COMPARATIVE EVALUATION OF ANTI-CANCER ACTIVITIES OF WILD AND *IN VITRO* PROPAGATED *OROXYLUM INDICUM* VENT.

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By

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CERTIFICATE

This is to certify that the thesis entitled **'Comparative evaluation of anti-cancer activities of wild and *in vitro* propagated *Oroxylum indicum* Vent.'** is a bonafide record of research work carried out by Mrs. Seema Menon, under my guidance and supervision at Amala Cancer Research Centre, Thrissur, Kerala and no part thereof has been presented for the award of any other degree, diploma or other similar titles. The contents of the thesis have been subjected to plagiarism check and the percentage of similar content was found to be within the acceptable maximum limit.

Place: Amala Nagar

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DECLARATION

This is to certify that the thesis entitled "**Comparative evaluation of anti-cancer activities of wild and *in vitro* propagated *Oroxylum indicum* Vent.**" is based on the original research carried out by me at Amala Cancer Research Centre, Thrissur, Kerala, India under the guidance of Dr. Jose Padikkala, Ph.D., Professor, Department of Biochemistry, Amala Cancer Research Centre, Thrissur, Kerala, India and no part thereof has been presented for the award of any other degree, diploma or other similar titles.

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Abbreviations

Ao	Acridine orange
b.wt.	Body weight
BA	Benzyl adenine
DLA	Dalton's Lymphoma Ascites
DMEM	Dulbecco's modified eagle medium
Dox	Doxorubicin
dw	Distilled water
EAC	Ehrlich Ascites Carcinoma
Eb	Ethidium bromide staining
e.g.	Example
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power assay
OIM	*Oroxylum indicum*70% hydro-methanolic extract
OCM	*Oroxylum indicum* callus 70% hydro-methanolic extract
OIMH	OIM high concentration
OIML	OIM low concentration
GE	Gallic acid equivalents
Hb	Hemoglobin
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
IAA	Indole –3 acetic acid

IARC	International Agency for Research on Cancer
IBA	Indole 3-butyric acid
LPO	Lipid peroxidation
MDA	Malondialdehyde
MS	Murashige Skoog’s
MeJa	Methyl jasmonate
NAA	Naphthalene acetic acid
NaF	Sodium fluoride
NSAID	Non-steroidal antiinflammatory drugs
PBS	Phosphate buffered saline
PI	Propidium iodide
QE	Quercetin equivalents
ROS	Reactive oxygen species
RPMI	Rosewell Park Memorial Institute medium
SD	Standard deviation
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reacting substance
TCA	Trichloroacetic acid
TLC	Thin Layer Chromatography
UV	Ultra Violet
WBC	White blood cells
WHO	World Health Organization

Chapter 1

Introduction and Review

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1.1. Introduction

A huge volume of research is still invested on finding herb-based remedies for combating cancer, despite the advent of synthetic or isolated compound-based chemotherapeutic procedures for treatment. This is a dreadful disease, whose incidence, progression, prevention and treatment are still continuing to be pivotal areas of world-wide clinical studies. Isolated compounds and medicinal preparations made from herbs are reported with anti-cancer properties, operating in various dimensions at tissue, cellular and molecular levels (Alam *et al.*, 2013).

Plants are repositories of bioactive secondary metabolites, whose structure, biosynthesis and pharmacological importance have been studied in detail. For exploration of the biological activities of plant resources, extraction of plant parts, pharmacological screening, isolation and characterization of bioactive compounds, toxicological evaluation and clinical evaluation are the important steps (Sasidharan *et al.*, 2011). Researchers' focus is also channelized on methods to enhance their production and availability by upgrading isolation techniques or by subjecting the source plant to conditions that favor the elicitation of secondary metabolite synthesis (Raja and Sreenivasalu, 2015). This holds more relevance because of the fact that many medicinal plants are overexploited, making them vulnerable to disappear from many areas where they were initially found in abundance (Mohammed and Kumar, 2012).

The present study is centered on the selected anti-cancer activities of *Oroxylum indicum*, a medium sized deciduous tree, coming under the family Bignoniaceae, various parts of which are used in Ayurveda, the indigenous system of medicine in India. The study also attempts to develop *in vitro* propagation techniques for raising this medicinally important plant in culture, as well as to figure out techniques to elicit the production of selected secondary metabolites from it.

1.2. Cancer

Any research focused on drug discovery is primarily based on the disease etiology. Cancer is characterized by an abnormal growth of cells having dreadful potential to invade other parts of the body. This abnormal behavior of cells roots from a deregulated balance of cell proliferation and cell death, which in turn, is caused by single or multiple changes in their gene expression (Ruddon, 2007c). The result is the development of significant morbidity, which certainly causes death of the affected person, if left untreated. Hence, cancer is disreputably regarded as a killer disease all over the globe, which is quite apparent from the disease statistics that follows.

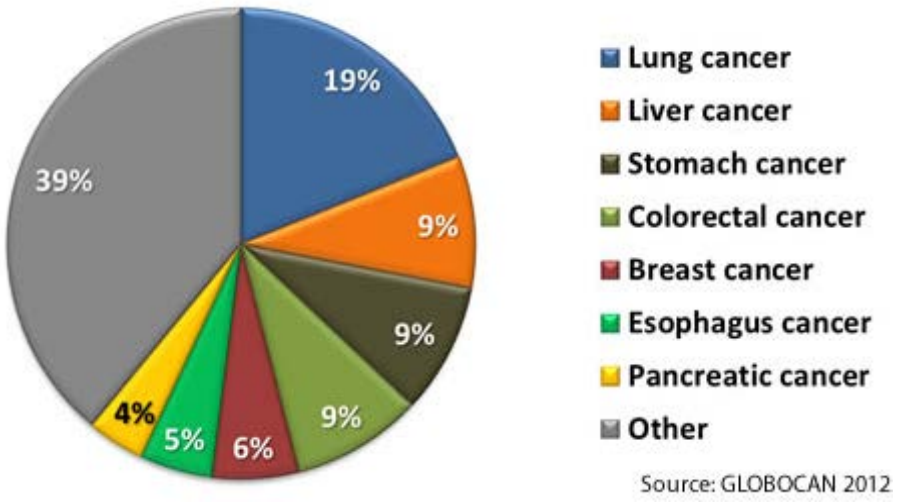
1.2.1. Cancer statistics

There is much focus given on cancer related research because of the resulting death tolls from this disease. More than being a fatal disease, cancer remains second only to cardiovascular diseases inflicting global disease mortality (Jemal *et al.*, 2007), causing every 1 in 6 disease related deaths. As defined in the Surveillance Research Program in the official website of National Institute of Health, USA, cancer incidence rate is the number of new cancers of a specific site/type occurring in a specified population during a year, which is usually expressed as the number of cancers per 100,000 individuals at risk (e-Article, 2017b). Statistical overviews of cancer incidence and cancer deaths, with reference to the type of cancer, the affected sex, age and time period are available from many sources. They are used as scales to determine the success of methods adopted for treatment and control.

A project namely 'Globocan' was launched in 2012 by the International Association of Cancer Registries with an aim to provide up-to-date estimates of the incidence, mortality and occurrence from 28 types of cancer in 184 countries globally. According to its report published by the World Health Organization in 2017, an alarming record of 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million affected people (within 5 years of

diagnosis) were accounted for the year 2012, around the globe. The incidence rate in men (0.205%) was found to be higher than women (0.165%). The most commonly diagnosed cancers in humans were lung (1.82 million), breast (1.67 million), and colorectal (1.36 million); the most common causes of cancer death were lung cancer (1.6 million deaths), liver cancer (745,000 deaths), and stomach cancer (723,000 deaths) (Ferlay *et al.*, 2015). With respect to sex, lung cancer was the most commonly diagnosed among men and breast cancer, among women (Figure 1.1).

Figure 1.1. Most common causes of global cancer deaths in 2012 (Ferlay *et al.*, 2015)



Focusing on Indian scenario, as per the latest update (8th May, 2017) of National Institute of Cancer Prevention and Research in their website, the estimated number of people living with cancer in the country is found to be approximately 2.5 million, new cases registered is over 0.7 million yearly and the number of cancer-related deaths is 0.56 million (Nandakumar, 2009). Cancers of oral cavity and lungs in males and cervix and breast in females account for over 50% of all cancer deaths in India (e-Article, 2001). The total

cancer cases in India are projected to increase from 979,786 to 1,148,757 cases between 2010 and 2020. The tobacco-related cancers for males are expected to go up from 190,244 to 225,241 for females from 75,289 to 93,563 during this time period. Gynecological-related cancers are estimated to go up from 153,850 to 182,602 in 2020 (Takiar *et al.*, 2010). In its projection, the Indian Council of Medical Research (ICMR) said in 2016 the total number of new cancer cases is expected to be around 1.45 million, which is likely to reach nearly 1.73 million in 2020 (P.T.I., 2016). According to the World Health Organization (WHO), death from cancer cases in India is projected to rise to 13.1 million by the year 2030.

Though the above reports are few adoptions from the huge volume of cancer statistical data available, all of those indicate the growing trend of cancer incidence in humans, despite contributing immense dimensions of work in cancer research.

1.2.2. Types of cancer

Hundreds of different tumor types arise from almost every tissue and in every organ, and hence, there are diverse types of cancer in humans. Their responses to therapeutic procedures are varying; so, an understanding of the diversity of cancer types is a pre-requisite in drug research. Irrespective of organ location, classification of cancers/tumors is based on the tissue of origin, due to the similarities in cellular structure and function among them (Pelengaris and Khan, 2006a). Tumors may be either liquid (eg. leukemias and lymphomas comprising neoplastic cells with motile precursors); or solid (comprising either epithelial or mesenchymal cells that are immobile). Pathologically, cancers are classified as: 1) carcinomas, (of epithelial origin); 2) sarcomas (of connective tissue origin like bone, cartilage, fat, muscle or blood vessels); 3) leukemias (originating in haematopoietic tissues such as the bone marrow), and causing large numbers of abnormal blood cells to be produced and enter the bloodstream; and 4) lymphomas (originates in the cells of the immune system).

Carcinomas typically represent over 80% of diagnosed human cancer each year as substantiated by the fact sheet published by the World Health Organization that says that the most common types of cancers accounted worldwide are those of lung (1.3 million deaths/year), stomach (803,000 deaths), colorectal tract (639,000 deaths), liver (610,000 deaths) and breast (519,000 deaths), all of which belong to the category of carcinomas (e-Article, 2009).

1.2.3. Cancer causes

Cancers arise from defects in DNA which may begin with a single cell mutation leading to an abnormally rapid rate of cell division. Proliferation gives rise to a clone of rapidly and abnormally dividing cells, which outgrow other normal cells. An additional mutation that arises in some of the clone's cells may further enhance their proliferative ability and they become dominant in the clone, which may in due course be overtaken by cells that contain still more mutations that facilitate faster and aggressive cell division. This process is called clonal evolution, whose rate depends on the frequency of mutagenesis (Pierce, 2011).

The transformation of a normal cell into cancerous cell is called oncogenic transformation, which arises from genetic damage (Lodish *et al.*, 2003) thereby leading to loss of cellular regulation. Mutations in two broad classes of genes have been identified in cancer onset: a) proto-oncogenes and b) tumor suppressor genes. Mutations in proto-oncogenes lead to their activation into oncogenes that make them excessively active in growth promotion, either through increased gene expression or production of a hyperactive product. Meanwhile, tumor suppressor genes normally restrain growth; hence their damage allows inappropriate growth. Genes falling in both these categories encode proteins that are pivotal in processes like: a) entry and progression through the cell cycle, b) programmed cell death by apoptosis and c) repair of DNA damage.

Mutations arise during a lifetime's exposure to carcinogens/cancer causing agents, which include certain chemicals and ultraviolet radiation. Cancer-causing mutations occur mostly in somatic cells, and hence, they are less likely to be inherited. In contrast, certain inherited mutations, developed in the germ line, increase the probability of cancer. Sometimes, in a destructive partnership, somatic mutations can combine with inherited mutations to cause cancer.

The four steps in carcinogenesis are tumor initiation, tumor promotion, malignant conversion, and tumor progression (Weston and Harris, 2003). Initiation is the genetic change/mutation arising spontaneously or induced by carcinogenic exposure, which leads to dysregulation of biochemical signaling circuits involved in cellular proliferation, survival, and differentiation. The rate of such alterations is determined by the rate and type of carcinogenic metabolism and the velocity of the DNA repair function. Promotion is a reversible process which comprises the selective clonal expansion of initiated cells, and is considered to be a pre invasive stage. It results from the altered expression of genes whose products induce high proliferation rate, tissue remodeling and inflammation (Abel and DiGiovanni, 2011). Malignant conversion is the transformation of a pre neoplastic cell into a malignant genotype, involving more genetic changes. The malignant phenotype expresses during tumor progression and the malignant cells become more aggressive and are characterized by genetic instability. The cells may undergo further mutations with invasive and metastatic potential. Metastasis involves the ability of tumor cells to secrete proteases that allow invasion of secondary sites through the bloodstream or the lymphatic system.

Oncogenesis or tumorigenesis, is an interplay between genetics and the environment. The cumulative effects of both genetic/epigenetic changes and exposure to environmental factors determine the likelihood of developing cancer (Pelengaris and Khan, 2006a). The environmental factors include: a) lifestyle-related risk factors, such as smoking, unhealthy diet, or unprotected

exposure to strong sunlight; b) factors not pertaining to the life style, such as high levels of exposure to radiation, cancer-causing chemicals or infections and c) natural processes, such as free radical generation, endogenous hormones, cosmic rays. The genetic factors include a) inherited monogenic disorders such as inheriting a mutant *p53* gene, or b) polygenic disorders.

The following are some of the common causes leading to cancer, as revealed from different reports.

1.2.3.1. Life style

The increase in the global incidence of cancer is attributed to worldwide transitions in economic and demographic factors. Life style aspects such as smoking, alcoholism, erratic diet patterns, physical inactivity, obesity, reproductive factors and hormonal factors contribute much to cancer incidence, and hence, changes in such aspects are in focus towards the prevention of cancer. Smoking is considered to be the prime risk factor leading to 20 to 30% of all incident cancers (Katzke *et al.*, 2015).

The role of tobacco and tobacco smoke has been identified by Working Groups of the International Agency for Research on Cancer as causative agents of tumors of the respiratory tract and of the upper digestive tract, malignant tumors of the bladder, renal pelvis and pancreas, cancers of the cervix and nasal cavity (Brinton *et al.*, 1984, I.A.R.C., 1985, I.A.R.C., 1986). Lung cancer, being the leading cause of cancer deaths in humans, it is noteworthy to realize that about 71% of lung cancer deaths are caused by tobacco use. The combined effects of tobacco use, low fruit and vegetable intake, urban air pollution, and indoor smoke from household use of solid fuels cause 76% of lung cancer deaths (Weiderpass, 2010).

Diet is a determinant factor in up to 20% of fatal cancers. A high saturated fat rich and fibre deficient diet is considered as a risk factor for colon and rectal cancers. Diets deficient in antioxidant vitamins link with risk of heart disease

and cancer. Oesophageal cancer risk is found to be high with excessive intake of hot drinks. Aflatoxin, a carcinogenic compound synthesized by *Aspergillus* species is associated with hepatocellular carcinoma which accesses human body through consumption of moldy grain and peanuts. Consumption of pickled fish and vegetables have been related with risk of nasopharyngeal and esophageal cancers in some studies (Pelengaris and Khan, 2006a).

Kalandidi *et al.* (1996) have found that excess calorific intake in early life, leading to higher attained physique, and excessive energy intake in later life and physical inactivity leading to higher body weight, increase the risk for endometrial cancer in women. Breast and prostate cancer risks are found to be influenced by diet because of its effect on circulating hormone levels; data have shown that the circulating estrogen, androgen and insulin/insulin like growth factor levels are regulated by dietary fat, fibre, phyto-oestrogen and alcohol (Willett, 2000, Key *et al.*, 2002, Key *et al.*, 2004). Some of the factors identified as potent risks for prostate cancer are high consumption of meat and dairy products, physical inactivity, obesity and high levels of circulating insulin-like growth factor 1 (IGF-1) (Mills *et al.*, 1989, Wolk, 2005).

Alcohol contributes to about 3% of cancer deaths, beyond posing a higher risk of liver (McKillop and Schrum, 2005), breast, mouth, larynx, pharynx, esophagus, stomach, breast and cancer(Adami *et al.*, 1992, Hedberg *et al.*, 1994, Dumitrescu and Shields, 2005), in a dose-dependent manner.

Identification of the risk factors of cancer development is very crucial in understanding and adopting life style patterns related to cancer prevention.

1.2.3.2. Exposure to radiation

Life time exposure to radiation, including both ionizing radiation and non-ionizing radiation damage DNA, some of which turn into oncogenic transformations, and thereby cancers arise; up to 10% of invasive cancers are related to radiation exposure. The risk of cancer is highest in tissues where

radiation exposure has been specifically concentrated, such as during diagnosis (Einstein *et al.*, 2007) or therapeutic exposure (Braunstein and Nakamura, 2013), or inadvertently following nuclear disasters (Fugazzola *et al.*, 1995).

Radiation, either singly, or synergistically (along with chemical carcinogens) produces carcinogenic changes by inducing mutations or altering gene expression patterns (without mutations) or by inducing oncogenic viruses, which can cause neoplasia. They have high ability to penetrate cells and to deposit energy within them randomly. All cells in the body are prone to damage by ionizing radiation; whose extent is determined by the radiation dose to which the cells are exposed. Radiation induces damage to nucleotide bases, point mutations due to misrepair deletions and chromosomal translocations, cross-linking and DNA single- and double-strand breaks (DSBs) (Little, 2000). DNA damage induced by radiation is mainly due to the generation of free radicals during the passage of radiation through tissues, which in turn depends on the energy of the radiation. X-rays and gamma rays have a low rate of linear energy transfer, sparse generation of ions and deep penetration into tissues, whereas protons and α particles, have a high linear energy transfer, higher generation of ions and have low penetration through tissues (Upton, 1982, Grosovsky *et al.*, 1988, Lutze *et al.*, 1992). Though all human tissues are vulnerable to the tumorigenic effects of ionizing radiation, the female breast, thyroid, and hematopoietic tissues are the most susceptible, whereas lung, certain organs of the gastrointestinal tract and bone are relatively less radiosensitive (Schottenfeld, 1983). The incidence of cancers due to radiation exposure has been well established as in the case of breast cancer (Golubicic *et al.*, 2008, Pijpe *et al.*, 2012), thyroid cancer (Hempelmann *et al.*, 1975), leukemia (Wakeford *et al.*, 2010) and colon cancer (Rapiti *et al.*, 2008).

1.2.3.3. Chemical carcinogens

The historical evidence for the carcinogenic role of chemical compounds has been obtained from data regarding the incidence of lung cancer in miners

(Luch, 2005), scrotal skin cancer among men exposed to chimney sweeps, nasal cancer in tobacco snuff users, skin cancer in workers exposed to tar and paraffin oils and urinary bladder cancer in aniline dye workers (Ruddon, 2007c). According to the list developed by the International Agency for Research on Cancer (IARC) and published by the American Cancer Society, more than 900 of the identified carcinogens have been assigned into five groups: a) Group 1: Carcinogenic to humans (e.g., Aflatoxins, Benzo[a]pyrene); b) Group 2A: Probably carcinogenic to humans (e.g., Dibenz[a,h]anthracene, N-Methyl-N-nitrosourea); c) Group 2B: Possibly carcinogenic to humans; d) Group 3: Unclassifiable as to carcinogenicity in humans; e) Group 4: Probably not carcinogenic to humans (e-Article, 2016a)

The most identified of human carcinogens include: a) aminoazo dyes (e.g., o-Aminoazotoluene, N,N-dimethyl-4-aminoazobenzene); b) aromatic amines and amides (e.g., 2-Naphthylamine, 4-Aminobiphenyl, 2-Acetylaminofluorene); c) aromatic hydrocarbons (e.g., Benzo[a]pyrene, 2,3,7,8-Tetrachlorodibenzo-p-dioxin, Polychlorinated biphenyls); d) some metals (e.g., arsenic, cadmium, nickel); e) N, nitroso compounds (e.g., N-Nitrosodimethylamine, 4-(Methylnitrosamino)-1- (3-pyridyl)-1-butanone); f) olefins (e.g., Ethylene oxide, Vinyl chloride, Trichloroethylene); g) paraffins and ethers (e.g., 1,2-Dichloroethane, Bis(chloromethyl) ether, Mustard gas (sulphur mustard), Nitrogen mustard); h) natural carcinogens (e.g., Aflatoxin B1, Asbestos); and i) a few anti cancer drugs (e.g., Melphalan, Thiotepa) (Ruddon, 2007c).

Most chemical carcinogens are not active by themselves, but require bio activation to electrophiles. If not detoxified, these will bind covalently to DNA or produce reactive oxygen species and induce mutations. The bio activation reactions include oxidation, reduction, thiol conjugation, acetyl transfer, sulfur transfer, methyl transfer, glucuronosyl transfer, and epoxide hydrolysis (Peter Guengerich, 1992). The enzymes involved in bio activation are usually phase I enzymes, while phase II enzymes mediate conjugation of the functional groups

leading to elimination of the carcinogen. A family of enzymes known as the cytochrome P-450s are involved in phase I reactions. (e.g., CYP1A1 in lung; CYP1A2, CYP2A6 and CYP2E1 in the liver). Hydrolases and the conjugative enzymes such as glucuronyltransferases, glutathione transferases, N-acetyltransferase, and sulfotransferase are the enzymes of phase II (Goldstein and Faletto, 1993). The phase II conjugative enzymes add a hydrophilic group such as sulfate, glucuronide or acetate to compounds, thus increasing their water solubility so as to facilitate their excretion. The pro carcinogens thus formed will interact with DNA (Pelengaris and Khan, 2006b). Both the phase I and phase II enzymes are highly inducible and their genomic responses determine whether bio activation or detoxification predominates (Penning, 2011). The specificity of action of carcinogens depends on differences in enzyme profiles between tissues and the cellular site of the reaction (Pelkonen and Vahakangas, 1980).

1.2.3.4. Free radicals

Reactive oxygen species (ROS), or low generally oxygen-free radicals (OFR), are produced in our body by normal cellular metabolism or from external sources such as exposure to X-rays, air pollutants, cigarette smoking, ozone and industrial chemicals (Bagchi and Puri, 1998), either enzymatically or non enzymatically. Free radicals are produced by enzymatic reactions in the respiratory chain, in phagocytosis, in prostaglandin synthesis, and in the cytochrome P-450 system. Non enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing reactions can also generate free radicals (Lobo *et al.*, 2010).

Oxidation products formed during metabolic processes in cells include superoxide, hydrogen peroxide, nitric oxide and hydroxyl radical (OH) (Ruddon, 2007c). These reactive oxygen and nitrogen species and their biological metabolites also play an important role in carcinogenesis. ROS induce DNA damage by DNA strand breaks, base modification and DNA

protein cross-links (Lobo *et al.*, 2010). A number of altered bases- hydroxymethyl uracil, thymine glycol, 8- ydroxyguanine - have been observed in cells undergoing oxidative stress.

Cells possess enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione-S-transferases to protect themselves from oxidative damage. In addition, cells have repair mechanisms to correct DNA damaged by oxidants, proteases to degrade oxidized proteins and glutathione peroxidases to destroy lipid peroxides. Nevertheless, some oxidative damage and misrepair may persist, and the ability to carry out these repair mechanisms decreases with aging (Ruddon, 2007c). The cell is said to undergo oxidative stress when the critical balance between free radical generation and antioxidant defenses is adverse and it inflicts damage to cellular lipids, proteins, and nucleic acids (McCord, 2000).

It is estimated that in one human cell is exposed to nearly 10^5 oxidative hits by free radicals in a day. These constant genetic changes accumulate and may contribute to the initiation step of tumorigenesis, either by the modulation of gene expression or by the stimulation of proliferation and growth signals by epigenetic effects on gene expression. Neoplastic progression can occur by chromosomal rearrangements resulting from loss of heterozygosity, alterations in gene expression, contributing to genetic amplifications and strand breakage misrepair. Active oxygen species can stimulate poly (ADP) ribosylation and protein kinase pathways, thereby affecting signal transduction. This will in turn lead to modulation of tumor promotion and proliferation genes (Pourahmad *et al.*, 2016).

The cell signaling pathways- mitogen-activated protein (MAP) kinase (Chang and Karin, 2001)/AP-1 (Karin, 1995) and NF-κB pathways- are most significantly influenced by oxidant reactions in a cell (Muller *et al.*, 1997). Carcinogens and tumor promoters including UV radiation, phorbol esters, asbestos, alcohol, and benzo(a)pyrene can activate NF-κB expression thereby

promoting cell proliferation (Baeuerle *et al.*, 1988, Li and Karin, 1998). Additionally, tumor cells from blood neoplasms, and cell lines from different cancers, including colon, breast, pancreas, and squamous cell carcinoma, have all been reported to constitutively express activated NF-κB (Bours *et al.*, 1994).

1.2.3.5. Endogenous hormones

Hormones play a significant role in the aetiology of cancers of the breast, prostate, endometrium, testis, ovary, bone and thyroid (Henderson *et al.*, 1982). Both endogenous and exogenous hormones drive cell divisions, control the differentiation of cells and increase the chance for random genetic errors (Henderson and Feigelson, 2000).

Breast cancer is the most frequently studied model for hormone dependent growth because prolonged exposure to female hormones starting from early onset of menarche, late menopause, hormone replacement therapy and postmenopausal obesity, increases its risk of incidence. Estrogens are postulated to induce the receptor-mediated stimulation of cellular proliferation, accumulation of genetic damage and stimulation of the synthesis of growth factors that act on the mammary epithelial cells via an autocrine or paracrine loop. Alternatively estrogens may also remove the effect of one or several inhibitory factors present in the serum, thereby inducing cell proliferation through negative feedback (Russo and Russo, 1998). In some cells that have undergone tumor initiation, estrogen could act as a promoter; and the period of exposure to the hormones increases the risk in a woman who has an inclination to develop breast cancer (Ruddon, 2007d). Along with estrogen, the combined effect of estrogen and progesterone is also hypothesized to increase breast cancer risk (Key and Pike, 1988). This is evident from the increase in breast cancer with early menarche, late menopause and oestrogen replacement therapy (Key, 1995). The breast cancer risk associated with body weight is thought to operate through the increased levels

of conversion of adrenal androgens to estrogen and lower levels of sex-hormone binding globulin in obese women (MacDonald *et al.*, 1978).

The influence of estrogen on endometrial cancer risk is also well established (Pike, 1987), and can be explained by the unopposed estrogen hypothesis, which states that mitotic activity of endometrial cells, number of DNA replication errors and somatic mutations resulting in malignant phenotype increases on exposure to estrogens unopposed by progesterone or synthetic progestins (Akhmedkhanov *et al.*, 2001).

Steroid hormones, particularly androgens are suspected to play a major role in human prostate carcinogenesis, which act as strong tumor promoters, which act through androgen receptor mediated pathways and enhance the carcinogenicity of endogenous carcinogens like reactive estrogen metabolites and estrogen and prostatitis generated reactive oxygen species, or some exogenous carcinogens. This is also modulated by factors like diet, hereditary vulnerability and polymorphic genes coding for steroid hormone receptors as well as enzymes taking part in steroid metabolism and mode of action (Gann *et al.*, 1996, Bosland, 2000).

1.2.3.6. Viruses

Tumorigenesis can be driven by the actions of specific tumor viruses, which contribute to the development of about 15% of all human cancers worldwide. Tumor viruses responsible for human cancers comprise two distinct types, viruses with DNA genomes (e.g. Epstein-Barr virus, human papilloma virus, hepatitis B virus, and human herpes virus-8) and those with RNA genomes (Human T lymphotrophic virus type 1 and hepatitis C viruses). About 80% of virus-originated cancers are hepatocellular carcinomas and cervical cancers. Cancer can develop either from primary infection, which, in fact is a small process, or from secondary factors like immune suppression, somatic mutations, genetic inclination, and carcinogenic exposure (zur Hausen, 1991).

Both EBV and HHV-8 (also known as Kaposi sarcoma herpes virus) contain genes encoding enzymes for DNA replication, repair and nucleotide biosynthesis (Liao, 2006). Epstein–Barr virus (EBV), is linked with the genesis of Burkitt's lymphoma and nasopharyngeal carcinoma. Human papilloma viruses (HPVs) are small non-enveloped DNA viruses that cause benign papillomas or warts in humans, some of whose high-risk subtypes are associated with the development of cervical cancer (Wallin *et al.*, 1999). Molecular studies have shown that the incorporation of Hepatitis B viral DNA into the host genome can act as a precursor to hepatocellular carcinoma (HCC) (Shafritz *et al.*, 1981). Meanwhile, hepatitis C virus is an enveloped RNA virus (Flavivirus family), causing both chronic hepatitis in humans later resulting in cirrhosis, which in turn can lead to primary hepatocellular carcinoma (Kew, 1994). HTLV-1 is a single stranded RNA retrovirus displaying a special affinity for CD4 cells, thereby causing adult T-cell leukemia.

Hence, alike cancer, which is of different types, the causes of cancer are also multifarious, which lead to fundamental defects in the regulation of cell division, and its study therefore has significance not only for public health, but also for our basic understanding of cell biology. A thorough understanding of cancer biology is therefore, the pre requisite in cancer related research.

1.2.4. Cancer biology

The growth and division of a normal cell is regulated by a delicate balance between stimulatory and inhibitory signals arising from internal and external sources. But, in a cancer cell, there occurs disruption of one or more of the signals, leading to an abnormally high proliferation rate. On losing their response to the normal controls, cancer cells gradually lose their regular shape and boundaries, eventually forming a distinct mass of anomalous cells forming a tumor (Pierce, 2011). The tumor is benign, if localized; and malignant, if the cells invade other tissues. Malignant tumors invade and destroy adjacent normal tissue; benign tumors grow by expansion, are usually encapsulated.

Benign tumors may, however, push aside normal tissue and may become life threatening if they press on nerves or blood vessels or if they secrete biologically active substances, such as hormones, that alter normal homeostatic mechanisms (Ruddon, 2007b).

Cancer cells are remarkably efficient in travelling to other sites in the body, where they establish secondary tumors; a property known as metastasis, probably the most dreaded of all properties.

Though the basic change in a cancerous cell underlies in its genetic alteration, it eventually leads to the development of a number of unique cytological, biochemical, behavioral and physiological transformations, most of which form the cytological criteria for cancer diagnosis.

1.2.4.1. Apparent morphological changes

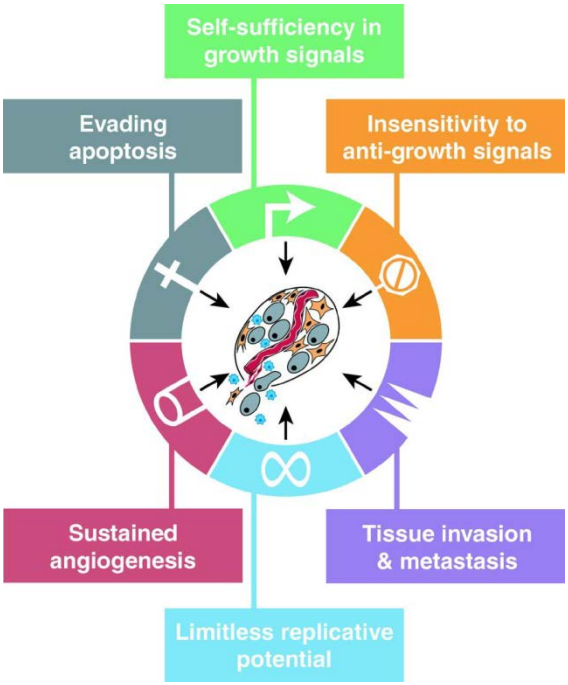
Cancer cells exhibit altered and variable morphology, compared to their normal counterpart cells from the same tissue, like; a) appearance as ‘‘giant cells’’, b) large, pleomorphic (variable size and shape) or multiple nuclei c) more apparent chromatin (‘‘hyperchromatic’’ condition), d) higher nucleo-cytoplasmic ratio, d) presence of prominent, large nucleoli and e) decreased cell adhesion (Ruddon, 2007b). These are noticeable changes in cancerous cells, but the reason behind these distorted characteristics lays in the alterations that have occurred in their basic cytological behavior.

1.2.4.2. Changes in basic cell behavior

Normal cells respond to growth factors, hormones, anti-growth signals, apoptotic signals and negative feedback mechanisms, which keep their proliferative turn over under check. According to Hanahan and Weinberg (2000), the hallmarks of cancer which make them different from normal cells are 1) self-sufficiency in growth signals, by which they can stimulate their own division by autocrine signaling, 2) insensitivity to anti-growth signals, 3) evasion of apoptosis or programmed cell death, 4) limitless replicative

potential, 5) sustained angiogenesis by which they recruit vasculature for nutrient supply, as well as 6) tissue invasion and metastasis which help them establish secondary tumors (Figure 1.2).

Figure 1.2. The hallmarks of cancer (Hanahan and Weinberg, 2000)



Pelengaris and Khan (2006a) have listed the inherent barriers/constraints which normal cells succumb to, but which are overcome by tumor cells that enables them to grow and progress:- a) growth factor dependence: cell proliferation requires tissue type- specific mitogenic and survival factors; if they are absent, apoptosis or programmed death is triggered; b) anchorage dependence: normal cells require transmembrane proteins called integrins to interact with components of the extracellular matrix; c) contact inhibition: normal cells show contact inhibition *in vitro*; that is, they respond to contact with other cells by ceasing cell division and cell movement; d) senescence: vertebrate somatic cells in culture can divide limited number of times (around 50–70 divisions for human cells) beyond which the cells cease to divide further – the Hayflick limit, a feature overcome by cancer cells; e) apoptosis: genetically programmed

death of cells, coupled with intracellular and extracellular signals; and f) nutrient and oxygen supply: in normal cells, increase of cell number may result in a tissue out-growing its blood supply, thereby resulting in deprivation of oxygen (hypoxia) or nutrients or accumulation of damaging waste products, all of which might in turn limit further tissue growth.

Research on early cancer detection and treatment is based on the molecular processes fundamental to such behavioral changes, as discussed below.

1.2.4.3. Autonomicity of growth signals

According to Hanahan and Weinberg (2000), for normal cells to move from a quiescent state into an active proliferative state, mitogenic growth signals are required. They get transmitted into the cell by transmembrane receptors when bound to diffusible growth factors, extracellular matrix components and cell-to-cell adhesion/interaction molecules. Tumor cells show a much reduced dependence on exogenous growth stimulation since they can generate many of their own growth signals. Acquired growth signal autonomy is a prominent factor in oncogenic transformation because of the prevalence of dominant oncogenes modulating it. Autonomy is achieved through the alteration of extracellular growth signals, transcellular transducers of those signals, or intracellular circuits that translate those signals.

Many types of cancer cells are known to have lost the requirement for specific growth factors to divide, a property proposed to be effected by: (a) autocrine activation (activation of autologous growth factor synthesis, (b) synthesis of altered growth factor receptor, or (c) activation of a post receptor pathway that bypasses the growth factor receptor requirement (Moses *et al.*, 1978, Kaplan *et al.*, 1982).

1.2.4.3.1. Autocrine activation

Autocrine signaling is the positive feedback signaling loop that enables cancer cells to synthesize growth factors to which they themselves are responsive,

contrary to the heterotypical growth factor signaling in normal cells where mitogenic growth factors synthesized by one cell type stimulates proliferation of another (Fedi *et al.,* 2000). The production of PDGF (platelet-derived growth factor) and TGF-α (tumor growth factor α) by glioblastomas and sarcomas, respectively, are two illustrative examples of autotypic growth factor production by cancer cells (Fedi *et al.,* 1999).

1.2.4.3.2. Synthesis of altered growth factor receptors

Over expression of transducer growth factor receptors on cell surface is a characteristic feature of cancer cells. Growth factor receptors, often carrying tyrosine kinase activities in their cytoplasmic domains, are over expressed in many cancers, which make them hyper-responsive to ambient levels of growth factors that normally would not elicit proliferation of cells (Fedi *et al.,* 1999). Certain malignant phenotypes including bladder cancer and brain tumors are found to over express the EGF (epidermal growth factor) trans membrane receptor, with intrinsic tyrosine kinase activity (Libermann *et al.,* 1984, Ro *et al.,* 1987). The enhanced ability of certain tumors to invade normal tissues and to metastasize (Neal *et al.,* 1985, Sainsbury *et al.,* 1985) is also associated with hyper expression of EGF receptor. There are further more examples, with many breast carcinomas showing aberrant signaling by the epidermal growth factor receptor EGFR (HER1, erbB1) and/or HER2/neu tyrosine kinases, which has been demonstrated by inhibition of HER2/neu (erbB2)- over expressing breast cancer cells using epidermal Growth Factor Receptor (HER1) tyrosine kinase inhibitors (Moulder *et al.,* 2001). Korc *et al.* (1992) has also reported that the EGF receptor, EGF, and TGF-α, which are found in acini and ducts of normal human pancreas, are expressed at higher levels in human pancreatic cancer tissues and the corresponding mRNA's encoding these proteins co-localize with their respective proteins.

Cancer cells are also known to possess altered extracellular (EC) matrix receptors (integrins), which transduce growth signals into the cell, on binding

with specific ligands in the EC matrix, leading to changes in cell behavior, such as motility, apoptotic resistance and entrance into the active cell cycle (Desgrosellier and Cheresh, 2010).

1.2.4.3.3. Activation of a post receptor pathway that bypasses the growth factor receptor requirement

A cytoplasmic circuitry exists in all cells, which receives and processes the signals elicited by ligand-activated growth factor receptors and integrins. The SOS-Ras-Raf-MAPK cascade is a key factor in signal transduction. In about 25% of human tumors, altered Ras proteins are known to release mitogenic signals into cells, bypassing ongoing stimulation by their normal upstream regulators (Medema and Bos, 1993). Recent studies also suggest that the p21 product of the Ras oncogene is mandatorily required as an intermediate in transducing the growth factor signal. Therefore, activation of Ras, in turn, activates the growth factor pathway evading the need for either a growth factor or its receptor (Goustin *et al.*, 1986).

The above mentioned factors relate to the autonomy of cancer cells in generation of growth signals, by which they acquire uncontrolled replication potential, the most significant of all characteristics.

1.2.4.4. Limitless replication potential

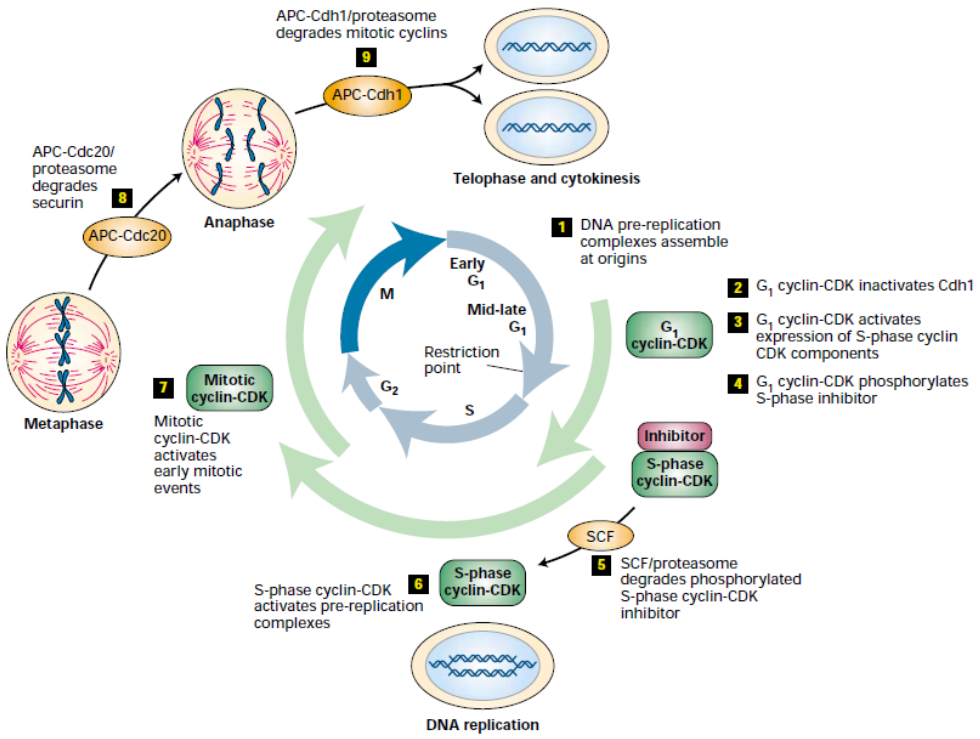
The cycle of cell replication is divided into four distinct phases: S phase /synthetic phase, characterized by the replication of chromosomal DNA, mitotic phase/M phase during which the partitioning of replicated chromosomes occurs, and two gap phases, prior to and following S phase, (referred to as G1 and G 2 respectively). Broadly, M phase in the cell cycle includes the various microscopically observed stages of nuclear division and cytokinesis (mitosis) and is itself divided into phases termed prophase, prometaphase, metaphase, anaphase, and telophase. Interphase encompasses G1, S, and G 2 phases of the cell cycle. The events of cell cycle are tightly

regulated and subject to precise quality-control steps (Figure 1.3). A cell commits to DNA synthesis only on exposure to a variety of growth-promoting and inhibitory factors; only if the balance favors growth promotion, G1/S transition takes place in normal cells. There also exist check points at different steps in the cell cycle, where the cell determines if an earlier event has occurred correctly before proceeding to the next (Pelengaris and Khan, 2006a).

Two important types of cell cycle control mechanisms are: a) a cascade of protein phosphorylations that convey a cell from one stage to the next, which is regulated by a family of kinases and b) a set of checkpoints that ensure completion of critical events and delay progression to the next stage, if necessary (Morgan, 1995). The kinases are otherwise called cyclin dependent kinases (CDKs), since their phosphorylating function becomes active only when they are bound to proteins known as cyclins present in the cells. Cyclin levels oscillate in the course of the cell cycle, and hence, the name. When bound to a CDK, cyclin specifies which proteins the CDK will phosphorylate; phosphorylation either activates a target protein or inactivates it. Each cyclin appears at a specific point in the cell cycle, usually because its synthesis and destruction are regulated by another cyclin (Pierce, 2011).

Cyclins fall under two major groups: a) G_1/S cyclins – controlling G1/S transition and b) G_2/M cyclins- regulating G_2/M transition. G_1/S cyclins are Cyclin A / CDK2, which is active in S phase; Cyclin D / CDK4, Cyclin D / CDK6, and Cyclin E / CDK2 which regulate transition from G_1 to S phase. Cyclin B / CDK1 is a G_2/M cyclin. Cyclins and CDKs come into action at different cell cycle check points (Karp, 2007). CDKs are also negatively regulated by phosphorylation events and in particular by a key group of proteins – the cyclin-dependent kinase inhibitors (CKIs), including p16INK4A, p21CIP1, and p27KIP1 (Pelengaris and Khan, 2006).

Figure 1.3. Cell cycle regulation (Lodish *et al.*, 2000)



G1/S transition is the point at which cells commit to replicate their DNA and enter the cell division cycle. The transcription factor E2F is required for expression of S-phase genes. G1/S transition is controlled by the RB (retinoblastoma) protein, the product of the tumor suppressor gene *RB*, acting as a switch off point for entry into S phase by binding E2F. In G1, cyclin D and cyclin E continuously increase in concentration and combine with their associated CDKs, after appropriate growth factor stimulation. Cyclin-D–CDK and cyclin-E–CDK both phosphorylate molecules of RB. The inhibitory effects of RB are removed by phosphorylation, which allows E2F to activate S-phase genes. Once this point is reached, even in the absence of growth factor stimuli, the cell cycle can proceed. RB or its regulatory pathways are frequently inactivated in many types of cancer, contributing to inappropriate and growth-factor independent cellular replication (Pelengaris and Khan, 2006a, Pierce, 2011). Cyclin D1 overexpression is detected in more than half of human breast cancers and is also responsible for mammary cancer in transgenic mice

(Casimiro *et al.*, 2012). Cyclin D1 plays an important role in cell cycle progression through the association with CDK4 and CDK6, which phosphorylate and inactivate the retinoblastoma protein pRb, leading to the expression of a subset of proliferation-associated E2F target genes (Musgrove *et al.*, 1994).

The expression of cyclin Es and their associated CDK2 kinase activity, shows maximal levels at the G1–S boundary after expression of cyclin D. Mutations to genes responsible for ubiquitin-mediated proteolysis of cyclin E are shown to cause certain types of tumors. An example is *hCDC4*, which has been found to be mutated in a number of primary cancers and cancer-derived cell lines, due to loss of cell cycle regulation of cyclin E (Reed *et al.*, 2004). Though correlations have been reported between the overexpression of cyclin E and breast cancer (Keyomarsi and Pardee, 1993, Keyomarsi *et al.*, 2002), there were contradictory studies also (Gladden and Diehl, 2003).

On cellular DNA damage, a tumor suppressor, p53 ensures that S phase is blocked, thus preventing the replication of mutated DNA until the damage has been repaired, or the cell eliminated through apoptosis (Pelengaris and Khan, 2006a). DNA from different human colon carcinomas generally contains mutations in p53 gene (Lodish *et al.*, 2003), amounting to about 75% of all cancers, as well as some oesophageal and skin cancers (Pelengaris and Khan, 2006a).

In the G2- to-M transition, cyclin B- CDK forms an inactive complex called *mitosis-promoting factor* (MPF), which gets activated by dephosphorylation. The amount of active MPF reaches a critical level towards the end of G2, which commits the cell to divide. Active MPF phosphorylates other proteins, and mediates nuclear-membrane disruption, spindle formation, and chromosome condensation. Cyclin B gets abruptly degraded at the end of metaphase, which lowers the amount of MPF eventually initiating anaphase, and ultimately closes mitosis (Pierce, 2011). Reports show that various human

tumors, such as breast cancer (Kawamoto *et al.*, 1997), head and neck squamous cell carcinoma (Hassan *et al.*, 2002), gastric cancer (Banerjee *et al.*, 2000), cervical cancer (Zhao *et al.*, 2006), colorectal cancer (Wang *et al.*, 1997) and non-small-cell lung cancer (Soria *et al.*, 2000) are characterized by over expression of cyclin B1.

The third check point is the spindle-assembly checkpoint, which plays in metaphase. It delays the onset of anaphase until the alignment of all chromosomes on the metaphase plate and attachment of sister kinetochores to spindle fibers from either poles is complete. Unless all chromosomes are correctly aligned, the checkpoint blocks cyclin B destruction, this keeps MPF active and maintains the cell in a mitotic state (Pierce, 2011). The spindle checkpoint inhibits the ubiquitin ligase activity of the anaphase-promoting complex or cyclosome (APC/C), thereby preventing precocious segregation of chromosomes. Aneuploidy, or abnormal chromosome number is a common form of genetic instability in human cancers, caused due to defects in spindle checkpoint (Bharadwaj and Yu, 2004). The gene *BUB1B*, encodes the mitotic spindle checkpoint protein BUBR1; when subject to mis-sense mutations it leads to mosaic variegated aneuploidy (Lengauer and Wang, 2004).

Besides succumbing to gene damages that deregulate the usual proliferative control mechanisms of a cell, it has been studied that many types of cancer cells carry mutations enabling them to escape the phenomenon of programmed cell death or apoptosis.

1.2.4.5. Evasion of apoptotic mechanisms

Apoptosis or programmed cell death) is a cell suicide mechanism that enables multicellular organisms to regulate cell number in tissues and to eliminate unneeded or aging cells during the developmental process (Ruddon, 2007b). The targets of apoptosis are those cells that have been subjected to DNA damage, hypoxia, nutrient limitation etc or displaced (cells that have moved out of their normal environment), in order to ensure proper tissue homeostasis. It is

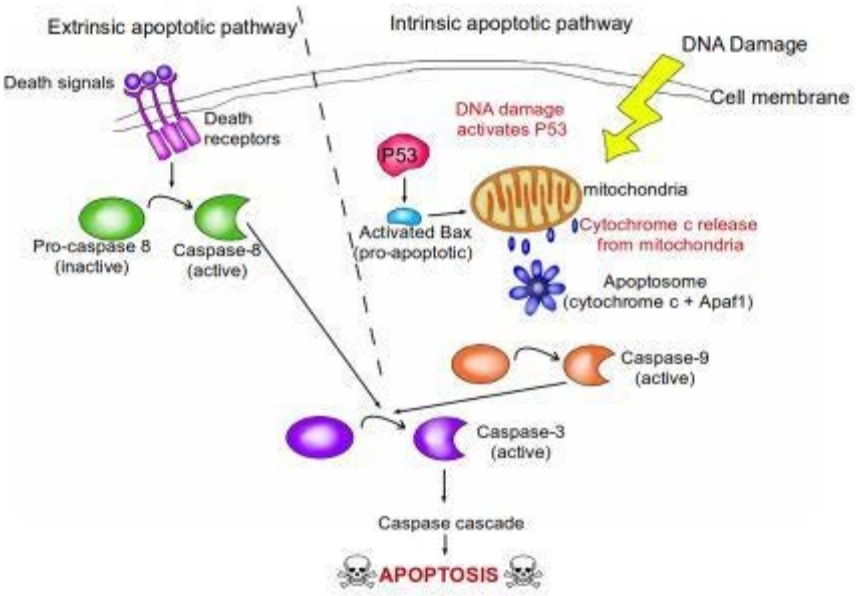
an ordered cell death process in which the entire cell is dismantled as contents of membrane-enclosed vesicles, thus preventing the release of intracellular components from the dying cell, taking care not to provoke an immune response. Macrophages of the immune system rapidly phagocytose the apoptotic cells and their membrane-bound apoptotic fragments, before leakage of any cell contents (Pelengaris and Khan, 2006a).

Cells subjected to acute injury undergo necrotic cell death, where there is typical swelling and subsequent bursting of the cell, thereby spilling their contents in the vicinity. This in turn leads to a potentially damaging inflammatory response. Contrary to this, apoptotic cell death doesn't impose damage on neighbouring cells; instead, there occurs cell shrinkage, condensation, collapse of the cytoskeleton, disassembly of the nuclear envelope and breakage of nuclear DNA into fragments. Alterations in the cell surface facilitate phagocytosis of the cell, either by a neighboring cell or by a macrophage. While necrosis inflicts damaging consequences, apoptosis doesn't impose any, but also allows recycling of the organic components of the dead cell by the phagocyte (Alberts *et al.*, 2002b).

There are two pathways that trigger apoptosis- a) the extrinsic pathway, otherwise known as the "death receptor pathway," and b) the intrinsic pathway or the " mitochondrial pathway"; the former being activated by the recruitment of death receptors on the cell surface, while the latter involving the release of cytochrome *c* (and other proteins) from the mitochondria (Figure 1.4). Both the pathways can lead to the activation of caspases, which are a family of aspartate-specific, cysteine proteases. They are primary mediators of apoptosis (Pelengaris and Khan, 2006a). The upstream caspase in the intrinsic pathway is caspase 9 while that of the extrinsic pathway is caspase 8. Both these pathways converge to caspase 3. Caspases are synthesized in the cell as inactive precursors, or *procaspases*. Their activation occurs by cleavage at aspartic acids by other caspases. On activation, these caspases cleave and activate other

procaspases, resulting in an amplifying proteolytic cascade. Some of the activated caspases then cleave other key proteins in the cell, like the nuclear lamins, leading to irreversible breakdown of the nuclear lamina; or sometimes a protein that normally holds a DNA-degrading enzyme (a DNAse) in an inactive form, thereby freeing the latter to fragment the DNA in the cell nucleus. This leads to dismantling of the cell and its debris is rapidly taken up and digested by another cell (Alberts *et al.*, 2002b).

Figure 1.4. Apoptotic pathways (Hanahan and Weinberg, 2000)



As reviewed earlier, the caspases are set into action either by the extrinsic pathway or the intrinsic pathway. The extrinsic death receptor pathway is initiated by binding of death ligands to a death receptor. The commonest death receptors include the type 1 tumor necrosis factor (TNF) receptor (TNFR1) and a related protein called Fas (CD95). The ligands which bind to them are the TNF and Fas ligand (FasL) respectively (Hengartner, 2001). On ligand binding, the intracellular death domain of these receptors recruits adapter proteins like TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD) (Schneider and Tschopp, 2000). The whole ligand-receptor-

adaptor protein complex is known as the death-inducing signalling complex (DISC) (O'Brien and Kirby, 2008). DISC then initiates the assembly and activation of pro-caspase 8. In extrinsic pathway, the activated form of the enzyme, caspase 8 is the initiator caspase, which initiates apoptosis by cleaving other downstream or executioner caspases (Karp, 2007).

The intrinsic pathway is initiated intracellularly, usually triggered by internal stimuli such as irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic Ca^{2+} and severe oxidative stress (Karp, 2007), and is characterized by increased mitochondrial permeability and the release of pro-apoptotic molecules such as cytochrome- c into the cytoplasm (Danial and Korsmeyer, 2004). This pathway is closely regulated by a group of proteins belonging to the Bcl-2 family which consists of two main groups of the Bcl-2 proteins, namely the pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1). The anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-c. Contrarily, the pro-apoptotic proteins act by promoting the release of cytochrome-c. Initiation of apoptosis is determined by a balance between the pro- and anti-apoptotic proteins (Reed, 1997). Cytochrome *c* then forms a multi-protein complex known as the 'apoptosome' and initiates activation of the caspase cascade through caspase 9. The apoptosome, which is made up of cytochrome c, Apaf-1 and caspase 9 activates caspase 3. Other apoptotic factors also get released from the mitochondrial inter membrane space into the cytoplasm. They include apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), Omi/high temperature requirement protein A (HtrA2) and direct IAP Binding protein with Low pI (DIABLO). On the other hand, Smac/DIABLO or Omi/HtrA2 promotes caspase activation by binding to inhibitor of apoptosis proteins (IAPs) which subsequently leads to disruption in the interaction of IAPs with caspase-3 or -9 (Kroemer *et al.*, 2007, LaCasse *et al.*, 2008).

Caspases are classified as a) initiator caspases (which cleave inactive pro-forms of effector caspases, thereby activating them and b) executioner caspases (which cleave other protein substrates within the cell, to trigger the apoptotic process). Caspases 8, and 9 are initiators and caspases 3, 6 and 7 are executioners (McIlwain *et al.*, 2013) in mammals. Executioner caspases cleave more than 100 different target cell proteins like: a) ICAD (Inhibitor of DNAse), an inhibitory protein on CAD (caspase activated DNAse); once activated, CAD translocates from the cytoplasm to the nucleus where it attacks DNA, severing it into fragments; b) nuclear lamins, which make up the inner lining of the nuclear envelope, whose cleavage leads to the disassembly of the nuclear lamina and shrinkage of the nucleus; c) protein kinases like FAK (focal adhesion kinase), PKB, PKC and *Raf1*; inactivation of FAK is presumed to disrupt cell adhesion leading to the detachment of the apoptotic cell from its neighbours; d) cytoskeletal proteins (actin, myosin, α actinin, tubulin, vimentin), whose cleavage by caspases leads to cell fragmentation, membrane blebbing etc; e) Golgi matrix proteins, when cleaved, leads to fragmentation of Golgi (Karp, 2007).

Avoidance of cell death is required for cells to form tumors; hence apoptosis is widely accepted as a tumor suppressive mechanism. Kerr *et al.* (1972) had associated apoptosis to the removal of potentially malignant cells, hyperplasia and tumour progression. Therefore, apoptosis resistance plays an important role in carcinogenesis. Apoptosis evasion occurs through 1) disrupted balance of pro-apoptotic and anti-apoptotic proteins, 2) reduced function of caspases and 3) incorrect death receptor signaling, as evident from the following:

Disrupted balance of pro-apoptotic and anti-apoptotic proteins

The Bcl-2 family of proteins consists of pro-apoptotic and anti-apoptotic proteins that play a key role in apoptosis regulation, especially initiated through the intrinsic pathway (Gross *et al.*, 1999). These are fundamental regulators of mitochondrial outer membrane permeabilization (MOMP), which is necessary

for cytochrome *c* release (Pelengaris and Khan, 2006a). Based on their function and the Bcl-2 homology (BH) domains the Bcl-2 family members are further divided into 3 groups: a) the anti-apoptotic proteins with all four BH domains, e.g., Bcl-2, Bcl-xL, Mcl-1, Bcl-w, A1/Bfl-1, and Bcl-B/Bcl2L10; b) the pro-apoptotic BH-3 only proteins, structurally restricted to the BH3 domain, e.g., Bid, Bim, Puma, Noxa, Bad, Bmf, Hrk, and Bik and which get activated during cellular stresses like DNA damage, growth factor deprivation and endoplasmic reticulum stress to initiate apoptosis c) the pro-apoptotic Bax subfamily containing all four BH domains in structure, e.g., Bax, Bak, and Bok/Mtd (Dewson and Kluck, 2010).

The first example showing the linkage of cancer to deregulated apoptotic protein gene expression was that of Bcl-2 upregulation due to t(14:18) chromosomal translocation in follicular B-cell lymphoma (Tsujimoto *et al.*, 1985). Later, over-expressed Bcl-2 has been detected in small cell lung cancer (Ikegaki *et al.*, 1994), chronic lymphocytic leukemia (Majid *et al.*, 2008), acute lymphoblastic leukemia (Gala *et al.*, 1994), prostate cancer (Catz and Johnson, 2003) and non Hodgkin's lymphoma (Monni *et al.*, 1997). Causes of Bcl-2 over expression include chromosomal translocation, DNA hypermethylation, and downregulation of the microRNAs that target Bcl-2. Mcl-1 overexpression is also associated with hematopoietic malignancies, such as multiple myeloma (Wuilleme-Toumi *et al.*, 2005) and chronic myeloid leukemia (Aichberger *et al.*, 2005), and with solid tumors, such as pancreatic cancer (Miyamoto *et al.*, 1999), prostate cancer (Reiner *et al.*, 2015) and melanoma (Strasser *et al.*, 1990, McKee *et al.*, 2013). Bcl-2 over expression has been found leading to inhibition of TNF-related apoptosis-inducing ligand (TRAIL) -induced apoptosis in neuroblastoma, glioblastoma and breast carcinoma cells (Fulda *et al.*, 2002).

Bak and Bax are pro-apoptotic proteins; but, in comparison with overexpression of the prosurvival /anti apoptotic proteins, relatively few

cancers are associated with mutations leading to Bak or Bax down regulation or loss-of-function. Both Bak and Bax are expressed in most cells, and their functions are found to overlap. Evasion of apoptosis requires loss or mutation in both alleles of Bak and both alleles of Bax, which is the presumable reason for their low relativity to cancer development. Yet, low levels of Bak or Bax, or a low ratio of proapoptotic protein to prosurvival protein, is found to correlate with increased incidence, poor prognosis, or resistance to chemotherapy in certain cases of colorectal cancer, melanoma, and chronic lymphocytic leukemia (Dewson and Kluck, 2010).

Reduced function of caspases

Malignancies can arise out of mutations within caspase family proteases (Jäger and Zwacka, 2010, Olsson and Zhivotovsky, 2011). Caspase-8 plays the key role in ligand-induced apoptosis of tumor cells triggered by cytotoxic T cells, more focus has been given on mutations and gene polymorphisms in caspase-8 and their association with cancer development than other caspases (Olsson and Zhivotovsky, 2011). Soung *et al.* (2005) have detected missense, deletion, and frameshift mutations within the caspase-8 gene in gastric cancers using single strand conformation polymorphism (SSCP). Caspase-8 mutation has also been reported to be linked to the development of breast cancer (Devarajan *et al.*, 2002b), meningioma, pancreatic cancer (Yang *et al.*, 2005), glioma (Schwartzbaum *et al.*, 2006, Martinez *et al.*, 2007) and glioblastoma multiforme relapse (Bethke *et al.*, 2008).

Many studies conducted to determine the link between caspase-9 deregulation and cancer development have shown, however that in many tumors, the corresponding gene is neither mutated nor silenced (Abel *et al.*, 2002, Soung *et al.*, 2006). Yet, there is report that CASP-9 promoter polymorphisms affect CASP-9 expression and increase the genetic vulnerability to lung cancer (Park *et al.*, 2006).

Caspase-3 is known to mediate apoptotic execution on activation by apoptotic signals from both death receptor and intracellular/mitochondrial pathways (Porter and Jänicke, 1999, Riedl and Shi, 2004). (Kurokawa *et al.*, 1999) has reported caspase-3 mutation in the MCF-7 breast cancer cell line. Some human cancers like colon and stomach cancer, Non-Hodgkin lymphoma (NHL), and hepatocellular carcinoma (Soung *et al.*, 2004) are found to have subjected to somatic caspase-3 mutations, though at very low frequencies. A study by Devarajan *et al.* (2002a) in breast tumors revealed that 75% of the tumor as well as morphologically normal peritumoral tissue samples lacked the caspase-3 transcript and caspase-3 protein expression, indicating that that the loss of caspases-3 expression is an important cell survival mechanism in breast cancer patients.

Caspase-7 is an important executioner caspase, functionally active especially in the cells with deficient or under-expressed caspase-3 (Kuribayashi *et al.*, 2006, Lakhani *et al.*, 2006). *Caspase-7* mutations were detected by Soung *et al.* (2003) in colon carcinomas, esophageal carcinomas and head/neck carcinomas. Downregulation of caspase-7 and -9 has been detected in colon cancer samples (Palmerini *et al.*, 2001). Hashimoto *et al.* (2008) detected periodic activation of caspases-7 and -8 during mitosis, and induction of mitotic arrest during specific interference with caspase-7 expression in cultured tumor cell lines, indicative of the role of caspase-7 for the *in vivo* proliferation of tumor cells.

Incorrect death receptor signaling

Death receptors and ligands of the death receptors are involved in the extrinsic pathway of apoptosis. The death domain on these receptors can bind to different molecules, when triggered by a death signal, resulting in the activation of a signalling cascade (Lavrik *et al.*, 2005). The extrinsic cell death pathway is triggered by ligand-receptor interactions at the cell surface. The ligands include FAS ligand-FAS/APO1, Tumor necrosis factor (TNF) receptors, and TNF-related apoptosis-inducing ligand (TRAIL) receptors.

Abnormalities such as loss of FAS expression, loss of TRAIL receptors DR4 and DR5, overexpression of TRAIL decoy TRID or overexpression of Fas decoy and overexpression of the caspase activation inhibitor have been identified in different human cancers (Ozoren and El-Deiry, 2003).

Several abnormalities in the death signalling pathways lead to evasion of the extrinsic pathway of apoptosis. Such abnormalities include down regulation of receptor gene expression, mutilation of receptor function as well as a reduced level in the death signals, resulting in impaired signaling and thereby a reduction of apoptosis. For example, downregulation of receptor surface expression has been found in some types of cancers as a mechanism of acquired drug resistance. Activation of the CD95 (APO-1/Fas) receptor/CD95 ligand (CD95/CD95-L) system has been identified to act in drug-induced apoptosis. In treatment-resistant leukaemia (Friesen *et al.*, 1997) or neuroblastoma (Fulda *et al.*, 1998) cells, CD95 downregulation was identified as a key mechanism. It has been postulated that cervical carcinogenesis can arise through the loss of Fas and the dysregulation of FasL, DR4, DR5, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in the cervical intraepithelial neoplasia (CIN)- cervical cancer sequence (Reesink-Peters *et al.*, 2005).

Hence, it is conclusive that apoptosis is a mechanism that restricts malignant cells from surviving and disseminating. Dissemination of cancer cells to other sites of the body is obviously, their most dreaded property, technically termed as metastasis. The success of the metastatic process relies on the degree of apoptosis evasion by malignant cells.

1.2.4.6. Metastasis

Metastasis is the seeding and growth of cancer cells in distant organs and accounts for death in around 90% of cancer patients (Pelengaris and Khan, 2006a, Pierce, 2011). Cancer metastasis is a stepwise process characterized by invasion, intravasation, dissemination, extravasation and colonization. During

invasion, cells show high motility due to alterations in cell-cell and cell-extra cellular matrix (ECM) interactions. Tumor cells then dislodge from the primary site and migrate into circulatory systems; this is called intravasation. During dissemination, malignant cells circulate to reach a capillary bed, and adhere to the vessel walls or get held at these sites due to size constraints. This is followed by extravasation in which tumor cells seep out of the vessels to enter the secondary/target organs. The final step is colonization, during which metastatic cells divide and form micrometastases or macrometastases (Chambers *et al.*, 2002, Horak *et al.*, 2007, Su *et al.*, 2015).

The ability to metastasize makes cancers less susceptible to surgical eradication or by localized irradiation. Dissemination depends upon their ability to loosen their adhesion to the neighbouring cells, dislodge from the primary site, invade other tissues to reach a blood vessel or a lymphatic vessel and cross the basal lamina and endothelial lining of the vessel so as to enter or exit from the circulation. For example, loss of adhesion to neighboring cells in an epithelium depends on reduced expression of the epithelial cell-cell adhesion molecule *E-cadherin* (selectin family), as demonstrated in some carcinomas. The ability to burrow through tissues depends on the production of proteolytic enzymes that can degrade extracellular matrix (Alberts *et al.*, 2002a), including serine proteases such as plasmin (activated by plasminogen activator), thiol proteases such as the cathepsins, and metalloproteases such as type IV collagenase. There is strong clinical and experimental evidence that the tumor-associated serine protease plasmin, its activator uPA (urokinase-type plasminogen activator), the receptor uPA-R (CD87), and the inhibitors PAI-1 and PAI-2 are linked to cancer invasion and metastasis. In cancer, increase of uPA, uPA-R, and/or PAI-1 is associated with tumor progression and with shortened disease-free and/or overall survival in patients afflicted with malignant solid tumors (Schmitt *et al.*, 1997).

Various other proteins have been implicated in the metastatic process, including cell members of the RHO family comprising RHO, RAC, and CDC42, which are involved in cytoskeletal organization. It is also postulated that metastatic potential is also attributed to reactivation of general embryonic pathways involved in morphogenesis and include mutations that deactivate E-cadherin and other cell adhesion molecules, those that activate transcription factors and signaling molecules such as NF-κB and TWIST, which might promote epithelial– mesenchymal transition. The correlation between epithelial– mesenchymal transition and metastatic potential has been established in various animal models systems (Pelengaris and Khan, 2006a).

The ligands binding to E-selectin expressed by vascular endothelial cells are carbohydrate determinants (sialyl Lewis A and sialyl Lewis X), very frequently expressed on human cancer cells. They facilitate cancer cell adhesion to vascular endothelium leading to hematogenous metastasis of cancer (Kannagi, 1997).

Metastatic property of cancer cells is tightly linked to their ability to promote blood vessel development, as discussed below.

1.2.4.7. Angiogenesis

Increased metabolic load and reduced oxygen availability to the tumor core necessitate angiogenesis. (Chambers *et al.*, 2002). Vascular growth is also required for the metastatic spread of tumor. Numerous stimulators of capillary endothelial cell growth, termed angiogenic factors (Folkman and Klagsbrun, 1987) or angiogenic activators, have been identified as, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin, transforming growth factor (TGF)-α, TGF-β, tumor necrosis factor (TNF)-α, platelet-derived endothelial growth factor, granulocyte colony-stimulating factor, placental growth factor, interleukin-8, hepatocyte growth factor, and epidermal growth factor (Nishida *et al.*, 2006). The production of VEGF and other growth factors by the tumor results in the formation of new

vasculature in and around the tumor, allowing it to grow exponentially; a phenomenon known as 'angiogenic switch'.

The most potent angiogenic factor is identified as vascular endothelial growth factor (VEGF), a homodimeric glycoprotein having endothelial cell specific mitogenic activity and the ability to stimulate in vivo angiogenesis, *in vivo* vascular permeability (Pelengaris and Khan, 2006a). VEGF directly stimulates endothelial cells, through its receptor VEGFR-2 (flk-1) or VEGFR-1 (flt-1) (Pepper *et al.*, 1992). VEGF is the principal mediator of angiogenesis in cancer, in which it is up-regulated by oncogene expression, influence of growth factors or hypoxia. Tumor vasculature formed under the influence of VEGF is irregularly shaped with dead ends, not organized into venules, arterioles and capillaries. VEGF hence is a rational target for anticancer therapy (Carmeliet, 2005). VEGF has been found to involve in the development of breast cancer, melanoma, oesophageal cancers, colorectal cancers, cancers of the central nervous system, pancreatic cancers and haematopoietic malignancies (Harmey, 2004).

The fibroblast growth factor (FGF) family consists of polypeptides that are potent mitogens and trophic factors for cultured endothelial cells with angiogenic properties. Fibroblast growth factors (FGFs) are involved in a variety of cellular processes, such as stemness, proliferation, anti-apoptosis, drug resistance, and angiogenesis. Katoh and Nakagama (2014) has reviewed that FGF receptor mutations are found to operate in lung cancer, estrogen receptor (ER)-positive breast cancer, diffuse-type gastric cancer, triple-negative breast cancer, 8p11 myeloproliferative syndrome, alveolar rhabdomyosarcoma, multiple myeloma, peripheral T-cell lymphoma, endometrial uterine cancer, melanoma, invasive bladder tumors and rhabdomyosarcoma.

If angiogenesis inhibitors are administered at levels that exceed those of angiogenesis promoters, tumor angiogenesis may be stopped, which open up a way in preventing tumor invasion.

1.2.5. Cancer metabolism

In comparison to normal cells, cancer cells exhibit altered or reprogrammed metabolic activities which support the attainment and maintenance of malignant properties. (DeBerardinis and Chandel, 2016). Altered metabolism enables them to gain essential nutrients from a nutrient-poor environment and make use of these nutrients to remain viable and maintain anabolic growth. Genetic alterations either directly or indirectly lead to the reprogramming of metabolism, and the resulting changes in intracellular and extracellular metabolites thus influencing transcription, translation, cellular differentiation and the tumor microenvironment. Pavlova and Thompson (2016) have categorized the tumor-associated metabolic changes into six hallmarks: 1) deregulated uptake of glucose and amino acids, 2) use of opportunistic modes of nutrient acquirement, 3) use of glycolysis/TCA cycle intermediates for biosynthesis and NADPH production, 4) increased demand for nitrogen, 5) alterations in metabolite-driven gene regulation, and 6) metabolic interactions with the microenvironment. Many tumors exhibit some of these, but few display all.

The hypothesis proposed by Otto Warburg in the 1920's, classically known as the Warburg effect or aerobic glycolysis is the best studied of all metabolic deregulations of cancer cells (Lunt and Vander Heiden, 2011). When normal cells resort to the glycolytic cycle as a physiological response to hypoxia, tumor slices and ascites cancer cells constitutively take up glucose and generate lactate, in spite of oxygen availability (Koppenol *et al.*, 2011), and show increased activity of enzymes of the glycolytic pathway, such as hexokinase, phosphofructokinase and pyruvate kinase. The credibility of this finding lies in the detection of hypoxic areas in the core of several tumors with predominant anaerobically metabolizing areas (Ruddon, 2007e). The end points of glycolysis- lactate and pyruvate- enhance tumor progression by the activation of hypoxia inducible factor-1 (HIF-1). HIF-1 is a transcription factor that in

turn up-regulates gene expression favoring glycolysis, glucose transport through GLUT-1, angiogenesis via VEGF, cell survival, and erythropoiesis. HIF-1 expression has been observed in cancers of the brain, breast, colon, lung, ovary, and prostate and their metastases, which was not found in the normal counterparts (Lu *et al.*, 2002). Several oncogenes (eg., ras, src and myc) enhance aerobic glycolysis by increasing the expression of glucose transporters and glycolytic enzymes (Gatenby and Gillies, 2004).

The proliferating cells meet their metabolic demands for macromolecule synthesis from subsidiary pathways utilizing glycolytic intermediates as well as tricarboxylic acid (TCA) cycle intermediates (Ahn and Metallo, 2015). In spite of the heterogeneity of tumors, there are some common anabolic, catabolic and redox balance reactions are induced in most of the tumors (Cantor and Sabatini, 2012), being a consequence of deregulation of signaling pathways like PI3K-AKT-mTOR (phosphatidylinositol 3-kinase- protein kinase B- mammalian target of rapamycin) network and the MYC pathway, which have been studied to promote glycolytic flux, glutaminolysis, serine metabolism and fatty acid synthesis (Yuan and Cantley, 2008, Dibble and Manning, 2013, Stine *et al.*, 2015).

The characteristic features of cancer cells mentioned above- including cytological, biochemical, metabolic and behavioral alterations- are manifestations of the basic changes that occur in the genetic make-up of the cell, which is acronymic to the term oncogenic transformation. The development of tumors in humans is attributed to alterations in more than 350 different genes as identified so far; the actual number is believed to be even much higher; and some of which have been discussed as follows.

1.2.6. Cancer genetics

As reviewed by Futreal *et al.* (2004), cancer arises from mutations in more than 1% of human genes, which mainly fall under two broad categories: a) oncogenes- dominant stimulatory genes whose mutations promote cell

proliferation and cancer development and: b) tumor suppressor genes, generally recessive, and when inactivated, lead to cancer. Cancer initiates by mutations in either one of these genes, or both, but the probability of acquiring mutations can in turn be increased by mutations in another group called DNA-repair genes (Pierce, 2011). A majority of cancer genes show somatic mutations (90%) and a few are subject to germline mutations (20%), while about 10% are prone to both (Futreal *et al.*, 2004).

1.2.6.1. Oncogenes

Oncogenes are the mutated versions of certain normal cellular genes regulating cell division, cell growth, survival, differentiation, or cell motility. The normal counterparts are called proto oncogenes, which are found to be related to viral oncogenes, as was first discovered by Michael Bishop, Harold Varmus and their colleagues in 1975. During viral infection, a proto-oncogene gets incorporated into the viral genome through recombination, which can then mutate to an oncogene within the viral genome. On insertion back into a cell, the gene leads to fast cell division and cancer. Since the proto-oncogenes have higher chances to undergo mutation or recombination within a virus, cancer and viral infection are often interlinked (Pierce, 2011).

Most of the proteins coded by oncogenes are participants of signaling pathways which regulate cell division and survival. They include polypeptide growth factors, growth factor receptors, elements of intracellular signaling pathways, and transcription factors. Based on the function of proteins encoded, oncogenes are grouped under five classes, viz., a) transcription factors, b) growth factors, c) growth factor receptors, d) signal transducers and e) apoptotic regulators (Pelengaris and Khan, 2006c), examples of which have been summarized in table 1.1.

Table 1.1. Types of oncogenes

Oncogene	Type of cancer	Method of activation	Protein involved
Transcription factors			
c-MYC	Multiple cancers, breast, stomach, lung, cervix, colon, neuroblastomas and glioblastomas. Burkitt's Lymphoma	Amplification Translocation Deregulation	Transcription factor
FOS	Osteosarcoma	Deregulated activity	Transcription factor AP1
JUN	Sarcoma	Deregulated activity	Transcription factor AP1
ERBA1	Erythroblastosis	Deregulated activity	T3 transcription factor
Growth factors			
v-sis	Glioma/ fibrosarcoma	Constitutive synthesis	B-chain PDGF
HST	Stomach carcinoma	Constitutive synthesis	FGF family
INT2	Mammary carcinomas	Constitutive synthesis	FGF family
KS3	Kaposi sarcoma	Constitutive synthesis	FGF family
Growth factor receptors			
Growth factor receptor tyrosine kinases			
HER1/EGFR	Squamous cell carcinoma	Gene amplification/ increased protein	EGF receptor
HER2/NEU	Neuroblastoma/ breast carcinoma	Gene amplification	Human EGFR-2
TRK	Colon/ thyroid carcinomas	DNA rearrangement/ constitutive activation	Fusion proteins
G protein coupled receptors			
MAS	Colon/thyroid carcinomas	DNA rearrangement/ constitutive activation (fusion proteins)	NGF receptor

Signal transducers			
Non-receptor tyrosine kinases			
ABL	CML	DNA rearrangement translocation (fusion proteins)	Tyrosine kinase
BCR-ABL	CML	Fusion proteins	Tyrosine kinase
SRC	Colon carcinoma	Constitutive activation	Tyrosine kinase
FGR	Sarcoma	Constitutive activation	Tyrosine kinase
Membrane-associated G proteins			
RAS	AML, thyroid carcinoma, melanoma	Point mutation	GTPase
GSP	Adenomas of thyroid	Point mutation	Gs alpha subunit
GTPase exchange factor (gef)			
DBL	Diffuse B-cell lymphoma	DNA rearrangement	Gef for rho and cdc42
Serine/threonine kinases: cytoplasmic			
RAF	Sarcoma	Constitutive activation	Protein kinase (Ser/Thr)
Cytoplasmic regulators			
PRAD-1	Breast		Cyclin D1
Apoptotic regulators			
BCL2	B-cell lymphomas	Constitutive activity	Anti apoptotic protein

Transcription factors belong to multigene families with common structural domains and they attain functional status, on interaction with other proteins. Oncogenes encode nuclear transcription factors such as *MYC*, *MYB*, *FOS*, *JUN*, *ERB* A and *REL*. Oncogenic mutations in these genes either leads to loss of negative regulatory elements (e.g., for *MYB*, *FOS* and *JUN*), or by dominant-negative mutation - loss of their active domains, producing a mutant protein that prevents the activity of the normal gene product (e.g., *ERB* A and *REL*) (Ruddon, 2007f).

Oncogene growth factors can trigger tumor cell proliferation under paracrine or autocrine mechanisms, but are less capable of maintaining the transformed phenotype, autonomously (Ruddon, 2007f). The best known GF oncogene is *SIS*, the oncogene incorporated into the genome of the simian sarcoma virus, and it encodes the platelet-derived growth factor PDGF-B chain (Pelengaris and Khan, 2006c), can abnormally auto stimulate division of cells that normally possess the PDGF receptor (Lodish *et al.*, 2000).

A majority of oncogenes encode growth factor receptors, many of which have associated tyrosine kinase activity. These include the *src* family of oncogenes, *erb* B (EGF receptor), and *fms* (CSF-1 receptor). These receptors get converted to oncogenic versions by changes in their amino-terminal domains, leading to aberrant binding of extracellular growth factors. Genes encoding G-protein coupled receptors often acting as oncogenes, the *MAS* gene, being the most noteworthy, which has been identified in mammary carcinomas, human epidermoid tumors, colon and thyroid carcinomas (Pelengaris and Khan, 2006c).

Since signal-transduction pathways regulate cell cycle events, defects in their components lead to cancer. The oncogenic mutations affecting the signaling cascades bring about malfunction of two functional gene groups- a) Genes encoding n*on* receptor membrane-associated tyrosine kinases and b) The RAS family. The SRC non-receptor tyrosine kinase was the first oncogene

discovered and was formerly recognized as the transforming agent (*v-src*) of the avian Rous sarcoma virus (RSV). The SRC protein phosphate groups to tyrosine residues on target proteins, thereby activating downstream signaling. When *c-SRC* (SRC) is found in low levels in normal cells, it may be over expressed or activated in cancer types, including neuroblastoma, small-cell lung carcinomas, colon carcinomas and breast cancers. Reduced expression of CSK (SRC tyrosine kinase), that inactivates SRC by phosphorylation leads to cancer. Meanwhile, protein tyrosine phosphatases (PTP) (e.g., PTP1B) that dephosphorylate the terminal tyrosine residue of SRC, thereby activating them, are found to be present in higher levels in breast cancer cell lines (Pelengaris and Khan, 2006c). The RAS superfamily is a group of monomeric G proteins, involved in normal cellular proliferation, motility and survival. These proteins bind GTP, have associated GTPases, and act as signal transducers for cell surface growth factor receptors. Activating mutations of *ras* genes or overexpression of Ras proteins are implicated in human tumorigenesis, probably by their effects such as deregulated cell proliferation, invasiveness and angiogenesis. 75% of tumors of the pancreas and 50% of those in the thyroid and colon have mutations in *ras* genes. Though mutant Ras proteins bind GTP, they fail to hydrolyze it to GDP, and hence, get retained in the activated form, consequently promoting cell division (Ruddon, 2007f, Pierce, 2011).

Genes encoding anti apoptotic proteins, on activation by mutation, can lead to uncontrolled cell proliferation and cancer. Anti apoptotic members, BCL-2, and its closest homologues BCL-x L and BCL-w, are potent inhibitors of apoptosis in response to many stress signals (Pelengaris and Khan, 2006c). It has been noted that during oncogenesis of certain cancers like human follicular lymphoma, diffuse large cell lymphoma and chronic lymphocytic leukemia, there occurs activation of constitutive expression of Bcl-2 in the B-cell clone and its progeny, by its chromosome translocation to the immunoglobulin heavy chain or light chain gene loci (Cory *et al.*, 2003).

It is generally a gain-of-function mutation that converts a proto-oncogene into an oncogene, which can occur by three mechanisms- a) synthesis of a constitutively acting protein product by point mutations in the gene; b) over expression of the protein product, by localized reduplication of a part of the genome including the proto-oncogene leading to its amplification; c) inappropriate gene expression resulting from chromosomal translocation that gets a growth regulatory gene under the control of another promoter. Such gain-of-function mutations leading to oncogenesis act dominantly; that is, cancer may be induced by mutation in at least only one of the two alleles (Lodish *et al.*, 2000).

1.2.6.2. Tumor suppressor genes

Tumor-suppressor genes, which make up about 10% of cancer-causing genes, follow recessive inheritance pattern, that is, both alleles must be mutated to turn into cancer, and so also, such genes are more difficult to be identified. Heterozygotes with one defective allele alone are inclined to cancer, since inactivation or loss of the wild allele totally eliminates the tumor-suppressor product, a process referred to as loss of heterozygosity, most commonly occurring by deletion mutations. The failure of tumor suppression eventually promotes runaway cell proliferation (Weinberg, 1993, Pierce, 2011). Many cellular processes including critical cellular checkpoints governing the mitotic cycle, signaling pathways involved in cell growth, other mechanisms such as DNA repair, transcription, apoptosis, and differentiation are supervised by tumor suppressors (Table 1.2). The imbalance created by their functional inactivation favors tumorigenesis (Roussel, 2006). When the tumor suppressor genes undergo mutation, some proteins limiting cell proliferation get inactivated. Out of the different tumor- suppressor proteins identified so far, the most elaborately studied are p53 and pRb (retinoblastoma); both take part in transcription and phosphorylation events necessary for G1 - S transition of cell cycle. Unless inactivated by cyclin-dependent kinases in late G1, the pRb

protein inhibits transcription factors and other proteins required for S phase, keeping G1 in suspension. Induction of p53 by DNA damage may act to cause cell cycle arrest or cell death by altering the transcription program of damaged cells (Hinds and Weinberg, 1994).

Table 1.2. Classification of tumor suppressor genes based on function (Roussel, 2006)

Gene	Name	Tumor type
Cell cycle regulators		
pRb/P53/TP 53	Retinoblastoma	Retinoblastoma, sarcomas, Lymphomas, sarcomas, brain and breast cancers
CDKN2A	Cyclin-dependent kinase inhibitory protein p16Ink4a	Melanoma, sarcomas, lymphomas
CDKN2C/ INK4C	Cyclin-dependent kinase inhibitory protein p18Ink4c	Testicular cancers
DNA repair		
BRCA1 and BRCA2	Breast cancer	Breast cancer and ovarian cancers
MSH2	Nonpolyposis colon cancer	Colon cancer
Transcription regulators		
WT1	Wilms' tumors	Kidney cancer (children)
VHL	Von Hippel–Lindau syndrome	Renal cancers, hemangioblastoma, pheochromocytoma
Signalling		
PTEN	Phosphatase and tensin homolog deleted on chromosome ten	Cowden disease; breast, brain, and prostate cancers; hyperkeratinosis
Cell cycle checkpoint regulation		
ATM	Ataxia-telangiectasia mutated	Cerebellar ataxia, cancer predisposition

1.2.7. Inflammation and cancer

It has been revealed from epidemiological studies that there is an inclination of chronic inflammation to cancer development. Tumor microenvironment often accumulates pro inflammatory mediators including proteases, eicosanoids, chemokines, cytokines and prostaglandins and inflammatory cells (tumor-associated macrophages) just as seen in chronic inflammatory responses. Many cytokines including the macrophage migratory inhibitory factor (MIF), TNF-α, IL-6, IL-17, IL-12, IL-23, IL-10, and TGF-β have been associated with both experimental and human cancers and are found to uphold or inhibit tumor development. The tissue remodelling and angiogenesis in cancer cells share the same mechanisms as that of tissue repair process (Candido and Hagemann, 2013).

The molecular and cellular circuits connecting inflammation and cancer can either be intrinsic or extrinsic. Oncogenic transformation triggers development of an inflammatory microenvironment in the former, while in the latter, inflammatory conditions lead to cancer development. The intrinsic pathway is demonstrated by oncogenes like those of tyrosine kinases, ras–raf, nuclear oncogenes and tumor suppressors who can set up proinflammatory circuits such as the angiogenetic switch or recruitment of myelo-monocytic cells. The extrinsic pathway is exemplified by the increased incidence of gastric cancer by infection from *Helicobacter pylori* for; cervical cancer by papilloma virus infection and liver carcinoma by hepatitis viruses (Colotta *et al.*, 2009).

Overviewed at the genetic level, it is known that NF-κB, which is an important regulator of inflammation, is seen in the aberrant mode in many cancers. It regulates tumour angiogenesis and invasiveness, and controls the ability of both pre-neoplastic and malignant cells to overcome apoptosis-based tumour surveillance in the cell. Hence, chemopreventive and chemotherapeutic investigations target signalling pathways that activate NF-κB (Karin, 2006).

The frequency of p53 mutations seen in tumours is also found to be similar to that of certain chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (Yamanishi *et al.*, 2002).

In the molecular level, it can be seen that cancer cells accumulate inflammatory mediator molecules such as chemokines, cytokines and interleukins (IL). Some tumour cells can regulate their chemokine expression to recruit inflammatory cells as well as to facilitate tumour growth and progression. In melanoma, certain chemokines like GROα/CXCL1, GROβ/CXCL2, GROγ/CXCL3 and IL-8/CXCL8 exercise autocrine control over cancer cell proliferation (Richmond and Thomas, 1986). The pro-inflammatory cytokine, TNF-α takes part in early events in tumours and is involved in the regulation of a cascade of cytokines, chemokines, adhesions, matrix-metalloproteases (MMPs) and pro-angiogenic events. TNF-α is thus responsible for the role of inflammation in promoting tumor growth (Balkwill, 2002).

Another finding supporting the cancer-inflammation link is the reduced cancer risk (colon, lung, oesophageal and stomach cancers) in users of non steroidal anti-inflammatory drugs (NSAIDs) (Baron and Sandler, 2000), which inhibit the cyclo-oxygenase enzymes - COX-1 and -2; the latter converts arachidonic acid to prostaglandins, the inflammatory mediators coming in action during tissue damage (Williams *et al.*, 1999). Some NSAIDs like flurbiprofen exert strong anti-metastatic effects by inhibiting platelet aggregation (Mamytbekova *et al.*, 1986).

The above findings in cancer biology have opened up new approaches to target inflammatory, apoptotic, metastatic and cell cycle regulatory pathways/processes in developing chemo-preventive and therapeutic agents against cancer, and the following section deals with the different modalities of cancer treatment.

1.2.8. Cancer treatment

The commonest modalities of cancer treatment are chemotherapy and radiotherapy; others being surgery, hormonal therapy and immunotherapy.

1.2.8.1. Surgery

Surgery can be applied to non hematological cancers; and is used to reduce the tumor burden in combination with other treatment modalities. The goals of surgery may be curative (to remove tumors), preventive (by excising pre-cancerous tissues), diagnostic (by collecting sample for testing), debulking (by removing only a portion of the cancerous organ), palliative (to relieve cancer related discomfort at later stages), preventive (e.g., prophylactic mastectomy in women with risk of breast cancer) or supportive (by complementing other therapies) (e-Article, 2011).

1.2.8.2. Chemotherapy

Chemotherapeutic agents are cytotoxic to cells, a property by which they destroy cancer cells. According to the cell cycle phase targeted by these agents, they're classified into: a) alkylating agents, b) anti metabolites, c) mitotic inhibitors and d) topoisomerase inhibitors. Apart from these, anti tumor antibiotics, enzymes and biologically targeted agents are also used in chemotherapy (Thomas *et al.*, 2006).

Alkylating agents form a diverse group of highly reactive molecules which react with DNA through the alkyl chemical group (R–CH2) to promote single- or double-strand DNA breaks and DNA cross-linking thereby leading to apoptosis or growth arrest (Colvin, 2003). The alkylating agents include nitrogen mustards (e.g., cyclophosphamide), alkyl sulfonates (e.g., busulfan), aziridines (e.g., thiotepa), nitrosoureas (e.g., BCNU) and metal salts (e.g., cisplatin, carboplatin). Platinum compounds like cisplatin inhibit DNA synthesis through the formation of intra-strand cross-links in the DNA and

formation of DNA adducts. Cisplatin is found to be effective in the treatment of testicular tumors, but its use is limited by renal toxicity.

Because of their structural resemblance with purines and pyrimidines, anti metabolites can either inhibit key enzymes of DNA synthesis or get incorporated into the DNA and RNA producing incorrect codes and cause strand breaks or premature chain terminations. Since they target DNA synthesis, the anti metabolites are S-phase specific. They include folate antagonists (e.g., methotrexate), arabinosides (e.g., Fludarabine), anti pyrimidines (e.g., 5 Flourouracil) and anti purines (e.g., 6 mercapto purine) (Scaife and Kerr, 2008).

Mitotic inhibitors bind to tubulin protein and prevent the mitotic spindle assembly required for chromosome segregation in mitosis. They include the vinca alkaloids - vincristine, vinblastine, and vinorelbine derived from the plant *Vinca rosea* or available in semisynthetic form. The vinca alkaloids are used in the treatment of lymphomas, leukemias, non small cell lung cancer and breast cancer, but in higher doses may lead to peripheral neuropathy and thrombocytopenia. The taxanes are another group of mitotic inhibitors that inhibit the disassembly of mitotic spindle by their preferential binding to microtubules. The taxanes target the protein, β-tubulin in the microtubule polymer. Paclitaxel, an extract from the bark of the pacific yew is an example. They are used in treating ovarian and breast cancer. They pose dose limiting side effects like bone marrow suppression and hypersensitivity reactions (Brown *et al.*, 2008).

Topoisomerase inhibitors inhibit the action of the cellular enzymes-topoisomerase I and II which control the structural changes in DNA by mediating the breaking and rejoining of the phosphodiester backbone of DNA strands, thus preventing the relegation of double-strand DNA breaks during normal cell cycle. This impedes DNA replication. The first topoisomerase 1 inhibitor to be discovered was Camptothecin. Another example is Irinotecan

which is widely used in colon cancer treatment, but is reported with side effects such as diarrhea, myelosuppression etc. Etoposide is the most widely used topoisomerase 2 inhibitor used for treating Hodgkin's disease, non-Hodgkin's lymphoma, small cell lung cancer, and testicular teratoma. But its major dose limiting side effect is myelosuppression, alopecia and mucositis (Thomas *et al.*, 2006).

Anti tumor antibiotics are cytotoxic antimicrobial molecules produced by cultured *Streptomyces* species. They include the anthracyclines, doxorubicin, daunorubicin, epirubicin and idarubicin, and the non-anthracycline antibiotics, bleomycin and actinomycin D. They not only act as alkylating agents but also as DNA intercalating agents and topoisomerase 2 inhibitors. They put up oxidative stress damage to cellular proteins, and inhibit their action. The main toxicity after anthracycline treatment is myelosuppression and dose dependent cardiotoxicity (Thomas *et al.*, 2006, Paz, 2008).

Cytotoxic enzymes like asparaginase are also used for chemotherapy, owing to their degradable action on asparagine, an essential amino acid for protein and nucleic acid synthesis. Tumor cells either lack or under-express asparaginase synthetase, and rely on exogenous supply of asparagine. Treatment with asparaginase therefore inhibits protein and nucleic acid synthesis. It is used mostly to treat acute leukemias, but the main side effect is that it evokes hypersensitivity reaction (Thomas *et al.*, 2006).

Therapeutic mechanisms targeting gene expression and signal transduction pathways characteristic only to cancer cells are also licensed for use in chemotherapy, as enlisted in table 1.3.

Table 1.3. Some chemotherapeutic agents targeting gene expression and signal transduction (Thomas *et al.*, 2006, Maemondo *et al.*, 2010, Rosen *et al.*, 2010, Boekhout *et al.*, 2011, Incorvati *et al.*, 2013, von Minckwitz *et al.*, 2017)

Drug	Cellular target	Molecular category	Tumor treated	Clinical status
Trastuzumab herceptin	HER-2	Anti-EGFR monoclonal antibodies	Breast, Gastric	Licensed
Cetuximab	EGFR/ HER-1	Anti-EGFR monoclonal antibodies	Colorectal	Licensed
Lapatinib	HER-1/ HER-2	Lapatinib ditosylate (TK inhibitor)	Breast cancer	Licensed
Pertuzumab	HER-2	Anti-EGFR monoclonal antibodies	Breast cancers, Solid tumors	Licensed
Gefitinib (ZD1839)	EGFR PO	Anti-EGFR TK inhibitors	Breast, NSCLC	Licensed
Bevacizumab (Avastin)	VEGF-A	Monoclonal antibody	Colorectal	Licensed

Radiotherapy is as important as chemotherapy in cancer treatment, which is discussed in the next section.

1.2.8.3. Radiotherapy

Radiotherapy is the application of ionizing electromagnetic radiation to treat cancer. The target is cellular DNA damage, whereby the proliferating cancer cells either die or get slowed down. Depending upon the source of radiation, the treatment can either be external beam therapy or internal beam therapy. When, in the former, a machine or a device emits the therapeutic dose of radiation; in the latter, a source of radiation is introduced into the patients' body. The biological effects of radiation result from absorption of energy derived from radiation. Since the dose is energy absorbed by unit mass, radiotherapy is prescribed in units of Grays; 1 Gray (GY) = 1 joule of energy

absorbed per kilogram of mass (Thomas *et al.*, 2006, e-Article, 2017a). External beam therapy can be carried out by machines such as X-ray tubes (which produce X-rays of energy range 80–300 kV for the radical treatment of skin cancer and the palliative treatment of skin and bone metastases), cobalt gamma ray machines (which release gamma rays equivalent to 2 MV X-rays) and linear accelerators (which use electromagnetic waves to accelerate electrons, which bombard a tungsten target resulting in the production of X-rays with energies of 4–25 MV). Internal beam therapy involves brachytherapy (placing of a radioactive source like caesium-137, iodine-125, or iridium-192 close to the tumor) and systemic therapy (which involves administration of unsealed sources like iodine-131, as oral dose used for treatment of thyrotoxicosis and thyroid cancers.

Radiotherapy damages the normal and healthy cells in the vicinity of the tumor, hence it poses side effects, and the effects are localized, unlike chemotherapy. General side effects include skin irritations, loss of hair or fatigue, but many localized effects are noticed, depending upon the body part exposed to radiotherapy (e-Article, 2017a). Acute side effects include mucositis, esophagitis (heartburn), nausea, vomiting, alopecia, transient worsening of neurological symptoms , diarrhea, dysuria, dry desquamation of skin etc (Thomas *et al.*, 2006). Epithelial and haematopoietic tissues are highly susceptible to radiation induced damage.

1.2.8.4. Hormone therapy

Hormone therapy is a part of medical oncology in which the endocrine system is manipulated through exogenous administration of hormones or hormone-antagonists. This results in changes in gene expression that ultimately lead to cell death or cessation of division. Breast cancer (de Cremoux, 2011), prostate cancer (Brawer, 2006), endometrial cancer (Markman, 2005) and cancers of the adrenal cortex (Wooten and King, 1993) are most responsive to hormone therapy. Tamoxifen is a selective estrogen receptor modulator which acts as a

competitive antagonist for estrogen receptors. Tamoxifen causes tumor regression in endocrine sensitive tumors in both premenopausal and postmenopausal women and is used for adjuvant therapy in women who have undergone surgery with or without radiotherapy as the primary treatment for their cancer. Androgen deprivation is the primary treatment for metastatic prostate cancer in men, and is achieved by orchidectomy or the use of GnRH analogs, which act as receptor antagonists (Thomas *et al.*, 2006).

1.2.8.5. Immunotherapy

Immunotherapy, has recently gained focus in the treatment of different types of cancers. It involves either the augmentation (activation immunotherapy) or suppression (suppression immunotherapy by using antibodies suppressing R cells) of the immune system to treat the disease. The use of antibodies can either release a brake on T cells, to trigger them tackle tumors or by infusing genetically engineered T cells (Pardoll, 2012, Couzin-Frankel, 2013). Another approach to activate anti tumor immunity is to block the immune checkpoints (that offer immune resistance to tumors) initiated by ligand–receptor interactions, by using specific antibodies.

1.2.9. Role of herbs in cancer treatment

Though chemotherapy and radiotherapy are among the common modalities of cancer treatment, they are known to pose toxic contra indications as discussed in previous sections. But these treatment modalities eliminate the rapidly proliferating hematopoietic bone marrow cells, hair follicles, digestive tract epithelial cells and reproductive tract cells, apart from their prime targets- the cancer cells. This poses serious side effects affecting vital organs like heart, lungs, kidney and digestive organs (e-Article, 2015, e-Article, 2017a). Hence, cancer research is being accelerated towards the investigation of plant-derived anticancer compounds/extracts- many of which have been used in traditional herbal treatments for centuries- owing to their effectiveness and manifestation of lesser contra-indications (Yance and Valentine, 1999).

Early in 1970's, one of the most important plant compounds was derived from the plant Pacific Yew, *Taxus brevifolia* and was tested by the National Cancer institute, US. The compound, taxol (paclitaxel), is approved worldwide for treatment of breast and ovarian cancers. The plant derived anti cancer agents act on diverse targets in the tumor cells. For example, the Vinca alkaloids disrupt the mitotic spindle assembly through interaction with the tubulin protein and inhibit mitosis. The plant-derived camptothecins (irinotecan, topotecan), isolated from the Chinese tree *Camptotheca acuminata* inhibit topoisomerase I; the plant-derived epipodophyllotoxins (etoposide and teniposide) and the microbial-derived anthracyclines (e.g. doxorubicin, epirubicin) inhibit topoisomerase II (Nobili *et al.*, 2009).

Besides isolated compounds from plants, plant extracts containing multiple compounds are also attributed with antitumor activities (Raveendran *et al.*, 2012, Solowey *et al.*, 2014, Akindele *et al.*, 2015, Engel *et al.*, 2016). Examples are *Baliospermum montanum, Andrographis paniculata, Annona atemoya, Phyllanthus niruri, Podophyllum hexandrum, Tinospora cordifolia, Semecarpus anacardium, Vitis vinifera, Pandanus odoratissimum, Pterospermum acerifolium, Raphanus sativus, Amorphopallus campanulatus, Moringa oleifera, Ficus bengalensis, Allium sativum,* etc, with reported anticancer properties (Jain *et al.*, 2010).

Herb-based drugs can be used as complementary treatment for cancer, along with chemotherapy and radiotherapy. For example, soy-derived phytoestrogens can complement tamoxifen-based breast cancer and postmenopausal symptoms in women (Fitzpatrick, 2003, He and Chen, 2013). Polyphenols like epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG), the soy isoflavones, β-carotene and lycopenes from plants can act as phytoestrogens in the treatment of prostate carcinoma (Nelson and Montgomery, 2003). For the treatment of lung cancer, plants like *Platycodon grandiflorum, Morus alba, Prunus armeniaca, Rhus verniciflua, Perilla*

frutescens, *Stemona japonica*, *Tussilago farfara*, and *Draba nemorosa* are known to be in traditional use (Liang *et al.*, 2011).

Plant extracts gain importance in clinical therapy because the presence of many compounds in the extract can buffer the toxic effects of a single component (Vickers, 2002). In the light of the importance of the use of plant extracts in cancer therapy, the present study is also focused on the exploration of anti cancer properties of *Oroxylum indicum* Vent., a plant belonging to the family Bignoniaceae. The plant is endowed with bioactive components of pharmaceutical importance, and most parts of it are acclaimed as valuable in pharmaceutical research, as will be discussed in the following sections.

1.3. *Oroxylum indicum* Vent.

1.3.1. Nomenclature

1.3.1.1. Taxonomic classification

Scientific name:		*Oroxylum indicum* Vent. .
Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Lamiales
Family	:	Bignoniaceae
Genus	:	*Oroxylum*
Species	:	*indicum*

Figure 1.5. *Oroxylum indicum* Vent.

1.3.1.2. Common names

Oroxylum indicum Vent. is known colloquially in different names as the following:-

Indian trumpet flower, Broken bones tree	:	English
Palakappayyani/ Vellappathiri	:	Malayalam
Shyonaka	:	Sanskrit
Bhut-vriksha	:	Hindi
Peiarlankei	:	Tamil

1.3.2. General characteristics

1.3.2.1. Description

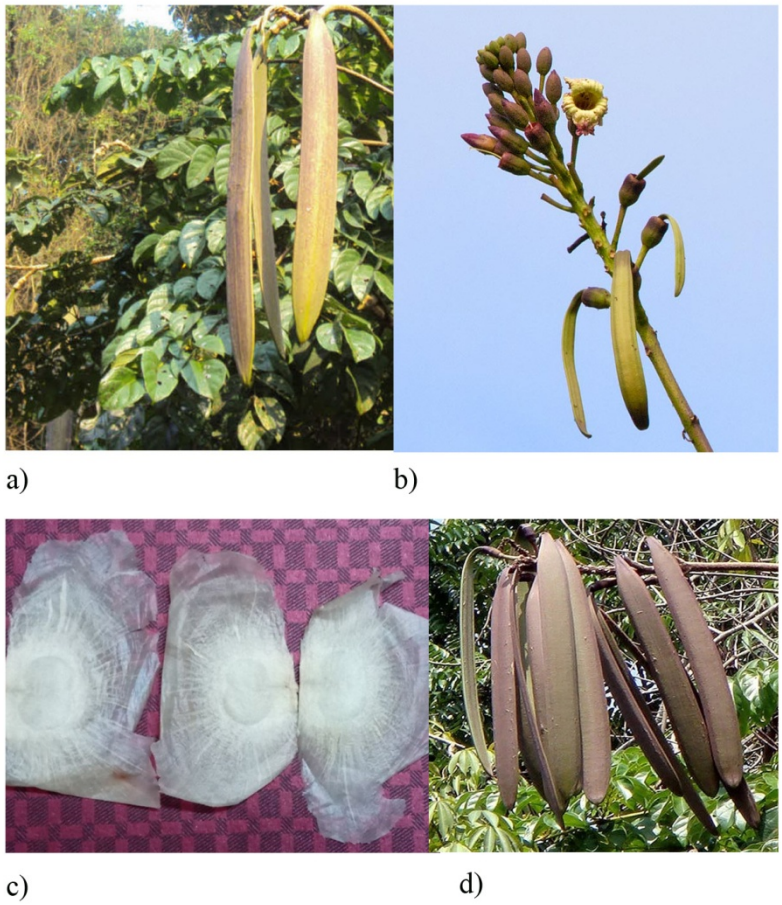
Oroxylum indicum Vent. is a small to intermediate sized tree (Figure 1.5.) belonging to the family Bignoniaceae. It emerges to a height of 12 metres and is most characterized by the presence of long sword shaped green fruit capsules (Figure 1.6.a.) which later get matured to brownish black seed pods (Figure 1.6.d). The grayish brown bark which was initially smooth and juicy later shrinks and adheres to the wood and seems to possess corky lenticels. The leaves are very large, ovate and acuminate in shape attaining 90-180 cm length, bi- or tripinnate in nature, with around 5 pairs of primary pinnae and swollen rachis. The flowers are coloured reddish purple outside and light pink to yellow inside, seen as large erect racemes (Figure 1.6.b). The fruits appear as long curved sword shaped flat capsules measuring to a length of 0.33 - 1m and a width of 5-10 cm, hanging down from the tree branches.

1.3.2.2. Habit

The tree is deciduous in habit; the large leaf stalks droop and fall off the tree and accumulate near the base of the trunk, like a pile of broken limb bones, giving the name “broken bones tree”. The plant flowers in June-July and bears fruits in November. The flowers are night- bloomers which release a strong, stinky smell attracting bats, favouring chiropterophily. The mature pods burst

open to release numerous flat papery winged seeds (Figure 1.6.c.) which either directly fall on to the ground or do so after traveling for a while.

Figure 1.6. *Oroxylum indicum* Vent., showing green fruits (a), inflorescence (b), papery winged seeds (c) and seed capsules (d).



1.3.2.3. Geographical distribution.

The species enjoys distribution throughout the Asian tropical and subtropical regions including countries like India, Sri Lanka, South China, Philippines and Malaysia and seem to grow in ranges from sea level to an altitude of 1200 m. In India, it is distributed in Himalayan foothills, Eastern and Western Ghats as well as North East India (Gokhale and Bansal, 2006, Padgilwar *et al.*, 2014).

1.3.3. Pharmacological properties

1.3.3.1. Traditional uses

Oroxylum indicum is one among the ten plants whose roots form ingredients of the widely used Ayurvedic formulation, Dashamoola. This combination often prescribed as Dashamoolarishta, Dashamoola Churna, Dashamoola Ghrita, Dashamoola Kalpa, Dashamoola Kwatha and Dashamoola Oil, is used commonly in Ayurveda for many nerve, muscle, bone and joint- related disorders owing to its strong anti-inflammatory, antioxidant and analgesic actions. (A.P.I., 2007, Singh *et al.*, 2011, Bhalerao *et al.*, 2015).

Various parts of the plant have been traditionally used in curing many ailments including certain types of cancers. Root bark is an ingredient of Dasamula preparations (Sharma, 2006). A decoction of the stem bark is used for the treatment of nasopharyngeal cancer and gastric ulcer by some ethnic groups in eastern India. They also treat scabies, arthritis, oral cancers and some skin diseases using a paste made from the bark (Mao, 2002). Some tribal groups in Tripura use the bark and fruit to make preparations to cure leucorrhoea and urinary problems (Hossan *et al.*, 2010). Besides their use as remedies for hypertension and throat infections, purgative properties are attributed to the seeds, when administered through oral route (Singh *et al.*, 2002). Reviews (Deka *et al.*, 2013, Preety and Sharma, 2016) on medicinal properties of this plant throw light on the traditional medicinal properties of its leaves, fruits and root bark. Leaves are prescribed as internal remedy for alleviating snake bite symptoms and used externally to treat splenomegaly, headaches, cough, bronchitis, rheumatic pain and ulcers. The fruits are used for de-worming, as appetite enhancers and expectorants, as well as for treating throat and cardiac diseases, haemorrhoids, bronchitis and leucoderma. The root bark is used in formulations against ailments such as fevers, bronchitis, intestinal worm trouble, vomiting, dysentery, leucoderma, asthma, inflammation, diarrhoea, dysentery, rheumatism etc.

1.3.3.2. Bioactive properties

Extracts and isolates from various parts of the tree, viz., leaves, stem bark, root bark and fruits are reported to possess significant biological properties in *in vitro* and *in vivo* biological systems. The aqueous extract of leaves showed potent anti-inflammatory activity in rats with carageenan induced paw edema (Upaganlawar *et al.*, 2009). Methanolic extract of the leaves of *O. indicum* Vent. is reported to possess hepatoprotective (Tenpe *et al.*, 2009), photocytotoxic (Ong *et al.*, 2009) as well as mice RBC membrane stabilizing effects (Chakma *et al.*, 2013).

Tree barks are repositories of endless bioactive substances. *In vitro* anti-arthritic activity in the presence of aqueous and ethanol extract of stem bark has been demonstrated in rat peritoneal leukocytes as indicated by release of myeloperoxidase enzyme (Laupattarakasem *et al.*, 2003). With reference to the bioactive properties of stem bark, there are reports of apoptosis-mediated cytotoxicity of petroleum benzene extract against HeLa cells (Moirangthem *et al.*, 2013), hepatoprotective effect of petroleum ether, chloroform, methanolic and aqueous extracts in CCl_4 induced rat models (Tripathy *et al.*, 2011), as well as membrane stabilizing effects of methanolic extract in mice erythrocytes (Chakma *et al.*, 2013).

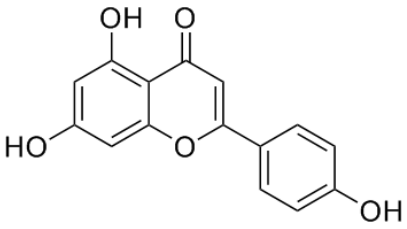
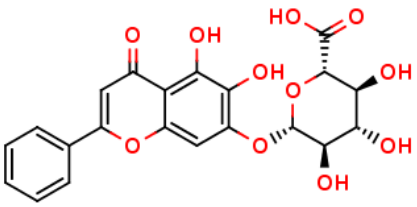
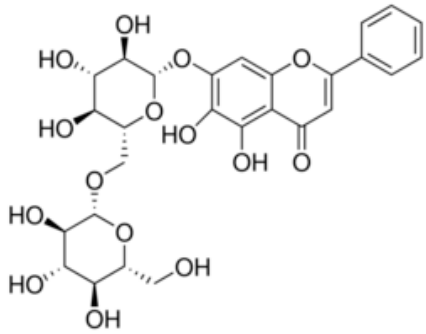
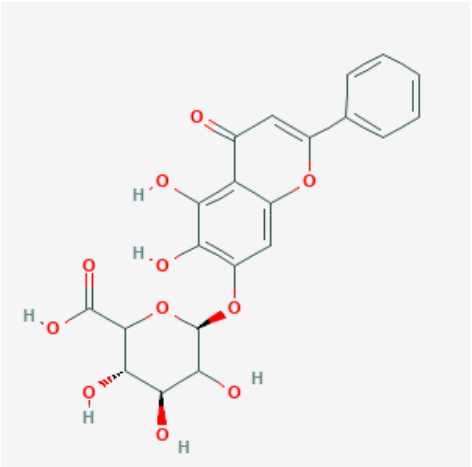
The roots of *O. indicum* Vent. are inevitable ingredients of Dasamoola preparations in Ayurvedic system of medicine. Studies showed that the petroleum ether, chloroform, ethyl acetate and n-butanol fractions of 50% alcoholic extract of root bark is gastroprotective against ethanol induced gastric mucosal damage (Khandhar *et al.*, 2006). Immunomodulatory (Zaveri *et al.*, 2006), *in vitro* cytotoxic (Dhru *et al.*, 2016) and hepatoprotective (Sastry *et al.*, 2011) properties have also been confirmed respectively in the n-butanol fraction, methanol extract and aqueous extract of *O. indicum* root bark.

Methanolic extract of the fruit of this plant was found to be anti-mutagenic against Trp-P-1 in an Ames test. The major anti mutagenic constituent was identified as baicalein which acted as a desmutagen by inhibiting the N-hydroxylation of Trp-P-2 (Nakahara *et al.*, 2001).

1.3.3. Phytochemistry

A flavone glucuronide named oroxindin has been isolated from the seeds of *Oroxylum indicum* Vent., extracted using ethanol by Nair and Joshi (1979). Chen *et al.* (2002) isolated five flavonoids from the seeds of *Oroxylum indicum* by high speed counter current chromatography and identified four of them as baicalein-7-O-glucoside, baicalein-7-O-diglucoside, baicalein and chrysin, using high-performance liquid chromatography–mass spectrometry and nuclear magnetic resonance. LC, MS and NMR have revealed the presence of chrysin, baicalein, baicalein-7-O-glucoside, baicalein-7-O-diglucoside, chrysin-7- O-glucuronide, baicalein-7-O-glucuronide, and a chrysin-diglucoside from the leaves of *O. indicum* Vent. methanol extract (Yuan *et al.*, 2008). The roots of this plant are reported to contain chrysin, baicalein, biochanin A and ellagic acid (Zaveri *et al.*, 2008). The methanolic extract of the fruits contain the flavonoid, baicalein (Roy *et al.*, 2007). Maungjunburee and Mahabusarakam (2007) isolated six flavonoids from the stem bark of *O. indicum* Vent., namely chrysin, 5,7-dihydroxy-3-methoxyflavone, 3,5,7- trihydroxyflavone, 5,7,4'-trihydroxy-3-methoxyflavone, 3,5,7,4'-tetrahydroxy flavone and 5,7,4-trihydroxyflavone. Table 1.4 shows the molecular structures and molecular weights of of various compounds isolated from *O. indicum* Vent.

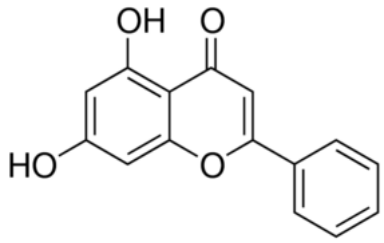
Table 1.4. Compounds isolated from *Oroxylum indicum* Vent. with molecular formula and molar mass

Baicalein $C_{15}H_{10}O_5$ Molar mass: 270.24 g·mol^{-1}	
Baicalein-7-O-glucoside $C_{21}H_{20}O_{10}$ Molar mass: 432.38 g·mol^{-1}	
Baicalein-7-O-diglucoside $C_{27}H_{30}O_{15}$ Molar mass: 594.52 g·mol^{-1}	
Baicalein-7-O-glucuronide $C_{21}H_{18}O_{11}$ Molar mass: 446.36 g·mol^{-1}	

Chrysin

$C_{15}H_{10}O_4$

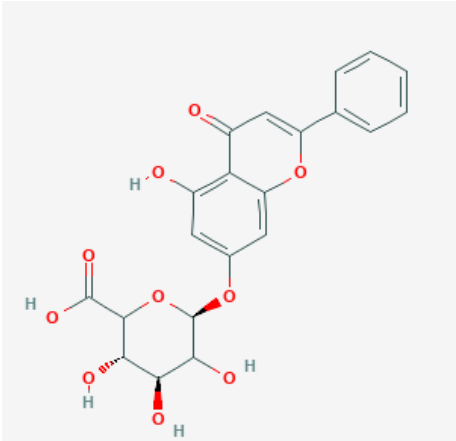
Molar mass: 254.241g·mol^{-1}



Chrysin-7-O-glucuronide

$C_{23}H_{22}O_{13}$

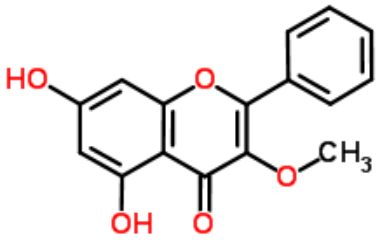
Molar mass: 254.241g·mol^{-1}



5,7- dihydroxy-3-methoxyflavone

$C_{16}H_{12}O_5$

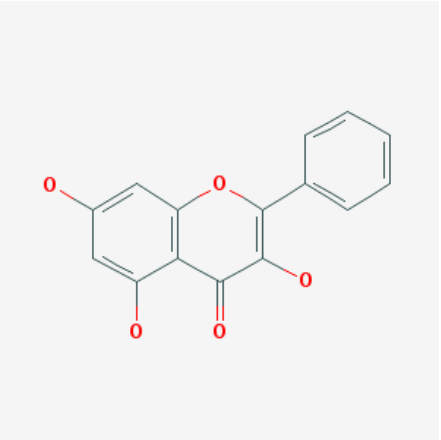
Molar mass: 284.26 g·mol^{-1}



3,5,7- trihydroxy flavone

$C_{15}H_{10}O_5$

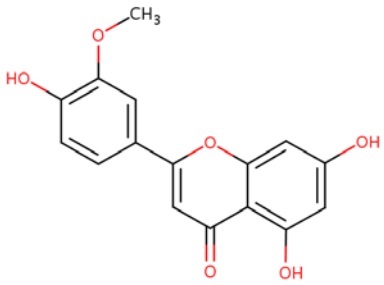
Molar mass: 270.24 g·mol^{-1}



5,7,4’- trihydroxy-3-methoxyflavone

$C_{16}H_{12}O_6$

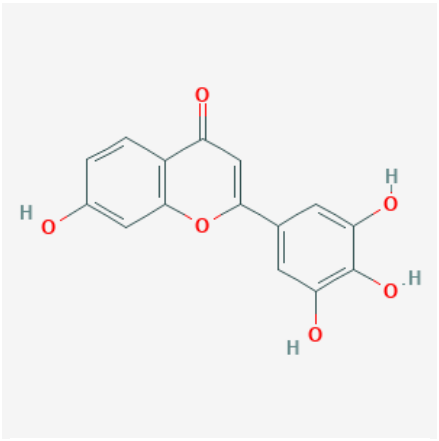
Molar mass: 300.26 g·mol^{-1}



3,5,7,4’- tetrahydroxy flavone

$C_{15}H_{10}O_6$

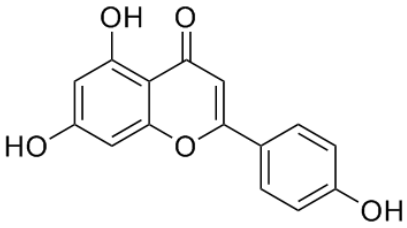
Molar mass: 286.24 g·mol^{-1}



5,7,4- trihydroxy flavone

$C_{15}H_{10}O_5$

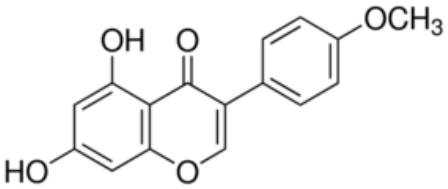
Molar mass: 270.24 g·mol^{-1}



Biochanin A

$C_{16}H_{12}O_5$

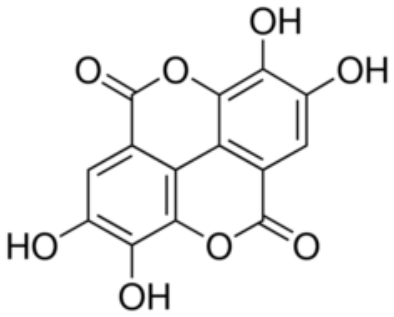
Molar mass: 284.26 g·mol^{-1}



Ellagic acid

$C_{14}H_6O_8$

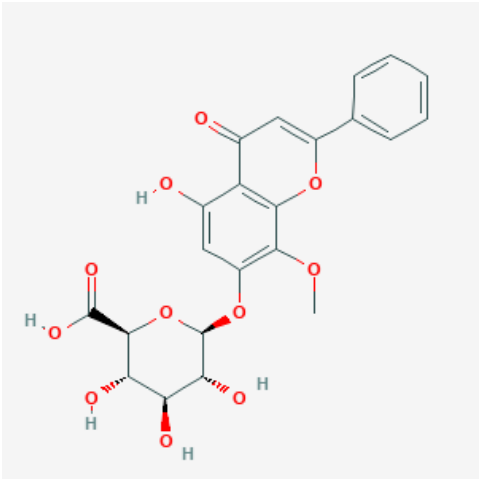
Molar mass: 302.20 g·mol^{-1}



Oroxindin

$C_{22}H_{20}O_{11}$

Molar mass: 460.39 g·mol^{-1}



1.3.4. Biodiversity status

Oroxylum indicum is a valuable medicinal tree appreciated for the pharmacological utility of all its parts, including leaves, stem, stem bark, roots, root bark, fruits and seeds. This had eventually led to the indiscriminate collection, over exploitation and uprooting of whole plants bearing roots. This valuable tree has been pushed to the vulnerable list in In Karnataka and Andhra Pradesh, the tree is listed as vulnerable, and in Kerala, Maharashtra, M.P. and Chhattisgarh, as endangered (Ravikumar and Ved, 2000). Owing to habitat destruction, engagement of destructive and/or non-sustainable collection methods and low regenerative ability, serious threat remains against the existence and availability of this highly useful tree (Yasodha *et al.*, 2004). Moreover, considerable rates of seed abortion of *O. indicum* (Gunaga *et al.*, 2012) as well as implementation of non-replenishable collection methods

(Mishra, 2011) add to its low abundance. So, there is an urgent need of its protection and regeneration through micropropagation.

1.3.5. Micropropagation

Tissue culture technology has opened extensive areas of research for biodiversity conservation. Standardised protocols of micropropagation have been developed for many endangered, rare and threatened medicinal plant species; e.g., *Picorrhiza kurroa, Tinospora cordifolia, Salaca oblonga, Glycyrrhiza glabra, Bacopa mooniera, Rauwolfia serpentina Aloe vera, Ocimum sanctum, Papaver somniferum* etc (Sharma *et al*., 2010; Verma *et al*., 2012).

During micropropagation, selection of the medium and the presence of growth regulators are critical in callusing, shoot and shoot development. The effects of plant growth regulators- auxins and cytokinins - on shoot and root multiplication in medicinal plants have been reported by Skirvin *et al.* (1990). The commonly used synthetic auxins are indole acetic acid, 1-naphthalene acetic acid and indole butyric acid. Kinetin and 6-benzylaminopurine are the commonly used synthetic cytokinins. Auxins, cytokinins and auxin-cytokinin interactions influence cell growth, cell division, tissue differentiation and organogenesis in plants (Evans *et al.*, 1981). Cytokinins stimulate cell division, release lateral bud dormancy and promote adventitious lateral bud formation. Auxins initiate cell division, promote root development and organisation of meristems to calluses (Aloni *et al.*, 2006). But, when used in combination, the requisite for each plant varies.

Development of a standard protocol is essential for successful regeneration of plant species through tissue culture techniques. Selection of the suitable explants, suitable medium and ideal supplements (plant growth regulators) is different for different plants. On realising the rarity and importance of *O. indicum* Vent., previous attempts have been made on establishing a suitable tissue culture system. Gokhale and Bansal (2009) had established a tissue

culture protocol for *O. indicum* Vent. using axillary and apical bud explants in the presence of growth regulators, benzylaminopurine (BAP) and kinetin in the medium. Rajurkar *et al.* (2011) has accomplished in *vitro* shoot induction and callus induction of *this plant* using apical and axillary bud and leaf midrib explants, with the growth regulators BAP for multiple shoot induction and 2, 4-D for root induction. In both the studies, Murashige and Skoog medium was used for propagation, and the results were indicative of the positive response of this tree on tissue culture endeavors, despite being a tree species. Hence, in the present study, following the above protocols with slight modification, micropropagation of the tree has been attempted. Though the goal of micropropagation is achieved, there is a need to ensure the quality of the tissue cultured counterparts with that of the wild, and hence, the study focuses on a comparison of selected biological activities, with emphasis to its anti cancer-related properties.

Plant secondary metabolites are considered as non-cytotoxic pharmacological agents to combat different types of cancer, established through prior works on the cancer chemopreventive effects of extracts in laboratory animal models (Kinghorn, 2000). Secondary metabolites are produced in plants as defense responses to the invasion of pathogens, pests and predators. They rarely take part in the primary metabolism of the source plant. They act as deterrents, owing to the unpalatable or toxic effects. There are pharmaceutically important secondary metabolites such as alkaloids, glycosides, flavonoids, volatile oils, tannins, resins etc. Many secondary metabolite classes are induced by infection, wounding or herbivory (Bennett and Wallsgrove, 1994). This basic principle can be applied to enhance the production of useful and pharmacologically important secondary metabolites in plants, by exposing them deliberately to conditions that mimic stress. This technique is termed as elicitation.

1.3.6. Elicitation techniques to enhance secondary metabolite production

Several plants known to contain valuable compounds are uncultivable or facing threats of disappearance due to overexploitation. Owing to structural complexity, there is a problem of low economic feasibility in their chemical synthesis. The application of biotechnological principles for secondary metabolite production in plant cell or organ cultures is a practical and feasible alternative to the extraction of whole plant. Though cell cultures can be established from plants, there is less assurance of obtaining sufficient amounts of the required secondary metabolites. At this context, it is noteworthy to mention the use of different types of elicitors in tissue cultures to increase secondary metabolite turnover (Namdeo, 2007).

Elicitors can be classified as abiotic or biotic, depending upon the source. Abiotic elicitors are categorized into physical, chemical, and hormonal elicitors. Light, osmotic stress, radiation, salinity, pH change, thermal stress etc are physical elicitors. Light is shown to stimulate gingerol and zingiberene production in *Zingiber officinale* callus cultures. (Anasori and Asghari, 2009). Proline, polyethylene glycol, sucrose etc are used as elicitors inducing osmotic stress. Exposure to salinity induces the production of phenols, terpenes, and alkaloids in plants (Naik and Al–Khayri, 2016). The production of stilbene from *Vitis vinifera* on UV exposure (Xu *et al.,* 2015) and that of Bacoside A from *Bacopa monnieri* using pH changes (Naik *et al.,* 2010) are previous examples of abiotic elicitation in plants. The use of metals such as Nickel, Silver, Iron and Cobalt are reportedly successful chemical elicitors (Zhao *et al.*, 2001). The most extensively used abiotic elicitors are the derivatives of jasmonic acid (JA) and salicylic acid (SA), since they play key roles in the plant defense response. JA is produced by plants in response to wound and pathogen infestation (Wasternack and Parthier, 1997). Along with its active derivative named methyl jasmonate (MeJA), it has been reported to enhance plant secondary metabolites such as rosmarinic acid in *Mentha piperita* (Krzyzanowska *et al.,* 2012), anthocyanin in *V. vinifera* (Curtin *et al.*, 2003), stilbene in *V. vinifera* cell cultures (Taurino *et al.,* 2015), as well as

withanolide A, withanone and withaferin A (Sivanandhan *et al.*, 2013). Besides this, the production of stilbene in the cell suspension of *V. vinifera* has also been elicited with the use of salicylic acid (Xu *et al.*, 2015).

Biotic elicitors include polysaccharides and other molecules of yeast, bacterial or fungal origin. For example, the use of the polysaccharide agropectin in cultured cells of *Lithospermum erythrorhizon* elicited the synthesis of the naphthoquinone shikonin (Fukui *et al.*, 1983) and similar effect was obtained by using the cell wall–derived elicitor oligogalacturonic acid in *Panax ginseng* cell suspension, to increase the saponin content (Hu *et al.*, 2003). The use of yeast extracts in enhancing ethylene biosynthesis in tomato (Felix *et al.*, 1991) and tanshinone in the root culture of *Perovskia abrotanoides* are examples of yeast mediated elicitation (Arehzoo *et al.*, 2015). The production of many phenylpropanoids or flavonoids has been reportedly enhanced by the use of pathogenic and nonpathogenic fungal preparations (Lattanzio *et al.*, Dixon *et al.*, 2002). An example for a bacteria-derived elicitors are coronatine (a phytotoxin from *Pseudomonas syringae)* successfully used for eliciting taxane synthesis in taxane media cell cultures (Onrubia *et al.*, 2013) and viniferins synthesis in the cell culture of *V. vinifera* (Taurino *et al.*, 2015).

Elicitation techniques can be attempted on medicinal plants to improve their pharmacological qualities. The present study, besides initiating calluses, shoots and roots of the rare medicinal tree, *O. indicum* Vent., also focuses on the use of elicitors **to** enhance the phytochemical profile of the tissue culture derivatives, as will be discussed in the forthcoming chapters.

Chapter 2

Materials and Methods

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2.1.1.2. Plant collection

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2.1.4. Instruments

2.1.5. Reagents and stains

2.1.6. Animals

2.1.7. Cell lines

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2.1. Materials

2.1.1. Plant material

2.1.1.1. Botanical classification

(As per section 1.3.1.1., Chapter 1)

2.1.1.2. Plant collection

The roots of *O.indicum* Vent. were collected in the month of August from the Ayurvedic Garden belonging to Amala Cancer Research Centre, Thrissur, Kerala, India. The plant was authenticated by Dr. P. Sujanapal, Scientist- B, Silviculture Department, KFRI, Peechi, Thrissur 680 653, Kerala (Voucher specimen No: KFRI/SILVA/GEN/06/11).

2.1.2. Chemicals

The chemicals used in the study, and the suppliers/providers is enlisted in table 2.1.

All other chemicals used for the study were of analytical grade.

2.1.3. Diagnostic kits

The diagnostic kits used in the study, and the suppliers/providers are enlisted in table 2.2.

2.1.4. Instruments

The procurement of instruments used in the study is enlisted in table 2.3.

Table 2.1. List of chemicals used

Sl No:	Chemical	Supplier
1.	Apigenin	Sigma-Aldrich Inc., St Louis, USA
2.	2,2‘- Azobis-2-amidinopropane) dihydrochloride (AAPH)	E-Merck India Pvt LtD, Mumbai, India
3.	2,2-Azobiz-3-ethylbenzthiazoline-6-sulfonic acid (ABTS)	Sigma-Aldrich Inc., St Louis, USA
4.	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
5.	Sodium dihydrogen phosphate	E-Merck India Pvt LtD, Mumbai, India
6.	5-5‘Dithiobis (2-nitrobenzoic acid) (DTNB)	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
7.	7,12- Dimethyl benz[a]anthracene (DMBA)	Sigma-Aldrich Inc., St Louis, USA
8.	Acetic acid	E-Merck India Pvt LtD, Mumbai, India
9.	Acetone	E-Merck India Pvt LtD, Mumbai, India
10.	Acetonitrile (HPLC grade)	E-Merck India Pvt LtD, Mumbai, India
11.	Acridine orange	E-Merck India Pvt LtD, Mumbai, India
12.	Agarose	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
13.	Arachidonic acid	Sigma-Aldrich Inc., St Louis, USA
14.	Ascorbic acid	E-Merck India Pvt LtD, Mumbai, India
15.	Baicalein standard (98%-	Sigma-Aldrich Inc., St Louis, USA

	HPLC grade)	
16.	Benzyl adenine (BA)	Sigma-Aldrich Inc., St Louis, USA
17.	Bovine serum albumin (BSA)	E-Merck India Pvt LtD, Mumbai, India
18.	Bromophenol blue	Sigma-Aldrich Inc., St Louis, USA
19.	Butanol	E-Merck India Pvt LtD, Mumbai, India
20.	Carboxymethyl cellulose	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
21.	Carrageenan	Sigma-Aldrich Inc., St Louis, USA
22.	Cetyl trimethylammonium bromide (CTAB)	E-Merck India Pvt LtD, Mumbai, India
23.	Chloroform	E-Merck India Pvt LtD, Mumbai, India
24.	Chrysin standard (≥98%-HPLC grade)	Sigma-Aldrich Inc., St Louis, USA
25.	Cisplatin	Sigma-Aldrich Inc., St Louis, USA
26.	Colchicine	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
27.	Croton oil	Sigma-Aldrich Inc., St Louis, USA
28.	Cyclophosphamide (i.p.) (Cytoxan)	Sigma-Aldrich Inc., St Louis, USA
29.	Deoxy ribose	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
30.	Dextran	Himedia Laboratories Pvt. Ltd, Mumbai, India
31.	Di Sodium hydrogen phosphate	Sigma-Aldrich Inc., St Louis, USA
32.	Dimethylbenz(a)anthracene (DMBA)	Sigma-Aldrich Inc., St Louis, USA
33.	Dimethyl sulfoxide (DMSO)	E-Merck India Pvt LtD, Mumbai, India

34.	Doxorubicin hydrochloride	Sigma-Aldrich Inc., St Louis, USA
35.	Dulbecco‘s modified eagle medium (DMEM)	Gibco, Thermo Fisher Scientific, USA
36.	Ethidium bromide	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
37.	Ethylene diamine tetra acetic acid (EDTA)	E-Merck India Pvt LtD, Mumbai, India
38.	Ferrric chloride (FeCl3)	E-Merck India Pvt LtD, Mumbai, India
39.	Fetal bovine serum (FBS)	Gibco, Thermo Fisher Scientific, USA, Thermo Fisher Scientific, USA, Thermo Fisher Scientific, USA
40.	Folin‘s reagent	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
41.	Formaldehyde	E-Merck India Pvt LtD, Mumbai, India
42.	Giemsa	E-Merck India Pvt LtD, Mumbai, India
43.	Glutathione reduced (GSH)	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
44.	Hemoglobin (Hb)	Sigma-Aldrich Inc., St Louis, USA
45.	HEPES buffer	Himedia Laboratories Pvt. Ltd, Mumbai, India
46.	High melting point agarose	Sigma-Aldrich Inc., St Louis, USA
47.	Hydrogen peroxide	E-Merck India Pvt LtD, Mumbai, India
48.	Indole –3 Acetic Acid (IAA)	Sigma-Aldrich Inc., St Louis, USA
49.	Indole 3-butyric acid (IBA)	Sigma-Aldrich Inc., St Louis, USA
50.	Isoamyl alcohol	E-Merck India Pvt LtD, Mumbai, India
51.	Isopropanol	E-Merck India Pvt LtD, Mumbai, India
52.	L-Glutamine	Himedia Laboratories Pvt. Ltd, Mumbai, India
53.	Lipopolysaccharide (LPS)	Sigma-Aldrich Inc., St Louis, USA

54.	Low melting point agarose	Sigma-Aldrich Inc., St Louis, USA
55.	Luteolin	Sigma-Aldrich Inc., St Louis, USA
56.	Mercuric chloride	E-Merck India Pvt LtD, Mumbai, India
57.	Methanol (HPLC grade)	E-Merck India Pvt LtD, Mumbai, India
58.	Methyl Jasmonate (MeJa)	Sigma-Aldrich Inc., St Louis, USA
59.	Murashige and Skoog Medium	Himedia Laboratories Pvt. Ltd, Mumbai, India
60.	Naphthalene acetic acid (NAA)	Sigma-Aldrich Inc., St Louis, USA
61.	Nitrobluetetrazolium (NBT)	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
62.	Penicillin	Himedia Laboratories Pvt. Ltd, Mumbai, India
63.	Phenol	E-Merck India Pvt LtD, Mumbai, India
64.	Potassium dihydrogen phosphate	E-Merck India Pvt LtD, Mumbai, India
65.	Potassium hydroxide (KOH)	E-Merck India Pvt LtD, Mumbai, India
66.	Propidium iodide	Sigma-Aldrich Inc., St Louis, USA
67.	Pyridine	E-Merck India Pvt LtD, Mumbai, India
68.	Riboflavin	Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.
69.	RNase A	Bangalore Genei Pvt LtD, Bangalore, India
70.	Rosewell park memorial institute medium (RPMI)	Gibco, Thermo Fisher Scientific, USA
71.	Sodium dodecyl sulfate	E-Merck India Pvt LtD, Mumbai, India
72.	Sodium flouride	E-Merck India Pvt LtD, Mumbai, India
73.	Streptomycin	Himedia Laboratories Pvt. Ltd, Mumbai, India

74.	Thiobarbituric acid (TBA)	Himedia Laboratories Pvt. Ltd, Mumbai, India
75.	Tris Buffer	E-Merck India Pvt LtD, Mumbai, India
76.	Triton X-100	E-Merck India Pvt LtD, Mumbai, India
77.	TRIzol reagent	Life technologies, UK
78.	Trypsin-EDTA	Himedia Laboratories Pvt. Ltd, Mumbai, India
79.	Tween 20	E-Merck India Pvt LtD, Mumbai, India
80.	Water (HPLC grade)	E-Merck India Pvt LtD, Mumbai, India

Table 2.2. List of diagnostic kits used

Sl No:	**Diagnostic kit**	**Supplier**
1.	Alkaline phosphatase (ALP)	Span Diagnostics Ltd., Gujarat.
2.	Creatine phosphokinase (CPK)	Agappe Diagnostics Ltd., Ernakulam
3.	Creatinine	Euro Diagnostic Systems Pvt. Ltd., Chennai.
4.	Glutamate oxaloacetate transaminase (GOT)	Span Diagnostics Ltd., Gujarat.
5.	Glutamate pyruvate transaminase (GPT)	Span Diagnostics Ltd., Gujarat.
6.	Hemoglobin (Hb)	Agappe Diagnostics Ltd., Ernakulam
7.	Lactate dehydrogenase (LDH)	Agappe Diagnostics Ltd., Ernakulam
8.	Total protein	Euro Diagnostic Systems Pvt. Ltd., Chennai.
9.	Urea	Euro Diagnostic Systems Pvt. Ltd., Chennai.

Table 2.3. List of instruments used

Sl No:	Instrument	Provider
1.	Deep freezer (-20ºC)	Remi Laboratory Instruments, Mumbai, India
2.	Deep freezer (-70ºC)	Eppendorf, Germany
3.	Electronic weighing balance	Contech Instruments Ltd, India
4.	Flow Cytometer	Beckman Coulter, U.S.A
5.	Fluorescent microscope	Leica, German Radicle, Ambala
6.	High speed cooling centrifuge	Remi Laboratory Instruments, Mumbai, India
7.	Horizontal Laminar flow hood	Cleanair, Chennai, india
8.	Hot air oven	Rotex Instruments Pvt. Ltd., India
9.	HPLC	Shimadzu Corporation, Japan
10.	HPTLC	CAMAG system, Switzerland
11.	Incubator	Rotex Instruments Pvt. Ltd., India
12.	Microcentrifuge	Tarsons Products Pvt. Ltd., Kolkata
13.	pH meter	Elico Limited, Hyderabad, India
14.	Phase contrast microscope	Magnus, INVI, New Delhi, India
15.	Upright research microscope	Meiji, Japan; Labex, Labovision
16.	UV/Visible spectrophotometer	PG instruments, UK
17.	60Co-Theratron-Phoneix teletherapy unit	Atomic Energy Ltd, Ottawa, Canada

2.1.5. Reagents and stains

a) Phosphate buffered saline (PBS)

NaCl - 8.00g

KCl - 0.20g

KH_2PO_4 - 0.20g

Na_2HPO_4. $2H_2O$ - 1.44g

The contents were dissolved in distilled water and made up to a final volume of 1000 ml with pH 7.2 and sterilized by autoclaving at 15 lbs for15 min.

b) Dragendorff‘s reagent (Potassium bismuth iodide solution)

Bismuth carbonate - 5.2 g

Sodium iodide - 4 g

Glacial acetic acid - 25 ml

The contents were mixed well, boiled for 3 minutes and kept at room temperature for 12 hrs, leading to the precipitation of sodium acetate crystals. The precipitate was filtered out and the resulting clear solution was mixed with 80 ml of ethyl acetate and 500 ml of distilled water. The stock solution, thus prepared was stored in a dark bottle.

10 ml of stock solution was mixed with 20 ml acetic acid and made up to a volume of 100 ml with distilled water to get the working solution.

c) Acridine orange/Ethidium bromide (Ao/Eb) staining solution

Acridine orange (Ao) - 5 mg/ml ethanol

Ethidium bromide (Eb) - 3 mg/ml ethanol

1 µl of Ao and 1 µl of Eb (both in 1 ml of phosphate buffered saline (PBS) were mixed to prepare a working solution of the stain. The stain is subjected to maturation for 2 weeks and used for the observation of apoptotic morphology in cells.

d) Sorensen‘s buffer (pH 6.8)

Na2HPO4 - 0.42g

KH2PO4 - 0.40g

The contents were dissolved in distilled water, making up a final volume of 100 ml.

e) Turk‘s fluid

Glacial acetic acid - 2 ml

1% crystal violet - 1 ml

The volume was made up to 100 ml with distilled water. The solution was stirred overnight, filtered and used for total WBC count analysis.

2.1.6. Animals

BALB/c mice (25 - 30 g) were purchased from the Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. The animals were housed at Amala Cancer Research Centre, Thrissur, Kerala in polypropylene cages with paddy husk as the bedding and stainless steel top grill with facilities for providing food and water. Standardized environmental conditions with temperature: 22 - 30ºC, relative humidity: 60 - 70% and 12 hrs of dark/light cycle, along with free access to standard rat feed (Lipton, India) and water *ad libitum* were maintained. All animal experiments conducted during the present study were subject to prior sanction from Institutional Animal Ethical Committee (as per approval No: ACRC/IAEC/15/02-(1) following the internationally accepted laboratory animal use and care guidelines and rules of CPCSEA (Approval no. of institution – 149/PO/Rc/S/99/ CPCSEA).

2.1.7. Cell lines

Dalton‘s Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells were procured from Adayar Cancer Institute, Chennai. The cells were cultured as transplantable tumors in the peritoneal cavity of BALB/c mice and

were maintained by weekly intra peritoneal inoculation. The cell lines namely, Vero (African green monkey kidney cells), HeLa (human cervical cancer cells), MDA - MB - 231 (human breast cancer cells), HCT 15 (human colon cancer cells) and Hep G2 (Human hepatoma cells), were procured from National Centre for Cell Science (NCCS), Pune and were cultured in DMEM as per the instructions of the supplier.

2.2. Methods

2.2.1. Extraction of plant material

The peeled bark was dried at 45-50^{o}C for a week in hot air oven, powdered and subjected to extraction using petroleum benzene, chloroform, ethyl acetate, methanol and water in Soxhlet apparatus. Further, the bark powder was subjected to crude extraction using 3 different solvents- 30% methanol, 50% methanol and 70% methanol - in a magnetic stirrer. The extracts were filtered and evaporated to get a dry residue, ensuring complete evaporation of any traces of solvent. A yield of 8.98%, 10.02% and 9.01% of the extract was obtained from extraction using 30% methanol, 50% methanol and 70% methanol respectively. This was dissolved in DMSO (50 mg of the extract in 1 ml DMSO) and used as stock solution. For *in vitro* cytotoxicity and antioxidant screening assays, the stock solution was appropriately diluted, ensuring that the concentration of DMSO in the final volume does not exceed 0.1%.

The 70% hydro methanolic extract (OIM) was re-dissolved in DMSO and distilled water respectively for further *in vitro* and *in vivo* studies.

2.2.2. Phytochemical analysis

2.2.2.1. Preliminary phytochemical study

Qualitative analysis of phytochemicals present in the extracts was done according to the standard procedures (Harborne, 1984, Sofowora, 1993, Trease and Evans, 2002).

2.2.2.1.1. Test for terpenoids

Salkowski test: Dry extract of weight, 1 g, was thoroughly mixed in vortex with 2 ml of chloroform. A volume of 2 ml of concentrated H_2SO_4 was then added along the sides of the test tube. The presence of terpenoids is indicated by the formation of a yellow ring at the interface between the two liquids.

2.2.2.1.2. Test for alkaloids

Dry extract weighing 0.5 g was mixed in vortex for 5 min with a volume of 8 ml HCl (1%) and filtered using Whatman no.1 filter paper. The filtrate was used for analyzing the presence of alkaloids.

Dragendorff‘s test: A volume of 1-2 ml of Dragendorff‘s reagent was added to 2 ml of this filtrate. The formation of yellow to orange precipitate indicates the presence of alkaloids.

2.2.2.1.3. Test for phenolic compounds

Ferric chloride test: Dry extract weighing 0.5 g was mixed with 5 ml of distilled water, followed by the addition of a few drops of 5% ferric chloride. The appearance of dark green color indicates the presence of phenolic compounds.

2.2.2.1.4. Test for tannins

Lead acetate test: Dry extract weighing 0.5 g was mixed with 5 ml of distilled water. A volume of 3 ml of the 10% lead acetate solution was added to this solution. The presence of tannins is indicated by the formation of bulky white precipitate.

2.2.2.1.5. Test for flavonoids

Dry extract weighing 0.5 g was mixed with 5 ml of distilled water. A volume of 5 ml of dilute ammonia solution was added to a small volume of the above mixture. Then, a few drops of concentrated H_2SO_4 was slowly added along the

sides of the test tube. The formation of yellow color indicates the presence of flavonoids.

2.2.2.1.6. Test for phytosterols

Libermann and Burchards test: Dry extract weighing 0.5 g was mixed with 2 ml of acetic anhydride. A few drops of concentrated H_2SO_4 were added along the sides of the test tube. The presence of violet to blue color indicates the presence of phytosterols.

2.2.2.1.7. Test for steroids

Dry extract weighing 0.5 g was mixed with 2 ml of chloroform. To this solution, a few drops of concentrated H_2SO_4 were added along the sides of the test tube. The formation of the brown ring indicates the presence of steroids.

2.2.2.1.8. Test for saponins

Foam test: Dry extract weighing 0.5 g was mixed with 5 ml of distilled water made up to a final volume of 20 ml. The suspension was shaken vigorously in a graduated cylinder for 15 min. Formation of 2 cm layers of foam that persist for 5 min indicate the presence of saponins.

2.2.2.1.9. Test for carbohydrates

Benedict‘s test: Dry extract weighing 1 g was mixed with 1 ml of distilled water. To 0.5 ml of this solution, equal volume of Benedict‘s reagent was added and the mixture was heated for 2 min on a boiling water bath. Formation of red colored precipitate indicates the presence of carbohydrates.

2.2.2.2. Total phenolic content

The total phenolic content was determined by according to the method of Ainsworth and Gillespie (2007) with minor modifications. A volume of 0.5 ml of the extract was mixed with 2 ml of the Folin-Ciocalteu reagent (with 1:10 dilution in de-ionized water) and was neutralized with 4 ml of sodium

carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min with periodic shaking for color development. Development of blue colour was observed, which was spectrophotometrically determined at 765 nm. The results were expressed as milligram of gallic acid equivalents (GAE)/gram of dry extract.

2.2.2.3. Total flavonoid content

Flavonoid determination was carried out by aluminum chloride colorimetric method (Chang *et al.*, 2002). A volume of 0.5 ml of extract was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride and 0.1 ml of 1 M potassium acetate, with the volume adjusted to 5 ml with distilled water. The reaction mixture was incubated at room temperature for 30 min and the absorbance of the reaction mixture was read at 415 nm. The results were expressed as milligram of quercetin equivalents (QE)/gram of dry extract.

2.2.2.4. HPTLC analysis

The extract was re-dissolved in methanol (30 mg/ml) and subjected to qualitative High performance thin layer chromatography (HPTLC) on pre-coated TLC plates of silica gel 60 F_{254} (Merck, India) (Sharma *et al.*, 2014). 10µl of the sample was loaded on pre-coated TLC plates (20 × 20 cm) of silica gel 60 F_{254} (Merck, India) using CAMAG automatic spotting device. The solvent system used was toluene: ethyl acetate: formic acid (5:5:0.5). The developed plates were scanned at 366 and 254 nm with CAMAG TLC Scanner 3. Derivatization of the plate was performed by spraying with anisaldehyde – sulphuric acid reagent and heating for 10 min in a hot air oven at 115ºC. The data were analyzed using CAMAG WinCat software.

2.2.2.5. HPLC analysis

High Performance Liquid Chromatography (HPLC) analysis was performed on Shimadzu SPD-10AVP HPLC system equipped with multi solvent delivery system and a UV-VIS detector. The column was purchased from Merck,

Germany with specifications- Purospher star reverse phase-18, 5 µm in diameter, and 250 × 4.60 mm in dimensions. Chromatographic separation was attained with mobile phase- acetonitrile: water (45:55) in isocratic elution mode, at flow rate was 1 ml/min. The volume of sample injected was 20 µl. The absorbance was read at 254 nm.

2.2.2.6. UPLC-Q-TOF-MS analysis

Ultra high pressure liquid chromatographic (UPLC) analysis of the extract was performed using an Acquity UPLC H class (Waters) system equipped with an autosampler and a diode-array detector (DAD). The mobile phase (methanol and 0.1% aqueous formic acid) was allowed to percolate through a BEH C18 column (with specifications, 50 mm × 2.1 mm × 1.7 µm, purchased from Waters, USA) using gradient elution (0-5 min, 5% acetonitrile; 5-7 min, 95% methanol; 8-9 min 5% methanol) at a flow rate of 0.3 ml/min. Detection was achieved at a wavelength of 210 - 400 nm. The MS and MS/MS data was retrieved from Xevo G2 (Waters, USA) Quadrapole – Time-of-Flight (Q-TOF) system. Mass spectrometric operations were carried out under a capillary voltage of 2.5 kV (negative ionization mode) and cone voltage of 30 V. The source temperature and desolvation temperature were maintained at 135^{o}C and 35^{o}C, respectively. The cone gas flow and desolvation gas flow were 50 and 900 l/h, respectively. The results were compared to previous MS/MS fragmentation reports to identify the compounds.

2.2.3. Cytotoxicity analysis

2.2.3.1. Short term cytotoxicity

2.2.3.1.1. Maintenance and isolation of DLA and EAC cells

Dalton's lymphoma ascites tumor cells (DLA) and Ehrlich ascites carcinoma (EAC) cell lines initially procured from Adayar Cancer Institute, Chennai and maintained in Amala Cancer Research Centre were used for the study. The cells were cultured as transplantable tumors in the peritoneal cavity of BALB/c

mice and were maintained by weekly intra peritoneal inoculation. For cytotoxicity analysis, the cells were aspirated aseptically on day 15 of inoculation, washed with phosphate buffered saline (PBS- 0.2 M, pH 7.4) and centrifuged for 3 min at 1500 rpm to remove any trace of blood. The pellet was re-suspended in PBS and subjected to the above the procedure thrice, finally making a cell suspension of count, 10^7 cells/ml and used for *in vitro* studies (Gothoskar and Ranadive, 1971).

2.2.3.1.2. Isolation of spleen cells

Spleen cells were used to probe the toxicity of the extract towards normal cells. Spleen isolated from healthy BALB/c mice was gently smashed through a fine nylon mesh using the plunger end of the syringe, into a half filled petri-dish containing PBS. The suspended spleen cells were centrifuged at 1500 rpm for 3 min. The cell pellet was re-suspended in PBS to get a final count of 1×10^7 cells/ml and used for *in vitro* toxicity analysis.

2.2.3.1.3. Short term *in vitro* cytotoxicity assay using trypan blue dye exclusion method

The underlying principle is the selective exclusion of trypan blue dye by viable (live) cells due to the presence of intact plasma membrane, while the permeable non-viable (dead) cells appear blue (Moldeus *et al.*, 1978). For the assay, 1×10^6 cells (DLA, EAC or spleen cells) and the extracts at various concentrations were made up into a final volume of 1 ml PBS and incubated for 3 hrs at 37^0C. Untreated cells served as control. 100 µl of 1% trypan blue was added to the tubes. After 2 min, the live and dead cell numbers were microscopically counted to calculate the % cell death according to the formula:-

Percentage cell death = (No: of dead cells/ Total no: of dead and viable cells) × 100

2.2.3.2. Long term cytotoxicity

2.2.3.2.1. Maintenance of cell lines

HeLa, MDA-MB-231, Hep G2, HCT 15 and Vero cell lines were maintained with filter sterilized Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in 25 ml culture flasks under 37°C incubation with 5% CO_2. Aseptic conditions were maintained throughout the study. The culture flasks were monitored regularly for any contamination, color change of medium or morphological changes of cells, with renewal of the medium either periodically or on appearance of yellow coloration, whichever earlier. The cells were subcultured on attainment of 70-80% confluence. Prior to this, fresh media, PBS and trypsin were allowed to reach a temperature of 37°C. After aspirating out the spent medium, the cells were washed thrice with PBS to remove any trace of media. Trypsinisation for cell detachment was carried out at 37°C using 1 ml trypsin (0.25%)/ EDTA (0.001%) solution and monitored under inverted microscope. A volume of 1 ml of fresh medium was then added for recovering the effect of trypsin. The cell suspension was mixed gently and thoroughly using a sterile pipette to prevent any clumping of cells, and thereafter equally dispensed into 5 new culture flasks.

2.2.3.2.2. Long term *in vitro* cytotoxicity study using MTT assay

Long term cytotoxicity of the extract was determined by MTT assay (Mosmann, 1983). The assay reflects the number of live cells based on the metabolic activity of the cell. In live cells, the microsomal NAD(P)H-dependent cellular oxidoreductase enzyme reduces the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) to insoluble purple formazan crystals, which can be measured spectrophotometrically, on dissolution.

Cells were seeded in 12 well plates to an approximate count of 1×10^6 per ml and incubated at 37^0C to facilitate adherence. Following this, the cells were

incubated with different concentrations of the extract for 48 hrs. Untreated cells served as control. Then, MTT (dissolved in PBS) was added to each well making a final concentration of 0.5 mg/ml and incubation was continued for another 4 hrs. The insoluble formazan crystals formed in the assay system were dissolved in 1 ml of solubilizing solution (5 ml of 10% Triton X-100, 0.43 ml of 0.1 N HCl and 50 ml isopropanol) by repeated aspiration. The absorbance was read at 570 nm and the percentage of cell viability was calculated according to the formula:-

Percentage cell viability = (Absorbance of treated/Absorbance of control) × 100

2.2.4. Estimation of hematological parameters

2.2.4.1. Determination of hemoglobin (Hb) content

The hemoglobin content was estimated by the method of Drabkin and Austin (1935).

Principle:

Hemoglobin is converted to cyanmethemoglobin upon reaction with potassium ferricyanide and potassium cyanide present in the cyanmeth reagent. The absorbance of cyanmethemoglobin is directly proportional to the hemoglobin concentration in the blood.

Procedure:

20 µl of fresh heparinized blood was incubated with 5 ml of the cyanmeth reagent after mixing and incubated for 5 min at room temperature. The absorbance was read at 546 nm. The absorbance of the standard solution equivalent to 60 mg/dl hemoglobin was also determined.

The hemoglobin content in blood was calculated using the formula:-

Hemoglobin (g/dL) = (Absorbance of the sample/Absorbance of Standard) × 60 × 0.251

2.2.4.2. Determination of total WBC count

The total WBC count was estimated by the method of Chesbrough and Arthur (1972).

Principle

Acetic acid present in the Turk‘s fluid will lyse enucleated red blood cells sparing all the nucleated cells to remain intact and stained with crystal violet.

Procedure

A volume of 20 µl of heparinized blood was diluted with 380 µl of Turk‘s fluid. The blood mixture was dispensed into a Neubauer counting chamber. The number of white blood cells in the corner squares was counted and the total WBC count was determined by the formula:-

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

2.2.5. Estimation of serum markers

2.2.5.1. Estimation of glutamate oxaloacetate transaminase (GOT) / aspartate aminotransaminase (AST) activity

The GOT/ AST activity was estimated by the method of Reitman and Frankel (1957).

Principle:

Aspartate aminotransferase (AST), also known as Glutamate Oxaloacetate Transaminase (GOT) catalyses the transamination between L-aspartate and α-ketoglutarate resulting in the formation of oxaloacetate and L-glutamate. In the assay system, the unstable oxaloacetate will get coupled to 2,4-Dinitrophenyl hydrazine (2, 4-DNPH) color reagent forming equivalent hydrazone. The

absorbance of the resultant brown colored complex can be determined at 505 nm under alkaline conditions.

Procedure:

Four reaction systems - blank, standard, test (for each serum sample) and control (for each serum sample) were maintained. To each test tube, 250 µl of buffered aspartate - α- ketoglutarate substrate (pH 7.4) was added. A volume of 50 µl of serum and 50 µl of working pyruvate standard (8 mM) were added into the test and standard respectively, and mixed well. All the tubes were incubated at 37ºC for 60 min, followed by the addition of 250 µl of 2,4-DNPH color reagent. Then, 50 µl distilled water and 50 µl of each serum sample was added to the blank and the serum control, respectively and incubated at room temperature for 20 min. Then, 2.5 ml of NaOH solution (0.4 N) was added to all tubes, mixed thoroughly and the absorbance was spectrophotometrically determined against distilled water as blank at 505 nm within 15 min. The enzyme activity was calculated using the formula:-

AST (GOT) activity (IU/L) = [(Absorbance of Test - Absorbance of Control)/ (Absorbance of Standard - Absorbance of Blank)] × Conc. of Standard

2.2.5.2. Estimation of glutamate pyruvate transaminase (GPT) / alanine aminotransferase (ALT) activity

The GPT/ ALT activity was estimated by the method of Reitman and Frankel (1957).

Principle:

Alanine aminotransferase/glutamate pyruvate transaminase (GPT) catalyses the formation of pyruvate and L-glutamate by the transamination between L-alanine and α-ketoglutarate. In the assay system, the unstable pyruvate formed will get coupled with 2,4 – Dinitrophenyl hydrazine (2, 4-DNPH) color reagent to form a corresponding hydrazone, The absorbance of the resulting brown

colored complex can be spectrophotometrically determined at 505 nm under alkaline conditions.

Procedure:

Four reaction systems - blank, standard, test (for each serum sample) and control (for each serum sample) were maintained. To each test tube, 250 µl of buffered alanine - α- ketoglutarate substrate (pH 7.4) was added. A volume of 50 µl of serum and 50 µl of working pyruvate standard (8 mM) were dispensed into the test and standard respectively, and thoroughly mixed. All the tubes were incubated at 37ºC for 30 min, followed by the addition of 250 µl of 2,4-DNPH color reagent. Then, 50 µl of distilled water and 50 µl of each serum sample were added to the blank and the serum control, respectively. The mixture was incubated at room temperature for 20 min. Then, 2.5 ml of NaOH solution (0.4 N) was added to all test tubes, mixed properly and the absorbance was spectrophotometrically determined against distilled water in a spectrophotometer at 505 nm within 15 min. The enzyme activity was calculated using the formula:-

ALT (GPT) activity (IU/L) = [(Absorbance of Test - Absorbance of Control)/(Absorbance of Standard - Absorbance of Blank)] × Conc. of Standard

2.2.5.3. Estimation of alkaline phosphatase (ALP) activity

The ALP activity was estimated by the method of Kind and King (1954).

Principle:

Alkaline phosphatase in serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. In alkaline medium, the phenol thus formed reacts with 4-aminoantipyrine in the presence of the oxidizing agent potassium ferricyanide. The absorbance of the resulting orange-red colored complex can

be measured at 510 nm. The color intensity is proportional to the enzyme activity.

Procedure:

The working solution for the substrate was prepared by reconstituting buffered substrate provided in the kit using 2.2 ml of water. Four reaction systems - blank, standard, test (for each serum sample) and control (for each serum sample) were maintained. A volume of 0.5 ml of working buffered substrate and 1.5 ml of distilled water was dispensed to all the test tubes, mixed well and incubated at 37ºC for 3 min. An amount of 50 µl serum and 50 µl of phenol (standard) were dispensed into the test and standard respectively, mixed well and incubated for 15 min at 37ºC. Thereafter, 1 ml of chromogen reagent was added to all tubes. A volume of 0.05 ml of serum each was added to the corresponding control tubes and mixed well. The absorbance was read against distilled water in a spectrophotometer at 510 nm within 15 min. The enzyme activity was calculated using the formula:-

ALP activity (KA/dL) = [(Absorbance of Test-Absorbance of Control)/(Absorbance of Standard-Absorbance of Blank)] × 10

Applying multiplication factor of 7.1, the activity (KA/dL) is expressed in IU/L.

2.2.5.4. Estimation of lactate dehydrogenase (LDH) activity

The LDH activity was estimated by the method of (McQueen, 1975)

Principle:

Lactate dehydrogenase catalyzes the reduction of pyruvate with NADH to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance, which is proportional to the LDH activity in serum.

Pyruvate + NADH + H^+ → L-lactate + NAD^+

Procedure:

Working reagent was prepared by mixing reagent 1 [Tris buffer (pH 7.4, 80 mM/l), pyruvate (1.6 mM) and sodium chloride (200 mM)] with reagent 2 [NADH (240 μM)] in 4:1 ratio. A volume of 10 μl of serum was added to 1 ml of the working reagent, mixed well and incubated for 1 min at 37^0C. The change in absorbance was measured per minute for 3 min at 340 nm. The enzyme activity was calculated using the formula:-

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

2.2.5.5. Estimation of creatine phosphokinase (CPK) or creatine kinase (CK)

Serum creatinine kinase levels were estimated according to the method of Tietz (1982).

Principle:

Creatine phosphokinase enzyme catalyzes the transfer of a phosphate group from creatine phosphate to adenosine diphosphate (ADP) yielding adenosine triphosphate (ATP). ATP thus formed is measured from two coupled reactions catalyzed by hexokinase (HK) and glucose-6-phosphate dehydrogenase (G-6-PDH) yielding ß-Nicotinamide adenine dinucleotide (reduced form) (NADH) from ß-Nicotinamide-adenine dinucleotide (NAD). The system monitors the rate of change in absorbance at 340 nm over a fixed time interval. The rate of change in absorbance is directly proportional to the activity of CPK in the sample.

Procedure:

Working reagent is prepared by mixing reagent 1 [with (imidazole (pH 6.7, 125 mM/L), D-glucose (25 mM/L), N-acetyl-L-cysteine (25 mM/L), magnesium acetate (12.5 mM/L), NADP (2.4 mM/L), EDTA (2.0 mM/L) and hexokinase (> 6800 U/L)] and reagent 2 [with Creatine phosphate (250 mM/ L), ADP (15.2

mM/ L), AMP (25 mM/ L), diadenosine pentaphosphate (103 µM/ L) and G-6-PDH (> 8800 U/ L)] in 4:1 ratio. A volume of 40 µl of serum was the mixed with 1 ml of the working reagent and incubated for 1 min at 37^0C. The change in absorbance was measured per minute for 3 min at 340 nm. The enzyme activity was calculated using the formula:-

CPK activity (U/L) = (Δ OD / min) × 4127

Creatine phosphate + ADP → Creatine + ATP (1)

ATP + D-Glucose → G-6-P + ADP (2)

G-6-P + $NADP^+$ → D-Glucose-6-phosphate + NADPH + H^+ (3)

Reactions 1, 2 and 3 are catalyzed by CPK, HK and G-6-PDH respectively.

2.2.5.6. Estimation of creatinine

Serum creatinine levels were estimated according to Jaffe‘s kinetic method.

Principle:

Creatinine is the catabolic product of creatinine phosphate formed by muscle contraction, which undergoes renal excretion. Therefore, renal dysfunction can elevate serum creatinine titre and hence, it is a measure of renal function. In the reaction system, picric acid will generate sodium picrate in alkaline condition which in turn reacts with creatinine to form an orange colored complex.

Creatinine + Sodium picrate → Creatinine-picrate complex

Procedure:

Working reagent (WR) was prepared by mixing equal volumes of picric acid reagent and alkaline buffer. Two reaction systems - standard and test – are maintained. Into 1 ml of WR, 50 µl of test serum and 50 µl of creatinine standard (2 mg/dL) were added to the test and standard respectively and mixed.

Absorbance of standard and test was read at 520 nm after 30 sec (A_0) and 90 sec (A_1). ΔA for standard (S) and Test (T) was determined as:-

$\Delta As = A_{S1} - A_{S0}$, where A_{S1} and A_{S0} is the absorbance of standard after 90 sec 30 sec respectively

$\Delta A_T = A_{T1} - A_{T0,}$ where, A_{T1} and A_{T0} is the absorbance of test after 90 sec 30 sec respectively.

The amount of creatinine present in serum was calculated using the formula:-

Serum creatinine (mg/dL) = (ΔA of Test/ΔA of Standard) × 2

2.2.5.7. Estimation of serum urea

Serum urea levels were estimated according to Berthelot enzymatic method.

Principle:

Urea titre in serum will increase during kidney failure and hence, serum urea estimation is a renal function test. In the reaction system, urea is converted quantitatively by urease into ammonia and carbon dioxide. The ammonium ions react with hypochlorite and salicylate to give a green colored complex. The color is enhanced by sodium nitroprusside. The intensity of color produced is directly related to the urea concentration in serum and is measured spectrophotometrically at 578 nm.

Procedure:

Working reagent (WR) was prepared by the addition of 25 ml of distilled water to urease and sodium salicylate reagent (R1). 1 ml of WR was dispensed to all the tubes. A volume of 10 µl of test serum and 10 µl of urea standard (40 mg/dl) were added into the respective samples and standard respectively. Blank was prepared by adding 10 µl of distilled water to 1 ml of WR. All mixtures were incubated at 37^0C for 5 minutes, followed by the addition of 1 ml of alkaline hypochlorite reagent (R2). The tubes were re-incubated for 5 min at

37^0C, and absorbance was read at 578 nm. The amount of urea present in serum was calculated using the formula:-

Urea (mgdL) = (Absorbance of Test/Absorbance of Standard) × 40

2.2.6. Estimation of bone marrow cellularity

Bone marrow cells from both femurs of the animals were flushed out with PBS containing 10% FBS. The total live cell count was determined using a hemocytometer and expressed as 'n' ($\times 10^6$)/femur.

2.2.7. Histological examination

After the sacrifice of animals, respective organs were excised using sterilized scissors/surgical blade and washed with normal saline (0.9% NaCl) for removing any traces of blood. Neutral buffered formalin at 10% concentration was used as the fixative. Dehydration was achieved by transit of the tissue through increasing alcohol grades (from 0-100%) and was cleared in xylene. Dehydrated and cleared tissue was embedded in paraffin. Embedded tissue was subjected to microtomy to obtain sections of thickness varying from 3-4 μm. The sections were de-paraffinised in xylene, followed by re-hydration in decreasing alcohol grades (100-0%). The rehydrated sections were stained first with nuclear stain hematoxylin, washed, followed by staining with cytoplasmic stain eosin (double staining). Excess stains were removed by washing with water. The stained sections were then mounted using DPX. The slides were observed under light microscope and photographed.

2.2.8. Analysis of tissue antioxidant parameters

2.2.8.1. Preparation of tissue homogenate

The organs were collected during animal sacrifice and rinsed thoroughly in ice cold saline, gently blotted between the folds of a filter paper and weighed in an analytical balance. The tissue was homogenized in 0.1M tris buffer (pH 7) using a polytron homogenizer. The homogenate was directly used for the

determination of total protein and tissue lipid peroxidation (LPO). For the estimation of total protein, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione (GSH), the supernatant obtained by centrifugation of the homogenate was used. Centrifugation was carried out for 60 min at 10,000 rpm in a cooling centrifuge for removing the unbroken cells, cell debris, nuclei and mitochondria.

2.2.8.2. Preparation of hemolysate from blood

The hemolysate was prepared by collecting the packed RBC from heparinized blood by centrifugation at 2,500 rpm. The packed RBC collected was washed with normal saline. The hemoglobin concentration of the packed RBC was determined as described in section 2.2.5.1. For hemolysis, 0.9 ml of cold water was added to 0.1 ml of the packed RBCs followed by the addition of 0.25 ml of chloroform and 0.5 ml of ethanol with vigorous shaking. The hemolysate was collected by centrifugation for 60 min at 10,000 rpm (4°C) and the supernatant was used for SOD and GSH estimation.

2.2.8.3. Estimation of superoxide dismutase (SOD) activity in blood and tissue

SOD enzyme activity was measured according to the method of (McCord and Fridovich, 1969)

Principle:

The assay principle lies in the ability of SOD to inhibit the reduction of nitro blue tetrazolium (NBT) by superoxide radical, generated during the reaction of photo reduced riboflavin with oxygen.

Procedure:

A volume of 100 µl of the test sample (hemolysate or tissue sample) was mixed with 200 µl of 0.0015 KCN in 0.1 M EDTA, 100 µl of 1.5 mM NBT and volume made up to 2950 µl with 67 mM K-Na phosphate buffer (pH 7.8). The

reaction mixture devoid of test sample served as control. 50 µl of 0.12 mM riboflavin was added and the absorbance of the reaction mixture was read at 560 nm and recorded as initial reading. The tubes were then uniformly illuminated for 15 min under an incandescent lamp. The absorbance was read and recorded as final reading. Percentage inhibition was calculated as the percentage change of absorbance of the test with that of control. The concentration of the sample required to scavenge 50% of the generated superoxide anion was considered as 1 unit of enzyme activity and was expressed as U/g Hb and U/mg protein for for blood and tissue samples respectively.

2.2.8.4. Estimation of glutathione peroxidase (GPx) activity in tissue

The method of Hafeman *et al.* (1974) was followed.

Principle:

Glutathione peroxidase depletes H_2O_2 in presence of glutathione (GSH). The remaining GSH is measured using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), which gives a colored complex.

Procedure:

A volume of 100 µl of 5 mM GSH, 100 µl of 1.2 mM H_2O_2 and 100 µl of 25 mM NaN_3 were added to 100 µl of tissue sample, and the volume was made up to 2.5 ml with 1M sodium phosphate buffer (pH 7.0). The reaction was incubated at 37ºC for 6 min and arrested using 2.0 ml of 1.65% HPO_3. The reaction mixture was centrifuged at 3000 rpm for 10 min. To 2.0 ml of the supernatant, 2.0 ml of 0.4 M Na_2HPO_4 and 1ml of 1 mM DTNB were added and incubated at 37ºC for 10 min. The intensity of yellow color formed was measured at 412 nm. The enzyme activity is expressed as units/mg protein.

2.2.8.5. Estimation of glutathione (GSH) content in blood and tissue

The method of Moron *et al.* (1979) was followed.

Principle:

GSH is measured by its reaction with DTNB, which yields a yellow colored complex with maximum absorption at 412 nm.

Procedure:

A volume of 500 µl of the test sample (hemolysate or tissue sample) was mixed with 125 µl of 25% TCA and cooled on ice for 5 min. To the mixture, 600 µl of 5% TCA was added and centrifuged at 2000 rpm for 10 min. 300 µl of the supernatant was mixed with 700 µl of 0.2 M sodium phosphate buffer (pH 8.0) and 2.0 ml of freshly prepared 0.6 mM DTNB. The absorbance was read at 412 nm. The GSH content of the sample was calculated from the standard graph plotted for different concentrations of GSH of range 10-50 nmoles. The results are expressed as nmol/ml blood or nmol/mg tissue protein.

2.2.8.6. Estimation of lipid peroxidation in tissue

The level of lipid peroxidation in tissue was measured as malondialdehyde (MDA) by the method of Ohkawa *et al.* (1979).

Principle:

Malondialdehyde (MDA) produced during peroxidation of polyunsaturated fatty acids (PUFAs) can react with thiobarbituric acid (TBA) reagent to form a pink colored product, with absorption maximum at 532 nm.

Procedure:

A volume of 400 µl of tissue sample was added to the reaction mixture containing 0.2 ml of 8% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA, making up a final volume of 4 ml in distilled water. The reaction mixture was incubated at 95°C for 1hr in a boiling water bath. This was cooled under running tap water and made up again to 5 ml. To this, 5 ml of pyridine:butanol (15:1) was added, mixed in a vortex and centrifuged at 3000

rpm for 10 min. The absorbance of the clear supernatant was read at 532 nm. The concentration of MDA was calculated from the standard graph plotted at concentrations ranging from1-10 nmol, and expressed as nmol of MDA/mg protein.

2.2.8.7. Estimation of total protein

Serum total protein was estimated according to Biuret method.

Principle:

In alkaline solution, proteins together with copper ions form a blue-violet colored complex.

Protein + Cu^{++} → Blue-violet colored complex

The intensity of the color formed is directly proportional to the amount of total proteins present in the sample.

Procedure:

To 1 ml of Biuret reagent, 10 µl of sample (serum or tissue sample) and 10 µl of protein standard (6 g/dl) were added into the test and standard reaction systems respectively, properly mixed and incubated at 37^0C for 5 min. The absorbance was measured at 555 nm within 60 min. The serum total protein was calculated using the formula:-

Total protein (g/dL) = (Absorbance of test/Absorbance of standard) × 6

2.2.9. *In vitro* antioxidant assays

2.2.9.1. Superoxide radical scavenging assay

The assay was done using the method of McCord and Fridovich (1969).

Principle:

In the reaction system, superoxide radicals generated from riboflavin during illumination reduces nitroblue tetrazolium (NBT). The ability of the extract to scavenge superoxide anions was determined in correlation with the extent of reduction of NBT.

Procedure:

The test substance (extract) was added at different concentrations to the reaction mixture containing 0.003 mg NaCN in 0.1 M EDTA, 0.12 mM riboflavin and 0.6 M phosphate (pH 7.8) into a final volume of 3 ml. The tubes containing the reaction mixture were continuously illuminated with incandescent lamp for 15 minutes to cause the photo-reduction of riboflavin. Reduced NBT was spectrophotometrically determined by the difference in optical densities taken before and after illumination of the tubes at a wavelength of 560 nm. The percentage of radical scavenging was calculated as:-

Scavenging effect (%) = $[(A_c - A_t) / A_c] \times 100$, where

A_c :- Absorbance of untreated control; A_t :- Absorbance of test.

2.2.9.2. Hydroxyl radical scavenging assay

The study was conducted using the thiobarbituric acid reacting substances (TBARS) method (Elizabeth and Rao, 1990).

Principle:

In the reaction system, the hydroxyl radicals generated from Fe^{2+}/ascorbate/H_2O_2 system produce TBARS by the degradation of deoxyribose (Fenton reaction). Hydroxyl radical scavenging activity was measured by the inhibiting ability of test compounds on hydroxyl radicals so generated.

Procedure:

The extract (dissolved in PBS) at required concentrations was added to the reaction mixture containing deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM) and KH_2PO_4-KOH buffer (20 mM, pH 7.4) in a final volume of 1 ml. The mixture was incubated at 37^oC for 1 hour, and the absorbance read at 532 nm. The percentage inhibition of hydroxyl radical generation at different concentrations was plotted and the IC_{50} was determined.

2.2.9.3. DPPH radical scavenging assay

The scavenging activity of the extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH) - a commercially available stable free radical soluble in methanol - was analysed by the method of (Aquino *et al.*, 2001).

Principle:

DPPH, in its radical form has purple colour, with an absorption peak at 515 nm. On reduction by an antioxidant compound, it disappears, and the solution undergoes transition from purple to yellow colour.

Procedure:

DPPH, in solution was incubated in the presence of the extract at different concentrations for 20 minutes at room temperature in the dark and absorbance was read at 515 nm.

2.2.9.4. ABTS radical scavenging assay

The study was conducted based on the method as described in Long *et al.* (2000).

Principle:

In this assay, the radical scavenging activity of the extract was determined by allowing it to react with $ABTS^+$, a model stable free radical derived from 2,2'-azino bis (3-ethyl benzothiazoline-6-sulphonic acid) (ABTS).

Procedure:

Ammonium persulphate (at 2.45 mM final concentration) was added to stock solution of ABTS (7 mM) and allowed to react for more than 16 hours in dark at room temperature. Reaction of ABTS with persulphate leads to its incomplete oxidation leading to generation of the free radical form. The ABTS radical solution was diluted to an absorbance of 0.75 at 734 nm in phosphate buffered saline (PBS). To 1 ml of this solution, the extract at different concentrations was added made up to a final volume of 1.1 ml with PBS. Decrease in OD of test sample was determined after 6 min of commencement of the reaction (at room temperature), with PBS as reference.

The scavenging activity of the extracts in the above assays was determined by the formula:-

Scavenging effect (%) = $[(A_c - A_t) / A_c] \times 100$, where

A_c :- Absorbance of untreated control; A_t :- Absorbance of test sample with extract.

2.2.9.5. Ferric Reducing Antioxidant Power (FRAP) assay

The study was conducted based on the method as described in Pulido *et al.* (2000) and Benzie and Strain (1996a).

Principle:

The assay is based on the reducing power of the extract to convert ferric complex in the reaction system to ferrous form.

Procedure:

The FRAP reagent for the assay was prepared fresh by mixing 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 2.5 ml of ferric chloride (20 mM $FeCl_3.6H_2O$) and 25 ml of acetate buffer (0.3 M, pH 3.6). To 0.9 ml of the reagent, the extract at different concentrations was added and incubated for

15 min at 37^{o}C. An intense blue coloured complex was formed when Fe^{3+} - TPTZ complex was reduced to the ferrous (Fe^{2+}) form, which could be measured as absorbance at 595 nm in a spectrophotometer. The absorbance of the samples was directly proportional to the increase in reducing power. The percentage increase in the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) was calculated by plotting a standard graph of $FeSO_4.7H_2O$ at concentrations ranging from 0.1 to 1 µM. The half maximal effective concentration (EC_{50}) was determined according to the formula:

EC_{50} = [(Absorbance of test – Absorbance of control)/Absorbance of test] × 100

All *in vitro* antioxidant assays were carried out in triplicate.

2.2.10. Establishment of plant tissue culture

Murashige and Skoog Medium (MS) (Murashige and Skoog, 1962) was used for micropropagation of *O. indicum*.

2.2.10.1. Preparation of culture media

All stock solutions were taken in appropriate proportions followed by the addition of sucrose [3% (w/v)] as carbon source. The volume was made up to 1 litre with double distilled water. For preparing solid medium, agar was used as the solidifying agent. Basal medium for seed germination contained no hormone supplements. For callus induction, shoot and root initiation studies, the media were supplemented with various phytohormones at varied study concentrations, as per requirement. pH of the medium was adjusted to 5.7-5.8 using 0.1N NaOH or 0.1N HCl before gelling with 0.75% agar (Hi-media, Mumbai). The media were then dispensed into culture tubes and plugged with non-adsorbent cotton and were steam sterilized at 121°C, 15 lb pressure for 20 min and allowed to cool at room temperature.

2.2.10.2. Collection of inoculates

The nodal sections from germinated seedlings were used for inoculation.

2.2.10.2.1. Seed germination

The dried pods of *O. indicum* Vent. were collected from the Ayurvedic Garden maintained in the Amala Cancer Hospital campus. Seeds were taken out by breaking the pods and soaked in distilled water for 30 minutes, followed by removal of their membranous papery wing covers. They were then surface sterilized with an aqueous solution of 0.1% mercuric chloride for 3 min, followed by repeated washing with sterile distilled water under sterilized laminar flow hood. The seeds were inoculated into basal MS media and maintained under at a temperature of 25 ± 2^0 C, in dark for a period of 2 weeks and thereafter for 4 weeks under a photoperiod (16-hour light/8 hour dark) with light intensity 1000-1500 lux.

2.2.10.2.2. Recovery of nodal explants

The nodal explants of length ranging from 10-15 mm were collected from the germinated seedlings for callus induction.

2.2.10.3. Callus induction

The nodal sections were inoculated into MS media supplemented with required combinations of plant growth regulators in combinations specified in the experiment. The cultures were incubated in dark at 25 ± 2^oC for four weeks. From the calluses initiated, (~) 500 mg (fresh weight) inoculates were cut out and subcultured into MS media fortified with cytokinin-auxin combinations as specified in the experiment. The cultures were maintained under a photoperiod (16-hour light/8 hour dark) with light intensity 1000-1500 lux.

2.2.10.4. Indirect organogenesis from callus

2.2.10.4.1. Induction of shoots

To induce multiple shoot regeneration, well-established compact calluses (~500 mg fresh weight) were transferred to MS basal medium supplemented with specified concentrations of auxin-cytokinin combinations. The cultures were incubated under a 16 hr photoperiod in cool white flourescent light of intensity 25μmol $m^{-2}s^{-1}$, and maintained at a constant temperature of 25 ± 2ºC.

2.2.10.4.2. Induction of roots

For root induction, calluses (~500mg fresh weight) were transferred into MS media fortified with the auxin, indole butyric acid (IBA) at concentrations as specified in the experiment.

2.2.10.5. *In vitro* root generation

For *in vitro* root generation, callus- generated shoots carrying three to four fully expanded leaves were transferred into MS media fortified with IBA at specified concentrations.

For all the above micro propagation experiments, medium without plant growth regulators used as control.

2.2.11. Establishment of cell suspension culture of *O. indicum* Vent.

Cell suspensions of *O. indicum* Vent. were established from callus cultures after two subsequent subcultures. 16 g of cells (fresh cell weight) was transferred into a 500 mL Erlenmeyer flask with 200 mL liquid MS medium with the same composition as that for agar containing medium. The cultures were constantly shaken at 110 rpm and maintained at 25°C with 16-h photoperiod. Suspensions were subcultured in 8 day intervals.

2.2.12. Elicitation of secondary metabolites from callus cultures

2.2.12.1. Elicitation using methyl jasmonate

Methyl jasmonate (Meja) stock solution (100 mM) was prepared by dissolving 22.43 mg in 1 ml of ethanol (70% v/v). For elicitation studies, further dilution

of this stock was done by using deionized water to get the appropriate concentrations in tissue culture medium. Solution was filtered through a microfilter (0.2 mm) before being dispensed into cell suspension cultures at required concentrations. Control cultures were treated with deionised sterile water, instead of elicitor.

2.2.12.2. Elicitation by irradiation

Cell suspension cultures in MS medium were exposed to 6 MV X-ray radiations at specified doses using in the phototherapy irradiation chamber at Amala Institute of Medical Sciences, Amalanagar, Thissur, Kerala, India. Non-irradiated cultures were kept as control.

Harvesting of calluses was performed periodically, as specified in the experimental protocol.

2.2.13. Preparation of extracts from tissues generated through micropropagation

The tissues harvested from micropropagation were lyophilized and ground into fine powder. This was extracted using 70% methanol by periodic (every 4 hours) stirring and warming at 45-50^{o}C. The extracts were filtered and evaporated to get a dry residue, until all traces of solvent was removed.

Chapter 3

Analysis of *in vitro* cytotoxic and anti proliferative activities of *Oroxylum indicum* Vent. root bark

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3.4. Discussion

3.1. Introduction

Drugs developed from plant origin are gaining accelerated demand for cancer therapy, mostly due to their non-toxic effects on normal cells, but simultaneous cytotoxic effects on cancer cells. The plant derived secondary metabolites such as polyphenols, flavonoids and brassinosteroids have been studied for their potential use as anticancer agents, in terms of antioxidant activity, cytotoxicity, inhibition of cancer cell proliferation and apoptosis induction (Greenwell and Rahman, 2015). Cancer prevention, suppression and treatment using natural plant products are also practiced in Ayurveda, the oldest and indigenous medicine system of India. It has been summed up from statistical data that plants with the basic Ayurvedic characteristics namely *Katu, Tikta, Kasāya Rasa* (bitter, pungent, and astringent taste), *Ushna Virya* (e.g., hot biopotency), *Katu Vipāka* (catabolic active metabolites) and dry, coarse, light, and sharp biophysical properties exert significantly high anticancer properties (Singh, 2004). *Oroxylum indicum* Vent., member of family Bignoniaceae is known for its use in the preparation of the Ayurvedic formulation, namely, *Dasamoola* and is attributed with the following properties- it tastes bitter, astringent (*tikta, kasaya rasa*); attributed with light/dry physical properties (*laghu, rooksha guna*) as well as hot potency (*ushna veerya*), and pungent post-digestion effect (*katu vipak*a) (H.P.I., 1998, Sharma, 2006). The plant parts also find use in folklore medicine to cure ailments such as urinary infections, bronchitis, leucoderma, diarrhea, nasopharyngeal cancers, oral cancers etc, as reviewed by Mao (2002), Deka *et al.* (2013) and Preety and Sharma (2016). The study, as a whole is focused on evaluation of the anti cancer activities of *O. indicum* root bark.

The most remarkable property of cancer cells is their rapid proliferative ability; hence, cytotoxicity assays are used as a presumptive way of screening anti cancer activities of drugs (Cragg and Newman, 2005, Shivakumar *et al.*, 2012 , Asirvatham *et al.*, 2013). The assays rely on changes in cell functions such as

enzyme activity, membrane permeability, cell adherence, ATP production and nucleotide uptake activity, characteristic of cancer cells. Except a report of cytotoxic effect of root bark in MCF-7 cells (Dhru *et al.*, 2016), the *in vitro* cytotoxic and anti proliferative activities or the underlying anti cancer mechanism of *O. indicum* root bark still remains unexplored. Hence, the study was focused on the short term cytotoxic and long term anti proliferative activities of *O. indicum* root bark on DLA/EAC cell lines and HeLa/HCT15/Hep G2/MDA-MB-231 cell lines respectively. The trypan blue dye exclusion method and MTT assay were used for evaluation of cytotoxicity and anti proliferation, respectively. The study also attempted to elucidate the mechanism of proliferative inhibition of cancer cells through analysis of cell cycle arrest and observation of apoptotic morphology in HeLa cells, in the presence of the extract. The studies of cell cycle arrest and apoptotic morphology were conducted using flow cytometric technique and microscopic examination (EtBr- acridine orange staining and Annexin-Hoechst staining) respectively.

As a forerunning step towards new drug discovery from plants, separation of medicinally active parts from inert parts is done using extraction with suitable solvents. One way of selecting the appropriate solvent is based on the solubility and polarity of the target compounds to be extracted, which in turn, depends on the phytochemical profile of the plant (Bergs *et al.*, 2013). An alternate approach for selection is the biological activity based screening of extracts prepared using different solvents, ranging from non polar solvents (petroleum ether, dichloromethane, acetone) to polar solvents (methanol, water) (Do *et al.*, Alothman *et al.*, 2009, Złotek *et al.*, 2016). Hence, to arrive at a suitable solvent system for the whole study, a preliminary screening of different extracts (chloroform, ethyl acetate, acetone, methanol, water and hydro-methanol combinations) of *O. indicum* root bark was carried out. Apart from cytotoxicity, an inter relationship has been established between the antioxidant and anti cancer activities of drugs since free radical induced damage is one of

the postulated mechanisms of cancer development (Abdel-Hady *et al.*, 2011, Shaikh *et al.*, 2014). Hence, in the present study, selection of suitable solvent system is based on screening of the extracts using short term *in vitro* cytotoxicity on Ehrlich's Ascites Carcinoma (EAC) cells and *in vitro* scavenging of superoxide radicals.

3.2. Materials and methods

3.2.1. S*creening of extracts of O. indicum (first stage)*

3.2.1.1. *Preparation of root bark extracts*

The collection and authentication of root bark of *O. indicum* was done as described in section 2.1.1, Chapter 2. Five different extracts were prepared using chloroform, ethyl acetate, acetone, methanol and water in a Soxhlet apparatus (method described in section 2.2.1, Chapter 2).

3.2.1.2. *Cell lines*

Ehrlich's Ascites Carcinoma cells and normal spleen cells were used for the study. These were procured (Chapter 2, section 2.1.7) and maintained as described in Chapter 2, sections 2.2.3.1.1 and 2.2.3.1.2 for studying short term *in vitro* cytotoxicity of the extracts.

3.2.1.3. *Cytotoxicity analysis*

The cytotoxic activity of root bark extracts was evaluated using trypan blue dye exclusion method on EAC cells and normal spleen cells (Chapter 2, section 2.2.3.1.3).

3.2.1.4. *Super oxide scavenging assay*

In vitro antioxidant property of the root bark extracts of *O. indicum* was evaluated based on the superoxide scavenging assay (Chapter 2, section 2.2.9.1).

3.2.2. *Screening of hydromethanolic extracts of O. indicum root bark (second stage)*

In the screening study conducted as per section 3.2.1, the methanol extract was superior to other extracts in its cytotoxicity to EAC cells. In superoxide scavenging assay based screening, the activity of methanol extract was second only to that of aqueous extract. Hence, a second stage screening was done with a combination of water and methanol in three different proportions, as follows:

3.2.2.1. *Preparation of extracts*

Three different hydromethanolic extracts of *O. indicum* root bark were prepared using 30%, 50% and 70% methanol as solvents in a magnetic stirrer (section 2.2.1, Chapter 2).

3.2.2.2. *Cell lines*

EAC cell lines were used for the study (as per section 3.2.1.2 above).

3.2.2.3. *Cytotoxicity analysis*

Trypan blue dye exclusion method was used (as per section 3.2.1.3 above) to study the toxicity of the extracts at concentrations ranging from 100-500 µg/ml.

3.2.2.4. *Super oxide scavenging assay*

Method referred to in section 3.2.1.4 was used to evaluate superoxide radical scavenging activity of various extracts of *O. indicum* root bark at assay concentrations ranging from 50-250 µg/ml.

3.2.3. Short term in vitro cytotoxicity of 70% hydromethanolic extract of O. indicum Vent. in DLA cell lines

3.2.3.1. Preparation of extract

Following screening, the 70% hydromethanolic extract of *O. indicum* Vent. (OIM) was selected for further studies. The extract was prepared as per the

method described in Chapter 2, section 2.2.1. An average yield of 9.01% was obtained in this system of extraction.

3.2.3.2. Cell line

The short term cytotoxicity of OIM at concentrations ranging from 100-500 µg/ml on DLA cell lines (procured and maintained as per Chapter 2, sections 2.2.3.1.1 and 2.2.3.1.2) was evaluated using the trypan blue dye exclusion method (3.2.1.3 above).

3.2.4. Anti proliferative ability of OIM

The anti proliferative ability of OIM extract was studied in cancer cell lines- HeLa, HCT15, Hep G2 and MDA-MB-231. Vero cell lines were used as control.

3.2.4.1. Cell lines

The cell lines were procured (Chapter 2, section 2.1.7) and maintained as described in section 2.2.3.2.1, chapter 2.

3.2.4.2. MTT assay

The anti proliferative ability of OIM extract at concentrations ranging from 40-200 µg/ml in HeLa, HCT15, Hep G2 and MDA-MB-231 cell lines were studied using MTT assay (section 2.2.3.2.2, chapter 2). Vero cell lines were used to analyse the toxicity of OIM extract to normal cells.

3.2.5. Analysis of cell cycle arrest using flow cytometry

The study of cell cycle stages in HeLa cells in the presence of OIM extract was conducted using flow cytometry.

The cells were incubated with the extract at two concentrations (50 and 100 µg/ml) for 24 hrs at 37^{o}C. Thereafter, they were harvested by trypsinization and centrifuged at 5000 rpm for 10 min at 4^{o}C in cold centrifuge. The pellet

was resuspended in 250 µl ice-cold PBS and centrifuged again at 10,000 rpm for 5 min. The resultant pellet was resuspended in 300 µl PBS. Cells were fixed by the dropwise addition of 700 µl ice-cold 70% ethanol and occasional stirring in vortex. The sample was incubated on ice for 30-60 min and centrifuged at 5,000 rpm for 10 min at room temperature. The pellet collected was resuspended in PBS and centrifuged at 5,000 rpm for 10 min at room temperature again. RNase (5µl) at concentration 10 mg/ml was added to the resuspended pellet (in 250 µl of PBS) and incubated for 1 hr at 37□C. Following this, propidium iodide (PI) (10 µl at concentration 1 mg/ml) was added to the suspension and filtered (40 µm pore size membrane filter). The cell suspended was stored in dark at 4^{o}C until it was analysed. Flow cytometry was used to reveal the percentage of cells in different stages on the basis of DNA concentration with propidium iodide staining, with reference to 10,000 events in each sample. Data was analyzed in Coulter Elite 4.5 Multicycle software 20.

3.2.6. Study of apoptotic morphology

The HeLa cells treated with OIM extract were studied to observe any characteristic morphological changes associated with apoptosis.

3.2.6.1. Dual Acridine orange/Ethidium bromide staining (Ao/Eb)

HeLa cells (count 2 × 10^{6} cells/ml) were seeded in a 6 well plate and incubated at 37^{o}C until attachment. The cells were treated with two concentrations of the extract (50 and 100 µg/ml) and incubated for 16 hrs. They were then washed with PBS, harvested by trypsinization and centrifuged at 2000 rpm for 10 min. The pellet was resuspended in 50 µl PBS. The cells were stained by mixing equal volumes of the suspension with dual fluorescent staining solution containing AO and EB (1:1) and subjected to fluorescent microscopy using a blue excitation filter (480 nm) and photographed.

3.2.6.2. Annexin-Hoechst staining

HeLa cells (count 2×10^4 cells/ml) were seeded on 96 well glass bottom plates. At 50 % confluence, the cells were treated with two concentrations of the extract (50 and 100 µg/ml) and incubated for 16 hrs. Cells were harvested and washed with Annexing binding buffer two times and incubated with annexin V Conjugate for 30 minutes at 1:100 dilution in binding buffer. Cells were then subjected to Hoechst33342 (Molecular probes H3570) staining and imaged with an epi fluorescent microscope Nikon TiE using Alexa 647 Filter sets from Semrock USA. Images were collected using CCD camera Retiga Exi (Q IMAGING) controlled through NIS element software (Nikon).

3.3. Results

3.3.1. Screening of O. indicum root bark extracts (1st stage)

3.3.1.1. In vitro cytotoxicity in EAC cells

Among the five different extracts screened for *in vitro* cytotoxicity in EAC cells, highest activity was obtained with methanol extract at the IC_{50} value, 249.94 ± 3.08 µg/ml concentration (Table 3.1.), which was found to be non toxic to normal spleen cells up to a concentration of 500 µg/ml. The aqueous and acetone extracts, which were also non toxic to normal cells, brought about 50% inhibition of EAC cells at 265.56 ± 1.54 and 272.15 ± 3.07 µg/ml respectively. The chloroform and acetone extracts were also found to be toxic to normal as well as cancer cells.

3.3.1.2. In vitro super oxide radical scavenging activity

Table 3.1 shows that the aqueous extract was superior to methanol, acetone, chloroform and ethyl acetate extracts in superoxide radical scavenging activity, with IC_{50} at a concentration of 59.15 ± 0.98 µg/ml. The methanol extract also caused 50% scavenging of superoxide radicals in the assay at a concentration of 70.12 ± 0.87 µg/ml.

Table.3.1. Screening of chloroform, ethyl acetate, acetone, methanol and aqueous extracts of *O.indicum* Vent. based on *in vitro* cytotoxicity in EAC cells and *in vitro* superoxide radicalscavenging activity. The values given are Mean ± SD for experiments run in triplicate.

Solvent used for extraction in Soxhlet	**IC_{50} (µg/ml) in EAC cells**	**IC_{50} (µg/ml) in normal spleen cells**	**IC_{50} (µg/ml) superoxide inhibition**
Chloroform	335.53 ± 2.43	397.16 ± 4.22	95.12 ± 1.11
Ethyl acetate	341.12 ± 2.22	335.15 ± 2.88	102.35 ± 2.01
Acetone	272.15 ± 3.07	> 500	73.54 ± 1.63
Methanol	**249.94** ± 3.08	**>500**	70.12 ± 0.87
Water	265.56 ± 1.54	> 500	**59.15** ± 0.98

3.3.2. Screening of hydro methanolic extracts of O. indicum root bark (2nd stage)

Based on the results (Table 3.2.) in the first stage screening, when the second stage was conducted with 30%, 50% and 70% hydromethanolic extracts, it was found that the activity of 70% methanol extract was superior to the other extracts in cytotoxicity induction (IC_{50} at 252.53±1.28 µg/ml) in EAC cells. The superoxide scavenging activity of the three different extracts, 30%, 50% and 70% showed less variation with IC_{50} at 62.99 ± 0.42, 63.12 ± 0.61 and 63.20 ± 0.51 µg/ml respectively. Hence, the 70% methanol extract was used for further studies.

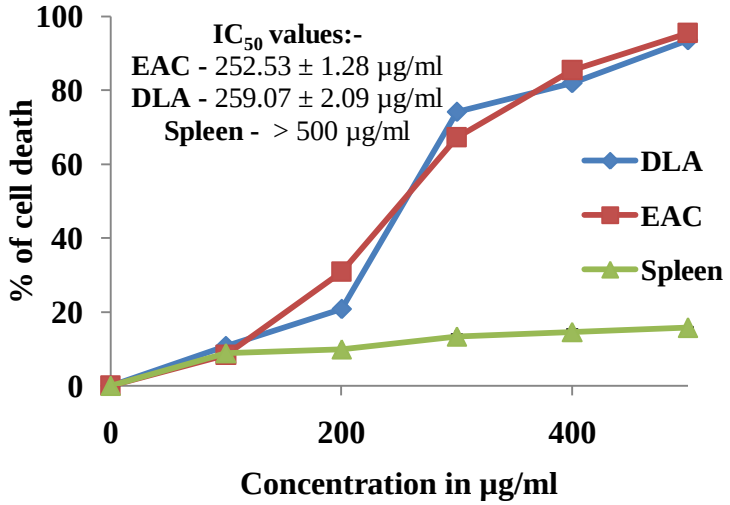
Table.3.2. Screening of 30%, 50% and 70% hydromethanolic extracts of *O. indicum* Vent. based on *in vitro* cytotoxicity in EAC cells and *in vitro* superoxide radical scavenging activity. The values given are Mean ± SD for experiments run in triplicate.

Solvent used for extraction	**IC_{50} (µg/ml) in EAC cells**	**IC_{50} (µg/ml) in normal spleen cells**	**IC_{50} (µg/ml) superoxide inhibition**
30% methanol	261.94 ±2.01	> 500	62.99 ±0.42
50% methanol	257.65 ±1.54	>500	63.12±0.61
70% methanol	**252.53 ±1.28**	**> 500**	63.20 ±0.51

3.3.3. Cytotoxicity of OIM in DLA cells

A concentration of 259.07 ± 2.09 µg/ml of OIM extract caused 50% toxicity in DLA cells. The cytotoxicity of OIM extract on DLA, EAC and normal spleen cells is given in figure 3.1.

Figure 3.1. Cytotoxicity of OIM extract on DLA, EAC and normal spleen cells with IC_{50} values (Mean ± SD for experiments run in triplicate).



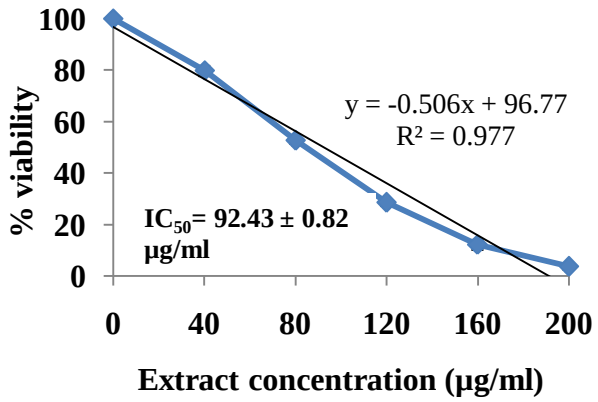
3.3.4. Anti proliferative activity of OIM

The study revealed that the OIM extract inhibited the proliferation of 50% of HeLa , HCT15 and MDA-MB-231 cells in the system at concentrations within 150 µg/ml (92.43 ± 0.82, 133.0 ± 1.2 and 112.84 ± 1.08 µg/ml respectively), with maximum activity in HeLa cell lines (Figure 3.2.). The morphology of HeLa, HCT15 and MDA-MB-231 on treatment with OIM extract at various concentrations is given in figure 3.3. But the extract could not induce 50% inhibition of Hep G2 cell growth up to a concentration of 200µg/ml, indicating that it was less toxic to this cell type. No signs of toxicity were found in Vero cell lines, which were maintained as control.

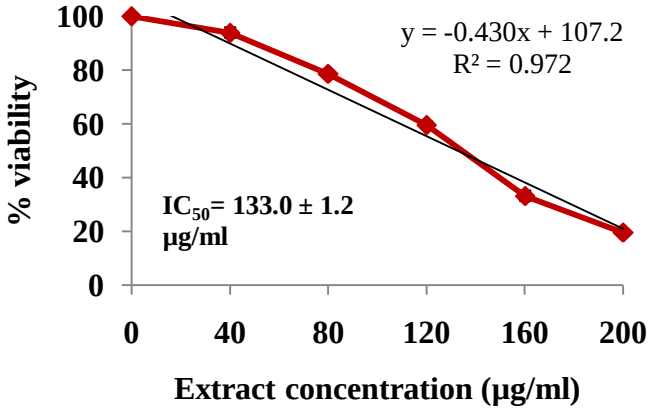
3.3.5. Analysis of cell cycle stages

As evident from the peaks (Figure 3.4.) obtained from flow cytometric analysis based on cellular DNA concentration with PI staining, OIM extract was to arrest HeLa cell cycle at the G1 phase. On treatment with OIM extract at concentrations- 50 and 100 µg/ml respectively, 79.7 and 86.0% of cells remained in G_1 phase of the cell cycle, compared to the untreated cells with only 71.9% of cells in G_1. Thus, OIM treatment brought about an increase of relative percentage of G_1 cells by 7.8 and 14.1%, in a dose dependent manner, suggesting cell accumulation in G_1/S transition phase. In support of the above finding, it was also observed that the relative percentage of cells in S phase was lesser (7.5%), in the presence of 100 µg/ml OIM, compared to the untreated cells (10.5%). Concomitant result was obtained for cells in G_2, which maintained a relative population of 8.2 and 4.6%, on treatment with 50 and 100 µg/ml OIM respectively, compared to untreated cells (16.7%). A higher proportion of cells- 1.8 and 1.9%- in sub-G_0 phase were observed in cell lines treated with 50 and 100 µg/ml OIM, but the relative percentage was 0.3% in untreated control. The relative cell percentage at various cell cycle stages is also depicted in figure 3.4.

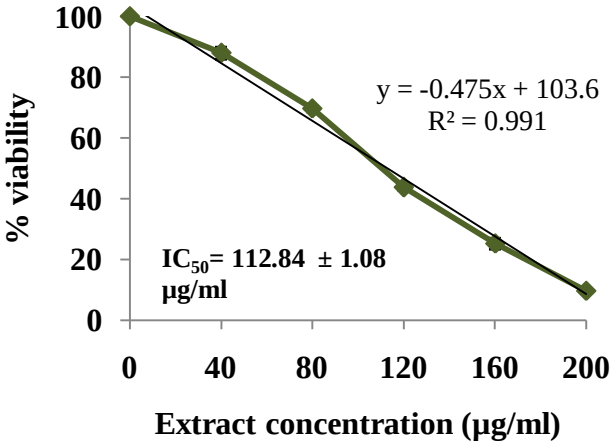
Figure 3.2. Anti proliferative activity of OIM extract on HeLa, HCT15 and MDA-MB-231 cells



a)

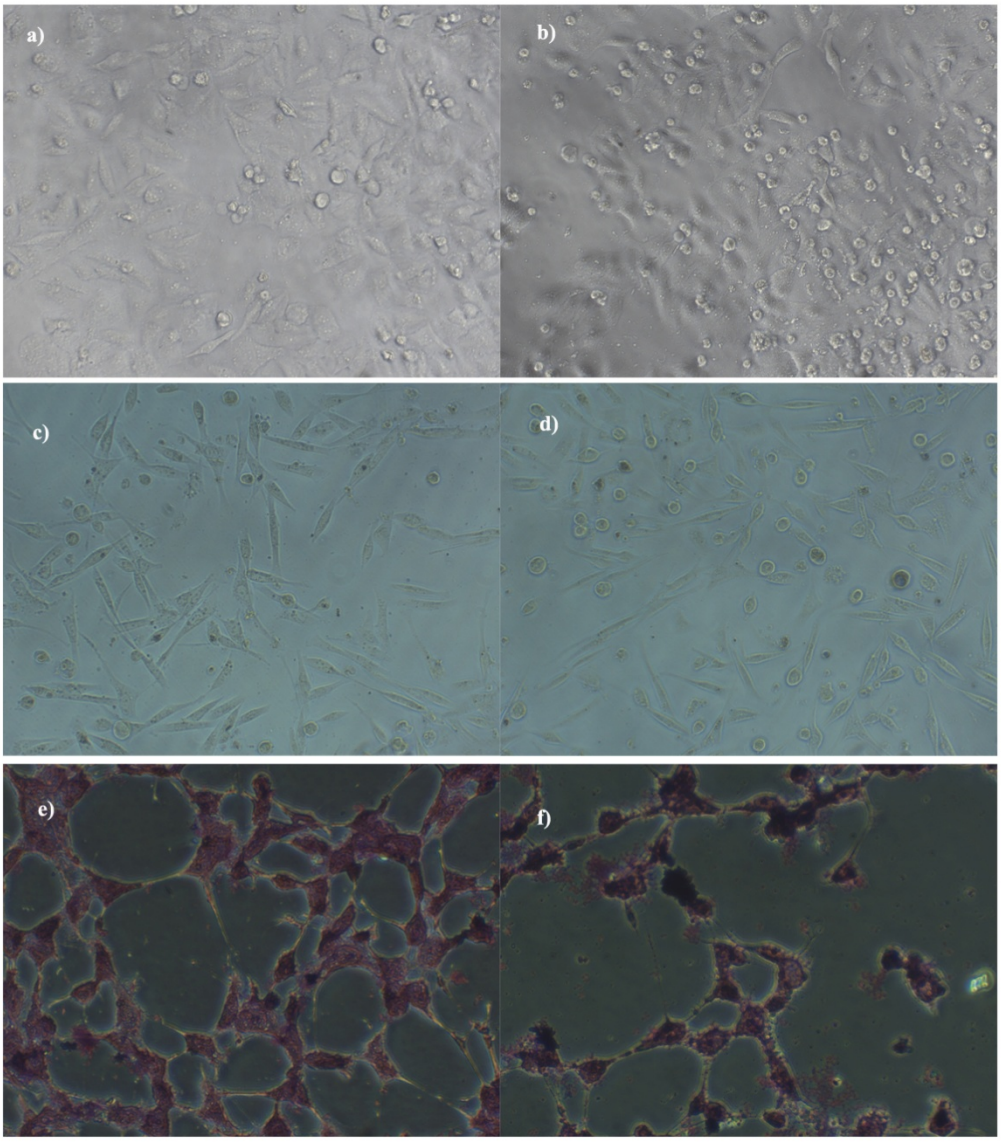


b)



c)

Figure 3.3. Morphology of HeLa, HCT15 and MDA-MB-231 incubated with OIM extract for 48 hours, as evident from the photomicrographs taken in a phase contrast inverted microscope (Magnus, INVI, Bangalore, India). a) Untreated HeLa cells, b) HeLa cells treated with 120 µg/ml OIM, c) Untreated MDA-MB-231 cells, d) MDA-MB-231 cells treated with 120 µg/ml OIM, (under 200× magnification) e) Untreated HCT15 cells and f) HCT15 treated with 120 µg/ml OIM (under 400× magnification).



3.3.6. Study of apoptotic morphology

3.3.6.1. Dual Ao/Eb staining

Flourescent microscopic observation revealed that HeLa cell sample incubated in the presence of 100 μg/ml OIM showed early and late apoptotic stages under Ao/Eb staining. The untreated cells showed live morphology and were uniformly stained green. The early apoptotic cells in the OIM treated sample also appeared green (in Ao nuclear stain), with bright green dots inside, characteristic of nuclear fragmentation. The late apoptotic cells with EtBr stain incorporated in nuclei appeared orange, but were with fragmented nuclei (Figure 3.5.).

3.3.6.2. Annexin-Hoechst staining

As revealed through the fluorescent microscopic images (Figure 3.6.), the untreated cells stained negative for annexin V, whereas HeLa cells treated with OIM showed annexin V positive staining. This indicated that no cell death occurred in untreated sample, but annexin V positive treated cells revealed death morphology. Hoechst staining exposed nuclear fragmentation in OIM treated samples, but no such noticeable nuclear morphology was present in untreated samples. In merged view of Annexin-Hoechst stained samples, it was observed that many of the annexin V positive cells of the treated sample correspondingly revealed nuclear fragmentation (evident from Hoechst staining). This is suggestive of apoptotic death of the cells, in the presence of OIM.

Figure 3.4. Effect of OIM exract treatment as revealed from flow cytometric analysis showing cell cycle stages in a) Untreated HeLa cells, b) and c) HeLa cells treated with OIM 50 and 100 µg/ml respectively and d) relative percentage of cells at various phases of cell cycle.

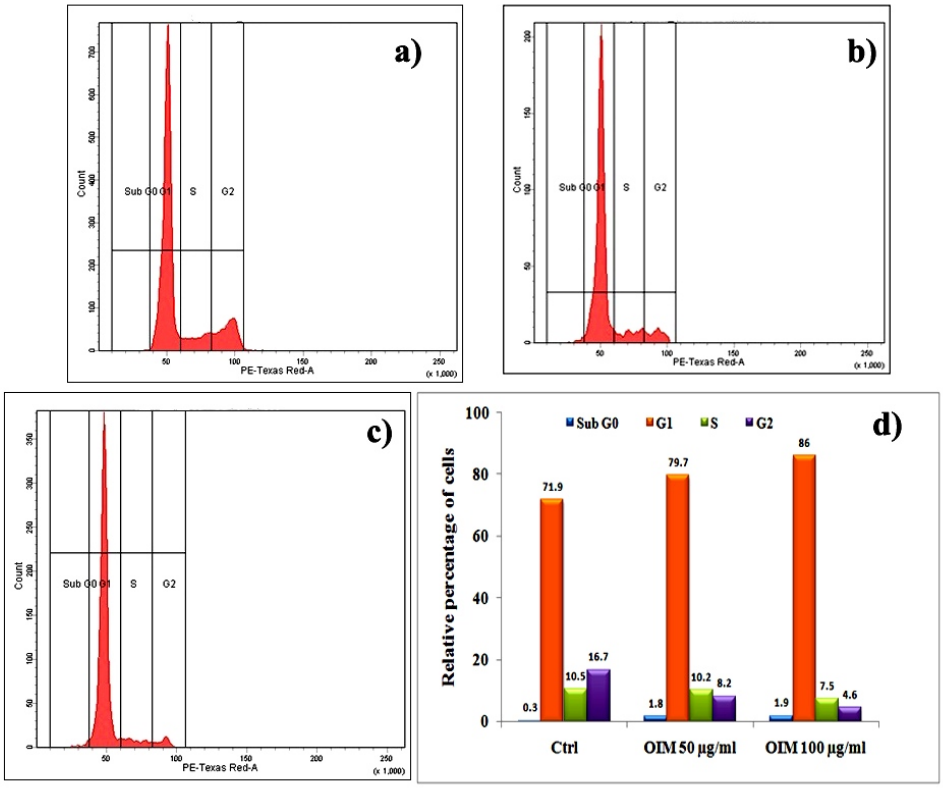


Figure 3.5. Effect of OIM extract treatment on morphology of HeLa cells with EtBr and acridine orange staining revealing apoptotic stages a) Untreated HeLa cells b) Cells treated with 100 µg/ml OIM showing early apoptotic (small arrow – b.1 & b.2) and late apoptotic (long arrow- b.2) stages.

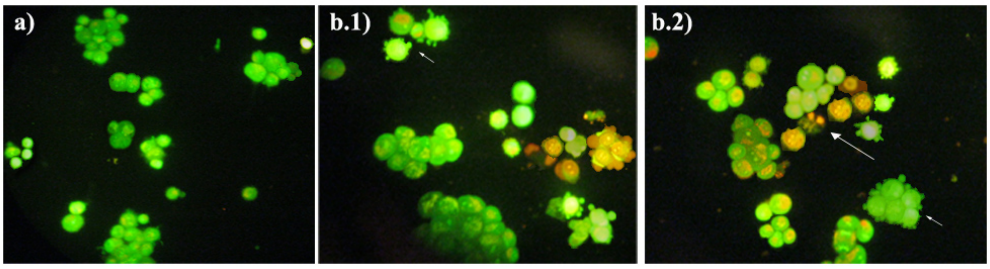
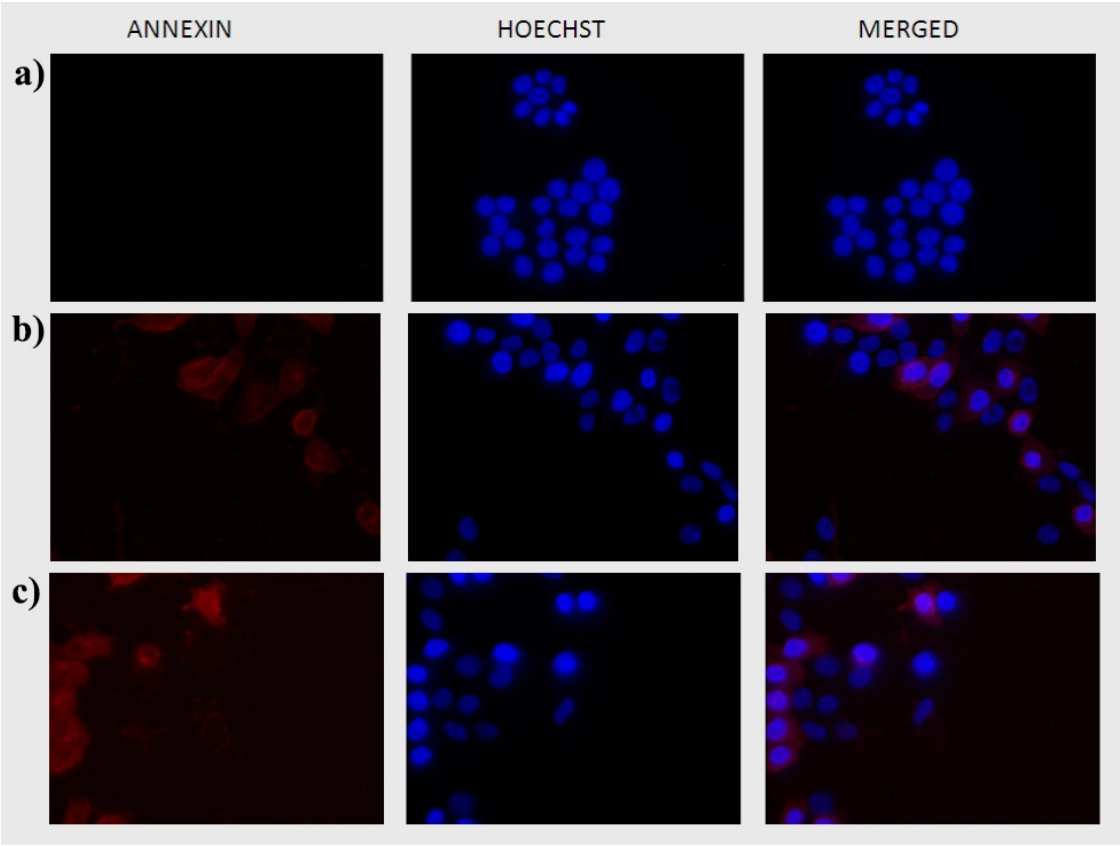


Figure 3.6. Effect of OIM extract treatment on morphology of HeLa cells with Annexin-Hoechst staining ; a)Untreated cells, b) and c) HeLa cells treated with OIM 50 and 100 µg/ml respectively.



3.4. Discussion

In the beginning of the study, preliminary screening of *O. indicum* root bark extract using short term cytotoxicity and superoxide scavenging activity was used as the basis to select appropriate solvent system in forthcoming investigations. Taking into consideration, the superior activities of methanol extract and aqueous extract in cytotoxicity and free radical scavenging respectively, a hydromethanolic combination (methanol and water in 70:30 ratios) was chosen for extraction of root bark powder. Since the bioactive constituents in plants possess different solubility properties, the extracts prepared from different solvents will vary in their pharmacological properties (Barchan *et al.*, 2014, Iloki-Assanga *et al.*, 2015). Since the study was focused on the anti cancer activities of the plant root bark, cancer cell toxicity and free radical scavenging activity in *in vitro* assay systems was used as the criteria for solvent selection.

Cell based screening assays are widely employed in drug discovery systems for determining the anti-cancer properties of both synthetic and plant derived compounds; and the viability measurements of cancer cell lines in the presence of the tested compound is a preliminary step in drug screening. In the study, we have found significant *in vitro* anti cancer property of OIM extract using short term cytotoxicity assay in DLA and EAC cell lines, where its non toxic effect was noticed in normal spleenocytes. The cytotoxic activity was also found to be concentration dependent. Similar cytotoxic effects on DLA and EAC cell lines have been studied with other plants (e.g., Scutellaria species (Salini *et al.*, 2013), Miliusa wayanadica (Favaz *et al.*, 2014) etc) with presence of flavonoids and phenolics detected in the active extracts. The root bark of *O. indicum* is rich in flavonoid (**chrysin, baicalein**, biochanin A) and phenolic compounds (ellagic acid) as studied earlier (Zaveri *et al.*, 2008, Joshi *et al.*, 2011). Detection limit of these constituents in the study (Zaveri *et al.*, 2008) were 0.656, 0.546, 3.13, 0.026 μg/ml for chrysin, baicalein, ellagic acid

biochanin-A respectively. This is supportive of the inference that the cytotoxicity of OIM extract may be due to the presence of such bioactive constituents, which is to be further ascertained in the forthcoming chapters.

The study also reported the anti proliferative potential of OIM extract on human cancer cell lines- HeLa (cervical cancer), HCT15 (colon cancer) and MDA-MB-231 (breast cancer). Anti proliferative ability of the methanolic extracts of fruits from *O. indicum* has been studied on HL-60 (promyelocytic leukemia) cell lines, with **baicalein** identified as an active principle in the extract (Roy *et al.*, 2007). The flavonoid is already reported to be present in the root bark (Zaveri *et al.*, 2008). Besides, many compounds reportedly present in the root bark are anti proliferative [e.g. **chrysin** (Samarghandian *et al.*, 2016), biochanin A (Kole *et al.*, 2011) and ellagic acid (Losso *et al.*, 2004a)], suggesting a correlation between the presence of cytotoxic compounds and biological activity of the extract. Meanwhile, the OIM extract was found less effective in inhibiting the proliferation of HepG2 (liver carcinoma) cell lines. The signaling pathways involved in cancer cell proliferation as well as proliferation inhibition are different (Evan and Vousden, 2001). The targeted signaling pathways of cytotoxic compounds are selective in different cancer cell types, as revealed earlier (Grunberger *et al.*, 1988, Pardhasaradhi *et al.*, 2005) as revealed from the differential cytotoxicity of OIM extract in cancer cell types. In the anti proliferative assays also, no evident toxic effect was produced by the extract on normal Vero (kidney epithelial cells derived from African green monkey) cell lines. These are aneuploid, immortalized and continuous cell lines used to confirm the safety of using cytotoxic anti cancer agents in cancer related drug discovery investigations (Vijayarathna and Sasidharan, 2012, Tugba Artun *et al.*, 2016). The results of the current study also suggest that OIM extract is safe on normal cells, while being toxic to certain cancer cell types.

It was found that OIM extract was most effective in inhibiting the proliferation of HeLa cells (IC_{50}: 92.43 ± 0.82 µg/ml), compared to other cell types used in the study. Hence, the investigation was extended to find out the cytological effects of OIM in HeLa cells using flow cytometric analysis and fluorescent microscopic examination. Cell cycle kinetics, as revealed through flow cytometric analysis suggests that in the presence of 100 µg/ml OIM extract, there was accumulation of cells in G_1 phase and concomitant depletion of cells in S phase and G_2 phase from the proliferative pool. It is therefore conclusive that OIM induces cell cycle arrest in the G_1/S transition phase. OIM treatment at this concentration developed a peak in the sub-G_0 phase, suggestive of proliferation arrest at this phase, a qualitative indicator of apoptosis (Tuschl and Schwab, 2004). Yet, the results were less evident in comparison with that of G_1/S phase arrest. The results obtained at this concentration of OIM can also be correlated to its IC_{50} value (92.43 ± 0.82 µg/ml) in the MTT assay. Cytotoxicity by anticancer agents is mediated through targeted mechanisms leading to cell cycle arrest and induction of apoptosis (Görgün *et al.*, 2010), and hence, regulation of cell cycle kinetics is correlated to the toxicity of many compounds in cancer cells (Jakubíková and Sedlák, 2006). Consistent with the findings of previous studies (Fofaria *et al.*, 2014, Kwan *et al.*, 2016), the results of the present work also links the proliferative inhibition of HeLa cells by OIM extract with its influence on mediating cell cycle arrest at G_1/S transition. Since there is a well established link between regulation of cell cycle events and apoptosis (Pucci *et al.*, 2000), the HeLa cells treated with 100 µg/ml OIM were subjected to the study of apoptotic morphology using Ao/Eb and annexin V-Hoechst staining methods.

In the discovery of anti cancer agents, apoptoic induction is a major hub because of the association between apoptotic dysregulation and cancer development (Choi *et al.*, 2008, Lu *et al.*, 2011). Loss of cell viability, fragmentation of DNA and condensation of DNA are apoptotic cell features which can be identified with their inability to exclude vital dyes,

electrophoretic patterns and nuclear staining. Basic morphological changes in apoptosis can be studied using dual Ao/Eb fluorescent staining which reveals differences among normal, early and late apoptotic as well as necrotic cells. Normal and early apoptotic cells with intact membranes are permeable to Ao, a nuclear stain that emits green flourescence. Only late apoptotic and dead cells with damaged membrane are permeated by Eb, emitting orange-red fluorescence. Necrotic cells also take up Eb and stain uniformly orange, fragmented apoptotic bodies are not seen in them (Ribble *et al.*, 2005, Liu *et al.*, 2015). In Ao/EtBr staining of cells treated with 100 µg/ml OIM, early and late apoptotis related morphological peculiarities were observed.

Phosphatidyl serine (PS) is a membrane phospholipid seen on the cytoplasmic side of the membrane in normal cells, but gets externalized in apoptotic cells so as to facilitate phagocytic recognition. This occurs prior to the nuclear changes in apoptosis. Annexin V can specifically bind to PS, because of its high affinity to the latter, and hence is a positive indicator of apoptosis. Hoechst stain is taken up by the nuclei of both live and dead cells, and emits blue fluorescence. In apoptotic cells, it reveals brightly stained appearance with condensed chromatin in the early stages, but shows fragmented nuclei, discernible as blue beads in late stages. Hence, in double staining, annexin V positive cells indicate death (apoptosis and necrosis) and Hoechst stain reveals nuclear fragmentation, if any, characteristic of only apoptotic cells, but not necrotic cells (Plesca *et al.*, 2008). In the results of the present work, fluorescent microscopic observation revealed corresponding apoptotic changes as above, but the number of cells revealing apoptotic morphology was less in both concentrations of OIM used.

From the study of cytological changes induced by OIM in HeLa cells, it is suggestive that the extract induces cytotoxicity through apoptosis and cell cycle arrest at G_1/S phase, but still higher concentrations of OIM may be required to produce significant results.

Chapter 4

Toxicological evaluation and study of *in vivo* anti cancer activities of *Oroxylum indicum* Vent.

4.1. Introduction

4.2. Materials and methods

4.2.1. Preparation of OIM extract

4.2.2. *Animals*

4.2.3. Cell lines

4.2.4. Diagnostic kits

4.2.5. Toxicity studies of OIM

4.2.6. In vivo anti cancer studies

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4.3. Results

4.3.1. Toxicity studies of OIM

4.3.2. Effect of OIM on ascites tumor

4.3.3. Effect of OIM on solid tumor

4.3.4. Effect of OIM and CTX on hematology

4.4. Discussion

4.1. Introduction

Animal models are important tools for studying the preclinical investigation of anti-cancer therapeutics and cancer prevention; data from such studies provide insight into the genetic mechanisms underlying malignant transformation and cancer progression. Mouse models recapitulate the human malignant diseases and serve as preclinical tools, which help in translating the basic knowledge into treatment and prevention of cancer in humans (Yee *et al.*, 2015). Anti cancer agents- of plant origin or otherwise - either bring about the inhibition/suppression of carcinogenesis, thereby acting as chemopreventive agents, or they pose toxicity to the cells in already developed tumors, thereby reducing tumor burden (Ruby *et al.*, 1995, Zhang *et al.*, 2014). In the previous chapter, the *in vitro* cytotoxic activity of OIM extract was investigated in DLA and EAC cell lines. The present study is extended to the evaluation of anti cancer activity of OIM in DLA and EAC induced transplantable solid and ascitis tumor models.

Ehrlich ascites carcinoma is a rapidly proliferating, experimental transplantable tumor maintained in outbred mice by a series of intraperitoneal passages. It was originally identified as a murine mammary adenocarcinoma, which later got adapted to ascites form (Jaganathan *et al.*, 2010, Ozaslan *et al.*, 2011), and has been exploited for many chemotherapeutic studies (Islam *et al.*, 2012, Gayatri *et al.*, 2015, Osman *et al.*, 2015). The antitumor activity of any agent against Ehrlich ascites can be assessed by cytological examination of the ascites cells post treatment, calculating the increase in survival time and/or by measuring the amount of ascites formed after treatment (Jaganathan *et al.*, 2010). Our study is relied on the evaluation of the anti cancer activity of *O. indicum* Vent. root bark, in EAC cells, in terms of its effect on increase in average life span.

Earlier models of evaluation of anti-tumor activity included murine models induced with ascitic leukemia using different types of cancer cell lines (Skipper *et al.*, 1964, Teicher and Beverly, 2006), but were not adequate enough for

identification of therapeutic agents against solid tumors (Schein and Scheffler, 2006, Talmadge *et al.*, 2007). Subsequent development of solid tumor models involved inoculation of tumor cells by intraperitoneal, subcutaneous or intravenous routes in animal models. In our study, the anti proliferative efficacy of the extract has been evaluated using the solid tumor model in mice, inoculated intramuscularly with the DLA cell line, which have been known to be in use for many similar investigations before (Babu *et al.*, 1995, Natesan *et al.*, 2007b).

In solid and ascites tumor studies with OIM, standard drug cyclophosphamide (CTX) was used as the positive control. In the context of serious side effects posed by chemotherapeutic agents used in cancer therapy such as CTX, a surge has been observed in developing complementary medication from plant sources, which pose lesser or no side effects. CTX is a common chemotherapeutic agent (Groopman and Itri, 1999), which is also known to cause myelosuppression and anaemia (Thews *et al.*, 2001a). Along with the comparison of CTX and OIM, the hematological changes (hemoglobin and total WBC count) of the animals were also tracked in the study.

Though natural plant products have been used as therapeutic agents from time immemorial, indiscriminate oral administration can pose adverse toxic effects, which is especially true, in the case of crude extracts from plants. So the toxicity/safety profile of plant extracts needs to the scientifically validated in animal models (Agbaje *et al.*, 2009, Olaniyan *et al.*, 2016). The current study also includes the acute and sub acute toxicity analysis of OIM in male and female BALB/c mice.

4.2. Materials and methods

4.2.1. Preparation of OIM extract

OIM extract was prepared according to the method given in Chapter 2, section 2.2.1., and was dissolved in distilled water and diluted to required concentrations for *in vivo* studies.

4.2.2. *Animals*

Male and female BALB/c mice (25-30 g) were procured and maintained as described in Chapter 2, section 2.1.6.

4.2.3. Cell lines

DLA and EAC cells lines were used to induce solid and ascites tumors respectively in animals. The procurement and maintenance of cells lines is explained in Chapter 2, section 2.1.7.

4.2.4. Diagnostic kits

Diagnostic kits for the estimation of Glutamate oxaloacetate transaminase (GOT), Glutamate pyruvate transaminase (GPT), Alkaline phosphatase (ALP), Creatinine, Urea and Hemoglobin were purchased as per list attached to Chapter 2, section 2.1.2.

4.2.5. Toxicity studies of OIM

Analysis of acute or sub acute toxicity, if any, of OIM extract was conducted in murine models.

4.2.5.1. Acute toxicity analysis

The *in vivo* anti cancer studies were preceded by acute toxicity analysis of OIM extract in male and female BALB/c mice. Animals were divided into six groups of three animals each, belonging to each sex. Prior to the experiment, all the animals were fasted overnight. A single dose of the extract was administered orally at dosages 0.05, 0.5, 1, 2, and 4 g/kg., b.wt. The animals were observed for mortality or behavioral changes, such as increased motor activity, tremors, lacrimation, diarrhea, muscle spasm etc., periodically for the

first 24 hours of the study, and then daily for subsequent 14 days according to guidelines of the Organization for Economic Cooperation and Development-423 (O.E.C.D., 2001). The change in body weight of the animals was recorded at weekly intervals.

The dose of OIM for *in vivo* studies was determined according to the observations in acute toxicity study.

4.2.5.2. Sub-acute toxicity analysis

Male and female BALB/c mice (25 - 30 g) were divided into four groups with 10 animals each belonging to each sex. Group I served as normal reference without drug administration; Group II, III, and IV were orally administered with OIM extract at daily dosages - 100, 200 and 400 mg/kg., b.wt. respectively for 14 consecutive days. The animals were observed for mortality or behavioral changes, if any. Changes in body weight of the animals were recorded periodically (every 3rd day). On day 15, the animals were sacrificed by ether anesthesia. Blood was collected through cardiac puncture. Non coagulated blood collected in heparinised tubes was used for the estimation of hemoglobin content (given in Chapter 2, section 2.2.4.1) and total WBC count (Chapter 2, section 2.2.4.2). The remaining coagulated blood was centrifuged at 2,500 rpm for 10 min to collect serum. Serum thus collected was used for the estimation of GOT/AST (Chapter 2, section 2.2.5.1), GPT/ALT (Chapter 2, section 2.2.5.2), ALP (Chapter 2, section 2.2.5.3), creatinine (Chapter 2, section 2.2.5.6) and urea (Chapter 2, section 2.2.5.7) using standardized kit procedures. Organo-somatic index/relative organ weight (ratio of organ weight to the whole body weight) of liver, kidney, spleen, heart and brain was recorded. Tissue sections from liver, kidney and small intestine were fixed in 10% buffered formalin and histologically examined under light microscope (400 x) (Chapter 2, section 2.2.7).

4.2.6. In vivo anti cancer studies

The anti cancer activity of OIM extract was studied in DLA induced solid tumor models and EAC induced ascites tumor models.

4.2.6.1. Dosage of OIM for in vivo studies

The dosage was determined based on acute toxicity analysis conducted as per OECD guidelines 423, which revealed that the OIM extract did not pose mortality up to a dose of 2 g/kg b.wt. Throughout the period of study, no toxicity symptoms were noticed in the dosage group up to 2 g/kg b.wt.- and the body weight of the animals showed no significant variation. LD_{50} value was not determinable even with the highest dose used- 4g/kg b.wt. For further studies, 400 mg/kg b.wt. of OIM ($1/10^{th}$ of the highest dose used in acute toxicity study) was selected as high dose (OIMH) and 200 mg/kg b.wt. of OIM (1/2 of OIMH) as low dose (OIML).

4.2.6.2. Effect of OIM extract on ascites tumor models

Ascites tumor was induced in BALB/c mice by intra-peritoneal injection of $1x10^6$ EAC cells/ animal. Animals were divided into four groups, each with 6 members. Group I served as untreated negative control. Group II served as the standard or positive control group treated with cyclophosphamide (CTX) (i.p.) at a dosage of 10mg/kg b.wt. Groups III and IV were the extract treated groups receiving oral administration of OIM extract at low (OIML- 200 mg/kg b.wt.) and high doses (OIMH- 400 mg/kg b.wt.) respectively. Drug administration started 24 hours after tumor inoculation and continued for 10 consecutive days. The death of the animals due to tumor burden was noted every day and the percentage of increase in lifespan (% ILS) was calculated using the formula [(T-C)/C] x 100, where 'T' and 'C' are the mean survival days of treated and control animals respectively (Mazumdar *et al.*, 1997).

4.2.6.3. Effect of OIM extract on solid tumor models

To induce solid tumor, DLA cells ($1x10^6$ cells/animal) were injected intramuscularly on the right hind limb of mice. Grouping of animals and drug

treatment protocol followed was the same as that of ascites tumor model. The tumor development was determined by measuring the diameter of growth in two perpendicular planes using vernier calipers at fixed intervals (on each 3rd day after induction, up to day 30) and the volume was calculated using the formula, $V= 4/3\ \pi\ r_1^2 \times r_2$, where r_1 and r_2 represent the minor and major radii of the tumor at two different planes. The percentage of inhibition of tumor volume in animals was calculated as:-

% of inhibition = (Vc-Vt)/Vc × 100 where, Vc and Vt are the tumor volumes of control and treated animals on 30th day (Ramnath *et al.*, 2002).

4.2.7. Comparison of hematological parameters between CTX and OIM treated animals

Total WBC count and hemoglobin level of the animals in solid tumor study were tracked on every 3rd day to compare the effect of CTX and OIM extract on hematological parameters of treated mice. Blood was collected from the caudal vein into heparinized tubes. The blood hemoglobin level total WBC count was determined by the methods described in Chapter 2, section 2.2.4.1 and section 2.2.4.2 respectively.

4.2.8. Statistical analysis

The values were expressed as mean ± SD of 3, 10 and 6 animals per group in acute toxicity study, sub-acute toxicity study and anti cancer studies respectively. Statistical evaluation of the data was done by one way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when p value was <0.05.

4.3. Results

4.3.1. Toxicity studies of OIM

4.3.1.1. Acute toxicity

The acute toxicity studies revealed that the administration of OIM extract was safe up to 2g/kg b.wt, with no toxicity or mortality (as described in section 4.2.5.1 of this chapter). The change in body weight of animals during the study period has been represented in figure 4.1.

4.3.1.2. Sub-acute toxicity

In the sub-acute toxicity study, no mortality was observed in any group. The parameters assessed- track of body weight gain/loss (figure 4.2.), total WBC count, hemoglobin level, serum titres of GOT, GPT, ALP, creatinine or urea (table 4.1.) showed no significant changes in the OIM treated groups, when compared to the normal reference. The organosomatic index of liver, heart, kidney, brain and spleen also showed no significant changes from normal (table 4.2.). Figure 4.3. reveals that the histological sections maintained normal micro architecture of liver, kidney, spleen and small intestine in the OIM treated groups.

Figure 4.1. The change in body weight of animals [a) male and b) female] in acute toxicity testing of OIM extract.

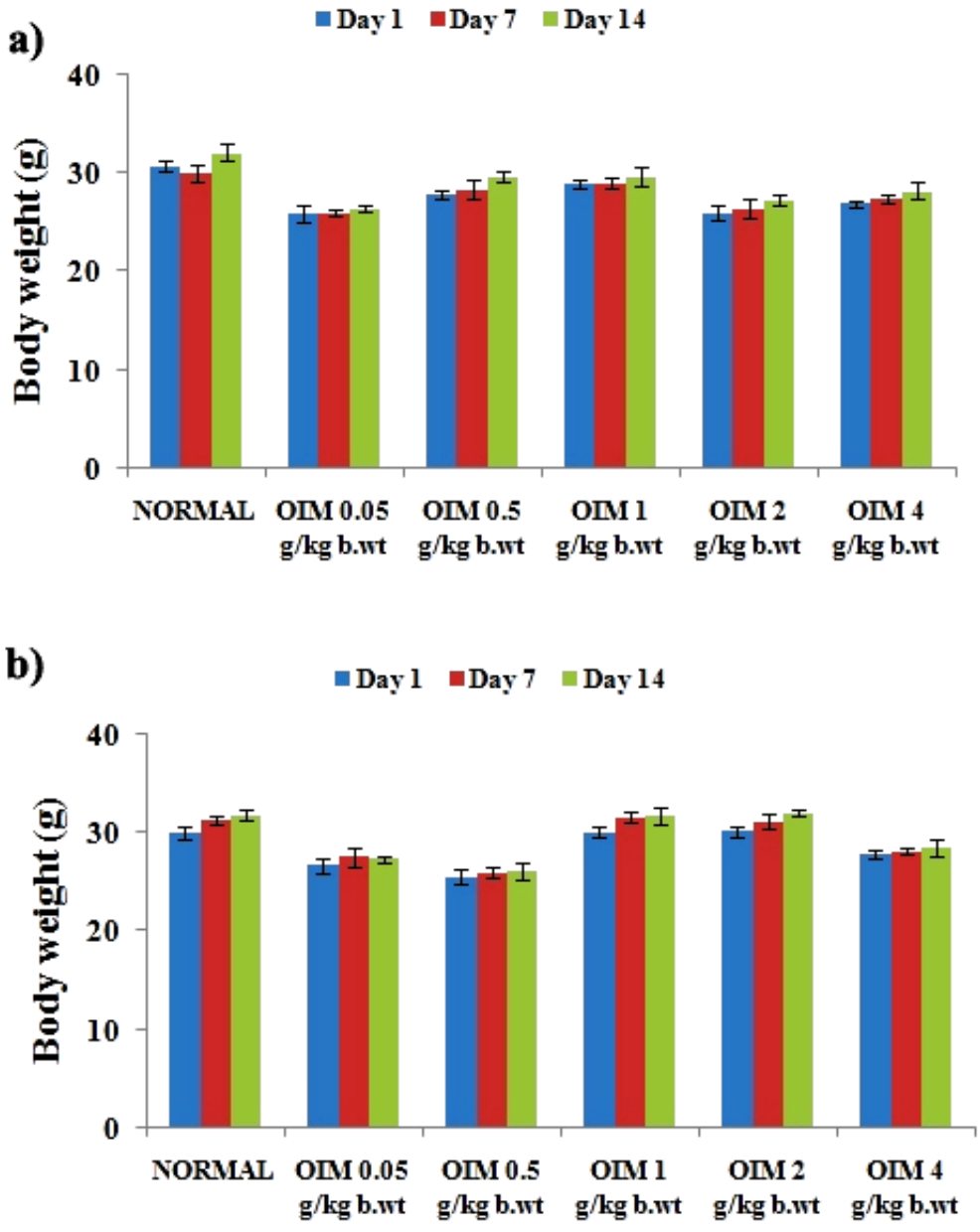


Figure 4.2. The change in body weight of animals [a) male and b) female] in sub-acute toxicity testing of OIM extract.

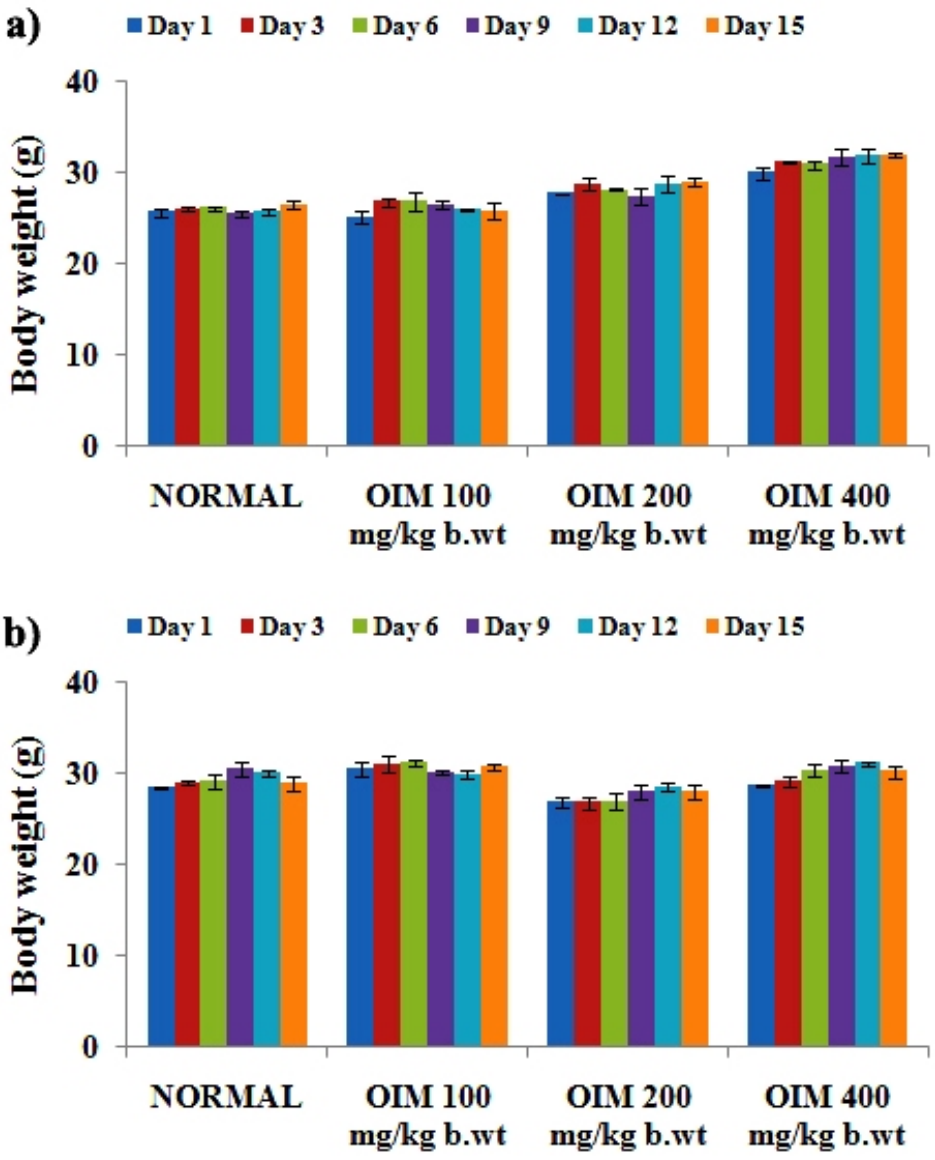


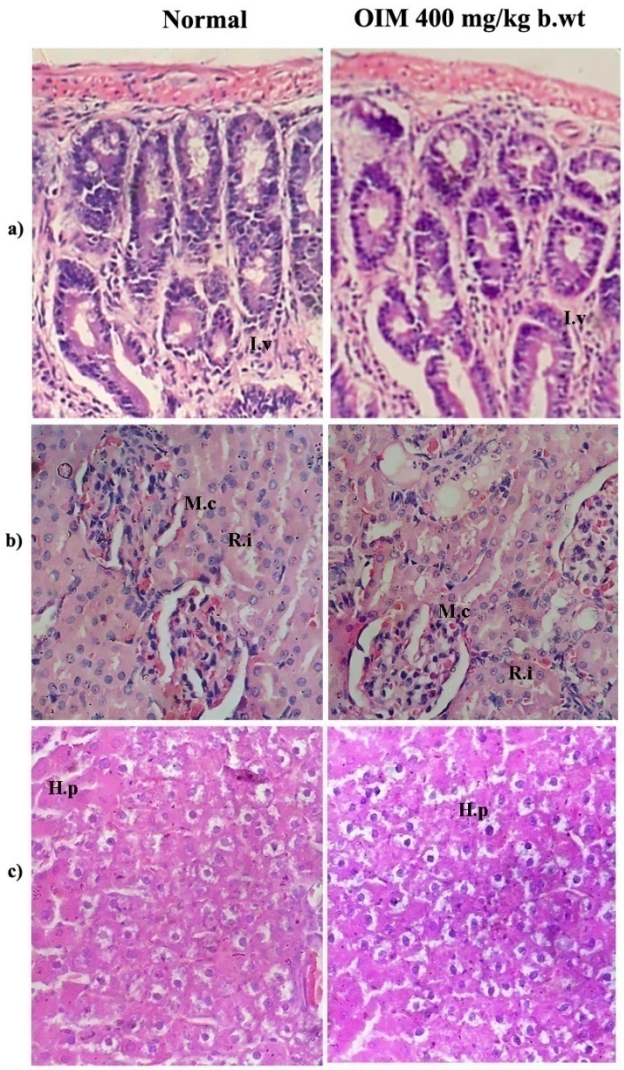
Table 4.1. Hematological parameters in sub-acute toxicity testing of OIM extract in male (M) and female (F) BALB/c mice

Dosage groups (mg/kg b.wt)	Sex	Total WBC count ('n' cells/mm3)	Hb (g/dL)	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	Creatinine (mg/dL)	Urea (mg/dL)
Normal	**M**	13833.3 ± 163.4	12.54 ± 0.64	105.8 ± 7.74	47.31 ± 9.51	58.018 ± 5.49	0.630 ± 0.015	14.65 ± 1.13
	F	14119.1 ± 203.2	12.94 ± 0.54	115.6 ± 8.13	38.17 ± 6.02	61.118 ± 3.99	0.642 ± 0.009	15.11 ± 1.09
OIM 100	**M**	13683.6 ± 133.1	13.41 ± 0.22	108.7 ± 7.54	35.53 ± 10.02	56.562 ± 6.06	0.633 ± 0.004	15.21 ± 0.86
	F	13442.4 ± 129.1	12.66 ± 0.37	112.1 ± 6.38	46.64 ± 5.04	53.86 ± 4.74	0.662 ± 0.020	14.79 ± 0.94
OIM 200	**M**	14001.2 ± 198.2	13.05 ± 0.54	121.8 ± 8.11	41.82 ± 7.27	57.64 ± 7.06	0.596 ± 0.013	14.44 ± 0.97
	F	13678.3 ± 148.5	13.14 ± 0.86	122.2 ± 6.84	39.98 ± 8.54	51.009 ± 8.77	0.625 ± 0.008	14.55 ± 1.06
OIM 400	**M**	14101.1 ± 153.6	12.79 ± 0.39	115.7 ± 7.94	41.18 ± 7.28	60.19 ± 5.85	0.638 ± 0.011	15.09 ± 1.20
	F	13771.3 ± 211.2	14.04 ± 0.70	107.1 ± 5.38	42.17 ± 8.35	54.58 ± 6.19	0.651 ± 0.010	15.48 ± 2.12

Table 4.2. The organosomatic index of liver, heart, kidney, brain and spleen in sub-acute toxicity testing of OIM extract in male (M) and female (F) BALB/c mice

Dosage groups (mg/kg b.wt)	Sex	LIVER	HEART	KIDNEY	SPLEEN	BRAIN
Normal	**M**	5.424 ± 0.11	0.589 ± 0.06	0.515 ± 0.09	0.331 ± 0.17	1.516 ± 0.14
	F	4.864 ± 0.04	0.610 ± 0.05	0.532 ± 0.11	0.305 ± 0.20	1.469 ± 0.09
OIM 100	**M**	4.653 ± 0.03	0.577 ± 0.05	0.544 ± 0.07	0.296 ± 0.11	1.499 ± 0.23
	F	5.316 ± 0.15	0.569 ± 0.09	0.526 ± 0.06	0.341 ± 0.23	1.501 ± 0.16
OIM 200	**M**	4.944 ± 0.23	0.607 ± 0.11	0.532 ± 0.09	0.306 ± 0.21	1.487 ± 0.08
	F	5.102 ± 0.18	0.595 ± 0.07	0.548 ± 0.12	0.289 ± 0.09	1.479± 0.12
OIM 400	**M**	5.008 ± 0.10	0.562 ± 0.06	0.519 ± 0.05	0.326 ± 0.08	1.515 ± 0.16
	F	4.861 ± 0.18	0.585 ± 0.04	0.532 ± 0.06	0.322 ± 0.15	1.487 ± 0.11

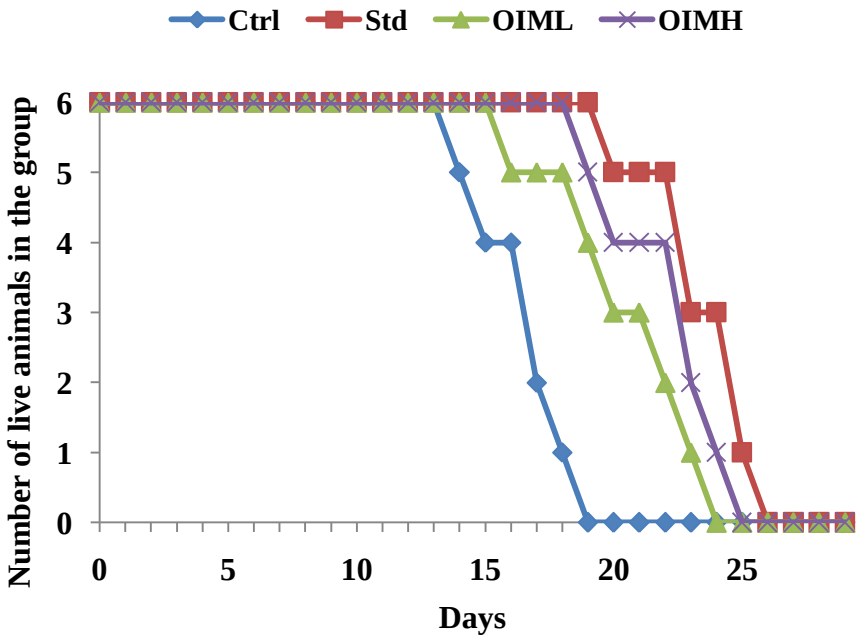
Figure 4.3. Histology of a) small intestine b) kidney and c) liver in sub-acute toxicity testing of OIM extract (400 mg/kg b.wt) by haematoxylin-eosin staining, viewed under 100 x magnification in compound light microscope. Intestinal sections of both normal and OIM treated animals represent normal structure of villi (I.v); the Malpighian corpuscles (M.c) and renal interstitial cells (R.i) in both normal and OIM treated animals show normal structure, with no evident lymphocyte infiltration; the hepatic parenchyma (H.p) of both normal and OIM treated group show similar architecture, with no profound devastating changes.



4.3.2. Effect of OIM on ascites tumor

All animals developed ascites tumor on receiving intra peritoneal inoculation of EAC cells. When the untreated control group sustained only for an average survival period of 16.67 ± 1.86 days, treatment with OIM extract at 400 and 200 mg/kg b.wt significantly increased the average life span of animals to 22.5 ± 2.43 ($p < 0.01$) and 20.67 ± 2.94 ($p < 0.05$) days respectively (figure 4.4.). OIMH and OIML treatments elevated the percentage increase in survival by 34.97 and 23.99% respectively. The animals under standard drug CTX survived for 23.67 ± 2.16 days (percentage increase in survival– 41.99%).

Figure 4.4. Effect of OIM extract treatment on average survival period of animals in ascites tumor study

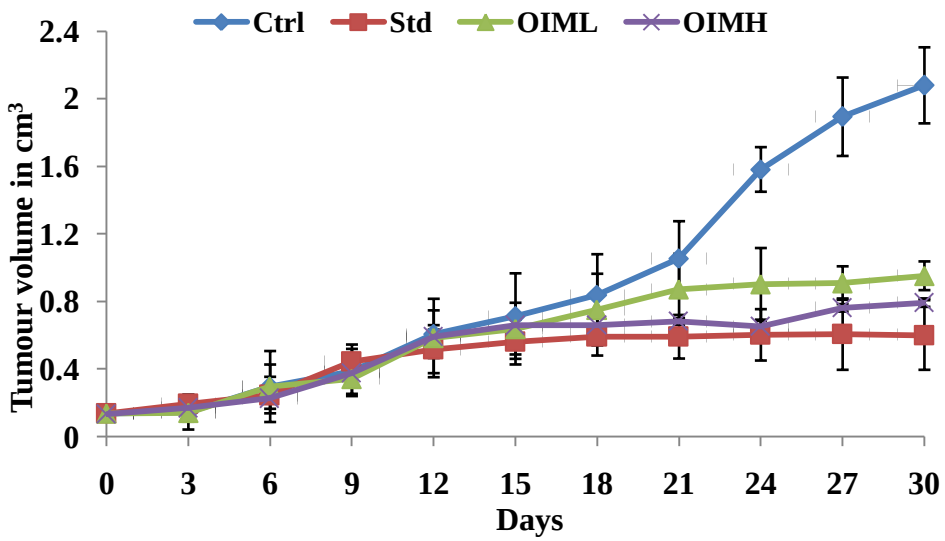


4.3.3. Effect of OIM on solid tumor

All animals having received intramuscular DLA injection developed tumors by the 6th day of induction, which grew in volume with successive days. Yet, compared to the final volume recorded on the 30th day in control group (2.07 ± 0.23 cm^3), OIM extract treated groups showed significant decrease ($p < 0.01$)

in tumor volume up to 0.79 ± 0.03 cm^3 in OIMH and 0.95 ± 0.09 cm^3 in OIML. OIMH and OIML treatment brought about 61.84% and 54.21% inhibition of tumor growth respectively, closer to that effected by CTX treatment in standard animal group (71.05%) (figure 4.5.).

Figure 4.5. Graph showing the effect of OIM extract treatment on growth pattern in solid tumor models.



4.3.4. Effect of OIM and CTX on hematology

Though it was evident that animals treated with CTX exhibited substantial anti-tumor activity in solid tumor models, there was gradual reduction in the hemoglobin concentration (Figure 4.6.) and total leucocyte count of the animals, which was not obviously seen in the control, OIML or OIMH groups (Figure 4.7.).

Figure 4.6. Effect of OIM extract treatment on change in hemoglobin concentration of solid tumor induced animals

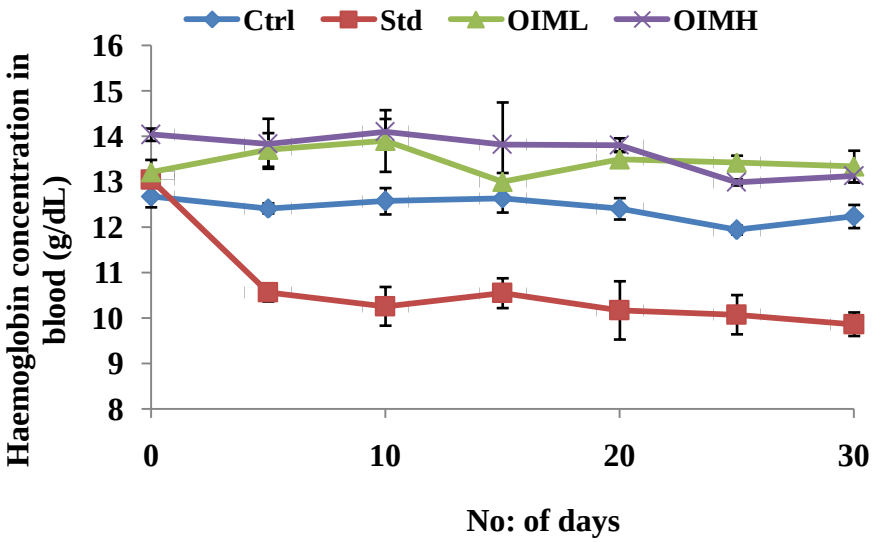
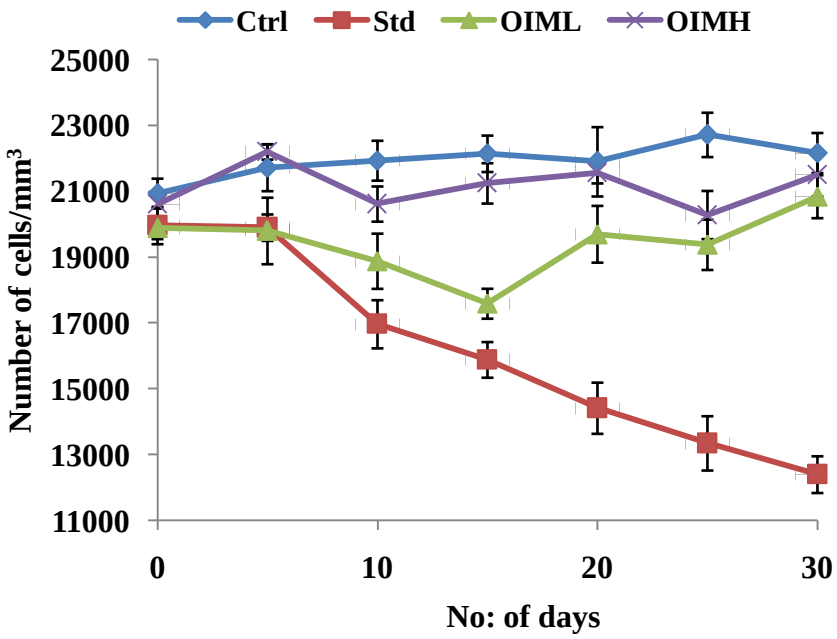


Figure 4.7. Effect of OIM extract treatment on change in total leucocyte count of solid tumor induced animals.



4.4. Discussion

Though plant derived products offer limitless capital for drug discovery, there exists only limited information about the toxic effects some of which may cause in living organisms. This is particularly true in the case of crude extracts which contain a cocktail of compounds with different biological effects, either useful or adverse. Hence, any study with plant extracts extrapolated to in vivo models should be preceded by standard toxicity analysis. The data derived from acute and subchronic toxicity studies is used to determine their safety to humans, particularly in the area of pharmaceutical research (Yuet Ping *et al.*, 2013). The observations made from acute and sub-acute toxicities of OIM indicated that it is safe to use in BALB/c mice up to a dose of 2 g/kg b.wt. This is also a positive indication which validates the use of *O. indicum* as an ingredient in Ayurvedic preparations (*Dasamoola*).

Prolongation of the life span of animals is a reliable criterion for adjudging an anticancer agent. Though death is inevitable, OIM in high dose showed significant increase in survival rate of mice, compared to untreated control. However, OIM extract at low dose (200 mg/kg b.wt) increased the life span of tumor bearing animals by merely 23.99%, but significantly different from the survival rate of untreated animals. Similar results of anti tumor activity in EAC induced tumors were previously reported by Samudrala *et al.* (2015) with *Alternanthera brasiliana* and Senthil Kumar *et al.* (2011) with *Prosopis glandulosa*. In EAC induced models, the survival time of mice and the final volume of the tumor are positively correlated, revealing its progressive growth pattern. Tumor growth is also enhanced by the amount of ascitic fluid in the mouse peritoneal cavity, which is an inflammatory exudate produced in response to EAC cell growth (Hartveit, 1961, Hartveit, 1966). In all EAC induced animals of our study, there was gradual increase noticed in ascites fluid volume, pertinent to the above concept.

Solid tumors (e.g., sarcomas, carcinomas, and lymphomas) are abnormal, localized masses of tissues devoid of cysts or liquid areas, of either benign or malignant nature (Mohan, 2002, Patil *et al.*, 2006). Dalton's lymphoma spontaneously originates in thymus of murine animals in transplantable form (Goldie and Dingman Felix, 1951, Koiri *et al.*, 2017), and hence, widely used in cancer research, with tumor volume measurement as a reproducible parameter. The current study also revealed that oral administration of OIM extract brought about gradual, but significant reduction in tumor volume which was evident from the 12^{th} day of induction onwards up to day 30, signifying the anti cancer property of the extract, consistent with previous anti cancer studies of plant extracts (Natesan *et al.*, 2007a, Jeena *et al.*, 2015) using DLA induced tumors.

In our study, it was observed that, though cyclophosphamide treatment increased the average life span in ascites tumor models and reduced solid tumors, the animals developed anemia and suffered a decrease in total leucocyte count, as revealed from the hematological examination. Contrarily, the study reveals that OIM extract exerts simultaneous anti tumor and myeloprotective activities similar to other plant extracts like *Melothria heterophylla* (Lour.) and *Piper betle* rich in bioactive compounds (Mondal *et al.*, 2013, Alam *et al.*, 2015), possibly due to the synergistic effect of these substances present in them.

The results of *in vivo* studies were also reflective of the cytotoxic effect of OIM in DLA and EAC cells, *in vitro*, as revealed from the previous chapter. With previously reported anti cancer properties (Peterson and Barnes, 1993, Losso *et al.*, 2004b, Khoo *et al.*, 2010, Peng *et al.*, 2011, Kasala *et al.*, 2015, Liu *et al.*, 2016) of flavonoids and phenolic compounds reportedly present in *O. indicum* root bark (Zaveri *et al.*, 2008), it is again conclusive that these bioactive constituents may be responsible for *in vitro* and *in vivo* anti cancer activities of the extract. This may be accomplished by the control of cancer cell survival and growth by modulation of key cell signalling pathways, thereby decreasing

tumor growth. However, it is also noteworthy that anti oxidant compounds such as polyphenols that can inhibit free radical generation and oxidative stress in cells, thereby acting as chemopreventive agents (Owen *et al.*, 2000). Moreover, a positive correlation has been established between inflammation and cancer; the former being an important trigger for tumor progression (Coussens and Werb, 2002). Hence, in the following chapter, we have extended the study for evaluating the anti oxidant, anti inflammatory and anti carcinogenic effects of OIM.

Chapter 5

Antioxidant, anti inflammatory and anti carcinogenic properties of *Oroxylum indicum* Vent.

5.1. Introduction

5.2. Materials and Methods

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5.2.2. Chemicals

5.2.3. Animals

5.2.4. In vitro antioxidant activity

5.2.5. In vivo antioxidant activity

5.2.6. In vivo anti inflammatory activity

5.2.7. Inhibition of papillomagenesis in DMBA-croton oil induced skin tumors

5.2.8. Statistical analysis

5.3. Results

5.3.1. In vitro antioxidant property of OIM extract

5.3.2. In vivo antioxidant activity of OIM extract

5.3.3. In vivo anti inflammatory activity of OIM extract

5.3.4. Inhibition of skin tumorigenesis by OIM extract in DMBA-croton oil induced papilloma

5.4. Discussion

5.1. Introduction

The previous chapters have revealed that the extract of *O. indicum* Vent. root bark (OIM) possesses anti-proliferative and cancer preventive activity, through *in vitro* and *in vivo* studies. The extract was found to be preventive against cancer cell proliferation, rather than being cytotoxic. This prompted an investigation on its antioxidant and anti inflammatory activities, which are the foci of this chapter. Overproduction of free radicals in cells can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, ageing, and other degenerative diseases in humans (Rahman, 2007). Consequently, a correlation has been derived between the anti oxidant nature of a wide array of biologically obtained compounds and their efficacy in cancer therapy (Pham-Huy *et al.*, 2008). A number of plants with characteristic anticancer activities have also been proved to yield compounds with remarkably high anti-oxidant properties. Hence, the study aims at an evaluation of the *in vitro* and *in vivo* antioxidant activities of OIM extract. The *in vitro* assays are based on the inhibition of production of oxidized products/free radical species in the presence of antioxidants in the assay system. This results in a reduction of the end point by scavenging free radicals. Electron acceptors like molecular oxygen readily react with free radicals to generate reactive oxygen species (ROS) such as superoxide anions (O^{+2}), hydrogen peroxide (H_2O_2) and hydroxyl radicals (^{+}OH). Antioxidant assays rely on the inhibition of superoxide and hydroxyl radicals generated in the respective reaction systems (Rafat Husain *et al.*, 1987, Kunchandy and Rao, 1990). Spectrophotometrically detectable free radicals, such as 2,2'-azino-bis (3-ethylbenzenthiazoline-6-sulphonic) acid radical ($ABTS^{+}$) and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{+}$) are also used for evaluating the antioxidant property of plant extracts or plant-derived compounds (Choi *et al.*, 2002). FRAP assay is an automated test to measure the "antioxidant power" or the ferric ion reducing ability of substances that depends on the principle that the reduction of ferric to ferrous

ion yields a colored ferrous-tripyridyltriazine complex in the presence of the antioxidant test substance in question (Benzie and Strain, 1996b). The present study evaluates the *in vitro* antioxidant activity of OIM extract using the superoxide scavenging, hydroxyl scavenging, DPPH scavenging, ABTS scavenging and FRAPS assays.

To evaluate *in vivo* the antioxidant activity of OIM extract, oxidative stress induced models induced with sodium fluoride (NaF) were used. Sodium fluoride intoxication is reported to generate free radicals in the body, thereby leading to inhibition of antioxidant enzyme systems such as catalase, glutathione peroxidase, superoxide dismutase, and decreases the glutathione content (Strunecka *et al.*, 2007). The study evaluated the ability of OIM extract in restoring antioxidant defense status in NaF-induced animals.

The inter-relationship between inflammation and cancer has been recognized for long, by experimental and statistical data. Inflammation has invariable role in the establishment, progression and aggressiveness of many malignancies. During inflammation, leukocytes secrete a variety of proliferative cytokines and angiogenic factors to the site of tissue damage for the mediation of proper wound healing, stimulation epithelial cell proliferation etc. However, if these get exaggerated, could lead to dysplasias and ultimately cancer. Tumor cells themselves also produce various cytokines and chemokines that attract leukocytes, which in turn produce cytokines and chemokines that stimulate further tumor cell proliferation (Landén *et al.*, 2016). Inflammation is considered to be an important component of tumor progression (Coussens and Werb, 2002). To seek the basis for the anti proliferative activity of the OIM extract, the study included its ability in mitigating inflammation induced in murine models induced with paw edema.

The anti inflammation-based ability of the OIM extract in preventing carcinogenesis was studied using the DMBA-croton oil-induced papilloma bearing animals. The major stages of chemical carcinogenesis have been

deduced over the past ~50 years, primarily from animal model studies (and particularly from studies using the mouse skin model); these stages are termed initiation, promotion, and progression. Tumor initiation begins when DNA in a cell or population of target cells is damaged by exposure to exogenous or endogenous carcinogens leading to mutations in critical target genes. The responsiveness of initiated cells to their microenvironment gives them a growth advantage relative to normal cells under certain conditions (Weston and Harris, 2003, Ruddon, 2007a). The tumor promotion stage is characterized by selective clonal expansion of the initiated cells, a result of the altered expression of genes whose products are associated with hyperproliferation, tissue remodeling, and inflammation. Tumor promoters are generally non mutagenic, are non carcinogenic by themselves, and do not often require metabolic activation to exert their biologic effects. A tissue, once exposed to a tumor initiator, the exposure to promoter reduces the latency period for tumorigenesis and increases the number of tumors formed in that tissue. In addition, they induce tumor formation in conjunction with a dose of an initiator that is too low to be carcinogenic alone. A widely used tumor promoter in murine skin carcinogenesis is croton oil, obtained from *Croton tiglium* seeds. Its most potent constituent is 12-otetradecanoylphorbol-13-acetate, which activates protein kinase C and promotes tumor development. Complete carcinogens are capable of both tumor initiation and promotion; e.g., benzo[*a*]pyrene and 4-aminobiphenyl (Yuspa *et al.*, 1996). The main features of progression are karyotypic instability and malignant growth (Pitot, 1993). During tumor progression, preneoplastic cells undergo malignant transformation through a process of selection that is facilitated by progressive genomic instability and altered gene expression. While the processes involved in each stage of experimental chemical carcinogenesis also appear to be involved in human carcinogenesis, the temporal nature of initiation, promotion, and progression events is more complex. In addition, multiple mutational events are involved in the formation of human tumors. Genetic background and nutritional status can

dramatically affect susceptibility to a carcinogenic exposure in both experimental animals and humans. An understanding of the multistage nature of carcinogenesis has led to the discovery of mechanism-based inhibitors that target events associated with specific stages. It is desirable that the action of chemopreventive agents is manifested within the initiation and promotion processes of carcinogenesis. Further study of the cellular, biochemical, and molecular mechanisms associated carcinogenesis induced by chemicals and other types of carcinogens will lead to identification of effective strategies for cancer prevention (Abel *et al.*, 2009). The two-step chemical tumorigenesis model using DMBA and croton oil has been widely used to study the malignant conversion of mouse skin tumors. In two-step-papillomagenesis, the initiation stage is achieved through a single topical application of 7, 12-dimethyl-benz[a]anthracene (DMBA). Promotion occurs by repeated topical application of a tumor promoter (i.e., phorbol ester 12-O-tetradecanoylphorbol-13 acetate (TPA), which causes a sustained hyperplasia and inflammation, and then a selective clonal expansion into benign papillomas. The third stage, progression, is the malignant conversion of benign papillomas to invasive squamous cell carcinoma (SCC). The evolution of human SCC has many similarities with the above (Indra *et al.*, 2007).

5.2. Materials and Methods

5.2.1. Preparation of OIM extract

OIM extract was prepared according to the method given in Chapter 2, section 2.2.1., and was dissolved in distilled water and diluted to required concentrations for *in vivo* studies.

5.2.2. Chemicals

The chemicals used for *in vitro* antioxidant assays- NBT, EDTA, Riboflavin, Deoxyribose, Potassium dihydrogen phosphate, ferric chloride and ABTS were purchased as per list in section 2.1.2., chapter 2. Sodium fluoride, ascorbic acid,

NBT, EDTA, riboflavin, DTNB, reduced glutathione, hydrogen peroxide, sodium dodecyl sulphate and thiobarbituric acid for *in vivo* antioxidant studies, carrageenan, dextran and formalin for *in vivo* anti inflammatory studies, and DMBA and croton oil for anti carcinogenic study were purchased as per list attached to Chapter 2, section 2.1.2. All other chemicals used were of analytical grade.

5.2.3. Animals

Sprague Dawley rats for *in vivo* antioxidant study, Swiss albino mice for *in vivo* anti inflammatory study and BALB/c mice for anti carcinogenic study were procured and maintained as per method described in section 2.1.6., Chapter 2.

5.2.4. In vitro antioxidant activity

The *in vitro* anti oxidant activities of OIM extract was determined by the superoxide scavenging assay, hydroxyl radical scavenging assay, DPPH radical scavenging assay, ABTS radical scavenging assay and Ferric reducing antioxidant power (FRAP) assay using the methods described in sections 2.2.9.1, 2.2.9.2, 2.2.9.3, 2.2.9.4 and 2.2.9.5 (Chapter 2) respectively.

5.2.4.1. Superoxide scavenging assay

OIM extract at concentrations ranging from 30-150 µg/ml was used to determine the superoxide radical scavenging activity as per method given in section 2.2.9.1, Chapter 2.

5.2.4.2. Hydroxyl radical scavenging assay

OIM extract at concentrations ranging from 20-100 µg/ml was used to determine the inhibition of hydroxyl radical formation *in vitro*, as per method given in section 2.2.9.2, Chapter 2.

5.2.4.3. DPPH radical scavenging assay

OIM extract at concentrations ranging from 25-125 µg/ml was used to determine the DPPH radical scavenging activity as per method given in section 2.2.9.3, Chapter 2.

5.2.4.4. ABTS radical scavenging assay

OIM extract at concentrations ranging from 3-15 µg/ml was used to determine the ABTS radical scavenging activity as per method given in section 2.2.9.4, Chapter 2.

5.2.4.5. FRAP assay

OIM extract at concentrations ranging from 4-20 µg/ml was used to determine the *in vitro* antioxidant activity by FRAP assay, as per method given in section 2.2.9.5, Chapter 2.

5.2.5. In vivo antioxidant activity

5.2.5.1. Grouping of animals

The antioxidant activity of OIM extract was determined against sodium fluoride mediated oxidative stress. Thirty rats were divided into five groups of 6 animals each. Group I was maintained as normal reference. Group II served as untreated negative control. Group III served as the standard or positive control group treated with ascorbic acid at a dose of 3 mM/day through drinking water. Groups IV and V were the extract treated groups receiving oral administration of OIM extract at low (OIML- 200 mg/kg b.wt.) and high doses (OIMH- 400 mg/kg b.wt.) respectively. The animals received pre-treatment for the first 7 days of the study as per the above protocol.

5.2.5.2. Oxidative stress induction

Oxidative stress induction was done in all groups except normal, from 8^{th} to 14^{th} day by administration of sodium fluoride (NaF) through drinking water at a concentration of 600 ppm/day.

5.2.5.3. Parameters assessed

The animals were sacrificed on the 15th day. The antioxidant status of the blood and liver tissue was determined as per method described in section 2.2.8., Chapter 2.

5.2.6. In vivo anti inflammatory activity

The *in vivo* anti inflammatory activity of OIM was determined using the carrageenan induced acute paw edema model, dextran induced acute paw edema model and the formalin induced acute paw edema model.

5.2.6.1. Grouping of animals

Animals for each study (acute- carrageenan/ acute- dextran/ chronic- formalin) were divided into four groups with 6 members each under the following treatment protocol for five consecutive days. Group I served as the untreated negative control. Group II was maintained as the standard or positive control group, treated with Diclofenac (i.p.) at a dose of 10mg/kg b.wt. Groups III and IV were treated with OIM extract, at dosages 200 (low dose - OIML) and 400 (high dose - OIMH) mg/kg b.wt.

5.2.6.2. Induction of acute paw edema

One hour after the last dose administration, acute paw edema was induced in all animals by sub-plantar injection of 0.02 ml of 1% carrageenan or 1% dextran in the right hand paw, after recording the paw volume. After induction, the paw volume was tracked hourly using vernier callipers up to the 6th hour (Winter *et al.*, 1962, Maity *et al.*, 1998).

The percentage of inhibition of acute paw edema was calculated as:-

% inhibition caused by the drug = $[(V_T - V_0)_{control} - (V_T - V_0)_{treated}]/ (V_T - V_0)_{control} \times 100$, where V_0 is the initial paw thickness and V_T is the paw thickness on the 3rd hour.

5.2.6.3. Induction of chronic paw edema

One hour after the last dose administration, chronic paw edema was induced in all animals by sub-plantar injection of 0.02 ml of 1% formalin in the left hand paw, after recording the paw volume. After induction, the paw volume was tracked every 24 hours using vernier callipers up to the day 6 (Chau, 1989).

The percentage of inhibition of chronic paw edema was calculated as:-

% inhibition caused by the drug = $[(V_T - V_0)_{control} - (V_T - V_0)_{treated}]/ (V_T - V_0)_{control} \times 100$, where V_0 is the initial paw thickness and V_T is the paw thickness on the 6^{th} day.

5.2.7. Inhibition of papillomagenesis in DMBA-croton oil induced skin tumors

5.2.7.1. Preparation of animals

Prior to the study, the animals were housed at a stocking rate of 3 members per cage. This evaded chances of fighting and consequent skin aberrations; aggressive members were maintained separately. A circular area of 2 cm diameter on the dorsal side of each mouse was shaved with a razor 2 days prior to the application of chemicals. The growth of hair follows a cyclic pattern with three phases- that proceeds from an active (anagen) phase through a regressive and shortening (catagen) phase to a resting (telogen) phase (Hardy, 1992). Those members showing a resting (telogen) phase in hair growth cycle were further selected for the experiment.

5.2.7.2. Grouping of animals and induction of skin papilloma

The animals were divided into five groups with six members each, based on the following topical application protocols. Group I was applied with the carcinogen alone, (DMBA) at a dose of 470 nmol (in 200 µL acetone) per mouse as single dose on day 1. Group II was applied with the promoter alone (1% croton oil in 200 µL acetone), 2 weeks after DMBA application, at a frequency of two times per week for 6 continuous weeks. Group III served as

the negative control group, treated with both DMBA (same regime as for group I) and croton oil (same regime as for group II). Group IV and V was also applied with both DMBA and croton oil (same regime as for group III) along with OIM extract at two doses respectively- low dose (OIML- 5% in 200 µL distilled water (d.w.) and high dose (OIMH)- 10% in 200 µL d.w., 30 minutes before each croton oil application.

Throughout the study period of 20 weeks duration, all animals were monitored for their food intake and toxicity symptoms, if any, such as weight loss or mortality. Skin tumor formation was recorded weekly and the percentage incidence of papilloma, cumulative count of papillomas developed, the number of papillomas developed per animal and the delay in tumour onset in each group were determined. Tumors >1 mm in diameter were included in the cumulative count if growth persisted for 2 weeks or more (Belman and Troll, 1972).

5.2.8. Statistical analysis

The values were expressed in mean ± SD of 3 independent experiments (for *in vitro* studies) or 6 animals per group (for *in vivo* studies). All groups were analysed for one way ANOVA by Dunnett's test using Graph Pad Instat software. The groups with $p<0.05$ were considered significant.

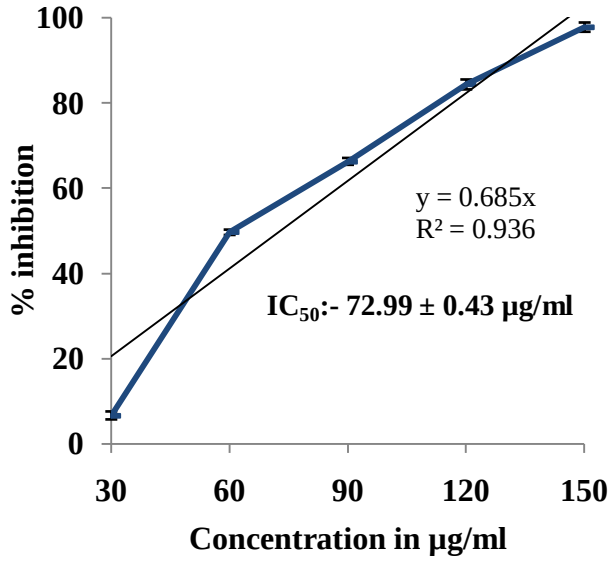
5.3. Results

5.3.1. In vitro antioxidant property of OIM extract

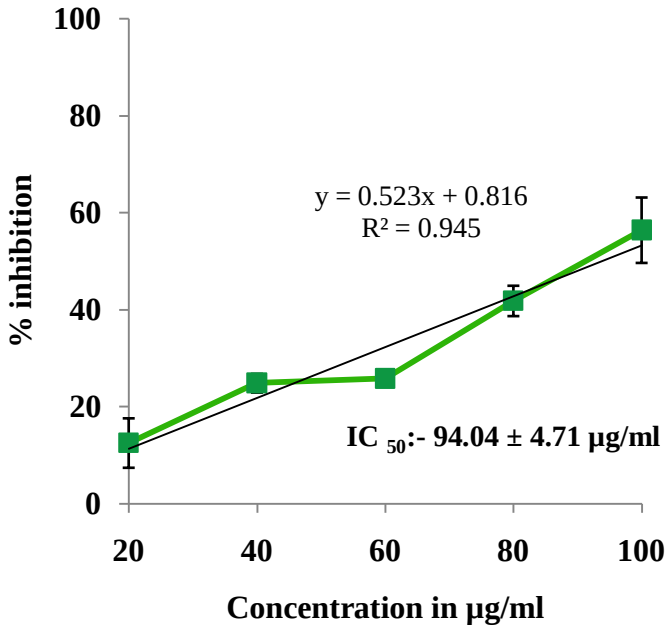
In the *in vitro* antioxidant assays, the OIM extract showed concentration-dependent activity, as represented in figure 5.1.a, b, c and d showing the percentage inhibition of superoxide, hydroxyl, DPPH and ABTS radicals by the extract at various concentrations. The IC_{50} values determined in the superoxide, hydroxyl, DPPH and ABTS scavenging assays were 72.99 ± 0.43, 94.04 ± 4.71, 60.26 ± 1.22 and 6.25 ± 0.09 µg/ml respectively. In the FRAP assay to determine the reducing power of OIM extract, the increase in reducing

equivalents of $FeSO_4.7H_2O$, determined from the standard graph (Figure 5.2.a) was found to be dependent on the concentration of OIM, as seen in figure 5.2.b. The EC_{50} value of OIM in FRAP assay was 13.13 ± 0.03 µg/ml.

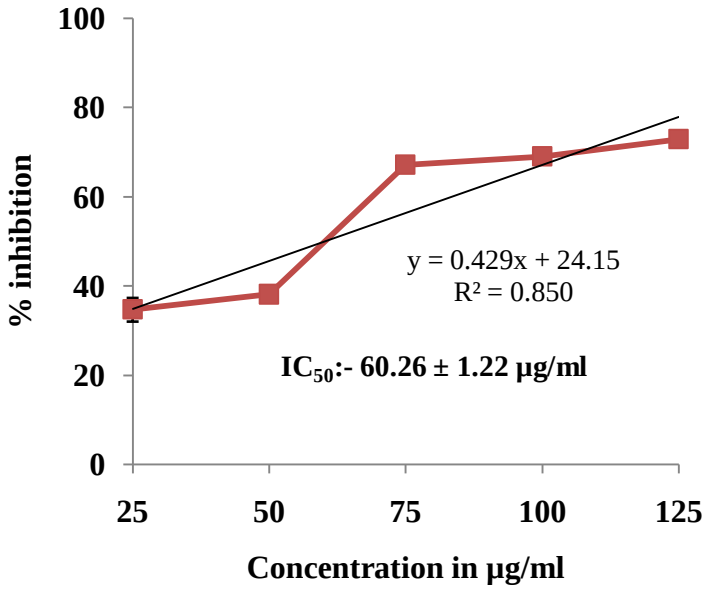
Figure 5.1. The percentage inhibition of superoxide, hydroxyl, DPPH and ABTS radicals (5.1.a, b, c, d) by OIM extract in *in vitro* antioxidant assays



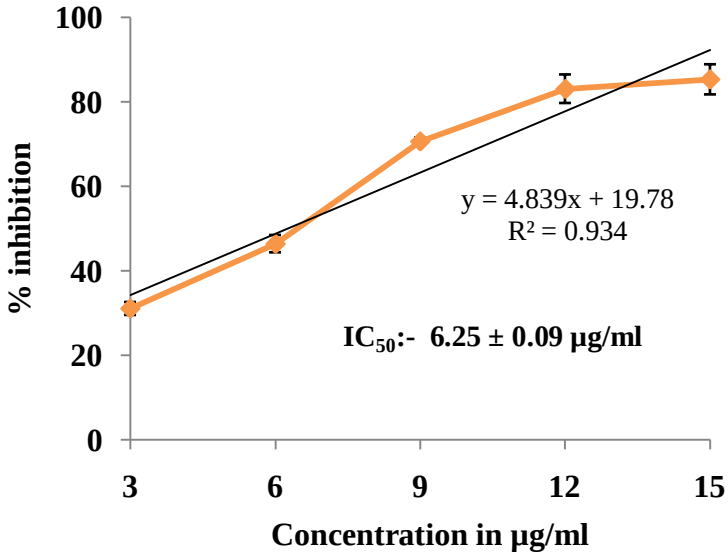
a)



b)

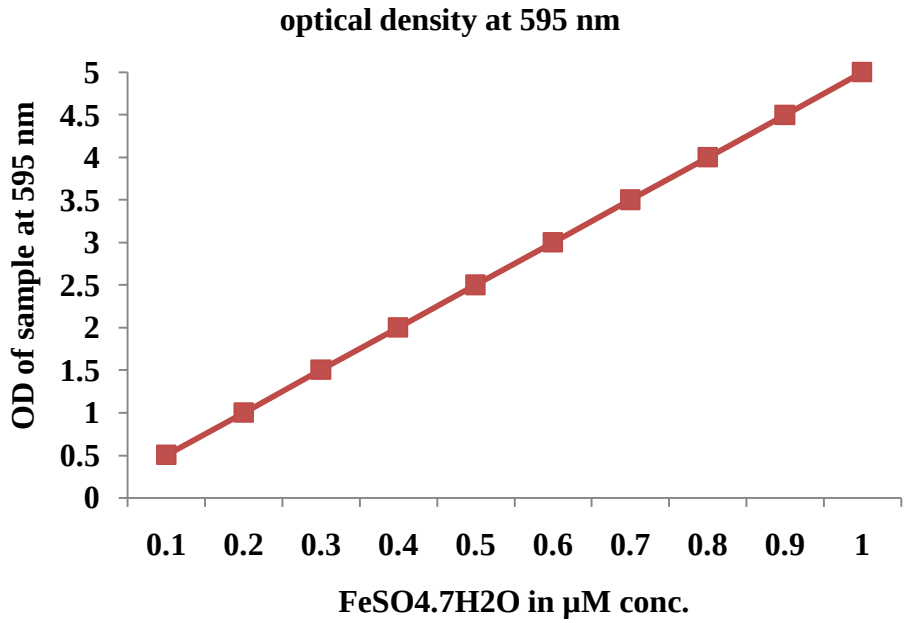

100
80
60
40
20
0
% inhibition
y = 0.429x + 24.15
R² = 0.850
IC50:- 60.26 ± 1.22 µg/ml
25
50
75
100
125
Concentration in µg/ml

c)

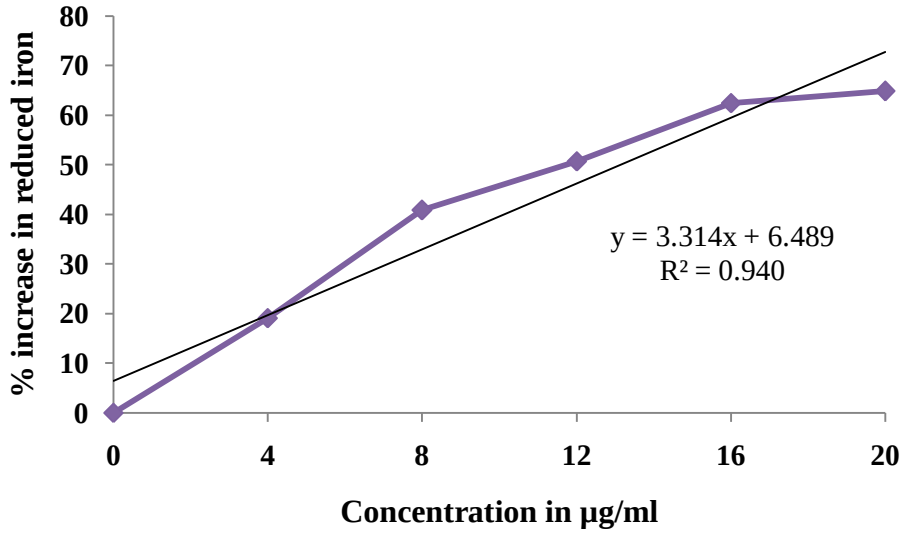

100
80
60
40
20
0
% inhibition
y = 4.839x + 19.78
R² = 0.934
IC50:- 6.25 ± 0.09 µg/ml
3
6
9
12
15
Concentration in µg/ml

d)

Figure 5.2. FRAP assay to determine the reducing power of OIM extract. (5.2.a. Standard graph representing the reducing equivalents of $FeSO_4.7H_2O$; 5.2.b. Graph showing reducing equivalents of $FeSO_4.7H_2O$ in the assay system, in the presence of various concentrations of OIM)



a)



b)

5.3.2. In vivo antioxidant activity of OIM extract

5.3.2.1. Anti-oxidant profile of blood

Compared to the normal reference animal group, the blood of untreated control rats induced with sodium fluoride showed significantly low ($p \leq 0.01$) levels of superoxide dismutase activity and glutathione concentration. Meanwhile, in the OIM administered animals, the anti-oxidant markers in blood showed significant elevation in a dose dependent manner, compared to the NaF stress induced control (Table 5.1). The effects of pre-treatment were similar to those shown by the ascorbic acid (standard/positive control) treated animals.

Table 5.1. Effect of OIM extract treatment on anti-oxidant profile of blood in sodium fluoride induced animals

Animal groups	SOD (U/g Hb)	GSH (nmoles/g Hb)
Normal	32.13 ± 2.99	12.13 ± 0.99
Control	25.13 ± 3.1[a]	8.13 ± 0.81[a]
Standard	32.13 ± 3.7[c]	11.73 ± 0.72[c]
OIML	28.13 ± 3.6[d]	12.09 ± 0.6[c]
OIMH	**31.13 ± 4.1[c]**	**12.16 ± 0.51[c]**

Values are expressed as mean ± SD for 6 animals.

[a]: $p < 0.01$ (control compared to normal),

[c]: $p < 0.01$, [d]: $p < 0.05$ (standard, OIML and OIMH compared to control)

5.3.2.2. Anti-oxidant profile of liver

Sodium fluoride induction had significant ($p \leq 0.01$) impact on antioxidant profile of liver in sodium fluoride induced untreated control group (Table 5.2), as evident from the decrease in reduced glutathione level, SOD activity and glutathione peroxidase activity. But, OIM pre-treated groups apparently restored near normal values of these parameters, despite sodium fluoride

exposure. There was notable decrease in the extent of lipid peroxidation in OIM treated animals (OIML and OIMH), (0.207 ± 0.016 and 0.203 ± 0.012 nmoles of MDA/mg protein, respectively) in comparison with the untreated control group (0.427 ± 0.006 nmoles of MDA/mg protein). Similar results were obtained in the standard/positive control treated with ascorbic acid (0.198 ± 0.008 nmoles of MDA/mg protein).

Table 5.2. Effect of OIM extract treatment on anti-oxidant profile of liver in sodium fluoride induced animals

Animal groups	**LPO (nmoles of MDA/mg protein)**	**SOD(U/mg protein)**	**GPx(U/mg protein)**	**GSH(nmoles/ mg protein)**
Normal	0.185 ± 0.005	0.213 ± 0.012	8.15 ±0.39	9.92 ± 0.133
Control	0.427 ± 0.006[a]	0.123 ± 0.010[a]	3.93 ± 0.30[a]	5.11 ± 0.102[a]
Standard	0.198 ± 0.008[c]	0.201 ± 0.007[c]	7.86 ± 0.73[c]	8.99 ± 0.110[c]
OIML	0.207 ± 0.016 [c]	0.165 ± 0.014[d]	8.14 ± 0.93[c]	7.36 ± 0.096[d]
OIMH	**0.203 ± 0.012 [c]**	**0.197 ± 0.009[c]**	**8.31 ± 1.01[c]**	**8.67 ± 0.167[c]**

Values are expressed as mean ± SD for 6 animals.

[a]: $p < 0.01$ (control compared to normal),

[c]: $p < 0.01$, [d]: $p < 0.05$ (standard, OIML and OIMH compared to control)

5.3.3. In vivo anti inflammatory activity of OIM extract

5.3.3.1. Acute paw edema

Figure 5.3 shows the effect of OIM administration on acute paw edema of animals injected with carrageenan. In animals treated with OIM extract at a dosage of 400 mg/kg body weight (OCMH), reduction of paw edema volume occurred by 41.28% in the 3rd hour, in comparison to the control. Meanwhile, a reduction of 35.46% was obtained in OIML group. The thickness of paw recorded in the 3rd hour for standard (0.282 ± 0.005 cm), OIMH (0.304 ± 0.008

cm) and OIML (0.315 ± 0.006 cm) groups was significantly different ($p<0.01$) from that of the untreated control (0.355 ± 0.005 cm). In dextran induced animals induced with acute paw edema, treatment with OIM extract brought about 23.14% and 14.85% reduction in paw volume with high (OIMH) and low (OIML) doses respectively. The thickness of paw recorded in the 3rd hour for standard (0.355 ± 0.007 cm), OIMH (0.376 ± 0.005 cm) and OIML (0.391 ± 0.002 cm) groups was significantly different ($p<0.01$) from that of the untreated control (0.432 ± 0.008 cm). Figure 5.4 shows the effect of OIM administration on acute paw edema of animals injected with dextran.

Figure 5.3. Effect of OIM extract administration on carrageenan induced acute paw edema

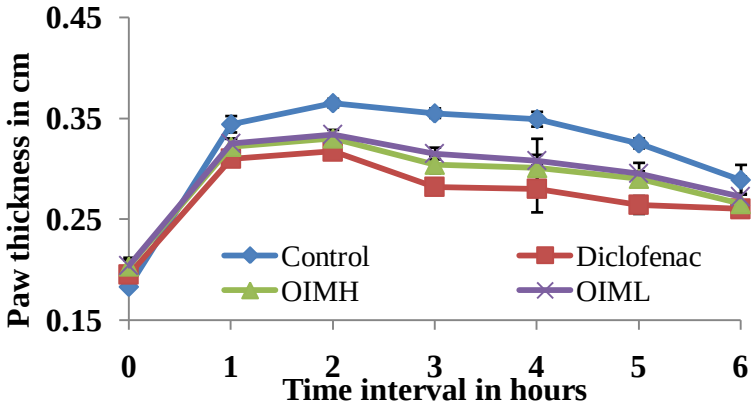
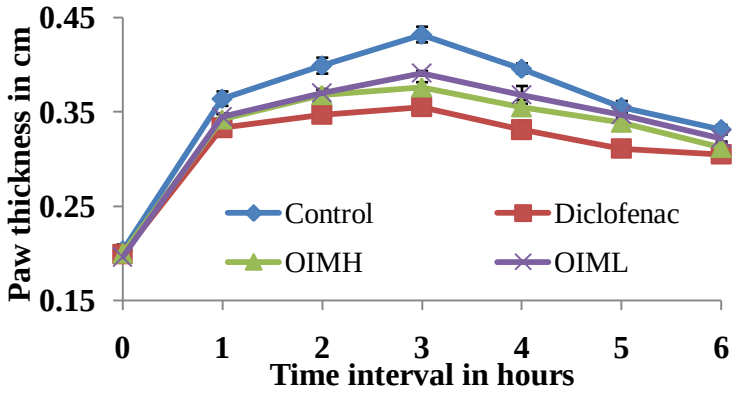


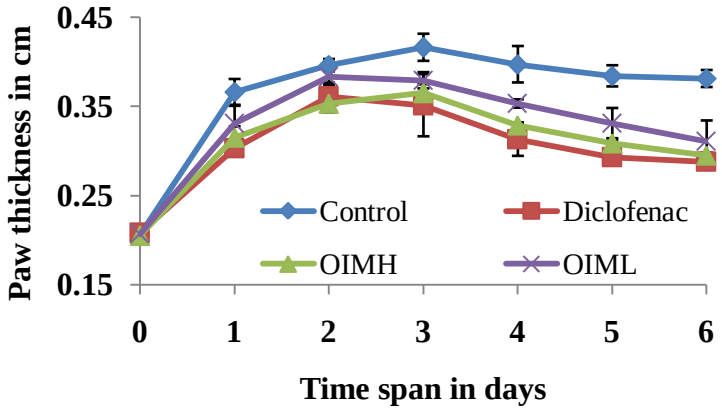
Figure 5.4. Effect of OIM extract administration on dextran induced acute paw edema



5.3.3.2. Chronic paw edema

In animals induced with chronic paw edema using formalin, a reduction of 48.57% and 40.57% in paw edema volume was observed in OIM extract treated animals with high (OIMH) and low (OIML) doses respectively. The thickness of paw recorded on the 6th day for standard (0.288 ± 0.003 cm), OIMH (0.295 ± 0.003 cm) and OIML (0.311 ± 0.023 cm) groups was significantly different ($p<0.01$) from that of the untreated control (0.381 ± 0.010 cm). Figure 5.5 shows the effect of OIM administration on chronic paw edema of animals injected with formalin.

Figure 5.5. Effect of OIM extract administration on formalin induced chronic paw edema.



5.3.4. Inhibition of skin tumorigenesis by OIM extract in DMBA-croton oil induced papilloma

Application of either DMBA or croton oil alone did not induce papilloma in any of the animals. Meanwhile, DMBA + croton oil application in untreated control animals led to full blown papilloma development (100% incidence). However, OIM application at low and high regimes reduced the incidence of tumor to 83.3 and 66.6% respectively (Table 5.3). Papilloma onset was registered in control group at the 6th week of topical application itself, whereas it was delayed in treated groups – 9th week in OIML and 12th week in OIMH

(Fig. 5.6 and 5.7). Table 5.4 shows that the average number of papillomas per animal was significantly ($p < 0.01$) lower in treated mice compared to the control. OIM treatment was found to inhibit papilloma development in low and high dose groups by 67.51% and 75.63% respectively.

Figure 5.6. Effect of OIM extract treatment on papillomagenesis by DMBA-croton oil application from the 5th week to 20th week.

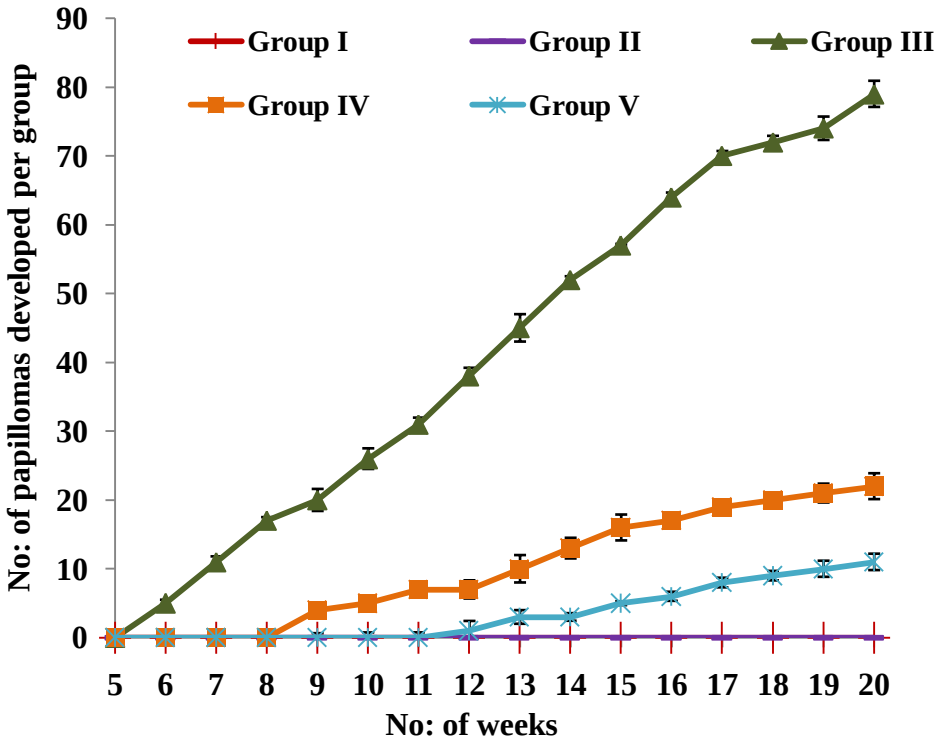
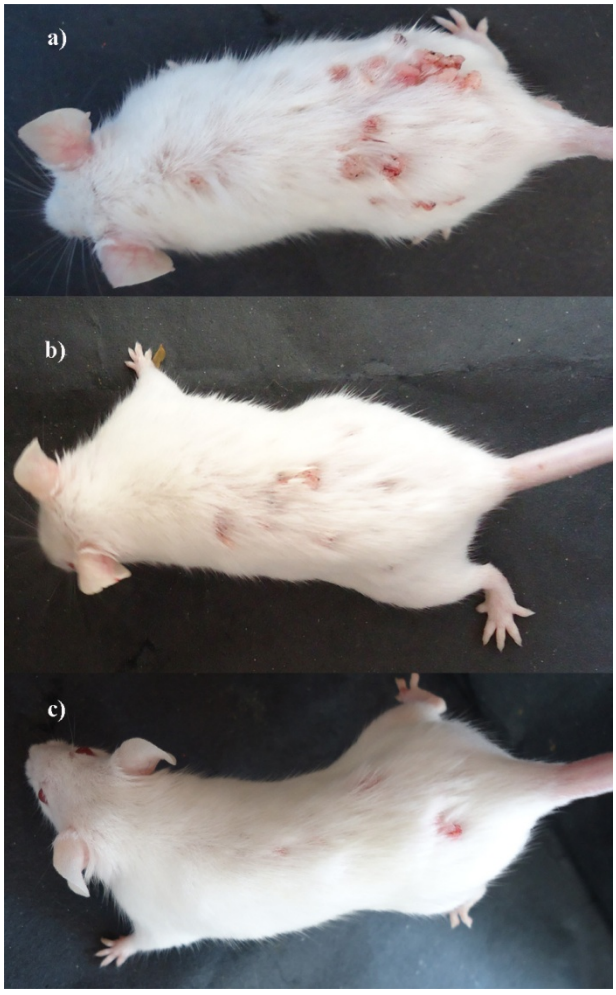


Table 5.3. Effect of OIM extract treatment on DMBA-croton oil induced papilloma

Groups	Treatment	% of incident papilloma	Cumulative count of papillomas	No: of papillomas per mouse	% inhibition of papilloma genesis
I	DMBA	0	0	0	-
II	Croton oil	0	0	0	-
III	DMBA + croton oil	100	79	11.32 ± 1.25	-
IV	DMBA + croton oil + OIM 5% in 200 µL dw	83.33333	22	3.67 ± 0.82[a]	67. 51
IV	DMBA + croton oil + OIM 10% in 200 µL dw	66.66667	11	2.75 ± 0.95[a]	75.63

Values are expressed as mean ± SD for six animals; [a]: $p \leq 0.01$ in comparison with negative control, group III; DMBA: Dimethylbenz(a)anthracene

Figure 5.7. Representative figure of the effect of OIM extract treatment on papillomagenesis in BALB/c mice by DMBA-croton oil application. a) Group III –control (DMBA + croton oil), b) DMBA + croton oil + OIM 5% in 200 µL dw and c) DMBA + croton oil + OIM 10% in 200 µL dw



5.4. Discussion

The supply of anti-oxidant compounds, either endogenous or exogenous, is undoubtedly necessary to endure the damages believed to be inflicted by free radical generation in tissues. This has created a need for searching natural sources for such compounds, from which they could be independently isolated or whose extracts contain combinations of them. Many plants used in traditional medicine, when investigated scientifically are sources of pharmaceutically important compounds with anti-oxidant, anti-inflammatory, cytotoxic, anti-cancer or organoprotective actions. One of the foci of the present study was the evaluation of *in vitro* and *in vivo* anti-oxidant properties of methanol extract of *O. indicum* root bark. The study revealed that the OIM extract inhibited/scavenged the superoxide, hydroxyl, DPPH and ABTS radicals in the assay system, in a concentration-dependent manner. Moreover, the extract also brought about reduction of ferric ion to ferrous form, as revealed through the FRAP assay, again in concentration-dependent pattern.

In the *in vivo* antioxidant study with OIM extract, when the untreated animals exposed to sodium fluoride (NaF) showed weakened antioxidant profile of both liver and blood tissues, it was observed that pre-treatment with *O. indicum* root bark extract significantly enabled them to withstand the stress. This is noticed from the enhanced levels of SOD, reduced glutathione and GPx in liver as well as SOD and reduced glutathione in blood, which were significantly elevated with reference to control. Formation of free radical by cascade mechanisms and glutathione-depleting agents favor the oxidation process in the cells leading to cellular damage. Formation of peroxyl radicals, superoxide radicals, singlet oxygen and hydroxyl radicals damage DNA. Though there are synthetic antidotes such as DMSA (meso-2, 3-dimercaptosuccinic acid), and BAL (2, 3-dimercapto-1-propanol) for fluoride toxicity, they may cause side effects when used singly. But, recent studies have shown that plant-derived polyphenols and flavonoids can ameliorate fluoride induced toxicity in cells (Pandiyan and

Prabu, 2013). *O. indicum* Vent. is already reported to be bestowed with antioxidant flavonoids such as baicalein, chrysin and biochanin A (Shieh *et al.*, 2000, Zhang *et al.*, 2011b, Kang *et al.*, 2012), whose presence may possibly have mitigated the oxidative stress, which, otherwise would have not been achieved in the absence of pre-treatment.

The study also revealed that *Oroxylum indicum* root bark has been found to mitigate the extent of both acute and chronic inflammation in carrageenan, dextran and formalin induced paw edema. Plant-derived compounds including carotenoids, flavonoids, phenolic acids, monoterpenes and sulfides are reportedly possessing anti inflammatory activity (Azab *et al.*, 2016). *O. indicum* Vent. root bark, presently used in the study is also detected with the presence of flavonoids and polyphenolic compounds, possibly contributing to the observation in the present study. The link between inflammation and cancer has led to the use of anti inflammatory agents for cancer prevention and therapy (Rayburn *et al.*, 2009). The nuclear factor-κB (NF-κB) transcription factor, the central mediator of the inflammatory process is also postulated to be involved in cancer development, thus linking suppression of inflammatory responses to prevention of carcinogenesis (DiDonato *et al.*, 2012). The previous chapters have revealed the anti-proliferative activity of OIM extract in cancer cell lines, reflecting its association with suppressing inflammatory reactions.

The revelation of anti inflammatory activity of OIM extract prompted an investigation of its anti-carcinogenic efficacy in suppressing DMBA and croton oil-induced papilloma-genesis. The inhibition of tumorigenesis, as revealed from the study, has occurred in the promotion stage. In the typical two-stage chemical carcinogenesis system in the mouse skin, *Hras1* mutation induced by a low dose of 7,12-dimethylbenz(a)anthracene gets promoted by repeated application of TPA, that acts as a tumor promoter. During the tumor promotion stage, due to altered expression of genes, the encoded protein products take part

in hyperproliferation, inflammation and tissue remodeling. The initiated cells are therefore subjected to selective clonal expansion, forming visible tumors (Abel and DiGiovanni, 2011). This facilitates the initiation and promotion stages to be distinctly viewed both operationally and mechanistically (Abel *et al.*, 2009). Topical application of OIM extract showed a dose-dependent inhibition of skin tumorigenesis, with a delay of three and six weeks respectively, in high and low dose groups, compared to the full blown skin tumors that developed in control mice, having received no external treatment. Chemoprevention, as stated by Wattenberg (1985), can occur either during initiation or during promotion; the mediators preventing the former are categorized as blocking agents, and those inhibiting the latter as suppressing agents. Chemoprevention can be best accomplished during the promotion stage of carcinogenesis because of its reversible nature that requires more time and higher incidence of exposure, unlike initiation or progression (Sporn, 1976, DiGiovanni, 1992). The relationship between inflammation and papilloma-genesis has been previously established. During human papilloma virus infection, host inflammatory response is postulated to promote lesion progression and through direct participation of inflammatory cells. Under the influence of the microbial genome or of epigenetic factors, somatic cells undergo changes mediated by autocrine and paracrine signals indicative of the association between chronic inflammation and cancer (Boccardo *et al.*, 2010, Fernandes *et al.*, 2015). Besides being a component of *Dashamoola* formulation, there is also mention of the usage of *O. indicum* Vent. as a single ingredient in treating rheumatoid arthritis and inflammation (Kirtikar and Basu, 1975, Chunekar and Pandey, 1999). There have also been previous demonstrations of the suppressive role of antioxidant and anti inflammatory mediators in chemoprevention of DMBA-croton oil induced papilloma in mice (Das *et al*., 2010, Henderson *et al*., 2011, Divya *et al*., 2016). In the present study discussed eartlier in the chapter, the antioxidant anti inflammatory activity of OIM extract was revealed. In the light of the above facts, it is

conclusive that the inhibitive action of OIM extract may be due to its suppressive effect on inflammation.

Chapter 6

Chemoprotective activities of

Oroxylum indicum Vent.

6.1. Introduction

6.2. Materials and methods

6.2.1. Preparation of OIM extract

6.2.2. Chemicals

6.2.3. Diagnostic kits

6.2.4. Animals

6.2.5. Grouping of animals

6.2.6. Induction of toxicity by chemotherapeutic agents

6.2.7. Parameters studied

6.2.8. Statistical analysis

6.3. Results

6.3.1. Effect of OIM extract on Dox induced toxicity

6.3.2. Effect of OIM extract on Cis induced toxicity

6.3.3. Effect of OIM extract on CTX induced toxicity

6.4. Discussion

6.1. Introduction

The antioxidant activity of *O. indicum* Vent. has been revealed from the results in previous chapters. In addition to this, it was also observed that the OIM extract, besides decelerating the growth of solid tumors in DLA induced animals, was superior to the positive control/standard drug cyclophosphamide in resisting the hematological damages inflicted by the latter. This prompted the evaluation of the chemoprotective activity of *O. indicum* Vent. root bark extract against the known toxicities induced by chemotherapeutic agents such as doxorubicin (Dox), cisplatin (Cis) and cyclophosphamide (CTX). As defined by the National Institute of Health, U.S., a chemoprotective agent protects normal tissues from the side effects caused by various anticancer drugs (e-Article, 2016b). Many antineoplastic agents create oxidative stress in living systems and may generate electrophilic aldehydes that attack cellular targets, thereby interfering with the ability of anticancer drugs to kill cancer cells. The use of anti-oxidants during chemotherapy can reduce oxidative stress induced by anticancer therapeutics (Conklin, 2004). In view of the antioxidant activity of OIM extract, the present study aimed at evaluating its chemoprotective activity against Doxorubicin, Cisplatin and Cyclophosphamide.

Doxorubicin, an anthracycline antibiotic is widely used in chemotherapy especially for haematological malignancies, some types of tissue sarcomas and carcinomas; yet is reported to cause heart damage, being its most serious contra-indication. Cardio-toxicity induced by doxorubicin is reported to be caused by oxidative stress, mitochondrial dysfunction, susceptibility of cardiac tissue to lipid peroxidation and the low levels of antioxidant defences associated with the heart tissue (Singal *et al.*, 2001, Schimmel *et al.*, 2004). In acute cases, doxorubicin cardiotoxicity may occur in 2–3 days after its administration with an approximate incidence rate of 11%, possibly due to doxorubicin-induced irreversible myocardial edema. The associated ECG changes include nonspecific ST-T changes, left axis deviation and decreased

amplitude of QRS complexes (Takemura and Fujiwara, 2007, Chatterjee *et al.*, 2010). Enhancement of antioxidant status of the heart muscle tissue can be achieved by the use of pharmacological agents attributed with oxidative stress-resistance. Hence, supplementation therapies with compounds of antioxidant nature are in demand, which neither interfere with the anticancer efficacy of doxorubicin nor impose serious myocardial damage (Rajalakshmy *et al.*, 2011, Vijay *et al.*, 2011). In the light of its *in vitro* and *in vivo* anti-oxidant properties, the present study aims at investigating the efficacy of the extract ameliorating doxorubicin mediated cardiotoxicity.

Another organ-toxic anti-neoplastic agent is cisplatin (*cis*-diamminedichloroplatinum(II), CDDP), which is used in the treatment of solid tumors of the head, neck, breast, bladder, lung (small-cell and non-small cell lung cancer) and gonads. Besides minor contraindications imposed on hematopoietic and enteric tissues, the most notable side effect of cisplatin is renal toxicity, which occurs in 20–30% of patients (Arany and Safirstein, 2003). Cisplatin is linked to glutathione and gets metabolized through gamma-glutamyl transpeptidase and cysteine S-conjugate β-lyase-dependent pathways into a reactive thiol acting as a potent nephrotoxin. The kidney gathers cisplatin to a higher degree than other organs by peritubular uptake. Unbound cisplatin is subjected to unrestricted glomerular filtration and is exported renal tubular cells by a transport-mediated route. The drug undergoes partial metabolism into toxic species that trigger intracellular effects, gene regulation, cytotoxicity, activation of mitogen-activated protein kinases (MAPKs), subsequent apoptosis induction, inflammation and fibrogenesis. This leads to tubular damage and dysfunction with wasting of ions like sodium, potassium, and magnesium. Generation of reactive oxygen species (ROS) and reactive nitrogen species (NOS) is postulated to be responsible for the cisplatin-induced renal tubular injury. Some of these stimulate changes extent of lipid peroxidation, chemical cleavage of DNA, proteins and a decline in cellular defenses by the oxidation of thiol pools in the cell (Yao *et al.*, 2007). Hence, cisplatin-induced oxidative

stress and inflammatory response in the kidney may partially be prevented by antioxidants of synthetic or natural origin. The antioxidant properties of OIM extract revealed in previous chapters has prompted the present study of evaluating its nephroprotective effect in cisplatin-induced animals.

Cyclophosphamide (CTX) is a common alkylating anti-neoplastic drug used for the treatment of breast cancer, lymphomas, leukemias, neuroblastoma, retinoblastoma, rhabdomyosarcoma,, and many solid tumors of breast, gonads and lung (Colvin, 2003). Still, it poses serious side effects like cardiac toxicity, hematopoietic depression, hemorrhagic cystitis, gonadal dysfunction, alopecia, nausea, gastrointestinal toxicity, renal toxicity, antidiuresis and vomiting (Slavin *et al.*, 1975). It has been cytotoxic to normal cells in humans as well as experimental animals (Fraiser *et al.*, 1991), with the haematopoietic compartment of bone marrow as the most affected part, leading to immunosuppression. CTX administration at high doses is also associated with the development of hepatotoxicity (Subramaniam *et al.*, 2013), nephrotoxicity and genotoxicity (Rehman *et al.*, 2012). CTX disrupts the redox balance of tissues, thereby succumbing them to biochemical and physiological damages and hence, agents with antioxidant properties can bring down cyclophosphamide-mediated organ injury (Mathew and Kuttan, 1997, Abarikwu *et al.*, 2012). Recent researches focus on the development of plant-based drugs that can ameliorate the toxicity induced by CTX, but at the same time, retain its antineoplastic effect (Kumar and Kuttan, 2005b). The chapter also includes an investigation on the chemoprotective activity of OIM extract in cyclophosphamide induced animals, by evaluating the ability of the extract in reducing myelosuppression, hepatic and renal injuries.

6.2. Materials and methods

6.2.1. Preparation of OIM extract

OIM extract was prepared according to the method given in Chapter 2, section 2.2.1., and was dissolved in distilled water and diluted to required concentrations for *in vivo* studies.

6.2.3. Chemicals and diagnostic kits

Doxorubicin (Dox), Cisplatin (Cis) and Cyclophosphamide were purchased as per list attached to Chapter 2, section 2.1.2. Diagnostic kits for the estimation of Glutamate oxaloacetate transaminase (GOT), Glutamate pyruvate transaminase (GPT), Alkaline phosphatase (ALP), Creatine phosphokinase (CPK), Lactate dehydrogenase (LDH), Creatinine, Urea and Hemoglobin were purchased as per list attached to Chapter 2, section 2.1.2. NBT, EDTA, riboflavin, DTNB, reduced glutathione, hydrogen peroxide, sodium dodecyl sulphate and thiobarbituric acid for estimating the tissue antioxidant parameters were purchased as per list attached to Chapter 2, section 2.1.2.

6.2.4. Animals

Female Sprague Dawley rats (200-220g) (for Dox and Cis induction) and male BALB/c mice (25-30 g) (for CTX administration) were procured and maintained as described in Chapter 2, section 2.1.6.

6.2.5. Grouping of animals

Five groups of 6 animals each were maintained for studying systemic toxicities using Dox and Cis, according to the treatment protocol. Group I served as normal reference group. Group II served as the untreated negative control group, exposed to Dox/Cis. Group III was maintained as the standard/positive control, which received either oral administration of Probucol at a dose of 20 mg/kg b.wt. for 14 consecutive days (for Dox inducing study) or Silymarin (i.p.) at a dose of 100 mg/kg .wt. for 5 consecutive days as pretreatment (for Cis inducing study). Groups IV and V were the extract treated groups receiving oral administration of OIM extract at low (OIML- 200 mg/kg b.wt.) and high

doses (OIMH- 400 mg/kg b.wt.) respectively, with the same pretreatment period as that of the standard.

Four groups of 6 animals each were maintained for studying the toxicity of CTX, according to the treatment protocol. Group I served as normal reference group. Group II served as the untreated negative control group, exposed to CTX. Groups III and IV were the extract treated groups receiving oral administration of OIM extract at low (OIML- 200 mg/kg b.wt.) and high doses (OIMH- 400 mg/kg b.wt.) respectively for 20 consecutive days. For each group, three sub-groups with 6 animals were maintained to enumerate the bone marrow cellularity on initial day, 7^{th} day and 4^{th} day.

6.2.6. Induction of toxicity by chemotherapeutic agents

6.2.6.1. Doxorubicin induced toxicity

Induction of toxicity started from day 11 day of the oral treatment, up to day 14, when all the groups (except I), received intra-peritoneal injections of Dox, making up a 4-day cumulative dose of 30 mg Dox/kg of animal body weight (Firdous and Kuttan, 2012b).

6.2.6.2. Cisplatin induced toxicity

Induction of toxicity was done, when all the groups except I, received intra-peritoneal injection of Cis, at a dose of 16 mg/kg of animal body weight as i.p. on day 6. Treatment procedure in Standard, OIML and OIMH groups continued 3 days post injection (Firdous and Kuttan, 2012a).

6.2.6.3. Cyclophosphamide induced toxicity

Induction of toxicity was done, when all the groups except I, received intra-peritoneal injection of CTX at a dose of 20 mg/kg of animal body weight as i.p. on consecutive days, from day 6 to 15 (Kumar and Kuttan, 2005a).

6.2.7. Parameters studied

6.2.7.1. Doxorubicin induced cardiotoxicity

6.2.7.1.1. ECG analysis

On the 15th day (24 hours after Cis injection), prior to sacrifice, ECG of the animals was recorded by anaesthetizing them with ketamine (80 mg/kg b.wt.: i.p.) (Van Pelt, 1977, Kushawaha *et al.*, 2011). Needle electrodes were inserted under the skin for the limb lead at position II and ECG tracings were recorded using BPL *Cardiart* 6108 T instrument.

6.2.7.1.2. Evaluation of serum marker enzymes

After ECG recording, rats were sacrificed; blood was collected by heart puncture into non-heparinised vials, allowed to clot and then centrifuged for 10 min at 5000 rpm to obtain clear serum. Serum cardiac markers – LDH, CPK and GOT were analyzed to evaluate the extent of heart tissue damage, as per commercial diagnostic methods described in sections 2.2.5.4., 2.2.5.5. and 2.2.5.1. of Chapter 2. Hepatic damage, if any, was also analyzed in terms of the liver marker enzyme GPT (method in section 2.2.5.2. of Chapter 2).

6.2.7.1.3. Evaluation of myocardial lipid peroxidation level and tissue antioxidant status

10% (w/v) homogenates of heart tissue were prepared in ice cold 0.1M Tris-HCl. Lipid peroxidation measured as MDA level in the heart tissue homogenate (10%) was analyzed according to the method described in section 2.2.8.6 of Chapter 2. The cytosolic fraction of the heart tissue homogenate was used for studying the antioxidant status based on SOD activity, GPx activity and reduced glutathione level according to the methods described in sections 2.2.8.3, 2.2.8.4 and 2.2.8.5 of Chapter 2, respectively.

6.2.7.1.4. Histopathological analysis

A small portion of the heart tissue recovered from one animal in each group was washed in phosphate buffered saline, fixed in 10% buffered formalin and

embedded in paraffin wax. Sections of 5 micron thickness were made and stained with hematoxylin-eosin and subjected to microscopic examination (as per method in Chapter 2, section 2.2.7).

6.2.7.2. Cisplatin induced renal toxicity

6.2.7.2.1. Evaluation of renal function markers in serum

On day 10 (72 hours after Cis injection), rats were sacrificed; blood was collected by heart puncture into non-heparinised vials, allowed to clot and then centrifuged for 10 min at 5000 rpm to obtain clear serum. Serum markers of renal function – creatinine and urea were analyzed to evaluate the extent of kidney tissue damage, as per methods described in sections 2.2.5.6 and 2.2.5.7 of Chapter 2.

6.2.7.2.2. Evaluation of hematological parameters

Blood collected in heparinised tubes was used for the estimation of hemoglobin content using commercial kit (method given in Chapter 2, section 2.2.4.1) and total WBC count (Chapter 2, section 2.2.4.2)

6.2.7.2.3. Evaluation of lipid peroxidation level and antioxidant status in renal tissue

10% (w/v) homogenates of renal tissue were prepared in ice cold 0.1M Tris-HCl. Lipid peroxidation measured as MDA level in the tissue homogenate was analyzed according to the method described in section 2.2.8.6 of Chapter 2. The cytosolic fraction of the renal tissue homogenate was used for studying the antioxidant status based on SOD activity, GPx activity and reduced glutathione level according to the methods described in sections 2.2.8.3, 2.2.8.4 and 2.2.8.5 of Chapter 2, respectively.

6.2.7.2.4. Histopathological analysis

The kidney recovered from one animal in each group was washed in phosphate buffered saline, fixed in 10% buffered formalin and embedded in paraffin wax. Sections of 5 micron thickness were made and stained with hematoxylin-eosin (as per method in Chapter 2, section 2.2.7) and subjected to microscopic examination.

6.2.7.3. Cyclophosphamide induced myelosuppression, hepatic and renal injuries

6.2.7.3.1. Tracking of periodic changes in body weight

The body weight changes of animals in all groups were tracked periodically in every 5^{th} day of the study.

6.2.7.3.2. Tracking of periodic changes in haemoglobin level and total leucocyte count

On every 5^{th} day of the study, blood was collected into heparinized tubes by caudal vein puncture to track the hematological parameters of blood (hemoglobin level as per method given in Chapter 2, section 2.2.4.1) and total leucocyte count as per method given in Chapter 2, section 2.2.4.2) periodically.

6.2.7.3.3. Tracking of periodic changes in bone marrow cellularity

On the initial day, 7^{th} day, 14^{th} day and the final day (immediately after sacrifice), bone marrow cells from both the femurs were collected by flushing out using PBS, and counted as per the procedure mentioned in Chapter 2, section 2.2.6.

6.2.7.3.4. Evaluation of renal and liver function markers in serum

On day 20 (120 hours after last Cis injection), mice were sacrificed; blood was collected by heart puncture into non-heparinised vials to collect serum. Serum markers of hepatic and renal function – GOT, GPT, ALP, creatinine and urea were analyzed to evaluate the extent of liver and kidney tissue damage, as per

methods described in sections 2.2.5.1, 2.2.5.2, 2.2.5.3, 2.2.5.6 and 2.2.5.7 of Chapter 2.

6.2.7.3.5. Evaluation of lipid peroxidation level and antioxidant status in hepatic and renal tissue

10% (w/v) homogenates of renal tissue and hepatic tissues were prepared in ice cold 0.1M Tris-HCl. Lipid peroxidation measured as MDA level in the tissue homogenate was analyzed according to the method described in section 2.2.8.6 of Chapter 2. The cytosolic fraction of the tissue homogenate was used for studying the antioxidant status based on SOD activity, GPx activity and reduced glutathione level according to the methods described in sections 2.2.8.3, 2.2.8.4 and 2.2.8.5 of Chapter 2, respectively.

6.2.7.3.6. Determination of organo-somatic index of spleen

The organo-somatic index of spleen was determined in animals of all groups.

6.2.8. Statistical analysis

The values were expressed as mean ± SD of 6 animals per group. Statistical evaluation of the data was done by one way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when p value was <0.05.

6.3. Results

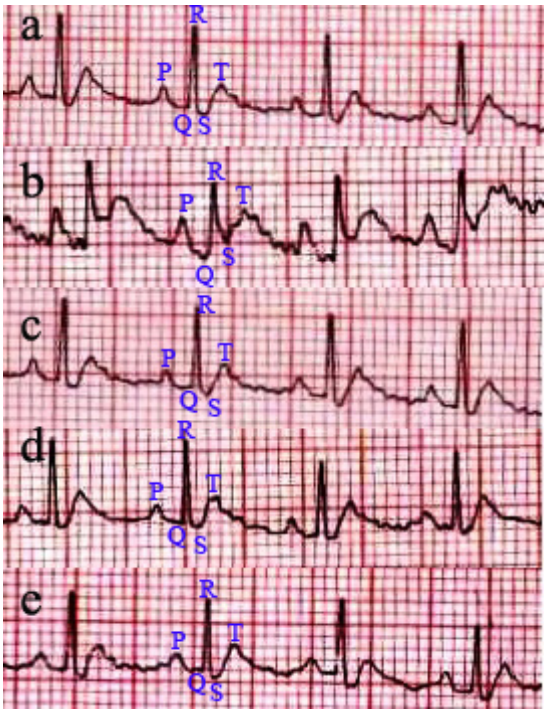
6.3.1. Effect of OIM extract on Dox induced toxicity

6.3.1.1. ECG

While the normal group (devoid of Doxorubicin induction) showed regular pattern of ECG (Figure 6.1.), the untreated Doxorubicin induced control animals presented clear ST segment depression, indicative of alterations in ventricular repolarisation phase. There were also changes such as reduction in QRS complex as well as prolongation of QT and PR intervals- changes, which were not visible

in treated animals. The standard and OIMH groups revealed normal ST segment, QRS complex, QT and PR interval patterns when compared to the control group.

Figure 6.1. Effect of OIM extract treatment on electrocardiogram of doxorubicin treated animals. a) ECG recording of normal group showed normal heart rate, ST deviation, PR, RR, ST and QT interval; b) Doxorubicin induced untreated control group showed arrhythmia, ST segment depression and prolonged PR and QT intervals; c) Rats treated with Probucol (standard) showed normal ECG pattern; Treatment with d) OIM extract low dose (200 mg/kg b.wt). and e) OIM extract high dose (400 mg/kg b.wt). showed normal electrocardiographic tracings.

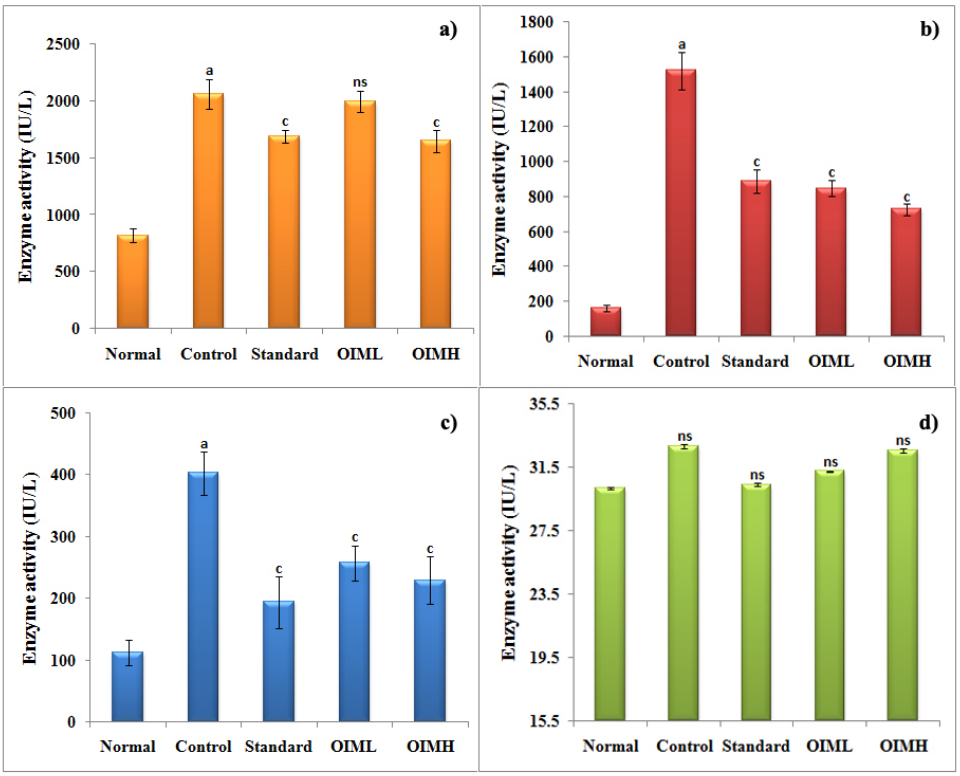


6.3.1.2. Serum marker enzyme levels

Significant ($p < 0.01$) increase in the level of cardiac marker enzymes - CPK, LDH and SGOT was discerned in the doxorubicin alone treated control group,

compared to normal. In the standard and OIMH treated group, though these were elevated, probably resulting from doxorubicin induction, they were inclined more towards normalcy, than a thorough devastating effect as seen in the control. But the levels of SGPT in all tested groups maintained a narrow range, revealing that hepatic damage was not imposed by Dox (Figure 6.2).

Figure 6.2. Effect of OIM extract treatment on serum cardiac markers in doxorubicin induced rats. 6.2.a, LDH: Lactate Dehydrogenase, 6.2.b., CPK: Creatine Phosphokinase, 6.2.c., GOT: Glutamate Oxaloacetate Transaminase and 6.2.d., Effect of OIM on serum liver marker, GPT: Glutamate Pyruvate Transaminase. [a]: p< 0.01, [b]: p<0.05 (control compared to normal), [c]: p< 0.01, [d]: p<0.05 standard, OIML and OIMH compared to control)



6.3.1.3. Myocardial lipid peroxidation level and tissue antioxidant status

The heart tissue isolated from control group, on biochemical evaluation, revealed high level of lipid peroxidation, when compared to the normal group. But this was significantly low in the standard group and OIM treated group, operational in a dose dependent manner. The antioxidant profile including SOD and GPx activities, as well as GSH levels were reduced in the control group, displaying deprived anti-oxidant defense status. The standard and the OIMH group, however disclosed a near normal antioxidant profile (Table 6.1).

Table 6.1. Effect of OIM extract treatment on myocardial lipid peroxidation level and tissue anti oxidant status in doxorubicin induced animals.

Animal groups	**LPO (nmoles of MDA/mg protein)**	**SOD (U/mg protein)**	**GPx (U/mg protein)**	**GSH (nmoles/mg protein)**
Normal	0.409 ± 0.005	0.223 ± 0.009	35.15 ± 1.39	4.92 ± 0.133
Control	0.527 ± 0.006[a]	0.073 ± 0.019[a]	22.53 ± 1.29[a]	3.11 ± 0.102[a]
Standard	0.443 ± 0.013[c]	0.181 ± 0.022[c]	32.66 ± 1.73[c]	4.69 ± 0.110[c]
OIML	0.460 ± 0.011 [c]	0.165 ± 0.014[c]	29.54 ± 1.09[c]	4.36 ± 0.096[c]
OIMH	**0.426 ± 0.011 [c]**	**0.197 ± 0.008[c]**	**31.31 ± 1.60[c]**	**4.67 ± 0.167[c]**

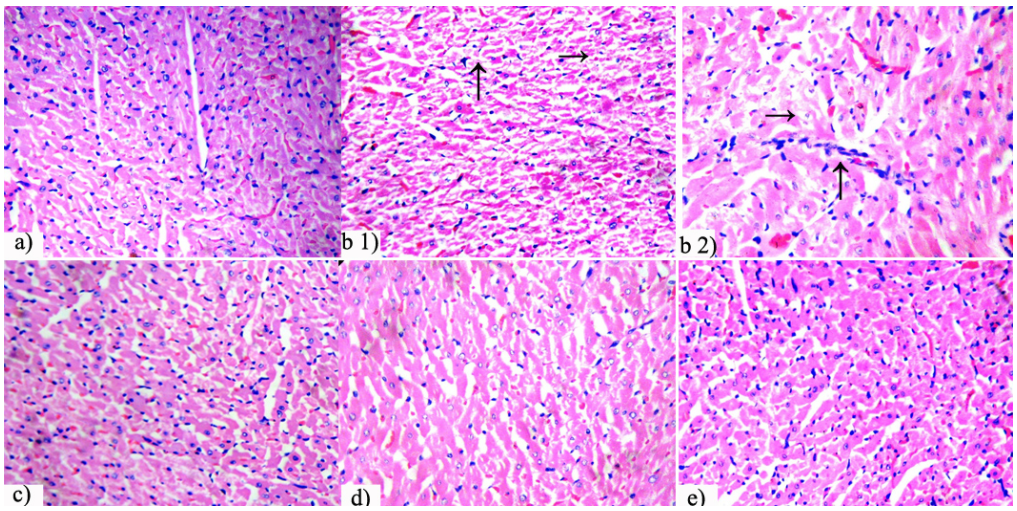
Values are expressed as mean ± SD for 6 animals. [a]: $p < 0.01$ (control, compared to normal), [c]: $p < 0.01$, (standard, OIML and OIMH, compared to control)

6.3.1.4. Histological findings

Light microscopic examination of normal heart stained with hematoxylin and eosin maintained a typical morphology (Figure 6.3.a), but doxorubicin induced control rats, having received no treatment developed cardiotoxicity signs such as focal loss of tissue and fragmentation, myofibrillar disorganization and lack of well defined boundaries of cardiac fibres indicating necrosis of cardiac tissue (Figure 6.3.b.1), scattered cytoplasmic vacuolation in myocytes as well as

lymphocyte infiltration (Figure 6.3.b.2). Amelioration of these effects has occurred in the Probucol (standard) treated rats (Figure 6.3.c) as well as OIM high dose groups (Figure 6.3.e).

Figure 6.3. Effect of OIM extract treatment on the histology of heart tissue (in light microscopic view- × 400) in doxorubicin induced rats. a) Cardiac histology of normal group with typical micro-architecture; (b) Doxorubicin induced untreated control group showing focal loss of tissue and fragmentation (small arrow to right), and lack of well defined boundaries of cardiac fibres indicating necrosis (big arrow pointed upwards) of cardiac tissue (b.1), cytoplasmic vacuolation (small arrow to right) and lymphocyte infiltration (big arrow pointed upwards) (b.2); c) Rats treated with Probucol (standard drug- 20 mg/kg bw) with normal histology; Treatment with d) OIM extract low dose (200 mg/kg bw) revealing minimal damage and e) OIM extract high dose (400 mg/kg bw) showing normal cardiac tissue architecture (with haematoxylin-eosin staining).



6.3.2. Effect of OIM extract on Cis induced toxicity

6.3.2.1. Serum markers of renal function

Cisplatin injection significantly ($p < 0.01$) increased the level of serum markers of renal injury - creatinine (5.14 ± 0.37 mg/dL) and urea (137.89 ± 9.59 mg/dL) - in the untreated negative control group, compared to normal (creatinine: 1.30 ± 0.18 mg/dL; urea: 18.82 ± 1.82 mg/dL). However, significantly low levels of creatinine (3.67 ± 0.51 and 4.09 ± 0.45 mg/dL) and urea (70.39 ± 5.25 and 87.83 ± 6.12 mg/dL) were maintained in OIMH and OIML groups respectively (Table 6.2).

6.3.2.2. Hematological parameters

The effect of cisplatin administration is most evident in the hemoglobin level (7.69 ± 1.53 g/dL) and total leucocyte count (3260 ± 642.7/mm^3 of blood) of the untreated negative control group, which is significantly reduced ($p < 0.01$) in comparison to the normal (Hb: 13.02 ± 0.71 g/dL; Total WBC: 11033.33 ± 1274.1/mm^3). It is seen from table 6.2. that the total leucocyte count of the OIML (6025 ± 727.4/mm^3) and OIMH (7400 ± 346.4/mm^3) groups were significantly high ($p < 0.01$), with reference to the negative control. Similarly, the high level of Hb in OIMH group (13.01 ± 2.93 g/dL), in comparison to the negative control was statistically significant ($p < 0.01$), despite no significant elevation in OIML (10.1 ± 0.85 g/dL).

Table 6.2. Effect of OIM extract treatment on renal function markers, total leucocyte count and haemoglobin level in cisplatin induced animals.

Animal group	**Urea (mg/dL)**	**Creatinine (mg/dL)**	**Total WBC (No: of cells/mm^3 of blood)**	**Hb (g/dL)**
Normal	18.82 ± 1.82	1.30 ± 0.18	11033.33 ± 1274.1	13.02 ± 0.71
Control	137.89 ± 9.59^b	5.14 ± 0.37^b	3260 ± 642.7^a	7.69 ± 1.53^a
Standard	45.22 ± 4.14^c	2.23 ± 0.42^c	7625 ± 1268.5^c	14.40 ± 0.84^c
OIML	87.83 ± 6.12^c	4.09 ± 0.45^c	6025 ± 727.4^c	10.1 ± 0.85
OIMH	**70.39 ± 5.25^c**	**3.67 ± 0.51^c**	**7400 ± 346.4^c**	**13.01 ± 2.93^c**

Values are expressed as mean ± SD for 6 animals. ^{a}: $p< 0.01$, ^{b}: $p<0.05$ (control, compared to normal), ^{c}: $p< 0.01$, (standard, OIML and OIMH, compared to control)

6.3.2.3. Lipid peroxidation level and antioxidant status in renal tissue

The level of lipid peroxidation was high (4.421 ± 0.97 nmoles of MDA/protein) in untreated negative control, when compared to the normal group (0.665 ± 0.37 nmoles of MDA/protein). But this was significantly low in the standard group (1.582 ± 0.21 nmoles of MDA/protein) and OIM treated groups (OIML: 2.117 ± 0.62 and OIMH: 1.355 ± 0.56 nmoles of MDA/protein), contrary to the control. The antioxidant profile including SOD and GPx activities, as well as GSH levels were reduced in the control group, displaying deprived anti-oxidant

defense status. The standard and the OIMH group, however disclosed a near normal antioxidant profile (Table 6.3).

Table 6.3. Effect of OIM extract treatment on renal lipid peroxidation level and tissue anti oxidant status in cisplatin induced animals.

Animal groups	LPO (nmoles of MDA/mg protein)	SOD(U/mg protein)	GPx(U/mg protein)	GSH(nmoles/mg protein)
Normal	0.665 ± 0.37	2.002 ± 0.14	34.2 ± 2.2	62.27 ± 3.77
Control	4.421 ± 0.97[a]	0.590 ± 0.26[a]	20.83 ± 3.57[a]	41.51 ± 1.07[a]
Standard	1.582 ± 0.21[c]	1.846 ± 0.19[c]	31.38 ± 2.79[c]	55.51 ± 2.68[c]
OIML	2.117 ± 0.62 [c]	0.165 ± 0.01[d]	21.92 ± 1.75[c]	44.36 ± 2.45[d]
OIMH	**1.355 ± 0.56 [c]**	**0.197 ± 0.01[c]**	**28.04 ± 3.57[c]**	**49.63 ± 2.21[c]**

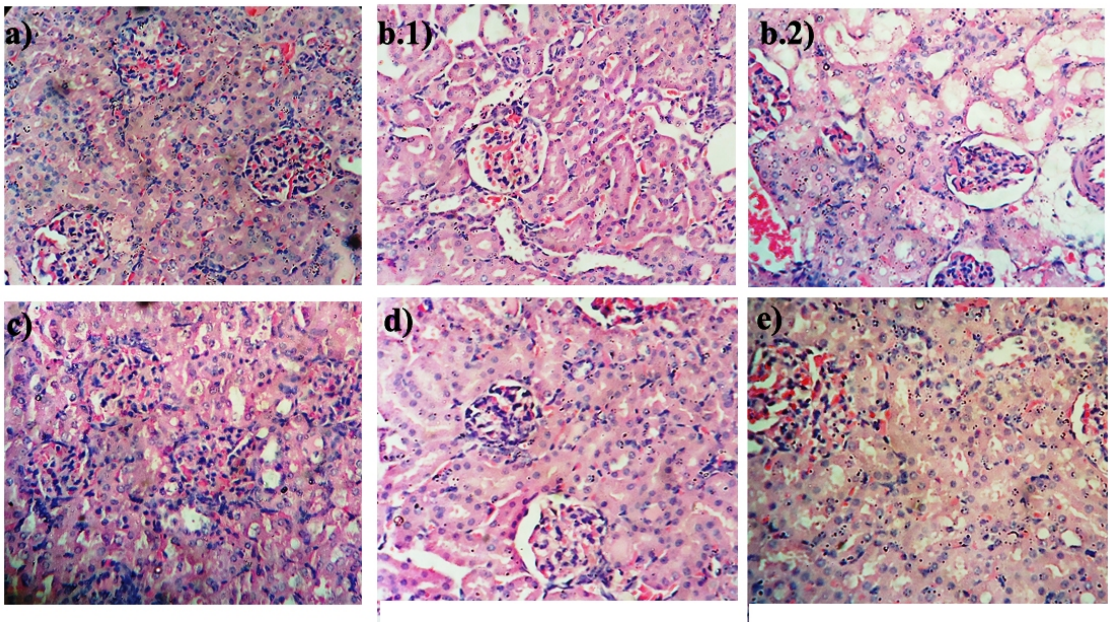
Values are expressed as mean ± SD for 6 animals. [a]: $p< 0.01$, [b]: $p<0.05$ (control, compared to normal), [c]: $p< 0.01$, [d]: $p< 0.05$ (standard, OIML and OIMH, compared to control).

6.3.2.4. Histological findings

The renal histology of normal group was characterized by typical micro-architecture with nephrons showing normal distribution of Bowman's capsule and instertitial tissue organisation. Meanwhile, in the cisplatin induced untreated control group, kidney damage was revealed by abnormal structure of Bowman's capsule, vacuolation in the cytoplasm of renal cells (b.1); and tissue with necrosis and haemorrhagic patches. (b.2). Animals who received

pretreatment with the standard drug, silymarin showed normal histology of kidney. Minimal damage was revealed in the OIML revealed minimal damage, indicative of the protective effect of OIM. The OIM group who received high dose (OIMH) of the extract showed normal architecture of the kidney as seen in microscopic view (Figure 6.4).

Figure 6.4. Effect of *O. indicum* Vent. extract (OIM) on the histology of renal tissue (in light microscopic view- × 400) in cisplatin induced rats. a) Renal histology of normal group with typical nephrons; (b) Cisplatin induced untreated control group with abnormal structure of Bowman's capsule and vacuolation in the cytoplasm of renal cells (b.1), tissue with necrosis and haemorrhagic patches (b.2); c) Rats treated with silymarin (standard drug- 100 mg/kg b.wt.) showed normal histology; Treatment with d) OIM extract low dose (200 mg/kg b.wt.) showing minimal damage and e) OIM extract high dose (400 mg/kg bw) with normal structure of tissue (with haematoxylin- eosin staining).

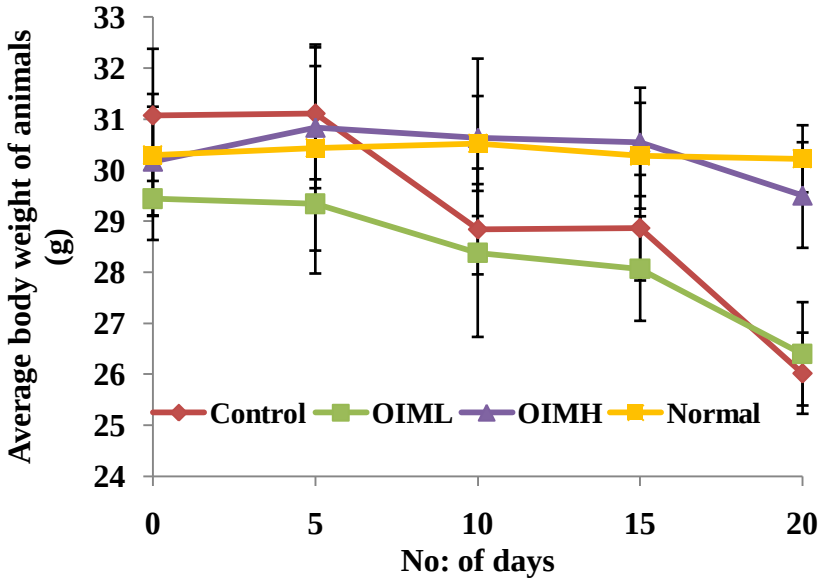


6.3.3. Effect of OIM extract on CTX induced toxicity

6.3.3.1. Changes in body weight

As seen from figure 6.5, progressive reduction was noticed in the average body weight of animals injected with cyclophosphamide alone (negative control group), from the initial day of CTX administration (31.08 ± 1.3 g) to the day of sacrifice (26.02 ± 0.8 g). The normal reference group showed no noticeable body weight loss. In the OIML group also, there was a slight decline of average body weight from day 5 (29.34 ± 1.4 g) to 20 (26.4 ± 1.0 g). But, in the OIMH group, only negligible change was recorded in body weight from day 5 (30.84 ± 1.2 g) to day 20 (29.514 ± 1.0).

Figure 6.5. Effect of OIM extract treatment on the average body weight of Cyclophosphamide induced animals

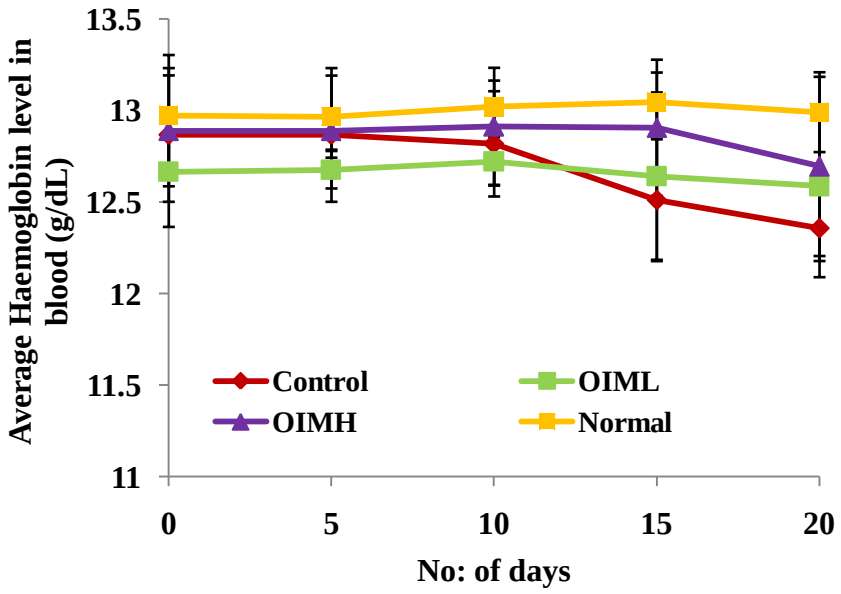


6.3.3.2. Changes in haemoglobin level and total leucocyte count

Figure 6.6 shows the change in hemoglobin level of blood in CTX induced animals over the period of study. The CTX induced control group, having received no pre-treatment, suffered a very slight reduction of blood Hb level

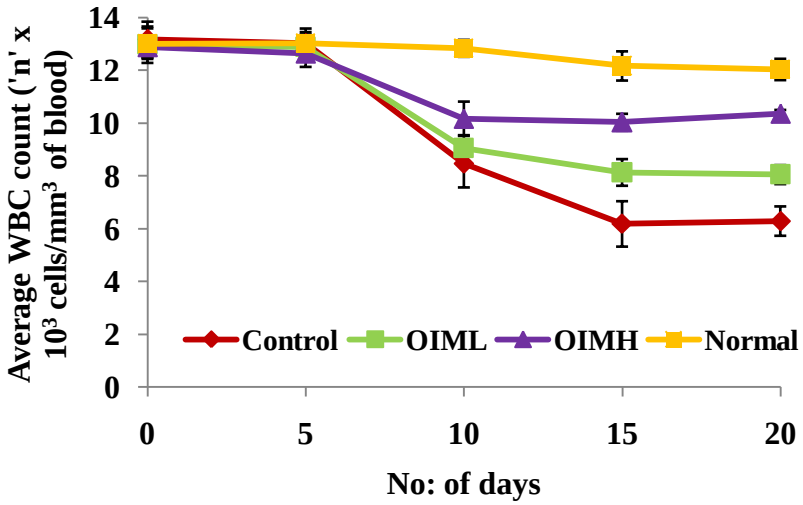
from the day of CTX induction (12.87 ± 0.37 g/dL) until sacrifice (12.36 ± 0.37 g/dL), though statistically non significant. However, the Hb values recorded in OIML (12.67 ± 0.1 to 12.59 ± 0.4 g/dL) and OIMH (12.89 ± 0.1 to 12.69 ± 0.5 g/dL) groups were within a narrower range recorded after CTX administration up to sacrifice.

Figure 6.6. Effect of OIM extract treatment on the average hemoglobin level of blood in Cyclophosphamide induced animals



The tracking of mean total leucocyte count in CTX administerd animals is shown in figure 6.7. When the normal reference group not exposed to CTX showed no significant changes, the untreated negative control suffered drastic and significant decline in WBC count from day 5 (13033/mm^3) to day 20 (6290/mm^3). OIML group also suffered such decline in WBC count from 12883/mm^3 to 8050/mm^3. But, treatment with OIM at high dose (OIMH), was demonstrated to withstand the myelosuppressive effect of CTX, as noted from non significant change in the total WBC count recorded from the 5^{th} day (12633/mm^3) to 20^{th} day (10360/mm^3).

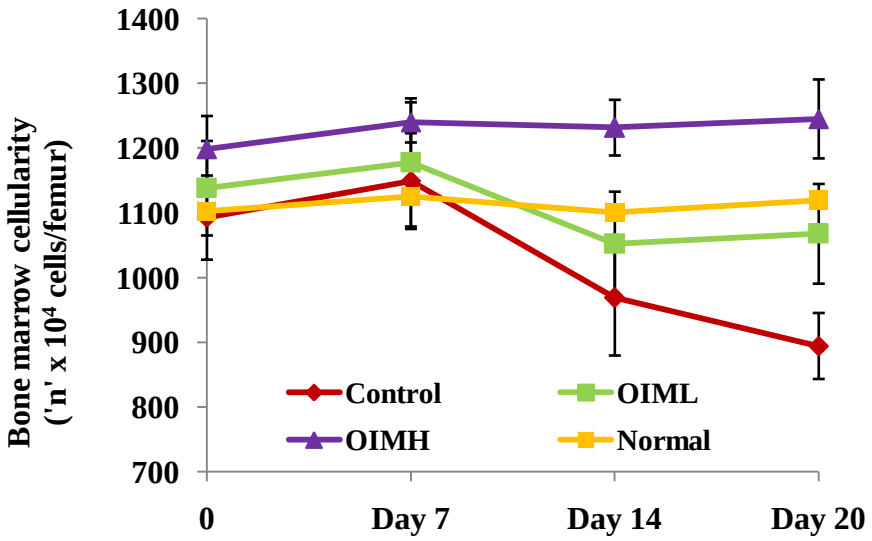
Figure 6.7. Effect of OIM extract treatment on the average total leucocyte count of blood in Cyclophosphamide induced animals



6.3.3.3. Changes in bone marrow cellularity

The effect of OIM extract on resisting the myelosuppressive effect of CTX administration was also analysed, based on the weekly bone marrow cellularity track record. The normal reference group showed no notable change. But the CTX administered untreated negative control suffered reduction in 2 week period after CTX administration, from day 7 (1149.22 × 10^4 cells/femur) to 20 (894.5 × 10^4). OIM treatment at high dose (OIMH) withstood the effect of CTX, as the values recoded from day 7 (1239.88 × 10^4) to 20 (1245.25 × 10^4), an effect better than what observed with lose dose (OIML) (1178.0 × 10^4 on day 7 and 1067.75 × 10^4 on day 20). The changes in bone marrow cellularity in various groups are given in figure 6.8.

Figure 6.8. Effect of OIM extract treatment on the average bone marrow cellularity in Cyclophosphamide induced animals



6.3.3.4. Renal and liver function markers in serum

Table 6.4 shows the levels of renal and liver function markers in the serum of CTX administered animals. Compared to the normal reference group, all serum markers in the untreated control– GOT (129.17 ± 8.37 U/L), GPT (82.2 ± 8.83 U/L), ALP (84.97 ± 6.63 U/L), urea (27.34 ± 4.81 mg/dL) and creatinine (1.25 ± 0.06 mg/dL) got significantly ($p < 0.01$) elevated, indicating hepatic and renal injury. But, these parameters were significantly ($p < 0.01$) lower in OIMH [GOT (114.29 ± 6.22 U/L), GPT (57.53 ± 6.35 U/L), ALP (63.75 ± 5.83 U/L), urea (17.47 ± 3.92 mg/dL) and creatinine (0.91 ± 0.036 mg/dL)] group. Meanwhile, in the OIM low dose group (OIML), the serum markers - GPT (69.22 ± 6.99 U/L), ALP (72.32 ± 4.62 U/L) and urea (20.55 ± 3.94 mg/dL)- showed significant reduction too.

Table 6.4. Effect of OIM extract treatment **on serum markers of liver and renal function in cyclophosphamide induced animals.**

Animal groups	GOT (U/L)	GPT (U/L)	ALP (U/L)	Urea (mg/dl)	Creatinine (mg/dl)
Normal	108.23 ± 6.19	42.98 ± 3.47	60.22 ± 4.74	17.87 ± 2.27	0.67 ± 0.03
Control	129.17 ± 8.37[a]	82.2 ± 8.83[a]	84.97 ± 6.63[a]	27.34 ± 4.81[a]	1.25 ± 0.06[a]
OIML	124.97 ± 7.29	69.22 ± 6.99[c]	72.32 ± 4.62[c]	20.55 ± 3.94[c]	1.201 ± 0.04
OIMH	**114.29 ± 6.22**[c]	**57.53 ± 6.35**[c]	**63.75 ± 5.83**[c]	**17.47 ± 3.92**[c]	**0.91 ± 0.036**[c]

Values are expressed as mean ± SD for 6 animals. [a]: $p < 0.01$ (control, compared to normal),

[c]: $p < 0.01$ (OIML and OIMH, compared to control).

6.3.3.5. Lipid peroxidation level and antioxidant status in hepatic and renal tissue

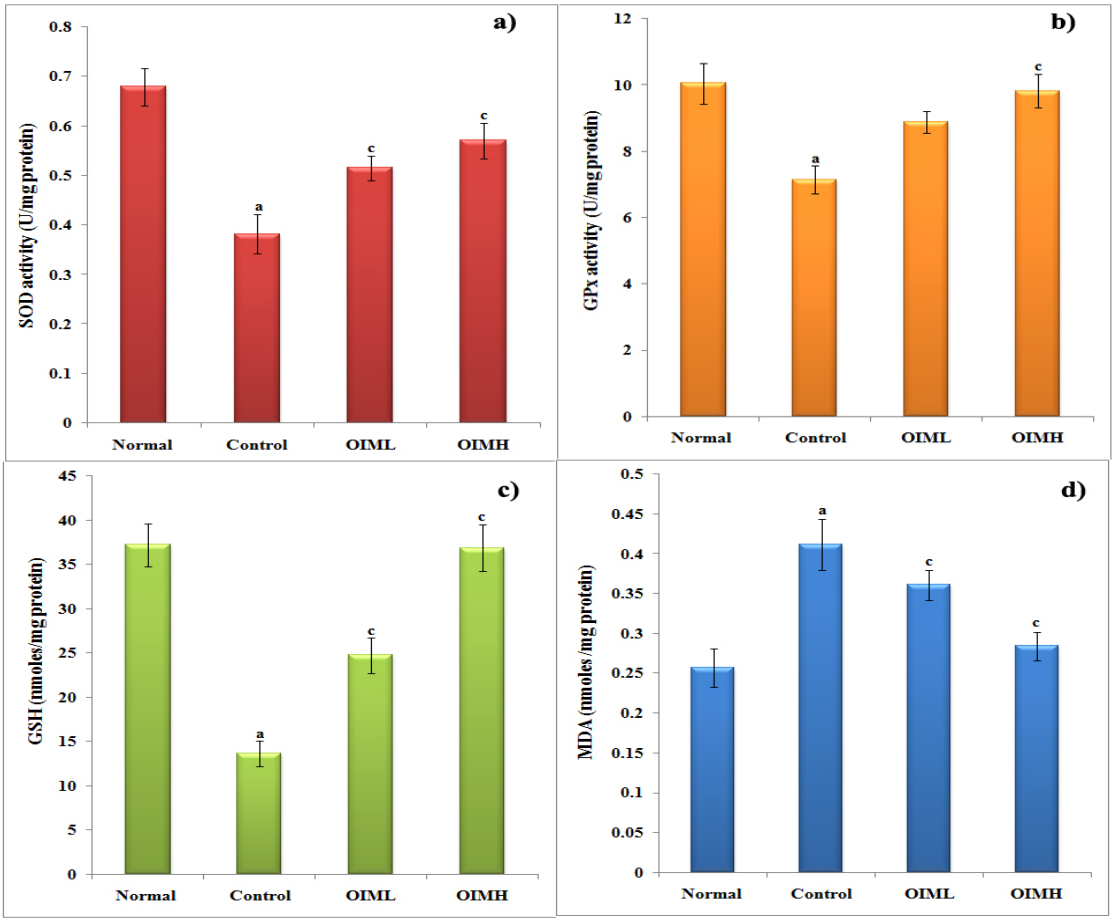
Treatment with OIM enhanced the antioxidant status of the liver and kidney in CTX induced animals as seen in figure 6.9 and 6.10. From figure 6.9, it can be seen that in CTX induced negative control group devoid of extract treatment, the levels of SOD (0.382 ± 0.04 U/mg protein), GPx (7.15 ± 0.43 U/mg protein) and GSH (13.63 ± 1.42 nmoles/mg protein) in liver were significantly lower than that of normal. Similar trend was observed in the levels of these parameters in the renal tissue (SOD - 0.577 ± 0.06, GPx - 6.75 ± 0.49 and GSH - 15.83 ± 2.12) (figure 6.10). But in OIM treated high dose group (OIMH), levels of liver SOD (0.571 ± 0.04), liver GPx (9.82 ± 0.51), liver GSH (36.88 ± 2.61), renal SOD (0.72 ± 0.07), renal GPx (10.2 ± 0.52) and renal GSH (27.24 ± 2.03) were elevated significantly higher than untreated control.

When CTX administration increased the lipid peroxidation activity in liver (0.412 ± 0.03 nmoles of MDA/mg protein) and kidney (0.42 ± 0.03) in untreated control, they were comparatively lower in OIMH (liver- 0.28 ± 0.02; kidney- 0.25 ± 0.02) and OIML (liver- 0.36 ± 0.02; kidney- 0.27 ± 0.02) groups, with statistical significance, $p < 0.01$.

6.3.3.6. Organo-somatic index of spleen

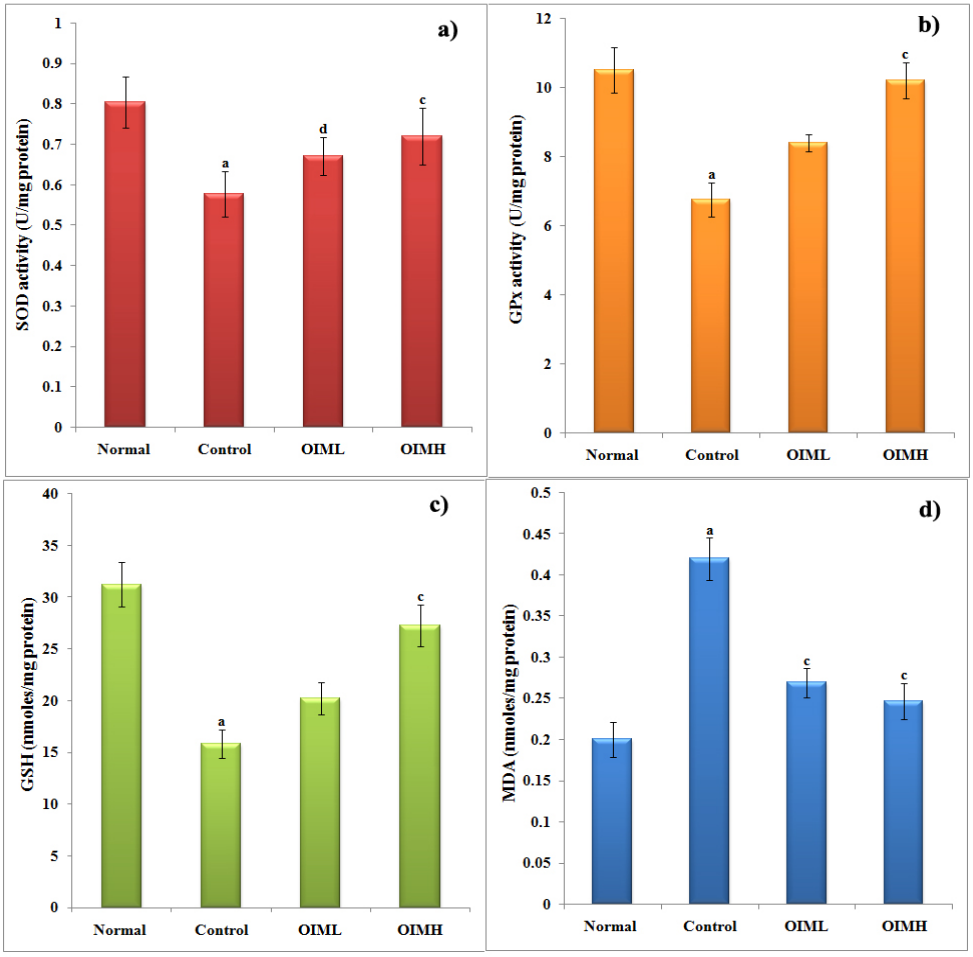
The impact of CTX administration on immune system was inferred on the basis of the organo somatic index of spleen, which was significantly very low in the untreated control (0.251 ± 0.016), with reference to normal; but was near normal in OIML (0.303 ± 0.016) and OIMH (0.318 ± 0.017) groups.

Figure 6.9. Effect of OIM extract treatment on liver lipid peroxidation level and tissue anti oxidant status in cyclophosphamide induced animals.



Values are expressed as mean ± SD for 6 animals; (a) $p< 0.01$, (b) $p<0.05$ compared to normal; (c) $p<0.01$, (d) $p<0.05$ compared to control.

Figure 6.10. Effect of OIM extract treatment on renal lipid peroxidation level and tissue anti oxidant status in cyclophosphamide induced animals.



Values are expressed as mean ± SD for 6 animals; (a) $p< 0.01$, (b) $p<0.05$ compared to normal; (c) $p<0.01$, (d) $p<0.05$ compared to control.

6.4. Discussion

The overall study has revealed the chemoprotective activity of *O. indicum* Vent. root bark against organ specific toxicities induced by doxorubicin, cisplatin and cyclophosphamide. Pre-treatment of the animals with OIM extract regained near normalcy in the tissue marker levels (SOD, GSH and GPx) of cardiac tissue in Dox induced animals, when compared to untreated negative control, attributing the cardioprotective role of *O. indicum* Vent. root bark extract to its enhancing effect on the tissue anti-oxidant status. The results are in consistency with some of the previous reports of cardioprotection (Swamy *et al.*, 2012, Rai *et al.*, 2015) by antioxidant rich plant extracts against the oxidative stress induced by doxorubicin. Likewise, antioxidant pool of the liver and kidney reportedly undergoes depletion under the effect of cisplatin and cyclophosphamide administration, as seen in earlier animal studies (Premkumar *et al.*, 2003, Song *et al.*, 2013, Sheweita *et al.*, 2016). Similar devastating effects on antioxidant defense were also observed in the untreated animals induced with Cis/CTX in the current study. But, on treatment with OIM, the antioxidant markers of liver, kidney and cardiac tissue maintained significantly higher levels than the negative control, seemingly resistant to the oxidative stress challenge posed by Dox, Cis or CTX. Though induction with Dox, Cis and CTX increases the susceptibility of cardiac tissue, renal and hepatic tissues to increased lipid peroxidation (Myers *et al.*, 1977, Sugihara *et al.*, 1987, Bhattacharya *et al.*, 2003, Stankiewicz and Skrzydlewska, 2003, Ray *et al.*, 2011), plant extracts rich in antioxidants are found to be ameliorative against this effect (Ingale *et al.*, 2013, Khan *et al.*, 2014, Devi and Mazumder, 2016). Concomitant effects were produced by OIM treatment in this study.

As reviewed by Nigam (2007), serum AST (GOT) activity is an important biochemical marker of myocardial injury, whose level increases 3-8 hours after the onset of the myocardial injury, summiting up at an average at 24 hours and finally returning to normal levels in 3-6 days. Other known markers include

lactate dehydrogenase and creatine kinase (its isoforms). An increase in serum LD activity is found following myocardial infarction beginning within 6-12 hours and reaching a maximum at about 48 hours and it remains elevated for 4-14 days before coming down to normal levels. Serum CK activity increases following myocardial injury beginning within 6 hours and peaking on an average at 24 hours and returning to normal within 2-3 days (Varley *et al.*, 1984). In OIM treated rats, GOT, CPK and LDH were significantly lower than that of control, representing amelioration of cardiac tissue injury. But, Dox induction was not seen to produce any elevation of serum GPT level, suggesting delay or absence of liver damage in our study. Such effects, though not always, are common in Dox induced injury because of its early devastating effects being inflicted on heart, compared to liver (Injac *et al.*, 2008, Sutejo and Efendi, 2017). But in Cis and CTX induction, elevation of serum GPT and GOT levels shot up significantly, which was alleviated by OIM treatment as well. This is suggestive of hepatic injury. Moreover, these two inducers were demonstrated to cause renal tissue damage, as evident from the elevation of serum urea and creatinine levels in the untreated control. Yet, an overall ameliorative effect was observed in these effects by OIM treatment, as revealed from the sera profile of treated animals.

Electrocardiographic abnormalities are indicators of myocardial injury. Transient electrocardiographic abnormalities such as nonspecific ST-T changes and QT prolongation are indicative of acute side effects of doxorubicin (Schimmel *et al.*, 2004). While studying the protective effect of *Lycium barbarum* on doxorubicin-induced cardiotoxicity, intra venous injection of doxorubicin significantly increased ST deviation and T-wave amplitude in electrocardiographic analysis (Xin *et al.*, 2007). ST segment depression with prolonged ST interval is constantly noticed in rat ECGs associated with studies of doxorubicin induced cardiac damage (Koti *et al.*, 2013, Warpe *et al.*, 2015a). The ECG records of the current investigation also presented similar effects with the doxorubicin induced control animals showing marked ST segment depression, reduction in QRS complex as well as prolongation of QT and PR intervals which were not

associated with the ECGs of the positive control (Probucol- standard) or drug treated groups.

Doxorubicin is reported to cause inter-fibrillar hemorrhages, congestion, and focal areas of disrupted cardiac muscle fibers (Shivakumar *et al.*, 2012) as well as cardiac muscular dysfunction (Hayward *et al.*, 2013). The heart tissue of OIM treated animals with a higher dose (400 mg/kg b.wt.) maintained normal histological architecture as was also observed in Probucol treated groups. Doxorubicin induced cardiotoxicity is associated with extensive myocardial degeneration manifested as the loss of myofibrils and focal cytoplasmic vacuolization, myocytic necrosis with moderate infiltration of lymphocytes and macrophages, granulated cytoplasm of myocytes etc (Khan *et al.*, 2005, Kelishomi *et al.*, 2008, Rashikh *et al.*, 2011, Vijay *et al.*, 2011). Comparable to the above, such devastating effects were also noticed in the doxorubicin treated control animals. The renal tissue damage inflicted by Cis administration can be viewed microscopically in stained sections of the tissue. Some of the common manifestations observed so are, necrosis, sloughing of renal epithelia, tubular dilation etc (Ramesh and Reeves, 2003, Atessahin *et al.*, 2005, Çetin *et al.*, 2006), as was also observed in the histopathological analysis of Cis administered animals in the present study. However, normal tissue architecture was revealed in OIM high dose (OIMH) treated groups.

Common side effects associated with cancer chemotherapy are myelosuppression, immunosuppression and anaemia, because rapidly dividing normal cells of the body also form targets for chemotherapeutic drugs, besides cancer cells (Spivak *et al.*, 2009). These effects have also been found true with cisplatin and cyclophosphamide in experimental animals (Wood and Hrushesky, 1995, Thews *et al.*, 2001b). The effects are manifested as drops in Hb and WBC count in blood, reduction in bone marrow cellularity and decrease in the relative weight of spleen (Begum and Anuradha, 2011, Ahmad *et al.*, 2013, Geetha *et al.*, 2015, Neboh and Ufelle, 2015, Sakthivel and Guruvayoorappan, 2015). In the

present study, cisplatin induction was found to inflict decline in Hb concentration and WBC count in control animals; meanwhile, CTX administration was found to affect WBC count significantly. CTX administration seemingly exerted its myelosuppressive effect on bone marrow of untreated CTX administered animals, evident from a significant and progressive reduction of bone marrow cellularity. Consequently, the relative organ weight of spleen, the secondary lymphoid organ was also found to be notably low in untreated control, in comparison with the normal. Such suppressive changes were evaded by the OIM treated animals, in a statistically significant magnitude.

The biological properties of plant extracts are attributed to the synergistic action of the multifarious compounds present in them. *O. indicum* Vent. root bark reportedly contains baicalein, chrysin, biochanin A and ellagic acid. Chrysin (Pushpavalli *et al.*, 2010, Veerappan and Senthilkumar, 2015), biochanin-A (Zhang *et al.*, 2011b) and ellagic acid (Warpe *et al.*, 2015a) are compounds attributed with antioxidant properties. There are proven studies of cardioprotective effects of chrysin (Mantawy *et al.*, 2014) as well as cardioprotective and chemoprotective properties of ellagic acid (Rehman *et al.*, 2012, Warpe *et al.*, 2015b). It is hence, conclusive that the presence of antioxidant compounds in the OIM extract may possibly have mediated the chemoprotective effects, through their combined action. Though it is not suggestive of an immediate clinical supplementation in humans undergoing chemotherapy for cancer, the study extends the scope of exploring active fractions from this plant extract for using it as an adjuvant in Dox/Cis/CTX-mediated chemotherapy, and thereby reduce the damaging side effects of these drugs, without compromising their cytotoxic activity.

The present as well as previous chapters have revealed the anti proliferative, antioxidant, anti inflammatory and chemoprotective activities of *O. indicum* Vent. root bark, which emphasizes the need of conservation or regeneration of this valuable, but vulnerable plant from the projected risk of biodiversity loss.

Hence, the forthcoming chapter discusses the attempts of producing roots, shoots and callus through its *in vitro* propagation and comparison of their activities with that of the wild plant.

Chapter 7
Comparative evaluation of pharmacological properties of micropropagated callus of *Oroxylum indicum* Vent. and wild root bark

7.1. Introduction

According to an article published in collaboration by the IUCN and TRAFFIC (the wildlife trade monitoring network), the population of many important species of medicinal plants have declined in India due to their overexploitation for meeting the demands of native and foreign drug markets (e-Article, 2008). Non sustainable collection methods, indiscriminate felling of trees and opportunistic marketing have led to the exhaustion of many plant resources (Schippmann *et al.*, 2006).

O. indicum Vent., which is also documented as a valuable medicinal tree species possessing pharmacologically important plant parts - leaves, roots, stem, fruits and seeds (Deka *et al.*, 2013) - has also fallen victim to overexploitation and subsequent population depletion (Yasodha *et al.*, 2004). This valuable tree got pushed to the vulnerable list in Karnataka and Andhra Pradesh and endangered in Kerala, Maharashtra, M.P. and Chhattisgarh and is feared to become endangered soon in other states too, as studied by the Foundation for Revitalisation of Local Health Traditions (FRLHT), Bangalore, India (Ravikumar and Ved, 2000). Natural propagation of the tree is limited by the low viability and high rate of abortion (30.56 to 47.62 %) of its seeds (Gunaga *et al.*, 2012). This necessitates an urgent need for its conservation and regeneration through micropropagation (Najar and Agnihotri, 2012). Moreover, *O. indicum* Vent. is also used as an ingredient of *Dasamoola*, the Ayurvedic formulation comprising roots of ten plants. The plants have been subjected to severe uprooting, with no concomitant replanting. Identifying the crisis, attempts of micropropagating some of these plants (*Gmelina arborea* and *Aegle marmelos*) were begun initially, – as reviewed by Yasodha *et al.* (2004) - followed by a few studies on *O. indicum* Vent. also (Dalal and Rai, 2004, Gokhale and Bansal, 2008, Gokhale and Bansal, 2009). Along with the benefit of regenerating medicinal plants facing biodiversity threats, tissue culture techniques can also be used for developing an alternative source for whole plant/parts or secondary metabolites (DiCosmo and Misawa, 1995, Mulabagal and Tsay, 2004). Along with propagation of tissue

cultured substitutes for pharmaceutical purposes, it is necessary that their pharmacological actions are to be compared with that of the wild plants; and if found effective, can be recommended for use in medicinal preparations, instead of uprooting the whole plants. This is particularly true for a tree species like *O. indicum* Vent. whose roots are extensively used in Ayurvedic preparations, and uprooting is the current method employed for collection of its roots. In the previous chapters, the wild root bark extract of *O. indicum* Vent. (OIM) was found to exhibit anti-proliferative, antioxidant and anti inflammatory properties. In this context, the current study is directed to the comparative evaluation of selected anti proliferative, anti oxidant and anti inflammatory properties of extract derived from tissue culture with that of OIM.

During *in vitro* propagation, selection of the culture medium and the presence of growth regulators is critical in callusing, shoot and root development. The effects of auxins and cytokinins on shoot multiplication of various medicinal plants have been reported by Skirvin *et al.* (1990). The commonly used synthetic auxins are indole acetic acid (IAA), 1-naphthalene acetic acid (NAA) and indole butyric acid (IBA). 6-benzyladenine (BA) and kinetin are the commonly used synthetic cytokinins. The study was initiated by the optimisation of conditions for callus induction, shoot initiation and root initiation in *O. indicum* Vent. through tissue culture techniques using MS medium with plant growth regulators- indole acetic acid, 1-naphthalene acetic acid, indole butyric acid and 6-benzylaminopurine.

7.2. Materials and methods

7.2.1. Chemicals

Murashige and Skoog medium, plant growth regulators - BA, IAA, IBA and NAA were used for micropropagation of *O. indicum* Vent.. These were purchased as per list in Chapter 2, section 2.1.2.

The chemicals used for *in vitro* antioxidant assays- NBT, EDTA, Riboflavin, Deoxyribose, Potassium dihydrogen phosphate, ferric chloride and ABTS were

purchased as per list in section 2.1.2., chapter 2. Carrageenan for *in vivo* anti inflammatory studies was purchased as given in Chapter 2, section 2.1.2.

All other chemicals used were of analytical grade.

7.2.2. Cell lines

EAC cells, DLA cells and normal spleen cells were used for short term *in vitro* cytotoxicity. These were procured (Chapter 2, section 2.1.7) and maintained as described in Chapter 2, sections 2.2.3.1.1 and 2.2.3.1.2.

Anti proliferative studies were conducted using HeLa, MDA-MB-231, Hep G2 and HCT 15 cell lines. Vero cell lines (kidney epithelial cell lines of African green monkey) were used as control. All cell lines were procured as per section 2.1.7 and maintained as per section 2.2.3.2.1 of chapter 2.

7.2.3. Animals

Swiss albino mice (25-30g) were used for *in vivo* anti inflammatory study. These were procured and maintained as per method given in section 2.1.6, Chapter 2.

7.2.4. Micropropagation of O. indicum Vent.

7.2.4.1. Preparation of culture medium

All micropropagation studies of *O. indicum* Vent. were conducted in MS medium, prepared as explained in Chapter 2, Section 2.2.10.1.

7.2.4.2. Collection of inoculates

Nodal sections from germinated seedlings of *O. indicum* Vent. were used for establishment of tissue culture (method described in Chapter 2, section 2.2.10.2.

7.2.4.3. Callus induction

The nodal sections were inoculated into MS media supplemented with combinations of the cytokinin, 6-benzyl aminopurine (BA) at a concentration of

1 mg/L and auxins, 1-naphthalene acetic acid (NAA- 0.5 mg/L) or indole acetic acid (IAA- 0.5 mg/L). The culture tubes were maintained under conditions mentioned in section 2.2.10.3, Chapter 2.

From the calluses initiated, (~) 500 mg (fresh weight) inoculates were cut out and subcultured into MS media supplemented with combinations of cytokinin (BA- 2 and 3 mg/L) and auxins (NAA- 0.5 and 1 mg/L or IAA- 0.5 and 1 mg/L). The increase in biomass in a time span of three weeks is assessed under culture conditions described in section 2.2.10.3, Chapter 2.

7.2.4.4. Indirect organogenesis from callus

The calluses generated from *O. indicum* Vent. nodal explants were further used for generation of shoots as well as roots.

7.2.4.4.1. Induction of shoots

To induce multiple shoot regeneration, well-established compact calluses (~500 mg fresh weight) were transferred to MS basal medium supplemented with different combinations of BA (2 and 3 mg/L) and NAA or IAA at concentrations 0.5 and 1mg/L and maintained for 8 weeks under conditions as described in Chapter 2, section 2.2.10.4.1. The shoot initiation percentage of calluses as well as the average number of shoot-buds induced on each callus inoculum was determined.

7.2.4.4.2. Induction of roots

For root induction, calluses (~500mg fresh weight) were transferred into MS media supplemented with the auxins, IAA/NAA/IBA at concentrations ranging from 0.5 to 2 mg/L. The percentage of root initiation and the numbers of roots initiated per inoculate was counted. Medium without plant growth regulators was used as control.

7.2.4.5. In vitro root generation

For *in vitro* root generation, callus- generated shoots carrying three to four fully expanded leaves were transferred into MS media fortified with IAA/NAA/IBA at concentrations ranging from 0.5 to 2 mg/L. The cultures were incubated in dark at 25 ± 2^{o} C for four weeks. The percentage of root initiation and the average number of roots initiated per inoculum was counted.

Control cultures were also maintained (section 2.2.10, Chapter 2) for all experiments.

7.2.5. Comparative evaluation of wild and in vitro propagated O. indicum Vent.

The comparative study was based on *in vitro* cytotoxicity, anti proliferative activity, antioxidant activity and *in vivo* anti inflammatory activity.

7.2.5.1. In vitro cytotoxicity of callus, in vitro shoots and roots

The *in vitro* cytotoxicity of callus (generated in BA3 IBA1 medium), callus-generated-shoots (in BA3 NAA 0.5 medium) and *in vitro* roots (in IBA2 medium) was compared. (Because of low yield of root number and biomass, the callus-generated-roots were not used).

7.2.5.1.1. Extract preparation

The callus, roots and shoots were extracted using 70% methanol as per method described in section 2.2.13., Chapter 2.

The percentage yield of extract obtained from callus (OCM), callus-generated-shoots (OSM), and *in vitro* roots (ORM) was 5.17, 4.92 and 2.97 respectively.

7.2.5.1.2. Screening based on cytotoxicity on EAC cells

The *in vitro* cytotoxic activity of OCM, OSM and ORM (at concentrations ranging from 400 to 1200 µg/ml) was determined using trypan blue dye exclusion method (section 2.2.3.1.3, Chapter 2). The toxicity of these extracts to normal spleen cells was also determined.

Based on the result of the assay, and considering the turn-over of biomass and percentage yield during extraction, the callus extract (OCM) was used for further comparative studies with OIM.

7.2.5.2. In vitro cytotoxicity on DLA cell lines

The cytotoxic activity of OCM (at concentrations ranging from 400 to 1200 µg/ml) on DLA cells was determined using the trypan blue dye exclusion method (section 2.2.3.1.3, Chapter 2). The result was compared with that of OIM.

7.2.5.3. In vitro anti proliferative activity on human cancer cell lines

The toxicity of OCM extract at concentrations upto 250 µg/ml on HeLa, MDA-MB-231, Hep G2 and HCT 15 was determined using MTT assay (section 2.2.3.2.2, Chapter 2). Vero cell lines were used as control. The result was compared with that of OIM.

7.2.5.4. In vitro antioxidant activity

The *in vitro* anti oxidant activities of OCM extract was determined by the superoxide scavenging assay, hydroxyl radical scavenging assay, DPPH radical scavenging assay, ABTS radical scavenging assay and Ferric reducing antioxidant power (FRAP) assay using the methods described in sections 2.2.9.1, 2.2.9.2, 2.2.9.3, 2.2.9.4 and 2.2.9.5 (Chapter 2) respectively.

7.2.5.4.1. Superoxide scavenging assay

OCM extract at concentrations ranging from 60-300 µg/ml was used to determine the superoxide radical scavenging activity as per method given in section 2.2.9.1, Chapter 2.

7.2.5.4.2. Hydroxyl radical scavenging assay

OCM extract at concentrations ranging from 40-200 µg/ml was used to determine the inhibition of hydroxyl radical formation *in vitro,* as per method given in section 2.2.9.2, Chapter 2.

7.2.5.4.3. DPPH radical scavenging assay

OCM extract at concentrations ranging from 50-150 µg/ml was used to determine the DPPH radical scavenging activity as per method given in section 2.2.9.3, Chapter 2.

7.2.5.4.4. ABTS radical scavenging assay

OCM extract at concentrations ranging from 10-50 µg/ml was used to determine the ABTS radical scavenging activity as per method given in section 2.2.9.4, Chapter 2.

7.2.5.4.5. FRAP assay

OCM extract at concentrations ranging from 8-24 µg/ml was used to determine the *in vitro* antioxidant activity by FRAP assay, as per method given in section 2.2.9.5, Chapter 2.

7.2.5.5. In vivo anti inflammatory activity

The *in vivo* anti inflammatory activity of OCM was determined using the carrageenan induced acute paw edema model. Prior to the study, acute toxicity of OCM extract was tested in Swiss albino mice (25-30 g). Animals were divided into six groups of three animals each, belonging to each sex. After overnight fasting, a single dose of OCM extract was administered orally at dosages 0.05, 0.5, 1, 2, and 4 g/kg., b.wt. The animals were observed for mortality or behavioral changes for subsequent 7 days according to guidelines of the Organization for Economic Cooperation and Development-423 with slight modifications (O.E.C.D., 2001).

Animals were divided into four groups with 6 members each under the following treatment protocol for five consecutive days. The dosage of OCM used was same as that of OIM, to facilitate comparison. Group I served as the untreated negative control. Group II was maintained as the standard or positive control group, treated with Diclofenac (i.p.) at a dose of 10mg/kg b.wt. Groups III and IV were

treated with OCM extract at dosages 200 (low dose - OCML) and 400 (high dose - OCMH) mg/kg b.wt. The induction of acute paw edema and subsequent recording of paw volume followed the same protocol as done for determining the anti inflammatory activity of OIM in carrageenan injected models (section 5.2.6.2, Chapter 5).

7.2.6. Statistical analysis

Values were expressed as mean ± SD for specified number (given in results) of replica culture tubes in tissue culture experiments. For *in vitro* cytotoxic, anti proliferative and antioxidant studies, values were expressed as mean ± SD for samples in triplicate. Results are represented as mean ± SD of 6 animals, for *in vivo* anti inflammatory study.

7.3. Results

7.3.1. Effect of growth regulators on callus induction

Nodal explants of *O. indicum* Vent. showed best callusing ability in MS media fortified with the growth regulator formulation BA3 NAA1 with an increase in biomass of 1.8 ± 0.48 g, as given in table 7.1. Stages of callus initiation are represented in figure 7.1.

Figure 7.1. Stages of callus initiation from nodal explants of *O. indicum* Vent. in MS media fortified with BA3 and IBA1. a) Callus initiation from nodal explant; b) subcultured callus; c) fully grown callus

a) b) c)

Table 7.1. Biomass (g) of callus generated from *O. indicum* Vent. nodal sections in MS media fortified with the growth regulators- BA, NAA, IAA and IBA in specified combinations. *Values expressed are mean ± SD for 72 culture tubes.

BA (mg/L)	**NAA (mg/L)**	**IAA (mg/L)**	**IBA (mg/L)**	**Increase in biomass(g) per inoculate**
2	0.5	-	-	0.79±0.13*
2	1	-	-	0.91±0.16
3	0.5	-	-	0.85±0.29
3	1	-	-	1.61±0.67
2	-	0.5	-	0.39±0.35
2	-	1	-	0.46±0.13
3	-	0.5	-	0.40±0.68
3	-	1	-	0.87±0.11
2	-	-	0.5	0.89 ± 0.32
2	-	-	1	0.89 ± 0.45
3	-	-	0.5	1.74 ± 0.51
3	**-**	**-**	**1**	**1.8 ± 0.48**

7.3.2. Effect of growth regulators on shoot initiation from callus

Highest shooting response was observed in BA3 NAA0.5 medium with 80.5% of the calluses developing shoots in 8 weeks (Figure 7.2.). An average number of 4.33 ± 0.76 shoots initiated per inoculum in this medium. Meanwhile, only 51.4% of shooting response was recorded in BA2 IBA0.5 medium. The percentage of shoot initiation and the number of shoots developed per inoculum in each medium is represented in table 7.2.

Figure 7.2. Shoots induced from *O. indicum* Vent. calluses in MS media fortified with BA and IBA in different combinations

BA3 IBA0.5 BA3 IBA1 BA2 IBA0.5 BA3 IBA1

Table 7.2. The percentage of shoot initiation and the number of shoots developed per 500 g callus inoculum of *O. indicum* Vent. in MS media fortified with the growth regulators- BA, NAA, IAA and IBA in specified combinations.

BA (mg/L)	**NAA (mg/L)**	**IAA (mg/L)**	**IBA (mg/L)**	**Percentage of shoot initiating calluses***	**Average number of shoots generated per responsive callus****
2	0.5	-	-	77.7	3.94±0.99
2	1	-	-	61.1	3.77±0.87
3	**0.5**	-	-	**80.5**	**4.33±0.76**
3	1	-	-	77.7	4.00±0.97
2	-	0.5	-	66.6	1.66±0.97
2	-	1	-	55.5	2.44±0.92
3	-	0.5	-	69.4	2.61±0.84
3	-	1	-	58.3	2.50±0.85
2	-	-	0.5	51.4	2.77±0.85
2	-	-	1	58.3	1.94 ± 0.99
3	-	-	0.5	58.3	1.46±0.97
3	-	-	1	61.1	1.37 ± 0.75

***** Values expressed are mean ± SD for 72 culture tubes; ****** Values expressed are mean ± SD for 36 culture tubes

7.3.3. Effect of growth regulators on root initiation from callus

Rooting responses of calluses were highest in IBA2 medium. 83% of the inoculated calluses developed roots in IBA2 medium (Figure 7.3.). Maximum number of roots (11.20 ± 1.55) was also initiated in the same medium (Table 7.3.). At the same time, there were no rooting responses obtained in the presence of NAA or IAA.

Figure 7.3. Roots induced from *O. indicum* Vent. calluses in MS media fortified with IBA2

Table 7.3. The percentage of root initiation and the number of roots developed per 500 g callus inoculum of *O. indicum* Vent. in MS media fortified with the growth regulators- NAA, IAA and IBA in specified combinations.

Conc. of growth regulator used		**% of root initiating calluses***	**Number of roots generated per responsive callus****
IBA (mg/L)	**0.5**	33 %	5.00±0.82
	1	75%	9.33±1.11
	2	**83%**	**11.20±1.55**
NAA (0.5, 1 and 2 mg/L)		No rooting responses found in the three combinations	
IAA (0.5, 1 and 2 mg/L)		No rooting responses found in the three combinations	

***** Values expressed are mean ± SD for 72 culture tubes

****** Values expressed are mean ± SD for 28 culture tubes

7.3.4. Effect of growth regulators on in vitro root initiation

Rooting responses of *in vitro* shoots were also found to be highest in IBA2 medium with 100% rooting (Figure 7.4.). In the medium, each inoculum developed 16.17 ± 2.51 roots (Table 7.4.). Media fortified with NAA or IAA was not found to support rooting from shoots.

Figure 7.4. Roots induced from *in vitro* shoots of *O. indicum* Vent. in MS media fortified with IBA2

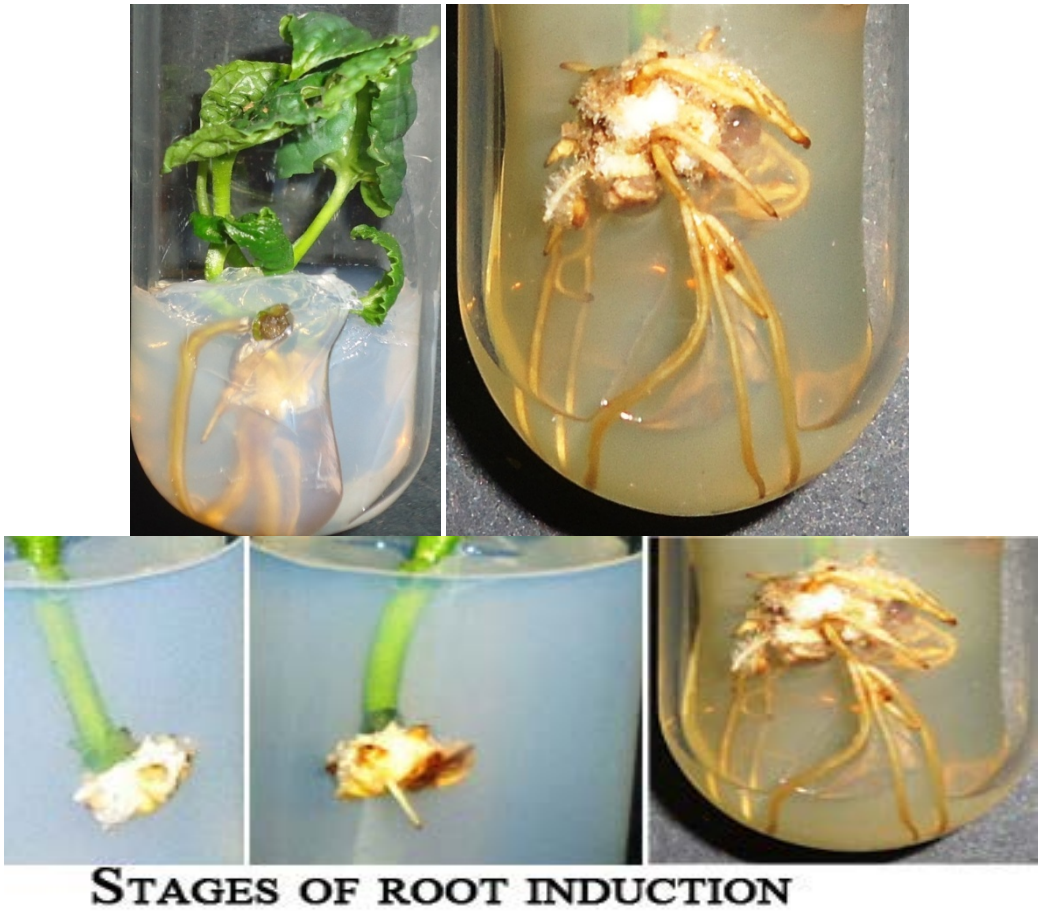


Table 7.4. The percentage of root initiation and the number of roots developed per *in vitro* shoot inoculum of *O. indicum* Vent. in MS media fortified with the growth regulators- NAA, IAA and IBA in specified combinations.

Conc. of growth regulator used		**% of root initiating shoots***	**Number of roots generated per responsive shoot****
IBA (mg/L)	**0.5**	41 %	4.20±0.84
	1	92 %	10.27±1.79
	2	**100%**	**16.17±2.51**
NAA (0.5, 1 and 2 mg/L)		No rooting responses found in the three combinations	
IAA (0.5, 1 and 2 mg/L)		No rooting responses found in the three combinations	

***** Values expressed are mean ± SD for 72 culture tubes

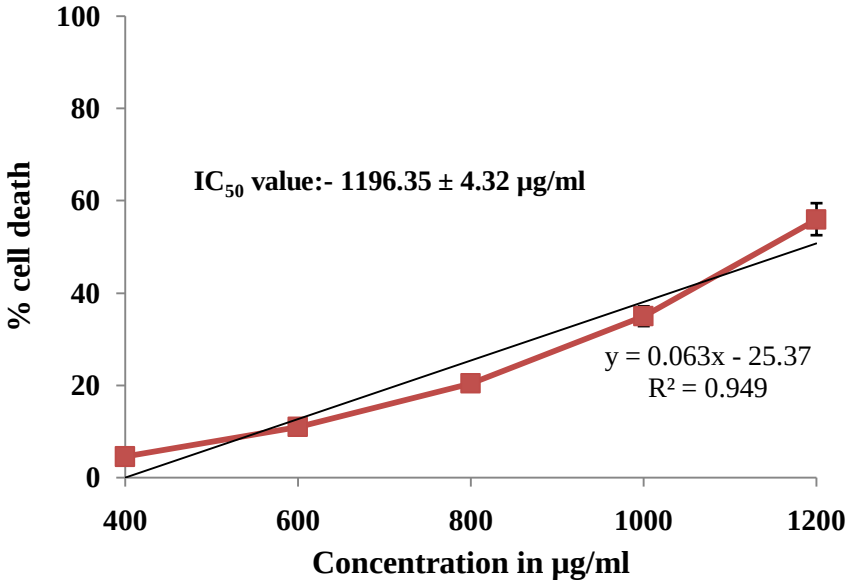
****** Values expressed are mean ± SD for 28 culture tubes

7.3.5. Comparison of in vitro cytotoxicity of callus, callus-generated-shoots and in vitro roots

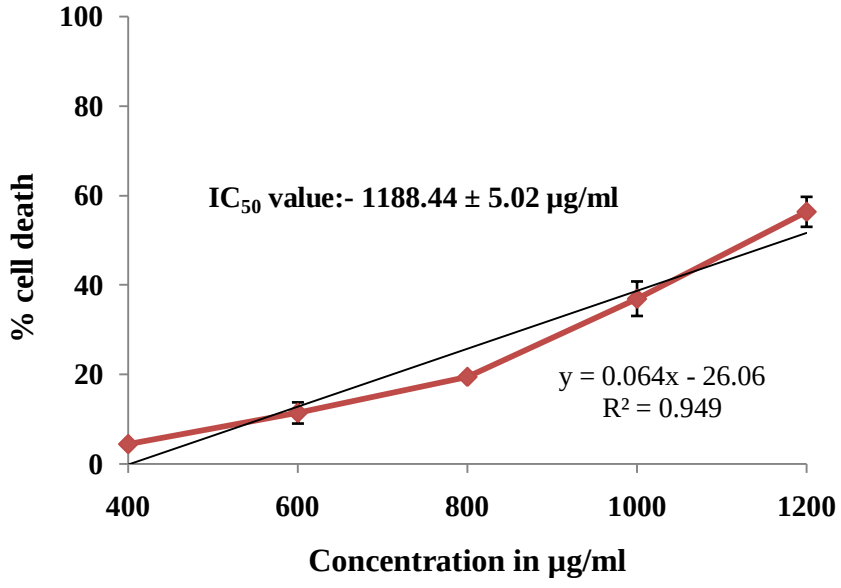
Out of the three extracts used in the study, the callus extract (OCM) and root extract (ORM) were found to be more cytotoxic to EAC cells, compared to the shoot extract (OSM). The IC_{50} of OCM and ORM were 1196.35 ± 4.32 and 1188.44 ± 5.02 µg/ml respectively, as represented in figure 7.5. Neither of the extracts was toxic to spleen cells. OSM extract did not show 50% toxicity to EAC cells up to a concentration of 1200 µg/ml.

However, the cytotoxic activities of OCM, ORM and OSM were lesser compared to that of the extract from wild root bark (OIM), with IC_{50} at 252.53 ± 1.28 µg/ml.

Figure 7.5. Comparison of *in vitro* cytotoxicity of the 70% hydromethanolic callus extract (OCM) (a) and70 % hydromethanolic *in vitro* root extract (ORM) (b) of *O. indicum* Vent. to EAC cells. Values are mean 5.02 ± SD for experiments run in triplicate.



a)



b)

7.3.6. Comparative evaluation of pharmacological activities of wild root bark extract and callus extract

The biomass of callus, *in vitro* shoots and *in vitro* roots generated per inoculum and the percentage of yield after solvent extraction is tabulated (Table 7.5.). The biomass and percentage yield of callus was comparatively higher than callus generated organs. Moreover, the cytotoxic activity of callus and root extracts (OCM and ORM respectively) on EAC cells did not show significant variation. So, further comparative studies with OIM, the callus extract (OCM) was used.

Table 7.5. The biomass of callus, callus-generated-shoots and *in vitro* roots generated per inoculum by micropropagation of *O. indicum* Vent. in MS media fortified with the growth regulators BA3 IBA1, BA3 NAA0.5 and IBA2 respectively. The percentage of yield after solvent extraction is also given.

Sample	**Biomass per inoculate***	**% yield of extract**
OCM	**1.8 ± 0.48***	**5.17%**
OSM	0.96± 0.07**	4.92%
ORM1	0.32± 0.09***	2.97%

*,** and *** Values expressed are mean ± SD for 72, 36 and 28 culture tubes respectively

7.3.6.1. In vitro cytotoxicity in DLA cells

The OCM extract posed 50% toxicity to DLA cells at a concentration of 981.17 ± 1.88 µg/ml (Figure 7.6.). The toxic effect was lesser than that of the wild extract (OIM), which induced DLA cell death with an IC_{50} value of 259.07 ± 2.09 µg/ml (Figure 7.7).

Figure 7.6. *In vitro* cytotoxicity of the 70% hydromethanolic callus extract (OCM) of *O. indicum* Vent. to DLA cells.

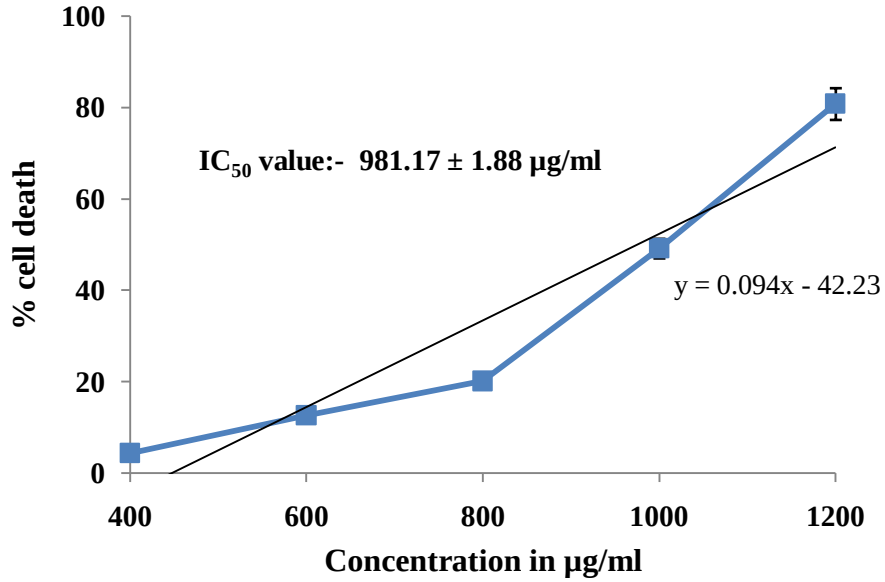
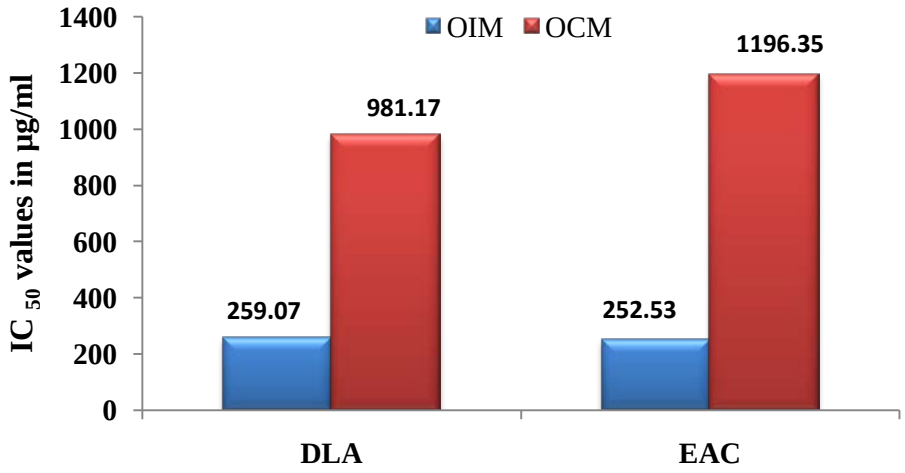


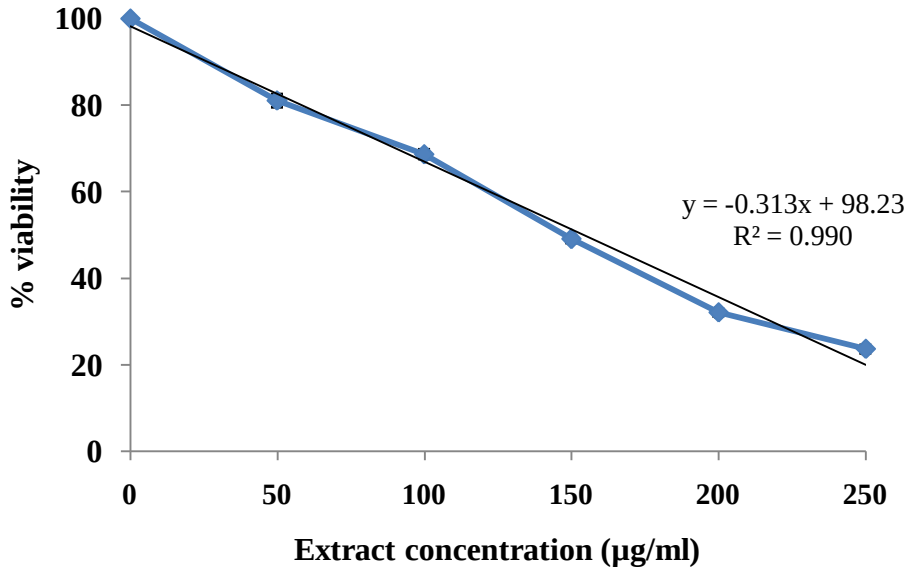
Figure 7.7. Comparison of *in vitro* cytotoxicity of the 70% hydromethanolic callus extract (OCM) and 70 % hydromethanolic wild root bark extract (OIM) of *O. indicum* Vent. to DLA and EAC cells.



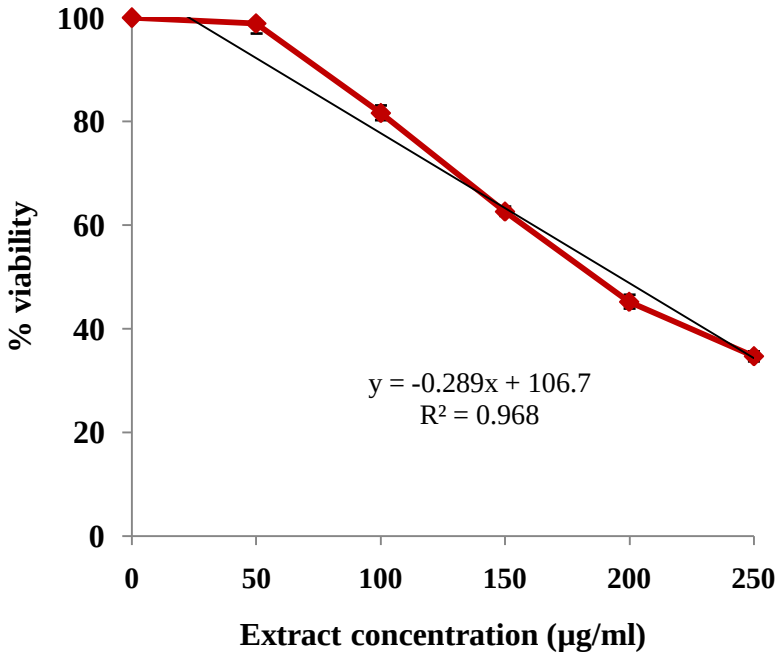
7.3.6.2. Anti proliferative activity

It was found that at concentrations of 154.09 ± 1.11, 196.2 ± 0.75 and 224.6 ± 0.49 µg/ml respectively, the OCM extract brought about 50% inhibition in the proliferation of HeLa, HCT15 and MDA-MB-231 cells (Figure 7.8.). However, IC_{50} values were indeterminable for Hep G2 cells, even up to a concentration of 250 µg/ml of OCM. No inhibition was effected by the OCM extract to the proliferation of Vero cells. The wild OIM extract was also found to be non-inhibitory to Vero cells. However, it exerted higher inhibiting activity on HeLa, HCT15 and MDA-MB-231 cell growth, with IC_{50} values- 92.43, 133.0 and 112.84 µg/ml respectively (Figure- 7.9.). The effect of both OIM and OCM was similar in that they could not bring about 50% proliferative inhibition of Hep G2 cells, at the highest concentrations used in the assays, indicating that they were less toxic to Hep G2 cells, in comparison with other cell lines.

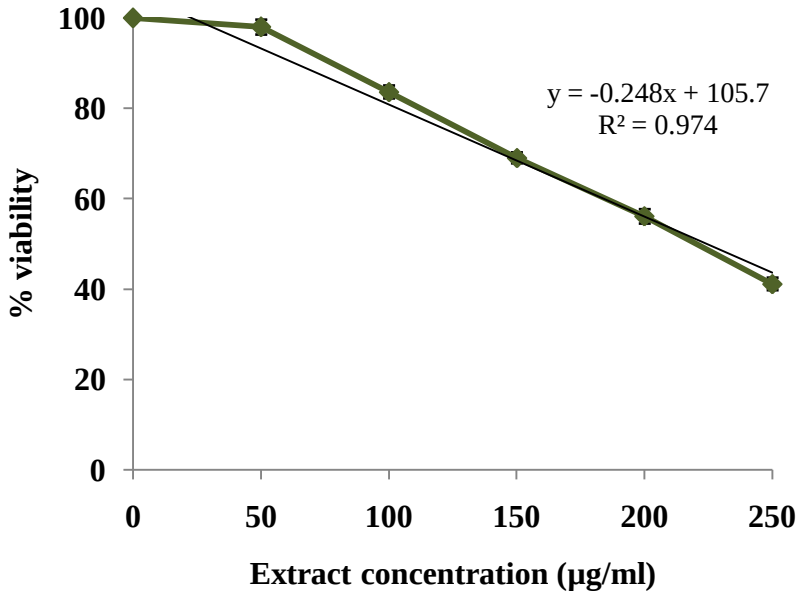
Figure 7.8. Anti proliferative activity of the 70% hydromethanolic callus extract (OCM) of *O. indicum* Vent. against HeLa (a), HCT15 (b) and MDA-MB-231 (c) cells



a)

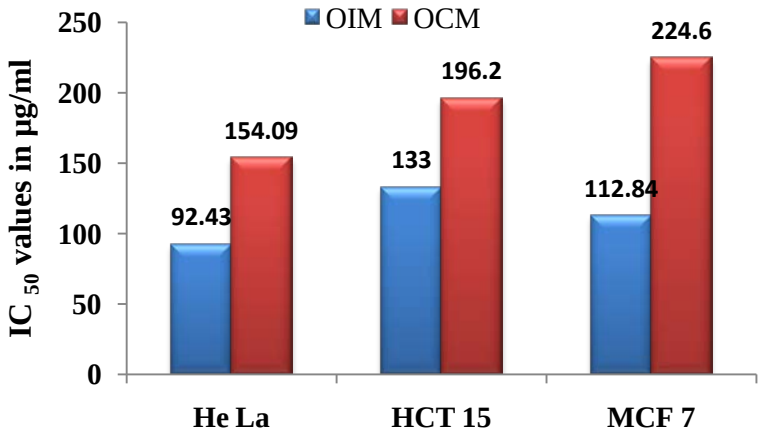

100
80
60
40
20
0
% viability
y = -0.289x + 106.7
R² = 0.968
0
50
100
150
200
250
Extract concentration (µg/ml)

b)


100
80
60
40
20
0
% viability
y = -0.248x + 105.7
R² = 0.974
0
50
100
150
200
250
Extract concentration (µg/ml)

c)

Figure- 7.9. Comparison of anti proliferative activity of the 70% hydromethanolic callus extract (OCM) and 70% hydromethanolic wild root bark extract (OIM) of *O. indicum* Vent. on HeLa, HCT15 and MDA-MB-231 cell growth.

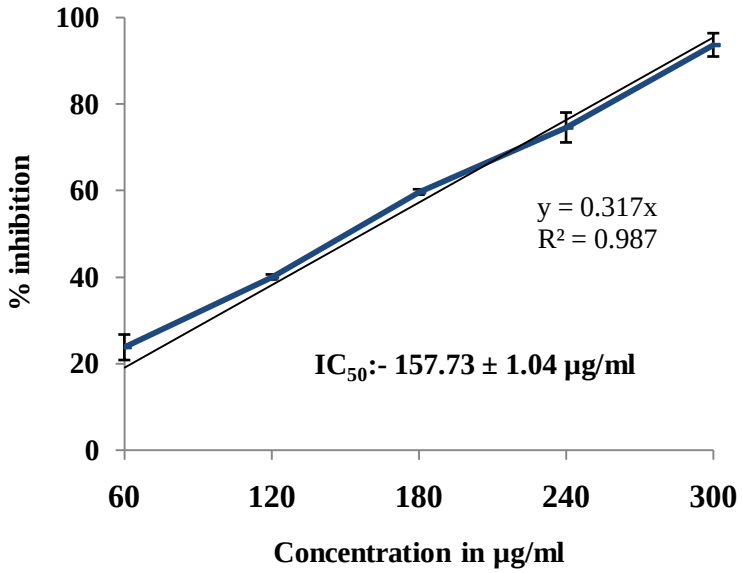


7.3.6.3. In vitro antioxidant activity

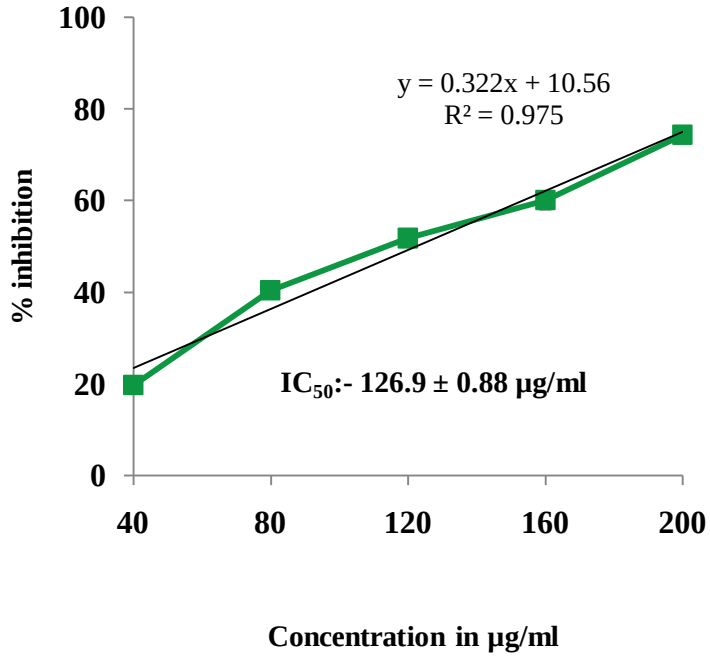
The percentage inhibition of superoxide, hydroxyl, DPPH and ABTS radicals by OCM extract at various concentrations is given in figure 7.10. a, b, c, d respectively. The IC_{50} values determined in the superoxide, hydroxyl, DPPH and ABTS scavenging assays were 157.73 ± 1.04, 126.9 ± 0.88, 124.84 ± 1.56 and 18.58 ± 0.04 µg/ml respectively. In the FRAP assay to determine the reducing power of OCM extract, the increase in reducing equivalents of $FeSO_4.7H_2O$, determined from the standard graph (Figure 7.11.a) was found to be dependent on the concentration of OCM, as seen in figure 7.11.b.

Comparing the IC_{50} values of OIM and OCM in radical scavenging/inhibiting assays (Figure 7.12), and the EC_{50} values of FRAP assay (Figure 7.13), it was found that the wild root bark extract exhibited higher *in vitro* antioxidant activity.

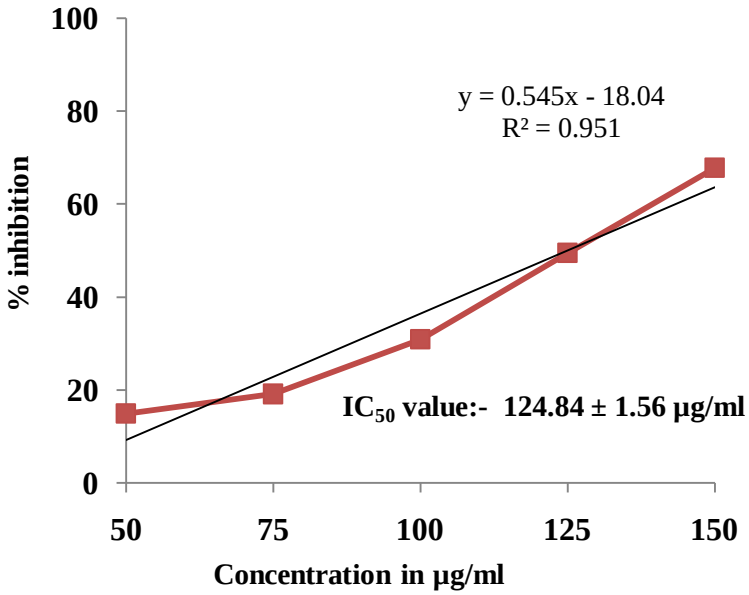
Figure 7.10. The percentage inhibition of superoxide, hydroxyl, DPPH and ABTS radicals (7.10.a, b, c, d) by 70% hydromethanolic OCM extract in *in vitro* antioxidant assays



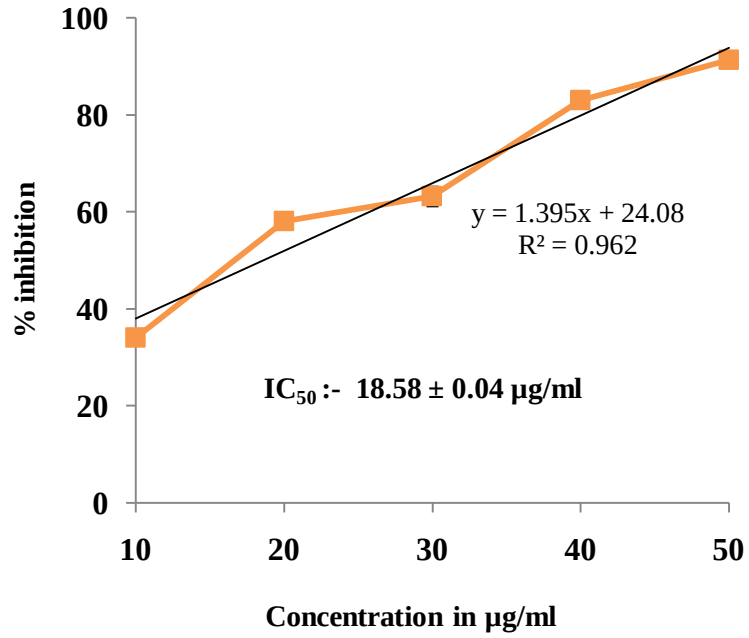
a)



b)


100
80
60
40
20
0
% inhibition
y = 0.545x - 18.04
R² = 0.951
IC50 value:- 124.84 ± 1.56 µg/ml
50
75
100
125
150
Concentration in µg/ml

c)


100
80
60
40
20
0
% inhibition
y = 1.395x + 24.08
R² = 0.962
IC50 :- 18.58 ± 0.04 µg/ml
10
20
30
40
50
Concentration in µg/ml

d)

Figure 7.11. FRAP assay to determine the reducing power of 70% hydromethanolic OCM extract

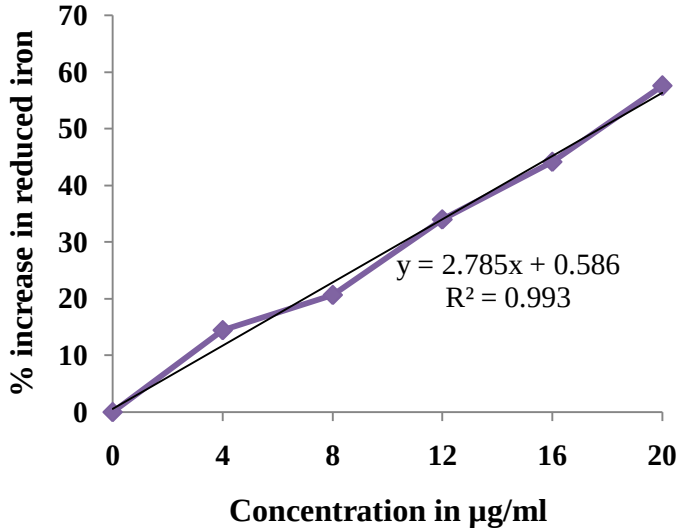


Figure 7.12. Comparison of antioxidant activities of 70% hydromethanolic callus extract (OCM) and 70% hydromethanolic wild root bark extract (OIM) of *O. indicum* Vent. OIM and OCM extracts in radical scavenging/inhibiting assays.

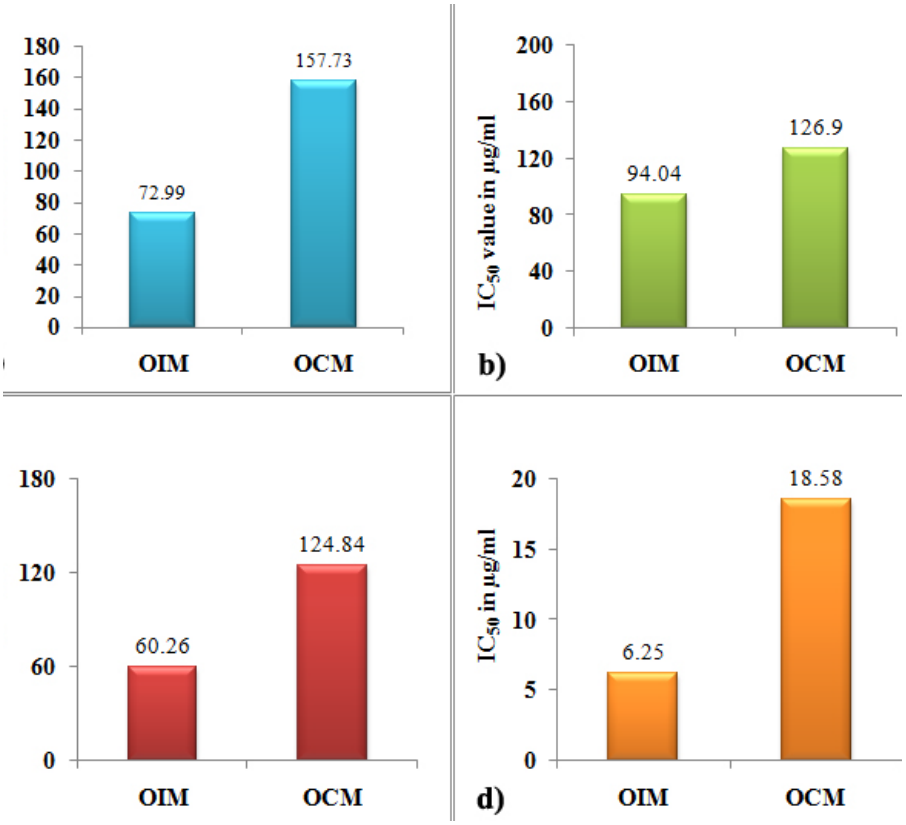
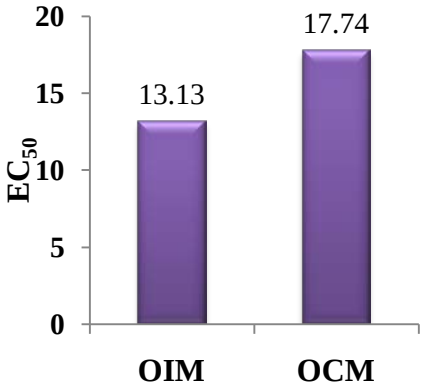


Figure 7.13. Comparison of EC_{50} values of 70% hydromethanolic callus extract (OCM) and 70% hydromethanolic wild root bark extract (OIM) of *O. indicum* Vent. in FRAP assay



7.3.6.4. In vivo anti inflammatory activity

Figure 7.14. shows the effect of OCM administration on paw edema of animals. In acute paw oedema models treated with OCM extract at a dosage of 400 mg/kg body weight (OCMH), a notable reduction of inflammation occurred by 33.14% in the 3rd hour, in comparison to the control. Meanwhile, a reduction of 31.98% was obtained in OCML group. The thickness of paw recorded in the 3rd hour for standard (0.282 ± 0.009 cm), OCMH (0.322 ± 0.015 cm) and OCML (0.332 ± 0.01 cm) groups was significantly different ($p<0.05$) from that of the untreated group (0.355 ± 0.021 cm). But with the same dosage administration of OIM in acute inflammation models, inhibition of paw edema was attained up to 41.28%, indicating higher anti inflammatory activity, compared to the callus (Figure 7.15).

Figure 7.14. Effect of 70% hydromethanolic *O. indicum* callus extract OCM administration on paw edema of animals in carrageenan induced acute paw oedema models

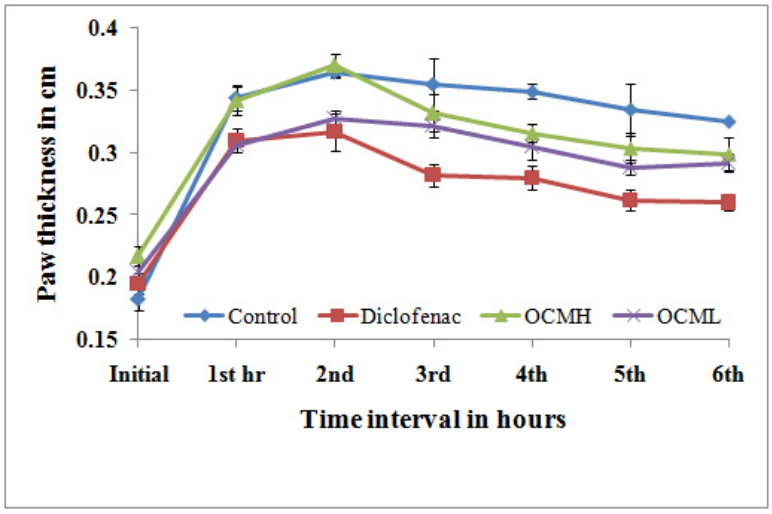
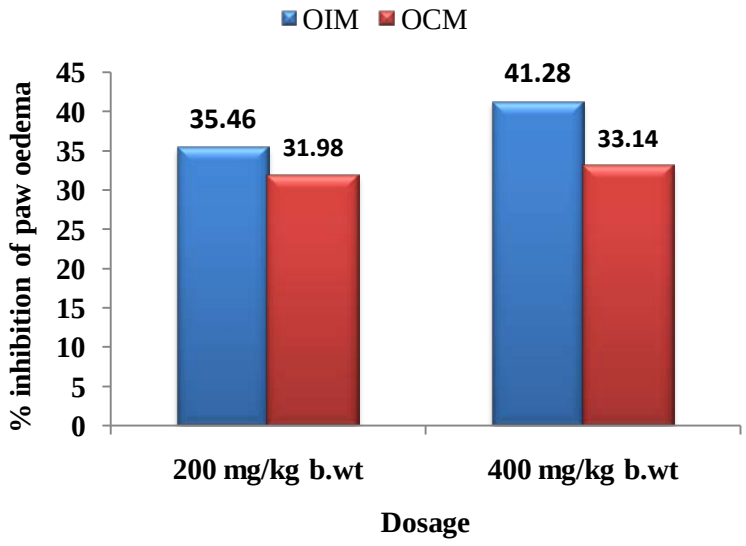


Figure 7.15). Comparison of 70% hydromethanolic callus extract (OCM) and 70% hydromethanolic wild root bark extract (OIM) of *O. indicum* Vent. in inhibition of paw edema in carrageenan induced acute inflammation models



7.4. Discussion

In the context of depleting medicinal plant biodiversity, the scope of *in vitro* regeneration is accelerating pace, thereby ensuring continuous supply of resources for producing plant based medicines (Tripathi and Tripathi, 2003); and the results of the present study suggest possibilities of re-establishing the biodiversity of *O. indicum* Vent. and ensuring its continuous supply. The standardization of culture conditions is a crucial step in tissue culture because the nutritional requirements, optimal growth conditions and responses of plants to growth regulators vary among species. In the present study, nodal explants from *O. indicum* Vent. showed callusing, shooting and rooting responses in the provided culture conditions. The nodal explants developed green friable calluses later turning compact, when kept in BA (cytokinin)-NAA (auxin) medium. Dark condition favoured callus initiation and growth. MS medium supplemented with BA (3 mg/L) and NAA (0.5 mg/L) was found to be superior for callus growth, compared to the other growth regulator combinations.

Auxins, cytokinins and their interactions influence cell growth, cell division, tissue differentiation and organogenesis in plants (Su *et al.,* 2011). Cytokinins stimulate cell division, release lateral bud dormancy and promote adventitious lateral bud formation. Auxins initiate cell division, promote root development and organisation of meristems to calluses (Aloni *et al.,* 2006, Müller and Leyser, 2011). But, when used in combination, the requisite for each plant varies. Here, with auxin concentrations kept unchanged (1 mg/L or 0.5 mg/L), increasing cytokinin concentration (3 mg/L) promoted higher growth rate of callus than 2 mg/L formulation. Similarly, keeping cytokinin concentrations constant (2 or 3 mg/L), it was found that 1 mg/L of the auxin NAA favoured better growth rate than 0.5 mg/L application. Hence it is conclusive that both auxins and cytokinins positively influence callus development and growth in *O. indicum* Vent. in tissue culture systems. Selection of concentration and combination of plant growth regulators is significant to shoot regeneration. Addition of cytokinin to the

medium greatly improves the regeneration and frequency of shoots as reported previously (Fatima and Anis, 2012). From the results of the current study also, it is demonstrated that the presence of higher amounts of cytokinins and lower amounts of auxins favour shoot initiation. Medium containing BA at 3 mg/L and NAA at 0.5 mg/L were superior to those with BA at 2 mg/L and NAA at 1 mg/L. The highest rooting percentage was obtained on medium containing IBA 2 mg/L, compared to lower concentrations. There are previous reports of root induction in *Bixa orellana* (Neto et al., 2003) and *Dioscorea zingiberensis* (Chen et al., 2003) by the addition of IBA. Consistent to these reports, the present investigation also showed higher rooting responses with increasing concentrations of IBA. In all the studies, control inoculums devoid of growth factor supplementation showed neither callusing nor shooting/rooting responses in the basal media. Hence, it is conclusive that the presence of auxins and cytokinins used in appropriate combinations can diversely generate organogenetic responses in *O. indicum* Vent. This also throws light on the scope of standardizing *ex situ* conservation techniques and regeneration of this highly valuable medicinal plant and protecting it from the threat of extinction.

The validation of pharmacological and phytochemical properties of tissue culture derived plant/plant parts is as important as their regeneration. The bioactive properties are attributed mainly to the presence of secondary metabolites in plants, whose production shows qualitative and quantitative variations in accordance with the growth stages and ambient conditions. Hence, to rely on tissue culture techniques for regeneration and use of medicinal plants, a comparison of wild and in vitro propagated plants is necessary (Subramaniam *et al.*, 2014). In previous chapters, it was found that the extract from wild root bark (OIM) showed cytotoxic and anti proliferative effects on cancer cells as well antioxidant and anti inflammatory properties. In this study, the preliminary screening of cytotoxic properties of callus, *in vitro* shoot and *in vitro* root extracts showed that the callus and roots were more toxic to EAC cells than shoot extracts. But the turn-over of biomass and yield of extract is crucial when tissue

culture is depended as an alternative source for plant-based drug development. Since the callus showed no significant variation from *in vitro* roots in EAC cell toxicity, and at the same time was superior to the *in vitro* shoots, further comparative studies were conducted using callus extract (OCM). Moreover, development of callus cultures has been identified as an alternative source for biologically important secondary metabolites (Rao and Ravishankar, 2002, Ali *et al.*, 2013), and therefore, can evade situations of sacrificing whole plants. This is particularly true for *O. indicum* Vent., which is being vastly uprooted for its utility in Ayurvedic and allied preparations.

Throughout the study aimed at comparison of biological activities of OCM and OIM, the results showed that the callus extract was less active, particularly in the anti proliferative and cytotoxic assays, where 50% inhibition of proliferation/toxicity was attained at much higher concentrations than OIM. This may be due to the undifferentiated stage of callus, when the rate of secondary metabolite production is less. Hence, the study necessitated the comparison of the phytochemical profile of root bark extract and callus extract, as discussed in the forthcoming chapter. Meanwhile, in the *in vitro* radical scavenging assays, the IC_{50} values were attained only at slightly higher concentrations than that of OIM. Similarly, in carrageenan induced paw edema models pre-treated with OCM in the same dose regime as that of OIM, the former reduced the edema volume, with significantly higher activity ($p<0.01$ for high dose; $p<0.05$ for low dose) with reference to the untreated group. This showed that the possibility of developing the callus as a substitute for wild plant parts cannot be ruled out; provided a biotechnological approach is adopted for enhancement of its biological activities. So, in the forthcoming chapter, the study is extended to the use of elicitors in tissue culture media for enhancing the phytochemical profile of callus extract, and analyzing the effects through HPLC based quantification.

Chapter 8

Enhancement of phytochemical profile of *Oroxylum indicum* Vent. callus through elicitation techniques

8.1. Introduction

Plants are rich sources of bioactive secondary metabolites that resist them against predators, pathogens and physical stress. A vast variety of them provide health benefits to humans. Different classes of secondary metabolites – phenolic compounds (Azmi *et al.*, 2006, Apostolou *et al.*, 2013), flavonoids (Ren *et al.*, 2003, Chahar *et al.*, 2011), terpenoids (Huang *et al.*, 2012), alkaloids (Wall and Wani, 1996), tannins (Marzouk *et al.*, 2007), saponins (Man *et al.*, 2010) and steroids (Bradford and Awad, 2007) - in plants have been proved to be potent anti cancer agents. Hence, the phytochemical evaluation of plant derived extracts is a pre-requisite for cancer drug discovery. In the previous chapters, the anti cancer, antioxidant, anti-inflammatory and chemoprotective properties of *O. indicum* Vent. was evaluated, thereby emphasizing the need of regeneration of this threatened plant through tissue culture. A comparison of the wild root bark extract with that of the regenerated callus showed that the latter is less active. So, the present study aimed at a comparative evaluation of the phytochemical properties of these two extracts- OIM (wild) and OCM (callus).

Since plant extracts are mixtures of different types of compounds, the separation and analysis of compounds is to be attained precisely, which involves chromatographic procedures like TLC, HPLC, HPTLC, column chromatography etc, as well as non-chromatographic procedures such as phytochemical screening assays and immunoassays, following solvent extraction (Sasidharan *et al.*, 2011). In this chapter, OIM and OCM extracts have been evaluated through qualitative and quantitative phytochemical methods. Qualitative comparative evaluation has been done using preliminary screening for different phytochemical classes, HPTLC profiling and UPLC-Q-TOF-MS analysis. Through HPLC, the reconfirmation of the presence of two already reported flavonoids - chrysin, baicalein (Zaveri *et al.*, 2008) - was done in OIM. OCM, on HPLC analysis also revealed the presence of these two compounds in the callus extract. The estimation of total flavonoid and phenol content was done in OIM and OCM.

Both extracts were then subjected to HPLC based quantification of baicalein and chrysin.

Consistent with the results of comparative pharmacological evaluation of OIM and OCM, their phytochemical comparison also revealed the richness of compounds in OIM, mainly with reference to the HPLC based quantification data of baicalein and chrysin. Hence, an attempt was made to enhance the phytochemical richness of the callus by elicitation techniques. Elicitors are biotic or abiotic signals that trigger stress response pathways in plants and enhance the formation of secondary metabolites. Exposure of plants to biotic elicitors (e.g, microbial enzymes, fungal and bacterial lysates, yeast extracts, microbial cell wall polysaccharides), chemical elicitors (e.g, methyl jasmonate, salicylates) and physical elicitors (gamma, UV and X-ray radiations) have been demonstrated to enhance secondary metabolite production in different plants (Rao and Ravishankar, 2002, Karuppusamy, 2009, Ramirez-Estrada *et al.*, 2016).

The present study involves the enhancement of phytochemical profile in *O. indicum* Vent. callus through two techniques - by introducing methyl jasmonate (MeJa- a chemical elicitor) in tissue culture medium and by exposing calluses to X-ray radiations. Usually, monitoring the enhancement of metabolite production is accomplished through qualitative (e.g., GC-MS) and quantitative analysis of the extracts (e.g., HPLC) (Staniszewska *et al.*, 2003, Srivastava *et al.*, 2016). In the present study, the effect of using elicitors is on *O. indicum* Vent. callus has been tracked through HPLC-based quantification of baicalein and chrysin.

The outcome of comparative evaluation of biological activities in the previous chapter was that the wild root bark extract was more active than the callus extract. This prompted the attempts for comparison of phytochemicals in the two extracts and enhancement of secondary metabolite through elicitation in the present chapter. Hence, to investigate for the effects of elicitation, the elicitor exposed callus extracts were further subjected to two assays of pharmacological

relevance- *in vitro* anti proliferative assay on HeLa cell lines and *in vitro* antioxidant assay by DPPH radical scavenging activity.

8.2. Materials and methods

8.2.1. Chemicals

Murashige and Skoog medium and plant growth regulators – BA and IBA for tissue culture; methyl jasmonate for elicitation; Baicalein - 98% (HPLC grade) and Chrysin ≥ 98% (HPLC) grade for HPLC; and DPPH for *in vitro* radical scavenging assay were purchased as listed in section 2.1.2., Chapter 2.

8.2.1. Cell lines

HeLa cell lines for anti proliferative assays were procured as per section 2.1.7 and maintained as per section 2.2.3.2.1 of chapter 2.

8.2.3. Phytochemical comparison

The 70% hydromethanolic extracts of wild root bark (OIM) as well as callus (OCM) of micropropagated *O. indicum* Vent. were subjected to phytochemical analysis and the results were compared.

8.2.3.1. Preparation of extracts

The extract of *O. indicum* Vent. root bark (OIM) was prepared using 70% methanol as solvent according to the method described in section 2.2.1, Chapter 2. The extract of *O. indicum* Vent. callus generated in MS medium (BA3 IBA1) was made in 70% methanol as per the method described in section 7.2.5.1.1, Chapter 7.

8.2.3.2. Preliminary phytochemical screening

The OIM and OCM extracts were tested for the presence of alkaloids, phenolic compounds, tannins, flavonoids, phytosterols, steroids, saponins and carbohydrates as per the method described in section 2.2.2.1, Chapter 2.

8.2.3.3. HPTLC profiling

The OIM and OCM extracts were subjected to qualitative high performance thin layer chromatography (HPTLC) according to the method described in section 2.2.2.4, Chapter 2. The number of bands formed in both lanes was determined. The R_f values were recorded.

8.2.3.4. UPLC-Q-TOF-MS analysis

The UPLC-Q-TOF-MS analysis of OIM and OCM extracts were carried out, according to the method described in section 2.2.2.6, Chapter 2. Some of the active constituents were identified based on previous MS/MS fragmentation reports.

8.2.3.5. Total phenol content

The total phenol content of OIM and OCM extracts was estimated according to the method given in section 2.2.2.2, Chapter 2. The experiment was run in triplicate. The results were compared.

8.2.3.6. Total flavonoid content

The total flavonoid content of OIM and OCM extracts was estimated according to the method given in section 2.2.2.3, Chapter 2. The experiment was run in triplicate. The results were compared.

8.2.3.7. HPLC based quantification of baicalein and chrysin

The OIM and OCM extracts were subjected to High pressure liquid chromatography (HPLC) as per method described in section 2.2.2.5, Chapter 2. The presence of baicalein (Bcl) and chrysin (Chr) in both extracts was detected by comparing with the peaks of standard compounds dissolved in HPLC grade methanol at a concentration of 0.05 mg/ml (50 ppm). The run time was maintained up to 20 minutes. The quantification of these two compounds

in OIM and OCM extracts was done based on the peak area obtained. The experiment was run in triplicate.

8.2.4. Elicitation of secondary metabolites

Elicitation of secondary metabolite production was done in the callus cultures of *O. indicum* Vent., maintained in MS medium fortified with BA (3 mg/L) and IBA (1 mg/L).

8.2.4.1. Maintenance of suspension cultures

Suspension cultures of *O. indicum* Vent. calluses were established as per method described in section 2.2.11, Chapter 2. Before the experiments, 250 ml flasks containing 50 ml medium were inoculated with 4 g wet weight cells taken from the previous subculture. For treatment of elicitors 18-day old cell cultures were used.

8.2.4.2. Elicitation using methyl jasmonate in the medium

Cell suspension cultures of *O. indicum* Vent. were treated with methyl jasmonate (MeJa) at concentrations 25 and 50 µM (section 2.2.12.1., Chapter 2). Control cultures were maintained by the addition of deionised sterile water, instead of MeJa. Calluses were harvested on the 1^{st}, 3^{rd} and 6^{th} day of MeJa addition. The experiment was run in triplicate.

8.2.4.3. Elicitation by X- ray irradiation

Cell suspension cultures of *O. indicum* Vent. were irradiated using 6 MV X-ray radiations as per method described in section 2.2.12.2., Chapter 2. The cultures were exposed to two doses of the radiation - 0.5 Gy and 1 Gy. Non-irradiated cultures were maintained as control. Calluses were harvested on the 1^{st}, 2^{nd} and 3^{rd} day of irradiation. The experiment was run in triplicate.

8.2.4.4. Preparation of extracts from elicited calluses

The harvested calluses were lyophilized, powdered and extracted using 70% methanol as solvent, according to the method described in section 2.2.13., Chapter 2.

8.2.4.5. HPLC-based quantification of baicalein and chrysin

The callus extracts were dissolved in HPLC grade methanol and subjected to HPLC analysis, as per method described in section 2.2.2.5, Chapter 2. Quantification of Bcl and Chr in the extracts was performed by comparing the peak area with that of standard compounds, prepared at 0.05 mg/ml (50 ppm) concentration.

8.2.5. In vitro assay-based screening of elicited callus extracts

The extracts from elicitation experiments were screened for *in vitro* anti proliferative activity in HeLa cell lines and free radical scavenging activity using DPPH radical scavenging assay.

Extracts prepared from calluses harvested on 3rd day of MeJa addition as well as calluses collected on 2nd day of X-ray irradiation were used for the above assays.

8.2.5.1. Anti proliferative activity

The anti proliferative activity of the extracts at concentrations up to 250 µg/ml on HeLa cell lines was determined using MTT assay (section 2.2.3.2.2, Chapter 2).

8.2.5.2. DPPH radical scavenging activity

The DPPH radical scavenging activity of the extracts at concentrations ranging from 50-150 µg/ml was determined (section, Chapter 2).

8.2.6. Statistical analysis

Values were expressed as mean ± SD of samples in triplicate used in HPLC-based quantification, estimation of total phenol content, estimation of total flavonoid content, elicitation studies, *in vitro* anti proliferative study and *in vitro* DPPH radical scavenging assay.

8.3. Results

8.3.1. Preliminary phytochemical screening

The presence of phytoconstituents in the wild root bark (OIM) and callus (OCM) extracts is given in table 8.1. OIM extract showed positive test result for flavonoids and phenols, which were detected in high amounts. OCM extract also tested positive, with moderate presence of phenols and flavonoids. Alkaloids were also detected in both, with moderate amounts in OIM and low in OCM. Both extracts showed positive results for carbohydrates, tannins and phytosterols, though detected in low amounts. When the OIM extract tested positive for terpenoids and sterols in low quantities, OCM extract reacted negative to the tests. Neither of the extracts showed any detectable amount of saponins.

8.3.2. HPTLC profile

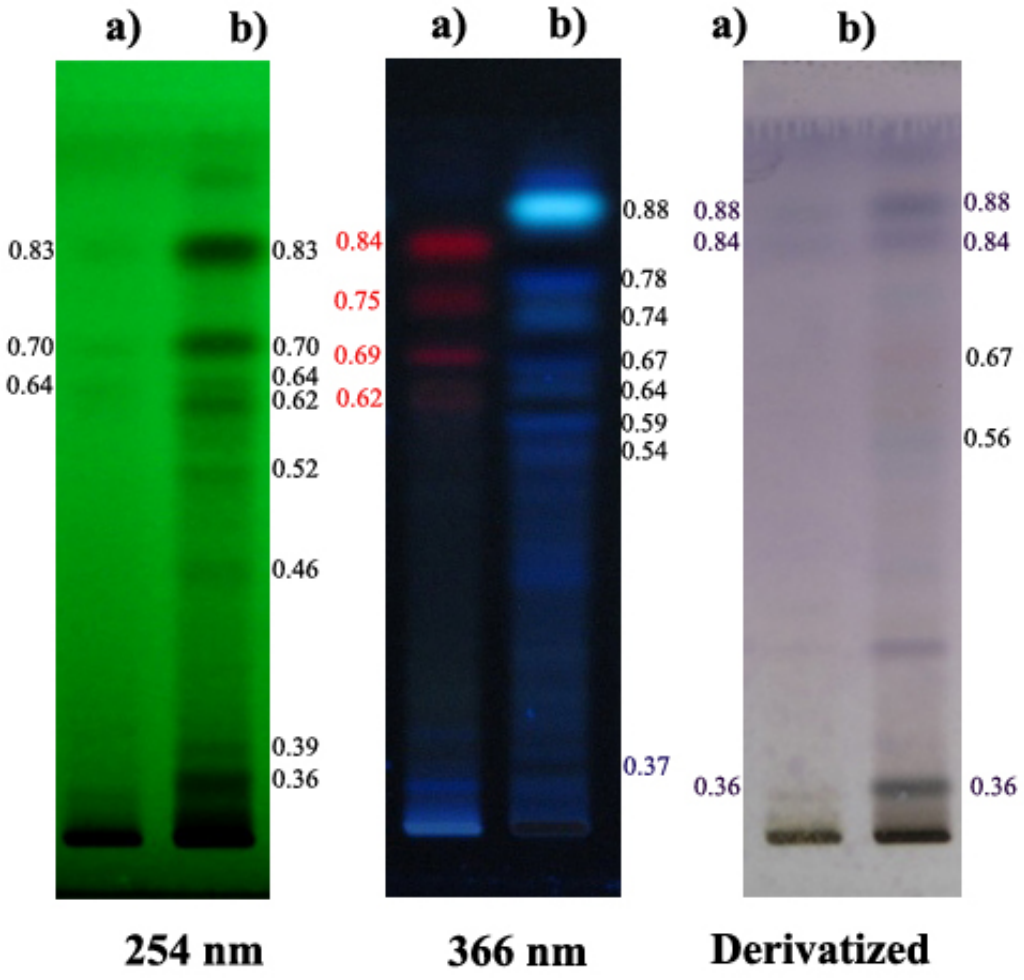
The HPTLC profile of OIM and OCM extracts is given in Figure 8.1., showing the bands detected at 254 and 366 nm scanning and by derivatization using spray reagent. At 254 nm, OIM lane revealed 8 bands (with R_f - 0.36, 0.39, 0.46, 0.52, 0.62, 0.64, 0.70 and 0.83); meanwhile 3 bands were detected in OCM lane (with R_f - 0.64, 0.70 and 0.83). Plates scanned at 366 nm revealed 8 blue bands from OIM (with R_f - 0.37, 0.54, 0.59, 0.64, 0.67, 0.74, 0.78, 0.88) and 4 red bands from OCM (with R_f - 0.62, 0.69, 0.75 and 0.89). Derivatized plate revealed 5 violet bands from OIM (with R_f - 0.36, 0.56, 0.67, 0.84 and 0.88) and 3 violet bands from OCM (with R_f - 0.36, 0.84 and 0.88).

Table 8.1. Phytoconstituents detected in *O. indicum* Vent. wild root bark (OIM) and callus (OCM) extracts

Phytoconstituent	**Detection (+/-) (presence /absence) in OIM**	**Detection (+/-) (presence /absence) in OCM**
Phenolics	+++	++
Flavonoids	+++	++
Alkaloids	++	+
Saponins	_	_
Terpenoids	+	_
Tannins	+	+
Carbohydrates	+	+
Phytosterols	+	+
Steroids	+	_

The presence of phytoconstituents in high, moderate and low amounts is indicated as '+', '++' and '+++' respectively. '_' indicates absence of a phytoconstituent.

Figure 8.1. HPTLC profile of *O. indicum* Vent. wild root bark (OIM) and callus (OCM) extracts on pre-coated silica gel 60 F_{254} TLC plates with toluene: ethyl acetate: formic acid (5:5:0.5) as solvent. The plates scanned at 366 and 254 nm as well as the derivatized plate sprayed with anisaldehyde – sulphuric acid reagent is shown in the figure. (R_f values of bands are shown).



8.3.3. UPLC-Q-TOF-MS analysis

Figure 8.2. shows the total ion chromatogram of the OIM extract subjected to *UPLC-Q-TOF-MS analysis*. The compounds provisionally identified based on previous fragmentation data from literature are enlisted in table 8.2. However, a few peaks could not be identified, as enlisted in table 8.3. Figure 8.3. shows the total ion chromatogram of the OCM extract. The compounds tentatively identified are enlisted in table 8.4. Table 8.5. lists the peaks obtained from *UPLC-Q-TOF-MS* chromatogram of OCM extract, which remained unidentified.

Figure 8.2. Total ion chromatogram of *O. indicum* Vent. wild root bark (OIM) extract subjected to *UPLC-Q-TOF-MS analysis* using Acquity UPLC H class (Waters) system with mobile phase (methanol and 0.1% aqueous formic acid) through BEH C18 column (50 mm × 2.1 mm × 1.7 µm) in gradient elution (0-5 min, 5% acetonitrile; 5-7 min, 95% methanol; 8-9 min 5% methanol). Detection wavelength set at 210 - 400 nm.

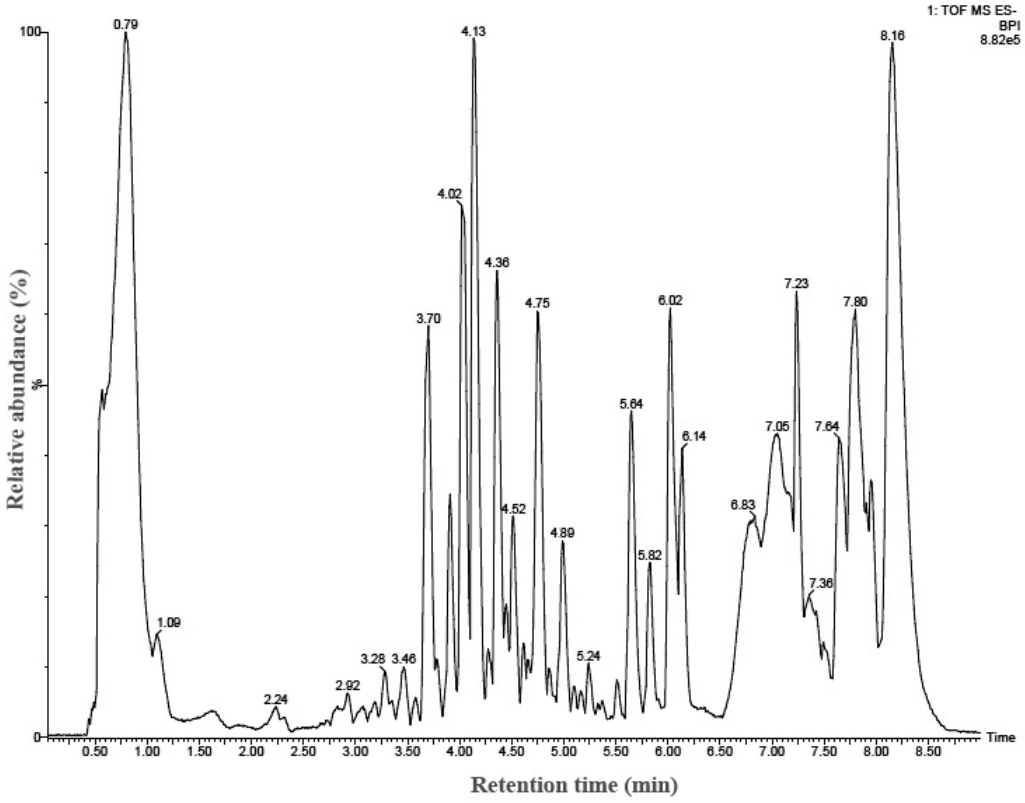


Table 8.2. The compounds tentatively identified from *O. indicum* Vent. wild root bark (OIM) extract by *UPLC-Q-TOF-MS analysis*

Sl. No:	**RT (Min)**	***m/z* and relative abundance %**	**Molecular weight (kDa)**	**Molecular formula**	**Name of the compound**
1	2.916	487.1551 (100%)	488.7	$C_{30}H_{48}O_5$	Asiatic acid
2	3.91	637.1891	638.184685	$C_{29}H_{34}O_{16}$	Demethoxycentaureidin 7-O-rutinoside
3	4.13	623.2098 (100%)	624.16903	$C_{28}H_{32}O_{16}$	Isorhamnetin-3-O-rutinoside (Narcissin)
4	4.35	607.2140 (100%)	608.174	$C_{28}H_{32}O_{15}$	Neodiosmin (Diosmetin-7-O-neohesperidoside)
5	4.75	651.2419	651.1965	$C_{28}H_{40}N_2O_9$	
6	4.89	445.1238 (20%)	446.08491	$C_{21}H_{18}O_{11}$	Baicalein-7-O-glucuronide (Baicalin)
7	5.64	269.0502 (100%)	270.05282	$C_{15}H_{10}O_5$	5,6,7-trihydroxyflavone (Baicalein)
8	5.82	327.2242	328.094688	$C_{18}H_{16}O_6$	3-Hydroxy-3',4',5'-trimethoxyflavone
9	6.14	283.0659	284.068473	$C_{16}H_{12}O_5$	5,7-Dihydroxy-3-(4-methoxyphenyl)chromen-4-one (Biochanin A)
10	7.05	299.2069	300.099774	$C_{17}H_{16}O_5$	4'-Hydroxy-5,7-dimethoxyflavanone
11	7.23	295.2332	296.104859	$C_{18}H_{16}O_4$	6-Ethoxy-3(4'-hydroxyphenyl)-4-methylcoumarin

Table 8.3. List of unidentified peaks of different retention time obtained from *O. indicum* Vent. wild root bark (OIM) extract by *UPLC-Q-TOF-MS analysis*

Sl. No:	**RT (Min)**	**m/z and relative abundance %**
1	3.701	639.2048 (100%)
2	4.02	653.2208 (100%)
3	4.508	547.1558 (100%)
4	4.75	651.2419 (100%)
5	4.75	300.0931
6	6.02	329.2394 (100%)
7	7.80	311.1749 (100%)
8	8.16	325.1904 (100%)

Figure 8.3. Total ion chromatogram of *O. indicum* Vent. callus (OCM) extract *O. indicum* Vent. subjected to *UPLC-Q-TOF-MS analysis* using Acquity UPLC H class (Waters) system with mobile phase (methanol and 0.1% aqueous formic acid) through BEH C18 column (50 mm × 2.1 mm × 1.7 µm) in gradient elution (0-5 min, 5% acetonitrile; 5-7 min, 95% methanol; 8-9 min 5% methanol). Detection wavelength set at 210 - 400 nm.

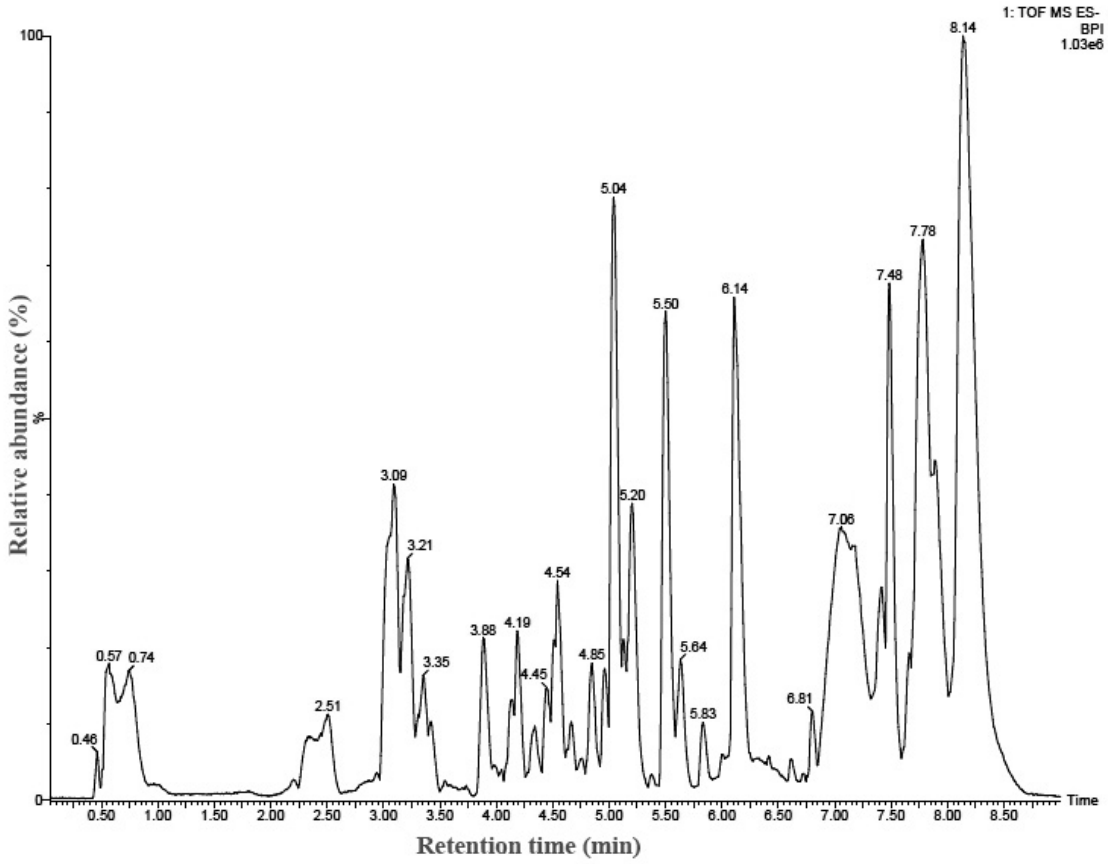


Table 8.4. The compounds tentatively identified from *O. indicum* Vent. callus (OCM) extract by *UPLC-Q-TOF-MS analysis*

Sl. No:	RT (Min)	*m/z* and relative abundance %	Molecular weight (kDa)	Molecular formula	Name of the compound
1	3.09	507.1807	508.12169	$C_{23}H_{24}O_{13}$	Syringetin-3-O-glucoside
2	3.88	163.0421	164.04734	$C_9H_8O_3$	2-Coumaric acid
3	4.85	445.0854	446.08491	$C_{21}H_{18}O_{11}$	Baicalein-7-O-glucuronide (Baicalin)
4	5.179	315.0562	316.05830	$C_{16}H_{12}O_7$	5,7-Dihydroxy-2-(4-hydroxyphenoxy)-6-methoxychromone (Capillarisine)
5	5.64	269.0502 (100%)	270.05282	$C_{15}H_{10}O_5$	5,6,7-trihydroxyflavone (Baicalein)
6	6.14	283.0658 (60%)	284.068	$C_{16}H_{12}O_5$	5,7-Dihydroxy-3-(4-methoxyphenyl)chromen-4-one (Biochanin A)
7	7.49	357.2137	358.214409	$C_{21}H_{20}O_{11}$	Luteolin-6-C-glucoside (Iso orientin)

Table 8.5. List of unidentified peaks of different retention time obtained from *O. indicum* Vent. callus (OCM) extract by *UPLC-Q-TOF-MS analysis*

Sl. No:	**RT (Min)**	***m/z*** **and relative abundance %**
1	2.51	197.0482 (100%)
2	4.19	523.1556 (100%)
3	4.54	185.0843 (100%)
4	4.54	141.0938
5	5.04	427.0747 (100%)
6	5.50	299.0612 (100%)
7	7.78	311.1745 (100%)
8	8.14	325.1910

8.3.4. Total phenol content

The total phenol content of OIM extract was 53.18 ± 0.26 mg of gallic acid equivalents (GAE)/gram of dry extract and that of OCM was 39.07 ± 0.12 mg of GAE/gram of dry extract.

8.3.5. Total flavonoid content

The total flavonoid content of OIM extract (53.88 ± 2.65 mg of quercetin equivalents (QE)/gram of dry extract) was significantly ($p < 0.01$) higher than that of OCM extract (33.34 ± 0.59 mg of QE/gram of dry extract).

8.3.6. HPLC-based quantification of Bcl and Chr

Baicalein and chrysin are flavonoids reportedly present in the root bark of *O. indicum* Vent. (Zaveri *et al.*, 2008). Through HPLC analysis, the presence of these flavonoids was re-confirmed in the OIM extract. OCM extract also revealed the presence of these two flavonoids in HPLC analysis in this study. Baicalein formed the detection peak at a retention time of 8.65 minutes and chrysin at 17.15 min. The HPLC chromatograms of the standard compounds are given in figure 8.4.

The concentration of baicalein as quantified was comparatively low in OCM (0.553 ± 0.094 mg/dry weight of callus) than OIM (3.363 ± 0.28 mg/dry weight of root bark). Similar tendency was recorded with the chrysin concentration in OCM (0.186 ± 0.076 mg/dry weight of callus) and OIM (1.111 ± 0.12 mg/dry weight of root bark) also. The HPLC chromatograms of OIM and OCM are given in figure 8.5.

Figure 8.4. The HPLC chromatograms of the standard compounds- baicalein and chrysin. Baicalein (Bcl) (0.1 mg/ml) was detected at a retention time of 8.65 min (fig. 8.4.a) and chrysin (Chr) (0.1 mg/ml) at 17.15 min (fig. 8.4.b). Figure 8.4.c. shows the chromatogram of mixture containing Bcl (0.05 mg/ml) and Chr (0.05 mg/ml).

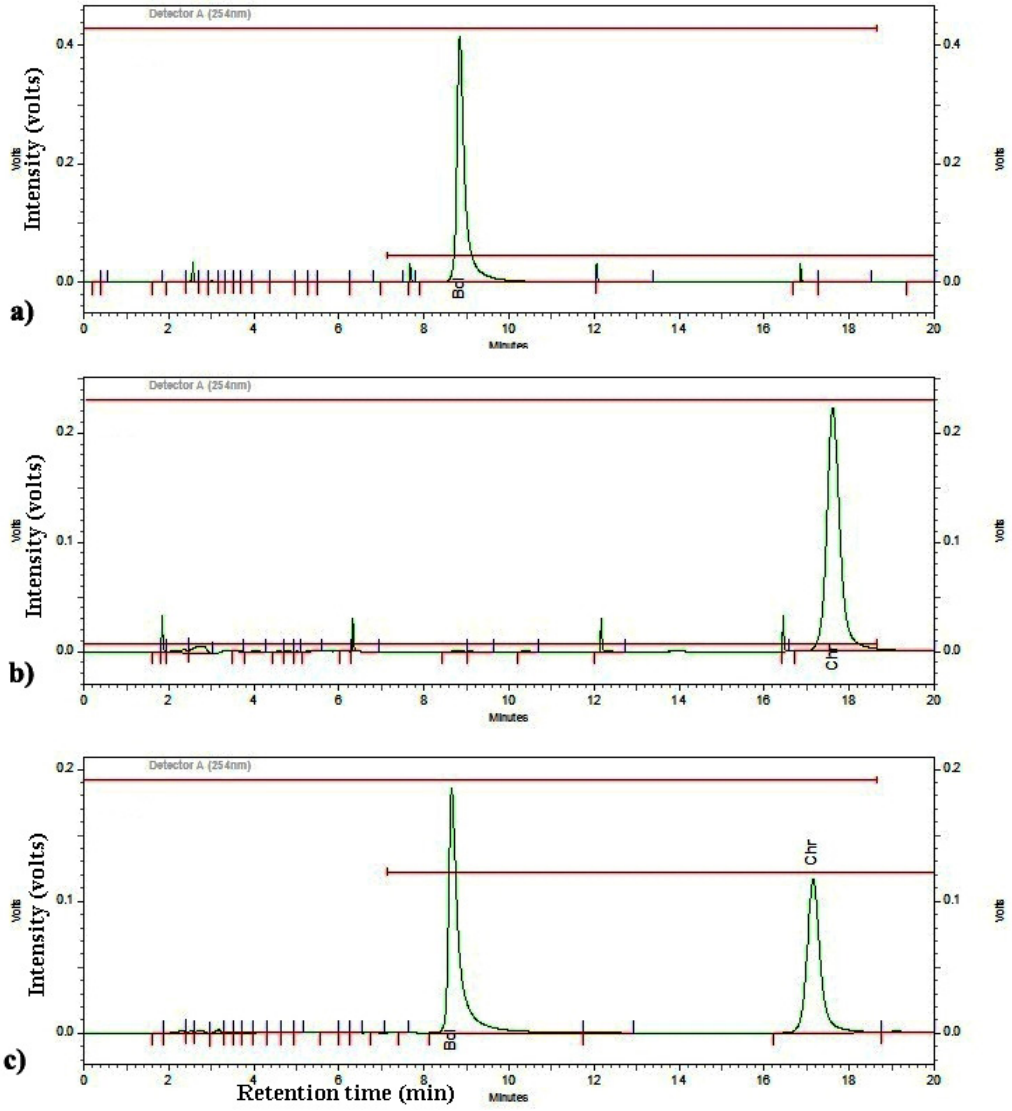
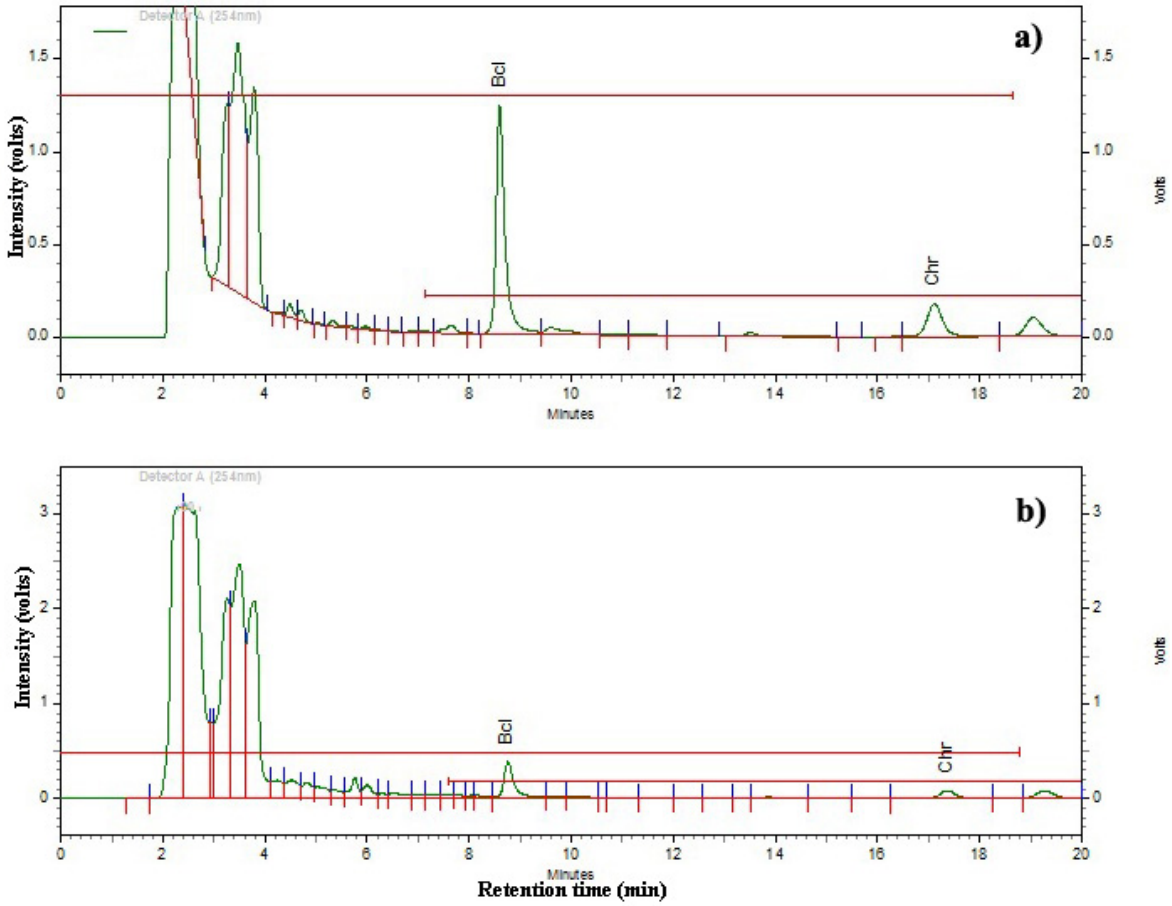


Figure 8.5. The HPLC chromatograms of wild extract (OIM) (Fig. 8.5.a) and callus extract (OCM) of *O. indicum* Vent. (Fig. 8.5.b). The flavonoids – baicalein (Bcl) and chrysin (Chr) have been identified and quantified, based on the retention time and peak area of the standard compounds.



8.3.7. Elicitation

8.3.7.1. Elicitation by methyl jasmonate

8.3.7.1.1. Effect of MeJa on Bcl concentration

HPLC quantification data showed that the addition of 50 µM methyl jasmonate to the medium had significant influence on the turn-over of Bcl. Figure 8.6 and table 8.6 show the concentration of Bcl in the callus cultures of *O. indicum* Vent. with the addition of 25 and 50 µM MeJa into the medium. The percentage increase in Bcl concentration was highest in medium enriched with 50 µM MeJa (77.51%) on the 3rd day and 6th day (74.62%), significantly higher ($p < 0.01$) than that of untreated control cultures (Table 8.9.). Though a slight increase was recorded in cultures treated with 25 µM MeJa, the increase was non-significant with reference to the control, both on day 3 and day 6 (23.17 and 20.46% respectively).

8.3.7.1.2. Effect of MeJa on Chr concentration

Similar results were observed in the concentration of chrysin, with a significant elicitation ($p<0.01$) brought about by the addition of 50 µM MeJa to the medium. Figure 8.7 and table 8.10 show the concentration of Chr in the callus cultures of *O. indicum* Vent. with the addition of 25 and 50 µM MeJa into the medium. A percentage increase of 54.79% was observed on day 3, which sustained up to 54.55% on day 6 (Table 8.9.). The change in Chr concentration was non-significant in callus cultures treated with 25 µM MeJa.

Figure 8.8 shows the HPLC chromatograms of calluses harvested on day 3, from 50 µM MeJa-added medium.

Figure 8.6. The concentration of Baicalein (Bcl) in the callus cultures of *O. indicum* Vent. with the addition of 25 and 50 µM MeJa into the medium

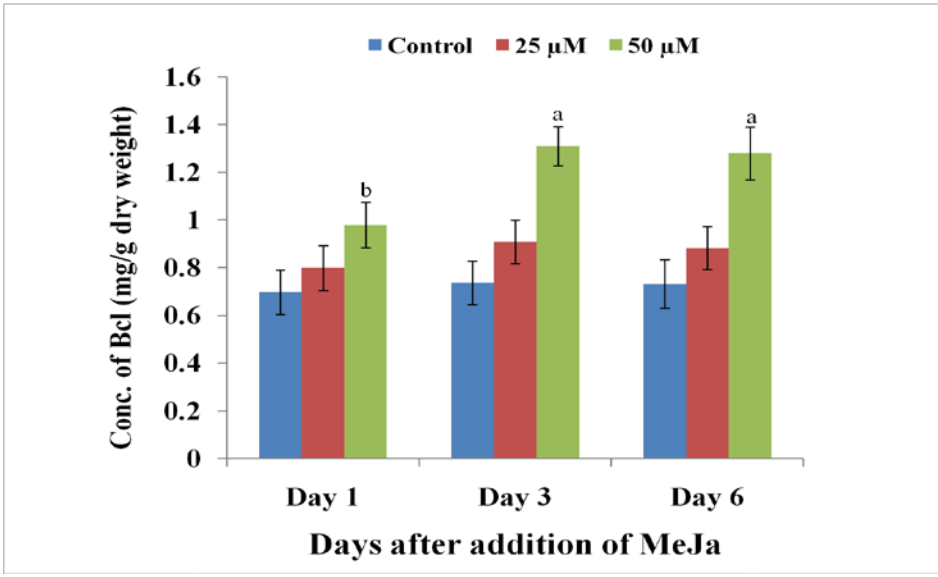


Table 8.6. The concentration of baicalein (Bcl) (mg/g dry wt. of powder) in the callus cultures of *O. indicum* Vent. by the addition of 25 and 50 µM MeJa into the tissue culture medium

Conc. of MeJa in the medium	**Conc. of Bcl (mg/g dry wt. of powder)**		
	Day 1	**Day 3**	**Day 6**
0 (Ctrl)	0.69 ± 0.09	0.73 ± 0.09	0.73 ± 0.10
25 µM	0.8 ± 0.09	0.90 ± 0.09	0.88 ± 0.08
50 µM	0.98 ± 0.10[b]	1.31 ± 0.08[a]	1.28 ± 0.10[a]

Values are expressed as mean ± SD for 3 replicates.

[a]: $p < 0.01$ and [b]: $p < 0.05$ compared to corresponding control

Table 8.7. The percentage increase of baicalein (Bcl) in the callus cultures of *O. indicum* Vent. by the addition of 25 and 50 µM MeJa into the tissue culture medium, compared to the untreated control

Concentration of MeJa in the medium	**% increase of Bcl conc.**		
	Day 1	**Day 3**	**Day 6**
25 µM	14.61	23.17	20.46
50 µM	40.40	**77.51**	74.62

Figure 8.7. The concentration of Chrysin (Chr) in the callus cultures of *O. indicum* Vent. with the addition of 25 and 50 µM MeJa into the medium

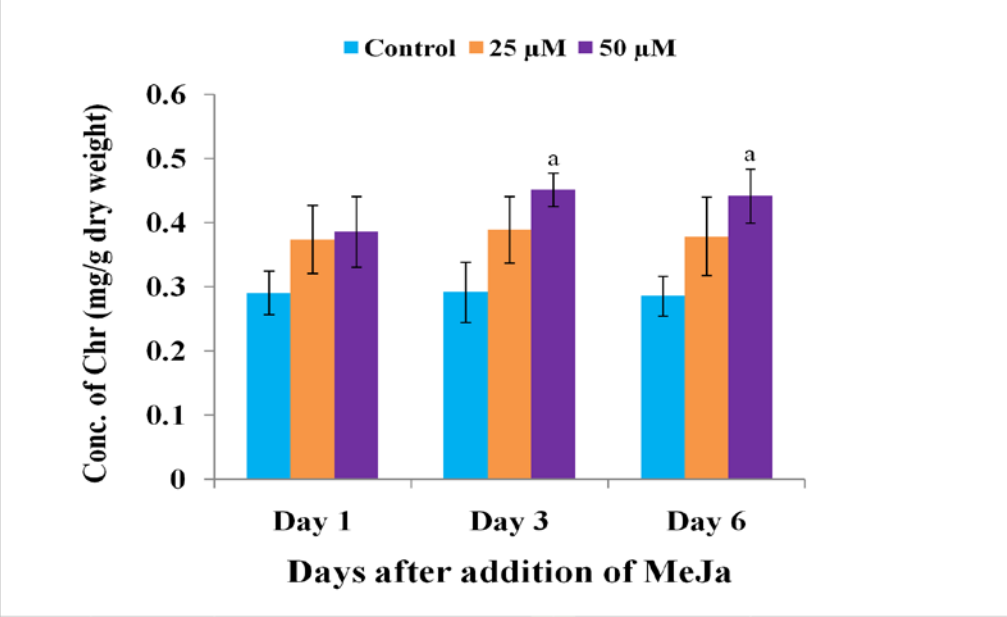


Table 8.8. The concentration of Chrysin (Chr) in the callus cultures of *O. indicum* Vent. by the addition of 25 and 50 µM MeJa into the tissue culture medium

Conc. of MeJa in the medium	**Conc. of Chr (mg/g dry wt. of powder)**		
	Day 1	**Day 3**	**Day 6**
0 (Ctrl)	0.29 ± 0.03	0.29 ± 0.04	0.29 ± 0.03
25 µM	0.37 ± 0.05	0.39 ± 0.05	0.38 ± 0.06
50 µM	0.39 ± 0.05	0.45 ± 0.03[a]	0.44 ± 0.04[a]

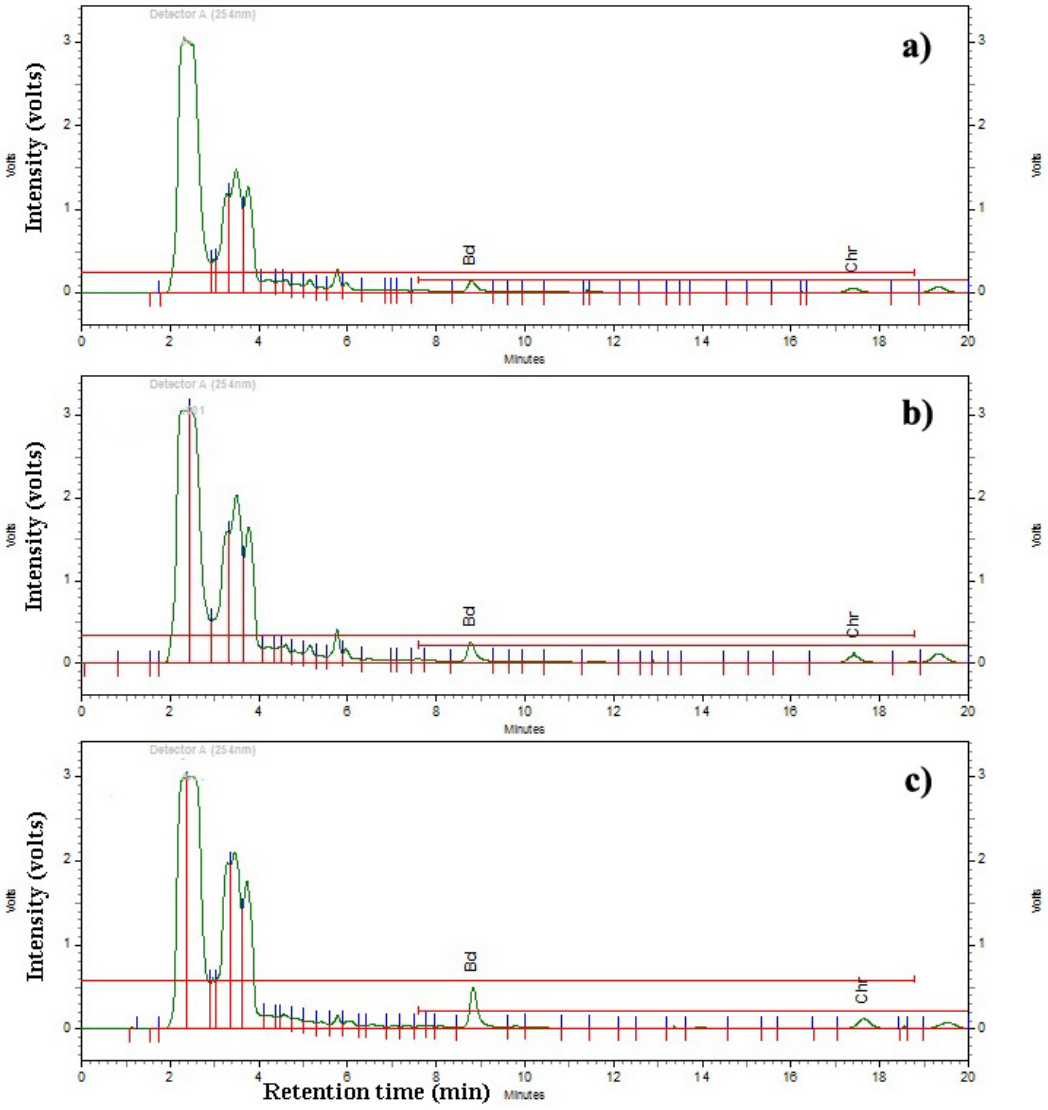
Values are expressed as mean ± SD for 3 replicates.

[a]: $p < 0.01$ and [b]: $p < 0.05$ compared to corresponding control

Table 8.9. The percentage increase of Chrysin (Chr) in the callus cultures of *O. indicum* Vent. by the addition of 25 and 50 µM MeJa into the tissue culture medium, compared to the untreated control.

Concentration of MeJa in the medium	**% increase of Chr conc.**		
	Day 1	**Day 3**	**Day 6**
25 µM	28.52	33.219	32.517
50 µM	32.65	**54.79**	54.55

Figure 8.8 The HPLC chromatograms of calluses of *O. indicum* Vent. harvested on day 3 after exposure to methyl jasmonate; Figure 8.8.a.) control (unexposed to MeJa), 8.8.b.) 25 µM and MeJa added medium and 8.8.c.) 50 µM MeJa added medium. The peaks of baicalein (Bcl) and chrysin (Chr) are also shown in the figure. The peaks were detected at wavelength of 254 nm.



8.3.7.2. Elicitation by X-ray irradiation

8.3.7.2.1. Effect of radiation on Bcl concentration

Exposure of callus cultures of *O. indicum* Vent. showed that 6 MV X-ray radiation at 0.5 and 1 Gy enhanced baicalein concentration significantly, as revealed from HPLC-based quantification data. Figure 8.9 and table 8.10 show the concentration of Bcl in the callus cultures of *O. indicum* Vent. with radiation exposure. The highest percentage of increase ($p < 0.01$) was recorded in cultures exposed to 0.5 Gy radiation on 2nd and 3rd day (83.23 and 81.29% respectively), compared to the non-irradiated control cultures. Though significant in its effect ($p<0.05$), exposure to 1 Gy radiation was not as effective as 0.5 Gy radiation, as revealed from HPLC data of Bcl which showed an increase of 43.0 and 42.99% on 2nd and 3rd day of exposure (Table 8.11.).

Figure 8.9. The concentration of Baicalein (Bcl) in the callus cultures of *O. indicum* Vent. exposed to 6 MV X-ray radiation at doses- 0.5 Gy and 1 Gy.

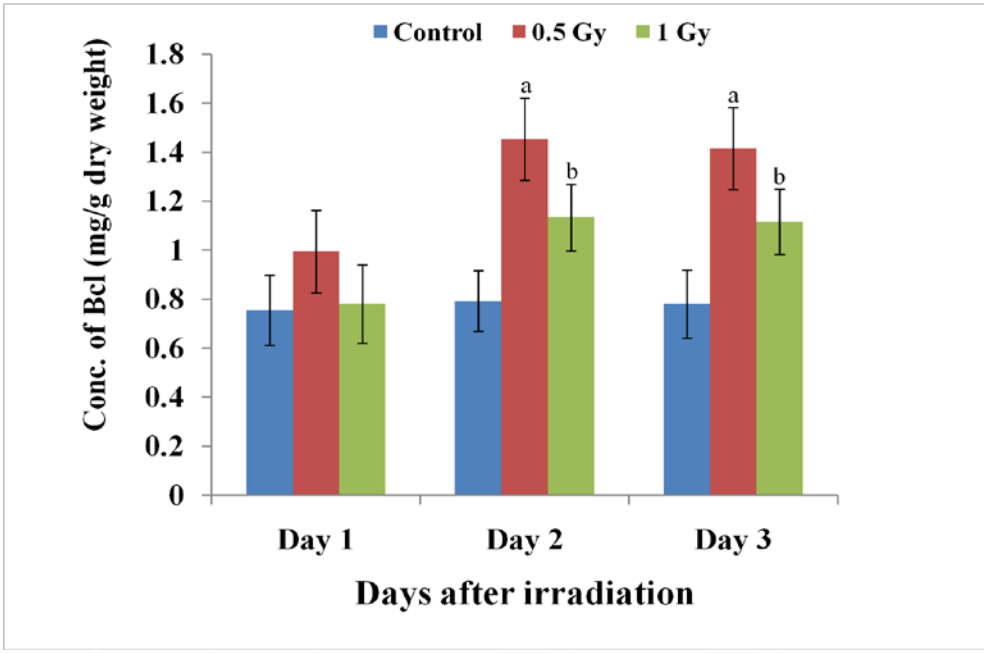


Table 8.10. The concentration of baicalein (Bcl) in the callus cultures of *O. indicum* Vent. exposed to 6 MV X-ray radiation at doses- 0.5 Gy and 1 Gy.

Radiation dose	**Conc. of Bcl (mg/g dry wt. of powder)**		
	Day 1	**Day 2**	**Day 3**
Ctrl	0.76 ± 0.14	0.79 ± 0.12	0.78 ± 0.13
0.5 Gy	1.00 ± 0.16	1.45 ± 0.17[a]	1.14 ± 0.17[a]
1 Gy	0.78 ± 0.16	1.13 ± 0.14[b]	1.12 ± 0.13[b]

Values are expressed as mean ± SD for 3 replicates.

[a]: $p < 0.01$ and [b]: $p < 0.05$ compared to corresponding control

Table 8.11. The percentage increase of baicalein (Bcl) in the callus cultures of *O. indicum* Vent., exposed to 6 MV X-ray radiation at doses- 0.5 Gy and 1 Gy, compared to the untreated control.

Radiation dose	**% increase of Bcl conc.**		
	Day 1	**Day 2**	**Day 3**
0.5 Gy	31.61	**83.23**	81.29
1 Gy	3.31	43.00	42.99

8.3.7.2.2. Effect of radiation on Chr concentration

Irradiation of cultures had significant ($p < 0.05$) effect on the concentration of chrysin, as indicated in figure 8.10 and table 8.12. Compared to the non-irradiated control cultures, the percentage increase of chrysin concentration was high with exposure to 0.5 Gy radiation, the 2nd day (42.15%) and 3rd day (42.40%), after exposure. Enhancement of chrysin concentration was also achieved with exposure to 1 Gy radiation, with an increase of 34.88% on day 2 and 34.80% on day 3 (Table 8.13). Figure 8.11 shows the HPLC chromatograms of calluses harvested on day 2, after irradiation with 6 MV X-ray at 1 Gy.

Figure 8.10. The concentration of Chrysin (Chr) in the callus cultures of *O. indicum* Vent. exposed to 6 MV X-ray radiation at doses- 0.5 Gy and 1 Gy

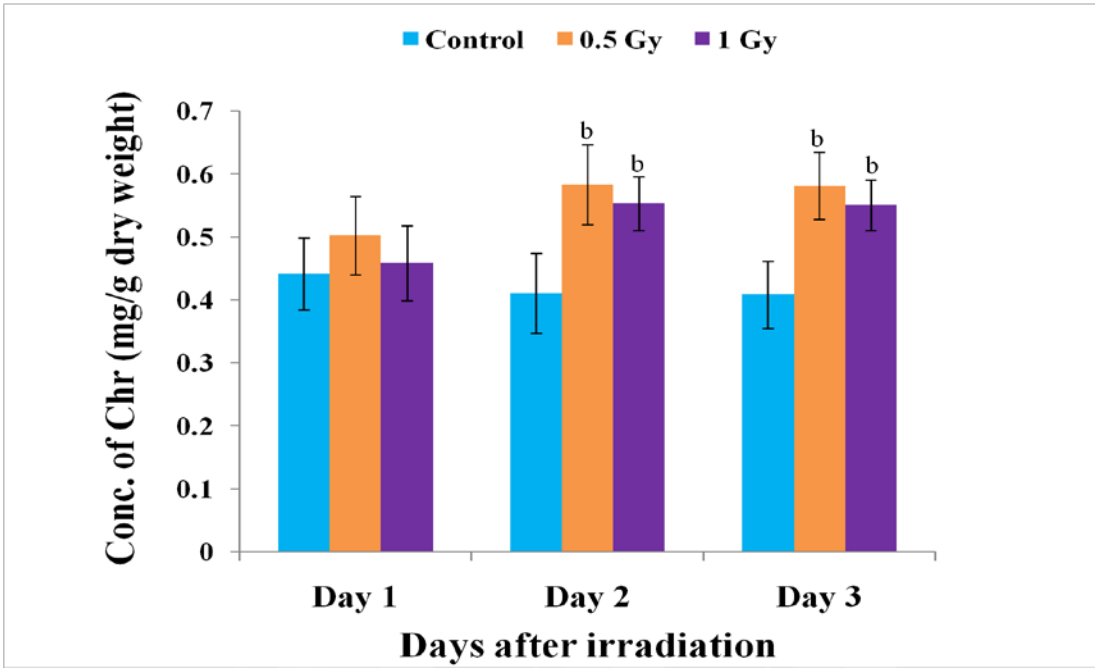


Table 8.12. The concentration of Chrysin (Chr) in the callus cultures of *O. indicum* Vent. exposed to 6 MV X-ray radiation at doses- 0.5 Gy and 1 Gy

Radiation dose	**Conc. of Chr (mg/g dry wt. of powder)**		
	Day 1	**Day 2**	**Day 3**
Ctrl	0.44 ± 0.05	0.41 ± 0.06	0.41 ± 0.05
0.5 Gy	0.50 ± 0.06	0.58 ± 0.06[b]	0.58 ± 0.05[b]
1 Gy	0.46 ± 0.05	0.55 ± 0.04[b]	0.55 ± 0.04[b]

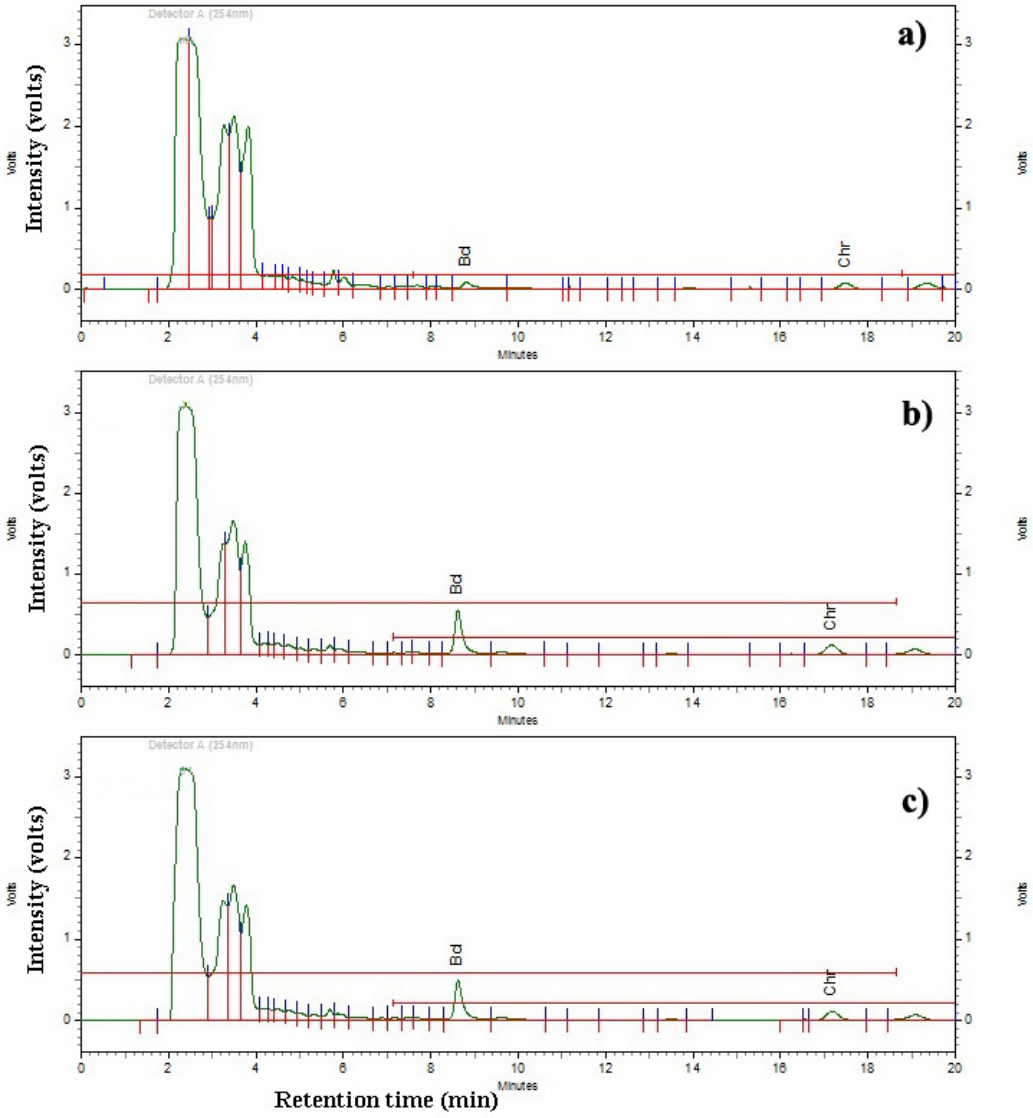
Values are expressed as mean ± SD for 3 replicates.

[a]: $p < 0.01$ and [b]: $p < 0.05$ compared to corresponding control

Table 8.13. The percentage increase of Chrysin (Chr) in the callus cultures of *O. indicum* Vent., exposed to 6 MV X-ray radiation at doses- 0.5 Gy and 1 Gy, compared to the untreated control.

Radiation dose	**% increase of Chr conc.**		
	Day 1	**Day 2**	**Day 3**
0.5 Gy	13.83	**42.15**	42.40
1 Gy	3.85	34.88	34.80

Figure 8.11. The HPLC chromatograms of calluses of *O. indicum* Vent., harvested on day 2 after exposure to 6 MV X-ray radiation; Figure 8.11.a.) unexposed control, 8.11.b.) calluses exposed to 0.5 Gy radiation and 8.11.c.) calluses exposed to 1 Gy radiation. The peaks of baicalein (Bcl) and chrysin (Chr) detected at a wavelength of 254 nm are also shown in the figure.

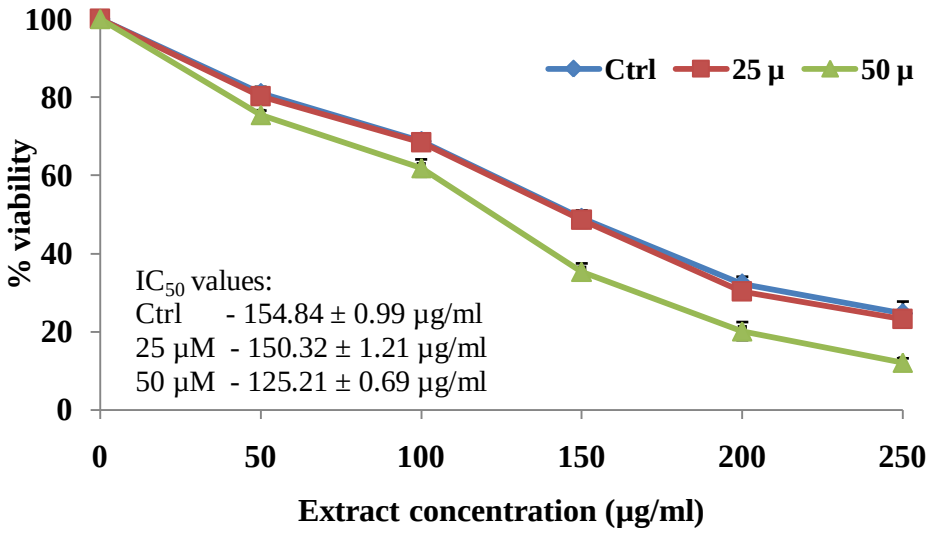


8.3.8. In vitro assay based screening

8.3.8.1. Anti proliferative activity on HeLa cells

Figure 8.12 shows the anti proliferative activity of callus extracts harvested on 3rd day of MeJa addition. The IC_{50} values revealed that the calluses grown in 50 µM MeJa-supplemented medium was most active (125.21 ± 0.69 µg/ml), compared to those in the presence 25 µM MeJa (150.32 ± 1.21 µg/ml) or untreated control cultures (154.84 ± 0.99 µg/ml).

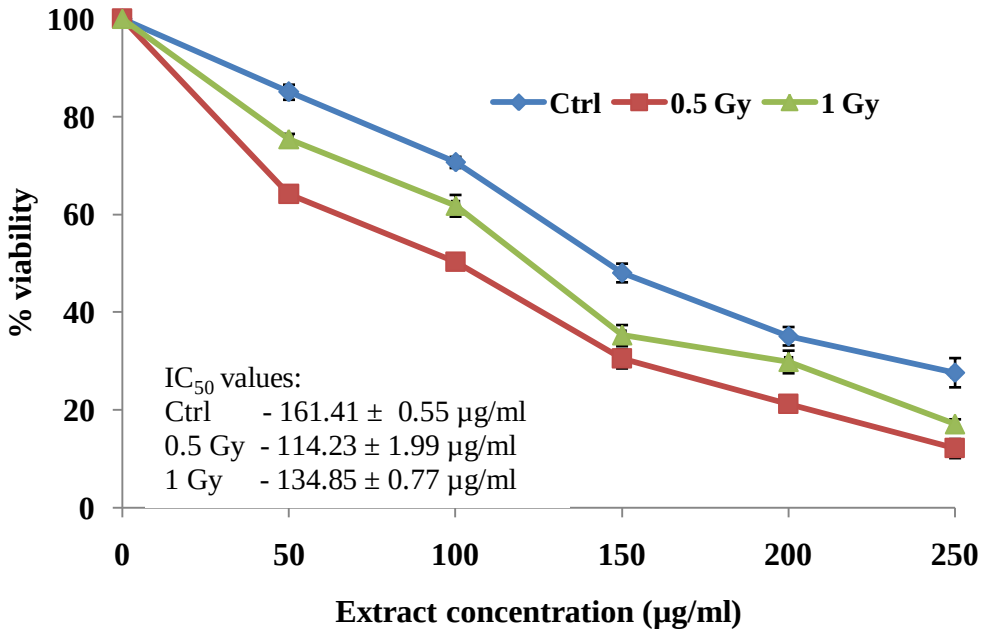
Figure 8.12. Anti proliferative activity of callus extracts of *O. indicum* Vent. harvested on 3rd day of MeJa addition.



The anti proliferative activity of callus extracts harvested on 2nd day of 6 MV X-ray radiations is given in figure 8.13. From the IC_{50} values determined from the curve, the most active extract was identified as that from calluses irradiated at a dose of 0.5 Gy (IC_{50}: 114.23 ± 1.99 µg/ml). Irradiated calluses exposed to 1 Gy X-rays were also more active (IC_{50}: 134.85 ± 0.77 µg/ml) than those of non-irradiated control cultures (IC_{50}: 161.41 ± 0.55 µg/ml).

However, none of these were found to be as active as the wild extract (OIM with IC_{50}: 92.43 ± 0.82 µg/ml).

Figure 8.13. Anti proliferative activity of callus extracts of *O. indicum* Vent. harvested on 2nd day of 6 MV X-ray irradiation.



8.3.8.2. DPPH radical scavenging activity

The DPPH scavenging activity of callus extracts harvested on 3rd day of MeJa addition is given I in figure 8.14. The calluses grown in 50 µM MeJa-supplemented medium was most effective (109.05 ± 0.57 µg/ml) than that of 25 µM MeJa-added medium (122.07 ± 0.97 µg/ml) or untreated control cultures (128.3 ± 1.04 µg/ml).

Irradiation at 6 MV X-ray radiations had profound effect on DPPH scavenging activity. The activity of callus extracts harvested on 2nd day of irradiation is given in figure 8.15. The IC_{50} values revealed from the curve showed that the most active extract identified was that obtained from irradiated calluses

exposed to 0.5 Gy radiation (IC_{50}: 105.35 ± 2.02 µg/ml). Irradiated calluses exposed to 1 Gy X-rays were also active (IC_{50}: 110.34 ± 0.63 µg/ml) compared to non-irradiated control cultures (IC_{50}: 133.08 ± 1.02 µg/ml).

Figure 8.14. DPPH radical scavenging activity of callus extracts of *O. indicum* Vent. harvested on 3rd day of MeJa addition.

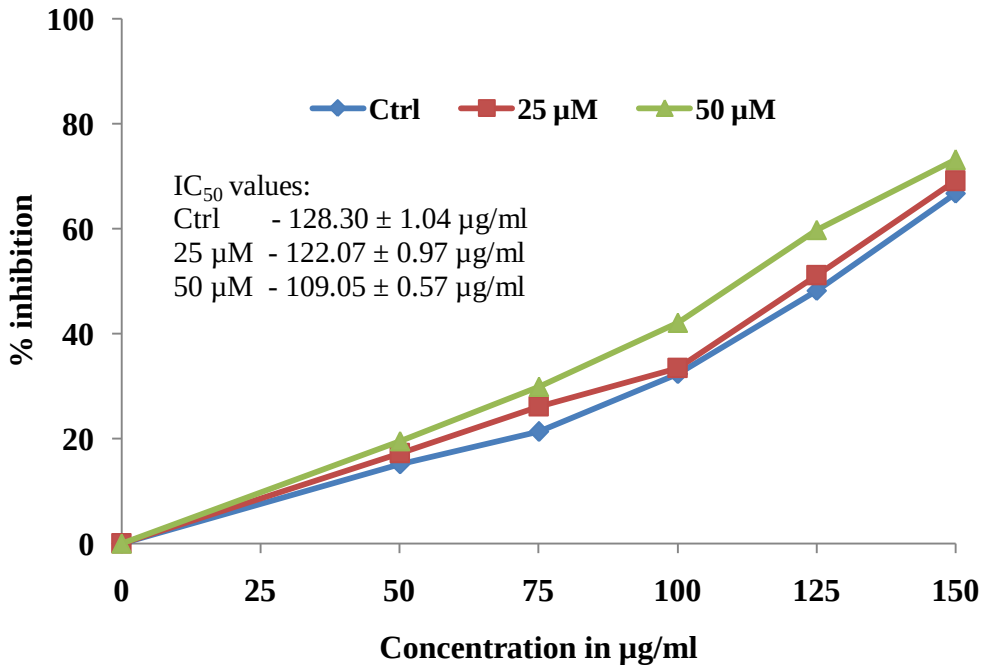
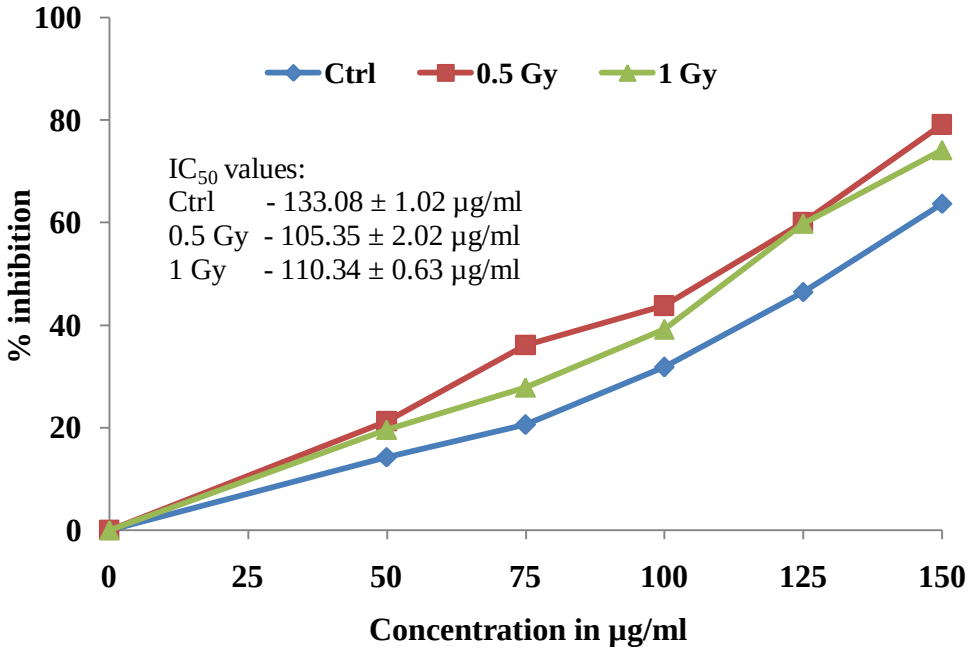


Figure 8.15. DPPH radical scavenging activity of callus extracts of *O. indicum* Vent. harvested on 2nd day of 6 MV X-ray irradiation.



8.4. Discussion

The comparative evaluation of *O. indicum* Vent. wild root bark extract (OIM) and callus extract (OCM), as revealed from the previous and present chapters suggest that the calluses established through micropropagation were active in anti proliferative, antioxidant and anti inflammatory studies, but may not be recommendable as competent substitutes for the natural root bark. Bioactive compound classes such as flavonoids and phenols, though detected in OCM, gave feeble positive tests than OIM, during preliminary phytochemical screening. The number of prominent bands revealed through HPTLC also was lesser in OCM, showing consistency with the preliminary tests.

UPLC-Q-TOF-MS fingerprint reconfirmed the presence of already reported flavonoids such as baicalein and biochanin A (Zaveri *et al.*, 2008), along with flavonoid compounds such as baicalin, demethoxycentaureidin 7-O-rutinoside, narcissin, methoxy flavones and methyl coumarin showing their tentative presence in the root bark. The presence of baicalein, baicalin and biochanin A was also provisionally detected in the callus extract, along with other compounds - syringetin-3-O-glucoside, capillarisine and isoorientin. The compounds which revealed their common presence in both root bark and calluses, are attributed with anti proliferative properties- biochanin A (Kole *et al.*, 2011), baicalin (Peng *et al.*, 2015) and baicalein (Roy *et al.*, 2007) against cancer cell lines. The anti oxidant properties of baicalein (Shieh *et al.*, 2000, Kang *et al.*, 2012) and biochanin A (Zhang *et al.*, 2011a) have been established previously, and is also discussed in earlier chapters. Baicalin, the glucoronide of baicalein is also antioxidant in nature (Peng-fei *et al.*, 2013). Most of the compounds identified in the UPLC-Q-TOF-MS analysis of both extracts were flavonoids, which are already appreciated for their anti inflammatory effects (Kim *et al.*, 2004, Serafini *et al.*, 2010), and therefore, the alleviation of paw edema by OIM and OCM treatment discussed in previous chapters remains explainable. As per earlier reports (Zaveri *et al.*, 2008), the root bark of *O.*

indicum Vent. contains a biologically active flavonoid – chrysin having anti proliferative (Samarghandian *et al.,* 2016), antioxidant and anti inflammatory properties (Veerappan and Senthilkumar, 2015). HPLC analysis of OIM extract in this study, using acetonitrile: water (45:55) as solvent system revealed the presence of chrysin (retention time (RT): 17.15 min), by extending the run time to 20 minutes, reconfirming its presence. The callus extract also produced a feeble peak for chrysin at the same RT. Baicalein (RT: 8.65 min) was also detected in both extracts using HPLC analysis with similar solvent system. A quantitative phytochemical estimation of these two compounds using HPLC revealed that the concentration of baicalein and chrysin were less in callus, as compared to root bark.

The comparative study of root bark and callus was aimed at the scope of evolving into the use of callus as a substitute in medicinal preparations, thereby reducing the chances of uprooting of this valuable, but threatened plant. Moreover, *O. indicum* Vent. shows positive responses to shooting, rooting and callusing triggers in tissue culture media, with calluses emerging with the highest turnover of biomass. Yet, it has often been found that (Wink *et al.*, 2005, Hussain *et al.,* 2012) callus cultures may fail to accumulate as much of secondary metabolites as their wild counterparts, thus remaining inferior to the latter in their biological activities. In this context, the use of elicitation techniques for enhancement of secondary metabolite synthesis gains significance (Zhaoa *et al.*, 2005, Naik and Al–Khayri, 2016). In the present study, addition of methyl jasmonate and X-ray irradiation was used as triggers to increase secondary metabolite production in callus cultures. Monitoring of elicitation was done using HPLC-based quantification to keep in track, the concentration of baicalein and chrysin at periodic intervals of the study. This is not only in respect of their reported biological activities, but also of the link between these two compounds in a common biosynthetic pathway. A study by Zhao *et al.* (2016) elucidated a new pathway in *Scutellaria baicalensis* for the synthesis of chrysin and wogonin, wogonoside, baicalein, and baicalin, in

which pinocembrin (4′-deoxyflavanone) acts as the key intermediate. The enzyme identified as flavone synthase (FNS) II-2 uses pinocembrin as the substrate to synthesise chrysin, which, in turn forms the intermediate compound in producing wogonin, wogonoside, baicalein, and baicalin.

The callus cultures of *O. indicum* Vent. exposed to 50 µM of methyl jasmonate (MeJa) showed an increase in concentration of baicalein (Bcl) by 77.51% and chrysin (Chr), by 54.79%, in comparison with the unexposed control. The effect was most notable on the third day after MeJa addition, compared to day 1 (40.4% for Bcl, and 32.65% for Chr) or day 6 (74.62% for Bcl, and 55.55% for Chr). Methyl jasmonate (MeJA), in different concentrations has been found to enhance secondary metabolite synthesis in different plant species grown under tissue culture (Zabala *et al.*, 2010, Wang *et al.*, 2015). Similar response was observed in *O. indicum* Vent. through the present study. MeJa induces signal transduction responses that mediate defensive responses in plants thereby enhancing secondary metabolite production in cell cultures (Walker *et al.*, 2002); probably this being the mechanism that recapitulated in *O. indicum* Vent. callus cultures also. The results, however derived from MeJa treatment at a concentration of 25 µM, were non-significant.

Apart from chemical elicitors, physical stress like wounding and irradiation also bring about stress responses in plants (Dörnenburg and Knorr, 1995). The effect of X-ray irradiation on enhanced plant growth, seed germination, synthesis of photosynthetic pigments, microbial resistance and metabolic readjustments against irradiation stress has been established before (Walker *et al.*, 2002, Al-Enezi *et al.*, 2012, De Micco *et al.*, 2014). In the present study, irradiated callus cultures of *O. indicum* Vent. exposed to 6 MV X-ray radiation at 0.5 Gy and 1 Gy contained higher concentration of Bcl (83.23%) and Chr (42.15%), compared to that of the non irradiated control cultures, when harvested on the 2nd day after irradiation. Cultures, harvested on day 3, however did not show significant variation, compared to that from previous

day. It was also found that 0.5 Gy exposed cultures contained higher amounts of the analytes, than those exposed to 1 Gy. The enhancement of these compounds is suggestive of the effect of X-ray irradiation on *O. indicum* Vent. callus cells in eliciting stress responses and activating biosynthetic pathways.

Further, calluses harvested after elicitation, were subjected to *in vitro* anti proliferative and DPPH scavenging assay and concomitant results were obtained. In anti proliferative assay against HeLa cells, maximum activity was shown by 0.5 Gy X-ray-irradiated calluses from day 2 harvest (IC_{50}: 114.23 ± 1.99 µg/ml), compared to the non irradiated control (IC_{50}: 161.41 ± 0.55 µg/ml). Similarly, calluses elicited with 50 µM MeJa were more active (IC_{50}: 125.21 ± 0.69 µg/ml) than the untreated control (IC_{50}: 154.84 ± 0.99 µg/ml). DPPH scavenging activity was also maximum in 0.5 Gy X-ray-irradiated calluses (IC_{50}: 105.35 ± 2.02 µg/ml) and 50 µM MeJa-exposed calluses (IC_{50}: 109.05 ± 0.57 µg/ml), compared to the respective control cultures (IC_{50}: 133.08 ± 1.02 µg/ml and 128.30 ± 1.04 µg/ml respectively). The anti proliferative and antioxidant nature of the enhanced compounds baicalein and chrysin, as discussed earlier in this section, support the findings of this study.

The results suggest that elicitation techniques can be applied to callus cultures of *O. indicum* Vent., with its possibility to evolve into a substitute for the wild root bark. Though the elicitation techniques have brought about changes in the phytochemical profile and pharmacological properties of *O. indicum* Vent. callus, it is also to be noted that it remains still, inferior to the wild root bark in its properties. Hence, before concluding into the results, elucidation of the molecular pathways involved in secondary metabolite production is a pre-requisite. Exploration of other chemical, biological and physical methods of elicitation needs to be carried out for producing better results.

Summary and conclusion

Cancer research is accelaerating towards the discovery of plant derived anti cancer agents, either directly used as therapeutics or are used as complements with other modalities of treatment. This is encouraged because they pose lesser side effects, compared to conventional chemotherapeutic agents. The plant selected for the study, *Oroxylum indicum* Vent. belongs to family Bignoniaceae. Different parts of *O. indicum* Vent. - leaves, roots, fruits, stem and seeds are acclaimed for their pharmacological and phytochemical properties, of which roots are the most extensively used. This had led to its indiscriminate uprooting and exploitation, eventually leading to its depletion in natural habitats. This calls for an urgent need for its conservation as well as regeneration through tissue culture techniques. Along with *in vitro* regeneration, it is recommendable that the plants/plant parts developed from micropropagation are as competent as their wild counterparts in bioactive properties, unless which they can't be substituted for the latter. The aim of the present study was to arrive at a comparative evaluation of anti cancer properties of wild and *in vitro* propagated *Oroxylum indicum* Vent.

The root bark of *O. indicum* Vent. is a regular ingredient of Dasamoola preparation in Ayurveda, most prescribed for inflammatory disorders. The root bark of this plant was selected for the present study for a thorough exploration of its anti cancer-related properties. *In vitro* cytotoxic and free radical scavenging assay-based screening showed that 70% methanol was ideal for extraction. The extract (OIM) showed concentration-dependent cytotoxicity to DLA and EAC cell lines and inhibition to the proliferation of HeLa, HCT 15 and MDA-MB-231 cells. Flow cytometric analysis indicated cell cycle arrest in HeLa cells treated with the extract at the G_1 phase. Further revelation of apoptotic induction, to a moderate extent was obtained through double acridine orange-ethidium bromide staining/Annexin-Hoecsht staining and fluorescent microscopy. Apart from being concentration-dependent, the cytotoxic and anti proliferative activities of the extract were attained at only at high concentrations.

Animal trials are inevitable in drug discovery, and are to be preceded by standard toxicity testing. Acute and sub-acute toxicity studies of OIM revealed that it is safe up to 2 g/kg b.wt. in acute dose administration and 400 mg/kg b.wt. in repeated dose administration. This could be appreciated as a positive indication, preceding any drug discovery. Further, the OIM extract was found to be effective as the standard drug, cyclophosphamide against DLA induced solid tumor and EAC induced ascites tumor, with reference to the statistical significance derived.

Cancer development is widely attributed to free radical formation, and endless compounds with antioxidant properties are found to be anti cancer in nature. The study revealed the *in vitro* and *in vivo* anti oxidant properties of OIM extract, emphasizing its ability to prevent cancer development. This was further substantiated by the *in vivo* anti inflammatory activity evaluated in acute and chronic paw edema models. Furthermore, significant resistance to papilloma development was observed in DMBA-croton oil induced animals, having received topical treatment with OIM extract. These studies highlight the anti carcinogenic activities of the extract.

Many plant derived extracts are suggested to be used in complementation therapies with chemotherapeutic agents, so that the toxic side effects of the latter can be ameliorated, without compromising its anti cancer property. OIM extract was found to mitigate the cardiotoxic, nephrotoxic and myelosuppressive effects of Doxorubicin, Cisplatin and Cyclophosphamide respectively. This is highly suggestive of the use of this plant in complementation therapies.

O. indicum Vent. has now come into the focus of plant tissue culturists, after realizing the threat it is facing in its habitat by succumbing to overexploitation. The callusing, shooting and rooting responses of this tree in the present study have increased the scope of its re-establishment through micropropagation As an attempt to recommend this as a substitute for wild root bark, a comparative

evaluation was carried out with its callus extract; callus cultures being common feasible sources for isolation of bioactive compounds. The callus extract (OCM) was found to be inferior to the OIM extract in its cytotoxic, anti proliferative and antioxidant activities. However, it exhibited significant anti inflammatory activity, offering scope for improving its pharmacological properties by using tissue culture technology.

The superior activity of OIM extract was further substantiated by comparison of HPTLC and HPLC profiles of OIM and OCM. Focus was turned on enhancing the phytochemical portfolio using elicitation techniques. The callus cultures responded positively to methyl jasmonate and X-ray irradiation, as monitored through the increase in concentration o two bioactive compounds-baicalein and chrysin. 70% hydromethanolic extracts from these calluses showed increased proliferation inhibition and DPPH scavenging activities than the unexposed normal calluses.

Through the study, some of the unreported properties – inhibition of papilloma, suppression of inflammation, amelioration of oxidative stress – of OIM extract were exposed. The reports are recommendable for its use in complementation therapy of cancer.

The response of the plant to micropropagation and elicitation has relieved the concern about the gradual population depletion of the plant. Yet, it is necessary that more elicitors are to be investigated for their ability to enhance the phytochemical and pharmacological properties of micropropagated *O. indicum* Vent., and are to be employed in developing substitutes for its wild parts. This can reduce the uprooting and felling of the trees, and thereby conserve this valuable medicinal tree.

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Appendix 1

List of publications as first author

1. **Seema Menon**, Lincy Lawrence, Noby John and Jose Padikkala **(2018)**. Chemoprotective activity of *Oroxylum indicum* Vent. root bark against cyclophosphamide mediated myelosuppression and organ injury. *International Journal of Advanced Research* **6 (3)**: 696-707.
2. **Seema Menon**, Lincy Lawrence, Vipin P. Sivaram and Jose Padikkala **(2017)**. Dasamula plant, *Oroxylum indicum* root bark extract prevents doxorubicin induced cardiac damage by restoring redox balance. *Journal of Ayurveda and Integrative Medicine* (Available online 03-02-2018).
3. **Seema Menon**, Lincy Lawrence, Vipin P. Sivaram and Jose Padikkala **(2015)**. Phytochemistry and evaluation of *in vivo* antioxidant and anti-inflammatory activities of *Oroxylum indicum* Vent. root bark. *Journal of Chemical and Pharmaceutical Research*. **7 (10):** 767-775.