Determination of the antineoplastic activity of Chilauni, *Schima wallichii* Korth in preclinical conditions

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K.Lalhminghlui, M.Sc

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Department of Zoology

Mizoram University

Aizawl-796004: North East India

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CERTIFICATE

This is to certify that **Kumari K. Lalhminghlui** carried out her research work under my supervision since 2014. The thesis entitled **"Determination of the Antineoplastic Activity of Chilauni, *Schima wallichii* Korth. In Preclinical Conditions"** is an original piece of work and has not been submitted for any other degree of any other university.

Place: Aizawl
Date: 1 June 2018

Prof. Ganesh Chandra Jagetia
Supervisor

MIZORAM UNIVERSITY
AIZAWL-796004

DECLARATION

I, **K.Lalhminghlui**, hereby declare that the subject matter of this thesis entitled **"Determination of the Antineoplastic Activity of Chilauni, *Schima wallichii* Korth. In Preclinical Conditions"** is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University, Aizawl for the award of the degree of Doctor of Philosophy in Zoology.

(K.LALHMINGHLUI)

(Head)
विभागाध्यक्ष
Head
जन्तुशास्त्र विभाग
Department of Zoology
मिजोरम विश्वविद्यालय
Mizoram University

(Prof. Ganesh Chandra Jagetia)
Supervisor

Ex/Ph.D./ZOOL

02

MIZORAM UNIVERSITY
AIZAWL

School of Life Sciences

Department of Zoology

Ph. D. Course Work Award-Sheet

The following is the assessment record of ***K. Lalhminghlui,*** *Roll No.* ***Zoo/CW/12/01*** *in the Ph. D. Course Work Examination held in March, 2013.*

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First Tabulator

Second Tabulator

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EVALUATION : *The following indicates the corresponding grades of grade points :*

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ABBREVIATIONS

DLA	Dalton's Lymphoma ascites
DNA	Deoxyribonucleic acid
DSB	Double strand break
g	Gram
h	Hour
mg	Milligram
MNBNC	Micronucleated binucleate cells
PBS	Phosphate Buffered Saline
Rf	Retention factor
SEM	Standard error of Mean
SPS	Sterile physiological saline
SWA	*Schima wallichii* aqueous extract
SWC	*Schima wallichii* chloroform extract
SWE	*Schima wallichii* ethanol extract
ug	Microgram

CHAPTER 1

General Introduction

INTRODUCTION

Cancers are a large family of diseases involving abnormal cell growth and having the potential to invade or spread into other parts of the body (WHO, 2014; National Cancer Institute, 2014). For the initiation of cancer, certain changes take place in the cells or group of cells and begin in cells (Croce CM, 2008). All tumor cells show six main properties or characters of cancer cell. These characteristics are the main requirements to produce a malignant tumor. They may be noted down as follows (Hanahan *et al*., 2000)

1) Self sufficiency of growth signals
2) Insensitivity to anti-growth signals
3) Evading apoptosis
4) Limitless replicative potential
5) Sustained Angiogenesis
6) Tissue Invasion and metastases

The prominent properties of cancer are: lack of differentiation of cells, local invasion of adjoining tissue, and often, metastasis (spread to distant sites through the bloodstream or the lymphatic system). The immune system likely plays a significant role in eliminating early cancers or premalignant cells because immunodeficiency states are associated with an increased incidence of various kinds of cancer, particularly those associated with viral infection, and tumors arising in the lymphatic system and the skin.

Cancer does not show clear sign and symptoms as they are great imitator. It is a very common mistake that people diagnosed with cancer are often treated for other disease. General symptoms of cancer may include unintentional weight loss, fever, excessive fatigue and changes in the skin

(O'Dell, 2009). Hodgkin disease, leukemias and cancers of the liver or kidney can cause a persistent fever (Bodel, 1974). People may become anxious or depressed post-diagnosis of cancer (Anguiano *et al*., 2012).

HISTORY

Cancer is known to exist since time immemorial. In 1,600 BC, the earliest written record regarding cancer was written by Edwin Smith Papyrus about breast cancer (Hajdu, 2011*)*. Hippocrates later described various types of cancer, and he referred them as the Greek word *karkinos* (crab or crayfish) (Hajdu, 2011). The name had been derived due to the appearance of the veins resembling the feet of the crab as seen on the cut surface of a solid malignant tumor (Majno et al., 2004). The Greek word *karkinos* was translated into the Latin word cancer by Celsus, which means crab and he even recommended surgery as the treatment. Tumor removal surgeries have been documented in ancient Egypt, hormone therapy and radiation therapy were developed in the late 19th Century. Chemotherapy, immunotherapy and newer targeted therapies have been introduced in the 20th century.

CAUSES

There can be many factors which can be said to cause cancers in human. It is reported that majority of cancers occurs due to environmental factors which contributes to 90 to 95% of cancers causing factors. The remaining 5 to 10 % cancer cases are genetically inherited (Anand *et al*., 2008). It cannot be said that a particular cancer is caused by a particular agent since there can be many factors which may cause cancer. The most common environmental factors which are known to cause cancer include tobacco, diet, obesity, infections, radiations, stress, poor physical activity and various environmental pollutants.

Chemicals and tobacco :

The main content of tobacco –nicotine though is not considered carcinogens, still tobacco smoke contains more than eighty known carcinogens including nitrosamine (Kuper *et al*., 2002; Hecht, 2003; Talhout *et al*., 2011; Bassiony *et al*., 2015). The substances which cause cancer are called carcinogens. It is reported that lung cancer death rate in men in the United States greatly shows how tobacco smoking is linked to lung cancer.

Diet, obesity and physical activity :

The role played by poor diet, less physical activity in relation to cancer is much more than one had in mind. Diet, obesity and physical activity are considered to contribute up to 30 to 35 % of cancer deaths. Physical inactivity contributes to cancer risk by its negative impacts on immune system and endocrine system (Kushi *et al*., 2006). The effect from diet comes from over nutrition since some foods are linked with some specific cancers. Azinomoto (monosodium glutamate) consumption in diet causes gastric cancer. Similarly, betel nut chewing can cause oral cancer (Park *et al*., 2008).

Infection and radiation :

Viruses are considered to be the most infectious agents which causes cancer even though some bacteria and parasites are also known to play a vital role in inducing cancer. *Oncovirus*es infection cause Kaposi's sarcoma, herpes virus hepatitis B and hepatitis C viruses, *Helicobacter pylori*, *Schistosoma haematobium* are some of the most common infectious agent (Pagano, 2004; Ljubojevic, 2014).

Heredity and physical agents:

Majority of the cancers are not hereditary. It can occur only when there is genetic defect. Less than 0.5 % are carriers of genetic mutation which can lead to cancers and these cause 3 to 10 % of familial cancers (Cunningham *et al*., 2010). Some of the physical cancer causing agents include long exposure to asbestos, rock wool, metallic cobalt (Maltoni, 2000), physical trauma, and chronic inflammation (John, 2000).

TYPES

Cancer can be classified depending on the type of origin of the tumour. The classification or types of cancer is done as follows:

Carcinoma : This group includes cancers which are derived from the epithelial cells. Most commonly occurring cancer includes those of the breast, prostate, lung, pancreas and colon.

Sarcoma : Cancers which arise from the connective tissue are included in this category. Connective tissues such as bone, cartilage, fat, nerves arising outside the bone marrow in the mesenchymal cells. Example : osteosarcoma.

Lymphoma and leukemia : These group include cancers arising from the haematopoietic cells which leave the bone marrow and matures in the lymph nodes and blood (Varricchio, 2004).

Germ cell tumor : Cancers which are derived from the pluripotent cells are classified under this group. They are mostly present in the testicle or the ovary.

Blastoma : Cancers arising from the immature precursor cells or embryonic tissue are included under this category. Example : lymphoblastoma.

STAGING

The development of cancer is a multistage process which involves initiation, promotion and progression at every stage of carcinogenesis. Staging explains the extent of spreading or extension of cancer at the time of diagnosis. The TNM Classification of Malignant Tumours (TNM) is a cancer staging system that describes the extent of cancer in a patient's body. The TNM staging system for all solid tumors was devised by Pierre Denoix between 1943 and 1952, using the size and extension of the primary tumor, its lymphatic involvement and the presence of metastases to classify the progression of cancer.

T describes the size of the tumor and whether it has invaded nearby tissue or not. N describes regional lymph nodes that are involved. M describes distant metastasis (spread of cancer from one body part to another). TNM is developed and maintained by the International Union Against Cancer (UICC) to achieve consensus on one globally recognized standard for classifying the extent or spread of cancer. The TNM classification is also used by the American Joint Committee on Cancer (AJCC) and the International Federation of Gynecology and Obstetrics (FIGO). In 1987, the UICC and AJCC staging systems were unified into a single staging system. Most of the common tumors have their own TNM classification. Not all tumors have TNM classifications, e.g, there is no TNM classification for brain tumors. The general outline for the TNM classification is below. The values in parenthesis give a range of what can be used for all cancer types, but not all cancers use this full range.

T (a, is,(0),1–4): size or direct extent of the primary tumor. **N**(0–3): degree of spread to regional lymph nodes. N0: tumor cells absent from regional lymph nodes. N1: tumor cells spread to closest or small number of regional lymph nodes (palpable, same-sided, non-fixed). N2: tumor cells spread to an extent between N1 and N3 (palpable, contralateral or bilateral, non-fixed). N3: tumor cells spread to most distant or numerous regional lymph nodes (anything fixed). **M**(0/1): presence of metastasis. M0: no distant metastasis. M1: metastasis to distant organs (beyond regional lymph nodes). Use of an "X" instead of a number or other suffix means that the parameter was not assessed.

Overall Stage Grouping is also referred to as Roman Numeral Staging. This system uses numerals I, II, III, and IV (plus the 0) to describe the progression of cancer.

Stage 0: Carcinoma in situ.

Stage I : Cancers are localized to one part of the body.

Stage II : Cancers are locally advanced, as are stage III.

Stage III : Whether a cancer is designated as Stage II or Stage III depends on the specific type of cancer; for example, in Hodgkin's disease, Stage II indicates affected lymph nodes on only one side of the diaphragm, whereas Stage III indicates affected lymph nodes above and below the diaphragm. The specific criteria for Stages II and III therefore differ according to diagnosis.

Stage IV : Cancers have often metastasized, or spread into other organs or throughout the body.

TREATMENT

Various types of treatments are indicated for cancer patients. The modern day cancer treatment strategies include surgery, radiotherapy, chemotherapy, immunotherapy, targeted therapy and palliative care either alone or in combination with one another (Yarbro, 1992; Sikora, 1999, Breugom *et al*., 2015). The effectiveness of the treatment depends greatly on the type, location,

grade and conditions of the patient's health. The treatment employed may be curative or may be not (palliative).

Surgery :

Surgery is the primary method of treatment in cancer mostly for isolated and solid tumours. Surgery may be open or minimally invasive. It is a local treatment in which only the part of cancer is treated. The effectiveness of surgery for treating cancer depends on the type of cancer and the advancement or the stage of the cancer. Surgery may be applicable for removing the entire tumour contained in an area, debulking a tumour and for removing tumour that can cause pain or pressure (National Cancer Institute, 2015). It may be combined with chemotherapy and radiation therapy in some cases. Few examples of surgical method for cancer involve prostatectomy for prostate cancer, and lung cancer surgery for non-small cell lung cancer. The main aim or goal of surgery can be either the removal of only the tumor, or the entire organ (Subotic *et al*., 2012). Apart from removal of the primary tumor, surgery is often employed for staging, e.g. to determine the extent of the tumour spread and whether it has metastasized to nearby organs or lymph nodes. Staging is a major indicator of prognosis and the reason for need of adjuvant therapy. Sometimes, it is very necessary to control various symptoms like spinal cord compression or bowel obstruction through surgery. Neoadjuvant is the term used for treatment before surgery which means surgery may be performed before or after other forms of cancer treatments. The survival rate of patients in breast cancer patients receiving neoadjuvant chemotherapy did not differ to those who are treated following surgery (Mieog *et al*., 2007).

Radiotherapy:

Radiotherapy is also known as radiation therapy, X-ray therapy or irradiation which involves the use of ionizing radiation for killing cancerous cells and shrinking tumour cells. Like surgery, the

effect of radiotherapy is also localized and treat the specific region. Radiotherapy is used to treat various types of solid tumours such as breast, cervix, lung etc as well as lymphoma and leukemia. For treatment, the radiation dose may differ depending on the sensitivity of the cancer type and the local and nearby areas of the cancer cell. For external administration of radiation, external beam radiotherapy (EBTR) is used and for internal administration brachytherapy is employed. Radiotherapy kills cancer cells by damaging their DNA while limiting minimum harm to the nearby areas or cells. The principle of radiotherapy lies on the ability to kill cancer cells by causing breaks in their DNA since cancer cells cannot repair damaged DNA while normal cells have the ability to repair the damages caused by radiation. Although, radiotherapy is considered to be effective treatment for cancer, however, it causes many side effects for many cancer patients specially in young patients like blindness and hearing loss.

Chemotherapy:

The treatment of cancer cells with antineoplastic drugs is known as chemotherapy and it involves the killing of cancer cells using this drugs. Chemotherapy drugs target rapidly dividing cells and interferes with cell division, the main property of cancer cell. The effectiveness of chemotherapy depends on the type of cancer and stage. The combination of surgery with chemotherapy has proved to be useful in cancer types such as breast cancer, colorectal cancer, pancreatic cancer, testicular cancer, ovarian cancer and certain lung cancers (NCI, 2014). Combination therapy is commonly chemotherapy method employed for treatment of cancer patients since some drugs work better together than individually. Therefore, combination therapy is applied by giving two or more drugs at the same time. Chemotherapy is known to cure some cancers, such as some leukemias (Nastoupil *et al*., 2012; Freedman *et al*., 2012) while it is considered to be ineffective in some brain tumors (Rampling *et al*., 2004).

Immunotherapy:

Immunotherapy refers to the use of therapeutic agents which can induce the patient's immune system so that the body may develope strong system to fight the cancer cell. Various methods used to generate immune responses in a body include intravesical BCG immunotherapy for bladder cancer. Use of interferons and cytokines to induce immune response in patients suffering from renal cell carcinoma and melanoma (Waldmann, 2003). Another form of immuonotherapy known as allogeneic hematopoietic stem cell transplantation which is bone marrow transplantation from a genetically non-identical donor. In this method, the donor's immune cells will often attack the tumor in a phenomenon known as graft-versus-tumor effect. This type of treatment is often used with the other modes of cancer treatment such as surgery, radiotherapy or chemotherapy and is called as Autologous Immune Enhancement Therapy (AIET) (Damodar *et al*., 2006; Sivaraman *et al*., 2008).

Targeted therapy:

Targeted therapy involves the use of specific agents for the deregulation of protein of the cancer cells. Small molecule targeted therapy drugs inhibit the enzymatic domains on mutated, overexpressed, or critical proteins within the cancer cell. Examples are tyrosine kinase inhibitors imatinib and gefitinib. In monoclonal antibody therapy the therapeutic agent is an antibody which specifically binds to a protein on the surface of the cancer cells. Photodynamic therapy (PDT) is another treatment for cancer involving a photosensitizer, tissue oxygen, and light (often using lasers) (Dolmans *et al*., 2003). It can be used as treatment for basal cell carcinoma (BCC) or lung cancer and in removing traces of malignant tissue after surgical removal of large tumors (Dolmans, 2003).

Palliative care:

Palliative care aims at treating and helping cancer patients to make them feel better. It may be combined with an attempt to treat the cancer. Palliative care involves various actions which can reduce physical, emotional, spiritual and psycho-social distress. The primary aim of palliative care is to improve quality of life of cancer patients. It may be applied to cancer patients who are displaying low performance status, having limited ability to take care of themselves, do not receive benefit from prior evidence-based treatments, not eligible to participate in any appropriate clinical trial, with no strong evidence that treatment would be effective (American Society of Clinical Oncology, 2012). Palliative care is practiced and adopted for cancer patients with a prognosis of less than 12 months of life after they are given aggressive treatment (Levy *et al*., 2006). From the above it is clear that lots need to be done to cure cancer despite availability of several treatment modalities since all these cause adverse effects during the treatment and thereafter. The natural products may be helpful in developing cancer treatment strategies that are non-toxic to the patients.

Schima wallichii (DC.) Korth. or Chilauni (Family:Theaceae) is an Asian species of evergreen tree, which is closely related to the genus Gordonia. *S. wallichii* is known to contain upto fifteen species but after the revision of *S. wallichi* and publication made by S. Bloembergen in 1952. It is now thought to be only one variable species since he placed all of them under *Schima wallichii* (DC.) Korth. The genus inhabits warm temperate to subtropical climates across southern and South East Asia, from the eastern Himalaya of Nepal to eastern India across Indochina, Southern China, Taiwan and the Ryukyu Islands. The flowers are white in colour and scented, 4 to 6 centimeters long across with five petals and numerous yellow stamens. These bloom on pedicels

1.5 centimeters long in the terminal axils of the spring growth and accompanied by delicate shiny red new leaves (Storrs, 1990). The blooming period is from the month of May to June. Its common name is needlewood tree and it usually grows up to 35 m high. However, in some places it may be seen only 40 ft high (Min *et al*., 2003). Locally, it is called "khiang" in Mizo language and it finds many medicinal uses.

The leaves and the stem bark are normally used for its medicinal properties. The bark is used as an antiseptic for cuts and wounds, vermicide, mechanical irritant and used to cure gonorrhea (Dewanjee *et al*., 2008). Decoction of bark is good for fever and is said to be effective against head lice (Gurung, 2002). The bark juice is given to disinfest the animal from liver flukes (Lalrinzuali, 2015). The sap from the stem is used for curing ear infection (Sam *et al*., 2004). Fruit decoction is used by the people of Western Mizoram, India against snakebite (Lalfakzuala *et al*., 2007; Lalrinzuali, 2015). The young plants, leaves and roots are also used medicinally against fever and the bark is anthelmintic and rubefacient (Gardner *et al*., 2000). The leaves of *Schima wallichii* are known to have antitumor and antimutagenic properties (Koshimizu *et al*., 1998; Subarnas *et al*., 2003). The astringent corollas are used to treat uterine disorders and hysteria (Paudel, 2014). Kaempferol-3-rhamnoside, a compound isolated from the leaves of *Schima wallichii* inhibited MCF-7 breast cancer cell proliferation through activation of the caspase cascade pathway (Diantini *et al*., 2012). The present study was undertaken to evaluate the ability of different extracts of *Schima wallichii* to scavenge various free radicals in vitro.

Therefore, the present study was carried out to determine the anticancer activity of *Schima wallichii* extracts *in vitro* and *in vivo* by carrying out the following studies:

1. Fraction guided preparation of *Schima wallichii* extracts.
2. Phytochemical analysis
3. Antioxidant activity
4. Evaluation of anticancer activity *in vitro* and *in vivo*.

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PLATE 1: The different parts of *Schima wallichii*. 1) Stem bark 2) Mature and Tender leaves 3) Mature tree 4) Flowers 5) Fruits

CHAPTER 2

Phytochemical analysis of the bark powder and various extracts of *Schima wallichii* in vitro.

ABSTRACT

The mature non-infected stem bark of *Schima wallichii* was collected, powdered and sequentially extracted with petroleum ether, chloroform, ethanol and distilled water in a Soxhlet apparatus. All the liquid extracts except petroleum ether were cooled, concentrated and stored at -80°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 different phytochemicals including, alkaloids, flavonoids, cardiac glycosides, saponins, steroids, terpenoids and tannins. The phlobatannins could not be detected in the bark powder. The systematic phytochemicals analysis of chloroform, ethanol and aqueous extracts led to the detection of alkaloids, tannins, flavonoids and cardiac glycosides. However, there was variation in the presence of alkaloids, flavonoids and saponins among the different extracts. The phlobatannins were completely absent in all the three extracts. The TLC study also showed the presence of different components as indicated by the different Rf values in different solvent systems.

1. INTRODUCTION:

A very diverse range of plants have been used as medicine from prehistoric times. Various natural products especially from plant sources have been investigated for their characteristics, medical properties and health effects. Plants and natural products have mainly contributed to the evolution of the effective and reliable traditional medicinal practices that have been employed for thousands of years in China, India and many other countries (Sneader, 2005). It was not until 19th century that humans began to isolate the active principles of medicinal plants and separation of quinine from *Cinchona* bark by the French scientists Caventou and Pelletier may be considered as landmark of discovery in this regard. The practice of alternative and complementary medicine received a boost when WHO recognized these traditional healthcare systems as one of the most important procedure of treatment for humans in the first decade of this century (WHO, 2002). The chemicals or phytochemicals produced by plants are regarded as secondary metabolites since the plants that manufacture them may have little need for them. These chemicals are synthesized in almost all different parts of the plant body including bark, stem, leaves, flower, root, fruits, seeds etc. indicating that any part of the plant body may be considered as a potential source of active compounds (Charles *et al*., 2013).

The relationship between the phytochemicals and the bioactivity of plant is desirable to know for the synthesis and manufacturing of compounds with specific activities to treat various health disorders and chronic disease (Pandey *et al*., 2013). It was recorded that more than plant produce more than 100,000 phytochemicals as secondary metabolites, which can be classified on the physical characteristics and chemical compositions and pathways that are involved in their formation (Qin *et al*., 2011). Despite this fact only 150 phytochemicals had undergone detailed study.

The primary constituents of plants include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's etc. which are essential for daily activities of plants. The secondary metabolites are those phytochemicals which may not be very essential but are still synthesized by plants. These secondary metabolites include alkaloids, flavonoids, terpenes, plant steroids, curcumines, saponins, phenolics, lignans and glucosides (Hahn, 1998). The numerous phytochemicals synthesized by plants that play an important role in preventing disease progression and promote healthcare have been studied to a large extent to establish their efficacy in treatment and also to understand the mechanism of their action. However, the physiological properties of a very few phytochemicals have been well studied, where many researches have focused on their possible role in preventing or treating cancer and heart diseases (Menger *et al*., 2012; Moudi *et al.,* 2013).

Schima wallichii (DC.) Korth, Chilauni or needlewood tree belongs to the tea family: Theaceae. It is an evergreen tree found in warm temperate to subtropical climates. It is distributed across Southern and South East Asia, the eastern Himalaya of Nepal to eastern India, Indochina, Southern China, Taiwan and the Ryukyu Islands. It is called "khiang" in Mizo language. *Schima wallichii* possesses many medicinal properties and usually its leaves and the stem bark are traditionally used for its medicinal properties. The bark is applied as an antiseptic to treat cuts and wounds. It is a vermicide, mechanical irritant and cures gonorrhea (Dewanjee *et al*., 2008). The bark decoction is a good remedy to treat fever and disinfect from head lice (Gurung, 2002). The stem bark juice of *S. wallichii* is used remove liver flukes infection in the animal (Lalrinzuali, 2015). The sap from the stem is utilized to cure ear infection (Sam *et al*., 2004). Fruit decoction is active against snakebite (Lalfakzuala *et al.,* 2007; Lalrinzuali, 2015). The young plants, leaves and roots are also used medicinally against fever and the bark is

anthelmintic and rubefacient (Gardner *et al*., 2000). Some preclinical studies have shown the antitumor and antimutagenic properties of *Schima wallichii* (Koshimizu *et al*., 1998; Subarnas *et al*., 2003). The astringent corollas are used to treat uterine disorders and hysteria (Paudel, 2014). The reports regarding its phytochemical investigation are scare. Therefore, the present study was undertaken to study the presence of various phytochemicals in *Schima wallichii* using standard procedures.

2. MATERIALS AND METHODS

1.1.*Chemicals and reagents*

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

1.2. Identification, collection and drying of plant material

1.3. *Schima wallichii* was identified and authenticated by Botanical Survey of India, Shillong BSI/ERC/Tech/Identification/2017/570. The non-infected, mature stem bark of *Schima wallichii* was collected from Bazar Veng, Lunglei, Mizoram, India during the months of April and May. The stem bark was thoroughly cleaned and shade dried at room temperature in clean and hygienic conditions. The dried stem bark was powdered in an electrical grinder.

1.4. Preparation of different extracts

1.4.1. Procedure I

Sequential extraction of the stem bark powder was carried out using four different solvents (petroleum ether, chloroform, ethanol and water) according to increasing polarity in order to fractionate the different compounds. The bark powder of *Schima wallichii* (250 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 bark powder was extracted with petroleum ether (B.P. 60-80°C) at 60°C for around nine cycles (about four hours) until the solvent acquired transparency and became colourless. The extract was dried at room temperature. The petroleum ether free powder of *Schima wallichii* was again extracted with chloroform at 60°C for around thirteen cycles and thereafter in absolute ethanol at 70°C for about thirty five cycles extensively until it became colorless. The last and 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 -80°C until further use.

1.4.2. Procedure II

The bark extract was prepared by weighing 10g powder of *Schima wallichii* and transferred 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 phytochemical analysis. The aqueous extract was kept in a refrigerator at 0^{o}C until use.

1.5. Phytochemical Screening

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

1.5.1. Alkaloids

The detection of alkaloids in *Schima wallichii* was done by employing the Dragendorff's test. Briefly, 0.1 g of different extracts of *Schima wallichii* 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).

Mayer's reagent was freshly prepared by dissolving a mercuric chloride (1.36g) and potassium iodide (5g) in water (100ml). The extract filtrates were treated with Mayer's reagent. Formation of yellow coloured precipitate indicated the presence of alkaloids.

The presence of alkaloids was further confirmed using Wagner's test, where filtrates were treated with Wagner's reagent (aqueous solution of iodine and potassium iodide) that led to the formation of brown reddish precipitate indicating the presence of alkaloids.

1.5.2. Flavonoids

The flavonoids were qualitatively estimated using alkaline reagent test, where 0.1 g of each extract of *Schima wallichii* was dissolved in appropriate solvents and a few drops of sodium hydroxide solution was added. 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).

1.5.3. Cardiac glycosides (Keller-Killani test)

0.1 g of samples were 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).

1.5.4. Saponins

Usually 0.1 g of the samples were 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).

1.5.5. Steroids

The presence of steroids in the samples was determined by Salkowski's test. Briefly 0.1 g of different samples 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).

1.5.6. Tannins

The presence of tannin was determined by Ferric chloride test. Usually 0.1 g of dried samples was dissolved in different 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).

1.5.7. Terpenoids (Salkowski test)

The terpenoids in aqueous extract of *Schima wallichii* 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 and allowed to form a layer. The formation of a reddish brown colour at the interface indicted presence of terpenoids.

1.5.8. Phlobatannins

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

1.4.9 Phenol ($FeCl_3$ test)

The samples were dissolved in water or water with ethanol and a few drops of dilute ferric chloride was added. Formation of red, blue, green or purple colour indicated the presence of phenol**.**

1.5.0 Phytosterols (Liebermann Burchard test)

About 2 mg of the sample was dissolved in 2 ml of acetic anhydride, heated to boil, cooled and 1 ml of concentrated sulphuric acid was added from the side of the tube gently. The appearance of a brown ring at the junction which turns the upper layer to dark green colour indicated the presence of phytosterols.

1.6.Quantification

1.6.1. Determination of Alkaloids

Five grams of the dried bark powder was weighed in a 250 ml beaker and 200 ml of 10 % acetic acid in ethanol was added 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 followed by the addition of concentrated ammonium hydroxide drop wise until complete precipitation. The mixture was 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 determined **(**Balandrin, 1985**)**.

1.6.2. 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).

1.6.3. Determination of Flavonoids

10 g of the extract was 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).

1.6 Determination of moisture content:

Determination of the amount of volatile matter (i.e. water drying off from the drug) in the drug is a measure of weight loss after drying of substances containing water as the only volatile constituent. The *Schima wallichii* powder was accurately weighed, placed (without preliminary drying) in a tared evaporating dish, dried at 105˚C for 5 hours, and weighed again. The percentage moisture content was calculated with reference to the initial weight. The moisture content was calculated using the following formula:-

Moisture content = Pw-Fw X 100 / W

Where Pw = Preweighed sample

Fw = Final weight of the dried sample

W = Total weight of the sample

1.7 Ash Content

The crude powder of *Schima wallichii* was weighed and heated at 500ºC in a Nabertherm muffle furnace for five hours. The final ash was collected and weighed and the ash contents were expressed in terms of percentage.

Total ash content = Pw-Fw X 100 / W

Where Pw= Preweighed crucible

Fw= Final weight of the crucible containing ash

W= Total weight of powdered plant material

1.8 Yield Percentage

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

2 Thin layer chromatography

Thin layer chromatography (TLC) was performed on the different extracts to visualize the separation of various phytochemical components. The TLC is a simple, less cumbersome and rapid technique that allows the identication and separation of a number of components present in any extract/organic mixtures. It also helps in finding a suitable solvent/s for separating the components by column chromatography as well as for monitoring reaction 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 TLC plates that were transferred into the mobile phase consisting of numerous combinations of solvent systems of different polarity such as chloroform:methanol (9:1, 8:2,7:3), pure chloroform, pure ethanol, ethyl acetate: butanol: water: formic acid (5:5:2:1), ethanol: chloroform and ethyl acetate: butanol: acetic acid: water(20:2.5:1.25:1.25) and allowed to move

on the adsorbent silica gel. The resultant spots were visualized under visible and ultra-violet light, and Dragendorff's reagent. 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 RESULT

The results of phytochemicals of *Schima wallichii* are shown in Tables 1-5.

Phytochemical Screening of the bark powder

Test for alkaloids: A reddish brown precipitate was formed on reaction with Dragendorff's reagent which indicated the presence of alkaloids in the bark powder **(Table 1)**.

Test for tannins: A brownish colour was observed which showed the presence of tannins in the bark powder of *Schima wallichii* **(Table 1)**.

Test for phlobatannins: No reddish precipitate was formed in the bark powder of *Schima wallichii* indicating absence of phlobatannins **(Table 1)**.

Test for Saponin: Formation of emulsion indicated the presence of saponin in the bark powder of *Schima wallichii* **(Table 1)**.

Test for flavonoids: Formation of yellow colour by the bark powder of *Schima wallichii* showed presence flavonoids **(Table 1)**.

Test for terpenoids: The formation of a reddish brown colour at the interface confirmed the presence of terpenoids in the bark powder **(Table 1)**.

Test for cardiac glycosides (Keller-Killani test): The appearance of a brown ring at the interface indicates the presence of cardiac glycosides in the bark powder of *Schima wallichii* **(Table 1).**

Test for steroids: The development of red colour at lower layer indicated the presence of steroids in the bark powder **(Table 1).**

Test for Phenol ($FeCl_3$ test): The formation of red colour after addition of dilute ferric chloride indicated the presence of phenol in the bark powder **(Table 1).**

Test for Phytosterols (Liebermann Burchard test): The appearance of a brown ring at the junction turning the upper layer to dark green colour indicated the presence of phytosterols in the bark powder **(Table 1).**

Phytochemical Screening of the different extracts of *Schima wallichii*

Tannins tests: The test for tannins showed that tannins were present in all the different extracts which was indicated by the appearance of blue colour (**Table 2)**.

Alkaloids tests: The presence of alkaloids was confirmed after treatment with Dragendorff's, Wagner's and Hager's reagents in both ethanol and aqueous extracts, indicated by the presence of reddish brown precipitate, however, alkaloid were distinctly absent from chloroform extract (Table 2).

Terpenoids test (Salkowski test): The formation of a reddish brown colour at the interface indicted presence of terpenoids (**Table 2)**.

Flavonoids: The flavonoids were present in all the extracts but the contents were highest in chloroform extract that was indicated by the formation of intense yellow colour in alkaline reagents test and red colour in zinc hydrochloride test (**Table 2)**.

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

Test for Phytosterols (Liebermann Burchard test): The appearance of a brown ring at the junction turning the upper layer to dark green colour indicated the presence of phytosterols in the ethanol extract **(Table 2).**

Saponins: The formation of emulsion in all the extracts indicated the presence of saponins. However, the emulsion was less in the chloroform extract as compared to the other two **(Table 2).**

Phenol test ($FeCl_3$ Test): The formation of red colour after addition of dilute ferric chloride indicated the presence of phenol in the all the extracts **(Table 2).**

Moisture and Ash contents: The moisture content of the crude bark powder was found to be 62.8% while the ash content was 12% **(Table 3)**.

Quantification of phytochemicals: The ethanol extract was found to contain 20.8 % alkaloids, 2.5 % flavonoids and 1.9 % saponins respectively **(Table 4).**

Yield percent

The extraction of *Schima wallichii* stem bark yielded 1.46% in chloroform,13.28 % in ethanol and 8.32% in aqueous **(Table 5)**.

TLC Analysis

The evaluation of various extracts of *Schima wallichii* showed the presence of different components as indicated by a varying number of spots and colours on the TLC plates using different solvent systems and visualization methods **(Table 6).**

DISCUSSION

Utilization of plants for their medicinal properties has been documented long back in human history. There can be many reasons why plants are very reliable healers to many ailments. The composition, contents of the plants are known to play important role in providing medicinal

activities and their effectiveness as a medicine. The discovery of drugs also has been proven to be successful with the knowledge of medicinal plant and the metabolites present in them. Therefore, the phytochemical and TLC analyses have been done to find out the various phytochemicals present in the stem bark of *Schima wallichii*.

Alkaloids are a group of naturally occurring chemical compounds having basic nitrogen atoms and are produced by a wide variety of organisms. They are known to possess several pharmacological properties which include antimalarial, analgesic, antibacterial, anticancer etc (Copenhaver *et al*., 2012; Cushnie *et al*., 2014). Among various organisms containing alkaloids several higher plants about 10 to 25 % contain alkaloids (Orekhov, 1955; Aniszewski, 2007). The bark powder sample as well as ethanol and aqueous extracts of *Schima wallichii* contain alkaloids whereas the alkaloids were conspicuous by their absence in the chloroform extract. The ethanol extract possesses the maximum quantity of alkaloids among all the extracts of *S. wallichi* screened. Therefore, the medicinal properties attributed to this plant may be due to the presence of alkaloids. Earlier observations supports this contentions, morphine and codeine as strong pain killers and quinine and several alkaloids have been reported to act as antimalarial and anticancer drugs (Veselovskaya, 2000; Kinghorn *et al*., 2016; Newman and Cragg, 2016).

Flavonoids are a class of secondary metabolites that are produced by plants and fungi. In higher plants, flavonoids are involved in UV filtration, symbiotic nitrogen fixation and floral and fruits' pigmentation (Petrussa *et al*., 2013; Brunetti *et al*., 2016). Fruits and vegetables are considered to be the main dietary sources of flavonoids for humans, along with tea and wine (Yao *et al*., 2004). The bark powder as well as all the different extracts of *Schima wallichii* contained flavonoids and the flavonoids percent was considered to be about 2.5 % in the ethanol

extract. The presence of flavonoid compound may be the reason for the medicinal properties of *Schima wallichii.* Many studies have shown that flavonoids act as medicine against a host to ailments including allergy, cancer, infection (bacterial and viral), cataract, diabetes, cardiac disease, hepatic disorder, osteoporosis, inflammation (Hegarty *et al*., 2000; Cushnie and Lamb, 2005; Chahar *et al*., 2011; Kumar and Pandey, 2013; Tanaka, 2013; Ivey *et al*., 2015).

Saponins have been originally separated from plants however theyhave been also found in marine organism like sea cucumber (Riguera, 1997). The saponins are glycosides synthesized by 100 plant families to protect themselves from herbivory and assault by pathogens (Man *et al*., 2010; Moses *et al*., 2014). The saponins possess soap-like property and produce a lather when mixed with water or when they are shaken. Saponins are known to support immune system, promote normal cholesterol level, produce an antioxidant effect, support bone strength and provides overall wellness (Shi *et al*., 2004; Sun *et al*., 2009). It is also reported that saponins have been found to exert anticancer effect in several preclinical model systems (Rao *et al*., 1995; Yan *et al*., 2009; Man *et al*., 2010; Yildirim and Kutlu, 2015). Saponins were also reported to offer therapeutic benefit against kidney or urinary stones (Chaudhary *et al*., 2010). The different extracts of *Schima wallichii* showed the presence of saponins and the quantification done from the ethanol extract gives more than one percent saponins contents. So, it may be stated that the medicinal and antioxidant property of the plant may be due to the presence of the phytochemical compound saponins.

Tannins are water-soluble polyphenols that are present in many plant foods, which contain sufficient hydroxyls and other suitable groups (such as carboxyls) and forms strong complexes with other macromolecules. Tannins are found in different species throughout the plant kingdom. They are commonly found in both gymnosperms and angiosperms and they are synthesized by

plants to protect themselves from biotic and abiotic stress (Simon, 1993; Piluzza *et al*., 2014). The antimutagenic, antimicrobial properties of tannins may be related to their antioxidant property. Tannins have also been reported to exert other physiological effects, such as to accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, produce liver necrosis, and modulate immunoresponses (Chung *et al*., 1998). The medicinal uses of *Schima wallichii* may be due to the presence of tannins. The tannins have been reported to be cytotoxic to neoplastic cells (Yildirim and Kutlu, 2015). The tannins have been reported to act as an antibacterial, antitumor and antiviral agents (Khanbabaee and Ree, 2001).

Cardiac glycosides are a class of organic compounds that increase the output force of the heart and decrease its rate of contractions by acting on the cellular sodium-potassium ATPase pump (Patel, 2016). They are commonly found as secondary metabolites and are known to have been suggested for anticancer effects (Riganti *et al*., 2011). Cardiac glycosides have been reported to be active against various tumors in preclinical studies (Felth *et al*., 2009; Slingerland *et al*., 2013). Due to their high toxicity, they may be used in low dosage or in conjunction with other treatments (Bullock *et al*., 2013). The different extracts as well as the bark powder of *Schima wallichii* have been found to contain cardiac glycosides.

Terpenoids are a large and diverse group of naturally occurring organic chemicals, which are derived from five-carbon isoprene units assembled and modified in number of ways. Plants synthesize more than 40,000 terpenoids with a diverse array of functions in humans (Bohlmann and Keeling, 2008; Lu *et al*., 2016). Terpenoids contribute about 60% of known natural products and are considered to be the largest group of natural products (Firn, 2010). Terpenoids act as antifeedant, repellent and attractants in plants and are used as antibiotics, pesticides, insecticides and fragrance by humans (Bohlmann and Keeling, 2008). Terpenes are known to be diuretics and

aid in relieving gastrointestinal spasms. They are added to creams and ointments to relieve pain and itching. Terpenes also possess antimicrobial properties which helps in fighting microorganisms resistant to antibiotics such as yeast and other fungi (Paduch *et al*., 2007; Santos *et al*., 2014). The medicinal properties of *Schima wallichii* may be due to the presence of terpenoids as one of its phytochemical constituents.

The various medicinal uses of *Schima wallichii* in cuts and wounds, snakebite, as vermicides (Dewanjee *et al*., 2008; Lalfakzuala *et al*., 2007; Lalrinzuali, 2015), its antitumour and anthelmintic properties may be due to the presence of various phytochemicals like alkaloids, flavonoids, saponins, tannins, cardiac glycosides, and terpenoids as observed in the present study. The medicinal activity of this plant may be due to the presence of one or more of these phytochemicals or may be due to the concerted action of all of these phytochemicals present in it.

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Table 1: Qualitative phytochemical analysis of the bark powder of *Schima wallichii.*

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

Legend Present (+), absent (-)

Table 2: Qualitative phytochemical analysis of various extracts obtained from *Schima wallichii.*

Phytochemicals	Tests	Solvent used for extraction		
		Chloroform	Ethanol	Aqueous
Alkaloids	Dragendroff's	-	+	+
	Mayer's	-	+	+
	Wagner's	-	+	+
Tannins	$FeCl_3$	-	+	+
Flavonoids	Alkaline reagent	+	+	+
	Lead acetate	+	+	+
Saponins	Froth	+	+	+
	Foam	+	+	+
Phytosterols	Liebermann Burchard's	-	+	-
Terpenoids	Salkowski's	+	+	+
Cardiac glycosides	Kellar kiliani's	+	+	+
Phenols	$FeCl_3$	+	+	+

Legend Present (+), absent (-)

Table 3: The percentage of moisture and ash content on the fresh stem bark of *Schima wallichii*

Moisture content			Ash content		
Initial weight (g) Wet weight	Final weight (g) Dry weight	Content (%)	Initial weight (g)	Final weight (g)	Content (%)
400	148.8	62.8	10	1.2	12

Table 4: Quantification of phytochemicals in the ethanol extracts of *Schima wallichi*

Phytochemicals	Initial weight (g)	Final weight (g)	Content (%)
Alkaloids	**5**	**1.04**	**20.8**
Flavonoids	**20**	**0.5**	**2.5**
Saponins	**20**	**0.38**	**1.9**

.

Table 5: The yield of different extracts of *Schima wallichi*

Extracts	Initial weight (g)	Final weight (g)	Yield (%)
Chloroform	**120**	**1.76**	**1.46**
Ethanol	**250**	**33.2**	**13.28**
Aqueous	**250**	**20.8**	**8.32**

Table 6 : TLC profile of the different extracts of *Schima wallichii* on pre-coated aluminium TLC plates.

Extracts	Solvent	Observations			
SWC	**$CHCl_3$: CH_3OH**	**Day light**	**UV 254**	**UV 365**	**Rf**
	9:1	**Pale yellow**	**Dark brown**	**UV active**	**0.5**
	8:2	**Yellow**	**Dark brown**	**UV active**	**0.605**
	7:3	**Light brown**	**Dark brown**	**UV active**	**0.609**
	6:4	**Light brown**	**Dark brown**	**UV active**	**0.64**
	5:5	**Yellow**	**Dark brown**	**UV active**	**0.404**
SWE	**Ethyl acetate: Butanol: Acetic acid: Water (20:2.5:1.25:1.25)**	**Light brown**	**Light green**	**Purple**	**0.84**
	Ethanol: $CHCl_3$(9:1, 8:2)	**Yellow**	**Brown**	**Purple**	**0.765, 0.833**
	Ethyl acetate: Butanol: Water: Formic acid(5:5:2:1)	**Three spots, light yellow and light brown**	**Brown**	**Purple**	**0.1, 0.56, 0.88**
SWA	**-**	**Not visible**	**Not visible**	**Not visible**	**0**

SWC: Chloroform extract, SWE: Ethanol extract, SWA: Aqueous extract

PLATE 2: Extraction process and phytochemical analysis of *Schima wallichii*. 1) Stem bark sample 2) Sample powder 3) Soxhlet apparatus 4) and 5) *S.wallichii* extracts 6) Alkaloid test 7) Flavonoid test 8) Phytochemical analyses 9) and 10) TLC analysis viewed under normal light and UV 254.

CHAPTER 3

Free radical scavenging and antioxidant activity of different extracts of *Schima wallichii* in vitro.

ABSTRACT

Free radicals are highly reactive species and can start a chain reaction once they are formed. The free radicals have the ability to damage important macromolecules including nucleic acid, proteins, lipids and the cell membrane.Therefore, any pharmacophore which is able to neutralize free radicals will be of important in healthcare. The ability of various extracts of *Schima wallchii* was evaluated for their ability to scavenge the generation of DPPH, hydroxyl ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$) nitric oxide ($NO^{\bullet}$), ABTS and FRAP free radicals *in vitro*. Total flavonoid and the total phenol contents were also determined for understanding their potential in free radical scavenging. The chloroform, ethanol, and aqueous extracts of *Schima wallchii* showed a concentration dependent inhibition in DPPH, $^{\bullet}OH$, $O_2^{\bullet-}$, $NO^{\bullet}$, $ABTS^{+\bullet}$ and FRAP free radicals. A maximum inhibition in the DPPH and $^{\bullet}OH$ free radicals was observed at 80 ug/ml, whereas the $NO^{\bullet}$ and $O_2^{\bullet-}$radicals were inhibited at a concentration of 100 µg/ml and 200 µg/ml, respectively. The highest inhibition of $ABTS^{+\bullet}$ and FRAP free radicals were observed at a concentration of 60 µg/ml and 200 µg/ml, respectively. The highest amount of total flavonoids were determined for 1000 µg/ml for all the extracts, whereas the maximum total phenols were detected for 1000 µg/ml at ethanol extract. The concentration dependent inhibition of the generation of DPPH, $^{\bullet}OH$, $O_2^{\bullet-}$, $NO^{\bullet}$, $ABTS^{+\bullet}$ and FRAP radicals of different extracts of *Schima wallchii* may be due to the presence of various polyphenols.

1.INTRODUCTION

Usage of traditional medicines and other medicinal plants in developing countries as therapeutic agents for maintaining proper health has been observed widely (UNESCO, 1996). Plants and other natural products are still in great need and demand due to various factors like their safety, dependability and lesser side effects (Shantabi *et al*., 2014) and 80 % of individuals in the developing countries still depend on plants to treat different diseases (Kim, 2005). The driving forces which lead to the use of alternative medicine for better cure in cancer treatment can be due to the greater side effects caused by many cancer drugs. About 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care which shows that the plant-based, traditional medicine systems continues to play an important part in human health care and recovery (Tilburt and Kaptchuk, 2008; Shantabi *et al*., 2015). The interest in medicinal plants as major breakthrough in health aid has been put forwarded into great milestone by the rising costs of prescription drugs for maintaining healthy conditions of a person and well being and the bioprospecting of new drugs derived from plants could be more economic (Lucy and Edgar, 1999).

The free radicals or simply ROS are highly reactive species which can be generated by cells during respiration, and cell-mediated immune functions (Chang *et al*., 2012; Ye *et al*., 2015). They are produced naturally in the body as they play an important role in many cellular functions however; if their production is quite high, the damages they can cause may play a role in the development of various human health disorders including cancer (Diplock *et al*., 1998; Valko *et al*., 2007). The excess free radicals produced during respiration and other activities would cause various severe damages eventually leading to loss of functionality to the organisms,

which may subsequently lead to death (Speakman and Selman, 2011). Antioxidants are molecules capable of inhibiting oxidation of other molecules and are helpful in reducing and preventing damage from free radical reactions because of their ability to donate electrons which can neutralize the radical without forming another (Halliwell and Gutteridge 1995; Halliwell 2007; Khlebnikov *et al*., 2007). Many plants synthesize secondary metabolites naturally including flavonoids and polyphenols which act as antioxidants and also plays major role in different biological activities (Middleton *et al*., 2000; Nichols and Katiyar, 2010; Carvalho *et al*., 2010). Therefore, plants and natural products could be a major source of antioxidants that can scavenge free radicals.

Schima wallichii (DC.) Korth.(Chilauni) is an Asian species of evergreen tree belonging to the tea family, Theaceae. The genus inhabits warm temperate to subtropical climates across southern and South East Asia, from the eastern Himalaya of Nepal to eastern India across Indochina, Southern China, Taiwan and the Ryukyu Islands. Its common name is needlewood tree and it usually grows up to 35 m high. However, in some places it may be seen only 40 ft high (Min *et al*., 2003). Locally, it is called "khiang" in Mizo language. *Schima wallichii* is known to possess several medicinal properties. Traditionally, the leaves and the stem bark are normally used for its medicinal properties. The bark is used as an antiseptic for cuts and wounds, vermicide, mechanical irritant and used to cure gonorrhea (Dewanjee *et al*.,2008). Decoction of bark is good for fever and is said to be effective against head lice (Gurung, 2002). The bark juice is given to disinfest the animal from liver flukes (Lalrinzuali, 2015). The sap from the stem is used for curing ear infection (Sam *et al*., 2004). Fruit decoction is used by the people of Western Mizoram, India against snakebite (Lalfakzuala *et al*., 2007; Lalrinzuali, 2015). The young plants, leaves and roots are also used medicinally against fever and the bark is anthelmintic and

rubefacient (Gardner *et al*., 2000). The leaves of *Schima wallichii* are known to have antitumor and antimutagenic properties (Koshimizu *et al*.,1998; Subarnas *et al.*,2003). Kaempferol-3-rhamnoside, a compound isolated from the leaves of *Schima wallichii* inhibited MCF-7 breast cancer cell proliferation through activation of the caspase cascade pathway (Diantini *et al*., 2012). Therefore, the present study was undertaken to evaluate the ability of different extracts of *Schima wallichii* to scavenge various free radicals in vitro.

2.MATERIALS AND METHODS

2.1. Chemicals and reagents

All the chemicals used were of analytical grade and Milli Q water was used for entire analysis. 1,1-Diphenyl-2-picrythydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), dimethyl sulfoxide (DMSO), ascorbic acid, nitrobluetetrazolium (NBT), phenazinemethosulphate (PMS), β-nicotinamide adenine dinucleotide (NADH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), sodium nitroprusside, and Griess reagent were procured from Sigma-Aldrich Co. Bangalore, India). Methanol, ethanol, sodium acetate, ferric chloride, Folin-Ciocalteau reagent, sodium carbonate, sodium hydroxide, sodium chloride, potassium chloride, disodium hydrogen phosphate (anhydrous), potassium dihydrogen phosphate, aluminium chloride, potassium acetate, gallic acid, ferrous ammonium sulphate, ammonium acetate, glacial acetic acid and acetyl acetone were supplied by Merck India, Mumbai.

2.2.Preparation of extracts

Schima wallichii was identified and authenticated by Botanical Survey of India, Shillong. The non-infected stem bark of *Schima wallichii* was collected from Bazar veng, Lunglei, Mizoram during the months of April and May. The bark was cleaned and shade dried at room temperature in clean and hygienic conditions. The dried bark was then powdered using an electrical grinder. The dried powder of *Schima wallichii* was extracted sequentially 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 them to dryness under reduced pressure. The concentrated extracts were stored at -80 until use.

2.2 Experimental protocol

The free radical scavenging activity of the different extracts of *Schima wallichii* was estimated according to standard protocols as given below.

2.3 DPPH free radical scavenging assay

The DPPH free radical scavenging activity of *Schima wallichii* was estimated according to Leong and Shui (2002). Various concentrations of extracts of *Schima wallichii* (0.5 ml each) were mixed thoroughly with 1 ml of methanolic solution of 0.1 mM 2, 2-diphenyl-1-picryl hydrazine (DPPH). The mixture was allowed to stand for 30 min in the dark. The absorbance was measured at 523 nm using a Eppendorf UV/VIS Spectrophotometer (Eppendorf, Germany). An equal amount of DPPH and methanol were used as a standard and blank, respectively. The scavenging activity was 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 assay

The hydroxyl radical scavenging activity of *Schima wallichii* was assayed according to the earlier described method (Halliwell *et al*., 1987) with minor modifications. The reaction mixture contained deoxribose (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) and different concentrations of *Schima wallichii* extracts in a final volume of 2 ml. The mixture was incubated at 37°C for 30 min followed by the addition of 2 ml of trichloroacetic acid (2.8% w/v) and thiobarbituric acid. The reaction mixture was kept for 30 min in a boiling water bath, cooled and the absorbance was measured at 532 nm in a UV-VIS spectrophotometer. Gallic acid was used as the standard and the results have been expressed as gallic acid equivalent.

2.5 Superoxide anion scavenging assay

Scavenging of the superoxide (O2•-) anion radical was measured using a modified method (Hyland *et al*., 1983). The reaction mixture contained 0.2 ml of NBT (1mg/ml of solution in DMSO), 0.6 ml extracts, 2 ml of alkaline DMSO (1 ml DMSO containing 5 mMNaOH in 0.1 ml H_2O) with a final volume of 2.8 ml. The absorbance was recorded at 560 nm using a UV-VIS spectrophotometer. The blank consisted of pure DMSO instead of alkaline DMSO. The results have been expressed as ascorbic acid equivalent which was used as a standard.

2.6 ABTS scavenging assay

ABTS scavenging activity of different extracts of *Schima wallichii* was determined as described earlier (Re *et al*., 1999). Briefly, 37.5 mg of potassium persulfate 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 so as to prepare ABTS solution. The ABTS solution was allowed to stand in the dark for about 15 hours at room temperature. The working solution was prepared by mixing 1 ml of ABTS solution with 88 ml of 50 % ethanol. 25 µl of different extracts of different concentration of *Schima wallichii* were mixed with 250 µl of the working solution and allowed to stand for 4 minutes. The absorbance was read at 734 nm in a UV-VIS spectrophotometer. The results have been expressed as ascorbic acid equivalent which was used as a standard.

2.7 Nitric oxide scavenging assay

The nitric oxide scavenging activity was estimated according to the method of Marcocci *et al*., (1994). Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with different concentrations of the various extracts of *Schima wallichii* and incubated at 25°C for 150 minutes. The samples were then mixed with Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% naphthylenediaminedihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with napththylenediamine was read at 546 nm using a UV-VIS spectrophotometer. The inhibition of nitric oxide formation was determined with respect to standard potassium nitrite in the same way with Griess reagent. The results have been expressed as potassium nitrite equivalent which has been used as a standard.

2.8. Ferric reducing antioxidant potential (FRAP) assay

The ability of *Schima wallichii* extracts to decrease ferric ions was measured as described earlier (Benzie and Strain, 1999) with minor modifications. 50 µl of various concentrations of the extracts were added to 3 ml of FRAP reagents (10 parts of 300 mM acetate buffer, pH 3.6, 1 part of TPTZ solution and 1 part of 20 mM $Fecl_3.6H_2O$ solution) and the reaction mixture was incubated at 37°C for 30 min. The increase in absorbance was measured at 593 nm using UV-VIS Spectrophotometer. The antioxidant activity of the extracts is based on their ability to reduce ferric ions and it has been expressed as mg ferrous sulphate equivalents/100 g of *Schima wallichii* extracts.

2.10 Determination of Total phenolic contents

The total phenolic contents of the *Schima wallichii* extracts were determined as described by (Singleton and Rossi, 1965). Briefly, 500 µl of different extracts of *Schima wallichii* were mixed with 1000 µl of 1:10 Folin-Ciocalteau's reagent and incubated at room temperature for 5 min followed by the addition of 900 µl saturated (7.5%) sodium carbonate solution. After 1 h of incubation at room temperature, the absorbance was recorded at 640 nm using UV-VIS Spectrophotometer. The total phenolic contents of the extracts have been expressed as gallic acid equivalents mg/100 g of the extracts.

2.11 Total Flavonoids Determination

The total flavonoids were determined by colorimetric method described by (Chang *et al*., 2002). 1 ml of various extracts of *Schima wallichii* was separately mixed with 1.5 ml of 95% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water and incubated for 30 min at room temperature. The absorbance of the reaction

mixture was recorded at 415 nm with a UV-VIS Spectrophotometer. The presence of flavonoids in *Schima wallichii* extracts were expressed as mg quercetin equivalent/100g of the extracts.

3. RESULTS

The results of free radical scavenging by different extracts of *Schima wallichii* are shown as mean ±SEM (Standard error of the mean in Figures 1-6, where as that of total phenols and flavonoids as figure 7-8).

1. DPPH free radical scavenging assay:

The chloroform, ethanol and aqueous extracts of *Schima wallichii* showed a concentration dependent rise in the scavenging of DPPH free radical and a maximum scavenging activity was recorded at a concentration of 160, 80 and 140 µg/ml respectively. Thereafter, the scavenging effect declined (Figure 1). The best extract was ethanol extract which required low concentration to scavenge higher amount of DPPH free radicals (Figure 1).

2. Hydroxyl radical scavenging assay

The scavenging of hydroxyl radical depends on the dose of extracts of *S. wallichii.* The chloroform, ethanol and aqueous extracts inhibited the generation of hydroxyl radical in a concentration dependant manner and a maximum inhibition in $^{\bullet}OH$ generation was observed at 80 µg/ml for ethanol, 100µg/ml for aqueous and 120 µg/ml for chloroform extracts (Figure 2).

3. Superoxide anion scavenging assay

The chloroform, ethanol and aqueous extracts of *S. wallichii* showed a concentration dependent increase in the inhibiton of superoxide generation and the highest scavenging activity for $O_2^{\bullet-}$, was observed at a concentration of 200 µg/ml for all the three extracts (Figure 3).

4.ABTS scavenging assay

Various extracts of *S. wallichii* showed a concentration dependent rise in the scavenging of the ABTS free radicals (Figure 4). The maximum activity for chloroform extract was recorded for 350 µg/ml, whereas ethanol and aqueous extracts showed maximum ABTS inhibitory action at 60 µg/ml and 100 µg/ml, respectively. The ethanol extract proved to be the best among all the three extracts as it has a maximum effect at lower concentration (Figure 4).

5. Nitric oxide scavenging assay

The analysis of nitric oxide scavenging activity also revealed a concentration dependent rise in its scavenging for chloroform, ethanol and aqueous extracts of *S. wallichii* (Figure 5). The greatest scavenging activity was discernible at 120 µg/ml, 100 µg/ml and 160 µg/ml for chloroform, ethanol and aqueous extracts, respectively and it declined thereafter (Figure 5).

6. Ferric reducing antioxidant potential (FRAP) assay

The FRAP radical scavenging activity of chloroform, ethanol and aqueous extracts of *S. wallichii* showed a concentration dependent rise up to 200 µg/ml, the highest concentration evaluated. All extracts were equally effective in scavenging the FRAP radical (Figure 6).

7. Determination of Total phenolic content

Total phenol contents of *S.wallichii* extracts showed a concentration dependant rise up to a concentration of 900 µg/ml for chloroform, 1000µg/ml for ethanol and 800 µg/ml for aqueous extracts (Figure 7).

8. Total Flavonoids Determination

The chloroform, ethanol and aqueous extracts of *S.wallichii* extracts showed a concentration dependant increase in the total flavonoid contents and maximum amount of flavonoids was estimated for 1000 µg/ml for chloroform, ethanol and aqueous extracts, respectively (Figure 8).

4. DISCUSSION

Free radical damage is closely associated with oxidative damage. Antioxidants are reducing agents, and limit oxidative damage to biological structures by passivizing free radicals (Herrling *et al*., 2008). When oxygen interacts with certain molecules, free radicals are formed. And when formed, the chief danger comes from the damage they can do when they react with important cellular components such as DNA or the cell membrane (Dröge, 2002). These free radicals are safely interacted with by the antioxidants, which can terminate them before damages occurs (Halliwell, 2012). Plants synthesize several compounds and many of them act as antioxidants. Therefore, the present study was undertaken to study the free radical scavenging ability of *Schima wallichii* in vitro.

DPPH **(**2,2-diphenyl-1-picrylhydrazyl) is a a dark-colored crystalline powder composed of stable free-radical molecules. Most notably, it is a common antioxidant assay and is a well known radical. DPPH radical has a deep violet color in solution, and it becomes colorless or pale yellow when neutralized and will be converted into DPPH-H (Goldschmidt and Renn, 1922). Many plants extracts have been reported to scavenge DPPH radical in vitro (Jagetia *et al*., 2003; Baliga *et al*., 2003; Wong *et al*., 2006; Shantabi *et al.*, 2014; Lalrinzuali *et al*., 2015a,b). The different extracts of *Schima wallichii* scavenged DPPH radical in a concentration dependant

manner. Other phytochemicals like mangiferin, and naringin have been reported to scavenge DPPH radical in a concentration dependent manner (Jagetia *et al*., 2003a; Jagetia and Venkatesha, 2005). The scavenging activity for ethanol extracts of *Schima wallichii* was 80µg/ml, 140µg/ml for aqueous extracts and twice the dose of ethanol extract(160µg/ml) for chloroform extracts. The DPPH scavenging activity of *Schima wallichii* may be due to the presence of flavonoids and other polyphenols in the extracts as indicated in the present study.

Hydroxyl radicals are highly reactive and consequently short-lived (Hayyan *et al*., 2016), and they are capable of inducing detrimental effects on the important macromolecules including proteins and nucleic acids. In the Haber-Weiss/Fenton reaction, hydroxyl radicals are generated in the presence of hydrogen peroxide and iron ions .(Floyd and Lewis, 1983; Michiels, 2014). The high reactivity of hydroxyl radicals lead to tremendous damage to the cell and its components and subsequently to the organisms whole (Dizdaroglu and Jaruga, 2012). Therefore, it is very important to remove hydroxyl radicals which cause detrimental effects. The different extracts of *Schima wallichii* inhibited the generation of hydroxyl free radicals in a concentration dependent manner. Similarly, many plants extracts have been found to scavenge hydroxyl free radicals in a concentration dependent manner (Jagetia *et al*., 2003 a,b; 2012; Jagetia and Venketasha, 2005; Shantabi *et al.*, 2014; Lalrinzuali *et al*., 2015a,b).

The $O_2^{\bullet-}$ generated in biological systems during cellular respiration and as such they are less toxic however, they are converted into highly reactive OH radical in the presence of iron (Lushchak, 2014). Moreover, superoxide anions produced as a result of incomplete metabolism of oxygen damage biomolecules directly or indirectly by forming H_2O_2, $^{\bullet}OH$, peroxy nitrite or singlet oxygen (Kirkinezosa and Morae, 2001; Lushchak, 2014**).** Therefore, the removal or neutralization of superoxide radical is necessary to protect the cells from its deleterious effect.

Various extracts of *Schima wallichii* inhibits the formation of superoxide anion radical in a concentration dependant manner. Other plant extracts and certain plant flavonoids have been found to scavenge superoxide free radical in a concentration dependent manner (Jagetia *et al*., 2003, ab, 2012; Jagetia and Venketasha, 2005; Shantabi *et al*., 2014; Lalrinzuali *et al*., 2015a,b).

Nitric oxide is an important cellular signaling molecule involved in many physiological and pathological processes. It is a powerful vasodilator with a short half-life of a few seconds in the blood (Hou *et al*,.1999;Lushchak, 2014) The nitric oxide radical ($NO^{\bullet}$) is toxic, after reaction with oxygen or superoxide anion radicals. Different extracts of *Schima wallichii* reduced the generation of $NO^{\bullet}$ in a concentration dependent manner. Several plant extracts and plant formulations have been reported to scavenge $NO^{\bullet}$ in a concentration dependent manner (Jagetia *et al*., 2003, Jagetia and Baliga 2004; Jagetia and Shetty, 2012; Shantabi *et al*., 2014, Lalrinzuali *et al*., 2015).

The $ABTS^{\bullet+}$ chromophore was produced through the reaction between ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and potassium persulfate which converts ABTS to its radical cation. This radical cation is blue in color and absorbs light at 734 nm.(Re *et al*., 1999). The $ABTS^{\bullet+}$ is reactive towards most antioxidants including phenolics, thiols and Vitamin C (Walker *et al*., 2009). Various extracts of *Schima wallichii* showed inhibition of ABTS radical production in a concentration dependant manner. A similar effect has been observed with the extract of *Syzygium cumini* and naringin and mangiferin (Jagetia *et al*., 2003; Jagetia and Venkatesha, 2005; Jagetia and Shetty, 2012). FRAP assay had been used to determine antioxidant activity as it is simple and quick method (Hodzic *et al*., 2009). The different extracts of *Schima wallichii* showed concentration dependant manner in inhibition of FRAP radical generation. Several plant extracts have been reported to exhibit antioxidant activity by

scavenging of FRAP radical in vitro (Wong *et al*., 2006; Aparadh *et al*., 2012; Shantabi *et al*., 2014, Lalrinzuali *et al*., 2015). *Cyanometra cauliflora* fruit and *Garcinia atrovirdis* fruit have been reported to possessed high FRAP value (Rabeta. and NurFaraniza, 2013).

The phytochemical analysis of *Schima wallichii* stem bark has shown the presence of phenols and flavonoids and their concentrations increased with the increase in the amount of extracts. The antioxidant activity of *Schima wallichii* may be due to the presence of various polyphenols and flavonoids

CONCLUSIONS

The present study showed that all the extracts of *Schima wallichii* showed a concentration dependent inhibition of free radicals and ferric reducing power. These activities of *Schima wallichii* may be due to the presence of various phenolic compounds and flavonoids. The ethanol extract showed maximum antioxidant activity followed by the aqueous extract, whereas the chloroform extract showed the least activity. The low doses of *Schima wallichii* may act as a good antioxidant.

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Table 1: Percentage scavenging activity of different extracts of *Schima wallichii* for DPPH, Hydroxyl and Superoxide free radicals.

Conc. (ug/ml)	DPPH			HYDROXYL			SUPEROXIDE		
	SWE	SWA	SWC	SWE	SWA	SWC	SWE	SWA	SWC
20	37.90±0.38	31.90±0.11	25.42±0.17	30.06±0.09	13.22±0.39	9.44±0.54	32.66±1.06	31.40±1.53	15.98±0.80
40	59.08±0.20	35.53±0.17	26.96±0.81	52.34±0.09	16.07±0.43	23.16±0.25	68.96±0.70	49.77±1.00	22.80±0.45
60	85.35±0.39	55.04±0.18	33.33±0.39	76.10±0.16	28.18±0.36	45.80±0.27	80.17±0.14	58.26±1.20	26.26±0.60
80	90.46±0.17	69.21±0.10	38.09±0.57	82.78±0.06	40.64±0.37	48.51±0.31	82.57±0.38	68.67±0.15	29.21±0.77
100	74.55±0.13	73.96±0.18	41.01±0.43	77.20±0.20	55.25±0.42	56.38±0.46	86.96±0.21	78.25±0.23	33.95±0.49
120	59.82±0.53	74.63±0.50	43.39±0.33	74.78±0.30	32.22±0.49	80.94±0.32	88.50±0.26	80.66±0.44	42.54±0.78
140	-	79.67±0.12	45.54±0.27	74.49±0.11	28.32±0.39	78.15±0.31	90.13±0.19	84.65±0.65	48.86±0.21
160	-	75.37±0.98	47.93±0.98	73.90±0.08	25.82±0.37	-	92.11±0.15	85.48±0.55	50.60±0.17
180	-	73.44±0.23	38.17±0.28	73.46±0.08	13.43±0.37	-	93.57±0.11	86.44±0.35	54.25±0.52
200	-	-	-	71.41±0.13	9.18±0.48	-	94.26±0.11	87.46±0.50	60.60±0.26

Table 2: Percentage scavenging activity of different extracts of *Schima wallichii* for Nitric, FRAP and ABTS free radicals.

Conc. (ug/m)	NITRIC			FRAP			ABTS		
	SWE	SWA	SWC	SWE	SWA	SWC	SWE	SWA	SWC*
20	32.90±0.16	21.36±1.14	8.01±1.40	47.96±0.32	25.20±0.39	8.97±1.39	24.42±0.25	17.41±0.30	10/12.47±0.45
40	35.46±0.82	24.08±0.97	19.95±0.75	54.75±0.39	38.59±0.59	22.52±0.30	42.49±0.45	23.60±0.31	50/20.44±0.48
60	39.48±0.38	30.10±0.11	34.57±0.68	67.44±0.47	45.03±0.23	31.87±0.88	59.24±0.08	34.87±0.27	100/34.23±0.68
80	53.65±0.59	32.70±0.80	44.51±0.75	69.03±0.39	51.19±0.18	42.85±0.60	71.59±0.37	40.31±0.42	150/36.52±0.05
100	57.49±0.30	39.37±0.19	48.68±1.07	74.58±0.61	58.80±0.44	40.59±0.35	78.60±0.16	48.93±0.14	200/54.40±0.37
120	41.86±0.22	42.85±0.78	51.46±0.99	76.70±0.32	61.65±0.14	52.28±0.70	83.60±0.24	57.03±0.19	250/64.37±0.54
140	30.25±0.41	45.03±0.51	36.66±0.65	80.91±0.20	59.44±0.31	52.42±0.15	-	60.61±0.16	300/70.19±0.85
160	10.41±0.68	48.74±0.58	20.14±1.31	82.70±0.21	64.44±0.25	53.20±0.08	-	67.61±0.24	350/71.44±0.64
180	47.62±0.57	-	-	86.57±0.16	65.20±0.37	52.21±0.60	-	76.92±0.16	400/65.21±0.66
200	14.16±0.98	-	-	87.00±0.15	69.13±0.27	56.12±0.52	-	83.05±0.06	-

Values were expressed as Mean± SEM, N=5.

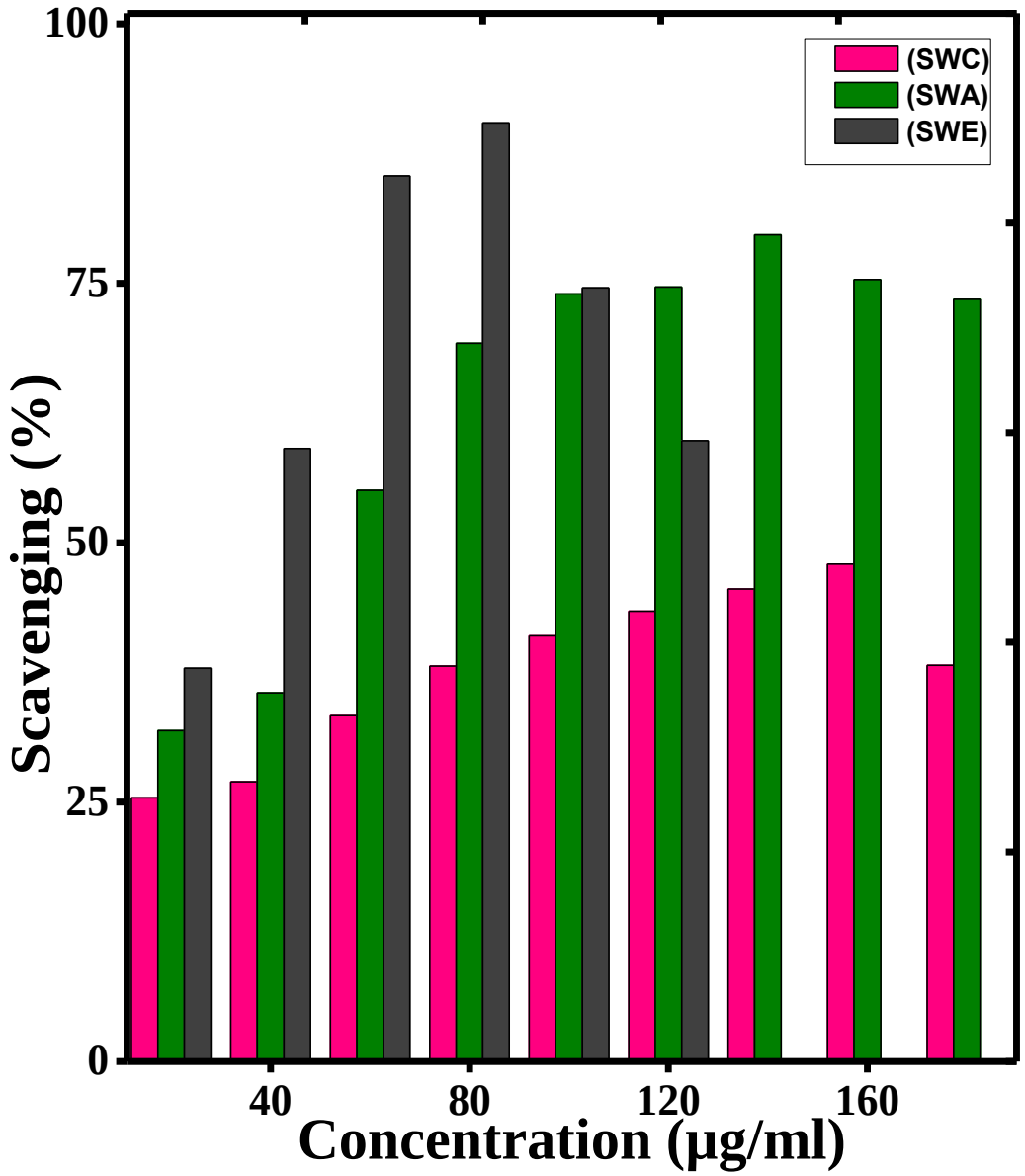

Figure 1: Different extract of *Schima wallichii* on DPPH radicals scavenging activity (20-180µg/ml).Values were expressed as Mean± SEM, n=5.

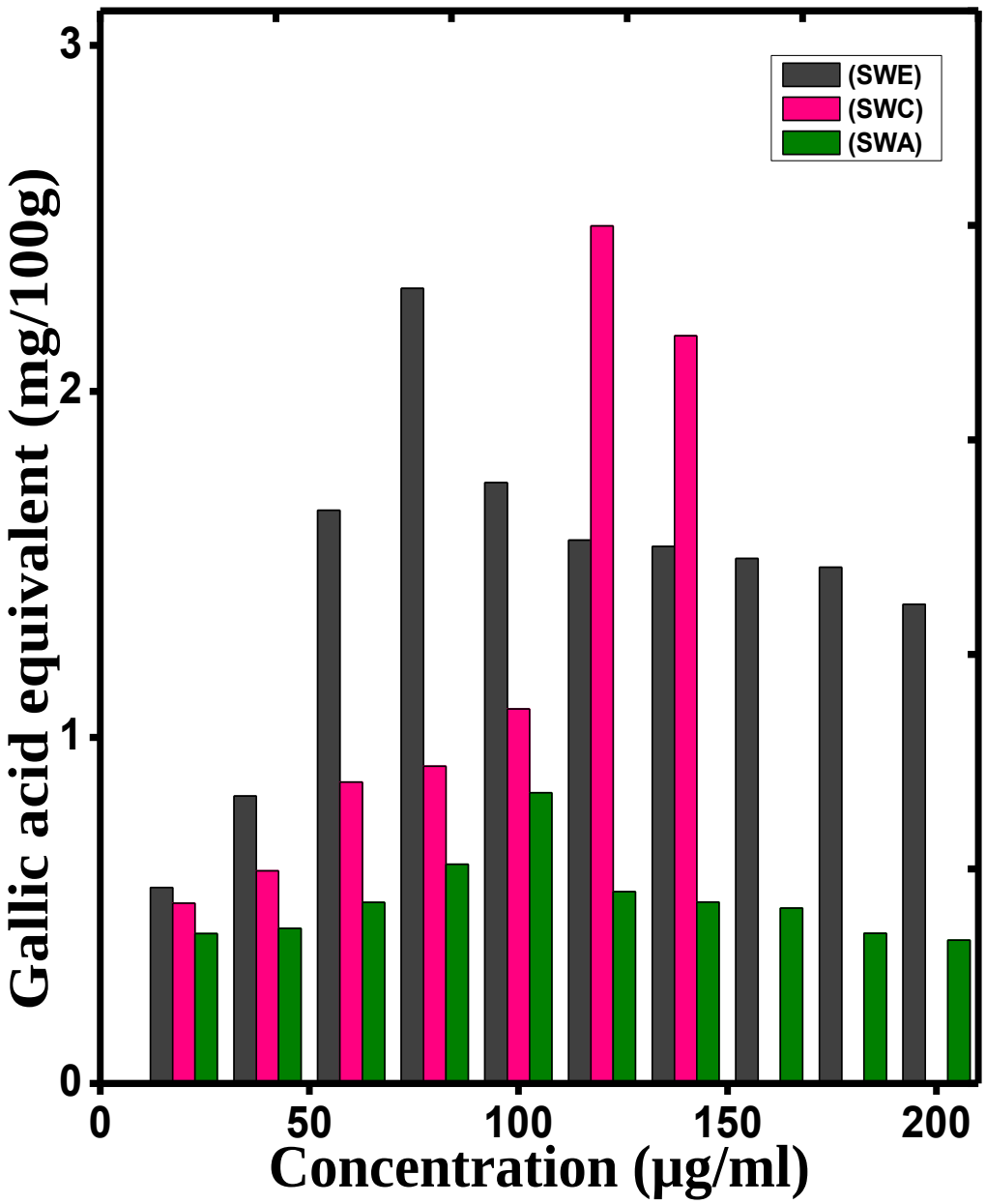

Figure 2: Hydroxyl radicals scavenging activity of different extracts of *Schima wallichii* expressed as gallic acid equivalent (20-200µg/ml). Values were expressed as Mean± SEM, n=5.

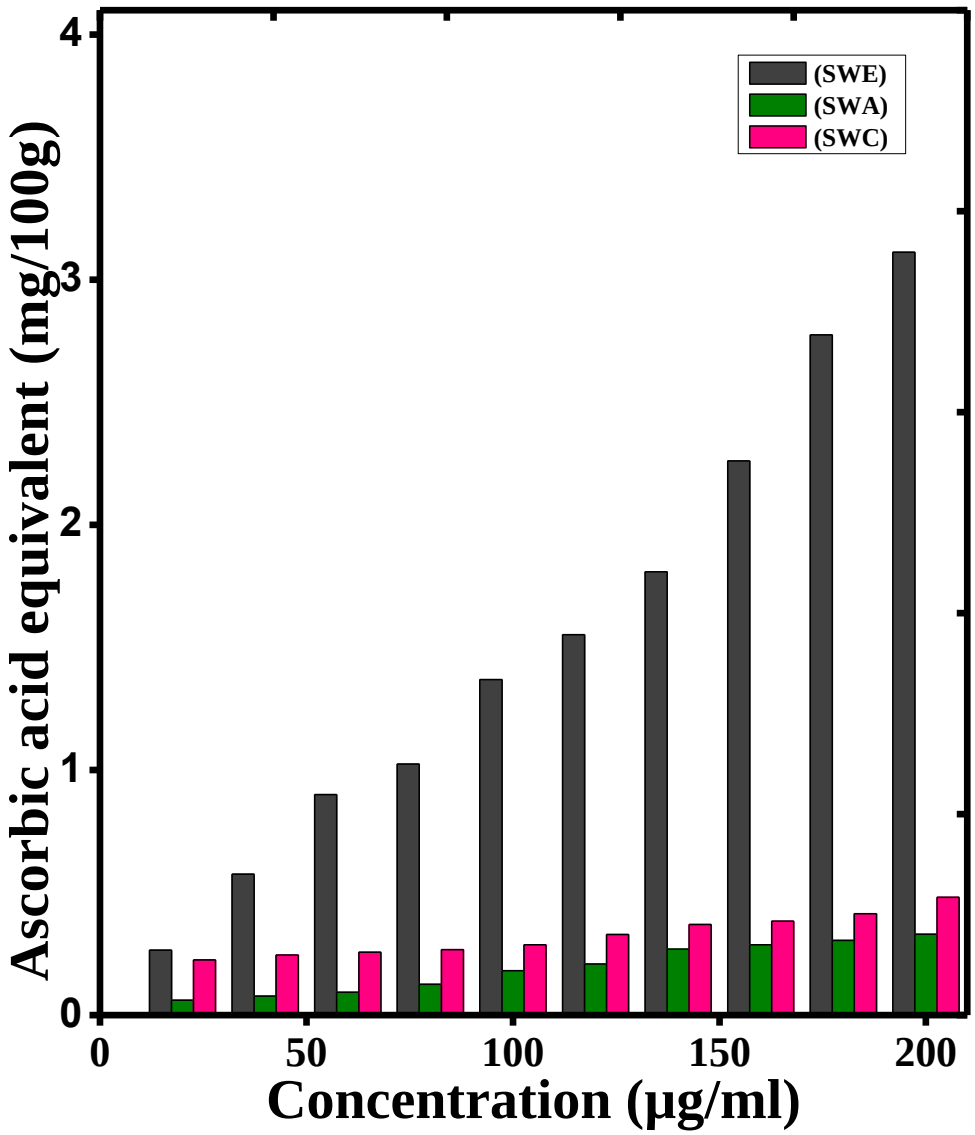

Figure 3: SO radicals scavenging activity of various extracts of *Schima wallichii* expressed as ascorbic acid equivalent (20-200µg/ml). Values were expressed as Mean± SEM, n=5.

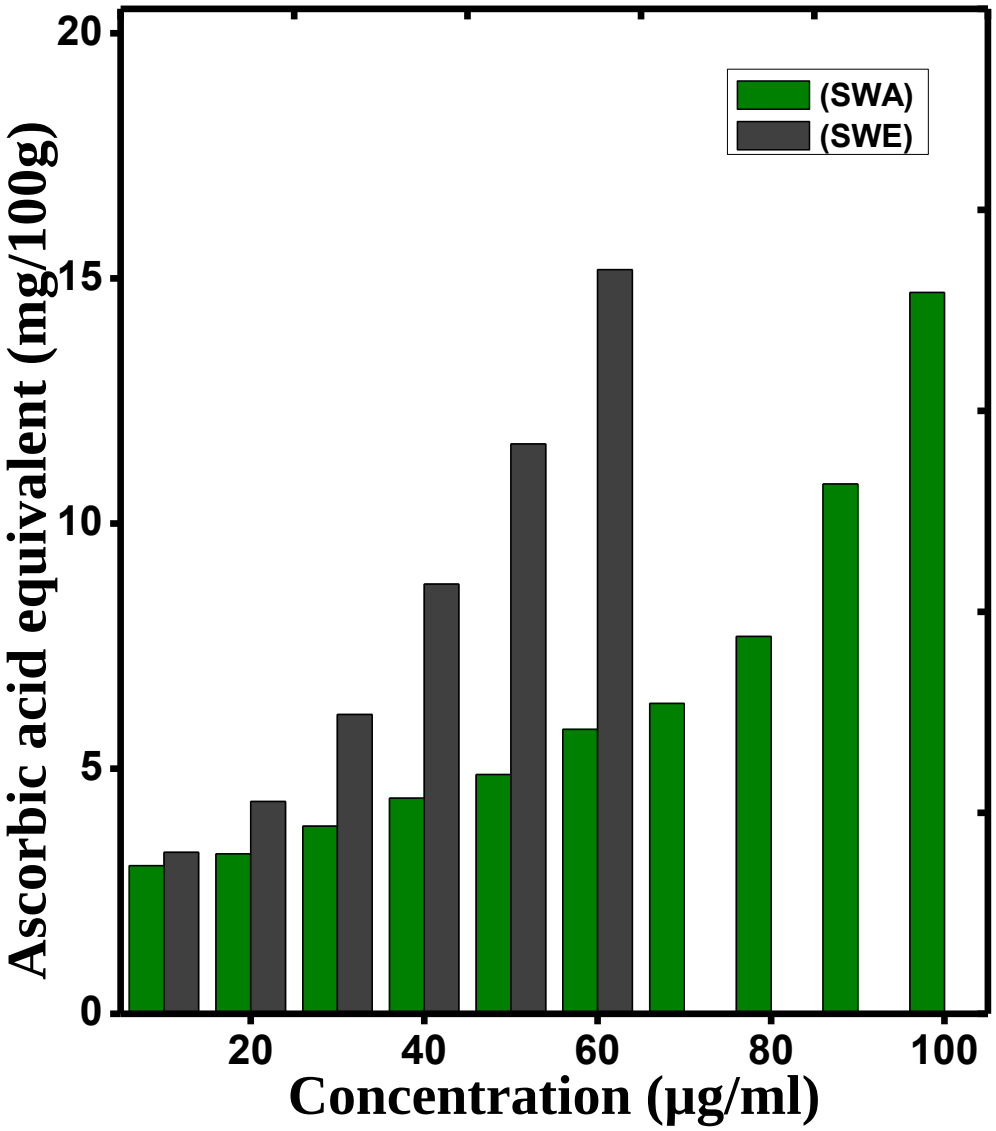

Fig.4(A): ABTS radicals scavenging activity of ethanol and aqueous extracts of *Schima wallichii* expressed as ascorbic acid equivalent (10-100µg/ml). Values were expressed as Mean± SEM, n=5.

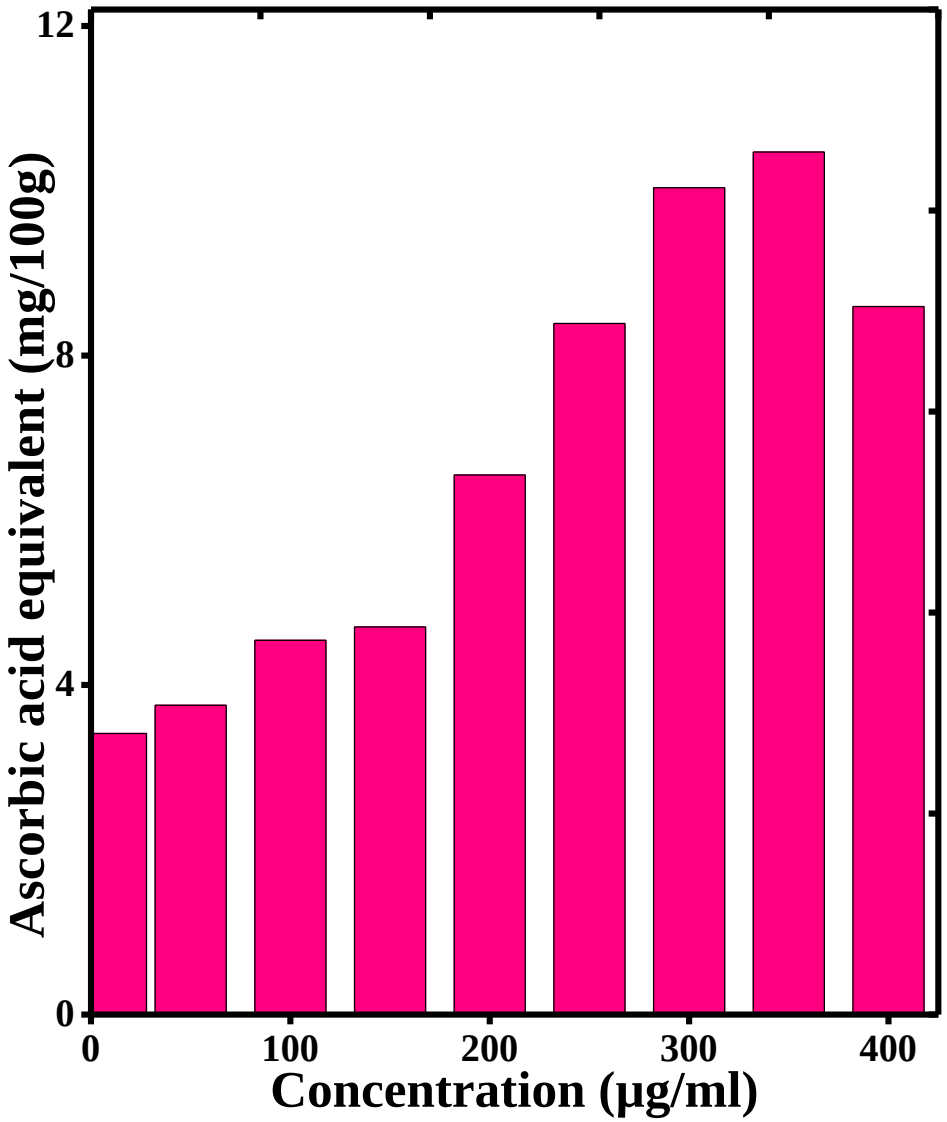

Figure 4(B): ABTS radicals scavenging activity of chloroform extracts of *Schima wallichii* expressed as ascorbic acid equivalent (10-400µg/ml). Values were expressed as Mean± SEM, n=5.

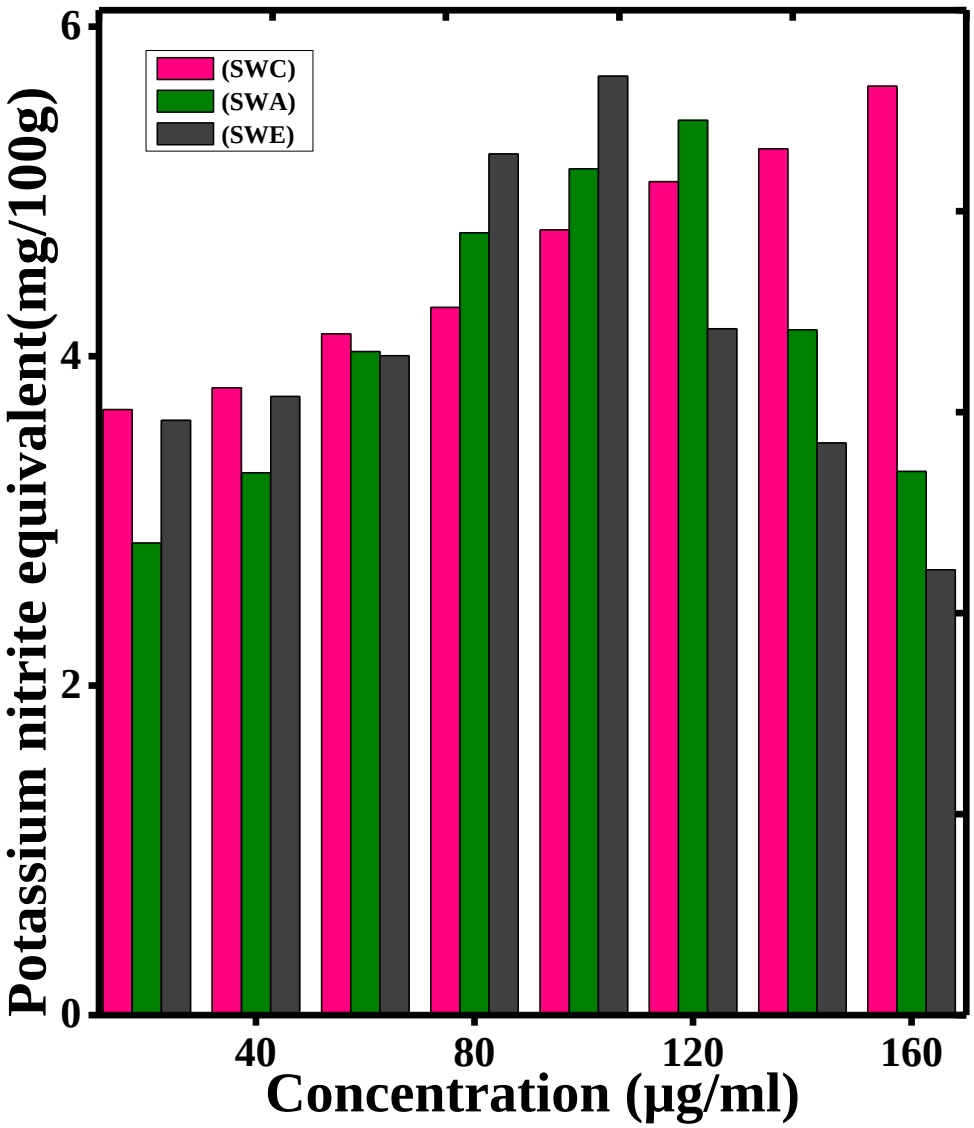

Figure 5: Nitric oxide radicals scavenging activity of various extracts of *Schima wallichii* expressed as Potassium nitrite equivalent (20-160µg/ml). Values were expressed as Mean± SEM, n=5.

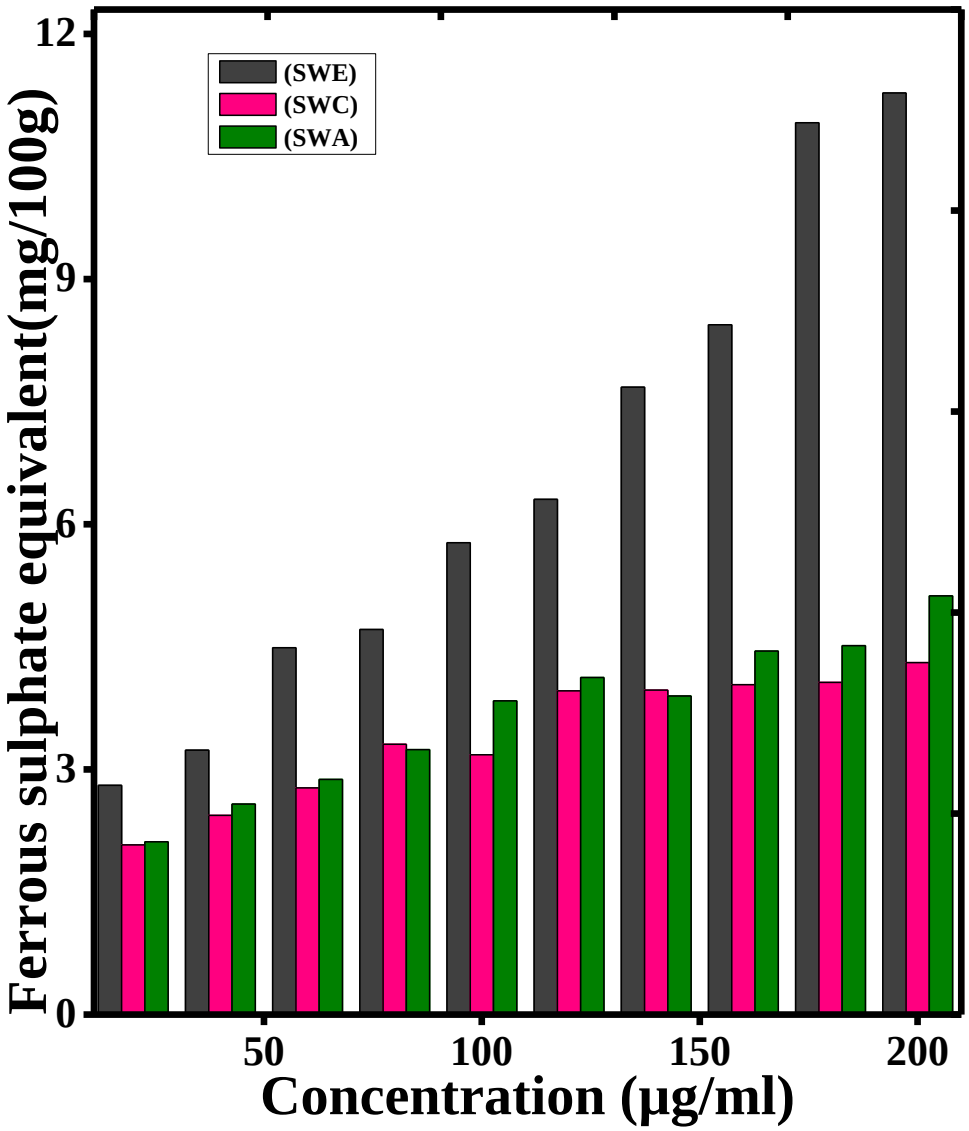

Figure 6: Inhibition of FRAP radical formation by various extracts of *Schima wallichii* expressed as Ferrous sulphate equivalent(20-200µg/ml) Values were expressed as Mean± SEM, n=5.

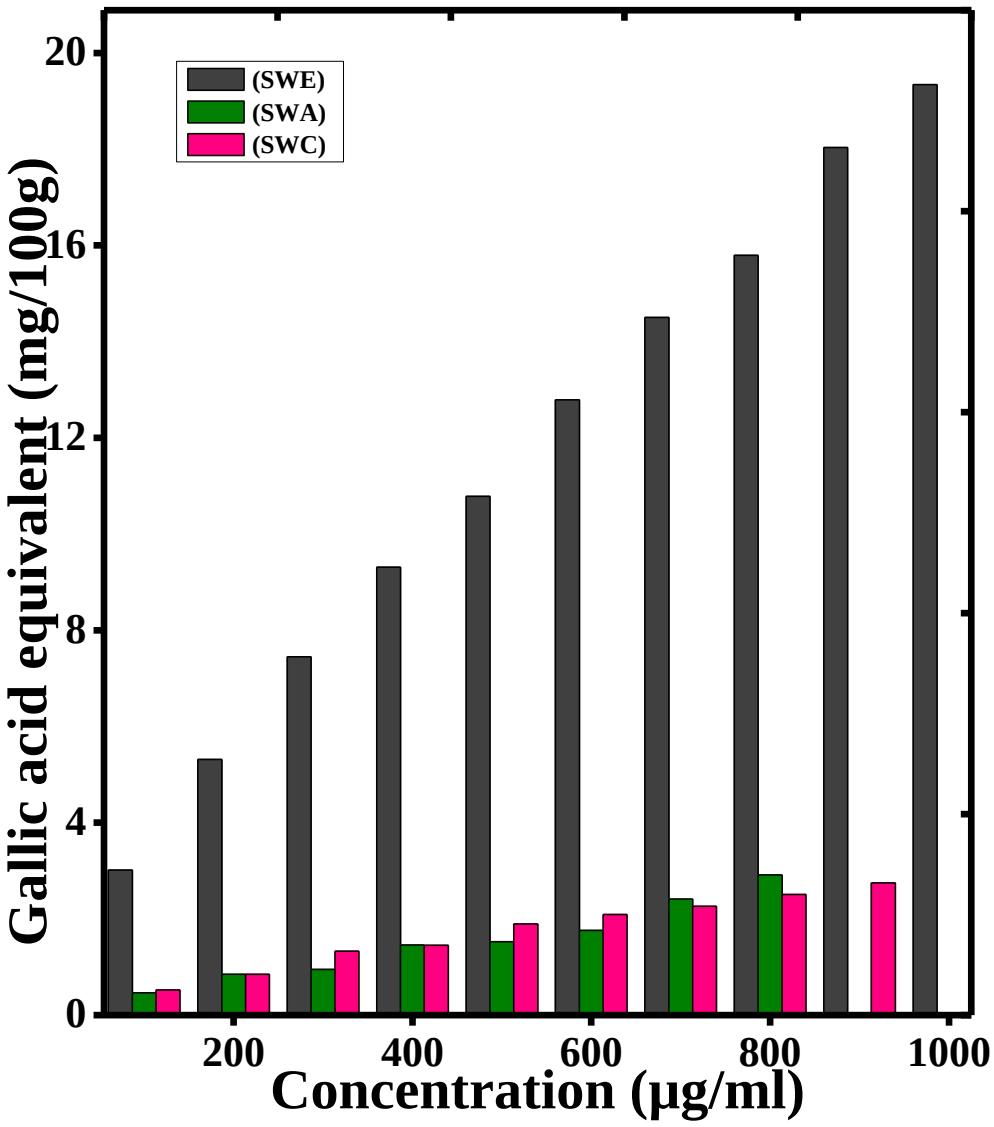

Figure 7: Total phenolic contents of the different extracts of *Schima wallichii* (100-1000µg/ml) determined as gallic acid equivalent. Values were expressed as Mean± SEM, n=5.

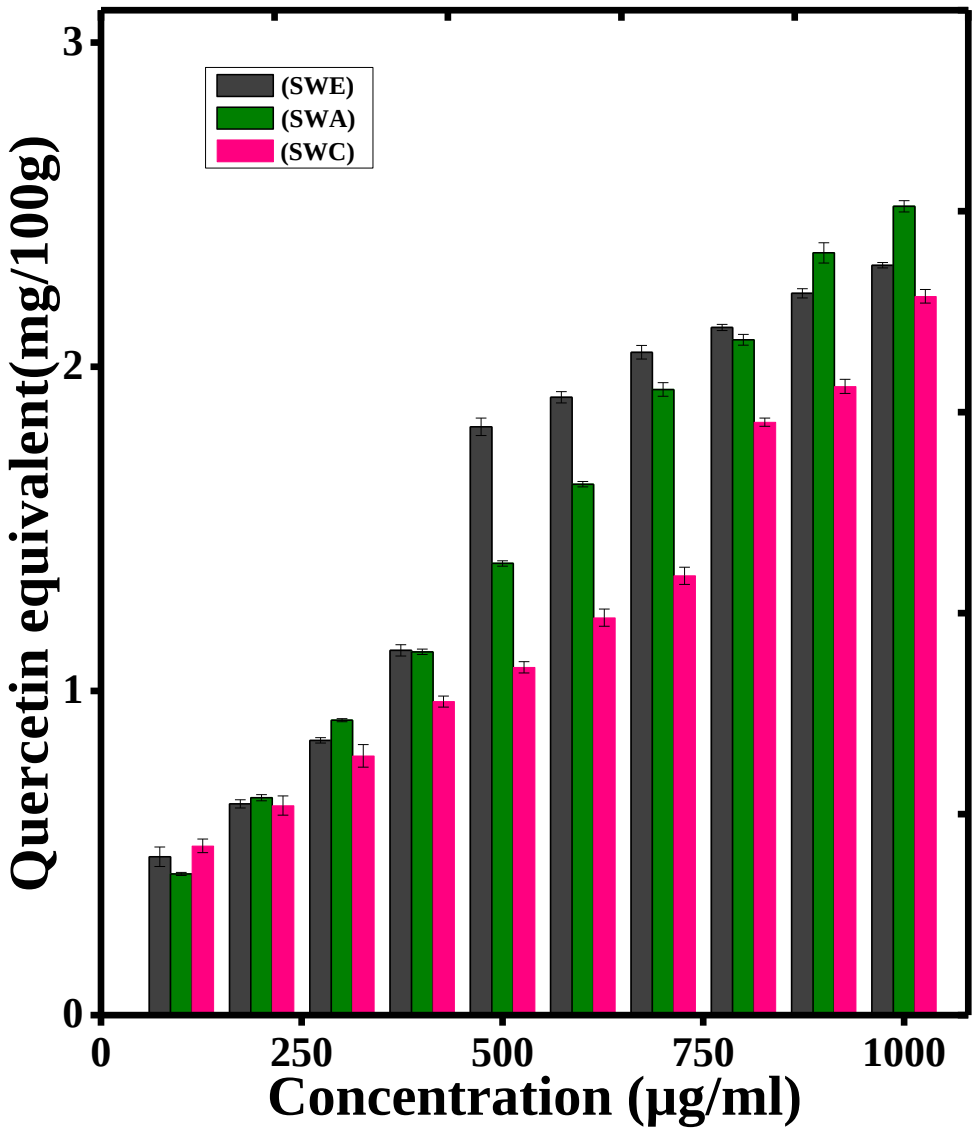

Fig.8. Total flavonoid contents of different extracts of *Schima wallichii* (100-1000µg/ml) determined as Quercetin equivalent. Values were expressed as Mean± SEM, n=5.

CHAPTER 4

Determination of the anticancer activity of *Schima wallichii* in cultured cell lines.

ABSTRACT

The cytotoxicity of the ethanol extract of *Schima wallichii* (SWE) was assessed in HeLa and V79 cells by MTT assay. The HeLa, and V79 cells treated with different concentrations of SWE showed a concentrations dependent increase in its cytotoxic effect. Treatment of cells with SWE for different durations also increased its cytotoxic effects in a time dependent manner. The results of MTT assay were confirmed by clonogenic assay in HeLa cells, where the cells were treated with different concentrations of SWE. Treatment of HeLa cells with various concentrations of SWE reduced the clonogenicity of cells in a concentration dependent manner. The ability of SWE to induce apoptosis was studied by determining the caspase 8 and 3 activities at different post- treatment times. The SWE treatment marginally increased activity of both capsase 8 and 3 in a time dependent manner. The effect of SWE treatment was studied on the lipid peroxidation, glutathione contents, glutathione-s-transferase, catalase and superoxide dismutase activites, where it was found to reduce the glutathione contents, and activites of glutathione-s-transferase, catalase and superoxide dismutase in a time dependent manner. Treatment of HeLa cells with ethanol extract of *Schima wallichii* increased the cytotoxic effect in a concentration dependent manner followed by a reduction in the the clonogenicity of HeLa cells. The cell killing effect of SWE may be due to the caspase activation and reduction in the glutathione contents, and activites of glutathione-s-transferase, catalase and superoxide dismutase.

1. INTRODUCTION

The natural products have formed a main source to cure different diseases including cancer since time immemorial (Atanasov *et al*., 2015; Balandrin *et al*., 1993; Newmann, 2014). The plants synthesize several secondary metabolites for different purposes and these are boon to human healthcare. These molecules are highly complex in structure and it is difficult to undertake their chemical synthesis. This has rekindled the interest of researchers and of pharmacological industries on the isolation of these secondary metabolites to develop them in to modern drug entities in recent years (Atanasov *et al*., 2015). The cancer has emerged has a major disease that has been putting higher financial burden on the healthcare of families and also of Governments who had to allocate higher financial resources to cancer centers. The fact remains that despite higher allocation and availability of most modern therapy for cancer patients the mortality rates remains higher in cancer patients (Siegel *et al*., 2017).

The chemotherapy has been the major treatment modality, especially in advanced stages of cancer. Several chemotherapeutic drugs isolated from plant/natural products are in the market (Balandrin *et al*., 1993; Newmann, 2014). The cytotoxic chemotherapeutic drugs are used alone or as an adjuvant therapy in patient to improve the survival (Morgan *et al*., 2004). Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer, and while the actual compounds isolated from the plant frequently may not serve as the drugs, they provide leads for the development of potential novel agents. The vinka alkaloids isolated from periwinkle plant *Catharanthus roseus* initially used to treat hematologic malignancies find their wide use to treat different types of solid neoplasia (Duflus *et al*., 2002; Moudi *et al*., 2013). Similarly,

epipodophyllotoxins are used to treat several malignant neoplasia (O'Dwyer *et al*., 1985). The taxols isolated from Pacific yew are also useful in the treatment of wide range of tumors (Rowinsky, 1997). The management of malignancies frequently requires the use of treatment modalities that are associated with significant toxic effects. The acceptability of specific therapy can be assessed by comparing its benefits with its potential cost in terms of toxicity (Miller, 1981). This indicates the need to continue the screening of natural products for treatment of cancer.

Schima wallichii(DC.) Korth.(Chilauni) belong to the tea family, Theaceae. It is an evergreen tree inhabiting warm temperate to subtropical climates. The tree is found across southern and South East Asia, and stretch from the eastern Himalaya of Nepal to eastern India across Indochina, Southern China, Taiwan and the Ryukyu Islands.

It is commonly known as needlewood tree and grows up to 35 m high. However, in some places it may be seen only 40 ft high (Min *et al*., 2003). Locally, it is called "khiang" in Mizo language. *Schima wallichii* is known to possess several medicinal properties. The leaves and the stem bark are normally used traditionally for its medicinal properties. The bark is used as an antiseptic for cuts and wounds. It acts as vermicide, mechanical irritant and as a cure against gonorrhea (Dewanjee *et al*., 2008). Decoction of bark reduces fever and is said to be effective against head lice (Gurung, 2002). The bark juice is given to disinfest the animal from liver flukes (Lalrinzuali 2015). The sap from the stem is used for curing ear infection (Sam *et al*., 2004). Fruit decoction is used by the people of Western Mizoram, India against snakebite (Lalfakzuala *et al*., 2007; Lalrinzuali, 2015). The young plants, leaves and roots are also used medicinally against fever and the bark is anthelmintic and rubefacient (Gardner *et al*., 2000). The leaves of

Schima wallichii are known to have antitumor and antimutagenic properties (Koshimizu *et al*., 1998; Subarnas *et al*., 2003). The astringent corollas are used to treat uterine disorders and hysteria (Paudel and Subba, 2014). The anticancer activity of *Schima wallichii* ethanol extract has not been studied in vitro therefore, the present study was undertaken to investigate its cytotoxic effect in vitro.

2. MATERIALS AND METHODS

2.1.Chemicals

Nitroblue tetrazolium (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), ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), sodium pyruvate, thiobarbituric acid (TBA) and crystal violet, were obtained from Sigma Chemical Co. (Bangalore, India). Sodium carbonate (Na_2CO_3), trichloroacetic acid (TCA), potassium chloride (KCl), hydrogen peroxide (H_2O_2), 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 were purchased from Merck India Limited, Mumbai, India. Trypsin EDTA 1X, MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide), minimum essential medium (MEM), 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 The Caspase kits were purchased from LabGills, Kolkata, India.

2.2.Preparation of the extract

The non-infected stem bark of *Schima wallichii* was collected from Bazar veng, Lunglei, Mizoram during the months of April and May. The authentication and identification of *Schima wallichii* was done by The Botanical Survey of India, Shillong The bark was cleaned and shade dried at room temperature in clean and hygienic conditions. The dried bark was powdered using an electrical grinder. The dried powder of *Schima wallichii* was extracted sequentially 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 them to dryness under reduced pressure. The concentrated extracts were stored at -80 until use. The ethanol extract was used for the study and it will be called as SWE henceforth.

2.3.Preparation of drug/s

The doxorubicin was freshly dissolved in distilled water, whereas the *Schima wallichii* ethanol extract was dissolved in 1% ethanol in water and diluted with MEM. The dissolved extract was filter sterilized before use.

2.4 Cell line and Culture

HeLa and V79 cells were procured from the National Centre for Cell Sciences, 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, and 1% L-glutamine 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 into several microplate wells and the cells were divided into the different groups depending on the experimental protocol:

MEM group: The cells of this group were used as negative control group.

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

DOX group: The cell were treated with 5, 10 or 20µg/ml of doxorubicin (DOX) and served as positive control.

MTT assay

The cytotoxic effects of 12.5, 25, 50, 100, 200, 300 and 400 µg/ml of SWE was studied by MTT assay in HeLa, and V79 cells as described by (Mosmann, 1983). Usually 10^4 cells were seeded into 96 well plates in 100 µl MEM. The cells were incubated at 37ºC 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. Thereafter, different concentrations of SWE or doxorubicin were added into each well of the microplate and incubated in the CO_2 incubator. After 48 hours of drug/s treatment, 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 lysis buffer and incubated once again for 4 hours after which the absorbance was measured at 560 nm using a micorplate reader (Biorad, USA). The cytotoxicity was calculated by the formula: Control-Treatment/Control X 100.

An another experiment was setup to study the effect of treatment duration where all the conditions were similar to that described above except that the cells were treated with 50, 100 or 200 µg/ml of SWE for 2, 4 and 6 h and processed for MTT assay as described above.

2.5.1 The Determination of clonogenic potential

The anticancer activity of SWE 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 served as negative control group.

SWE group: This group of cells was treated with different concentrations of 50, 100 or 200µg/ml SWE for 6h.

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 medium from each flask were decanted and the flasks were washed twice with sterile PBS, subjected to trypsin EDTA treatment and processed for clonogenic assay (Puck and Marcus, 1955).

Usually 200 HeLa cells were seeded into several individual petridishes containing 5 ml MEM, left undisturbed and allowed to grow for another 11 days. The resultant colonies of cells were stained with 1 % crystal violet in methanol and scored. The clone containing a minimum number of 50 cells was considered as a colony. The plating efficiency (PE) of the cells was determined and surviving fraction (SF) calculated according to the following formulae:-

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

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

2.5.4 Apoptosis assay

For the study of apoptosis, the cells were terminated at 6, 12 and 24 hours post drug treatment. The activity of caspase 8 and 3 was determined according to the manufacturer's protocol after treatment of HeLa cells with 100 µg/ml ethanol extract of *Schima wallichii.*

2.6 Biochemical assays

A separate experiment was performed to estimate the effect of SWE on the activities of various antioxidant enzymes in HeLa cells, where grouping and other conditions were essentially

similar to that described for anticancer activity except that the cell cultures were terminated at 2, 6 and 12 hours 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 ultra sonicator (PCI Analytics Pvt. Ltd., Mumbai, India).

2.6.1 Glutathione estimation

Glutathione was estimated as described earlier (Moron *et al*., 1979). The concentration of glutathione was measured by its reaction with DTNB (Ellman's reagent) to give a compound that absorbs light at 412 nm. Briefly, 1.8 ml of 0.2M Na_2HPO_4 was mixed with 40 µl 10 mM DTNB and 160 µl 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, 1974). Briefly, 0.5 ml of 0.1 M phosphate buffer pH 6.5, 0.1ml of 20mM CDNB, and 8.8 ml distilled water were mixed and 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 recorded at 340 nm at 1 min intervals for 6 minutes using 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 activity was estimated as described by (Fried, 1975). Briefly, 100 µl of cell homogenate, 100 µl of 186 µM phenazenemethosulfate, 300 µl of 3.0 mMnitrobluetetrazolium, 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 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.

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.

3. RESULTS

The results are expressed in tables 1-8 and figures 1-11 as mean±standard error of the mean.

3.1 Determination of Cytotoxicity

Treatment of HeLa, and V79 cells with different concentrations of SWE caused a concentration dependent increase in the cytotoxicity and the maximum cytotoxic effect was observed at the highest concentrations of SWE used. Treatment of HeLa cells with different concentrations of SWE induced highest toxic effect at a concentration of 400µg/ml which was 62.21% (Table 1). Similarly, SWE induced maximum cytotoxicity of 73.62% at 400 µg/ml in

V79 cells. However, 50% cytotoxicity was found at 100 µg/ml for the extract for both of the cells used. The standard drug DOX was used as the positive control (Table 1, Figure 1 and 2).

3.2 Determination of optimum exposure time for cytotoxicity

The optimum exposure time for cytotoxic effect of SWE was also evaluated by MTT assay. The SWE treatment resulted in a time dependent increase in the cytotoxicity in HeLa, and V79 cells and maximum cytotoxic effect was observed in the cells treated with SWE for 6 h (Table 2). The HeLa and V79 cells showed 50% cytotoxicity at the maximum exposure time which was selected for further experimentation.(Figure 3 and 4).

3.3 Clonogenic Assay

Treatment of HeLa cells with different concentrations of SWE caused a concentration dependent decline in the clonogenicity of cells. A maximum decline in the clonogenicity was observed for 200 µg/ml SWE, where extreme reduction in the cell survival (0.15) was observed. (Table 3). The reduction in clonogenic potential by SWE was comparable to positive control DOX except the fact that the doses required by SWE were ten times greater than the DOX (Figure 5).

3.4 Apoptosis

3.4.1 Caspase 8

Treatment of HeLa cells with different concentrations of SWE caused a statistically significant and concentration dependent increase in the activity of Caspase 8 at different post treatment time. The maximum activity was observed at 48h post drug treatment. (Table 4, Figure 6).

3.4.2 Caspase 3

Treatment of Hela cells with different concentrations of SWE caused a statistically significant and concentration dependent increase in the activity of Caspase 3 at different post treatment time. The maximum activity was observed at 48h post drug treatment. (Table 5, Figure 7).

3.5 Glutathione

Treatment of HeLa cells with different concentrations of SWE caused a statistically significant and concentration dependent reduction in glutathione contents at all the post-treatment times (Figure 8). The concentration of glutathione also declined in a similar fashion in DOX treated group (Table 6). The glutathione concentration also showed a time dependent reduction and maximum decline was observed for 200 µg/ml SWE at 12 h post treatment.

3.6 Glutathione-s-transferase

The GST activity declined in a concentration dependent manner in HeLa cell exposed to 50-200 µg/ml SWE (Figure 9). The GST activity also reduced with assay time and a nadir was observed at 12 h post-drug treatment (Table 7). The decline in GST activity in SWE group was comparable to DOX treatment.

3.7 Catalase

The assay of catalase activity showed a concentration dependent alleviation in HeLa cells with increasing concentration of SWE. The catalase activity also declined with post-treatment assay time and the lowest catalase activity was recorded at 12 h post SWE treatment (Table 8). There was an abrupt decline in the catalaseactivity at 6 h post treatment when compared to 2 h

after SWE treatment. The DOX treatment also resulted in an identical decline in catalase activity at all post treatment times (Figure 10).

3.8 Superoxide dismutase

The exposure of HeLa cells to different concentrations of SWE caused a concentration dependent but gradual reduction in the SOD activity (Figure 11). The SOD activity also showed a time dependent decrease with a maximum reduction in the SOD activity at 12 h post – treatment (Table 8). The positive control DOX also showed a pattern similar to that of SWE treatment

DISCUSSION

Several studies have been focused on natural anticarcinogenic agents. Many natural products have been identified to treat malignant neoplasia (Balandrin *et al*., 1993; Newmann, 2014). The importance of natural products in medicine cannot be underestimated. The Federal Drug Administration, USA has approved approximately 547 products from natural resources or their derivatives for clinical use (Patridge *et al*., 2016). This indicate that plants still forms the major source for drug development and the screening of plants provides a major avenue for new drug discovery. Therefore the present study was designed to evaluate the antineoplastic action of *Schima wallichii* in vitro.

The MTT assay is a rapid and standard technique to determine the cytotoxicity of any drug and the treatment various cultured 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. The treatment of HeLa and V79 cells with different concentrations of SWE caused a concentration dependent rise in the cytotoxicity of ethanol extract of *Schima wallichii*. MTT assay has been used to test cytotoxicity in various

cell lines earlier (Mosmann, 1983). The cytotoxicity of ethanol extract of *Consolida orientalis* L, *Ferula assa-foetida* L, *Coronilla varia* L,*Orobanche orientalis* G. Beck on HeLa cells were also found to show concentration dependent manner (Nemati *et al*., 2013). The other plants like *Alstonia scholaris, Consolida orientalis, P. pellucidum, Tinospora cordifolia*, *Ferula assa-foetida* and *Coronilla varia* extract have been shown to exert cytotoxicity in cultured HeLa cells earlier (Jagetia *et al*., 1994; Jagetia and Baliga, 2005; Jagetia and Rao, 2006; Widowati *et al*., 2013; Jagetia and Venkaatesha, 2016).

The clonogenic assay is the most confirmatory test, which indicates the reproductive integrity and the extent of cell survival and it is also a long-term assay which takes cares of the delayed effects induced by drug treatments (Franken *et al*., 2006; Bunel *et al*., 2014). The cytotoxic effect of SWE was further confirmed by clonogenic assay where SWE treatment caused a concentration dependent decline in the clonogenicity of HeLa cells. There are no reports where SWE has been used to evaluate the reproductive potential of any cell line earlier. However, other plants including *Alstonia scholaris, Aphanamixis polystachya* and *Tinospora cordifolia* have been reported to reduced the Clonogenic potentials of HeLa cells earlier (Jagetia *et al*., 1994; Jagetia and Rao, 2006; Jagetia and Venkatesha, 2016).

The cancer cells show elevated oxidative stress however, excess oxidative stress also kills cancer cells by eliciting various mechanisms of cell death and agents than can induce greater amount of oxidative stress may be useful in killing the cancer cells (Ryter *et al*., 2007; Chen *et al*., 2008; Panieri and Santoro, 2016). Therefore, effect of SWE on oxidative stress was also studied in HeLa cells.

Glutathione (γ -glutamylcysteinyl glycine) is the most abundant non-protein thiolin in the cell. It plays various physiological roles including counter balancing the excess free radicals

produced in the cells during numerous physiological processes (Lushchak, 2012; Schumacher, 2015). Glutathione occurs in two forms namely the reduced form (GSH) and the oxidized form (GSSG). 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, its higher amount is indicated in tumor microenvironment-related aggression, apoptosis evasion, colonizing ability, and multidrug and radiation resistance (Obrador *et al*., 2002; Franco *et al*., 2009; Ortega *et al*., 2010). Increased concentration of GSH in the tumor cells have been known 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, glutathione attrition in cancer cells may enhance the cytotoxic effects of chemotherapeutic agents (Mayer *et al*., 2007). A similar connection is true in the present study, where SWE reduced the glutathione concentration in a concentration and time dependent manner. Earlier, *Alstonia scholaris* has been reported to reduce the glutathione contents in tumor cells (Jaetia and Baliga, 2016). The glutathione-S-transferasesare a multi-gene family of enzymes, which carry out detoxification and activation of certain chemicals (Eaton and Bammler, 1999). The GSTs are overexpressed in a wide variety of tumors and their negative modulation has emerged as a promising therapeutic target as they have been implicated in the resistance to cancer therapy. The augmented activityof GST in tumor cells is associated with suppression of tumor cell kill by apoptosis (McIlwain *et al*., 2006; Zeng *et al*., 2014). The SWE treatment has decreased the GST activity significantly and that may be one of the reasons of increased cell killing effect in the present study. Several GST inhibitors have been shown to

reduce drug resistance by sensitizing tumor cells to anticancer drugs and bring effective cell killing (Townsend and Tew, 2003; Laborde, 2010).

The catalase is involved in the detoxification of H_2O_2 into water and molecular oxygen. However, it also plays a crucial role in various other processes. High levels of catalase have been found in patients with lung cancer, whereas the patients suffering from breast cancer, head and neck cancer, gynaecological cancer, lymphoma, prostate cancer and urological cancer showed decreased levels of catalase (Kodydková *et al*., 2014). The higher activity of catalase has been linked to suppression of apoptosis in tumor cells undergoing chemotherapy (Bechtel, 2009). The untreated HeLa cells have shown higher catalase activity, whereas SWE treatment reduced the activity of catalase indicating that SWE action may be mediated by reducing catalase activity in HeLa cells. Superoxide Dismutase (SOD) present in all oxygen metabolizing cells and it catalyzes the dismutation of the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Weydert and Cullen, 2010). Four different forms of SOD are known until now (Fridovich *et al*., 1974) of which the Cu-Zn SOD is the most abundant and comprises approximately 90% of total SOD activity in a eukaryotic cell (Rolke *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, 1979; Scandalios, 1993). Malignant cells have been reported to show elevated Cu-Zn SOD activity (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 (Che *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 refractory to therapy. The SWE treatment reduced SOD activity in HeLa cells, which may account for its higher cell killing activity.

The exact mechanism of action of SWE is not known. It may have utilized different pathways to kill the cells. The present observations indicate that SWE treatment reduced the levels of GSH, GST, catalase and SOD, which may have increased the oxidative stress and stimulated the mechanisms that may have brought apoptotic and non-apoptotic form of cell death. The analysis of caspase 8 and 3 in HeLa cells indicate that part of the cell death seems to mediated by apoptosis and increased oxidative stress indicate SWE also induced non-apoptotic form of cell death. The increased oxidative stress by SWE may have triggered events that may have damged the cellular genome thereby bringing cell death in the present study. It is plausible that SWE may have increased the activation of P53 and related proteins to stimulate apoptotic form of cell death. The SWE may have inhibited the transcriptionof NF-κB, COX-II and Nrf2 elements which are responsible for tumor cell proliferation and therapy resistance (Sobolewski *et al*, 2010; Lu and Stark, 2015; Park *et al*., 2011). In fact the down modulation of these proteins have been reported to enhance cell killing (Xu *et al*., 2014; Pozdeyev *et al*., 2015; Menegon *et al*., 2016). The SWE may have also used some other unknown mechanisms to kill the HeLa cells in the present study.

CONCLUSIONS

It is concluded that SWE administration caused effective killing of HeLa cells and the anticancer activity of SWE may due to reduction in the GSH and other antioxidant enzymes including GST, CAT and SOD. SWE may have also retarded the transcriptional activation of Nrf2, NF-κB and COX-II which may have contributed in killing the HeLa cells. The stimulation of caspase 8 and 3 in the present study indicates that SWE induced apoptotic mode of cell death in some of the cells if not all. Our results demonstrates that SWE act as an anticancer agent.

However, further studies are required to ascertain its potential as an anticancer agent in different tumor models and understanding its mechanism of action.

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Table 1 :The cytotoxic effect of different concentrations of ethanol extract of *Schima wallichii* in HeLa and V79 cell lines by MTT assay. p<0.05

Cell line	Treatment(µg/ml)	Dose	Cytotoxicity (%)±SEM
HeLa	DOX	5	60.92 ± 0.14
		10	70.44 ±0.16
		20	83.38 ±0.11
	SWE	12.5	17.76 ±0.38
		25	25.00 ± 0.12
		50	34.31 ± 0.12
		100	51.52 ± 0.17
		200	57.07 ±0.12
		300	59.44 ± 0.08
		400	62.21 ± 0.15
V79	DOX	5	68.67 ± 0.11
		10	78.26 ± 0.19
		20	81.42 ± 0.17
	SWE	12.5	20.41 ± 0.18
		25	34.88 ± 0.15
		50	44.30 ± 0.08
		100	54.27 ± 0.17
		200	62.13 ± 0.18
		300	67.20 ± 0.16
		400	73.62 ± 0.16

Table 2: Effect of treatment duration on the cytotoxic effects of ethanol extract of *Schima wallichii* (SWE) and DOX in HeLa and V79 cell lines by MTT assay.

Cell line	Treatment	Dose	Cytotoxicity (%)±SEM		
			Post-treatment time (h)		
			2	4	6
Hela	DOX	5	14.92 ± 0.18	20.45 ± 0.16	31.68 ± 0.16
		10	20.27 ± 0.17	36.67 ± 0.11	52.64 ± 0.13
		20	33.84 ± 0.15	49.83 ± 0.15	57.95 ± 0.13
	SWE	50	12.02 ± 0.1852	20.11 ± 0.33	30.00 ± 0.14
		100	18.57 ± 0.14	33.47 ± 0.22	53.74 ± 0.15
		200	26.74 ± 0.15	45.22 ± 0.34	56.73 ± 0.15
V79	DOX	5	19.82 ± 0.29	22.89 ± 0.12	26.03 ± 0.15
		10	27.38 ± 0.28	31.82 ± 0.18	38.93 ± 0.14
		20	39.71 ± 0.22	45.76 ± 0.20	52.06 ± 0.12
	SWE	50	17.00 ± 0.22	19.35 ± 0.14	22.73 ± 0.15
		100	24.76 ± 0.20	30.11 ± 0.16	43.56 ± 0.11
		200	37.36 ± 0.27	40.76 ± 0.14	51.87 ± 0.09

N=8, $p<0.05$.

Table 3: Effect of different concentrations of the ethanol extract of *Schima wallichii* or doxorubucin (DOX) treatment on the clonogenicity of HeLa cells.

Treatment	Dose (µg/ml)	Surviving Fraction± SEM
Control(MEM)	**0**	**1.125 ±0.095**
DOX	**5**	**0.329 ± 0.020***
	10	**0.072 ± 0.009***
	20	**0.035 ± 0.006***
SWE	**50**	**1.082 ±0.024***
	100	**0.393± 0.0223***
	200	**0.150 ± 0.014***

N=3.*p<*0.05 when treatment groups are compared to control group (MEM).*

Table 4: Effect of 100 µg/ml ethanol extract on the Caspase activity in HeLa cells at different post treatment times.

	Post treatment time (h)	Treatment (µg/ml)		
		Control	SWE	DOX
Caspase 8	12	0.807 ±0.016	0.824 ±0.018	1.004 ±0.012*
	24	0.815 ±0.002	0.831 ±0.036*	1.035 ±0.114*
	48	0.838 ±0.004	0.856 ±0.011*	0.939 ±0.011*
Caspase 3	12	0.217 ±0.001	0.230 ±0.008*	0.246 ±0.006*
	24	0.236 ±0.005	0.252 ±0.008*	0.260 ±0.004*
	48	0.244 ±0.007	0.259 ±0.006*	0.278 ±0.016*

N=3, p<*0.05*

Table 5: Effect of 100 µg/ml ethanol extract of *Schima wallichii* on the glutathione contents in HeLa cells at different post-treatment assay times.

Post treatment time (h)	µmol/mg protein±SEM						
	Control (MEM)	Treatment (µg/ml)					
		Schima wallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	2.86 ±0.005	2.22 ±0.002*	2.01 ±0.001*	1.77 ±0.006*	1.65 ±0.002*	1.28 ±0.002*	0.88 ±0.008*
6	2.38 ±0.029	2.08 ±0.003*	1.62 ±0.008*	1.36 ±0.004*	1.25 ±0.002*	0.96 ±0.003*	0.68±0.003*
12	2.18± 0.002	1.85± 0.003*	1.31± 0.007*	1.18 ±0.002*	0.87 ±0.005*	0.655 ±0.001*	0.55 ±0.003*

N=5. p<0.05

Table 6: Alterations in the glutathione-s-transferase activity of HeLa cells treated with different concentrations of *Schima wallichii* or doxorubicin.

	nmol/mgprtoein±SEM						
Post treatment time (h)	Control (MEM)	Treatment (µg/ml)					
		Schima wallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	0.214 ±0.001	0.148 ±0.005*	0.118 ±0.004*	0.08 ±0.004*	0.09 ±0.006*	0.072 ±0.006*	0.034 ±0.004*
6	0.189 ±0.002	0.122 ±0.004*	0.092 ±0.005*	0.066 ±0.0058*	0.072 ±0.004*	0.046 ±0.002*	0.030 ±0.003*
12	0.169 ±0.003	0.088 ±0.002*	0.068 ±0.004*	0.046 ±0.002*	0.05 ±0.003*	0.032 ±0.004*	0.016 ±0.004*

N=5, $p<0.05$

Table 7: Alterations in the Catalase activity of HeLa cells treated with different concentrations of *Schima wallichii* ethanol extract (SWE) or doxorubicin (DOX).

Post treatment time (h)	Unit/ mg protein±SEM						
	Control (MEM)	Treatment (µg/ml)					
		Schimawallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	8.36 ±0.03	7.19 ±0.02*	5.28 ±0.01*	2.87 ±0.005*	5.91 ±0.03*	3.85 ±0.02*	3.09 ±0.009*
6	8.71 ±0.03	4.25 ±0.02*	3.47 ±0.81*	1.19 ±0.003*	4.25 ±0.02*	2.19 ±0.02*	1.73 ±0.003*
12	7.46 ±0.02	2.55 ±0.02*	1.20 ±0.003*	0.77 ±0.006*	2.56 ±0.03*	1.15 ±0.02*	0.095 ±0.01*

N=5, $p<0.05$

Table 8: Alterations in the SOD of HeLa cells treated with different concentrations of *Schima wallichii* ethanol extract (SWE) or doxorubicin (DOX).

	Unit/ mg protein±SEM.						
Post treatment time (h)	Control (MEM)	Treatment (µg/ml)					
		Schima wallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	3.27 ±0.02	2.34 ±0.03*	2.16 ±0.02*	1.85 ±0.03*	2.18 ±0.02*	1.92 ±0.02*	1.79 ±0.03*
6	2.90 ±0.03	2.15 ±0.03*	1.64 ±0.03*	1.26 ±0.03*	1.55 ±0.02*	1.41 ±0.03*	1.25 ±0.03*
12	2.46 ±0.03	1.75 ±0.02*	1.36 ±0.02*	1.09 ±0.02*	1.17 ±0.03*	1.09 ±0.02*	0.9 ±0.03*

N=5. p<0.05

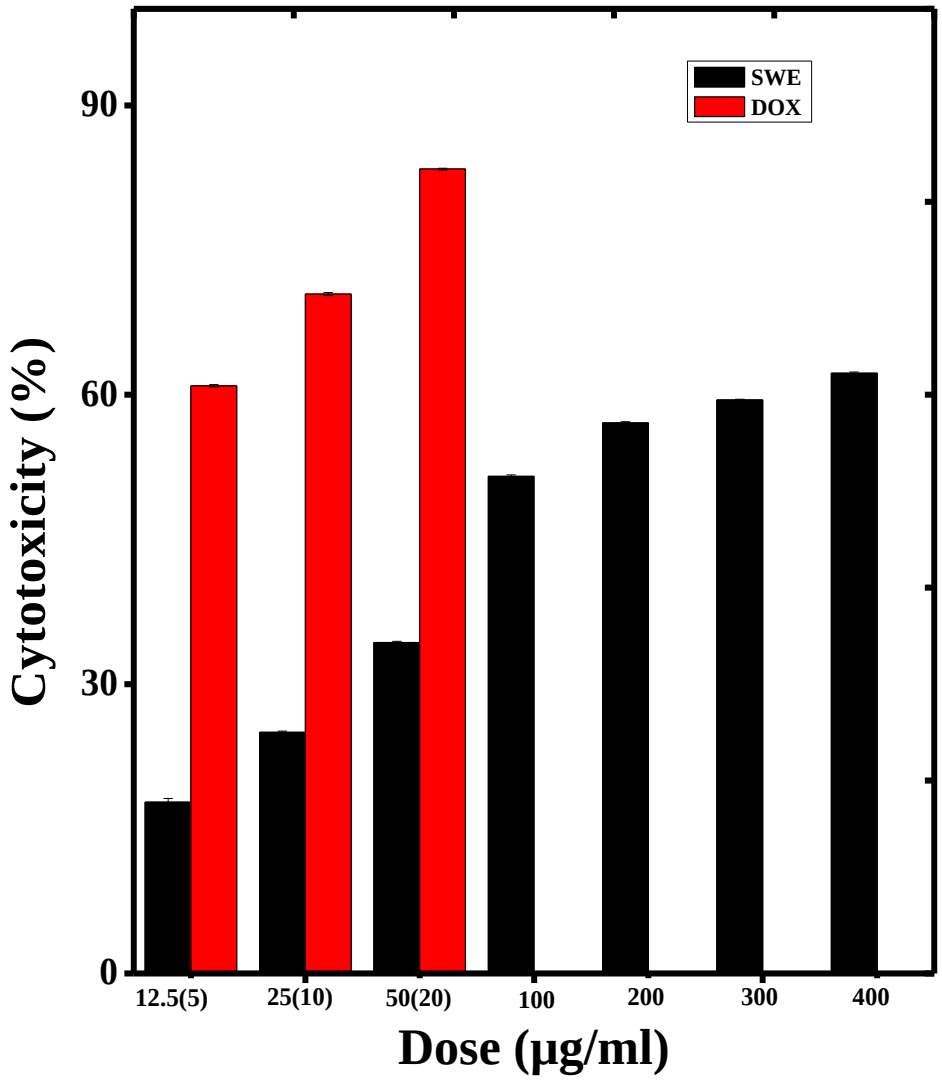

Figure 1: Cytotoxic effects of ethanol extract of *Schima wallichii* on HeLa cells evaluated by MTT assay. N=8;p<0.05

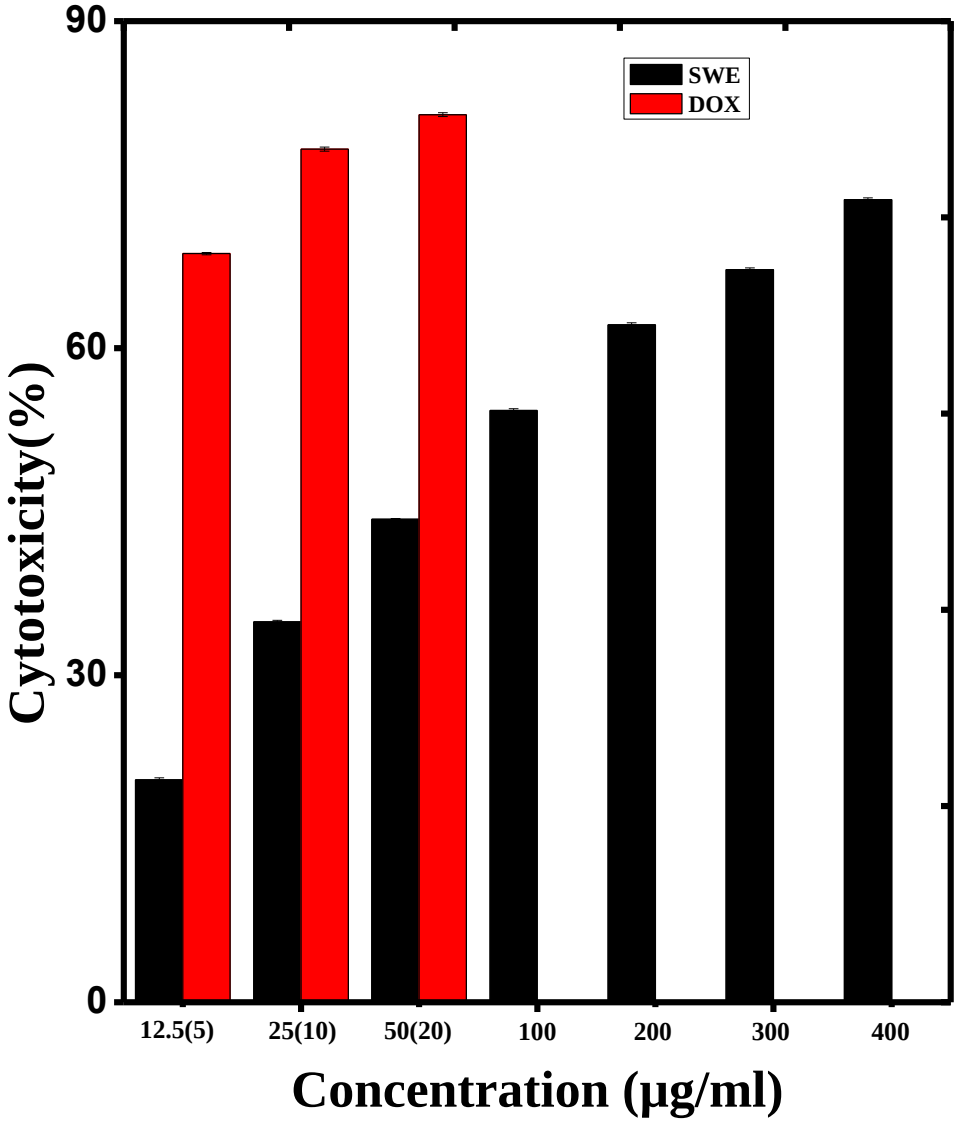

Figure 2: Cytotoxic effects of ethanol extract of *Schima wallichii* on V79 cells evaluated by MTT assay. N=8; p<0.05

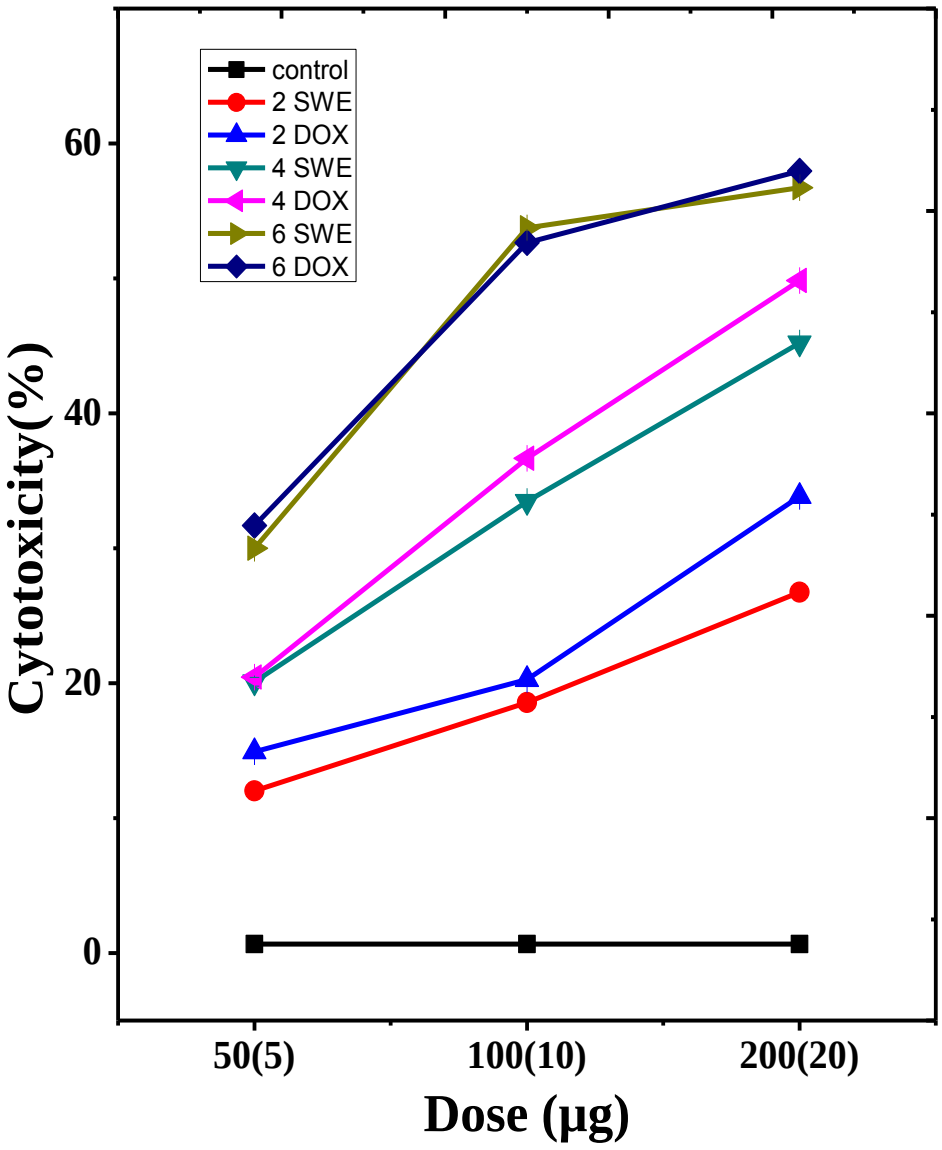

Figure 3: Cytotoxic effects of ethanol extract of *Schima wallichii* or DOX at different exposure time on HeLa cells by MTT assay. N=8; p<0.05.

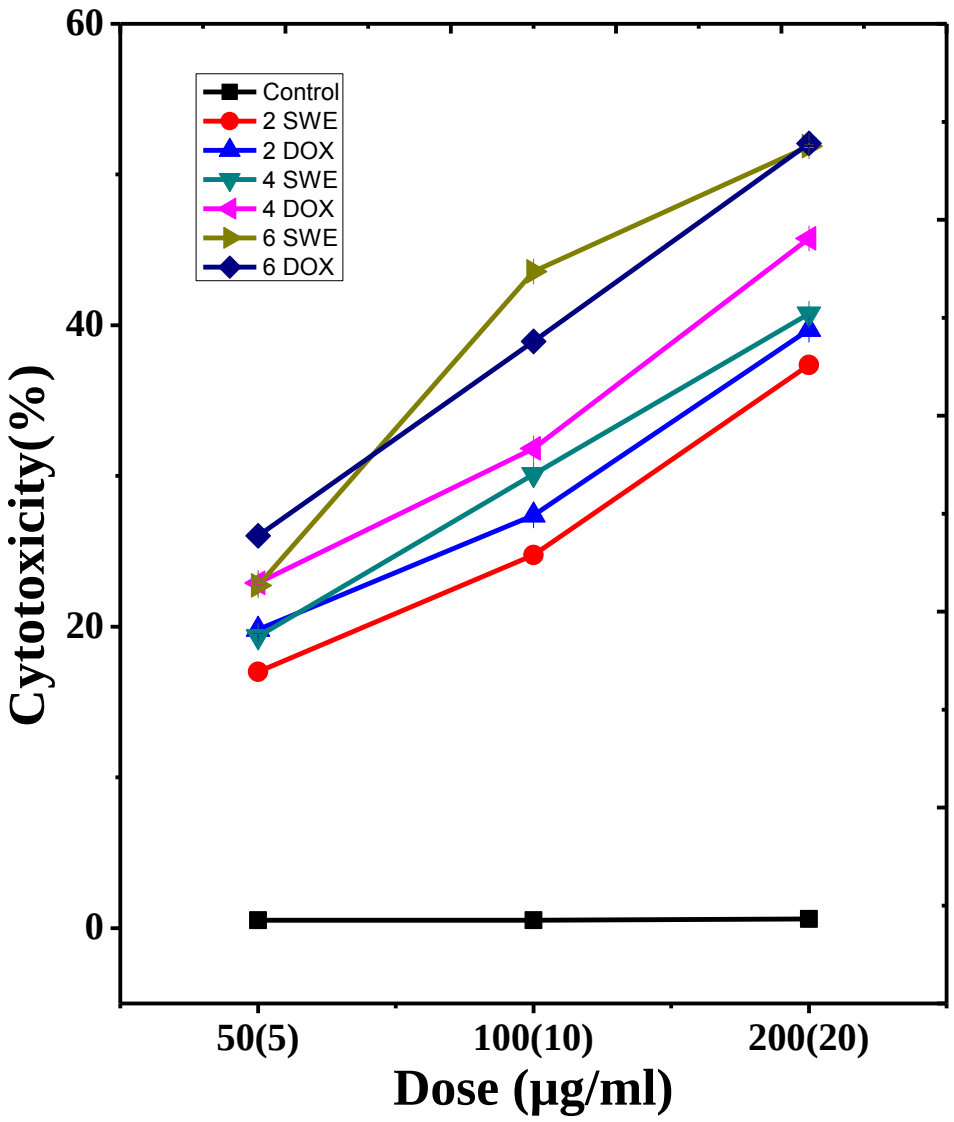

Figure 4: Cytotoxic effects of ethanol extract of *Schima wallichii* or DOX onV79 cells at different exposure times by MTT assay. N=8; p<0.05.

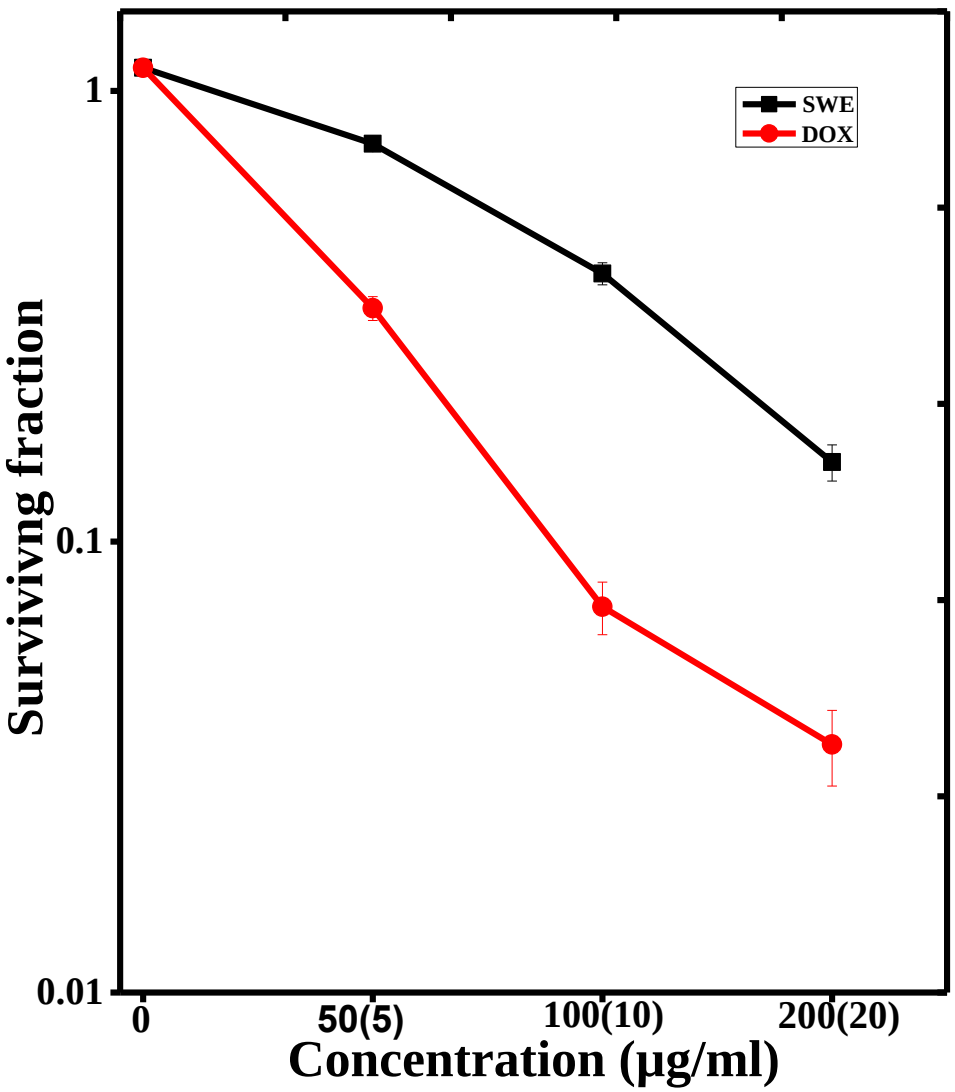

Figure 5: Effect of different concentrations of the ethanol extract of *Schima wallichii* or doxorubucin (DOX) treatment on the survival of HeLa cells. N=3.

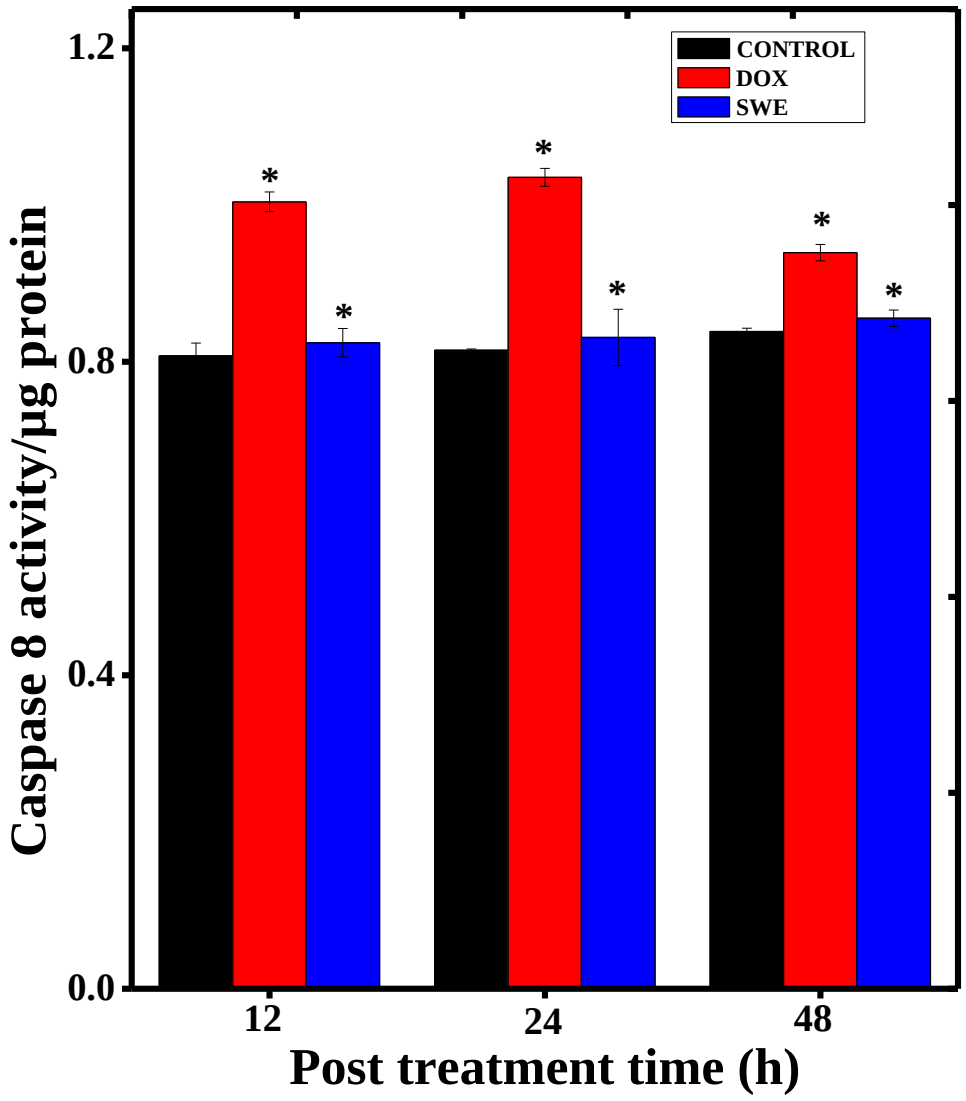

Figure 6: Determination of the caspase8 activity in HeLa cell treated with *Schima wallichii* (SWE) or doxorubicin (DOX) at different post treatment times. N=3. p<0.05

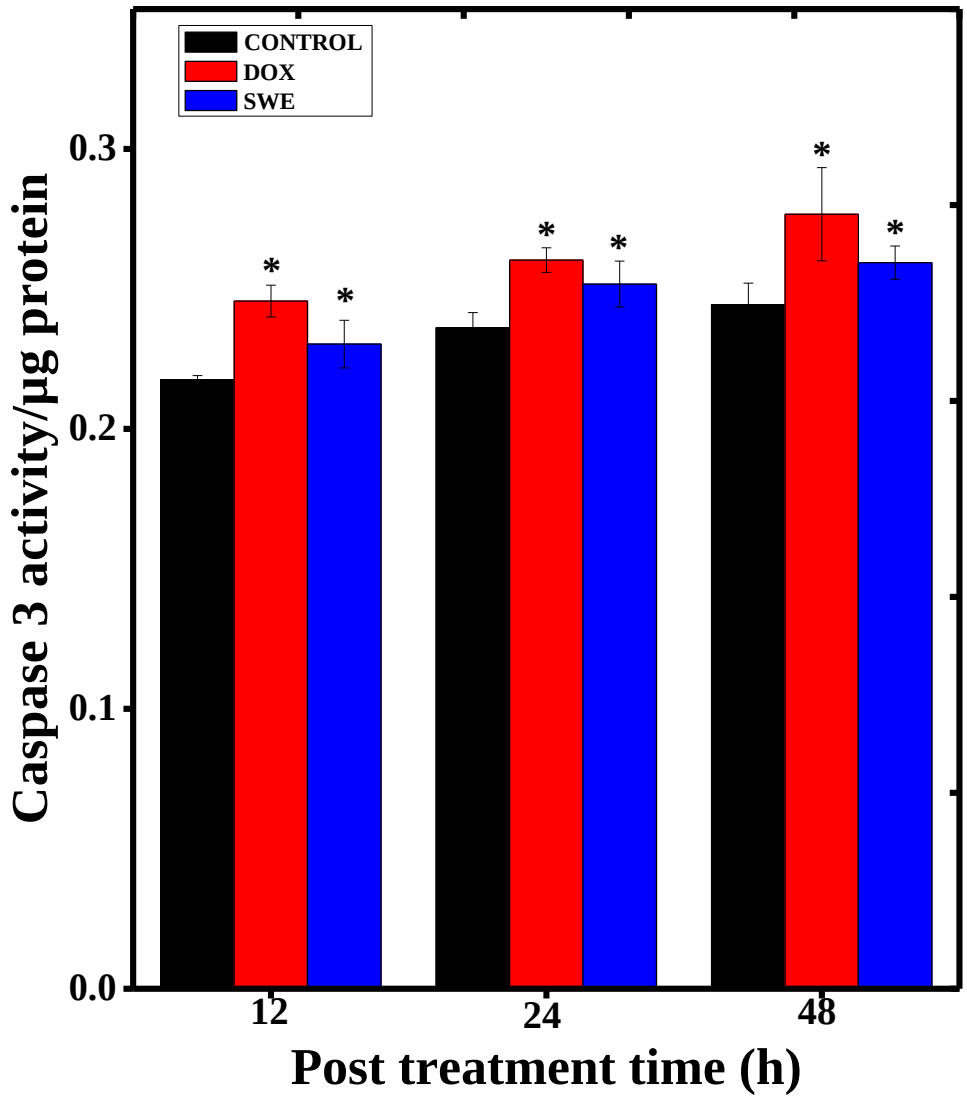

Figure 7: Determination of the caspase 3 activity in HeLa cell treated with *Schima wallichii* (SWE) or doxorubicin (DOX) at different post treatment times. N=3. p<0.05

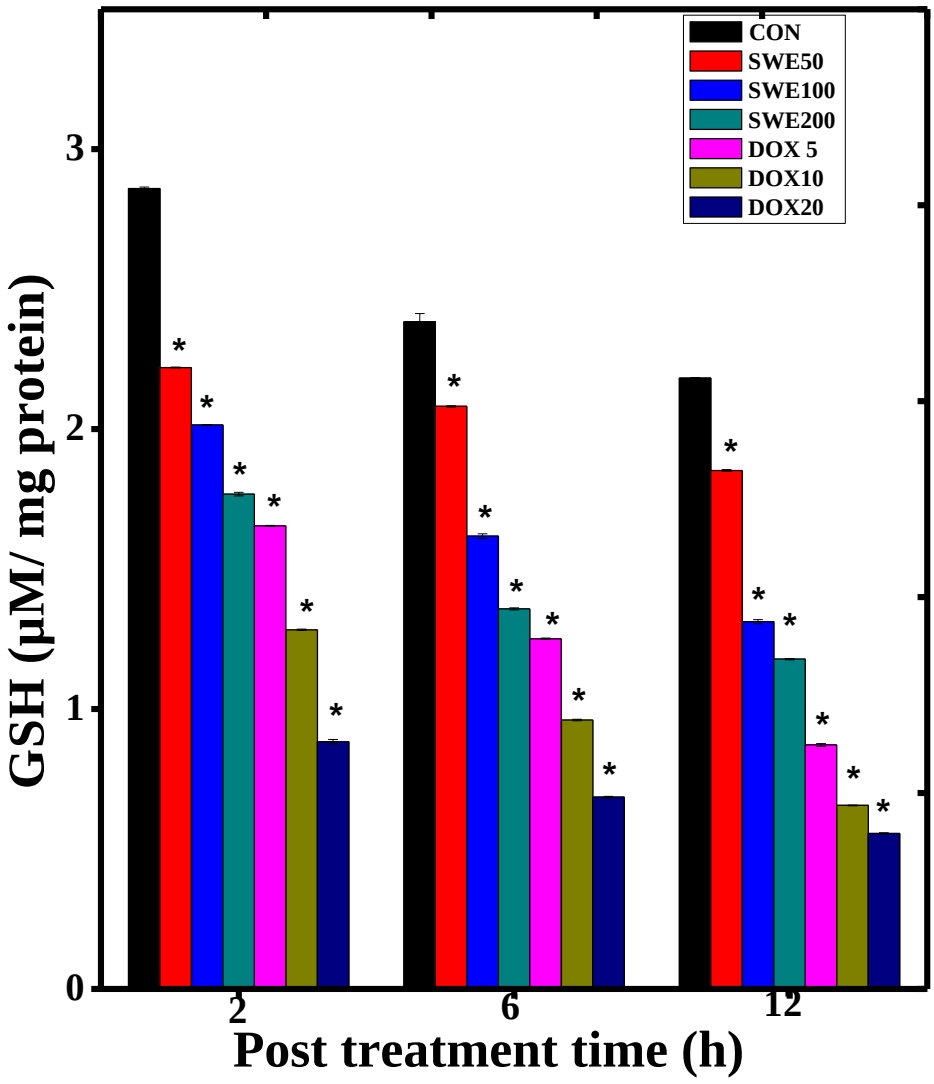

Figure 8 :Alteration in the glutathione contents in HeLa cells treated with various concentrations of *Schima wallchii* ethanol extract (SWE)or doxorubicin (DOX) at different post treatment times. N=5;*$p<0.05$ when treatment are compared with concurrent control (SPS) group.

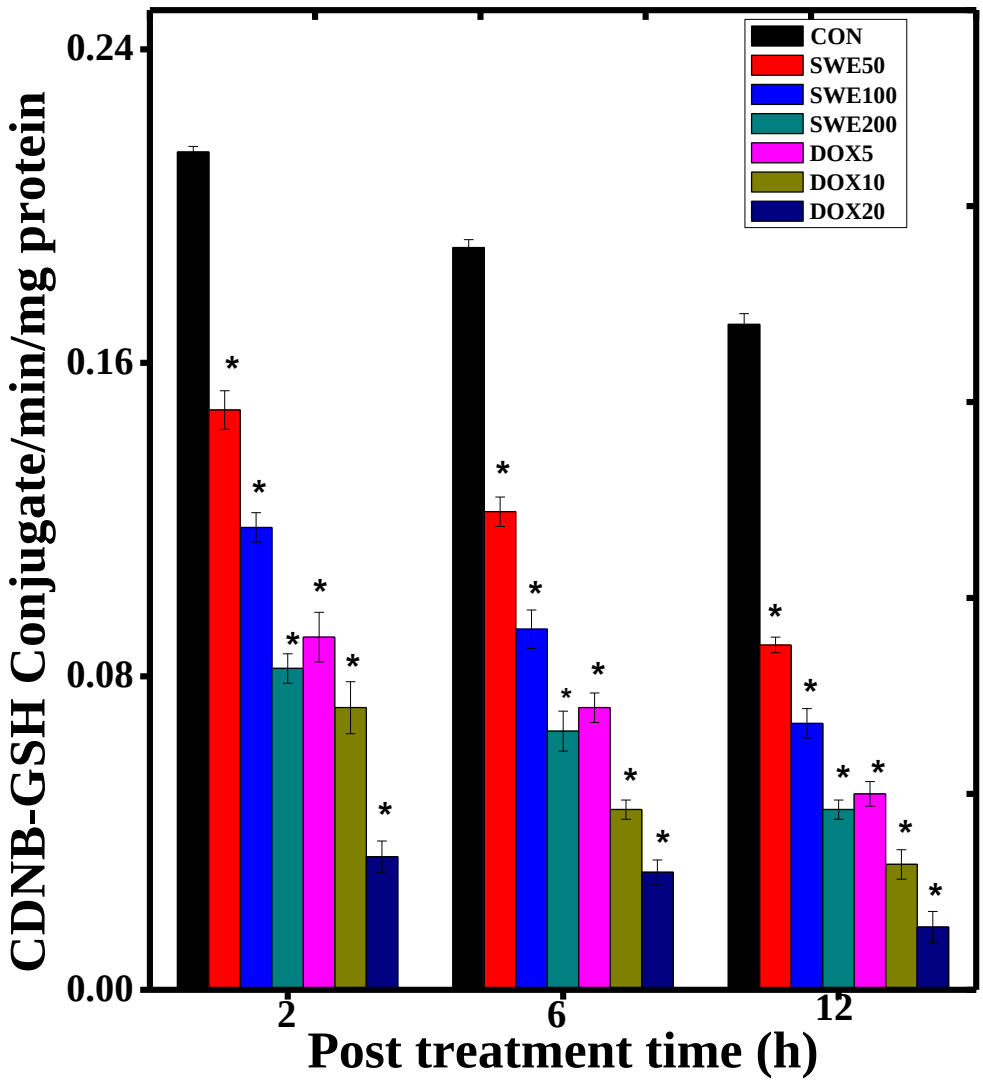

Figure 9 :Alteration in the glutathione-s-transferase (GST) activity in HeLa cells treated with various doses of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;*$p<0.05$when treatment are compared with concurrent control (SPS) group.

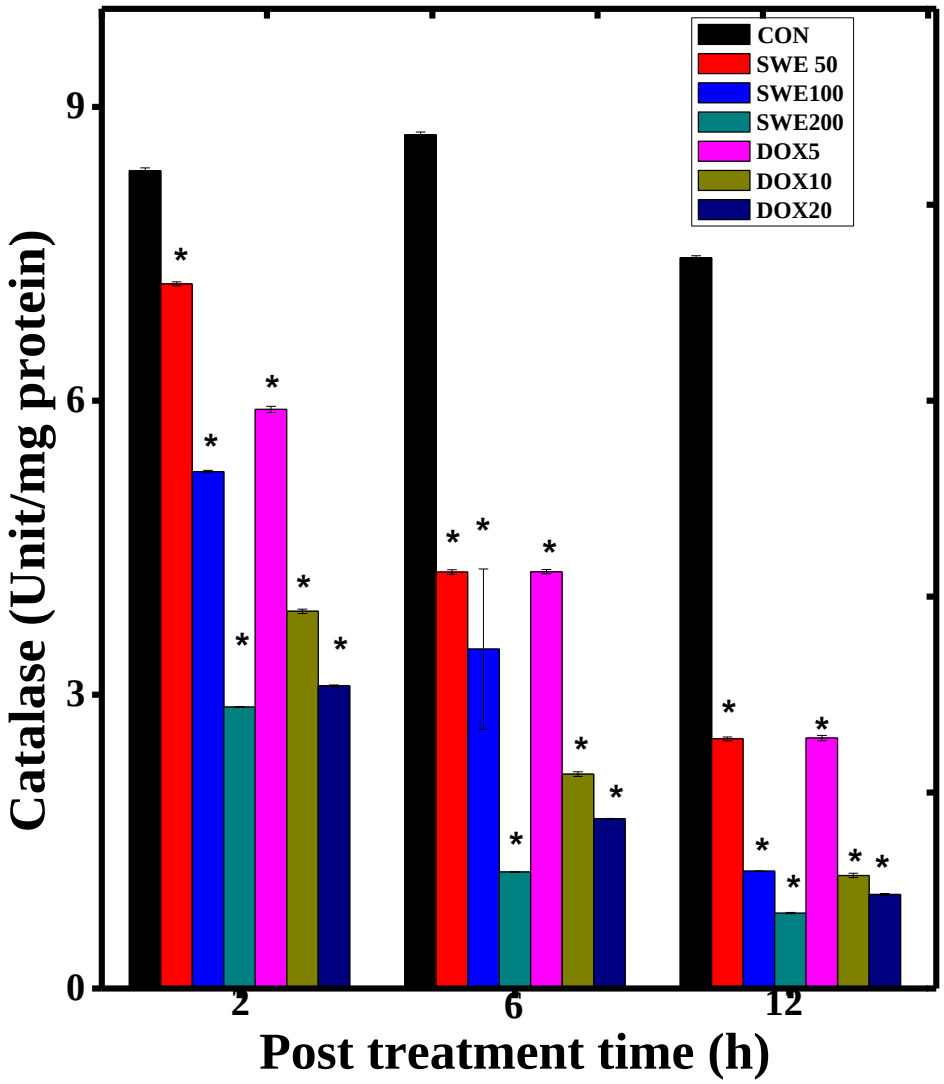

Figure 10 :Alteration in the Catalase activity on HeLa cells treated with various doses of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;*$p<0.05$when treatment are compared with concurrent control (SPS) group.

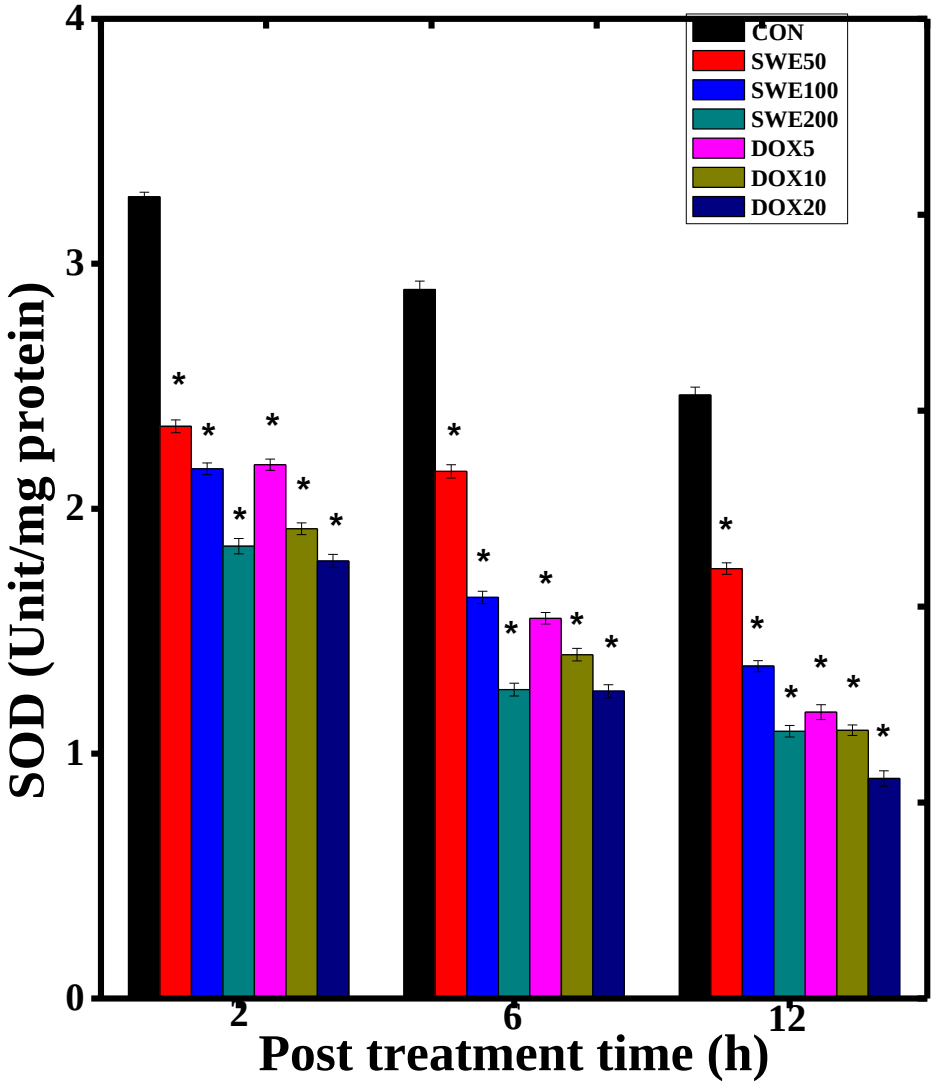

Figure 11 : Alteration in the Catalase activity on HeLa cells treated with various doses of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;.*$p<0.05$ when treatment are compared with concurrent control (SPS) group.

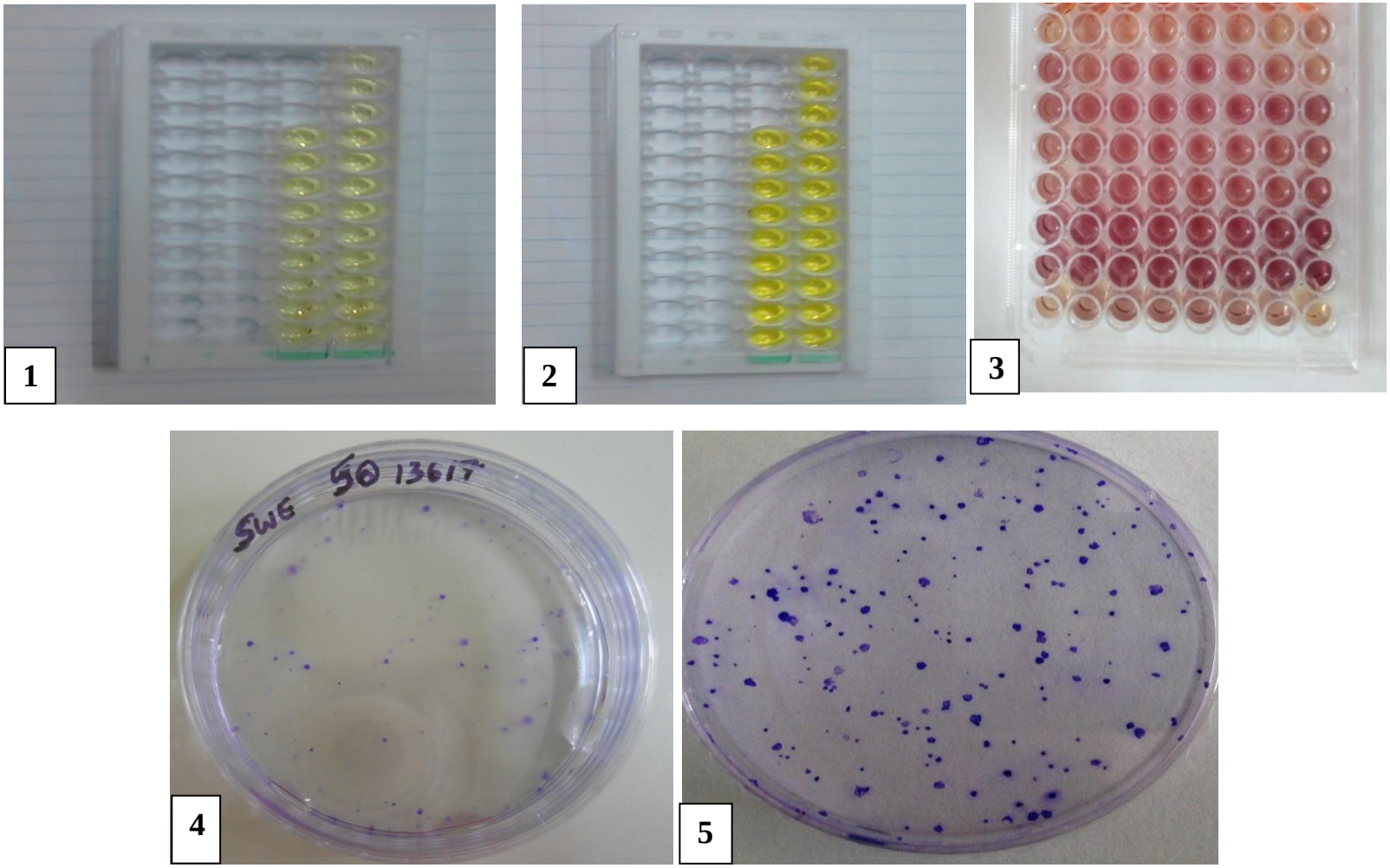

PLATE 3: 1) Caspase 8 activity 2) Caspase 3activity 3) MTT assay 4) and 5) Clonogenic assay

CHAPTER 5

Investigation of the antineoplastic activity of the different extracts of *Schima wallichii* in vivo.

ABSTRACTS

The effect of various doses of chloroform, ethanol and aqueous extracts of *Schima wallichii* was studied in Dalton's lymphoma tumor bearing mice. The acute toxicity was determined by oral and intraperitoneal administration in normal non tumor bearing mice. The oral administration studies have revealed that the chloroform and aqueous extracts of *Schima wallichii* were non-toxic up to 4g/kg body weight, whereas the ethanol extract was non-toxic upto 2g/kg body weight. The intraperitoneal administration of various doses of different extracts showed signs of toxicity in mice and LD50 of 500,100 and 500 mg/kg body weight was recorded for chloroform, ethanol and aqueous extract, respectively. The administration of 10- 250 mg/kg b.wt. chloroform, ethanol and aqueous extracts of *Schima wallichii* to tumor bearing mice resulted in a dose dependent increase in the tumor free survival and maximum effect was observed for 10 mg/kg ethanol extract, which increased the tumor free survival by 40% beyond 120 days, whereas chloroform and aqueous extracts were not that effective. However, a 20 and 40 % long term tumor free survivors were observed up to 60 days for chloroform and aquous extract at 150 and 100 mg/kg, respectively. The administration of 10mg/kg b. wt. ethanol extract resulted in an increase in the AST up to 64.81 days (IALS, 204.272%) and MST upto72.6 days (IMLS, 224.14 %). Therefore, 10 mg/kg body weight of the ethanol extract was considered as an optimum dose for its antineoplastic activity and further investigations were carried out using this dose. The administration of 10 mg/kg body weight ethanol extract into tumorized mice resulted in a time dependent rise in the micronuclei in both mononucleate and binucleate cells up to 24 h. The analysis of apoptotic and necrotic index also showed a time dependent increase and the maxium rise was observed at 36 h post treatment in the tumorized mice receiving 10 mg/kg body weight of ethanol extract of *S. wallichii*. 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 *Schima wallichii* 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.

INTRODUCTION

Cancer is a severe threat to human health and affects the lives of millions of people around the world. It drains financial and emotional resources of a family in which cancer is detected. It is the second largest cause of death succeeding cardiovascular diseases (Siegel *et al*., 2015). Globally, the number of cancer cases diagnosed are 12.7 million and both sexes are affected equally. The mortality due to cancer is projected to increase from 7.1 million in 2002 to 11.5 million in 2030 with 21 million diagnoses (Mathers and Loncar,2006; Vinay *et al*., 2015). Man power, natural and many material resources are spent for research purpose and in the development of new drugs for prevention and treatment of cancers each year worldwide. The impact of cancer on economic is significant and is rising to a high level. It has been estimated that the total annual economic cost of cancer in 2010 was approximately US$ 1.16 trillion (Stewart BW, 2014). The present cancer treatments include radiotherapy, chemotherapy and chemically derived drugs. The application of chemotherapy can put cancer patients under a lot of pressure as it may be responsible for further serious damage to their health. Therefore, using an alternative treatments and therapies against cancer is the main aim for developing anticancer agents with plants being the desired source (Cancer Research UK, 2014).

Natural products have been used for centuries for the treatment of several ailments. Many bioactive compounds have been discovered from plants, animals and microbes, which synthesize natural products and secondary metabolites for various purposes. These products and secondary metabolites serve a major source of drugs to treat different diseases including cancer (Kliebenstein, 2004; Zhao *et al*., 2013; Fulda and Efferth, 2015). However, research on this aspect has been limited, and more and more pharmaceutical industries are interested in examining potential of natural products and plant secondary metabolites as sources of novel medicinal compounds (Song *et al*., 2014). In the 21st century, finding and developing new

drugs from natural plants and marine life have attracted more and more attention (Wang *et al*., 2012; Kinghorn *et al*., 2016; Newman and Cragg, 2016). Different medicines from plant and health products have been accepted by people from all over the world, looking forward to improving the quality of life, disease prevention and treatment of chronic diseases and geriatric diseases as well as western medicine with helpless mysterious illness (Song *et al*., 2014). New therapeutic strategies are not only a question of eliminating cancer cells by induction of apoptosis, but also include targeting the tumor microenvironment, avoiding angiogenesis, modulating the immune response or the chronic inflammation which are often associated with cancers (Feitelson *et al*., 2015; Pitt *et al*., 2016;Cui and Guo, 2016; Law *et al*., 2017). Plants have a long history of use in the treatment of cancer and have played an important role as a source of effective anti-cancer agents, and it is significant that over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms ((Hartwell, 1982; Cragg *et al*., 2005; Newman *et al*., 2003; Newman and Cragg, 2016, Kinghorn, 2016).

The first agents to advance into clinical use were the vinca alkaloids, vinblastine (VLB) and vincristine (VCR), isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae), which was used by various cultures for the treatment of diabetes (Gueritte and Fahy, 2005). Plants did also provide several other modern chemotherapeutic molecules including podophyllotoxins, taxols, camptotheicins, doxorubicin and beomycins (Kinghorn *et al*., 2016 and Newman and Cragg, 2016) that are in frequent clinical use to treat different type of malignant neoplasia. However, the adverse effects of modern isolated molecules are severe and their use has been associated with second malignancies (Akin *et al*., 2010; Morton *et al*., 2014; Leeuwen and Ng, 2016; Zhang *et al*., 2017). The search for novel drugs is still a priority target for cancer

therapy due to the fact that chemotherapeutic drug resistance is becoming more and more frequent (Edelman and Mao, 2013; Housman *et al*., 2014).

Schima wallichii (DC.) Korth.or Chilauni belongs to the tea family, Theaceae. It is an evergreen tree growing luxuriously in the warm temperate to subtropical climates. It is widely found across southern and South East Asia, and stretch from the eastern Himalaya of Nepal to eastern India across Indochina, Southern China, Taiwan and the Ryukyu Islands. It is commonly known as needlewood tree and may grows up to 35 m higher even 40 ft tall (Min *et al*., 2012). Locally, it is called "khiang" in Mizo language. *Schima wallichii* is credited to possess several medicinal properties. The leaves and the stem bark are normally used traditionally for it as a medicine. The bark is used as an antiseptic for cuts and wounds. It is a vermicide, mechanical irritant and used to cure gonorrhea (Dewanjee *et al*., 2008). Decoction of bark is used to cure fever and is said to be effective against head lice (Gurung, 2002). The bark juice is given to disinfest the animal from liver flukes (Lalrinzuali, 2015). The sap from the stem is used for curing ear infection (Sam *et al*., 2004). Fruit decoction is used by the people of Western Mizoram, India against snakebite (Lalfakzuala *et al*., 2007; Lalrinzuali, 2015). The young plants, leaves and roots are also used medicinally against fever and the bark is anthelmintic and rubefacient (Gardner *et al*., 2000). The leaves of *Schima wallichii* are known to have antitumor and antimutagenic properties (Koshimizu *et al*., 1998; Subarnas *et al*., 2003). The astringent corollas are used to treat uterine disorders and hysteria (Paudel and Subba, 2014). The antineoplastic activity of *Schima wallichii* has not been investigated systematically until now, therefore the present study was undertaken to obtain an insight into the antineoplastic activity of *Schima wallichi* in the Swiss albino mice transplanted with Dalton's Lymphoma.

MATERIALS AND METHODS

1.1. Chemicals

Dimethyl sulphoxide (DMSO), nitrobluetetrazolium (NBT), phenazinemethosulphate (PMS), 5, 5'dithio 2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitronezene (CDNB),. 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), triton X-100, ethylenediaminetetra-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, and hydrogen peroxide (H_2O_2), 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. Reduced nicotinamide adenine dinucleotide (NADH) waspurchased from HiMedia, Mumbai, India. Doxorubicin, was requisitioned from Getwell Pharmaceuticals, Gurgaon, India..

1.2. Collection and Preparation of the plant extract

The non-infected stem bark of *Schima wallichii* was collected from Bazar veng, Lunglei, Mizoram during the months of April and May. The authentication and identification of *Schima wallichii* was done by Botanical Survey of India, Shillong (BSI/ERC/Tech// Identification/2017/570). The bark was washed with water to remove dust and other extraneous material and shade dried at room temperature in clean and hygienic conditions. The dried bark was then powdered using an electrical grinder. The dried powder of *Schima wallichii* was

extracted sequentially with petroleum ether, chloroform, ethanol and distilled water in order of increasing polarity using a Soxhlet apparatus. The liquid extracts were filtered and concentrated by evaporating their liquid contents to dryness under reduced pressure. The concentrated extracts were stored at -80 until use.

1.3. Preparation of Drug and mode of administration

The various extracts of *S. wallichii* were dissolved in appropriate solvent before use. The chloroform (SWC) extract was dissolved in 5% ethanol in distilled water, 1% absolute ethanol was used for dissolving the ethanol extract (SWE) whereas doubled distilled water was used for dissolving aqueous extract (SWA) and doxorubicin. The weight of the animals from different groups were taken and recorded. According to the body weight of the animals, each animal received treatments orally and intraperitoneally depending on the experimental design.

1.4. *Animal care handling*

The guidelines issued by the World Health Organization (WHO), Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India) were strictly followed for handling and care of animals. Swiss albino mice purchased from Pasteur Institute, Shillong were bred and maintained in a controlled environment of temperature (24-25ºC), 50% humidity and a light and dark cycle (12 h each). About five animals were housed in a sterile polypropylene cage which contained sawdust (procured locally) as bedding material. For experimentation, normally six to eight weeks Swiss albino mice of both genders weighing around 25-30 g were selected. The animals were fed with commercially available food pellets and with free access to water. The 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.MZU/IAEC/14-15/10 Aizawl, India.

1.5. Determination of acute toxicity

The acute toxicity of all extracts of *S.wallichii* was determined by administering 0, 2 or 4 g/kg b. wt. of chloroform, ethanol or aqueous extracts of *S.wallichii* orally or 0.1, 0.5, 2, 3, 4 or 5 g/kg b. wt. of chloroform, ethanol or aqueous extracts of *S.wallichii* intraperitoneally according to the guidelines of Organization for Economic Co-operation and Development (OECD). The mice of both sexes (5 males and 5 females) were categorized into different groups by random sampling technique. Usually ten animals were utilized for each dose of each extract. The animals were fasted for 18 hours (both food and water withdrawn) prior to adminstration of different extracts of *S.wallichii*. The weights of the animals were recorded before and after fasting to estimate their weight loss. The selected animals were divided into four groups according to the extracts administered. The SWC group received chloroform extract, the SWE group received ethanol extract, the SWA group received aqueous extract. The control group received sterile physiological saline (SPS). The animals were provided with food immediately after administration of different extracts.

The animals under treatment were observed for first two hours and every 6 hours until 24 hours, and daily thereafter for a total period of 14 days for the development of toxic symptoms. If mortality was observed in 3-4 animals, then the dose administered was assigned as toxic dose. The behavior of the animals was observed and recorded and the LD_{50} for each extracts was calculated using probit analysis.

1.6. Tumor Model

A Dalton's lymphoma ascites (DLA) tumor was used as it provides most convenient model system to study antitumor activity within a short time (Shanker *et al*., 2000; Koiri *et al*., 2017). DLA was procured from North-Eastern Hills University (NEHU), Shillong and was

maintained in 4-6 weeks old Swiss albino mice by serial intraperitoneal transplantation. Usually one million viable DLA cells were inoculated intraperitoneally into each animal under aseptic conditions and the day of inoculation was taken as day 0.

1.7. Experimental design

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

1.7.1. Control groups: This group received 0.01 ml/kg body weight of sterile physiological saline (SPS) and served as the negative control group.

1.7.2. DOX groups: In this group, the animals intraperitoneally received 0.5 mg/kg body weight of doxorubicin, a standard anticancer drug and served as positive control.

1.7.3. SWC groups: The animals of this group were administered with 50, 100, 150, 200 and 250 mg/kg body weight of the chloroform extract of *Schima wallichii* intraperitoneally.

1.7.4. SWE groups: The animals in this group were intraperitoneally injected with10, 20, 30, 40 and 50 mg/kg body weight of the ethanol extract of *Schima wallichii*.

1.7.5. SWA groups: This group of animals was given 50, 100, 150, 200 and 250 mg/kg body weight of the aqueous extract of *Schima wallichii* intraperitoneally.

The tumor bearing animals were given treatment once daily on 1 day after tumorization and 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) 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

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

1.8. Micronucleus Assay

The ability of SWE to induce DNA damage in Dalton's lymphoma was studied by performing experiments where 1 x 10^6 Dalton's lymphoma cells were transplanted into 5 – 8 weeks old mice and allowed to develop the tumor for 1day. Thereafter, these animals were given a nine days treatment of 10 mg/kg body weight of *Schima wallichii* ethanol extract (SWE) or 0.5 mg/kg body weight doxorubicin intraperitoneally. One hour after the last drug/s administration, each of the tumorized mouse was injected with 150 µg of cytochalasin B so as to suppress cytokinesis in the proliferating tumor cells. The mice were euthanized at 6, 12, 24 and 48 h post-drug treatment 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 treated with mild hypotonic solution(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).

1.9. Apoptosis Assay

The ability of SWE to induce apoptosis in Dalton's lymphoma cells was performed to investigate induction of DNA damage, where grouping and other conditions were exactly similar to that described for micronucleus assay except that tumor bearing mice were euthanized at 2, 6, 12, 24 and 48h 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.

1.10. Biochemical assays

A separate experiment was conducted to study the various biochemical changes in the SWE 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 2, 4, 8, 12 and 24 hours. The animals were perfused with isotonic cold saline transcardially. Both the treated and untreated livers of the animals were removed under aseptic conditions. The livers were weighed and 10% homogenate was prepared in cold sterile PBS (pH 7.4) and used for the estimation of glutathione (GSH), glutathione-s-transferase(GST), catalase, superoxide dismutase (SOD) and lipid peroxidation (LOO).

1.10.1. 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 tissue homogenate and allowed to stand for 2 minutes. The absorbance was read against the blank at 412 nm in a Eppendorf UV/VIS Spectrophotometer (Eppendorf India Ltd., Kolkata). The blank consisted of distilled water instead of tissue homogenate.

1.10.2. 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 20mM 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 tissue 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.

1.10.3. Catalase Assay

Catalase was assayed according to technique described by Aebi (1984). In a 3 ml cuvette, 20 µl of tissue 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.

1.10.4. 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 earlier (Fried, 1975). 100 µl of tissue homogenate, 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitrobluetetrazolium, 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.

1.10.5. 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 thoroughly mixed with 2 ml of TCA-

TBA-HCl reagent, 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 tissue homogenate substituted with distilled water.

Determination of Liver and Kidney function

For the estimation of the toxicity of SWE in the liver and kidney of the tumorized mice, the tumor bearing mice were injected with 10 mg/kg b. wt. of ethanol extract one day after transplantation of Dalton's lymphoma for nine days subsequently. After the treatment was over, the mice were sacrificed, perfused with cold isotonic saline transcardially and the livers were collected at different post treatment times. The livers were weighed and 10% homogenate was prepared in cold sterile PBS (pH 7.4) and used for the estimation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and uric acid at 2, 4, 8, 12 and 24 h after the last administration of the extract. Commercially available kits were used to measure AST, ALT, creatinine and uric acid according to the manufacturer's protocol.

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, whereas Mann Whitney "U" test was applied 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.

2. RESULTS

The results have been expressed as the mean ± standard error of the mean (SEM), and are presented in Tables 1-15and Figures 1-11.

2.1. Acute toxicity

The administration of the different extracts of *Schima wallichii* orally showed no signs of toxicity up to 4g/kg b. wt. for chloroform and aqueous extracts while administration of 2g/kg b.wt.of ethanol extract also did not reveal any toxic effect (Table 1). The acute toxicity assay after the intraperitoneal administration was carried out by up and down method. This mode of administration exerted toxic effects at 2 g/kg b.wt. for chloroform extract where 30% animals succumbed to death (Tables2-5). Administration of ethanol extract was highly toxic as 30% animals died at 500 mg/kg b. wt. The intraperitoneal administration of 3 g/kg b. wt. of aqueous extract led to a 30% mortality and this was least toxic when compared to other extracts (Table 5). The probit analysis resulted into the LD50 of 100mg/kg b.wt. for ethanol extract, whereas it was 500 mg/kg b. wt. for the chloroform and aqueous extracts, respectively (Tables 2-5).

2.2. Change in the body weight

The transplantation of DLA cells into mice resulted in continuous gain in the body weights until the survival of mice and there was no sign of tumour regression in the negative control group. The DLA mice treated with 50, 100, 150, 200 and 250 mg/kg body weight for chloroform and aqueous extracts and 10, 20, 30, 40 and 50 mg/kg body weight of ethanol extract of *Schima wallichii* showed an increase in the body weight however, this gain in body weight was lesser when compared to negative control group. However, this increase was insignificant up to 21st day of tumor transplantation as compared with day 0 within all the treated groups. The comparison of *Schima wallichii* extract treated groups with negative control revealed a

considerable decrease in the body weight due to inhibition of cell propagation (Table 6, Figure 1).

2.3. Anticancer activity

Dalton's lymphoma transplanted intraperitoneally into mice developed speedily with no signs of regression and all the untreated tumorized mice died within 24 days (Table 6). The AST and MST for this group were 21.3 and 21 days, respectively (Table 8).

The administration of 50, 100, 150,200 and 250 mg/kg body weight of chloroform extract significantly increased in the number of survivors when compared to negative control group ($p<0.05$). The maximum survival of tumour bearing mice was observed at 150mg/kg where 20% of the animals survived upto 60 days post tumour transplantation (Table 8). The AST of 48.4 days, MST of 47.5 days, IMLS of 124.34 % and an IALS of 127.49 % were determined for this dose (Figure 4).

Treatment of Dalton's lymphoma bearing mice with 50, 100, 150, 200 and 250 mg/kg body weight of the aqueous extract resulted in a dose dependent rise in the survival of mice up to a dose of 250 mg/kg SWE when compared to SPS control ($p<0.05$) (Table 4, Figure 4). A maximum number of tumor free survivors was observed at 100 mg/ kg body weight SWA where the 40% long term tumor free survivors were recorded up to 60 days and 20% of the animals did survive up to 85 days (Table 7). The AST of 54.73 days and MST of 55.8 days were recorded for 100 mg/kg with an IMLS of 165.71 % and an IALS of 156.948 %, respectively (Table 8, figure 4).

The treatment of tumor bearing mice with 10, 20, 30, 40 and 50 mg/kg body weight of the ethanol extract resulted in a rise in the survival and maximum number of tumor free survivors (40%) was observed at 10 mg/kg body where animals survived up to 120 days with no evidence

of disease. The administration of 20 mg/kg b. wt of ethanol extract resulted in a 60% tumor free survivors up to 90 days however, no survivors could be recorded up to 120 days (Table 7). The administration of 10 mg/kg body weight SWE resulted in an AST of 64.81 days, MST of 72.6 days, IMLS of 224.14 % and an IALS of 204.27 %, respectively (Table 8, Figure 3). Since 40% animals survived at 10 mg/kg SWE until 120 days or more it was regarded as the best anticancer dose and further investigations were carried out using this dose.

2.4. Micronucleus Assay

The frequency of micronuclei bearing mononucleate (MNMNC) and binucleate cells (MNBNC) has been represented separately (Table 9). Treatment of Dalton's lymphoma bearing mice with SWE or DOX showed a time dependent rise in the frequency of micronuclei ($p<0.05$) up to 24 h post-drug treatment and decline thereafter (Figure 5).The administration of 10 mg/kg SWE also induced cells bearing two micronuclei in both mono and binucleate cells which were 10 or more fold higher than the negative control (Table 9).

Apoptosis Assay

The administration of SWE or DOX induced apoptotic and necrotic cells in Dalton's lymphoma cells as early as 2 h post drug treatment that continued to rise up to 48 h post treatment (Figure 6). The number of apoptotic and necrotic cells in SWE or DOX treated DLA cells significantly ($p<0.05$) increased when compared to concurrent negative control group at all the post drug treatment times (Table 10). The apoptotic cells increased by 6 to 10 folds in SWE treated group when compared with the negative control group (Table 10).

Biochemical Assays

2.4.1. Glutathione content

The treatment of DLA mice with10 mg/kg b. wt SWE caused a significant decrease in the glutathione contents in a time dependent manner up to 24 h post treatment (Figure 7). The maximum reduction in GSH concentration was observed at 24 h ($p<0.05$). A similar pattern was observed for doxorubicin treatment (Figure 7). The SWE treatment reduced the GSH contents comparable to DOX treatment (Table 11).

2.4.2. Glutathione - S – Transferase (GST)

The administration of SWE or DOX resulted in a significant ($p<0.05$) reduction in the GST activity at all the post treatment times (Table 12). The GST activity declined in a time dependent manner and the highest decrease in GST activity was observed at 24 h post treatment (Figure 7).

2.4.3. Catalase (CAT) activity

Administration of SWEor DOX into Dalton's lymphoma bearing mice led to a gradual and time dependent attrition ($p<0.05$) in the catalase activity until 24 h post treatment (Figure 8). The pattern of decline in catalase activity in DOX group was almost similar except that it was marginally lower than SWE treatment (Table 13).

2.4.4. Superoxide dismutase (SOD) activity

Treatment of tumorized mice with 10 mg/kg b. wt SWE resulted in a significant reduction in the SOD activity (Table 14). The SOD activity declined in a time dependent manner after SWE or DOX treatment until 24 h post treatment where a greatest reduction in SOD

activity was observed after SWE administration, which was at par with DOX treatment (Table Figure 10).

2.5. Lipid peroxidation

The SWE caused a significant ($p<0.05$) rise in the lipid peroxidation as early as 2 h post – treatment in the tumorized mice administered with 10 mg/kg of SWE (Figure 11). Increase in assay time resulted in a further rise in LOO and a highest elevation in LOO was recorded at 12h post treatment in the SWE group (Table 15). The pattern of LOO after DOX treatment was almost similar except that the rise was greater than SWE treatment (Figure 11). The LOO declined thereafter without reaching to SPS treatment level (Table 15).

2.6. Liver and Kidney function tests

The intraperitoneal administration of 10 mg/kg b. wt SWE and DOX for consecutive 9 days did not significantly alter aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities in the liver of tumorized mice (Table 16). The level of the AST and ALT in the serum of nine days treated mice showed a slight rise at 8 h post treatment when compared to concurrent control group ($p<0.05$) (Figure 10). Despite this marginal rise the activities of AST and ALT were within normal range.

The administration of SWE resulted in an elevation in creatinine and uric acid, especially at 12 and 24 h post treatment (Figure 11). 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/dL (Bone *et al*., 1945) and 3.4-7 mg/dL (Fossati and Prencipe, 1980) for creatinine and uric acid, respectively, indicating the safety of SWE for the kidney (Table 16).

DISCUSSION

The realization of cancer as a disease in human stimulated several investigation and there has been a constant endeavor to fight against the disease by evolving various modalities. The Chemoterapy has emerged as one of the most important and promising modalities of cancer treatment however, it also affects normal cells of different organs leading to many adverse effects (Leeuwen and Ng, 2016; Zhang *et al*., 2017). The cancer mortality is approximately 63% globally despite availability of state of the art treatment strategies, which indicates the need of alternative strategies to contain or reduce the cancer related mortalities. The history of use of plants and natural products for healthcare is as old as the human civilization (Greenwell and Rahman, 2015; Jagetia, 2017). The plants contain several molecules and use of plants for cancer treatment may prove most useful as they may attack cancer cells through multiple mechanisms (Singh *et al*., 2016). The plant-derived anticancer agents may be effective inhibitors of cancer cells (Sivaraj *et al*., 2014). Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products (Gordaliza, 2007; Newman and Cragg, 2016) and the plant kingdom has been the most significant source of these drugs. In addition, the emergence of resistance to cancer chemotherapy has stimulated researchers to turn to natural products of plant or marine origin. Many herbs have been evaluated in clinical studies and are currently being investigated to understand their tumouricidal properties against various cancers (Balachandran, 2005). 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, the present study was undertaken to assess the ability of *Schima wallichii* to exterminate the Dalton's lymphoma cells transplanted in mice.

The oral acute toxicity studies have shown that 4 g/kg b. wt for chloroform and aqueous extracts, 2g/kg b.wt for ethanol extracts of *S.wallichii* were non-toxic in the normal mice whereas the intraperitoneal administration caused toxicity and the LD_{50} was 500 mg/kg b.wt for chloroform and aqueous extracts and 100 mg/kg b.wt for ethanol extract. The acute toxicity studies revealed that the ethanol extract has highest toxicity level when administered intraperitoneally and the toxicity level of ethanol extract is five times higher as compared to chloroform and aqueous extracts. There are no reports regarding the acute toxicity of *S wallichi*. However other plants like *Alstonia scholaris and Nigella sativa* were found to exhibit toxic effect beyond 1000 mg/kg after intraperitoneal administration (Baliga *et al*., 2004; Jagetia and Ravikiran, 2014). Oral administration of *Pericampylus glaucus*or *S. alata* did not show any toxicity up to 4 and 3 g/kg b. wt in mice (Kifayatullah *et al*., 2015; Roy *et al*., 2016).

Evaluation of antineoplastic activity of *S.wallichii*on Dalton's lymphoma transplanted in the peritoneum of Swiss albino mice showed that the mice without any treatment developed tumorspeedily and all the untreated control mice died within 24 days after tumor inoculation with an average survival time (AST) and median survival time (MST) of 21.3 and 21 days, respectively. The tumorized mice receiving different extract of *S.wallichii* significantly enhanced the life span of tumorized mice due to regression of tumors, which caused increased in the life span up to 60, 90 and 120 days for chloroform, aqueous and ethanol extracts, respectively. The most potent extract proved to be the ethanol extracts where 40% of the tumor free survivors were observed beyond 120 days indicating its efficacy in killing the Dalton's lymphoma cells. The studies on the anticancer activity of *S.wallichii* are unavailable. However extracts of *Alstonia scholaris, Aphnamixis polystachya, Ervatamia heyneana, Hygrophila spinosa, Podyphyllum hexandrum, Rubia cordifolia, Tinospora cordifolia* and *Tylophora indica* have been found to

increase the tumor free survivors 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 triggering of DNA damage is one of the important aspects to induce cytotoxicity in tumor cells. The analysis of micronuclei provides an indirect way to study the DNA damage as the micronuclei arise as a result of defective cell division, mis-segregation of chromosomes, DNA exchanges and faulty or suppressed DNA repair leading to cell death (Countryman and Heddle, 1977; 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 that leads to DSB (Dianov *et al*., 1991), which are converted into micronuclei after a cell undergoes division. 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 ability of ethanol extract of *S. wallichii* to induce DNA damage was studied by micronucleus assay, where administration of 10 mg/kg b. wt of *S.wallichii* in tumor bearing mice resulted in a significant increase in the micronuclei frequency in the mononulceate as well as binucleate DLA cells indicating that ethanol extract of *S.wallichii* efficiently induced DNA damage. Treatment of Dalton's lymphoma bearing mice with SWE showed a time dependent elevation in the frequency of micronuclei up to 24 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 other plant extract from *Tinospora cordifolia* and *Aphnamixis polystchya have been*

reported to kill tumor cell by inducing DNA damage in the form of micronuclei (Jagetia *et al*., 1998; Jagetia and Venkatesha, 2016). The berberine a secondary plant metabolite has been reported to kill tumor cells by inducing molecular damage to cellular genome earlier (Jagetia and Rao, 2015 a,b). The peak frequency of micronuclei at 24 h may be due to the fact that these frequencies are observed immediately after first cell division and thereafter the reduction in micronuclei may be due to the division of micronucleated and other cells that will reduce the micronuclei frequency. The SWE induced not only one micronuclei but also cells with two micronuclei indicating that it induced complex multiply site of DNA damage that would have repressed the DNA damage repair leading to tumor cell death and increase in tumor free survivors. 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 remission of tumor and increase in tumor free survivors by SWE may be due to the induction of apoptosis, which will be able to remove the tumor cells efficiently. The treatment of tumor bearing mice with SWE induced apoptosis in a time dependent manner leading to increased tumor free survivors in the present study. The infliction of DNA damage in the tumor cells by SWE had triggered a cascade of biochemical and molecular events that triggered 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). The various plant extract have been reported to induce apoptotic mode of cell death in different cultured cell lines earlier (Thuncharoen *et al*., 2013; Bhatia *et al*., 2015; Badmus *et al*., 2015).

Determination of the activity of transaminase enzyme can provide valuable information on toxicity (Dufour et.al, 2000). The intraperitoneal administration of SWE (10 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 tissue enzyme. 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/dL (Bone *et al*., 1945) and 3.4-7 mg/dL (Fossati and Prencipe, 1980) for creatinine and uric acid, respectively, indicating the safety of SWE administration.

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). The elevated levels of glutathione are responsible for chemoresistance and lead to treatment failure in cancer patients (Ramsay and Dilda, 2014), whereas alleviation in GSH causes enhanced oxidative stress that make tumor cells more amenable to chemotherapy or radiotherapy (Ortega *et al*., 2012; Rocha *et al*., 2014). The treatment of SWE decreased the glutathione contents in the Dalton's lymphoma cells in a time dependent manner, and this reduction in GSH concentration may have elevated the oxidative stress and killed the DLA cells leading to tumor regression. Similarly, treatment of Dalton's lymphoma with SWE 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 SWE causing increased tumor free survival in the present study. The other important effect of SWE was the induction of lipid peroxidation in

DLA cells, which has certainly increased the oxidative stress in tumor cells and killed them in the present study. The lipid peroxidation is involved in the non-apoptotic form of cell death and its role in triggering cell death is now well understood (Magtanong *et al*., 2016; Gaschler and Stockwell, 2017). The SWE treatment has increased lipid peroxidation in a time dependent manner and this increase in the lipid peroxidation may have contributed in its own to the death of DLA cells and subsequently increased the tumor free survivors. The *Alstonia scholaris* has been reported to kill Ehrlich tumor cells by increasing lipid peroxidation earlier (Jagetia and Baliga, 2016). The mechanism of action by which SWE induced cytotoxicity in the tumor cells in the present study is not clearly known. The SWE may have used multiple putatative mechanisms 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. The reduction in GSH, GST, catalase and SOD seems to be another mechanism that may have initiated a cascade of events leading to cell kill in the present study. The increased lipid peroxidation may have initiated non-apoptotic form of cell death thus increasing the tumor free survivors. The SWE may have also utilizied molecular pathways by suppressing the transcriptional activation of NF-κB, COX-II, and Nrf2 which are overexpressed in the tumor cells and give them survival advantage (Sobolewski *et al*., 2010; Lu and Stark, 2015; Park *et al*., 2011). The triggering of apoptosis may have been due to the alleviated expression of Bcl_{xL}, survivin, IAP, c-FLIP and IKK (Hu *et al*., 2006; Li *et al*., 2009). The SWE may have also stimulated the activation of caspase to kill tumor cells.

Conclusions

The different extract of *Schima wallichii* were found to be non-toxic up to 2 g when administered orally however, the intraperitoneal administration resulted in a LD_{50} of 500 mg/kg body weight

for chloroform and aqueous extracts, whereas it was only 100 mg/kg body weight for ethanol extract. The intraperitoneal administration of chloroform, aqueous and ethanol extract led to increase in the tumor free survivors for 60, 90 and beyond 120 days, respectively. The ethnol extract was most potent and its mechanism of action seems to be due to increased micronuclei frequency, apoptosis and lipid peroxidation. The attrition in the GSH concentration and activities of GST, catalase and SOD had also contributed in their own ways to kill tumor cells. The inhibition of NF-κB, COX-II, and Nrf2 may have triggered events that led to increased cell death by SWE. The reduced expression of Bcl_{xL}, survivin, IAP, c-FLIP,and IKK may have also contributed to bring out cell death by apoptosis. The activation of caspase cascade may have also killed tumor cells in the present study.

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Table 1: Acute toxicity of different extracts of *Schima wallichii* administered orally in Albino mice with different concentrations in both sexes.

Extract/ Group	Sex	Dose (g/kgb.wt.)	Body weight (g)			Survival
			Before fasting	After fasting	Loss (18 h)	
Control (SPS)	M	0	30	27	3	> 14 days
			32	29.8	2.2	> 14 days
			28.2	25.0	3.2	> 14 days
	F		30	25.9	4.1	> 14 days
			25.8	22.2	3.6	> 14 days
			27	24	3	> 14 days
Chloroform	M	4	31.6	29.9	1.7	> 14 days
			35	31	4	> 14 days
			29.6	27	2.6	> 14 days
			33	30.2	2.8	> 14 days
			30.3	27.4	2.9	> 14 days
	F		25	22	3	> 14 days
			28.5	26.7	1.8	> 14 days
			29.4	27.3	2.1	> 14 days
			25.7	22.8	2.9	> 14 days
			25.3	24	1.3	> 14 days
Ethanol	M	2	35.2	33.3	1.9	> 14 days
			35.5	33.2	2.3	> 14 days
			31.6	29.9	1.7	> 14 days
			27.2	25.0	2.2	> 14 days
			32.2	29.8	2.4	> 14 days
	F		25.8	23.7	2.1	> 14 days
			26.4	23.5	2.9	> 14 days
			25.8	23.7	2.1	> 14 days
			29.3	27.0	2.3	> 14 days
			27.0	25.4	1.6	> 14 days
Aqueous	M	4	35.2	32.6	2.6	> 14 days
			32.5	30.4	2.1	> 14 days
			35	31.6	3.4	> 14 days
			27.2	23.8	3.4	> 14 days
			25.9	24.1	1.8	> 14 days
	F		30	27.5	2.5	> 14 days
			28.8	25.2	3.6	> 14 days
			30	27.5	2.5	> 14 days
			29.5	26.7	2.8	> 14 days
			33.0	30.5	2.5	> 14 days

N=10.

Table 2: Acute toxicity of chloroform extract of S*chima wallichii* after intraperitoneal administration in mice.

Dose (g/kg)	Mortality(%) on different days														Total	Remarks
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
5	100	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Paralysis, lethargy, died within 1h.
4	100	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Lethargy, died within 3h.
3	-	-	20	20	-	-	20	-	-	30	-	-	-	-	90	Inactive,died before day 10.
2	-	-	-	-	20	-	30	-	-	20	-	-	-	-	70	Inactive, died within day 10.
1	-	-	-	-	-	20	20	-	-	-	-	20	-	-	60	Active,4 survived after day 12
0.5	-	-	-	-	-	-	-	10	-	10	-	20	10	-	50	Active, 5 died within day 13.

N=10.

Table 3: Acute toxicity of ethanol extract of S*chima wallichii* after intraperitoneal administration in mice.

Dose (g/kg)	Mortality(%) on different days														Total	Remarks
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
5	100	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Paralysis, lethargy, semiconscious, died within 1h.
4	100	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Paralysis, lethargy, died within 4h.
3	80	-	-	20	-	-	-	-	-	-	-	-	-	-	100	Lethargic, dullness, died within day 4.
2	50	-	20	-	-	20	-	-	-	-	-	-	-	-	90	Lethargic, dullness, died before day 7.
1	20	-	20	-	-	40	-	-	-	-	-	-	-	-	80	Dullness, died before day 7.
0.5	20	-	-	-	-	-	-	-	20	-	20	10	-	-	70	Inactive, died within day 12.
0.25	-	20	-	-	-	-	20	-	-	-	-	20	-	-	60	Inactive, died within day 12.
0.1	-	-	-	-	-	-	10	-	-	40	-	-	-	-	50	Active and only 5 died.

N=10

Table 4: Acute toxicity of aqueous extract of S*chima wallichii* after intraperitoneal administrationin mice.

Dose (g/kg)	Mortality(%) on different days														Total	Remarks
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
5	100	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Paralysis, lethargy, died within 1h.
4	100	-	-	-	-	-	-	-	-	-	-	-	-	-	100	Paralysis, lethargy, died within 3h.
3	-	-	20	-	-	40	-	-	10	-	-	-	-	-	70	Loss of appetite,inactive,died within day 9..
2	-	20	-	-	-	40	-	-	-	-	-	-	-	-	60	Inactive, died before day 7.
1	-	-	-	20	-	-	-	-	20	-	-	20	-	-	60	Inactive, died within day 12
0.5	-	-	-	-	-	-	-	-	-	30	-	20	-	-	50	Active,5 died on day 12.

N=10

Table 5: The LD50 for different extracts of *Schima wallichii* after intraperitoneal administration in mice (determined using probit analysis).

Extracts	Dose (mg/kg)	Survival %	LD_{50} (mg/kg)
Chloroform	5000	0	500
	2000	30	
	500	50	
Ethanol	5000	0	100
	500	30	
	100	50	
Aqueous	5000	0	500
	3000	30	
	500	50	

N=10

Table 6: Change s in body weight of Dalton's lymphoma bearing Swiss albino mice after intraperitoneal administration with different extracts.

Treatment (*S.Wallichii*)	Dose (mg/kg bwt)	Mean body weight (g±SEM)								
		Post tumour transplanted time(day)								
		0	1	3	6	9	12	15	18	21
SPS		26.37±0.32	26.88±0.35	27.39±0.37	28.13±0.37	29.08±0.39	31±0.51	33.26±0.53	35.24±0.39	36.82±0.48
Chloroform	50	25.77±0.43	26.16±0.43	26.64±0.42	27.24±0.41	27.82±0.40	29.91±0.43	31.88±0.48	33.18±0.27	34.27±0.25
	100	26.12±0.33	26.48±0.31	27.13±0.44	27.53±0.44	28.45±0.48	29.62±0.46	31.34±0.44	32.63±0.43	33.97±0.35
	150	25.86±0.28	26.21±0.31	26.72±0.29	27.41±0.28	28.27±0.31	29.72±0.36	31.46±0.39	33.32±0.26	34.54±0.22
	200	26.06±0.26	26.46±0.27	26.98±0.27	27.58±0.32	28.27±0.31	29.41±0.34	30.93±0.34	32.89±0.31	34.48±0.20
	250	26.03±0.27	26.48±0.22	27±0.21	27.72±0.21	28.66±0.28	29.73±0.32	31.56±0.38	32.84±0.33	34.02±0.30
Ethanol	50	25.98±0.77	26.37±0.78	27.09±0.76	27.78±0.72	28.58±0.69	29.84±0.71	31.275±0.72	32.4±0.67	35.8±0.63
	40	26.29±0.37	26.7±0.35	27.15±0.34	27.73±0.33	28.58±0.36	29.54±0.33	30.94±0.30	32.83±0.22	34.19±0.20
	30	26.66±0.39	26.37±0.39	26.92±0.39	27.53±0.36	28.11±0.36	29.68±0.33	30.69±0.30	31.62±0.33	32.69±0.33
	20	26.66±0.45	27±0.44	27.52±0.45	28.11±0.47	28.68±0.47	29.41±0.47	30.16±0.41	31.02±0.38	31.78±0.34
	10	25.98±0.55	26.31±0.56	25.76±0.57	25.87±0.59	26.42±0.57	27.3±0.55	28.37±0.51	29.49±0.39	30.36±0.37
Aqueous	50	26.25±0.29	26.73±0.29	27.43±0.29	28.02±0.28	28.59±0.29	29.83±0.20	31.59±0.25	33.24±0.24	33.96±0.20
	100	26.08±0.36	26.67±0.39	27.29±0.37	28.19±0.40	29.02±0.44	30.26±0.42	31.45±0.54	32.21±0.59	33.54±0.65
	150	25.84±0.29	26.4±0.30	27.32±0.28	28.53±0.29	29.84±0.30	31.66±0.33	32.9±0.29	34.37±0.37	36.06±0.51
	200	26.35±0.37	26.72±0.37	27.19±0.34	27.77±0.33	28.31±0.33	29.06±0.35	30.44±0.32	33±0.44	34.79±0.39
	250	26.42±0.35	26.78±0.38	27.23±0.36	27.73±0.36	28.24±0.35	29.27±0.33	30.67±0.42	31.93±0.40	33.63±0.30

N=10

Table 7: Effect of different extracts of *Schima wallichii* on the Survival of Dalton's lymphomas ascites bearing miceafter intraperitoneal administration.

Post tumor transplant time (day	Tumor free survival %															
	SPS (Control)		Chloroform					Ethanol					Aqueous				
			Dose(mg/kg b. wt.)					Dose(mg/kg b. wt.)					Dose(mg/kg b.wt.)				
			50	100	150	200	250	10	20	30	40	50	50	100	150	200	250
0	100		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
10	100		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
20	90											80					
25	50			60	90	70		90		80	90	50	80		70	70	70
30	0		70		80	50	70	80	80	70		20	50	90	40		
35				50		30	40			60	60	20	10			60	
40			50		70	10	10			50		0	0	80			60
45			30	30	50	0	0									50	
50			10	20	40					30	50			70	20	40	40
55			0	10											10		30
60				0	20			60		20	40			40	0	20	10
65					0					10						10	
70										0	0					0	
75																	
80											0			30			
85														20			
90								50	60								
100									20					0			
120								40	0								

N=10.

Table 8: Effect of different extracts of *Schima wallichii* 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,.

Treatment	Dose(mg/kg b. wt.)	MST	AST	IMLS	IALS
SPS(control)	-	21	6.3	0	0
Chloroform	50	41.444±0.212*	41.466±0.118*	97.354±1.007*	94.68±0.553*
	100	41.389±0.298*	38.6±0.033*	97.09±1.418*	81.221±0.156*
	150	47.111±0.321*	48.555±0.103*	124.339±1.525*	127.49±0.483*
	200	34.5±0.204*	32.244±0.058*	64.285 ±0.972*	51.382±0.272*
	250	36.222±0.222*	35.122±0.148*	72.487±1.058*	64.893±0.698*
Ethanol	10	72.6±0.145*	64.81±0.205*	224.137±7.38*	204.272±0.963*
	20	67.85±0.211*	85.02±0.156*	206.964±6.783*	299.155±0.733*
	30	47.25±0.227*	44.33±0.161*	119.375±3.901*	108.122±0.757*
	40	44.7±0.281*	48.97±0.17*	104.881±4.301*	129.906±0.798*
	50	25.45±0.189*	24.11±0.103*	16.637±2.433*	13.192±0.482*
Aqueous	50	32.25±0.227*	31.8± 0.146*	53.571±1.079*	49.296±0.685*
	100	55.8±0.226*	54.73 ±0.268*	165.714±1.076*	156.949±1.262*
	150	42.8±0.226*	38.3 ±0.097*	103.809±1.076*	79.812±0.459*
	200	46.95±0.273*	48.21 ±0.162*	123.571±1.302*	126.338±0.759*
	250	44.05±0.263*	45.74 ±0.324*	109.762±1.252*	114.742±1.521*

*$p<0.05$, when treatment are compared to spontaneous control group.

N=10

Table 9: Frequency of micronuclei in the Dalton's lymphoma ascites bearing mice treated with 10mg/kgb.wt. ethanol extract of *Schima wallichii* (SWE) or 0.5mg/kgb.wt. doxorubicin (DOX) at different post treatment times.

Cell type	Post treatment time (h)	Micronuclei/1000±SEM								
		SPS			SWE- 10			DOX- 0.5		
		One Mn	Two Mn	Total	One Mn	Two Mn	Total	One Mn	Two Mn	Total
Mono nucleate cell	6	5.1±0.28	0.6±0.22	5.7±0.3	31.7±0.63	0.7±0.26	32.4±0.73	36.8±1.17	3.3±0.58	40.1±0.91
	12	6.5±0.27	0.6±0.16	7.1±0.31	69.5±0.82	6.8±1.37	76.3±1.71	77.6±1.34	5.8±0.78	83.4±1.69
	24	8.8±0.57	0.9±0.31	9.7±0.45	105.3±1.54	10.7±0.84	116±2	124.4±1.22	8.6±0.64	133±1.32
	48	8.1±0.23	0.7±0.21	8.8±0.33	91.9±1.58	10±0.70	101.9±1.66	119.1±1.43	8.9±0.61	128±1.61
Binucleate cell	6	6.8±0.2	1±0.33	7.8±0.33	33.2±0.51	0.8±0.25	34±0.61	38.9±1.49	4.7±0.82	43.6±1.55
	12	7.6±0.22	0.5±0.17	8.1±0.23	71.9±0.56	7±1.26	78.9±1.56	84.4±1.36	7.7±0.58	92.1±1.39
	24	10.3±0.3	0.6±0.22	10.9±0.38	109.5±1.96	12.1±0.75	121.6±1.91	132.2±1.12	12.6±0.96	144.8±1.59
	48	8.9±0.43	0.7±0.21	9.6±0.56	99.8±2.10	9.4±1.00	109.2±1.93	121.6±1.17	8.2±0.66	129.8±1.39

N=5

Table 10: Induction of apoptosis and necrosis in Dalton's lymphoma ascites bearing mice treated with 10mg/kgb.wt. ethanol extract of *Schima wallichii* (SWE) or 0.5mg/kgb.wt. doxorubicin (DOX) at different post treatment times.

Post treatment time(h)	Mean±SEM					
	SPS		SWE 10		DOX 0.5	
	Apoptotic	Necrotic	Apoptotic	Necrotic	Apoptotic	Necrotic
2	0.72±0.03	0.36±0.03	5.08±0.11	2.21±0.07	5.57±0.13	3.53±0.15
6	0.95±0.05	0.55±0.03	8.97±0.14	4.06±0.12	10.58±0.14	5.43±0.11
12	1.17±0.04	0.78±0.03	11.44±0.14	5.5±0.12	13.43±0.12	6.49±0.13
24	1.36±0.05	0.8±0.03	14.92±0.17	6.59±0.12	16.69±0.19	8.55±0.14
48	2.22±0.05	0.82±0.03	13.16±0.14	6.41±0.09	18.13±0.13	8.99±0.11

N=5.

Table 11: Changes in the glutathione contents of mice bearing Dalton's lymphoma ascites treated with 10 mg/kg b. wt. *Schima wallichii* ethanol extract (SWE) or doxorubicin(DOX).

Post Treatment Time(h)	µM/mg protein ± SEM		
	SPS	SWE	DOX
2	14.92± 0.005	11.21 ± 0.16*	9.72 ± 0.05*
4	14.76 ± 0.03	9.88 ± 0.22*	9.12 ± 0.03*
8	14.50± 0.01	9.42 ± 0.11*	8.50 ± 0.04*
12	14.04± 0.02	9.16± 0.04*	7.80 ± 0.008*
24	13.75± 0.03	8.95 ± 0.006*	7.26 ± 0.02*

p<0.05 when treatment are compared with concurrent control (SPS) group.
No symbol= no significance.
N=10

Table 12: Changes in the glutathione-s-transferase activity in mice bearing Dalton's lymphoma treated with *Schima wallichii* ethanol extract (SWE) or doxorubicin(DOX).

Post Treatment Time (h)	U/mg protein ± SEM		
	SPS	SWE	DOX
2	0.082± 0.002	0.050 ±0.002*	0.047 ±0.003*
4	0.080 ±0.001	0.049 ±0.003*	0.045 ±0.002*
8	0.079 ±0.002	0.046 ±0.004*	0.038 ±0.003*
12	0.077 ±0.001	0.041 ±0.003*	0.035 ±0.002*
24	0.074 ±0.005	0.040 ±0.002*	0.032 ±0.002*

p<0.05 when treatment are compared with concurrent control (SPS) group.
No symbol= no significance.
N=10

Table 13: Alterations in the catalase activity of mice bearing Dalton's lymphoma treated with *Schima wallichii* ethanol extract (SWE) or DOX.

Post Treatment Time (h)	U/mg protein ± SEM		
	SPS	SWE	DOX
2	19.79± 0.11	16.70± 0.16*	14.62 ±0.18*
4	19.56 ±0.11	15.47 ±0.20*	13.36 ±0.09*
8	19.49± 0.08	13.62 ±0.13*	11.69 ±0.14*
12	19.39± 0.12	12.71± 0.18*	10.35 ±0.11*
24	19.52± 0.16	11.46± 0.21*	10.42 ±0.17*

p<0.05 when treatment are compared with concurrent control (SPS) group.
No symbol= no significance.
N=10

Table 14: Changes in the superoxide dismutase activity in mice bearing Dalton's lymphoma treated with *Schima wallichii* ethanol extract (SWE) or DOX.

Post Treatment Time (h)	U/mg protein ± SEM		
	SPS	SWE	DOX
2	1.81 ± 0.05	1.62 ± 0.028 *	1.50 ± 0.06*
4	1.804 ± 0.03	1.41 ± 0.02*	1.35 ± 0.01*
8	1.814 ± 0.04	0.91 ± 0.06*	0.78 ± 0.04*
12	1.79 ± 0.069	0.70 ± 0.09*	0.51 ± 0.01*
24	1.81 ± 0.07	0.83 ± 0.05*	0.49 ± 0.03*

p<0.05 when treatment are compared with concurrent control (SPS) group.
No symbol= no significance.
N=10

Table 15: Alterations in the lipid peroxidation in mice bearing Dalton's lymphoma treated with *Schima wallichii* ethanol extract (SWE) or DOX.

Post Treatment Time (h)	Lipid peroxidation (MDA) Mean ± SEM, (nmol /mg protein)		
	SPS	SWE	DOX
2	0.32 ±0.005	3.27± 0.005*	4.09 ±0.010*
4	0.43 ±0.020	3.61 ±0.015*	5.69 ±0.022*
8	0.50± 0.003	4.55 ±0.110*	7.87 ±0.006*
12	0.51 ±0.001	4.18 ±0.018*	6.59 ±0.144*
24	0.56± 0.013	3.92 ±0.010*	5.94 ±0.007*

p<0.05 when treatment are compared with concurrent control (SPS) group.
No symbol= no significance.
N=10

Table 16: Effect on the liver and kidney toxicity of Dalton's lymphoma bearing mice treated with *Schima wallichii* ethanol extract (SWE) or DOX.

Treatment	Time (h)	Mean±SEM			
		AST	ALT	Creatinine	Uric acid
control	2	212.3± 0.76	40.55± 0.09	0.34± 0.04	2.62± 0.12
	4	223.85± 0.71	41.24± 0.05	0.38 ±0.05	2.96 ±0.31
	8	228.5 ±0.99	42.26± 0.07	0.42± 0.06	3.3 ±0.35
	12	231.12± 0.39	42.79 ±0.03	0.46± 0.05	3.54± 0.28
	24	235.65 ±0.52	43.44 ±0.06	0.5 ±0.07	3.74± 0.19
Dox 0.5	2	244.62± 0.59	51.54± 0.03	0.24 ±0.02	2.4 ±0.09
	4	247.61± 0.29	51.86 ±0.02	0.28 ±0.05	2.9 ±0.28
	8	250.87 ±0.34	51.87± 0.15	0.32± 0.02	3.14 ±0.29
	12	255.22 ±0.31	52.38 ±0.02	0.34 ±0.04	3.34 ±0.26
	24	257.89± 0.65	52.83± 0.02	0.36± 0.02	3.48± 0.25
SWE 10	2	251.26± 0.29	54.55± 0.77	0.32± 0.05	2.4 ±0.12
	4	275.1 ±0.31	63.78± 0.57	0.38± 0.06	2.56± 0.35
	8	367.23± 0.62	73.34 ±0.48	0.44± 0.04	2.9 ±0.36
	12	333.79 ±0.82	45.25 ±0.62	0.52± 0.06	3.1± 0.43
	24	330.93± 0.33	35.62± 0.64	0.54 ±0.02	3.28± 0.16

N=10

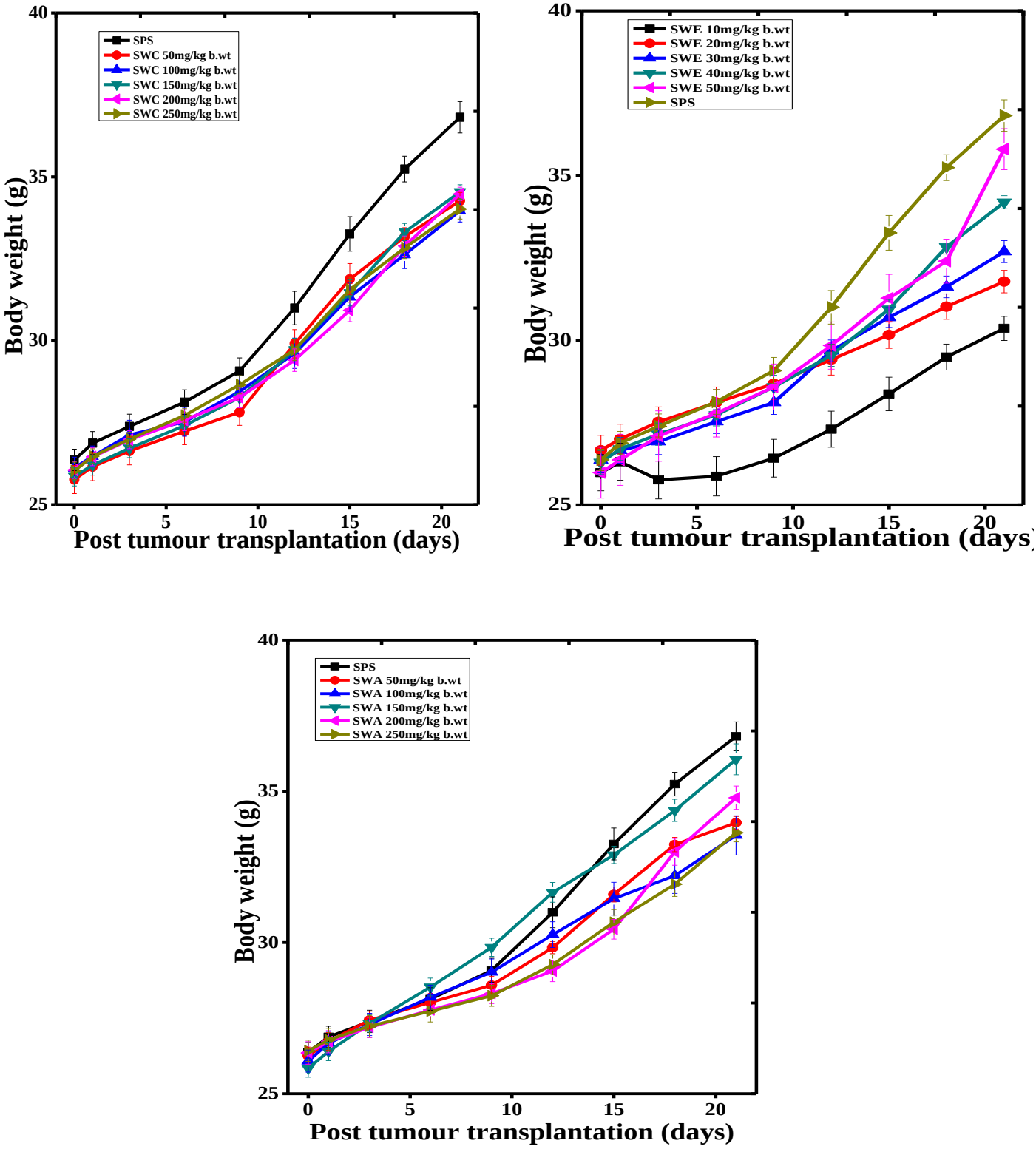

Figure 1: **Change in body weight of Dalton's lymphoma bearing Swiss albino mice after treatment with different concentrations of the various extract of *Schima wallichii.* Left: Chloroform ; Right: Ethanol ; Middle: Aqueous. The data are expressed as Mean ±SEM ,N=10.**

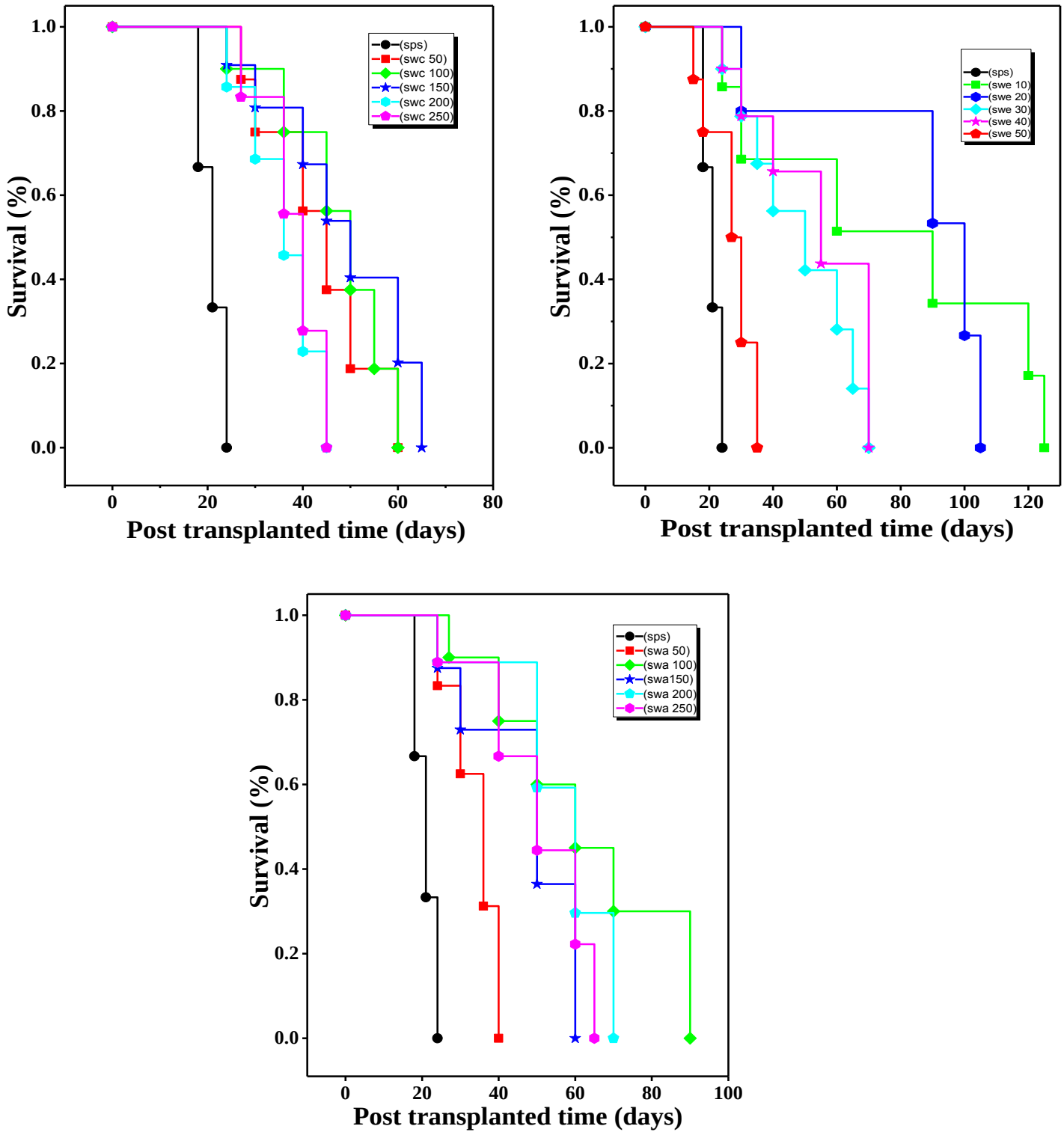

Figure 2: Effect on the survival of Dalton's lymphoma ascites bearing mice treated with various extracts of *Schima wallichii* for 9 days consecutive. Left: Chloroform, Right: Ethanol, Middle: Aqueous extracts of *Schima wallichii*. N=10/dose of each extract.

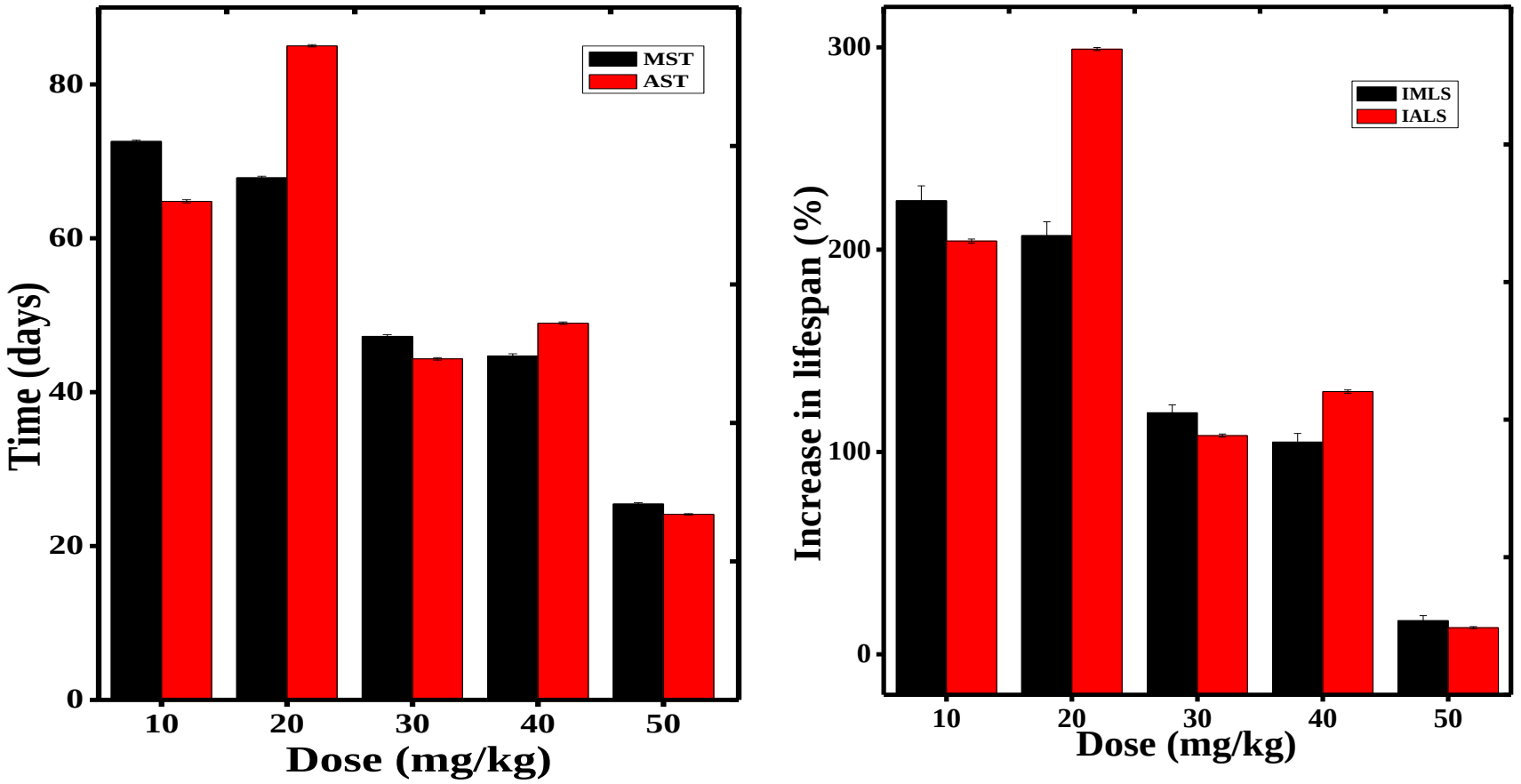

Figure 3: Effect of ethanol extract of *Schima wallichii* on Dalton's lymphoma ascites bearing mice on the tumor response. Left: Median survival time (MST), Average survival time (AST) ; Right: Increase in median life span (% IMLS) and Increase in average life span (% IALS).The results are expressed as Mean ± SEM, N=10

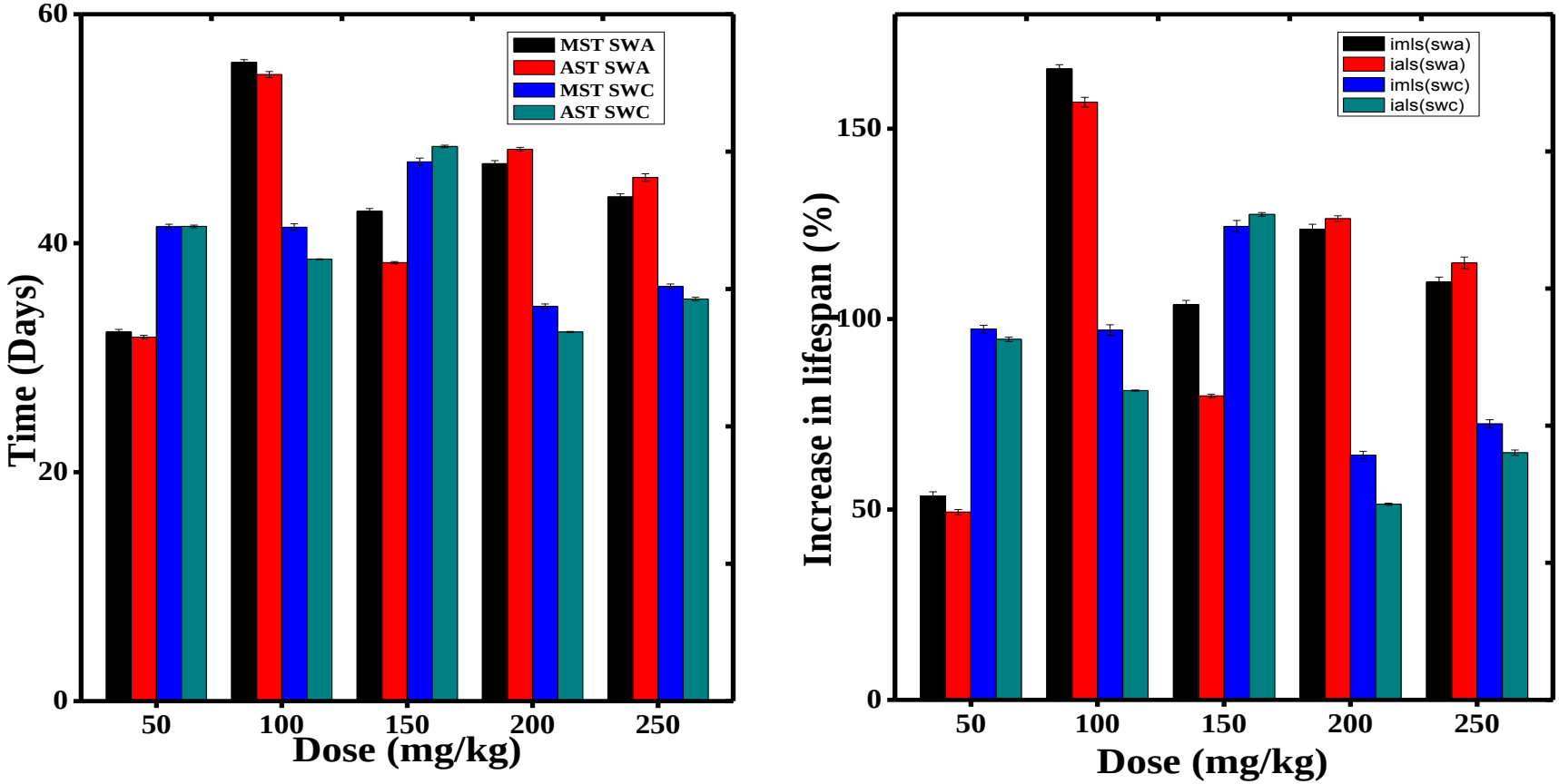

Figure 4 : Effect of chloroform and aqueous extracts of *Schima wallichii* on Dalton's lymphoma ascites bearing mice on the tumor response assessment. Left: Median survival time (MST), Average survival time (AST) , Right: Increase in mean life span (% IMLS), increase in average life span (% IALS).The result expressed as Mean ± SEM, N=10.

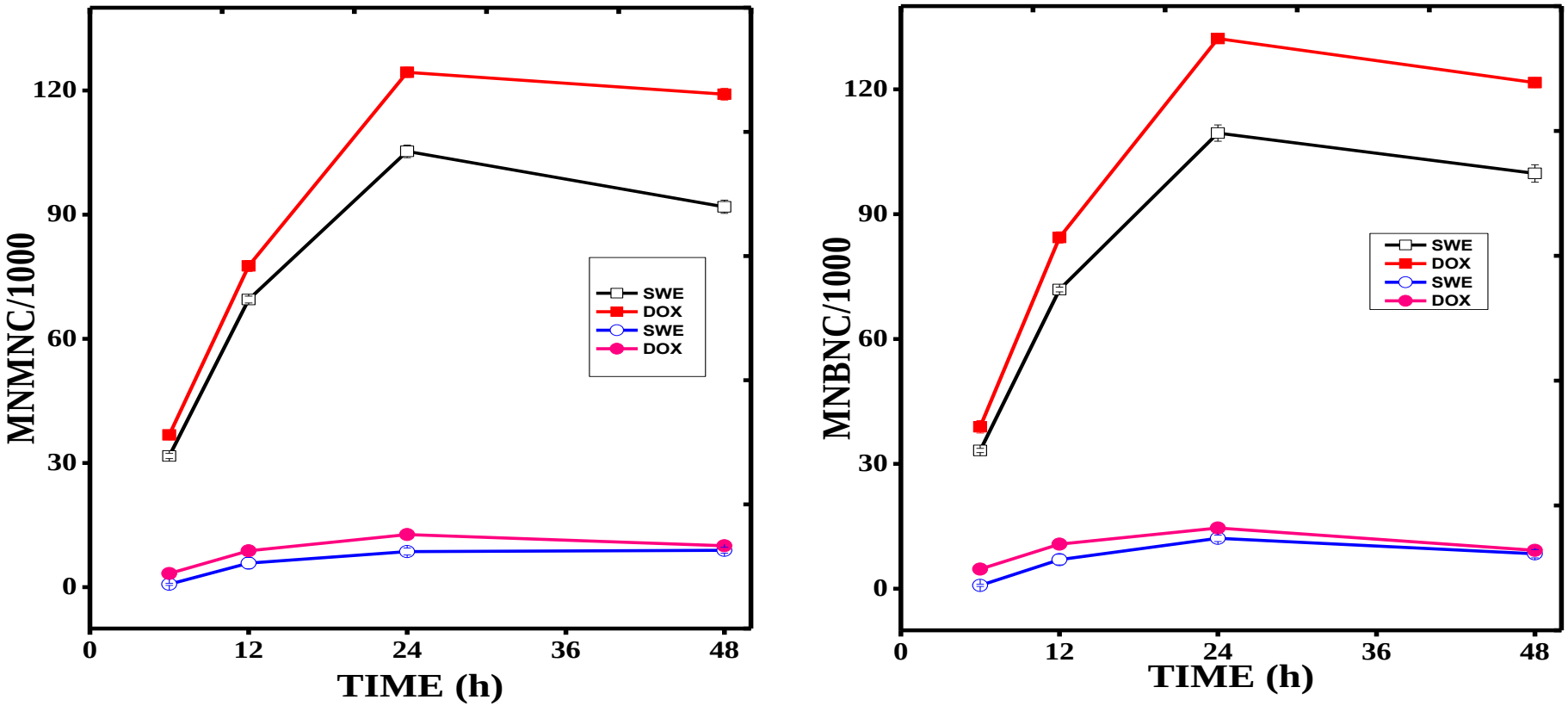

Figure 5 : Induction of micronuclei in Dalton's lymphoma ascites bearing mice by treated with 10mg/kgb.wt. ethanol extract of *Schima wallichii* (SWE) or 0.5mg/kg b.wt. doxorubicin (DOX) at different post treatment times. Left: Micronucleated mononucleate cells and Right: Micronucleated binucleate cells. Closed squares: One micronuclei (DOX); Open squares: One micronuclei (SWE) and Closed circles: Two micronuclei (DOX); open circles: two micronuclei (SWE). N =5 for each assay time.

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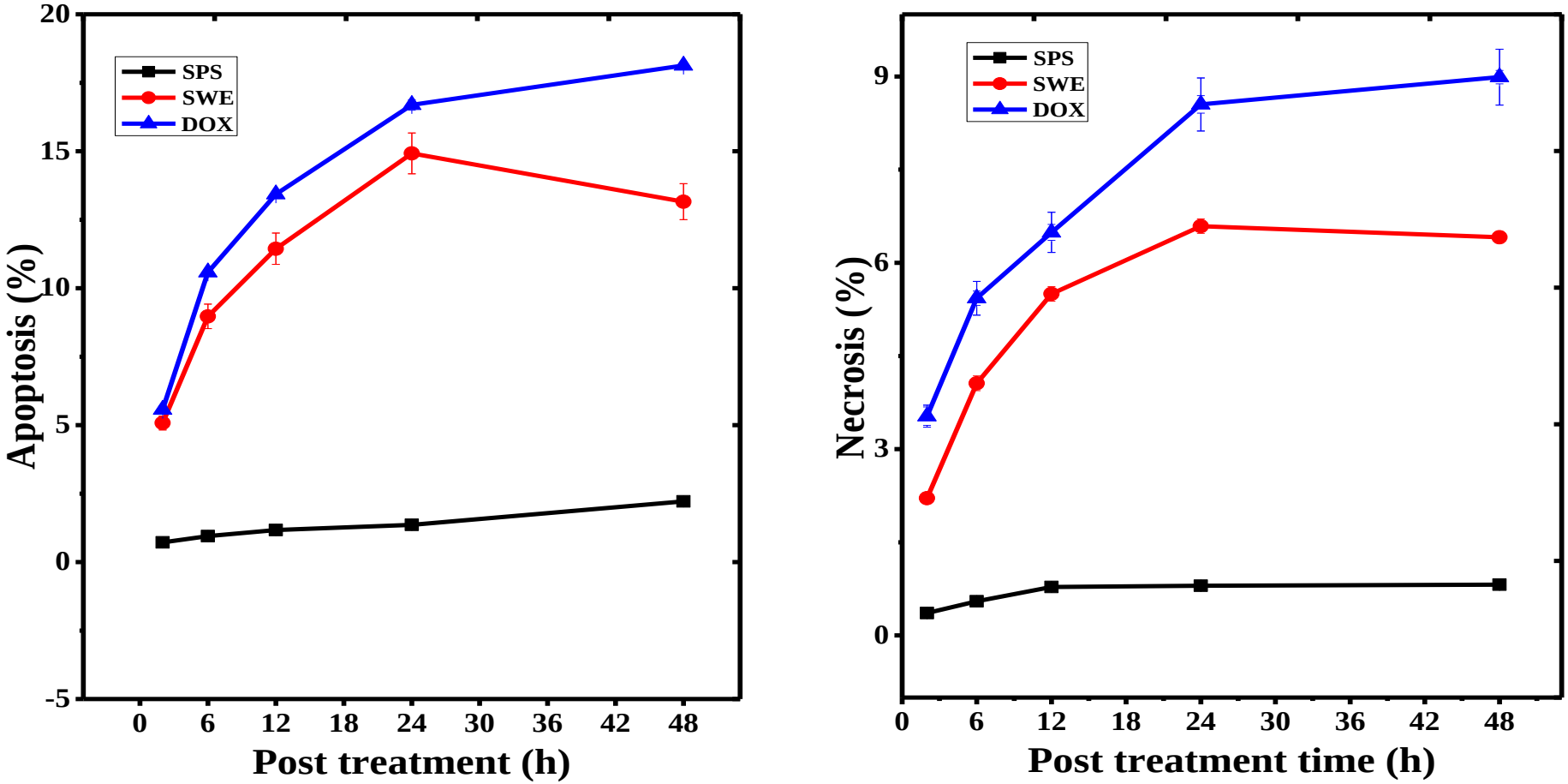

Figure 6 : Apoptosis and necrosis index in Dalton's lymphoma ascites bearing mice treated with 10mg/kgb.wt. ethanol extract of *Schima wallichii* (SWE) or 0.5mg/kgb.wt. doxorubicin (DOX) at different post treatment times. Left: Apoptosis and Right: Necrosis. N=5 for each assay time.

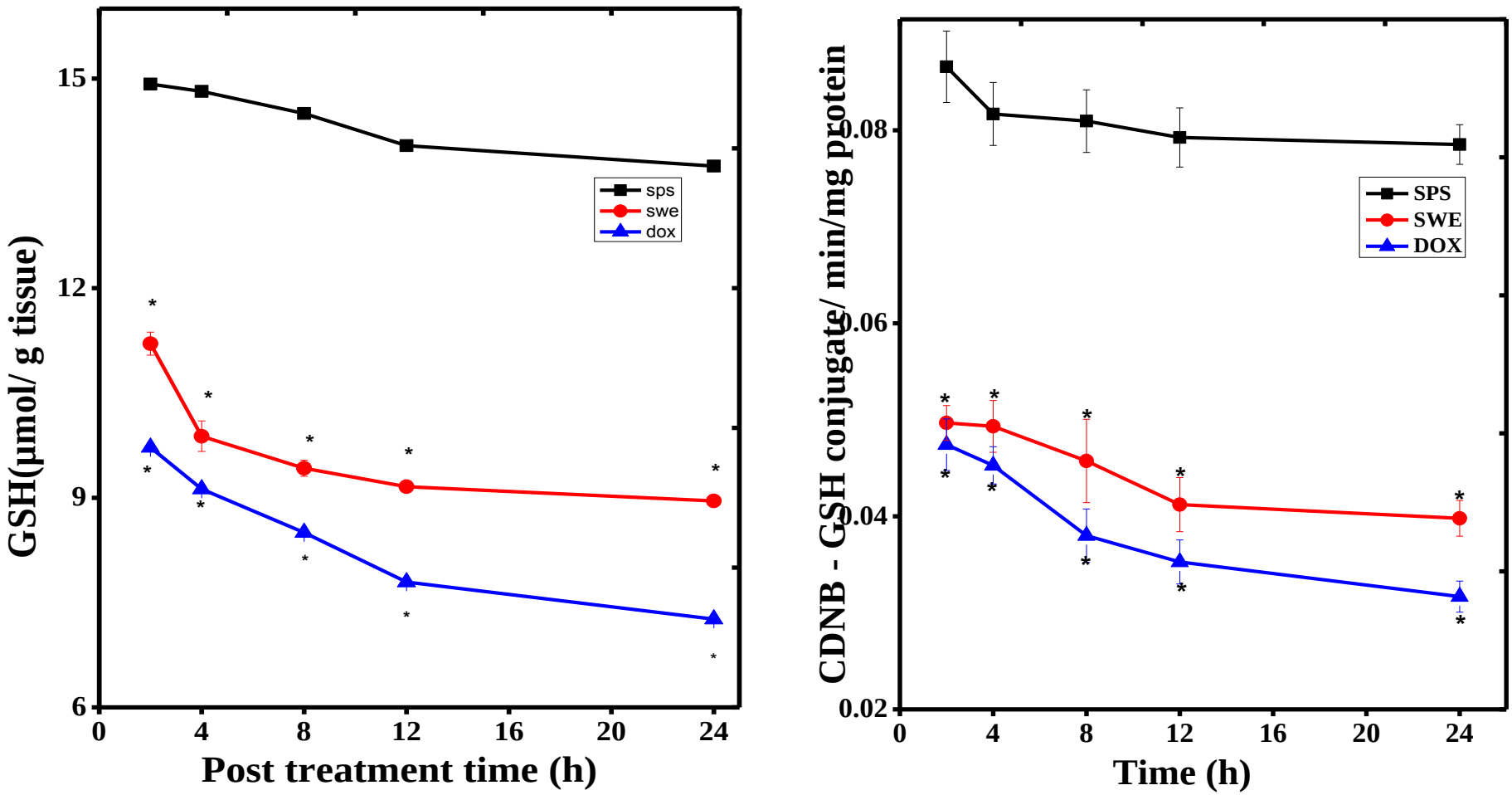

Figure 7 : Alteration in the glutathione content (left) and glutathione-s-transferase (GST) activity (right) of mice bearing Dalton's lymphoma ascites treated with various doses of *Schima wallchii* ethanol extract (SWE)and doxorubicin (DOX). The results expressed as the Mean ± SEM, n=10. **p<0.05when treatment groups are compared with concurrent control (SPS) group. No symbol= no significance.*

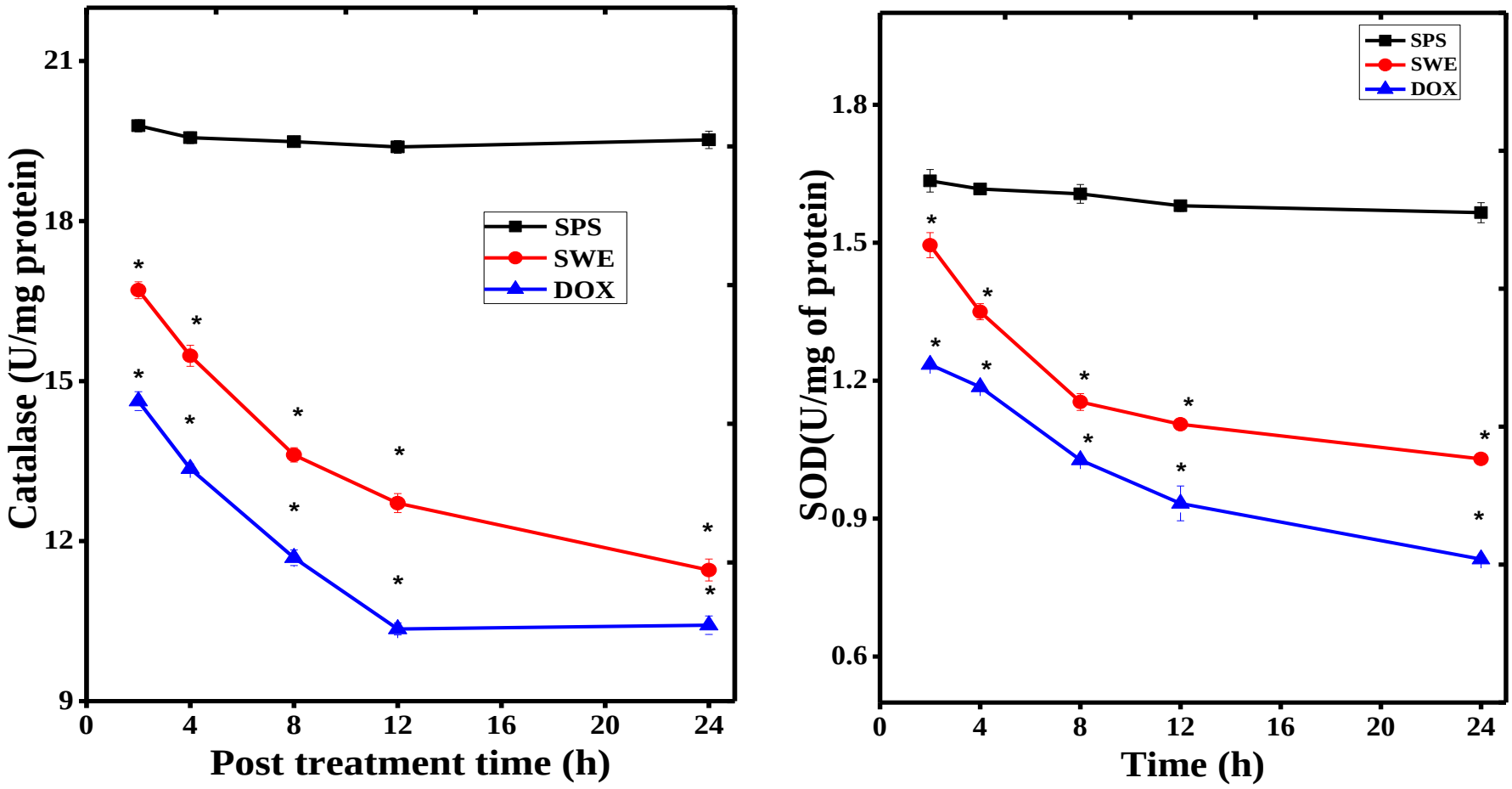

Figure 8: Alteration in the Catalase (left) and Superoxide dismutase (SOD) (right) activities of mice bearing Dalton's lymphoma ascites treated with various doses of *Schima wallchii* ethanol extract (SWE) and doxorubicin (DOX). The results expressed as Mean ± SEM, n=10. **p<0.05when treatment are compared with concurrent control (SPS) group. No symbol= no significance*

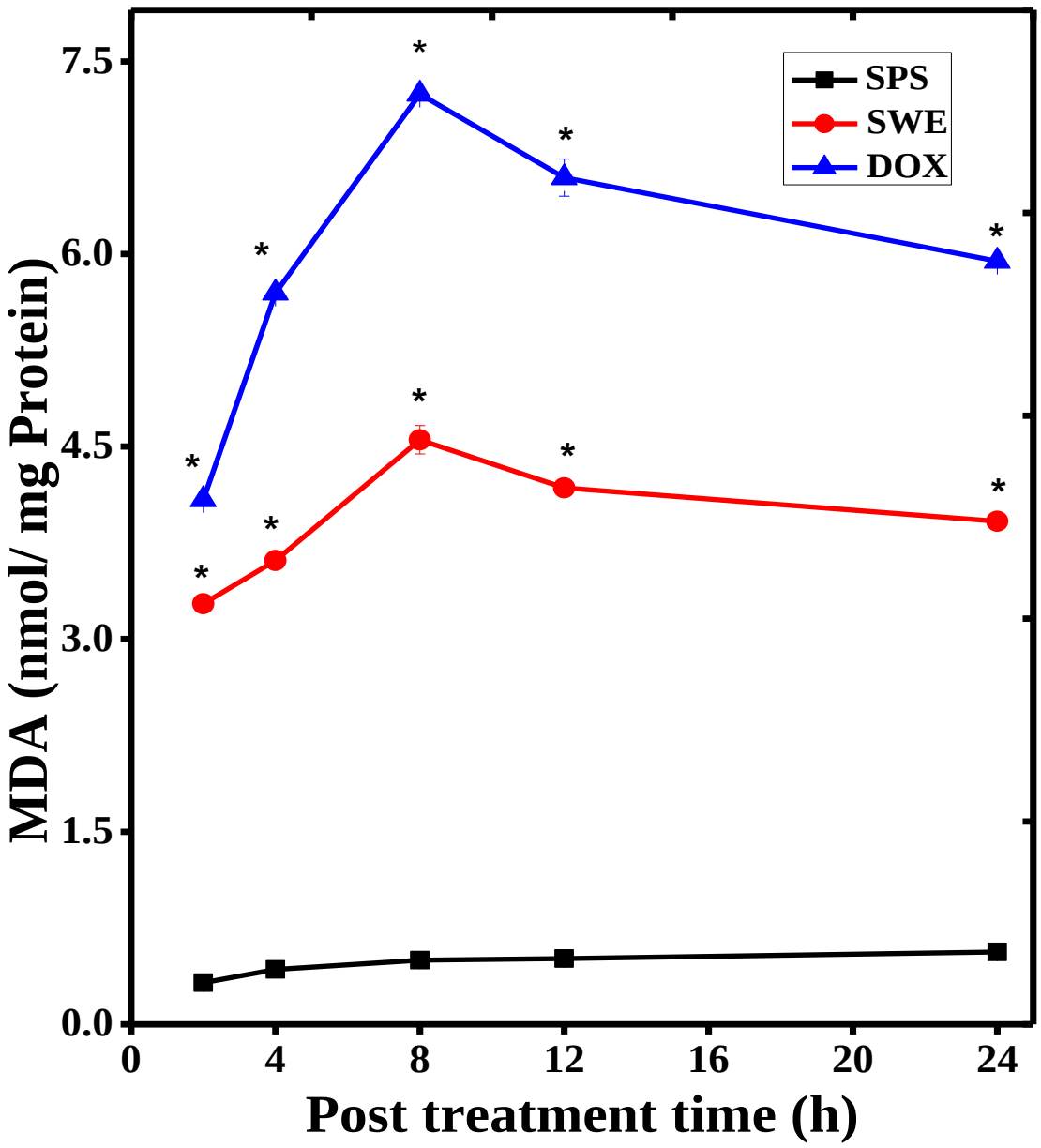

Figure 9: Alteration in the Lipid peroxidation (LOO) level of mice bearing Dalton's lymphoma ascites treated with various doses of *Schima wallchii* ethanol extract (SWE) and doxorubicin (DOX). The results expressed as the Mean ± SEM, n=10.p<0.05when treatment are compared with concurrent control (SPS) group.***
No symbol= no significance

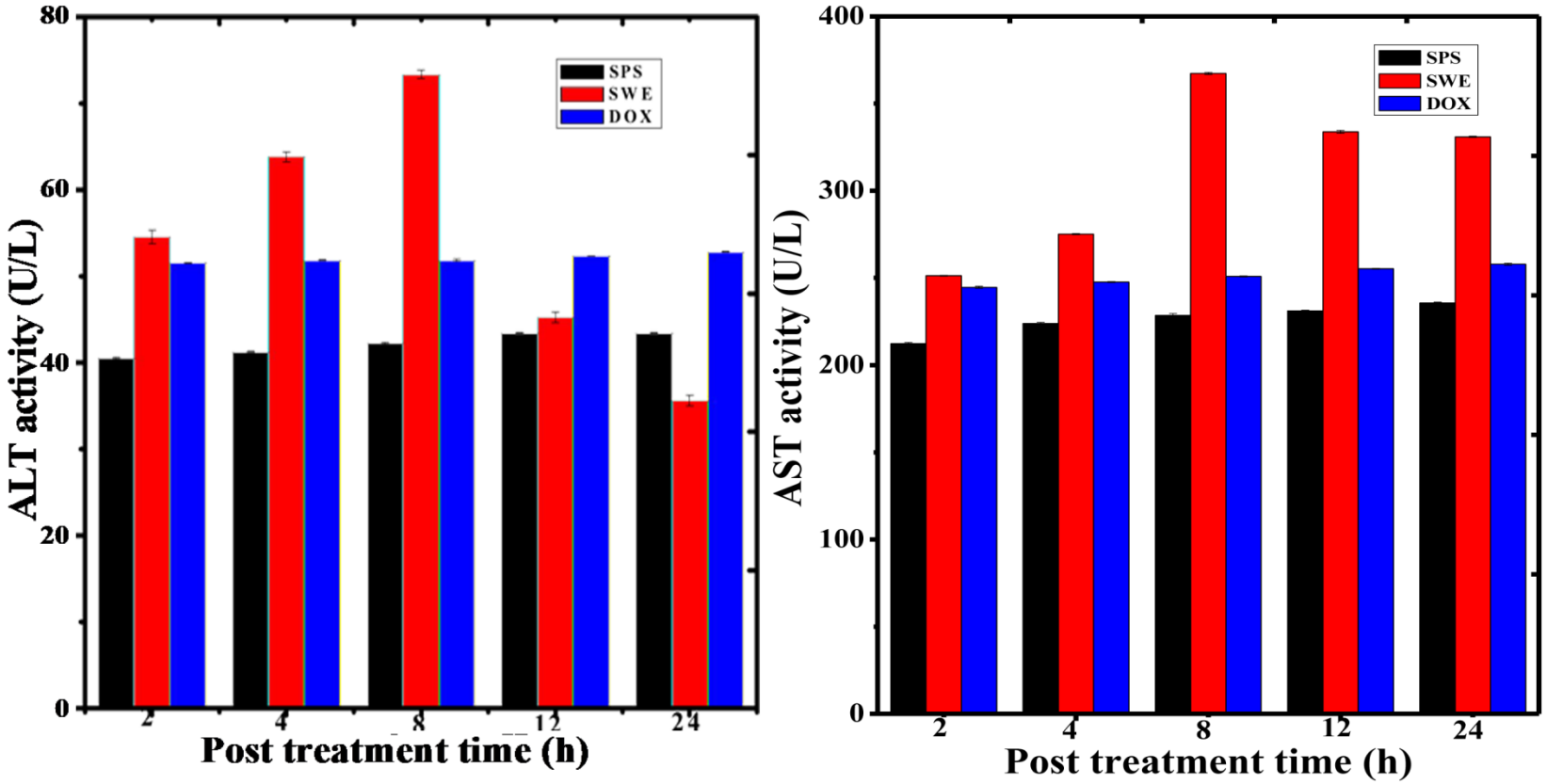

Figure 10 : Determination of ALT activity (left) and AST activity (right) by10 mg/kg b.wt of SWE and 0.5 mg/kgb.wt of DOX on Dalton's lymphoma ascites bearing mice. The results present the Mean ± SEM, N=10. $p<0.05$.

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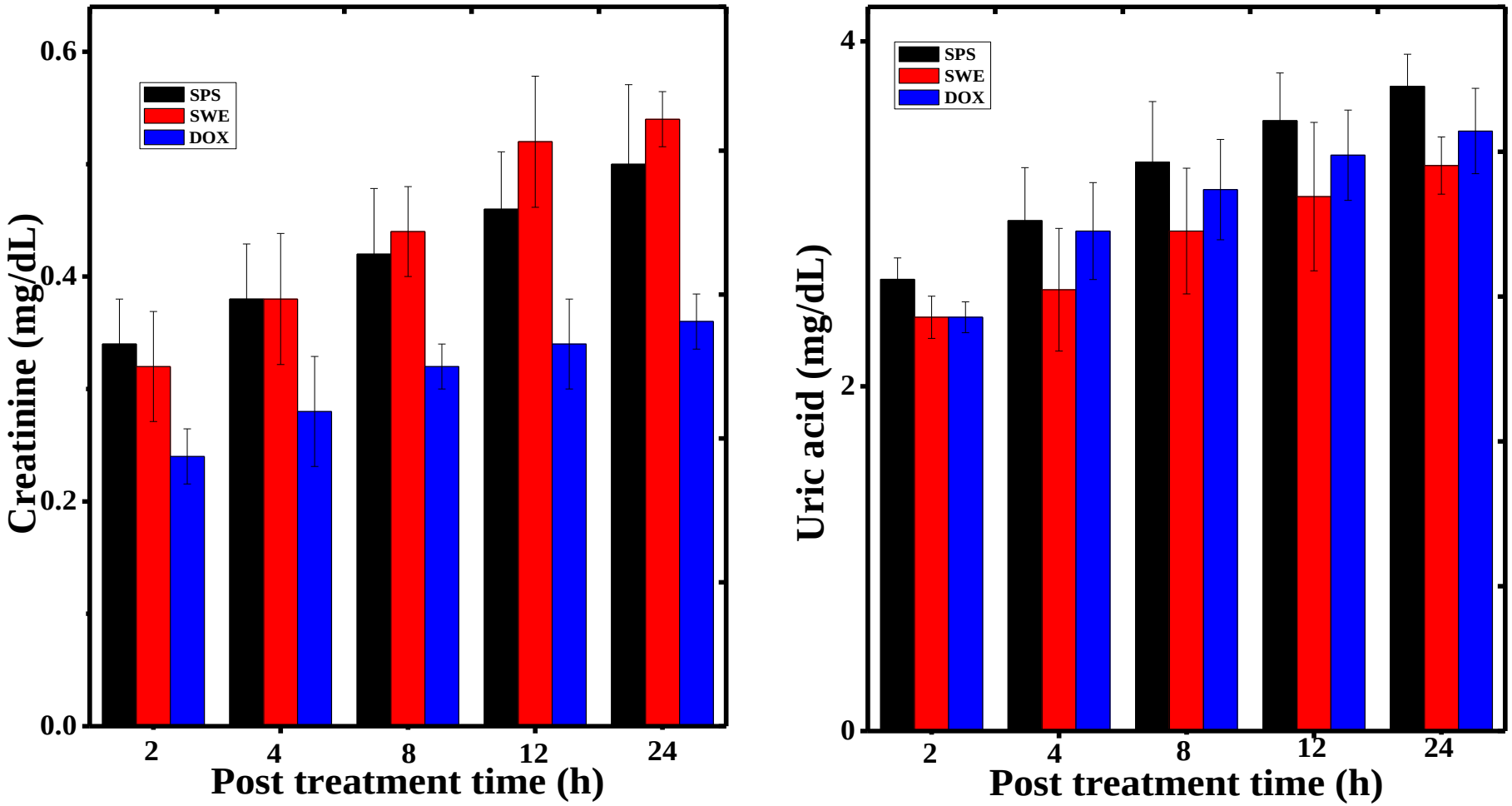

Figure 11 :Determination of Creatinine level (left) and Uric acid level (right) by10 mg/kg b.wt of SWE and 0.5 mg/kgb.wt of DOX on Dalton's lymphoma ascites bearing mice. The results present the Mean ± SEM, N=10. p<0.05.

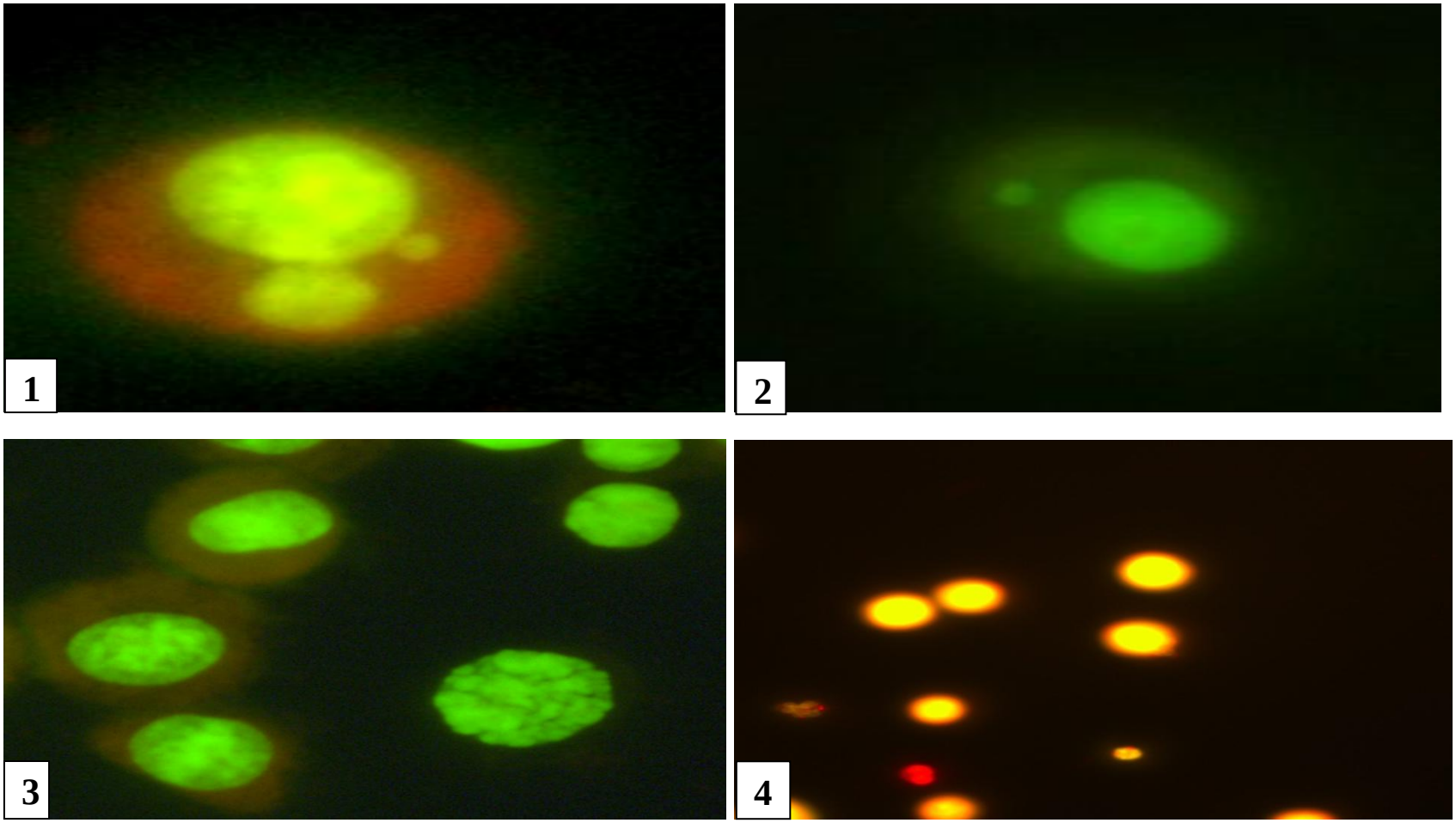

PLATE 4: 1) One micronucleus binucleated cell 2) One micronucleus mononucleated cell 3) Apoptotic cell 4) Necrotic cell

CHAPTER 6

Summary and conclusions.

Chapter 6

INTRODUCTION

Cancer is a severe threat to human health and affects the lives of millions of people around the world. It drains financial and emotional resources of a family in which cancer is detected. It is the second largest cause of death succeeding cardiovascular diseases (Siegel *et al*., 2015). Cancers are a large family of diseases involving abnormal cell growth and having the potential to invade or spread into other parts of the body.(WHO,2014; National Cancer Institute, 2014). All tumor cells show six main properties or characters of cancer cell. These characteristics are the main requirements to produce a malignant tumor.

The prominent properties of cancer are: lack of differentiation of cells, local invasion of adjoining tissue, and often, metastasis (spread to distant sites through the bloodstream or the lymphatic system). The immune system likely plays a significant role in eliminating early cancers or premalignant cells because immunodeficiency states are associated with an increased incidence of various kinds of cancer, particularly those associated with viral infection and tumors arising in the lymphatic system and the skin.

Cancer does not show clear sign and symptoms as they are great imitator. It is a very common mistake that people diagnosed with cancer are often treated for other disease. General symptoms of cancer may include unintentional weight loss, fever, excessive fatigue and changes inthe skin (O'Dell, 2009). Hodgkin disease, leukemias and cancers of the liver or kidney can cause a persistent fever (Bodel, 1974). People may become anxious or depressed post-diagnosis of cancer (Anguiano *et al*., 2012). Man power, natural and many material resources are spent for research purpose and in the development of new drugs for prevention and treatment of cancers each year worldwide. The impact of cancer on economic is significant and is rising to a high level. It has been estimated that the total annual economic cost of cancer in 2010 was approximately US$

1.16 trillion (Stewart BW, 2014). The present cancer treatments include radiotherapy, chemotherapy and chemically derived drugs. The application of chemotherapy can put cancer patients under a lot of pressure as it may be responsible for further serious damage to their health.

Plants and natural products have mainly contributed to the evolution of the effective and reliable traditional medicinal practices that have been employed for thousands of years in China, India and many other countries (Sneader, 2005). Different medicines from plant and health products have been accepted by people from all over the world, looking forward to improving the quality of life, disease prevention and treatment of chronic diseases and geriatric diseases as well as western medicine with helpless mysterious illness (Song *et al*., 2014). The interest in medicinal plants as major breakthrough in health aid has been put forwarded into great milestone by the rising costs of prescription drugs for maintaining healthy conditions of a person and well being and the bioprospecting of new drugs derived from plants could be more economic (Lucy and Edgar, 1999).

Schima wallichii (DC.) Korth. or Chilauni (Family:Theaceae) is an Asian species of evergreen tree. The genus inhabits warm temperate to subtropical climates across southern and South East Asia, from the eastern Himalaya of Nepal to eastern India across Indochina, Southern China, Taiwan and the Ryukyu Islands. Its common name is needlewood tree and it usually grows up to 35 m high. However, in some places it may be seen only 40 ft high (Min *et al*., 2003). Locally, it is called “khiang” in Mizo language and it finds many medicinal uses. *Schima wallichii* has been reported to possess several medicinal properties (Dewanjee *et al*., 2008; Gurung, 2002; Lalrinzuali, 2015; Sam *et al*., 2004; Lalfakzuala *et al*., 2007; Gardner *et al*., 2000; Koshimizu *et al*., 1998; Subarnas *et al*., 2003; Diantini *et al*., 2012; Paudel, 2014).

AIM OF THE STUDY

Schima wallichii has been reported to possess several medicinal properties in which the bark and the leaves are normally used. The leaves are reported to have antitumor and antimutagenic properties (Koshimizu *et al*., 1998; Subarnas *et al*., 2003) and a compound isolated from the leaves inhibited MCF-7 breast cancer cell proliferation through activation of the caspase cascade pathway (Diantini *et al*., 2012). However, the antineoplastic activity of the bark extract is not reported. Therefore, the present study was carried out to determine the anticancer activity of *Schima wallichii* extracts *in vitro* and *in vivo* by carrying out the following studies:

1. Fraction guided preparation of *Schima wallichii* extracts.
2. Phytochemical analysis
3. Antioxidant activity
4. Evaluation of anticancer activity *in vitro* and *in vivo*.

CHAPTER 1

This chapter refers to the general account of cancer, its history, types, causes and the different stages of cancer. The various kinds of cancer treatment modalities, importance of palliative care and the harmful effects are also highlighted and mentioned. It also gives the different medicinal uses of *Schima wallichii* and the general information about the plant used in this research work. Detail description on the aim and scope of the thesis is clarified in the following chapters.

CHAPTER 2

This chapter deals with the phytochemical screening and the TLC profiling of *Schima wallichii* and the extraction process. The non-infected stem bark of *Schima wallichii* was collected from Bazar veng, Lunglei, Mizoram during the months of April and May and identification was done at BSI, Shillong. The bark was cleaned and shade dried at room temperature in clean and hygienic conditions. The dried bark was then powdered using an electrical grinder. The dried powder of *Schima wallichii* was extracted sequentially 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 them to dryness under reduced pressure. The concentrated extracts were stored at -80°C until use. Qualitative phytochemical analysis was performed on the bark powder as well as the different extracts excluding petroleum ether. The bark powder was found to contain various phytochemicals other than phlobatannins. Flavonoids, saponins, cardiac glycoside, phenols are present in all the extracts whereas tannins and alkaloids are absent in the chloroform extracts. The quantitative phytochemical analysis showed the differences in the alkaloids, saponins and flavonoids content among the different extracts. The yield percentage, ash content and moisture content of the plant powder was also

determined. The TLC profiling also showed the presence of different components as indicated by the different Rf values in different solvent systems.

CHAPTER 3

Chapter 3 gives an account on the free radical scavenging activity and the antioxidant potential of the different extracts of *Schima wallichii in vitro*. The free radicals or simply ROS are highly reactive species which can be generated by cells during respiration, and cell-mediated immune functions. They are produced naturally in the body as they play an important role in many cellular functions however; if their production is quite high, the damages they can cause may play a role in the development of various human health disorders including cancer. However, the plants and natural products could be a major source of antioxidants that can scavenge free radicals. The antioxidant activity was estimated by investigating the ability of the different extracts of *Schima wallichii* to inhibit the generation of DPPH, superoxide anion, hydroxyl, nitric oxide, ABTS, FRAP free radicals using standard protocols. The total phenols and total flavonoids were also evaluated. The various extracts of *Schima wallichii* inhibited the production of DPPH, superoxide anion, hydroxyl, nitric oxide, ABTS, FRAP free radicals in a concentration dependant manner. The free radical scavenging activity increased upto a certain concentration which remained unaltered thereafter. The ethanol extracts was found to show the highest free radical scavenging activity among the three extracts. The amount of total phenols and flavonoids also increased with increasing concentration and the maximum total phenol contents was recorded for ethanol extract while total flavonoids was observed at aqueous extract. Therefore, the antioxidant and free radicals scavenging activities of *Schima wallichii* may be due the presence of various phytochemicals mostly alkaloids which is the highest phytochemical content determined.

CHAPTER 4

In this chapter, *in vitro* anticancer activity of the ethanol extract of *Schima wallichii* was determined using different assay following standard protocols. The cytotoxicity of the ethanol extract of *Schima wallichii* (SWE) was assessed in HeLa and V79 cells by MTT assay. The HeLa and V79 cells treated with different concentrations of SWE showed a concentrations dependent increase in its cytotoxic effect. Treatment of cells with SWE for different durations also increased its cytotoxic effects in a time dependent manner. The results of MTT assay were confirmed by clonogenic assay in HeLa cells, where the cells were treated with different concentrations of SWE. Treatment of HeLa cells with various concentrations of SWE reduced the clonogenicity of cells in a concentration dependent manner. The ability of SWE to induce apoptosis was studied by determining the caspase 8 and 3 activities at different post- treatment times. The SWE treatment marginally increased activity of both capsase 8 and 3 in a time dependent manner. The effect of SWE treatment was studied on the lipid peroxidation, glutathione contents, glutathione-s-transferase, catalase and superoxide dismutase activities, where it was found to reduce the glutathione contents, and activities of glutathione-s-transferase, catalase and superoxide dismutase in a time dependent manner. Treatment of HeLa cells with ethanol extract of *Schima wallichii* increased the cytotoxic effect in a concentration dependent manner followed by a reduction in the clonogenicity of HeLa cells. The cell killing effect of SWE may be due to the caspase activation and reduction in the glutathione contents, and activities of glutathione-s-transferase, catalase and superoxide dismutase.

CHAPTER 5

In this chapter, the acute toxicity of *Schima wallichii* was evaluated both orally and intraperitoneally in which the oral administration did not showed toxicity up to 2g/kg b.wt for ethanol extract while 4g/kg b.wt.for chloroform and aqueous extracts, respectively. However, the LD_{50} was found to be 100mg/kg b.wt. for ethanol extract and 500mg/kg b.wt for chloroform and aqueous extracts, respectively when these extracts were administered intraperitoneally. The administration of 10- 250 mg/kg b.wt. chloroform, ethanol and aqueous extracts of *Schima wallichii* to tumor bearing mice resulted in a dose dependent increase in the tumor free survival and maximum effect was observed for 10 mg/kg ethanol extract, which increased the tumor free survival by 40% beyond 120 days. 20 and 40 % long term tumor free survivors were observed up to 60 days for chloroform and aqueous extracts at 150 and 100 mg/kg, respectively. The administration of 10mg/kg b. wt. ethanol extract resulted in an increase in the AST up to 64.81 days (IALS, 204.27%) and MST up to 72.6 days (IMLS, 224.14 %). Therefore, 10 mg/kg body weight of the ethanol extract was considered as an optimum dose for its antineoplastic activity and further investigations were carried out using this dose. The administration of 10 mg/kg body weight ethanol extract into tumorized mice resulted in a time dependent rise in the micronuclei in both mononucleate and binucleate cells up to 24 h. The analysis of apoptotic and necrotic index also showed a time dependent increase and the maximum rise was observed at 36 h post treatment in the tumorized mice receiving 10 mg/kg body weight of ethanol extract of *S. wallichii*. 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 *Schima wallichii* 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.

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BIODATA

NAME: **K. LALHMINGHLUI**

FATHER'S NAME: **K. REMSANGA**

GENDER: **FEMALE**

ADDRESS: **BAZAR VENG, LUNGLEI**

QUALIFICATION: **M.Sc (Zoology)**

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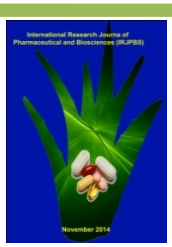

Evaluation of the anticancer activity of Chilauni, *Schima wallichii* (DC.) Korth. in vitro

K. Lalhminghlui and Ganesh Chandra Jagetia*

Department of Zoology, Mizoram University, Aizawl -796004, India.

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***Corresponding author:**
gc.jagetia@gmail.com

Abstract

The cytotoxicity of the ethanol extract of *Schima wallichii* (SWE) was assessed in HeLa and V79 cells by MTT assay. The HeLa, and V79 cells treated with different concentrations of SWE showed a concentrations dependent increase in its cytotoxic effect. Treatment of cells with SWE for different durations also increased its cytotoxic effects in a time dependent manner. The results of MTT assay were confirmed by clonogenic assay in HeLa cells, where the cells were treated with different concentrations of SWE. Treatment of HeLa cells with various concentrations of SWE reduced the clonogenicity of cells in a concentration dependent manner. The ability of SWE to induce apoptosis was studied by determining the caspase 8 and 3 activities at different post- treatment times. The SWE treatment marginally increased activity of both caspase 8 and 3 in a time dependent manner. The effect of SWE treatment was studied on the glutathione contents, glutathione-s-transferase, catalase and superoxide dismutase activities, where it was found to reduce the glutathione contents, and activities of glutathione-s-transferase, catalase and superoxide dismutase in a time dependent manner. Treatment of HeLa cells with ethanol extract of *Schima wallichii* increased the cytotoxic effect in a concentration dependent manner followed by a reduction in the clonogenicity of HeLa cells. The cell killing effect of SWE may be due to the caspase activation and reduction in the glutathione contents, and activities of glutathione-s-transferase, catalase and superoxide dismutase.

Keyword: *Schima wallichii*, MTT, Clonogenic, Caspase.

INTRODUCTION

The natural products have formed main source to cure different diseases including cancer since time immemorial [1-3]. The plants synthesize several secondary metabolites for different purposes and these are boon to human healthcare. These molecules are highly

complex in structure and it is difficult to undertake their chemical synthesis. This has rekindled the interest of researchers and of pharmacological industries on the isolation of these secondary metabolites to develop them in to modern drug entities in recent years [1]. The cancer has emerged has a major disease that has been putting higher financial burden on the healthcare of families and also of Governments who had to allocate higher financial resources to cancer centers. The fact remains that despite higher allocation and availability of most modern therapy for cancer patients the mortality rates remains higher in cancer patients [4].

The chemotherapy has been the major treatment modality, especially in advanced stages of cancer. Several chemotherapeutic drugs isolated from plant/natural products are in the market [2, 3]. The cytotoxic chemotherapeutic drugs are used alone or as an adjuvant therapy in patient to improve the survival [5]. Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer, and while the actual compounds isolated from the plant frequently may not serve as the drugs, they provide leads for the development of potential novel agents. The vinca alkaloids isolated from periwinkle plant *Catharanthus roseus* initially used to treat hematologic malignancies find their wide use to treat different types of solid neoplasia [6, 7]. Similarly, epipodophyllotoxins are used to treat several malignant neoplasia [8]. The taxols isolated from Pacific yew are also useful in the treatment of wide range of tumors [9]. The management of malignancies frequently requires the use of treatment modalities that are associated with significant toxic effects. The acceptability of specific therapy can be assessed by comparing its benefits with its potential cost in terms of toxicity [10]. This indicates the need to continue the screening of natural products for treatment of cancer.

Schima wallichii (DC.) Korth. (Chilauni) belong to the tea family, Theaceae. It is an evergreen tree inhabiting warm temperate to subtropical climates. The tree is found across southern and South East Asia, and stretch from the eastern Himalaya of Nepal to eastern India across Indochina, Southern China, Taiwan and the Ryukyu Islands.

It is commonly known as needle wood tree and grows up to 35 m high. However, in some places it may be seen only 40 ft high [11]. Locally, it is called “khiang” in Mizo language. *Schima wallichii* is known to possess several medicinal properties. The leaves and the stem bark are normally used traditionally for its medicinal properties. The bark is used as an antiseptic for cuts and wounds. It acts as vermicide, mechanical irritant and as a cure against gonorrhea [12]. Decoction of bark reduces fever and is said to be effective against head lice [13]. The bark juice is given to disinfest the animal from liver flukes ([14]. The sap from the stem is used for curing ear infection [15]. Fruit decoction is used by the people of Western Mizoram, India against snakebite [16, 14]. The young plants, leaves and roots are also used medicinally against fever and the bark is anthelmintic and rubefacient [17]. The leaves of *Schima wallichii* are known to have antitumor and antimutagenic properties [18, 19]. The astringent corollas are used to treat uterine disorders and hysteria [20]. The anticancer activity of *Schima wallichii* ethanol extract has not been studied in vitro therefore, the present study was undertaken to investigate its cytotoxic effect in vitro.

MATERIALS AND METHODS

Chemicals

Nitroblue tetrazolium (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), ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), sodium pyruvate, thiobarbituric acid (TBA) and crystal violet, were obtained from Sigma Chemical Co. (Bangalore, India). Sodium carbonate (Na_2CO_3), trichloroacetic acid (TCA), potassium chloride (KCl), hydrogen peroxide (H_2O_2), were procured from SD Fine Chemicals, Mumbai, India, whereas sodium hydroxide (NaOH), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), sulphuric acid (H_2SO_4), hydrochloric acid (HCl), n-butanol were purchased from Merck India Limited, Mumbai, India. Trypsin EDTA MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide), minimum essential medium (MEM), 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. The caspase kits were purchased from LabGills, Kolkata, India.

Preparation of the extract

The non-infected stem bark of *Schima wallichii, Chilauni* (DC.) Korth. was collected from Bazar veng, Lunglei, Mizoram during the months of April and May. The authentication and identification of *Schima wallichii* was done by the Botanical Survey of India, Shillong. The bark was cleaned and shade dried at room temperature in clean and hygienic conditions. The dried bark was powdered using an electrical grinder. The dried powder of *Schima wallichii* was extracted sequentially 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 them to dryness under reduced pressure. The concentrated extracts were stored at -80 until use. The ethanol extract was used for the study and it will be called as SWE henceforth.

Preparation of drug/s

The doxorubicin was freshly dissolved in distilled water, whereas the *Schima wallichii* ethanol extract was dissolved in 1% ethanol in water and diluted with MEM. The dissolved extract was filter sterilized before use.

Cell line and Culture

HeLa and V79 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, and 1% L-glutamine at 37°C in an atmosphere of 5% CO_2 in humidified air in a CO_2 incubator (Eppendorf AG, Hamburg, Germany).

Experimental Design

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

MEM group: The cells of this group were used as negative control group.

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

DOX group: The cell were treated with 5, 10 or 20µg/ml of doxorubicin (DOX) and served as positive control.

MTT assay

The cytotoxic effects of 12.5, 25, 50, 100, 200, 300 and 400 µg/ml of SWE was studied by MTT assay in HeLa, and V79 cells as described [21]. Usually 10^4 cells were seeded into 96 well plates in 100 µl MEM. The cells were incubated at 37°C 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. Thereafter, different concentrations of SWE or doxorubicin were added into each well of the microplate and incubated in the CO_2 incubator. After 48 hours of drug/s treatment, 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 lysis buffer and incubated once again for 4 hours after which the absorbance was measured at 560 nm using a micorplate reader (Biorad, USA). The cytotoxicity was calculated by the formula: Control-Treatment/Control X 100.

An another experiment was setup to study the effect of treatment duration where all the conditions were similar to that described above except that the cells were treated with 50, 100 or 200 µg/ml of SWE for 2, 4 and 6 h and processed for MTT assay as described above.

The Determination of clonogenic potential

The anticancer activity of SWE 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 served as negative control group.

SWE group: This group of cells was treated with 50, 100 or 200µg/ml SWE for 6h.

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 medium from each flask decanted and the flasks were washed twice with sterile PBS, subjected to trypsin EDTA treatment and processed for clonogenic assay [22].

Usually 200 HeLa cells were seeded into several individual petridishes containing 5 ml MEM, left undisturbed and allowed to grow for another 11 days. The resultant colonies of cells were stained with 1 % crystal violet in methanol and scored. The clone containing a minimum number of 50 cells was considered as a colony. The plating efficiency (PE) of the cells was determined and surviving fraction (SF) calculated according to the following formulae:-

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

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

Apoptosis assay

For the study of apoptosis, the cells were terminated at 6, 12 and 24 hours post drug treatment. The activity of caspase 8 and 3 was determined according to the manufacturer's protocol after treatment of HeLa cells with 100 µg/ml ethanol extract of *Schima wallichii.*

Biochemical assays

A separate experiment was performed to estimate the effect of SWE on the activities of various antioxidant enzymes in HeLa cells, where grouping and other conditions were essentially similar to that described for anticancer activity except that the cell cultures were terminated at 2, 6 and 12 hours post drug treatment. The drug containing media were removed; the cells were washed with sterile PBS and 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 ultra sonicator (PCI Analytics Pvt. Ltd., Mumbai, India).

Glutathione estimation

Glutathione was estimated as described earlier [23]. The concentration of glutathione was measured by its reaction with DTNB (Ellman's reagent) to give a compound that absorbs light at 412 nm. Briefly, 1.8 ml of 0.2M Na_2HPO_4 was mixed with 40 µl 10 mM DTNB and 160 µl 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).

Glutathione - S – transferase estimation

Glutathione-s-transferase activity was estimated by the method of [24]. Briefly, 0.5 ml of 0.1 M phosphate buffer pH 6.5, 0.1ml of 20mM CDNB, and 8.8 ml distilled water were mixed and 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 recorded at 340 nm at 1 min intervals for 6 minutes using UV-VIS Biospectrophotometer.

Catalase estimation

Catalase was assayed according to technique described [25]. 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.

Superoxide dismutase estimation

SOD activity was estimated as described [26]. Briefly, 100 µl of cell homogenate, 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitrobluetetrazolium, 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 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.

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.

RESULTS

The results are expressed in tables 1-8 and figures 1-11 as mean±standard error of the mean.

Determination of Cytotoxicity

Treatment of HeLa, and V79 cells with different concentrations of SWE caused a concentration dependent increase in the cytotoxicity and the maximum cytotoxic effect was observed at the highest concentrations of SWE used. Treatment of HeLa cells with different concentrations of SWE induced highest toxic effect at a concentration of 400µg/ml which was 62.21% (Table 1). Similarly, SWE induced maximum cytotoxicity of 73.62% at 400 µg/ml in V79 cells. However, 50% cytotoxicity was found at 100 µg/ml for the extract for both of the cells used. The standard drug DOX was used as the positive control (Table 1, Figure 1 and 2).

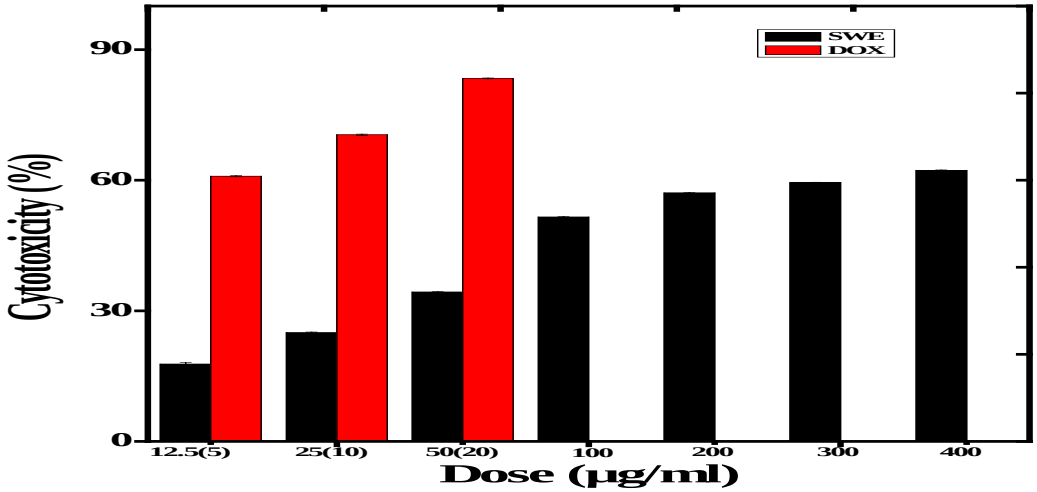

Figure 1: Cytotoxic effects of ethanol extract of *Schima wallichii* on HeLa cells evaluated by MTT assay. N=8;p<0.05

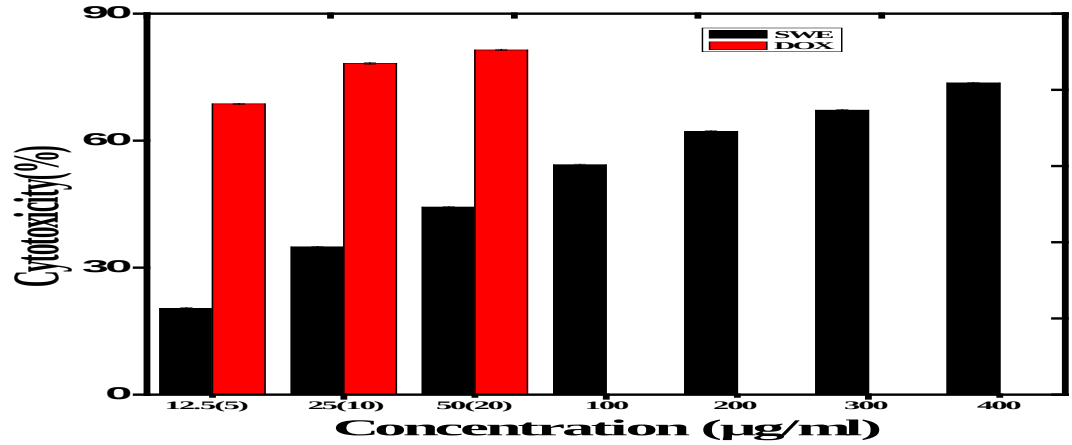

Figure 2: Cytotoxic effects of ethanol extract of *Schima wallichii* on V79 cells evaluated by MTT assay. N=8; p<0.05

Determination of optimum exposure time for cytotoxicity

The optimum exposure time for cytotoxic effect of SWE was also evaluated by MTT assay. The SWE treatment resulted in a time dependent increase in the cytotoxicity in HeLa, and V79 cells and maximum cytotoxic effect was observed in the cells treated with SWE for 6 h (Table 2). The HeLa and V79 cells showed 50% cytotoxicity at the maximum exposure time which was selected for further experimentation (Figure 3 and 4).

Table 1 :The cytotoxic effect of different concentrations of ethanol extract of *Schima wallichii* in HeLa and V79 cell lines by MTT assay. p<0.05

Cell line	Treatment	Dose	Cytotoxicity (%)±SEM
Hela	DOX	5	60.92 ± 0.14
		10	70.44 ±0.16
		20	83.38 ±0.11
	SWE	12.5	17.76 ±0.38
		25	25.00 ± 0.12
		50	34.31 ± 0.12
		100	51.52 ± 0.17
		200	57.07 ±0.12
		300	59.44 ± 0.08
		400	62.21 ± 0.15
V79	DOX	5	68.67 ± 0.11
		10	78.26 ± 0.19
		20	81.42 ± 0.17
	SWE	12.5	20.41 ± 0.18
		25	34.88 ± 0.15
		50	44.30 ± 0.08
		100	54.27 ± 0.17
		200	62.13 ± 0.18
		300	67.20 ± 0.16
		400	73.62 ± 0.16

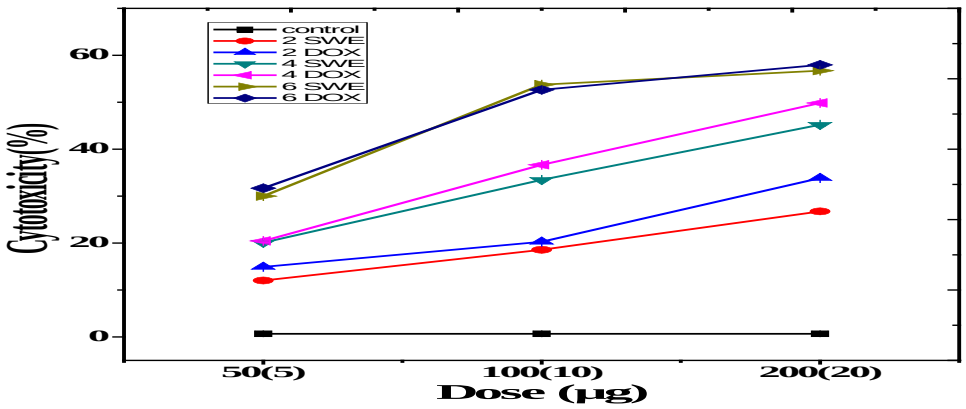

Figure 3: Cytotoxic effects of ethanol extract of *Schima wallichii* or DOX at different exposure times on HeLa cells by MTT assay. N=8; p<0.05.

Table 2: Effect of treatment duration on the cytotoxic effects of ethanol extract of *Schima wallichii* (SWE) and DOX in HeLa and V79 cell lines by MTT assay.

Cell line	Treatment	Dose	Cytotoxicity (%)±SEM		
			Post-treatment time (h)		
			2	4	6
Hela	DOX	5	14.92 ± 0.18	20.45 ± 0.16	31.68 ± 0.16
		10	20.27 ± 0.17	36.67 ± 0.11	52.64 ± 0.13
		20	33.84 ± 0.15	49.83 ± 0.15	57.95 ± 0.13
	SWE	50	12.02 ± 0.1852	20.11 ± 0.33	30.00 ± 0.14
		100	18.57 ± 0.14	33.47 ± 0.22	53.74 ± 0.15
		200	26.74 ± 0.15	45.22 ± 0.34	56.73 ± 0.15
V79	DOX	5	19.82 ± 0.29	22.89 ± 0.12	26.03 ± 0.15
		10	27.38 ± 0.28	31.82 ± 0.18	38.93 ± 0.14
		20	39.71 ± 0.22	45.76 ± 0.20	52.06 ± 0.12
	SWE	50	17.00 ± 0.22	19.35 ± 0.14	22.73 ± 0.15
		100	24.76 ± 0.20	30.11 ± 0.16	43.56 ± 0.11
		200	37.36 ± 0.27	40.76 ± 0.14	51.87 ± 0.09

N=8, p<0.05.

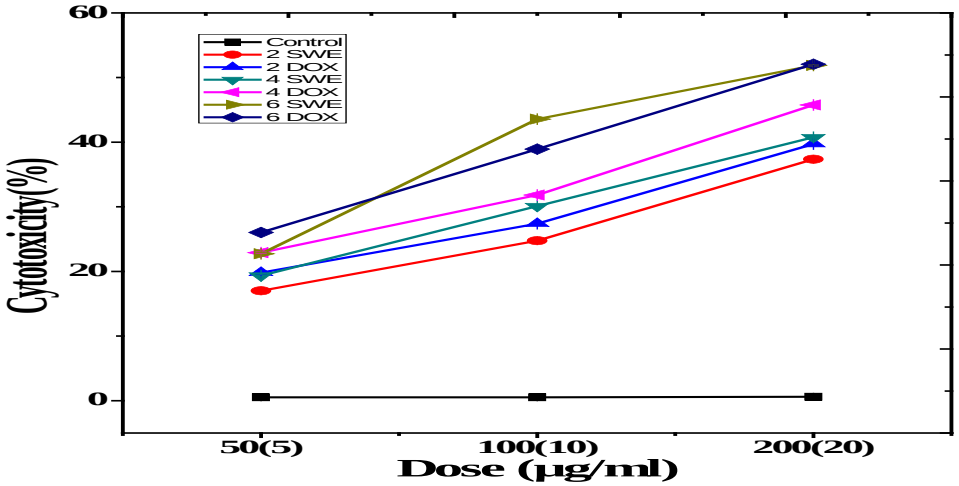

Figure 4: Cytotoxic effects of ethanol extract of *Schima wallichii* or DOX onV79 cells at different exposure times by MTT assay. N=8; p<0.05.

Clonogenic Assay

Treatment of HeLa cells with different concentrations of SWE caused a concentration dependent decline in the clonogenicity of cells. A maximum decline in the clonogenicity was observed for 200 µg/ml SWE, where extreme reduction in the cell survival (0.15) was observed. (Figure 5). The reduction in clonogenic potential by SWE was comparable to positive control DOX except the fact that the doses required by SWE were ten times greater than the DOX (Figure 5).

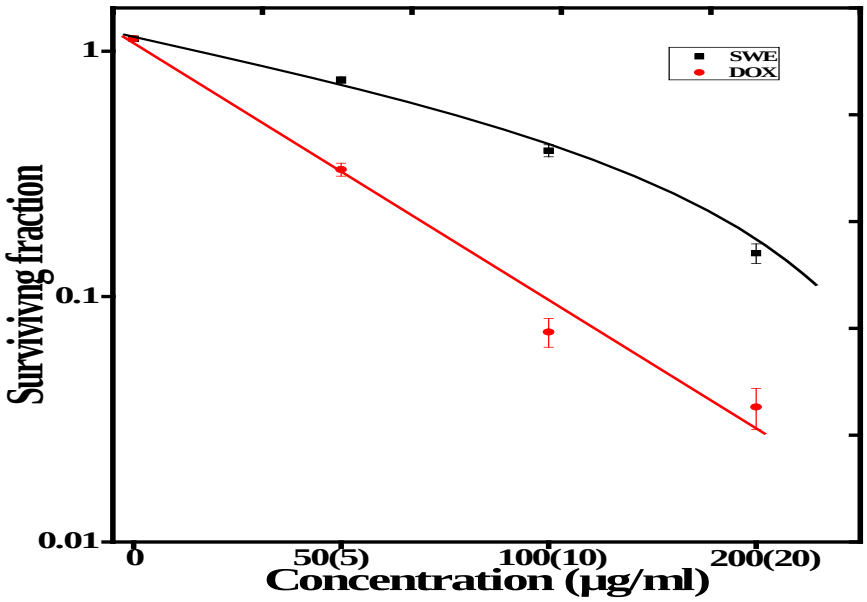

Figure 5: Effect of different concentrations of the ethanol extract of *Schima wallichii* or doxorubucin (DOX) treatment on the survival of HeLa cells. N=3.

Apoptosis

Caspase 8

Treatment of HeLa cells with different concentrations of SWE caused a statistically significant and concentration dependent increase in the activity of caspase 8 at different post treatment time. The maximum activity was observed at 48h post drug treatment. (Table 3, Figure 6).

Table 3: Effect of 100 µg/ml ethanol extract on the caspase activity in HeLa cells at different post treatment times.

	Post treatment time (h)	Treatment (µg/ml)		
		Control	SWE	DOX
Caspase 8	12	0.807 ±0.016	0.824 ±0.018	1.004 ±0.012*
	24	0.815 ±0.002	0.831 ±0.036*	1.035 ±0.114*
	48	0.838 ±0.004	0.856 ±0.011*	0.939 ±0.011*
Caspase 3	12	0.217 ±0.001	0.230 ±0.008*	0.246 ±0.006*
	24	0.236 ±0.005	0.252 ±0.008*	0.260 ±0.004*
	48	0.244 ±0.007	0.259 ±0.006*	0.278 ±0.016*

N=3

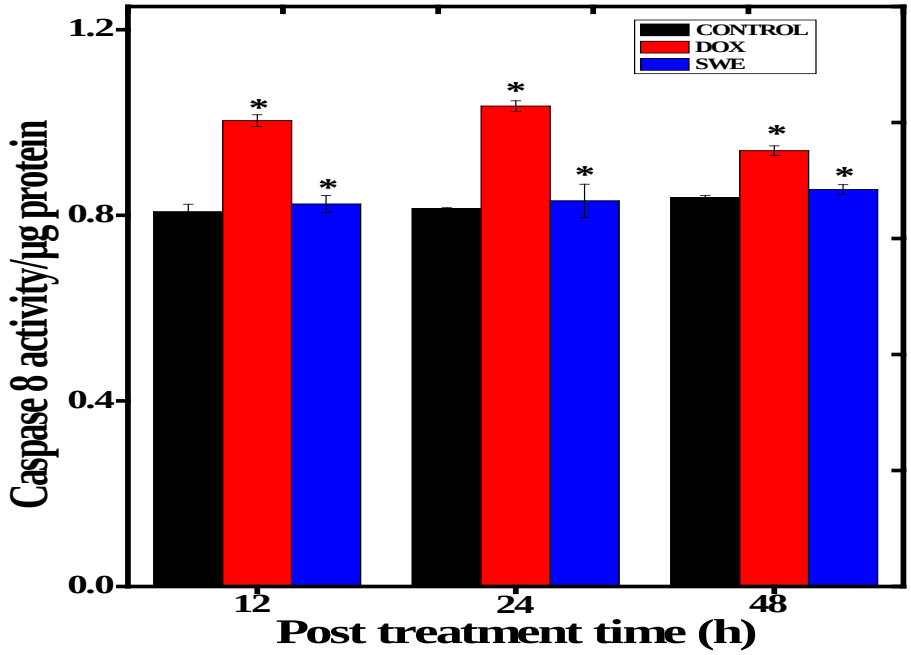

Figure 6: The caspase 8 activity in HeLa cell treated with *Schima wallichii* (SWE)or doxorubicin (DOX) at different post treatment times. N=3. p<0.05

Caspase 3

Treatment of HeLa cells with different concentrations of SWE caused a statistically significant and concentration dependent increase in the activity of caspase 3 at different post treatment time. The maximum activity was observed at 48h post drug treatment. (Table 3, Figure 7).

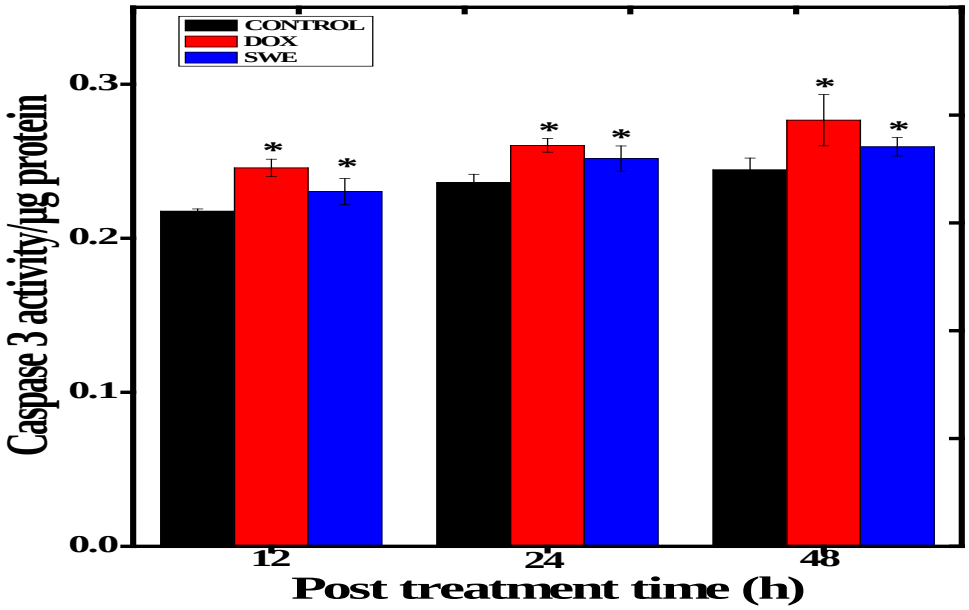

Figure 7: The caspase 3 activity in HeLa cell treated with *Schima wallichii* (SWE) or doxorubicin (DOX) at different post treatment times. N=3. p<0.05

Glutathione

Treatment of HeLa cells with different concentrations of SWE caused a statistically significant and concentration dependent reduction in glutathione contents at all the post-treatment times (Figure 8). The concentration of glutathione also declined in a similar fashion in DOX treated group (Table4). The glutathione concentration also showed a time dependent reduction and maximum decline was observed for 200 µg/ml SWE at 12 h post treatment.

Glutathione-s-transferase

The GST activity declined in a concentration dependent manner in HeLa cell exposed to 50-200 µg/ml SWE (Figure 9). The GST activity also reduced with assay time and a nadir was observed at 12 h post-drug treatment (Table 5). The decline in GST activity in SWE group was comparable to DOX treatment.

Catalase

The assay of catalase activity showed a concentration dependent alleviation in HeLa cells with increasing concentration of SWE. The catalase activity also declined with post-treatment assay time and the lowest catalase activity was recorded at 12 h post SWE treatment

Table 4: Effect of 100 µg/ml ethanol extract of *Schima wallichii* on the glutathione contents in HeLa cells at different post-treatment assay times.

Post treatment time (h)	µmol/mg protein±SEM						
	Control (MEM)	Treatment (µg/ml)					
		Schima wallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	2.86 ±0.005	2.22 ±0.002*	2.01 ±0.001*	1.77 ±0.006*	1.65 ±0.002*	1.28 ±0.002*	0.88 ±0.008*
6	2.38 ±0.029	2.08 ±0.003*	1.62 ±0.008*	1.36 ±0.004*	1.25 ±0.002*	0.96 ±0.003*	0.68±0.003*
12	2.18± 0.002	1.85± 0.003*	1.31± 0.007*	1.18 ±0.002*	0.87 ±0.005*	0.655 ±0.001*	0.55 ±0.003*

N=5. p<0.05

Table 5: Alteration in the glutathione-s-transferase activity of HeLa cells treated with different concentrations of *Schima wallichii* or doxorubicin.

	nmol/mg protein ±SEM						
Post treatment time (h)	Control (MEM)	Treatment (µg/ml)					
		Schima wallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	0.214 ±0.001	0.148 ±0.005*	0.118 ±0.004*	0.08 ±0.004*	0.09 ±0.006*	0.072 ±0.006*	0.034 ±0.004*
6	0.189 ±0.002	0.122 ±0.004*	0.092 ±0.005*	0.066 ±0.0058*	0.072 ±0.004*	0.046 ±0.002*	0.030 ±0.003*
12	0.169 ±0.003	0.088 ±0.002*	0.068 ±0.004*	0.046 ±0.002*	0.05 ±0.003*	0.032 ±0.004*	0.016 ±0.004*

N=5, p<0.05

(Table 5). There was an abrupt decline in the catalase activity at 6 h post treatment when compared to 2 h after SWE treatment. The DOX treatment also resulted in an identical decline in catalase activity at all post treatment times (Figure 10).

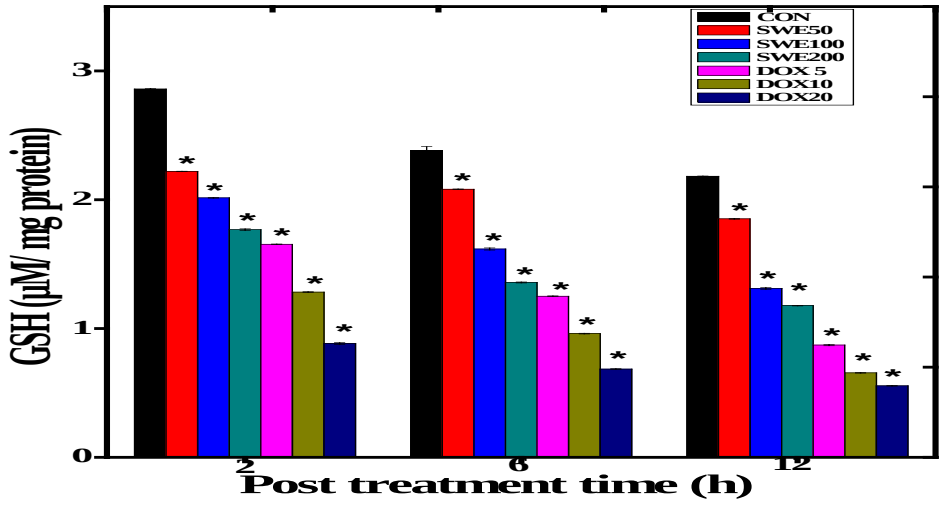

Figure 8: Alteration in the glutathione contents in HeLa cells treated with various concentrations of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;p<0.05 when treatment groups compared with concurrent control (SPS) group.***

Table 6: Alterations in the catalase activity of HeLa cells treated with different concentrations of *Schima wallichii* ethanol extract (SWE) or doxorubicin (DOX).

Post treatment time (h)	Unit/ mg protein±SEM						
	Control (MEM)	Treatment (µg/ml)					
		Schima wallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	8.36 ±0.03	7.19 ±0.02*	5.28 ±0.01*	2.87 ±0.005*	5.91 ±0.03*	3.85 ±0.02*	3.09 ±0.009*
6	8.71 ±0.03	4.25 ±0.02*	3.47 ±0.81*	1.19 ±0.003*	4.25 ±0.02*	2.19 ±0.02*	1.73 ±0.003*
12	7.46 ±0.02	2.55 ±0.02*	1.20 ±0.003*	0.77 ±0.006*	2.56 ±0.03*	1.15 ±0.02*	0.095 ±0.01*

N=5, p<0.05

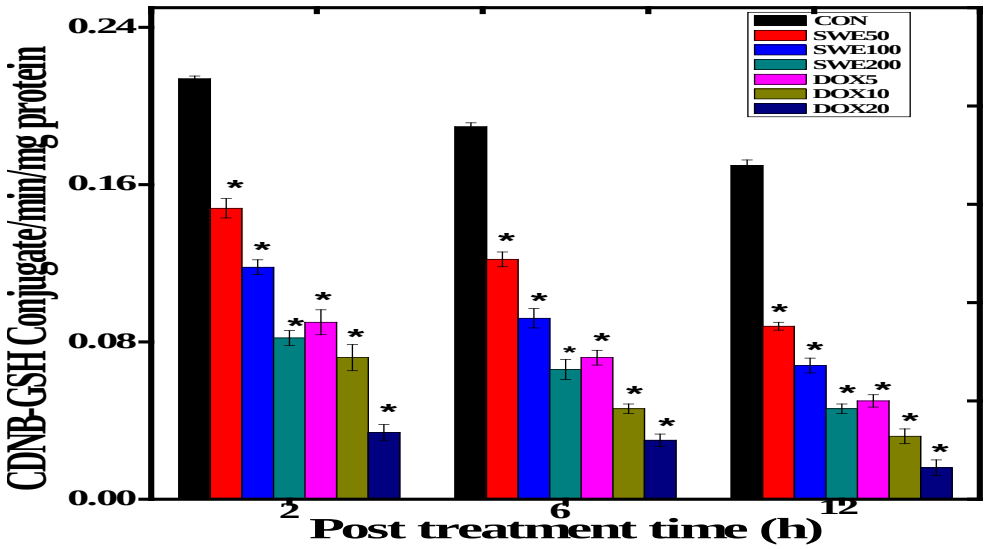

Figure 9 :Alteration in the glutathione-s-transferase (GST) activity in HeLa cells treated with various doses of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;p<0.05 when treatment groups compared with concurrent control (SPS) group.***

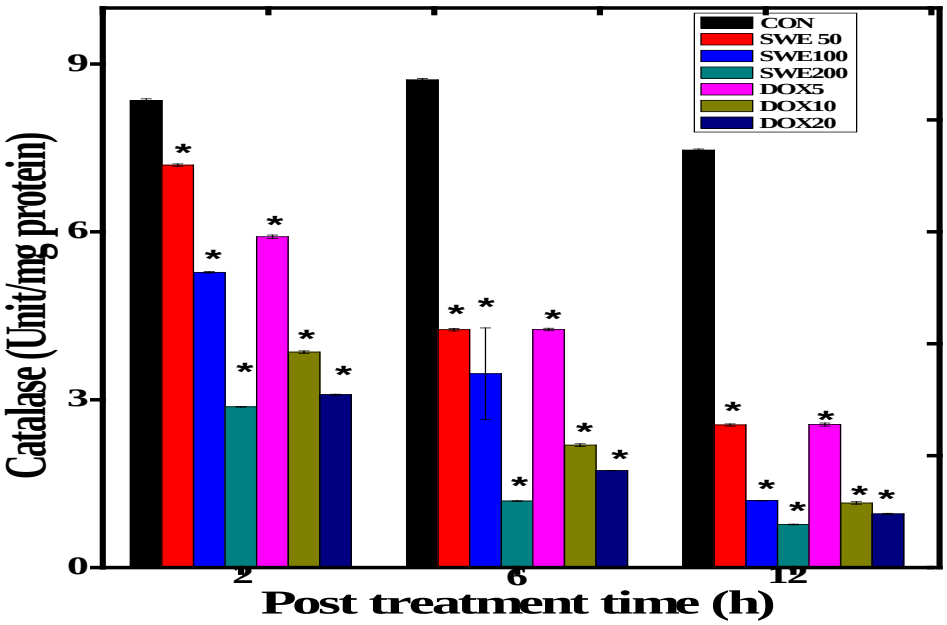

Figure 10 : Alteration in the catalase activity on HeLa cells treated with various doses of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;p<0.05 when treatment groups compared with concurrent control (SPS) group.***

Superoxide dismutase

The exposure of HeLa cells to different concentrations of SWE caused a concentration dependent but gradual reduction in the SOD activity (Figure 11). The SOD activity also showed a time dependent decrease with a maximum reduction in the SOD activity at 12 h post – treatment (Table 7). The positive control DOX also showed a pattern similar to that of SWE treatment

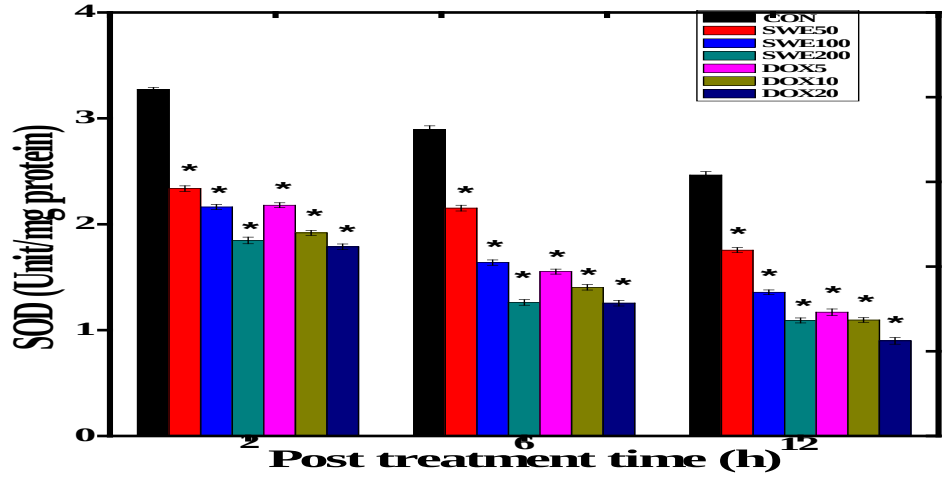

Figure 11 : Alteration in the Catalase activity on HeLa cells treated with various doses of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;.p<0.05 when treatment are compared with concurrent control (SPS) group.***

Table7: Alteration in the SOD activity HeLa cells treated with different concentrations of *Schima wallichii* ethanol extract (SWE) or doxorubicin (DOX).

	Unit/ mg protein±SEM.						
Post treatment time (h)	Control (MEM)	Treatment (µg/ml)					
		Schimawallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	3.27 ±0.02	2.34 ±0.03*	2.16 ±0.02*	1.85 ±0.03*	2.18 ±0.02*	1.92 ±0.02*	1.79 ±0.03*
6	2.90 ±0.03	2.15 ±0.03*	1.64 ±0.03*	1.26 ±0.03*	1.55 ±0.02*	1.41 ±0.03*	1.25 ±0.03*
12	2.46 ±0.03	1.75 ±0.02*	1.36 ±0.02*	1.09 ±0.02*	1.17 ±0.03*	1.09 ±0.02*	0.9 ±0.03*

N=5. p<0.05

DISCUSSION

Several studies have focused on natural anticarcinogenic agents. Many natural products have been identified to treat malignant neoplasia [2, 3]. The importance of natural products in medicine cannot be underestimated. The Federal Drug Administration, USA has approved approximately 547 products from natural resources or their derivatives for clinical use [27]. This indicate that plants still forms the major source for drug development and the screening of plants provides a major avenue for new drug discovery. Therefore the present study was designed to evaluate the antineoplastic action of *Schima wallichii* in vitro.

The MTT assay is a rapid and standard technique to determine the cytotoxicity of any drug in various cultured 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. The treatment of HeLa and V79 cells with different concentrations of SWE caused a concentration dependent rise in the cytotoxicity of ethanol extract of *Schima wallichii*. MTT assay has been used to test cytotoxicity in various cell lines earlier [21]. The cytotoxicity of ethanol extract of *Consolida orientalis* L, *Ferula assa-foetida* L, *Coronilla varia* L, *Orobanche orientalis* G. Beck on HeLa cells increased in a concentration dependent manner [28]. The other plants like *Alstonia scholaris, Consolida orientalis*, *P. pellucidum, Tinospora cordifolia*, *Ferula assafoetida* and *Coronilla varia* extract have been shown to exert cytotoxicity in cultured HeLa cells earlier [29-33].

The clonogenic assay is the most confirmatory test, which indicates the reproductive integrity and the extent of cell survival and it is also a long-term assay which takes cares of the delayed effects induced by drug treatments [34,35]. The cytotoxic effect of SWE was further confirmed by clonogenic assay where SWE treatment caused a concentration dependent decline in the clonogenicity of HeLa cells. There are no reports where SWE has been used to evaluate the reproductive potential of any cell line earlier. However, other plants including

Alstonia scholaris, Aphanamixis polystachya and *Tinospora cordifolia* have been reported to reduce the clonogenic potentials of HeLa cells earlier [29, 31,33].

The cancer cells show elevated oxidative stress however, excess oxidative stress also kills cancer cells by eliciting various mechanisms of cell death and agents than can induce greater amount of oxidative stress may be useful in killing the cancer cells [36-38]. Therefore, effect of SWE on oxidative stress was also studied in HeLa cells.

Glutathione (γ -glutamylcysteinyl glycine) is the most abundant non-protein thiol in the cell. It plays various physiological roles including counter balancing the excess free radicals produced in the cells during numerous physiological processes [39,40]. Glutathione occurs in two forms namely the reduced form (GSH) and the oxidized form (GSSG). 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, its higher amount is indicated in tumor microenvironment-related aggression, apoptosis evasion, colonizing ability, and multidrug and radiation resistance [41-43]. Increased concentration of GSH in the tumor cells have been known 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 [44, 45]. Therefore, glutathione attrition in cancer cells may enhance the cytotoxic effects of chemotherapeutic agents [46]. A similar connection is true in the present study, where SWE reduced the glutathione concentration in a concentration and time dependent manner. Earlier, *Alstonia scholaris* has been reported to reduce the glutathione contents in tumor cells [47]. The glutathione-S-transferases are a multi-gene family of enzymes, which carry out detoxification and activation of certain chemicals [48]. The GSTs are overexpressed in a wide variety of tumors and their negative modulation has emerged as a promising therapeutic target as they have been implicated in the resistance to cancer therapy. The augmented activity of GST in tumor cells is associated with suppression of tumor cell kill by apoptosis [49,50]. The SWE treatment has decreased the GST activity significantly and that may be one of the reasons of increased cell killing effect in the present study. Several GST inhibitors have been shown to reduce drug resistance by sensitizing tumor cells to anticancer drugs and bring effective cell killing [51,52]

The catalase is involved in the detoxification of H_2O_2 into water and molecular oxygen. However, it also plays a crucial role in various other processes. High levels of catalase have been found in patients with lung cancer, whereas the patients suffering from breast cancer, head and neck cancer, gynecological cancer, lymphoma, prostate cancer and urological cancer showed decreased levels of catalase [53]. The higher activity of catalase has been linked to suppression of apoptosis in tumor cells undergoing chemotherapy [54]. The untreated HeLa cells have shown higher catalase activity, whereas SWE treatment reduced the activity of catalase indicating that SWE action may be mediated by reducing catalase activity in HeLa cells. Superoxide dismutase (SOD) present in all oxygen metabolizing cells and it catalyzes the dismutation of the superoxide radical (O_2) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) [55]. Four different forms of SOD are known until now [56] of which the Cu-Zn SOD is the most abundant and comprises approximately 90% of total SOD activity in a eukaryotic cell [57]. 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 [58, 59]. Malignant

cells have been reported to show elevated Cu-Zn SOD activity [60]. The role of SOD in cancer is controversial as it is found to be overexpressed in some cancers, whereas other cancer show reduced expression [61]. The SOD are overexpressed in late stage of cancer, especially metastatic tumors [61] indicating that high levels of SOD in tumors may make them refractory to therapy. The SWE treatment reduced SOD activity in HeLa cells, which may account for its higher cell killing activity.

The exact mechanism of action of SWE is not known. It may have utilized different pathways to kill the cells. The present observations indicate that SWE treatment reduced the levels of GSH, GST, catalase and SOD, which may have increased the oxidative stress and stimulated the mechanisms that may have brought apoptotic and non-apoptotic form of cell death. The analysis of caspase 8 and 3 in HeLa cells indicate that part of the cell death seems to be mediated by apoptosis and increased oxidative stress indicate SWE also induced non-apoptotic form of cell death. The increased oxidative stress by SWE may have triggered events that may have damaged the cellular genome thereby bringing cell death in the present study. It is plausible that SWE may have increased the activation of P53 and related proteins to stimulate apoptotic form of cell death. The SWE may have inhibited the transcription of NF-κB, COX-II and Nrf2 elements which are responsible for tumor cell proliferation and therapy resistance [62-64]. In fact the down modulation of these proteins have been reported to enhance cell killing [65-67]. The SWE may have also used some other unknown mechanisms to kill the HeLa cells in the present study.

It is concluded that SWE administration caused effective killing of HeLa cells and the anticancer activity of SWE may due to reduction in the GSH and other antioxidant enzymes including GST, CAT and SOD. SWE may have also retarded the transcriptional activation of Nrf2, NF-κB and COX-II which may have contributed in killing the HeLa cells. The stimulation of caspase 8 and 3 in the present study indicates that SWE induced apoptotic mode of cell death in some of the cells if not all. Our results demonstrates that SWE act as an anticancer agent. However, further studies are required to ascertain its potential as an anticancer agent in different tumor models and understanding its mechanism of action.

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Research Article

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Evaluation of the free-radical scavenging and antioxidant activities of Chilauni, *Schima wallichii* Korth *in vitro*

K Lalhminghlui[‡,1] & Ganesh Chandra Jagetia*[,‡,1]

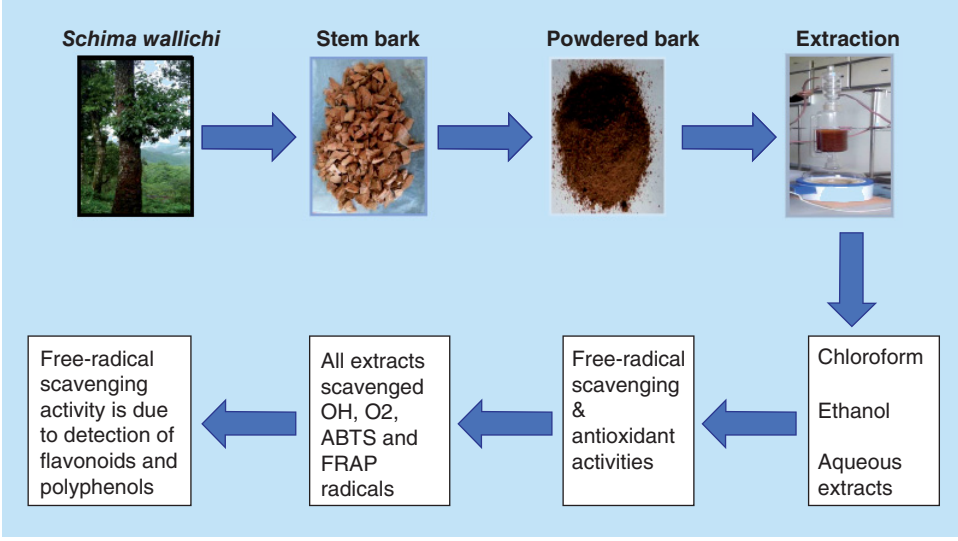
[1]Department of Zoology, Mizoram University, Tanhril, Aizawl-796 004 Mizoram, India

* Author for correspondence: Tel.: +91 011 389 233724; Fax: +91 011 389 233227; gc.jagetia@gmail.com

‡ Authors contributed equally

Aim: Free radicals are an outcome of various metabolic activities and their excess production leads to many diseases. Therefore, it is necessary to neutralize excess free radicals. **Materials & methods:** Free-radical scavenging activity of various extracts of *Schima wallichii* was evaluated using standard protocols. **Results:** Chloroform, ethanol and aqueous extracts of *S. wallichii* scavenged DPPH, hydroxyl, superoxide, nitric oxide and ABTS free radicals and increased ferric-reducing antioxidant potential in a concentration-dependent manner. A total of 1000 μg/ml of all the extracts and ethanol extract showed highest total flavonoids and phenol contents, respectively. **Conclusion:** The different extracts of *S. wallichii* scavenged different free radicals efficiently due to the presence of flavonoids and polyphenols and may be helpful in free radical-induced diseases.

Lay abstract: Free radicals induce several health disorders including cancer, and their excess generation needs to be controlled. Our study demonstrates that *Schima wallichii* scavenged DPPH, hydroxyl, superoxide, nitric oxide, ABTS free radicals and increased ferric-reducing antioxidant potential in a concentration-dependent manner. It could be one of the agents able to mitigate excess free-radical generation and ward off free radical-induced inflammation and diseases.



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Keywords: antioxidant • DPPH • flavonoid • hydroxyl • *Schima wallichii*

FUTURE SCIENCE

Usage of traditional medicines and other medicinal plants as therapeutic agents for maintaining proper health has been practiced widely in developing countries [1]. Plants and other natural products are still in great demand due to various factors like their safety, dependability and lesser side effects [2]. The greater adverse side effects caused by many cancer chemotherapeutic drugs may have been the main driving force to the use of alternative medicine in the hope of a better cancer cure. Approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary healthcare indicating that plant-based, traditional medicine systems will continue to play a major role in human healthcare in the future [3–5]. The interest in medicinal plants in healthcare has been rekindled recently due to the rising costs of prescription drugs for maintaining the proper health of an individual and their well being. The bioprospecting of new drugs derived from plants could be more economical with lesser side effects or no toxicity at all [6].

Free radicals are molecules or fragments of molecules that contain an unpaired electron in their atomic or molecular orbitals or simply reactive oxygen species, which in addition also contain other oxygen species including hydrogen peroxide that are highly reactive moieties and are generated by cells during respiration, and cell-mediated immune functions [7,8]. They are produced naturally in the body as they play an important role in many cellular functions. However, their high production induces molecular and cellular damage leading to the development of various human health disorders including cancer [9,10]. The excess free radicals produced during respiration and other activities could cause various damages leading to loss of function and eventually death of the organism [11]. Reactive oxygen species-induced damage can be alleviated using certain substances known as antioxidants, which are molecules capable of inhibiting oxidation of other molecules. The antioxidants are helpful in reducing and preventing damage from free-radical reactions because of their ability to donate electrons that can neutralize the radical formation [12–14]. Many plants synthesize secondary metabolites naturally, including flavonoids and polyphenols which act as antioxidants and also play a critical role in different biological activities [15–17]. Therefore, plants and natural products could be a major source of antioxidants that can scavenge free radicals and protect from excess oxidative stress-induced ailments.

Schima wallichii (DC) Korth, Chilauni or the needle wood tree, is an Asian species of evergreen tree belonging to the tea family, Theaceae. The genus inhabits warm temperate to subtropical climates across southern and Southeast Asia, ranging from the Eastern Himalaya of Nepal to eastern India across Indochina, southern China, Taiwan and the Ryukyu Islands. It usually grows up to 35 m in height and in some places, it may be 40 feet tall [18]. Locally, it is called 'khiang' in the Mizo language. *S. wallichii* is known to possess several medicinal properties. Traditionally, the leaves and the stem bark are normally used. The bark is used as an antiseptic for cuts and wounds, and as a cure for gonorrhea. It acts as a vermicide and a skin irritant [19]. Decoction of bark is good for fever and is effective against head lice infection [20]. The bark juice of Chilauni is used in animals as a liver fluke disinfesting agent [21]. The sap from its stem is used for curing ear infection [22]. Fruit juice of Chilauni is used by the people of western Mizoram, India against snakebite [21,23]. Its young plants, leaves and roots are also used medicinally against fever. The bark of *S. wallichii* is anthelmintic and rubefacient [24]. The leaves of *S. wallichii* are known to have antitumor and antimutagenic properties [25,26]. Kaempferol-3-rhamnoside, a compound isolated from the leaves of *S. wallichii* inhibited MCF-7 breast cancer cell proliferation through activation of the caspase cascade pathway [27].

Cancer cells are always at elevated oxidative stress, which offers a survival advantage to them, therefore we reasoned that if *S. wallichii*, which is used ethnomedicinally in traditional systems to treat various disorders would possess antioxidant potential, could be useful as an anticancer agent. Keeping this in mind we have evaluated the free-radical scavenging activity of various extracts of *S. wallichii in vitro*.

Materials & methods

Chemicals & reagents

Analytical grade chemicals and Milli Q water were used for the entire analyses. Ascorbic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethylenediaminetetraacetic acid (EDTA), β-nicotinamide adenine dinucleotide (NADH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), trichloroacetic acid (TCA), sodium nitroprusside and (N-(1-naphthyl)ethylenediamine dihydrochloride (NED or Griess reagent) were supplied by Sigma-Aldrich Chemical Co (Bangalore, India). Aluminum chloride, ethanol, methanol, ferric chloride, Folin-Ciocalteu reagent, potassium chloride, sodium acetate, sodium carbonate, sodium

hydroxide, sodium chloride, disodium hydrogen phosphate (anhydrous), potassium dihydrogen phosphate, potassium acetate, gallic acid, ferrous ammonium sulfate, ammonium acetate, glacial acetic acid and acetyl acetone were requisitioned from Merck (Mumbai, India).

Preparation of extracts

S. wallichii (family: Theaceae) was identified by the Department of Horticulture Aromatic and Medicinal Plants, Mizoram University, Aizawl, India and authenticated by the Botanical Survey of India, Shillong (BSI/ERC/Tech//Identification/2017/570). The noninfected and matured stem bark of *S. wallichii* was collected from Bazar Veng, Lunglei, Mizoram, India during the months of April and May. The bark was cleaned and shade dried at room temperature in clean and hygienic conditions. The dried bark was powdered using an electrical grinder and was extracted sequentially 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 them to dryness under reduced pressure. The concentrated extracts were stored at -80°C until use.

Experimental protocol

The free-radical scavenging activity of different extracts of *S. wallichii* was estimated according to standard protocols as described below.

DPPH free-radical scavenging assay

The DPPH free-radical scavenging activity of *S. wallichii* was estimated as described earlier [28]. Various concentrations of different extracts of *S. wallichii* (0.5 ml each) were mixed thoroughly with 1-ml methanol solution of 0.1 mM DPPH. The mixture was allowed to stand for 30 min in the dark. The absorbance was measured at 523 nm using a UV/VIS Spectrophotometer (Eppendorf India Limited, Kolkata, India). An equal amount of DPPH and methanol were used as standard and blank, respectively. The scavenging activity was calculated using the following formula:

$$\text{Scavenging (\%)} = (A_{control} - A_{sample})/A_{control} \times 100,$$

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

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity of *S. wallichii* was assayed according to the earlier described method [29] with minor modifications. 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) and different concentrations of *S. wallichii* extracts in a final volume of 2 ml. The mixture was incubated at 37°C for 30 min followed by the addition of 2 ml of trichloroacetic acid (2.8% w/v) and thiobarbituric acid. Thereafter it was kept for 30 min in a boiling water bath, and cooled. The absorbance was recorded at 532 nm in a UV–VIS spectrophotometer. Gallic acid was used as the standard and the results have been expressed as gallic acid equivalent.

Superoxide anion scavenging assay

Scavenging of the superoxide ($O_2^{\bullet-}$) anion radical was measured using a modified method [30]. The reaction mixture contained 0.2 ml of NBT (1 mg/ml of solution in DMSO), 0.6 ml different extracts, 2 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml H_2O) in a final volume of 2.8 ml. The absorbance was recorded at 560 nm using a UV–VIS spectrophotometer. The blank consisted of pure DMSO instead of alkaline DMSO. The results have been expressed as ascorbic acid equivalent which was used as a standard.

ABTS scavenging assay

ABTS scavenging activity of different extracts of *S. wallichii* was determined as described earlier [31]. Briefly, 37.5 mg of potassium persulfate was dissolved in 1 ml of distilled water. A total of 44 µl of this solution was added to 9.7 mg of ABTS dissolved in 2.5 ml of distilled water so as to prepare ABTS solution. The ABTS solution was allowed to stand in the dark for about 15 h at room temperature. The working solution was prepared by mixing 1 ml of ABTS solution with 88 ml of 50% ethanol. A total of 25 µl of different concentrations of chloroform, ethanol or aqueous extract of *S. wallichii* were mixed with 250 µl of ABTS working solution and allowed to stand for 4 min. The absorbance was read at 734 nm in a UV–VIS spectrophotometer. The results have been expressed as ascorbic acid equivalent which was used as a standard.

Nitric oxide scavenging assay

The nitric oxide scavenging activity was estimated according to the earlier described method [32]. Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with different concentrations of the chloroform, ethanol or aqueous extract of *S. wallichii* and incubated at 25°C for 150 min. The samples were then mixed with Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with NED was read at 546 nm using a UV–VIS spectrophotometer. The inhibition of nitric oxide formation was determined with respect to standard potassium nitrite in the same way with Griess reagent. The results have been expressed as potassium nitrite equivalent which has been used as a standard.

Ferric-reducing antioxidant potential assay

The ability of different *S. wallichii* extracts to decrease ferric ion production was measured as described earlier [33] with minor modifications. A total of 50 µl of various concentrations of chloroform, ethanol or aqueous extract were added to 3 ml of ferric-reducing antioxidant potential (FRAP) reagent (ten parts of 300 mM acetate buffer, pH 3.6, one part of TPTZ solution and one part of 20 mM $Fecl_3.6H_2O$ solution) and the reaction mixture was incubated at 37°C for 30 min. The increase in absorbance was measured at 593 nm using UV–VIS spectrophotometer. The antioxidant activity of the extracts is based on their ability to reduce ferric ions and it has been expressed as milligram ferrous sulfate equivalents/100 g of *S. wallichii* extracts.

Determination of total phenolic contents

The total phenolic contents of the *S. wallichii* extracts were determined as described earlier [34]. Briefly, 500 µl of different extracts of *S. wallichii* were mixed with 1000 µl of 1:10 Folin-Ciocalteu's reagent and incubated at room temperature for 5 min followed by the addition of 900 µl saturated (7.5%) sodium carbonate solution. After 1 h of incubation at room temperature, the absorbance was recorded at 765 nm using UV–VIS spectrophotometer. The total phenolic contents of the extracts have been expressed as gallic acid equivalents mg/100 g of the extracts.

Total flavonoids determination

The total flavonoids were determined by colorimetric method described earlier [35]. 1 ml of chloroform, ethanol or aqueous extract of *S. wallichii* was individually mixed with 1.5 ml of 95% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water and thereafter incubated for 30 min at room temperature. The absorbance of the reaction mixture was recorded at 415 nm with a UV–VIS spectrophotometer. The presence of flavonoids in *S. wallichii* extracts was expressed as milligram quercetin equivalent/100 g of the extract/s.

Results

The results of free-radical scavenging by different extracts of *S. wallichii* are shown as mean ± standard error of the mean in Figures 1 and 2, whereas that of total phenols and flavonoids in Figure 3.

DPPH free-radical scavenging

The chloroform, ethanol and aqueous extracts of *S. wallichii* showed a concentration-dependent rise in the scavenging of DPPH free radicals and a maximum scavenging activity was recorded at a concentration of 160, 80 and 140 µg/ml chloroform, ethanol and aqueous extracts, respectively. Thereafter, the scavenging effect declined (Figure 1). The ethanol extract was best as its low concentration scavenged higher amount of DPPH free radicals (Figure 1).

Hydroxyl radical scavenging

The scavenging of hydroxyl radicals depended on the dose of extracts of *S. wallichii.* The chloroform, ethanol and aqueous extracts inhibited the generation of hydroxyl radicals in a concentration-dependent manner and a maximum inhibition in •OH generation was observed at 80 µg/ml for ethanol, 100 µg/ml for aqueous and 120 µg/ml for chloroform extracts (Figure 1).

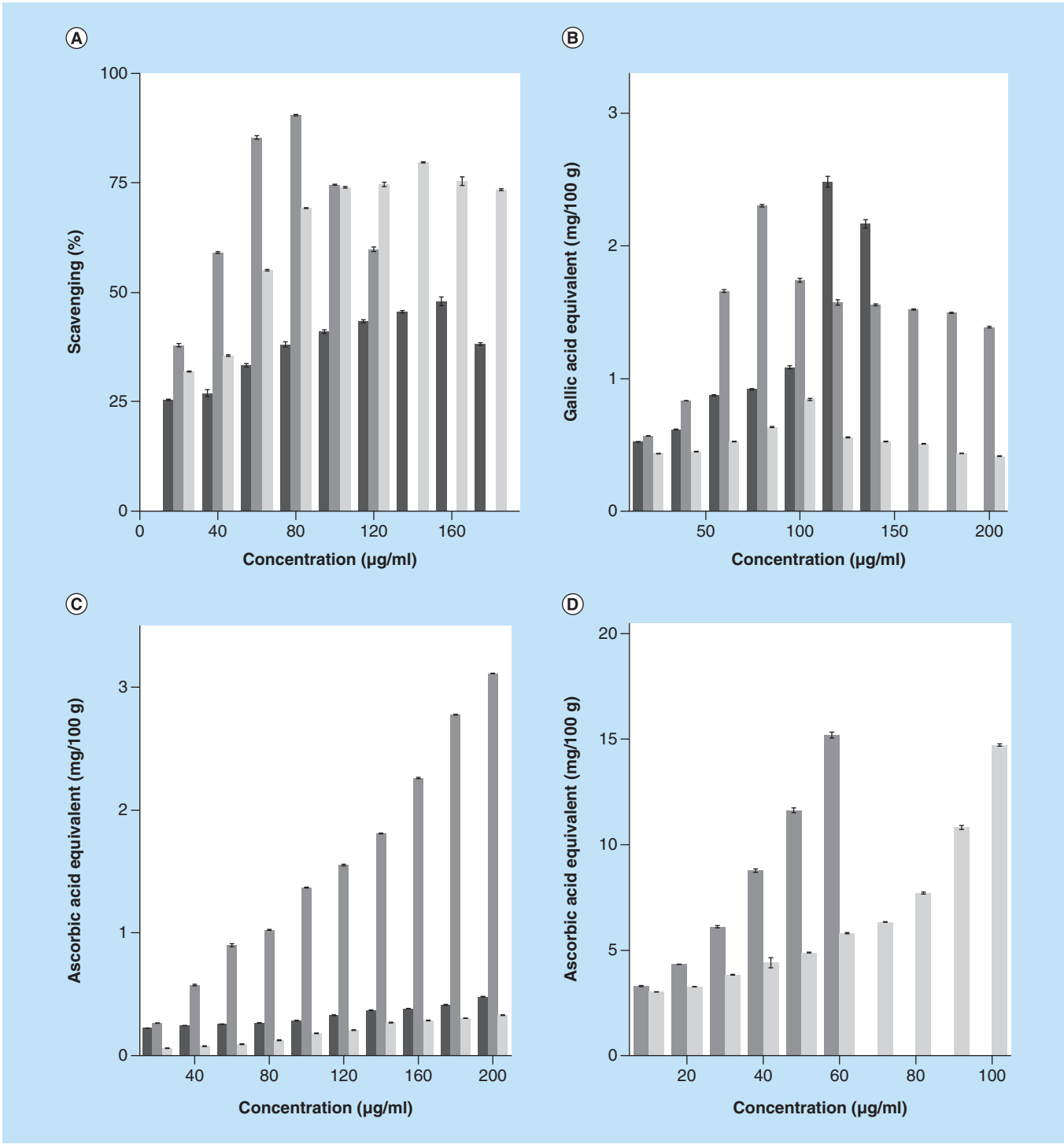

Figure 1. The free-radical scavenging activity of different stem extracts of *Schima wallichii*. (A) DPPH, **(B)** hydroxyl, **(C)** superoxide and **(D)** ABTS radicals. Dark gray: chloroform extract; gray: ethanol extract; and light gray: aqueous extract. The data are expressed as mean ± standard error of the mean; n = 5.

Superoxide anion scavenging

The chloroform, ethanol and aqueous extracts of *S. wallichii* showed a concentration-dependent increase in the inhibition of superoxide generation and the highest scavenging activity for $O_2^{\bullet-}$, was observed at a concentration of 200 µg/ml for all the three extracts (Figure 1).

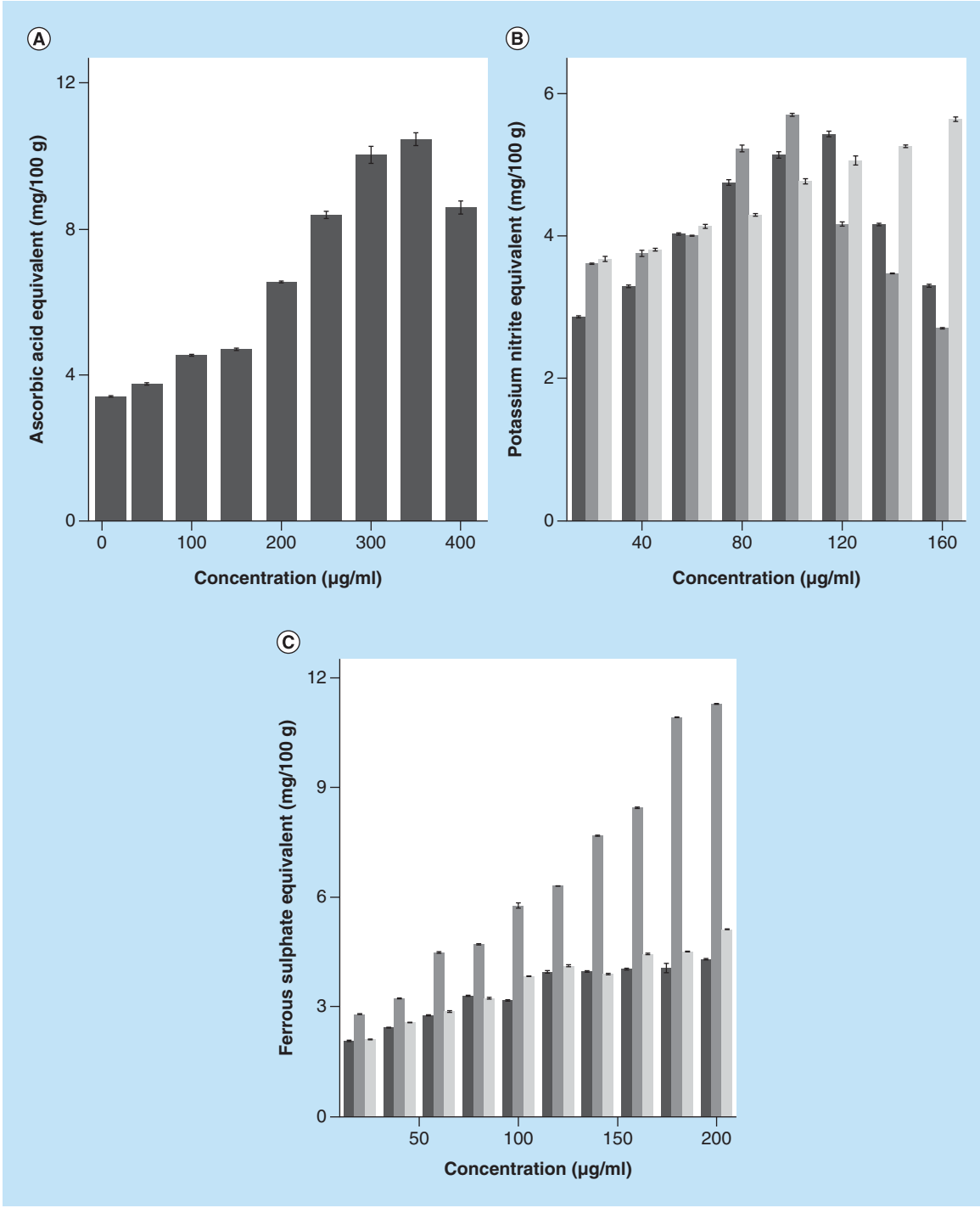

Figure 2. The free-radical scavenging activity of different stem extracts of *Schima wallichii*. **(A)** ABTS ($CHCl_3$), **(B)** Nitric oxide, **(C)** Ferric-reducing antioxidant potential (FRAP) radicals. Dark gray: chloroform extract; gray: ethanol extract; and light gray: aqueous extract. Values are expressed as mean ± standard error of the mean; n = 5.

ABTS scavenging

Various extracts of *S. wallichii* showed a concentration-dependent rise in the scavenging of the ABTS free radicals (Figures 1 & 2). The maximum activity for chloroform extract was recorded for 350 µg/ml (Figure 2), whereas ethanol and aqueous extracts showed maximum ABTS inhibitory action at 60 and 100 µg/ml, respectively (Figure

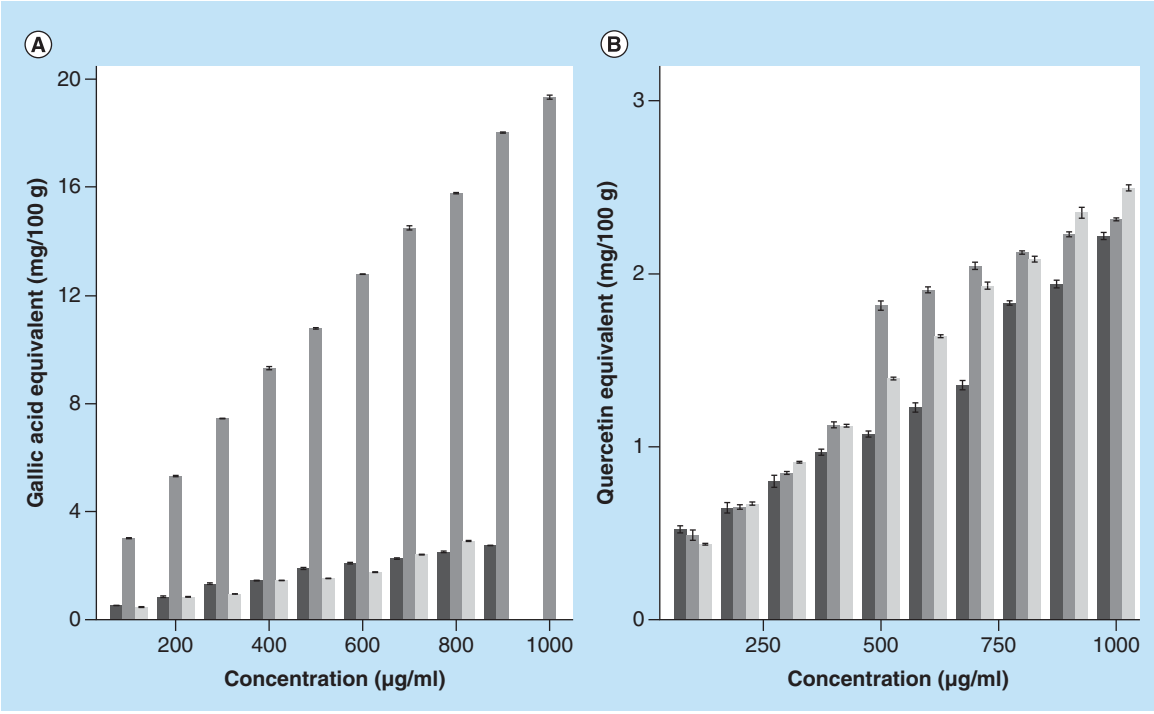

Figure 3. The total phenol and flavonoid contents of different extracts of *Schima wallichii* (100–1000 µg/ml). The data are expressed as mean ± standard error of the mean; n = 5. **(A)** Total phenols and **(B)** flavonoids.

1). The ethanol extract proved to be the best among all the three extracts as it has maximum effect at a lower concentration (Figures 1 & 2).

Nitric oxide scavenging

The analysis of nitric oxide scavenging activity also revealed a concentration-dependent rise in its scavenging by chloroform, ethanol and aqueous extracts of *S. wallichii* (Figure 2). The greatest scavenging activity was discernible at 120, 100 and 160 µg/ml for chloroform, ethanol and aqueous extracts, respectively, which declined thereafter (Figure 2).

Ferric-reducing antioxidant potential

The FRAP of chloroform, ethanol and aqueous extracts of *S. wallichii* showed a concentration-dependent rise up to 200 µg/ml, the highest concentration evaluated. All extracts were equally effective in scavenging the FRAP radical (Figure 2).

Determination of total phenolic contents

Total phenol contents of *S. wallichii* extracts showed a concentration-dependent rise up to a concentration of 900 µg/ml for chloroform, 1000 µg/ml for ethanol and 800 µg/ml for aqueous extracts (Figure 3).

Total flavonoids contents

The chloroform, ethanol and aqueous extracts of *S. wallichii* showed a concentration-dependent increase in the total flavonoid contents. The maximum quantity of flavonoids was estimated for 1000 µg/ml for chloroform, ethanol and aqueous extracts, respectively (Figure 3).

Discussion

Free radicals are closely associated with oxidative damage and antioxidants are reducing agents, which limit oxidative damage to biological structures by donating electrons to free radicals and passivating them [36]. The interaction of oxygen with certain molecules leads to the formation of free radicals and once formed, the chief danger comes from the damage they can inflict when they react with important cellular components including DNA, proteins and the

cell membrane [37]. These free radicals interact with the antioxidants, which can eventually neutralize them before damages are initiated [38]. Plants synthesize several compounds as secondary metabolites and many of them act as antioxidants. Therefore, the present study was undertaken to study the free-radical scavenging ability of *S. wallichii in vitro*.

DPPH is a dark-colored crystalline powder composed of stable free-radical molecules. Most notably, it is a common antioxidant assay and is a well-known radical. DPPH radical has a deep violet color in solution, and it becomes colorless or pale yellow when neutralized and converted into DPPH-H [39]. Many plant extracts have been reported to scavenge DPPH radicals *in vitro* [2,21,40–43]. Different extracts of *S. wallichii* scavenged DPPH radicals in a concentration-dependent manner. Similarly, different tea extracts containing a number of polyphenols have been reported to scavenge DPPH free radicals [44]. Kaempferol present in several plants including *S. wallichii* has been reported to scavenge DPPH free radicals earlier with an IC50 value of 0.004349 $mg{\cdot}ml^{-1}$ [45–47]. Other phytochemicals like mangiferin and naringin have been reported to scavenge DPPH radicals in a concentration-dependent manner [40,48]. The scavenging activity for ethanol extracts of *S. wallichii* was 80 and 140 μg/ml for aqueous extracts and twice the dose of ethanol extract (160 μg/ml) for chloroform extract. The DPPH scavenging activity of *S. wallichii* may be due to the presence of flavonoids and other polyphenols in the extracts as indicated in the present study.

Hydroxyl radicals are highly reactive and are short-lived [49]. They are capable of inducing detrimental effects on the important macromolecules including proteins and nucleic acids. In the Haber-Weiss/Fenton reaction, hydroxyl radicals are generated from hydrogen peroxide in the presence of iron ions [50,51]. The high reactivity of hydroxyl radicals lead to tremendous damage to the cell and its components and subsequently to the organisms as a whole [52]. Therefore, it is very important to remove hydroxyl radicals which cause detrimental effects. The different extracts of *S. wallichii* inhibited the generation of hydroxyl free radicals in a concentration-dependent manner. Kaempferol flavonoid present in *S. wallichii* scavenged OH radicals in an earlier study [45]. Similarly, many plant extracts and flavonoids including mangiferin, and naringin have been found to scavenge hydroxyl free radicals in a concentration-dependent manner [2,21,40,43,48]. Several flavonoids synthesized by different plants as secondary metabolites have been reported to scavenge OH radicals earlier [53,54].

The $O_2^{\bullet-}$ are generated in biological systems during cellular respiration and as such they are less toxic; however, they are converted into highly reactive OH radical in the presence of iron [55]. Moreover, superoxide anions produced as a result of incomplete metabolism of oxygen damage biomolecules directly or indirectly by forming H_2O_2, $^{\bullet}OH$ and peroxynitrite or singlet oxygen [55,56]. Therefore, the removal or neutralization of superoxide radicals is necessary to protect the cells from their deleterious effects. Various extracts of *S. wallichii* inhibited the formation of $O_2^{\bullet-}$ in a concentration-dependent manner. Kaempferol has been found to scavenge $O_2^{\bullet-}$ in an earlier report [45]. Other plant extracts and certain plant flavonoids including mangiferin, naringin, quercetin, myricetin and rutin have been found to scavenge superoxide free radical in a concentration-dependent manner [2,21,40,43,48,57].

Nitric oxide is an important cellular signaling molecule involved in many physiological and pathological processes. It is a powerful vasodilator with a short half-life of a few seconds in the blood [55,58]. The nitric oxide radical ($NO^{\bullet}$) is toxic, after reaction with oxygen or superoxide anion radicals. Different extracts of *S. wallichii* reduced the generation of $NO^{\bullet}$ in a concentration-dependent manner. Several plant extracts and plant formulations have been reported to scavenge $NO^{\bullet}$ in a concentration-dependent manner [2,21,40,59]. Similarly, betanin, phyllocactin and betanidin have been reported to scavenge NO radical in a concentration-dependent manner [60]. Kaempferol, myricetin, epigallocatechin gallate, catechin, epicatechin and resveratrol have been reported to scavenge NO radicals [58]. Various flavonoids including delphinidin, pelargonidin, malvin mangiferin and naringin have been found to neutralize NO radicals in earlier studies [40,45,48,54,61–63].

The $ABTS^{\bullet+}$ chromophore is produced through the reaction between ABTS and potassium persulfate which converts ABTS into its radical cation. This radical cation is blue in color and absorbs light at 734 nm [31]. The $ABTS^{\bullet+}$ is reactive towards most antioxidants including phenols, thiols and vitamin C. [64]. The various extracts of *S. wallichii* showed inhibition of ABTS radical production in a concentration-dependent manner. A similar effect has been observed with the extract of *Syzygium cumini*, naringin and mangiferin earlier [40,48,59]. The presence of kaempferol has been reported to scavenge ABTS radicals earlier [45]. FRAP assay had been used to determine antioxidant activity as it is a simple and quick method [65]. The different extracts of *S. wallichii* showed a concentration-dependent rise in FRAP. Several plant extracts have been reported to exhibit antioxidant activity by exhibiting high FRAP values *in vitro* [2,21,42,66]. Likewise, fruits of *Cynometra cauliflora* and *Garcinia atroviridis*

have been also reported to possess high FRAP value [67]. Flavonoids from 19 different plants have been found to scavenge ABTS radicals and showed higher FRAP in an earlier study [68].

The exact mechanism of free-radical scavenging by different extracts of *S. wallichii* is not known. However, the phytochemical analysis of *S. wallichii* stem bark has shown the presence of phenols and flavonoids and their concentrations increased with the increase in the amount of extracts. Therefore, the free-radical scavenging and antioxidant activities of *S. wallichii* may be due to the presence of various polyphenols and flavonoids. The presence of kaempferol-3-rhamnoside may have been also responsible for the free-radical scavenging and antioxidant activities of *S. wallichii*.

Conclusion

The present study demonstrates that all the extracts of *S. wallichii* caused a concentration-dependent inhibition of free radicals and increased ferric-reducing antioxidant power. These activities of *S. wallichii* may be due to the presence of various phenolic compounds and flavonoids. The ethanol extract showed maximum antioxidant activity followed by the aqueous extract, whereas the chloroform extract showed the least activity. Our study showed that *S. wallichii* possesses antioxidant potential and it might be useful against free radical-induced disorders.

Future perspective

Inflammation is one of the most important phenomena implicated in various diseases including cardiovascular disorders, diabetes and cancer. The use of antioxidants is helpful in neutralizing free radicals, the main causative factor of inflammatory disorders, and subsequently could be able to prevent free radical-induced ailments. *S. wallichii* use might be helpful in inflammatory disorders and could act as a healthcare aid. However, future studies are required to isolate the active principles. The activity guided isolation of different phytochemicals will be purposeful to establish their antioxidant potential and other disease curing ability in different preclinical models.

Summary points

- Free radicals are necessary to carry out various physiological functions in the body; however, their excess production may lead to different health disorders due to triggering of the inflammatory cascade.
- The excess of free radicals may be neutralized by the use of certain exogenous antioxidants.
- Plants synthesize several phytochemicals as secondary metabolites including flavonoids that provide different colors to flowers and fruits and have been consumed by humans since time immemorial.
- *Schima wallichii* a tree belonging to the family Theaceae, which is ethnomedicinally used to treat fever, gonorrhoea, cuts, wounds and lice infection.
- The stem bark powder of *S. wallichii* was extracted in chloroform, ethanol and water and their free-radical scavenging potential was determined.
- The chloroform, ethanol and aqueous extracts of *S. wallichii* scavenged DPPH, hydroxyl, superoxide and nitric oxide radicals in a concentration-dependent manner.
- The chloroform, ethanol and aqueous extracts of *S. wallichii* also showed antioxidant potential as they inhibited the generation of ABTS radical and increased FRAP in a dose-dependent manner.
- The phytochemical analysis of chloroform, ethanol and aqueous extracts of *S. wallichii* showed presence of flavonoids and polyphenols, which increased with increasing concentration.
- The flavonoid contents were maximum at 1000 μg/ml whereas total phenols increased in a concentration-dependent manner up to 900 μg/ml in chloroform, 1000 μg/ml in ethanol and 800 μg/ml in aqueous extracts.
- The free-radical scavenging activities of different extracts may be due to the presence of flavonoids and other polyphenols.
- Our study demonstrates the antioxidant potential of *S. wallichii*, and that its use could be helpful in inhibiting inflammatory health disorders.

Financial & competing interests disclosure

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Determination of the antineoplastic activity of Chilauni, *Schima wallichii* Korth in preclinical conditions

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K.Lalhminghlui, M.Sc

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Department of Zoology

Mizoram University

Aizawl-796004: North East India

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INTRODUCTION

Cancer is a severe threat to human health and affects the lives of millions of people around the world. It drains financial and emotional resources of a family in which cancer is detected. It is the second largest cause of death succeeding cardiovascular diseases (Siegel *et al*., 2015). Cancers are a large family of diseases involving abnormal cell growth and having the potential to invade or spread into other parts of the body.(WHO,2014; National Cancer Institute, 2014). All tumor cells show six main properties or characters of cancer cell. These characteristics are the main requirements to produce a malignant tumor.

The prominent properties of cancer are: lack of differentiation of cells, local invasion of adjoining tissue, and often, metastasis (spread to distant sites through the bloodstream or the lymphatic system). The immune system likely plays a significant role in eliminating early cancers or premalignant cells because immunodeficiency states are associated with an increased incidence of various kinds of cancer, particularly those associated with viral infection and tumors arising in the lymphatic system and the skin.

Cancer does not show clear sign and symptoms as they are great imitator. It is a very common mistake that people diagnosed with cancer are often treated for other disease. General symptoms of cancer may include unintentional weight loss, fever, excessive fatigue and changes inthe skin (O'Dell, 2009). Hodgkin disease, leukemias and cancers of the liver or kidney can cause a persistent fever (Bodel, 1974). People may become anxious or depressed post-diagnosis of cancer (Anguiano *et al*., 2012). Man power, natural and many material resources are spent for research purpose and in the development of new drugs for prevention and treatment of cancers each year worldwide. The impact of cancer on economic is significant and is rising to a high level. It has been estimated that the total annual economic cost of cancer in 2010 was approximately US$

1.16 trillion (Stewart BW, 2014). The present cancer treatments include radiotherapy, chemotherapy and chemically derived drugs. The application of chemotherapy can put cancer patients under a lot of pressure as it may be responsible for further serious damage to their health.

Plants and natural products have mainly contributed to the evolution of the effective and reliable traditional medicinal practices that have been employed for thousands of years in China, India and many other countries (Sneader, 2005). Different medicines from plant and health products have been accepted by people from all over the world, looking forward to improving the quality of life, disease prevention and treatment of chronic diseases and geriatric diseases as well as western medicine with helpless mysterious illness (Song *et al*., 2014). The interest in medicinal plants as major breakthrough in health aid has been put forwarded into great milestone by the rising costs of prescription drugs for maintaining healthy conditions of a person and well being and the bioprospecting of new drugs derived from plants could be more economic (Lucy and Edgar, 1999).

Schima wallichii (DC.) Korth. or Chilauni (Family:Theaceae) is an Asian species of evergreen tree. The genus inhabits warm temperate to subtropical climates across southern and South East Asia, from the eastern Himalaya of Nepal to eastern India across Indochina, Southern China, Taiwan and the Ryukyu Islands. Its common name is needlewood tree and it usually grows up to 35 m high. However, in some places it may be seen only 40 ft high (Min *et al*., 2003). Locally, it is called “khiang” in Mizo language and it finds many medicinal uses. *Schima wallichii* has been reported to possess several medicinal properties (Dewanjee *et al*., 2008; Gurung, 2002; Lalrinzuali, 2015; Sam *et al*., 2004; Lalfakzuala *et al*., 2007; Gardner *et al*., 2000; Koshimizu *et al*., 1998; Subarnas*et al*., 2003; Diantini *et al*., 2012; Paudel, 2014).

AIM OF THE STUDY

Schima wallichii has been reported to possess several medicinal properties in which the bark and the leaves are normally used. The leaves are reported to have antitumor and antimutagenic properties (Koshimizu *et al*., 1998; Subarnas *et al*., 2003) and a compound isolated from the leaves inhibited MCF-7 breast cancer cell proliferation through activation of the caspase cascade pathway (Diantini *et al*., 2012). However, the antineoplastic activity of the bark extract is not reported. Therefore, the present study was carried out to determine the anticancer activity of *Schima wallichii* extracts *in vitro* and *in vivo* by carrying out the following studies:

1. Fraction guided preparation of *Schima wallichii* extracts.
2. Phytochemical analysis
3. Antioxidant activity
4. Evaluation of anticancer activity *in vitro* and *in vivo*.

CHAPTER 1

This chapter refers to the general account of cancer, its history, types, causes and the different stages of cancer. The various kinds of cancer treatment modalities, importance of palliative care and the harmful effects are also highlighted and mentioned. It also gives the different medicinal uses of *Schima wallichii* and the general information about the plant used in this research work. Detail description on the aim and scope of the thesis is clarified in the following chapters.

CHAPTER 2

This chapter deals with the phytochemical screening and the TLC profiling of *Schima wallichii* and the extraction process. The non-infected stem bark of *Schima wallichii* was collected from Bazar veng, Lunglei, Mizoram during the months of April and May and identification was done at BSI, Shillong. The bark was cleaned and shade dried at room temperature in clean and hygienic conditions. The dried bark was then powdered using an electrical grinder. The dried powder of *Schima wallichii* was extracted sequentially 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 them to dryness under reduced pressure. The concentrated extracts were stored at -80°C until use. Qualitative phytochemical analysis was performed on the bark powder as well as the different extracts excluding petroleum ether. The bark powder was found to contain various phytochemicals other than phlobatannins. Flavonoids, saponins, cardiac glycoside, phenols are present in all the extracts whereas tannins and alkaloids are absent in the chloroform extracts. The quantitative phytochemical analysis showed the differences in the alkaloids, saponins and flavonoids content among the different extracts. The yield percentage, ash content and moisture content of the plant powder was also

determined. The TLC profiling also showed the presence of different components as indicated by the different Rf values in different solvent systems.

CHAPTER 3

Chapter 3 gives an account on the free radical scavenging activity and the antioxidant potential of the different extracts of *Schima wallichii in vitro*. The free radicals or simply ROS are highly reactive species which can be generated by cells during respiration, and cell-mediated immune functions. They are produced naturally in the body as they play an important role in many cellular functions however; if their production is quite high, the damages they can cause may play a role in the development of various human health disorders including cancer. However, the plants and natural products could be a major source of antioxidants that can scavenge free radicals. The antioxidant activity was estimated by investigating the ability of the different extracts of *Schima wallichii* to inhibit the generation of DPPH, superoxide anion, hydroxyl, nitric oxide, ABTS, FRAP free radicals using standard protocols. The total phenols and total flavonoids were also evaluated. The various extracts of *Schima wallichii* inhibited the production of DPPH, superoxide anion, hydroxyl, nitric oxide, ABTS, FRAP free radicals in a concentration dependant manner. The free radical scavenging activity increased upto a certain concentration which remained unaltered thereafter. The ethanol extracts was found to show the highest free radical scavenging activity among the three extracts.The amount of total phenols and flavonoids also increased with increasing concentration and the maximum total phenol contents was recorded for ethanol extract while total flavonoids was observed at aqueous extract. Therefore, the antioxidant and free radicals scavenging activities of *Schima wallichii* may be due the presence of various phytochemicals mostly alkaloids which is the highest phytochemical content determined.

CHAPTER 4

In this chapter, in vitro anticancer activity of the ethanol extract of *Schima wallichii* was determined using different assay following standard protocols. The cytotoxicity of the ethanol extract of *Schima wallichii* (SWE) was assessed in HeLa and V79 cells by MTT assay. The HeLa and V79 cells treated with different concentrations of SWE showed a concentrations dependent increase in its cytotoxic effect. Treatment of cells with SWE for different durations also increased its cytotoxic effects in a time dependent manner. The results of MTT assay were confirmed by clonogenic assay in HeLa cells, where the cells were treated with different concentrations of SWE. Treatment of HeLa cells with various concentrations of SWE reduced the clonogenicity of cells in a concentration dependent manner. The ability of SWE to induce apoptosis was studied by determining the caspase 8 and 3 activities at different post- treatment times. The SWE treatment marginally increased activity of both capsase 8 and 3 in a time dependent manner. The effect of SWE treatment was studied on the lipid peroxidation, glutathione contents, glutathione-s-transferase, catalase and superoxide dismutase activities, where it was found to reduce the glutathione contents, and activities of glutathione-s-transferase, catalase and superoxide dismutase in a time dependent manner. Treatment of HeLa cells with ethanol extract of *Schima wallichii* increased the cytotoxic effect in a concentration dependent manner followed by a reduction in the clonogenicity of HeLa cells. The cell killing effect of SWE may be due to the caspase activation and reduction in the glutathione contents, and activities of glutathione-s-transferase, catalase and superoxide dismutase.

CHAPTER 5

In this chapter, the acute toxicity of *Schima wallichii* was evaluated both orally and intraperitoneally in which the oral administration did not showed toxicity up to 2g/kg b.wt for ethanol extract while 4g/kg b.wt.for chloroform and aqueous extracts, respectively. However, the LD_{50} was found to be 100mg/kg b.wt. for ethanol extract and 500mg/kg b.wt for chloroform and aqueous extracts, respectively when these extracts were administered intraperitoneally. The administration of 10- 250 mg/kg b.wt. chloroform, ethanol and aqueous extracts of *Schima wallichii* to tumor bearing mice resulted in a dose dependent increase in the tumor free survival and maximum effect was observed for 10 mg/kg ethanol extract, which increased the tumor free survival by 40% beyond 120 days. 20 and 40 % long term tumor free survivors were observed up to 60 days for chloroform and aqueous extracts at 150 and 100 mg/kg, respectively. The administration of 10mg/kg b. wt. ethanol extract resulted in an increase in the AST up to 64.81 days (IALS, 204.27%) and MST Up to72.6 days (IMLS, 224.14 %). Therefore, 10 mg/kg body weight of the ethanol extract was considered as an optimum dose for its antineoplastic activity and further investigations were carried out using this dose. The administration of 10 mg/kg body weight ethanol extract into tumorized mice resulted in a time dependent rise in the micronuclei in both mononucleate and binucleate cells up to 24 h. The analysis of apoptotic and necrotic index also showed a time dependent increase and the maximum rise was observed at 36 h post treatment in the tumorized mice receiving 10 mg/kg body weight of ethanol extract of *S. wallichii*. 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 *Schima wallichii* 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.

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