ANTI-INFLAMMATORY AND ANTICANCER ACTIVITIES OF BIOACTIVES FROM MOREL MUSHROOMS, *MORCHELLA* SPECIES FROM KASHMIR.

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By

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I, hereby declare that thesis entitled **"ANTI-INFLAMMATORY AND ANTICANCER ACTIVITIES OF BIOACTIVES FROM MOREL MUSHROOMS, *MORCHELLA* SPECIES FROM KASHMIR"** is a based on the original research work done by me, under the guidance of Dr. K.K.Janardhanan, PhD, FNABS, Professor at Amala Cancer Research Centre, Thrissur and co-guidance of Dr. Fathimathu Zuhara K, PhD, Professor, University of Calicut, Thenjippalam for the partial fulfilment for the award of Doctor of Philosophy in Microbiology. No part of this work has formed the basis for the award of any degree, diploma or other similar titles of any other university.

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Abstract

Cancer is the one of the major causes of death among children and adults worldwide. Despite the discovery of several anticancer drugs, mortality rate is increasing at an alarming rate. Among the treatment options for cancer, chemotherapy is considered as the most effective option. However, drug resistance and dose limiting toxicities are some of the limitations of chemotherapy. A large number of chemotherapeutic drugs derived from synthetic as well as natural sources have been employed in the treatment of cancer for a long time. Most of the chemotherapy drugs presently in use have severe side effects in patients, hence search for chemotherapy drugs with least toxicity to the host , is continued. Discovery of safe and nontoxic chemotherapeutic agents would benefit millions suffering from cancer. Mushrooms are functional food containing numerous bioactive compounds. Several medicinal mushrooms and their bioactive compounds have demonstrated anticancer, anti-inflammatory, antiviral, immune-modulatory and antimicrobial properties. Fruiting bodies of mushrooms show great potential for developing drug and neutraceutical products. A number of mushroom derived preparations such as tablets, capsules or powders are sold as drugs and food supplements. Extensive studies were conducted on medicinal properties of various mushrooms such as *Lentinus edodes, Schizophyllum commune, Cordyceps sinensis, Ganoderma lucidum, Flammulina velutipes, Pleurotus ostreatus, Trametes versicolor, Grifola frondosa, Agaricus blazei, Phellinus linteus*, etc. The biological activities shown by these mushrooms are antioxidant, anticancer, antifungal, anti-diabetic, anti-inflammatory, cardio-protective, hepato-protective and immune-modulatory activities. During our search for bioactive natural products from higher fungi, we investigated the medicinal properties of fruiting bodies of an excellently edible mushroom, *Morchella elata* (Fr) Pers occurring in Kashmir Himalayas. *Morchella elata* is highly nutritious morel mushroom. However, medicinal properties of this mushroom are not investigated adequately. Current investigation was undertaken to evaluate the antioxidant, anti-inflammatory, anticancer and genoprotective activities of the bioactive ethyl acetate extract (EAE) of the fruiting of *M. elata* .

Fruiting bodies of *M.elata* were collected from Gulmarg and Mammer, Kashmir and dried at 40-50^{0}C. The powdered mushroom samples (100g) were extracted with various solvents based on their polarity. Sequential extraction was carried out using petroleum ether (PTE), chloroform (CHE), ethyl acetate (EAE),methanol (MEE) and aqueous (AQE).The yields of

petroleum ether (PTE), chloroform (CHE), ethyl acetate (EAE), methanol (MEE), and aqueous extract (AQE) were 1.23, 0.828, 1.33, 2.63, and 1.95 % respectively.

The *in vitro*-free radical scavenging activities of PTE, CHE, EAE, MEE and AQE were examined by DPPH radical, $ABTS^{+}$ radical, hydroxyl radical, FRAP and, nitric oxide radical scavenging assays. The extracts were found to scavenge DPPH radicals, hydroxyl radicals and ABTS radicals by donating electrons to corresponding oxidizing agents. Similarly the extracts reduced ferric ions in the FRAP assay. In addition, the extracts prevented lipid peroxidation indicating the antioxidant activity. The *in-vitro* antioxidant assays showed that the extract possessed significant free radical scavenging activity. Since EAE showed higher antioxidant activity than all the other extracts, it was selected for further studies. *In vivo* antioxidant activity was examined in Swiss albino mice. The normal antioxidant capacity of serum was found to be 19.26µM trolox equivalent/20µL serum which was reduced to 14.86µM trolox equivalent by the administration of paracetamol. EAE at 500 mg/Kg b. wt dose raised antioxidant capacity of serum back to 17.05µM trolox equivalent. All these experiments under *in vitro* as well as *in vivo* revealed that *M.elata* is an excellent source of antioxidants.

Many antioxidants can effectively reduce adverse effect of inflammatory reaction due to close link between oxidative stress and inflammation. Current experiment results reveal that EAE of morel mushroom, *M.elata* showed profound anti-inflammatory activity. In this study, anti-inflammatory activity of *M.elata* was investigated using animal model. EAE was orally administered to mice bearing carrageenan induced acute paw edema. The highest dose administered was 500 mg/Kg b.wt. At this dose EAE reduced paw edema by 49.2% compared to control. Chronic inflammation is more prolonged and damaging than acute inflammation. Persistence of it can cause a variety of diseases, especially cancer. The chronic anti-inflammatory activity of EAE was studied using induced chronic inflammation mouse model. The oral administration of EAE at 500 mg/Kg b. wt dose reduced inflammation by about 53.5%.

In an additional model, inflammation was induced on skin by topical application of croton oil and EAE was topically applied prior to croton oil application. The extract prevented

inflammation to a significant level as evident from reduced skin thickness and leukocyte infiltration compared to the untreated control.

To find out possible mechanism of action of EAE in relieving inflammation, the effect of the extract on prevention of cyclooxygenase (COX) enzyme activity was examined. EAE significantly inhibited COX-2 activity as compared to control indicating the profound anti-inflammatory activity of *M.elata*. a t doses of100, 50 and 25 µg/mL inhibited COX -2 enzyme activity by 51.8, 46.91 and 34.53% respectively. The standard drug diclofenac at 100 µg/mL concentration showed an inhibition of 72.5%

Leukocyte infiltration into the inflammatory sites plays a crucial role in induction of inflammation. Hence the effect of EAE in preventing leukocyte infiltration was also examined. Carrageenan induced leukocyte infiltration into the peritoneal cavity of mice was inhibited by the extract by 30%. The EAE also showed marked NF-kB inhibiting activity. This indicated the ability of the extract to inhibit the pro-inflammatory signaling molecules. The results suggest the significant anti-inflammatory effect of bioactive ethyl extract of *M.elata*. Since *M.elata* shows excellent anti-inflammatory property, consumption of this edible mushroom might be useful to provide relief from inflammation and arthritis.

Several studies have shown that cancer is associated with oxidative stress and inflammation. It is also confirmed that the probability for prolonged inflammation ending up in cancer is very high. Having demonstrated that *M.elata* possessed significant antioxidant and anti-inflammatory activities, the study was extended to find out it's possible anticancer activity. Cytotoxic activity of the EAE was evaluated by MTT assay using DLA cells. EAE was found to possess significant cytotoxic effect at 80µg/mL concentration EAE caused 60% DLA cell death. This was followed by the study of antitumor activity of EAE. The extract significantly inhibited the development of solid tumor induced by DLA cell line in mice. The EAE showed antitumor activity with a tumor inhibition level of 86% at a dose of 500mg/kg b.wt. The standard drug cyclophosphamide at a dose of 25mg/kg body weight inhibited 94% tumor growth. However, cyclophosphamide caused drastic reduction in body mass of treated animals. Mortality rate in cyclophosphamide administered group was also very high.

Another carcinogenesis model, skin carcinoma induced by DMBA and promoted by croton oil was studied. Topical application at a doses of 50 mg of EAE prior to croton oil application increased the latency period and reduced the average number of papilloma formed on mouse skin. These studies revealed the profound anticancer activity of the extract of *M.elata*.

Angiogenesis has been confirmed to play a significant role in tumor growth. Hence antiangiogenic activity of EAE was studied. Angiogenesis was induced on the walls of peritoneal cavity of mice by the injection of DLA cells to the peritoneal cavity. EAE was given for seven days. The extract treatment caused marked decrease in the angiogenesis compared to that in the extract untreated control. Anti-angiogenic activity of EAE was studied on one more model. In this model, solid tumor was induced on mouse thigh using DLA cells. Tumor formation induced angiogenesis. The extract administration resulted in the prevention of angiogenesis.

Cancerous cells are caused by genetic damage in its genome. Carcinogens are known to cause DNA damage. Hence, prevention of genetic damage is extremely important. Most of this damage is caused by reactive oxygen species. Since EAE showed excellent *in vivo* antioxidant activity, the effectiveness of EAE as a geno protective agent was examined. DNA damage was induced by hydrogen peroxide in bone marrow cells in the presence and absence of EAE. The presence of EAE significantly reduced DNA damage.

Identification of bioactive components is essential for drug development. EAE was analyzed by TLC, column chromatography, HPTLC, and LC-MS for the identification of active principle/s. Chemical examination of the EAE was found to contain steroids, terpenoids, alkaloids, coumarins, phenols, tannins, saponins and carbohydrate. The HPTLC analysis of extract showed 10 peaks in the chromatogram. EAE fractions from the column was separated into three major fractions (F1, F2, F3). Each fraction was examined for their anticancer activity by MTT assay. Fraction (F2) which showed highest anticancer activity was analyzed by LC-MS to identify the active compounds. The major bioactive compounds in EAE of *M.elata* were identified as Celastrol, Convallotoxin, Cucurbitacin A, Madecassic acid. These

compounds belong to the group of limonoids which have been reported to possess anti-inflammatory and anticancer activities.

A number of anticancer drugs have been rejected due to their high toxicity. So, EAE of *M.elata* though possessed excellent anticancer and anti-inflammatory activity, was tested for its toxicity. Acute toxicity of EAE was studied in male Swiss albino mice by orally administrating a single dose as high as 5000 mg/Kg b. wt. None of the animals showed mortality or any other abnormal behavior indicating that even at a large dose the extract did not induce acute toxicity. In the sub acute toxicity study mice were orally administered up to a dose of 500 mg/Kg b. wt for one month and haematological parameters and renal toxicity were examined. EAE did not cause any toxic symptoms until the end of the experiment.

In conclusion the results of the current study reveal that *Morchella elata* possessed profound antioxidant, anti-inflammatory, anticancer and genoprotective activities. The investigations indicated that *M.elata*, excellently edible mushrooms possessed significant anti-inflammatory and anticancer properties. The results of investigations thus suggest the promising therapeutic use of fruiting bodies of *M. elata*.

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Abbreviations

$ABTS^{+}$	2, 2'-azinobis (3-ethyl benzothiazolin -6- sulphonic acid)
AIDS	Acquired Immuno Defficiency Syndrome
ALDH	Alcohol Dehydrogenase
ALP	Alakaline Phosphatase
AQE	Aqueous extract
BRMs	Biological response modifiers
CAM	Complementary and alternative medicine
CAT	Catalase
CHE	Chloroform extract
COX	Cyclooxygenase
DLA	Dalton's Lymphoma Ascites
DNA	Deoxy nucleic acid
DPPH	1, 1-diphenyl -2- picryl hydrazyl
EAE	Ethylacetate extract
EBV	Epstein Barr virus
FB	Fruiting bodies
FRAP	Ferric Reducing Antioxidant Power
GSH	Reduced glutathione
GST	Glutathione S transferase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HHV8	Human herpes virus 8
HPTLC	High Performance Thin Layer Chromatoghraphy
HPV	Human papilloma virus
HR LCMS	High Resolution-Liquid Chromatography Mass Spectrometry
IL	Inter Leukins
MCPyV	Merkel cell polyomavirus
MDA	Malondialdehyde
MEE	Methanol extract
NAD	Nicotinamide Adenine Dinucleotide
NF	Nuclear Factor
NfkB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
$NO^{\cdot}$	Nitric oxide

NSAID	Nonsteroidal anti-inflammatory drugs
$O_2^{\cdot}$	Superoxide anion
OD	Optical Density
PAF	Platelet Activating Factor
PTE	Petroliem ether extract
RA	Rheumatoid Arthritis
RBC	Red Blood Cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SGOT	Serum Glutamate Oxaloacetate Transaminase
SGPT	Serum Glutamate Pyruvate Transaminase
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
UV	Ultra Violet

Chapter 1: Introduction

Cancer continues to be a major cause of mortality of people of all ages. It was reported to be one among the leading causes of death. It is an anticipated to rise to the thirteen million in 2030 (Ferlay et al., 2010; Torre et al., 2015). The wrestle against cancer has intensified in the past decades with multidirectional loom including behavioral and dietary change, chemotherapy, radiotherapy, surgery, and in recent times, immunotherapy. Regrettably, these approaches are not null and void of serious side effects spanning from recurrence and weakened immune system to reduced quality of life of patients. This has rattled scientists, leading to combined efforts of finding better therapies that, apart from managing the cancerous cells, boosting the immune system to battle cancer and other related diseases (Chen et al., 2004). Among the above therapies, complementary and alternative medicine (CAM) has been fronted as a substitute due to its potential of holistic treatment including augmentation of immune system. Most of the CAMs are plant-derived, including algae and mushrooms that have been used extensively in most parts of the world, where they are considered as biological response modifiers (BRMs) and immunoceuticals (Kidd, 2000). Among these, mushrooms, the most obvious form of certain fungi have also contributed to the growing list of natural products that have medicinal value. For several years, mushrooms have been associated with nutritional and medicinal properties including immune modulation and antitumor properties (Lull et al., 2005; Vetvicka et al., 2008; Reis et al., 2017). They contain variety of secondary metabolites including phenolic compounds, polyketides, terpenes, tannins, alkaloids, steroids etc. Some of these compounds have tremendous importance to humankind in that they display a broad range of useful antibacterial, antiviral, and other pharmaceutical activities as well as less toxic effect.

There are more than fourteen thousand mushroom species but only about three thousand are edible, with approximately seven hundred exhibiting medicinal properties and one percentage being poisonous (Borchers et al., 2008). Still among the known mushrooms, only a few mushrooms have been studied adequately for their therapeutic potential. They include *Lentinus edodes*, *Ganoderma lucidum*, *Trametes versicolor, Schizophyllum commune, Phellinus linteus, Nonotus obliquus, Agaricus blazei, Morchella esculenta*, *Cordyceps sinesis, Pleurotus spp* etc. These mushrooms possess a wide range of biological activities such as antioxidant, antitumour, antifungal, antibacterial antiviral, anti-inflammatory,

Immunomodulatory, anti-hyperlipidemic, cardiovascular, anti-ageing, anti-diabetic activities etc.

Most commonly used anti-neoplastic drugs are known to produce toxicity in organs.Several antitumor agents are associated with toxic effects such as bone marrow (doxorubicin), nephrotoxicity (cisplatin) and pulmonary fibrosis (bleomyin). One of the main challenges for medical oncology is to expand therapeutic process that will avoid toxicity induced by anti-neoplastic treatment without impairing their antitumor effect. Edible mushrooms, are believed to strengthen the immune system by deploying their effects on cellular activities, secondary chemical compounds that heighten the immune system, and helping to treat diseases and regain cell immunity destroyed by radiation and chemotherapy. Active compounds in majority of mushrooms are glucans. Beta-glucans are water-soluble polysaccharide that augments immune cells, proteins, macrophages, T cells, natural killer cells, and cytokines that molest tumor cells (Ooi et al., 2000; Vetvicka et al., 2008). Mushroom-derived polysaccharides and polysaccharide-protein complexes are considered as one of the major sources of therapeutic agents for immunomodulatory and antitumor effect (Gao et al., 2002; Mizuno, 2002).

Free radicals play important roles in metabolic functions. However, when they are overproduced they have a negative impact, because of their involvement in the causation of various diseases such as inflammation, cancer, neurodegenerative diseases. Free radical formation is controlled by various bioactive compounds known as antioxidants. Antioxidants are capable of stabilise, or deactivate free radicals before the latter attack cells and biological targets. Reactive oxygen species that are involved in inducing oxidative stress that play important role in inflammatory responses**.** Inflammation has a helpful value if the response is at optimum level, inflammatory responses exceed the limit resulting in damage to tissues and leading to causation of cancer. A large number of investigations have demonstrated that mushroom bioactives are good anti-inflammatory agents. While the mediators and effectors of oxidative stress and inflammation share many signaling molecules, Thus it appears rational to study anti-inflammatory and antioxidant activity vis-a vis-a antineoplastic activity.

In our search for biologically active natural products from higher fungi for anticancer bioactives, we evaluated the medicinal properties of an excellently edible mushroom,

Morchella elata which is found growing in Kashmir and Himachal Pradesh. *Morchella* species, popularly known as morel mushrooms are excellently edible and highly nutritious. They are in used for a long time for various health benefits in the hill regions of Himalayas. Morels have been reported to contain substantial quantity of high quality of protein, vitamins and minerals (Magrati, et al., 2011) and they have been recognized as source of biologically active, physiologically beneficial and non-toxic medicines. Regular consumption of this mushroom may help in the enhancement of cognition abilities and memory in old age and provide nutrition. Many members of the genus *Morchella* have yielded pharmacologically active compounds. A number of previous studies have shown various extracts *Morchella* species possess antioxidant and antimicrobial activities. However, to the best of our knowledge no significant investigation on the medicinal properties of *Morchella elata* has been carried out. The main aim of the current investigation is to determine the antioxidant, anti-inflammatory, anticancer, and genoprotective properties of this edible mushroom and to identify the bioactive molecules.

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2.1 Mushrooms

The mushroom species on earth are estimated approximately 140,000, of which nearly 10% are identified. Of this, more than 2000 mushroom species are found to be safe to consume and 700 species are known to have significant pharmacological properties (Chang, 1996: Wasser and Weis, 1999; Reshetnikov et al., 2001). Mushrooms, rich source of non-starchy carbohydrates, with a high content of dietary fiber, minerals, vitamins, moderate quantities of proteins and essential amino acids, have attracted much attention (Croan, 2004).

Mushrooms play a significant role in the areas of foods, biopharmaceuticals and they have a great potential in the production of useful bioactive metabolites, as they are prolific resource of drugs. Mushrooms have profound nutritional properties, medicinal values and great for the diversity of bioactive components (Chang, 1996; Wasser, 2002; Borchers et al 1999: Rai et al., 2005). The Greek look on mushrooms as sources of brawn for warriors in battles field. The Pharaohs gifted mushrooms as delicacies and the Roman regarded as the food of the Gods, and served only in festive occasions.

The nutritional value of mushrooms is in between meat and vegetables. They are rich sources of proteins, vitamins, minerals, low in fat content and high proportion of polyunsaturated fatty acids (2-8%). Mushrooms contain 19-35% protein containing essential amino acids rich in lysine, methionine, valine and leucine. Fresh mushrooms have abundance of carbohydrate and their range varies from 51-88%. Carbohydrates present in mushrooms include chitin, glycogen, trehalose, and mannitol. Glucose, mannitol and trehalose are common in edible mushrooms, while fructose and sucrose are in limited amounts. Additionally, they contain fiber, hemicelluloses and pectin. Minerals present in mushrooms are calcium, magnesium, potassium, iron, zinc and copper and their value is 4-20%. Mushroom are excellent source of riboflavin (vitamin B2), niacin, folates and traces of vitamin C, B1, B12, D and E.

2.1.1 Medicinal value of mushrooms

Mushrooms are functional food and source of physiologically beneficial and non-toxic medicines. Mushrooms were used in folk medicine throughout the world since ancient time (Wasser and Weis., 1999). Mushrooms have been used for extracting many pharmaceutical substances with potent medicinal properties. Conventional Chinese drug extracts from medicinal mushrooms have been used for the cure of diseases,

Modern scientific and medical studies have proved this. The reported medicinal properties are anticancer, antitumor anti-inflammatory, lowering of cholesterol, blood pressure, heapto-protective, anti-fibrotic, anti-diabetic and antimicrobial activity. The therapeutic effects of mushrooms are being explored worldwide to learn their biotechnological potential.

2.1.2 *Morchella elata*

Morchella elata (Fr) is a widely distributed excellently edible morel mushroom belonging to Ascomycotina division. In India, it is abundantly found in Jammu and Kashmir and Himachal Pradesh forests. Members of the genus *Morchella* (Morchellaceae) are highly priced edible mushrooms in the world. They are characterized by a hollow fruiting body consisting of a pitted cap with an intergrown stipe (Arora, 1986; Gursoy et al., 2009) (Fig 2.1). *Morchella* species have been used in traditional Chinese medicine for the treatment of various diseases. They contain a wide variety of biomolecules, which are bioactive with nutritional properties and used in healthcare by traditional hill societies (Prasad et al., 2002). *Morchella* mushrooms play an important role in the treatment of indigestion, excessive phlegm and for treatment of asthma. Powder of *Morchella* sp act as an antiseptic. *Morchella* sp helps in healing the wounds and used for the treatment of stomach-ache. Morels are known as Guchhi in India. Immunostimulatory properties mediated by the activation of macrophages were reported by the crude extract and galactomannan isolated from the fruiting body of *Morchella esculenta* (Duncan et al., 2002). Morel mycelium is being produced on a commercial scale in United States and the product is successfully competing with the fruiting bodies.

Proteins from the fruiting body of *Morchella* are parallel to vegetative protein and can be used as an excellent source of protein supplement. The studies showed that mycelia contain 22-51% protein, which is composed of the presence of 17 aminoacids including with essential aminoacids (Janardhanan, 1970). Fruiting bodies of *Morchella* sp possess a wide range of active compounds including tocopherols, carotenoids, organic acids and phenolic compounds. Recently, it has been proven that morel possess anti-inflammatory, antitumor, antioxidant and antimicrobial activities (Mau, et al., 2004; Nitha et al., 2007; Nitha et al.,, 2008; Nitha, et al, 2013).

Figure 2.1. Fruiting body of *Morchella elata*

2.2 Cancer

Cancer is a group of diseases characterized by uncontrolled cell division and the ability of these cells to spread either by direct growth into adjacent tissue through invasion, or by implantation into distant sites by metastasis (Simi, 2018). Cancers are abnormal cells triggered by gene mutations. Among the possible causes of cancer, damage to DNA and other cellular molecules, by reactive oxygen species, ranks high as a major culprit in the onset and development of the disease. Inflammation and exposure to exogenous sources pollutants, smoking, certain drugs and radiation, can induce cancer causing mutations.

2.2.1 . Carcinogenesis

Carcinogenesis is a multi-stage process characterized by cumulative action of multiple events occurring in a single cell and can be described by three stages: initiation, promotion, and progression

- Initiation (Phase I): ROS may produce DNA damage by introducing gene mutation and structural alteration of DNA.
- Promotion (Phase II): The damaged cell goes into uncontrolled growth.
- Progression (Phase III): The tumor cells invades the surrounding tissue and leads to oncogenesis (Fig 2.3) (De Ruyck et al., 2015).

Initiation is the first stage, ROS causes gene mutations which results in DNA damage. In the promotion stage, ROS can contribute to abnormal gene expression, blockage of cell-to-cell communication, and modification of second messenger systems, thus resulting in an increase of cell proliferation or a decrease in apoptosis of the initiated cell population. The final stage in carcinogenesis is development of a pre-malignant lesion (benign) into malignant accompanied by more rapid growth, invasiveness, metastasis and increased genetic instability (Ames, 1998: Weisburger et al.,1975).

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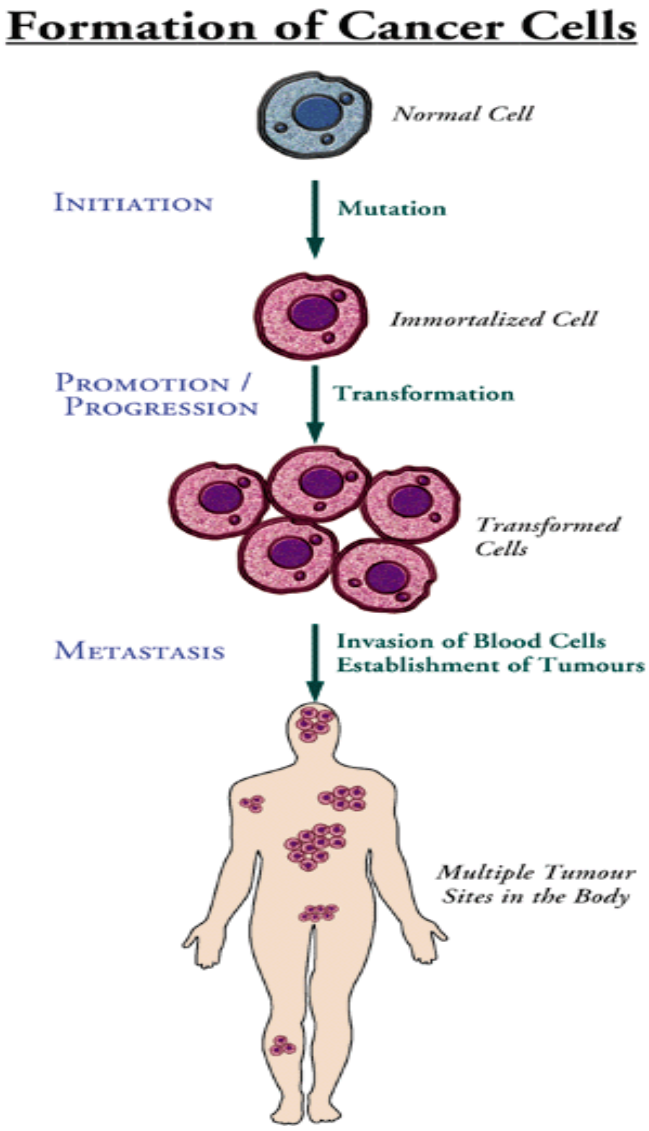

Figure 2.2. Formation of cancer cells or carcinogenesis

2.2.2 Etiology of cancer

Carcinogenesis can be caused by different types of agents or their combination and are stated below (Aquino et al, 2001)

2.2.2.1 Physical agents

Physical carcinogens, such as X-ray, Gamma-ray and UV-ray may cause mutations and genomic instability. Ionizing radiations such as X-rays and gamma-ray can eject electrons

from atoms forming electrically charged ions or free radicals. Within the cells these ions can cause DNA, damage (Hei et al., 1998). Ultravioiet radiation occurring in sunlight catalyzes the formation of pyrimidine cyclobutane dimers formed between adjacent thymine bases and can cause base substitution mutations if DNA is not repaired. The involvement of dimer formation in carcinogenesis is strongly supported by studies on the genetic disease Xeroderma pigmentosum, which is characterized by deficient excision repair of ỤV induced pyrimidine dimmers. Because UV radiation is absorbed by epidermis, it does not reach the germline but is responsible for high skin cancer incidence.

2.2.2.2 Chemical agents

Many human cancers arise from environmental factors, mainly chemicals. Epidemiological studies show that about 80% of human cancer is caused by chemical carcinogens in the tobacco smoke, in the work place. Some chemical carcinogen that are known to cause cancer are alkylating agents, polycyclic, heterocyclic, and aromatic hydrocarbons, aflatoxin B, tobacco, arsenic,vinyl chloride chromium, nickel, and asbestos (Baan et al.,2009).

2.2.2.3 Biological agents

Biological agents include several viruses, bacteria and some parasites. The viruses that cause cancer are termed as oncogenic viruses. Among different type of cancers, twenty percentage is reported to be caused by viruses. These include hepatitis B and hepatitis C virus (HBV & HCV), Epstein Barr virus (EBV), human papilloma virus (HPV), human herpes virus 8 (HHV8), Merkel cell polyomavirus (MCPyV), and HTLV-1. The traced mechanism by which viruses induce cancer is extremely complex. Briefly, they include chronic inflammation, interruption of genetic integrity, interfering with DNA repair, activating signaling pathways, varying the expression of genes, nullifying tumor suppressor and pro apoptotic genes etc (Meyer-ter-Vehn et al., 2000; Chang et al., 2010).

Viruses are responsible for about 20% of human cancers. These include cervical cancer, Burkitt's lymphoma, hepatocarcinoma and Kaposi's sarcoma. Viruses are involved in cancers because they can either carry a copy of oncogenes or can alter the expression of the cells copy of proto oncogenes or tumor suppressor genes. Viruses often act as initiators of the neoplastic

process, it acts as cofactor providing some of the steps required to generate maliganant cells (Luo et al., 2015).

2.2.3 Therapy strategies for cancer

The goal of a *primary treatment* is to completely erase the cancer from your body or kill all the cancer cells. Any cancer treatment can be used as a primary treatment, but the most common primary cancer treatment for the most common types of cancer is surgery. If a cancer is particularly sensitive to radiation therapy or chemotherapy, the patient receives either of those therapies as your primary treatment. The aim of *adjuvant therapy* is to kill any cancer cells that still remain after primary treatment in order to reduce the chances of cancer recurrence. Any of the known cancer treatments can be used as an adjuvant therapy. Common adjuvant therapies include chemotherapy, radiation therapy and hormone therapy. Neoadjuvant therapy is similar, but treatments are used before the primary treatment in order to make the primary treatment easier or more effective. *Palliative treatments* may help lessen side effects of treatment or signs and symptoms caused by cancer itself. Surgery, radiation, chemotherapy or hormonal therapy can all be used to relieve symptoms. Other medications may mitigate symptoms such as pain and shortness of breath. The third mode can be used at the same time as other treatments intended to cure cancer (Roffe et al., 2005: Porzsolt, 1993)

2.2.3.1 Surgery

Surgical procedures are used mainly for the physical removal of malignant tissue, and it remains one of the most important mode of treatment for malignant tumours. It is also a first-rate way of decreasing tumour burden in some advanced malignant diseases (Aruoma, 1994). Surgery also plays a role in cure, prevention; diagnosis, staging and the evident examples include mastectomy for breast cancer and prostatectomy for prostate cancer. The Grail of the surgery can be either the removal of only the tumour, or it can be the entire organ (King, 1993).

2.2.3.2 Chemotherapy

Chemotherapy is the application of chemical substances in the cancer treatment. In its recent-day use, it specifies primarily the cytotoxic drugs that are used for treating cancer. The quantum leap in antibacterial chemotherapy was followed by the discovery of sulfa drugs

inspired research that added more chances into cancer chemotherapy. There are certain known chemotherapeutic drugs that are utilized for the medicaments of cancer. Most chemotherapeutic drugs wield their action on cell multiplication and tumour growth by impairing mitosis effectively targeting fast dividing cells. Most of these types bring into play their action on cell multiplication and tumour growth by impairing mitosis (Dong et al., 1997). Lack of selectivity or specificity of cyototoxic drugs for tumour cells, conflict of cells towards drugs, and the stimulated growth of tumour cells after treatment are the major constrain of chemotherapeutic drugs (Ghafourifar et al., 2001). The tolerance level of normal tissues to the chemotherapeutic drugs usually regulates whether curative chemotherapy is feasible and applicable at that instant. Another curb is intrinsic or acquired resistance to cytostatic drugs (Balkwill et al., 2001). The advent of resistant cancer cells after repeated courses of chemotherapy is a major hindrance to cancer treatment and is aggravated by the development of multi-drug resistance. Another drawback of chemotherapy is the accelerated growth of tumour cells after treatment with cytostatic drugs, this phenomenon was documented by experimental therapists. It is probably caused by enrolment of resting stem cells in to the cycle. Destructive side effects limit the doses of most chemotherapeutic agents that can be used.

2.2.3.3 Hormonal therapy

Some types of cancer are powered by body's hormones. Examples include breast cancer and prostate cancer. Removing those hormones from the body or blocking their effects may cause the cancer cells to stop growing; *Targeted drug treatment* bedrocks on specific abnormalities within cancer cells that allow them to survive; *Cryoablation* kills cancer cells with cold. During this procedure, a thin, wandlike needle (cryoprobe) is inserted through your skin and directly into the cancerous tumor. A gas is pumped into the cryoprobe in order to freeze the tissue and then the tissue is allowed to thaw. The freezing and thawing process is repeated various times during the same treatment session in order to kill the cancer cells; Radiofrequency ablation allows electrical energy to heat cancer cells, causing them to die. During radiofrequency ablation, a thin needle is guided through the skin or through an incision and into the cancer tissue.

High-frequency energy streams through the needle and causes the surrounding tissue to heat up, killing the nearby cells; *Clinical trials* are studies to investigate new ways of treating cancer. Thousands of cancer clinical trials are underway (Kue et al., 2018; Ortiz et al., 2012; Sabel et al., 2004; Curley, 2003; Wagner et al., 2010)

2.2.4 Role of free radicals and oxidative stress in cancer

Free radicals are any molecular species that are capable of independent existence that contains an unpaired electron in an atomic orbital. Most of the free radicals are unstable and are highly reactive. They can either donate an electron or accept an electron from other molecules, and act as oxidants. The most significant oxygen-containing free radicals in many diseases are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical. These are proved to be highly reactive species, causing damage of membranes and in the important molecules such as DNA, proteins, carbohydrates , and lipids . Targets or the quarries of free radicals include all kinds of molecules in the body. Lipids, nucleic acids, and proteins are the most among them (Chapple et al., 2007).

Free radicals may be either oxygen derived or nitrogen derived termed as Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) (Matés et al., 2000). ROS the most vital class of radical species generated in living systems. They are hydrogen peroxide (H_2O_2), singlet oxygen, and hypochloric acid etc. Molecular oxygen (dioxygen) has a unique electronic configuration and itself is a radical (Khatun et al., 2012).

Nitric oxide is most important nitrogen derived free radical and one of the smallest molecules in nature with molecular weight 30Da (Masella et al., 2005). Peroxy nitrate is another dominant nitrogen derives oxidant that interacts with a wide range of targets to cause tyrosine nitration, thiol oxidation, lipid peroxidation, DNA strand break and the cell death. The reaction of peroxy nitrite $ONOO^-$ with excess NO generates NO_2, which then combines with more NO to form N_2O_3 to cause nitrosative stress (Jurado et al., 1999).

Free radicals can be formed from both endogenous and exogenous substances (Chang et al., 1996). If free radicals are not inactivated, their chemical reactivity can have the capability to damage all cellular macromolecules including proteins , carbohydrates, lipids and

nucleic acids. Free radical damage to DNA is also implicated in the causation of cancer (Shinde et al., 2012). The beneficial role of free radicals in cancer treatment is also studied and verified. Some free radicals are reported to kill cancer cells. In actual fact, certain cancer drugs aim in increasing the free radical amount in body (Horton, 2003).

2.2.5 Generation of reactive species in the body

Free radicals can be formed from both endogenous and exogenous factors. They are continuously forming in cell and environment (Phaniendra et al., 2015).

2.2.5.1 Endogenous factors

- UV radiations, X-rays, gamma rays and microwave radiation.
- Metal-catalyzed reactions.
- Inflammation initiates neutrophils and macrophages to produce ROS and RNS.
- In mitochondria- catalyzed electron transport reactions, oxygen free radicals produced as by product.
- ROS formed from several sources like mitochondrial cytochrome oxidase, xanthine oxidases, neutrophils and by lipid peroxidation

2.2.5.2 Exogenous factors

- ROS generated by the metabolism of arachidonic acid, platelets, macrophages and smooth muscle cells.
- Interaction with chemicals, automobile exhausts fumes, smoking of cigarettes, cigars, beedie etc.
- Oxygen free radicals in the atmosphere considered as pollutants.
- Burning of organic matter during cooking, forest fires, volcanic activities.
- Industrial effluents, excess chemicals, alcoholic intake, certain drugs, asbestos certain pesticides and herbicides, some metal ions, fungal toxins and xenobiotics

2.2.6. Some of the commonly used anticancer drugs

1. Alkylating Agents

- Nitrogen mustard: Melphalan, Cyclophosphamide, Ifosfamide.
- Nitrosoureas
- Alkylsulfonates
- Ethyleneiminies
- Triazene
- Methyl hydrazines
- Platinum coordination complexes: cisplatin, carboplatin

2. Antimetabolites

- Folate antagonists: Methotrexate
- Purine antagonists
- Pyrimidine antagonists: 5-Fluorouracil, cytarabibe

3. Natural products

1. Plant products

- Vinca alkaloids: Vincristine, vinblastine
- Taxanes: Paclitaxel, Docetaxel
- Epodophyllotoxins: Etoposide
- Camptothecins: Irinotecan

2. Microorganism products

- Antibiotics : Doxorubicin, Bleomyci
- Enzymes: L-asparaginase

2.3 Inflammation

Inflammation is an important process in the body's defense system, which acts to remove and repair damaged tissue or to neutralize harmful agents (Ferrero et al., 2006; Maslinska et al.,1998**).** Inflammation can be classified into two categories: acute inflammation and chronic inflammation.

Acute inflammation is the initial response of the immune system against pathogens and tissue injury. It is a rapid self-limiting process, mediated by eicosanoids and vasoactive amines, which increase the movement of plasma and leukocytes into infected site **(**Serhan et al., 2008**).** Acute inflammation helps the body ward off infections; it lasts for short period and generally is regarded as therapeutic inflammation **(**Aggarwal et al., 2009; Lin et al., 2007**).** Early in the inflammatory response, pro-inflammatory mediators such as prostaglandins and leukotrienes play an important role **(**Samuelsson et al., 1987**).** Neutrophils, a type of granulocyte, are the first leukocytes to appear at the injured site (Fig 2.3). These cells phagocytose and kill invading microorganisms through the release of superoxide radicals, hypochlorite, and hydroxyl radicals. These ROS kill pathogens as well as adjacent cells, sick and healthy alike.

In chronic inflammation, various cytokines and growth factors are released, resulting recruitment of higher order immune cells such as leukocytes, lymphocytes and fibroblasts. Macrophages and other leukocytes release ROS and proteases that destroy the source of inflammation; however, damage to the body's own tissues often results. Chronic inflammation is abnormal and does not benefit the body, in fact, Chronic inflammation are found to be the leading causes of a large number of diseases which include cancer (Verma et al., 2010: Aggarwal et al.,2006: Sanchez et al., 2015). Thus at times it is necessary to control or prevent inflammation by medical intervention.

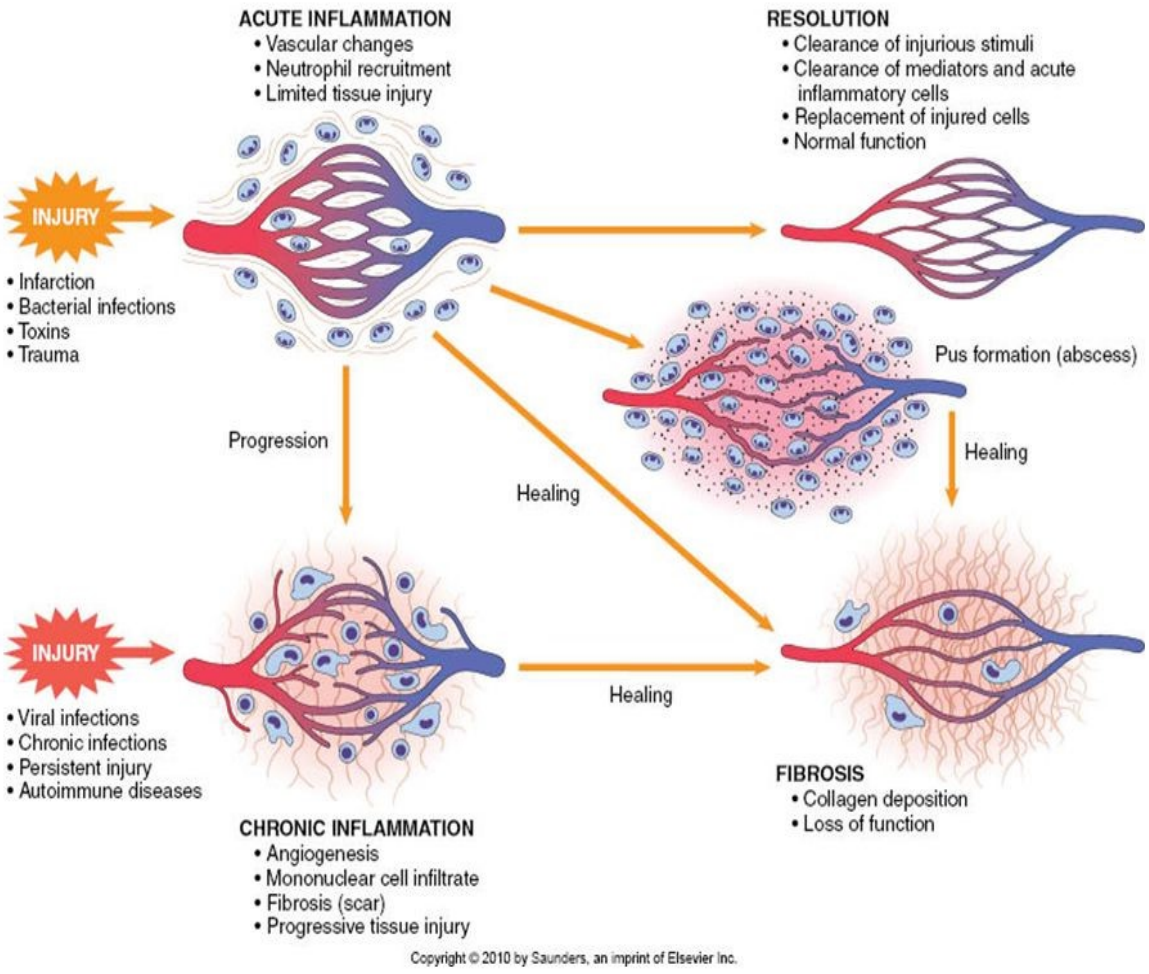

Figure 2.3 Types of inflammation: acute inflammation and chronic inflammation

2.3.1 Inflammation and cancer

Certain biological, chemical, and physical factors cause chronic inflammation, which will result in a high probability of risk escalation causing human cancers (Fig 2.4). Epidemiological and experimental data recommend the connection of inflammation with cancer. Effectiveness in cancer prevention and treatment is conveyed through many therapies. The risk of cancer will be high if the inflammation occurs for a longer period (Marx, 2004: Savill, 2000: Philip et al., 2004).

Metabolites of arachidonic acid, cytokines, chemokines, and free radicals involved as inflammatory mediators, which may lead to increased cell proliferation, mutagenesis,

oncogene activation, and angiogenesis. The result will be cell proliferation in which cells have lost normal growth control. Certain experimental evidence with animal models shows that inflammation can promote cancer in some possible mechanisms.

By the signaling pathways activated by ROS, and factors such as oxidation, nitration, halogenation of nuclear DNA, RNA, and lipids may lead to an initiation of carcinogenesis succeeding an inflammatory stimulus. H_2O_2, a part of ROS may be generated either by a process of simultaneous oxidation and reduction from superoxide anion or voluntarily in peroxisomes from molecular oxygen. As of reactivity of H_2O_2 is lesser than that of ROS, H_2O_2 plays an important role in carcinogenesis as it is capable of diffusing throughout the mitochondria and across cell membranes and will give rise to many types of cellular injury.

The very unstable electron structure hydroxyl radical (•OH) results in the main injurious effects of ROS in mammalian cells. Because of hydroxyl radical (•OH) is a very unstable electron structure, it is unable to diffuse more than one or two molecular diameters before it reacts in practice with any cellular component.

Certain signaling pathways are activated by ROS and they come up with tumor development through the regulation of cellular proliferation, angiogenesis, and metastasis. By activating AP-1, nitrosative stress plays a critical role in inflammation-associated carcinogenesis, which is involved in cell transformation and proliferation as a representative redox-sensitive transcription factor (Coussens et al, 2002).

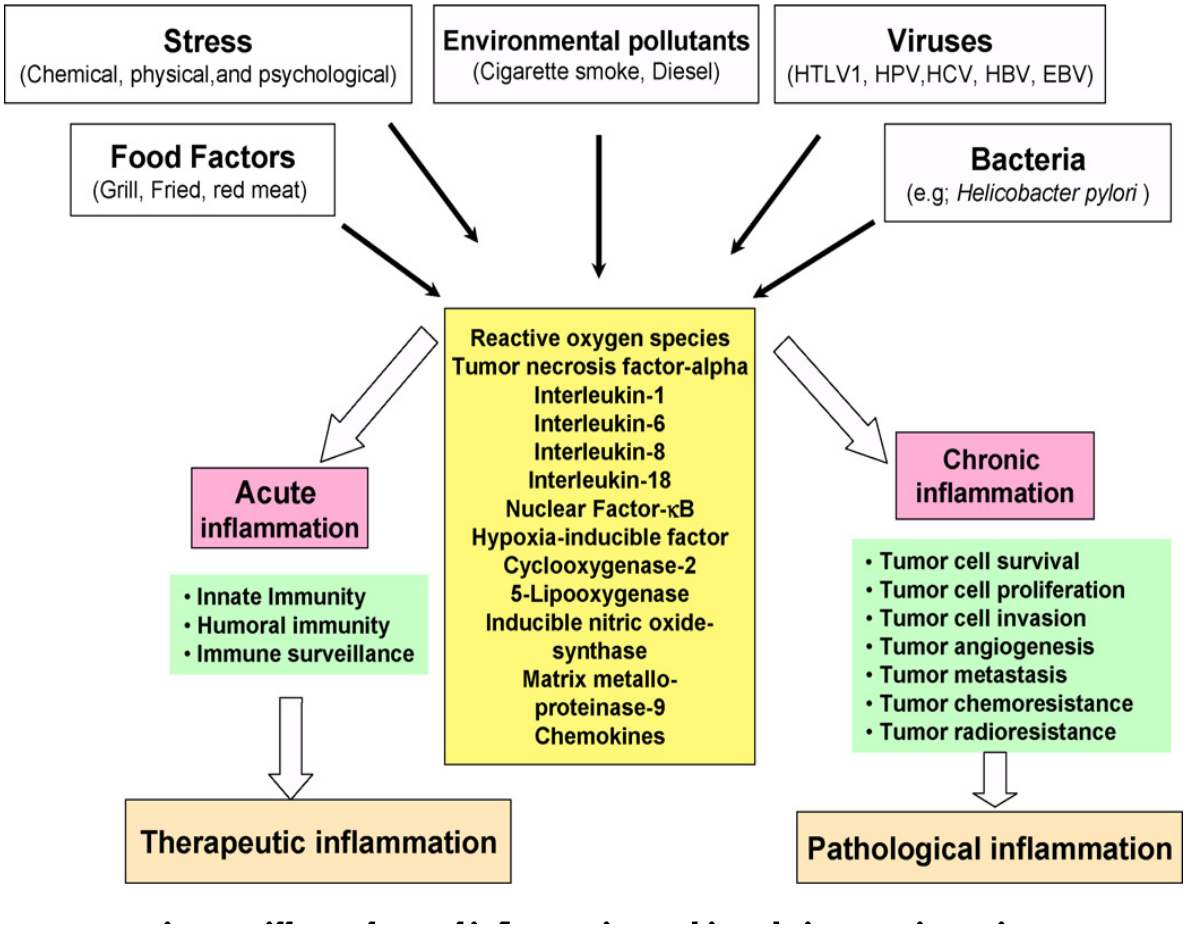

Figure 2.4 Different faces of inflammation and its role in carcinogenesis

2.4 Antioxidants

An antioxidant (reductant or reducing agent) can be classified as a compound capable of preventing the pro-oxidation process, or biological oxidative damage (Cao et al.,1998). According to halliwell (Haliwell, et al 1990 and 1995), antioxidants are agents, when present in low concentration, significantly prevents or delays oxidation of an oxidizable substrate. Antioxidants neutralize free radicals or their actions. It can be classified into enzymatic and non enzymatic antioxidants. Examples for enzymatic antioxidants include glutathione peroxidase, catalase and superoxide dismutase and that for nonenzymatic antioxidants include vitamin C and E, glutathione, thioredoxin, lipoic acid, melatonin, carotenoids, natural flovonoids etc.

2.4.1Classification of antioxidants

Classified as two types

- Enzymatic antioxidants
- Non-enzymatic antioxidants.

2.4.1.1Enzymatic Antioxidants

Enzymatic antioxidants act by breaking down and removing free radicals. The antioxidant enzyme convert dangerous oxidative products to hydrogen peroxide and then convert to water. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), Catalase (CAT) (Fernandez et al., 2007; Sies et al., 1995).

2.4.1.1.a Glutathione peroxidase (GPx)

There are two forms of this enzyme, one is selenium-dependent and the other is selenium-independent. The differences are due to the number of subunits, catalytic mechanism, and the binding of selenium at the active centre, and glutathione metabolism is one of the most important antioxidative defense mechanisms present in the cells. There are four different Se-dependent glutathione peroxidases present in humans and these are known to add two electrons to reduce peroxides by forming selenoles (Se-OH) and the antioxidant properties of these seleno-enzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction. Selenium-dependent glutathione peroxidase acts in association with tripeptide glutathione (GSH), which is present in high concentrations in cells and catalyzes the conversion of hydrogen peroxide or organic peroxide to water or alcohol while simultaneously oxidizing GSH. It also competes with catalase for hydrogen peroxide as a substrate and is the major source of protection against low levels of oxidative stress. However, the most important H_2O_2-removing enzymes in human cells are glutathione peroxidises (GSHPX), enzymes that require selenium (has selenocysteine at the active site) for their action. GSHPX enzymes remove H_2O_2 by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase, an FAD containing enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power (Hayes et al., 2005; Sharma et al., 2004).

2.4.1.1.b Superoxide dismutase

SOD are a class of closely related superoxide dismutase. All of these enzymes catalyze the conversion of superoxide anion into oxygen and hydrogen peroxide. This group of enzymes is present in almost all aerobic organisms (Zelko et al., 2002: Johnson et al.,2005)

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extra cellular SOD (EC-SOD)

2.4.1.1.c Catalase

This enzyme is present in the peroxisome of aerobic cells and is very efficient in promoting the conversion of hydrogen peroxide to water and molecular oxygen (Chelikani et al., 2004: Zamocky et al., 1999). Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute

2.4.1.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants act by terminating chain reaction of free radicals external. Examples of the non-enzymatic antioxidants are vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione (Huyut et al., 2017).

2.4.1.2.a Vitamin C (ascorbate)

Vitamin C is a water-soluble radical-scavenging agent (Percival, 1997). Ascorbic acid is one form of vitamin C. This antioxidant are obtained from diet and not produced in human body. Some animals produce this antioxidant in their body. Vitamin C helps to reduce oxidative damage caused by free radicals. So they act as a reducing agent (Smirnoff et al., 2001).

2.4.1.2.b Vitamin E (tocopherol)

Tocopherol is a fat-soluble vitamins with antioxidant properties (Herrera et al, 2001). There are eight forms of vitamin E and α-tocopherol is the most important lipid soluble antioxidant. Tocopherols are found mainly in palm oil, cereal grains,and kale. The most important function of δ--tocopherol is as a signaling molecule, with this molecule having no significant role in antioxidant metabolism. γ-tocopherol is a nucleophile that may react with electrophilic mutagens, and tocotrienols is important in protecting neurons from damage (Ruperez et al., 2001: Packer et al., 1995)

2.4.1.2.c Glutathione

Glutathione, an important water-soluble antioxidant, is synthesized from the amino acids glycine, glutamate, and cysteine. These aminoacids are found in plants, animals, fungi, bacteria etc. Glutathione directly quenches ROS such as lipid peroxides, and also plays a major role in xenobiotic metabolism (Andreson, 1998).

2.4.1.2. d Melatonin

Melatonin is a powerful antioxidant that can easily cross cell membranes and the blood–brain barrier (Pierrefiche et al.,1993). This is an indoleamine neurohormone that is synthesized mainly in the pineal gland and has many effects on a wide range of physiopathological functions. One major function of melatonin is to scavenge free radicals in oxygen metabolism, thereby potentially protecting against free radical-induced damage to DNA, proteins and membranes, thus it has the potential to play an important role in the reduction of free radical mediated diseases.

2.4.1.2.e Uric acid

It is present in human plasma is a powerful scavenger of hydroxyl radicals and singlet oxygen. It can scavenge reactive oxygen species (Becker et al., 1991).

2.4.1.2.f Polyphenolic compounds

Polyphenols consist of lignans, lignins, quinoids and phenolic compounds of small molecule. These polyphenolic compounds can act through terminating chain reactions and chelating redox-active metal ions (Machado et al., 2013)

2.4.1.2.f.g Flavonoids

It has antiviral, anti-allergic, anti-inflammatory, antithrombogenic and anticarcinogenic effects in vitro. It act as antioxidant by chelating reactive elements like iron or inhibiting oxidative enzymes (Braca et al., 2002).

2.4.1.2.h Bilirubin

Biliverdin and bilirubin are powerful scavengers of different oxidants in vitro, although several factors need to be considered when attempting to extrapolate this activity to a potential *in vivo* antioxidant property of the pigments. In mammals, for example, biliverdin does not usually accumulate to an appreciable concentration because of its rapid conversion to bilirubin by biliverdin reductase. In human plasma, normal bilirubin concentrations are 5 –20 mM, and essentially all of the pigment is bound by albumin. Albumin bound bilirubin can synergize with lipoprotein associated δ-tocopherol and, by doing so, effectively inhibit LDL lipidoxidation, particularly if the latter process is caused by lipid soluble radical oxidants. Bilirubin is also an effective inhibitor of protein oxidation (Asad et al., 2001)

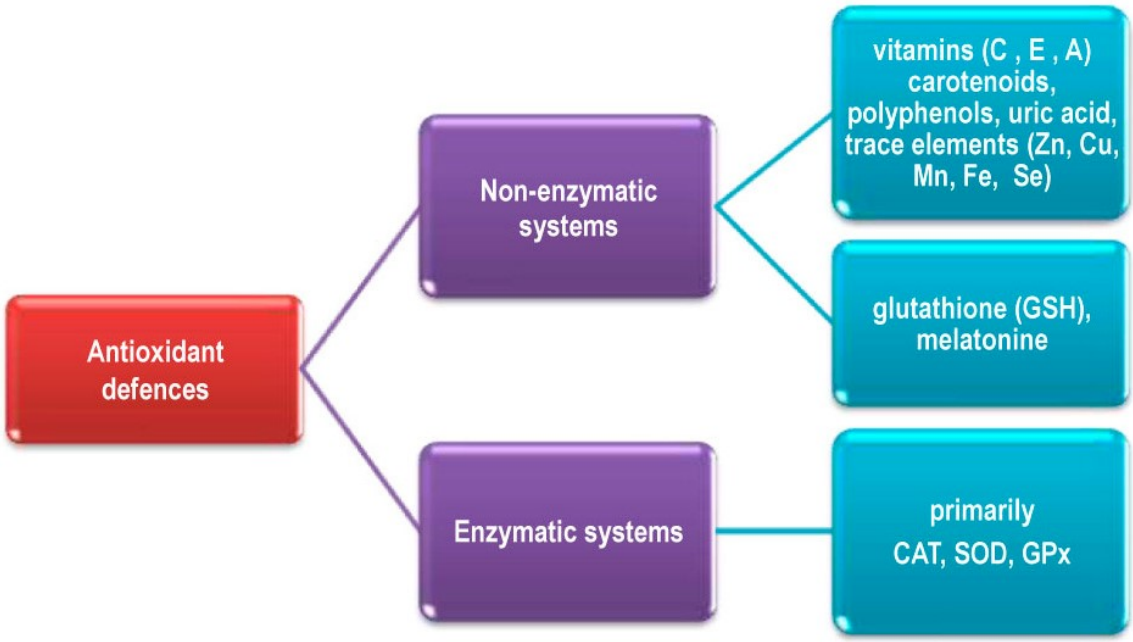

Figure 2.5: Enzymatic and nonenzymatic antioxidants

2.4.2 Antioxidants and anti-inflammatory properties of mushrooms

The antioxidant property of mushroom has been reported for many species of mushrooms, such as *Lentinus edodes, Ganoderma lucidum*, *Trametes versicolor, Schizophyllum commune, Phellinus linteus, Agaricus blazei, Morchella esculenta*, *Cordyceps sinesis, Pleurotus spp., Flammulina velutipes, Pholiota nameko, Tricholoma matsutake, Auricularia auricular-judae, Grifola frondosa,Tremella fuciformis, Denropolyporus umbellatus, Hericium erinaceus, Inonotus obliquus, Boletus asiaticus Agrocyhe bisporus* etc (Muszyńska et al., 2018: Lindequist et al., 2005, Sheena et al.,2003; Ferreria et al.,2009: Puttaraju et al.,2006). A number of the antioxidant compounds are present in mushrooms such as cinnamic acid, p-hydroxy-benzoic acid, protocatechuic acid, caffeic acid, asiaticusin A and B, flavoglaucin and Polysaccharides (Kozarski et al.,2015, Teleszko et al.,2015)

Some of the major classes of compounds with anti-inflammaory property are polysaccharides, terpenes, sterols, luteolin, quercetin, luteolin 7-glucoside, genistein, pinoresinol, woorenoside,

lariciresinol glycoside,cynaropicrin, reynosin, santamarine (Table 2.1). Most of them assert their effect by modulating the cytokine system.

2.4.3Anticancer activity of mushrooms

Some of the mushrooms that show noteworthy antitumor activities are *Ganoderma, Trametes*, *Cordyceps*, *Schizophyllum*, *Phellinus*, *Pleurotus*, *Agaricus*, *Clitocybe*, *Antrodia*, *Xerocomus*, *Calvatia*, *Flammulina*, *Suillus*, *Inonotus*, *Inocybe*, *Funlia*, *Lactarius*, *Albatrellus*, *Russula*, *Fomes and Lentinus* (Blagodatski et al., 2018: Roupas et al., 2012). Investigation also proves that they have significant anti mutagenic and anti carcinogenic activities. Indian medicinal mushrooms are potential sources of antioxidant, anticancer compounds delineated the antitumor, antioxidant, and anti-inflammatory activities of polysaccharides isolated from *Phellinus rimosus* (Ajith et al, 2007: Meera, 2009: Jose et al., 2002). Medicinal mushrooms are able to produce a variety of biologically active anticancer compounds such as polysachrrides, lectins, terpenoids, phenols, aminoacids, vitamins, minerals, flavonoids etc (Fig 2.6 and Table 2.2).

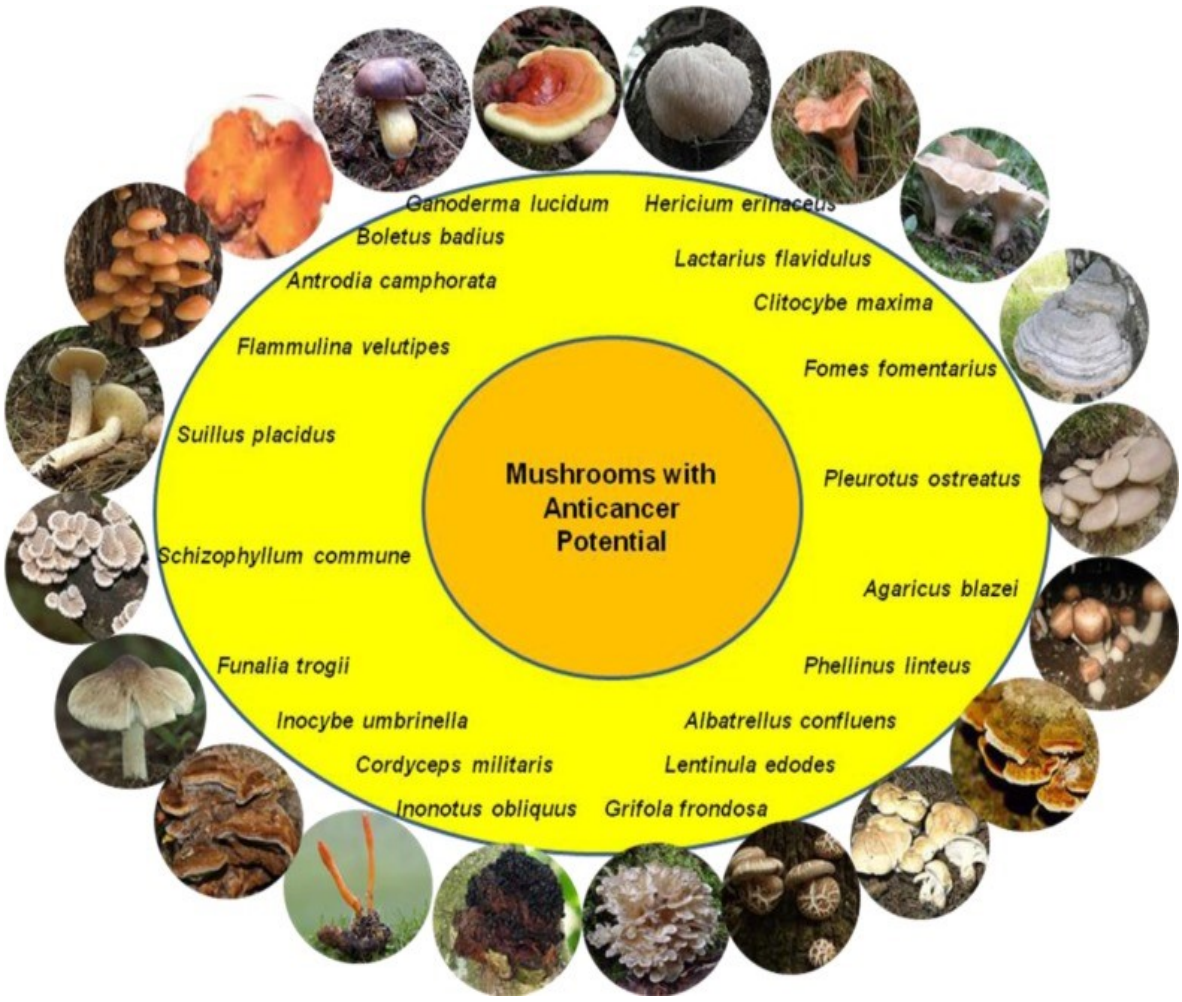

Figure 2.6. Mushrooms with anticancer potential.

Table 2.1 Anti-inflammatory compounds of mushrooms

Mushroom species	**Bioactive compounds**
Agaricus blazei	Polysaccharides
Pleurotus pulmonarius	Polysaccharides (1→3), (1→6)-linked *β*-glucan
Phellinus linteus	Polysaccharides (proteoglycan)
Lentinus edodes	Heterogalactan (fucomannogalactan) with main chain of (1→6)-linked *α*-D-glactopyranosyl unit
Grifola frondosa	Agaricoglycerides
Ganoderma lucidum	Triterpenes Lucidenic acid, ganoderic acid
Geastrum saccatum	*Geastrum saccatum*
Inonotus obliquus	Ergosterols, lanosterol, inotodiol, trametenolic acid
Lactarius deliciosus	Pyrogallol, flavonoids
Daldinia childiae	Benzophenones (daldinals A-C)
Fomitopsis pinicola	Polysaccharides
Pholiota nameko	Polysaccharides

Table 2.2 Antitumor properties of mushrooms

Fungus	**Glucans**	**Linkages**	**Medicinal Properties**
Lentinula edodes	*Lentinan*	(1-3)- β D	Anti inflammatory, Antiviral, Hypolipidemic
Schizophyllum commune	*Schizophyllan*	(1-6)- β D	Immunomodulating, Anti inflammatory
Poria cocos	*Pachymaran*	(1-3)- β D	Anti inflammatory
Ganoderma lucidum	*Ganoderma* glucans	(1-3)- β D	Anti inflammatory, Kidneytonic,Blood pressure regulation, Immunomodulating
Agrocybe cylindracea	*Agrocybe* glucan	(1-3)- β D	Hypolipidemic
Volvariella volvacea	*Volvariella* glucan	(1-3)- β D	Hypolipidemic
Auricularia auricular	*Auricularia* glucan	(1-3)- β D	Blood pressure regulation

2.4.5. Novel approaches of cancer treatment using mushroom products

Vaccinotherapy

Vaccine preparations with defensive properties against liver and cervical cancers associated with hepatitis B and human papillomatosis infections were developed in Belgium and USA (Ivanonova et al., 2014). Simultaneously, to date there is no vaccine on commercial scale aimed to cure existing tumors, metastasis or relapses. Toting up of immunomodulating substances of natural and synthetic origin to vaccines can sufficiently enhance their anticancer properties. Thus, there were corroborated doses and schemes of *L. edodes* polysaccharide fraction administration along and design of it combination with vaccine on the basis of autological glycopeptides of Ehrlich's carcinoma, Sarcoma 37, LLC, L1210, and B16 cell lines. Such preparations enhance cytolytic activity of lymphocytes, metabolic activity of peritoneal macrophages, and cytolytic activity of blood serum in the presence of complement in intact animals and in Sarcoma 37 bearing animals.

An immunomodulating protein Ling Zhi-8 from mycelia of *G. lucidum* with stimulatory activity on dendritic cells was recently identified (Chu et al., 2012). The extensive amplification of recombinant protein Ling Zhi-8 has been achieved in a patented yeast system. It was shown that above mentioned protein significantly increases the efficacy of a cancer DNA vaccine in a preclinical tumor model.

Nano-vectors for drug delivery

In this day and age, preparation of nanoparticles using "green" chemistry and bioprocess approach is beneficial over physical and chemical methods owing to its environmental significance. In this aspect, living organisms are highly potent for the production of nanoparticles. Irregular shaped gold nanoparticles were incorporated by photo-irradiation technique using *Pleurotus florida* as a reductant (Bhat et al., 2013). It was suggested that flavins (flavo proteins) present in the mushroom extract are responsible for the reduction of ions into nanoparticles: when exposed to sunlight flavins absorb photons of energy and can act in reduction-oxidation reactions. Acquired nanoparticles showed cytoto xicity against A-549, K-562, HeLa, and MDA-MB cancer cells and no effect against Vero normal cells.

Many of the recent cytoprotective agents capable of protecting normal tissues against radiation-damage have some undesirable and serious side effects that limit their therapeutic applications (Smina et al., 2011). However, mushroom products have been proven to exhibit radio-protective effects in normal cells and enhance the recovery of cellular immune-competence from gamma–irradiation (Chen et al., 1995). In conclusion, mushrooms may represent a practical and promising approach for cancer prevention and cancer treatment based on current available data from *in vitro* and *in vivo* studies. Nevertheless, further experimental, epidemiological, and clinical studies need to be conceded out to identify other molecular targets; resolve the relationships between mushroom intake and cancer risks; and explore the optimum dosing, efficacy, and safety alone and in amalgamation with chemotherapy/radiotherapy (Kao et al., 2013).

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Chapter 3: Materials and Methods

3.1 MATERIALS

3.1.1 Animals

3.1.2 Chemicals

3.1.3 Cell Line

3.2 METHODS

3.2.1 Preparation of extracts

3.2.2. Use of the extract in experiment

3.2.3 Preparation of tissue homogenates.

3.2.4 Determination of tissue lipid peroxidation

3.2.5 Determination of serum glutamate oxaloacetate transaminase (SGOT) activity

3.2.6 Determination of serum glutamate pyruvate transaminase (SGPT) activity

3.2.7 Determination of serum alkaline phosphatase (ALP) activity

3.2.8. Determination of haemoglobin (Hb) in blood

3.2.9. Determination of total white blood cell (WBC) count

3.2.10 Statistical analysis

3.1 MATERIALS

3.1.1Animals

Male Swiss albino mice weighing 25±3 g and 6-8 weeks old purchased from Small Animal Breeding Station, Agricultural University, Mannuthy, Thrissur, Kerala, India, were maintained under standard environmental conditions and fed with standard mice feed and water *ad libitum*. Animal experiments were conducted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and with the approval of the Institutional Animal Ethics Committee (IAEC)(ACRC/IAEC/16/06/03).

3.1.2 Chemicals

Petroleum ether, Methanol, Ethanol, Ethyl acetae, Chloroform, 2, 2- Diphenyl-1-picrylhydrazyl (DPPH), Ammonium per sulfate, Deoxyribose, Ferric chloride ($FeCl_3$), Ethylenediamine tetra acetic acid (EDTA), Hydrogen peroxide (H_2O_2), Acetic acid, Sodium dodecyl sulfate (SDS), Butanol, Pyridine, Ascorbic acid, Potassium chloride (KCl), Sodium acetate, Hydro chloric acid (HCl), 2, 4, 6-Tri Pyridyl-s-triazine(TPTZ), Ascorbate, Thiobarbituric acid(TBA), 2, 2-azino-bis 3-ethyl benzothiazoline-6-sulfonic acid (ABTS), Formaldehyde, Sodium chloride (NaCl), Low melting agar(LMA), High melting agar(HMA), Triton-X, Propidium iodide. Potassium hydroxide, Tris-HCl, Ammonium ferrous sulfate, Diclofenac, Carrageenan, Carboxymethyl cellulose, Croton oil, Acetone, Glutathione, Hemoglobin, Trichloroacetic acid, , Hematoxylin-oesin stain, Potassium mercuric iodide, Potassium iodide, Sodium carbonate, Sodium citrate, Copper(II) sulphate pentahydrate, Potassium sodium tartrate, Sodium hydroxide(NaOH),, Acetic anhydride, Sulphuric acid, Lead acetate, Propidium iodide, Ethidium bromide, Acridine orange, 7,12 – dimethyl benz [a] anthracene (DMBA), Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Cyclophosphamide, Agar-agar, Vanillin, Anisaldehyde, Dimethylsulfoxide (DMSO).

3.1.3 Cell Line

Dalton's lymphoma ascites (DLA) cell line was obtained from Cancer Institute, Adayar, Chennai. The cells were maintained in mice by intraperitoneal inoculation of 1×10^6 viable cells.

3.1.4 Instruments

Electronic balance	:-Contech instrument company, Mumbai.
Hot air oven	:-Beston, India.
P^H meter	:-Eutech instruments
BOD incubator	:-Narang scientific works Pvt.Ltd, New Delhi.
Water bath	:-Betson, India.
Autoclave	:-Narang scientific work Pvt.Ltd, New Delhi, India.
Cyclomixer	:- Remi equipments private limited
Cooling Centrifuge	:- Remi, R8C Laboratory centrifuge
Lyophilizer	:-Lobconco, corporation Missouri
Rotary vaccum evaporated	:-Buchi-R210, Switzerland
UV- VIS double beam spectrometer	:-Systronic, India
Laminar airflow hood	
Plethysmometer	:Orchid scientific
Electrophoresis apparatus	: Genie, india
Fluorescent microscope	: Olympus, Japan and TIE, Nikon
HPTLC scanner	:Camag TLC scanner
Deep freezer	:U410 premium, New Brunswick
Tissue homogeniser	: Remi laboratory instruments, India
Bench top centrifuge	: Remi laboratory instruments, India
Hot plate	: Rotek industries, India
CO_2 incubator	: SANYO
Magnetic Stirrer -	: REMI Equipments

3.2 METHODS

3.2.1 Preparation of extracts

Fruiting bodies of *Morchella elata* collected from Gulmarg and Mammar were dried at 50 °C and powdered. One hundred gram (100 g) samples of powdered material were extracted with petroleum ether (PTE), chloroform (CHE), ethyl acetate (EAE), methanol (MEE), and aqueous extracts (AQE) sequentially for 8-10 hours. The extracts were filtered through Whatman No.1 filter paper. The filtrates were concentrated using a rotary vacuum evaporator and solvents completely evaporated at 40ºC. The yields of petroleum ether (PTE), chloroform (CHE), ethyl acetate (EAE), methanol MEE), and aqueous extract (AQE) were 1.23, 0.828, 1.33, 2.63, and 1.95 % respectively.

3.2.2 Use of the extract in experiment

In all the animal experiments, the extract was administered to animals orally. The EAE which was used for most of the experiments, was only partially soluble in water. Hence, the extract was administered to the animals as a fine suspension in water.

3.2.3 Preparation of tissue homogenates

Animals were sacrificed after the end of experiments. Brain, skin and liver were excised and rinsed thoroughly in ice cold saline to remove the blood. They were then gently blotted using filter paper and then weighed in an analytical balance. 10% of homogenate was prepared in 0.05M phosphate buffer (pH 7) using a polytron homogenizer and then stored at 4^0C.

3.2.4 Determination of tissue lipid peroxidation

The level of lipid peroxidation was measured as malondialdehyde (MDA) according to the method of Ohkawa et al (1979).

Procedure

4 ml of reaction mixture containing 0.4 ml of the tissue homogenate, 1.5 ml of 0.8 % TBA, 1.5 ml of acetic acid (20 %, pH 3.5) and distilled water was kept for 1 hour in a boiling water bath at 95°C. After 1 hr, the reaction mixture was removed from the water bath, cooled and added 1 ml of distilled water. 5 ml of butanol: pyridine mixture (15:1) was added to the

reaction tube, mixed carefully and centrifuged at 3000 rpm for 10 min. Absorbance of the clear supernatant was measured at 532 nm against butanol: pyridine mixture.

3.2.5 Determination of serum glutamate oxaloacetate transaminase (SGOT) activity

The serum SGOT level was determined using Agappe diagnostic kit.

Procedure

100µl of the serum sample was mixed immediately with 1000µl of the working reagent containing (tris buffer (70.40mM, pH – 7.8), L-aspartate (208mM), lactate dehydrogenase (1200U/L), malate dehydrogenase (720U/L),α ketoglutarate (2.4mM) and NADH (0.048mM)) and incubated at 37ºC for 1 minute and read the absorbance per minute at 340nm against distilled water for the first three minutes. The enzyme activity was expressed as units per liter using the relation: SGOT activity = (ΔOD/minute) X 1745.

3.2.6 Determination of serum glutamate pyruvate transaminase (SGPT) activity

The serum SGPT level was assayed using Agappe diagnostic kit

Procedure

100µl of serum was adde with 1000µl of the buffered substrate (tris buffer (88mM, pH7.5), L-alanine (480mM), lactate dehydrogenase (1200U/L), α- ketoglutarate (3.2mM) and NADH (0.048mM).The reaction mixture was incubated at 37ºC for 1 minute and decrease in absorbance per minute was read at 340 nm against distilled water for the first three minutes. The activity of the enzyme was expressed as units per liter and calculated as

SGPT activity = (ΔOD/minute) X 1745

3.2.7 Determination of serum alkaline phosphatase (ALP) activity

ALP level in the serum was assayed using Agappe diagnostic kit.

Procedure

20µl of the sample serum was mixed with 1000µl of the working reagent containing diethanolamine buffer (100mM, pH-10.2), Magnesium chloride (0.5mM) and P- nitrophenyl phosphate (10mM). The reaction mixture was incubated at 37ºC for 1 minute and

increase in absorbance per minute was read at 405nm against distilled water for the first three minutes. The activity of the enzyme was expressed as units per litre using the following relation

ALP activity (U/L) = (ΔOD/minute) X 2750

3.2.8. Determination of haemoglobin (Hb) in blood

Haemoglobin was determined according to the method of Drabkin and Austin (1932) using Agappe diagnostic kit.

Principle

Haemoglobin reagent contains potassium dihydrogen phosphate, potassium cyanide and potassium ferrricyanide. When blood was added to it, ferricyanide forms methhaemoglobin, which is converted to cyanmethhaemoglobin. The optical density was read at 546 nm and OD is directly proportional to the amount of haemoglobin present in the blood.

Procedure

0.02ml of blood was mixed with 5ml of the cyanmeth reagent. It was then incubated at room temperature for 5 minutes and then the OD was read at 546 nm against reagent blank. The standard (60mg/dl) was treated in the same manner was used for calculation of concentration of haemoglobin in the blood.

Calculation

$$\text{Haemoglobin (g/dl)} = \frac{\text{Optical Density of test} \times 60 \times 0.251}{\text{Optical Density of standard}}$$

3.2.9. Determination of total white blood cell (WBC) count

Total WBC count was estimated.

Principle

The whole blood was diluted in a dilutent which hemolyses red blood cells. The number of white blood cells in a known volume and known dilution was counted using haemocytometer.

Procedure

0.02 ml of blood was mixed with 0.38ml of diluting fluid. Well mixed fluid was then added to a haemocytometer. Total number of white blood cells in the four large corner squares of chamber was counted after 3-4 minutes.

Calculation

Total WBC Count = Number of cells counted × 50 /mm^3

3.2.10 Statistical analysis

The experimental results were statistically analyzed. All the values were expressed as mean ±SD. Statistical significance compared to control was tested by One-way ANOVA. If the P value is <0.05, the result was considered significant.

Chapter 4: Antioxidant activity of Morchella elata

4.1. INTRODUCTION

4.2. MATERIALS AND METHODS

4.2.1. Preparation of the extracts

4.2.2. *In vitro* antioxidant assay

4.2.2.1. DPPH radical scavenging activity

4.2.2.2. ABTS radical scavenging activity

4.2.2.3. Ferric Reducing Antioxidant Power (FRAP) Assay

4.2.2.4. Nitric oxide scavenging activity

4.2.2.5. Hydroxyl radical scavenging activity

4.2.2.6. Inhibition of lipid peroxidation

4.2.3. *In vivo* antioxidant assay

4.2.3.1. Activity against croton oil induced lipid peroxidation in skin

4.2.3.2 Total antioxidant assay

4.3. RESULTS

4.4. DISCUSSION

4.1 INTRODUCTION

A large number of free radicals and other reactive oxygen species are produced in human body during the normal metabolic process. Overproduction of free radicals results in damage to various biomolecules and the condition is oxidative stress. Oxidative stress has been recognized as the causative agent of several chronic diseases such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation and other degenerative diseases. This realization has prompted to control and prevent diseases by reducing oxidative stress (Pham-Huy et al., 2008). Since a number of antioxidants are found to have scavenging activity of these free radicals, several studies have tried the efficacy of the use of antioxidants in preventing as well as in curing diseases resulting from oxidative stress. The antioxidants extensively used for varied purposes can be divided in two categories, natural and synthetic. Glutathione, selenium, vitamin E, vitamin C, vitamin A, different kinds of phenolic compounds, etc. are examples of natural antioxidants while butylatedhydroxyaniosole (BHA), butylatedhydroxytoluene (BHT), propyl gallate and tert-butyl hydroxyl quinone are examples of synthetic antioxidants. However, BHA and BHT are suspected to be carcinogenic and to cause liver damage. So use of antioxidants from natural sources has gained much interest.

Most of the natural antioxidants are derived from either plants or animal sources. Though mushrooms have been in use as food and medicine for thousands of years, only recently they are recognized as an excellent source of strong antioxidants. A large number of medicinal mushrooms have recently been reported to possess significant antioxidant activity (Jose et al., 2002, Nitha et al., 2008). Many of these mushrooms are known to be non toxic. A variety of compounds with strong antioxidant activity are now known to present in mushrooms. They include phenolic compounds, vitamin C, carotenoids, L-ergothioneine, flavonoids, tocopherols, polysaccharides etc.

Morchella species, popularly known as morel mushrooms are excellently edible and highly nutritious. They are used in traditional medicine for a long time for various health benefits in hill area (Negi, 2006). Morels have been reported to contain substantial quantity of high quality of protein, vitamins and minerals and they have been recognized as a source of biologically active, physiologically beneficial and non-toxic medicines (Magrati et al, 2011).

Given the well established relation between oxidative stress and several diseases, especially inflammation and cancer, studies of the medicinal properties of various compounds also began to examine their antioxidant activities. Hence studies on the anti-inflammatory and anticancer activity of *Morchella elata,* was initated by examining the antioxidant activity of its extracts. Different solvents based on the polarity extracted the bioactive components *M.elata.* The antioxidant activities of these extracts were evaluated.

4.2 MATERIALS AND METHODS

4.2.1. Preparation of extracts

Various extracts of fruiting bodies of *Morchella elata* were prepared as described in section 3.2.1

4.2.2 *In vitro* antioxidant Assay

4.2.2.1 DPPH$^{\cdot}$ radical scavenging assay

In this method, a commercially available and stable free radical DPPH (2,2-diphenyl-1-picryl hydrazyl) which is soluble in methanol was used**.** 100 µL of different concentrations of the extracts were added to freshly prepared DPPH solution (150 µM) to make a final volume of 2 mL and kept under dim light. After 20 minutes, absorbance of the test samples was measured at 517nm against methanol. The ability of the extracts to scavenge DPPH radical was calculated by comparing the absorbance values of the control with that of treated. Tubes without extracts were used as control (Aquino et al., 2001).

4.2.2.2 ABTS$^{\cdot+}$ radical scavenging assay

In this assay the extracts were allowed to react with $ABTS^{+}$, a model stable- free radical derived from 2,2'-azinobis (3-ethylbenzothiazolin-6-sulphonic acid). Ammonium persulphate (2.45 mM, final concentration) was added to a solution of ABTS (7 mM) and allowed to react for more than 16 hrs in dark at room temperature. ABTS and persulphate react with each other leading to the incomplete oxidation of ABTS to generate $ABTS^{\cdot-}$ radical. The $ABTS^{\cdot-}$ radical solution was diluted to an absorbance of 0.75 at 734 nm using ethanol. Different concentrations of the extracts were added to 2 mL of $ABTS^{\cdot-}$ radical solution. The decrease in

the absorbance was measured against ethanol by a spectrophotometer after 6 minutes of initial mixing (Pino et al.,2005).

4.2.2.3 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing ability of the extracts was measured at low pH**.** The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6) and 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl and ferric chloride (20 mM) in a ratio of 10:1:1. Different concentrations of the extracts were added into the reagent solution and incubated in dark for 30 minutes. The intense blue color developed was measured at 593 nm. The reducing powers of the extracts were calculated from a standard graph made by using different concentrations of $FeSO_4$. $7H_2O$ (10-100 µM in distilled water) (Benzie et al., 1996).

4.2.2.4 Nitric oxide scavenging activity

Nitric oxide radical inhibition can be estimated by the use of Griess Illosvoy reaction (Garratt, 2012). Sodium nitroprusside (10 mM) was prepared in phosphate buffer saline (pH 7.4) and 3 mL of the solution was incubated with various concentrations of the extracts at 25 °C for 150 min. After incubation, 0.5 mL of the reaction mixture was mixed with 1 mL of Sulphanilamide solution (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min at 25°C for completing diazotization. Then 1mL of naphthyl ethylene diamine dihydrochloride was added, mixed, and allowed to stand for 30 min at 25°C. A pink colored chromophore is formed. The absorbance of these solutions was measured at 540 nm against the corresponding blank (PBS buffer) solution.

4.2.2.5 Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by Fenton's reaction. The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), K_2HPO_4-KOH buffer (20 mM, pH 7.4), EDTA (0.1 mM), H_2O_2 (1.0 mM), ascorbic acid (0.1 mM) and various concentrations of the extracts of *M.elata* in a final volume of 1 mL. The hydroxyl radical would attack deoxyribose and results in the formation of TBARS. Scavenging activity of extracts was determined by measuring TBARS by the method of (Ohkawa et al., 1979)**.** Briefly, the reaction mixture

was incubated at 37ºC for 60 min. After incubation, 0.4mL of the reaction mixture was treated with 0.2 mL of SDS (8.1%), 1.5 mL of TBA (0.8%) and 1.5 mL of acetic acid (20% V/V, pH 3.5). The total volume was made up to 4mL using distilled water and kept in a boiling water bath for 60 minutes. The reaction mixture was cooled by dipping the tubes in tap water. 1mL of distilled water and 5mL of butanol:pyridine mixture (15:1, V/V) were added to the reaction mixture. The tubes were vortexed and centrifuged at 2750 g for 10 minutes. The OD of the organic layer was measured at 532 nm in a double beam spectrophotometer. The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treated (Kunchandy et al.,1990).

4.2.2.6 Inhibition of lipid peroxidation

Lipid peroxidation was induced by Fe^{2+}-ascorbate system in the rat liver homogenate in the presence and absence of extracts to form TBARS. The reaction mixture contained 0.1 mL of rat liver homogenate (25%, w/v) in Tris-HCl buffer (20 mM, pH 7), KCl (30 mM), $FeSO_4(NH_4)_2SO_4.6H_2O$ (0.16 mM), ascorbate (0.06 mM) and various concentrations of the extracts of *M.elata* in a final volume of 0.5 mL. The TBARS formed is measured according to the method of Ohkawa et al (1979).

4.2.3 *In vivo* Antioxidant Activity

4.2.3.1 Activity against croton oil induced lipid peroxidation in skin

Lipid peroxidation was induced in skin by the application of croton oil and the activity of EAE to prevent the lipid peroxidation (MDA) was estimated by the method described in the section 3.2.4.

4.2.3.2 Total antioxidant assay

Male swiss albino mice weighing 25±4 g were used for the study. The animals were divided into 5 groups of 6 animals in each group. The treatment schedule is described as follows,

Group I : Normal (Distilled water)

Group II : Paracetamol (250 mg/kg body weight)

Group III : Paracetamol (250mg/Kg b.wgt) + EAE (500 mg/kg body weight)

Group IV : Paracetamol (250mg/Kg b.wgt) + EAE (250 mg/kg body weight)

Group V : Paracetamol (250mg/Kg b.wgt) + EAE (100 mg/kg body weight).

EAE was orally administered to three groups at three different doses (500, 250, 100 mg/Kg b. wt) for seven consecutive days. In alternative days paracetamol (250 mg/Kg b. wt) was orally administered to four groups in which one group that not received the extract served as paracetamol control. The enduring one group treated without any treatments was kept as untreated control. At the end of the 8th day, the animals were sacrificed serum was collected and assayed for total antioxidant capacity of the serum. The antioxidant capacity of serum samples were expressed in micromolar trolox equivalents using a trolox standard curve.

4.3. RESULTS

4.3.1.*In vitro* antioxidant activity

4.3.1.1DPPH radical scavenging activity

The three extracts, CHE, EAE and MEE showed DPPH radical scavenging activity in a concentration dependent manner while PTE and AQE extracts did not show scavenging activity against this radical up to a concentration of 1000 µg/mL. Among the three active extracts, EAE was found to have strongest activity with 50% of radical scavenging at 500 µg/mL concentration (Fig 4.1).

4.3.1.2 ABTS radical scavenging activity

Though all the extracts were able to reduce ABTS radicals in a concentration dependent manner, EAE, MEE and AQE extracts showed strong activity compared to PTE and CHE extracts. EAE, was the most efficient to reduce ABTS radicals and caused more than 80% reduction of $ABTS^{+}$ at a concentration of 500 µg/mL (Fig 4. 2).

4.3.1.3 Ferric reducing antioxidant power

All the extracts were shown to possess the ability to convert ferric ions to ferrous ions. Five hundred micrograms of EAE, which possessed the highest activity, reduced around 45 nanomoles of $Fe3^{+}$ ions (Fig 4.3).

4.3.1.4 Nitric oxide scavenging activity

All the extracts showed moderate to strong nitric oxide scavenging activity. EAE was found to have higher activity than all other extracts. EAE showed more than 60% NO radical inhibition at 500µg/mL concentration (Fig4.4)

4.3.1.5 Hydroxyl radical scavenging activity

All the extracts were able to scavenge the hydroxyl radicals generated in the reaction mixture. MEE was the most efficient scavenger among the five extracts tested followed by EAE, PTE, CHE and AQE extracts respectively (Fig4.5). Different concentrations of the extracts scavenged hydroxyl radicals to a varying extend.

4.3.1.6 Lipid peroxidation inhibiting activity

All the extracts were able to reduce lipid peroxidation though the activity was considerably low. There was considerable overlap among the results of different extracts. EAE, which gave the highest activity, inhibited almost 28% of lipid peroxidation at 500 µg/mL concentration. PTE, CHE and AQE extracts were shown to have similar range of activities (Fig 4.6).

Since EAE was found the most active extract, further studies were carried out using this extract.

4.3.2 *Invivo* antioxidant activity

4.3.2.1 Inhibition of croton oil induced lipid peroxidation in skin

The level of lipid peroxidation in the skin treated with croton oil was found twice higher than that of untreated animals. EAE at 50 mg dose caused 78% reduction in croton oil induced lipid peroxidation (Fig 4.7).

4.3.2.2 Total antioxidant activity

Paracetamol decreasing capacity of serum from the normal level which is about 19.26µM trolox equivalent 20µL of serum 0+66 of serum to 14.86µM trolox equivalent in paracetamol control group. EAE at 500 mg/Kg b. wt dose raised antioxidant capacity of serum back to 17.05µM trolox equivalent (Fig 4.8)

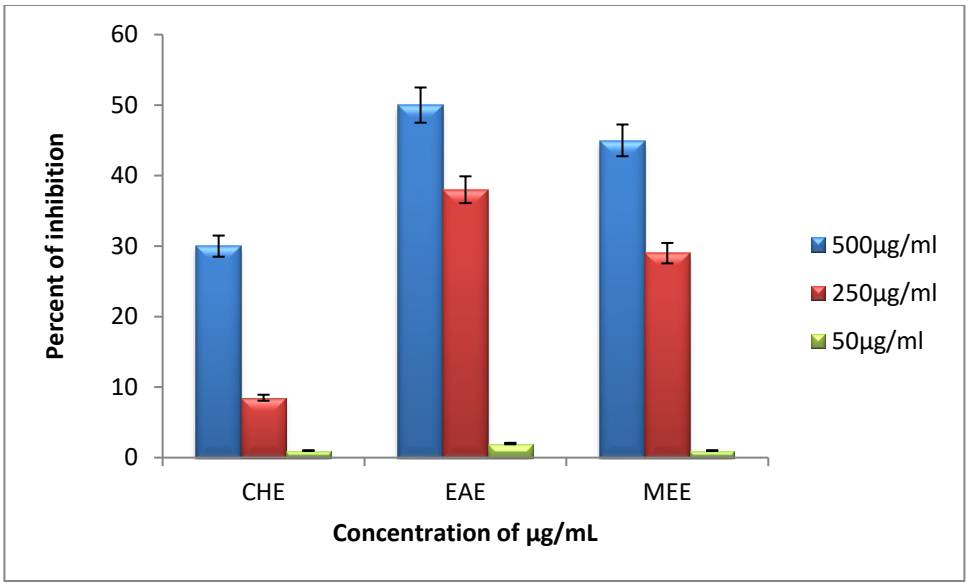


Figure 4.1: DPPH. radical scavenging activity of *Morchella elata* extracts: The quantity of DPPH. radicals scavenged is shown in percentage CHE- chloroform extract, EAE-ethyl acetate extract, MEE- methanol extract.

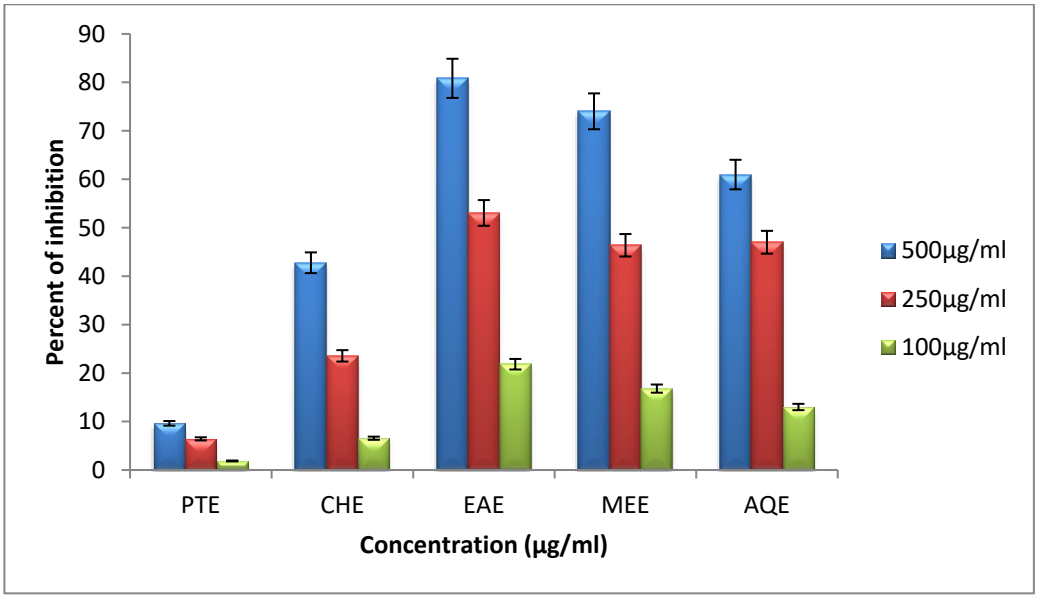


Figure 4.2: ABTS.+ radical scavenging activity of *Morchella elata*: The amount of radicals scavenged by the extracts is expressed in percentage. PTE-Petroleum ether extract , CHE-chloroform extract, EAE-ethyl acetate extract, MEE- methanol extract, AQE- aqueous extract

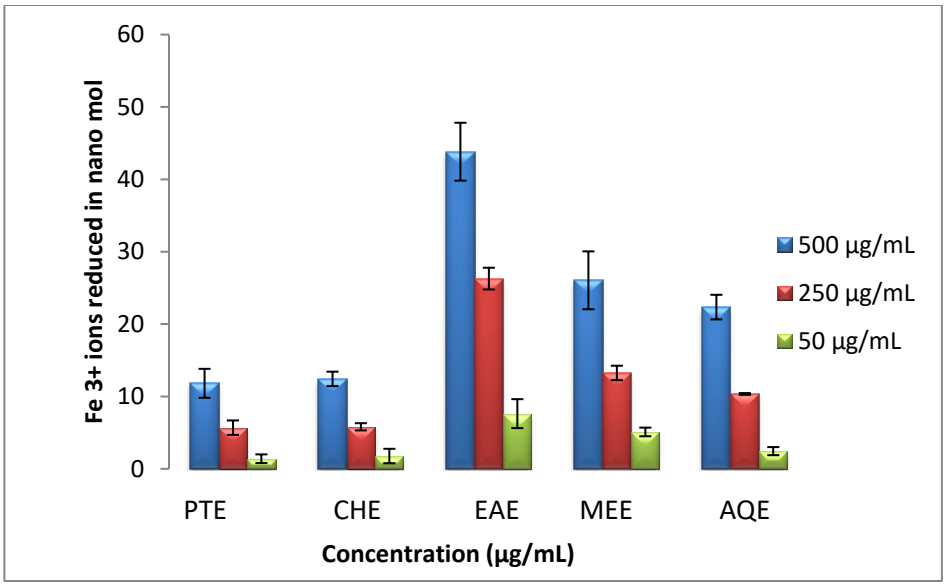


Figure 4. 3**:** Ferric reducing antioxidant power of *Morchella elata* extracts: The number of Ferric ions reduced at various concentration of the extracts is expressed in nano Mol. PTE-Petroleum ether extract , CHE- chloroform extract, EAE-ethyl acetate extract, MEE-methanol extract, AQE-aqueous extract.

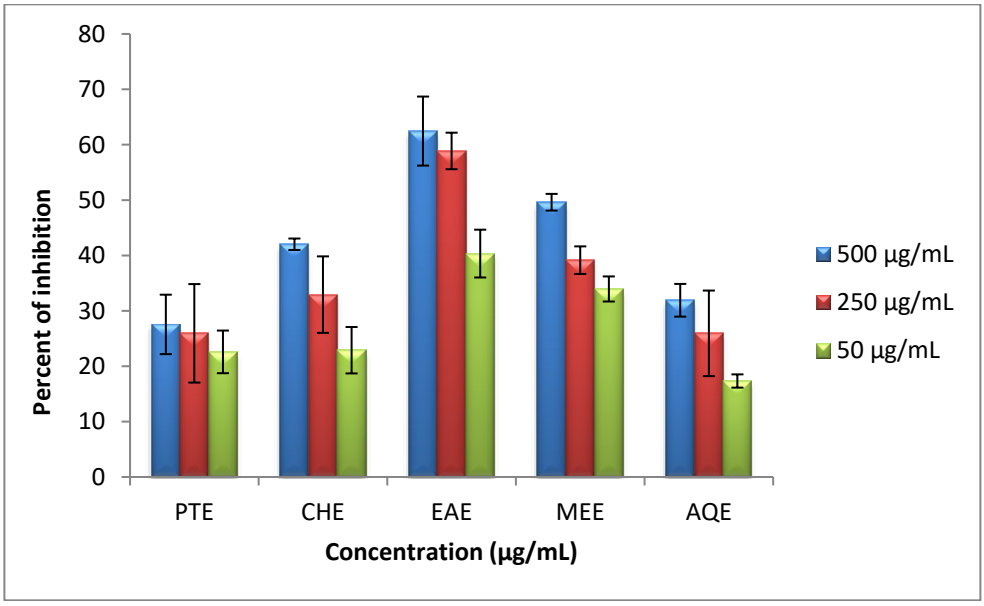


Figure 4. 4: Nitric oxide radical scavenging activity of *Morchella elata.* Nitric oxide radicals scavenged by the extracts is shown in percentage. PTE-Petroleum ether extract , CHE-chloroform extract, EAE-ethyl acetate extract, MEE- methanol extract, AQE- aqueous extract.

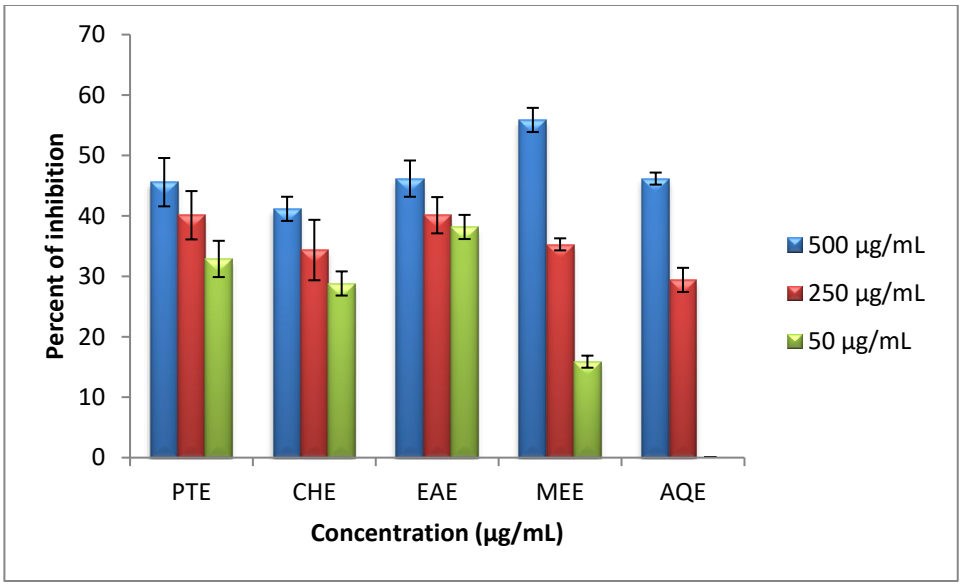


Figure 4.5: Hydroxyl radical scavenging activity *Morchella elata* : PTE-Petroleum ether extract , CHE- chloroform extract, EAE-ethyl acetate extract, MEE- methanol extract, AQE-aqueous extract

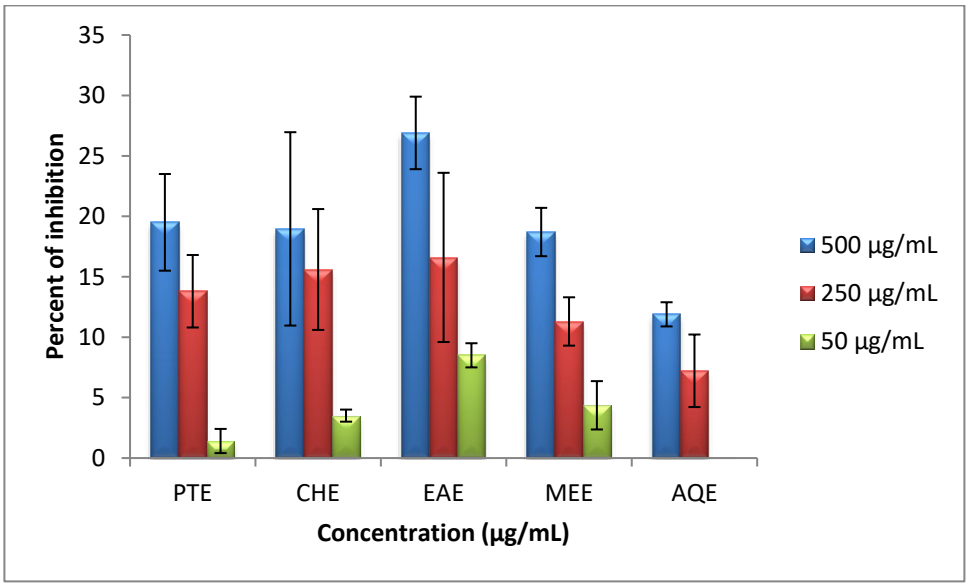


Figure 4.6; Lipid peroxidation inhibition activity : PTE-Petroleum ether extract , CHE-chloroform extract, EAE-ethyl acetate extract, MEE- methanol extract, AQE- aqueous extract

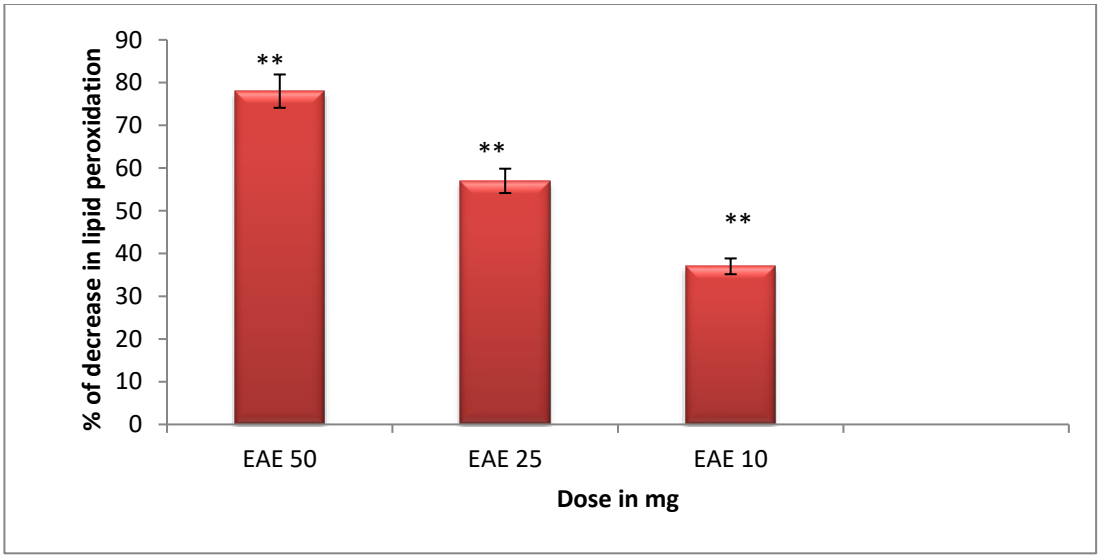


Figure 4.7: Inhibition of lipid peroxidation in skin by EAE of *Morchella elata.*

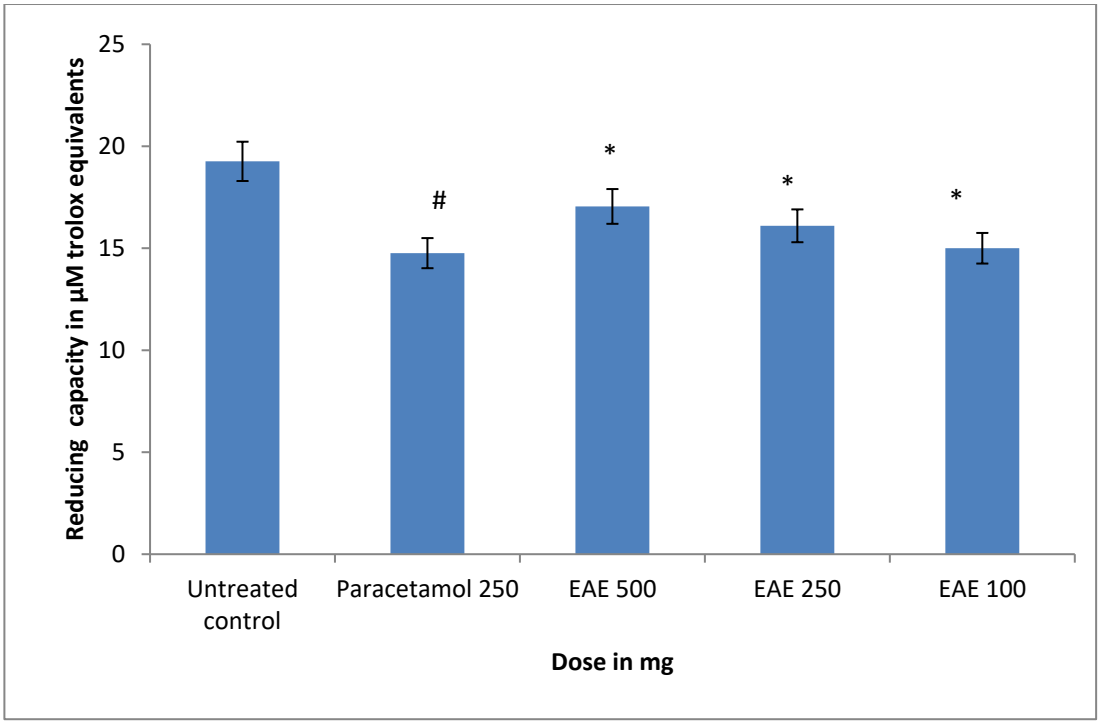


Figure 4.8:- Anti-oxidant capacity of serum was enhanced on EAE of *M.elata* administration. Value for each group was presented as mean±sd. * indicates p value < 0.05 Compared to paracetamol control. # indicates p value < 0.05 compared to normal

4.4 DISCUSSION

Mushrooms contain a large variety of bioactives which can influence various biochemical reactions occurring in human body. As a result, mushrooms possess therapeutic potential against a wide range of diseases. In the present study, *in vitro* and *in vivo* antioxidant activities of petroleum ether (PTE), chloroform (CHE), ethyl acetate (EAE), methanol (MEE), and aqueous (AQE) extracts of the fruiting bodies of *Morchella elata* (Fr) collected from Kashmir Himalaya were evaluated.

The existence of free radicals and their relevance in pathophysiology of diseases are extensively investigated in recent years. Under normal conditions healthy body has a balance between free radical and antioxidant defence. When this balance is disturbed, the condition leads to oxidative stress. Oxidative stress which can cause injury to biomolecules resulting to numerous ailments including cancer and cardiovascular diseases. Reactive oxygen species and increased levels of blood lipid are key to the pathogenesis of atherosclerosis, one of major causes of mortality in recent years. Antioxidants are major defence against radical mediated toxicity by protecting damages caused by free radicals and reactive oxygen species (ROS). Protection of biomolecule from ROS mediated damage by natural products is considered to be promising. Phenolics and flavonoids contents of natural products contribute to the antioxidant activity. Several edible and medicinal mushrooms have been reported to possess significant antioxidant activity.

All antioxidant assays are based on either the tendency of various compounds to release electrons to an oxidant or their ability to make the oxidants unable to freely exist and react with other molecules. DPPH radical scavenging activity assay, ABTS+ radical scavenging activity assay and FRAP assay are coming under the first category. In our study it was found that PTE and AQE extracts were unable to donate electrons to the DPPH radical while CHE, EAE and MEE extracts reduced the DPPH, all of these assays include electron transfer reactions. The reason for such a variation in response from assay to assay may be because the following two reasons: One is the chemical property of the oxidant and the second is the influence of the chemical environment under which these reactions are taking place.

Lipid peroxidation inhibition, hydroxyl and nitric oxide scavenging assay belong to the second category, which evaluates the scavenging ability of various compounds on free radicals.

Some extracts prevent hydroxyl menace by acting as an alternative target for hydroxyl radicals and others prevent the production of these radicals itself by inhibiting Fenton reaction. Fenton reaction requires iron for the production this radical. *Morchella* extracts are known for their iron chelating property. In the present study, all the extracts were able to reduce the level of OH radicals present in the reaction mixture. Steroids with hydroxyl groups, coumarins, terpenoids and phenolic compounds are proven class of compounds with hydroxyl radical scavenging activity. All these compounds are present in the extracts.

In the lipid peroxidation inhibition assay, lipid peroxidation is induced by generating hydroxyl radicals by Fenton reaction. Hence the compounds that can scavenge hydroxyl radicals are expected to have lipid peroxidation activity. As in the case of hydroxyl radical assay, all the extracts showed to have lipid peroxidation inhibition activity. Availability of π electrons in the compounds present in the extract might be a determining factor in the strength of lipid peroxidation inhibition activity because such a condition can mimic the double bonds present in the target sites of lipid. As we know phenols, coumarins, alkaloids and terpenes all are rich in π electrons. The result of the current experimental studies reveal that morel mushroom , *Morchella elata* from Kashmir Himalaya possessed profound antioxidant activity. The various solvent extract of the this mushroom showed marked DPPH, ABTS, hydroxyl , nitric oxide scavenging and lipid peroxide inhibiting activities *in vitro*. In the two *in vivo* antioxidant assays the capacity of the EAE to reduced lipid peroxidation was found significantly high. The ethyl acetate extract (EAE) showed overall higher antioxidant activity than all the other extracts, hence it was selected for further studies.

Chapter 5: Antiinflammatory activity of Morchella elata

5.1. INTRODUCTION

5.2. MATERIALS AND METHODS

5.2.1. Preparation of the extract

5.2.2. Animals

5.2.3. Carrageenan induced acute paw edema

5.2.4. Formalin induced chronic paw edema

5.2.5. Croton oil induced skin inflammation model

5.2.6 Inhibition of leukocyte migration induced by carrageenan

5.2.7. *In vitro* analysis of Cyclooxygenase enzyme activity

5.2.8 Assay for NF-kB inhibition activity

5.3. RESULTS

5.4. DISCUSSION

5.1 INTRODUCTION

Reactive species that are involved in oxidative stress play an important role in inflammatory responses**.** Inflammation has a useful value when the response is at optimum level. But in many cases, inflammatory responses exceed the limit resulting damage to tissues and even act as an important cause leading to cancer. Therefore, it is crucial to regulate or reduce inflammatory responses. This is often achieved by anti-inflammatory drugs. Several mushrooms components have proved to be effective anti-inflammatory agents. (Ruparelia et al., 2017: Matczak et al., 2018). Since the mediators and effectors of oxidative stress and inflammation share many signaling molecules, it appears logical to study anti-inflammatory activities of different compounds and their relation to antioxidant activity. However, inflammation is one of the target area of biomedical research in recent years.

Natural products are excellent resources for development of therapeutic compounds with anti-inflammatory potential with low toxic effects. Several studies report that mushroom derived bioactives such as phenolics, polysaccharides, indolic and flavonoids show anti-inflammatory activity due to their ability to diminish the production of inflammatory mediators by downregulation of the gene expression of different inflammatory mediators such as interleukins, IL,1β, IL-6,IL-8), tumor necrosis factor (TNF-α) and prostaglandin E2 (PGE2)and COX-2. Attempts are now focused on efforts to find out bioactive compounds that can suppress these inflammatory mediators.

The anti-inflammatory effect of certain mushroom, such as *Ganoderma lucidum, Agaricus bisporus,* and their bioactive are used as effective and safe anti-inflammmatory agents. Since, the ethyl acetate extract (EAE) of *M.elata* showed overall higher antioxidant activity than other extracts, it was selected for evaluating anti-inflammatory activity. In this Chapter, studies on the anti-inflammatory activity of bioactive ethylacetate extract (EAE) of a morel mushrooms, *M.elata* is described.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of extracts

EAE was prepared as described in section 3.2.1

5.2.2 Animals

Female Swiss albino mice weighing 25±4 g were used for the study.

5.2.3 Carrageenan induced acute paw odema

Animals were divided into five groups of six animals each. The treatment schedule is described as follows.

Group I : Untreated (Distilled water)

Group II : Diclofenac (10 mg/kg body weight)

Group III : EAE (500 mg/kg body weight)

Group IV : EAE (250 mg/kg body weight)

Group V : EAE (100 mg/kg body weight)

Acute inflammation was induced in all groups by intradermal injection of 1% suspension of carrageen in carboxymethyl cellulose on the right hind paw of mice (Lin et al., 1999). The paw thickness was measured using Plythismometer before and after carrageen in each group. The increase in paw thickness was measured after 3hrs and percentage inhibition were calculated as described in the earlier experiment.

5.2.4 Formalin induced chronic paw edema

The animals were divided into five groups of six animals in each group and experiment was performed as follows.

Group I : Untreated control (Distilled water)

Group II : Diclofenac (10 mg/kg body weight)

Group III : EAE (100 mg/kg body weight)

Group IV : EAE (250 mg/kg body weight)

Group V : EAE (500 mg/kg body weight)

Edema was induced on the right hind leg paw of all the animals of groups by subcutaneous injection of 2% formalin. Paw thickness of all the animals was measured before formalin injection using a Plythismometer and was taken as initial paw thickness. Group 1 was maintained as untreated control which received no drug. Group 2 was administered with standard drug diclofenac orally (10 mg/Kg b. wt). EAE was given orally to all the animals in the remaining groups at dose of 500, 250 and 100 mg/Kg dose once daily for six days.

Six days after formalin injection, paw thickness was again measured. The inhibition of paw edema was quantified by comparing the difference in paw thickness of treated animals with that of untreated animals and expressed as percentage of reduction using the formula, (UG – TG)/UG) × 100, where UG is mean value of net paw thickness of the untreated group, TG is mean value of net paw thickness of treated group (Ajith and Janardhanan, 2001).

5.2.4 Anti-inflammatory activity assay: Croton oil induced skin inflammation model

Animals were divided into seven groups of six animals each. The treatment schedule is as follows.

Group I : EAE (50 mg)

Group II : EAE (25 mg)

Group III : EAE (10 mg)

Group IV : Diclofenac (1mg)

Group V :Croton oil control

Group VI : Acetone control

Group VII : Untreated (Distilled water)

Fur from the dorsal side of male swiss albino mice is removed. The animals were grouped into ten groups, each group consist of six animals. After 48 hours of fur removal, 0.1 mL of 50% of croton oil in acetone (v/v) was applied on the fur removed area of skin of all the animals of five groups twice at an interval of 24 hours between two applications. Of the croton oil applied groups, three groups received three different doses (50, 25, 10mg) of topical application of EAE in 0.1 mL acetone thirty minutes before of each croton oil application.

Fourth group received standard drug diclofenac gel (1 mg) instead of extract. The fifth group did not receive any kind of medication and served as croton oil control. The sixth and seventh groups were kept as acetone (50%) and untreated controls. One hour after the second application of croton oil, animals were sacrificed, and sections of equal area (9 mm diameter) of skin were removed from all groups, to determine the skin thickness, skin mass, and level of lipid peroxidation among the different groups (Section 3.2.4) (Shin et al., 2010)

5.2.4.a Histopathological examination

Skin samples of all the group of experimental animals of croton oil induced inflammation model were fixed in 10% formalin and then embedded in paraffin. Microtome sections of the samples were taken and stained with hematoxilin-oesin. The stained sections were observed under a light microscope.

5.2.5 Inhibition of leukocyte migration induced by carrageenan

Animals were divided into five groups with six animals in each group.

Group I : Control (Distilled water)

Group II : Diclofenac (10 mg/kg body weight)

Group III : EAE (500 mg/kg body weight)

Group IV : EAE (250 mg/kg body weight)

Group V : EAE (100 mg/kg body weight)

Group I injected with Carrageenan alone and Group II treated with Diclofenac (10 mg/Kg b. wt) were maintained as control and standard drug treatment respectively. The remaining groups III-V were orally treated with EAE at doses of (500, 250, 100 mg/kg b. wt). After one hour of extract administration, animals of six of five groups were injected with 0.25 mL of 0.75% of carrageenan (i.p). After four hours of carrageenan administration, the mice were sacrificed and 10 mL of PBS was injected into the peritoneal cavity and the fluid was drained back from the peritoneal cavity and the leukocytes were counted (Al Amin et al., 2012).

5.2.6 *In vitro* Cyclooxygenase (COX) enzyme inhibition assay

The Cyclooxygenase enzyme activity was assayed by the method (Walker and Gierse, 2010). Briefly, RAW 264.7 cells obtained from National Cell Science Centre, for cell science Pune, India were grown to 60% confluence followed by activation with 1 µL lipopolysaccharide (LPS) (1 µg/mL). LPS stimulated RAW cells were exposed to different concentrations of the extract (100, 50 and 25 µg/mL) and incubated for 24 hours. After incubation, 100 µL cell lysate was incubated in 1 mL reaction mixture containing Tris – HCl buffer (p^H-8), glutathione 5mM and hemoglobin 5mM for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid 200 mM, incubated for 20 minutes at 37°C and terminated by the addition of 200 µL of 10% trichloroacetic acid in 1 N HCl. Centrifuged and the supernatant was treated with 200 µL of 1% thiobarbiturate and the tubes were boiled for 20 minutes. The reaction mixture was then cooled, centrifuged and absorbance was measured at 632 nm. Diclofenac was used as standard and the reaction mixture without any extract served as control.

COX activity inhibition was expressed in percentage using the following relation

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

5.2.7 Assay for NF-kB inhibition activity

NF-kB inhibiting activity was assayed using Lentix-293T P65 Ds Red stable cell line developed by the laboratory of Dr T.R Santhoshkumar , Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, India. Briefly, cells were seeded in 96-well optical bottom plates (Greiner Bio-One, GmbH). After attaining 60–70% confluence, the medium was removed and 50 µg/mL ethyl acetate extract of *M.elata* in 5 % FBS containing DMEM was added . TNF-α (100ng/mL) was added as inflammatory stimulus . The Lentix-293T P65 Ds Red cells were incubated for 24 hrs in 5 % CO_2 incubator at 37^o C. Plates were imaged at 0 hr and 24 hr, with BD Pathway™ 435 Bioimager (Becton Dickinson Biosciences, USA). Ds Red p65, 562/40 nm excitation and emission at 620/40 nm were collected. Images for each well was acquired in the respective channels using a dry 20X objective with NA 0.75. As the cells have a high and homogeneous expression, an average exposure time of

100–200 ms was sufficient to acquire the images. P65 RFP was detected by flow cytometry and ratio analysis by FACS was performed in a FACSAria III (BD Biosciences) equipped with a 561 nm laser line. Briefly, cells seeded in 12-well plates were treated with 50 µg/mL ethyl acetate extract of *M. elata* . TNF-α (100 ng/ml) was added as inflammatory stimulus. After 24 hr incubation in 5 % CO_2 at 37^{o} C , the cells were trypsinized, washed twice with PBS and passed through a 40 µm sieve (BD Biosciences) before analysis. The cells were excited with 561 nm laser and the emission was collected at 620 nm.

5.3 RESULTS

5.3.1 Anti-inflammatory activity against carrageen induced acute paw edema

EAE significantly inhibited the acute inflammation induced by carrageenan at a dose of 500, 250,100 mg/Kg b.wt was able to decrease the paw edema by 49.2%, 27.5%, 15% respectively. The standard drug diclofenac at a dose of 10mg/kg body weight showed 55.3% reduced the paw edema (Fig 5.1).

5.3.2 Anti-inflammatory activity against formalin induced chronic paw edema

The EAE showed significant effect against chronic inflammations. The anti-inflammatory effect of the extract was found dose dependent. The EAE at a dose of 500, 250,100 mg/Kg b.wt was able to reduce the paw edema by 53.2, 39.5,18.2% respectively as compared to that of control (Fig 5.2).

5.3.3 Inhibition of croton oil induced skin inflammation

The effect of EAE on skin inflammation caused by croton oil was assessed by monitoring skin thickness, skin mass, and by evaluating histopathological parameters.

Skin thickness: Croton oil treatment resulted in increase in skin thickness. Croton oil application almost doubled the skin thickness compared to the normal skin thickness. The topical application of EAE at three different doses, 50, 25, and 10 mg, prevented inflation of skin thickness to varying extends. Diclofenac caused 79% decrease in thickness compared to the croton oil control group (Fig 5.3).

Skin Mass : The mass of the skin from the croton oil treated group was found to be markedly increased. EAE application reduced the gain of mass, an inflammatory response, significantly (Fig 5.4)

Histopathology of skin sections

Histopathology of croton oil applied skin showed extensive leukocyte infiltration, a well established feature of inflammated tissue. Sections taken from EAE treated animals showed infiltration to a lesser extent (Fig 5.5) indicating the anti-inflammatory effect of the extract.

5.3.4 Leukocyte migration inhibition activity

In the EAE and diclofenac administered groups of animals, the number of infiltrated cells were comparatively less than that in carrageenan control group. The EAE at a dose of 500 mg/Kg b.wt reduced infiltration of leukocytes by 30%. The inhibitory effect of EAE was higher than that of diclofenac at 10 mg/Kg b.wt (Fig 5.6).

5.3.5 COX2 inhibition enzyme activity

Ethyl acetate extract (EAE) at three different concentrations, 100, 50 and 25 µg/mL inhibited COX2 enzyme activity by 51.8, 46.91, 34.53% respectively (Fig 5.7). The standard drug diclofenac at 25 µg/mL concentration showed an inhibition of 72.5%.

5.3.6 NF-kB inhibiting activity

High throughput imaging and flow cytometry assay revealed that treatment of Lentix-293T P65 Ds Red cells with 50 µg/mL EAE after 24 hr showed significant reduction in P65 Ds red expression *in vitro*. The results indicated the ability of the extract to inhibit NF-kB activity significantly (Fig 5.8).

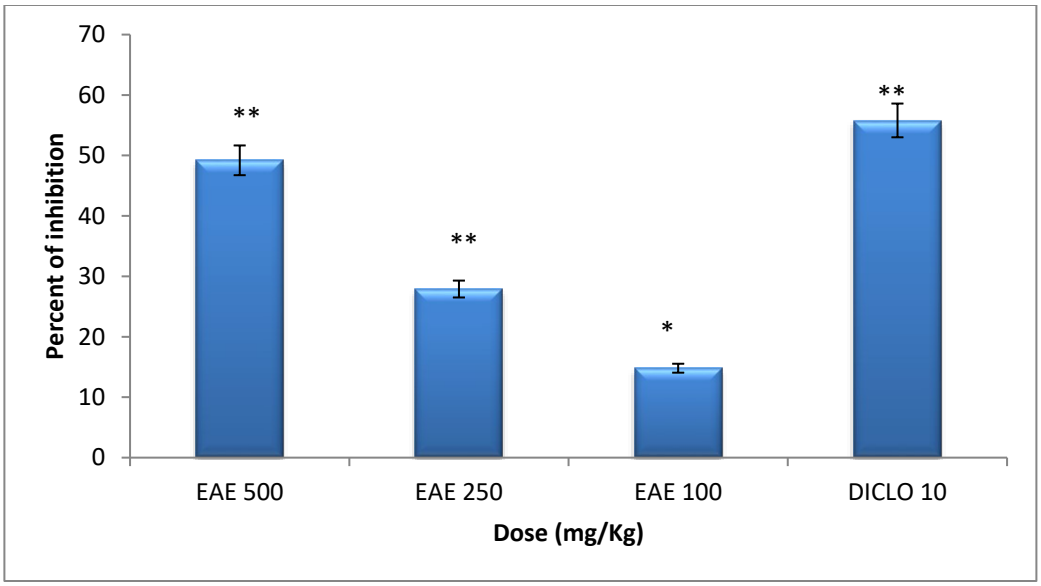


Figure 5.1: Effect of EAE against acute paw edema* $p < 0.05$ significantly different from carrageenan control group whose inflammation was taken as 100%.

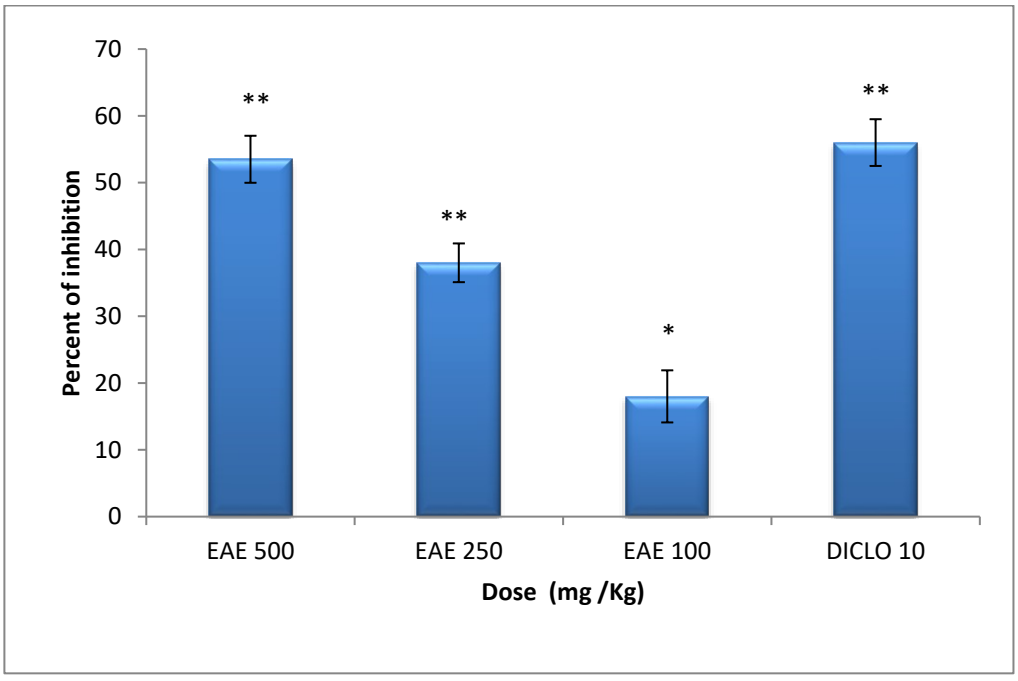


Figure 5.2 ; Effect of EAE against chronic paw edema* $p < 0.05$ significantly different from formalin control group whose inflammation was taken as 100%.

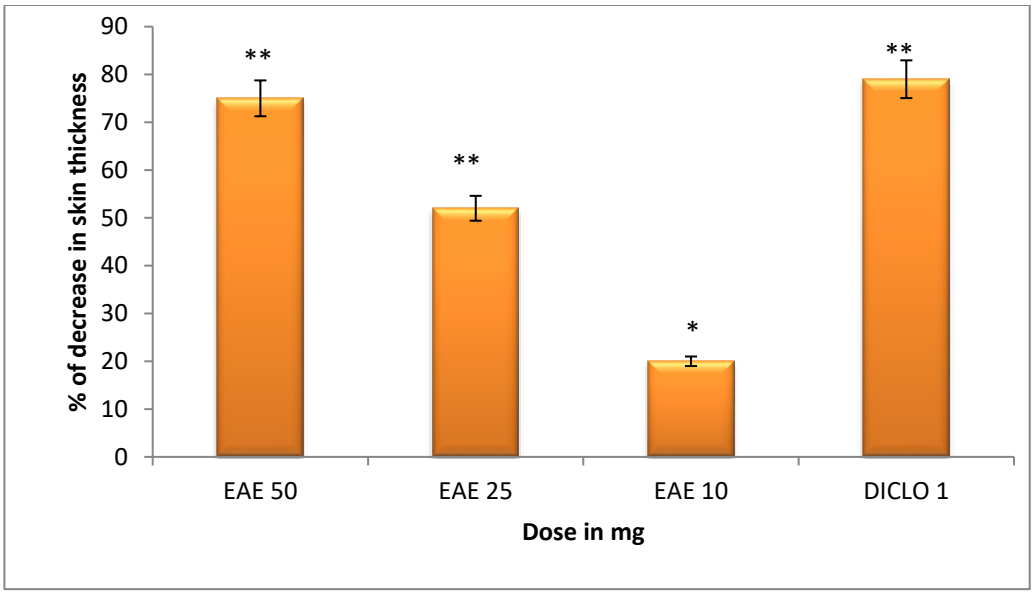


Figure 5.3: - Reduction in croton oil induced skin thickness on *M.elata* of EAE treatment. Value for each group was presented as mean±sd.* indicates p value < 0.05 compared to croton oil control group whose inflammation was taken as 100%.

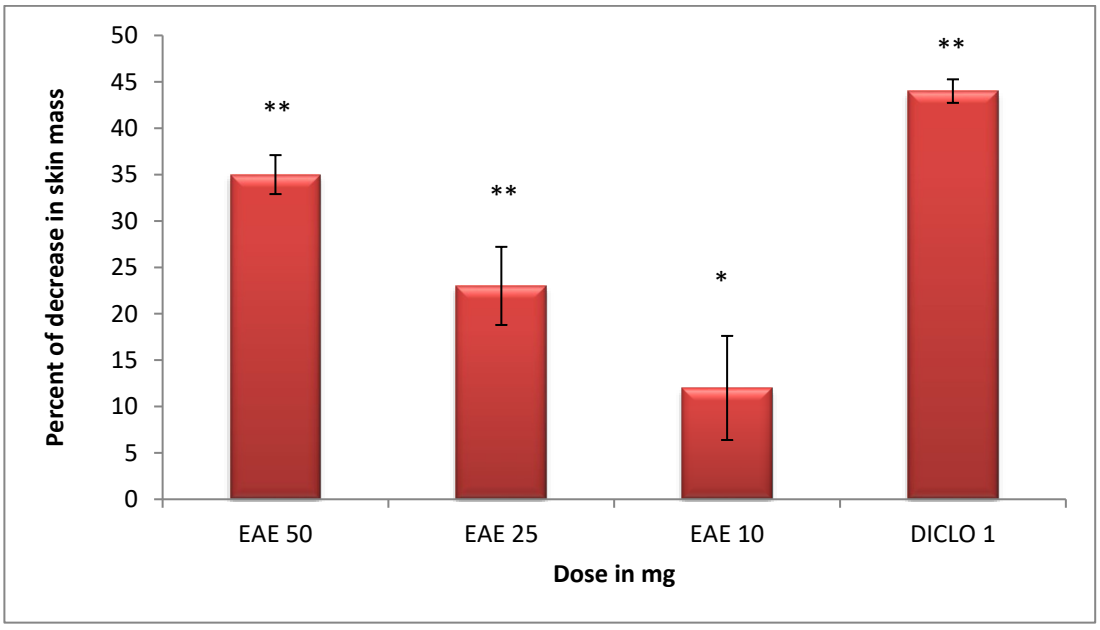
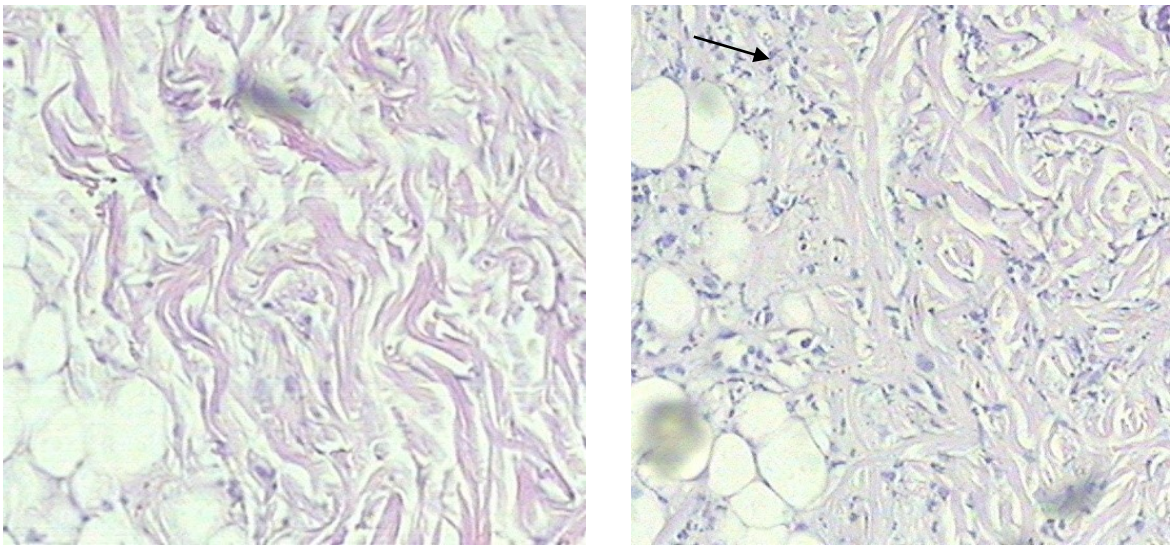
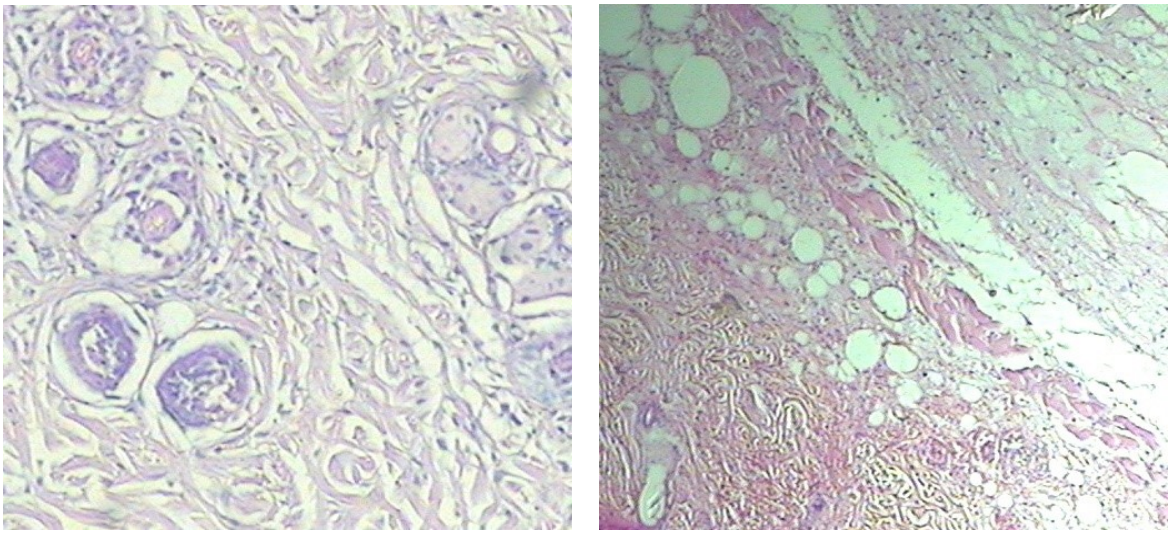


Figure 5.4:. Effect of EAE: Reduction in skin mass. Value for each group was presented as mean±sd.* indicates p value < 0.05 compared to croton oil control whose inflammation was taken as 100%.



A. Normal skin B. Croton oil treated



C. Acetone control D. EAE 50mg

Figure 5.5: Photomicrograph of skin sections showing anti-inflammatory effect of EAE of *Morchella elata*. A) Normal control, B) Croton oil applied positive control, C) Acetone control D) EAE (50 mg) plus croton oil . Arrow heads indicate epidermal infiltration of leukocyte. (H&E staining with magnification 10X).

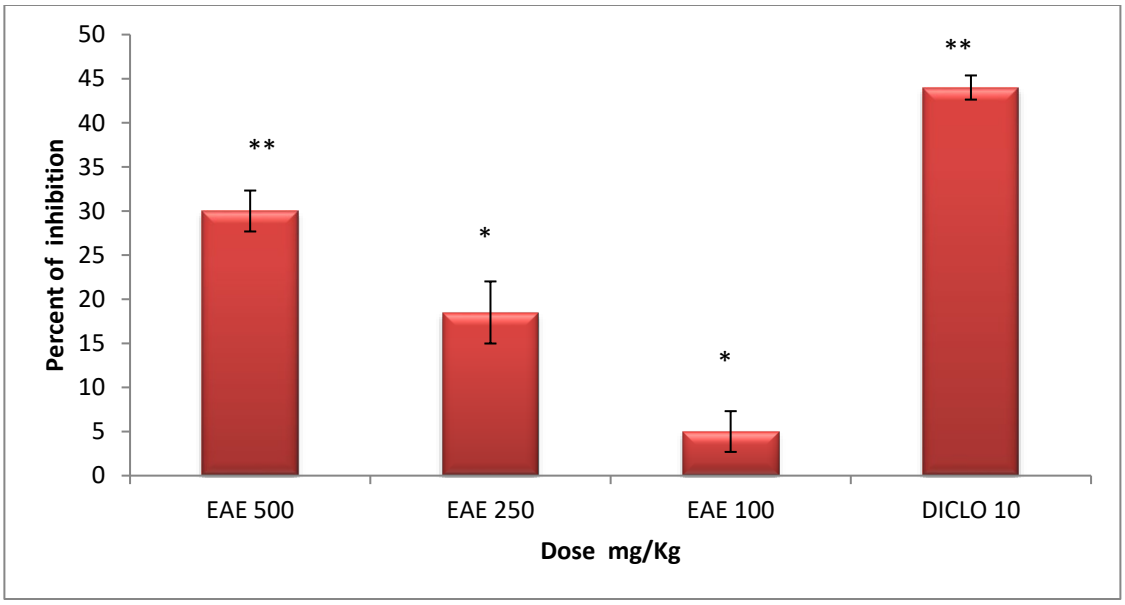


Figure 5.6: Inhibition of leukocyte migration by *Morchella elata* of EAE expressed in percentage. Value for each group was presented as mean±sd*. Indicates p value <0.05 compared to carrageenan control.

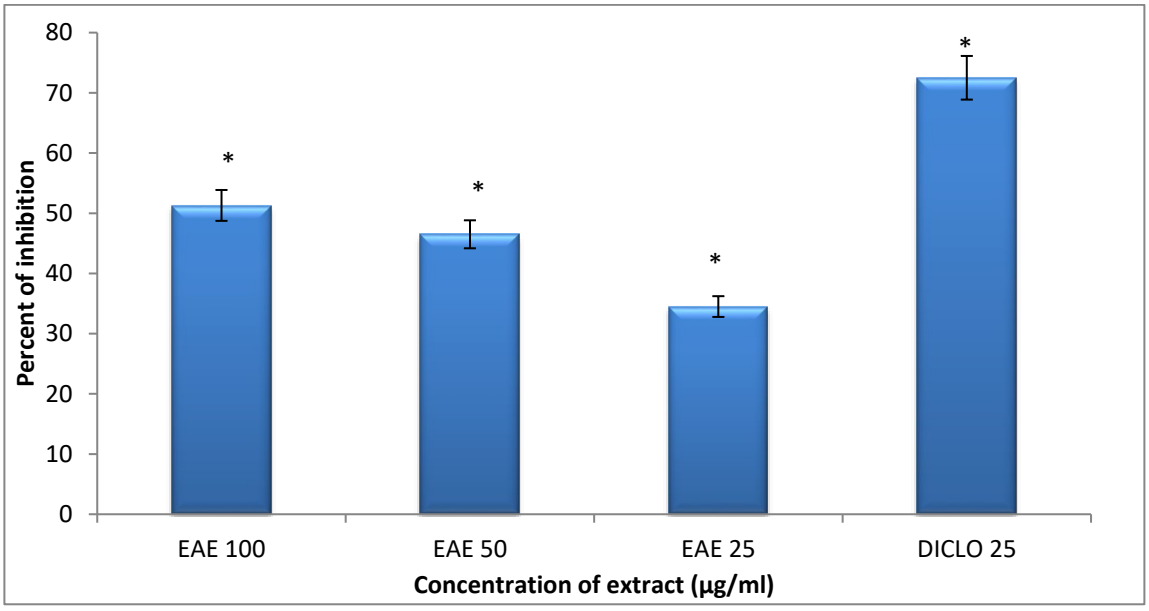


Figure 5.7: COX2 inhibition assay : Effect of various concentrations of EAE on COX enzyme activity. Value for each group was presented as mean±sd. * indicates p value < 0.05 compared to diclofenac control.

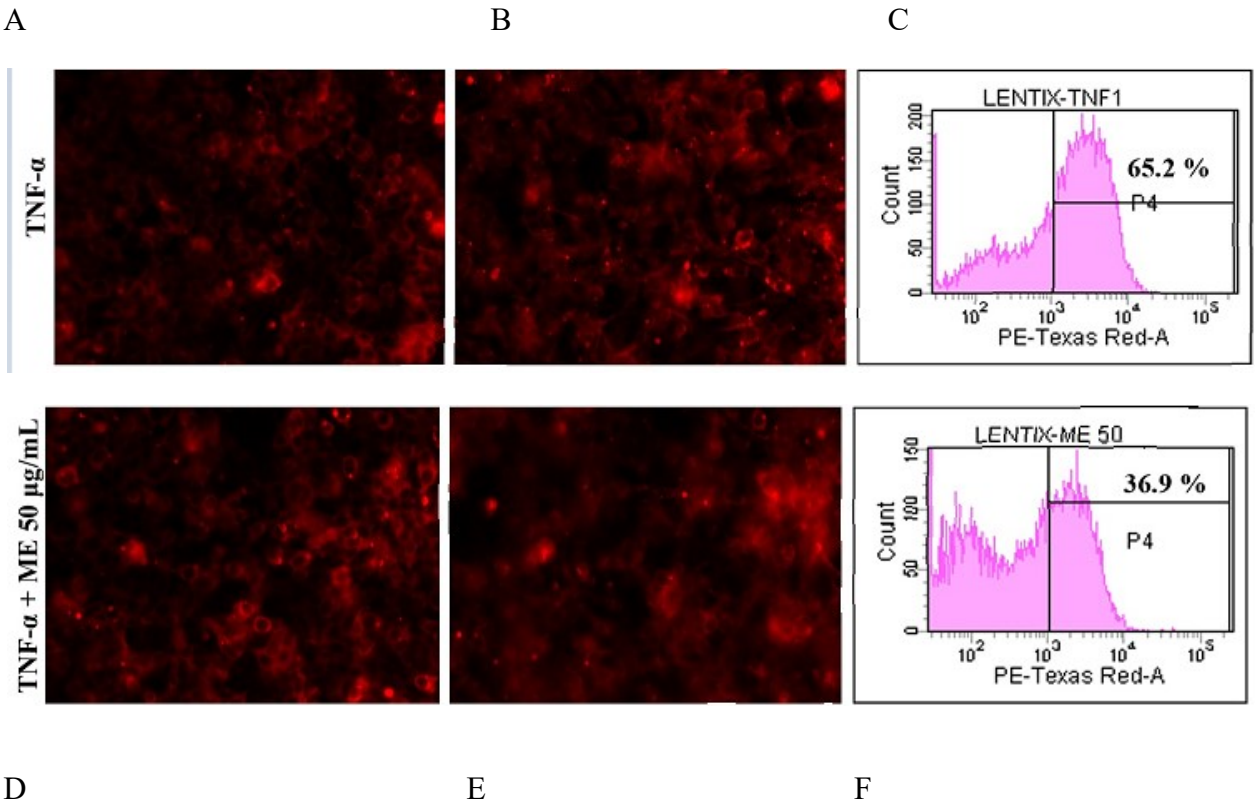


Figure 5.8. Flow cytometry analysis of NF-kB inhibiting activity by ethyl acetate extract(EAE) of *M. elata*. (A) Lentix-293T P65 Ds Red cells incubated with TNF-a at A) 0 h, (B) 24 h, and (C) Histogram of control (TNF-a). Cells incubated with TNF-a þ ethyl acetate extract (EAE) (50 mg/mL) at (D)0 h, (E) 24 hr and (F) histograms of TNF-a þ ethyl acetate extract (EAE) (50 mg/mL).

5. 4 DISCUSSION

Mushrooms contain a large diversity of biomolecule with nutritional and bioactive properties. Morels, *Morchella* species are excellently edible and one of the most highly priced and economically important beneficial wild mushrooms. They have been used in traditional medicine for centuries (Zaidman et al., 2005). Morels are precious medicinal fungi with high nutritional value containing many biological active compounds such as carotenoids, tocopherols, phenolic compounds ,water soluble polysaccharides, protein, trace element dietary fibers, vitamin etc. (Yang et al., 2015). Morels health benefits are attributed mainly to polysaccharide, phenolic compounds, tocopherol, ascorbic acid and vitamin D (Tietel et al., 2018).

Inflammation is a long chain cellular and molecular reaction characterized by sign of warmth, redness, pain, and swelling. Chronic inflammation is involved in a large number disease conditions including cancer, cardiovascular disease, diabetes, neurological, and autoimmune disorders etc. Thus inflammation is one of the target areas of biomedical research (Arulselvan et al., 2016). The complex processes of inflammation include cells of blood vessels and immune system such as lymphocytes, macrophages, monocytes, and plasma cells. When the immune cells are activated, ROS with other inflammatory mediators such as chemokines, cytokines, and prostaglandins are generated (Ahmed et al., 2017). These mediators induce classical pathways such as NF-kB, mitogen activated protein kinase, and janus activated kinase-signal signal transducers and activators of transcription (Reuter et al., 2010; Kaulmann et al., 2014). NF-kB activation can result in intracellular adhesion COX-2 and inhibition of antioxidant defense. Therefore, attenuation of oxidative stress by the *Morchella elata* extract in turn inhibits NF-kB activation. Furthermore, direct inhibition of NF-kB and COX-2 can render protection from the inflammation (Fig 5.9). Development of effective non-steroidal anti-inflammatory drugs with a little or no side effects is an area of significant importance in pharmaceuticals industry. Frequently prescribed drugs such as aspirin and diclofenac for treatment of inflammation have been reported to have severe side effect. Due to this, natural products-based anti-inflammatory agents are becoming popular in recent years (Kataoka et al., 2012). Abnormal NF-kB activation and regulation have been evidenced in arthritis. Arthritis is a highly prevalent disease characterized by nonspecific inflammation. The inflammatory

reaction is a major concern of arthritis patients. Visual arthritis index was used to evaluate severity of arthritis.

Arthritis is a highly prevalent disease characterized by nonspecific inflammation. The inflammatory reaction is a major concern of arthritis patients. Visual arthritis index was used to evaluate severity of arthritis (Weinblatt et al., 1985). Determination of paw edema is a simple and sensitive method to assess the therapeutic effect. Formalin-induced paw edema is a suitable test procedure to screen chronic anti-inflammatory agents as it closely resembled arthritis. The effect of formalin is biphasic, an early neurogenic component followed by tissue-mediated response (Ahemad et al., 2005, Wheeler-Aceto et al., 1991).

Many antioxidants can effectively reduce adverse effect of inflammatory reaction due to close link between oxidative stress and inflammation. Current experimental results reveal that ethyl acetate extract of *M. elata* showed profound anti-inflammatory activity. The observed anti-inflammatory activity of this mushroom could be attributed to the hydroxyl and nitric oxide radicals scavenging effects of the extracts. Besides the chemical constituents such as phenols, terpenes, and alkaloid present in *M. elata* also might be contributing to its anti-inflammatory effect. In addition, EAE also showed marked cyclooxygenase (COX-2) inhibiting activity. COX-2 is a marker enzyme that mediates inflammatory reactions and promotes inflammation process. The EAE also showed marked NF-kB inhibiting activity. This indicated the ability of the extract to inhibit the proinflammatory signaling molecules. Current study reveals that the EAE of *Morchella elata* possessed significant anti inflammatiory activities. Several lines of experimental evidence support these findings. Since morel mushrooms are excellently edible the findings are of significant importance. The findings thus, suggest the potential therapeutic use of *M. elata* as an anti-inflammatory agent.

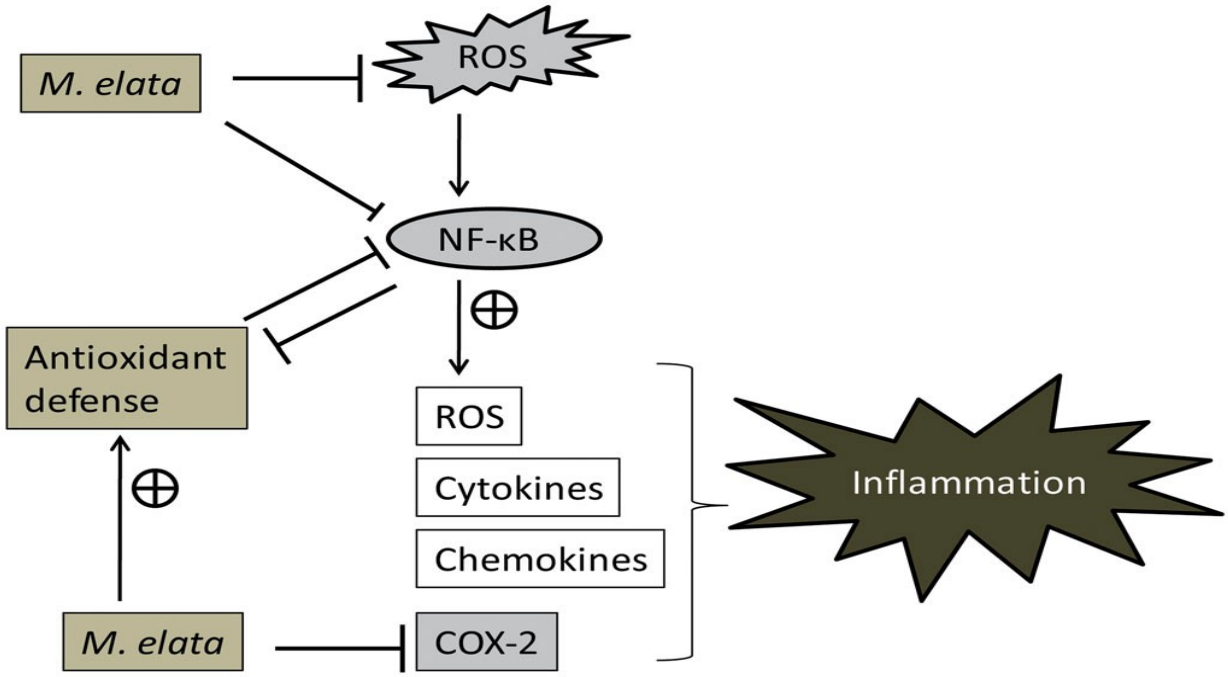


Figure 5.9. Schematic representation of possible mechanism of *M. elata.* Reactive oxygen species (ROS) generated from various sources can activate the NF-kB, which in turn inhibits the antioxidant defense, increase various cytokines, ROS and augment the inflammation. *M. elata* by its antioxidant and direct NF-kB and cyclooxygenase-2 (COX-2) inhibitory activity attenuates the inflammation.

Chapter 6:Anticancer activity of Morchella elata

6.1 INTRODUCTION

6.2 MATERIALS AND METHODS

6.2.1 Preparation of the extract

6.2.2 Cell line

6.2.3 Animals

6.2.4 Cytotoxicity against DLA cell line

6.2.4.1 MTT assay

6.2.5 Determination of antitumor activity

6.2.5.1 Solid tumor model

6.2.6 Anti- carcinogenic activity against DMBA induced skin carcinoma

6.2.7 Determination of anti-angiogenic activity

6.2.7.1 Assay for Antiangiogenic activity of EAE using DLA cells in peritoneal cavity

6.2.7.2 Assay for Anti angiogenic activity of EAE using DLA cells induced solid tumor model.

6.2.8 Apoptotic detection by double staining of acridine orange-ethidium bromide

6.3 RESULTS

6.4 DISCUSSION

6.1 INTRODUCTION

Cancer is one of the most serious health problems worldwide. It is a set of diseases, uncontrolled cellular growth with normal cancer cells invasion to different body parts and spreading to other organs, a process called metastasis. Metastasis is the major cause of cancer related mortality. In modern medicine chemotherapy, radiotherapy and surgery are the major therapeutic choices of cancer treatment. Chemotherapy plays an essential role in the clinical treatment of cancer patients (Wong, 2014). However, drug resistance and dose limiting toxicities are some of the limitations of chemotherapy. A large number of chemotherapeutic drugs derived from synthetic as well as natural sources have been used in the treatment of cancer for a long time. Most of the chemotherapy drugs currently in use have severe side effects in patients, hence search for chemotherapy drugs with least toxicity to the host cells, is continued. Discovery of safe and nontoxic chemotherapeutic agents would benefit millions suffering from cancer.

Some examples of natural product derived anti-cancer drugs include vincristine, vinblastine, methotrexate, paclitaxel, and doxorubicin. Among natural resources, mushrooms are one of the main potential sources of lead molecules for anticancer drug discovery. Mushrooms have attracted great deal of interest as a source of biopharmaceuticals in recent years. They have been demonstrated to possess great potential in developing useful anticancer bioactives (Wasser et al., 2000 and Hobbs, 2002). A number of bioactive substances derived from mushrooms such as triterpenes, polysaccharides, phenolics, polypeptides, steroids and lectins have been demonstrated to possess health-enhancing benefit. These compounds have been found to possess anticancer, anti-angiogenic antimicrobial, anti-inflammatory, antidiabetic, immunomodulator and antioxidant properties (Grienke et al., 2014). In our previous studies, EAE of *Morchella elata* showed higher antioxidant and anti-inflammatory activities. Since oxidative stress and inflammation have a causative role in the initiation and progression of cancer, EAE was examined for antitumor, anticarcinogenic, and anti-angi-ogenic activities.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of the extract

EAE of *Morchella elata* was prepared as described in section 3.2.1

6.2.2 Cell line

Daltons lymphoma cell line s were obtained from Cancer institute , Adayar, Chennai and were maintained in mice by intraperitoneal inoculation of 1×10^6 viable cells.

6.2.3 Animals

Male Swiss Albino mice weighing (20 ± 2) were used for the experiments

6.2.4 Cytotoxicity against DLA cell line

6.2.4.1 MTT assay

Around 1×10^6 cells were transferred into 96 well plate with RPMI 1640 medium supplemented with 10% FBS. The cells were incubated with different concentrations of EAE at 37^0C in a CO_2 incubator. After 24hrs of incubation, 20µL MTT solution(5mg/mLwas added to each well and incubated for an additional 4hrs. The MTT formazan blue product formed in the wells was dissolved in DMSO. The absorbance was measured at 570nm using an ELISA plate reader. The OD of untreated control and extract treated were compared and expressed as percentage of endurance at different concentrations of the extract (Arunkumar et al., 2012).

6.2.5 Determination of antitumor activity

6.2.5.1 Solid tumor model

Animals were divided into five groups of six animals each

Group I - DLA Cells (control)

Group II - DLA Cells + EAE 500mg /kg body weight

Group III - DLA Cells + EAE 250mg /kg body weight

Group IV - DLA Cells + EAE 100mg /kg body weight

Group V - DLA Cells+ Cyclophosphoamide, 25mg /kg body weight

Viable DLA Cells 1×10^6 in 0.1ml PBS were transplanted subcutaneously into the hind limb of mice. EAE at concentration of (500, 250, 100 mg/kg body weight) orally 24 hours after tumor implantation and continued for 10 consecutive days. The control group received only the DLA cell line. Cyclophosphamide (25mg /kg body weight, orally) was used as the standard reference drug. The tumor development on animals in each group was determined by measuring the diameter of tumor growth in two perpendicular planes using vernier callipers twice a week for 5weeks.The tumor volume was calculated using the formula $V=4/3\ \pi r_1^2 r_2^2$, where, r_1 is the minor radius and r_2 is the major radius. At the end of the fifth week, animals were sacrificed under anaesthesia, tumor extirpated and weighed. The percent inhibition was calculated using the formula (1-T/C) ×100; Where, C is the average tumour weight of the control group and T that of the treated group (Chihara et al., 1970).

6.2.6 Anti- carcinogenic activity against DMBA induced skin carcinoma

Male Swiss albino mice weighing 25±3g were shaved on their back using surgical clippers 2 days before the experiment. Animals with complete hair growth arrest were grouped into five groups of six animals each.

Group I : Untreated (Distilled water)

Group II : DMBA + croton oil (Control)

Group III : DMBA + croton oil + 50mg EAE

Group IV : DMBA +croton oil+ 25mg EAE

Group V : DMBA + croton oil+ 10mg EAE

The skin tumor was initiated with a single topical application of 390 n mol of 7,12 – dimethyl benz anthracene (DMBA) in 200 µl acetone on the fur removed area of all the mice of all the groups except one group which was kept as DMBA untreated control. One week after tumor initiation , the promotion was induced by topical application of 10% of freshly isolated croton oil in acetone. (v/v) twice weekly for 8 weeks on the same area. The EAE (50mg, 25mg, 10 mg in 200 µl acetone /mouse) was applied topically 40 minutes before each croton oil application. The animals were observed for 16 weeks for papilloma formation. Average number of

papilloma and latency period were recorded to assess anticarcinogenic activity of EAE (Patil et al., 2016)

6.2.7 Determination of anti-angiogenic activity

6.2.7.1 Assay for Antiangiogenic activity of EAE using DLA cells in peritoneal cavity

The study was carried out in Swiss albino mice. Five groups of animals were used for the study. Viable DLA cells were injected into the peritoneal cavity of the animals of four groups. The first group acted as untreated control. The second group was not injected with the DLA cells was served as normal. Groups III-V received EAE in three different dosages (500, 250 and 100 mg/Kg b. wt). The extract was administered once in a day for seven consecutive days.

Group I : Untreated control (Distilled water)

Group II : Normal

Group III : EAE (500 mg/kg body weight)

Group IV : EAE (250 mg/kg body weight)

Group V : EAE (100 mg/kg body weight)

24 hours after the administration of last dose of the extract, the animals were sacrificed, the peritoneal cavity was cut open, ascetic fluid was drained off washed well. The capillaries visible on interior wall of the peritoneal cavity were detected and photographed.The capillary formation was examined in treatment and compared with control (Ravikumar et al., 2021).

6.2.7.2 Assay for Anti angiogenic activity of EAE using DLA cells on solid tumor model.

Animals were divided into five groups with six animals in each group. Viable DLA cells $1x10^6$ in 0.1ml PBS was injected subcutaneously into the thigh of right hind limb of all the mice of seven groups.

Group I : Untreated control (Distilled water)

Group II : Normal

Group III : EAE (500 mg/kg body weight)

Group IV : EAE (250 mg/kg body weight)

Group V : EAE (100 mg/kg body weight)

The first group acted as untreated control. The second group was not injected with the DLA cells was served as normal. The remaining six groups orally received the EAE at doses of (500, 250 and 100 mg/kg b. wt) respectively. The extract was administered and once daily for ten consecutive days starting from the day of DLA cell implantation. After three weeks after tumor induction, the animals were sacrificed and the skin over the tumors was removed so that the underlying capillaries are intact. The number and density of capillaries present on the tumor was observed and evaluated the activity.

6.2.8 Apoptotic detection by double staining of acridine orange-ethidium bromide

DLA cells obtained from mice were washed with PBS, and ($1x10^6$ cells/ mL) cells suspended in phosphate buffered saline (PBS). Various concentration of EAE 500,250,100µg/mL was incubated with cells at 37^0C for four hours. 25µL of the cell suspension was transferred onto glass slides after the incubation period. One microlitre of acridine orange and ethidium bromide double fluroscent staining solution (both dye 100µg/mL) was added to the cell suspension and next covered with a coverslip. After 3minutes the cell morphology was analyzed, photographed and a fluorescent microscope used to count 500 cells within 20minutes (Olympus, Japan, blue filter was used) (Ciniglia, et al., 2010).

6.3 RESULTS

6.3.1 Cytotoxicity

MTT assay showed that EAE showed significant cytotoxic effect against DLA cells. EAE at a concentrations of 80, 60 ,and 40µg/ml, caused 60%, 43% , 28% of cell death (Fig 6.1). This indicated that EAE of *M. elata* possessed marked cytotoxic activity against DLA cell line.

6.3.2 Antitumor activity of extract

The EAE of *Morchella elata* drastically reduced the DLA- induced solid tumor. The EAE showed higher antitumor activity with a tumor inhibition level of 86% at 500mg/kg body weight dose (Fig 6.2 and 6.3). The standard reference drug cyclophosphamide at a dose of 25mg/kg body weight inhibited 94% tumor growth. However, cyclophosphamide caused drastic reduction in body weight of treated animals. Mortality rate in cyclophosphamide-administered group was very high.

6.3.3 Anticarcinogenic effect against DMBA induced skin papilloma

Though EAE showed marked activity in all experiments, its anticarcinogenic activity was further studied against DMBA induced carcinogenesis. The EAE interfered either with the initiation of cancer cells or with their promotion achieved by the application of croton oil. This was evident from the delay to develop papilloma and also from the reduced size and number of found on the extract treated animals compared to the untreated control. On an average, DMBA- croton oil control mice had four cancerous lesions compared to the EAE treated group(50mg, the maximum dose) was 2. (Fig 6.4) The anticancer activity was found dose dependent. Another finding was that the extract also increased the latency period (Fig 6.5). In DMBA-croton oil control group, lesions began to appear in the 11^{th} week, while in the extract treated group lesion occurred in the 12^{th} week.

6.3.4 Anti-angiogenic activity against DLA induced malignant ascites

The growth of DLA cells in the ascetic fluid induced angiogenesis on the peritonieal cavity surface. A large number of capillaries were present in the DLA control group and the blood vessels was found to have a significantly increased diameter compared to the normal control group. EAE was found to possess profound anti-angiogenic activity against the formation of blood capillaries. In order to provide a quantitative calculation, an attempt was made to count the capillaries, but it was found to be impractical (Fig 6.6).

6.3.5 Activity against angiogenesis associated with DLA induced on solid tumor

The solid tumor induced capillary formation in its surrounding area. In the DLA control tumor, a large number of capillaries present. Increased number of capillaries, and the width

of blood vessels were found increased compared to normal. Treatment with EAE was highly effective in inhibiting the capillary formation (Fig 6.7).

6.3.6 Detection of apoptosis by acridine orange –ethidium bromide double staining

Acridine orange-ethidium bromide staining showed to indication of apoptosis. The cells showed membrane blebbing and chromatin condensation following incubation with the EAE. The presence of EAE treated cells in red color indicated that the extract had caused enough membrane damage to the entry of ethidium bromide (Fig 6.8)

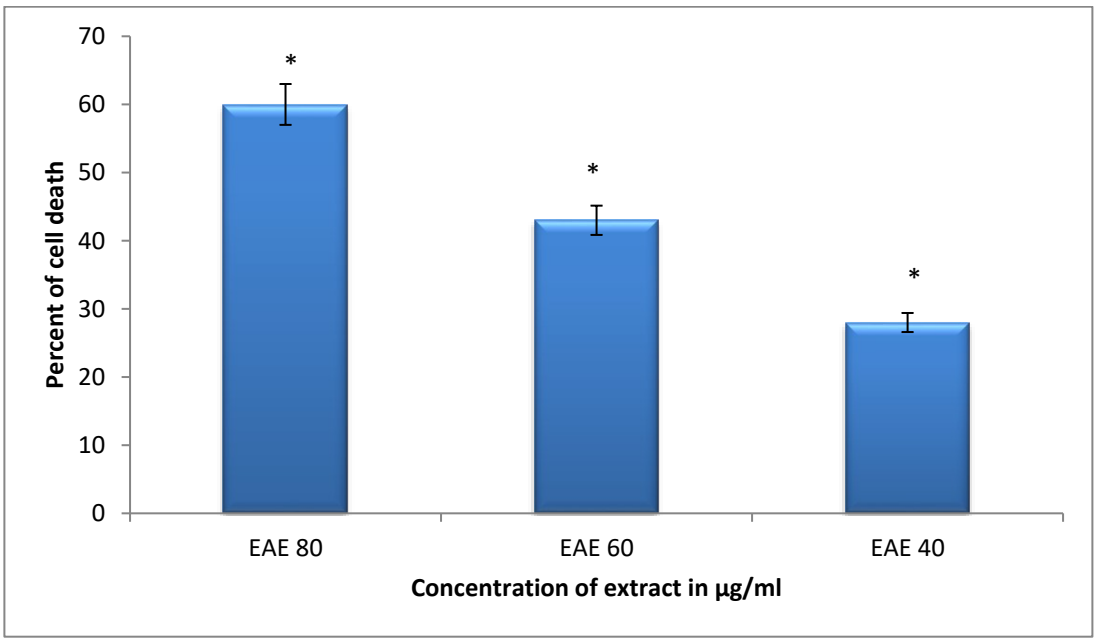


Figure 6.1 Cytotoxicity of EAE of *Morchella elata* against DLA cell as determined by MTT assay. Values obtained from triplicate tests for each concentration were presented as mean±sd. * indicates p value < 0.05 compared to untreated control.

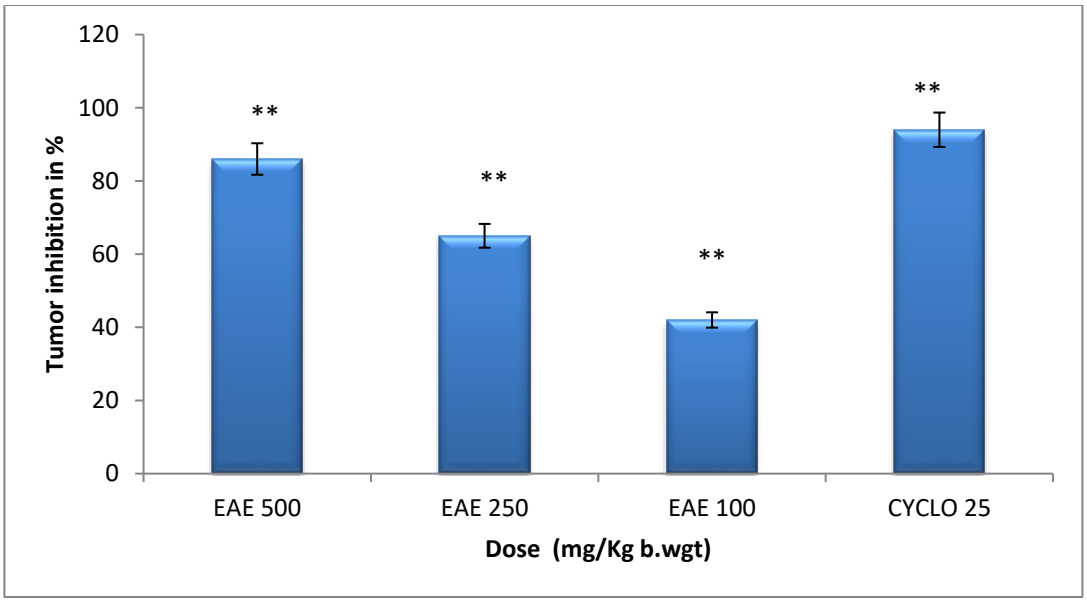


Figure 6.2. Reduction in tumor mass expressed in percentage. Value for each group was presented as mean±sd. * indicates p value < 0.05 compared to untreated control whose tumor mass was taken as 100%.

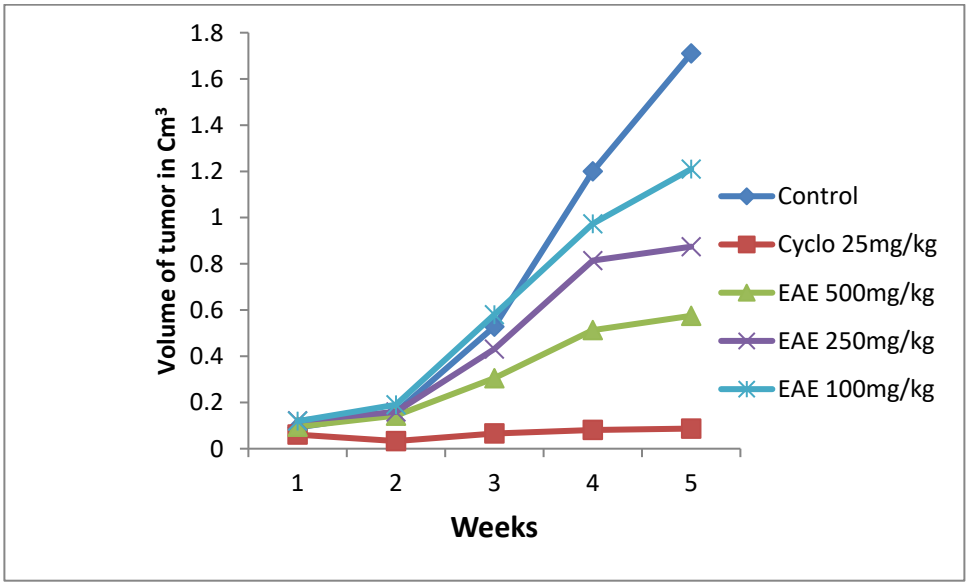


Figure 6.3 Effect of EAE of *Morchella elata* on DLA induced solid tumor model.

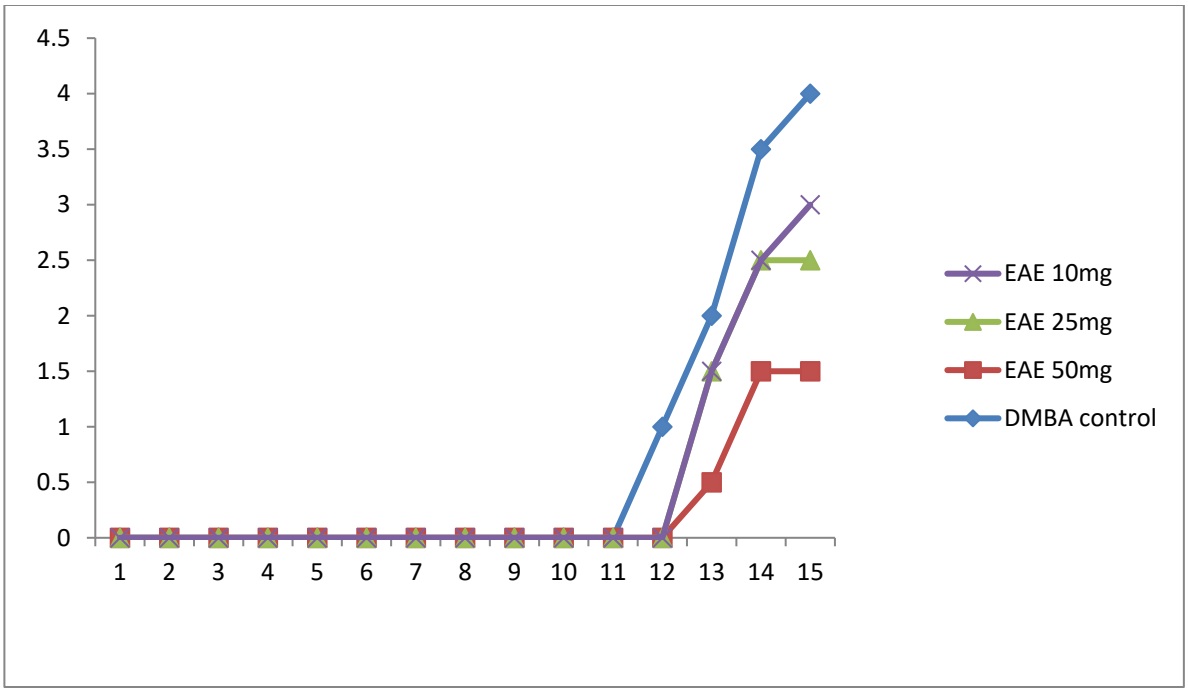


Figure 6.4 Average number of papilloma lesions on mice and corresponding latent period in various groups.

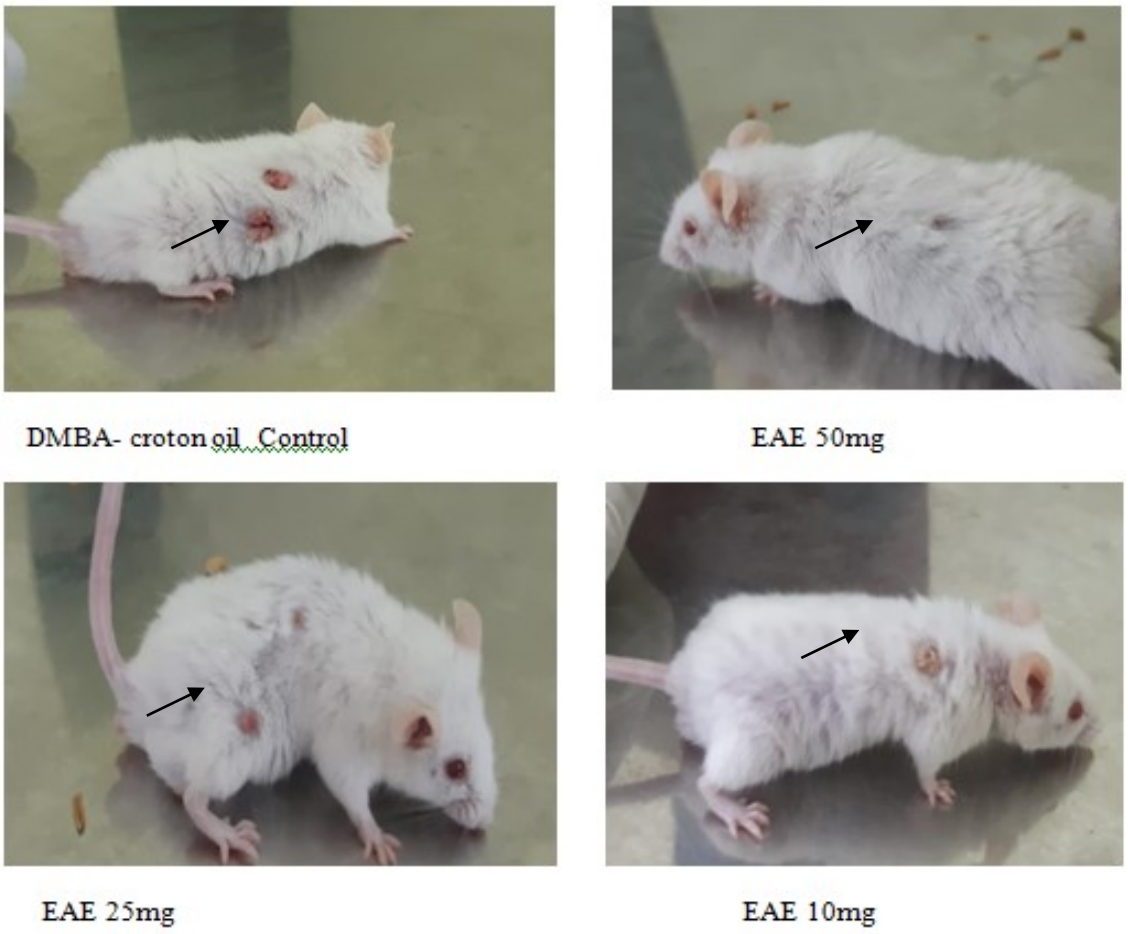


Figure 6.5 Effect of EAE on DMBA induced and croton oil promoted skin papilloma in mice.

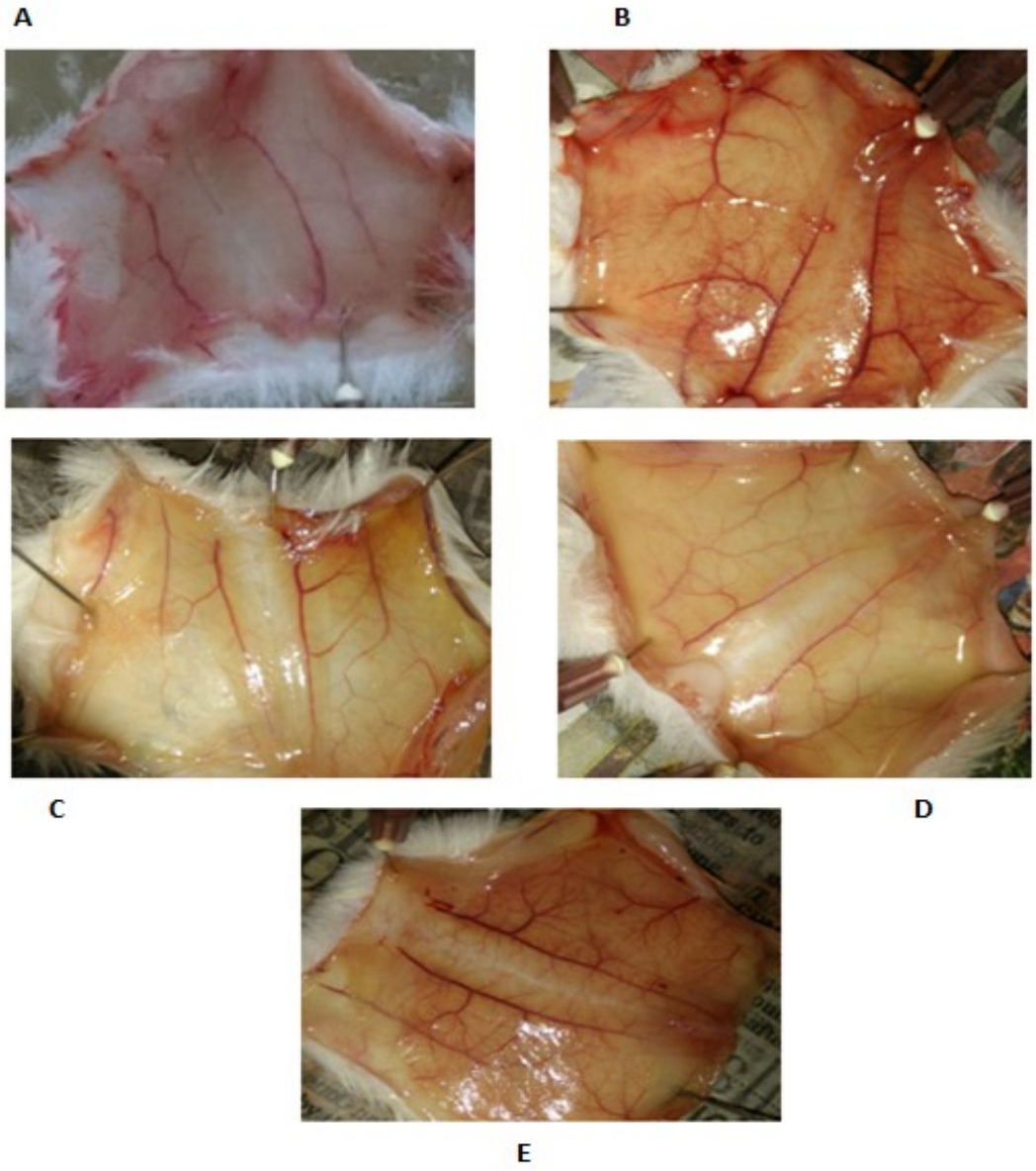
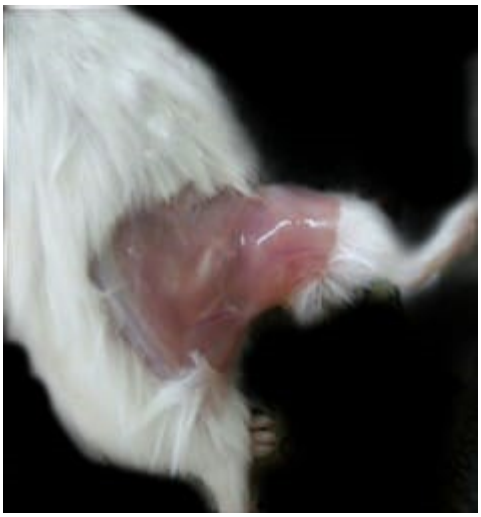
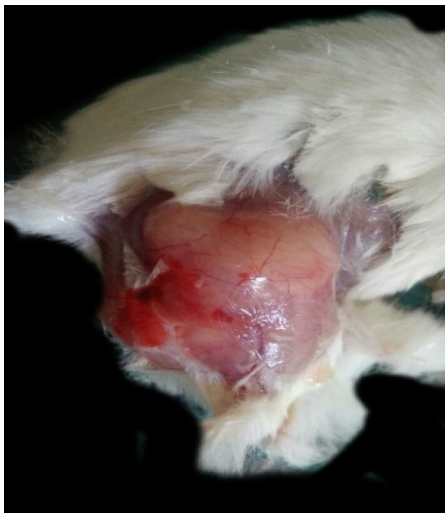


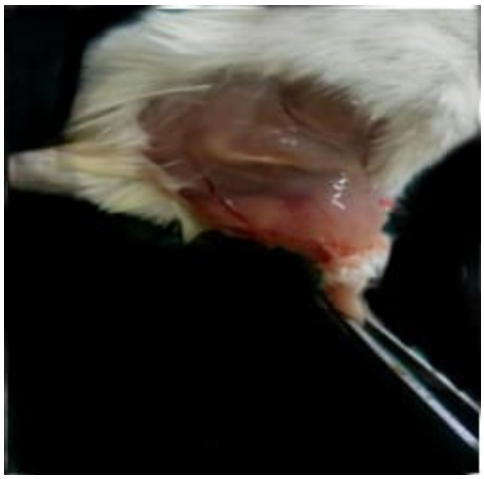
Figure 6.6: Anti Angiogenesis effect of EAE - DLA induced angiogenesis on peritoneal cavity wall A) Normal control, B) Untreated control C) EAE 500 mg/kg D) EAE 250mg/kg E) 100mg/kg.



Normal

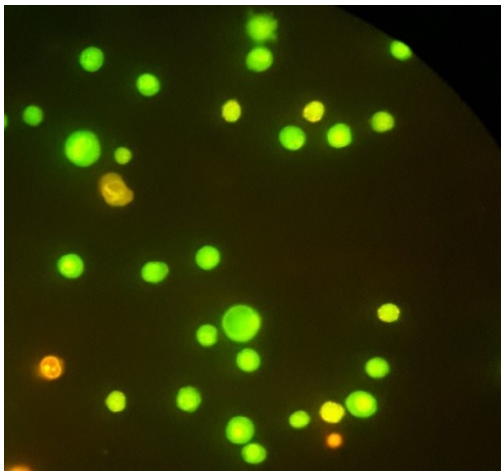


Untreated control

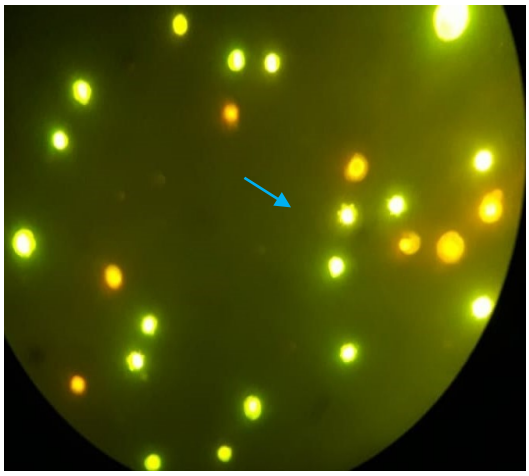


EAE 500mg/Kg

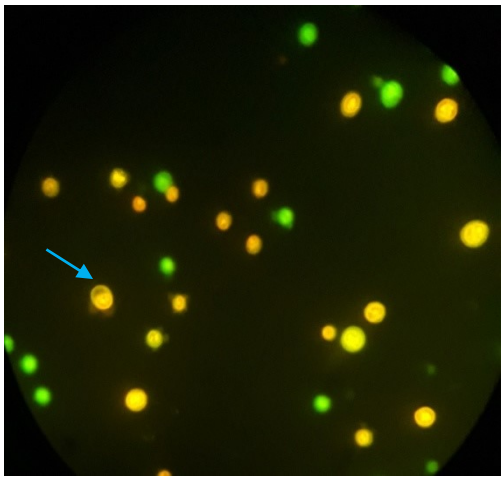
Figure 6.7 Anti Angiogenesis effect of EAE -DLA induced angiogenesis in solid tumor



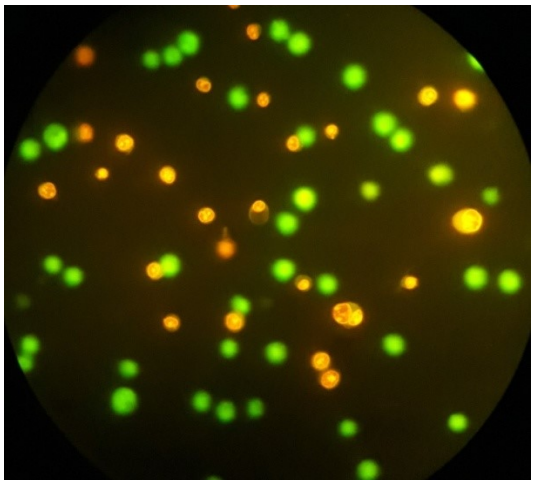
Untreated



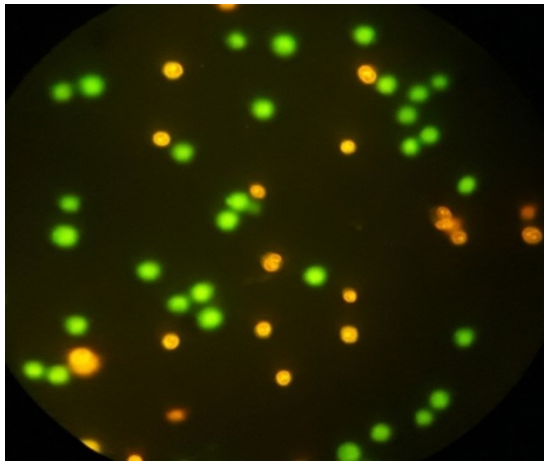
H_2O_2 treated (100 µM)



EAE 500 µg/mL



EAE 250 µg/mL



EAE 100 µg/mL

Figure 6.8: Acridine orange-ethidium bromide staining shows indications of apoptosis. The arrow indicates membrane blebbing

6.4. DISCUSSION

A number of medicinal mushrooms derived products are used in traditional medicines for the treatment of diseases such as hypertension, arthritis, diabetes, hypercholesterolemia, hepatitis and cancer. Among the medicinal mushrooms used in traditional therapy, *Morchella* is the most interesting because of its attractive therapeutic effects. This mushroom contains a wide variety of bioactive constituents such as terpenoids, steroids, phenols, glycoproteins, and polysaccharides etc.

Cytotoxicity is one of the chemotherapeutic targets of antitumor effect. Most of the clinically used antitumor agents possess significant cytotoxic activity. The higher cytotoxic activity of the extract partially explains its significant antitumor activity against solid tumors. The MTT assay showed that EAE extract was highly effective in inducing cell death to the DLA cells. Therefore, higher cytotoxicity of the extract explains its significant antitumor activity against induced solid tumor. EAE extract of morel mushroom showed significant antitumor activity. At a dose of 500 mg/kg body weight these extract showed remarkable reduction in both volume and weight. Experimental results from DMBA induced skin papilloma indicate that the application of the extract croton oil before each application drastically reduced the tumor prevalence and the number of animals with tumor as compared to control group. Experimental results indicated that *M.elata* possessed significant antitumor and anticarcinogenic activity.

Angiogenesis has a critical role in the growth and spread of cancer. In advanced stages, anti-angiogenic therapy is identified as a targeted prognosis to cure cancer .Yet diverse side effects are caused by the use of synthetic drugs. The advancement of cancer followed by neovascularization has drawn significant attention. Two models were evaluated to study the anti-angiogenic activity: the DLA-induced malignant ascites model, and the solid tumor model. The result of the experiment in both models showed that EAE of *M.elata* inhibited capillary formation indicating the anticancer effect of the mushroom extracts. The current experimental results strongly suggest the potential therapeutic use of *M.elata* as an adjuvant in the treatment of cancer.

Chapter 7:Genoprotective activity of Morchella elata

7.1 INTRODUCTION

7.2 MATERIALS AND METHODS

7.2.1 Preparation of the extract

7.2.2 Animals

7.2.3 Prevention of DNA damage induced by H_2O_2

7.3. RESULTS

7.4 DISCUSSION

7.1 INTRODUCTION

Cancer is considered as a genetic disease. Every type of cancer is linked to a genetic change. The transformation from a normal cell to a malignant is caused by changes to DNA which can be attributed to mutation. Oxidative stress causes damage to DNA as well as strand breaks in DNA, which lead to cause disruptions in normal mechanisms of cellular signalling. Genomic damage plays a major role in mutagenesis, carcinogenesis and aging. Free radicals are highly reactive atoms or molecules that possess one or more unpaired electrons in its outer orbit (Cuzzocrea et al., 2001). They are produced by exogenous chemicals or endogenous metabolic processes in the human body. They are inherently unstable, since they contain extra energy. To reduce their energy load, they react with biomolecules in the body and interfere with the normal functions of cells. The oxidants or free radicals have very short half life and damaging activity towards many molecules within the cells like proteins, lipids and DNA. They are associated with the development of many acute and chronic diseases like cancer, diabetes, atherosclerosis, inflammation, cirrhosis etc and also responsible for aging and tissue damage.

DNA is the most important biomolecule and any damage to it leads to serious consequences. Majority of free radicals react with DNA by adding to the double bonds of bases forming base radicals. Therefore, inhibition of oxidative DNA damage may be one of the strategies in a number of clinical disorders. There are various compounds derived from natural sources that offer protection against DNA damage. In case of genoprotective agents, antioxidants have a significant importance. Several lines of studies have demonstrated that genoprotective agents are efficient antioxidants. Important genoprotective agents are δ-tocopherol, resveratrol, amifostine, ascorbic acid, catechins, gallic acid and thymoquinone.

Mushrooms are potential sources of antioxidant and genoprotective compounds. Several mushrooms *Agaricus bisporus, Agaricus blazie, Agrocybe cylindracea, Ganoderma lucidum,* have been reported to possess significant genoprotective activity. While EAE of *M.elata* showed high antioxidant activity both *in vitro* and *in vivo* conditions, it was selected to investigate genoprotective activity. All these activities of EAE indicate the possibility that EAE might possess tremendous genoprotective activity. Hence genoprotective effect of EAE extract was examined and the results are reported in this chapter.

7.2 MATERIALS AND METHODS

7.2.1 Preparation of the extract

EAE of *Morchella elata* fruiting bodies was prepared as described in section 3.2.1

7.2.2 Animals

Male Swiss albino mice weighing (25±2g) were used for the study

7.2.3 Prevention of DNA damage induced by H_2O_2

Genoprotective activity of the extract against H_2O_2 induced DNA damage was studied by comet assay. This test is used to determine the ability of the drug to repair the damage caused to DNA by an agent. This assay is used to identifying the genoprotective effect of any bioactive compounds. DNA damage was induced by treating DNA samples with 100 µM H_2O_2.

Principle

Comet assay is a versatile and sensitive method for measuring the breaks in DNA strand. Relaxation of supercoiled DNA in nucleoids by single DNA break releases the loop to extend in to halo and to be pulled towards the anode under electrophoretic field. Comets from undamaged DNA have tightly packed, supercoiled DNA and no tail. Damage that leads to DNA breaks relaxes the supercoiling in loops with breaks, theses loop relax, and are pulled in to a tail under electrophoresis.

Procedure

Bone marrow cells were collected from Swiss albino mice, washed in PBS and the cells were distributed to different test tubes at a cell density of 1×10^7 cells/ml. EAE was added in to the tubes at different concentrations (500µg/ml, 250µg/ml, and100µg/ml). One tube was kept without any extract as untreated control. H_2O_2 was added at 100 µM concentration and incubated for one hour at ambient temperature. Then the cells were collected and comet assay was done to evaluate DNA damage.

Bone marrow cells containing the stock solution were mixed with 200 µl of low melting point agarose (1%) for slide preparation on microscope slides precoated with high melting point

agarose. Stock solution without drug was kept as negative control. Following cell lysis (60 min, 4°C in a lysis solution) and electrophoresis (30 min, 5°C, 25v), slides were dipped in neutralizing solution (10 min, 25°C) and washed with distilled water. Then the solutions of DNA on slides were stained with propidium iodide.

Slides were viewed under 10x objective of fluorescence microscope equipped with camera connected to a computer image analyzer. Nuclei of the cells were observed to assess the level of DNA damages in the nucleus.

7.3.RESULTS

Genoprotective activities

H_2O_2 at 100µM concentration caused considerable DNA breaks in bone marrow cells. The addition of EAE of *M.elata* reduced these double strand breaks to a marked degree. As a result of genoprotective activity of EAE the tail length – an indicator of DNA damage was reduced significantly in the samples treated with the extract. The result thus indicates that the EAE possessed significant genoprotective activity against DNA damage (Fig 7.1).

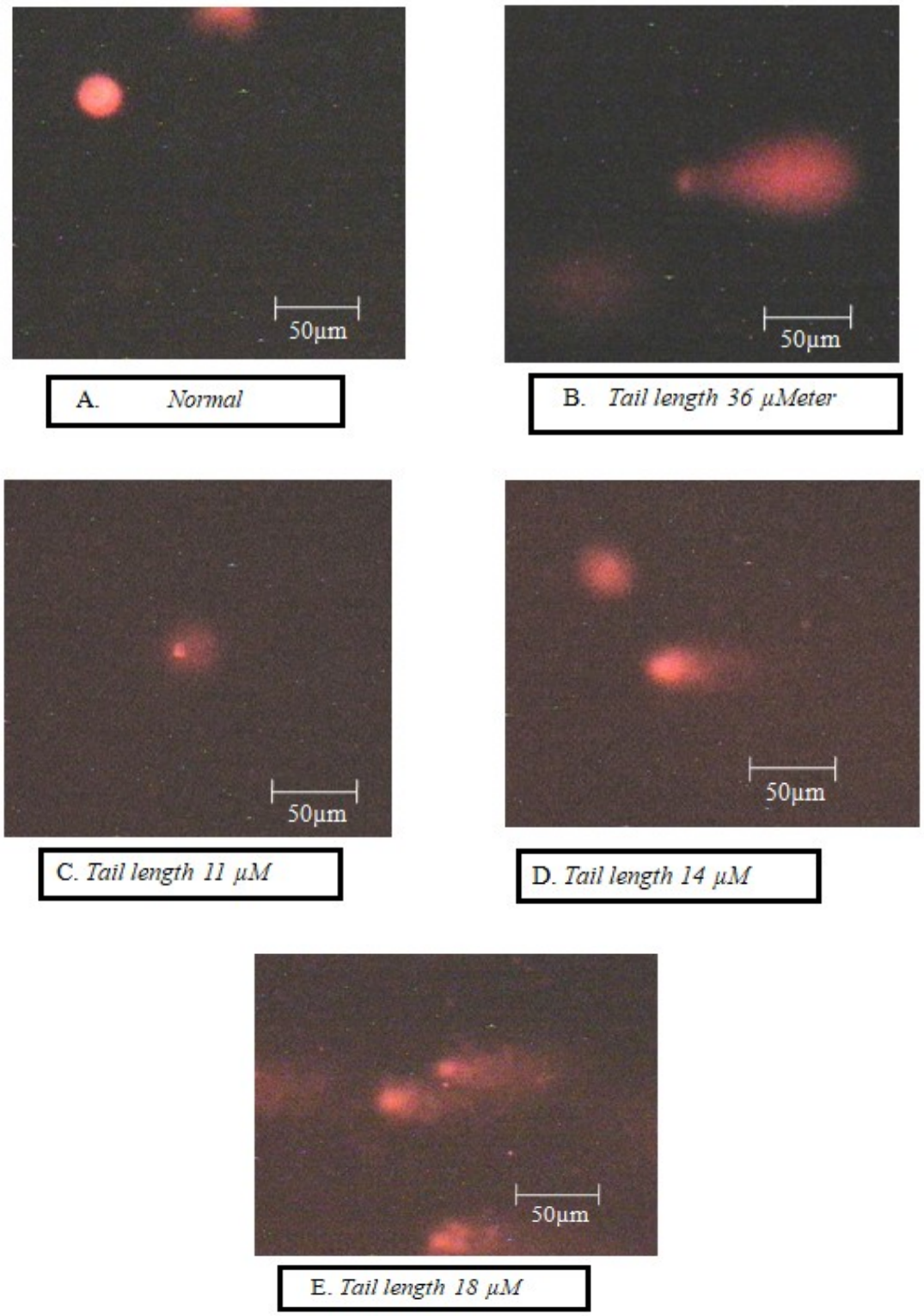


Figure 7.1.: H_2O_2 induced DNA damage and its inhibition by EAE as demonstrated in comet assay.**A**.Untreated group, **B**.Untreated Control H_2O_2 (100 µM), **C**. EAE 500 µg/ml, **D**. EAE 250 µg/ml, **E**. EAE 100µg/ml.

7.4 DISCUSSION

The integrity of DNA is crucial to the health. If so preventing DNA damage or increasing efficiency of repair is beneficial. Nutraceuticals that protect DNA from oxidative damage may have potential health benefits and help lower risk of age related diseases.

The protective effect of mushroom metabolites against DNA damages have been investigated by “comet assay ”(single cell gel electrophoresis). The comet assay is a versatile and sensitive method for measuring single and double strand breaks in DNA, capable of giving information about various kinds of DNA damage present in a cell and also about the cell’s ability to repair the damage. Ostling and Johanson first described the behavior of DNA from single cells under an electric field, detecting breaks introduced by ionizing radiation. Some studies revealed that the highest genoprotective effects were obtained with cold and hot water extracts of *Agaricus bisporus* and G*anoderma lucidum*. These findings indicate that some edible mushrooms represent a valuable source of biologically active compounds with potential for protecting cellular DNA from oxidative damage.

Cancerous cells are caused by genetic damage in its genome. Carcinogens are agents that cause changes in DNA. Mutation is caused by when mutated DNA replicates and transfer the mutated copies to daughter cells. So the mutagen cause the cell to be live, divisible state, so that mutation result in cancer.

Mutation are caused by several chemicals which are categorized as potential carcinogens based on their ability. Different mutagens employs different mechanism of action to bring about DNA damage, offer by generation of ROS. Ionizing radiation, metals such as arsenic, chromium, iron, nickel and various infections cause the generation of ROS by interacting with DNA and resulting in chemical modification of DNA. ROS cause the activation of several chemicals such as aflatoxin B1, aromatic amines and polycyclic aromatic compound as a result carcinogens are formed. Free radical scavenging property of *M.elata* play an important role in genoprotection.

In this study EAE of *M.elata* showed high antioxidant activity in both *in vivo* and *in vitro* EAE also showed significant protective activity in genoprotection. H_2O_2 has been reported to cause single and double strand breaks in DNA. Once H_2O_2 enter the cells, it generates

$OH^{\cdot}$ radicals by Fenton reaction. Hydroxyl radicals attack the sugar unit of DNA resulting sugar unit modification that leads to DNA strand breaks (Hemnani et al., 1998). EAE reduced DNA strand break significantly in comet assay. The finding suggests the potential genoprotective effect of *M. elata* bioactives.

Chapter 8: Phytochemical analysis of Morchella elata extracts and Identification of bioactives compounds

8.1. INTRODUCTION

8.2. MATERIALS AND METHODS

8.2.1. Preparation of the extracts.

8.2.2. Phytochemical analysis of the extracts.

8.2.2.1. Steroids/ terpenoids

8.2.2.1.a. Liebermann-Burchard Test

8.2.2.2. Test for Alkaloids

8.2.2.2a Mayer's test

8.2.2.2.b Dragendorff's test

8.2.2.2.c Wagner's test

8.2.2.3. Coumarins

8.2.2.4. Test for Phenolic Compounds

8.2.2.5. Test for Tannins

8.2.2.6. Test for Saponins:

8.2.2.7. Test for Carbohydrates.

8.2.2.8. Test for antraquinones

8.2.2.9 Test for flavanoides (Shinodas test)

8.2.3 Thin layer chromatography

8.2.3.a TLC plates analysis of extracts

8.2.3.b Spray reagent: Vanilin – sulphuric acid reagent

8.2.4 HPTLC analysis

8.2.5. Column Chromatography

8.2.6 HR LCMS analysis

8.3 RESULTS

8.4 DISCUSSION

8.1 INTRODUCTION

Once medicinal properties of a biological material are identified, it is quint-essential to find out the active compounds which impart its activity. Any bioactive extract of a natural product will be composed of hundreds of different compounds. Of these, only a tiny fraction may be responsible for the observed bioactivities. So, it is desired to isolate the active compounds and characterize them. This will facilitate to understand the chemical nature and to attempt its synthesis. For this purpose, structural elucidation of the compound is necessary. The benefits of knowing the structure of the active compound have immense value.

Higher ascomycetes, particularly mushrooms such as *Morchella* species contain important therapeutic bioactive components. Bioactive components present in these mushrooms include polysaccharides, lipopolysaccharides, proteins, peptides, phenols, glycoproteins, carbohydrates, alkaloids, nucleosides, triterpenoids, lectins, lipids, and their derivatives (Duncan et al., 2002). Most of the biologically active compounds in mushrooms have medicinal value. The medicinal mushrooms are known to possess anticancer, antiviral, hepatoprotective, immune protecting, and hypocholestice agents. EAE of fruiting bodies of *M.elata* possessed significant anti-inflammatory and anticancer activities. Investigations were carried out to examine the major chemical constituents of extracts of *Morchella elata* and the findings are presented in this chapter.

8.2 MATERIALS AND METHODS

8.2.1 Preparation of the extract

EAE of *Morchella elata* was prepared as described in section 3.2.1

8.2.2 Phytochemical screening of the extract

Phytochemical screening of extract was done by standard analytical methods (Tiwari 2011 and Yemm., 1954).

8.2.1.1Steroids/ terpenoids

8.2.1.a.Liebermann-Burchard Test

Few milligrams of extracts were dissolved in chloroform and an ice-cold mixture of acetic anhydride and sulphuric acid (4:1) is added. A green colour indicated the presence of steroids and a pink colour for terpenoids.

8.2.2.2 Test for Alkaloids

8.2.2.2a Mayer's test

One or two drops of Mayer's reagent were added to the acidified extract. A white precipitate indicated the presence of alkaloids.

8.2.2.2b Dragendorff's test

The Drangendroff''s reagent was prepared by mixing solution A (containing 0.6g of bismuth subnitrate in 2ml of conentraction HCL and 10ml of distilled water) solution B (containing 6g of potassium iodide in 10ml of distilled water) mixed together with 7ml of concentration HCL and 15ml distilled water and the whole solution was diluted to 100ml with distilled water to form drangendroff's reagent. The extracts were treated with drangendroff''s reagent. Formation of orange red precipitate indicates the presesnce of alkaloids.

8.2.2.2.cWagner's test

The extracts were treated Wanger's reagent containing 1.27g of iodine and 2g of potassium iodide are dissolved in 5ml of distilled water and made upto 100ml with distilled water. Formation of brown flocculent precipitate indicates the presence of alkaloids.

8.2.2.3.Coumarins

A small amount of the material dissolved in methanol/ethanol was added to alcoholic KOH/NaOH. A yellow colour was formed which disappeared on adding conc.HCl.

8.2.2.4 Test for Phenolic Compounds

Detection: A small amount of the extract was added to 10% alcoholic ferric chloride. And the colour formation was noted.

Catechol's (easily oxidizable) - green colour

Salicilaldehyde (compounds with H-bonded OH groups) - purple colour

4- hydroxyl benzoic acid (with highly acidic phenolic OH groups) - brown colour

8.2.2.5 Test for Tannins

A small amount of extracts was dissolved in 2ml distilled water and a few drops of lead acetate solution was added. Formation of white precipitate indicates the presence of tannins.

8.2.2.6. Test for Saponins:

A small amount of the extracts was mixed well with water and the formation of froth in the test tube, which persist for a few minutes, showed the presence of saponins

8.2.2.7. Test for Carbohydrates

Extracts were dissolved in distilled water and a few drops of Molish's reagent was added. To this mixture concentrate sulphuric acid was added slowly through the sides of the test tube. The formation of violet ring indicates the presence of carbohydrate.

8.2.2.8Test for antraquinones

The extracts were dissolved in chloroform and added magnesium acetate solution. The formation of a pink color indicates the presence of antraquinons.

8.2.2.9 Test for flavanoides(Shinodas test)

Extracts were dissolved in methanol and magnesium turnings were added followed by Con. HCl. Pink colour indicates the presence of flavonoids.

8.2.3 Thin layer chromatography

EAE was analyzed by TLC using silica gel G(MERK,Germany:)) 25g of silica gel G was suspended in 40ml distilled water. The slurry formed was poured uniformly on to clean TLC plates air dried and then kept in hot air oven for 1 hour at 110^0C for activation. The sample was spotted on TLC plates 2cm above the base the of plates. Solvent system (Chloroform : methanol 9:1) was poured into a TLC jar and left undistributed for half hour for saturation .The plate was the placed in the solvent at 45^0 angles and allowed the solvent to run. After complete solvent run the plates were removed from the jar, the solvent front was marked and allowed to dry at room temperature.

8.2.3a Examination of TLC plates

The plates were examined under UV for detecting fluroscent spots. The plates were then sprayed with about 10ml spray reagent heated at 100^0C for 5-10minutes then observed in visible light for the detection of the organic compounds.

8.2.3.b Spray reagent: Vanilin – sulphuric acid reagent

10% Ethanolic sulphuric acid (Solution I)

1% Ethanolic vanilin (Solution II)

The plate was sprayed vigourosly with solution I, followed immediately by solution II. After heating at 110^0C for 5-10minutes under, the plates were observed and evaluated for the detection of organic compounds .

8.2.4 HPTLC analysis

Ethyl acetate extract was dissolved in methanol 10mg/mL and was applied to silica gel 60 F254 TLC plate (E. Merck, Germany: 7cm x 10cm) using Linomat V sample applicator. Plate was then developed upto 80mm in a twin trough glass chamber using the mobile phase chloroform- methanol- water (30:4:1) solvent system. After solvent run , the plate was derivatised using vanilin – sulphuric acid reagent. Then the plates were heated at 110^0C for

10 minutes and then scanned denstiometrically at 580nm using TLC scanner 3 equipped with Wincats software.

8.2.5.Column Chromatography

The EAE (3g) was loaded onto a silica gel column (3cmx60cm) and eluted with petroleum ether (F 1-5), Chloroform: methanol 9:1 (F 6-13), Methanol (F 14-22). Fractions of 50ml were collected and analyzed by silica gel TLC. After TLC run , anisaldehyde reagent was used for visulalization and fractions which gave similar spots on TLC were pooled and the various fractions from the column was separated into three major fractions. Each fraction was examined for their cytotoxic activity by MTT assay (Section 6.2.4).

8.2.6 HR LCMS analysis

The fraction (F2) was analyzed by LC-MS. Acq method: 30minutes+-ESI-10032014.M. Acquisiton SW: 6200 series. Version : Q-TOF B.05>01(B5125) and the components were identified with the help of computer literary.

8.3 RESULTS

8.3.1. Phytochemical analysis of extracts

Preliminary phytochemical analysis showed the presence of polysaccharides, terpenoids, steroids, phenolics, tannis and alkaloids (Table 8.1).

8.3.2 TLC Analysis

TLC analysis of EAE showed presence of more than ten major compounds. Chloroform – methanol (9:1) solvent system was found to have better separation (Fig 8.1).

8.3.3 HPTLC analysis

The HPTLC analysis of EAE showed 10 peaks in the chromatogram. This indicated that EAE is composed of at least 10 different compounds with the 8^{th} and 10^{th} peaks occupying 42.2% and 33.14% area respectively (Fig 8.2, 8.3 and Table 8.2)

8.3.4 Purification by column chromatography

The fraction from the column was separated into three major fractions (F1, F2, F3). Each fraction was examined for their cytotoxicity activity by MTT assay.

8.3.5. MTT Assay

MTT assay showed that EAE fractions showed significant cytotoxic effect against DLA cells. The fractions (F1, F2, F3) at a highest concentrations of 100µg/ml, caused 34%, 80% and 39.2% of cell death (Fig 8.4A, 8.4B, 8.4C). This indicated that EAE fractions of *M. elata* possessed marked cytotoxic activity against Dalton lymphoma ascites cell line. So, the fraction (F2) which showed highest cytotoxic activity was analyzed by LC-MS analysis to identify the active compounds.

8.3.6 HR-LCMS analysis

The LC-MS analysis revealed the presence of a number of compounds. Nevertheless, LC-MS is vulnerable to the production of irregular molecular masses due to the formation of molecular artefacts. However, four major compounds were identified (1)Celastrol (RT 9.504, $C_{29}H_{38}O_4$, MW 450.27) (2) Convallatoxin(RT 9.60, $C_{29}H_{42}O_{10}$, MW 550.27) (3)Cucurbitacin A (RT 11.97, $C_{32}H_{46}O_9$, MW 574.71) (4) Madecassic acid(RT 14.35, $C_{30}H_{48}O_6$, MW 504.70) *(*(Fig 8.5A and Fig 8.5 B).

Table 8. 1. Qualitative analysis of phytochemicals in *Morchella elata* extracts. (+++ strongly present, ++ present, + weekly present, -absent

Extracts	**Phenolics**	**Coumari**	**Alkal**	**Terp**	**Steroids**	**Flava**	**Tann**	**Sapo**	**Anthraqui**	**Carbohyd**
Petroleum ether extract(PTE)	-	-	+	-	+++	-	-	-	-	+
Chloroform extract(CHE)	++	+	-	-	++	-	-	-	-	+
Ethyl acetate extract(EAE)	+++	+++	-	++	+++	-	-	-	-	++
Methanol extract(MEE)	+	+	+	-	-	-	++	-	-	++
Aqueous extract(AQE)	-	+	+	-	-	-	++	+	-	+++

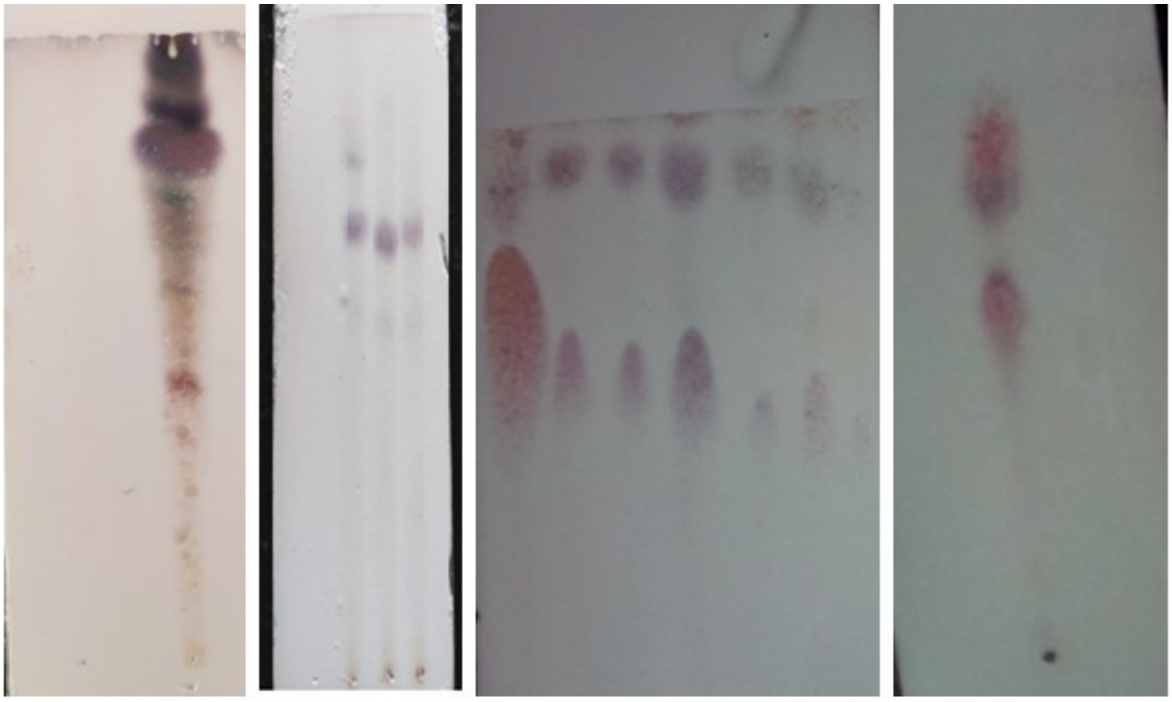


Figure 8.1 :A. TLC of EAE of *Morchella elata* extract. B, C, D: Some of the fractions collected from extract.

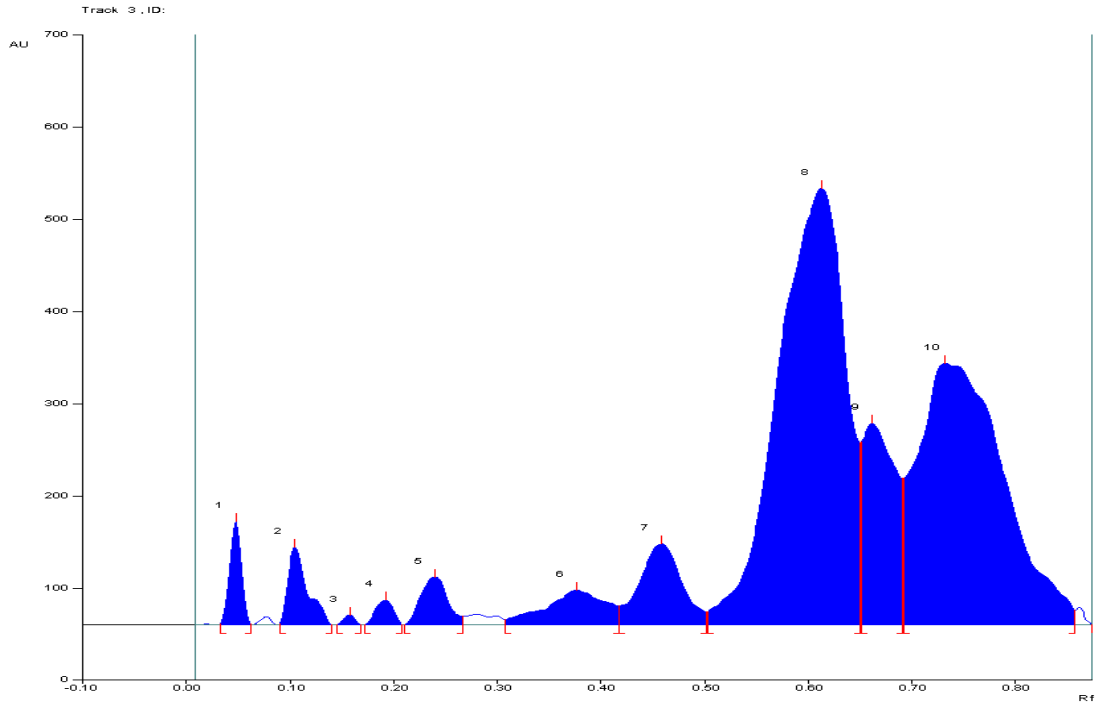


Figure 8.2 HPTLC finger print profile of EAE of *Morchella elata*.

Track 3, ID:

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.04 Rf	1.0 AU	0.05 Rf	112.2 AU	8.09 %	0.07 Rf	0.7 AU	1230.2 AU	1.81 %
2	0.09 Rf	1.2 AU	0.11 Rf	84.2 AU	6.07 %	0.14 Rf	0.2 AU	1510.7 AU	2.22 %
3	0.15 Rf	0.1 AU	0.16 Rf	10.7 AU	0.77 %	0.17 Rf	0.2 AU	109.9 AU	0.16 %
4	0.18 Rf	0.2 AU	0.20 Rf	26.7 AU	1.92 %	0.21 Rf	0.2 AU	423.8 AU	0.62 %
5	0.21 Rf	0.0 AU	0.24 Rf	51.9 AU	3.74 %	0.27 Rf	9.1 AU	1270.7 AU	1.87 %
6	0.31 Rf	5.9 AU	0.38 Rf	37.6 AU	2.71 %	0.42 Rf	20.2 AU	2041.6 AU	3.00 %
7	0.42 Rf	20.3 AU	0.46 Rf	87.6 AU	6.32 %	0.51 Rf	13.8 AU	3362.8 AU	4.94 %
8	0.51 Rf	14.2 AU	0.62 Rf	473.3 AU	34.14 %	0.65 Rf	37.6 AU	28947.2 AU	42.56 %
9	0.65 Rf	198.1 AU	0.67 Rf	218.6 AU	15.77 %	0.70 Rf	58.6 AU	6581.8 AU	9.68 %
10	0.70 Rf	158.9 AU	0.74 Rf	283.7 AU	20.46 %	0.86 Rf	15.5 AU	22542.4 AU	33.14 %

Table 8.2: Peak area report of the anisaldehyde sulphuric acid derivatised plate scanned densitometrically at 580 nm.

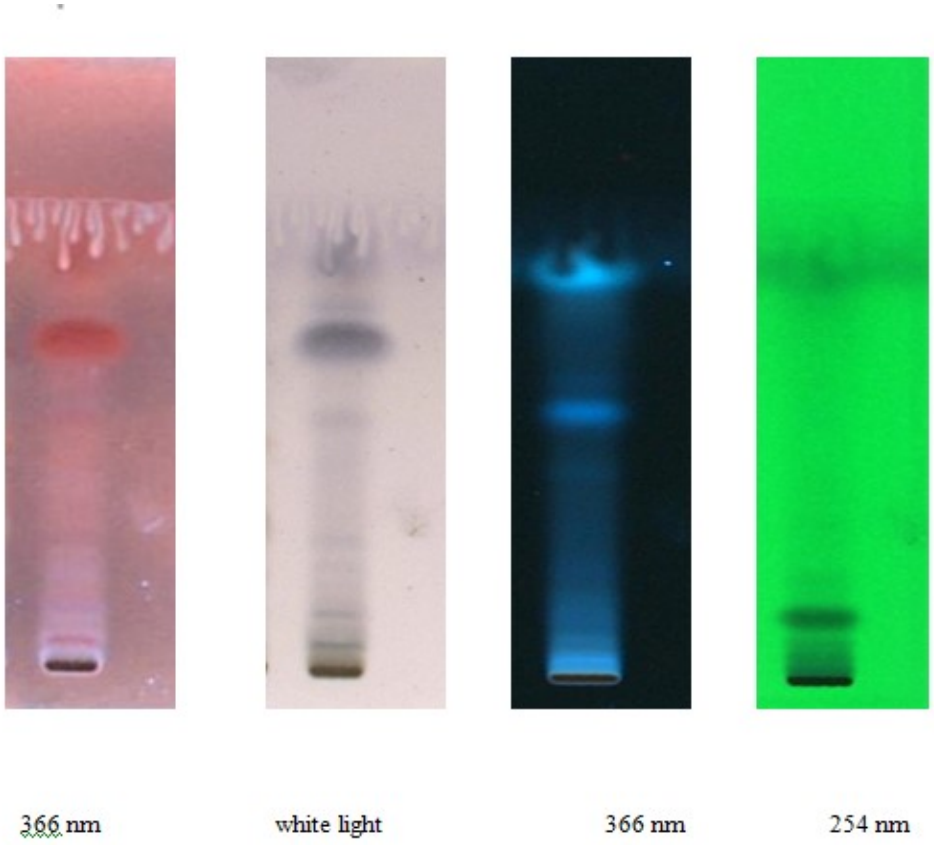


Figure 8.3 : Derivatized plates photographed using light of various wave lengths

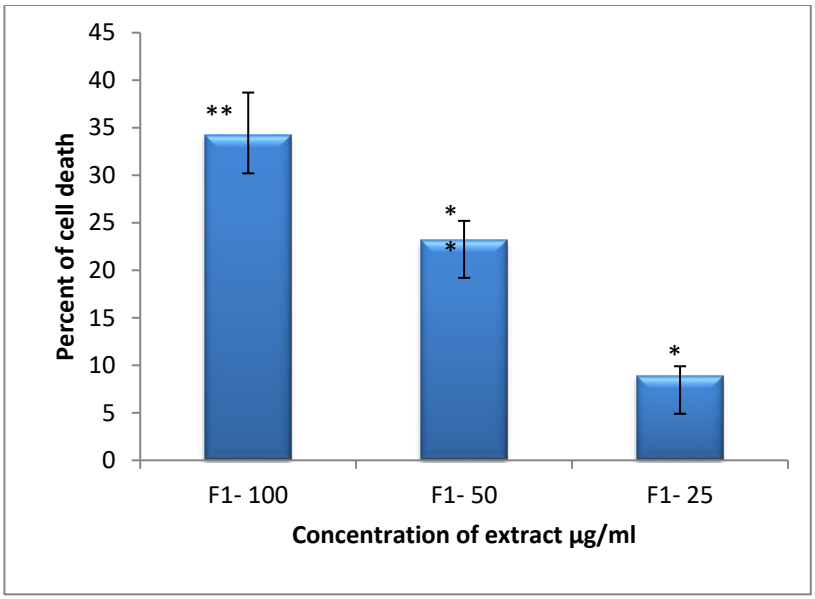


Figure 8.4 A. Cytotoxicity of F1 (Fraction 1) of *Morchella* species against DLA cells as determined by MTT assay.Values obtained from triplicate tests for each concentration were presented as mean±sd. * indicates p value < 0.05 compared to untreated control.

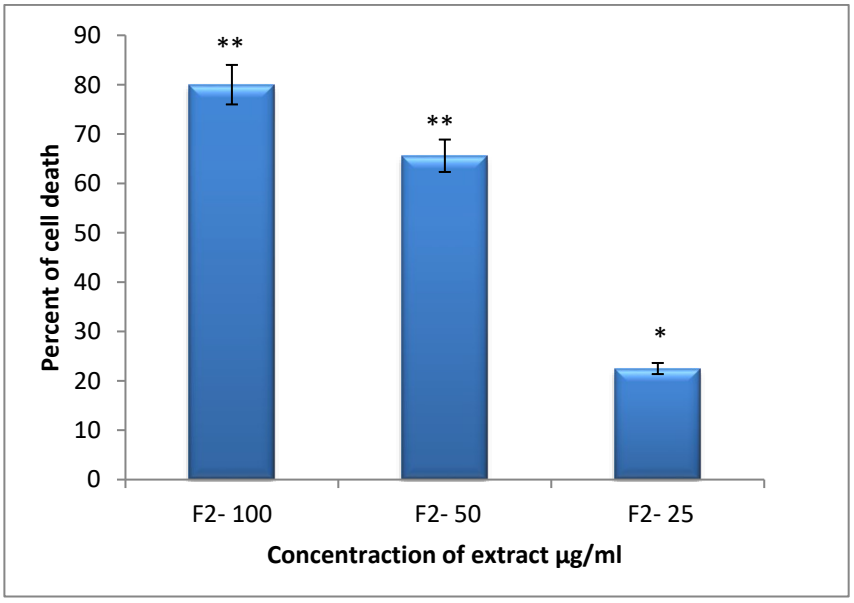


Figure 8.4 B. Cytotoxicity of F2 (Fraction 2) of *Morchella* species against DLA cells as determined by MTT assay Values obtained from triplicate tests for each concentration were presented as mean±sd. * indicates p value < 0.05 compared to untreated control.

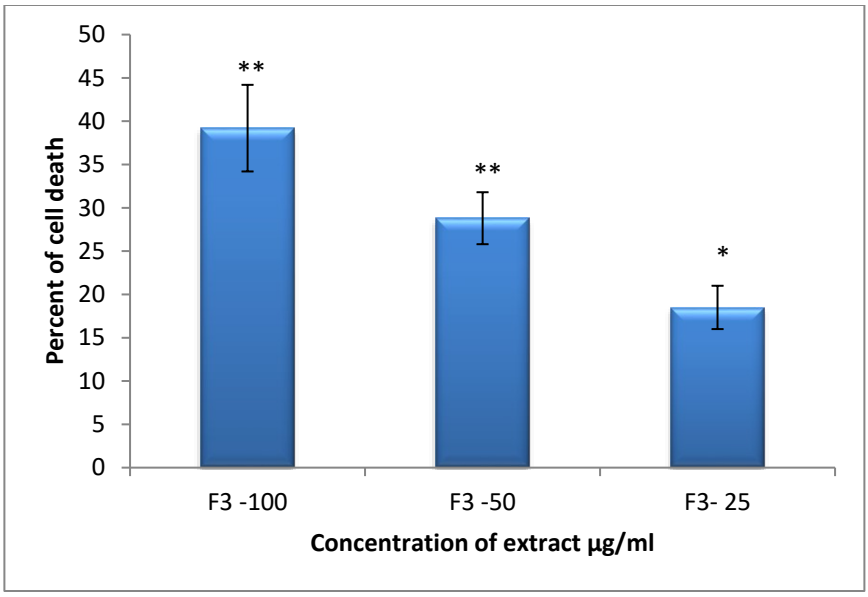


Figure 8.4 C. Cytotoxicity of F3 (Fraction 3) of *Morchella* species against DLA cells as determined by MTT assay. Values obtained from triplicate tests for each concentration were presented as mean±sd. * indicates p value < 0.05 compared to untreated control.

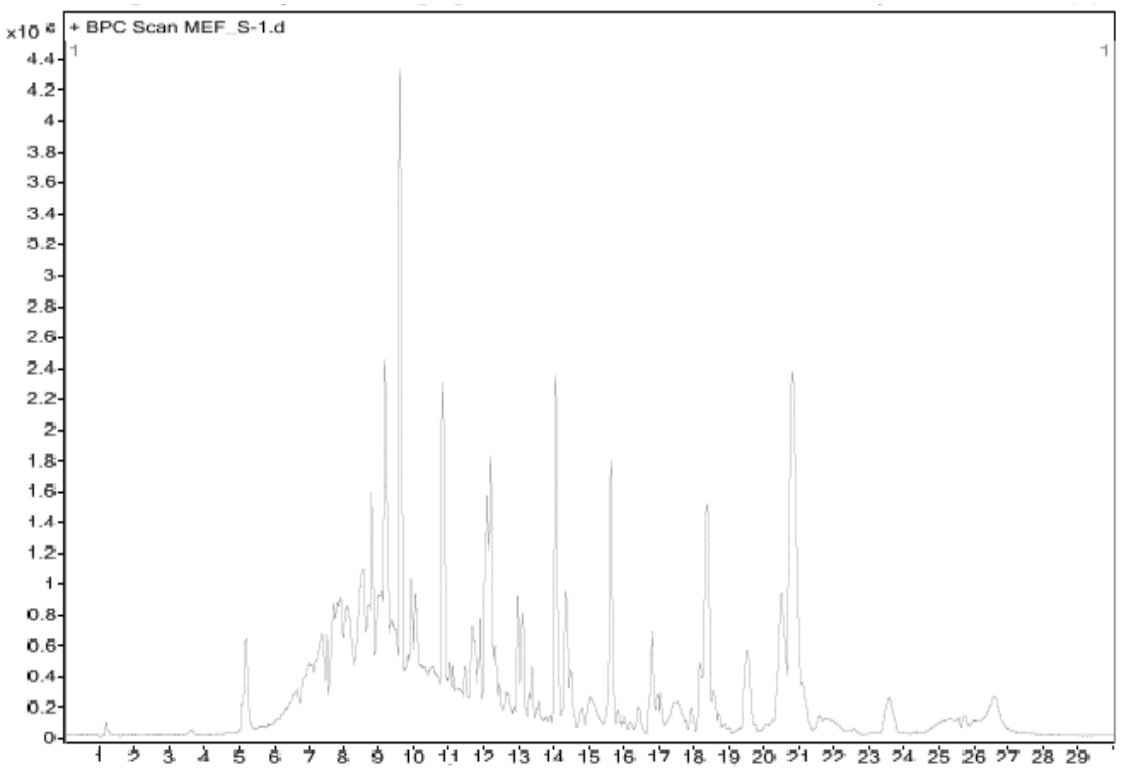
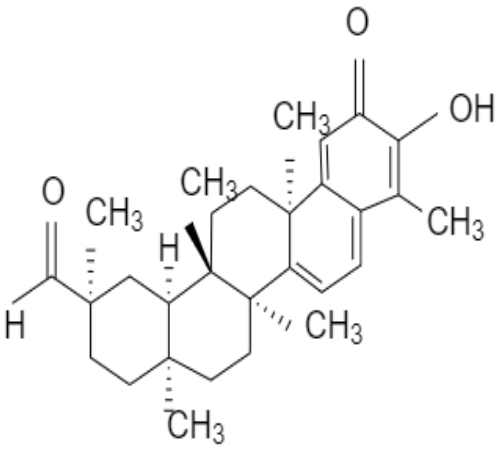


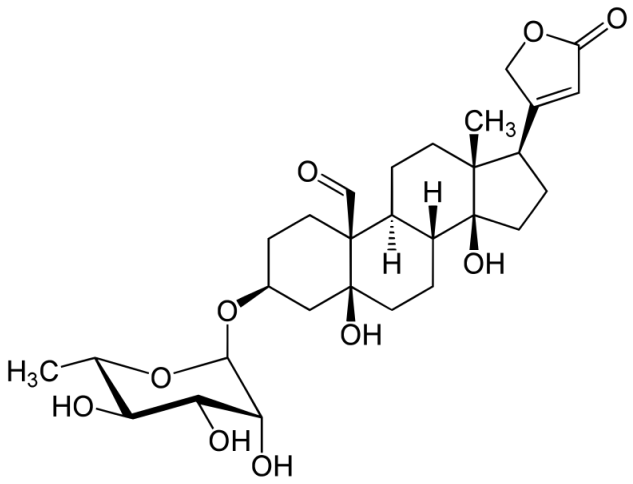
Figure 8.5: A. LC-MS spectrum of partially purified fraction from EAE . The four peaks at retention times of 9.50, 9.60, 11.97,and 14.35 shows Celastrol, Covallotoxin, Cucurbitacin A, Madecassic acid respectively.



Celastrol

Chemical formula: $C_{29}H_{38}O_4$

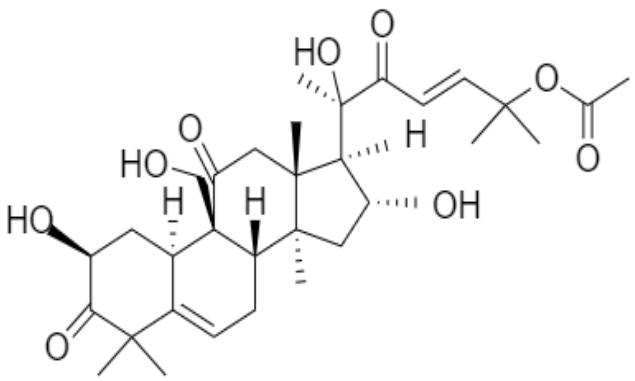
Molecular weight: 450.27g/mol



Convallatoxin

Chemical formula: $C_{29}H_{42}O_{10}$

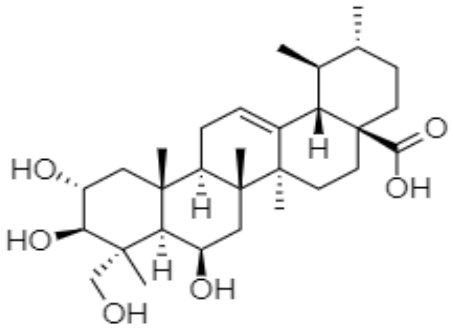
Molecular weight: 550.27g/mol



Cucurbitacin A,

Chemical formula $C_{32}H_{46}O_9$

Molecular weight 574.711 g/mol



Madecassic acid

Chemical formula: $C_{30}H_{48}O_6$

Molecular weight: 504.708g/mol

Figure 8.5 B, Structure of celastrol, convallatoxin, cucurbitacin A, and madecassic acid

8.4 DISCUSSION

Preliminary phytochemical analysis was carried out to acquire more information about the bioactive compounds. Analytical methods such as TLC, column chromatography, HPTLC and LC-MS were done for this purpose.

Preliminary chemical examination of the extracts of *M. elata* showed the presence of steroids, alkaloids, coumarins, phenols, tannins, saponins, and carbohydrates. EAE showed to possess more number of compounds. This might be the reason for the higher *invivo* and *invitro* activity of EAE compared to other extracts.

In HPTLC analysis, when the plates were derivatized with anisaldehyde sulphuric acid reagent, EAE showed 10 peaks in the chromatogram. This indicated that EAE is composed of at least 10 different compounds with the 8^{th} and 10^{th} peaks occupying 42.2% and 33.14% area respectively. These two components appeared to be the major compounds in the extract. EAE fractions from the column was separated into three major fractions (F1, F2, F3). Each fraction was examined for their anticancer activity by MTT assay. Fraction (F2) which showed highest anticancer activity was analyzed by LC-MS to identify the active compounds. The major bioactive compounds in EAE of *M.elata* were identified as Celastrol, Convallotoxin, Cucurbitacin and Madecassic acid. Cucurbitacin and Madecassic acid are triterpenes and have been reported to possess anticancer activity (Alghasham, 2013: Han et al., 2020). Celastrol and Convallatoxin were shown to have anticancer and anti-inflammatory activity (Allison et al., 2001: Kaushik et al., 2017: and Anderson et al., 2017).

Chapter 9:Toxicity studies

9.1. INTRODUCTION

9.2. MATERIALS AND METHODS

9.2.1. Preparation of the extracts

9.2.2. Animals

9.2.3. **Determination of toxicity of *Morchella elata***

9.2.3.1 Acute toxicity study

9.2.3.2. Subacute toxicity study

9.3. RESULTS

9.4. DISCUSSION

9.1 INTRODUCTION

Toxicity tests of drugs and medicines before their use need to be evaluated. The aim of the toxicity study is to determine the toxic effects prior to therapeutic use. Mushrooms are known as valuable source of therapeutically useful compounds. Uses of mushroom in folk medicine and traditional medicines are well documented.

M.elata is an excellently edible and nutritious mushrooms. Investigations in our laboratorary showed *M.elata* possessed significant antioxidant, anti-inflammatory and anticancer properties. Since it is an edible mushroom the possibility of toxicity is a rare chance. However, toxicity studies would be desirable before it is recommended for pharmaceutical use or development of dietary supplements. In this chapter, results of acute and subacute toxicity studies of ethyl acetate extract of *Morchella elata* are presented.

9.2. MATERIALS AND METHODS

9.2.1 Preparation of extract

EAE of fruiting bodies of *M.elata* was prepared as described in section 3.2.1.

9.2.2 Animals

Male Swiss albino mice weighing 25+2g were used for the toxicity studies.

9.2.3 Determination of toxicity

Toxicity of EAE of *Morchella elata* was determined by acute and subacute toxicity models. Toxicity study of the formulation was conducted as per OECD guideline.

9.2.3.1 Acute toxicity study

Animals were divided into four groups of six animals each. A single dose of the EAE was administered orally as follows (Walum, 1998). The animals were observed for 72 hours for toxic symptoms and mortality.

Group I: Normal (Distilled water)

Group II: EAE 1000mg/kg body weight

Group III: EAE 2500mg/Kg body weight

Group IV: EAE 5000 mg/kg body weight

9.2.3.2 Subacute Toxicity

Animals were divided into four groups of 6 animals each. The ethyl acetate extract was administered orally once daily for 30 days (Parchment, 1998)

Group I : Normal (Distilled water)

Group II: EAE 500mg/kg body weight

Group III : EAE 250mg/Kg body weight

Group IV : EAE 100 mg/kg body weight

During the period, animals were observed for behavioral changes diarrhea, immobility and adverse reactions. The changes in body weight were recorded weekly with repeated observation of toxic symptom and mortality. Twenty four hours after the last dose the animals were sacrificed. The blood was collected from heart puncture for routine test including hameglobin , total erythrocyte count, and differential count, liver and renal function tests such as SGOT,SGPT ALP, urea and creatinine were done as mentioned in the section 3.2.6 to 3.2.10.

Section of the liver tissues of the treated animals were fixed 10% neutral buffered formalin for histopathological analysis. The section were stained with hematoxylin-eosinand observed under the light microscope.

9.3 RESULTS

9.3.1 Acute toxicity

The animals administered with EAE extract of *Morchella elata* did not show any symptoms of toxicity or mortality upto to the dose of 5000mg/Kg body weight orally (Table 9.1).

9.3.2 Subacute toxicity

Administeration of different doses (500, 250,100mg/Kg) of ethyl acetae extract were given orally to the animals for 30 days and no significant change in the haematogical (TC,DC) (Table 9.2) and biochemical parameters (SGOT,SGPT and ALP) were observed. Body weight of the animals remained almost constant throughout the period of study. No mortality rate was observed in at all of the group (Table 9.3).

9.3.2.1 Effect on liver function

The SGPT and SGOT activities in the normal animals were found 57.4±5.6, 62.5±7.2 IU/L. Treatment with EAE extract of *Morchella elata* at doses of (500, 250,100mg/Kg) exhibited no significant reduction of SGOT and SGPT level (Table 9.4). The renal function indicators, urea and creatinine levels, were not found changed in the extract administrated groups compared to the untreated animals (Table 9.5). Treatment with the extract for 30 days did not produce any significant changes in the liver function test parameters compared to the normal group of animals.

9.3.2.2 Histopathology of liver

Histopathological examination of liver of treated animals with EAE did not show any pathological manifestations as compared to the normal (Fig 9.1)

Table 9.1 Effect of *Morchella elata* EAE on the body weight and mortality rate of animals.

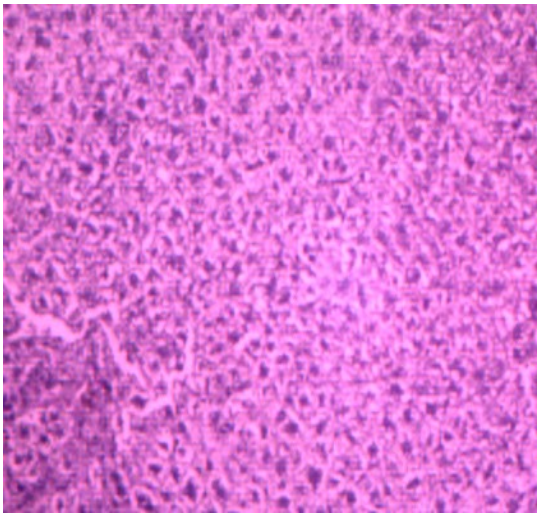
Treated groups (mg/Kg)	**Initial body weight (g)**	**Final body weight (g)**	**Number of dead animals**
Acute toxicity			
Normal	28.9 ±2.5	29.3±3.5	0
5000	27.6 ±2.7	27.9 ±4.4	0
2500	29.0 ±2.9	28.3 ±2.9	1
1000	28.7 ±2.5	28.9 ±3.4	0
Sub acute toxicity			
Normal	27.4 ±2.0	28.3 ±3.1	0
500	26.8 ± 1.2	27.9 ±2.7	0
250	27.2 ±2.5	28.3 ±2.2	0
100	26.5 ±1.0	25.2 ±2.6	1

Table 9.2 : Effect of EAE on the hematological parameters of treated animals

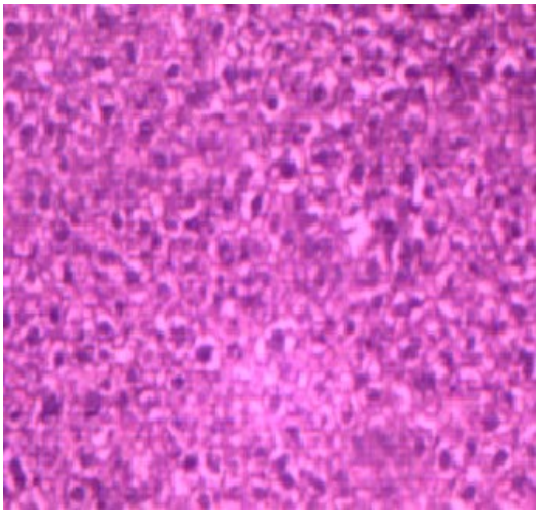
Treatments (mg/Kg)	**Hb (g/dl)**	**WBC (cells/mm^3)**	**RBC (Million/mm^3)**	**Neutrophils (%)**	**Lymphocytes (%)**	**Eosinophils (%)**
Normal	15.65±3.41	7400±282.2	5.4±0.14	56±4.20	64±2.3	5±0.30
EAE 500	14.9.0±1.124	7200±280.8	5.3±0.15	53±3.2	66±1.7	4±0.32
EAE 250	12.25±0.92	6800±197.5	5.1±0.16	51±1.28	56±2.0	4±0.05
EAE 100	12.81±1.15	6200±141.4	4.9±0.19	47±1.39	53±2.8	3±0.14

Table 9.3 Effect of EAE on the activity of liver function enzymes and renal markers

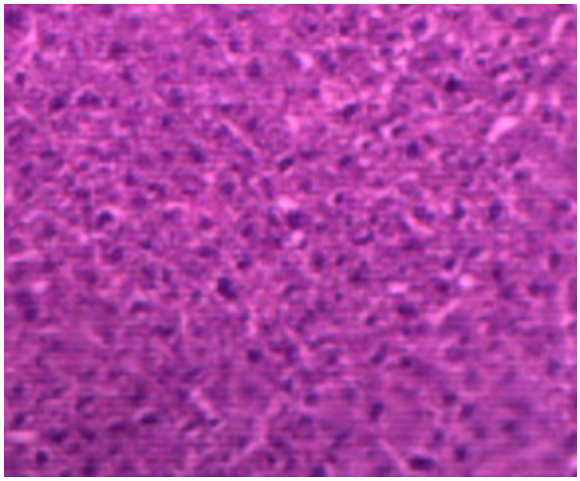
Treatments (mg/Kg)	**SGPT (IU/L)**	**SGOT (IU/L)**	**ALP (IU/L)**	**Urea (mg/dl)**	**Creatinine (mg/dl)**
Normal	57.4±5.6	62.5±7.2	258±36.20	41.2±4.1	0.5±0.2
EAE 500	53.5±4.00	55.2±3.27	253±32.1	37.3±3.5	0.4±0.01
EAE 250	50.8±3.8	53.8±2.86	247±37.2	35.5±2.9	0.39±0.03
EAE 100	49.3±3.1	50.2±2.3	243±2.90	33.8±3.0	0.3±0.12



Normal group



EAE 500mg/kg



EAE 100mg/kg

Figure 9. 1: Histopathological analysis of liver from EAE treated mice (Staining Hematoxylin- Eosin, Magnification 10X)

9.4 DISCUSSION

The acute toxicity study by oral administeration of EAE extract at dose up to 5000, 2500,1000mg/Kg body weight showed no toxic sign on tested mice. Sub acute toxic study by oral administration of EAE extract at (500, 250,100mg/Kg body weight) for 30 days caused no mortality, behavioral abnormality, and no significant change in the haematogical (TC,DC) parameters. Liver and renal function tests showed no significant change in the liver function enzymes (SGOT, SGPT and ALP)and serum urea and creatinine levels in the extract treated group from normal group.The results supported the safety on the extract.

In conclusion the present study reveals that EAE is absolutely non toxic. Fruiting bodies of *Morchella* has been consumed for many years by people in several parts of the world and no human toxicity has been reported so far. The current studies show that the EAE of *Morchella elata* is safe at tested doses. The finding suggests the potential use of this mushroom is safe and can be considered as a nutraceutical or food supplements with therapeutic properties.

Summary and Conclusion

Morchella species, commonly known as morels are excellently edible and economically important traditionally used medicinal mushrooms in Kashmir Himalayas. Since, the information on the medicinal properties of these mushrooms is inadequate, a species of this mushroom was selected for the study. The objective of the current investigation was to evaluate the anti-inflammatory and anticancer activities of a morel mushroom, *Morchella elata.* During the current study antioxidant, anti-inflammatory, antitumor, antiangiogenic, and genoprotective activities of *M. elata* were examined. Attempt was also made to identify the major bioactive compounds present in this mushroom.

The extracts of this mushroom were found to be excellent scavengers of free radicals. The highly active EAE reduced oxidative stress induced tissue damages in mice. Significant free radical scavenging activity both *in vitro* and *in vivo* by the extract was observed. Antioxidants can effectively reduce adverse effect of inflammatory reaction due to close link between oxidative stress and inflammation.

Inflammation is a cellular response resulting from long chain molecular reaction characterized by sign of warmth, redness, pain, and swelling which often lead to loss of function. There has been a new awareness about its role in a wide variety of diseases, including cancer. Thus inflammation is one of the target areas of biomedical research (Arulselvan et al, 2016). Development of effective and economical nonsteroidal anti-inflammatory drugs with a little or no side effects is an area of significant importance in pharmaceuticals industry. Frequently prescribed drugs such as aspirin and diclofenac for treatment of inflammation have been reported to have serious side effects. Due to this natural products based anti- inflammatory agents are becoming popular in recent years (Kataoka et al., 2012). Chronic inflammation is a major concern of the global cancer burden. Therefore, therapeutic agents against inflammation play significant role in defending many diseases. Although acute inflammation is a part of the defense response, chronic inflammation can lead to cancer, diabetes, cardiovascular, pulmonary, and neurological diseases. EAE also showed marked cyclooxygenase (COX2) inhibiting activity. COX2 is a marker enzyme that mediate inflammatory reactions and promotes inflammation process. EAE also showed marked NF-kB inhibiting activity. This indicated the ability of the extract to inhibit the pro-inflammatory signaling molecules. The extract showed a significant protection against both

acute and chronic inflammation. The results suggest the significant anti-inflammatory effect of edible morel mushroom *M.elata*. Since *M.elata* is an excellent edible mushroom, the medicinal properties of this mushroom have great practical application. The antioxidant properties of this mushroom make it as a choice of interest for treating oxidative stress mediated diseases. Anti-inflammatory activity of this mushroom also will be advantageous, as long term consumption of this mushroom will be able to provide relief to patients suffering from inflammation and arthritis.

The link between inflammation and cancer was illustrated by epidemiologic and clinical studies. The fact that continuous irritation over long periods of time can lead to cancer. Cancer is a group of diseases characterized by uncontrolled cell division with ability to metastasize. Although several drugs are currently available for the treatment of cancer, they also produce number of side effects and have relatively low efficacy. Therefore, identification of new molecules with high efficacy and low side effects for the treatment of cancer -related diseases is currently of great interest. Cytotoxicity is one of the chemotherapeutic targets of antitumor activity. Most of the clinically used antitumor agents possess significant cytotoxic activity . The higher cytotoxic activity of the extract moderately explains its significant antitumor activity against tumors. The EAE significantly inhibited the development of solid tumor induced by DLA cell line in mice.

The EAE interfered either in the initiation of cancer cells or their promotion by croton oil. EAE treatment resulted in marked delay in the development of papilloma, reduced size and numbers compared to the control. The most significant activity of this mushroom was its ability to suppress cancer cell induced angiogenesis, because anti-angiogenic activity is important attribute in cancer treatment. Prevention of neoplasm due to DNA damage can contribute to the prevention of cancer. The EAE was found to prevent DNA damage in mice as evident from comet assay. Thus the current investigations indicate that EAE of *M.elata* possessed anticancer activity. Finally, identification of the major bioactive compounds were accomplished by LCMS analysis. The major bioactive compounds in EAE of *M.elata* were identified as Celastrol, Convallotoxin, Cucurbitacin and Madecassic acid. Cucurbitacin and Madecassic acid are triterpenes and have been reported to possess anticancer activity. Celastrol and Convallatoxin were shown to have anticancer and anti-inflammatory activity.

The acute and subacute toxicity of the extract was carried out in mice. The EAE did not produce any toxic effect.

In conclusion, the results of the present investigations indicate that ethyl acetate extract of *M.elata* fruiting bodies possessed profound antioxidant, anti-inflammatory, anticancer, and genoprotective activities. The findings, thus suggest the potential therapeutic use of this mushroom as anti-inflammatory and anticancer agent. Being an excellently edible mushroom it has great promise in therapeutic use.

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- **Ramya, H**., Ravikumar, K. S., Fathimathu, Z., Janardhanan, K. K., Ajith, T. A., Shah, M. A. & Reshi, Z. A.. Morel mushroom, *Morchella* from Kashmir Himalaya: a potential source of therapeutically useful bioactives that possess free radical scavenging, anti-inflammatory, and arthritic edema-inhibiting activities. *Drug and Chemical Toxicology,* 2021, 1-10, DOI:10.1080/01480545.2021.1894750,.(**Impact factor**: **3.34**).

- Veena R K, Joy Carmal E, **Ramya H,** Ajith A, Wasser S P, Janardhanan K K. Caterpillar medicinal mushroom, *Cordyceps militaris* (Acomycetes) , mycelia attenuates Doxorubicin-induced oxidative stress and upregulates Kerbs cycle dehydrogenases activity and ATP level in rat brain. *International Journal of Medicinal Mushrooms*, 2020; 22:593-604, DOI: 10.1615/IntJMedMushrooms.2020035093, **Impact factor : 1.94**)

- Chen X, Veena R K, **Ramya H**, Janardhanan K K, George V. G. Gano oil: A novel antinociceptive agent from *Ganoderma lucidum* inhibits paw oedema and relieves pain by hypnotic and analgesic actions of fatty acid amides. *Journal of Ethnopharmacology*, 2020; 263: 113144, https://doi.org/10.1016/j.jep.2020.113144 (**Impact factor : 4.360**)

- Ravikumar K S, **Ramya H**, Ajith A, Shah MA, Janardhanan K K . Bioactive extract of *Fomitopsis pinicola* rich in 11- alpha- actoxykhivorin mediates anticancer activity by cytotoxicity, induction of apoptosis, inhibition of tumor growth, angiogenesis and cell cycle progression. *Journal of Functional Foods*, 2021: 78:104372, https://doi.org/10.1016/j.jff.2021.104372 (**Impact factor : 4.451**)

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- Ravikumar K S, **Ramya H**, Ajith A, Shah MA, Janardhanan K K . Antioxidant and anti-inflammatory activities of bioactive extracts of a polypore mushroom, fomitopsis pinicola (sw.:fr.) p. karst, from kashmir. *International journal of advanced research*. 2008 6(8) 1144-1156, DOI:10.21474/IJAR01/7624.

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