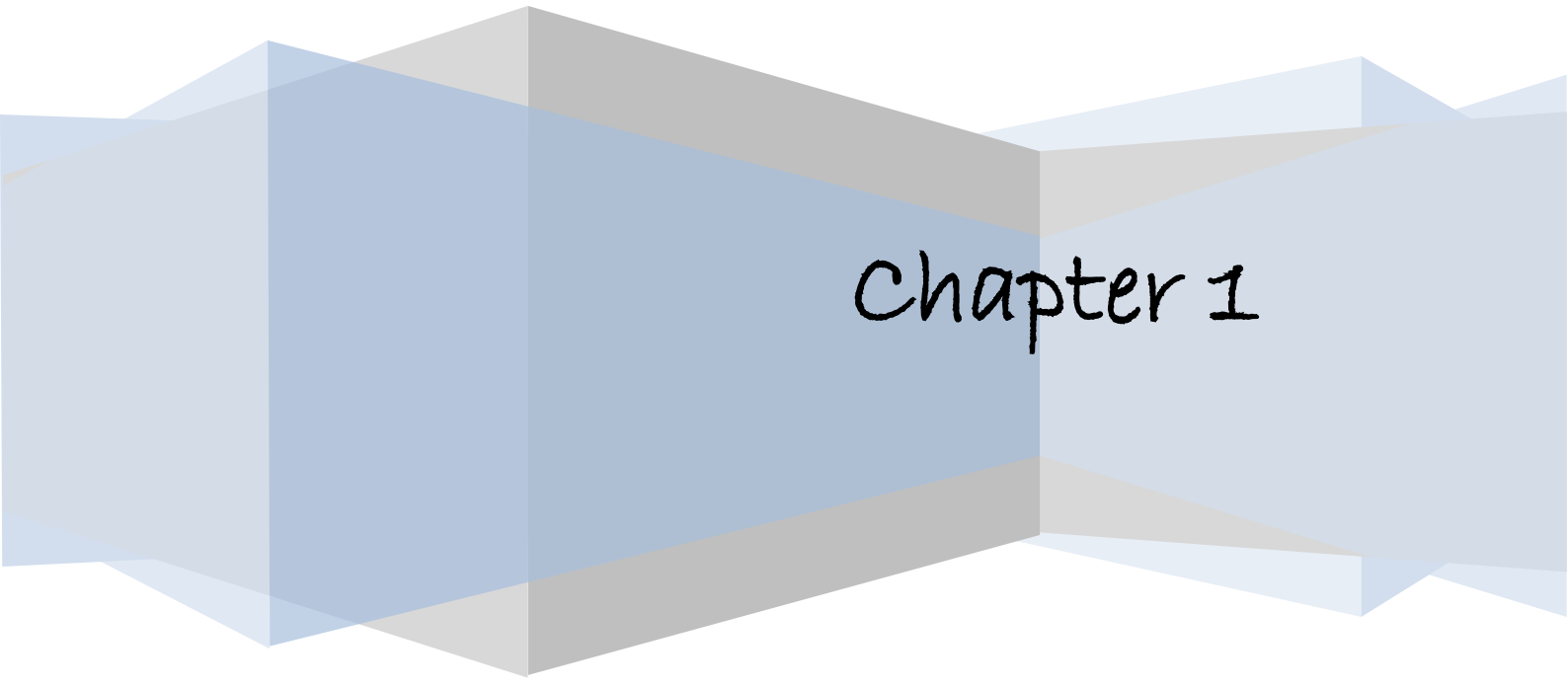
General Introduction



Chapter 1

Cancer is a group of diseases that comprises mutation or dynamic changes in the genome of the cell producing proteins that disturb the normal cellular balance leading to the uncontrolled proliferation of cells (Bishop and Weinberg, 1996; Hejmadi, 2010; American Cancer Society, 2015). Though the proliferation of cells is indispensable for embryogenesis, growth and the proper function of several adult tissues, it can also lead to tumorigenesis and eventually to death if its controlling mechanisms are lost or dysregulated (DeBerardinis *et al*., 2008). In normal condition, the cells enter the active proliferative phase only after receiving the mitogenic growth signals, and cannot multiply in the absence of these signals. However, cancer cells have lost the need of these stimulatory signals and therefore can proliferate whether these signals are present or not (Hanahan and Weinberg, 2010). Cancer cells have the ability to produce their own growth factors mimicking the normal growth factors which make them independent of the normal growth factors (Fedi *et al*., 1997).

Though there are more than 100 different kinds of cancer and different subtypes even from a single origin, there are six characteristics that most cancers share called the six hallmarks of cancer. Self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg in 2000). The ability to reprograme energy metabolism and evade immune destruction were later added to the six hallmarks of cancer (Sonnenschein and Soto, 2013).

A normal adult human body constitutes approximately 10^{15} cells, of which approximately 10^{12} are formed, divide and differentiate each day to replace the dead

and worn out cells. Even in some organs which exhibit low division of cells for example, the liver, a significantly high cell division can be initiated due to trauma or infection. Despite this huge production of cells per day, the human body maintains an unchanged body weight for years due to the homeostatic mechanism (Bertram, 2001).

Though cancer can arise due to mutation, a single mutation is not enough to cause cancer. Amplification of the accumulated DNA mutation is required for the development of cancer (Hejmadi, 2010). The evolutionary concept "Survival of the fittest" applies in case of carcinogenesis as well due to the multiple checks and balances that exist in stem cells to limit excess cell division. Cancer cells must accumulate multiple mutations in the key cellular genes in order to attain the properties of autonomous replication and invasion. To become cancerous the cells require at least five successful gene mutations and with each mutation creating a cell increasingly well adapted for autonomous growth in the host organism. The neoplastic cells acquire this by selecting the mutations that activates the oncogenes or inactivates the tumor suppressor genes (Bertram, 2001).

History

Cancer of human as well as animals has been recorded throughout history and doctors have written about it since the olden times. The Edwin Smith Surgical Papyrus, one of the eight Egyptian medical papyri, is believed to be the first document containing a record of human cancer. This text is believed to be written by Imhotep, the physician-architect around 3000–2500 BC, the pyramid age of Egypt. It recorded cancer as a disease without any treatment except palliative care (Breasted, 1984; Donegan, 2006; Sudhakar, 2009). Cancer is assumed to be older than human as it has been identified in the bones of dinosaurs from the Jurassic period (Greaves, 2000). However, the earliest evidence of human cancer found till date is a metastatic

carcinoma in a young man from ancient Nubia (presently an archaeological site of Amara West in modern Sudan, situated on the left bank of the Nile river) which dated back to 1200 BC (Binder *et al*., 2014). Besides this, fossilized bone tumors, human mummies of ancient Egypt as well as olden scripts indicated the presence of cancer in the past. Evidence of sarcoma, a bone cancer as well as damage to the skull bone characteristics of head and neck cancer has been found in ancient mummies (American Cancer Society, 2010).

Galen of Pergamum (129–200 BC) compared the dilated veins that radiated from carcinomas to the legs of a crab; as a result, the crab became a symbol for cancer. Leonides, a surgeon of the Alexandrian school had also shared this view but rather he related the firm adherence to the surrounding tissues to the pinchers of crab. Hippocrates (460–375 BC) has described cases of breast cancer in detail in his legacy, *Corpus Hippocraticum.* He believed one can maintain an optimum health by upholding a balance between blood, phlegm, yellow bile, and black bile, the four bodily fluids, as was assumed. He linked breast cancer with menopause and even tried to restore menstruation in younger women with breast cancer (Donegan, 2006). With the advent of microscope in the nineteenth century, the work of Schleiden and Schwann shed propounded that the human body is composed of cells and Virchow dictated that cancer is also made up of cells. The metastatic ability of cancer was suspected by Müller which later was confirmed by Thiersch and Waldeyer (De Moulin, 1983; Virchow, 1978). In 1846, Virchow coined the terms ‘‘hyperplasia’’ and ‘‘metaplasia’’ and recognized that both conditions are potential precursors of cancer, and that cancer cells have marked difference both in size and shape as compared to benign cells. In 1877, Julius Cohnheim (1839- 1884), a Berlin pathologist, in his *Vorlesungen uber allgemeine Pathologie* anticipated that tumors

develop either from nests of cells that are out-of-place during development or those that have retained embryonal characteristics. He assumed that these misplaced cells are dispersed all over the body and can develop into cancer due to changes in angiogenesis or the genetic makeup of a person. Twenty years later, Moritz Wilhelm Hugo Ribbert (1855-1920), a Zurich pathologist, added to the embryonal theory of Cohnheim that mechanical irritation like chronic inflammation and trauma can also lead to the development of cancer especially the epithelial and connective tissue cells (Cohnheim, 1877; Ribbert, 1904). The development in microbiology and the discovery of new organisms and parasite led researchers to assert them as causative agents for cancer without sufficient evidence. However, *Schistosoma haematobium* which causes bladder cancer was the only parasite linked to any type of cancer for several decades until the discovery of *Clonorchis sinensis*, a causative factor for bile cancer (Harrison, 1889). Before the discovery of radiation for treatment, surgery was the only standard treatment for cancer (Hajdu, 2005). Since surgery cannot treat advanced cancer cases William B. Coley made an attempt to treat these cases with bacterial toxins which was effective particularly for soft tissue and bone cancers but was later discontinued due to its high toxicity (Coley, 1894).

Causes

Many factors contribute to the cause of cancer whether directly or indirectly which can be broadly classified as environmental, lifestyle and behavioral exposures (Steward and Wild, 2014). Tobacco, diet, stress, obesity, radiations, infections, lack of physical activity and environmental pollutants are some of the common environmental factors leading to cancer (Anand *et al*., 2008). Cigarette smoking has been estimated to cause 90% of male and 75%–80% of female lung cancer deaths in the United States each year and is the major cause of lung cancer accounting for about

80 % of all lung cancers. Though the most addictive component, nicotine itself is not a carcinogen, about 70 carcinogens have been identified in tobacco smoke (Biesalski *et al*., 1998; Hecht, 1999). Physical inactivity increases cancer risk not only through obesity but also through its negative effect on the hormonal balance as well as the immune system (Kushi *et al.*, 2006) and has been linked with increased risk of cancer of the breast, colon, prostate, and pancreas and of melanoma (Booth *et al*., 2002). In the United States, 14% of men and 20% of women cancer death is attributed to higher body mass index (Drewnowski and Popkin, 1997). Diet contributes to about 39-35 % of all cancers in the USA (Doll and Peto, 1981). High consumption of animal fat and red meat is linked with cancers such as breast, colon, and prostate (Armstrong and Doll, 1975). Folate deficiency, one of the most common vitamin deficiencies can cause chromosomal breaks due to the deficient methylation of uracil to thymine thereby increasing the risk of cancer (Ames and Gold, 1998). A diet rich in salt is linked to gastric cancer especially in Japan. The consumption of alcohol contributes to oral, esophageal, liver and breast cancer and chewing of betel nut is also linked to oral cancer. It has also been observed that immigrants often develop risk to cancer prevalent in the country where they migrate suggesting the relationship between diet and cancer (Buell and Dunn, 1965; Park *et al*., 2008).

Infection of virus, bacteria and parasites accounts for 18 % of cancer death in the world. Deposition of the eggs of *Schistosoma japonica* and *S. haematobium* in the colonic mucosa and bladder causes cancer of the colon and bladder respectively by causing inflammation. In China, *Chlonorchis sinensis* infection is linked with increased risk of biliary tract cancer. Among the viruses, hepatitis viruses and the human papilloma virus are also known to increase the risk of liver and cervical cancer, respectively. Epstein–Barr virus is related to B-cell lymphoproliferative

disease and nasopharyngeal carcinoma and the Kaposi's sarcoma. Herpes virus is linked to Kaposi's sarcoma and primary effusion lymphomas. The viruses that cause cancer are called as the oncoviruses. The bacterium *Helicobacter pylori* is known to cause stomach cancer in humans (Ames and Gold, 1998; Pagano *et al*., 2004; Anand *et al*, 2008).

Ionizing radiations are potent carcinogens and exposure to radiation is known to cause 10 % of all cancers. Cancers induced by radiation include some types of leukemia, lymphoma, thyroid cancers, skin cancers, sarcomas, lung and breast carcinomas. Ionizing radiation usually cause cancer by inducing double strand breaks in the DNA. A cell that is heavily damaged normally die or lose its reproductive ability and do not cause cancer, however, lighter damage may lead to cells that are only partly functional but stable and capable of division which may later become cancerous especially if the tumor suppressor genes are damaged (Acharya 1975; 1976; 1977; Little, 2000; Anand *et al*., 2008).

Staging

Staging describes the extent or spread of cancer at the time of diagnosis. It is necessary in order to determine the treatment plan and in assessing prognosis. The stage of a cancer is established from the size or extent of the primary tumor and whether it has spread to nearby lymph nodes or other areas of the body. A number of different staging systems are used to classify cancer. The term in situ is used when the cancer cells are present only in the place of its origin. However, if it has breached past its origin, it become invasive and is categorized as local, regional, or distant based on the extent of spread. The American Joint Committee on Cancer (AJCC) and the International Union for Cancer control (IUCC) maintain the TNM classification system and is used by clinicians for solid tumors. The T gives information about the

primary tumor whereas the N and M is used for information regarding whether the cancer has spread to the nearby lymph nodes or to distant parts of the body. Some cancers like leukemia cannot be staged using this system since it is present throughout the body. Once the TNM values are determined, Roman Numeral Staging is done to assign the overall staging. Stage 0 is when there is only the primary tumor and has not spread to other sites. The difference between stage I, II and III depends upon the size of the primary tumor as well as the extent it has spread into nearby tissues. Stage IV is the highest and most advanced stage where the cancer has spread to distant parts of the body (Webber et al., 2014; American Cancer Society, 2015).

Treatment

Cancer treatment usually involves one or more of surgery, radiotherapy and systemic therapy. Low risk patients with early stage cancer are often cured with only surgery; however, many cases require combination treatment, where one or more treatment modalities are combined to cure cancer. Systemic therapies include hormonal therapy, targeted therapy, and chemotherapy. It is the only option once the disease has metastasized since delivery through the bloodstream is required for cancer cells at different sites (Caley and Jones, 2012)

Surgery

Surgery is the oldest form of treatment for cancer and is still used in the modern world for solid tumors. Surgery can be used as a cure if the cancer is detected in the early stages. It is known to be most effective and gives higher success rate for treatment of cancer than the other form of treatment when used alone (Harvey, 1974; Caley and Jones, 2012). However, surgery also has its own limitations. Some of the cancer cells maybe missed during surgery while removing further of the surrounding tissues so as to clear all the cancer cells can lead to loss or reduced functioning of the

organ. Besides, it cannot be used for the treatment of later stages of cancer that has already metastasized (Greene, 2002).

Radiation

The use of radiation to kill cancer cells is known as radiation therapy. Though it is believed that radiation can cause cancer, research over the last century indicates that low doses of ionizing radiation can have beneficial effects. Evidences also suggested that total or half-body low-dose irradiation may cure cancer or significantly delay its progression, leading to a reduction in cancer mortality without symptomatic side effects. The use of radiation as cancer treatment became common in the 1950s when cobalt-60 gamma radiation became available followed by particle accelerators in the 1970s (Cuttler and Pollycove, 2003). Radiotherapy may be used either alone or in combination with surgery or chemotherapy to cure cancer, where it may eliminate the cancer or prevent the recurrence of cancer. It may also be given with palliative intent where the purpose is not to cure but to relieve the symptoms caused by cancer. The radiation given before surgery to shrink the tumor is called neoadjuvant therapy whereas the radiation used after surgery to destroy the microscopic tumor cells after surgery is called adjuvant therapy. The ionizing radiation kills or cause genetic alterations in the cells it passes through by depositing energy in the form of ions which damages DNA causing single or double or strand breaks (Lomax *et al*., 2013). This DNA damage occurs not only in tumor cells but also to normal cells which are adjacent or nearby to the tumor. This changes the fidelity of genome causing cancer recurrence. However, cancer cells are less efficient than normal cells in repairing damage resulting in differential cancer killing (Begg *et al*., 2011). A major limitation of radiotherapy is the tumor cells that are in a low-oxygen state called as hypoxia which are 2 to 3 time more resistant to radiation damage as compared to those

growing in a normal oxygen environment (Harrison *et al*., 2002). Usually combination therapy is incorporated in such situations.

Chemotherapy

Chemotherapy is the treatment of cancer using drugs to destroy cancer cells. The drugs used for chemotherapy differs in their mode of action. The first alkylating agent was described by Paul Ehrlich, the father of chemotherapy in 1898 (Mann, 1999). He was also the first person to document the effectiveness of *in vivo* models to screen a series of chemicals for their potential activity against diseases, an accomplishment that had major ramifications for cancer drug development (DeVita and Chu, 2008). There are more than 100 different types of chemotherapeutic drugs for the treatment of different types of cancers, which are used either alone or in combination. The alkylating agents directly damage the DNA of cancer cells and prevent them from reproducing. They can be used to treat different types of cancer since they kill the neoplastic cells in any stages of the cell cycle. Nitrogen mustards, nitrosoureas, alkyl sulfonates, triazines and ethyleneamines are different types of alkylating agents (Colvin, 2003). Unlike the alkylating agents, the antimetabolites, work only during the S-phase of the cell cycle and drive the cell to programmed cell death. They hinder the synthesis of DNA or RNA by inhibiting the enzymes necessary for DNA synthesis thereby preventing mitosis. They can also incorporate themselves into the DNA since many of them have structures similar to the nucleotides. The anti-folates, fluoropyrimidines, deoxynucleoside analogues and thiopurines are the different types of anti-metabolites, which are used in clinics for the treatment of cancer (Lind, 2008; Parker, 2009). The drug that can cause microtubule dysfunction can be divided into two main types: those that can prevent the assembly of microtubules namely the vinca alkaloids and those that block the disassociation of the

microtubules, the taxanes. Though their mode of action is opposite, both of them eventually induce apoptosis by preventing mitosis. Vincristine and vinblastine isolated from *Catharanthus roseus* are examples of vinca alkaloids whereas paclitaxel extracted from *Taxus brevifolia* is an example of taxanes (Lind, 2008; Yue *et al*., 2010; Liu et al., 2007). Another other group is cytotoxic antibiotics, whcih interrupt cell division by intercalating into the DNA. The doxorubicin an anthracycline and bleomycin are the subgroups in the antibiotic category. They are used in the treatment of different types of neoplasia like breast, ovary, bladder and lung cancers, and lymphomas and sarcomas (Chabner and Longo, 2001). Chemotherapy drugs are given repeatedly at a regular time intervals known as treatment cycles. Each cycle kills a fraction of cells, and not a constant number of cells. Since normal cells have more capacity to repair as compared to tumor cells, the repeated cycles allow normal cells to repair and repopulate while tumor cells constantly decrease in numbers (Caley and Jones, 2012). A detectable cancer usually has gone through over 30 doublings and contains 10^8-10^9 cells (Price *et al*., 2008). Therefore, even though not detected after treatment there is still considerable number of cells which can cause relapse of the disease (Caley and Jones, 2012).

Chemotherapy also has its own limitations as it does not specifically target tumor cells. Since the chemotherapeutic agents hamper cell division or inhibit enzymes involved in DNA replication or metabolism, they also damage the normal dividing cells especially the rapidly regenerating tissues, such as those of the bone marrow, gut mucosa and hair follicles (Wu *et al*., 2008). Besides, it can also lead to the development of drug resistant cells and many of the drugs that kill tumors can cause mutations that transform normal cells to cancer (Aqeilan *et al*., 2009). Another problem with chemotherapy is that the non-homogenous cancer stem cells are not

affected by chemotherapy and therefore cannot be eliminated even if all the cancer cells die. These cancer stem cells thereby can cause cancer again (Cetin and Topcul, 2012). Combination chemotherapy is often used to provide maximum cell kill at lower toxicity to the host and to prevent the development resistance (Page and Takimoto, 2004).

Targeted therapy

Targeted cancer therapy means the use of anticancer drugs designed to interfere with a specific molecular target, usually a protein with a critical role in tumor growth or progression. This has been a promising strategy since it has come to light that many diseases are regulated by the abundance of proteins such as receptors and hormones (Meiyanto *et al*., 2012). This therapy makes use of targeting the pathways and molecules involved in the cancer formation and progression such as monoclonal antibodies, antiangiogenic agents, hormones and hormone receptors, inhibitors of PARP, tyrosine kinase, proteasome, cyclin dependent kinases, Raf kinase, farnesyl transferase, matrix metalloproteinase, protein kinase, mTOR (mammalian target of rapamycin), glutathione-S-transferase etc. (Topcul and Cetin, 2014).

Phytoceuticals

Plants have been the major source for several drugs and it is well known that 75% of the modern chemotherapeutic drugs have their origin in plants or natural products (Cragg and Newman, 2013; Harvey et al., 2015). Several phytoceuticals have been screened for their anticancer activities earlier. *Alsotnia scholaris, Aegle marmelos, Aphanmixis polystychya, Solanum khasianum, Tinospora cardiofolia* have been found to possess anticancer activity in differenct preclinical systems (Jagetia et al., 1998; Jagetia and Baliga, 2005; Jagetia et al., 2005, Jagetia and Venkatesha, 2012;

Rosangkima and Jagetia, 2015). Extracts from *Urtica membranacea, Artemesia monosperma,* and *Origanum dayi post* have also been reported to exert anticancer activity (Solowey et al., 2014). Therefore plants and natural products still provide a major avenue for screening and developing of new nontoxic molecules including drugs for cancer treatment.

Oroxylum indicum (*O. indicum*) is a deciduous tree which grows at an altitude of 1200 m mainly in ravines, in damp and moist places in the forest. It is commonly called the tree of Damocles or Indian caper (Chauhan, 1999). It is found in India, Srilanka, China, Thailand, Philippines and Indonesia of the Asian continent. In India, it is distributed in Himalayan foothills, Eastern and Western Ghats and North East India (Kritikar and Basu, 2001). The tree is small to medium sized up to 12 m in height with soft light brown or grayish brown bark. It has very large leaves about 90-180 cm long which when whither and fall off near the tree looks like a pile of broken limb bones hence the name broken bones. The flowers are reddish purple outside and pale, pinkish-yellow within, which blooms at night. The flowers emit a strong, stinky odor which attracts bats and therefore is pollinated by bats (Padgilwar et al., 2014).

Traditionally, the root tonic is useful in dropsy, cough, sprains, neuralgia, hiccough, asthma, bronchitis, anorexia, dyspepsia, flatulence, colic, diarrhea, dysentery, strangury, gout, vomiting, leucoderma, wounds, rheumatoid arthritis and fever. Root bark is used in stomatitis, nasopharyngeal cancer and tuberculosis. The leaves are used as stomachic, carminative and flatulent. It is also prescribed for snake bite. The decoction of the leaf is used to treat rheumatic pain, enlarged spleen, ulcer, cough, and bronchitis. The fruits are useful in pharyngodynia, cardiac disorders, gastropathy, bronchitis, haemarrhoids, cough, piles, jaundice, dyspepsia, smallpox, leucoderma and cholera. The seeds are used as purgative and the dried powder is used

by women to induce conception. It is also used in the form of paste for quick relief of tonsil pain after grounded with fire soot. The stem bark decoction is taken for curing gastric ulcer and a paste made of the bark powder is applied for mouth cancer, scabies and other skin diseases (Nadkarni, 1982; Khare, 2007; Bhattacharje, 2005).

The plant is used as one of the important ingredient in most commonly used Ayurvedic preparations like Dasamularistha, Syonaka putapaka, Syonaka sidda ghrta, Brhatpancamulyadikvatha, Amartarista, Dantyadyarista etc. (Zaveri et al., 2008). A man of about 50 years of age from Maram Naga village, Manipur was diagnosed with nasopharyngeal cancer and was treated with chemotherapy but could not be cured. He was informed by the doctor that he may live about 6 months i.e., until November 1996. However, he learned about someone with similar condition who got cured by taking the decoction of the stem bark of *Oroxylum indicum*. He too started to take the decoction three times a day. His condition improved and he was still surviving, living a normal life until the year 2000 (Mao, 2002). Besides, local sayings indicate that a man from Hnahlan, Mizoram was also cured from cancer after taking the decoction of this plant. Therefore, the present study was carried out to obtain an insight into the anticancer activity of *Oroxylum indicum* extracts *in vitro* and *in vivo* by carrying out the following studies:

1. Fraction guided preparation of *Oroxylum indicum* extracts and its phytochemical analysis.
2. Antioxidant activity
3. Anti-inflammatory and analgesic activities
4. Evaluation of anticancer activity *in vitro* and *in vivo*.
5. Wound healing activity.
6. Isolation of active principle.

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***Oroxylum indicum* tree**

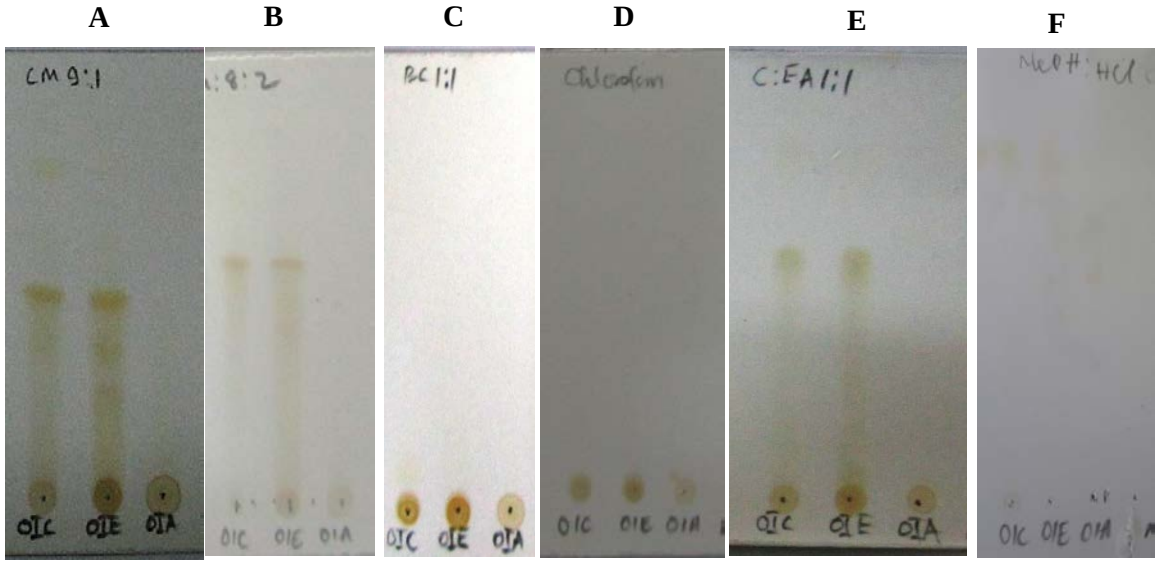


Figure 1: TLC profile of different extracts of *O. indicum* using different solvent systems observed under normal light to detect phytochemicals present in the extracts.

A). CHC_{l3}:CH_3OH (9:1) B) $CHCl_3$:CH_3OH (8:2) C) C_6H_6:$CHCl_3$ (1:1)
D) $CHCl_3$ E) $CHCl_3$:$C_4H_8O_2$ (1:1) F) CH_3OH:HCl (9:1)
OIC – O. indicum chloroform extract, OIE – O. indicum ethanol extract, OIA – O. indicum aqueous extract.

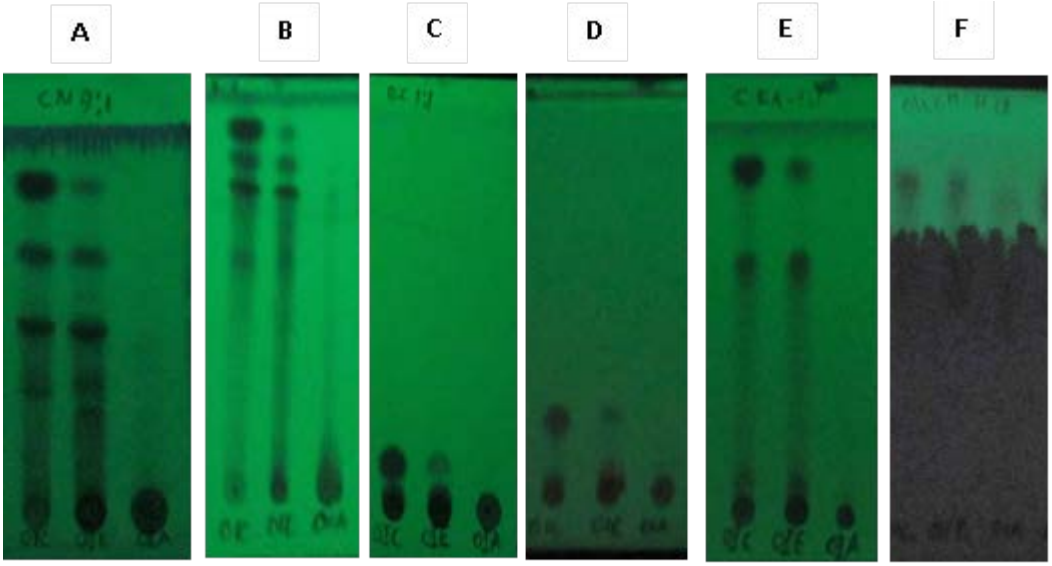


Figure 2: TLC profile of *O. indicum* on different solvent system observed under UV 254 nm to detect phytochemicals present in the extracts.

A). $CHCl_3$:CH_3OH (9:1) B) $CHCl_3$:CH_3OH (8:2) C) C_6H_6:$CHCl_3$ (1:1)
D) $CHCl_3$ E) $CHCl_3$:$C_4H_8O_2$ (1:1) F) CH_3OH:HCl (9:1)
OIC – O. indicum chloroform extract, OIE – O. indicum ethanol extract , OIA – O. indicum aqueous extract.

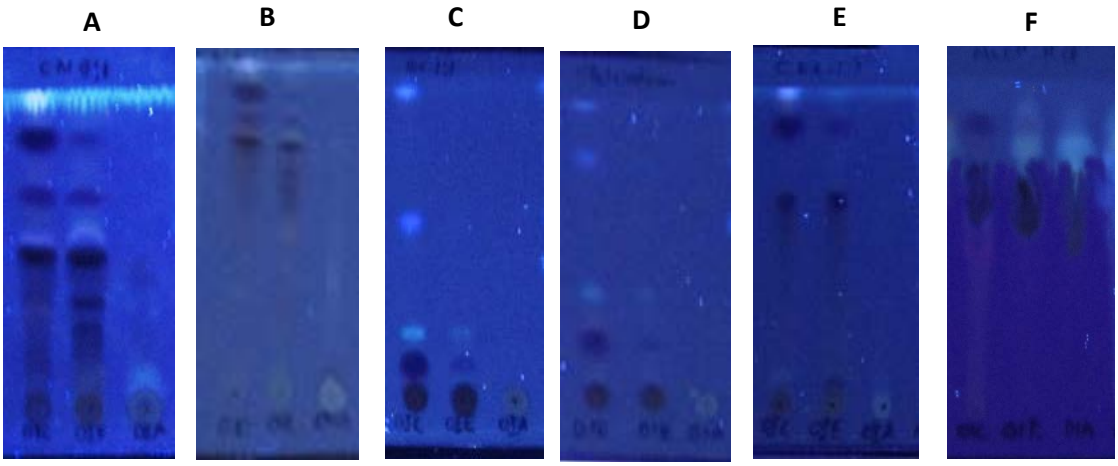


Figure 3. TLC profile *of O. indicum* on different solvent systems observed under UV 365 nm to detect phytochemicals present in the extracts.

A). $CHCl_3$:CH_3OH (9:1) B) $CHCl_3$:CH_3OH (8:2) C) C_6H_6:$CHCl_3$ (1:1)
D) $CHCl_3$ E) $CHCl_3$:$C_4H_8O_2$ (1:1) F) CH_3OH:HCl (9:1)
OIC – *O. indicum* chloroform extract OIE – *O. indicum* ethanol extract
OIA – *O. indicum* aqueous extract

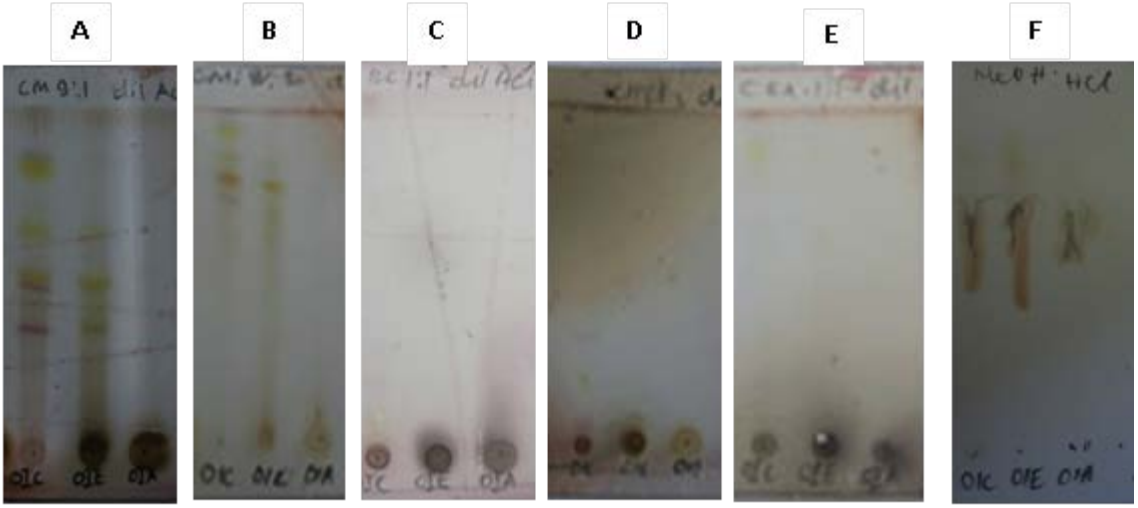


Figure 4: TLC profile of *O. indicum* using different solvent systems sprayed with dil H_2SO_4 to detect phytochemicals present in the extracts.

A). $CHCl_3$:CH_3OH (9:1) B) $CHCl_3$:CH_3OH (8:2) C) C_6H_6:$CHCl_3$ (1:1)
D) $CHCl_3$ E) $CHCl_3$:$C_4H_8O_2$ (1:1) F) CH_3OH:HCl (9:1)
OIC – *O. indicum* chloroform extract OIE – *O. indicum* ethanol extract
OIA – *O. indicum* aqueous extract

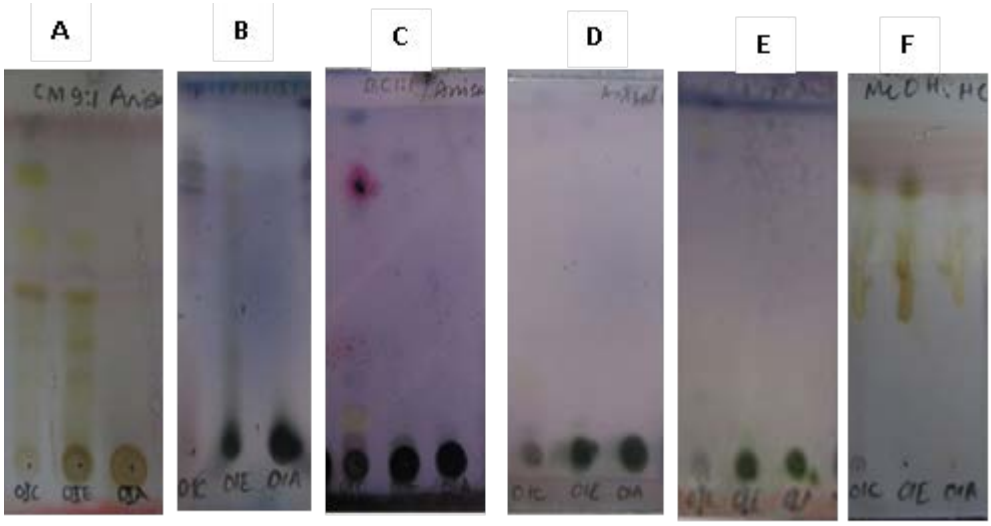


Figure 5: TLC profile of *O. indicum* using different solvent systems sprayed with anisaldehyde to detect phytochemicals present in the extracts.

A). $CHCl_3$:CH_3OH (9:1) B) $CHCl_3$:CH_3OH (8:2) C) C_6H_6:$CHCl_3$ (1:1)
D) $CHCl_3$ E) $CHCl_3$:$C_4H_8O_2$ (1:1) F) CH_3OH:HCl (9:1)
OIC – *O. indicum* chloroform extract OIE – *O. indicum* ethanol extract
OIA – *O. indicum* aqueous extract

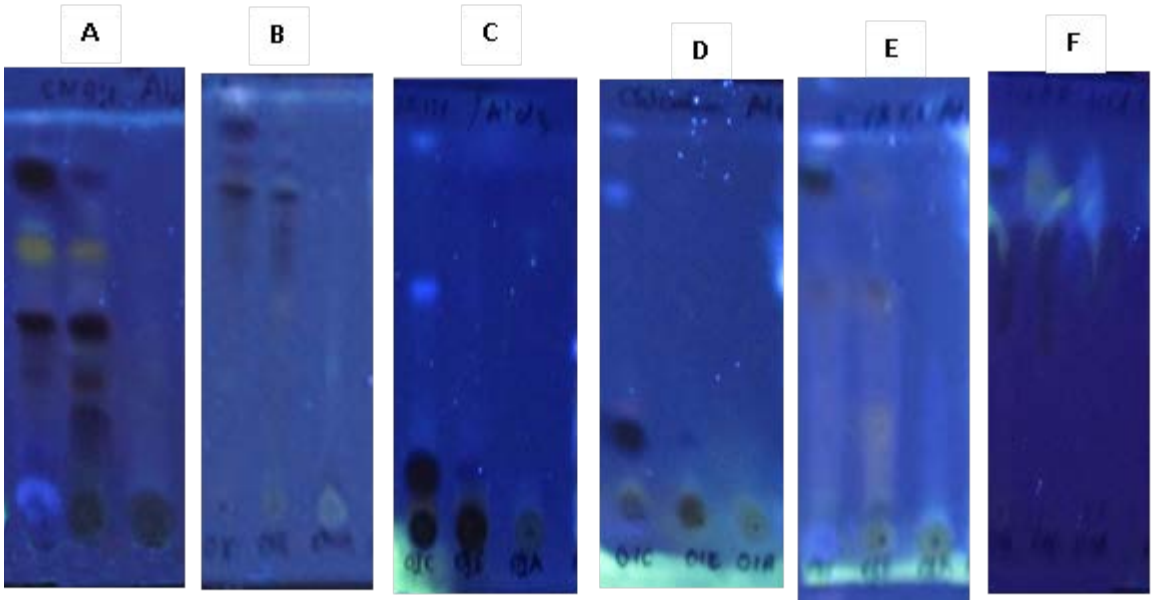


Figure 6: TLC profile of *O. indicum* using different solvent systems sprayed with Aluminium chloride and observed under UV 365 nm to detect phytochemicals present in the extracts.

A). $CHCl_3$:CH_3OH (9:1) B) $CHCl_3$:CH_3OH (8:2) C) C_6H_6:$CHCl_3$ (1:1)
D) $CHCl_3$ E) $CHCl_3$:$C_4H_8O_2$ (1:1) F) CH_3OH:HCl (9:1)
OIC – *O. indicum* chloroform extract OIE – *O. indicum* ethanol extract
OIA – *O. indicum* aqueous extract

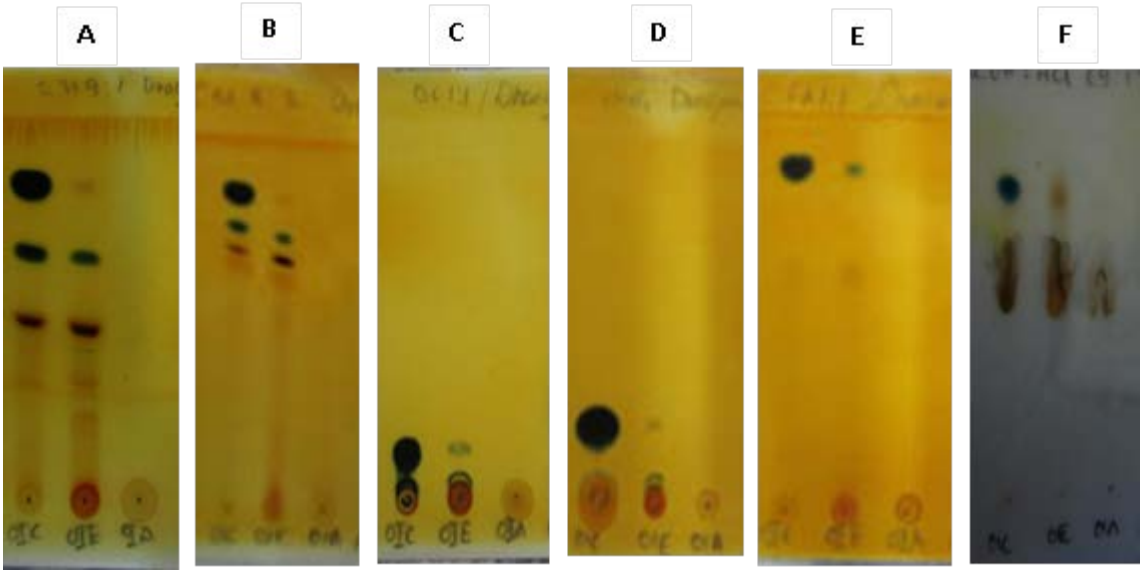
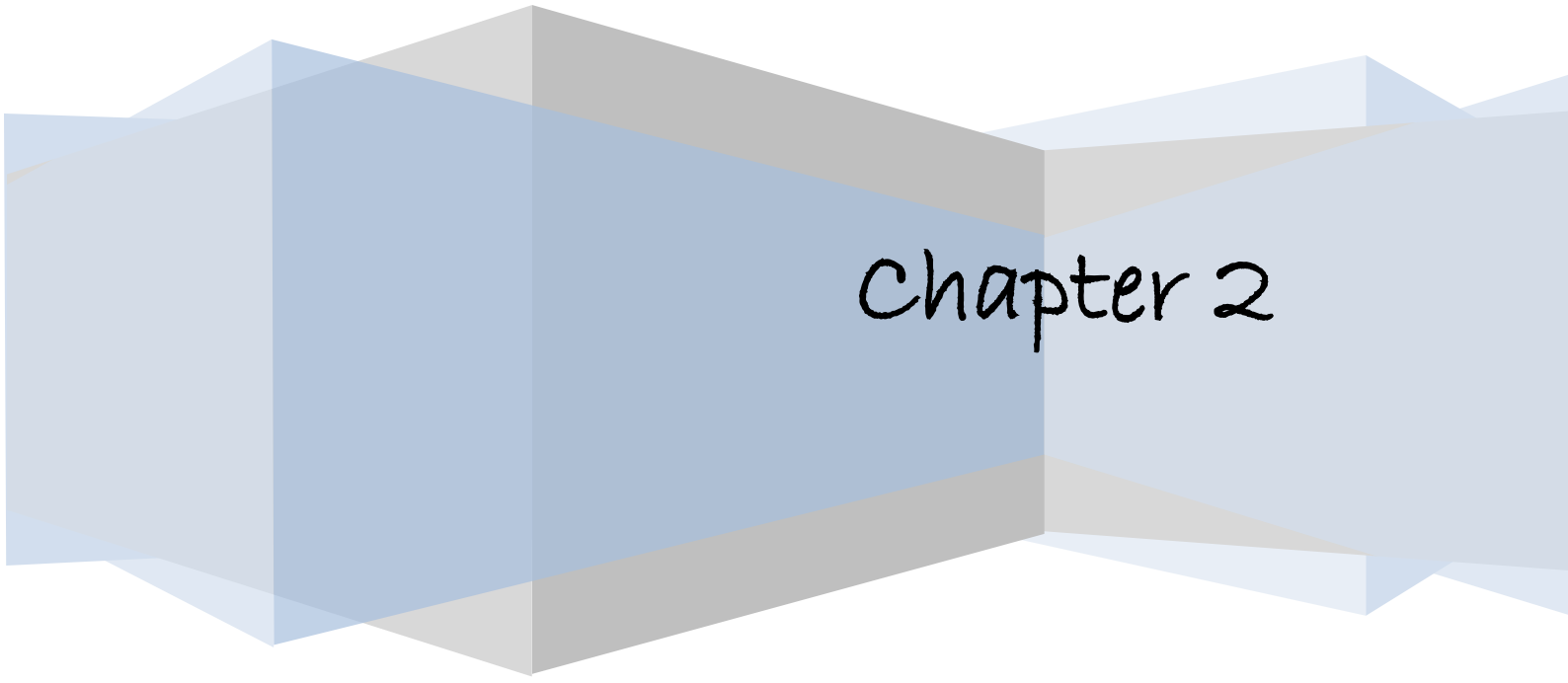


Figure 7: TLC profile of *O. indicum* using different solvent systems sprayed with Dragendorff's reagent and observed under daylight to detect phytochemicals present in the extracts.

A). $CHCl_3$:CH_3OH (9:1) B) $CHCl_3$:CH_3OH (8:2) C) C_6H_6:$CHCl_3$ (1:1)
D) $CHCl_3$ E) $CHCl_3$:$C_4H_8O_2$ (1:1) F) CH_3OH:HCl (9:1)
OIC – *O. indicum* chloroform extract OIE – *O. indicum* ethanol extract
OIA – *O. indicum* aqueous extract

Phytochemical and TLC profiling of *Oroxylum indicum*

(Published in Plant Biochemistry & Physiology, 2015; 3:3)



Abstract

The non-infected stem bark of Oroxylum indicum was collected, powdered and sequentially extracted with petroleum ether, chloroform, ethanol and distilled water using a Soxhlet apparatus. All the liquid extracts except petroleum ether were cooled, solidified and stored at -70°C for further use. The bark powder as well as the different extracts was subjected to different phytochemical analyses using standard protocols. The TLC profiles of various extracts were obtained using different solvent systems. The bark powder contained various phytochemicals including, alkaloids, flavonoids, cardiac glycosides, saponins, steroids, terpenoids and tannins. The phlobatannins were absent in the bark powder. The systematic phytochemical analysis of chloroform, ethanol and aqueous extracts revealed the presence of tannins, flavonoids and cardiac glycosides. However, there was variation in the presence of alkaloids, steroids and saponins among the different extracts. The phlobatannins were completely absent in all the three extract. The TLC study also showed the presence of different components as indicated by the different Rf values in different solvent systems.

1. INTRODUCTION

The use of plants as medicine has been recorded throughout the human history in different parts of the world. The writings of ancient Chinese and the Egyptian papyrus around 3000 BC could be the first written record of plants as medicine (Ampofo et al., 2012). Though the use of medicinal plants is declining in the modern world especially in the West due to the development of easily available synthetic drugs, many developing countries still continue to benefit from their rich traditional knowledge on the use of plants for healthcare. This is clearly shown by the continuance of Siddha and Ayurveda medicines in India, Kampo Medicine in Japan, traditional Chinese medicine (TCM), and Unani medicine in the Middle East and South Asia (Mosihuzzaman and Choudhary, 2008). These plant based medicines are not only economical but also safer than the modern synthetic drugs. The fact is that around 80% of the world's populations still rely on plants for their healthcare (WHO, 2010). Besides, they are produced from renewable resources with eco-friendly processes and can bring about economic prosperity to the masses growing these raw materials (Kashaw et al., 2011).

The plants usually synthesize many chemicals, which are either product of metabolism or intentionally for nutrition, defence, pollination and against stress and predators (Cseke et al., 2006). The phytochemicals synthesized by plants can be mainly grouped into primary and secondary metabolites (Irchhaiya et al., 2015). The primary metabolites include phytosterols, acyl lipids, amino acids and organic acids that have shared biological function across all plant species (Waterman, 1992). The primary metabolites are responsible mainly for growth, development and other metabolic activities of the plants (Croteau et al., 2000). The metabolism of primary metabolites generates secondary metabolites, which are not involved in any of the

metabolic activity of plants (Irchhaiya et al., 2015). The properties of these phytochemicals have been under investigation since the 1850s and they have been used as dyes, polymers, fibers, glues, oils, waxes, flavoring agents, perfumes, and even as drugs (Croteau et al., 2000). It is now fairly well established that the synthesis of secondary metabolites plays an important role in the survival of plants and other activities (Li et al., 1993). The plants usually synthesize these phytochemicals in specialized cells during particular developmental phase making their extraction and purification difficult (Shula et al., 2009). The various phytochemicals synthesized by plants as secondary metabolites have been found to exert various physiological effects in mammals including humans and hence they are also called the active principles of that plant (Shula et al., 2009). The phytochemicals produce various biological activities, and this has been the reason that plants have been used to treat several ailments in traditional medicine since the time immemorial. It is also known that almost 70% of the modern medicines have a direct or indirect origin in plants (Newmann and Cragg, 2014). The phytochemicals derived from plants include antibiotic, antifungal and antiviral, antitumor and antigerminative compounds, which helps plants to protect from plant pathogens, insects and predators. The plants also synthesize important UV absorbing compounds, to safeguard the leaves against the damaging effect of UV light from sunlight (Li et al., 1993; Qin et al., 2011). The phytochemicals synthesized by plants are usually complex and it is sometimes difficult to synthesize them in the laboratory therefore phytochemicals will continue to play crucial role in the new drug discovery.

Oroxylum indicum (family Bignoniaceae), sona patha is a deciduous tree distributed throughout Asia and grows at an altitude of 1200 m mainly in ravines, in damp region and moist places in the forests. In India, it is distributed in the

Himalayan foothills, Eastern and Western Ghats and North East India (Kritikar and Basu, 2001). *O. indicum* lives in relationship with an actinomycete *Pseudonocardia oroxyli*, a gram positive bacterium (Gu et al., 2006) that has the capacity to produce many secondary metabolites exhibiting a wide variety of biological activity (Qin et al., 2011). Almost every part of this tree possesses medicinal properties and has been used in several traditional Ayurvedic and folk medicines (Sastry et al., 2011). *O. Indicum* has been reported to possess several medicinal properties including analgesic, antibacterial, anti-inflammatory, anticancer, antioxidant (Rasadah et al., 1998; Ong et al., 2009; Kumar et al., 2010; Hosen et al., 2011; Talari et al., 2013). Therefore, an attempt has been made to study the phytochemical constituents of *Oroxylum indicum.*

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Potassium iodide, bismuth nitrate, sulphuric acid, ferric chloride, hydrochloric acid, aluminium chloride, ammonium hydroxide, glacial acetic acid, chloroform, ethanol, olive oil, Silica gel G were procured from Sd fine Chemical Ltd., Mumbai, India. The TLC plates were commercially procured from Merck India, Mumbai.

2.2. Identification, collection and drying of plant material

Oroxylum indicum, Sonapatha, an important medicinal plant in Ayurvedic medicine was selected for the present study. The plant was collected from Champhai, Mizoram and its identity was confirmed by the Botanical Survey of India, Shillong as *Oroxylum indicum* (Family: Bignoniaceae). It is a deciduous, medium sized tree and has been reported to possess several medicinal properties. The bark was collected during the dry season. The non-infected bark was peeled off from the trunk and chopped into about a feet each. It was then washed with water and scrubbed properly

to remove all the dirt and other extraneous materials. The washed stem bark was spread into stainless steel trays and allowed to shade dry at room temperature in dark, clean and hygienic conditions to avoid entry of insects, animals, fungus, and extraneous terrestrial materials. The exhaust and free air circulation was allowed. Drying of the collected stem bark took about three to four months and required periodic observation for contamination if any. After the bark was completely dried, it was chopped into small pieces and ground to powder using an electrical grinder at room temperature.

2.3. Preparation of different extracts

2.3.1. Procedure I

Sequential extraction of the bark powder was carried out using three different solvents according to increasing polarity in order to fractionate the different compounds. The bark powder of *O. indicum* (170 g) was packed in a funnel (20 cm in length) made up of Whatman filter paper No.1. The funnel was transferred into a Soxhlet apparatus and the powdered material was extracted with petroleum ether (B.P. 60-80°C) at 60°C for around seven cycles (about three hours) until the solvent was transparent and colourless. The extract was dried at room temperature. The petroleum ether free powder of *O. indicum* was subsequently extracted with chloroform at 60°C for around thirteen cycles and thereafter in absolute ethyl alcohol at 70°C for twenty five cycles extensively until transparency. The final extraction was carried out with distilled water at 100°C extensively until it became colourless. The cooled liquid extracts were concentrated by evaporating their liquid contents using rotary evaporator. Each extract, except petroleum ether was dried *in vacuo* and stored at -70°C until further use.

2.3.2. Procedure II

The bark extract was prepared by weighing 5 g powder of *O. indicum* and transferring it into a beaker containing 200 ml of distilled water. The mixture was heated on a hot plate with continuous stirring at 60º- 80ºC for 30 minutes. The water extract was filtered through filter paper and the filtrate was used for the phytochemical analysis. The aqueous extracts were kept in refrigerator at 0^{o}C until use.

2.4. Phytochemical Screening

The phytochemical screening was performed in the bark powder and different extracts prepared using procedure I and II.

2.4.1. Alkaloids

The presence of alkaloids in *O. indicum* was confirmed by employing the Dragendorff's test. Briefly, 0.1 g of different extracts of *O. indicum* or *M. pachycarpa* was mixed with 0.5 ml of Dragendorff's reagent. The development of reddish brown precipitate indicates the presence of alkaloids (Harborne, 1998; Kokate et al., 2006; Doughari, 2012).

2.4.2. Flavonoids

The flavonoids were qualitatively estimated using alkaline reagent test, where 0.1 g of each extract of *O. indicum* was dissolved in appropriate solvents and mixed with a few drops of sodium hydroxide solution. The formation of intense yellow colour, which turned colourless on addition of a few drops of dilute acid indicated the presence of flavonoids (Sofowara, 1993; Harborne, 1998; Kokate et al., 2006; Doughari, 2012).

2.4.3. Cardiac glycosides (Keller-Killani test)

0.1 g of *O. indicum* was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution with an under laying of 1 ml of concentrated sulphuric acid. The appearance of brown ring at the interface indicated the presence of deoxysugar, which is a characteristic of cardenolides (Harborne, 1998; Doughari, 2012).

2.4.4. Saponins

Usually 0.1 g of the extracts of *O. indicum* was mixed with 3 drops of olive oil and shaken vigorously for a few minutes. The formation of a fairly stable emulsion indicated the presence of saponins (Sofowara, 1993; Harborne, 1998; Doughari, 2012).

2.4.5. Steroids

The presence of steroid in various extracts of *O. indicum* was determined by Salkowski's test. Briefly 0.1 g of various extracts of *O. indicum* and *M. pachycarpa* dissolved in different solvents were mixed with a few drops of concentrated sulphuric acid. The development of red colour at lower layer indicated the presence of steroids, whereas the formation of yellow colour indicated the presence of triterpenoids (Harborne, 1998; Kokate et al., 2006; Doughari, 2012).

2.4.6. Tannins

The presence of tannin was determined by Ferric chloride test. Usually 0.1 g of dried samples of each extract of *O. indicum* was dissolved in their respective solvents and a few drops of 0.1% ferric chloride were added. The formation of brownish green or a blue-black colour indicated the presence of tannins (Harborne, 1998; Kokate et al., 2006; Doughari, 2012).

2.4.7. Test for terpenoids (Salkowski test)

The terpenoids in aqueous extract of *O. indicum* were detected by mixing 5 ml of each extract with 2 ml of chloroform with the careful addition of 3 ml concentrated H_2SO_4 so as to allow the formation of a layer. The formation of a reddish brown colour at the interface confirmed the presence of terpenoids.

2.4.8. Phlobatannins

The different extracts of *O. indicum* were boiled in 1% aqueous hydrochloric acid and deposition of a red precipitate indicated the presence of phlobatannins (Harborne, 1998; Doughari, 2012).

2.5. Quantification

2.5.1. Determination of Alkaloids

Five grams of the dried powder was weighed in a 250 ml beaker and 200 ml of 10 % acetic acid in ethanol was added. The mixture was covered and allowed to stand for 4 hours and Filtered. The extract was concentrated on a water bath until its final volume was reduced to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until complete precipitation, allowed to stand and the precipitate was collected. The precipitate was washed with dilute ammonium hydroxide and filtered. The residue was rich in the alkaloids. It was dried, weighed and the percentage of alkaloids was calculated **(**Balandrin, 1985**)**.

2.5.2. Determination of Saponins

20 g of the extract was placed into a conical flask and 100 ml of 20 % aqueous ethanol was added. The sample was heated over a hot water bath for 4 h with continuous stirring at 55 °C. The mixture was filtered and the residue was re-extracted with another 200 ml of 20 % ethanol. The combined extracts were reduced to 40 ml over water bath at 90 °C. The concentrate was transferred into a 250 ml separating

funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated And 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight and saponin contents were calculated as percentage (Brinda et al., 1981).

2.5.3. Determination of Flavonoids

10 g of the extract repeatedly extracted with 100 ml of 80 % aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No 41. The filtrate was allowed to evaporate to dryness over a water bath and weighed **(**Hagerman et al., 2000).

2.6. Ash Content

The crude powder as well as the different extracts of *O. indicum* were weighed and heated at 500ºC in a Nabertherm muffle furnace for five hours. The final ash was collected and weighed and the ash contents are expressed in terms of percentage.

$$\text{Total ash content} = \frac{\text{Pw-Fw x 100}}{\text{W}}$$

Where Pw= Preweighed crucible

Fw= Final weight of the crucible containing ash

W= Total weight of powdered plant material

2.7. Yield Percentage

The percentage yield was calculated by weighing the powder before extraction and the final yield after completion of the cycles.

2.8. Thin Layer Chromatography

2.8.1. TLC on preparative plates

Thin layer chromatography (TLC) was performed on the different extracts to allow the separation of various phytochemical compounds present in the *Oroxylum indicum* extracts. TLC plates were prepared on 75x2.5 mm glass slides by coating them with aqueous silica gel G as an adsorbent. The resultant plates were dried and activated by heating in a hot air oven for ten minutes at 110°C. A small amount of different extracts was applied onto the plate, about 0.5 centimeters above from the bottom of plates. The plates were placed in closed different chambers containing different solvent systems in order to identify the various compounds present in the extracts. Petroleum ether: Acetone (9:1; 8:2; 7:3; 5:5), Chloroform: Methanol (9:1; 8:2; 7:3), Forestal (Acetic acid: Conc.HCl:Water) in the ratio of 30:3:10 and 15:1.5:5, BEW (Butanol: Ethanol: Water), Benzene: Chloroform (20:20), Butanol:Propanol, Butanol: Ethyl acetate, and Methanol: NH_4OH (200:3) were used as solvent system for the chloroform extract. The ethanol extract was also subjected to Methanol: HCl (1:9; 2:8; 3:7; 4:6; 5:5), Ethyl acetate: Butanol: Water: Formic acid (10:10:4:2), Chloroform: Acetone (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7,2:8), Forestal (Acetic acid: Conc. HCl: Water) (15:1.5:5). The resultant spots were observed under visible as well as ultra-violet light. The value of the retention factor (Rf) was calculated using the formula:-

Rf = Distance travelled by solute/ Distance travelled by solvent

2.8.2. TLC on pre-coated aluminium plates

2.8.3. Thin layer chromatography

Thin layer chromatography (TLC) was performed on the different extracts to visualize the separation of various phytochemical components as it is a simple, less

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

cumbersome and rapid technique. The TLC can identify and separate a number of components present in any extract/organic mixtures and it also helps in finding a suitable solvent/s for separating the components by column chromatography as well as for monitoring reactions progress. Pre-coated TLC plates (Silica gel 60 F_{254}) procured from Merck India, Mumbai were used as an adsorbent. A small amount of each of the different extracts was applied as 1 mm diameter, 5 mm above the bottom of the plates. The TLC plates were transferred into the mobile phase consisting of numerous combinations of solvent systems of different polarity such as chloroform:methanol (9:1, 8:2) benzene:chloroform (1:1), pure chloroform, chloroform: ethyl acetate (1:1) and methanol:hydrochloric acid (9:1) and allowed to move on the adsorbent silica gel. The resultant spots were observed under visible and ultra-violet light, dilute acid (H_2SO_4), anisaldehyde, aluminium chloride and Dragendorff's stain. The measure of the distance a compound travelled is considered as the retention factor (R_f), which was calculated using the following formula:-

R_f = Distance travelled by solute/Distance travelled by solvent

3. RESULTS

The results of phytochemicals of *Oroxylum indicum* have been shown in Tables 1-7 and Figures 1-7.

3.1. Phytochemical Screening of the bark powder (Table 1):

3.1.1. Test for alkaloids: The formation of a reddish brown precipitate on reaction with Dragendorff's reagent indicated the presence of alkaloids in the bark powder.

3.1.2. Test for tannins*:* A brownish colour was observed indicating that *Oroxylum indicum* bark powder contains tannin.

3.1.3. Test for phlobatannins: The test was negative due to the absence of a red precipitate in the bark powder of *Oroxylum indicum*.

***3.1.4. Test for Saponin*:** Formation of emulsion indicated the presence of saponin in the bark powder of *Oroxylum indicum*.

3.1.5.* T*est for flavonoids: A yellow colour was observed indicating that the bark powder of *Oroxylum indicum* contained flavonoids.

***3.1.6. Test for terpenoids*:** The formation of a reddish brown colour at the interface confirmed the presence of terpenoids in the bark powder.

3.1.7. Test for cardiac glycosides (Keller-Killani test): The appearance of a brown ring at the interface indicates the presence of cardiac glycosides in bark powder of *Oroxylum indicum*.

3.2. Phytochemical Screening of the different extracts of Oroxylum indicum

3.2.1. Tannins tests: - Tannins were present in all the different extracts which was indicated by the appearance of blue colour (Table 2).

3.2.2. Alkaloids tests: - The presence of alkaloids was confirmed with Dragendorff's reagent in both ethanol and chloroform extracts, indicated by the presence of reddish brown precipitate but absent in aqueous extract. The others reagents like Mayer's reagent, Wagner's reagent and Hager's reagent also showed positive for alkaloids in ethanolic and chloroform extract (Table 2).

***3.2.3. Steroid and Tripertenoids*:-** A red colouration at the lower layer indicated the presence of steroids in the ethanolic extract while it was absent in chloroform and aqueous extracts (Table 2).

***3.2.4. Flavonoids*:-** The flavonoids were present in all the extracts but the contents was highest in chloroform extracts which was indicated by the formation of

intense yellow colour in alkaline reagents test and red colouration in zinc hydrochloride test (Table 2).

***3.2.5. Cardiac glycoside*:-** The cardiac glycoside was present in all the extracts which was indicated by the presence of brown ring at the interface (Table 2).

***3.2.6. Saponins*:-** The formation of emulsion in both the ethanol and aqueous extracts indicating the presence of saponins in both the extracts. However, the emulsion was absent in the chloroform extract indicating the absence of saponins (Table 2).

***3.2.7. Phlobatannins*:-** The analysis for phlobatannins revealed that the phlobatannin was absent in all the extracts (Table 2).

3.3. Ash content

The ash content of the crude bark powder was found to be 9.4 % while the chloroform, ethanol and aqueous extracts were found to contain 0.146 %, 4.8 % and 7.2 % respectively (Table 3).

3.4. Extract Yield

The extraction of *O. indicum* stem bark yielded 5.6% in ethanol and 1.825 % in chloroform Table 3).

3.5. Quantification of phytochemicals

The ethanol extract was found to contain 2.4 % alkaloids, 30.9 % flavonoids and 1.05 % saponins respectively (Table 4).

3.6. TLC Analysis

The evaluation of various extracts of *Oroxylum indicum* showed the presence of different components as indicated by a varying number of spots and colours on a TLC plates using different visualization methods (Table 5-7; Figure 1-7).

4. DISCUSSION

Use of botanicals for healthcare is as old as human civilization. They are considered non-toxic and safer than other exotic pure chemicals. This may be due to the fact that phytoceuticals origin is biological and also they have been experimented since the advent of human history (Jagetia and Venkatesha, 2005; Shantabi et al., 2014). The wide spread use of medicinal plants in healthcare entails that their systematic phytochemical evaluation shall be undertaken for the reasons of safety and medicinal use. Therefore, an attempt has been made to analyse phytochemical constituents of *Oroxylum indicum*.

The alkaloids are the class of nitrogenous compounds and a diverse array of alkaloids are produced by numerous plants as secondary metabolites. They are usually produced by plants for defence, harbivory, and to protect from pathogenic organisms and harmful insects (Kutchan, 1995). More than 10,000 alkaloids are known to be produced by plants. Many of the alkaloids synthesized by plants are highly toxic to humans and they have been found to exert dramatic physiological activities in humans and hence they have been widely used as medicines to treat several human disorders (Yang and Stöckigt, 2010). The plant alkaloids have been reported to be active against hypertension, arrhythmia, malaria, cancer cardiovascular disorders and HIV (Wink et al., 1998; Hagel and Facchini, 2013; Pan et al., 2013; Amoa Onguéné et al., 2013; Xing 2014; Chaves Valadão, 2015). The stem bark powder as well as the chloroform and ethanol extracts showed the presence of alkaloids and this presence of alkaloids shows that the medicinal activities of this plant in humans may be in part due their alkaloid contents. A similar observation has been made earlier (Shantabi et al., 2014).

Flavonoids are present in all vascular plants and about ten classes of them are recognized (Harborne, 1998). Approximately 8000 flavonoids have been reported in

different plant species. The flavonoids are mainly responsible for the beautiful colours of flowers along with anthocyanins (Iwashina, 2015). Many of the flavonoids serve as copigment/s contributing to variation in the flower colouration. The flavonoids are also essential in stimulation, protection, flavouring, pigmentation and in plant-microorganism communication in the plants (Ghasemzadeh and Ghasemzadeh, 2011). The flavonoids are valued as antioxidants in plants as well as humans. They also protect plants against stress and aid in their development (Brunetti et al., 2016). Consumuption of flavonoids have been reported to exert several beneficial effects in humans (Ivey et al., 2015). Flavonoids have been reported to possess a diverse array of activities in humans. They act as antiallergic, anticancer, hepatoprotective, cardioprotective, anticatatactogenic, antiosteoporotic, antidiabetic, antibacterial, antiinflammatory, and antiviral (Hegarty et al., 2000; Cushnie and Lamb, 2005; Chahar et al., 2011; Kumar and Pandey, 2013; Tanaka, 2013). The bark powder as well as the all the different extracts contained flavonoids and various medicinal properties of this plant may due to the presence of flavanoids and polyphenolic compounds in them.

Cardiac glycosides are another class of phytochemicals synthesized by plants, which have several medicinal properties. The anticancer effects of cardiac glycosides were realized as early as 1967 and the recent preclinical investigation in HT29, HCT116, and CC2 colon cancer cell lines proved their cytotoxic action in vitro (Jagetia and Reddy, 2014). The cardiac glycosides have also been found to act against, melanoma, breast, lung, prostate, pancreatic cancers, leukaemia, neuroblastoma and renal adenocarcinoma (Felth et al., 2009). The administration of a cardiac glycoside digoxin in combination with chemotherapy has been reported to increase the overall survival of patients receiving chemotherapy for the treatment of colorectal, breast,

head and neck, and hepatocellular carcinoma (Prassas and Diamandis, 2008). The cardiac glycosides have been used in the treatment of cardiovascular diseases (Menger et al., 2012). All the extracts as well as the bark powder contained cardiac glycosides and many of the medicinal properties may be related to these phytochemicals.

The bark powder, the ethanol and aqueous extracts have shown the presence of saponins that are a vast group of glycosides, differentiated by their surface active properties from other group of glycosides. They possess the property of foam formation as well as detergent ability, which account for their soap like behaviour in water and hence they can be used as detergents (Oleszek, 2002; Chen et al., 2010; Couraud et al., 2014). They are allelopathic and act as defense against insects and pathogens in plants (Vincken et al., 2007). Saponins have been found to be antimicrobial, antimalarial, antiplasmodial, antiproliferative, antipsoriatic, antiallergic, antiatherosclerosis, antidiabetic, insecticidal, molluscicidal, anti-inflammatory and anticancer activities. The have also been reported to be active against obesity (Takagi et al.,1980; Mert-Türk, 2006; Man et al., 2010; Dinda et al., 2010; Elekofehinti, 2015; Mroczek, 2015). Some of the medicinal activities exhibited by the plant may be attributed to the presence of saponins in them.

Terpenoids, detected in the bark powder, are classified into different types based on the number of union of their C5 isoprene units. They are the primary constituents of the essential oils in many plants and they are synthesized for defence or as signals against indirect defence including herbivory and other enemies (Maatalah et al., 2012). Plant synthesize nearly 40,000 terpenoid molecules as secondary metabolites and they have diverse applications as industrial chemicals,

flavouring agents, pharmaceuticals, fragrance, pesticides and disinfectants (Cheng et al., 2007).

The terpenoids have been reported to show a diverse array of medicinal activities including, antiviral, antibacterial antimalarial, antiinflammatory, anticancer and chemopreventive. They have been found to inhibit cholesterol synthesis (Mahato and Sen, 1997; Wen et al., 2007; Bohlmann and Keeling, 2008; Sarala et al., 2011; Thoppil and Bishayee, 2011). The medicinal properties shown by the bark powder may also be due to its terpenoid contents.

Tannins are complex polyphenolic phytochemicals, which are synthesized by numerous plants as secondary metabolites (Khanbabaee and van Ree, 2001; Lu et al., 2012). The tannins protect plants against the attack by herbivores and insects by decreasing the availability of proteins or inducing toxicity (Robbins et al., 1987; Frutos et al., 2004). They act as a barrier for microorganisms and protect the plants due to their ability to form complexes with protein, starches and other macromolecules (Barbehenn and Constabel, 2011). Tannins have been reported to act as antioxidants in vertebrates, and prooxidants in the presence of oxygen (Robbins et al., 1987). The tannins have been reported to act as astringent, antibacterial, antiulcerogenic, antiviral, antitumour, antithrombogenic, and anti-inflammatory (Takechi and Tanaka, 1987; Banso and Adeyemo, 2007; Clinton, 2009; Ashok and Upadhyaya, 2012). These observations confirm the medicinal uses of *Oroxylum indicum* which may be due to the presence of tannins.

Phlobatannins are coloured phenolic compounds and could not be detected in the bark powder as well as the different extracts. They have been reported to possess wound healing, anti-inflammatory, antioxidant and analgesic activities (Okwu and Okwu, 2004; Ayinde et al., 2007; Kumari and Jain, 2015).

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Table 1: Qualitative phytochemical analysis of the bark powder of *O. indicum*.

Phytocompounds	**Bark powder**
Alkaloids	+
Tannins	+
Phlobatannins	-
Saponins	+
Flavonoids	+
Steroids	+
Terpenoids	+
Cardiac glycosides	+

Legend Present (+), absent (-)

Table 2: Qualitative phytochemical analysis of various extracts obtained from *O. indicum*.

Phytochemicals	Test	Solvent used for extraction		
		Chloroform	Ethanol	Aqueous
Tannins	$FeCl_3$	+	+	+
Alkaloids	Dragendroff's	+	+	-
	Mayer's	+	+	-
	Wagner's	+	+	-
	Hagar's	+	+	-
Flavonoid	Alkaline Reagent test	+	+	+
	Zinc hydrochloride test	+	+	+
Steroids and Triterpenoids	Salkowski	-	+	-
Cardiac glycoside		+	+	+
Phlobatannin		-	-	-
Saponin		-	+	+

Legend Present (+), absent (-)

Table 3: The Yield and Ash content of the different extracts of *Oroxylum indicum*

Plant samples	**Yield (%)**	**Ash content (%)**
Bark powder	-	9.4
Chloroform	1.825	0.146
Ethanol	5.6	4.8
Aqueous	-	7.2

Table 4: Quantification of phytochemicals in the ethanol extracts of *O. indicum*.

Phytochemicals	Initial weight (g)	Final Weight (mg)	Content (%)
Alkaloids	5	120	2.4
Flavonoids	10	3000.09	30.9
Saponins	20	210	1.05

Table 5: TLC profile of chloroform extract using manually prepared TLC plates.

Solvent system	Observation	Rf
Petroleum ether: Acetone (9:1)	Two spots- Yellow and light brown (under visible light)	0.28 0.26
Petroleum ether: Acetone (8:2)	Light yellow and dark green (under visible light)	0.41 0.83
Petroleum ether: Acetone (7:3)	Light yellow and dark green (under visible light)	0.33 0.68
Petroleum ether: Acetone (5:5)	Light yellow and greenish(dark) spots (under visible light)	0.09 0.57
Chloroform: Methanol (9:1)	Two dark green spots (After staining with iodine iodide)	18 0.66
Chloroform: Methanol (8:2)	Two dark green spots (After staining with iodine iodide)	0.15 0.42
Chloroform: Methanol (7:3)	Three dark green spots (After staining with iodine iodide)	0.15 0.18 0.51
Forestal (Acetic acid:Conc.HCl:Water) (15:1.5:5)	Pink spot after staining with iodine iodide	0.3
BAW (Butanol:Acetic acid:Water) (4.5:0.5:5)	Yellow colour spot with tailing, turns light grey after staining	0.375
Forestal (Acetic acid: Conc. HCl: Water) (30:3:10)	Under visible light- light brown, deep brown and light green colour observed wheres under U.V, it gives purple colour.	0.83 0.714 0.595

BEW (Butanol: Ethanol: Water)	Under visible light- light yellowish green and dark green spots which gives dark brown colour under U.V	0.96
BEW (Butanol: Ethanol: Water)	Under visible light- light yellowish green and dark green spots which gives dark brown colour under U.V	0.64
Benzene: Chloroform (20:20)	Yellow and green spots under visible light	0.925
Benzene: Chloroform (20:20)	Yellow and green spots under visible light	0.7
Butanol:Propanol	Yellow, dark green and brown spots	3.3
Butanol:Propanol	Yellow, dark green and brown spots	2.9
Butanol: Ethyl acetate	Yellow, light grey and dark grey spots	0.9
		0.48
Butanol: Ethyl acetate	Yellow, light grey and dark grey spots	0.407
Methanol: NH_4OH (200:3)	Under visible light- Pale yellow and light grey spots.	0.296
		0.62
Methanol: NH_4OH (200:3)	Under U.V- Pale green colour.	0.35

Table 6: TLC profile of ethanol extract using manually prepared TLC plates.

Solvent system	Oservation	*Rf*
Methanol: HCl (1:9)	Light orange coloured spot with tailing observed in all the cases.	0.27
Methanol: HCl (2:8)		0.26
Methanol: HCl (3:7)		0.22
Methanol: HCl (4:6)		0.21
Methanol: HCl (5:5)		0.15
Ethyl acetate: Butanol: Water: Formic acid (10:10:4:2)	A dark brown and black spot observed.	1
		0.10
Chloroform: Acetone 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7,2:8.	No proper spots except in 7:3 (Rf= 0.96 and 0.23) where a yellow and grey spot was observed. Yellow/brown spot with lot of tailing in 8:2 and 6:4.	
Forestal (Acetic acid: Conc. HCl: Water) (15:1.5:5)	Single spot	0.18

Table 7: TLC profile of the different extracts of *Oroxylum indicum* on pre-coated aluminium TLC plates.

Extracts	Solvent	Day light	R_f	UV 254	R_f	UV 365	R_f	Dil. H_2SO_4	R_f	Anisal-dehyde	RF	$AlCL_3$ UV	R_f	Dragen-dorff	R_f
OIC	$CHCl_3$:CH_3OH 9:1	3 light yellow	0.369 0.492 0.861	4black	0.329 0.487 0.682 0.878	3black, 1 uv active	0.543 0.574 0.659 0.851	1 purple, 3 yellow	0.347 0.495 0.661 0.826	1red 2yellow 3blue green	0.263 0.354 0.463 0.636 0.818	1yellow 2black	0.475 0.655 0.852	2 reddish brown, 2blue green	0.495 0.371 0.666 0 .886
OIE		2 light yellow	0.365 0.486	4black	0.325 0.486 0.681 0.876	5 black 1 uv active	0.234 0.34 0.543 0.574 0.659 0.851	3Yellow	0.345 0.495 0.661	3 yellow	0.236 0.445 0.472	4 black 1yellow	0.229 0.327 0.459 0.655 0.851	2 reddish brown, 1 blue green	0.486 0.370 0.643
OIA		not visible	0	not visible	0	not visible	0	not visible	0	not visible	0	not visible	0	not visible	0
OIC	$CHCl_3$:CH_3OH-8:2	2 yellow	0.639 0.819	4black	0.59 0.77 0.836 0.934	4 black	0.591 0.771 0.836 0.934	3 yellow	0.591 0.773 0.835 0.934	2 purple 2 yellow	0.592 0.778 0.836 0.935	4 light yellow, 1brown, 1yellow 1black	0.158 0.301 0.396 0.666 0.73 0.825 0.968	2 black	0.687 0.734 0.812
OIE		2 yellow	0.635 0.815	4black	0.59 0.77 0.836 0.935	4 black	0.589 0.769 0.835 0.932	3 yellow	0.59 0.771 0.832 0.931	2 yellow	0.590 0.771	4yellow	0.666 0.73 0.825 0.968	1 reddish brown, 1 black	0.684 0.731
OIA		not visible	0	not visible	0	not visible	0	not visible	0	not visible	0	not visible	not visible	not visible	0
OIC	C_6H_6:$CHCl_3$-1:1	not visible	0	1black	0.109	1 deep blue, 3 uv active	0.115 0.393 0.571 0.964	1yellow, 2 black	0.098 0.215 0.980	2 purple, 1 yellow	0.04 0.12 0.96	1 black, 2yellow	0.104 0.541 0.916	1 black	0.123
OIE		not visible	0	1black	0.108	1 blue, 1 uv active	0.178 0.39	not visible	0	not visible	0	1 black	0.104	1 black	0.12
OIA		not visible	0	not visible	0	not visible	0	not visible	0	not visible	0	not visible		not visible	0
OIC	$ChCl_3$	not visible	0	1black	0.219	4 uv	0.200	3yellow	0.200	1 yellow	0.218	2yellow	0.816	blue green	0.218

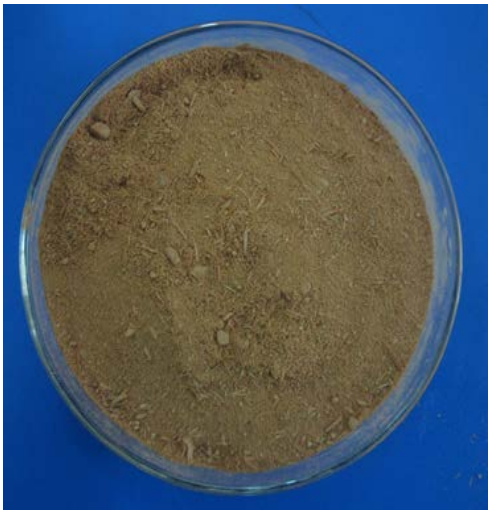
						active	0.342 0.742 0.914		0.342 0.742 0.914				0.915		
OIE		not visible	0	1black	0.218	not visible	0	not visible	0	not visible	0	not visible	0	blue green	0.216
OIA		not visible	0	not visible	0	not visible	0	not visible	0	not visible	0	not visible	0	not visible	
OIC	$CHCl_3$:$C_4H_8O_2$-1:1	2 yellow	0.596 0.865	2black	0.594 0.878	2black, 1active	0.638 0.833 0.888	yellow	0.842	yellow	0.842	3yellows	0.666 0.861 0.972	deep black	0.846
OIE		2 yellow	0.594 0.864	2black	0.592 0.876	2 black	0.631 0.829 0.885	yellow	0.842	yellow	0.842	3yellows	0.270 0.666 0.861	black	0.846
OIA		not visible	0	not visible		not visible	0	not visible	0	not visible	0	not visible	0	not visible	0
OIC	CH_3OH:HCl (9:1)	Green and brown mixed	0.694	black	0.846	2 black	0.846 0.641	1Yellow, 1 light brown	0.833 0.694	yellow	0.861	yellow UV active	0.861	blue green, dark brown	0.857 0.628
OIE		Green and brown mixed	0.694	black	0.846	light green, black	0.820 0.641	2 light brown	0.833 0.556	light brown	0.833	yellow UV active	0.833	light brown, orange	0.8 0.6
OIA		Green and brown mixed	0.722	black	0.82	light green	0.82	2 dark brown	0.639	no proper spots	0	yellow UV active	0.833	light brown	0.571

OIA- Aqueous extracts; OIC- Chloroform extract; OIE- Ethanol extracts

Oroxylum indicum tree

Stem of *Oroxylum indicum*

Stem bark of *Oroxylum indicum*



Stem bark powder of *Oroxylum indicum*

Plate I: The collection and preparation of plant sample (*Oroxylum indicum*).

Table 1: Percentage scavenging activities of different extracts of *Oroxylum indicum* in cell free system by various concentrations.

Conc. (µg/ml)	Hydroxyl radical			Superoxide			Nitric oxide			ABTS		
	$CHCl_3$	EtOH	H_2O	$CHCl_3$	EtOH	H_2O	$CHCl_3$	EtOH	H_2O	$CHCl_3$	EtOH	H_2O
1	3.40±0.04	2.10±0.02	0.40±0.01	12.73±0.26	12.57±0.68	12.83±0.31	0.30±0.01	0.64±0.04	1.50±0.13	13.45±0.31	16.01±0.24	20.52±0.28
10	09.32±0.08	3.40±0.01	0.70±0.02	13.50±0.35	13.18±0.77	12.27±0.74	0.75±0.02	0.85±0.01	1.52±0.05	20.21±0.52	21.94±0.36	21.18 0.19
50	11.79±0.03	6.20±0.02	0.95±0.01	17.42±0.19	15.82±0.23	12.45±0.53	2.16±0.05	1.32±0.05	1.71±0.09	37.13 ±080	46.62±0.38	23.42±0.38
100	18.03±0.13	12.75±0.05	02.22±0.08	23.49±0.19	19.31±0.95	13.66±0.60	3.36±0.04	2.09±0.05	1.91±0.03	51.48±0.77	68.14±0.31	27.13±0.50
200	20.23±0.13	15.79±0.03	13.50±0.09	30.21±0.85	28.26±0.55	14.44±0.37	4.84±0.05	3.06±0.10	2.38±0.05	71.72±0.56	89.03±0.01	39.49±0.41
500	25.97±0.32	27.56±0.04	17.10±0.03	47.38±0.76	41.43±0.33	16.77±0.68	15.74±0.02	9.68±0.13	8.94±0.07	88.27±0.03	89.22±0.02	88.91 ±0.3
1000	36.19±0.52	37.49±0.01	21.10±0.05	72.29±0.92	64.48±0.8	23.64±0.35	35.93±0.10	19.13±0.13	11.42±0.51	87.98±0.07	89.39±0.03	88.68±0.08
2500	84.49±0.02	66.34±0.05	27.94±0.07	85.88±1.97	81.09±0.91	32.32±0.19	59.84±0.53	49.73±0.32	24.42±0.08	85.91±0.13	87.62±0.02	88.04±0.02
5000	68.87±0.10	76.09±0.05	34.67±0.51	86.57±0.71	77.37±0.74	34.48±0.82	77.41±0.32	55.29±0.52	41.93±0.03	77.55±0.08	88.06±0.03	87.59±0.01

The results are expressed as Mean standard error of the mean (SEM), n=5

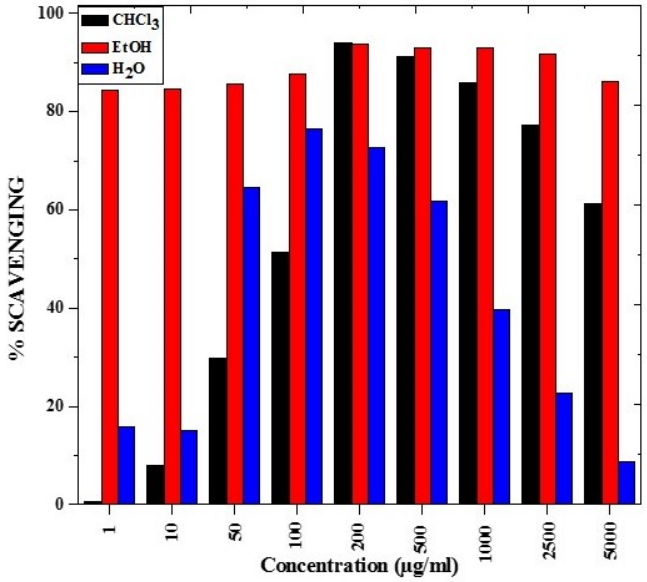


Fig.1. Different extract of *Oroxylum indicum* on DPPH radicals scavenging activity (1-5000µg/ml). Values were expressed as Mean± SEM, n=5.

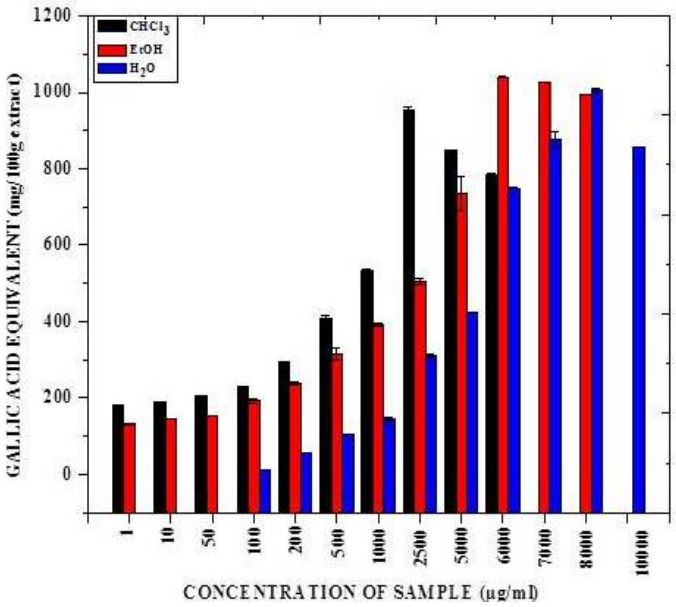


Fig.2. Hydroxyl radicals scavenging activity of different extracts of *Oroxylum indicum* expressed as gallic acid equivalent (1-10000µg/ml). Values were expressed as Mean± SEM, n=5.

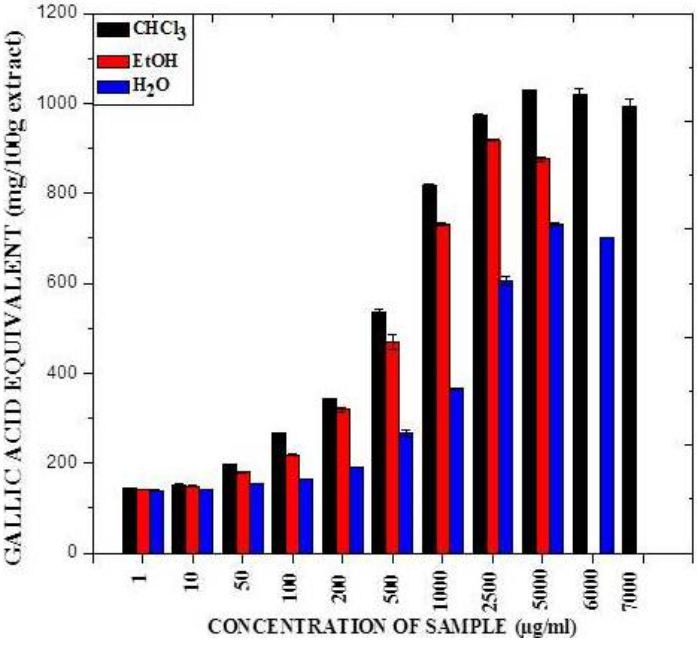


Fig.3. Gallic acid equivalent SO radicals scavenging activity of different extracts of *Oroxylum indicum* (1-7000µg/ml). Values were expressed as Mean± SEM, n=5.

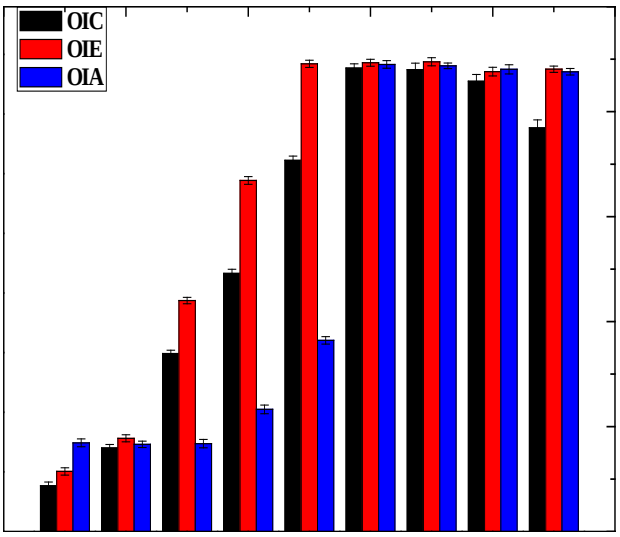


Fig.4. ABTS radicals scavenging activity of different extracts of *Oroxylum indicum* expressed as trolox equivalent (1-5000µg/ml). Values were expressed as Mean± SEM, n=5.

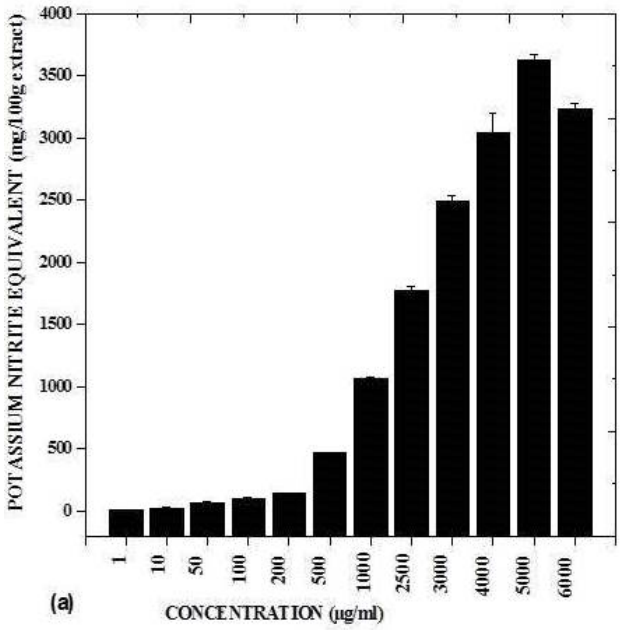


Fig.5a. Nitric oxide radicals scavenging activity of chloroform extract of *Oroxylum indicum* expressed as gallic acid equivalent (1-5000µg/ml). Values were expressed as Mean± SEM, n=5.

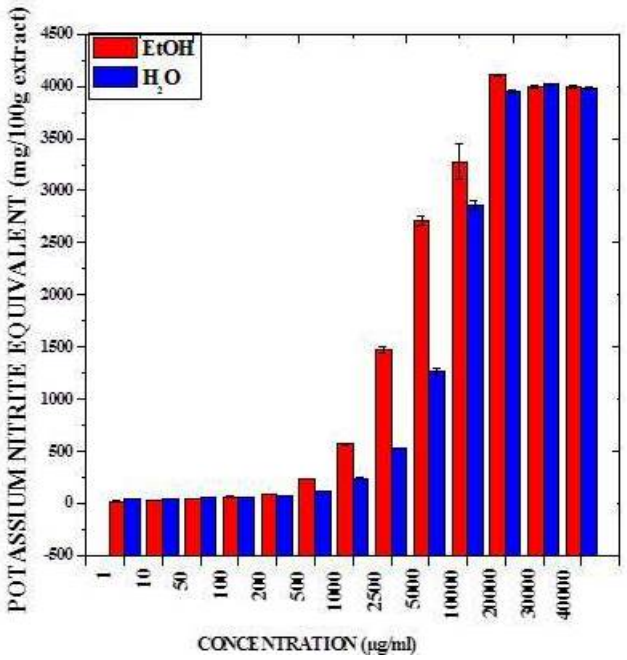


Fig.5b. Nitric oxide radicals scavenging activity of ethanol and aqueous extracts of *Oroxylum indicum* expressed as gallic acid equivalent (1-40000µg/ml). Values were expressed as Mean± SEM, n=5.

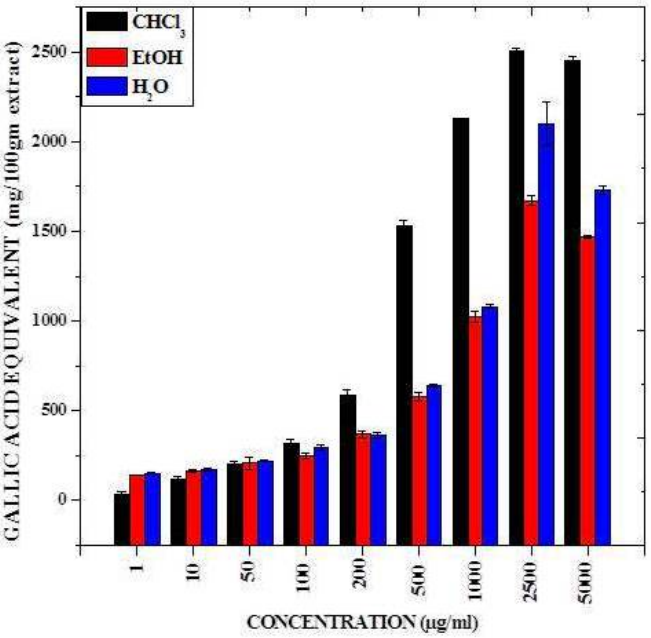


Fig.6. Reducing power of the different extracts of *Oroxylum indicum* expressed as gallic acid equivalent (1-5000µg/ml). Values were expressed as Mean± SEM, n=5.

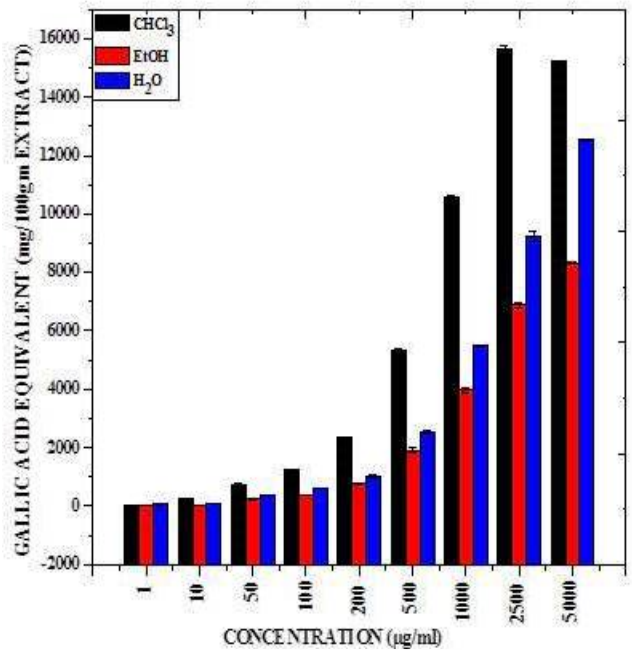


Fig.7. Total phenolic contents of the different extracts of *Oroxylum indicum* (1-5000µg/ml) determined as gallic acid equivalent. Values were expressed as Mean± SEM, n=5.

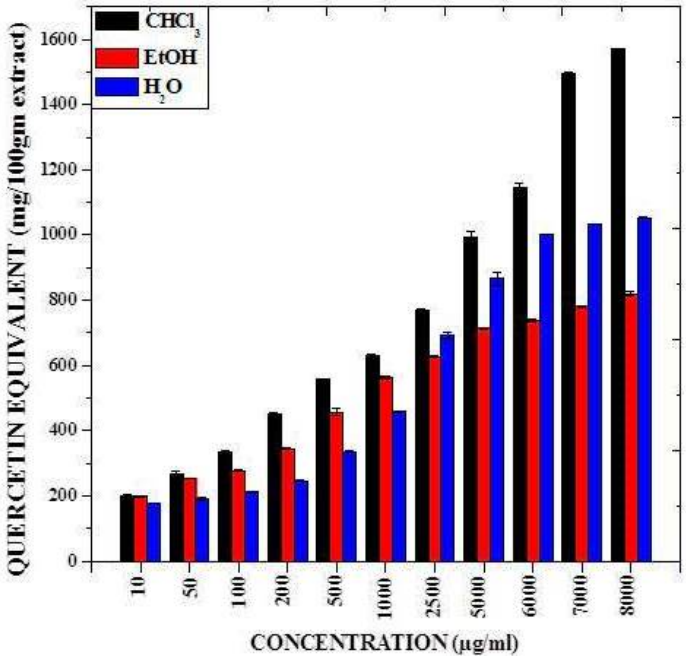


Fig.8. Total flavonoid contents of different extracts of *Oroxylum indicum* (1-8000µg/ml) determined as gallic acid equivalent. Values were expressed as Mean± SEM, n=5.

Free radical scavenging and antioxidant potential of different extracts of *Oroxylum indicum in vitro*

(Published in Advances in Biomedicine and Pharmacy, 2015; 2(3): 120-130)

Chapter 3

Abstract

Free radicals are necessary evil as they are produced during respiration and as a defense against the pathogenic attack. However, excess of free radicals generation produce oxidative stress, inflammation and various diseases and any agent that can reduce the oxidative stress will be useful in treating oxidative stress related disorders. Therefore, the aim of present study was to investigate the free radical scavenging activity of different extracts of Oroxylum indicum in vitro. The non-infected stem bark of O. indicum, an indigenous medicinal plant of Mizoram was collected, shade dried and sequentially extracted with petroleum ether, chloroform, alcohol and water using a Soxhlet apparatus. The ability of various O. indicum extracts to inhibit the generation of DPPH, superoxide anion, hydroxyl, nitric oxide as well as Fe^{3+} radicals was studied in cell free system using standard procedures. Simultaneously, the presence of total phenols and flavonoids were also estimated. The various extracts of O. indicum inhibited the generation of DPPH, ABTS superoxide anion, hydroxyl, nitric oxide and Fe^{3+} radicals in a concentration dependent manner and this ability depended on the type of extract, the concentration and the species of the free radical. The free radical scavenging activity increased up to a certain concentration and remained unaltered thereafter. However, the scavenging activity for all the free radicals was highest for the chloroform extract. The amount of total phenols and flavonoids also increased with increasing concentration and the maximum amount was recorded for the chloroform extract. The findings of the present investigation revealed that all extracts scavenged the free radicals in a concentration dependent manner and the chloroform extract was more potent than the ethanol and the aqueous extracts. This free radical scavenging ability of O. indicum may be due to the presence of flavonoids.

1. INTRODUCTION

The plants have been used by mankind since the advent of human history for healthcare. They are still used for the treatment of various ailments and several systems of medicine are especially devoted to the use of medicinal plants for healthcare. Apart from other systems of medicine, the Indian and Chinese systems of healthcare have systematically described the use of plants and other natural products for the treatment of various ailments. Despite innumerable advances made in the modern system of allopathic medicine the plant and natural products continue to play a major role in the human healthcare as 80 % of human population worldwide mainly depend on traditional medicines that are derived from plant and natural products for their primary healthcare (Cordell, 2011). Natural products and plants have formed the basis for the treatment of several diseases and many modern drugs have been isolated from plants before they were synthesized chemically (Cragg and Newman, 1830). Plants contain several phytochemicals that they synthesize for numerous purposes including pollination and defense (Larsonn, 1995). Further, it is believed that plant based products are less toxic, safer and even more reliable than the synthetic compounds used for healthcare (Benli et al., 2008). Plants synthesize complex polyphenols and other chemicals as secondary metabolites that are useful to humans as medication or antioxidants (Larson, 1988). Earlier history reflects the use of secondary compounds as curing agents for many diseases (Rao and Kingston, 1982). The most common natural antioxidants are flavonoids and phenolic acids and the medicinal properties of plants are generally due to the presence of these secondary metabolites, which occupy the prime place as they exhibit multiple biological activities (Middleton et a., 2000; Nichols and Katiyar, 2010; Carvalho et al., 2010). Different plant parts such as seeds, leaves and stem bark and root contain substantial

amounts of phytoconstituents including phenolics, flavonoids, tannins which have the ability to inhibit the free radicals and hence can act as antioxidants (Samatha et al., 2012). The secondary metabolites of plants have gained importance as natural antioxidants in recent years (Aliyu et al., 2010).

O. indicum is a small to medium sized deciduous tree with light grayish brown, soft, spongy bark and large, flat, sword shaped capsules full of many flat and papery thin seeds with broad silvery wings (Tiwari et al., 2007). It is found in ravine and moist places of forests of India, Sri Lanka, Philippines and Indonesia (Anonymous, 1972; Bennet at al., 1992). In India, it is distributed throughout the Eastern, Western Ghats and North East region up to an altitude of 1200 m and has been categorized as vulnerable medicinal plant by the government of India (Ravikumar and Ved, 2000). All parts of *O. indicum* possess medicinal properties (Ahmad and Ghafoor, 2002). It is an important herb in Ayurvedic medicine and indigenous medical system and has been used for human healthcare for thousands of years (Joshi et al., 1977) It is recognized as a multipurpose medicinal tree by Ayurveda and it is used in many formulations such as Shyonakapatpak, Bruhatpanchanulayadikwath, Dashmula and Chyawanprash (Vaidya, 1975). The stem bark and leaves of *O. indicum* is reported to contain flavonoids namely, chrysin, oroxylin-A, scutellarin, baicalein (Sankara and Nair, 1972a; 1972b). It is used as an astringent, carminative, diuretic, stomachic, aphrodisiac and has high potential for stimulating digestion, curing fevers, coughs and preventing other respiratory disorders (Gokhale and Bansal, 2006; Zaveria and Jain, 2010). Therefore, considering the importance of natural product-based antioxidants in treating several human ailments, the present study was undertaken to evaluate the antioxidant property of *O. indicum in vitro*.

2. MATERIALS AND METHODS

2.1. Preparation of the extract

The *Oroxylum indicum* was identified and authenticated by the department of Horticulture and Aromatic Medicinal Plants, Mizoram University, Aizawl. The non-infected stem bark of *O. indicum* was collected from Champhai, Mizoram during the dry season, in the month of January. The bark was washed and allowed to shade dry at room temperature in the dark in clean and hygienic conditions. The dried bark was powered using an electrical grinder at room temperature.

The powdered bark of *O. indicum* was sequentially extracted with petroleum ether, chloroform, ethanol and distilled water according to increasing polarity using a Soxhlet apparatus at their respective boiling points until they became colourless. The liquid extracts, were filtered and concentrated by evaporating their liquid contents using rotary evaporator. Each extracts, except petroleum ether was concentrated *in vacuo* and stored at -70°C until further use.

2.2. Experimental protocol

The antioxidant activities of the different extracts of *O. indicum* were determined using standard protocols described below.

2.3. DPPH radical scavenging activity

The test was carried out according to Leong and Shui (2002) with modification. To different concentrations of various extracts of *O. indicum* (0.5 ml each), 1 ml of methanol solution of 0.1 mM DPPH was added. After thorough mixing, the mixture was allowed to stand in the dark for 30 min and the absorbance was measured at 523 nm using UV-VIS double beam spectrophotometer (Model 2201, Systronics, Ahmedabad, India). Methanol was utilized for the baseline correction. The results have been compared with that of the control prepared as above without sample.

Radical scavenging activity has been expressed as a percentage and calculated using the following formula:-

% Scavenging= ($A_{control}$-A_{sample})/ $A_{control}$ X 100.

Where A_{sample} is the absorbance of the test sample and $A_{control}$ is the absorbance of the control.

2.4. Hydroxyl radical scavenging activity

Scavenging of the hydroxyl ($^{\bullet}OH$) free radical was determined by the earlier described method (Halliwell, 1987). Briefly, the reaction mixture contained deoxyribose (2.8 mM), KH_2PO_4-NaOH buffer, pH 7.4 (0.05 M), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM) and different concentrations of OIE in a final volume of 2 ml. The reaction mixture was incubated for 30 min at ambient temperature followed by the addition of 2 ml of trichloroacetic acid (2.8% w/v) and thiobarbituric acid. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was read at 532 nm in a UV-VIS double beam spectrophotometer. The results have been expressed as gallic acid equivalent which was used as a standard.

2.5. Superoxide anion scavenging activity

Superoxide scavenging activity was estimated by the method as described earlier (Hyland et al., 1983). To the reaction mixture containing 0.2 ml of NBT (1 mg/ml of solution in DMSO), 0.6 ml extract, 2 ml of alkaline DMSO (1 ml DMSO containing 5 mM NAOH in 0.1 ml H_2O) was added to give a final volume of 2.8 ml. The absorbance was recorded at 560 nm using a UV-VIS double beam spectrophotometer. The blank consisted of pure DMSO instead of alkaline DMSO. Gallic acid was used as the standard and the results have been expressed as gallic acid equivalent.

2.6. ABTS scavenging activity

ABTS scavenging activity of different extracts of *O. indicum* was carried out as described earlier (Re et al., 1999). Briefly, 37.5 mg of potassium persulphate was dissolved in 1 ml of distilled water. 44 µl of this solution was added to 9.7 mg of ABTS dissolved in 2.5 ml of distilled water to prepare ABTS solution. The ABTS solution was allowed to stand in the dark at room temperature for 12-16 hours. The working solution was prepared by mixing 1 ml of ABTS solution with 88 ml of 50% ethanol. 25 µl of different concentrations (1- 5000 µg/ml) of the different extracts (Chloroform, ethanol and aqueous) of *Oroxylum indicum* was mixed with 250 µl of the working ABTS solution and allowed to react for 4 minutes. The absorbance was then measured at 734 nm in a UV-VIS Softmax spectrophotometer. Trolox was used as the standard antioxidant and the activity was expressed as trolox equivalent. The percentage scavenging activity was calculated as follows:

% Scavenging = Control O.D – Sample O.D/ Control O.D x 100.

2.7. Nitric oxide scavenging activity

The nitric oxide scavenging activity was estimated by spectrophotometric method (Marcocci et al., 1994). Briefly, sodium nitroprusside (5 mM) in phosphate buffer saline was mixed with different concentrations of the various extracts of *Oroxylum* indicum and incubated at 25ºC for 150 min. The samples were mixed with Greiss reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% napthylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with napthylenediamine was read at 546 nm using a UV-VIS double beam spectrophotometer. The inhibition of nitric oxide formation was determined with respect to standard potassium nitrite treated in

the same way with Greiss reagent. The results have been expressed as potassium nitrite equivalent which has been used as a standard.

2.8. Reducing power

The reducing power of the extracts was determined as described earlier (Oyaizu, 1986). Various extracts of *O. indicum* (1 µg/ml to 5 mg/ml) in distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50ºC for 20 min and subsequently 2.5 ml of trichloroacetic acid (10%) was added to this mixture It was centrifuged at 3000 rpm for 10 min and the supernant (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 1%). The absorbance was measured at 700 nm with a double beam UV-VIS double beam spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power.

2.9. Total phenolic contents

The total phenolic contents were estimated by Folin-Ciocalteau reagent (McDonald et al., 2001). A diluted extract of *O. indicum* (0.5 ml) or gallic acid (standard phenolic compound) was mixed with Folin-Ciocalteau reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1M). The mixture was allowed to stand for 15 minutes and the total phenolic contents were measured at 756 nm with a UV-VIS double beam spectrophotometer. The total phenol contents are expressed in terms of gallic acid equivalent (mg/100 g of extracts), which is a common reference compound.

2.10. Total flavonoid contents

The total flavonoid contents were estimated using Aluminum chloride method as described earlier (Chang et al., 2002). Different concentrations of *O. indicum* extract was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1

ml of 1M potassium acetate and 2.8 ml distilled water and incubated at room temperature for 30 min. The absorbance was recorded at 415 nm with a double beam spectrophotometer. Quercetin was used as the standard and the results have been expressed as quercetin equivalent.

3. RESULTS

3.1. DPPH radical scavenging activity

Various extracts of *Oroxylum indicum* showed a concentration dependent increase in the scavenging of DPPH radicals as indicated by the discolouration of DPPH which is purple in colour. Maximum scavenging was observed at a concentration of 200 µg/ml for ethanol and chloroform extracts that plateaued thereafter (Figure 1), whereas the aqueous extract showed the highest DPPH scavenging activity at 100 µg/ml that declined thereafter and reached a nadir at 5000 µg/ml (Figure 1).

3.2. Hydroxyl Radical Scavenging activity

Different extracts of *O. indicum* inhibited the generation of hydroxyl radical in a concentration dependent manner and a maximum inhibition in OH generation was observed at 2500 µg/ml for chloroform extract, whereas this concentration was 5000 µg/ml for ethanol extract, which almost remained unaltered even at 6000 µg/ml (Table 1). Similarly, aqueous extract of *O. indicum* showed a concentration dependent scavenging of OH radical up to 5000 µg/ml the maximum concentration studied (Table 1). When the efficiency of scavenging was determined with respect to gallic acid equivalent the maximum scavenging concentration was 2500 µg/ml for the chloroform extract, whereas this concentration was 6000 µg/ml and 8000 µg/ml for ethanol and aqueous extract, respectively (Figure 2).

3.3. Superoxide anion scavenging activity

The chloroform, ethanol and aqueous extracts of *O. indicum* showed a concentration dependent inhibition of superoxide radical generation up to a concentration of 2500 µg/ml for ethanol extract and 5000 µg/ml for chloroform and aqueous extracts, the highest concentration of these extracts evaluated (Table 1). The gallic acid equivalent inhibitory activity for superoxide radical for different extract was 2500 µg/ml for ethanol extract whereas it was 5000 µg/ml for chloroform and aqueous extracts, respectively (Figure 3).

3.4. ABTS scavenging activity

The different extracts of *Oroxylum indicum* showed a concentration dependent rise in the scavenging activity of the ABTS free radicals up to 200 µg/ml for ethanol extract and remained almost same with increasing concentration up to 5000 µg/ml, whereas the chloroform and aqueous extracts showed maximum ABTS inhibitory action at 500 µg/ml and marginal decline thereafter for the chloroform extract and remained almost unaltered for the aqueous extract (Table 1). The ethanol extract was considered to have highest activity since it showed high activity at low concentration as compared to the other extracts (Figure 4). The estimation of scavenging activity as trolox equivalent also showed almost similar results, where the scavenging activity of *Oroxylum indicum* extracts was almost comparable to trolox (Figure 4).

3.5. Nitric oxide scavenging activity

Various extracts of *O. indicum* showed a concentration dependent increase in the scavenging activity of nitric oxide radicals and a highest scavenging of NO was observed for 5000 µg/ml for chloroform, ethanol and aqueous extracts, respectively (Table 1). The determination of potassium nitrite equivalent NO activity showed that the maximum scavenging activity was observed at 5000 µg/ml for chloroform extract

that declined thereafter, this concentration was 20000 µg/ml for ethanol and 30000 µg/ml for aqueous extracts (Figure 5a & b).

3.6. Reducing power

The reducing power of *O. indicum* extracts increased in a concentration dependent manner and the maximum reduction was reported for 1000 µg/ml for chloroform and aqueous extracts, whereas it was 2500 µg/ml for ethanol extract (Table 1). The estimation of reducing power of different extracts of *O. indicum* equivalent to gallic acid showed a concentration dependent increase in reducing power and a maximum activity was observed for 2500 µg/ml for chloroform, ethanol and aqueous extract, respectively (Figure 6*).*

3.7. Total phenolic contents

The presence of phenolic compounds in the extract was estimated as total phenol contents that increased in a concentration dependent manner up to 5000 µg/ml for chloroform, ethanol and aqueous extracts, respectively equivalent to gallic acid contents (Figure 7).

3.8. Total flavonoid contents

The total flavonoid contents in chloroform, ethanol and aqueous extract of *O. indicum* increased in a concentration dependent manner up to 8000 µg/ml equivalent to quercetin (Figure 8).

4. DISCUSSION

Oxygen is one of the essential elements for aerobic life and utilization of oxygen by organism comes has been associated with a cost in the form of generation of toxic free radical (Harman, 1956). The use of oxygen for energy production during respiratory pathways generates oxygen derived free radicals, especially the superoxide ($O2^{\bullet-}$) and hydroxyl ($^{\bullet}OH$), which in turn generate oxidative stress if not handled

carefully by the cells (Cadenasa and Davies, 2000). Similarly, neutrophils also use free radicals to combat infection. The nitric oxide (NO) is another free radical which finds its use in defense and nerve conduction. This indicates that generation of free radicals is a necessary evil. The controlled production of free radicals keeps the individual healthy. However, excess production of free radicals is harbinger of several diseases including autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular disorders, neurodegenerative diseases and cancer (Valko et al., 2007). This indicates that it is essential to screen newer paradigms that can neutralize the excess free radical production and protect humans from the deleterious effect of free radicals or free radical-induced oxidative stress. Plants produce numerous biomolecules to protect from various harmful organisms such as insects, pests, and fungi and other environmental stresses. These biomolecules may be of great value for human health as they will be biocompatible due to their biologic origin and would not have adverse effect at their maximum tolerated doses (Jagetia et al., 2003a; 2003b; Jagetia, 2007). Therefore the present study was under taken to evaluate the free radical scavenging activity of various extracts of *Oroxylum indicum in vitro.*

Estimation of DPPH scavenging is a convenient method to study the antioxidant activity of any pharmacological agent. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants. DPPH possesses a free electron and is violet coloured and it will change its colour to yellow once it accepts an electron from any antioxidant and will be converted into DPPH-H (Goldschmidt and Renn, 1922). The various stem bark extracts of *Oroxylum* inhibited the generation of DPPH free radical. The methanol extract of different parts of *Oroxylum* has been reported to

scavenge DPPH free radicals *in vitro* (Mishra et al., 2010; Moirangthem et al., 2013). Other plant extracts have been reported to scavenge DPPH radical earlier (Narayanaswamy and Balakrishnan, 2001; Jagetia et al., 2003a; Baliga et al., 2003; Wong et al., 2006; Jagetia et al., 2012; Aparadh et al., 2012). Similarly, flavonoids like naringin, and mangiferin have also been reported to scavenge DPPH free radicals in a concentration dependent manner (Jagetia et al., 2003a; Jagetia and Venkatesha, 2005). The free radicals scavenging activity was maximum at 100 µg/ml for aqueous extract, whereas chloroform and ethanol extract required twice the dose of aqueous extract for similar activity. The DPPH scavenging activity may be due to the presence of flavonoids and other poly phenols in the extracts.

The hydroxyl free radical is highly reactive and it reacts in the close vicinity of its formation (Pastor et al., 2000). It is produced during metabolic processes like respiration and it may be converted into H_2O_2, a highly toxic and strong oxidizing agent. Despite the fact that H_2O_2 is less reactive, it becomes dangerous in the presence of metal as it is capable of generating hydroxyl radical by Haber Weiss and/or Fenton reaction (Halliwell, 2006; Valko et al., 2007). The hydroxyl radical has higher propensity to react and it is capable of inducing detrimental effect on the important macromolecules including proteins and nucleic acids. It reacts with DNA leading to base and sugar damages (Tsunoda, 2010). Thus, neutralization of hydroxyl radical is crucial to protect cells from its deleterious effects. The various extracts of *Oroxylum* have scavenged the hydroxyl free radicals in a concentration dependent manner. *Oroxylum* has been reported to scavenge hydroxyl radical earlier (Mishra et al., 2010) and it may be a useful agent to neutralize this radical *in vivo*. Many other plant extracts and flavonoids have been found to scavenge hydroxyl free radicals in a

concentration dependent manner earlier (Jagetia et al., 2003a; 2003b; Jagetia and Venkatesha, 2005; Jagetia et al., 2012).

The superoxide anion free radical is an intermediate during cellular respiration it is produced as a result of incomplete metabolism of oxygen (Kirkinezosa and Morae, 2001). The superoxide anion produces H_2O_2, which in turn generates hydroxyl free radicals in the presence of metals (Turrens, 2003). Thus, neutralization of superoxide radical will inhibit the chain of ROS generation and protect the cells from the oxidative stress. Various extracts of *Oroxylum* have been found to inhibit the production of superoxide radical in a concentration dependent manner. Other plant extracts and some flavonoids have been reported to scavenge the superoxide anion free radical earlier (Jagetia et al., 2003a; 2003b; Jagetia and Venkatesha, 2005; Jagetia et al., 2012).

The $ABTS^{\bullet+}$ chromophore was produced through the reaction between ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and potassium persulfate indicated by blue/green colouration. The addition of antioxidants to the pre-formed radical cation reduces ABTS, indicated by the discoloration of the blue/green colour. The extent of decolorization was expressed as the percentage inhibition of the $ABTS^{\bullet+}$ (Re et al., 1999). This trapping of ABTS derived radical cation ($ABTS^{\bullet+}$) by free radical scavengers is a commonly employed method to evaluate the total charge of antioxidants present in complex mixtures (Aliaga and Lissi, 1998).

The nitric oxide radical ($NO^{\bullet}$) is a labile molecule and it is generated in mammalian cells as byproduct of respiration. It is also used by neutrophils to eliminate invading bacteria (Valko et al., 2007). $NO^{\bullet}$ also plays an important role in signal transduction and nerve conduction. However, excess production of $NO^{\bullet}$ is toxic, especially after reaction with oxygen or superoxide anion radicals. The reaction

products of NO• including NOx and ONOO- (peroxynitrite) are able to inflict severe cellular damage (Beckman et al., 1990; Radi et al., 1991; Lipton et al., 1993). Different extracts of *Oroxylum* reduced the generation of NO• in a concentration dependent manner. Several plant extracts and plant formulations (Jagetia et al., 2003b; 2012) have been reported to scavenge NO• in a concentration dependent manner. Similarly, some of the plant flavonoids including naringin and mangiferin have been reported to scavenge nitric oxide free radical in a concentration dependent manner earlier (Jagetia et al., 2003a; 2012; Jagetia nad Venkatesha, 2005).

Reducing power is a simple technique to evaluate the antioxidant property of any agent. Various extracts of *Oroxylum* exhibited a concentration dependent elevation in the Ferric ion reducing power. Several plant extracts have been reported to exhibit antioxidant activity by increasing Ferric ion reducing power *in vitro* (Wong et al., 2006; Aparadh et al., 2012). The phytochemical analysis of *Oroxylum* has shown the presence of phenols and flavonoids in the stem bark and their concentrations increased with the elevation in the amount of extracts. The presence of various polyphenols and flavonoid may be responsible for the antioxidant activity of *Oroxylum.*

The exact mechanism of free radical scavenging by *Oroxylum* is not known. However, the free radical scavenging and antioxidant activity of *Oroxylum* may be due to the presence of various phytochemicals like polyphenols and flavonoids, which may be able to donate or accept electron thus neutralizing their oxidative effects. Plants produce phenolic compounds and flavonoids in particular as secondary metabolites that help plants in pollination, to ward off against fungal attacks and also give attractive colours to flowers (Middleton and Chithan, 1993; Harborne and Baxter, 1999; Harborne and Williams, 2000). These flavonoids have been reported to

exert a conducive effect on human health owing to their free radical scavenging ability and antioxidant nature.

5. CONCLUSIONS

The present study showed that all the extracts of *Oroxylum* showed a concentration dependent inhibition of free radicals, and ferric reducing power. These activities of *Oroxylum* may be due to the presence of various phenolic compounds and flavonoids. The chloroform extract showed maximum antioxidant activity followed by the ethanol extract, whereas the aqueous extract showed the least activity which is in accordance with their flavonoid as well as phenolic contents.

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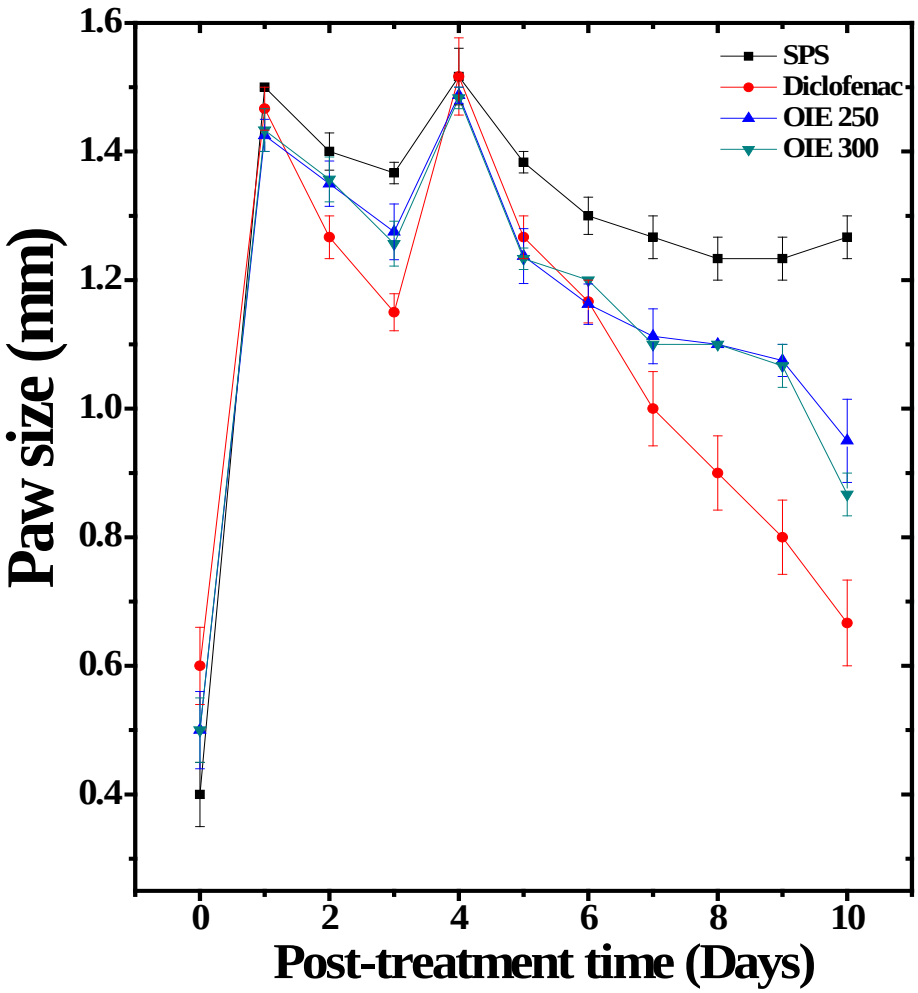

Figure 1: Effect of ethanol extract of *Oroxylum indicum* on the formalin induced inflammation in mice paw.

Investigation of the antiinflammatory and analgesic activities of ethanol extract of stem bark of *Oroxylum indicum*, Sonapatha *in vivo*

(Published in International Journal of Inflammation, 2016)

Chapter4

Abstract

Inflammation is all a pervasive phenomenon, which is elicited by the body in response to obnoxious stimuli as a protective measure. However, sustained inflammation leads to several diseases including cancer. Therefore it is necessary to neutralize inflammation. Sonapatha (Oroxylum indicum), a medicinal plant is traditionally used as a medicine in Ayurveda and other folk systems of medicine. It is commonly used to treat inflammatory diseases including rheumatoid arthritis and asthma. Despite this fact its anti-inflammatory and analgesic effects are not evaluated scientifically. Therefore, the anti-inflammatory and analgesic activities of sonapatha (Oroxylum indicum) were studied in Swiss albino mice by different methods. The hot plate, acetic acid and tail immersion tests were used to evaluate the analgesic activity whereas, xylene-induced ear edema and formalin-induced paw edema tests were used to study the anti-inflammatory activity of sonapatha. The administration of mice with 250 and 300 mg/kg b. wt. of O. indicum reduced pain and inflammation indicating that sonapatha possesses analgesic and anti-inflammatory activities. The maximum analgesic and anti-inflammatory activities were observed in mice receiving 300 mg/kg b. wt. of O. indicum ethanol extract. Our study indicates that O. indicum possesses both anti-inflammatory and analgesic activities and it may be useful as an anti-inflammatory agent in the inflammation related disorders.

Chapter 4

1. INTRODUCTION

The inflammation is a sequence of events that occurs in response to noxious stimuli, infection, trauma or injury in the living tissues (Calixto et al., 2004). The inflammation is initiated by a cascade of events including enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair processes (Risso et al., 2010). The inflammation releases white blood cells as a protective measure against injury. These white blood cells synthesize several biomolecules and release them after injury leading to swelling and redness. The inflammation is characterized by induction of pain, redness and rashes (Ghislaino, 1998). Prostaglandins are one of the important biomolecules, which play a key role in the induction of inflammatory response as their biosynthesis is significantly increased during inflammation (Ricciotti et al., 2011). The inflammatory responses are elicited as a defense mechanism by an organism or tissues; however, sustained inflammation can lead to undesired health effect as consequence of interplay of various biomolecules that are secreted during the process of inflammation. Inflammation has been indicated in several diseases including cancer (Sergei et al., 2010; Moore et al., 2010). The agents that contain or block inflammation may play an important role in treating pathologies associated with inflammatory reactions (Sosa, 2002).

Natural products have a long history of use as a folk remedy for inflammatory conditions including fevers, pain, migraine and arthritis. Many of the diseases in the modern world are thought to be due to inflammation therefore, anti-inflammatory agents, anti-inflammatory food and food products are of great interest to contain or reduce inflammation-induced health disorders (Yuan et al., 2006). Fossil records indicate the use of natural products, especially, the plants as medicine since Middle Paleolithic (approximately 60,000 years) age (Fabricant and Farnsworth, 2001). The

modern allopathic drugs are single active chemical molecules and target one specific pathway, whereas herbal medicines contain pleiotropic molecules that work on an orchestral approach that are able to target many elements of the complex cellular pathway (Durmowicz, 1999). The pain and inflammatory conditions are usually managed by either steroidal (corticosteroids) or non-steroidal (aspirin) drugs, which induce toxic side effects at different levels including allergic reactions, occasional hearing loss and renal failure. These drugs also increase the risk of hemorrhage by negatively altering platelet function (Thomas, 2000). The medicinal plants have been a major source of a wide variety of biologically active compounds for many centuries and have been used extensively in crude form or as pure isolated compounds to treat various disease conditions including inflammation (Newman and Cragg, 2014).

The inflammatory conditions can be cured using plant or plant derived products effectively. *Cleodendron inerme* has been reported to exhibit anti-inflammatory activity *in vitro* (Sangeetha et al., 2011). *Hydrocotyle umbellate* and several other plants have been reported to possess anti-inflammatory activity in different study systems (Florentino et al., 2013; Kumar et al., 2013). Sonapatha or *Oroxylum indicum* belongs to Family Bignoniaceae and it is characterized by brown bark and large pinnate leaves. It is a medium sized, deciduous tree, distributed in India, Sri Lanka, Malaysia, China, Thailand, Philippines and Indonesia. In India, *Oroxylum* is found in Eastern and Western Ghats and also in the North-East regions (Bennet et al., 1992). The existence of *Oroxylum indicum* (L) Vent. in natural population is highly threatened and it has been categorized as endangered medicinal plant by the Govt. of India. Various parts of *Oroxylum indicum* are utilized for medicinal purposes (Ravi Kumar and Ved, 2000). It has been used in Ayurveda and other traditional medicinal health systems since centuries (Warrier et al., 1995). The decoction of the bark is used

to cure gastric ulcers and the bark paste is useful in treating mouth cancer, scabies and other skin diseases. The bark paste is applied to the wounds of animals to kill maggots. Poultice of the bark is topically applied to treat rheumatism, sprains, inflammations and skin diseases (Sawmliana, 2003). The bark decoction of *Oroxylum* is also a useful remedy to deworm cattles (National Innovation foundation-India). Apart from this, *Oroxylum* species are reported to have a variety of medicinal properties like anticancer, antiulcer, antidysenteric, antimicrobial and anti-inflammatory (Raghu et al., 2013). It has been shown to be antibacterial, antioxidant, hepatoprotective, and immunomodulatory (Ahad et al., 2012). From the above it is clear that the systematic evaluation of anti-inflammatory and analgesic activities of *Oroxylum indicum* is lacking, which stimulated us to obtain an insight into the anti-inflammatory and analgesic activities of *Oroxylum indicum* in Swiss albino mice.

2. MATERIALS AND METHODS

2.1. Preparation of Extract

The non-infected and matured stem bark of *Oroxylum. indicum* (Family: Bignoniacae) was collected from Champhai (23.456°N latitude and 93.329°E longitude), Mizoram, India during the month of January. The plant was identified by the Department of Horticulture and Aromatic and Medicinal Plants, Mizoram University, Aizawl, India. The bark of *O. indicum* was thoroughly rinsed with clean water and shade dried at room temperature in the dark in clean and hygienic conditions. The dried bark was powdered in an electrical grinder at room temperature. The stem bark powder of *O. indicum* was sequentially extracted in petroleum ether, chloroform, ethanol and distilled water according to increase in polarity using a Soxhlet apparatus until the solvents became colourless (Suffnes and Douros, 1979). The ethanol extract was

concentrated using rotary evaporator and stored at -70°C until further use. Henceforth the ethanol extract will be referred to as OIE throughout the manuscript.

2.3. Animal care and handling

The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Usually, 6 to 8 weeks old healthy male Swiss albino mice weighing 30-35 g were selected from an inbred colony maintained under the controlled conditions of temperature (25±2ºC) and humidity (55–60%) with 12 hours of light and dark cycle, respectively. The animals were housed in a sterile polypropylene cage containing paddy husk (procured locally) as bedding material. The animals had free access to standard rodent diet and water. All animal experiments were carried out according to NIH and Indian National Science Academy, New Delhi, India guidelines. The study was approved by the Institutional Animal Ethics Committee of the Mizoram University, Aizawl, Mizoram: India vide letter No. MZU/IAEC/4503.

2.4. Experimental

The anti-inflammatory and analgesic activities were determined by dividing the animals into the following groups:

2.4.1. SPS group: The animals of this group did not receive any treatment except the sterile physiological saline (SPS).

2.4.2. DIF Group*:* The animals of this group were injected with 20 mg/kg b. wt. of diclofenac sodium intraperitoneally.

2.4.3. OIE group*:* The animals of this group were administered with 250 and 300 mg/kg b. wt. of ethanol extract of *Oroxylum indicum* intraperitoneally.

The analgesic and anti-inflammatory activities were determined 30 minutes after the administration of SPS or diclofenac (DIF) or ethanol extract of *Oroxylum indicum*.

2.5. Analgesic Activity

The analgesic activity of OIE was determined by carrying out the following tests:

2.5.1. Hot-plate test

A separate experiment was conducted to determine analgesic activity of OIE by the hot plate test as described by (Asongalem et al., 2004), where the grouping and other conditions were essentially similar to that described above. The hotplate contained metallic surface (diameter 20 and 10 cm high) and its temperature was set at 55ºC. Briefly, each mouse was placed onto the hotplate and covered with a glass beaker to avoid heat loss. Each mouse also acted as its own control. The time taken to lick the fore paws or jumping was recorded. The latency is defined as the reaction time taken by each mouse to respond to licking of the fore paws or jumping. Untreated animals exhibiting latency of 5–20 s were selected. The latency period for all groups was recorded thirty minutes after administration. Usually 10 mice were used for each group.

The percent inhibition was calculated as follows:

Post-treatment latency (s) - Pre-treatment latency (s) x 100/Pre-treatment latency (s).

2.5.2 Acetic Acid Induced Writhing Test

A separate experiment was performed to evaluate the analgesic activity by acetic acid-induced writhing test, which was carried out as described earlier (Hosseinzadeh et al., 2000). The grouping and other conditions were essentially similar to that described earlier. The mice were administered intraperitoneally with

0.7% v/v acetic acid (volume of acetic acid did not exceed 10 µl/ g b. wt.). Immediately after acetic acid administration, the mice were individually placed into glass beakers and five min were allowed to elapse. The number of writhes produced in these animals was counted up to 30 min. For scoring purposes, a writhe is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. Usually10 mice were used for each group.

Inhibition of writhing (%) was calculated as follows:

$$\frac{\text{Control-Treated}}{\text{Control}} \times 100.$$

2.5.3. Tail-immersion test

A separate experiment was conducted to evaluate the analgesic activity of sonapatha ethanol extract by tail immersion test according to the procedure described elsewhere (Aydin et al., 1998). The grouping and other conditions were essentially similar to that described above in the experimental section. The tail-immersion test was carried out in a hot water bath set at a temperature of 55 ± 0.5ºC, where 3 cm of animal tail was immersed into the hot water and tail withdrawal reaction was recorded as time in seconds in all groups using a digital stopwatch. A minimum of two observations were collected for each animal in control group, immediately and 10 min after the initial reading. The tail withdrawal test was carried out in the treatment groups periodically at 0, 0.5, 1, 2, 3, 4 and 6 hours after administration of OIE, or diclofenac. Usually10 mice were used for each group.

2.6. Antiinflammatory Activity

The anti-inflammatory activity was studied by xylene-induced ear edema and formalin-induced paw edema in mice.

2.6.1. Xylene-induced ear edema

A separate experiment was carried out to evaluate the anti-inflammatory activity by xylene-induced ear edema as described earlier (Guillen et al., 1997). The grouping and other conditions were essentially similar to that described above in the experimental section. Mice were divided into five groups of 10 animals each. The mice were intraperitoneally administered either with distilled water (10 µl/g b. w.) or diclofenac (20 mg/kg b.wt.) or OIE (250–300 mg/ kg b. wt.). Thirty minutes after administration the inner surface of right ear of each animal was applied with 0.03 ml of xylene for the induction of ear edema and the left ear served as the control. Fifteen minutes after the application of xylene, the mice were killed under ketamine anesthesia. Circular sections of both the ears were taken, using a cork borer of diameter of 6 mm, and weighed.

$$\text{Inhibition (\%)} = \frac{\text{Difference in ear weight (control) - Difference in ear weight (test)}}{\text{Difference in ear weight (control)}} \times 100.$$

2.6.2. Formalin induced inflammation

A separate experiment was conducted to evaluate the anti-inflammatory activity by formalin induced inflammation. The grouping and other conditions were essentially similar to that described above in experimental section. The anti-inflammatory activity was assessed as described earlier (Saxena et al., 1984). Swiss albino mice were divided into groups of ten. The inflammation was produced by subaponeurotic injection of 0.1 ml of 2% formaldehyde in the right hind paw of the mice on the first and third day. The animals were treated daily with the OIE and diclofenac intraperitoneally for 10 days. The daily changes in paw size were measured by wrapping a piece of cotton thread round the paw and measuring the circumference with a meter rule. Usually10 mice were used for each group.

3. Statistical analysis

The data were analyzed by one-way ANOVA, followed by application of Tukey test (Pro 8 SRO v8.0724 (B724), Northampton, MA, USA. A P value of < 0.05 was considered to statistically significant.

4. RESULTS

The results of analgesic and anti-inflammatory activities are presented in table 1-4 and figure 1.

4.1. Analgesic Activity

4.1.1. Hot-plate test

The analgesic activity was assessed using the hot plate method (Table 1). The administration of ethanol extract (OIE) showed a significant analgesic activity for both 250 and 300 mg/kg and 300 mg/kg exhibited the highest activity (62.5% inhibition) as compared to 250 mg/kg b. wt. (52.63% inhibition) as indicated by pain attenuation. The positive control diclofenac showed higher analgesic activity than OIE (76.31% inhibition) at a dose of 20 mg/kg b. wt. (Table 1).

4.1.2. Acetic acid induce writhing test

The results of acid writhing test are depicted in Table 2. Administration of acetic acid to control mice produced 66.2±1.16 writhes within 30 minute observation period. Pretreatment with the OIE at 250 and 300 mg/kg b. wt. reduced the number of writhes up to 22.5±1.12 (66.0% inhibition) and 20.8±0.74 (68.58 % inhibition), respectively. The standard drug diclofenac reduced the number of writhes to 10.8±0.74 (83.68 % inhibition) at a dose of 20 mg/kg b. wt. (Table 2)

4.1.3. Tail immersion test

Analgesic activity was also estimated using tail immersion. The tail immersion test indicated that both the doses of the extract as well as the positive controls showed

a significant inhibition in tail immersion rest when compared to the negative control (Table 3). The maximum analgesic effect was recorded for 300 mg/kg b. wt. OIE. The diclofenac (positive control) showed better activity as compared to the OIE.

4.2. Anti-inflammatory activity

4.2.1. Xylene induced ear edema.

The results of the anti-inflammatory study using xylene induced ear edema are shown in table 4. SPS treated control mice showed an increase in ear weight up to 13.98±0.60 mg and the OIE administration has inhibited this weight gain by 68.097 % (4.46±0.89 mg) and 71.3877 % (4.00±0.24 mg) for 250 and 300 mg/kg b. wt. of OIE, respectively (Table 4). The positive control diclofenac showed 52.72 % (6.61±0.49 mg) inhibition at 20 mg/kg b. wt. which was lower as compared to both the doses of OIE (Table 4).

4.2.2. Formalin induced inflammation

Treatment of mice with OIE gradually reduced diameters of the paw with time of both the treated and positive control groups (Figure 1). The OIE reduced the inflammatory reactions when compared to the SPS control group as indicated by the significant reduction in the paw diameter (Figure 1). However, the effect was more pronounced for 300 mg/kg OIE treatment (Figure 1).

5. DISCUSSION

Inflammation is well orchestrated response to deleterious stimuli including tissue injury, and infection (Lumeng and Saltiel, 2011). It is elicited to restore normal condition of tissue or body. Classically inflammation is characterized by increase in the blood flow, reddening of the affected part due to increase erythrocyte accumulation and edema (Punchard, 2004). Physiologically inflammation results in the secretion of numerous cytokines, acute phase proteins, and recruitment of

leucocytes to the site of injury (Lumeng and Saltiel, 2011). Inflammation has been indicated as a major cause in the development of several diseases in humans including neurological, cardiovascular, intestinal, dental and renal disorders. Inflammation is also linked to ageing, diabetes, obesity, ankylosing spondylitis, multiple sclerosis, pancreatitis and cancer (Kuek et al., 2006; Grivennikov et al.,2010; Lumeng and Saltiel, 2011; Jenny, 2012; Hoque et al., 2012: Marchant et al., 2012; Wyss-Coray and Rogers, 2012). The strategies to combat inflammation will be useful in reducing the inflammation related disorders. Therefore, the present study was undertaken to evaluate the analgesic and anti-inflammatory activities of *Oroxylum indicum* in mice.

The analgesic activity of *Oroxylum indicum* was studied by the hot plate, tail immersion and acetic acid tests, which are standard procedures to evaluate central and peripheral nervous system acting analgesics (Bartolini et al., 1987; Hiruma-Lima et al., 2000). The acetic acid is known to trigger the production of noxious substances within the peritoneum resulting in writhing response (Bartolini et al., 1987). It is a simple, rapid and reliable model and especially suitable to evaluate peripheral type of analgesic action of any drug (Shinde et al., 1999). The administration of *Oroxylum indicum* extract showed a significant analgesic activity indicating that it has some analgesic effect on both the central and peripheral nervous systems as indicated by reduced pain by hot plate method and suppression of acetic acid-induced writhing. Several plant extracts including *Adhatoda vasica*, *Acacia hydaspica*, *Boswellia serrate*, *Glaucium grandiflorum*, and *Landolphia owariensis* have shown analgesic activity *in vivo* (Owoyele et al., 2001; Morteza-Semnani et al., 2004; Sharma et al., 2010; Mulla et al., 2010; Afsar et al.,2015). The tail immersion test has been used as a standard procedure to study the analgesic activity of pharmacological agents (Luttinger, 1985; Dykstra et al., 1987), which was originally devised by (Janssen et

al., 1963). The withdrawal latency is unusually determined once or twice to limit the conditioning effect (Dykstra et al., 1987). The increase in the tail withdrawal latency is good a measure of analgesia induced by any chemical agent. Treatment of mice with *Oroxylum indicum* extract increased tail withdrawal latency confirming to its analgesic effects.

The anti-acute inflammatory activity of any agent can be determined by xylene-induced ear edema or formalin induced paw edema tests (Zanini et al., 1992; Wang et al., 2011). The formalin administration elicits behavioral effects stimulated by nociceptors. The inflammatory phase induced pain evokes a combination of stimuli, including inflammation of peripheral tissues and mechanisms of central sensitization (Shibata et al., 1989; Le Bars et al., 2001). The central nervous system acting drugs including opioids, suppresses both phases equally, however drugs that act on peripheral nervous system such as NSAIDs and corticosteroids, only inhibit the second phase (Shibata et al., 1989). Our findings indicate that *Oroxylum indicum* extract acts as anti-inflammatory agent as it reduced the xylene induced ear edema as well as formalin induced paw edema in treated mice. *Oroxylum indicum* extract is effective on both the central and peripheral nervous systems since it is able to desensitize neurons of both central and peripheral nervous systems equally as indicated by the attenuation of pain and inflammation. Many plants have been reported to possess anti-inflammatory activity in various study systems (Kumar et al., 2013; Bhagyasri et al., 2015). Similarly, *Adhatoda vasica, Acacia hydaspica, Boswellia serrate, Glaucium grandiflorum, and Landolphia owariensis, Harpagophytum procumbens, Rosa canina, Oenothera biennis, Ribes nigrum, Borago officinalis, Zingiber officinale, Nigella sativa* and *Folium eriobotryae* have been reported to act as anti-inflammatory agents in different study systems (Owoyele et al.,

2001; Morteza-Semnani et al., 2004; Chrubasik et al., 2007; Sharma et al., 2010; Mulla et al., 2010; Afsar et al.,2015; Zhang et al., 2015).

The exact mechanism of suppression of inflammation by *O. indicum* is not known. However, it contains flavonoids and other phenolic compounds that may have contributed to its analgesic and anti-inflammatory actions. *O. indicum* has been found to scavenge DPPH, superoxide anion, hydroxyl, nitric oxide and Fe^{3+} radicals, which are major players in eliciting the inflammatory response (Lalrinzuali et al., 2015). Apart from free radical scavenging, it may have also reduced activation of cytokines like NF-κB, TNFα, IL-1β, and IFNγ. Inflammation has been reported to stimulate the activation of these cytokines (Souza et al., 2008; Hoesel and Schmid, 2013). Biochanin-A present in the root bark of *O. indicum* has been reported to inhibit TNFα (Knight and Eden, 1996). Chrysin has been isolated form the ethanol extract of *O. indicum* (data not shown) has been reported to suppress the transcriptional activation of NF-κB and Cox-II (Yao et al., 2014). The observed anti-inflammatory action of *O. indicum* may also be due to its inhibitory action on cyclooxygenase which are involved in prostaglandin synthesis (Clària, 2003).

6. CONCLUSION

Our study demonstrates that the *O. indicum* acts as an analgesic and anti-inflammatory agent. The analgesic and anti-inflammatory activities of *O. indicum* may due to its ability to neutralize free radicals which are the main players in inflammation. It may have also suppressed the activation of proinflammatory cytokines including NF-κB, TNFα, IL-1β, and IFNγ and the activity of cyclooxygenase enzymes which are involved in inflammation. The anti-inflammatory and analgesic activities of *O. indicum* may be due to the presence of flavonoids and other polyphenols. The *O. indicum* may be used to reduce inflammation; however, further studies are required to understand molecular mechanisms of action against inflammation.

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Table 1: Effect of *Oroxylum indicum* on the analgesic activity in mice by hot plate test.

Treatment	Dose (mg/kg b.wt.)	Mean ±SEM		Increase in latency period (%)
		Pre-treatment reaction latency(s)	Post-treatment reaction latency(s)	
Control	0	7.60±0.58	7.60±0.45	0
OIE	300	8.00±0.32	13.20±1.07*	62.5
OIE	250	7.60±0.89	11.60±1.04*	52.63
Diclofenac	20	7.60±0.55	13.40±0.84*	76.31

N = 10.

**p< 0.05 when treatments are compared to SPS treated control*

Table 2: Alteration in the analgesic activity by acetic acid induced writhing in mice treated with different doses of *Oroxylum indicum*.

Treatment	Dose (mg/kg bwt)	Mean ±SEM No. of writhings	Percentage inhibition of writhing (%)
Control	0	66.2±1.16	0
OIE	300	20.8±0.74*	68.58
	250	22.5±1.12*	66.01
Diclofenac	20	10.8±0.74*	83.68

N = 10.

**p< 0.05 when treatments are compared to SPS treated control*

Table 3: Alteration in the response time in mice treated with *Oroxylum indicum* before subjecting them to tail immersion test.

Treatment	Dose (mg/kg b. wt.)	Response time in seconds ±SEM							
		Assessment time (h)							
		0	**0.5**	**1**	**2**	**3**	**4**	**5**	**6**
Control	0	4.30±.05	4.2±0.20	4.03±0.12	4.6±0.35	4.81±0.51	4.62±0.42	4.31±0.37	4.30±0.40
OIE	300	5.67±0.20	6.33±0.15* (11.64)	6.78±0.51* (19.57)	8.80±0.06* (55.20)	7.56±0.05* (33.33)	6.98±0.15* (23.104)	6.80±0.20* (19.92)	6.61±0.27* (16.57)
	250	4.69±0.50	4.72±0.08* (1.91)	4.97±0.30* (5.97)	4.78±0.46* (19.18)	7.14±0.54* (52.23)	5.65±0.65* (20.46)	4.90±0.15* (4.47)	4.80±0.10* (2.35)
Diclofenac	20	4.29±.08	4.43±0.20* (3.26)	5.03±0.11* (17.29)	6.76±0.5* (57.57)	7.31±0.57* (70.39)	6.87±0.48* (60.13)	6.39±0.47* (48.95)	4.50±0.70* (4.89)

Inhibition (%) is shown in brackets.

N = 10.

**p< 0.05 when treatments are compared to SPS treated control*

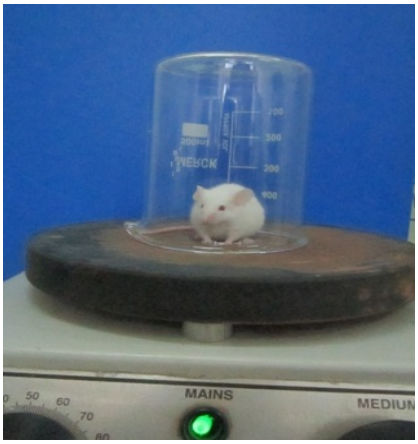
Table 4: Effect of ethanol extract of *Oroxylum indicum* on xylene induced ear edema in mice.

Treatment	Dose (mg/kg b. wt.)	Mean increase in ear weight (mg) ±SEM	Inhibition (%)
Control	0	13.98±0.60	-
OIE	300	4.00±0.24*	71.3877
	250	4.46±0.89*	68.097
Diclofenac	20	6.61±0.49*	52.7182

N = 10.

**p< 0.05 when treatments are compared to SPS treated control*

(a) Acetic acid induced writhing



(b) Hot Plate test

(c) Xylene induced ear edema

(d) Formalin induced inflammation

Plate I: Effect of *Oroxylum indicum* extract on analgesic and anti-inflammatory activity. Analgesic activity test (a & b); anti-inflammatory (c & d).

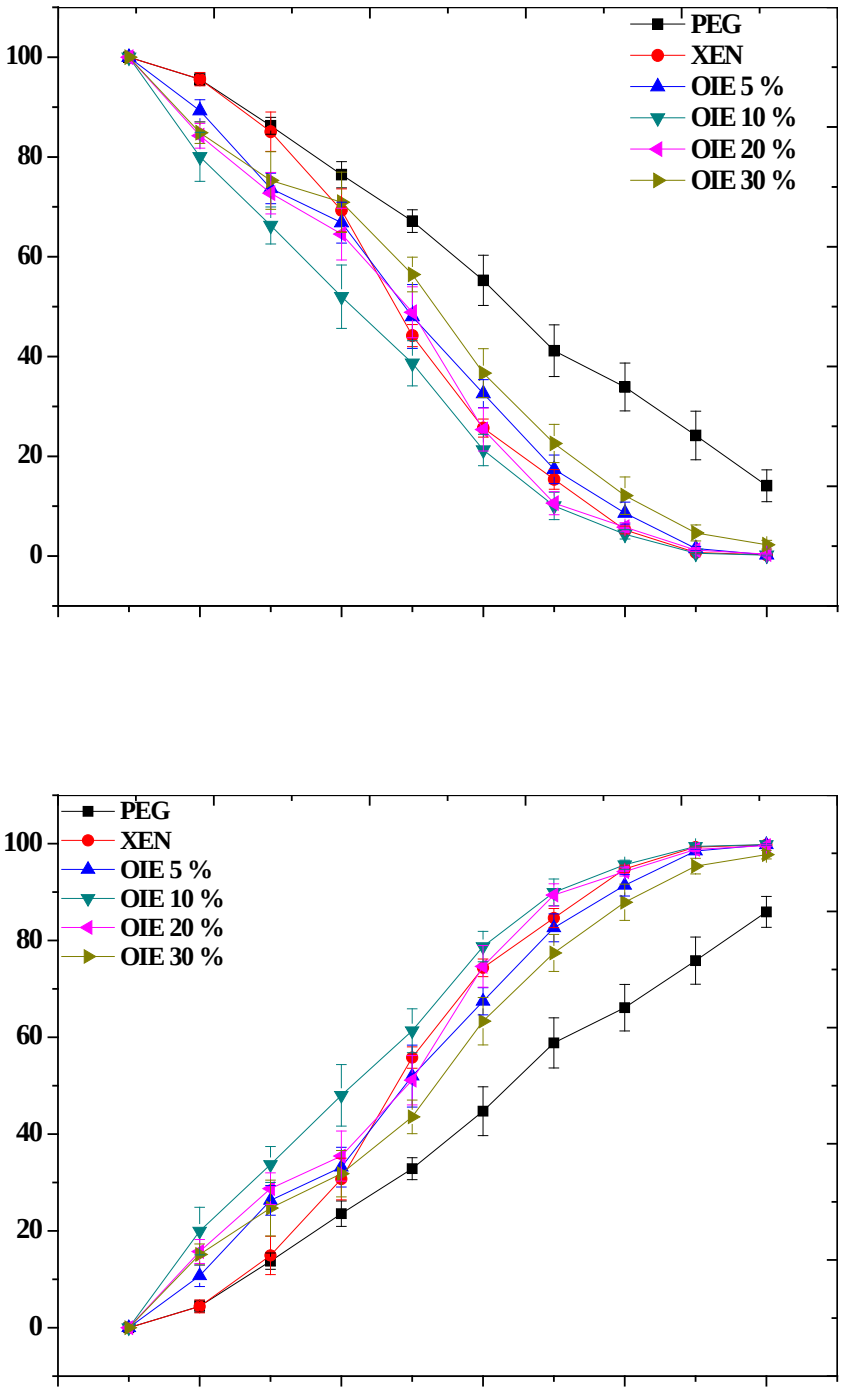


Figure 1: Effect of topical application of various concentrations of *Oroxylum indicum* extract on the contraction of excision wound in mice. The data are expressed as Mean± SEM, n=10, **P<0.05*.

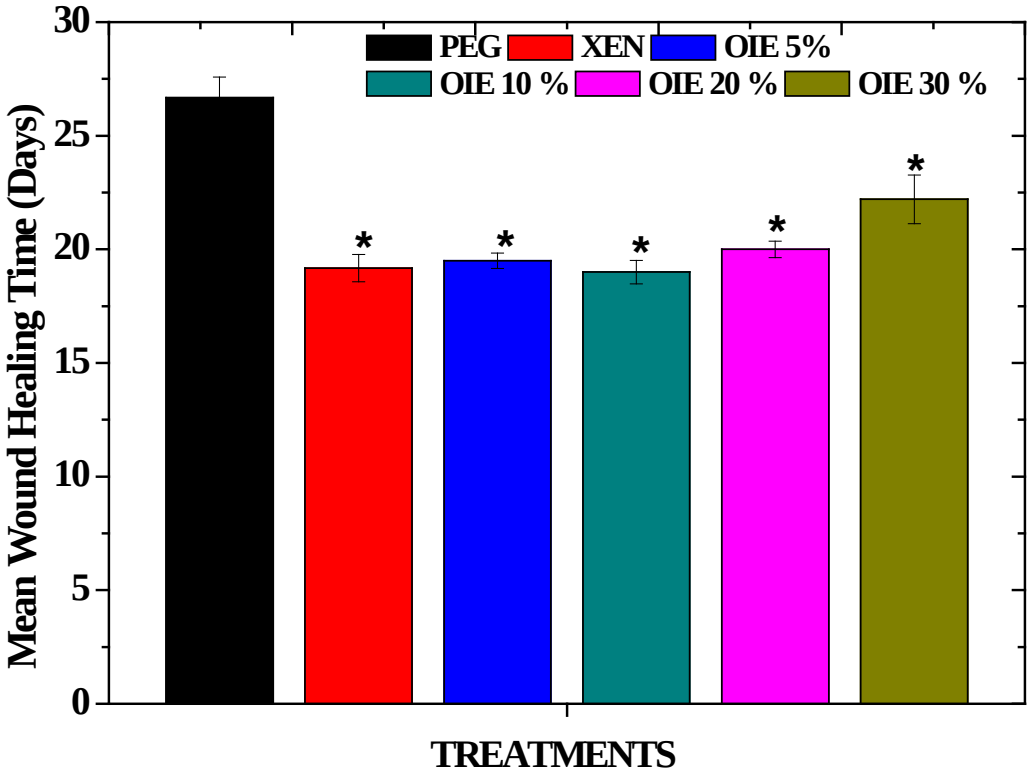


Figure 2: Effect of various concentrations of topical application of *Oroxylum indicum* extract on the determination of mean wound healing time of excision wound in mice. The data are expressed as Mean± SEM, n=10, **P<0.05.*

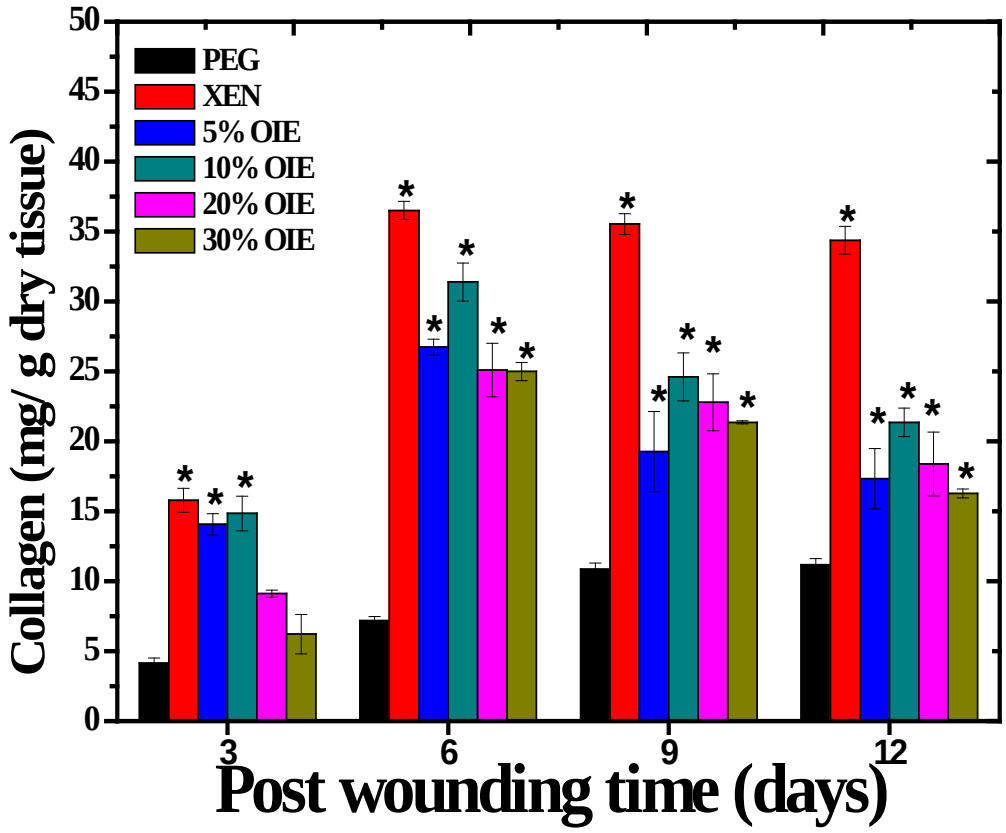


Figure 3: The effect of different concentration of *Oroxylum indicum* extract on the biosynthesis of collagen on the regenerating excision wounds of mice at different post wounding days. Values are expressed as Mean ± SEM. N=5. **P<0.01* when treatment groups are to compare polyethylene group.

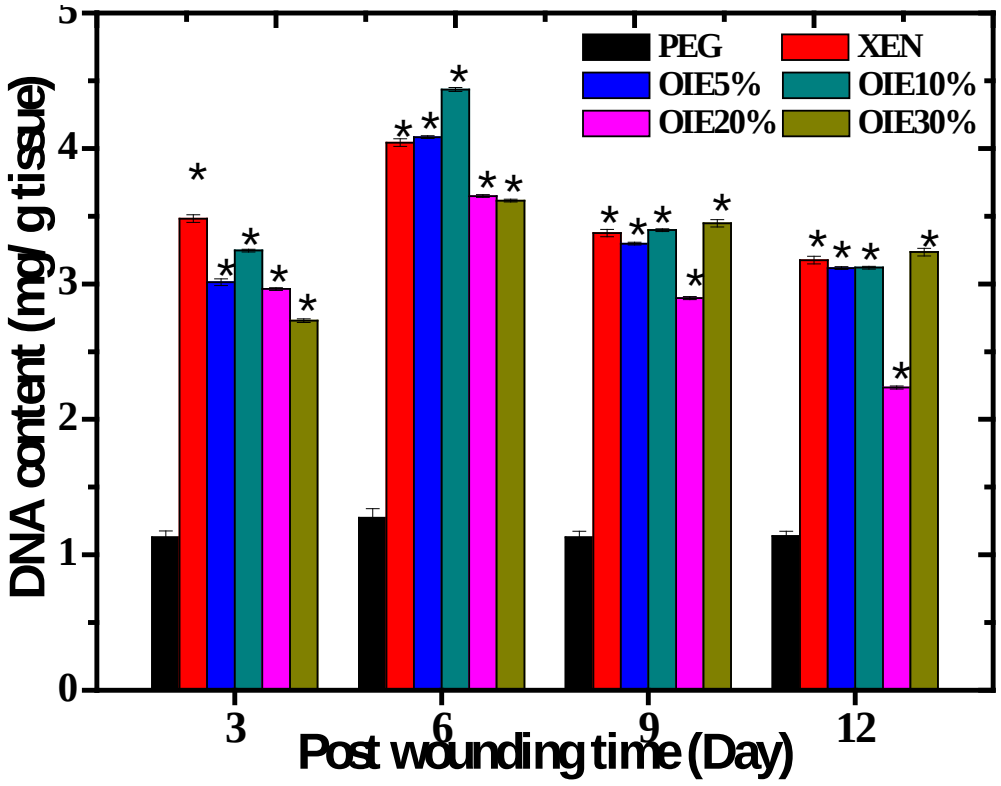


Figure 4: The effect of topical application of the different concentrations of *Oroxylum indicum* extract on biosynthesis of deoxyribose nucleic acid on excised of mice. Values are expressed as Mean ± SEM. N=5. *$P<0.01$ when treatment groups are to compare polyethylene group.

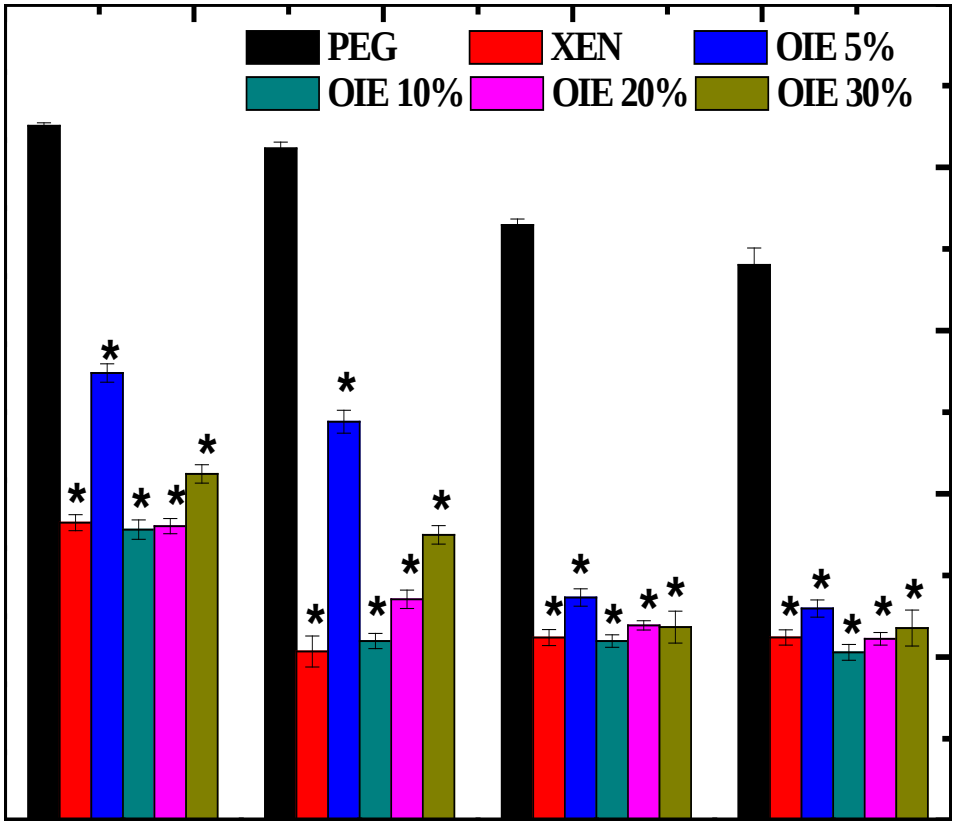


Figure 5: Effect of different concentration of *Oroxylum indicum* extract on lipid peroxidation level in the skin of mice excised wound. The results are shown as Mean ± SEM, N=5, *$P < 0.01$ when treatment groups are compared to polyethylene groups.

Table 1: Effect of *Oroxylum indicum* extract on acceleration of wound healing time in days. The results are expressed as Mean ± Standard error.

Treatment	Mean Wound Healing Days
PEG	26.67 ±0.91
XEN	19.17 ±0.60*
OIE 5%	19.5 ±0.34*
OIE 10%	19 ±0.51*
OIE 20%	20 ±0.36*
OIE 30%	22.2 ± 0.97*

*N=5. *p<0.01, when treatment groups are compared to polyethylene group.*

Table 2: The effect of different concentration of *Oroxylum indicum* extract on biosynthesis of collagen on excised of mice. The values are expressed as Mean ± SEM.

Treatment time (Days)	Collagen (mg/g tissue)					
	PEG	XEN	OIE 5%	OIE 10%	OIE 20%	OIE 30%
3	4.24 ± 0.57	14.97 ± 0.86*	14.07 ± 0.76*	14.84 ± 1.24*	9.11 ± 0.27	6.22 ± 1.40
6	7.27 ± 0.19	38.81 ± 0.65*	26.74 ± 0.56*	31.39 ± 1.36*	25.09 ± 1.91*	24.99 ± 0.64*
9	10.94 ± 0.56	36.74 ± 0.75*	19.27 ± 2.86*	24.61 ± 1.71*	22.79 ± 2.04*	21.36 ± 0.10*
12	11.07 ± 0.48	33.77 ± 0.98*	17.33 ± 2.17*	21.35 ± 1.01*	18.38 ± 2.27*	16.28 ± 0.33*

*N=5. *p<0.01 when treatment groups are compared to polyethylene group.*

Table 3: The effect of different concentration of *Oroxylum indicum* extract on biosynthesis of deoxyribose nucleic acid on excised of mice. The values are expressed as Mean ± SEM.

Treatment time (Days)	DNA (mg/g tissue)					
	PEG	XEN	OIE 5 %	OIE 10 %	OIE 20 %	OIE 30 %
3	1.14 ± 0.06	3.68 ± 0.03*	3.01 ± 0.03*	3.25 ± 0.01*	2.96 ± 0.01*	2.73 ± 0.01*
6	1.67 ± 0.057	4.03 ± 0.02*	4.08 ±0.01*	4.43 ± 0.02*	3.65 ± 0.01*	3.62 ± 0.01*
9	1.14 ± 0.08	3.39 ± 0.03*	3.29 ±0.01*	3.39 ± 0.01*	2.89 ± 0.01*	3.45 ± 0.03*
12	1.13± 0.07	3.19 ± 0.03*	3.12 ± 0.01*	3.12 ± 0.01*	2.23 ± 0.01*	3.23 ± 0.03*

*N=10. *p<0.01 when treatment groups are compared to polyethylene group.*

Table 4: Effect of different concentration of *Oroxylum indicum* extract on lipid peroxidation level in the skin of mice excised wound. The results are shown as Mean ± SEM.

Treatment time (Days)	MDA (nmol/ g tissue)					
	PEG	XEN	OIE 5 %	OIE 10 %	OIE 20 %	OIE 30 %
3	51.05 ± 0.22	21.87 ± 0.58*	32.88 ± 0.67*	21.36 ± 0.71*	21.62 ± 0.56*	25.46 ± 0.67*
6	49.38 ± 0.46	12.41 ± 1.14*	29.30 ± 0.83*	13.18 ± 0.56*	16.24 ± 0.68*	20.98 ± 0.67*
9	43.76 ± 0.44	13.43 ± 0.58*	16.37 ± 0.64*	13.17 ± 0.46*	14.33 ± 0.34*	14.20 ± 1.17*
12	40.82 ± 1.24	13.44 ± 0.56*	15.57 ± 0.63*	12.34 ± 0.58*	13.34 ± 0.46*	14.13 ± 1.32*

N=10, *p < 0.01 when treatment groups are compared to polyethylene groups.

Effect of topical application of ethanol extract of *Oroxylum* indicum on deep dermal excision wound in Swiss albino mice.

Chapter 5

Abstract

The effect of different concentrations of ethanol extract of Oroxylum indicum (OIE) was studied on the deep dermal excision wound in mice by evaluating wound contraction, mean wound healing time (MHT), collagen and DNA syntheses in the regenerating wound granulation tissues. Topical application of 5%, 10%, 20% or 30% gel resulted in a concentration dependent rise in the wound contraction and the highest wound contraction was recorded for 10% OIE. The topical application of OIE also reduced the MHT in a concentration dependent fashion and the maximum reduction in MHT was observed for 10% OIE. The topical application of different concentrations of OIE increased the DNA and neocollagen syntheses in a dose dependent manner at all post wounding days and the greatest acceleration in DNA and neocollagen syntheses was observed for 10 % OIE. The evaluation of lipid peroxidation (LOO) showed a dose dependent decline in LOO, which was lowest for 10% OIE. The present study demonstrates that OIE accelerated the wound healing, which may be due to reduced lipid peroxidation and increased collagen and DNA syntheses.

Chapter 5

1. INTRODUCTION

Nature has been and always will remain an important source for the production of drugs for different diseases, especially the plants. Many important modern drugs have been prepared from plants, which form the therapeutic basis for the treatment of several health related disorders (Cragg and Newmann, 2013). The artifacts of Egypt illustrates the use of plants as medicine around 3000 BC and more than 340 drugs from plants have been archived in the Charak-Samhila, written around 1000 BC which is believed to be one of the earliest pieces on Indian medicine (Bhishagratna, 1907; El-Assal and George, 1972)

The drugs which are currently in use for healing wounds are synthetic which not only are expensive but also have several side effects indicating the need to screen newer cost-effective substitutes with minimal side effects (Sai and Babu, 1998; Jagetia et al, 2007; Logeeswari and Sripathi, 2012). Plants are known to be potent agents to heal wounds since they promote the repair mechanisms in the natural way (Sharma et al., 2013). Approximately 70% of the wound healing Ayurvedic drugs are derived from plants (Biswas and Mukherjee, 2003) and 80% of the compounds derived from plants have been utilized for the same or related purpose it served in the traditional medicinal systems (Farnsworth et al., 1985). Not only that the herbal preparations are more effective than synthetic medicines but they can be taken for an extended period due to their non-toxic nature (Vinothapooshan and Sundar. 2010). They are also abundantly available, cheap and do not become resistant to microorganism unlike antibiotics (Farahpour and Habibi 2012).

Oroxylum indicum (Family:Bignoniaceae), a deciduous tree which grows at an altitude of 1200 m is distributed throughout Asia (Kritikar and Basu, 2001) and is found in the Eastern and Western Ghats as well as the North-East region of India

(Jayaram and Prasad, 2008). This plant lives in a mutualistic relationship with an actinomycete *Pseudonocardia oroxyli*, a gram positive bacterium (Gu et al., 2006) that has the capacity to produce many secondary metabolites exhibiting a wide variety of biological activity (Qin et al., 2011) which have been used for treating several diseases since olden times (Kingston and Rao, 1982). Different parts of this plant are used in Ayurveda and other traditional systems of medicine for treating various health related disorders. *Oroxylum indicum* is even categorized under the endangered medicinal plant by the Govt. of India (Warrier et al., 1995; Ravi Kumar and Ved, 2000; Sastry et al., 2011). The bark decoction of *Oroxylum indicum* is used to cure gastric ulcers, diarrhea and to deworm cattles. The bark paste is useful in treating mouth cancer, scabies, and other skin diseases. It is also applied to the wounds of animals to kill maggots. Poultice of the bark is topically applied to treat rheumatism, sprains, inflammations and skin diseases (Sankara and Nair, 1972a; Sankara and Nair, 1972b; Lalrinzuali et al., 2015). The bark powder and paste is topically applied on wounds and burns respectively (Gaur and Sharma, 2011). Different studies have shown that the stem bark possess numerous biological activities such as antioxidant, anti-inflammatory and anticancer (Lotufo et al., 2005; Narisa et al., 2006; Kalaivani et al., 2009; Mishra et al., 2010; Kumar et al., 2011; Brahma et al., 2011; La lrinzuali et al., 2015; Doshi et al., 2011; Lalrinzuali et al., 2016). However, the systematic scientific evaluation on the wound healing activity of *Oroxylum indicum* is lacking. Therefore, the aim of this study was to assess the wound healing ability of *Oroxylum indicum* in mice inflicted with deep dermal excision wound.

2. MATERIALS AND METHODS

2.1. Chemicals

Poly ethylene glycol, Xenaderm (XEN) was procured from Health point Ltd., Fort Worth, Texas, USA. Hydroxyproline (catalog No: H5534), chloramine-T (catalog No: C9887), deoxyribonucleic acid (catalog No: D4522), diphenylamine (catalog No: D2385), N-(1-naphthyl) ethylenediamine dihydrochloride (catalog No: N5889) p-dimethylamino-benzaldehyde (catalog No: 42363–0250) and thiobarbituric acid (TBA) were procured from Sigma Chemical Co., St. Louis, MO, USA, while ethanol, trichloroacetic acid, perchloric acid, sodium hydroxide, hydrochloric acid and sodium chloride were requisitioned from SD fine-chemicals Ltd., Mumbai, India.

2.2. Preparation of the Extract

The non-infected and matured stem bark of *Oroxylum indicum* (Family: Bignoniacae) was collected from Champhai, Mizoram, India during the month of January and was identified by the Department of Horticulture and Aromatic and Medicinal Plants, Mizoram University, Aizawl, India. The bark of *O. indicum* was thoroughly washed and shade dried at room temperature in clean and hygienic conditions in the dark. The dried bark was powdered and it was sequentially extracted in petroleum ether, chloroform, ethanol and distilled water according to increasing polarity using a Soxhlet apparatus until the solvents turned colourless (Suffness and Douros, 1979). The ethanol extract was concentrated using rotary evaporator and stored at -70°C until further use. Hence forth ethanol extract will be called as OIE.

2.3. Animal care and handling:

The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Usually, 6 to 8 weeks old healthy male Swiss albino mice weighing 30-35 g were selected from an inbred colony maintained under the controlled conditions of temperature (25±2ºC), humidity (55–

60%) and 12 hours of light and dark cycle, respectively. The animals were housed in a sterile polypropylene cage containing paddy husk (procured locally) as bedding material. The animals had free access to standard rodent diet and water. All animal experiments were carried out according to NIH and Indian National Science Academy, New Delhi, India guidelines. The study was approved by the Institutional Animal Ethics Committee of the Mizoram University, Aizawl, Mizoram: India.

2.4. Experimental

The wound healing activity of ethanol extract was evaluated in the deep dermal excision wound created in mice.

2.4.1. Preparation of drug and mode of administration

Different concentrations viz: 5%, 10%, 20% and 30% of the ethanol extract of *Oroxylum indicum* were prepared in polyethylene glycol (PEG). The PEG or OIE was topically applied on the excision wound one day after wound creation until completing healing of the wounds.

2.4.2. Production of full-thickness skin wound.

The fur of the dorsum of each animal was removed with a cordless electric mouse clipper (Wahl Clipper Corporation, Illinois, USA). Briefly, the animals were anaesthetized using ketamine and the entire body was cleaned and decontaminated by wiping with 70 % ethanol. The cleared dorsal surface of the skin was marked with a sterile rectangular (2.5 x 1.5 cm) acrylic stencil. A full thickness wound was created by excising the full thickness skin flap in an aseptic environment under a vertical laminar flow apparatus using sterile forceps and scissors. Each wounded animal was housed in a separate sterile polypropylene cage until the termination of experiments.

The wound healing activity was determined by dividing the animals into the following groups:

PEG group (Negative control): The animals of this group did not receive any treatment except the polyethylene glycol (PEG) which was used as a vehicle to prepare the OIE gel.

XEN group (Positive control): The animals of this group received xenaderm, a standard wound healing ointment topically.

OIE group (Treatment): The animals of this group received topical application of 5%, 10%, 20% or 30% of OIE gel prepared in PEG.

2.4.3. Measurement of wound contraction

The PEG, OIE or XEN was topically applied usually at 11 AM to each deep dermal excision wound of each animal of respective groups once every day from day 1 post wounding so as to cover the whole area of the wound until the complete healing of the wounds. The contraction of the wound was measured every alternate days starting from day 1 post wounding by superimposing the transparent grid containing several squares of 1 mm^2. The wound area was determined by counting the number of the squares, which superimposed the whole wound area.

2.4.4. Mean wound healing time

The animals of each group were also monitored until complete healing of wounds and the day at which each wound healed in each group was recorded. Mean of all healed wounds was determined and has been expressed as mean wound healing time (MHT) in days.

2.5. Biochemical Analysis

A separate experiment was conducted to study the effect of topical application of PEG, OIE or XEN, where grouping and other conditions were essentially similar to that described for wound contraction except that the granulation tissue from each

animal of the respective groups was collected on 3, 6, 9 and 12 days post wounding for the estimation of the collagen and DNA contents.

2.5.1. Collagen

Hydroxyproline (an indication of collagen formation) concentration was determined as described by Woessner, (1961). The weighed granulation tissues were hydrolysed in 6 N HCl for 3 hours at 130 ºC, neutralised to pH 7 with 2.5 N NaOH and diluted with Milli-Q water. The diluted solution was mixed with chloramine-T reagent and incubated for 20 minutes at room temperature followed by the addition of freshly prepared ρ-dimethylaminobenzaldehyde (Ehrlich's reagent). The whole mixture was incubated for 15 minutes at 60 ºC. The absorbance of each sample was measured at 550 nm using a double beam ultraviolet (UV)-visible spectrophotometer (Shimadzu UV-260, Shimadzu Corporation, Tokyo, Japan). The amount of hydroxyproline was determined by comparing with the standard curve. Total collagen from hydroxyproline analysis was determined by multiplying with a factor of 6.94 (Reference). Collagen contents of granulation tissue have been expressed as milligram per gram dry tissue weight.

2.5.2. Deoxyribonucleic acid

Estimation of DNA gives an indication of cell proliferation and its concentration was measured by homogenizing the dry granulation tissue from each wound from respective groups in 5% TCA followed by centrifugation. The pellets were washed with 10% TCA, resuspended in 5% TCA and incubated at 90 ºC for 15 minutes. The contents were centrifuged again and the resultant supernatant was used for the estimation of DNA by the method of Burton (1956). The DNA was hydrolysed with 60% perchloric acid at 80ºC for 20 minutes followed by the addition of Burton's diphenylamine reagent and overnight incubation at room temperature. Thereafter,

95% ethanol was added and absorbance was read at 600 nm using a double beam UV-visible spectrophotometer. The amount of DNA was determined by comparing with the standard curve and has been expressed as mg/g dry tissue weight.

2.5.3. Lipid Peroxidation Assay

Lipid peroxidation assay was carried out following the method of Buege and Aust (1978). 1 ml of granulation tissue homogenate was mixed with 2 ml of TCA-TBA-HCl reagent thoroughly. The solution was heated in a boiling water bath for 15 minutes and cooled immediately at room temperature. After cooling, the solution was centrifuged at 1000 rpm for 10 min and supernatant was collected and its absorbance was read at 535 nm against blank in a UV-VIS Spectrophotometer. The blank contained all the reagents minus the cell homogenate substituted with distilled water. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56x10^{6}M^{-1}cm^{-1}$.

3. RESULTS

Periodic estimation of wound area is a good measure of wound healing and regeneration (Jagetia and Rajanikant, 2012; Jagetia and Ravikiran, 2015). The topical application of different concentrations of OIE on excision wound daily showed acceleration in wound healing as evidenced by a constant contraction of the wound with elapse of time (Figure 1). The OIE increased the wound contraction up to 10% where the greatest wound contraction was observed. The 10% OIE was the most efficient dose for topical application that accelerated wound healing; thereafter the effect was almost similar with increasing concentrations of OIE, except 30% OIE application, which reduced wound contraction, when compared to other concentrations. Despite this fact the wound contraction was greater, when compared to PEG application alone (Figure 1). The wound contraction for all concentrations of

OIE was almost similar to the positive control XEN whereas 10% OIE was even better than XEN application (Figure 1). The measurement of mean wound healing time (MHT) indicated that PEG application completely healed the wound within 27 days (Table 1 and Figure 2). Application of different concentrations of PEG reduced the MHT by approximately eight days for 5 and 10 % OIE when compared to PEG application alone. The increasing concentration of OIE resulted in a marginal elevation in MHT and it was 7 and 5 days less than the PEG control for 20 and 30% OIE respectively (Table 1 and Figure 2).

3.1. Collagen

Application of different concentrations of OIE gel on the regenerating wound resulted in a concentration dependent rise in neocollagen synthesis up to 10% OIE, which declined significantly for 20 and 30 % OIE when compared to 10 % OIE (Table 2 and Figure 3). Despite that the collagen synthesis was significantly higher than PEG application alone at these OIE concentrations (Table 2 and Figure 3). The collagen synthesis increased with evaluation time and a maximum collagen synthesis was reported on day 6 that declined thereafter up to 12 days post wounding. This rise in collagen synthesis was almost 3.5 fold higher for 5, 20 and 30% OIE whereas it was 4.3 fold higher for 10% OIE when compared to PEG application alone (Table 2 and Figure 3). The 10 % OIE was most comparable to the standard drug as compared to the other concentrations.

3.2. DNA

The DNA synthesis is increased significantly with regeneration of wound and almost more than 2.5 fold rise was observed in the DNA synthesis for all the concentrations of OIE except 30% on day 3, 6 and 20% on day 6 and 12, when compared to PEG treatment alone (Table 3). The DNA synthesis increased with time

up to day 6 and declined thereafter for all the groups and it remained least for the PEG treated group when compared to all other groups (Figure 4).

3.3. Lipid Peroxidation

Wounding has increased lipid peroxidation many folds when compared to the spontaneous level (Figure 5). The topical application of different concentrations of OIE alleviated lipid peroxidation and a maximum attrition in lipid peroxidation was observed for 10% OIE when compared to PEG treatment alone (Figure 5). Application of 10 and 20% OIE reduced the lipid peroxidation approximately by 3 fold on all post wounding days except day 3, where it was approximately 2.4 folds lower than PEG application alone (Table 4).

4. DISCUSSION

The normal wound healing is a dynamic process, which follows a definite sequence of events and has been conveniently divided in to four phases including hemostasis, inflammatory, proliferative and remodeling phases (Gurtner, 2007; Janis and Harrison, 2014). Acute wounds progress through the normal wound healing process within the expected time frame, which usually lasts for 3-4 weeks. It is a sequence of overlapping and well-coordinated events that involve platelet aggregation, phagocytosis, chemotaxis, mitogenesis, and synthesis of different components of the extracellular matrix (Enoch., 2007). Wounding is very common phenomenon and several remedies are used from house hold to sophisticated depending on the severity of the wounds. However, screening of new paradigms for wound healing is necessary in the hope of getting early and scarless wound healing. Therefore, present study was designed to investigate the effect of topical application of different concentrations of ethanol extract gel of *Oroxylum indicum* on the deep dermal excision wound in mice.

The immediate response after wounding is to restrict/stop bleeding. The injury causes platelets to aggregate in the wound bed and initiate the processes that help in the wound debridement and lay the foundation for epithelialization of the wounds. The neutrophils, leucocytes, macrophages migrated into the wound milieu and secretes various growth factors and cytokines to initiate the healing cascade (Barrientos et al., 2008). The oxidative activity is increased due to production of reactive oxygen species (ROS) and an action of NADPH oxidase has been reported (Darr and Fridovich, 1994; Bedard and Krause, 2007). The wounds have shown increased amount of H_2O_2 and superoxide radical production at the wound site (Schafer and Werner, 2008). Earlier studies have shown increased lipid peroxidation after wounding (Ojha et al., 2008; Loo et al., 2012). Though these reactive oxygen species are required for defence, cell signaling and angiogenesis, excess amount leads to oxidative stress which eventually causes chronic and non-healing wounds (Schafer and Werner, 2008). The increased level of reactive oxygen species may retard the wound healing process (Hallberg et al., 1996). Wounding induces the activation of different cytokines which lead to an increase in the reactive oxygen species and removal of excess free radicals/ toxins could be one of the most important factors for wound healing (Steiling et al., 1999; Behm et al., 2011). The topical application of different concentrations of OIE has enhanced wound contraction and also curtailed mean wound healing time. Which may be due to the controlling effect of OIE on oxidative stress as it may have kept the oxidative stress to minimum that is essential for wound healing. Several other natural products like ascorbic acid, curcumin, *Nigella sativa* extract have been reported to enhance wound contraction in earlier studies (Jagetia et al., 2003; Jagetia and Rajanikant, 2005; Jagetia and Ravikiran, 2015). The topical application of other plant extracts including Pupalia. Lappacea and

Euphorbia nerifolia have been reported to enhance wound contraction in wounded rats (Yadav et al., 2012; Pattanaik et al., 2014; Udegbunam et al., 2014). Earlier studies from this laboratory has shown that the *Oroxylum indicum* neutralizes various free radicals (Lalrinzuali et al., 2015) and contains many flavonoids like chrysin, baicalein, oroxylin and scutellarin (Sankara and Nair, 1972a; Sankara and Nair, 1972b) which could be responsible for the wound healing activity in the present study.

The proteins have been reported to play a major role in wound healing since their deficiency has been reported to retard several important aspect of wound healing including inflammation, fibroplasia, synthesis of proteoglycans and collagen, angiogenesis and wound remodeling (MacKay and Miller, 2003). The OIE application had increased the synthesis of collagen and DNA synthesis indicating rise in the fibroblast proliferation. The fibroblasts play a major role in wound contraction as they are differentiated into contractile myofibroblasts, which are essential in matrix deposition and wound contraction (Micallef et al., 2012). This would have led to earlier closure of the wound and reduced wound healing time in the present study. Earlier studies from this laboratory has shown increased synthesis of collagen and DNA with increased fibroplasia in the regenerating wounds of mice receiving ascorbic acid, curcumin or *Nigella sativa* extract (Jagetia et al., 2003; Jagetia and Rajanikant, 2005; Jagetia and Ravikiran, 2015).

The exact mechanism of augmentation of wound healing by OIE is not well understood. The OIE contains several phytochemicals which may have acted in concert with each other by employing several possible mechanisms. Wounding produces free radicals and excess free radicals are known to derail the normal wound healing process. The application of OIE may have neutralized the excess production of free radicals keeping the normal wound healing processes in order. OIE has been

found to scavenge free radicals earlier (Lalrinzuali et al., 2015). OIE also reduced the production of LOO, which would have positive impact on cell proliferation, cytokine secretion and other processes involved in wound repair and regeneration. The increased collagen and DNA syntheses would have been a result of increased fibroplasia that accelerated the healing of regenerating wounds leading to early closure and reduced MHT. Although no attempt has been made to study the molecular mechanism of action of OIE in wound healing there is no reason to believe that OIE may have not stimulated the vasculoendothelial growth factor (VEGF), TGF β, PDGF which are essential for the closure of regenerating wounds and inhibited the activation of NF-κB, COX-II and LOX (Zhou et al., 1999; Gaddipati et al., 2003), which have negative impact on wound contraction. Chrysin a flavonoid present in OIE has been reported to inhibit proinflammatory cytokines like TNF α, IL-1β, IL-4, and IL-6 and NF-κB (Bae et al., 2011). OIE has been recently reported to inhibit NF-κB (Trans et al., 2015).

5. CONCLUSIONS

The present study demonstrates conclusively that topical application of the OIE accelerated healing of regenerating wounds and reduced the MHT significantly. It also increased the syntheses of collagen and DNA and reduced LOO in the regenerating wounds. The increased wound healing ability of OIE may be due to its ability to scavenge free radicals, and increase cell proliferation which would have been able raise the collagen synthesis leading to early closure of the wound. It may have reduced the expression of genes related to proinflammatory cytokines including TNF α, IL-1β, IL-4, and IL-6 and NF-κB in the regenerating wounds.

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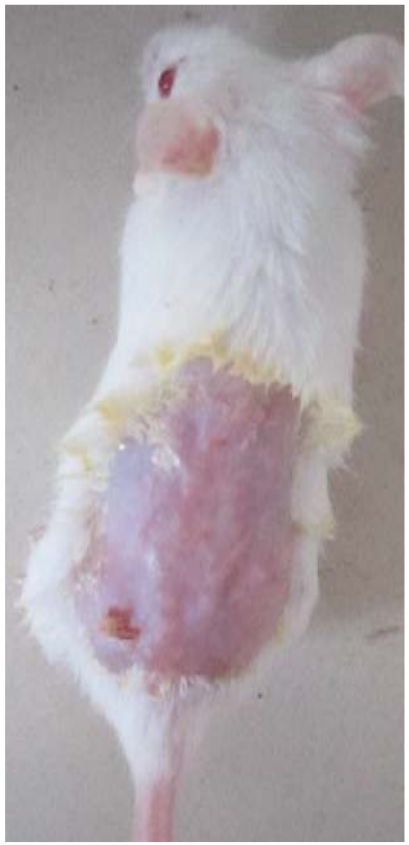

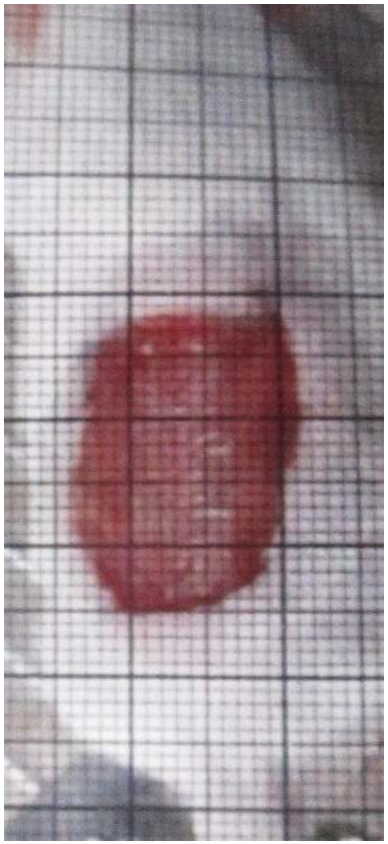

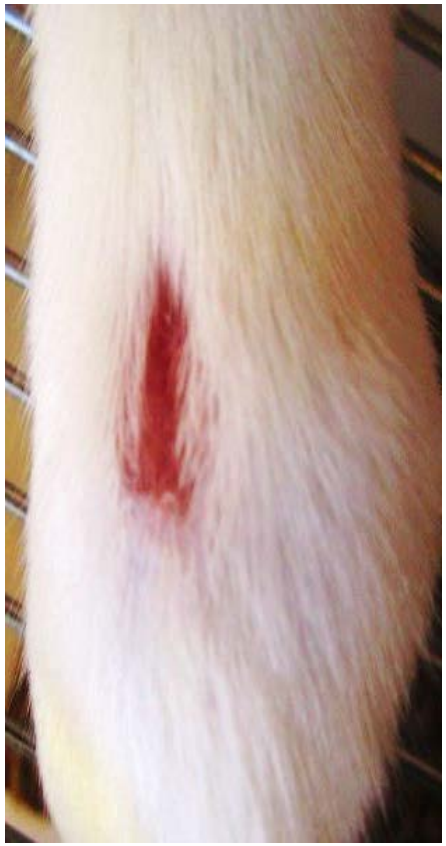

Day 1 **Day 2** **Day 18**

Plate I: Effect of *Oroxylum indicum* extract treatment on deep dermal wound of Swiss albino mice.

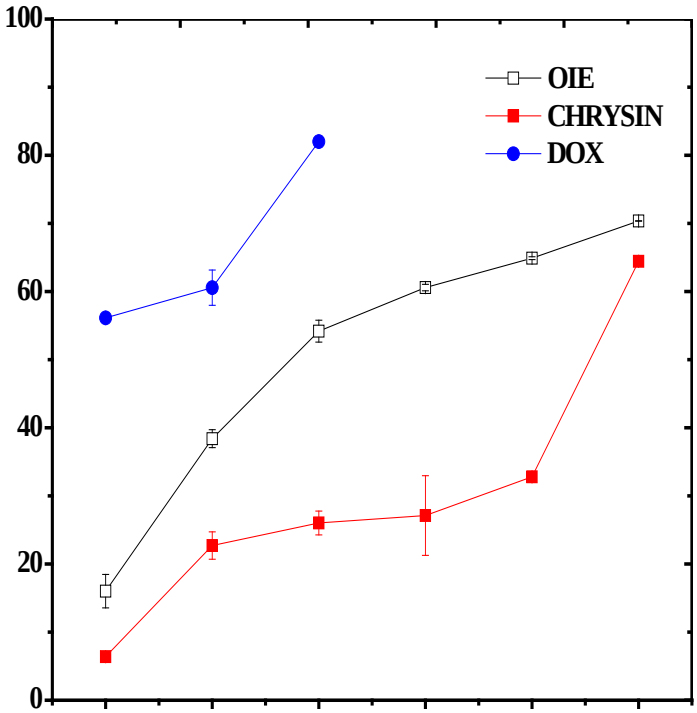

Figure 1: The cytotoxic effect of different concentration of ethanol extract of *Oroxylum indicum*, chrysin and doxorubicin on HeLa cells assessed by dose dependent MTT assay. OIE- Ethanol extract of *Oroxylum indicum*, DOX- Doxorubicin, CHR- Chrysin. Figures in brackets on X- axis indicate concentration of doxorubicin.
The data represent Mean ±SEM, N=5, $p<0.05$.

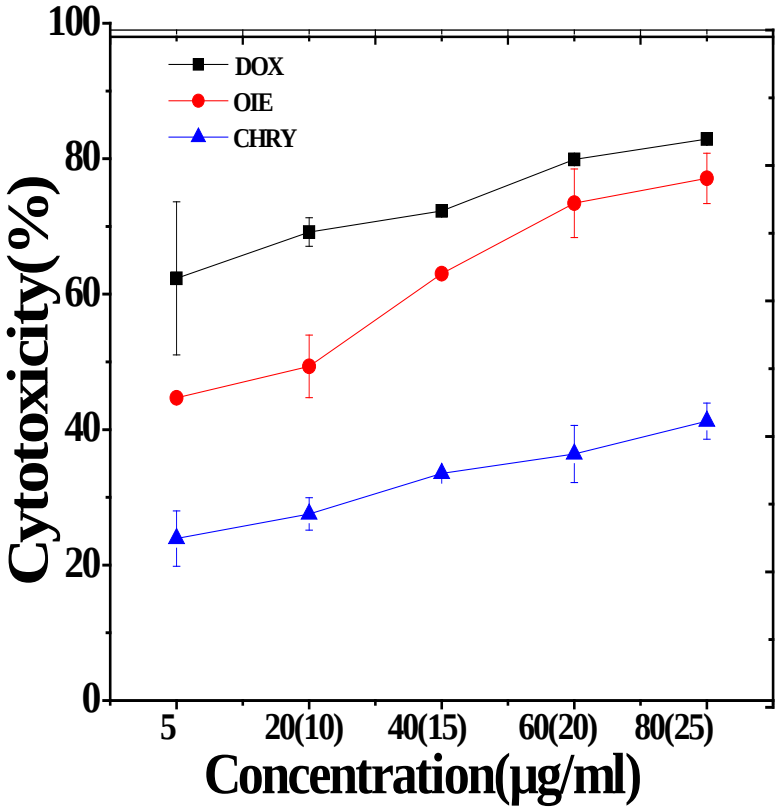

Figure 2: The cytotoxic effect of different concentration of ethanol extract of *Oroxylum indicum*, chrysin and doxorubicin on HepG2 cells assessed by dose MTT assay. OIE- Ethanol extract of *Oroxylum indicum*, doxorubicin (DOX), chrysin (CHR). Figures in brackets on X-axis ndicate concentration of doxorubicin. The data represent Mean ±SEM, N=5, $p<0.05$.

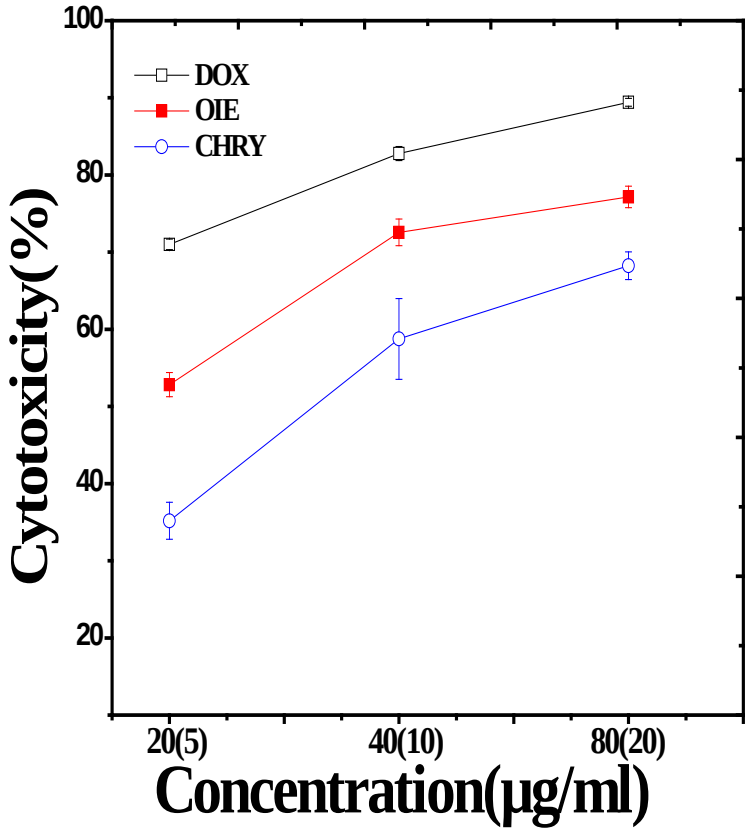

Figure 3: The cytotoxic effect of different concentration of ethanol extract of *Oroxylum indicum*, chrysin and doxorubicin on MCF-7 cells assessed by concentration dependent MTT assay. OIE- Ethanol extract of *Oroxylum indicum, d*oxorubicin (DOX), chrysin (CHR). Figures in brackets on X- axis indicate concentration of doxorubicin. The data represent Mean ±SEM, N=5, $p<0.05$.

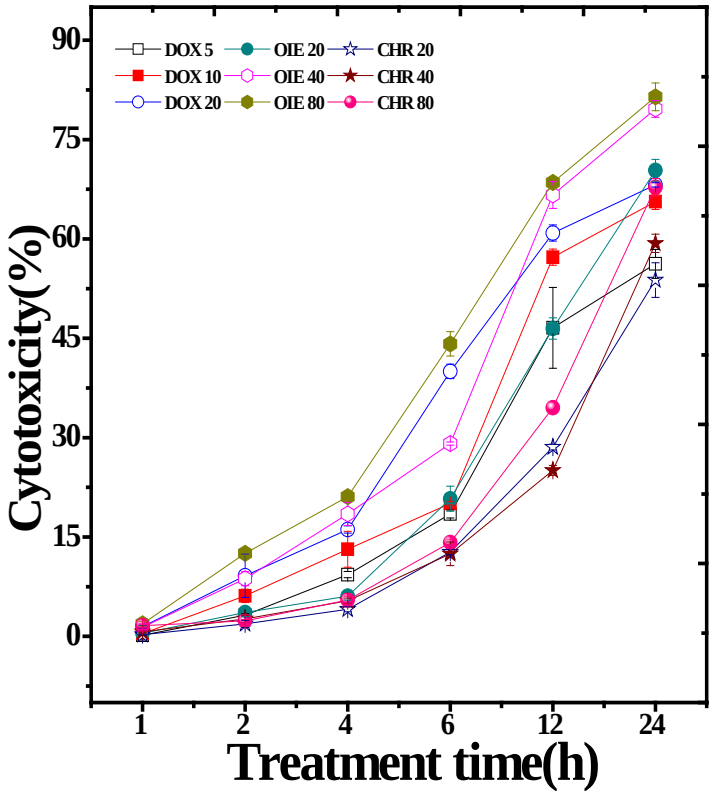

Figure 4: The cytotoxic effect of different concentration of ethanol extract of *Oroxylum indicum*, chrysin and doxorubicin on HeLa cells assessed by Time dependent MTT assay. OIE- Ethanol extract of *Oroxylum indicum*, doxorubicin (DOX), chrysin (CHR). The data represent Mean ±SEM, N=5, $p<0.05$.

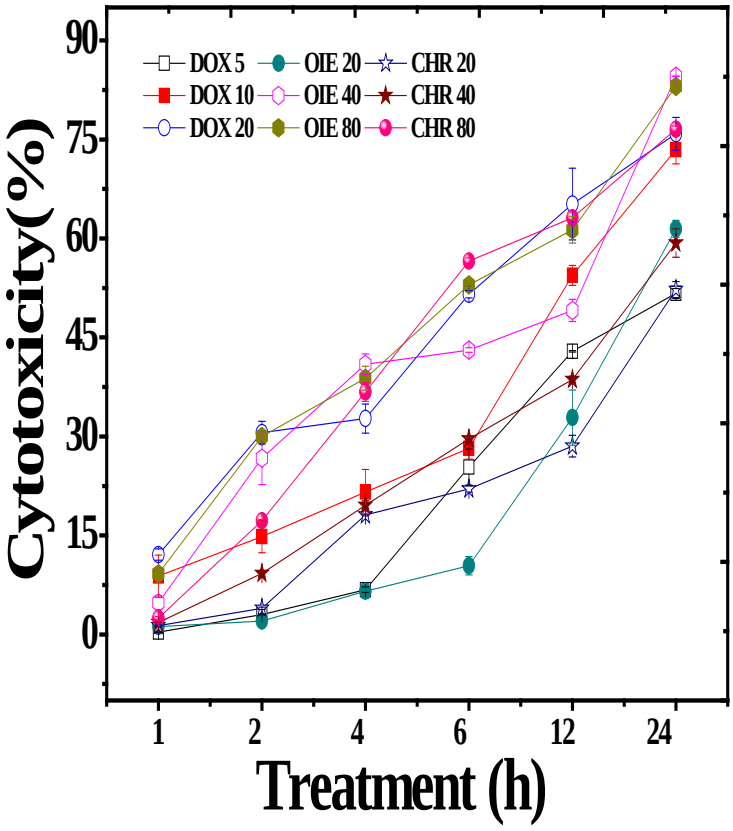

Figure 5: The cytotoxic effect of different concentration of ethanol extract of *Oroxylum indicum*, chrysin and doxorubicin on HepG2 cells assessed by Time dependent MTT assay. Ethanol extract of *Oroxylum indicum* (OIE), doxorubicin (DOX), chrysin (CHR). The data represent Mean ± SEM, N=5, $p<0.05$.

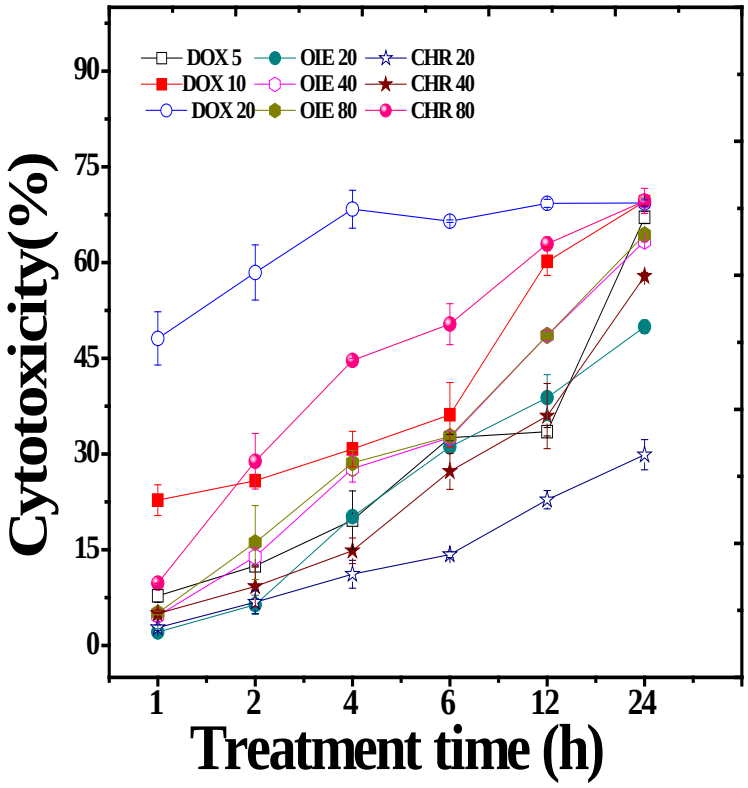

Figure 6: The effect of different concentration of the ethanol extract of *Oroxylum indicum*, chrysin and doxorubicin on MCF-7 cells determined by Time dependent MTT assay. - Ethanol extract of *Oroxylum indicum* (OIE), *d*oxorubicin (DOX), chrysin (CHR).

The data represent Mean ±SEM, N=5, $p<0.05$.

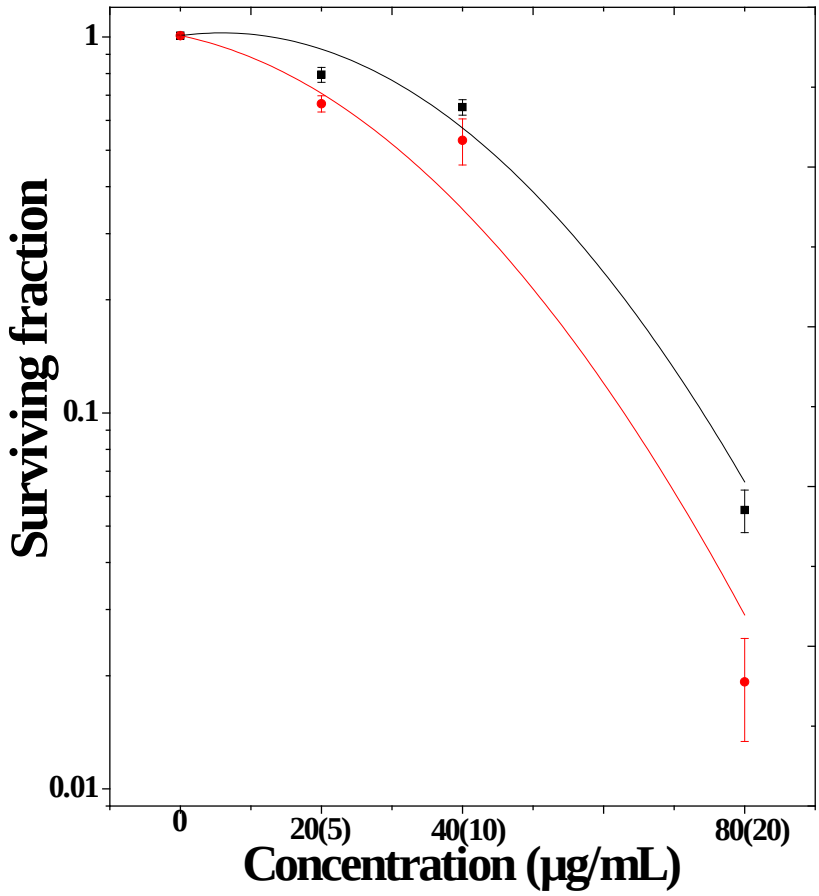

Figure 7: Effect of the different concentrations of OIE and DOX on the reproductive integrity of HeLa cells assessed by clonogenic assay. Figures in brackets on X- axis indicate concentration of doxorubicin. The data represent Mean ± SEM, N=5, $p<0.05$.

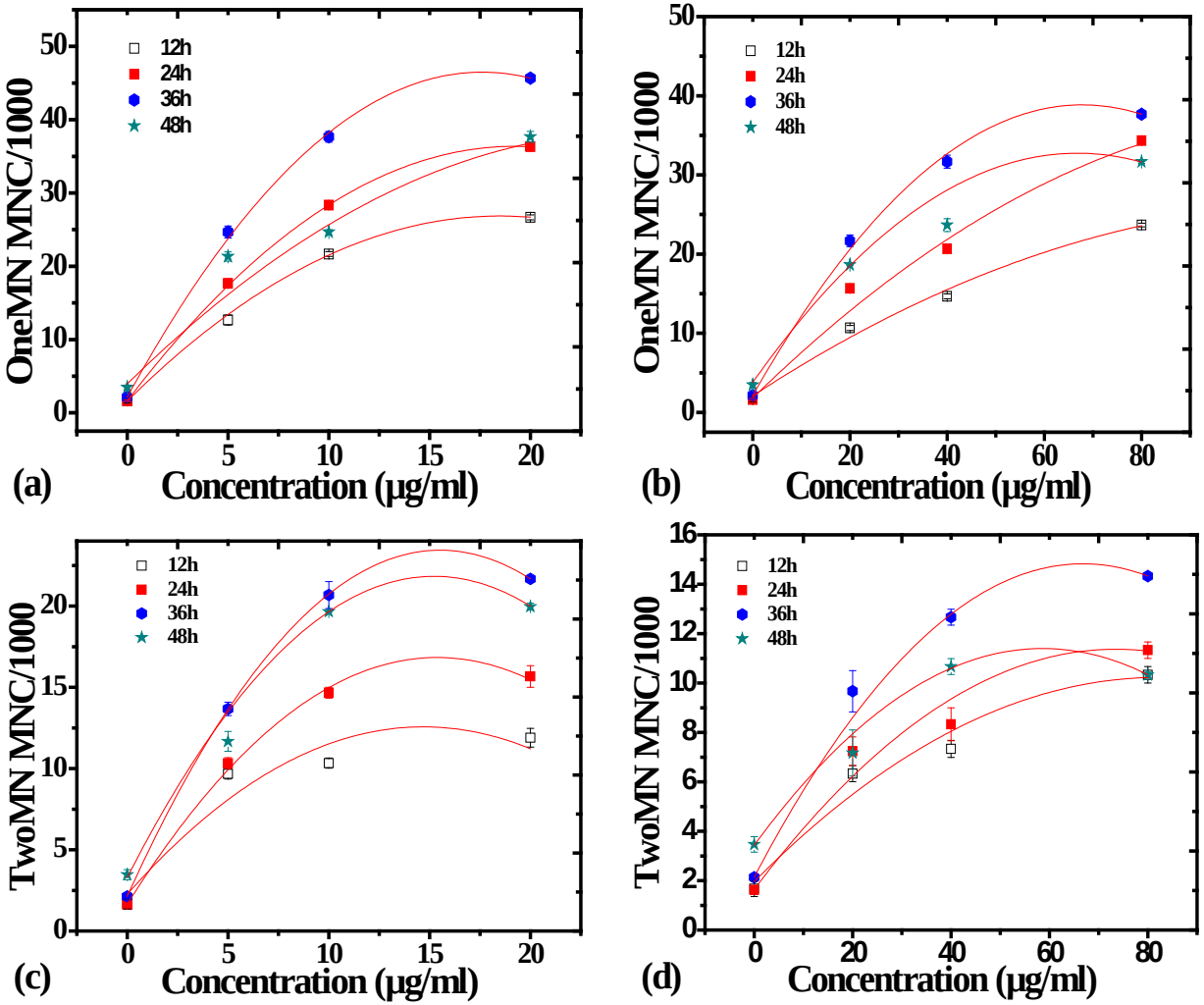

Figure 8: Induction of micronuclei in HeLa by treatment with different concentration of OIE and DOX at various dose dependent treatments. The data represent Mean SEM, N=5, p<0.01.

Mononucleated cell bearing one micronucleus (MN): (a) DOX (b) OIE and two micronuclei: (c) DOX (d) OIE.

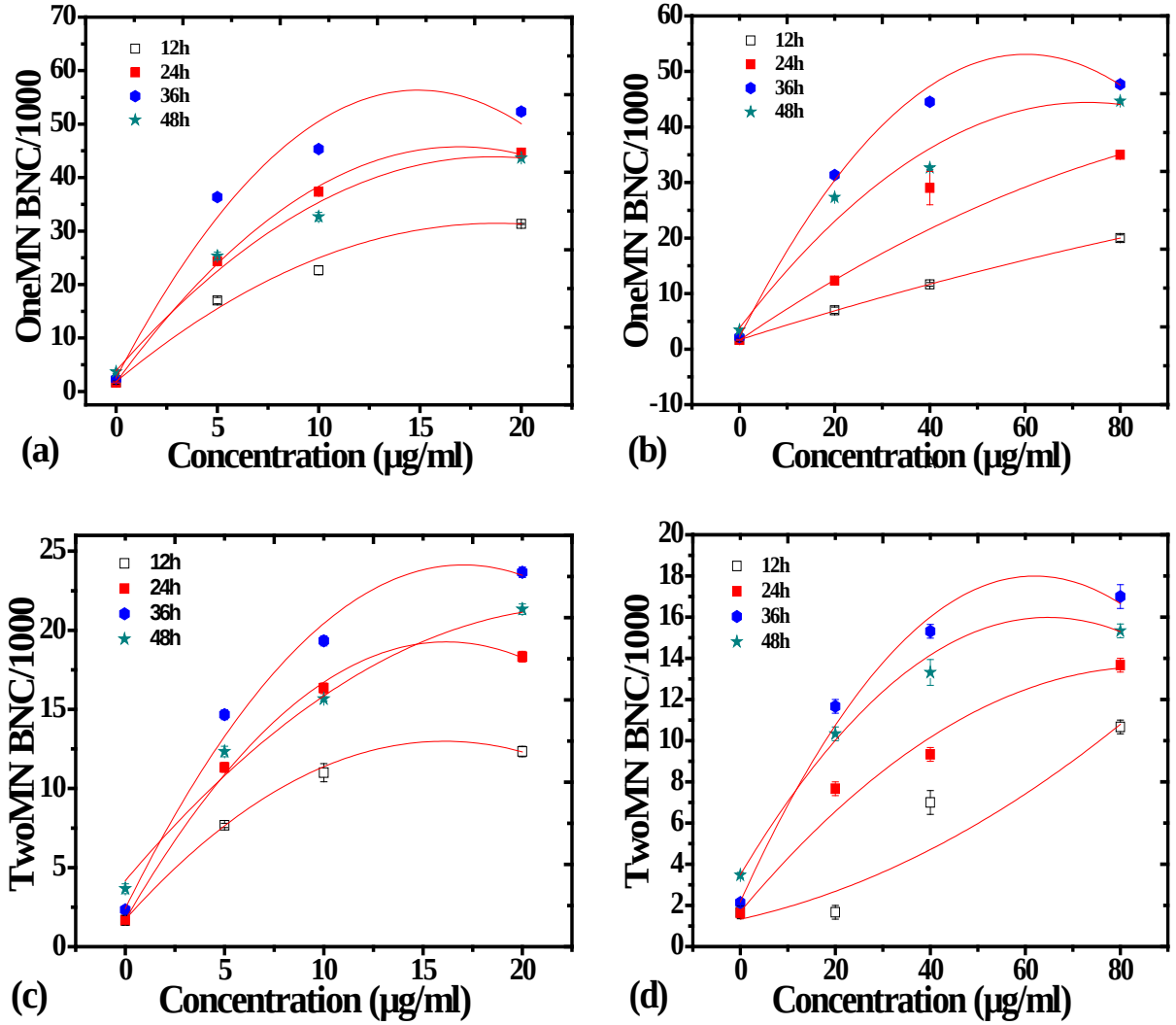

Figure 9: Induction of micronuclei in HeLa cells by treatment with different concentration of OIE and DOX at various dose dependent treatments. The data represent Mean SEM, N=5, p<0.01.

Binucleated cell bearing one micronucleus (MN): (a) DOX (b) OIE and two micronuclei: (c) DOX (d) OIE.

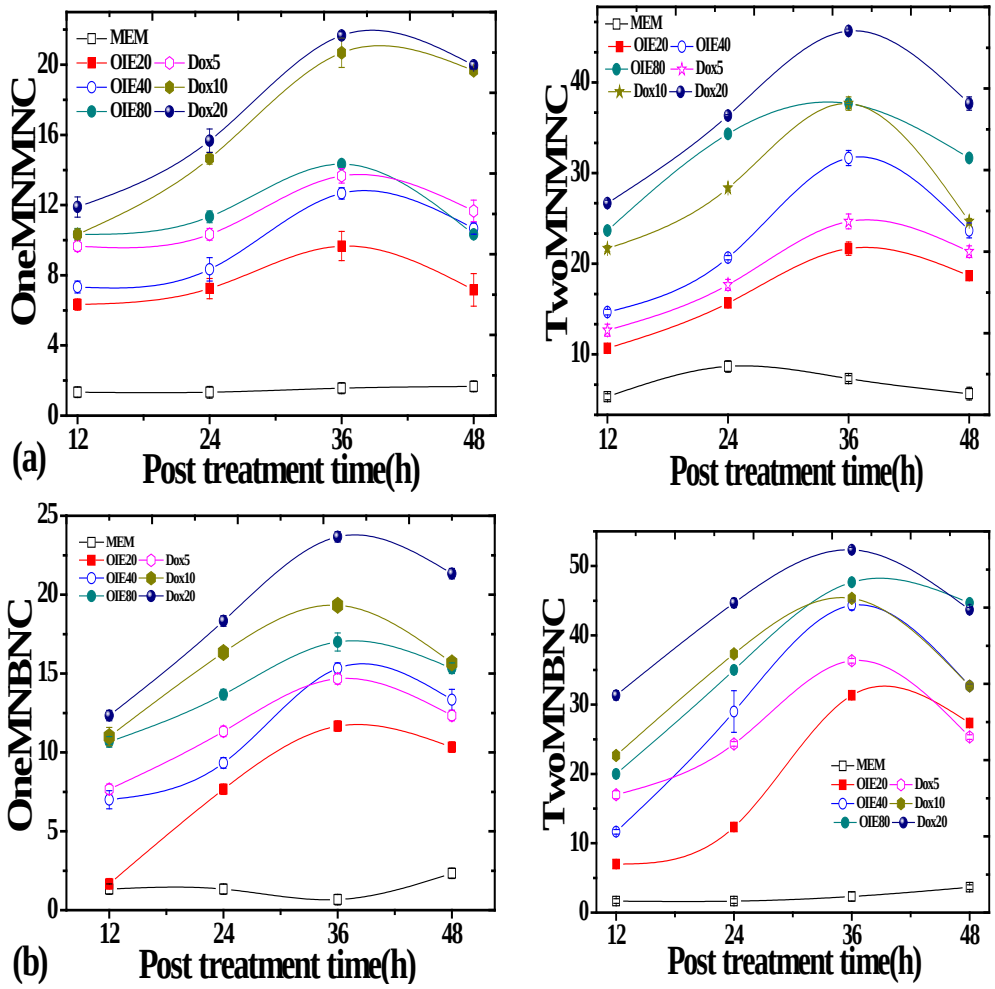

Figure 10: Induction of micronucleus in cultured HeLa cells treated with different concentration of OIE and DOX at different post treatment time.

(a) Mononucleated cells bearing micronucleus: Left- one micronucleus and Right-two micronuclei.

(b) Binucleated cells bearing micronucleus : Left- one micronucleus and Right-two micronuclei. The data represent Mean ±SEM, N=5, *p<0.01.

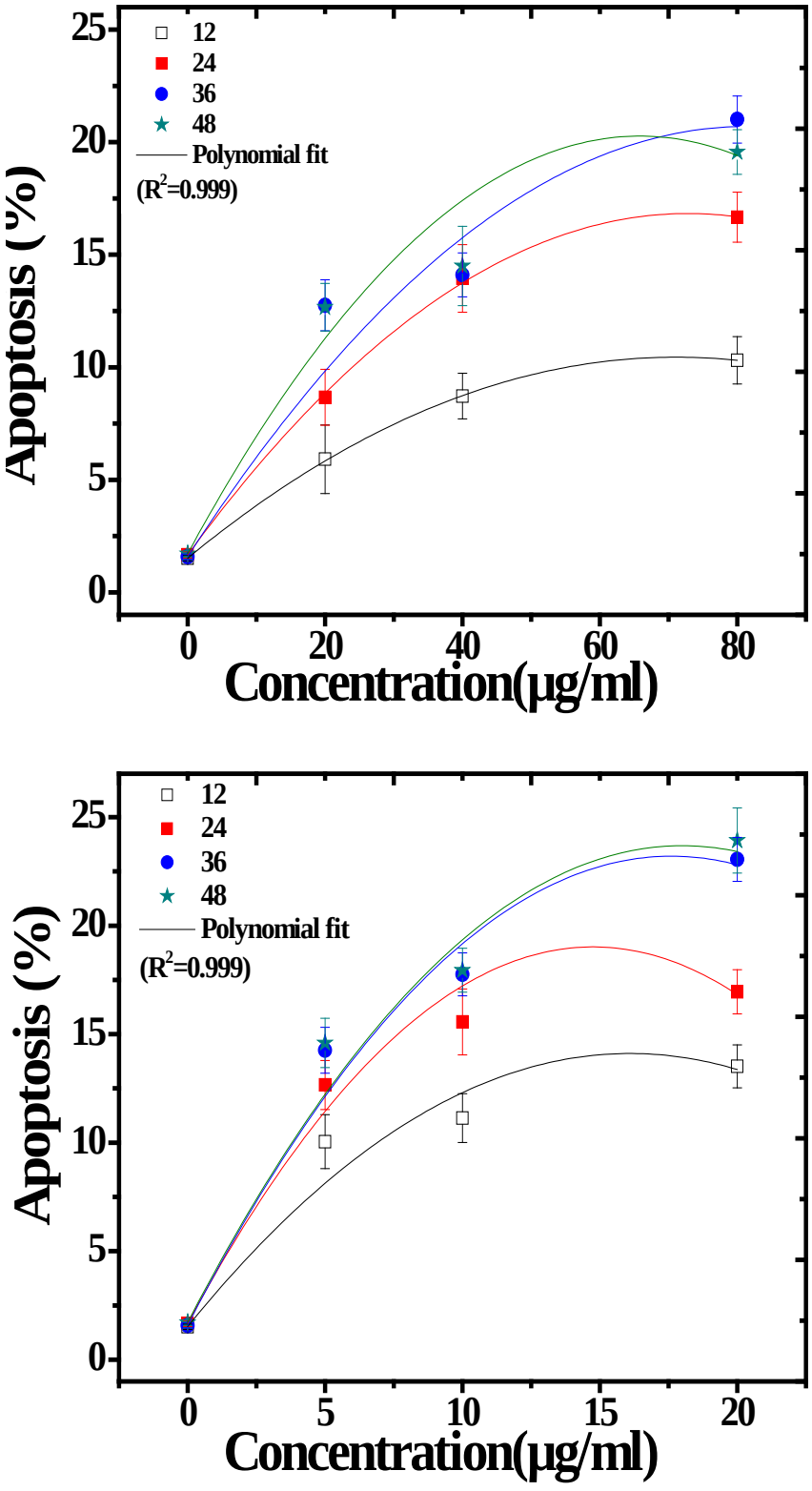

Figure 11: Induction of apoptosis by treatment with different concentrations of OIE and DOX on HeLa cell at different post treatment time. Above: OIE (0-80µg/ml) Below: DOX (0-20µg/ml). The data represent Mean ±SEM, N=3.

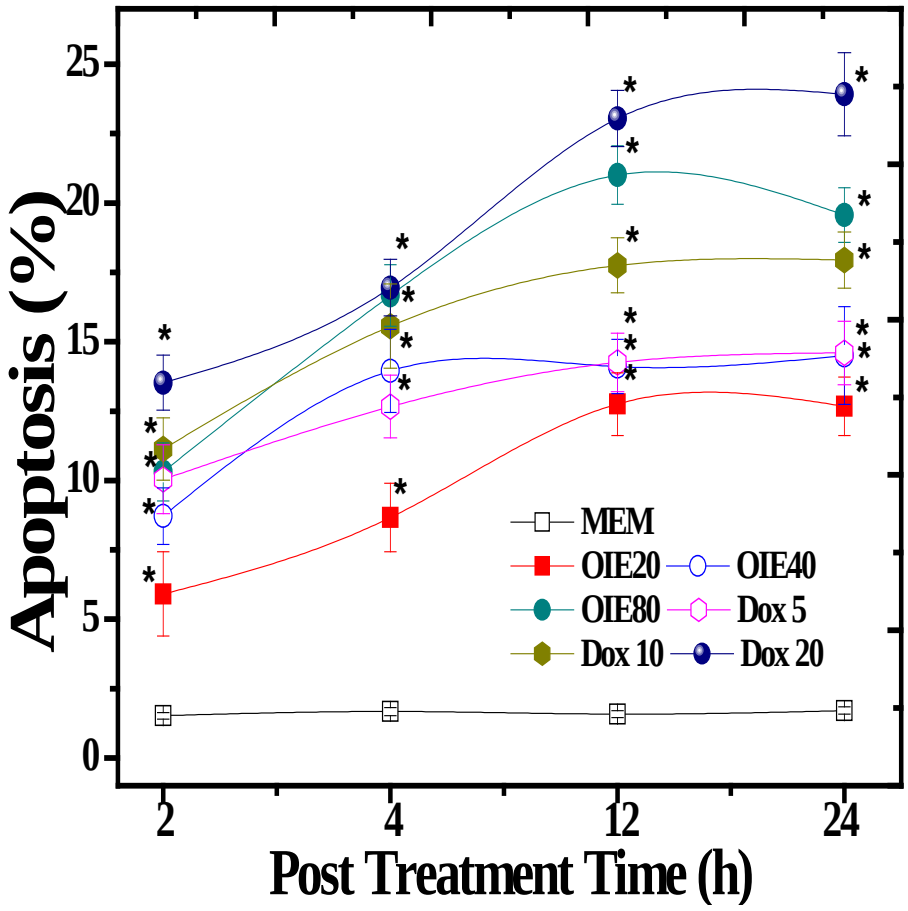

Figure 12: Induction of apoptosis in HeLa cells treated with different concentrations of OIE or DOX at different post treatment times. The represent Mean ±SEM, N=3.

***P<0.01 when treatment groups are compared to MEM group.**

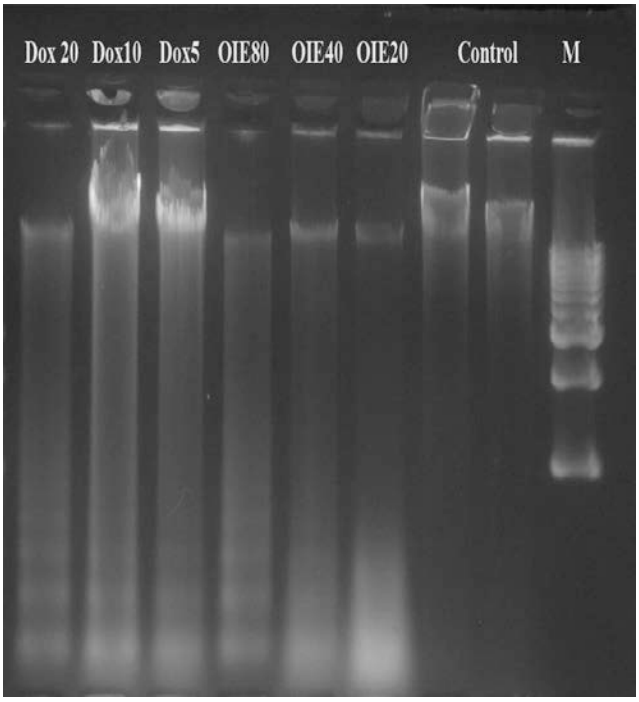

Figure 13: DNA fragmentation induced in HeLa cells by different concentrations of OIE or DOX visualized by agarose electrophoresis.

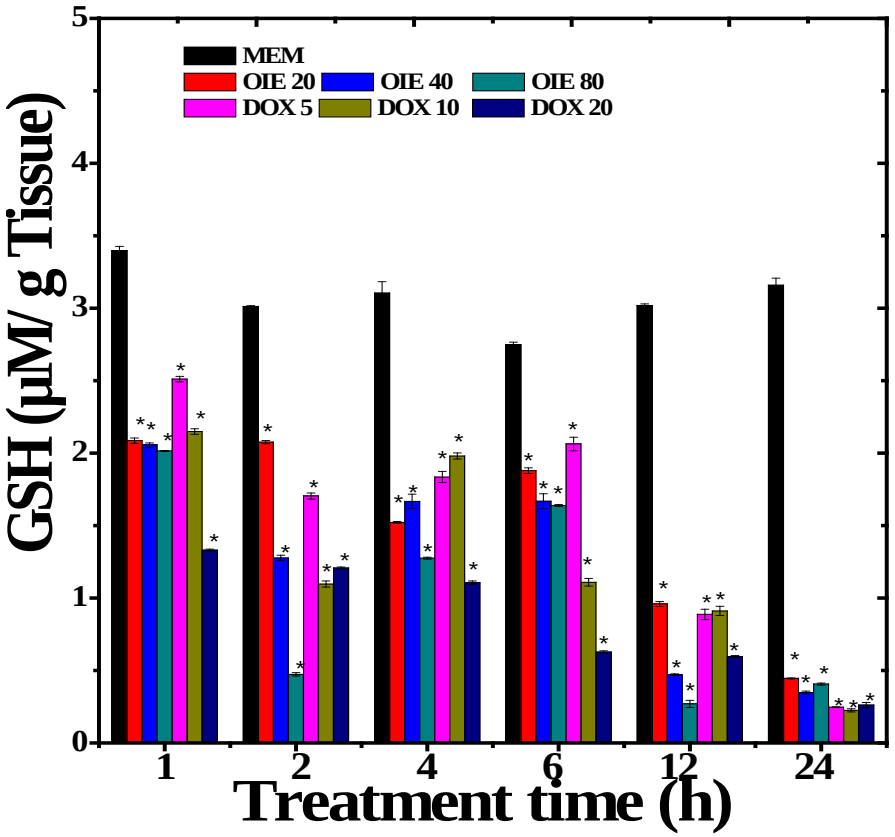

Figure 14: Alteration in the GSH content of HeLa cells treated with different concentrations of OIE and DOX. Minimum essential media (MEM), Ethanol extract of *Oroxylum indicum* (OIE), doxorubicin (DOX). The data represent Mean± SEM, N=5.

***P<0.01 when treatment are compared to MEM group.**

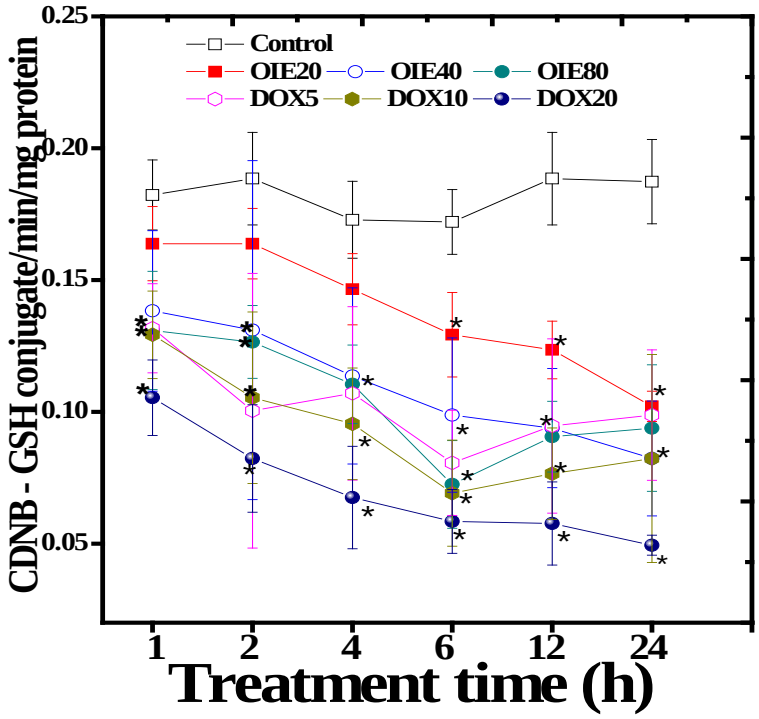

Figure 15: Alteration in the GST activity of cultured HeLa cells treated with different concentrations of OIE and DOX. Minimum essential media (MEM), Ethanol extract of *Oroxylum indicum* (OIE), doxorubicin (DOX). The data represent Mean± SEM, N=5. *P<0.01 when treatment are compared to MEM group. Standard error of the mean (SEM).

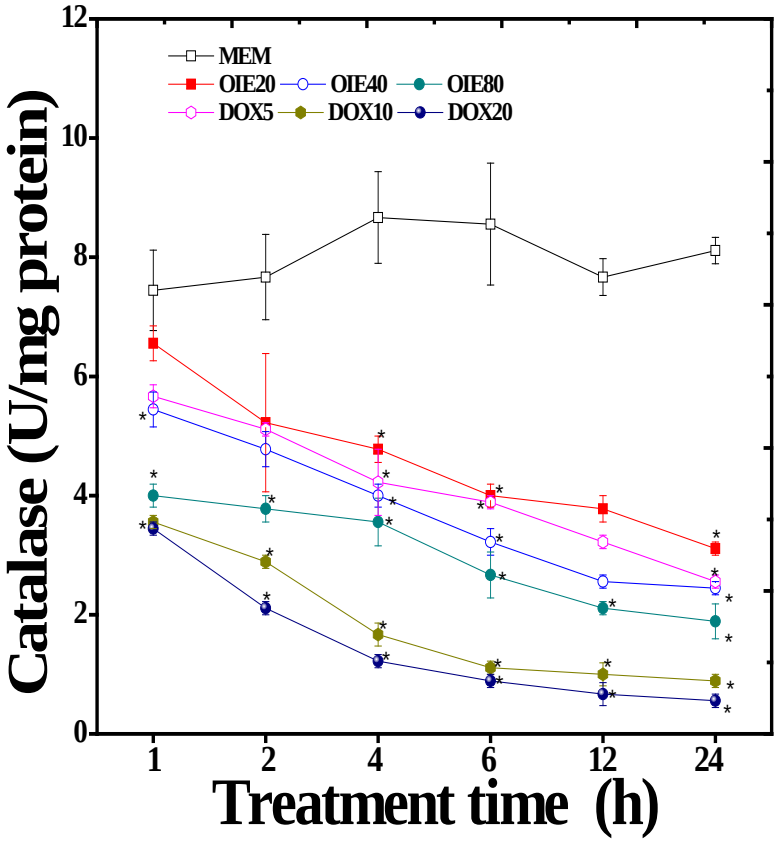

Figure 16: Alteration in the catalase activity of cultured HeLa cells treated with different concentrations of OIE and DOX. Minimum essential media (MEM), Ethanol extract of *Oroxylum indicum* (OIE), doxorubicin (DOX). The data represent Mean± SEM, N=5.
p<0.01 when treatment are compared to MEM group.
Standard error of the mean (SEM).

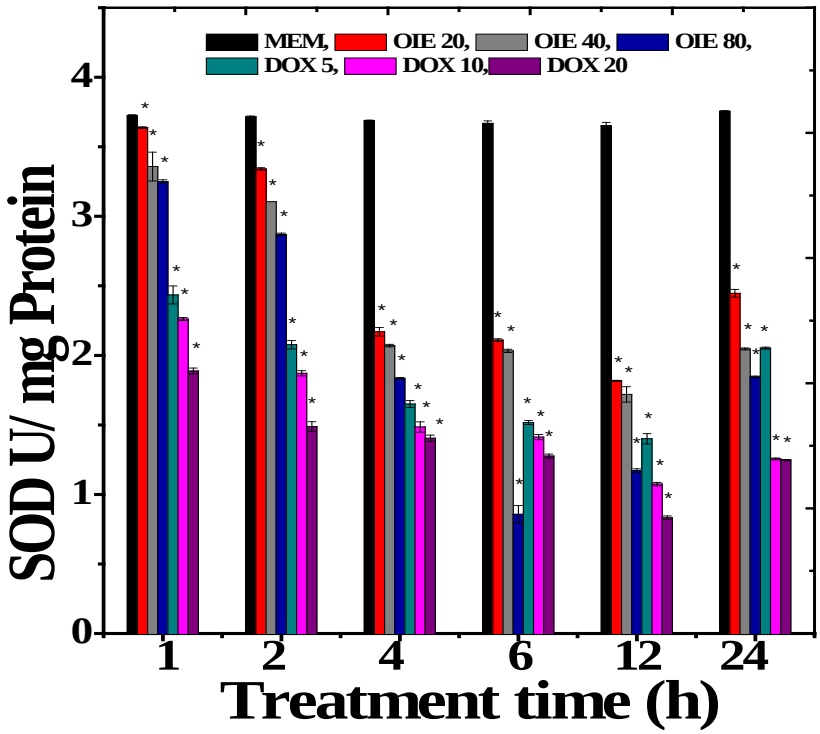

Figure 17: Alteration in the SOD activity of cultured HeLa cells treated with different concentration of OIE and DOX. Minimum essential media (MEM), Ethanol extract of *Oroxylum indicum* (OIE), doxorubicin (DOX). The data represent Mean± SEM, N=5.
p<0.01 when treatment are compared to MEM group.
Standard error of the mean (SEM).

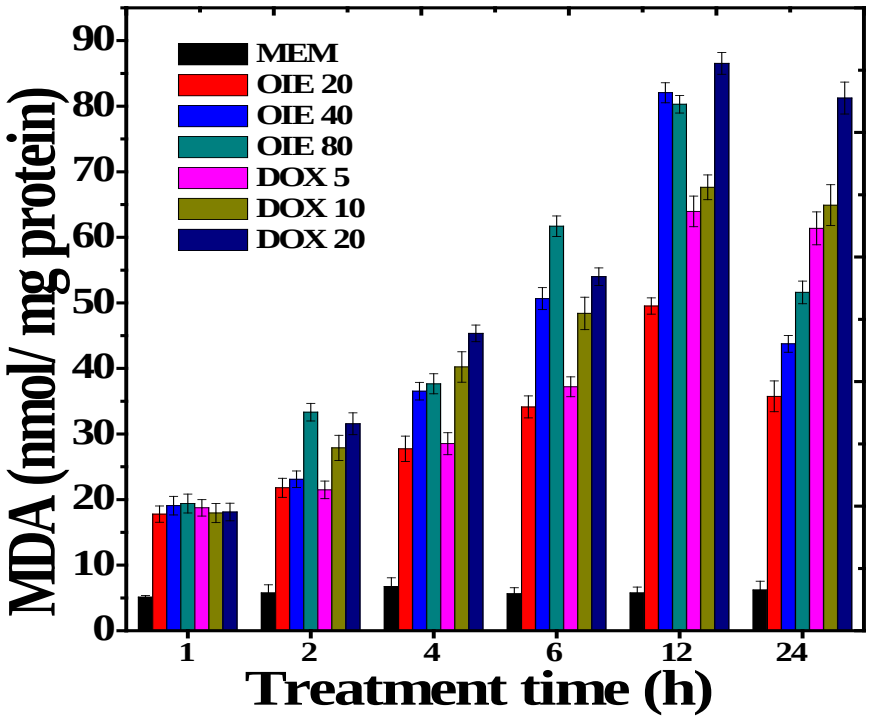

Figure 18: Changes in the lipid peroxidation of cultured HeLa cells treated with different concentrations of *Oroxylum indicum* and doxorubicin. Minimum essential media (MEM), Ethanol extract of *Oroxylum indicum* (OIE), doxorubicin (DOX). The data represent Mean± SEM, N=3.

p<0.01 when treatment are compared to MEM group.

Standard error of the mean (SEM).

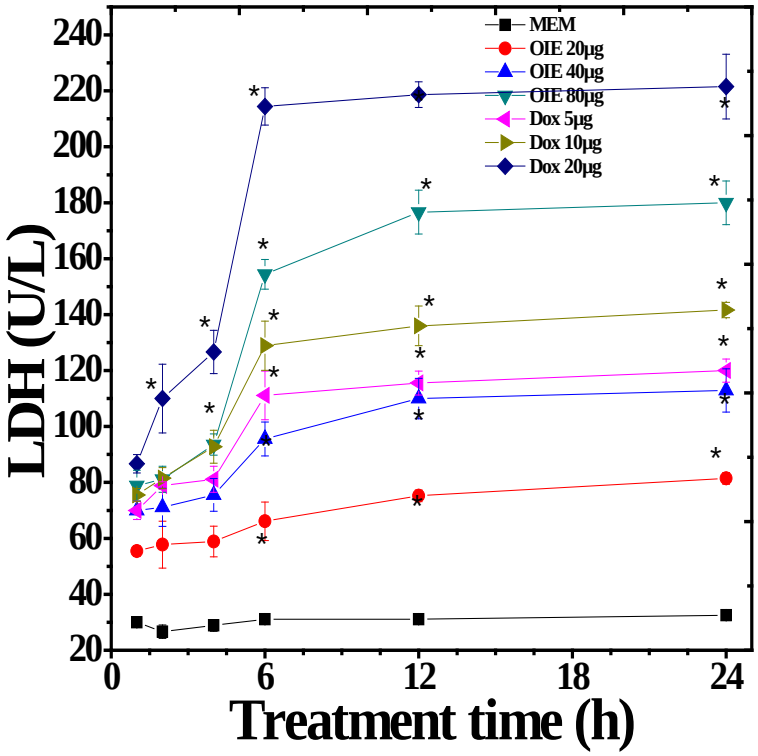

Figure 19: Alteration in the LDH release by HeLa cells treated with various concentrations of OIE and DOX on dose dependent treatment at different exposure times. The data represent Mean± SEM, N=3. Minimum essential media (MEM), Ethanol extract of *Oroxylum indicum* (OIE), doxorubicin (DOX).

p<0.01 when treatment groups are compared to MEM group.

Standard error of the mean (SEM).

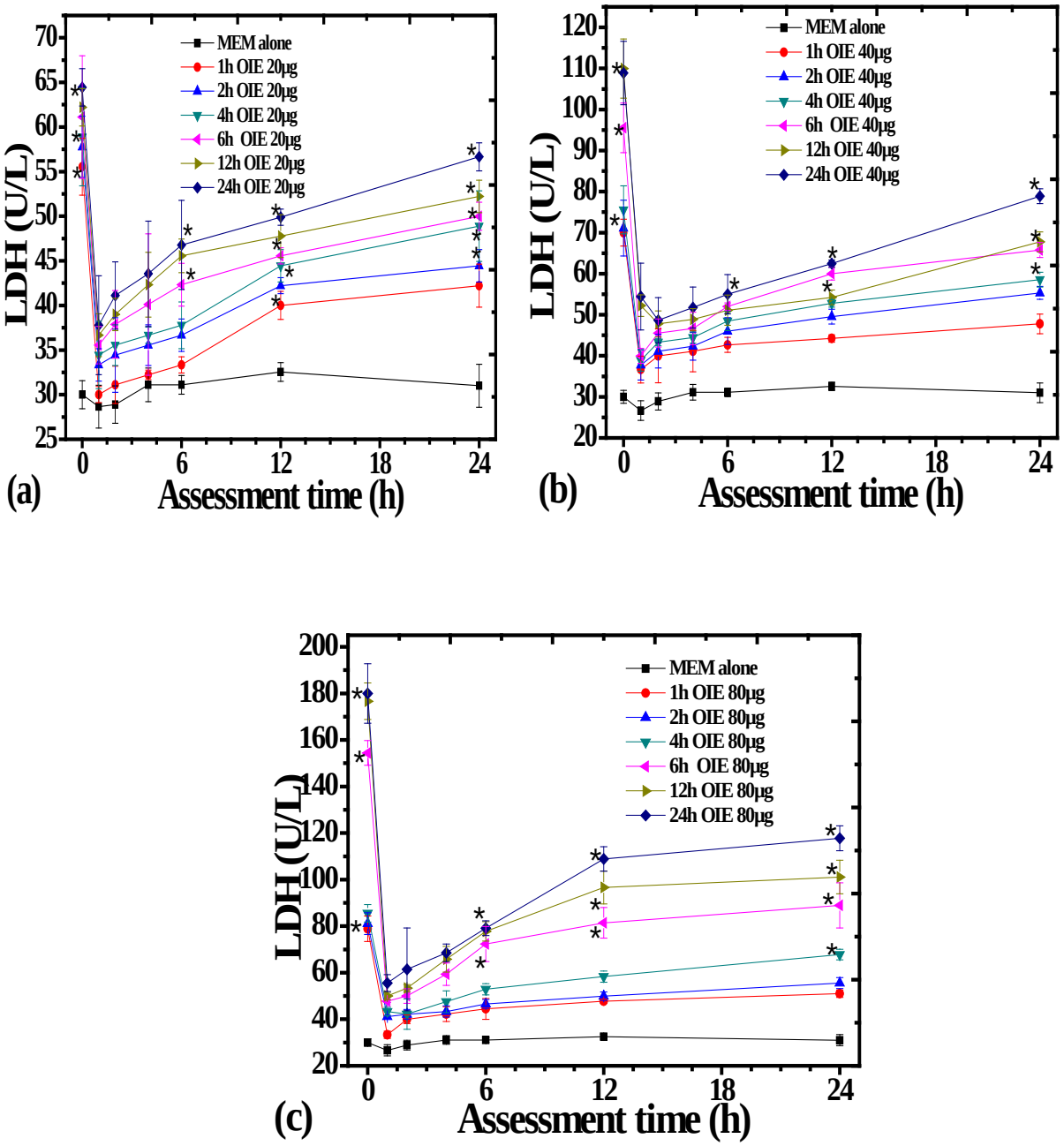

Figure 20: Alteration in the LDH release by HeLa cells treated with various concentrations of OIE at different treatment time and assessment times. (a) OIE 20µg/ml (b) OIE 40µg/ml (c) OIE 80µg/ml. The data represent Mean± SEM, N=3. Minimum essential media (MEM), Ethanol extract of *Oroxylum indicum* (OIE), doxorubicin (DOX).

p<0.01 when treatment groups are compared to MEM group.

Standard error of the mean (SEM).

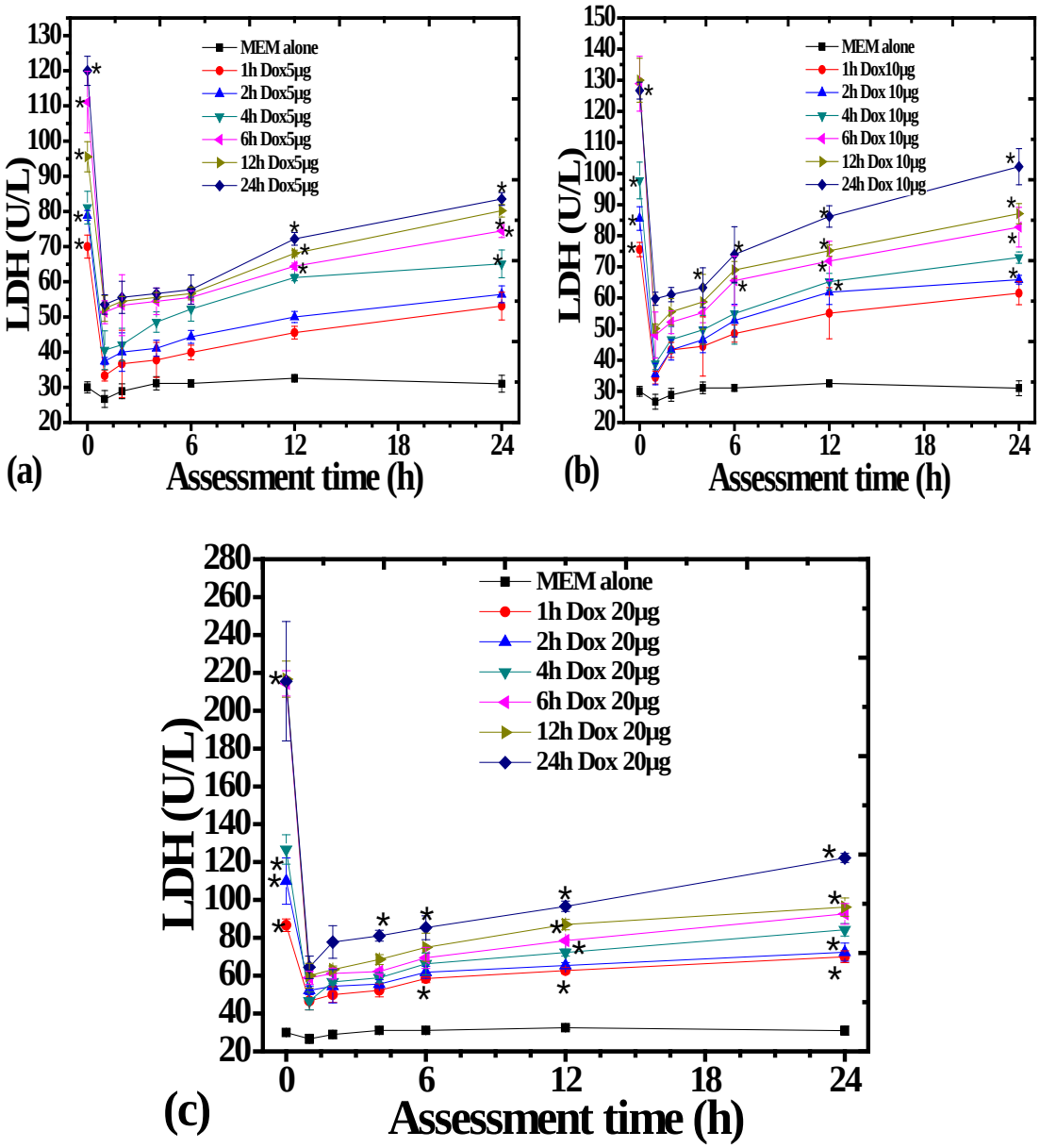

Figure 21: Alteration in the LDH release by HeLa cells treated with various concentrations of DOX at different treatment time and assessment times. (a) DOX 5µg/ml (b) DOX 10 µg/ml (c) DOX 20 µg/ml. The data represent Mean± SEM, N=3. Minimum essential media (MEM), Ethanol extract of *Oroxylum indicum* (OIE), doxorubicin (DOX).

p<0.01 when treatment groups are compared to MEM group.

Standard error of the mean (SEM).

Investigation of the anticancer activity of

Oroxylum indicum in cultured neoplastic cells

Chapter 6

Abstract

The cytotoxicity of the ethanol extract of Oroxylum indicum (OIE) and chrysin, a flavonoid present in it was assessed in HeLa, MCF-7 and HepG2 cells by MTT assay. The cells were treated with different concentrations of OIE or chrysin. Treatment of HeLa, MCF-7 and HepG2 cells with different concentrations of OIE caused a concentration and time dependent increase in the cytotoxic effect of OIE and chrysin. A separate experiment was conducted to evaluate the clonogenic potential of HeLa cells treated with OIE, where OIE was found to reduce the clonogenicity of cells in a concentration dependent manner. The ability of OIE to induce DNA damage and subsequent cytotoxicity was studied by conducting individual experiments on micronuclei and apoptosis induction, where OIE was found to increase the frequency of micronuclei in a concentration and assay time dependent manner. A maximum frequency of micronucleated mononulceate and binuceate cells was observed at 36 h post drug treatment. The OIE treatment caused a concentration dependent increase in the HeLa cells undergoing apoptosis, which increased with screening time up to 36 h post treatment and remained unaltered thereafter. In still another experiment OIE reduced the content of GSH and activities of the GST, SOD and catalase in a concentration and time dependent manner accompanied by an increased the lipid peroxidation and lactate dehydrogenase activity. The present study demonstrates that OIE induced cytotoxic effects, which may be due increased DNA damage and apoptosis accompanied by an elevated lipid peroxidation, LDH and reduced glutathione and GST, SOD and catalase activities.

1. INTRODUCTION

Cancer is as old as recorded human history but is still one of the leading causes of death all over the world both in the developed as well as in the less developed countries (Torre et al., 2012). In the developed countries, every fourth citizen is estimated to be cancer stricken sometime during his/her life and approximately 400 new incidents emerge for every 100,000 individuals diagnosed annually (Siegel et al., 2015). Despite lot of advances made with tremendous efforts to fight against the cancer, the cancer mortality still has not declined appreciably (Siegel et al., 2015). This indicates that there is still a need to find better and newer pharmacological agents to develop them as drugs for human healthcare including cancer cure (Pan et al., 2012; Newman and Cragg, 2014). Plants have always been used as medicine since time immemorial and many of the modern drugs are derived from plants either directly or indirectly (Newman and Cragg, 2014). Many drugs of today for the treatment of different diseases including cancer are obtained from natural products (Mathieu et al., 2015). In fact, about 75 % of the registered small anticancer molecules since the 1940s have originated from natural products (Newmann and Cragg, 2014).

Natural products including flowering plants have been the major source of many chemotherapy agents, before their actual chemical synthesis began. The vinca alkaloids vinblastine and vincristine isolated from the Madagascar periwinkle, *Catharanthus roseus* are used in combination with other drugs for the treatment of different types of cancers including leukemias, testicular cancer and both Hodgkin and non-Hodgkin lymphomas (Moudi et al., 2013). The taxol is another anticancer compound isolated from the bark of the Pacific Yew, *Taxus brevifolia* (Rowinsky et al., 1995; Creemers et al., 1996; Bertino, 1997) and is indicated in the treatment of

endometrial cancer, cervical carcinoma, breast cancer, non-small-cell lung cancer, and bladder cancer (Hajek et al., 2005; Khanna et al., 2015). Another class of cytotoxic drug derived from plants are podophyllotoxins the etoposide and teniposide, which have been isolated from *Podophyllum peltatum* and *Podophyllum hexandrum*. Clinically they are used in the treatment of testicular cancer, leukemias and small cell lung cancer either alone or in combination with other chemotherapeutic drugs (Liu et al., 2016.). The other chemotherapeutic drug originated from plant has been camptothecin, which was isolated from *Camptotheca acuminata* in 1966; however, its insolubility was a major concern. This problem was solved by synthesizing its water soluble derivatives topotecan and irinotecan, which are used in the treatment of ovarian cancer, cervical cancer small-cell lung cancer and colorectal cancer (Venditto and Simanek, 2010). The major drawback of these modern synthetic compounds has been their high toxicity and development of drug resistance leading to treatment failure (DeVita and Chu, 2008; Housman et al., 2014).

The cancer treatment is always a multimodality treatment where surgery, radiation, and chemotherapy are combined to cure cancer. The radiation and chemotherapy are toxic to normal cells and their therapeutic use is associated with the development of second malignancies in the survivors (Kumar, 2012; Pendelton et al., 2014; Murray et al., 2015). This indicates that plants can still provide newer molecules which may have curative effect on cancer. The secondary metabolites of plants are considered to be a very promising source for cancer cure which fuels research on traditionally used medicinal plants all over the world (Demain and Vaishnav, 2011).

Oroxylum indicum is a medium sized deciduous tree distributed throughout the Southeast Asia and some parts of India (Mao, 2002). Ayurveda, the oldest Indian

traditional system recognizes this plant as a multipurpose medicinal source and therefore is used in many of its formulations (Vaidya, 1975). The different parts of this plant are considered to possess medicinal properties (Ahmad and Ghafoor, 2002). The decoction of the root and stem bark is used for stomach ulcer and diarrhea (Neeti Mahanti, 1994; Lalramnghinglova, 2003; Lalrinzuali et al., 2015). Decoction of the root bark is used in fever, colic, stomach ulcer, constipation, indigestion, intestinal worms, strangury, asthma, cough, hiccough, diarrhea, dysentery etc. Decoction of the leaves is also useful in headaches, flatulence, ulcers, etc. and the fruits for colic, cough, diseases of the heart, bronchitis, dyspepsia, leucoderma, piles etc. Poultice of the bark is applied to rheumatism, sprains, inflammations, and other skin diseases (Sawmliana, 2013 Lalrinzuali et al., 2015). The dried seed powder is used by women to induce conception in ethnic communities (Gokhale and Bansal, 2006; Lalrinzuali et al., 2015). The bark paste is applied to kill maggots on the wounds of animals and the decoction is given to animals for deworming. The paste of bark is also applied to mouth for cancer, scabies and tonsil pain. Either the fruit or branch is used to kill crabs in wet paddy fields by the farmers (Lalrinzuali et al., 2015). The decoction of *Oroxylum indicum* bark could cure nasopharyngeal cancer and is also used for curing gastric ulcers (Mao, 2002). The earlier study from this laboratory has shown that it synthesizes several secondary metabolites including alkaloid and flavonoids (Lalrinzuali et al., 2015), which suggests that it may have potential to be developed as an anticancer drug. Therefore, the present study was undertaken to investigate the anticancer activity of *Oroxylum indicum* in various cell lines *in vitro*.

2. MATERIALS AND METHODS

2.1. Chemicals

Nitroblue tetrazolium salt (NBT), phenazine methosulphate (PMS), 5,5'dithio 2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitronbezene (CDNB), DMSO (Dimethyl sulphoxide). 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), triton X-100, ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), sodium pyruvate, thiobarbituric acid (TBA), agarose, ethidium bromide, acridine orange, crystal violet, chrysin and cytochalasin B were obtained from Sigma Chemical Co. (Bangalore, India). Sodium carbonate (Na_2CO_3), trichloroacetic acid (TCA), potassium chloride (KCl), potassium sodium-tartrate, hydrogen peroxide (H_2O_2), and Folin-Ciocalteu reagent were procured from SD Fine Chemicals, Mumbai, India, whereas sodium hydroxide (NaOH), sodium chloride (NaCl), disodium hydrogenphosphate (Na_2HPO_4), sulphuric acid (H_2SO_4), hydrochloric acid (HCl), n-butanol, Tris buffer (Tris (hydroxymethyl) aminomethane and ammonium oxalate were purchased from Merck India Limited, Mumbai, India. Trypsin EDTA 1X, MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide), MEM medium, phenol-chloroform-isoamyl alcohol (PCI), fetal bovine serum (FBS), and reduced nicotinamide adenine dinucleotide (NADH) were purchased from HiMedia, Mumbai, India. Doxorubicin was supplied by Biochem Pharmaceutical Industries, Mumbai, India and LDH kit was purchased from Coral Clinical Systems, Verna Industrial Estate, Verna, Goa, India.

2.2. Preparation of the extract

The details of identification, authentication and extraction of *Oroxylum indicum* are given elsewhere (Lalrinzuali et al., 2015b Advances in Biomed Pharm). Briefly, the non-infected stem bark of *O. indicum* was collected from Champhai,

washed, shade dried and powered using an electrical grinder at room temperature. The powdered bark of *O. indicum* was sequentially extracted with petroleum ether, chloroform, ethanol and distilled water according to increasing polarity using a Soxhlet apparatus. The liquid extracts, were filtered and concentrated by evaporating their liquid contents using rotary evaporator. Each extracts, except petroleum ether was concentrated in vacuo and stored at -70°C until further use. The ethanol extract was used for the entire study and henceforth it will be called as OIE.

2.3. Dissolution of drug/s

The doxorubicin was freshly dissolved in MEM and the *Oroxylum indicum* ethanol extract and chrysin were dissolved in DMSO and diluted with MEM and filter sterilized before use. The concentration of DMSO never exceeded 0.2%.

2.4. Cell line and Culture

HeLa S3, HepG2 and MCF-7 cells were procured from the National Centre for Cell Science, Pune, India. The cells were routinely grown in 25 cm^2 culture flasks (HiMedia, Mumbai, India) containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 µg/ml gentamicin sulfate with loosened caps at 37°C in an atmosphere of 5% CO_2 in humidified air in a CO_2 incubator (Eppendorf AG, Hamburg, Germany).

2.5. Experimental Design

A known amount of cells were inoculated and the cells were divided into the different groups depending on the experimental protocol:

2.5.1. Determination of Cytotoxicity

2.5.1.1 MTT assay

MEM group: The cells of this group were treated with 2 µl/ml of DMSO.

OIE group: This group of cells was treated with different concentrations of OIE.

CHY group: The cells of this group of were treated with 5, 20, 40, 60, and 80 µg/ml of chrysin (CHY).

DOX group: The cell cultures of this were treated with 5, 20, 40, 60, and 80 µg/ml of doxorubicin (DOX) that served as positive control.

The cytotoxic effects of different concentrations of ethanol extract of *Oroxylum indicum* and chrysin was studied by MTT assay in HeLa, HepG2 and MCF-7 cells as described by Mosmann (1983). Usually 10^4 cells were seeded into 96 well plates in 100 µl minimum essential medium (MEM). The cells were incubated at 37 ºC in a CO_2 incubator in an atmosphere of 5% CO_2 in 95 % humidified air. The cells were allowed to attach for 24 hours. Different concentrations of OIE or chrysin or doxorubicin were added into each well of the microplates and incubated in the CO_2 incubator. After 48 hours, 20 µl of MTT was added into each well and the microplates were incubated for another 2 hours. The drug containing media were removed and the insoluble purple formazan formed was dissolved with DMSO and incubated once again for 4 hours after which the absorbance was measured at 560 nm. The cytotoxicity was calculated by using the formula Control-Treatment/Control X 100. The IC50 was also calculated using GraphPad Prism 6 software.

2.5.2. Determination of optimum exposure time for cytotoxicity

A separate experiment was conducted to study the effect of treatment time of OIE on the cytotoxicity, where grouping and other conditions were essentially similar to that described above except that the cells were exposed to OIE for different times and the cytotoxicity was determined by MTT assay as described above.

2.5.3. The Determination of anticancer activity

The anticancer activity of OIE was determined by inoculating 10^6 exponentially growing HeLa cells into several culture flasks. The cells were allowed to attach for 24 h and were divided into the following groups:

MEM group: The cells of this group were treated with 2 µl/ml of DMSO.

OIE group: This group of cells was treated with different concentrations of 20, 40, or 80 µg/ml OIE for 6 h.

DOX group: The cell cultures of this were treated with 5, 10 or 20 µg/ml DOX and served as a positive control.

After 6 hours of drug treatment the media were removed and the flasks were washed twice with sterile PBS, subjected to trypsin EDTA treatment and the following studies were conducted.

2.5.4. Clonogenic Assay

Usually 200 HeLa cells were seeded into several individual petridishes containing 5 ml MEM and allowed to grow for another 11 days. The resultant colonies of cells were stained with 1 % crystal violet in methanol and scored. Plating efficiency (PE) of the cells was determined and surviving fraction (SF) calculated.

PE = (Number of colonies counted x 100) / (Number of cells seeded)

SF = (Number of colonies counted) / (Number of cells seeded) x (mean plating efficiency).

2.5.5. Micronucleus Assay

The $5X10^5$ cells remaining after clonogenic assay were seeded in triplicate for each concentration and allowed to grow for 12, 24, 36 and 48 h. The micronuclei were prepared according to the modified method of Fenech and Morley, (1985). Briefly, the cells were allowed to attach for 6 h to attach, after which 5 µg/ml of cytochalasin-

B was added to inhibit cytokinesis. The cells were left undisturbed and terminated at 12, 24, 36 and 48 h post-drug-treatment, where the media containing cytochalasin-B were removed. The cells were washed with PBS, dislodged by trypsin-EDTA treatment and pelleted by centrifugation at 1000 rpm for 5 min. The cell pellet was disturbed and subjected to mild hypotonic treatment (0.75% ammonium oxalate) at 37°C, centrifuged again and the resultant cell pellet was fixed in Carnoy's fixative 3:1 (methanol: acetic acid). After centrifugation, the cells were resuspended in a small volume of fixative and spread on to pre cleaned coded slides to avoid observer's bias. The slides containing cells were stained with 0.025% acridine orange (BDH, England, Gurr Cat. no. 34001 9704640E) in Sorensen's buffer (pH 6.8) and subsequently washed twice in the buffer to remove excess stain. The slides mounted in Sorensen's buffer were observed under a fluorescence microscope (DM-2500, Leica Microsystems, Wetzlar, Germany) equipped with 450–490 nm BP filter set with excitation at 453 nm using a 20 X N Plan objective. A minimum of one thousand binucleate cells with well-preserved cytoplasm was scored for each concentration and post-treatment time. The frequency of mononucleated cells bearing micronuclei as well as binucleated cell bearing micronuclei was determined. The micronucleated cells were scored according to the criteria of Kirsch-Volders et al., (2003) and Fenech et al., (2003).

2.5.6. Apoptosis Assay

The $4X10^6$ HeLa cells remaining after clonogenic and micronucleus assay were inoculated in several six well culture plates (Himedia, Mumbai, India) and the cultures from all the groups were terminated at 2, 4, 12, and 24 h post-drug treatment. The ability of the drugs to induce apoptosis was studied using standard protocol. Briefly, the cells were labeled with nucleic acid-binding dye mix of 25 µg/ml acridine

orange and 25 µg/ml ethidium bromide in the ratio of 1:1:1 in PBS (Cohen, 1993). The cells were examined under a fluorescence microscope (DM-2500, Leica Microsystems, Wetzlar, Germany). For each sample, at least 1000 cells were scored, and apoptotic cell index was determined as follows:

Apoptotic index (%) = Number of apoptotic cells scored X 100/Total number of cells counted.

2.5.7. DNA fragmentation assay

Since DNA fragmentation is a biochemical marker of apoptosis, the ability of OIE to induce apoptosis in HeLa cells was studied by conducting a separate experiment, where grouping and other conditions were essentially same as described above except that 1×10^6 exponentially growing HeLa cells were inoculated into individual wells of six well plates and treated with OIE or DOX. The cells were harvested at 2, 4, 12 and 24 h post-drug treatment and the DNA ladder formation was detected by SDS/proteinase-K/RNase method which allowed the isolation of only fragmented DNA without contamination with RNA (Herrmann et. al., 1994). Briefly, the cells were pelleted, washed in cold PBS, lysed in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.2 % Triton X-100 and kept at 4°C for 20 min, and the contents were centrifuged at 14,000 g for 15 min. The supernatant was collected and mixed with proteinase K (0.5 mg/ml) and incubated for 1 h at 37°C followed by the RNase-A (0.5 mg/ml) treatment for 1 h at 50°C. The DNA was extracted with phenol, chloroform and isoamyl alcohol (1:1:1) and precipitated with ammonium acetate (3 M) and chilled isopropanol. Various samples were loaded on to 1 % agarose gel placed in a horizontal electrophoresis tank containing TBE (44.6 mM Tris, 44.5 mM boric acid and 1 mM EDTA) buffer. The DNA was resolved at 100

Volts and 40 mA and the resultant DNA fragmentation ladder was visualized under 265 nM UV light by staining the gel with ethidium bromide (0.5 µg/ml).

2.6. Biochemical assays

A separate experiment was carried out to estimate the effect of OIE on the activities of various antioxidant enzymes, and lactate dehydrogenase and lipid peroxidation in HeLa cells, where grouping and other conditions were essential similar to that described for anticancer activity except that the cell cultures were terminated at 1, 2, 4, 6, 12 and 24 h post drug treatment. The drug containing media were removed; the cells were washed with sterile PBS and the dislodged using trypsin EDTA treatment. The cells were pelleted and weighed and 5% homogenate of the cells was prepared in PBS (pH 7.4) using sonicator (PCI Analytics Pvt. Ltd., Mumbai, India).

2.6.1. Glutathione estimation

Glutathione was estimated as described earlier (Moron et al., 1929). Glutathione concentration was measured by its reaction with DTNB (Ellman's reaction) to give a compound that absorbs light at 412 nm. Briefly, 1.8 ml of 0.2 M Na_2HPO_4 was mixed with 40 µl 10 mM DTNB and 160 µl of cell homogenate. The blank consisted of distilled water instead of cell homogenate. The mixture was allowed to stand for 2 minutes and the absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India).

2.6.2. Glutathione - S – transferase estimation

Glutathione-s-transferase activity was estimated by the method of Habig et al., (1974). Briefly, 0.5 ml of 0.1 M phosphate buffer pH 6.5, 0.1ml of 20 mM CDNB, and 8.8 ml distilled water were incubated at 37°C for 10 min. After incubation, 0.5 ml of 20 mM GSH and 0.1 ml of cell homogenate were added. The absorbance was read

against the blank (distilled water) at 340 nm at 1 min intervals for 6 minutes in UV-VIS Biospectrophotometer.

2.6.3. Catalase estimation

Catalase was assayed according to technique described by Aebi (1984). Briefly, in a 3 ml cuvette, 20 µl of sample was diluted with 1.98 ml of 50 mM phosphate buffer (pH 7.0). The reaction (maintained at 20ºC) was started by adding 1 ml of 30 mM H_2O_2 and the decrease in absorbance was monitored at 240 nm for 60 seconds in a UV-VIS Biospectrophotometer.

2.6.4. Superoxide dismutase estimation

SOD is an enzyme that catalyzes dismutation of two superoxide anions (O_2^-) into hydrogen peroxide and molecular oxygen and its activity was estimated as described by Fried (1975). Briefly, 100 µl of cell homogenate, 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitroblue tetrazolium, 200 µl of 780 µM NADH were incubated for 90 seconds at 30°C. The reaction was terminated by adding 1000 µl of acetic acid and the addition of 4 ml n-butanol. The absorbance was recorded at 560 nm using a UV-VIS Biospectrophotometer. The percent inhibition was calculated by measuring the absorbance of blank without SOD enzyme and the test with SOD enzyme samples using the formula (Blank-Sample)/Blank X 100.

2.6.5. Estimation of Lipid peroxidation

Lipid peroxidation (LOO) assay was carried out following the method of Buege and Aust (1978). Briefly, 1 ml of tissue homogenate was mixed with 2 ml of TCA-TBA-HCl reagent and mixed thoroughly. The reaction mixture was heated in a boiling water bath for 15 minutes, cooled immediately to room temperature, centrifuged at 1000 rpm for 10 min and supernatant was collected and its absorbance was read at 535 nm against blank in a UV-VIS Biospectrophotometer. The blank

contained all the reagents minus the cell homogenate substituted with distilled water. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56x10^{6}M^{-1}cm^{-1}$.

2.6.6. Lactate dehydrogenase estimation

A separate experiment was conducted to study the activity of the enzyme lactate dehydrogenase as described earlier (Decker and Lohmann-Matthes, 1988) using LDH kit purchased from Coral Clinical Systems. Briefly, $1x10^{5}$ cells were seeded in several twelve well plates and treated with different concentrations (20, 40 and 80 µg/ml) of OIE for 1, 2, 4, 6, 12 and 24 h. After each exposure time was over, the whole medium was removed, washed with sterile PBS and replaced with a new medium of the same volume. This was counted as 0 hour for each exposure time. 50 µl of the medium was removed and replaced with a new medium at 1, 2, 4, 6, 12 and 24 h. 50 µl of the media was mixed with 1 ml of working reagent prepared by mixing buffer reagent and starter reagent in the ratio of 8:2 according to the manufacturer's protocol. The activity of LDH was estimated by recording the absorbance of the medium every minute for four minutes at 25°C in a UV-VIS Biospectrophotometer and the mean absorbance change per minute (ΔA/ min) was calculated. The activity of LDH has been expressed as unit per liter using the formula LDH activity in U/L = ΔA/ min. x 3333.

3. Statistical Analysis

The statistical analyses were performed using Origin Pro 8. All the results are expressed as mean ± standard error mean (S.E.M). Experimental data were analyzed by one way ANOVA followed by Tukey's test for multiple comparisons for different parameters between the groups. A P value of < 0.05 was considered as significant.

4. RESULTS

The results are expressed in table 1-14 and figure 1-21 as mean ± standard error of the mean.

4.1. Determination of Cytotoxicity

Treatment of HeLa, HepG2 and MCF-7 cells with different concentrations of OIE caused a concentration dependent increase in the cytotoxicity and the maximum cytotoxic effect was observed at the highest concentrations of OIE used. Treatment of HeLa cells with different concentrations of OIE induced highest toxic effect at a concentration of 40 µg/ml and increasing concentrations did not increase the cytotoxic effect significantly as the difference between 40 µg/ml was statistically non-significant (Figure 1). Similarly, OIE induced maximum cytotoxicity at 40 µg/ml in HepG2 cells and increasing concentrations up to 100 µg/ml resulted in approximately 10 and 14% rise in cytotoxic effect for 80 and 100 µg/ml OIE, respectively (Figure 2). The breast cancer MCF-7 cells were most sensitive to OIE treatment, where 52% cytotoxicity was recorded for 20 µg/ml and a maximum of 77% cytotoxicity in the cells treated with 80 µg/ml OIE (Figure 3). The chrysin, a flavonoid present in OIE was also screened for its cytotoxic effect and it has also shown a dose dependent cytotoxicity, where the pattern of cytotoxicity was almost similar to OIE except that it was less cytotoxic at similar concentrations than the OIE. (Table 1 Figure 1-3). The positive control DOX was the most cytotoxic drug when compared to other two (Figure 1-3).

4.2. Determination of optimum exposure time for cytotoxicity

The optimum exposure time for cytotoxic effect of OIE and chrysin was also evaluated by MTT assay. The OIE treatment resulted in a time dependent increase in the cytotoxicity in HeLa, HepG2 and MCF-7 cells and maximum cytotoxic effect was

observed in the cells treated with OIE or chrysin for 24 (Figure 3-6). Interestingly the OIE and chrysin have shown higher or comparable toxic effect at early times than DOX. The HeLa and HepG2 cells were equally sensitive; however, MCF-7 cells showed less cytotoxicity than the HeLa and HepG2 cells (Figure 3-6). The 6 h drug treatment time was selected for further experimentation as the cytotoxic effect was almost 50%.

4.3. Clonogenic Assay

Treatment of HeLa cells with different concentrations of OIE caused a concentration dependent decline in the clonogenicity of cells. A maximum decline in the clonogenicity was observed for 80 µg/ml OIE, where a drastic reduction in the cell survival was observed. (Figure 7). The reduction in clonogenic potential by OIE was comparable to positive control DOX except the fact that the doses required by OIE were four times greater than the DOX (Figure 7). IC50 was found to be 50 µg/ml for OIE treatment. The data fitted on a linear quadratic model with an r square of 0.999.

4.4. Micronucleus

The occurrence of OIE induced micronuclei formation was scored in mononucleate as well as binucleate HeLa cells treated with different concentrations of OIE. The frequency of mononucleate cell bearing one and two micronuclei (MNMNC) increased in a concentration dependent fashion in the OIE treated group (Table 4 and Figure 8). The frequency of mononucleate cells bearing micronuclei significantly increased at 12 h post treatment and continued to rise with scoring time up to 36 h post OIE treatment, where a maximum frequency of MNMNCs was recorded. Thereafter the frequency of MNMNC declined at 48 h post treatment (Table 4 and Figure 8-10). The lowest concentration d 20 µg resulted in a 3 fold rise in MNMNCs at 12 h, whereas this rise was 6.4 folds for 80 µg/ml OIE. The number of

binuleated cells bearing one and two micronuclei (MNBNC) elevated in a concentration and scoring time dependent manner up to 36 h post treatment where the frequency of MNBNCs was greatest and it declined thereafter by 48 h post treatment (Table 4 and Figure 9-10). This increase in MNBNC at 36 h was 13.8, 19 and 20.7 fold greater for 20, 40 and 80 µg/ml OIE respectively (Table 4).

4.5. Apoptosis

The exposure of HeLa cells to different concentrations of OIE resulted in a concentration and time dependent rise in the number of apoptotic cells undergoing apoptosis. The apoptosis was characterized by chromosome condensasation, DNA fragmentation and membrane blebbing. The apoptotic cells showed a gradual rise with scoring time and a maxima was achieved by 12 h post treatment (Figure 12), thereafter this rise was almost same as that of 12 h (Table 5 and Figure 11). Treatment of HeLa cells with various concentrations resulted in 8, 9 and 13 fold rise in the number of apoptotic cells undergoing apoptosis at 12 h post treatment for 20, 40 and 80 µg/ml OIE. The apoptosis induction was comparable to positive control doxorubicin (Table 5 and Figure 11). The morphological findings of apoptosis were confirmed by DNA laddering shown in Figure 13.

4.6. Glutathione

Treatment of HeLa cells with different concentrations of OIE caused a statistically significant and concentration dependent reduction in glutathione contents at all the post-treatment times (Figure 14). A maximum attrition of 11.7 fold in glutathione concentration was observed for 80 µg/ml OIE at 24 h, when compared to MEM group (Table 6). The concentration of glutathione also declined in a similar fashion in DOX treated group (Table 6 and Figure 14).

4.7. Glutathione-s-transferases

The GST activity declined in a concentration dependent manner in HeLa cell exposed 0-80 µg/ml OIE (Figure 15). The GST activity also reduced with assay time and a nadir was observed at 24 h post-drug treatment (Table 7, Figure 15). It was 2.7 fold lower after 80 µg/ml OIE treatment when compared to MEM treatment at 24 h post-drug treatment. The decline in GST activity in OIE group was comparable to DOX treatment (Table 7 and Figure 15).

4.8. Catalase

The assay of catalase activity showed a gradual but dose dependent alleviation in HeLa cells with increasing concentration of OIE (Figure 16). The catalase activity also declined with post-treatment assay time and the highest decrement in catalase activity was recorded at 24 h post OIE treatment, where it was 4.3 fold lower than MEM group in cells exposed to 80 µg/ml OIE (Table 8). The DOX treatment also resulted in an identical decline in catalase activity at all post treatment times (Table 8, Figure 16).

4.9. Superoxide dismutase

The exposure of HeLa cells to different concentrations of OIE caused a concentrate dependent reduction in the SOD activity (Figure 17). The SOD activity also showed a time dependent decrease with a maximum attrition at 24 h post – treatment, where it was 4.4 fold lower in 80 µg/ml OIE treated group than MEM group (Table 9). The positive control DOX also showed a pattern similar to that of OIE treatment (Figure 17).

4.10. Lipid Peroxidation

The treatment of HeLa cells with different concentrations of OIE induced LOO efficiently as indicated by a concentration dependent rise in the LOO at all post-

treatment time (Figure 18). The maximum LOO was estimated at 12 h post –treatment in all the groups, where it was 8.6, 9 and 14 folds greater after 20, 40 and 80 µg/ml OIE treatment than MEM group (Table 10). The LOO registered a decline at 24 h post drug treatment however it was still 5.8, 7 and 8 fold higher at 2, 40 and 80 µg.ml OIE, when compared to MEM treatment (Table 10). The DOX treatment also showed a pattern similar to that of OIE (Figure 18).

4.11. Lactate dehydrogenase estimation

The LDH release in the medium of HeLa cells increased with increasing concentrations of OIE (Figure 19). The LDH activity also increased with increase in drug/s treatment time and post- treatment assay time (Figure 20). The maximum increase in LDH activity was recorded at 0 h post treatment time as media was collected after duration of treatment was over and subsequent measurement were at different post drug treatment times (Table 11). The maximum LDH release was observed in the cells that were in contact with the drug/s for 24 h and also at 24 h after removal of the drugs (Table 12-14).

5. DISCUSSION

The metastatic cancer was treated using chemotherapy in 1956 and since then several synthetic chemical molecules have been successfully used in the treatment of cancer either alone or in combination with radiation and surgery. However, normal toxicity has been a major concern as most of the chemotherapeutic drugs act by damaging the cellular DNA and the long term use of the chemotherapeutic drugs has been found to induce second malignancies in the survivors (Pendelton et al., 2014). The number of modern chemotherapeutic drugs has been derived initial from plants before their actual chemical synthesis (Cragg and Newman, 2014). The plant still forms the major source for new drug development. It is also necessary that chemotherapy drugs do not

produce systemic toxicity and shall be well tolerated by the patients. The screening of plants provides a major area for new drug discovery. Therefore the present study was designed to evaluate the antineoplastic action of *Oroxylum indicum in vitro*.

The MTT assay is a rapid and standard technique to determine the cytotoxicity of any drug and the treatment of MCF-7, HeLa and HepG2 cells caused a concentration dependent rise in the cytotoxicity of ethanol extract of *Oroxylum indicum* and chrysin due the reduction of MTT by mitochondrial succinate dehydrogenase by these cells. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. Hence, the weaker the color formed, the more are the dead cells. MTT assay has been used to test cytotoxicity in various cell lines earlier (Mossmann, 1983). The results indicate that chrysin was less cytotoxic than OIE in HeLa, MCF and HepG2 cells. The butanol extract of *Oroxylum indicum* root has been reported to increase the cytotoxicity in a concentration dependent manner in MCF-7 cells (Dhru et al., 2016). The cytotoxic effect of OIE was further confirmed by clonogenic assay where OIE treatment caused a concentration dependent decline in the clonogenicity of HeLa cells. A far as authors are aware the anticancer activity of OIE has not been tested using clonogenic assay however it has been used to test cytotoxic effects of extracts of *Tinospora cordifolia*, and *Aphanmixis polystchya* and doxorubicin, daunorubicin and cytarabine earlier (Jagetia and Nayak, 1994; Williams et al., 2010; Jagetia and Rao, 2011, 2015; Jagetia and Venkatesha, 2016). It has been considered as gold standard in in radiobiological studies.

Most of the anticancer agents kill neoplastic cells by inflicting severe DNA damage, where recovery is almost impossible. The OIE induced micronuclei in a concentration dependent manner in HeLa cells. The micronuclei induction was

equally efficient in mononucleate as well as binucleate cells. Further OIE also efficiently induced cells bearing two or more micronuclei indicating its effectiveness in triggering complex multiply site of DNA damage in HeLa cells. Analysis of micronuclei provides an indication of mechanisms of cells death as they arise due induction of damaged DNA including DNA double strand breaks, DNA exchanges, multiply sites of DNA damage, chromosome damage, and spindle dysfunction (Lau et al., 2009; Sage and Harrison, 2011; Jagetia and Rao, 2015: Jagetia and Venkatesha, 2016). The multi-micronucleated cells are produced due to the production of complex multiply sites of DNA damage (Sage and Harrison, 2011). The earlier studies have indicated that micronuclei bearing cells are dying cells and a direct correlation has been reported between cell death and micronuclei formation (Jagetia and Aruna, 2000; Jagetia and Venkatesh, 2015, Jagetia and Venkatesha, 2016). Micronuclei assessment gives information similar to the clonogenic assay in a shorter time (Jagetia and Aruna, 2000; Jagetia and Venkatesh, 2015, Jagetia and Venkatesha, 2016) and the present study also supports it as there has been a direct correlation between the decline in clonogenicity of cells and micronuclei formation. Higher was the formation of micronuclei lower was the clonogenicity of HeLa cells.

Apoptosis is another mechanism that is triggered by the damaged cells to exclude it from the system and it is also one of the ways by which chemotherapeutic agents kill cancer cells. Apoptosis is characterized by a set of morphological alterations such as chromatin condensation, nuclear fragmentation, membrane blebbing, and cell shrinkage that stimulate biochemical and molecular events leading to death of the cells (Khan et al., 2007; Alberts et al., 2010; Nikoletopoulou et al., 2013). Certain plant extracts including *Acacia hydaspica* and *Avicennia marina* have been reported to kill various cancer cells in vitro by triggering apoptosis (Afsar et al.,

2016; Huang et al., 2016). Similarly, artichoke polyphenols were found to bring cell death in several cultured cell lines by induction of apoptosis (Mileo et al., 2016).

The increase oxidative stress may kill anticancer cells by eliciting various mechanisms of cell death. Cells possess effective antioxidant systems that can either protect or recover from oxidative injury. This system includes low molecular weight antioxidant like glutathione, vitamins, and antioxidant enzymes such as catalase, superoxide dismutase etc. (Halliwell and Gutteridge, 1984; Singh, 1992; Witt et al., 1992). Therefore, effect of OIE on oxidative stress was also studied in HeLa cells.

Glutathione (γ -glutamylcysteinyl glycine) is the most abundant non-protein molecule in the cell and has a number of physiological roles including neutralization of excess free radicals produced in the cells during numerous physiological processes (Lushchak, 2012; Schumacker, 2015). Glutathione occurs in two forms namely the reduced form (GSH) and the oxidized form (GSSG). Under normal physiological conditions mammalian cells maintain more than 98% of glutathione in the reduced form (GSH). When the oxidative stress is elevated, the reduced form of GSH is converted into the oxidized form GSSG, which can be reverted back by the action of enzyme glutathione reductase (Ramsay and Dilda, 2014). The major function of GSH is the detoxification of xenobiotics or their metabolites. In normal conditions, GSH protects the cells against the damaging effects of free radicals, xenobiotics, ionizing radiations and some cytokines. It also regulates DNA synthesis, cell proliferation and the carcinogenic mechanisms. In cancer cells, it is indicated in tumor microenvironment-related aggression, apoptosis evasion, colonizing ability, and multidrug and radiation resistance (Obrador, 2002; Franco et al., 2009; Ortega et al., 2010; 2011). Increased concentration of GSH in the tumor cells have been reported to make the tumor refractory to treatment, while depletion of glutathione has been

reported to enhance the cell death and apoptosis of the tumor cells along with the loss of essential sulfhydryl groups that result in an alteration of the calcium homeostasis and eventually loss of cell viability (Neal et al., 2003; Ramsay and Dilda, 2014). Therefore, decreasing the level of glutathione in cancer cells is one way to enhance the cytotoxic effects of chemotherapeutic agents (Mayer et al., 1987). In the present study, OIE was able to reduce the glutathione level which may be accountable for its anticancer activity and increased cell death.

The glutathione-S-transferases, discovered in the early 1970s, are a multi-gene family of enzymes that are mainly involved in the detoxification and activation of certain chemicals (Eaton and Bammler, 1999). GSTs catalyze the nucleophilic attack of glutathione (GSH) on electrophilic substrates by binding with glutathione on its hydrophilic G-site and its adjacent H-site with the electrophilic substrates to bring them in a close proximity. They also activate the sulfhydryl group on GSH, thereby allowing for nucleophilic attack of GSH on the electrophilic substrate (Armstrong, 1997). The GSTs have emerged as a promising therapeutic target because specific isozymes are overexpressed in a wide variety of tumors and may play a major role in the resistance to cancer therapy. Elevated levels of GST in tumor cells are associated with increased resistance to apoptosis (McIlwain et al., 2006; Zeng et al., 2014). Various GST inhibitors have been shown to modulate drug resistance by sensitizing tumor cells to anticancer drugs. The alleviated activity of GST by OIE could also be responsible for its cancer activity in the present study as drug inhibiting GST activity have been found to sensitize the cells (Townsend and Tew, 2003; Laborde, 2010).

The principal role of catalase is to detoxify H_2O_2 into water and oxygen but is also involved in various other processes. The reports regarding the expression of catalase in cancers are heterogeneous. High levels of catalase have been reported in

patients with lung cancer, whereas decreased levels of catalase were indicated in breast cancer, head and neck cancer, gynaecological cancer, lymphoma, prostate cancer and urological cancer (Kodydková et al., 2014). The HeLa cells have shown raised catalase activity, whereas OIE administration decreased the activity of catalase indicating that OIE action may be mediated by reducing catalase activity in HeLa cells. The over expression of catalase has been reported to reduce the apoptosis in tumor cells after chemotherapy (Bechtel and Bauer, 2009).

Superoxide Dismutase (SOD) present in all oxygen metabolizing cells, catalyzes the dismutation of the superoxide radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2) (Weydert and Cullen, 2010; Che et al., 2016). To date, four different forms of SOD are known (Fridovich, 1974) of which the CuZnSOD is the most abundant and comprises approximately 90% of total SOD activity in a eukaryotic cell (Liu et al., 2004). All the SODs are very efficient scavengers of the superoxide radical and cell damage occurs when there is not enough SOD to handle the flux of O_2^{-} (Oberley and Bueftner, 1979; Scandalios, 1993). Malignant cells accumulate several differences from normal cells during transformation and the Cu-Zn SOD activity was found to be increased several fold after transformation in SV4O-transformed cells (Yamanaka and Deamer, 1974). The role of SOD in cancer is controversial as it is found to be overexpressed in some cancers, whereas other cancer show reduced expression (Chen et al., 2016). The SOD are overexpressed in late stage of cancer, especially metastatic tumors (Che et al., 2016) indicating that high levels of SOD in tumors may make them resistance to therapy. The OIE treatment reduced SOD activity in HeLa cells, which may account for its higher cell killing activity.

The oxidative degradation of lipids caused by free radicals by stealing electrons is known as lipid peroxidation. Malondialdehyde (MDA) is a major product of lipid peroxidation (Rice-Evans and Burdon, 1993). MDA has the ability to react with nucleic acid bases and form adducts to dG, dA, and dC (Marnett, 2002). Lipid peroxidation has been implicated in the pathogenesis of a number of diseases including cancer due to its ability to damage DNA and subsequent mutations in the tumor suppressor genes (Cejas et al., 2004; Zhong and Yin, 2015). This property of lipid peroxidation may be useful in killing tumor cells by those therapeutic modalities that increase LOO in the tumor cells. The HeLa cells treated with OIE showed enhanced lipid peroxidation that may be responsible for its increased cell killing effect.

The exact mechanism by which *Oroxylum indicum* killed HeLa cells is not clearly understood. However, putative mechanisms responsible for this effect could be several. The most important of them is the increase in DNA damage and apoptosis by OIE in the present study. The increased lipid peroxidation by OIE in HeLa cells may have been responsible in elevating the DNA damage. The reduction in GSH, GST, CAT and SOD by OIE may have led to this increase in oxidative stress causing greater cell kill by increasing apoptosis. The DNA damage in HeLa cells may have been also caused by inhibition of topoisomerase II enzyme by OIE. Chrysin one of the flavonoids present in OIE has been reported to act as topoisomerase II poison in an earlier study (Bandele and Osheroff, 2007). The depletion of antioxidants may be due to the downregulation of Nrf2 transcription by OIE. Chrysin has been reported to suppress the transcription of Nrf2 gene by inhibiting PI3K-Akt and ERK pathway earlier (Gao et al., 2013). NF-κB is another transcription factor which is overexpressed by most of the tumor cell lines and inhibition of NF-κB and COX-II

transcription by OIE may have been responsible for greater cell kill in the present study. The dichloromethane extract of the stem bark of Oroxylum has been reported to inhibit NF-κB activation recently (Tran et al., 2015). Chrysin present in OIE has been also reported to inhibit NF-κB and COX-II transcription (Woo et al., 2005).

6. CONCLUSIONS

It is concluded that OIE administration caused effective killing of HeLa cells and the anticancer activity of OIE may due to increased DNA damage and apoptosis, which may have been due to attrition in the GSH and other antioxidant enzymes including GST, CAT and SOD. The decline in antioxidant has increased the lipid peroxidation that would have brought effective cell kill by inducing DNA damage after OIE treatment. The OIE may have also inhibited the activity of topoisomerase – II inducing greater DNA damage in the HeLa cells. OIE may have also retarded the transcriptional activation of Nrf2, NF-κB and COX-II, which may have contributed in their own way in killing the HeLa cells.

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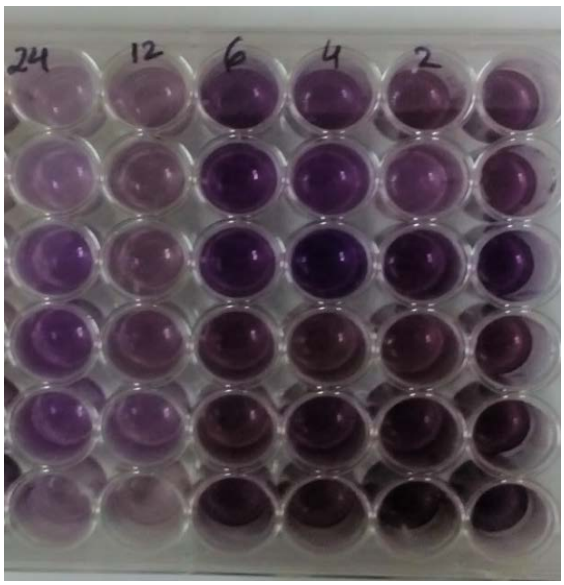
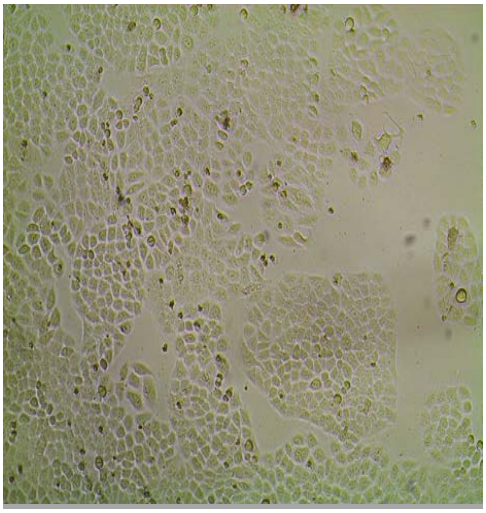
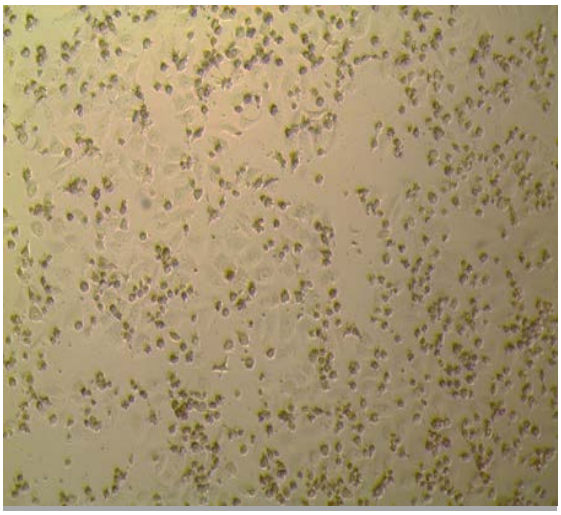
Cultured cells at 37˚C in 5% CO_2



MTT Assay

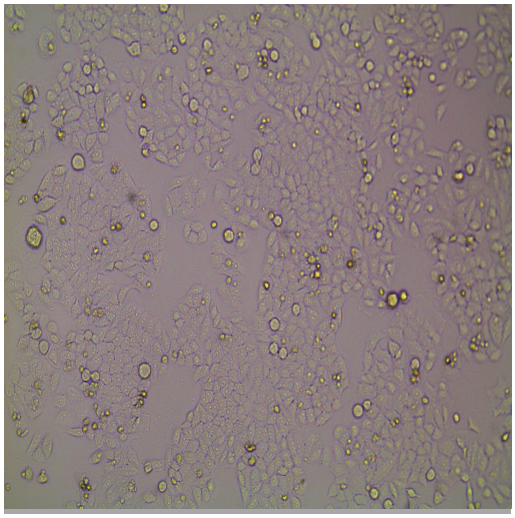


Untreated Hela Cells

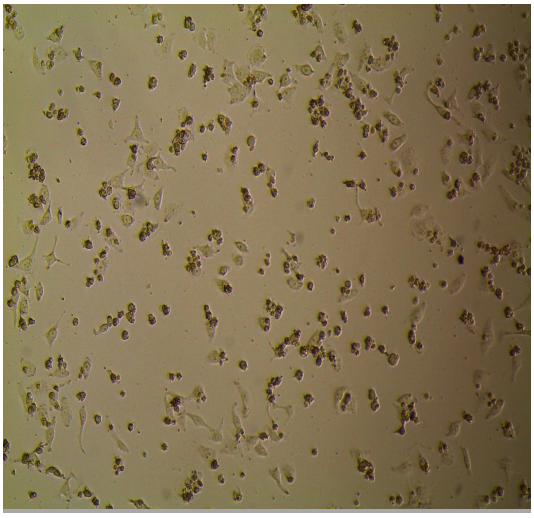


Hela Cells treated with OIE

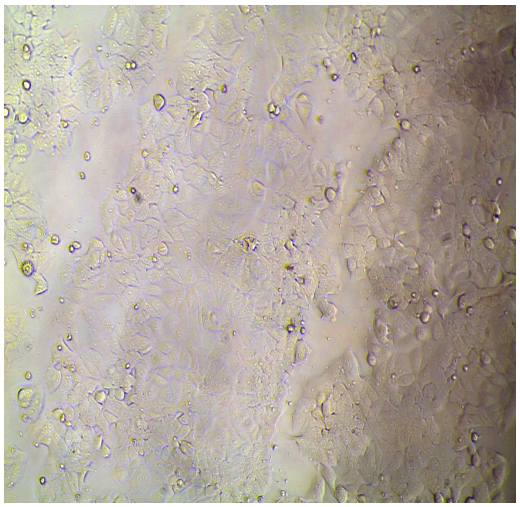
Plate 1: Cervical cancer HeLa cells treated with *Oroxylum indicum* extract (OIE).



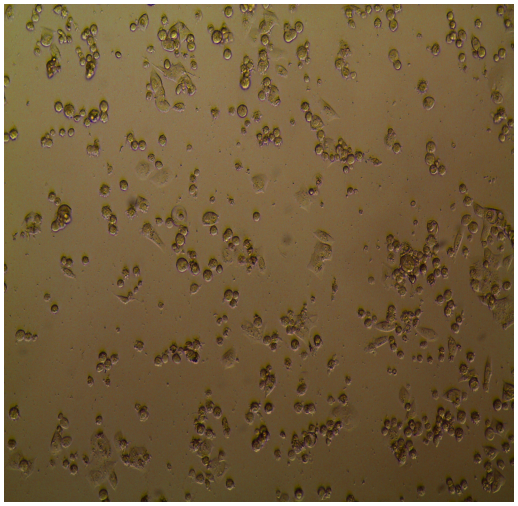
Untreated HepG2 Cells



HepG2 cells treated with OIE



Untreated MCF-7 Cells



MCF-7 cells treated with OIE

Plate II: Liver cancer (HepG2) cells and breast cancer (MCF-7) exposed to *Oroxylum indicum* extracts.

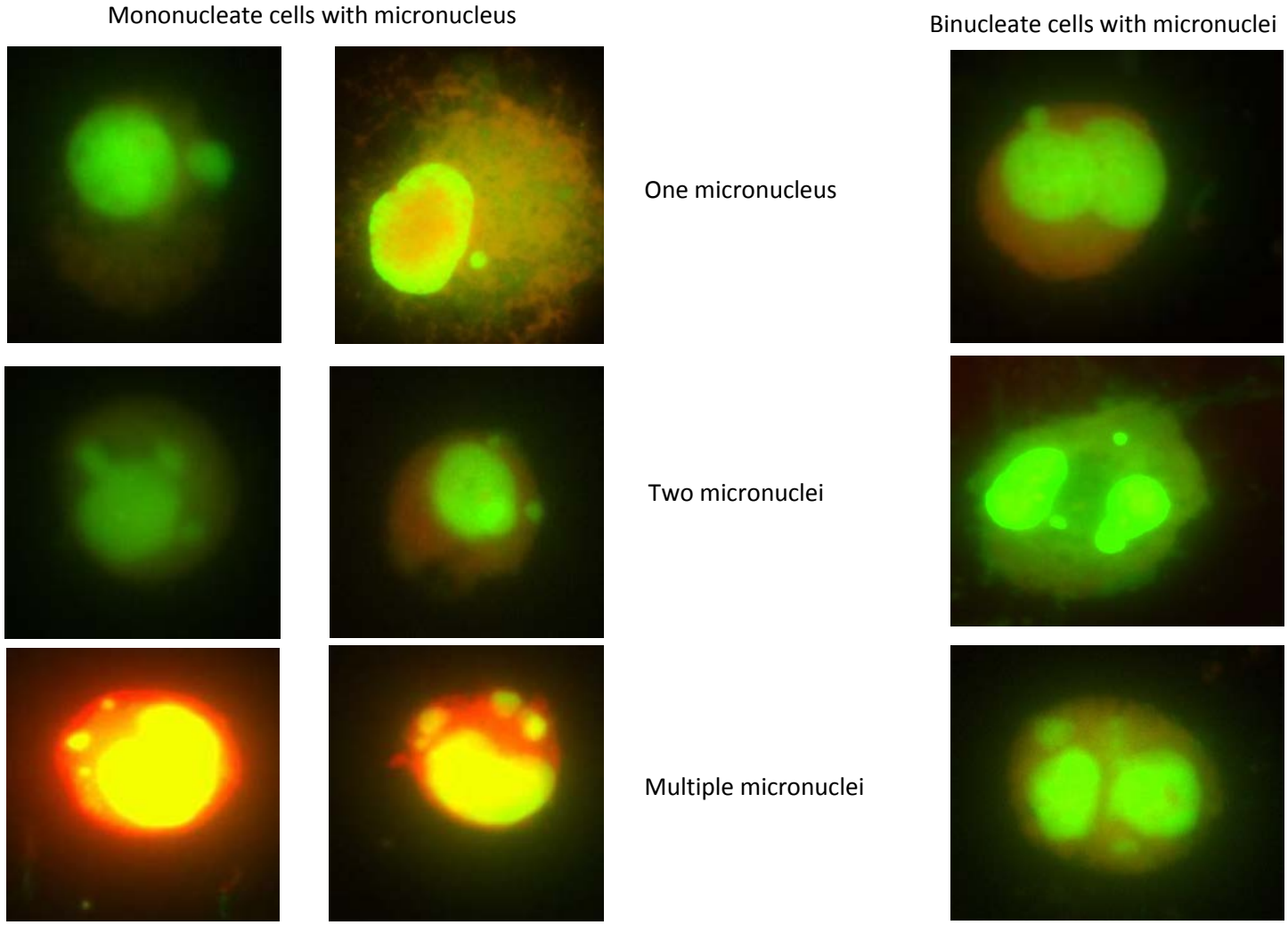
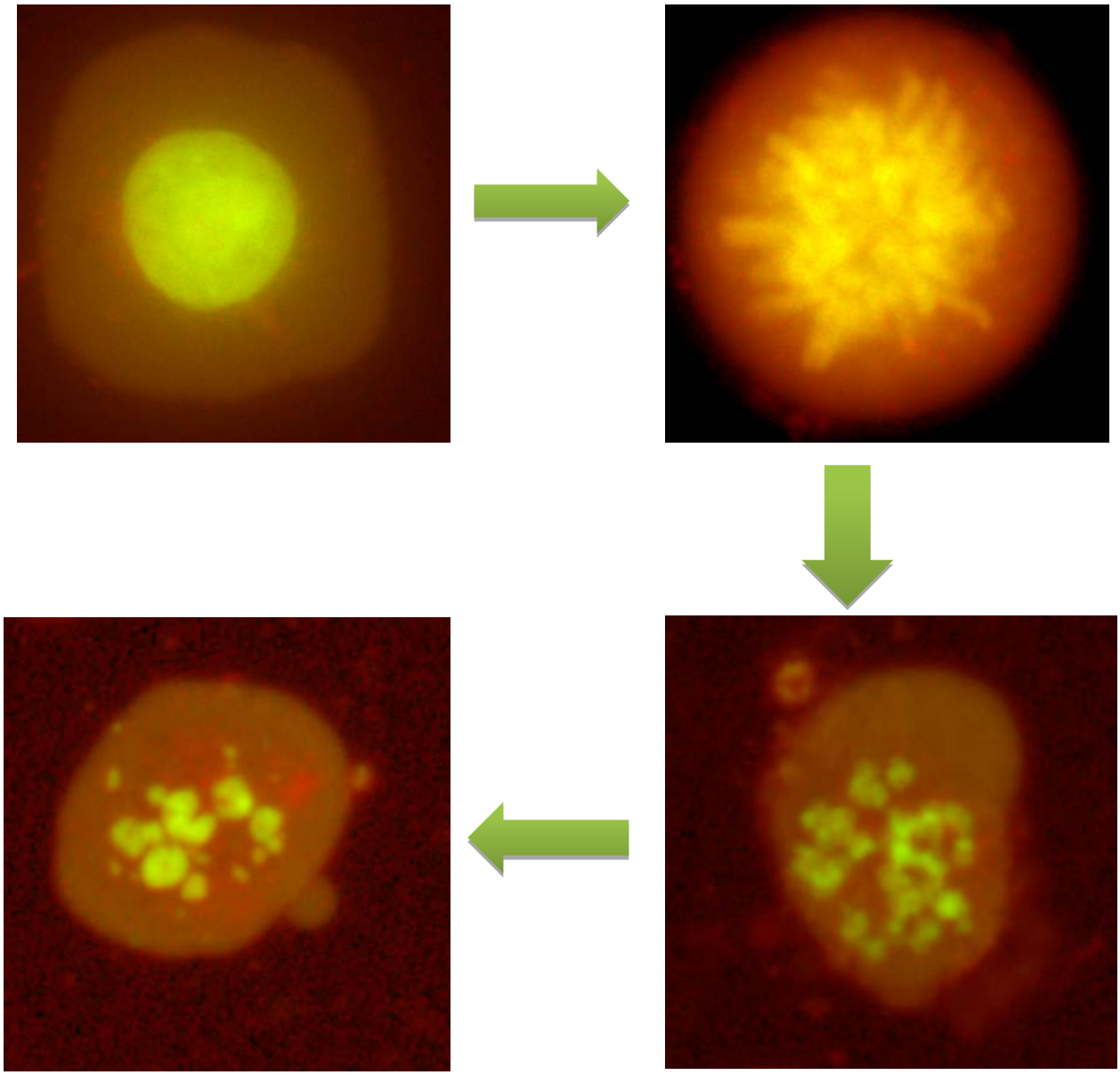


Plate III: Induction of micronuclei in HeLa cella. Left: Mononucleate cells bearing micronuclei and Roght: Binucleate cells bearing micronuclei



late IV: Progressive stages of apoptosis in HeLa cells induced by *Oroxylum indicum.*

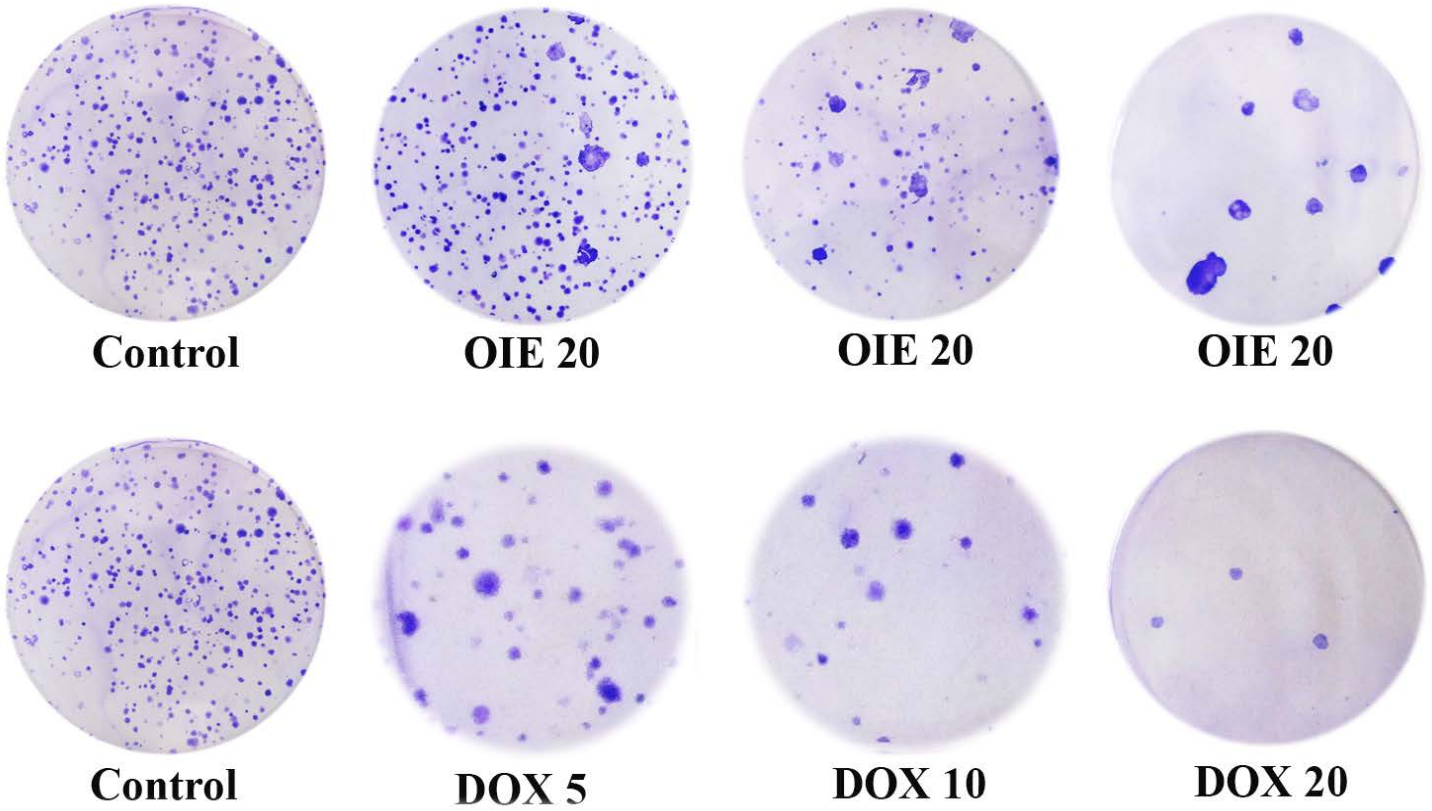


Plate V. Effect of *Oroxylum indicum* extract (OIE) treatment on the reproductive integrity of HeLa cells assessed by Clonogenic assay.

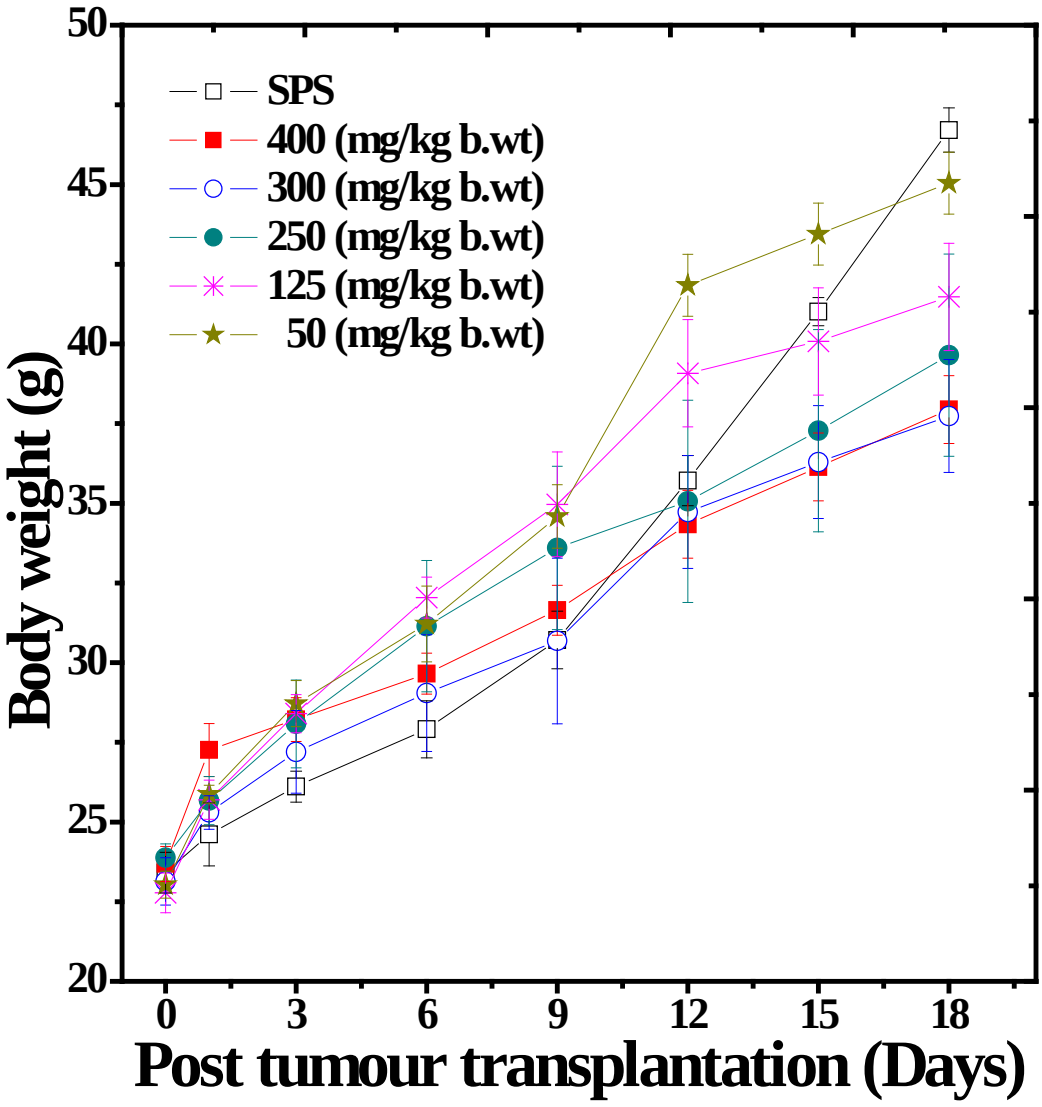


Figure 1: Body weight change of Dalton's lymphoma ascites bearing Swiss Albino mice by treating with chloroform extract of *Oroxylum indicum*. Square open: SPS control; Square red: 400mg/kg. b.wt.; Open circle: 300mg/kg.b.wt.; Closed circle: 250mg/kg.b.wt.; Asterix: 125mg/kg.b.wt.; Star: 50mg/kg.b.wt. The result shown as Mean± standard error mean (SEM), n=10.

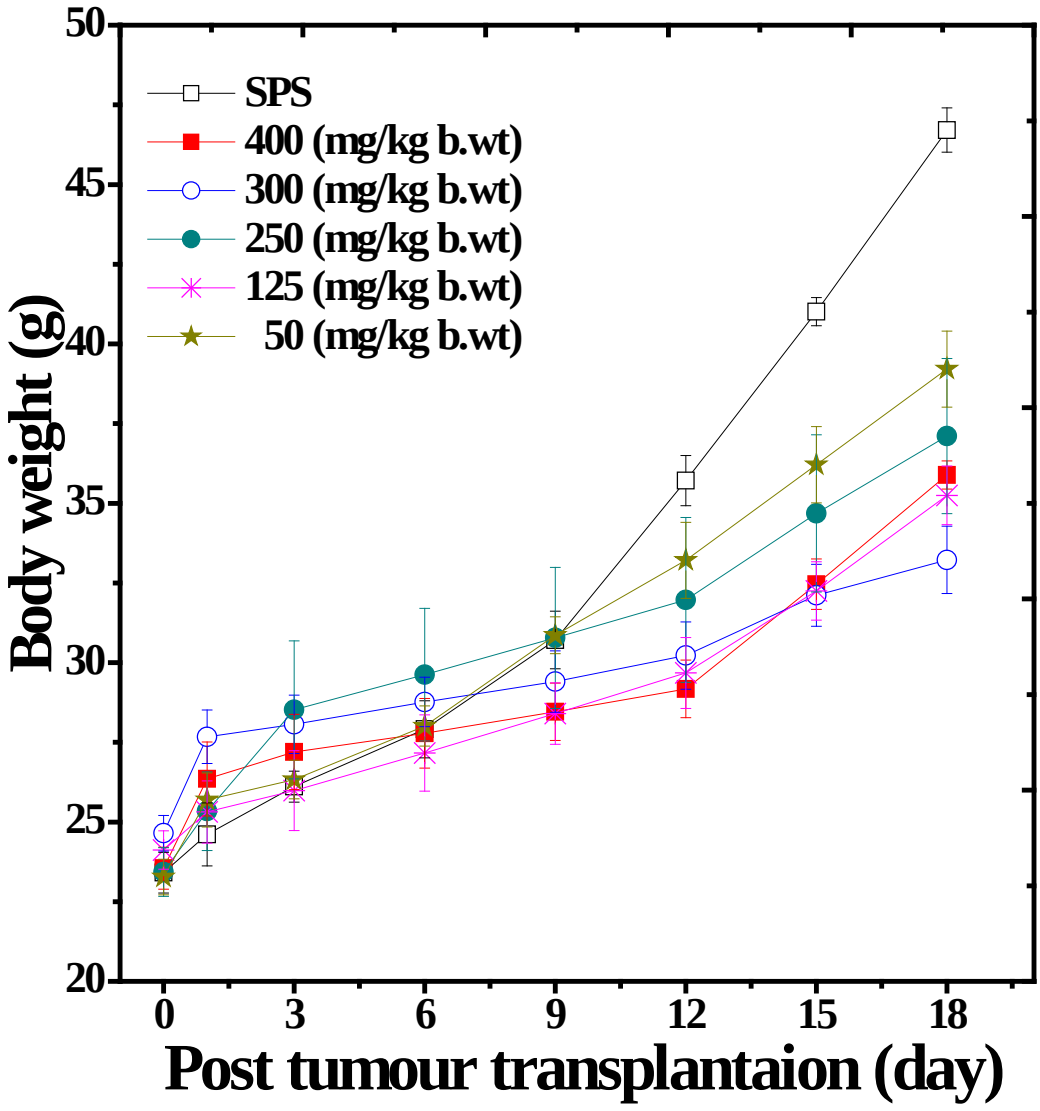


Figure 2: Body weight change of Dalton's lymphoma ascites bearing Swiss Albino mice by treating with ethanol extract of *Oroxylum indicum*. Square open: SPS control; Square red: 400mg/kg. b.wt.; Open circle: 300mg/kg.b.wt.; Closed circle: 250mg/kg.b.wt.; Asterix: 125mg/kg.b.wt.; Star: 50mg/kg.b.wt. The result shown as Mean± standard error mean (SEM), n=10.

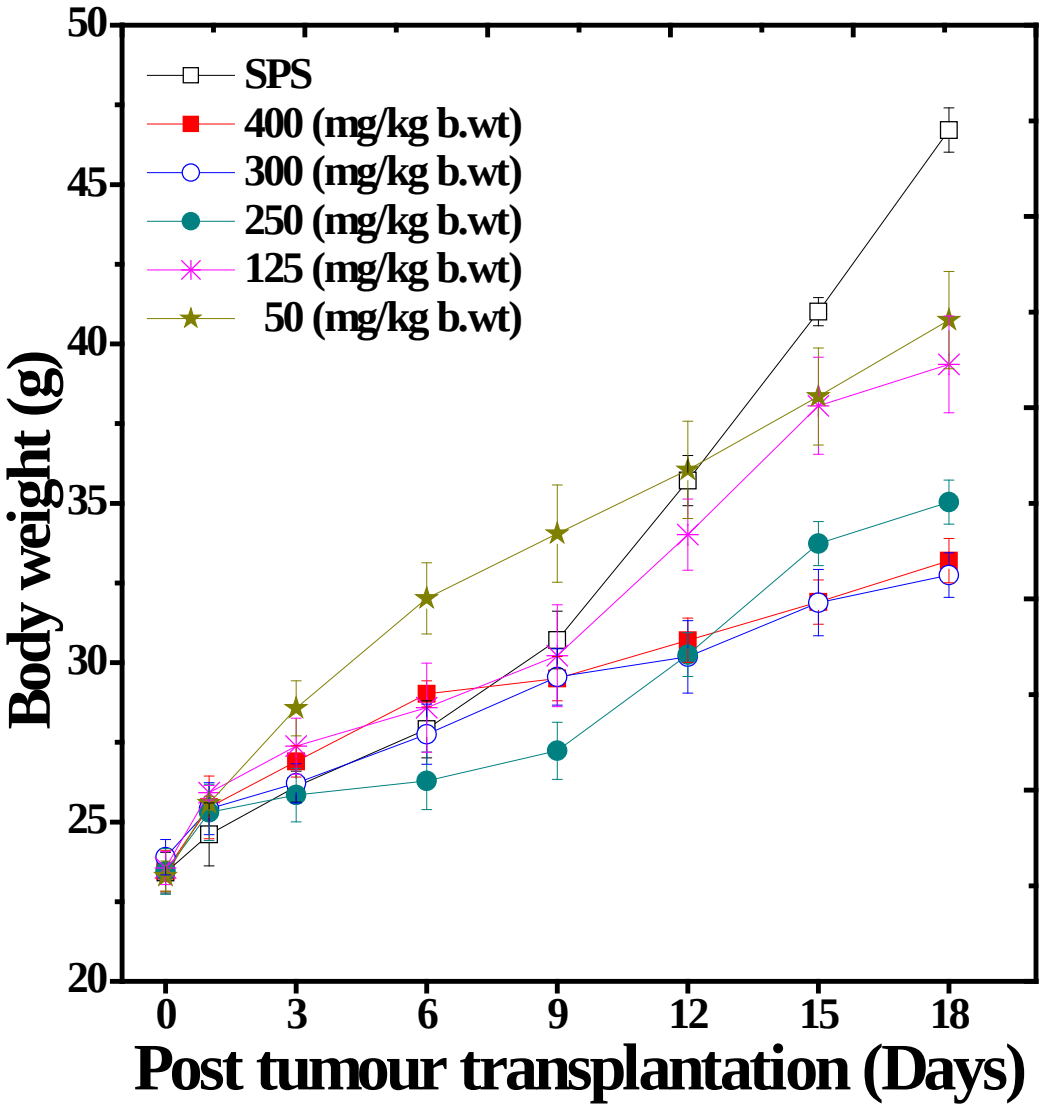


Figure 3: Body weight change of Dalton's lymphoma ascites bearing Swiss Albino mice by treating with aqueous extract of *Oroxylum indicum*. Square open: SPS control; Square red: 400mg/kg. b.wt.; Open circle: 300mg/kg.b.wt.; Closed circle: 250mg/kg.b.wt.; Asterix: 125mg/kg.b.wt.; Star: 50mg/kg.b.wt. The result shown as Mean± standard error mean (SEM), n=10.

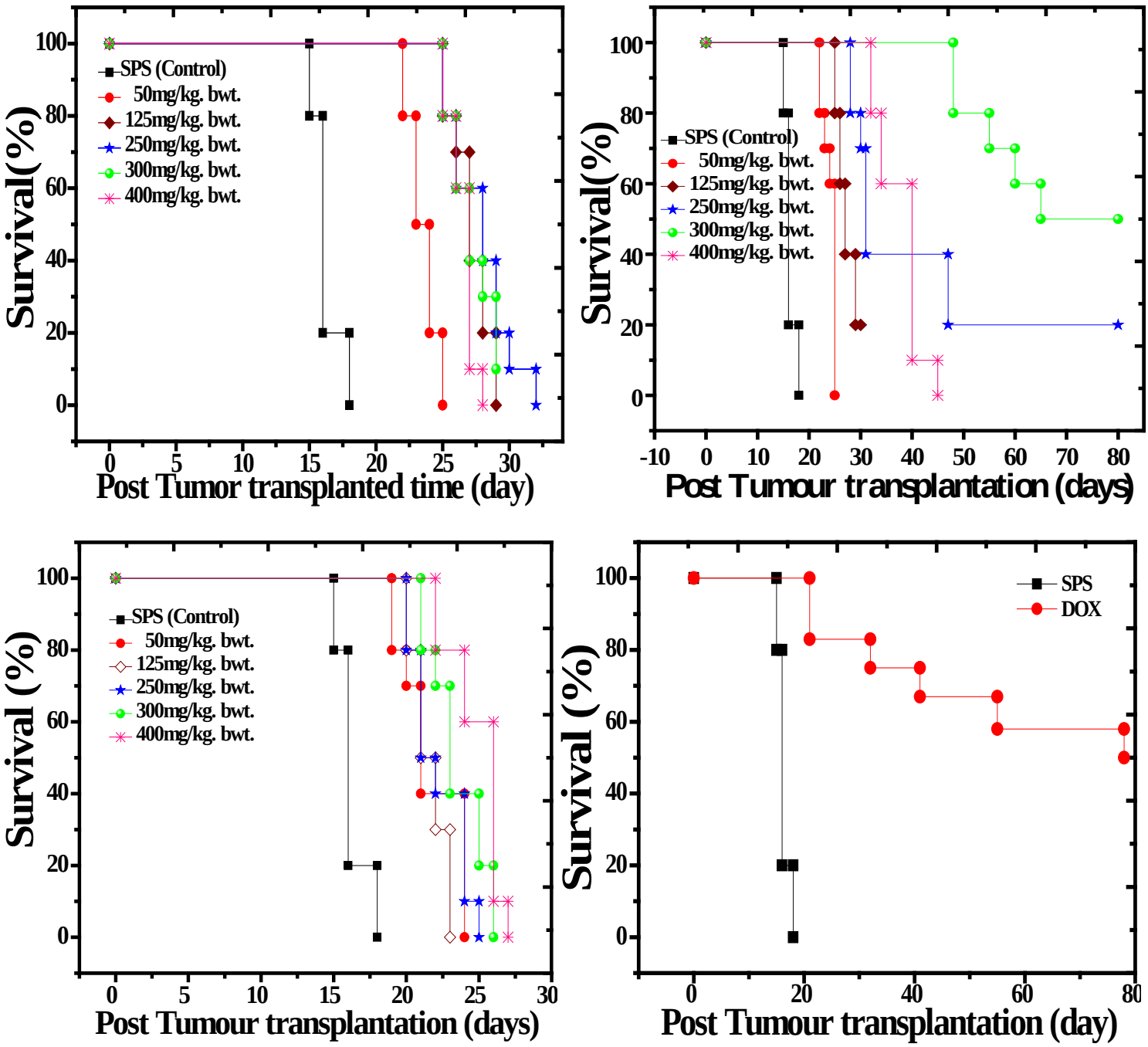


Figure 4: Effect on the survivality of Dalton's lymphoma ascites bearing mice by treatment for 9 days consecutive with various extract of *Oroxylum indicum in vivo*. Above Left: Chloroform extract with various doses. Above Right: Ethanol extract with different doses. Below Left: Aqueous extract with various doses. Below Right: Doxorubicin, standard drug. The result indicates percent survival, n=10.

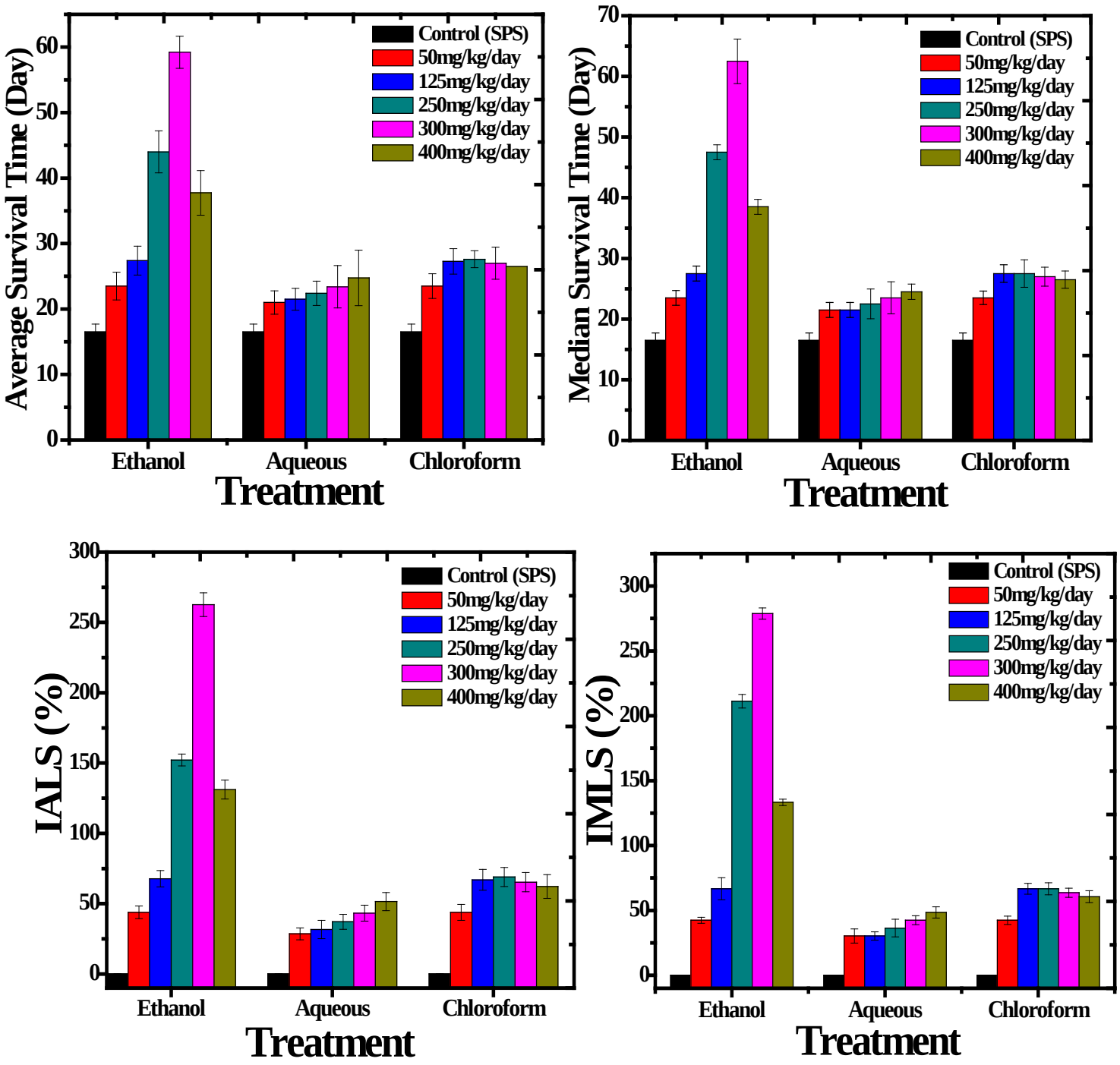


Figure 5: Effect of different extract of *Oroxylum indicum* in Dalton's lymphoma ascites bearing mice on the tumor response assessment based on median survival time (MST) and average survival time (AST), increase in mean life span (% IMLS) and increase in average life span (% IALS). Above Left: Chloroform extract with various doses. Above Right: Ethanol extract with different doses. Below Left: Aqueous extract with various doses. Below Right: Doxorubicin, standard drug. The result expressed as Mean ± SEM, n=10.

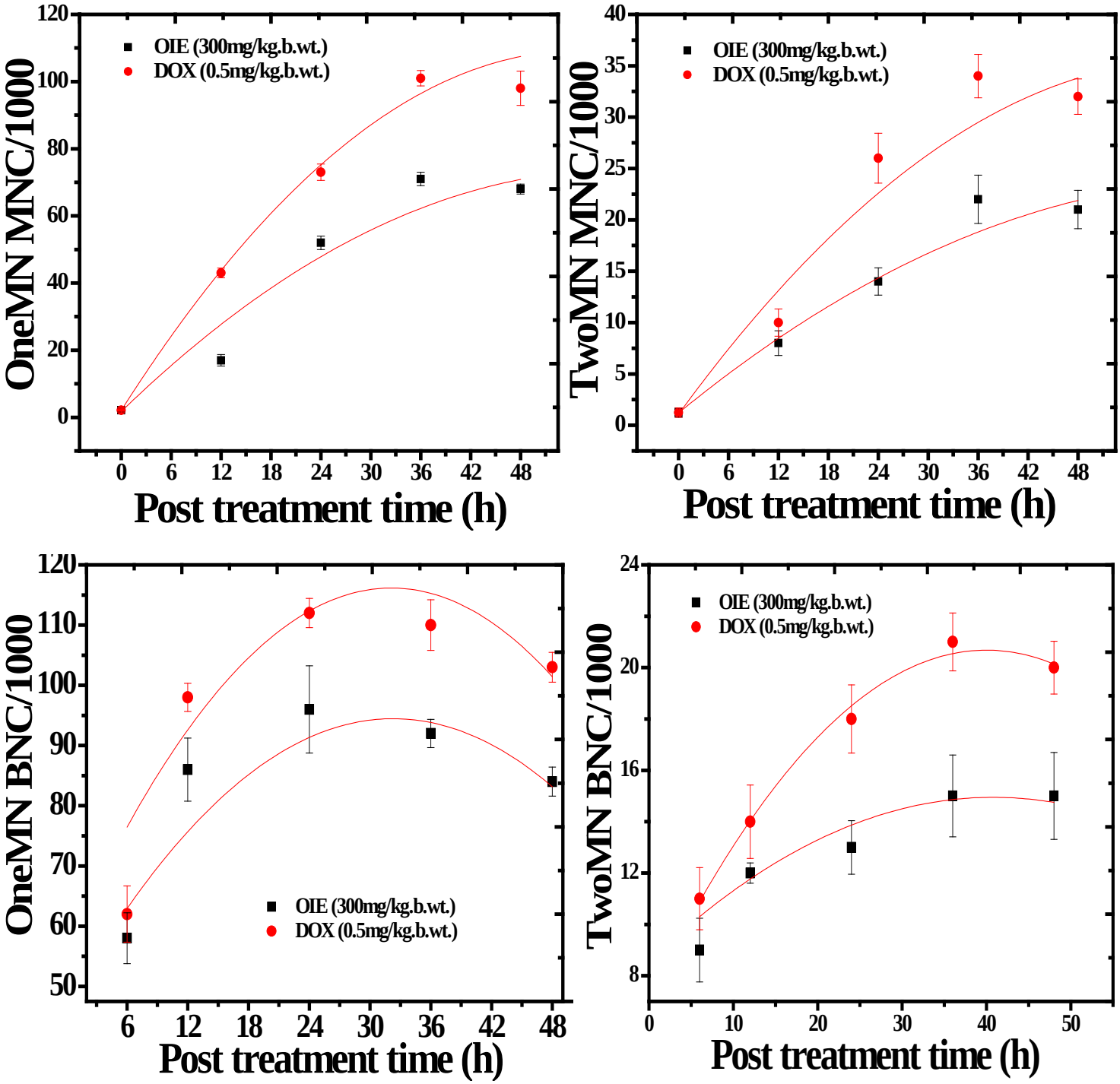


Figure 6: Induction of micronuclei in Dalton's lymphoma ascites bearing mice by treated with 300mg/kg.b.wt. ethanol extract of *Oroxylum indicum* (OIE) and 0.5mg/kg.b.wt. doxorubucin (DOX) at different post treatment time. Above left: One micronucleus in mononucleated cells. Above Right: Two micronucleus in mononucleated cells. Below Left: One micronucleus in binucleated cells. Below Right: Two micronucleus in binucleated cells. The results were determined as frequency of Micronuclei/1000 cells and expressed as the Mean ± SEM, n=10.

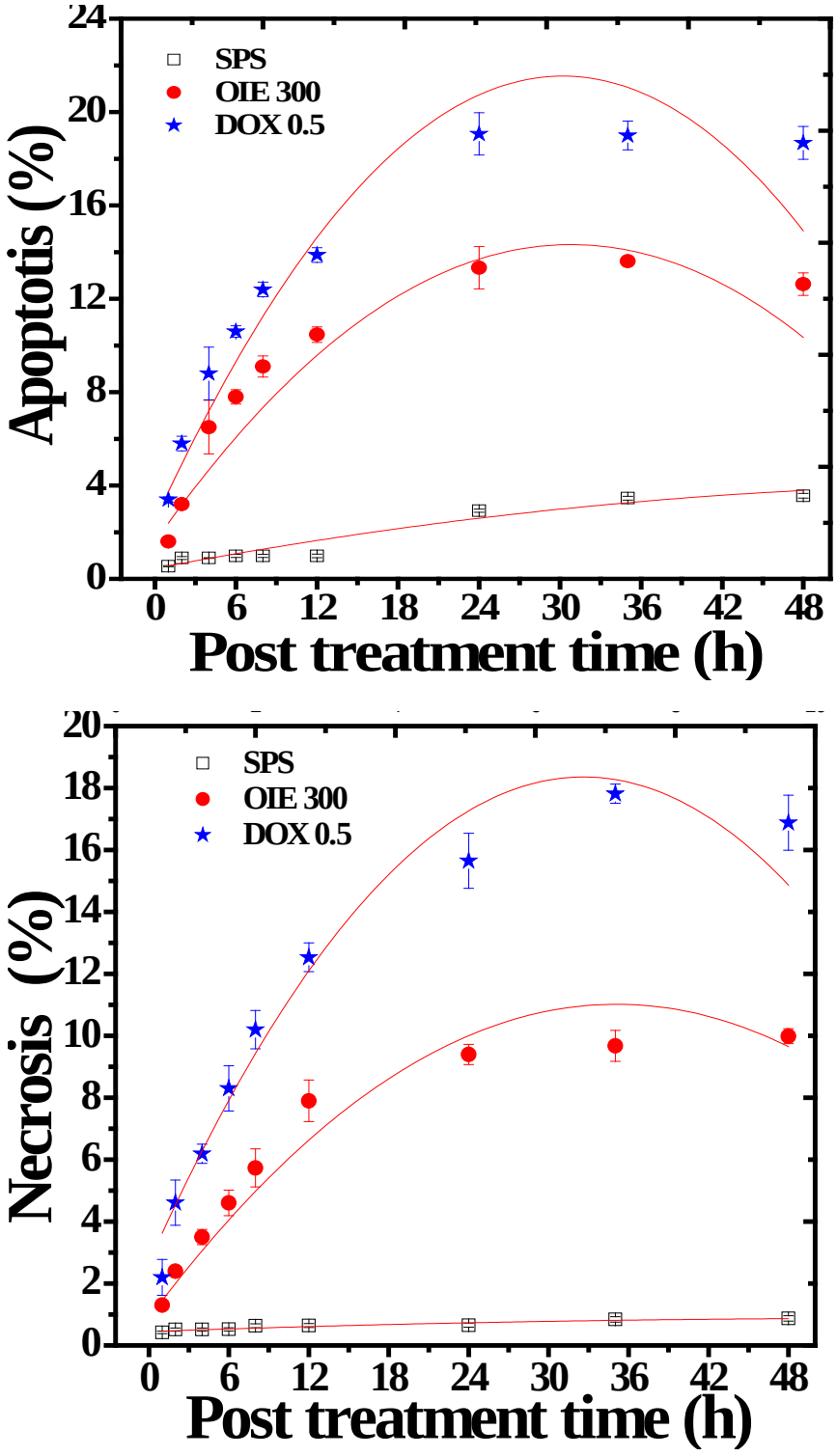


Figure 7: Induction of apoptosis and necrosis in Dalton's lymphoma ascites bearing mice by treated with 300mg/kg.b.wt. ethanol extract of *Oroxylum indicum* (OIE) and 0.5mg/kg.b.wt. doxorubucin (DOX) at different post treatment time. The results determined as percent index and expressed as the Mean ± SEM, n=10.

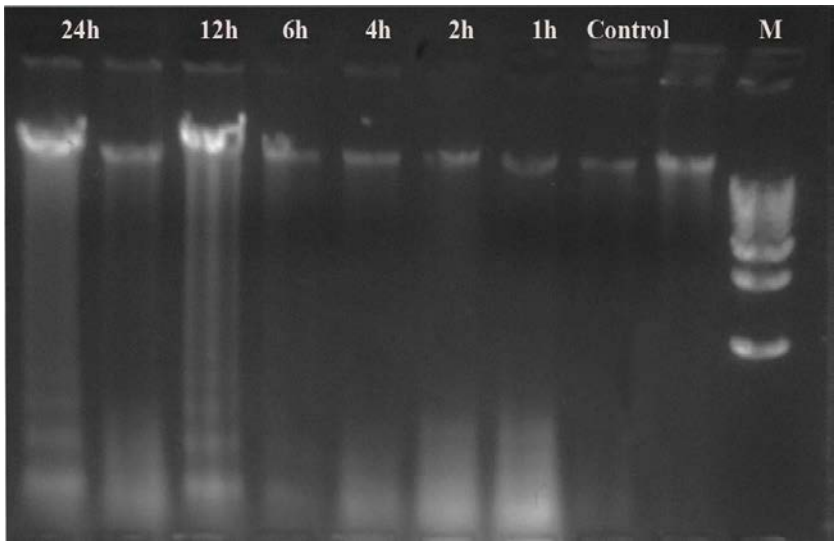


Figure 8: Induction of DNA fragmentation in Dalton's Lymphoma cells by OIE (300 mg/kg) at different time intervals.

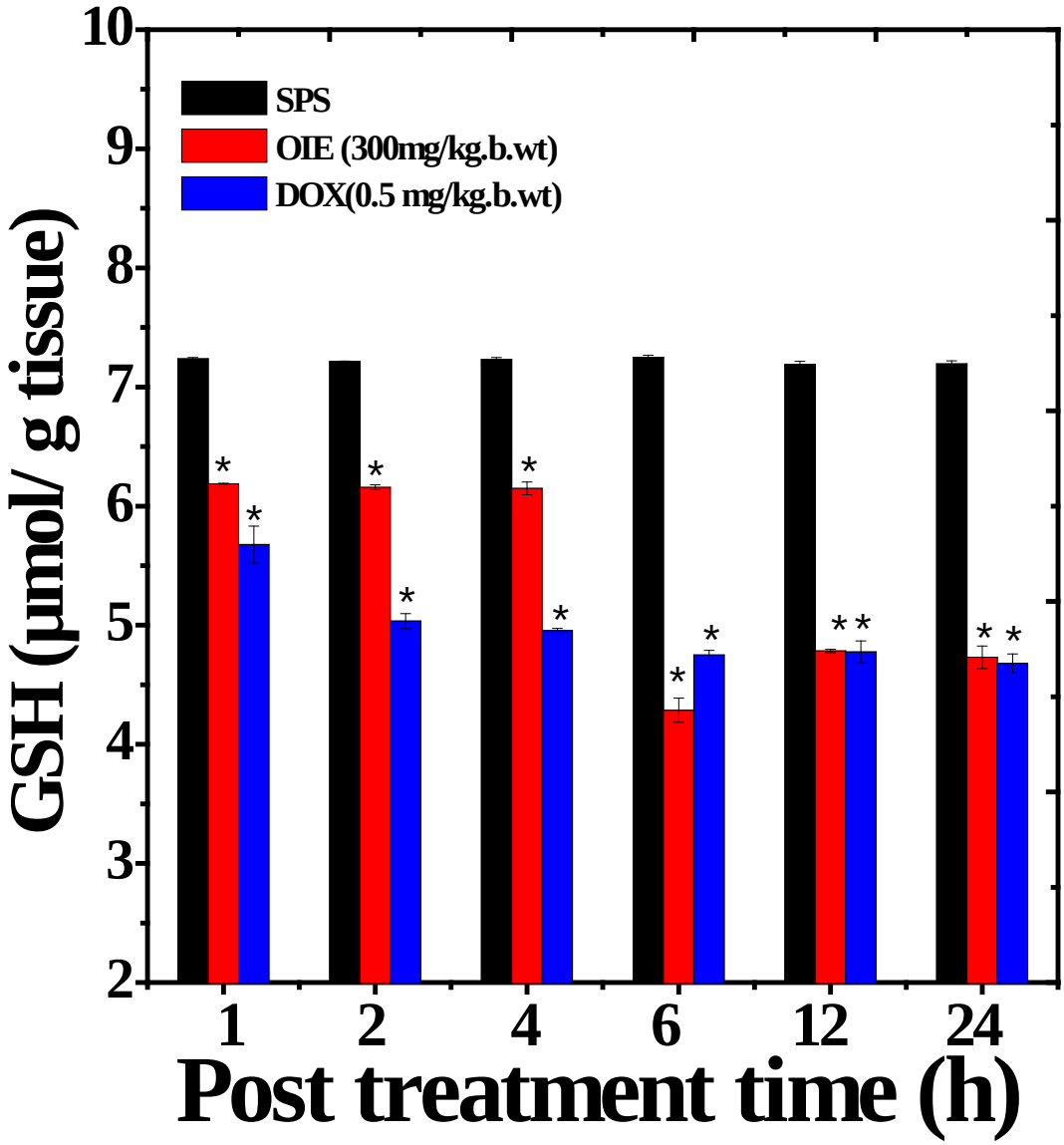


Figure 9: Alteration in the glutathione content of mice bearing dalton's lymphoma ascites treated with various doses of *Oroxylum indicum* extract (OIE) and doxorubicin (DOX). The results expressed as the Mean ± SEM, n=10.

p<0.001when treatment are compared with concurrent control (SPS) group.

No symbol= no significance.

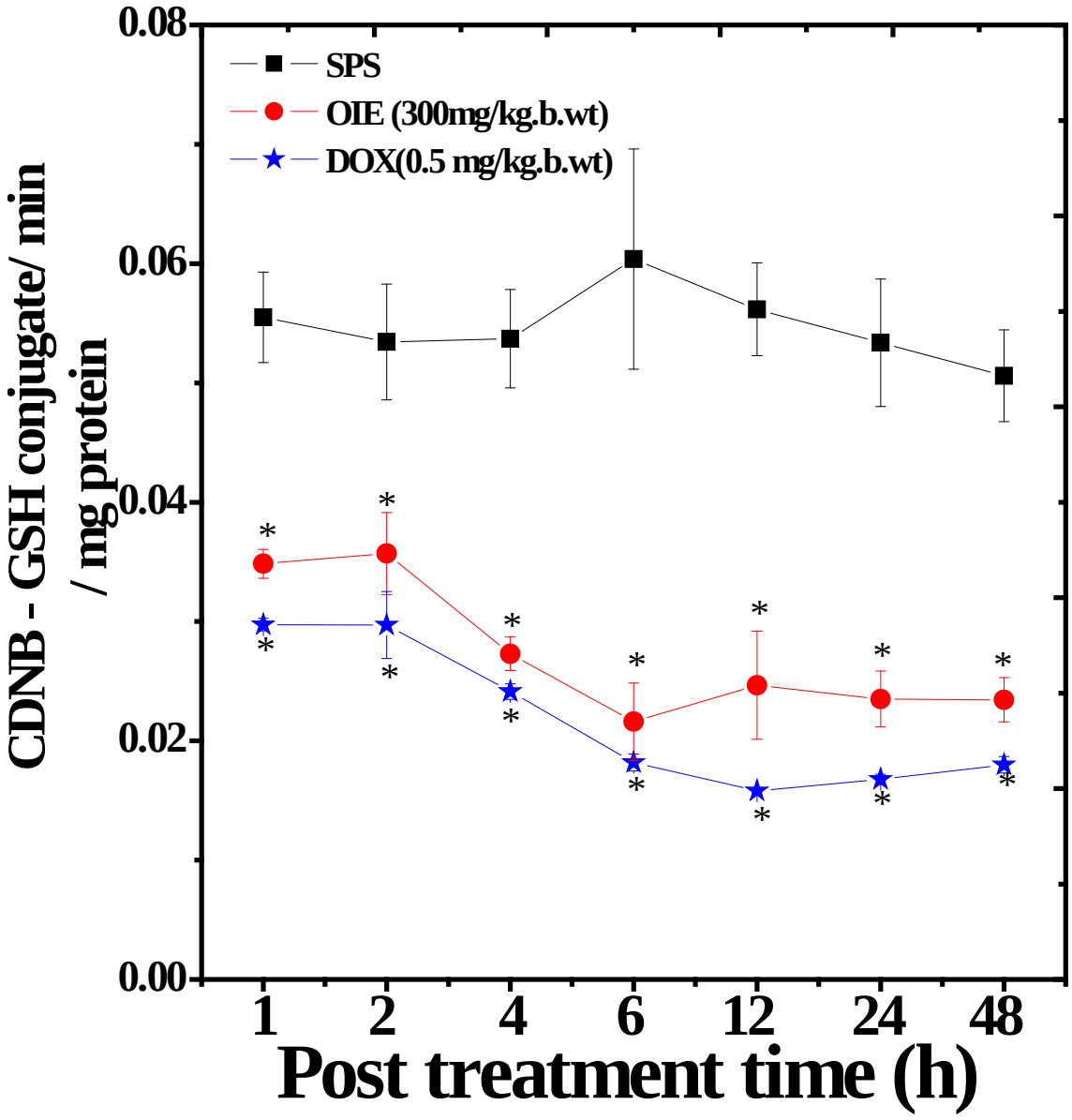


Figure 10: Alterations in the glutathione-s-transferase (GST) activity of Dalton's lymphoma ascites bearing mice treated with 300 mg/ kg. body weight of *Oroxylum indicum* (OIE) and Doxorubicin (DOX). The results expressed as the Mean ± SEM, n=10.

p<0.001when treatment are compared with concurrent control (SPS) group.

No symbol= no significance.

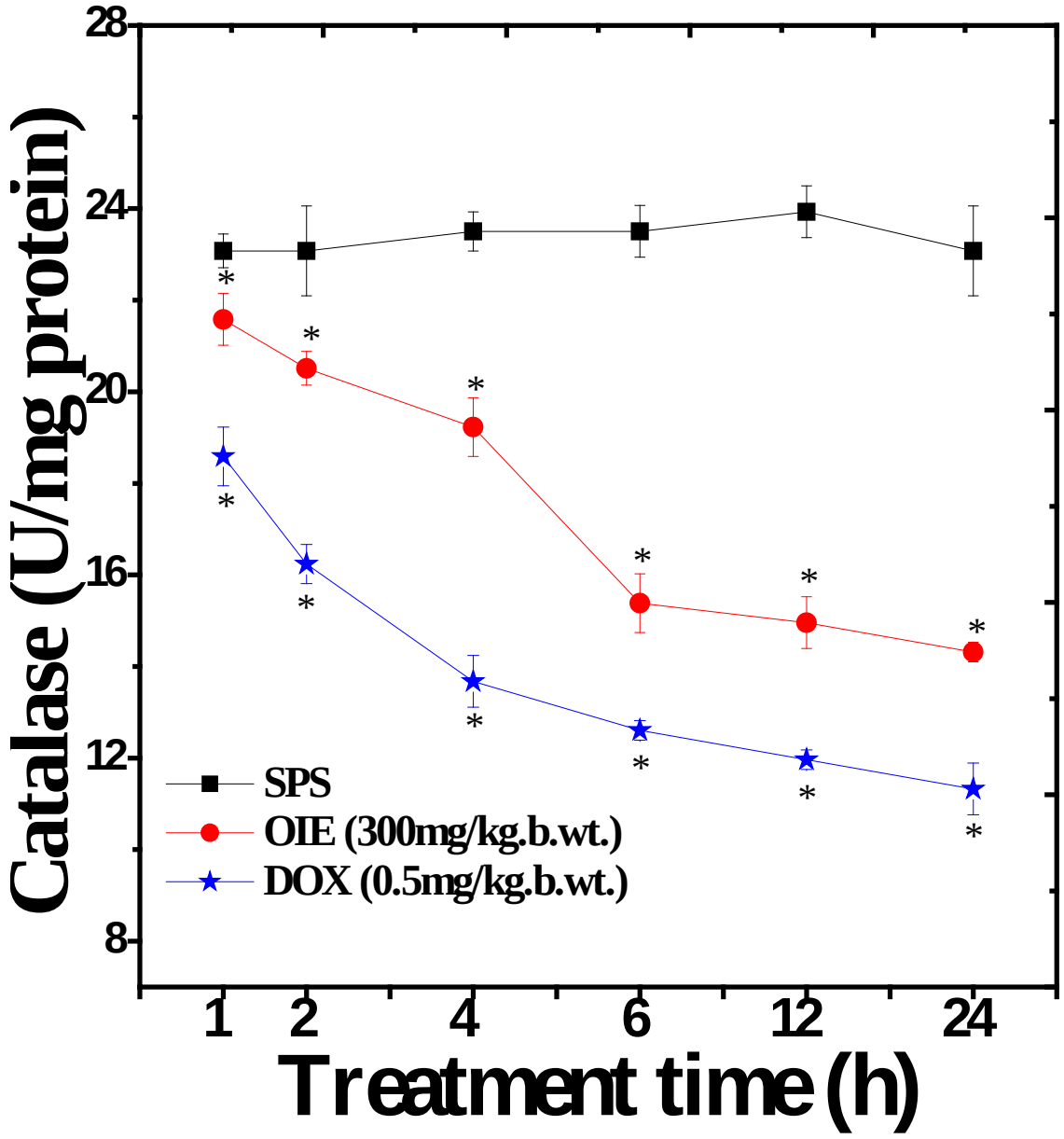


Figure 11: Alterations in the catalase (CAT) activity of Dalton's lymphoma ascites bearing mice treated with 300 mg/ kg. body weight of *Oroxylum indicum* (OIE) and Doxorubicin (DOX). The results shown the Mean ± SEM, n=10.

p<0.001when treatment are compared with concurrent control (SPS) group.

No symbol= no significance.

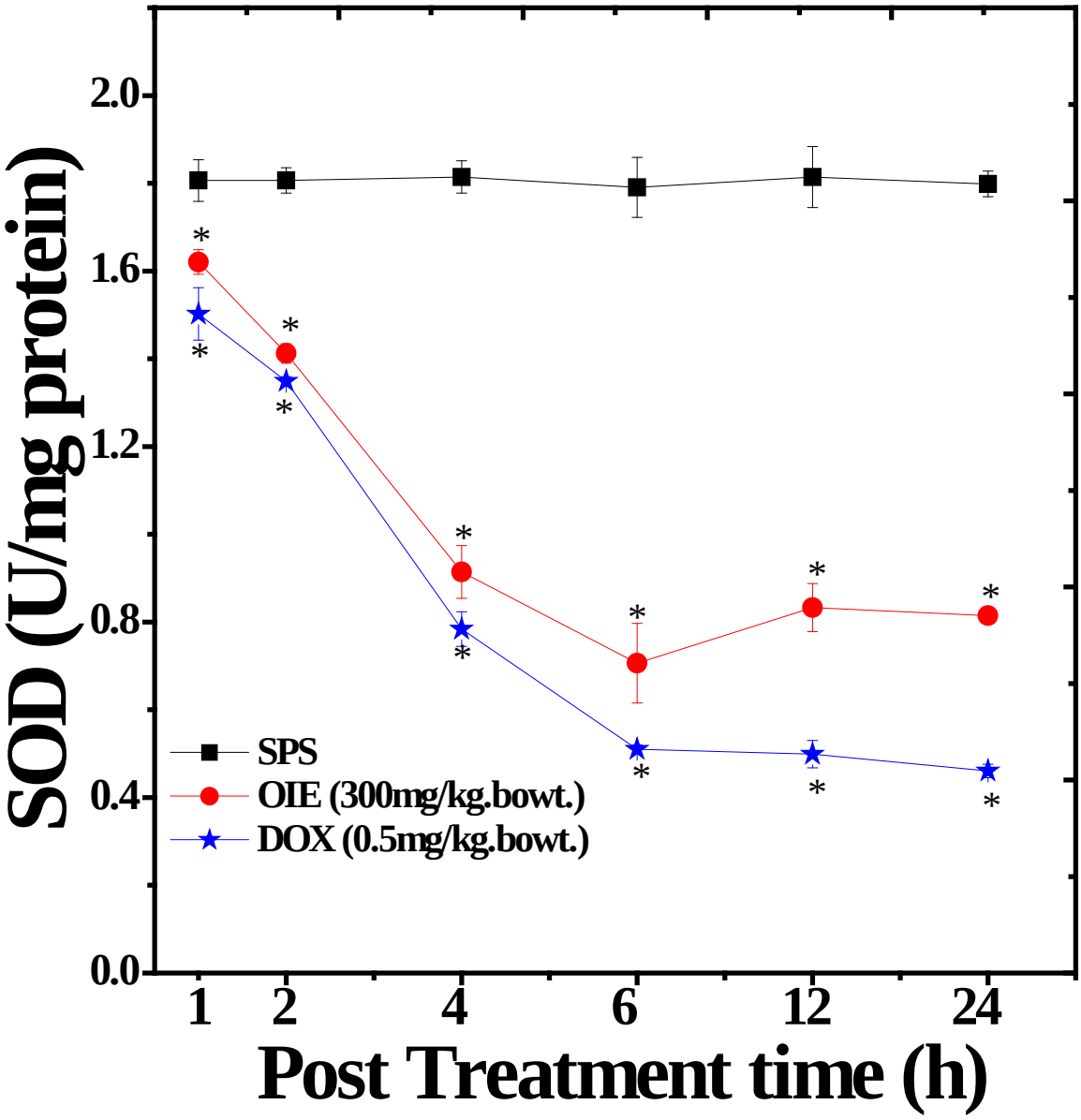


Figure 12: Alterations in the superoxide dismutase (SOD) activity of Dalton's lymphoma ascites bearing mice treated with 300 mg/ kg. body weight of *Oroxylum indicum*(OIE) and Doxorubicin (DOX). The results expressed as the Mean ± SEM, n=10.
p<0.001when treatment are compared with concurrent control (SPS) group.
No symbol= no significance.

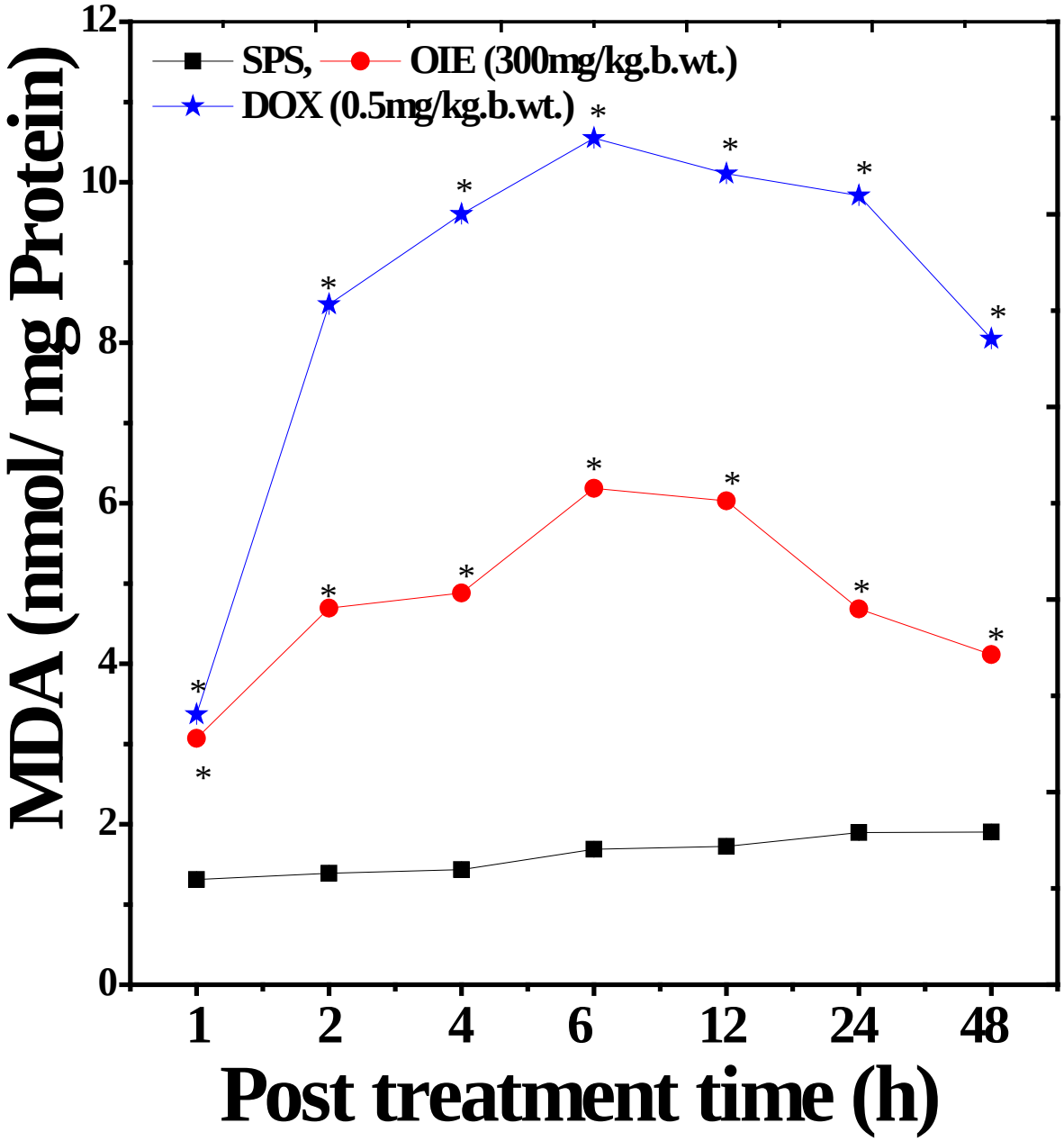


Figure 13: Alterations in the lipid peroxidation (LOO) level in the Dalton's lymphoma ascites bearing mice treated with 300 mg/ kg. body weight of *Oroxylum indicum* (OIE) and Doxorubicin (DOX). The results present the Mean ± SEM, n=10.
p<0.0001when treatment are compared with concurrent control (SPS) group.
No symbol= no significance.

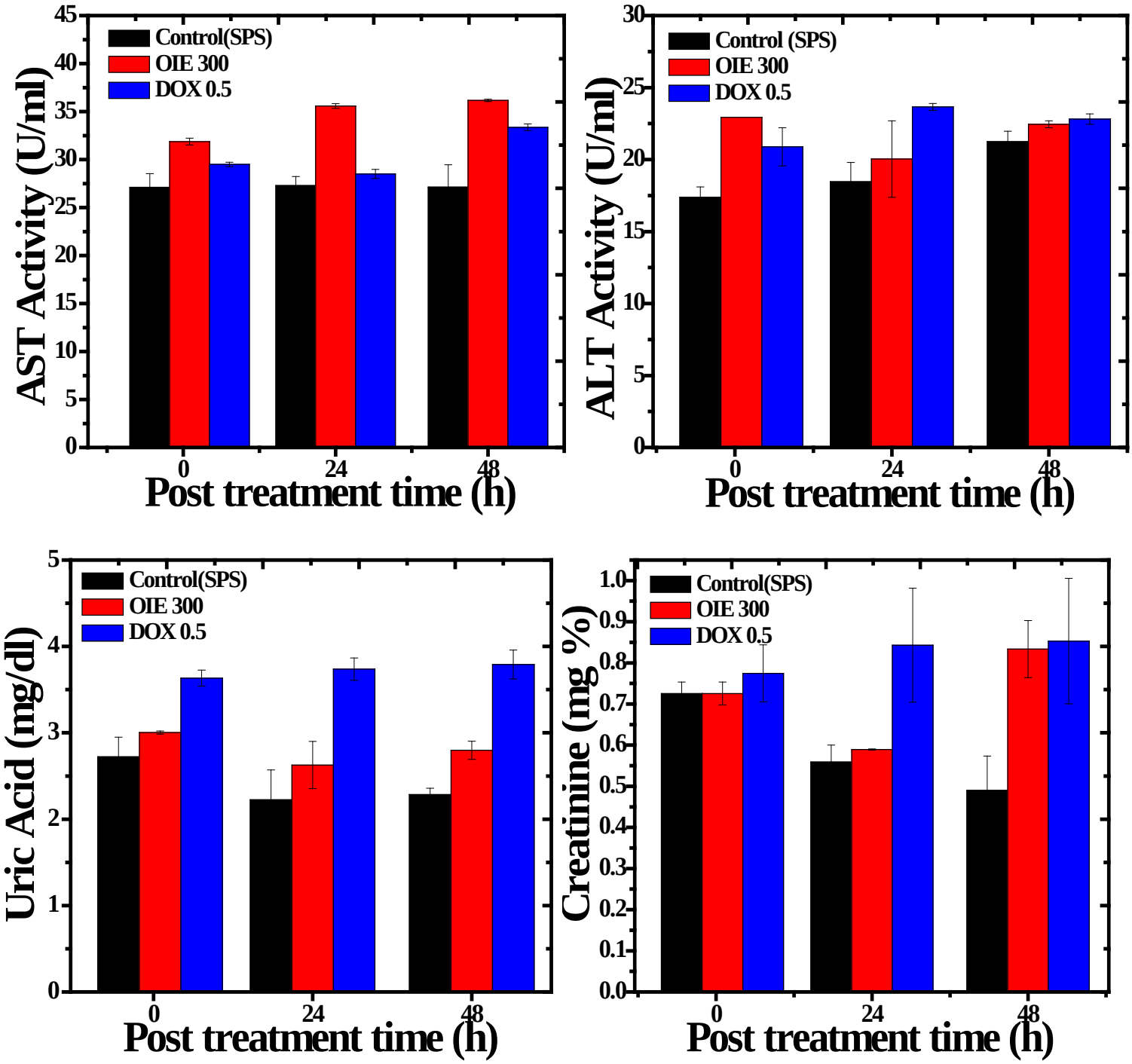


Figure 14: Effect on the liver and kidney function by treated with 300 mg/kg.b.wt of OIE and 0.5 mg/kg.b.wt of DOX on Dalton's lymphoma ascites bearing mice. The results present the Mean ± SEM, n=10. P<0.05.

Assessment of the antitumor activity of the different extracts of *Oroxylum indicum* in mice transplanted with Dalton's lymphoma

Chapter 7

Abstracts

The effect of different doses of chloroform, ethanol and aqueous extracts of Oroxylum indicum was studied in Dalton's lymphoma tumor bearing mice, whereas their acute toxicity was determined after oral and intraperitoneal administration in normal mice. The chloroform, ethanol and aqueous extracts of Oroxylum were non- toxic up to 3 g/kg. body weight when administered orally. The intraperitoneal administration of various doses of different extracts to mice showed signs of toxicity with LD50 of 520, 700 and 2000 mg/kg. body weight for chloroform, ethanol and aqueous extracts, respectively. The administration of 50- 400 mg/kg b .wt chloroform, ethanol and aqueous extracts of Oroxylum indicum in tumor bearing mice resulted in a dose dependent increase in the tumor free survival and maximum effect was observed for 300 mg/kg ethanol extract, which has increased the tumor free survival by 40% beyond 120 days, whereas chloroform and aqueous extracts were not that effective. The administration of 300 mg/kg b. wt. ethanol extract resulted in an increase in the AST of 59.22 days (IALS, 262.59 %) and MST of 62.5 days (IMLS, 278.79 %). Therefore, 300 mg/kg body weight of the ethanol extract was considered as an optimum dose for its antineoplastic activity and was selected for further investigations, where 300 mg/kg body weight ethanol extract induced micronuclei in both mononucleate and binucleate cells in a time dependent manner up to 36 h. The apoptosis increased continuously up to 24 h post treatment. The biochemical studies revealed a significant decline in the glutathione, glutathione-s-transferase, superoxide dismutase and catalase accompanied by elevated lipid peroxidation. The cytotoxic effect of ethanol extract of O. indicum may be due to its ability to induce DNA damage and apoptosis and alleviate glutathione, glutathione-s-transferase, superoxide dismutase and catalase accompanied by a rise in the lipid peroxidation.

1. INTRODUCTION

The use of plants as medicine is as old as human civilization (Fabricant and Farnsworth, 2001). The use of different parts of plants as medicine has been listed in Sumerian clay tablets, which are about 5000 years old, the Ebers Papyrus and the Chinese book Pen T'Sao written around 1550 and 2500 BC, respectively (Petrovska, 2012). Besides this, archaeological studies indicated use of plant medicines approximately 60,000 years ago in Iraq (Gourhan, 1975). Plants and natural products are still in great demand due to their safety, efficacy and lesser side effects (Thillaivanan and Samraj, 2014) and about 80 % individuals in the developing countries still depend on plants to treat different diseases (Kim, 2005). Also, 25 % of modern drugs are obtained from plants and 70% of the drugs introduced in the United States for the past 25 years have their origin in plants (Rates, 2001; Kinghorn et al., 2011). Plants contain many phytochemicals which work in a synergistic mode of action in such a way that their uses can complement or damage others or neutralize their possible negative effects (Hassan, 2013). The use of multicompounds is preferred over the use of single drug for the treatment of several diseases including cancer, AIDS, diabetes, etc. due to their beneficial effects (Pan et al., 2014).

Cancer is an international health burden shared throughout the world affecting both the rich and poor. It is the second most common cause of death next only to heart diseases in the USA (Jemal et al., 2005; American cancer society, 2015; Siegel et al., 2015) and was the single cause of the death of more than 8.2 million patients in the year 2012 (Ferlay et al., 2015). Even though remarkable progress has been achieved towards the understanding the hallmarks of cancer, the progress in cancer treatment modalities did not reduce the mortality rates significantly. Cancer is still responsible for one in eight deaths globally (Garcia et al., 2007; Center et al., 2011). The currently

used chemotherapy for cancer treatment has several side effects and therefore the need for better therapy with lesser side effects (Lotfi-Jam et al., 2008). Besides, the high cost as well as lack of effectiveness of the current conventional therapies (chemotherapy and radiation), especially to solid tumors use of plants for cancer treatment may be acceptable (Wood-Sheldon et al., 1997). The side effects due to most cancer drug toxicity also act as a driving force to the use of alternative medicine for better cure (Rao et al., 2008). Plants are not only safe for long term therapy but also provide nutrition and reduce the side effects of conventional cancer therapy. The high cost and negative impact of conventional therapy, low-cost and safety of plants has been drawing increased attention towards plants and plant derived products for cancer cure (Jagetia and Venkatesha, 2012). The search for anticancer agents from plants has led to the development of four classes of plant derived anticancer agents such as the vinca alkaloids, the epipodophyllotoxins, the taxanes and the camptothecin derivatives (Kinghorn, 2001; Cragg and Newman, 2013). Though substantial progress has been made in this field, still continuous efforts are required to scientifically explore traditionally used plants for the development of anticancer drugs.

Oroxylum indicum or sonapatha is a deciduous tree widely used in many traditional systems especially in India. The Ayurvedic traditional system recognizes it as a multifunctional medicinal tree, which finds its use in single form as well as many composite formulations (Vaidya, 1975; Bhattacharje, 2000). It is mainly found in ravines and moist places of the forest and is distributed throughout the foothills of the Himalaya, Eastern and Western Ghats as well as in the Northeast region of India (Bennet et al., 1992; Kritikar and Basu, 2001; Jayaram and Prasad, 2008). Traditionally, the bark decoction of *Oroxylum indicum* has been used to cure

nasopharyngeal cancer, gastric ulcer, hepatobiliary diseases and cardiac problems (Lalrinzuali et al., 2015). The bark paste is also applied to mouth to treat cancer, scabies etc. (Mao, 2002; Tangjang et al., 2011). In Peninsular Malaysia, the bark of *O. indicum* has been used in dysentery (Burkill, 1966). The roots are used as a refrigerant, antiinflammatory, anodyne, aphrodisiac, expectorant, appetizer, carminative, digestive, anthelmintic, diaphoretic, diuretic, antiarthritic, antidiabetic and febrifuges. The tonic of the root is useful in dropsy, cough, hiccough, asthma, bronchitis, anorexia, dyspepsia, flatulence, colic, diarrhea, dysentery, strangury, gout, vomiting, leucoderma, wounds, neuralgia, sprains, rheumatoid arthritis and fever. The root bark is used in stomatitis, nasopharyngeal cancer and tuberculosis (Bhattacharje, 2005). The root bark is one of the components of a popular Ayurvedic formulation known as Dasamula that comprises of ten different roots which is in high demand due to its multiple uses for different medicinal purposes. Owing to the high demand of this formulation, uprooting of the whole plant and over exploitation led to its reduced availability and now it has been categorized as vulnerable medicinal plant by the Govt. of India (Darshan and Ved, 2003; Jayram and Prasad, 2008). The Ayurvedic Pharmacopoeia of India (API) advocates the use of stem bark of *O. indicum* in place of the root bark (Anonymous, 2007). Our recent study has confirmed its anti-inflammatory activity *in vivo* (Lalrinzuali et al., 2015). Therefore, taking all these into account, it was desired to study the anticancer activity of the stem bark of *Oroxylum indicum* in mice transplanted with Dalton's lymphoma cells.

2. MATERIALS AND METHODS

2.1. Chemicals

Nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), 5, 5'dithio 2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitronezene (CDNB), dimethyl sulphoxide

(DMSO), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), triton X-100, ethylenediamine tetra-acetic acid (EDTA), sodium pyruvate, thiobarbituric acid (TBA), pyruvic acid, ethidium bromide, acridine orange, crystal violet, and cytochalasin B were obtained from Sigma Aldrich Chemical Co. (Kolkata, India). Sodium carbonate (Na_2CO_3), trichloroacetic acid (TCA), potassium chloride (KCl), potassium sodium-tartrate, hydrogen peroxide (H_2O_2), and agarose were procured from SD Fine-Chem Ltd., Mumbai, India, whereas sodium hydroxide (NaOH), sodium chloride (NaCl), disodium biphosphate (Na_2HPO_4), sulphuric acid (H_2SO_4), hydrochloric acid (HCl), n-butanol, Tris (hydroxymethyl) aminomethane (Tris buffer) and ammonium oxalate were procured from Merck India Limited, Mumbai. Phenol-chloroform-isoamyl alcohol (PCI), and reduced nicotinamide adenine dinucleotide (NADH) were purchased from HiMedia, Mumbai, India. Doxorubicin, was requisitioned from a local pharmacy.

2.2. Collection and Preparation of the Extract

Oroxylum indicum (L.) Benth ex Kurz (family: Bignoniaceae) is commonly called as Sonapatha was collected from Champhai, Mizoram, India during the dry season and its identity was authenticated by the Botanical Survey of India, Shillong as *Oroxylum indicum*. The non-infected stem bark of Sonapatha was carefully peeled off from the trunk of a matured tree and chopped into pieces of about one foot each. It was then washed with clean water and scrubbed properly to remove all the dirt and other extraneous material. The washed stem bark was spread into stainless steel trays and allowed to shade dry at room temperature in dark, clean and hygienic condition with periodic observation for contamination if any. The exhaust and free air circulation was allowed. The non-infected dried stem bark was chopped into smaller pieces and ground to powder using an electrical grinder at room temperature. The

powdered bark of *O. indicum* was sequentially extracted in petroleum ether, chloroform, ethanol and distilled water according to increasing polarity using a Soxhlet apparatus at their respective boiling points until the solvents became colourless. The liquid extracts were filtered and concentrated by evaporating their liquid contents using rotary evaporator. Each extract, except petroleum ether was concentrated in vacuo and stored at -70°C until further use.

2.3. Preparation of Drug and mode of administration

The different extracts of *Oroxylum indicum* were dissolved in appropriate solvent immediately before use, Chloroform (OIC) and ethanol (OIE) extracts were dissolved in sterile physiological saline (SPS) and Tween 20 (9.5:0.5 ml), whereas doxorubicin and aqueous extract (OIA) were dissolved in sterile physiological saline (SPS) alone. Each animal from each group received different treatments according to body weight intraperitoneally.

2.4. Animal care and handling

The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Swiss albino mice were purchased from Pasteur Institute, Shillong and were bred before use in a controlled environment of temperature (24-25ºC), 50% humidity and light and dark (12 h each) cycle. Usually 5-6 animals were housed in a sterile polypropylene cage containing paddy husk (procured locally) as bedding material. Usually, six to eight weeks old male and female Swiss albino mice weighing 20-30 g were selected from the inbred colony for experimentation. The animals were fed with commercially available food pellets and water ad libitum. All animal experiments were carried out according to NIH and Indian National Science Academy, New Delhi, India guidelines. The Institutional

Animal Ethics Committee of Mizoram University, approved the entire study vide letter no. IAEC/4504, Aizawl, India.

2.5. Acute Toxicity Test

The acute toxicity study of all extracts was performed as per Organization for Economic Co-operation and Development (OECD) guidelines both orally and intraperitoneally. Albino mice selected by random sampling technique (n=10) of both sexes (5 males and 5 females) were used per dose. The animals were fasted for 18 hours (both food and water withdrawn) prior to intraperitoneal injection of different extracts of *Oroxylum indicum*. The control group received the vehicle used for dissolving the extracts. The animals were weighed before and after fasting to estimate their weight loss. If mortality was observed in 2-3 animals then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxicity. The LD_{50} for each extracts was calculated using probit analysis.

2.6. Tumor Model

Dalton's lymphoma ascites (DLA) tumor was procured from the Department of Zoology, North- Eastern Hills University, Shillong and was maintained in 10-12 weeks old mice by serial intraperitoneal transplantation of 1×10^6 viable tumor cells per animal (in 0.25 ml PBS, pH 7.4) under aseptic conditions.

2.7. Experimental

Dalton's lymphoma tumor bearing mice were divided into the following groups:

2.7.1. Negative Control groups: This group received 0.01 ml/kg body weight of respective vehicle used for dissolving the extract/s. Each extracts had its own control

group. The negative control group for the chloroform and ethanol received SPS + Tween 20. However, control group for aqueous extracts received SPS alone.

2.7.2. DOX groups: This group of animals were injected with 0.5 mg/kg body weight of doxorubicin, a standard anticancer drug and served as positive control.

2.7.3. OIC groups: The animals of this group were administered with 50, 125, 250, 300 or 400 mg/kg body weight of the chloroform extract of *Oroxylum indicum*.

2.7.4. OIE groups: This group of animals received 50, 125, 250, 300 and 400 mg/kg body weight of the ethanol extract of *Oroxylum indicum*.

2.7.5. OIA groups: The animals were injected with 50, 125, 250, 300 and 400 mg/kg body weight of the aqueous extract of *Oroxylum indicum*.

The tumor bearing animals were given treatment once daily 1 day after tumorization for subsequent 9 days (Geran et al., 1972). Each group consisted of ten animals for each extract dose and 170 animals were used for this experiment. The animal survival was monitored daily up to 120 days, since the survival of animals up to 120 days is approximately equivalent to 5 years in humans (Nias, 1990). The deaths, if any, of the tumor bearing mice were recorded daily and the survival was determined. The tumor response was assessed by calculating median survival time (MST) and average survival time (AST). The MST and AST were calculated from the animals dying within 120 days and those surviving beyond 120 days were excluded from the study (Geran et al., 1972). The increase in median life span (% IMLS), increase in average life span (% IALS) and the increase in life span (%ILS) was also calculated using the formulae:

MST= First death + Last death in the group/2

AST= Sum of animals dead on different days/No. of animals

%IMLS= MST of treated mice – MST of control x 100/MST of control

%IALS = AST of treated mice – AST of control x 100/AST of control

% ILS = (T/C x 100) - 100

Where, T is the mean survival days of treated mice and C is that of the control mice.

The optimum dose for each extract was determined and the optimum dose as well as extract which increased the longest tumor free survival was selected for biochemical assays.

2.8. Micronucleus Assay

The ability of OIE to induce DNA damage in Dalton's lymphoma was studied by performing a separate experiment, where 1 x 10^6 Dalton's lymphoma cells were transplanted into 5 – 8 weeks old mice and allowed to develop the tumor for 9 days. Thereafter, these animals were given a single dose treatment of 300 mg/kg body weight of OIE or 0.5 mg/kg body weight doxorubicin intraperitoneally. One hour after the drug/s administration each of the tumorized mouse was injected with 150 µg of cytochalasin B so as to suppress cytokinesis in proliferating tumor cells. The mice were euthanized at 6, 12, 24, 36 and 48 h post-drug administration and the tumor cells were collected in individual tubes. The tumor cells were washed with ammonium chloride to lyse erythrocytes and centrifuged at 1000 rpm. The micronuclei were prepared according to the modified method of Fenech and Morley, (1985). In brief, cells were washed with sterile PBS and pelleted by centrifugation at 1000 rpm for 5 min. The cell pellet was disturbed and subjected to mild hypotonic treatment (0.75% ammonium oxalate) at 37°C, centrifuged once again and the resultant cell pellet was allowed to fix in Carnoy's fixative 3:1 (Methanol: Acetic acid) overnight. The cells were centrifuged and the resultant pellet was resuspended in a small volume of fixative. The cells were spread on to pre cleaned coded slides to avoid observer's bias.

The cells were stained with 0.025% acridine orange (BDH, England, Gurr Cat. no. 34001 9704640E) in Sorensen's buffer (pH 6.8) and subsequently washed twice in the buffer to remove excess stain. The slides were mounted in Sorensen's buffer and observed under a DM-2500 fluorescent microscope (Leica Microsystems, Wetzlar, Germany) equipped with 450–490 nm BP filter set with excitation at 453 nm using a 20 X N Plan objective. Usually one thousand mononucleated or binucleated cells with well-preserved cytoplasm were scored for each post-treatment time in each group. The frequency of mononucleated cells bearing micronuclei (MNMNC) as well as binucleated cell bearing micronuclei (MNBNC) was determined. The micronucleated cells were scored according to the criteria of Kirsch-Volders et al., (2003) and Fenech et al., (2003).

2.9. Apoptosis Assay

Another experiment was conducted to investigate the ability of *Oroxylum indicum* to induce apoptosis in Dalton's lymphoma cells, where grouping and other conditions were exactly similar to that described for micronucleus assay except that tumor bearing mice were euthanized at 1, 2, 4, 6, 8, 12, 24, 36, 48 h post drug treatment. The tumor cells were aspirated and washed with ammonium chloride to lyse the erythrocytes and cells were pelleted by centrifugation. The cells were washed again with sterile PBS and spread on to clean coded slides and stained with freshly prepared ethidium bromide and acridine orange (1:1) (Sigma Aldrich Chemical Co. Bangalore, India) stain and observed under a DM-2500 fluorescent microscope (Leica Microsystems, Wetzlar, Germany). The number of live, necrotic and apoptotic cells were counted. A total of 1000 cells were counted for each slide and a total of 5000 cells were counted for each group. The percentage of apoptotic, and necrotic cells was calculated as follows:

Apoptotic index (%) = Number of apoptotic cells scored X 100/Total number of cells counted.

2.10. DNA fragmentation assay

The induction of DNA damage by OIE in tumorized mice was studied by DNA fragmentation assay where grouping and the dose of the drugs used were essentially same as described for apoptosis assay. The tumor bearing mice were killed by euthanasia and Dalton's lymphoma cells were collected at 2, 4, 12 and 24 h post-drug treatment and the DNA ladder formation was detected by SDS/proteinase-K/RNase method that allowed the isolation of only fragmented DNA without contamination with RNA (Herrmann et. al., 1994). Briefly, the cells were centrifuged, washed with ammonium chloride and lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.2 % Triton X-100), kept at 4°C for 20 min, and centrifuged at 14,000 g for 15 min. The supernatant was collected and mixed with proteinase K (0.5 mg/ml) and incubated for 1 h at 37°C followed by the RNase-A (0.5 mg/ml) treatment for next 1 h at 50°C. The DNA was extracted with phenol, chloroform and isoamyl alcohol (1:1:1) and precipitated with ammonium acetate (3M) and chilled isopropanol. The samples were loaded on to 1 % agarose gel placed in a horizontal electrophoresis tank containing TBE (44.6 mM Tris, 44.5 mM boric acid and 1 mM EDTA) buffer. The DNA was resolved at 100 Volts and 40 mA and the resultant DNA fragmentation ladder was visualized under 265 nM UV light after staining the gel with ethidium bromide (0.5 µg/ml).

2.11. Biochemical Assays

A separate experiment was conducted to study the various biochemical changes in the OIE treated Dalton's lymphoma cells in vivo. The grouping and other conditions were essentially similar to that described for apoptosis assay. The animals

were sacrificed after nine days of drug/s treatment at an interval of 1, 2, 4, 6, 12 and 24 hours. Both the treated and untreated Dalton's lymphoma cells were aspirated under sterile conditions, washed with ammonium chloride followed by sterile phosphate buffer saline and pelleted. The cell pellets were weighed and 5% homogenate was prepared in cold sterile PBS (pH 7.4) and used for the estimation of various antioxidant and lipid peroxidation.

2.11.1. Estimation of Glutathione

Glutathione was estimated as described earlier (Moron et al., 1929). Glutathione was measured by its reaction with DTNB (Ellman's reaction) to give a compound that absorbs light at 412 nm. In brief, 1.8 ml of 0.2 M Na_2HPO_4 was mixed with 40 µl of 10 mM DTNB and 160 µl of cell homogenate and allowed to stand for 2 minutes. The absorbance was read against the blank at 412 nm in a Systronic UV-VIS double beam spectrophotometer (Systronics India Ltd., Ahmedabad, India). The blank consisted of distilled water instead of cell homogenate.

2.11.2. Estimation of Glutathione - S – Transferase

Glutathione-s-transferase was determined by the method of Habig et al., (1987). Usually, 0.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.1 ml of 20 mM CDNB, and 8.8 ml distilled water were incubated at 37°C for 10 min followed by the addition of 0.5 ml of 20 mM GSH and 0.1 ml of cell homogenate. The absorbance was read at 340 nm with a UV-VIS double beam spectrophotometer at 1 min intervals for 6 minutes. Distilled water was used as a blank.

2.11.3. Catalase Assay

Catalase was assayed according to technique described by Aebi (1984). In a 3 ml cuvette, 20 µl of cell homogenates was mixed with 1.98 ml of 50 mM phosphate

buffer (pH 7.0). The reaction (maintained at 20ºC) was started by adding 1 ml of 30 mM H_2O_2 and the decrease in absorbance was monitored at 240 nm for 60 seconds.

2.11.4. Superoxide Dismutase Assay

SOD is an enzyme that catalyzes the dismutation of two superoxide anions ($O_2^{\bullet-}$) into hydrogen peroxide and molecular oxygen and the activity of SOD was estimated as described by Fried (1975). 100 µl of cell homogenate, 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitroblue tetrazolium, 200 µl of 780 µM NADH were incubated for 90 seconds at 30°C. The reaction was terminated by adding 1000 µl of acetic acid followed by the addition of 4 ml n-butanol. The absorbance was recorded at 560 nm using UV/VIS double beam spectrophotometer. The percent inhibition was calculated by measuring the absorbance of blank without SOD enzyme. The SOD activity was calculated using the formula (Blank-Sample)/Blank X 100.

2.11.5. Lipid Peroxidation Assay

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids, serve as a convenient index for determining the extent of peroxidation reaction of lipids. MDA has been identified as the product of lipid peroxidation (LOO) that reacts with thiobarbituric acid to give a red species absorbing at 535 nm. LOO assay was carried out following the method of Buege and Aust, 1978. One ml of tissue homogenate was added to 2 ml of TCA-TBA-HCl reagent and was mixed thoroughly. The solution was heated in a boiling water bath for 15 minutes, cooled immediately at room temperature and centrifuged at 1000 rpm for 10 min. The supernatant was collected and its absorbance was read at 535 nm against blank in a UV-VIS double beam spectrophotometer. The blank contained all the reagents minus the cell

homogenate substituted with distilled water. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56x10^{6}M^{-1}cm^{-1}$.

3. Determination of Liver and Kidney function

3.1. Collection of serum

A separate experiment was conducted to estimate the toxicity of the OIE in the liver and kidney of the tumorized mice. The tumor bearing mice were injected with 300 mg/kg b. wt. of ethanol extract one day after transplantation of Dalton's lymphoma until nine subsequent days. After the treatment period was over, the blood was collected at different time intervals from the retro orbital sinuses using a capillary tube after anesthetizing the mice with ketamine. The collected blood was allowed to stand for 30 min at 4ºC so as to separate the serum. The samples were centrifuged at 1000 rpm for 5 min and the serum was collected to estimate AST, ALT, creatinine and uric acid at 1, 24 and 48 hours after the last drug administration. Commercially available kits were used to measure AST, ALT, creatinine and uric acid. The reactions for SGPT/ALT and SGOT/AST do not follow Beer's law hence the requirement of standard curve (Reitman & Frankel, 1957) which was plotted as described by Reitman & Frankel (1957) following manufacturer's protocol.

3.2. Preparation of the standard calibration curve for AST and ALT

Different amounts (0, 0.05, 0.10, 0.15, 0.20 ml) of the pyruvate standard were added to different amounts (0.50, 0.45, 0.40, 0.35, 0.30 ml) of the substrate reagent to make a final volume of 0.50 ml. 0.10 ml of distilled water was added to each tubes followed by 0.50 ml of DNPH reagent. The solution was mixed well and allowed to stand at room temperature for 20 minutes. 5 ml of working NaOH reagent was added, mixed well and allowed to stand at room temperature for 10 minutes. The absorbance

was measured at 505 nm in a UV-VIS double beam spectrophotometer.and the standard graph was prepared.

3.2.1. Estimation of Aspartate transaminase (AST)

3.2.2. AST Assay

The AST was assayed according to the protocol provided by the manufacturer, In brief, 0.50 ml of the substrate reagent from the kit was taken in a clean test tube, incubated at 37ºC for 3 minute and 0.10 ml of serum was added to the sample tubes. The tubes were mixed well and incubated at 37ºC for 60 minute followed by the addition of 0.50 ml of DNPH reagent. The tubes were allowed to stand at room temperature for 20 minute followed by the addition of 0.10 ml of distilled to the blank tubes. 5 ml of working sodium hydroxide reagent was added, mixed and allowed to stand at room temperature for 10 minute. The absorbance of the tests was measured against the blank at 505 nm in a UV visible double beam spectrophotometer and the activity of AST was calculated form the calibration curve plotted earlier.

3.2.3. ALT Assay

The ALT activity in the serum was determined according to manufacturer's protocol, where 0.50 ml of the substrate reagent was transferred into a clean test tube and incubated at 37ºC for 3 minute. Thereafter, 0.10 ml of serum was added to the sample tubes, mixed well and incubated at 37ºC for 30 minute with the addition of 0.50 DNPH reagent and allowed to stand at room temperature for 20 minute followed by the addition of 0.10 ml of distilled to the blank tubes. 5 ml of working sodium hydroxide reagent was added, mixed and allowed to stand at room temperature for 10 minute. The absorbance of the samples was measured against the blank at 505 nm with a UV visible double beam spectrophotometer and the activity of the tests was read form the calibration curve plotted earlier.

3.2.4. Estimation of Creatinine

The creatinine test was performed as described by Bones et al. (1945). In brief, 2 ml of picric acid was added to 0.2 ml of serum sample and centrifuged at 2500 rpm for 10 minutes to obtain a clear supernatant. Then, 1.1 ml of the supernatant was collected in a clean test tube. The blank consisted of 1 ml of picric acid reagent 0.1 ml of distilled. The standard consisted of creatinine and 1 ml of picric acid. This was followed by the addition of 0.1 ml of buffer reagent to sample, blank and standard. All the tubes were mixed well incubated at room temperature for 20 minute. The absorbance of the standard and samples were measured against the blank at 520 nm in a UV visible double beam spectrophotometer. The amount of creatinine was calculated using the following formula:

Creatinine in mg % = Absorbance of test/Absorbance of standard x 2.0.

3.2.5. Estimation of Uric Acid

The amount of uric acid was measured as described by Fossati (1980). Usually 1 ml of working reagent was pipetted in a clean and dry test tube and 0.02 ml sample or distilled water, or uric acid standard were added to the sample, blank, and the standard tubes, respectively. All the tubes were vortexed to mix the contents well and incubated at 37ºC for 5 min. The absorbance of the standard and the samples were read against the blank at 520 nm in UV visible double beam spectrophotometer. The amount of uric acid was calculated using the following formula:

Uric acid in mg/dl = absorbance of test/ Absorbance of sample x 8.

4. Statistical Analyses

The statistical analyses were carried out using Origin Pro 8 SRO v8.0724 (B724), Northampton, MA, USA. The significance for survival analysis was determined by Kaplan Meier test and student's 't' test was applied for biochemical

studies and followed by Tukey's post -hoc tests for multiple comparisons, wherever necessary. The Wilcoxson's signed rank test was utilized for micronucleus and apoptosis assays. The results were confirmed by repetition of the experiments. Test of homogeneity was applied to determine any statistical differences between the repeat experiments. Since no significant differences were observed the data of all experiments were combined and expressed as mean ± standard error of the mean (SEM). A p value of < 0.05 was considered statistically significant.

5. RESULTS

The results have been expressed as the mean ± standard error of the mean (SEM), wherever required and are presented as Tables 1-13 and Figures 1-14.

5.1. Acute toxicity

Oral administration of the different extracts of *Oroxylum indicum* showed no signs of toxicity up to 3 g/kg b. wt. and the testing of higher doses was not possible due to problem in dissolving the higher amounts of the extracts. The acute toxicity assay after the intraperitoneal mode of administration was carried out by up and down method. Based on the toxicity, the chloroform extract showed the maximum toxicity followed by the ethanol extract with an LD_{50} of 520 mg/kg b. wt. and 700 mg/kg b. wt. respectively, whereas the aqueous extract showed the least toxicity with an LD_{50} of 2 g/kg b. wt. (Table 1-2).

5.2. Change in the body weight

The DLA bearing mice gained weight continuously until their survival and there was no sign of tumour regression in the negative control group. The treatment of DLA mice with 50, 125, 250, 300 and 400 mg/kg body weight of all extracts of *Oroxylum indicum* exhibited slight elevation in the body weight of all treatment groups. However, this increase was insignificant up to 18^{th} day of tumor

transplantation when compared with 0 day within groups. The comparison of *Oroxylum indicum* extract treated groups with negative control revealed a significant reduction in the body weight due to alleviated cell proliferation (Table 3, Figure 1-3).

5.3. Anticancer activity

Dalton's lymphoma transplanted in the peritoneum of mice developed rapidly with no signs of regression and all the untreated tumorized mice died within 18 days (Table 4). The AST and MST for this group were 16.33 and 16.5 days, respectively (Table 5; Figure 4).

The administration of 50, 125, 250, 300 and 400 mg/kg body weight of chloroform extract resulted in a significant rise in the number of survivors when compared to spontaneous control group ($p<0.001$) (Table 4; Figure 4). A 20% rise in survival was observed in animals treated with 50 mg/kg of chloroform extract by 20 days. Time of survival increased with increasing dose up to 250 mg/kg where 20% animals survived up to 30 days and all animals succumbed to death after 32 days (Table 4). However, 10% DLA bearing survived when treated with 250 mg/kg body weight of chloroform extract beyond the 32nd day of tumor post transplantation, (Table 8 and 9; Figure 4).The AST of 27.60 days, MST of 27.52 days, IMLS of 66.67 % and an IALS of 68.98 % , respectively were determined for this dose (Table 5; Figure 5).

Treatment of Dalton's lymphomas bearing mice with 50, 125, 250, 300 and 400 mg/kg body weight of the ethanol extract resulted in a dose dependent rise in the survival of mice up to a dose of 300 mg/kg OIE when compared to SPS control ($p<0.001$) (Table 4, Figure 4). Treatment of tumor bearing mice with 400 mg/kg b. wt. also increased long term survivors by 60% up to 4) days (Table 4). A maximum number of tumor free survivors was observed at 300 mg/ kg. body weight OIE where

40% animals survived up to 120 days with no evidence of disease. AST of 59.22 days and MST of 62.5 days were found for 300 mg/kg with an IMLS of 278.79% and an IALS of 262.59%, respectively (Table5, figure 5). Since 40% animals survived at 300 mg/kg OIE until 120 days or more it was regarded as the best anticancer dose and it was investigated further (Table 5, Figure 5).

The treatment of tumor bearing mice with different doses of 50, 125, 250, 300 and 400 mg/kg body weight of the aqueous extract resulted in a dose dependent rise in the survival and maximum number of survivors (60%) was observed at 400 mg/kg body until 26 days. The administration of 400 mg/kg body weight OIA resulted in an AST of 24.75 days, MST of 24.55 days, IMLS of 48.48% and an IALS of 51.53%, respectively (Table 5, Figure 5).

5.4. Micronucleus Assay

The frequency of micronuclei bearing mononucleate (MNMNC) and binucleate cells (MNBNC) has been represented separately (Table 6, Figure 6). Treatment of Dalton's lymphoma bearing mice with OIE or DOX showed a rise in the frequency of micronuclei ($p<0.001$) in a time dependent manner up to 36 h post-drug treatment that declined thereafter in mononucleate cells, whereas this rise was highest at 24 h in the binucleate cells (Figure 6). The OIE treatment not only induced mononucleated and binucleated cells bearing one micronuclei but also the cells bearing two or more than two micronuclei thereafter (Figure 6).

5.5. Apoptosis Assay

The administration of OIE or DOX did induce apoptosis in Dalton's lymphoma cells as early as 1 h post drug treatment (Figure 7). The number of apoptotic cells in OIE or DOX treated DLA cells significantly ($p<0.001$) increased when compared to concurrent control group at all the post drug treatment times (Table

7). The analysis of apoptotic cells at different time revealed that OIE treatment increased the frequency of apoptotic cells with increasing assay time and the maximum number of apoptotic cells was scored at 24 h post drug treatment (Table 7 and Figure 7). The pattern of apoptosis was exactly similar in the DOX treated DLA cells (Figure 7). The necrotic cells also increased in time dependent manner and highest number of necrotic cells was observed at 36 h post –treatment (Figure 7).

5.6. DNA Fragmentation

The induction of apoptosis was further confirmed by DNA fragmentation assay in the form of DNA ladder formation. Administration of OIE or DOX in DLA mice caused fragmentation of DNA and formed a ladder like pattern characteristic of apoptosis at different post drug treatment times (Figure 8).

5.7. Biochemical Assays

5.7.1. Glutathione content

The treatment of DAL mice with 300 mg/kg b. wt. OIE caused a significant decrease in the glutathione contents in a time dependent manner up to 24 h post treatment (Figure 9). The maximum reduction was observed at 6 h where the glutathione contents were reduced by 1.68 fold ($p<0.001$). The difference in this alleviation in GSH contents between 6, 12 and 24 h was non-significant despite the fact that there was a marginal rise in GSH contents after 6 h. A similar pattern was observed for doxorubicin treatment (Figure 9). The OIE treatment reduced the GSH contents comparable to DOX treatment (Table 8).

5.7.2. Glutathione - S – Transferase (GST)

The administration of OIE or DOX resulted in a reduction in the GST activity significantly (Table 9) at all the post treatment times ($p<0.001$) and the highest decrease in GST activity was observed at 6 h post treatment (Figure 10) where this

decline was 2.8 fold ($p<0.001$). The activity of GST showed a minor rise at 12 and 24 h post treatment however, it was not significantly higher than 6 h value (Table 9).

5.7.3. Catalase (CAT) activity

Administration of OIE and DOX into Dalton's lymphomas bearing mice led to a gradual and time dependent decline ($p<0.001$) in the catalase activity until 24 h post treatment (Figure 11), where it was 1.5 fold lower than the SPS treatment and time (Table 10). The pattern of decline in catalase activity in DOX group was almost similar except that it was marginally higher than OIE treatment (Figure 11).

5.7.4. Superoxide dismutase (SOD) activity

The SOD activity declined in a time dependent manner after OIE or DOX treatment until 6 h post treatment where a greatest reduction in SOD activity was observed after OIE administration (Figure 12). This decline in SOD activity was 2.5 fold when compared to concurrent control ($p<0.001$) at 6 h post treatment and an elevation thereafter (Table 11).

5.8. Lipid peroxidation

The OIE caused a significant ($p<0.0001$) rise in the lipid peroxidation as early as 1 h post –treatment (Figure 13), where it was 2.34 fold higher than SPS group. Increase in assay time resulted in a further rise in LOO and 3.7 fold elevation was recorded at 6 h post treatment in the OIE group (Table 12). The pattern of LOO after DOX treatment was almost similar except that the rise was greater than OIE treatment (Figure 13). The LOO declined thereafter without reaching to SPS treatment level (Figure 13).

5.9. Liver and Kidney function tests

The intraperitoneal administration of OIE (300 mg/kg. b. wt.) and DOX (0.5 mg/kg. b. wt.) for consecutive 9 days did not significantly alter aspartate

aminotransferase (AST), and alanine aminotransferase (ALT), (Table 13 and Figure 14). The level of the AST and ALT in the serum of nine days treated mice showed a slight rise at 48 h post treatment when compared to concurrent control group ($p<0.05$). Despite this rise, the changes were within the normal range 8-40 U/ml and 7-35 U/ml, respectively (Reitman and Frankel, 1957). Therefore, the treatment of 300 mg/kg body weight of OIE did not cause undesirable effect on the liver.

The levels of creatinine and uric acid also followed same pattern as that of serum enzyme. Though a minor change was observed in both creatinine and uric acid levels, these changes did not cross the normal level i.e. 0.6-1.2 mg% (Bone et al., 1945) and 3.4-7 mg/dl (Fossati and Prencipe, 1980) for creatinine and uric acid, respectively; indicating the safety of OIE for the kidney (Table 13 and Figure 14).

6. DISCUSSION

The occurrence of cancer has been increasing even though there is availability of numerous modern treatment modalities. The cancer treatment involves surgery, radiation or chemotherapy or their combinations in different forms depending on the disease. The chemotherapy is the most preferred modality, especially when patients have metastasis. The term ‘Chemotherapy’ was coined by German chemist Paul Ehrlich in the 1900s since then it is being used in medical science to treat various disease states in humans. The cancer chemotherapy started in 1946 with the use of nitrogen mustards and antifolate drugs to treat cancer (Morrison, 2010). The development of cancer chemotherapy received a fillip with the development of transplantable tumors in rodents that provided a tool to evaluate different chemicals in preclinical setting (DeVita and Chu, 2008). Chemotherapy has three major roles in cancer: 1. as the primary treatment, 2. as an adjuvant to the primary treatment (to prevent or delay relapse), or 3. as palliative therapy to improve symptoms and prolong

survival following primary treatment failure (Devita and Chu, 2008). The chemotherapeutic agents primarily damage proliferating neoplastic cells and non-specificity of cytotoxic agents is a major drawback and potential to damage normal tissues which means that cure with chemotherapy is not often achieved (Corrie, 2008). This leads to the development of second neoplasia in the survivors (Morton et al., 2014). This clearly indicates that the incidence of cancer may be reduced by evolving treatment strategies/modalities, which will not allow the cells to get transformed into the malignant phenotype. The natural products may play an important role by killing neoplastic cells and not allowing the normal cells to transform into the malignant phenotype. The advantage of natural products is that they are natural in origin and hence most biocompatible with minimum side effects in comparison to chemical synthetic products (Jagetia, 2007). Therefore, present study was undertaken to evaluate the ability of *Oroxylum indicum* to kill the Dalton's lymphoma cells transplanted in mice.

Different extracts of *Oroxylum indicum* administered with a single oral dose showed no signs of any toxicity at 3 g/kg b. wt. in Swiss albino mice, which is in conformation to similar observations reported recently (Deori and Yadav. 2016). However, the intraperitoneal mode of administration revealed significant toxicity for both chloroform and ethanol extracts except aqueous extract, where the toxicity was higher than 2 g/kg body weight. Based on the toxicity study, the chloroform extract showed higher toxicity than ethanol extract with LD_{50} of 520 mg/kg b. wt. and 700 mg/kg b. wt., respectively after intraperitoneal administration. Systematic report on the toxicity of *Oroxylum indicum* stem extracts is lacking. The acute toxicity of 85% ethanol extract of *Alsonia scholaris* varied with season of stem bark collection and summer collection showed the highest toxicity and oral administration was less toxic

than the intraperitoneal treatment (Baliga et al., 2004). In an earlier study the LD_{50} for interaperitoneal administration was found to be lower than oral admiration for leaf extract of *Blighia Unijugata* (Frédéric et al., 2013).

Assessment of antitumour activity on Dalton's lymphoma transplanted in the peritoneum of mice indicated that the mice without any treatment developed DLA cells rapidly and all the untreated control mice died within 18 days with average survival time (AST) and median survival time (MST) of 16.33 and 16.5 days respectively. However, OIC treatment prolonged the survival of DLA bearing mice with an AST of 27.60 and MST of 27.52 days at the concentration of 250 mg/kg body weight for chloroform extract. Treatment of DAL mice with different doses of OIE increased the survival of mice in a dose dependent manner and a maximum effect was observed at a dose of 300 mg/kg b. wt. with a 40% tumor free survival beyond 120 days. The aqueous extract of *Oroxylum* was also not very effective in increasing the tumor free survival. The increase in tumor free survival have been reported for the stem bark extract of *Alstonia scholaris, Aphnamixis polystachya, Ervatamia heyncana, Hygrophila spinosa, Podyphyllum hexandrum, Rubia cordifolia, Tinospora cordifolia* and *Tylophora indica* earlier (Chitnis et al., 1971; 1972; 1979; Adwankar et al., 1980; Mazumdar et al., 1997; Jagetia et al., 1997; 2006; Goel et al., 1998; Jagetia 2008; Jagetia and Venkatesha, 2012).

The infliction of DNA damage is one of the important aspects to induce cytotoxicity in tumor cells. The ability of ethanol extract of *Oroxylum indicum* was tested in the DAL cells transplanted in mice and it was found that OIE triggered DNA damage by increasing the formation of micronuclei in mononucleated as well as binucleated cells effectively. Treatment of Dalton's lymphoma bearing mice with OIE showed a time dependent elevation in the frequency of micronuclei up to 36 h post

treatment and a decline thereafter. A similar effect has been observed earlier (Adiga and Jagetia, 1999; Jagetia and Venkatesh, 2015; Jagetia and Venkatesha, 2016). The maximum number of micronuclei is observed after first cell division and the frequency is reduced with subsequent division due to dilution. The OIE induced not only one micronuclei but also cells with two or more micronuclei indicating that it induced complex DNA damage in the form of multiply damaged sites that would have repressed the DNA damage repair causing higher cell death. A number of studies have indicated that the cells expressing micronuclei are dying cells and correlation between cell killing and micronuclei has been established (Jagetia et al., 2007; Jagetia and Venkatesh, 2015; Jagetia and Venkatesha, 2016). The analysis of micronuclei provide an indirect way of DNA damage assessment as the micronuclei also arise as a result of defective cell division, mis-segregation of chromosomes, DNA exchanges and faulty or suppressed DNA repair leading to cell death (Jagetia et al., 2007; Fenech, 2011; Sage and Harrison, 2011; Jagetia and Rao, 2011, 2015; Yates and Campbell, 2012; Zhang et al., 2015; Jagetia and Venkatesh, 2015; Jagetia and Venkatesha, 2016;). The formation of DNA DSBs and micronuclei is often the consequence of simultaneous excision repair of damages, wrong base incorporation and failure of the appropriate gap-filling event leads to DSB (Dianov et al., 1991). This may happen only if the level of DSBs exceeds the repair capacity of dividing cells, which is mainly due to either the misrepair of DSBs by the dysfunctional homologous recombination (O'Donovan and Livingston, 2010).

The cytotoxic effect of *Oroxylum indicum* may be due to the induction of apoptosis as OIE induced apoptosis in a time dependent manner leading to increased tumor free survivors in the present study. The infliction of DNA damage in the cells by OIE had triggered a cascade of biochemical and molecular events inducing

apoptosis, which was characterized by chromosome condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies and cell death (Cotter, 2009; Nikoletopoulou et al., 2013). Chrysin present in the stem bark of OIE has been reported to induce apoptosis by reducing the expression of Bcl_{xL}, survivins, IAP, c-FLIP; IKK and NF-κB and by increasing the caspase 3 activity (Pal-Bhadra et al., 2012; Sawicka et al. 2012). The other flavonoid Oroxylin A, present in *Oroxylum indicum* has been also reported to induce apoptosis in HeLa cells (Hu et al., 2006; Li et al., 2009; Hu et al., 2010). *Oroxylum indicum* has certainly induced DNA fragmentation in DLA cells exhibited by the formation of DNA ladder, which may have been due the activation of endonucleases that degrades DNA into multiple fragments of 180-200 base pairs that can be easily visualized in agarose gel (Wyllie, 1980; Bortner et al., 1995) and also micronuclei, which are large DNA fragment of the genome.

The intraperitoneal administration of OIE (300 mg/kg. b. wt.) for consecutive 9 days was safe as it did not significantly alter aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and uric acid. The level of creatinine and uric acid level also followed the pattern similar to serum enzymes. Though there was minor change in both creatinine and uric acid level, these changes did not cross the normal level i.e. 0.6-1.2 mg% (Bone et al., 1945) and 3.4-7 mg/dl (Fossati and Prencipe, 1980) for creatinine and uric acid, respectively; indicating the safety of OIE for host.

The glutathione is an important biomolecules synthesized by cells, which is essential in the elimination and detoxification of toxins¸ cell differentiation, proliferation and apoptosis however, reduced GSH levels cause oxidative stress (Meister and Anderson, 1983; Ganesaratnam et al., 2004; Lushchak, 2012; Traverso

et al., 2013; Schumacker, 2015). A reduction in GSH leads to several pathologic conditions in humans (Colell et al., 1998; Lluis et al., 2005). The elevated levels of glutathione are responsible for chemoresistance and lead to treatment failure (Ramsay and Dilda, 2014), whereas alleviation in GSH leads in the enhanced oxidative stress that sensitizes cells during chemotherapy or radiotherapy (Ortega et al., 2012; Rocha et al., 2014). The treatment of OIE decreased the glutathione contents in the Dalton's lymphoma cells in a time dependent manner, which may have increased the oxidative stress and killed the DAL cells effectively. Similarly, treatment of Dalton's lymphoma with OIE had a negative effect on the activities of GST, Catalase and SOD, which are also involved in resistance to chemotherapy (Kodydková et al., 2014; Zeng et al., 2014; Che et al., 2016). This depletion in their activities may have made tumors more amenable to the cytotoxic effect of OIE causing increased tumor free survival in the present study. The other important effect of OIE was the induction of lipid peroxidation in DAL cells, which has certainly increased the oxidative stress in tumor cells and killed them in the present study.

The mechanism by which OIE induced cytotoxicity in the tumor cells in the present study is not fully understood. The OIE may have used several pathways to induce cell death in the tumor bearing mice. First and foremost important action seems to be the induction of DNA damage in the tumor cells, which is corroborated by increased frequency of micronuclei and apoptosis. Electrophoretic study confirmed that OIE brought the DNA fragmentation in DAL cells, a hallmark of apoptosis. The apoptosis may have been induced by alleviated expression of Bcl_{xL}, survivin, IAP, c-FLIP; IKK and NF-κB. The stem bark extract of *Oroxylum* has been found to inhibit the NF-κB activity (Tran et al., 2015). Chrysin a flavonoid present in the *Oroxylum* has been actually reported to suppress the expression of all these transcription factors

and stimulation of caspase 3 activity (Pal-Bhadra et al., 2012; Sawicka et al. 2012). *Oroxylum indicum* may have stimulated preapoptotic pathway including Bax and p21 leading to cell death (Hu et al., 2006; Li et al., 2009).The reduction in GSH, GST, catalase and SOD and rise in the lipid peroxidation by OIE would have increased the oxidative stress in the DAL cells bringing effective cell killing in the present study. Some other unknown mechanisms may also have contributed to the cytotoxic effect of OIE.

The OIE killed tumor cells and increased the tumor free survival, which may be due to its ability to fragment DNA, induce micronuclei and apoptosis. The apoptosis may have been triggered by the activation of proapoptotic genes including Bax and p21 and caspase 3, and blockage of antiapoptotic cascade including Bcl_{xL}, survivin, IAP, c-FLIP; IKK and NF-κB. The reduction in GSH, GST, catalase and SOD and elevation in lipid peroxidation may have played a major role in inducing DNA damage and stimulating apoptotic pathway that finally killed the DAL cells and increased the tumor free survivors in OIE treat mice beyond 120 days.

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Table 1: Acute toxicity of different solvent extracts of *Oroxylum indicum* administered orally in Albino mice with concentration of 2g/kg. body weight in both sexes, n=10.

Extract/ Group	Sex	Dose (g/kg.bwt.)	Body weight (g)			Survival
			Before fasting	After fasting	Loss (18 h)	
Control (SPS)	M	0	39	34	5	> 14 days
			28	25.3	2.7	> 14 days
			28	25.3	2.7	> 14 days
	F		29	25.7	2.86	> 14 days
			22	20.5	1.5	> 14 days
			22	20.5	1.5	> 14 days
Chloroform	M	2	34	29	5	> 14 days
			33	31	2	> 14 days
			34	29	5	> 14 days
			33	31	2	> 14 days
			34	32	2	> 14 days
	F		25	22	3	> 14 days
			24	22	2	> 14 days
			25	22	3	> 14 days
			24	22	2	> 14 days
			25	22	3	> 14 days
Ethanol	M	2	31.6	29.9	1.7	> 14 days
			37.5	33.2	4.3	> 14 days
			31.6	29.9	1.7	> 14 days
			37.5	33.2	4.3	> 14 days
			37.5	33.2	4.3	> 14 days
	F		25.8	23.7	2.1	> 14 days
			23.1	21.5	1.6	> 14 days
			25.8	23.7	2.1	> 14 days
			25.8	23.7	2.1	> 14 days
			23.1	21.5	1.6	> 14 days
Aqueous	M	2	35	31.6	3.4	> 14 days
			35.7	32	3.7	> 14 days
			35	31.6	3.4	> 14 days
			35.7	32	3.7	> 14 days
			35.7	32	3.7	> 14 days
	F		30	27.5	2.5	> 14 days
			33	29.5	3.5	> 14 days
			30	27.5	2.5	> 14 days
			33	29.5	3.5	> 14 days
			33	29.5	3.5	> 14 days

Table 2: Effect of different solvent extracts of *Oroxylum indicum* on acute toxicity administered various doses intraperitoneally in Swiss albino mice. The LD50 was determined using probit, n=10.

Extract type	Dose (mg/kg b. wt.)	Survival (%)	LD_{50} (mg/kg)
Chloroform	400	100	**520**
	500	50	
	1000	20	
	1500	0	
	2000	0	
Ethanol	400	100	**700**
	500	70	
	1000	30	
	1500	10	
	2000	0	
Aqueous	400	100	**2000**
	500	100	
	1000	100	
	1500	100	
	2000	50	

Table 3: Change in body weight of Dalton's lymphoma bearing Swiss albino mice. The data are expressed as Mean ±SEM , n=10.

Treatment	Dose (mg/kg.b.wt)	Post tumour transplanted time (day)							
		0	1	3	6	9	12	15	18
SPS	0	23.41±0.64	24.61±0.98	26.11±0.49	27.91±0.89	30.71±0.91	35.71±0.78	41.01±0.45	46.71±0.68
Chloroform	50	23.30±0.47	25.59±0.21	28.56±0.86	32.02±1.11	34.05±1.51	36.05±1.53	38.35±1.52	40.75±1.50
	125	23.55±0.51	25.92±0.23	27.38±0.87	28.58±1.3	30.22±1.59	34.02±1.11	38.06±1.52	39.35±1.52
	250	23.45±0.72	25.30±0.88	25.84±0.84	26.29±0.90	27.23±0.89	30.25±0.68	33.73±0.69	35.03±0.68
	300	23.86±0.55	25.42±0.81	26.21±0.62	27.75±0.94	29.55±0.88	30.18±1.13	31.88±1.04	32.75±0.69
	400	23.46±0.64	25.45±0.98	26.90±0.49	29.02±0.41	29.50±0.69	30.70±0.68	31.90±0.70	33.20±0.71
Ethanol	50	23.27±0.54	25.69±0.84	26.33±0.61	28.01±0.63	30.85±0.58	33.21±1.19	36.21±1.18	39.21±1.19
	125	24.12±0.60	25.31±0.97	25.98±1.24	27.16±1.19	28.40±0.96	29.68±1.11	32.24±0.91	35.25±0.92
	250	23.43±0.76	25.34±1.23	28.51±2.16	29.62±2.07	30.78±2.21	31.96±2.58	34.68±2.46	37.11±2.43
	300	24.65±0.55	27.67±0.83	28.06±0.91	28.76±0.77	29.41±0.96	30.22±1.05	32.11±0.97	33.22±1.06
	400	23.54±0.65	26.35±1.16	27.20±1.18	27.78±1.09	28.45±0.89	29.17±0.90	32.46±0.78	35.89±0.44
Aqueous	50	23.03±0.42	25.87±0.27	28.71±0.73	31.21±1.18	34.58±0.99	41.84±0.97	43.44±0.92	45.04±0.98
	125	22.77±0.62	25.69±0.63	28.40±0.59	32.04±0.64	34.97±1.63	39.08±1.68	40.07±1.65	41.48±1.66
	250	23.88±0.43	25.67±0.75	28.08±1.37	31.14±2.06	33.60±2.56	35.06±3.17	37.27±3.16	39.65±3.14
	300	23.14±0.74	25.29±0.52	27.20±1.29	29.04±1.83	30.68±2.59	34.72±1.76	36.29±1.72	37.74±1.76
	400	23.68±0.56	27.26±0.83	28.21±0.68	29.65±0.64	31.64±0.78	34.34±1.06	36.14±1.06	37.94±1.07

Table 4: Effect of different solvent extracts of *Oroxylum indicum* on Survival of Dalton's lymphomas ascites bearing mice treated with various doses administered intraperitoneally. n=10.

Post tumor transplant time (day)	SPS (Control)	Survivality (%)														
		Chloroform Extract					Ethanol Extract					Aqueous Extract				
		Dose (mg/kg.b.wt)					Dose (mg/kg.b.wt)					Dose (mg/kg.b.wt)				
		50	125	250	300	400	50	125	250	300	400	50	125	250	300	400
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
18	0	100	100	100	80	100	100	100	100	100	100	100	100	100	100	100
20	0	90	100	100	80	90	90	100	100	100	100	100	90	80	100	100
22	0	80	90	90	70	80	80	100	100	100	100	80	80	70	80	100
24	0	20	80	80	60	50	60	80	100	100	100	30	50	50	70	80
26	0	0	40	60	30	20	0	60	100	100	100	0	0	10	20	60
28	0	0	20	40	20	10	0	50	80	100	100	0	0	0	0	10
30	0	0	0	20	10	0	0	40	70	100	100	0	0	0	0	0
32	0	0	0	10	0	0	0	0	60	100	80	0	0	0	0	0
40	0	0	0	0	0	0	0	0	50	100	60	0	0	0	0	0
44	0	0	0	0	0	0	0	0	40	100	10	0	0	0	0	0
46	0	0	0	0	0	0	0	0	30	90	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	20	80	0	0	0	0	0	0
54	0	0	0	0	0	0	0	0	10	70	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	10	60	0	0	0	0	0	0

Table 5: Effect of different extracts of *Oroxylum indicum* on Dalton's lymphoma ascites bearing mice and the tumor response assessment based on median survival time (MST) and average survival time (AST). Increase in mean life span (% IMLS) and increase in average life span (% IALS). The results were expressed as percent (%) ± SEM, n=10.

Treatment	Dose (mg/kg.b.wt.)	MST	AST	IMLS (%)	IALS (%)
Control (SPS)	**0**	16.5±0.53	16.33±**0.52**	0.05±0.00	0.05±0.00
Chloroform	**50**	23.45±1.23*	23.50±1.11*	42.42±1.08$^{\alpha}$	43.88±2.88$^{\alpha}$
	125	27.50±1.41*	27.27±1.24*	66.47±1.34$^{\alpha}$	66.94±2.44
	250	27.52±1.22*	27.60±1.41*	66.67±1.82$^{\alpha}$	68.98±2.42$^{\alpha}$
	300	27.00±2.13*	27.00±0.94*	63.64±1.95$^{\alpha}$	65.31±1.83$^{\alpha}$
	400	26.50±1.72*	26.50±0.58*	60.61±2.16$^{\alpha}$	62.24±1.35$^{\alpha}$
Ethanol	**50**	23.50±1.25*	23.50±0.64*	42.42±1.24$^{\alpha}$	43.88±1.94$^{\alpha}$
	125	27.25±1.45*	27.40±0.85*	66.67±1.56$^{\alpha}$	67.76±2.14$^{\alpha}$
	250	47.50±1.08*	44.00±0.42*	211.27±1.89$^{\alpha}$	152.16±2.19$^{\alpha}$
	300	62.55±1.05*	59.22±0.85*	278.79±2.58$^{\alpha}$	262.59±2.09$^{\alpha}$
	400	38.50±2.15*	37.75±0.45*	133.33±2.46$^{\alpha}$	131.12±2.47$^{\alpha}$
Aqueous	**50**	21.50±1.02*	21.00±0.25*	30.30±1.73$^{\alpha}$	28.57±1.09$^{\alpha}$
	125	21.25±1.95*	21.50±0.48*	30.30±1.34$^{\alpha}$	31.63±0.85$^{\alpha}$
	250	22.50±2.014*	22.40±0.59*	36.36±2.45$^{\alpha}$	37.14±1.23$^{\alpha}$
	300	23.40±1.08*	23.42±1.52*	42.42±2.82$^{\alpha}$	43.27±2.12$^{\alpha}$
	400	24.55±1.44*	24.75±1.55*	48.48±2.21$^{\alpha}$	51.53±2.45$^{\alpha}$

*$p<0.001$, $^{\alpha}$,$p<0.0001$ when treatment are compared to spontaneous control group.

Table 6: Induction of micronuclei in Dalton's lymphoma ascites bearing mice by treatment with 300mg/kg.b.wt. ethanol extract of *Oroxylum indicum* (OIE) and 0.5mg/kg. b.wt. doxorubucin (DOX) at different post treatment time. The results were determined as frequency of Micronuclei/1000 cells and expressed as the Mean ± SEM, n=10.

Cell type	Post Treatment time (h)	Frequency of Micronuclei (Mean ±SEM)								
		SPS			OIE 300			DOX 0.5		
		One MN	Two MN	Total	One MN	Two MN	Total	One MN	Two MN	Total
Mononucleated cell	6	4.62±1.42	0.82±0.06	**5.44±0.65**	2.12±0.5	1.24±0.45	3.36±**0.98**	4.24±0.50	1.24±0.56	**5.48±0.57**
	12	5.09±0.95	1.13±0.02	**6.04±1.66**	17.01±1.73	8.89±1.21	**25.90±1.47**	43.00±1.45	10.00±1.32	**53.00±1.26**
	24	5.65±1.53	1.13±0.01	**6.98±1.52**	52.00±2.00	14.21±1.32	**66.21±0.85**	73.00±2.42	26.00±2.43	**99.00±1.84**
	36	5.88±0.86	1.52±0.05	**7.40±1.68**	71.00±1.80	22.35±2.35	**93.35±1.45**	101±2.31	34.00±2.11	**135.00±2.18**
	48	6.14±1.75	1.21±0.05	**3.35±1.24**	68.00±1.50	21.42±1.87	**89.43±2.05**	98.00±2.5.11	32.00±1.73	**130.00±3.64**
Binucleated cell	**6**	5.12±1.22	1.02±0.07	**6.24±0.58**	58.12±4.21*	9.25±1.24*	**67.37±2.72**	62.22±4.67*	11.43±1.2*	**73.65±2.94**
	12	6.05±0.67	1.23±0.09	**7.28±1.54**	86.24±5.23*	12.42±0.39*	**98.66±2.81**	98.35±2.35*	14.32±1.4*	**112.67±1.89**
	24	6.14±1.23	1.25±0.08	**7.39±1.22**	96.11±7.25*	13.22±1.04*	**109.33±4.14**	112.11±2.43*	18.22±1.3*	**130.33±1.88**
	36	6.15±1.52	1.62±0.04	**7.79±1.42**	92.72±2.34*	15.27±1.59*	**107.85±1.96**	110.51±4.21*	21.26±1.1*	**131.77±2.67**
	48	6.34±1.83	1.21±0.08	**7.55±1.54**	84.52±2.41*	15.13±1.47*	**99.65±1.94**	103.38±2.46*	20.28±1.0*	**123.66±1.75**

**p<0.001when treatment are compared with concurrent control (SPS) group.*

No symbol= no significance.

Table 7: Induction of apoptosis and necrosis in Dalton's lymphoma ascites bearing mice by treatment with 300mg/kg.b.wt. ethanol extract of *Oroxylum indicum* (OIE) and 0.5mg/kg.b.wt. doxorubucin (DOX) at different post treatment time. The results were determined as percent index and expressed as the Mean ± SEM, n=10.

Post treatment time(h)	Apoptosis & necrosis (% ±SEM)					
	SPS		OIE 300		DOX 0.5	
	Apoptotic	Necrotic	Apoptotic	Necrotic	Apoptotic	Necrotic
1	0.54±0.02	0.42±0.04	1.60±0.17*	1.30±0.07[a]	3.40±0.11 [a]	2.20±0.57 [a]
2	0.89±0.06	0.51±0.05	3.20±0.21 [a]	2.40±0.15[a]	5.80±0.31 [a]	4.61±0.73 [a]
4	0.89±0.02	0.51±0.04	6.50±1.15 [a]	3.50±0.24[a]	8.80±1.12 [a]	6.19±0.31 [a]
6	0.98±0.07	0.52±0.05	7.80±0.31 [a]	4.60±0.41[a]	10.60±0.25[a]	8.30±0.73 [a]
8	0.98±0.06	0.63±0.06	9.10±0.45 [a]	5.73±0.62[a]	12.40±0.31[a]	10.20±0.61[a]
12	0.99±0.08	0.64±0.06	10.46±0.33[a]	7.90±0.67[a]	13.87±0.31[a]	12.53±0.46[a]
24	2.92±0.07	0.65±0.05	13.33±0.91[a]	9.39±0.32[a]	19.07±0.91[a]	15.65±0.88[a]
36	3.45±0.08	0.84±0.03	13.61±0.15[a]	9.67±0.50[a]	18.99±0.62[a]	17.82±0.31[a]
48	3.56±0.10	0.87±0.07	12.63±0.48[a]	9.98±0.25[a]	18.67±0.70[a]	16.88±0.86[a]

**p<0.05, [a]p<0.001 when treatment are compared with concurrent control (SPS) group.*

No symbol= no significance.

Table 8: Changes in the glutathione contents of Mice bearing Dalton's lymphoma ascites treated with *Oroxylum indicum* extract (OIE) and doxorubicin (DOX). The results were expressed as the Mean ± SEM, n=10.

Post Treatment Time (h)	Glutathione (GSH) (µM/mg protein), Mean ± SEM		
	SPS	OIE	DOX
1	7.24 ± 0.01	6.19 ± 0.002*	5.68 ± 0.15*
2	7.21 ± 0.01	6.16 ± 0.019*	5.03 ± 0.06*
4	7.23 ± 0.02	6.15 ± 0.053*	4.95 ± 0.02*
6	7.25 ± 0.02	4.29 ± 0.12*	4.75 ± 0.04*
12	7.19 ± 0.03	4.78 ± 0.01*	4.78 ± 0.09*
24	7.19 ± 0.03	4.73 ± 0.09*	4.68 ± 0.08*

p<0.001when treatment are compared with concurrent control (SPS) group. No symbol= no significance.

Table 9: Changes in the glutathione-s-transferase activity of Mice bearing Dalton's lymphoma treated with *Oroxylum indicum* extract (OIE) and DOX. The results were expressed as the Mean ± SEM, n=10.

Post Treatment Time (h)	Glutathione-S-transferase (GST) (U/mg protein), Mean ± SEM		
	SPS	OIE	DOX
1	0.056 ± 0.004	0.035 ± 0.001*	0.03 ± 0.0005*
2	0.053 ± 0.005	0.035 ± 0.003*	0.029 ± 0.0028*
4	0.054 ± 0.004	0.027 ± 0.001*	0.024 ± 0.006*
6	0.06 ± 0.009	0.021 ± 0.003*	0.018 ± 0.007*
12	0.056 ± 0.004	0.025 ± 0.005*	0.0158 ± 0.001*
24	0.053 ± 0.005	0.024 ± 0.002*	0.017 ± 0.004*

p<0.001 when treatment are compared with concurrent control (SPS) group.
No symbol= no significance.

Table 10: Alterations in the catalase activity of Mice bearing Dalton's lymphoma treated with *Oroxylum indicum* extract (OIE) and DOX. The results shown are the Mean ± SEM, n=10.

Post Treatment Time (h)	Catalase (CAT) (U/mg protein), Mean ± SEM		
	SPS	OIE	DOX
1	23.08 ± 0.37	21.58 ± 0.56	18.59 ± 0.64*
2	23.07 ± 0.98	20.51 ± 0.37	16.24 ± 0.43*
4	23.51 ± 0.43	19.23 ± 0.64*	13.68 ± 0.56*
6	23.50 ± 0.58	15.38 ± 0.64*	12.60 ± 0.21*
12	23.93 ± 0.57	14.96 ± 0.57*	11.97 ± 0.21*
24	23.07 ± 0.98	14.32 ± 0.21*	11.32 ± 0.57*

p<0.001 when treatment are compared with concurrent control (SPS) group.
No symbol= no significance.

Table 11: Changes in the superoxide dismutase activity of Mice bearing Dalton's lymphoma treated with *Oroxylum indicum* extract (OIE) and DOX. The results were expressed as the Mean ± SEM, n=10.

Post Treatment Time (h)	Superoxide dismutase (SOD) (U/mg protein), Mean ± SEM		
	SPS	OIE	DOX
1	1.81 ± 0.05	1.62 ± 0.028 *	1.50 ± 0.06*
2	1.804 ± 0.03	1.41 ± 0.02*	1.35 ± 0.01*
4	1.814 ± 0.04	0.91 ± 0.06*	0.78 ± 0.04*
6	1.79 ± 0.069	0.70 ± 0.09*	0.51 ± 0.01*
12	1.81 ± 0.07	0.83 ± 0.05*	0.49 ± 0.03*
24	1.79 ± 0.03	0.81 ± 0.01*	0.46 ± 0.02*

p<0.001when treatment are compared with concurrent control (SPS) group. No symbol= no significance.

Table 12: Alterations in the lipid peroxidation level of Mice bearing Dalton's lymphoma treated with *Oroxylum indicum* extract (OIE) and DOX. The results were presented as the Mean ± SEM, n=10.

Post Treatment Time (h)	Lipid peroxidation (MDA) Mean ± SEM, (nmol /mg protein)		
	SPS	OIE	DOX
1	1.31 ± 0.012*	3.07 ± 0.005*	3.37 ± 0.017*
2	1.39 ± 0.01*	4.69 ± 0.02*	8.48 ± 0.015*
4	1.43 ± 0.03*	4.88 ± 0.01*	9.60 ± 0.028*
6	1.69 ± 0.02*	6.19 ± 0.02*	10.59 ± 0.03*
12	1.72 ± 0.01*	6.03 ± 0.01*	10.11 ± 0.02*
24	1.89 ± 0.02*	4.68 ± 0.05*	09.84 ± 0.02*

p<0.0001when treatment are compared with concurrent control (SPS) group. No symbol= no significance.

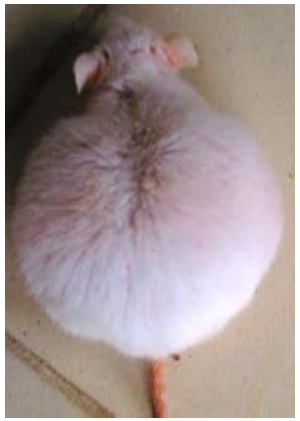
Table 13: Effect on the liver and kidney toxicity of Dalton's lymphoma bearing mice treated with *Oroxylum indicum* extract (OIE) and DOX. The results were presented as the Mean ± SEM, n=10.

Treatment	Dose (mg/kg.b.wt.)	Post treatment time (h)	Liver function test		Kidney function test	
			AST (U/ml)	ALT (U/ml)	Creatinine (mg%)	Uric acid (mg/dl)
Control (SPS)	0	1	27.11±1.42	17.36±0.72	0.73±0.02	2.72±0.23
		24	27.28±0.94	18.46±1.32	0.56±0.04	2.22±0.35
		48	27.12±2.32	21.24±0.72	0.49±0.08	2.29±0.07
OIE	300	1	31.88±0.35	22.93±0.34	0.73±0.02	3.00±0.02
		24	35.58±0.23	20.03±2.65	0.58±0.01	2.63±0.17
		48	36.16±0.12*	22.44±0.24	0.83±0.06	2.79±0.11
DOX	0.5	1	29.49±0.23	20.88±1.32	0.77±0.06	3.63±0.09
		24	28.50±0.47	23.65±0.25	0.84±0.03*	3.74±0.13*
		48	33.36±0.35*	22.81±0.36	0.85±0.05*	3.79±0.17*

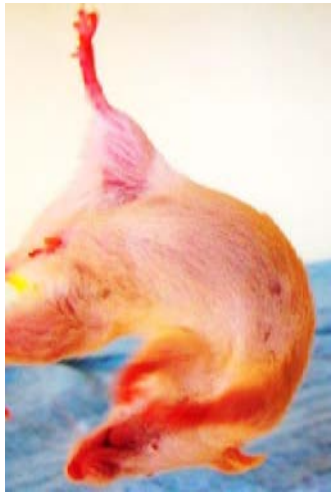
**p<0.05 when treatment group are compared with concurrent control group.*

No symbol= no significance.

Normal Swiss albino mice



Dalton's transplanted mice



DLA bearing mice Treated with OIE

DLA cells stained with Trypan blue dye

Plate I: Effect of *Oroxylum indicum* extract (OIE) treatment on Dalton's lymphoma ascites bearing Swiss albino mice.

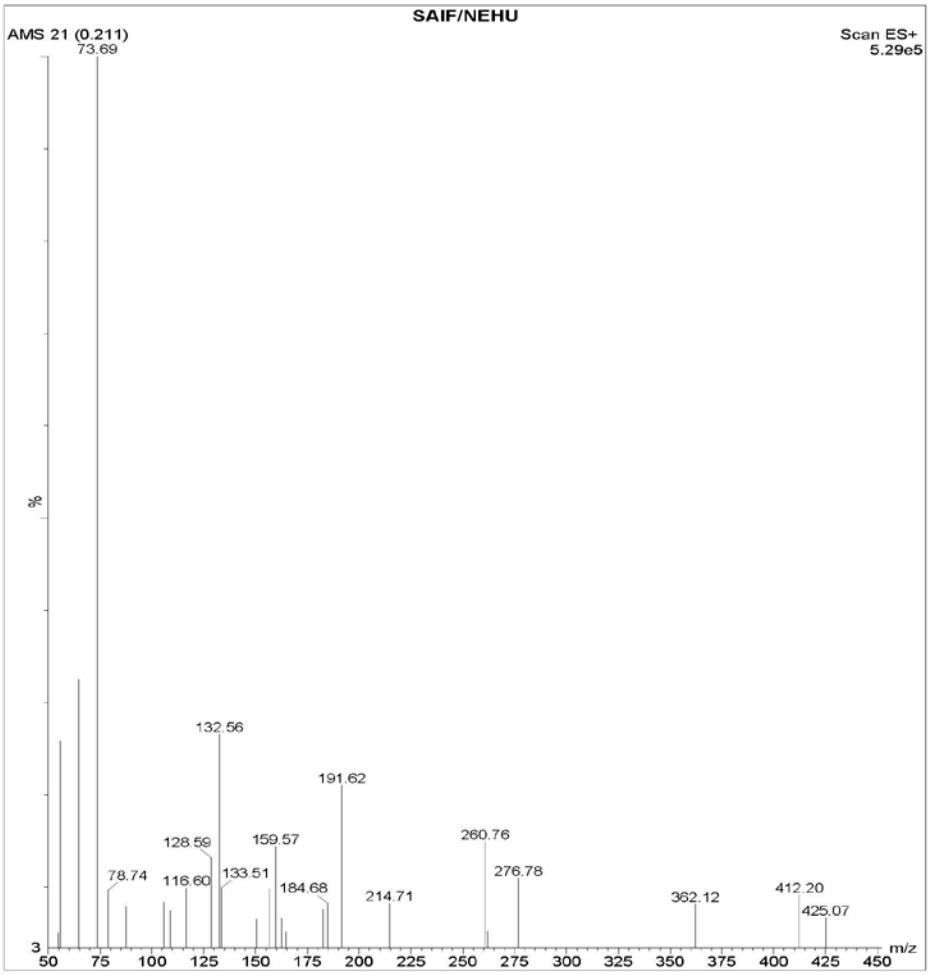


Figure 1: Mass spectrum of column fraction (A) performed in NEHU-SAIF for LC-MS ESI (+) for determining the molecular weight (LC-MS Waters ZQ-4000.

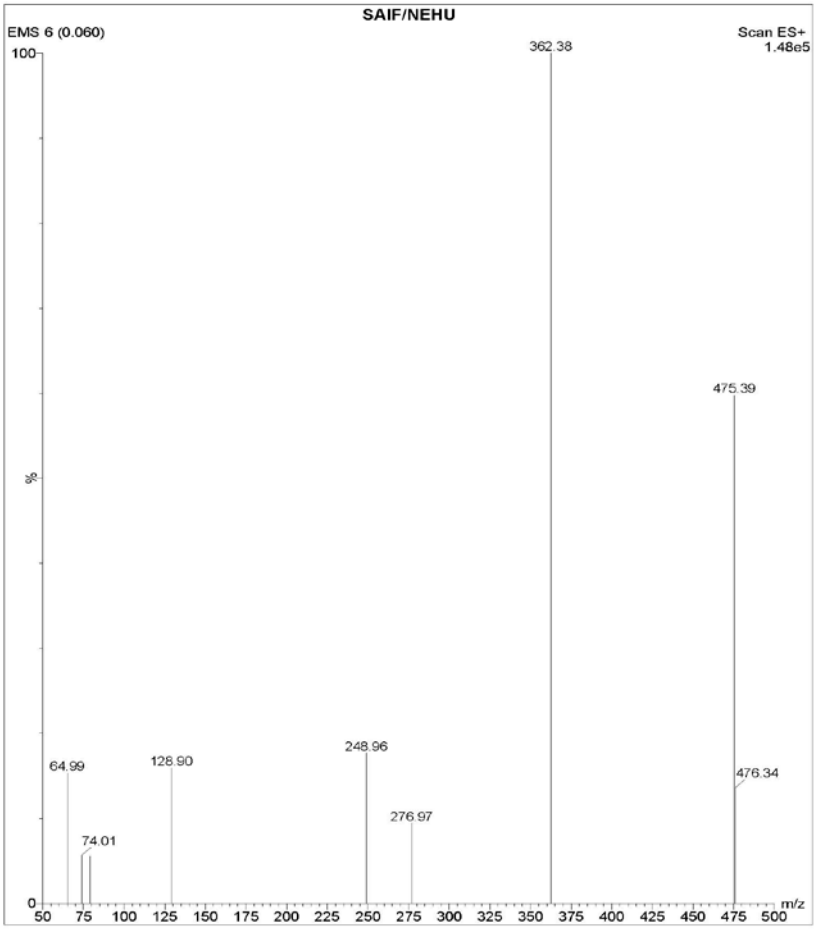


Figure 2: Mass spectrum of column fraction (E) performed in NEHU-SAIF for LC-MS ESI (+) for determining the molecular weight (LC-MS Waters ZQ-4000).

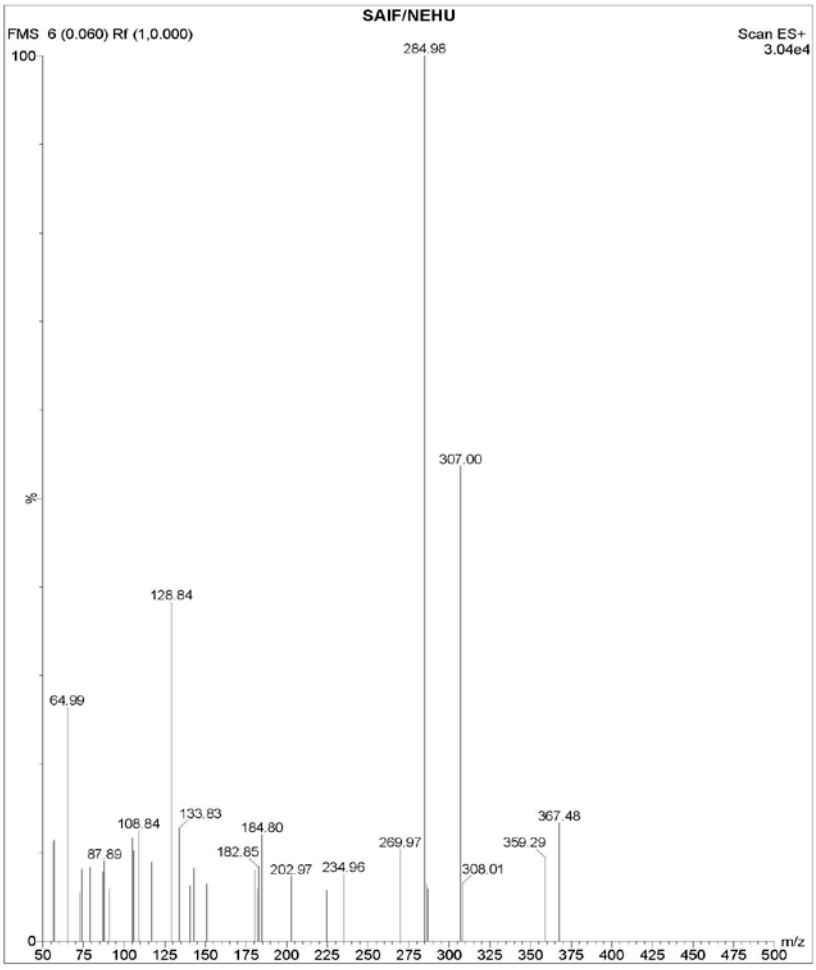


Figure 3: Mass spectrum of column fraction (F) performed in NEHU-SAIF for LC-MS ESI (+) for determining the molecular weight (LC-MS Waters ZQ-4000.

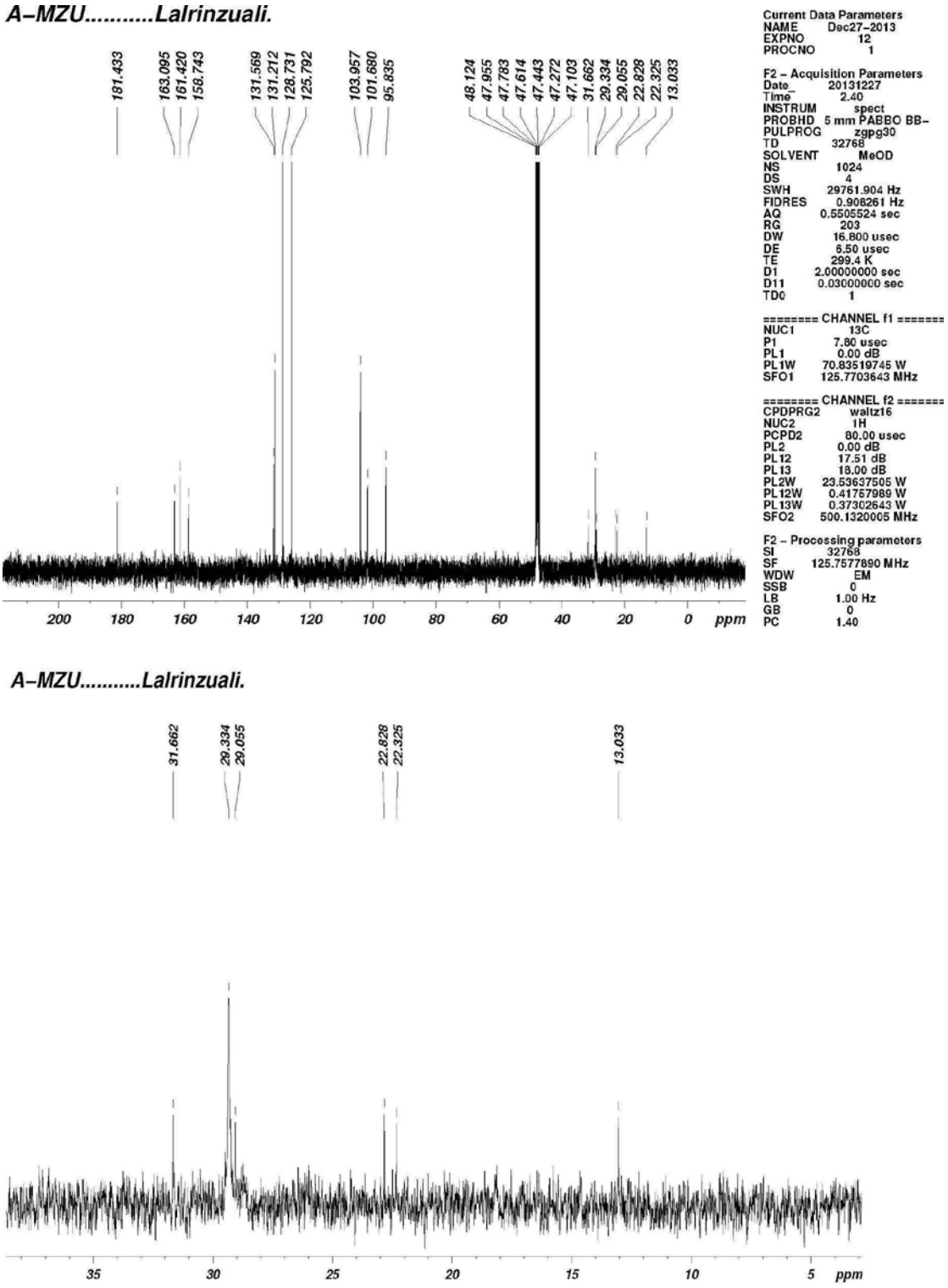


Figure 4: Nuclear magnetic resonance (NMR) spectrum of isolated compound fraction (A) for the detection of Carbon number performed at SAIF-IIT Chennai (Bruker AV III, 500MHz FT NMR Spectrometer).

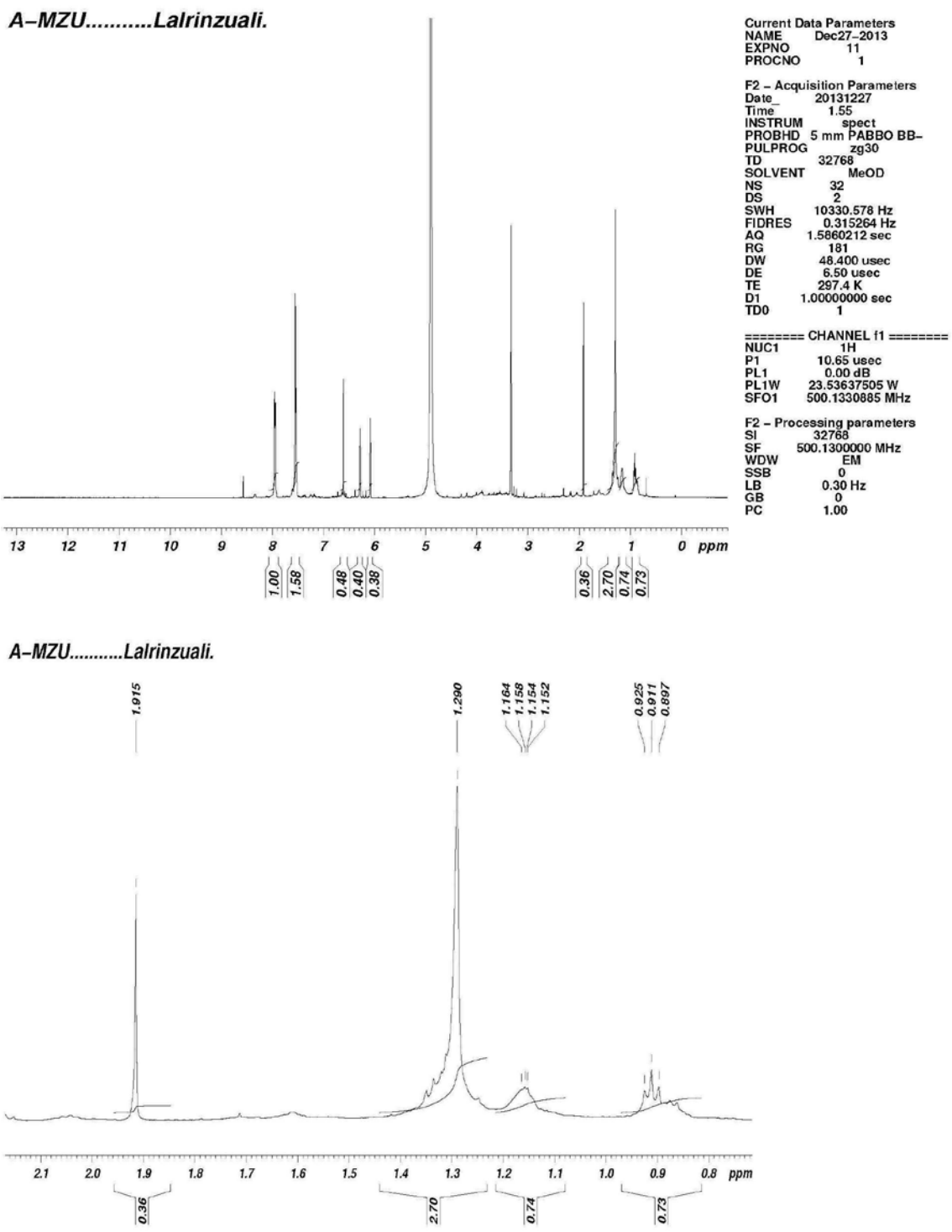


Figure 5: Nuclear magnetic resonance (NMR) spectrum of isolated compound fraction (A) for the detection of proton number performed at SAIF-IIT Chennai (Bruker AV III, 500MHz FT NMR Spectrometer).

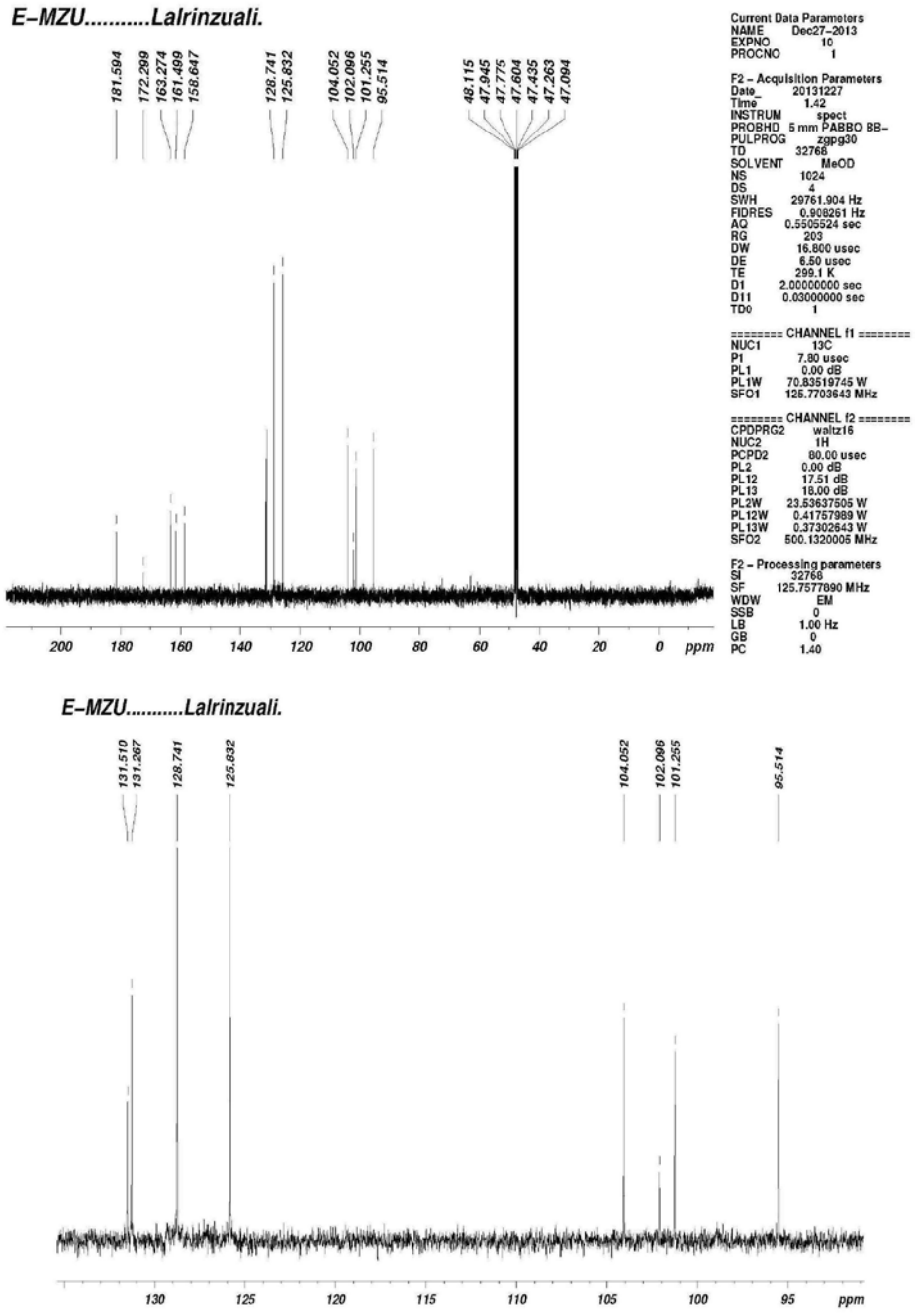


Figure 6: Nuclear magnetic resonance (NMR) spectrum of isolated compound fraction (E) for the detection of carbon number performed at SAIF-IIT Chennai (Bruker AV III, 500MHz FT NMR Spectrometer).

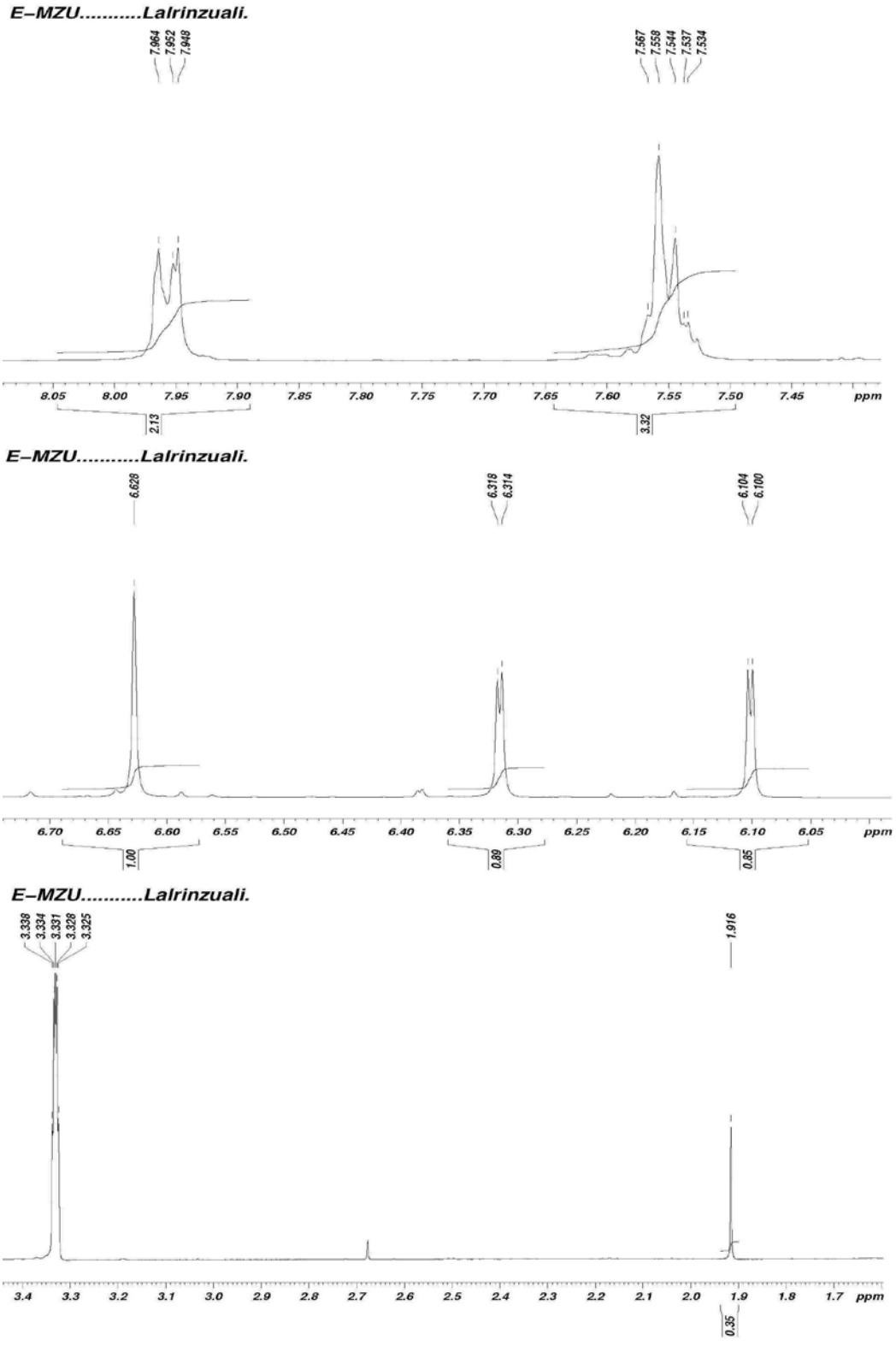


Figure 7: Nuclear magnetic resonance (NMR) spectrum of isolated compound fraction (E) for the detection of proton number performed at SAIF-IIT Chennai (Bruker AV III, 500MHz FT NMR Spectrometer).

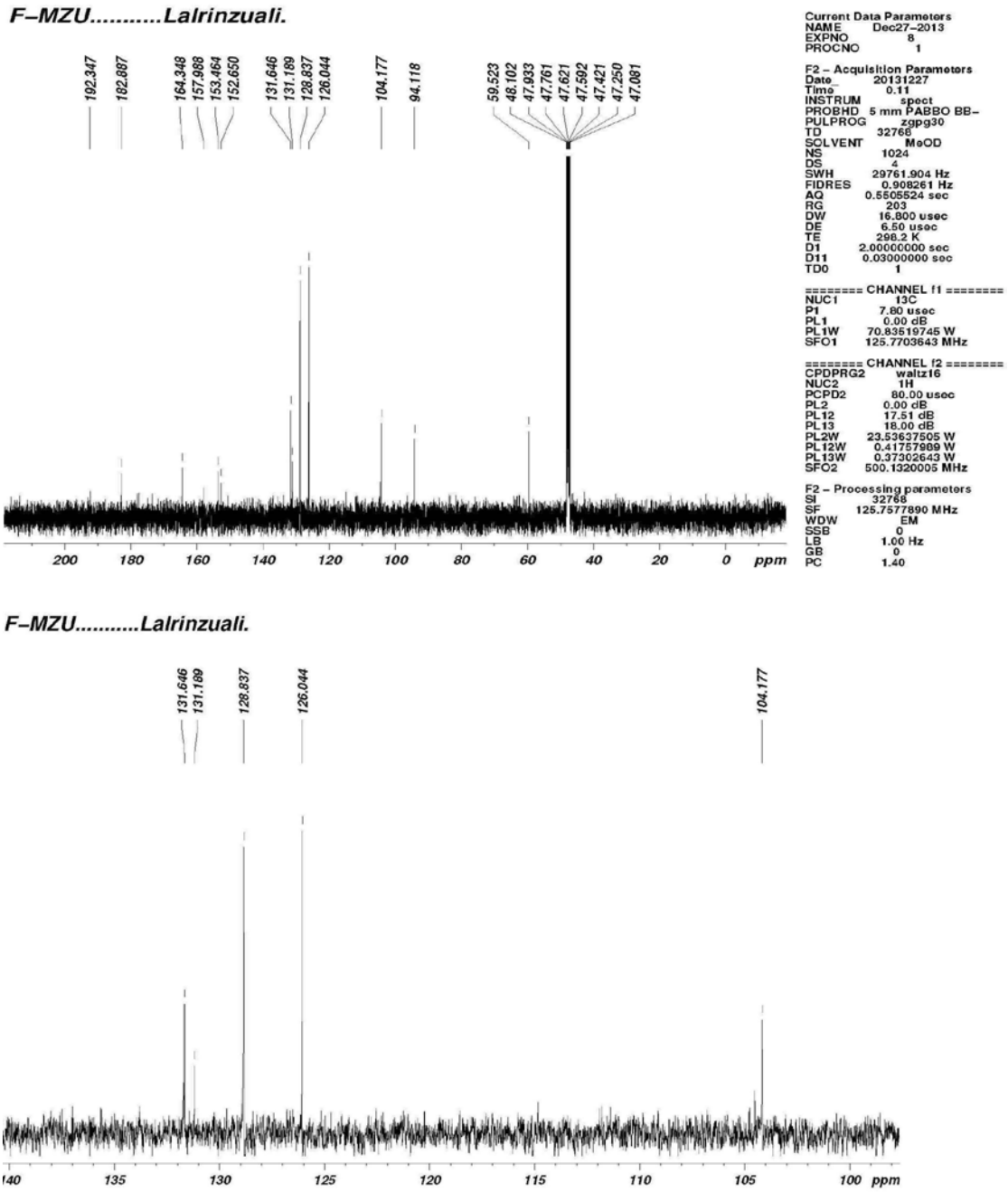


Figure 8: Nuclear magnetic resonance (NMR) spectrum of isolated compound fraction (F) for the detection of carbon number performed at SAIF-IIT Chennai (Bruker AV III, 500MHz FT NMR Spectrometer).

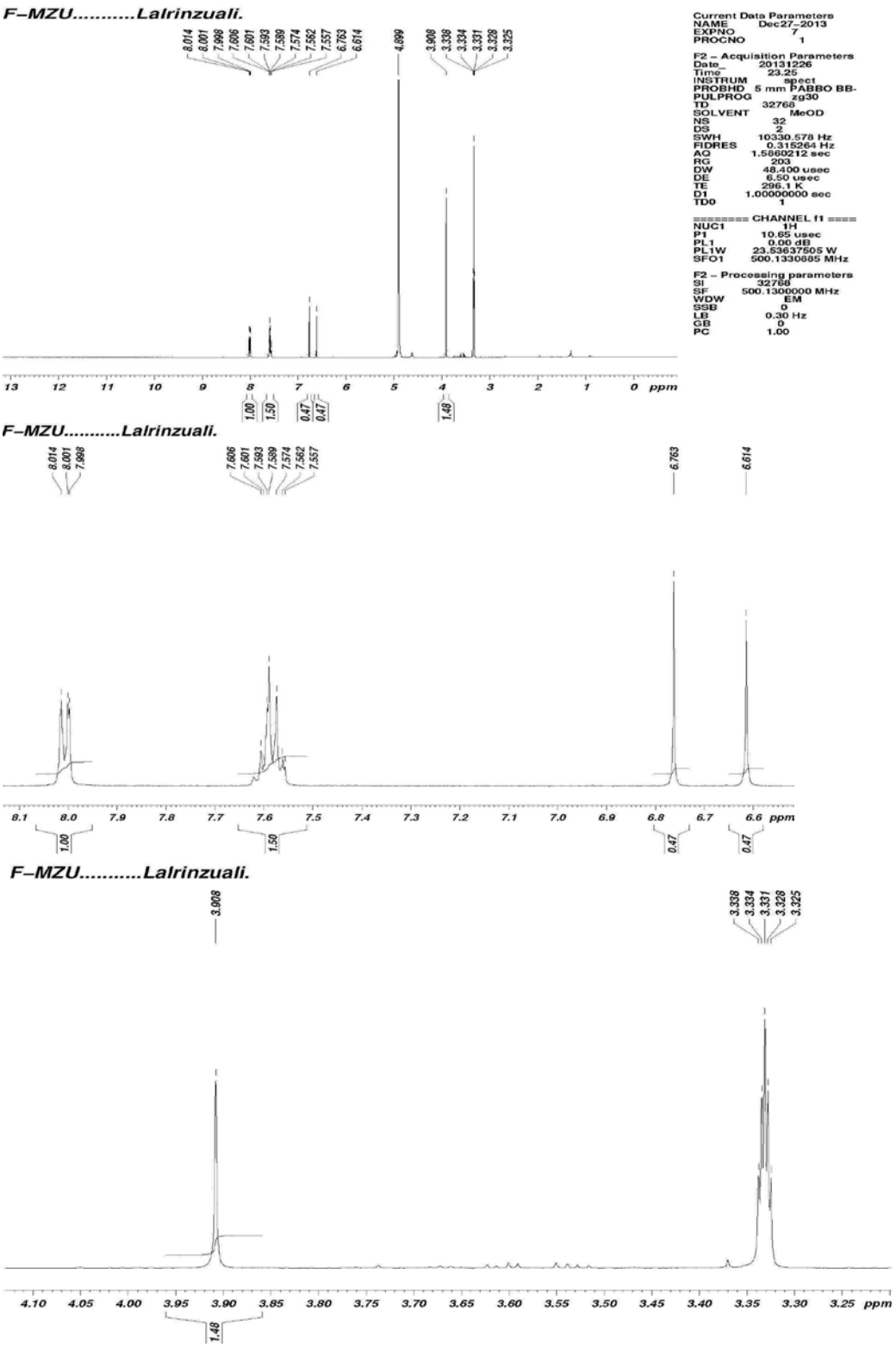


Figure 9: Nuclear magnetic resonance (NMR) spectrum of isolated compound fraction (F) for the detection of carbon number performed at SAIF-IIT Chennai (Bruker AV III, 500MHz FT NMR Spectrometer).

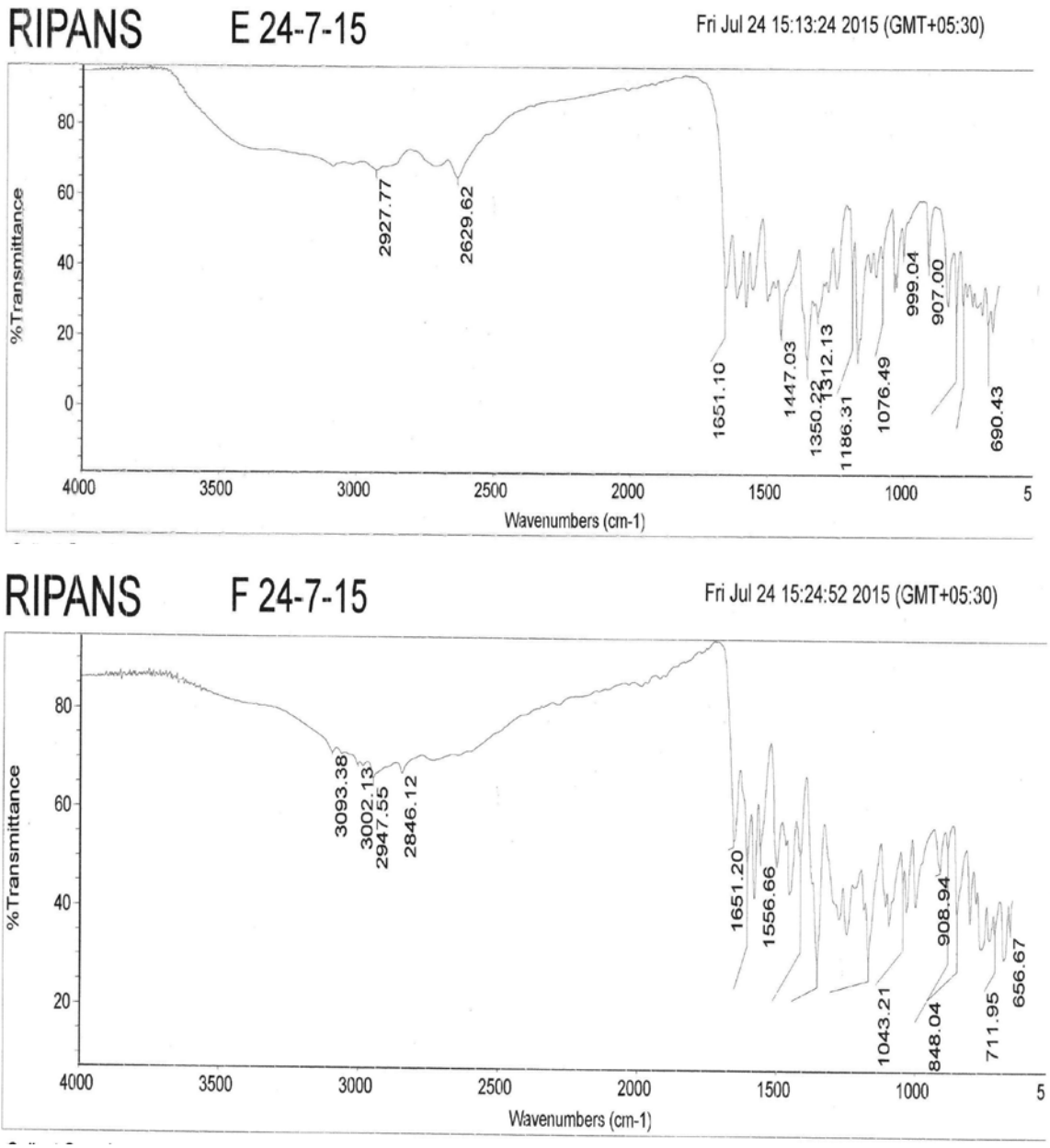
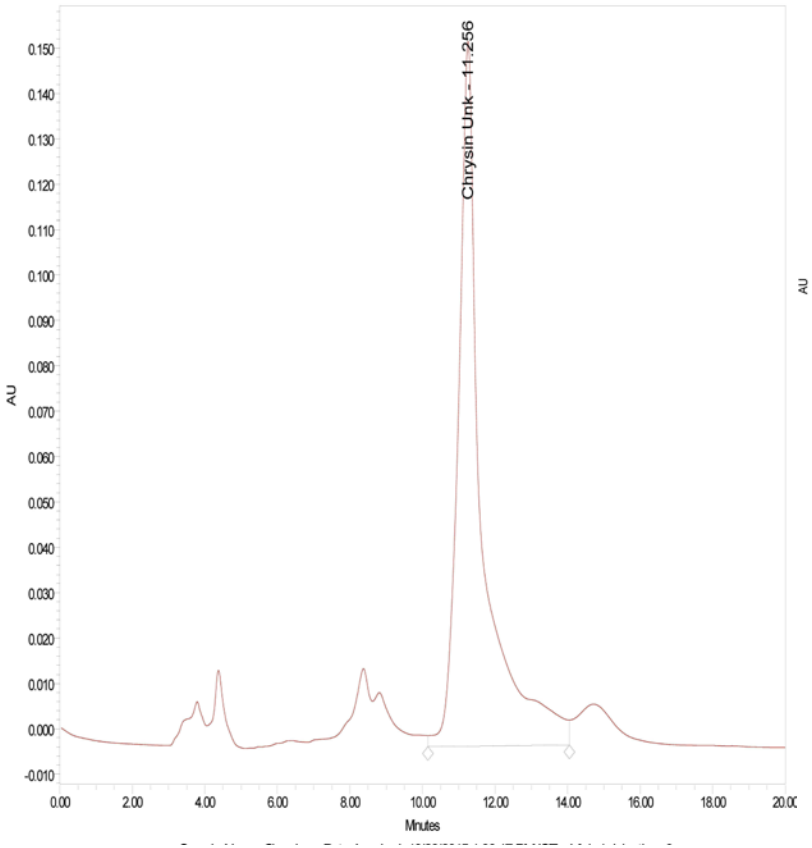
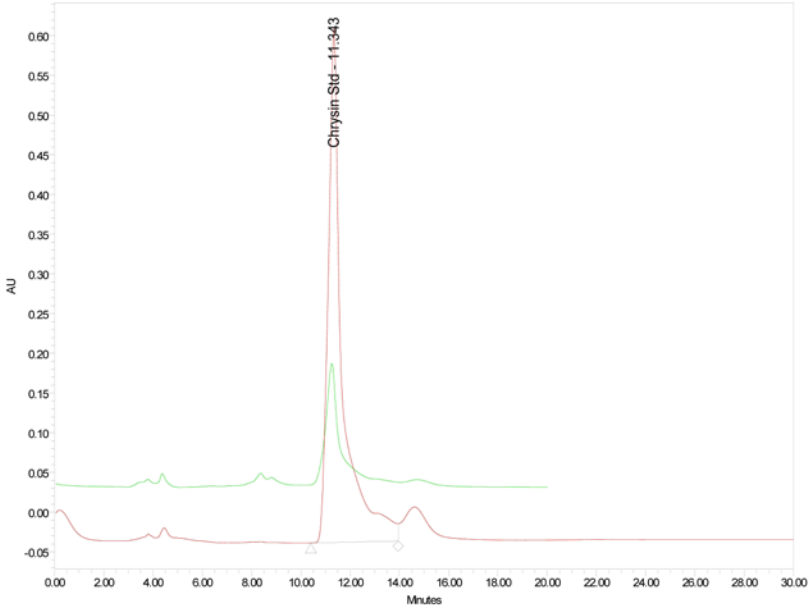


Figure 10: FTIR spectrum of fraction E and F.



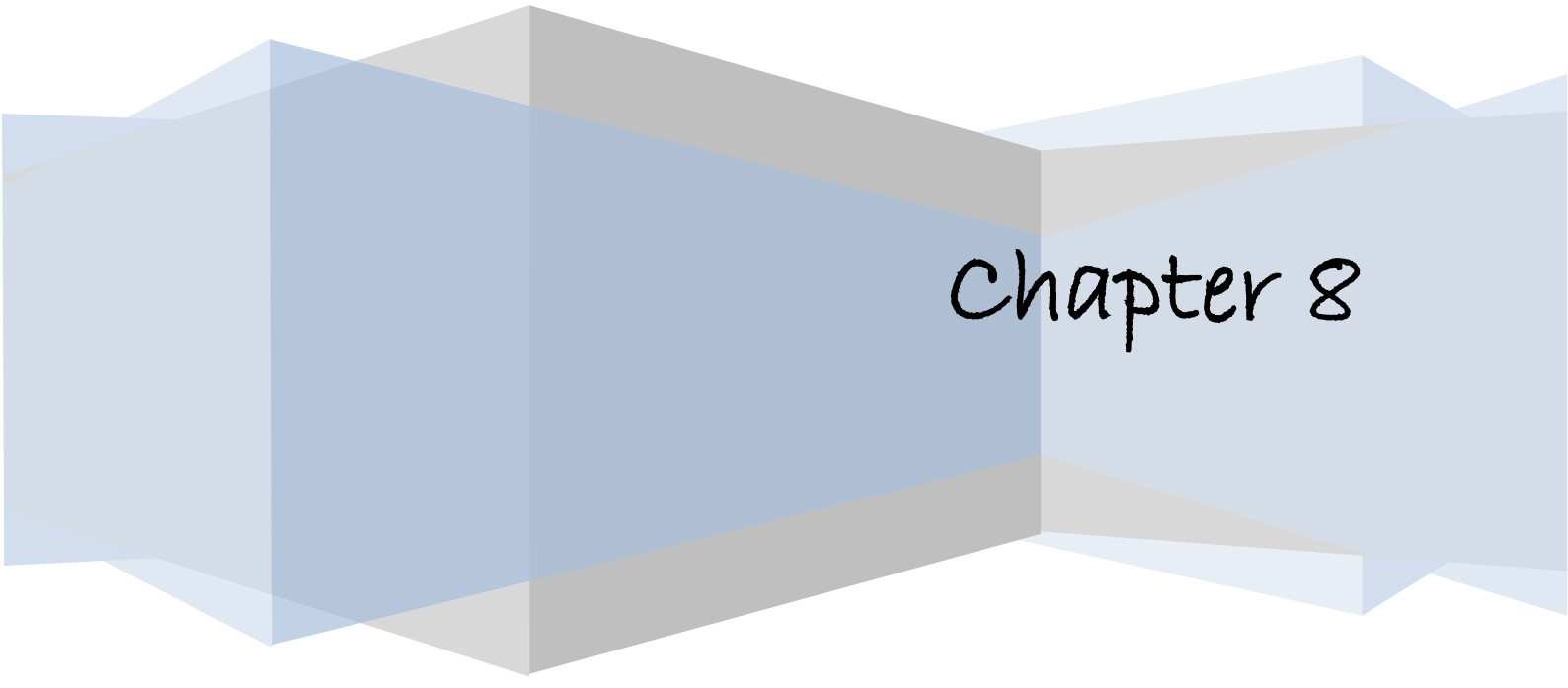


Peak Summary with Statistics
Name: Chrysin Std

	Sample Name	Vial	Inj	Name	Retention Time (min)	Area	% Area	Height	Amount	Units
1	Chrysin Std 50	1	4	Chrysin Std	11.343	26119585	100.00	647637	50.0	mcg/mL
2	Chrysin Unk 20	1	6	Chrysin Unk	11.256	7049618	100.00	155389	13.5	mcg/mL
Mean					11.299					
Std. Dev.					0.062					

Figure 11: The High performance liquid chromatography (HPLC) of isolated compound (Chrysin) with Standard chrysin. Left: Isolated compound (Unknown); Right: Overlayed chromatogram of isolated compound (fraction E) with Standard Chrysin

Isolation and identification of Chrysin and Oroxylin from the stem bark of Sonapatha, *Oroxylum indicum* Vent.



Abstracts

Plants synthesize numerous phytochemicals, out of which several chemicals turned into clinically useful anticancer agents. Our earlier studies have shown that ethanol extract of Oroxylum indicum had potent anticancer activity in vitro and in vivo. Therefore it was decided to isolate bioactive components responsible for this activity. The isolation of bioactive components was carried out by thin layer chromatography, vacuum liquid chromatography in chloroform and ethanol solvents followed by repeated column chromatography. Finally, the sephadex gel chromatography was used for separation of the bioactive components. The isolates were subjected to mass spectroscopy, NMR, and HPLC. The structural elucidation indicated the presence of two flavonoids Chrysin and Oroxylin A, which was further confirmed by HPLC and melting point determinations.

1. INTRODUCTION

Utilization of plants for healthcare is as old as the human civilization (Samuelsson, 2004; Shantabi et al., 2014; Lalrinzuali et al., 2015). These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations (Balick and Cox, 1997; Samuelsson, 2004). The specific plants to be used and the methods of application for particular ailments were passed down through oral history. Eventually information regarding medicinal plants was recorded in herbals. In more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century (Kinghorn, 2001; Samuelsson, 2004). Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use (Newman et al., 2000; Butler, 2004; Samuelsson, 2004). Isolation and characterization of pharmacologically active compounds from medicinal plants continue today.

The first synthetic drug, aspirin was derived from a plant *Spiraea ulmaria,* prior to which humans depended solely on the crude plant materials for the treatment of different ailments and the plant itself was also used in crude form for fever and swelling in Egypt (Jack, 1997; Pan et al., 2014). Both the raw materials as well as the extracts of plants contain many phytochemicals such as fatty acids, steroids, alkaloids, flavonoids, glycosides, saponins, tannins, lignans, and terpenes as well as some other small molecules like peptides and oligosaccharides. It is therefore difficult to know that which of the component is the bioactive (Kinghorn et al., 2011; Doughari, 2012). Many bioactive compounds have been isolated from plants, animals and microbes, and some have been developed into modern drugs to treat several diseases including

cancer. Over 60% of the clinical use of anti-cancer drugs originated from natural products (Seelinger et al., 2012). Some of the drugs initially isolated form plants before their chemical synthesis include vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, and paclitaxel (taxol) (Cragg and Newman, 2013).

Oroxylum indicum is a deciduous tree with a soft, light brown or greyish brown bark (Dalal and Rai, 2004). It has been reported to possess several medicinal properties (Lalrinzuali et al., 2015). It has been reported to cure fevers, gastritis, hypertension, inflammation liver disorders, cancer, headache, epilepsy, muscular sprain, intestinal worms, vomiting, dysentery, leucoderma, asthma, and general weakness (Kala, 2005; Khumbongmayum et al., 2005; Deka1 et al., 2013). A paste of the stem bark is applied to cure scabies and to treat arthritis (Grampurohit et al., 1994; Laupattarakasem et al., 2003). A decoction of the stem bark taken three times a day has been reported to cure nasopharyngeal cancer (Mao, 2002). Our earlier studies have shown that the ethanol extract was cytotoxic to HeLa cells and also exerted antineoplastic action *in vivo*. This stimulated us to undertake the isolation of its bioactive components. Therefore, it was desired to isolate bioactive compound from *Oroxylum indicum* that may have been responsible for antitumour activity.

2. MATERIALS AND METHODS

2.1. Chemicals

Silica gel F_{254}, Silica gel 60, Silica gel G (TLC grade), methanol, ethyl acetate, were purchased from Merck India Limited, Mumbai, India. Hexane, petroleum ether, chloroform, ethanol were procured from SD Fine Chemicals, India. Magnesium ribbon, HPLC grade methanol, acetonitrile and HPLC grade water were obtained

from HiMedia, Mumbai, India. Sephadex LH-20 and Chrysin were supplied by Sigma Chemical Co. (Bangalore, India).

2.2. Procurement of plant material and extraction

The identification and authentication of *Oroxylum indicum* (family: Bignonaceae) was done by the Botanical Survey of India, Shillong. The non-infected stem bark of *O. indicum* was collected from Champhai region of Mizoram during the dry season. The bark was thoroughly washed and shade dried at room temperature in the dark in clean and hygienic conditions. The powder of the dried bark was prepared using mortar and pestle and a grinder at room temperature.

The bark powder of *O. indicum* was sequentially extracted with petroleum ether, chloroform, ethanol and distilled water according to increasing polarity using a Soxhlet apparatus at their respective boiling points until the solvents became colourless. The ethanol extract was filtered and concentrated by evaporating its liquid contents using rotary evaporator.

2.3. Vacuum Liquid Chromatography

The column was prepared in a Buchner funnel using TLC grade silica gel. The sample was fractionated with pure chloroform to separate the non-polar compounds until the eluting solvent was clear followed by the addition of 10 % methanol to increase the polarity of the solvent. The polarity of the solvent was then gradually increased until 50 %, in order to separate the different compounds according to their polarity. It was then washed with pure methanol to separate all the remaining compounds. Each eluting gradient was collected, allowed to dry and used for column chromatography.

Alkaloid test was performed for the different VLC fractions according to Dragendorff's test, where the formation of reddish brown precipitate indicates the

presence of alkaloids. The 80 % + 20 % ($CHCl_3$:CH_3OH) and the 60 % + 40 % ($CHCl_3$:CH_3OH) showed the presence of alkaloids. TLC was also performed on the different fractions.

2.4. Column and Thin Layer Chromatography (TLC)

The column chromatography was carried out in 10% methanol + 90% chloroform fraction from VLC and was designated as the main column. 14 gram sample was recovered from the fraction. The wet method of column chromatography was used for separation.

Fractionation of the sample was carried out with pure chloroform to separate the non-polar compounds until the eluting solvent was clear followed by the addition of 10 % methanol to increase the polarity of the solvent. The solvent polarity was increased gradually until 50 % (100 % chloroform, chloroform: methanol- 95:5, 90:10, 80:20, 70:30, 60:40, 50:50) in order to separate the different compounds according to their polarity. It was washed with pure methanol to separate all the remaining compounds. Each eluting gradient was collected allowed to dry and stored for further analysis. More than 100 fractions were collected from the column and TLC was performed for all the fractions. Similar fractions were combined based on the TLC profile.

2.5. Separation of A fraction and identification

The fraction 36-39 from the main column contained yellow spot on TLC. Therefore, this yellow spot was targeted and subjected to column chromatography using petroleum ether, chloroform and methanol as solvent systems. The fractions 6-10, 11-20, 21-29 (100% chloroform) on TLC contained the spot and was purified further using hexane:ethyl acetate (8:2) as solvent systems which was passed through

sephadex LH-20 for further purification. The fractions 4-24 from sephadex were pooled together based on TLC profile to yield the compound A.

2.6. Separation of Compound E fraction and identification

The fraction 17-19 from main column turned into crystal of somewhat needle shaped structures, and a small amount of which was dissolved in chloroform, whereas the other in methanol. The separation using their solubility property could not result in individual components. Therefore, they were combined with fraction 20-23 due to similarity in TLC profiles. Preparative TLC was carried out on pre-coated readymade TLC plates (silica gel 60 F_{254}) using 9.6:0.4 ($CHCL_3$:CH_3OH) as solvent system which gave two main yellow spots. TLC showed presence of some impurities therefore upper spot was purified by running it on preparative TLC using pure chloroform, whereas 9.4:0.6 ($CHCL_3$:CH_3OH) was used for the lower one. Manually prepared preparative TLC was employed for further purification where silica gel G (TLC grade) was used as the stationary phase. The spots were scraped off from the plate and dissolved in methanol and filtered in order to separate the compounds from the gel. Flavonoid test was done on both the compounds according to Trease and Evans (2002) where ferric chloride was added to 1 ml of the solution. Both the solutions turned into dark blue colour whose original colour was light yellow indicating presence of flavonoids.

The lower spot was again subjected to Sephadex LH-20 using methanol as solvent system. TLC was performed on all the fractions and the fractions 14-34 eluted from Sephadex column were pooled together based on similar TLC profiles.

2.7. Separation of F fraction and identification

The fractions 40 to 55 from main column were combined based on TLC and were subjected to column chromatography for recolumn using chloroform and

methanol as solvent system. Silica gel of mesh size 200-400 (column chromatography grade) was used as stationary phase, which was further purified using preparative TLC. The yield from preparative TLC was further purified using Sephadex LH-20 with chloroform and methanol (1:1). The fractions 8-19 from the sephadex column were pooled together based on similar TLC profiles.

2.8. Magnesium ribbon test

Flavonoid test was performed on the isolated compounds using magnesium ribbon method where a small piece of magnesium ribbon was added to different extracts dissolved in methanol, followed by the drop wise addition of concentrated hydrochloric acid. Colours varying from orange to red indicated flavones, red to crimson indicated flavonols, crimson to magenta indicated flavonones (Krishnaswamy, 2003).

2.9. Spectroscopic Characterization

After getting only a single spot on TLC, the isolated compounds were sent to NEHU-SAIF, NEHU, Shillong, India for LC-MS ESI (+) for determining the molecular weight (LC-MS Waters ZQ-4000). ^{1}C-NMR and ^{13}H-NMR were also performed using deuterated methanol (MeOD) as solvent system at SAIF-IIT, Chennai, India (Bruker AV III, 500MHz FT NMR Spectrometer) to elucidate their structures. Tetramethyl silane (TMS) was employed as internal standard.

2.10. High Performance Liquid chromatography (HPLC)

The isolated compound E was subjected to high-pressure liquid chromatography (Waters 515 HPLC pump, a valve type injector, Waters 2489 UV/Visible Detector), Symmetry® C18 (250×4.6 nm) column with a particle size of 5 µm (Waters, Singapore). Acetonitrile and water (HPLC grade, Merck India) in the ratio of 60:40 were used as the solvent system. An amount of 20 µl was injected with

a flow rate of 0.5 ml/min at room temperature. The detection was carried out at 254 nm. The sample had retention time of 11 minutes (Figure 1). The purity of the isolated compound was also calculated and was found to be 67.5 %. The compound E showed characteristic similar to chrysin. Therefore, chrysin was incorporated as standard and both the sample and as well as chrysin was subjected to HPLC using similar conditions.

3. RESULTS

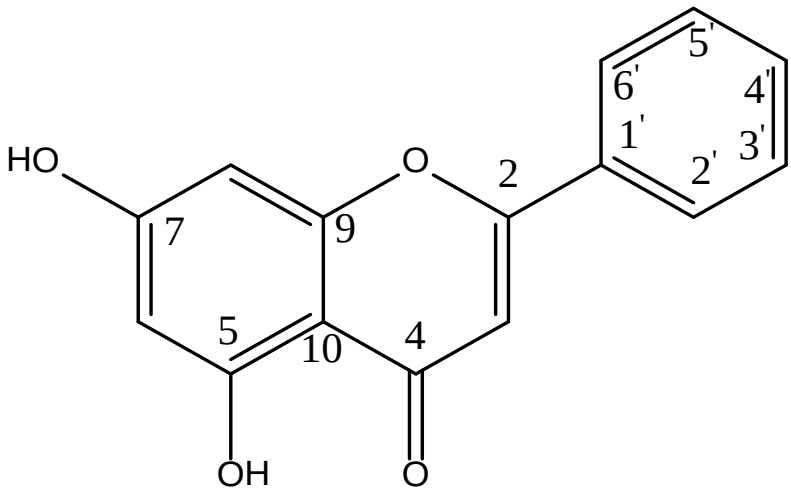
3.1. Magnesium ribbon test

The magnesium ribbon test on the isolated compounds A, E and F gave an orange colour which indicated that the compounds are flavones in nature having molecular formula $C_{10}H_{15}O_2$.

3.2. Identification of Chrysin

In mass spectroscopy, the molecular ion peaks for compound A and E were observed at 276.78 and 276.97 respectively. The molecular weight of chrysin is 254. These were the expected peaks for chrysin in ESI (+) mode for $[M+Na]^+$ since the solvent used was methanol-d4 (Figure 1 & 2). In the ^{13}CNMR, the presence of carbon atoms in the range of 120-160 δ indicated the aromatic nature of the compound. The presence of peaks between 100-150 δ showed the presence of double bonded carbon atoms. The peak observed between 150-160 δ indicated that the compound is a phenolic compound. Carbon chemical shifts were observed at 95.5 ppm, 101.2 ppm, 104.1 ppm, 125.8 ppm, 128.7 ppm, 131.3 ppm, 131.5 ppm, 158.6 ppm, 161.5 ppm, and 163.3 ppm for the positions 8, 6, 3, 10, (2', 6') (3', 5'), 4, 1', 9, 5 and (2, 7) respectively (Figure 4 & 6). The chemical shift at 181.6 ppm could correspond to the 4-oxo arrangement since the C=O is mainly found in the shifts between 180-200 ppm. The chemical shifts of the compounds A and E for H-NMR are of 7's (6.5-8.4) which

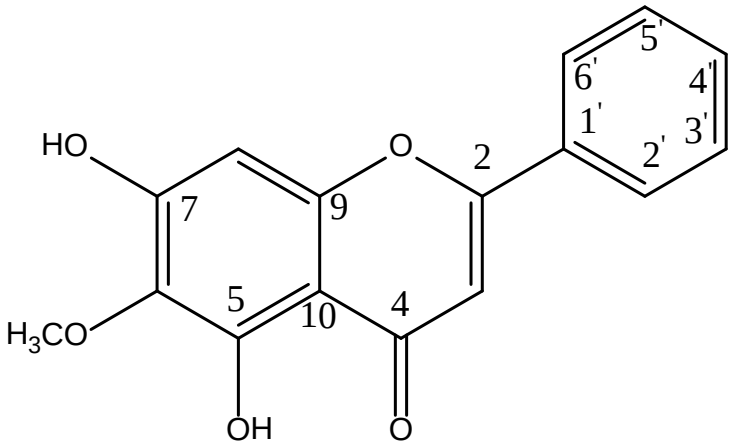
indicate that the compound is aromatic (Figure 5 & 7). Based on the HNMR spectral data, a singlet was observed for the hydrogens positioned at 6.10 ppm, 6.31 ppm and 6.63 ppm, respectively. A multiplet was observed at 7.55 ppm for positions 3', 4', 5'. The peak at 7.96 ppm was also observed as a multiplet for the hydrogen positioned at 2' and 6'. Very small peaks below 2 ppm were expected to be of fatty acid derivatives (Figure 5 & 7). The FTIR spectrum of the fraction E showed two peaks which could correspond to the two OH groups of the compound (Figure 10). These spectral data confirmed that the compounds A and E are chrysin (5, 7-dihydroxyflavone) with the molecular formula of $C_{15}H_{10}O_4$, whose molecular structure is given below and its melting point is found to be 284˚C.



3.3. Identification of Oroxylin A

The carbon peaks in the range of 150-160 ppm indicated that the compound is phenolic. The presence of peaks in the range of 120-160 δ in C-NMR also indicates the aromatic nature of the compound. In the LC-MS, the molecular weight was found to be 284, therefore the peaks are expected at 285 and/or 307 in ESI (+) mode due to the solvent used (Figure 3). The chemical shift for carbon atoms were observed at 94.1 ppm, 104.2 ppm, 126.0 ppm, 128.8 ppm, 131.2 ppm, 131.6 ppm, 152.7 ppm,

153.5 ppm, 158.0 ppm, 164.3 ppm for the positions 8, (3, 10) (2', 6'), (3', 5'), (1', 6), 4', 5, 9, 7 and 2 respectively. The carbon chemical shift at 182.9 corresponds to the C=O at position 4 (Figure 8). The H-NMR chemical shifts are of 7's (6.5-8.4) which indicated that the compound is aromatic. The HNMR spectrum gave a singlet peak at 6.61 ppm and 6.76 ppm for the hydrogens at position 8 and 3 respectively (Figure 9). The carboxylic group was also observed at 3.91 ppm. A multiplet comprising three hydrogen peaks was observed at 7.59 ppm for the hydrogens at 3', 4' and 5'. Another multiplet peak at 8.01 gave rise to two hydrogen atoms positioned at 2' and 6'. The 13C-NMR shift in the range of 50-60 ppm (59) indicated that there is an H_3C-O group in the compound. The H-NMR chemical shift at the 3's (3.91) also indicated the presence of oxygenated hydrogen atoms. The peaks on the FTIR spectrum of fraction F could be the two OH group and OCH_3 (Figure 10). Based on these spectral data, the sample F was therefore identified as 5, 7-dihydroxy-6-methoxyflavone (Oroxylin A), with a molecular formula of $C_{16}H_{12}O_5$ whose molecular structure is depicted below its melting point is found to be 196˚C.



4. DISCUSSION

The isolation of bioactive components from plant extract is an essential part of drug discovery. The column chromatography resulted in the separation of fraction A, E and F. Further analyses by mass spectroscopy and NMR revealed that fraction A and E are similar and contained chrysin, whereas fraction F was dissimilar and had different structural composition when compared to chrysin and it was oroxylin A. Their identity was further confirmed by melting point determination. The melting point of chrysin was 284˚C, whereas of oroxylin A was 196˚C. Chrysin has been isolated earlier from *O. indicum* seeds and roots (Chen et al., 2003; Yadav et al., 2013). Similarly, oroxylin A has been isolated from *O. indicum* earlier (Yadav et al., 2013; Ranjith et al., 2013).

A C2-C3 double bond in a flavonoid is important to increase the anticancer activity (Lazaro, 2002). This arrangement is found in both the isolated compounds. There is also evidence that the unsaturated 2,3-bond and 4-oxo arrangement of flavones may promote the formation of ROS induced by divalent copper in the presence of oxygen (Cao et al., 1997). In an epidemiological study, high intake of flavones has been related with reduced risk of colorectal cancer with an odd ratio of 0.78. An inverse relation of flavones with renal cancer and breast cancer was also found with an odd ratio of 0.68 and 0.81 respectively (Rossi et al., 2010). Therefore, the isolated compounds chrysin and oroxylin A could be responsible for the anticancer activity discussed in the previous chapters.

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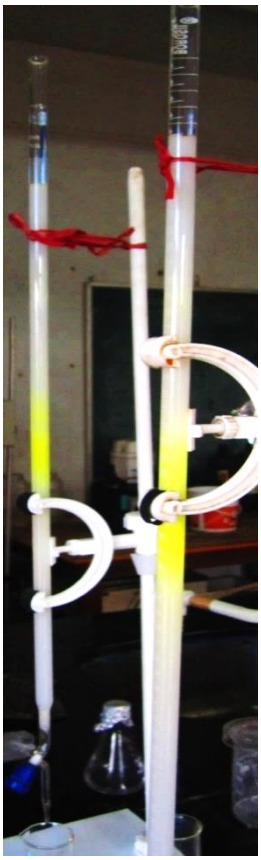
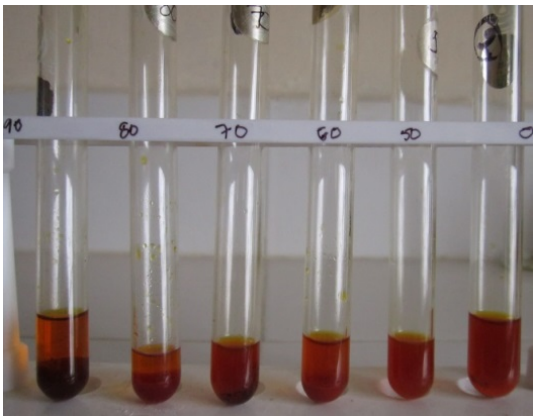
Vacuum Liquid chromatography

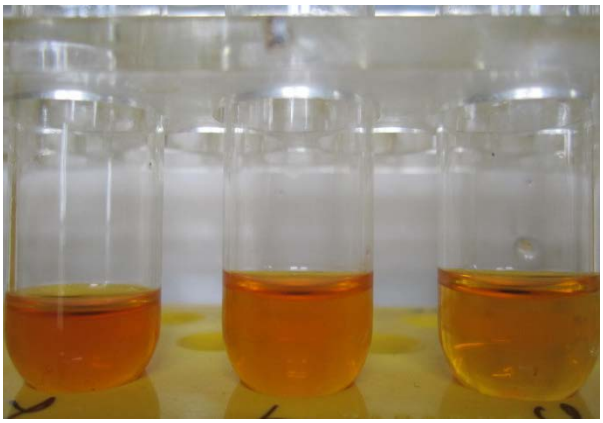


Column chromatography **Sephadex LH 20**

Plate I: Extraction and isolation of bioactive compound from stem bark of *Oroxylum indicum* at various processing stages.



Alkaloid test on different VLC fractions



Positive flavone test using magnesium ribbon

Preparative TLC

TLC of Chrysin standard and isolated (E)

Plate II: Extraction and isolation of bioactive compounds from stem bark of *Oroxylum indicum* at various processing stages.

Summary & Conclusion

INTRODUCTION

Cancer is a group of diseases that comprises mutation or dynamic changes in the genome of the cell producing proteins that interrupt the normal cellular balance which leads to the uncontrolled proliferation of cells (Bishop and Weinberg, 1996; Hejmadi, 2010; American Cancer Society, 2015). Cancer cells lack the requirement of stimulatory signals for division and therefore can proliferate even in the absence of division signals (Hanahan and Weinberg, 2000). Though there are several types of cancers, they all share six common characteristics such as self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. These six characteristics are called as the hallmarks of cancer (Hanahan and Weinberg in 2000). Besides these, the ability to reprogram energy metabolism and evade immune destruction were later added to these six hallmarks of cancer (Sonnenschein and Soto, 2013). Due to the multiple checks and balances that exist in stem cells to limit excess cell division, a cell must attain at least five successful gene mutations to be able to grow autonomously in the host organism. The neoplastic cells acquire this by selecting the mutations that activates the oncogenes or inactivates the tumor suppressor genes (Bertram, 2001).

Cancer is assumed to be older than human as it has been identified in the bones of dinosaurs from the Jurassic period (Greaves, 2000). The earliest evidence of human cancer found till date is a metastatic carcinoma in a young man from ancient Nubia which dated back to 1200 BC (Binder et al., 2014). Many earlier scientists such as Hippocrates, Virchow, Cohnheim, Ribbert etc. pioneered and wrote about cancer in the past and their numerous works and records shed light on the current knowledge of the characteristics of cancer. There are many factors that can cause

cancer either directly or indirectly which can be broadly classified as environmental, lifestyle and behavioral exposures (Steward and Wild, 2014). Tobacco, diet, stress, obesity, radiations, infections, lack of physical activity and environmental pollutants are some of the common environmental factors leading to cancer (Anand et al., 2008). Several parasites, bacteria and viruses also contribute to the cause of different types of cancer (Ames and Gold, 1998; Pagano et al., 2004; Anand et al., 2008).

The staging system is used to classify cancer which is based on the extent of spread. The American Joint Committee on Cancer (AJCC) and the International Union for Cancer control (IUCC) maintain the TNM classification system and is used by clinicians for solid tumors. This system cannot be applied to some cancers like leukemia since it is present throughout the body. Stage 0 is when there is only the primary tumor and has not spread to other sites. The difference between stage I, II and III depends upon the size of the primary tumor as well as the extent it has spread into nearby tissues. Stage IV is the highest and most advanced stage where the cancer has spread to distant parts of the body (Webber et al., 2014; American Cancer Society, 2015; Global Cancer facts and figures, 2015).

The treatment of cancer usually involves combination or single use of surgery, radiotherapy and systemic therapy. Earlier stages of cancer can be cured with surgery alone however systemic therapies is the only option once the disease has metastasized since delivery through the bloodstream is required for cancer cells at different sites. The systemic therapy includes hormonal therapy, targeted therapy and chemotherapy (Caley and Jones, 2012). The currently used different types of cancer treatments have their own limitations. During surgery, some cancer cells maybe missed while removing further of the surrounding tissues so as to clear all the cancer cells can lead

to loss or reduced functioning of the organ. Besides, it cannot be used for the treatment of later stages of cancer that has already metastasized (Greene et al, 2002).

The ionizing radiation used during radiotherapy damage DNA of not only the cancer cells but also the normal adjacent cells eventually leading to recurrence of cancer (Begg et al., 2011). Besides, it is less effective to hypoxic tumors (Harrison et al., 2002). Several types of chemotherapeutic drugs with different mode of action are available which could be used either alone or in combination. Drugs such as the antimetabolites hinder the synthesis of DNA or RNA while some drugs like alkylating agents directly damage the DNA of cancer cells. Another mechanism exhibited by some anticancer drugs as the vinca alkaloids and taxanes is by causing microtubule dysfunction (Colvin, 2003; Liu et al., 2007; Lind, 2008; Yue et al., 2010). Chemotherapy also has its own limitations as it does not specifically target tumor cells. Since the chemotherapeutic agents hamper cell division or inhibit enzymes involved in DNA replication or metabolism, they also damage the normal dividing cells especially the rapidly regenerating tissues, such as those of the bone marrow, gut mucosa and hair follicles (Wu et al., 2008). Therefore, it is desirable to search for new drugs, which are less toxic and do not produce undesired changes in the normal cells (Newman and Cragg, 2014).

Plants have been the major source for several drugs and it is well known that 75% of the modern chemotherapeutic drugs have their origin in plants or natural products (Cragg and Newman, 2013; Harvey et al., 2015). Several phytoceuticals have been screened for their anticancer activities earlier. *Alsotnia scholaris, Aegle marmelos, Aphanmixis polystychya, Solanum khasianum, Tinospora cardiofolia* have been found to possess anticancer activity in different preclinical systems (Jagetia et al., 1998; Jagetia and Baliga, 2005; Jagetia et al., 2005, Jagetia and Venkatesha, 2012;

Rosangkima and Jagetia, 2015). Extracts from *Urtica membranacea, Artemesia monosperma,* and *Origanum dayi post* have also been reported to exert anticancer activity (Solowey et al., 2014). *Artocarpus obtusus, Blumea balsamifera, Boerhaavia diffusa, Calotropis procera, Citrus maxima, Emblica officinalis, Moringa oleifera, Panax ginseng, Pfaffia paniculata, Rheum officinale, Saxifraga stolonifera, Vitex negundo, Withania somnifera,* and *Zingiber officinale* have also been found to possess anticancer activity under different conditions (Merina et al., 2012). Therefore plants and natural products still provide a major avenue for screening and developing of new nontoxic molecules including drugs for cancer treatment.

Oroxylum indicum Sonapatha is a deciduous tree which grows at an altitude of 1200 m mainly in ravines, in damp and moist places in the forest. It is commonly called the tree of Damocles or Indian caper (Chauhan, 1999). It is found in India, Srilanka, China, Thailand, Philippines and Indonesia of the Asian continent. In India, it is distributed in Himalayan foothills, Eastern and Western Ghats and North East India (Kritikar and Basu, 2001). The tree is small to medium sized up to 12 m in height with soft light brown or grayish brown bark. It has very large leaves about 90-180 cm long which when whither and fall off near the tree looks like a pile of broken limb bones hence the name broken bones. The flowers are reddish purple outside and pale, pinkish-yellow within, which blooms at night. The flowers emit a strong, stinky odor which attracts bats and therefore is pollinated by bats (Padgilwar et al., 2014).

Traditionally, the root tonic is useful in dropsy, cough, sprains, neuralgia, hiccough, asthma, bronchitis, anorexia, dyspepsia, flatulence, colic, diarrhea, dysentery, strangury, gout, vomiting, leucoderma, wounds, rheumatoid arthritis and fever. Root bark is used in stomatitis, nasopharyngeal cancer and tuberculosis. The leaves are used as stomachic, carminative and flatulent. It is also prescribed for snake

bite. The decoction of the leaf is used to treat rheumatic pain, enlarged spleen, ulcer, cough, and bronchitis. The fruits are useful in pharyngodynia, cardiac disorders, gastropathy, bronchitis, haemarrhoids, cough, piles, jaundice, dyspepsia, smallpox, leucoderma and cholera. The seeds are used as purgative and the dried powder is used by women to induce conception. It is also used in the form of paste for quick relief of tonsil pain after grounded with fire soot. The stem bark decoction is taken for curing gastric ulcers and a paste made of the bark powder is applied for mouth cancer, scabies and other skin diseases (Nadkarni, 1982; Bhattacharje, 2005; Gokhale and Bansal, 2006; Khare, 2007).

AIM OF THE STUDY

The plant is used as one of the important ingredients in most commonly used Ayurvedic preparations like Dasamularistha, Syonaka putapaka, Syonaka sidda ghrita, Brhatpancamulyadikvatha, Amartarista, Dantyadyarista etc. (Zaveri et al., 2008). A man of about 50 years of age from Maram Naga village, Manipur was diagnosed with nasopharyngeal cancer and was treated with chemotherapy but could not be cured. He was informed by the doctors that he may live about 6 months i.e., until November 1996. However, he learned about someone with similar condition who got cured by taking the decoction of the stem bark of *Oroxylum indicum*. He too started taking the decoction three times a day. His condition improved and he was still surviving, living a normal life until the year 2000 (Mao, 2002). Besides, local sayings indicate that a man from Hnahlan, Mizoram was also cured from cancer after taking the decoction of this plant. Therefore, the present study was carried out to obtain an insight into the anticancer activity of *Oroxylum indicum* extracts *in vitro* and *in vivo*.

CHAPTER 1

This chapter gives the history, causes and different stages of cancer. It also gives an account on the present knowledge of different types of treatment modalities and their harmful effects. A brief description on the aim and scope of the thesis is enlisted at the end of this chapter.

CHAPTER 2

In this chapter, an account has been given on the phytochemical screening and TLC profile of the different extracts of *Oroxylum indicum.* The non-infected stem bark of *Oroxylum indicum* was collected cleaned properly, shade dried and powdered. The powdered stem bark was sequentially extracted with petroleum ether,chloroform, ethanol and distilled water using Soxhlet apparatus and the liquid extracts were concentrated with rotary evaporator and stored at -70°C until further use. Qualitative phytochemical analysis was carried out on the bark powder as well as on all the extracts except petroleum ether which was discarded. The bark powder contains all the secondary metabolites screened except phlobatanins. All the different extracts contained tannins, flavonoids and cardiac glycosides while the phlobatanins were absent in all of them. However, there was variation in the alkaloids, steroids and saponin contents among the different extracts. The TLC study also showed the presence of different components as indicated by the different Rf values in different solvent systems.

CHAPTER 3

This chapter describes the *in vitro* antioxidant activity of the different extracts of *Oroxylum indicum.* Free radicals are necessary evils as they are produced during normal metabolism of the body as well as a defense against the pathogenic attack. However, excess of free radical generation produce oxidative stress, inflammation and

various diseases and any agent that can reduce the oxidative stress will be useful in treating oxidative stress related disorders. Therefore, it was necessary to investigate the free radical scavenging activity of different extracts of *Oroxylum indicum in vitro.* The antioxidant activity was studied by evaluating the ability of various *O. indicum* extracts to inhibit the generation of DPPH, superoxide anion, hydroxyl, nitric oxide as well as Fe^{3+} radicals using standard procedures in cell free system. The total phenols and flavonoids were also estimated. The various extracts of *O. indicum* inhibited the generation of DPPH, ABTS, superoxide anion, hydroxyl, nitric oxide and Fe^{3+} radicals in a concentration dependent manner and this ability depended on the type of extract, concentration and species of the free radical. The free radical scavenging activity increased up to a certain concentration and remained unaltered thereafter. However, the scavenging activity for all the free radicals was highest for the chloroform extract. The amount of total phenols and flavonoids also increased with increasing concentration and the maximum amount was recorded for the chloroform extract. Therefore, the antioxidant activities of *Oroxylum indicum* may be due to the presence of flavonoids.

CHAPTER 4

This chapter gives an insight into the anti-inflammatory and analgesic activity of the ethanol extract of stem bark of *Oroxylum indicum.* Inflammation is a naturally occurring process in the body in response to noxious stimuli; however, persistent inflammation in the body can lead to several diseases including cancer. Therefore, it was desired to screen the *Oroxylum indicum* for anti-inflammatory activities in Swiss albino mice. The hot plate, acetic acid and tail immersion tests were employed for the evaluation of the analgesic activity whereas, xylene-induced ear edema and formalin-induced paw edema tests were used to study the anti-inflammatory activity of *O.*

indicum. The administration of mice with 250 and 300 mg/kg b. wt. of *O. indicum* reduced pain and inflammation indicating that sonapatha possesses analgesic and anti-inflammatory activities. The maximum analgesic and anti-inflammatory activities were observed in mice receiving 300 mg/kg b. wt. of *O. indicum* ethanol extract.

CHAPTER 5

Chapter 5 gives an account on the wound healing activity of the different concentrations of ethanolic extract of stem bark of *Oroxylum indicum* (OIE). Full thickness excision wound of 2.5x1.5 cm was inflicted on the dorsum of mice and the ethanol extract was topically applied once a day on the wound area. The contraction of the wound was measured by superimposing a transparent graph sheet containing 1 mm^2 grid squares. All the different concentrations of OIE increased the wound contraction and reduced the mean wound healing time when compared to the negative control. The greatest contraction was observed for the 10% OIE. The syntheses of collagen and DNA were also significantly increased whereas the lipid peroxidation was reduced in the regenerating wounds by OIE. The increased wound healing ability of OIE may be due to its antioxidant activity and capacity to increase cell proliferation which would have been able raise the collagen synthesis leading to early closure of the wound. It may have reduced the expression of genes related to proinflammatory cytokines including TNF α, IL-1β, IL-4, and IL-6 and NF-κB in the regenerating wounds.

CHAPTER 6

This chapter deals with the cytotoxicity of the ethanol extract of *O. indicum* and chrysin on HeLa, HepG2 and MCF-7 cells using MTT assay where OIE or chrysin treatment increased the cytotoxicity in a time as well as dose dependent manner. It also gives an account on the effect of different concentrations of the ethanol extract on

the clonogenicity, OIE treatment also augmented DNA damage, apoptosis and antioxidant status in cultured HeLa cells. The OIE reduced the contents of GSH and activities of SOD, GST and catalase of HeLa cells in a concentration and time dependent manner. However, a rise in the lipid peroxidation and lactate dehydrogenase release was observed. The incidence of micronuclei and apoptosis was also increased in a concentration dependent manner which could be accountable for the cytotoxic effect. DNA fragmentation was also induced by the treatment which could be due to the decline in the different endogenous enzymes along with the increase in the level of lipid peroxidation and the release of lactate dehydrogenase.

CHAPTER 7

In this chapter, the acute toxicity was assessed both orally and intraperitoneally where the oral administration of the different extracts of *Oroxylum indicum* did not exhibit toxicity even at 3 g/kg b. wt. However, when the route of administration was altered to intraperitoneal, the extracts showed toxicity at higher doses with LD_{50} of 520, 700 and 2000 mg/kg. body weight for the chloroform, ethanol and aqueous extracts, respectively. The evaluation of anticancer activity of different extracts of *Oroxylum indicum* resulted in a dose dependent increase in the survival of tumor bearing mice as compared to the untreated control. A maximum increase in tumor free survivors was observed for the 300 mg/kg body weight of ethanolic extract which resulted in 40% of tumor free survivors beyond 120 days and was therefore selected for further studies. The 300 mg/kg body weight of the ethanol extract induced micronuclei in both mononucleate and binucleate cells in a time dependent manner up to 36 h and it had also increased the apoptosis in a time dependent manner until 24 h post treatment. The biochemical studies revealed a significant decline in the GSH contents and activities of GST, catalase and SOD accompanied by elevated lipid peroxidation. The cytotoxic

effect of ethanol extract of *O. indicum* may be due to its ability to induce DNA damage and apoptosis and alleviate GSH, GST, catalase and SOD and increase lipid peroxidation.

CHAPTER 8

This chapter deals with the isolation of the bioactive principles of the ethanol extract. Thin layer chromatography, vacuum liquid chromatography, column chromatography and sephadex were employed during the isolation procedure. The isolated compounds were subjected to mass spectroscopy, C-NMR, H-NMR, FTIR, and HPLC for the structural elucidation. Based on these spectral data, the isolated compounds were identified as chrysin and oroxylin A. Both the isolated compounds were flavones with C2-C3 double bond necessary to increase anticancer activity. Therefore, the anticancer activity observed in the previous chapters could be due to the presence of these compounds.

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