflammation, free radicals and antioxidants. *Nutr*. 1996; 12: 274-277.
[23] Halliwell B and Gutteridge JM. The importance of free radicals and catalytic metal ions in human diseases. Molecular Aspects of Medicine, 1985; 8(2):89–193
[24] Azab,A., Nassar, A., Azab, AN, Anti-Inflammatory Activity of Natural Products. Molecules, 2016; 21, 1321; doi:10.3390/molecules21101321
[25] Hwang S, Lam ML, Shen T. Release of platelet activating factor and its involvement in the first phase of carrageenan rat foot oedema. *Eur J Pharmacol*. 1986; 120: 33–41.
[26] Mansouri MT, Hemmati AA, Naghizadeh B, Mard SA, Rezaie A, Ghorbanzadeh B. A study of the mechanisms underlying the antiinflammatory effect of ellagic acid in carrageenan-induced paw edema in rats. Indian J Pharmacol, 2015;47:292-8.
[27] Madhuri AS, Mohanvelu R, Ramabhimaiah S. Evaluation of anti- inflammatory activity of aqueous extract of Mangifera indica leaves in albino rats. Int J Basic Clin Pharmacol, 2016;5:635-8.
[28] Panthong A, Kanjanapothi D, Taesotikul T, Phankummoon A, Panthong K, Reutrakul V. Anti-inflammatory activity of methanolic extracts from *Ventilago harmandiana* Pierre. *J Ethnopharmacol*. 2004; 91: 237-42.
[29] Lo TN, Almeida AP, Beaven MA. Dextran and carrageenan evoke different inflammatory response in rat with respect to composition of infilterates and effect of indometacin. *J Pharmacol Exp Ther*. 1982; 21: 221–261.
[30] Greenwald RA. Animal model for evaluation of arthritic drugs. *Methods Find Exp Clin Pharmacol*. 1991; 13: 75-83.
[31] Zhao , J., Maitituersun, A., Li,C., Li,Q., Xu,F., Liu, T, Evaluation on Analgesic and Anti-Inflammatory Activities of Total Flavonoids from *Juniperus Sabina*. Evidence-Based Complementary and Alternative Medicine,2018,1-9.
[32] Yin Z N, Wu W J, Sun C Z, Liu H F, Chen W B, Zhan Q P, Lei Z G, Xin X, Ma J J, Yao K, Min T, Zhang M M, And Wu H. Antioxidant and Anti-inflammatory Capacity of Ferulic Acid Released from Wheat Bran by Solid-state Fermentation of *Aspergillus niger, Biomed Environ Sci, 2019; 32(1): 11-21*.
[33] Ma Y, H Huang. Characterisation and comparison of phenols, flavonoids and isoflavones of soymilk and their correlations with antioxidant activity. Int J Food Sci Tech, 2014; 49, 2290-8.
[34] Dhingra AK, Chopra B, Bonthagarala B. Natural Anti-Inflammatory Agents: Recent Progress and Future Perspectives. Ann Pharmacol Pharm. 2018; 3(5): 1158.