Investigation of the anticancer potential of Pasaltakaza, *Helicia nilagirica* Bedd.

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Submitted by
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CERTIFICATE

This is to certify that **Kum. Jennifer Zoremsiami** carried out her research work under my supervision since 2014. The thesis entitled **"Investigation of the anticancer potential of Pasaltakaza, *Helicia nilagirica Bedd.*"** is an original piece of work and has not been submitted for any other degree of any other university.

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DECLARATION

I, Jennifer Zoremsiami, hereby declare that the subject matter of this thesis entitled **"Investigation of the anticancer potential of Pasaltakaza, *Helicia nilagirica Bedd.*"** 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.

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Ph. D. Course Work Award-Sheet

The following is the assessment record of ***Jennifer Zoremsiami,*** *Roll No.* ***Zoo/CW/12/02*** *in the Ph. D. Course Work Examination held in March, 2013.*

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Grade Point	Grade	Grade Point	Grade	Grade Point	Grade			
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First Tabulator

Second Tabulator

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

'O'	'A'	'B'	'C'
7.00-10.00	6.00-6.99	5.00-5.99	Below 5.00

The performance of the scholar shall be evaluated in the following grades : ***'O'*** *– Outstanding,* ***'A'*** *and* ***'B'*** *with grade point valuation in the 10 point scale, i.e., 7.00-10.00 corresponding to* ***'O'*** *grade, 6.00-6.99 corresponding to* ***'A'*** *grade, 5.00-5.99 corresponding to* ***'B'*** *grade. Those securing less than 5.00 points shall be graded as* ***'C'****.*

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ABBREVIATIONS

µg	Microgram
ABTS	2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)
BNMn	Binucleated micronucleus
CDNB	1-Chloro 2,4-Dinitrobenzene
CMC	Carboxy methyl cellulose
DLA	Dalton's lymphoma ascites
DOX	Doxorubicin
DPPH	2, 2-diphenyl-1-picryl hydrazine
DTNB	5-5'-dithiobis[2-nitrobenzoic acid]
EDTA	Ethylenediamine tetra-acetic acid
HNE	*Helicia nilagirica* extract
MTT	(3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide)
NADH	b-nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
PMS	Phenazine methosulphate
R_f	Retention factor
ROS	Reactive oxygen species
TBA	Thiobarbituric acid
TCA	Tricarboxylic acid
TLC	Thin layer chromatography

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1

Cancer can be defined as a multifactorial disease in which a group of abnormal cells grow uncontrollably defying all the normal rules and regulatory mechanisms of cell growth and division (Hejmadi 2010). The word cancer is derived from Latin, which literally means crab and the Greek word is *karkinos* which also means crab**.** The term originated from the observation of Hippocrates (the father of modern medicine) that the blood vessels around malignant tumors looked like the claws of a crab, and hence named it cancer. The advancement in cancer research provided an insight into cancer cell's autonomy, and now the cancer has been defined as a disease which involved changes or mutation into the cell genome. The mutated cell genome produced proteins which disrupt the cellular balance leading to their uncontrolled division and subsequently form cancer.

HISTORY

Paleopathologic findings have indicated that tumors already existed in animals in prehistoric times, long before men appeared on the Earth. The earliest written description of cancer, a breast cancer, was found in the Edwin Smith Papyrus that was written approximately 3000 BC. The Ebers Papyrus, dated circa 1500 BC, contains the first reference to a soft-tissue tumor, a fatty tumor, and includes reference to possible cancers of the skin, uterus, stomach, and rectum (Ebbell 1937). The Sumerians, Chinese, Indians, Persians, and Hebrews of the same epoch were used herbal remedies such as tea, fruit juices, figs, and boiled cabbage to treat cancer, but in advanced cases, they did not hesitate to resort to solutions and pastes of iron, copper, sulfur, and mercury to control the disease. Many of these concoctions remained in external and internal use, in various concentrations, for more than 3000 years (Wolff 1928; Castiglioni 1931). Although sporadic application of chemical agents was introduced by the Egyptians and Greeks before the Common Era (BCE), the earliest systematic therapeutic use of

chemicals was initiated in the 16th century by Paracelsus (1493-1541) who introduced mercury, lead, sulfur, iron, zinc, copper, arsenic, iodine, and potassium as internal remedies (Paracelsus 1562). The word metastasis was introduced by Joseph Recamier (1774-1852), a French gynecologist, in 1829. By watching the growth and spread of cancers, he was able to identify blood vessel invasion by cancer with the naked eye. In 1838 Johannes Muller described cancers as special groupings of abnormal cells and stroma. He attributed cancer to the formation of new cells in diseased organs with potential to be destructive and to spread to other parts of the body by vascular invasion. He associated cancer with aging and identified tumor necrosis (apoptosis) as a sign of regression. He microscopically distinguished epithelial and connective tissue tumors. Muller divided malignant epithelial tumors for carcinoma simplex (squamous carcinoma), carcinoma alveolare (adenocarcinoma), carcinoma fasciculatum (spindle cell carcinoma), carcinoma medullare (medullary carcinoma), and carcinoma melanodes (malignant melanoma). With regards to malignant connective tissue tumors, Muller described infiltrating fibrous tumors (desmoid tumor and fibrosarcoma), cystosarcoma of the breast, chondrosarcoma, and osteosarcoma of bones. In 1846, Virchow coined the terms ‘‘hyperplasia’’ and ‘‘metaplasia’’ and recognized that both conditions are potential precursors of cancer, and that cancer cells have marked difference both in size and shape as compared to benign cells. The embryonal characteristics were proposed by Julius Cohnheim in 1877. In addition twenty years later, Moritz Wilhelm Hugo Ribbert, a Zurich pathologist, proposed that mechanical irritation like chronic inflammation and trauma can also lead to the development of cancer especially in the epithelial and connective tissue cells (Cohnheim 1877; Ribbert 1904). *Schistosoma haematobium* was accused to be the only microorganism which caused cancer (bladder) for decades until the discovery of *Clonorchis sinensis*, a causative factor for bile cancer (Harrison 1889). Despite the

fact that the field of medicine had made great strides in some ancient civilizations, the progress in cancer treatment has been meager, which is even dreaded today due to the persistent view that cancer is an incurable disease (Diamandopoulus 1996; Kardinal 1979).

CHARACTERISTICS OF CANCER AND CANCER CELLS

In normal condition, adult human body constitutes approximately 10^{15} cells, of which approximately 10^{12} are formed, divide and differentiate each day to replace the dead and worn out cells, these cells will enter the active proliferative phase only after receiving the mitogenic growth signals, and cannot multiply in the absence of these signals. However, cancer cells have lost the need of these stimulatory signals and therefore can proliferate whether these signals are present or not and produce their own growth factors mimicking the normal growth factors which make them independent of the normal growth factors (Fedi *et al*. 1997; Hanahan and Weinberg 2000).

Till date there are more than 100 distinct types of cancers and tumors and each specific organ has subtypes, in spite of this diversity, human cancers share several fundamental properties. A set of six characteristic properties of cancers has been proposed by Hanahan and Weinberg and called it the 'hallmarks of cancer' (Hanahan and Weinberg, 2000). These capabilities comprised of

i. **Self-sufficiency in growth signals**: Tumor cells show a greatly reduced dependency on exogenous growth stimulation as they generate their own growth signals by altering the extracellular growth signals, transducers of those signals and intracellular circuits that translate those signals into action thereby disrupting the normal homeostatic mechanism within a tissue. Examples include the production of PDGF (platelet-derived growth factor) and TGFα (tumor growth factor α) by glioblastomas and sarcomas, respectively.

ii. **Insensitivity to anti-growth signals:** Tumor cells are able to evade the antiproliferative signals by disrupting the retinoblastoma protein (pRb) pathway which blocks the cells from advancing through G1 phase of the cell cycle, thereby allowing the cells to proliferate, rendering cells insensitive to antigrowth factors (Weinberg 1995).

iii. **Evasion of apoptosis:** Cancer cells can acquire resistance to apoptosis through mutation of the p53 tumor suppressor gene which is evident in more than 50% of human cancers (Harris 1996).

iv. **Limitless replicative potential:** The three common acquired capabilities of cancer cells such as the growth signal autonomy, insensitivity to antigrowth signals and resistance to apoptosis can lead to an uncoupling of a cell's growth program from signals in its environment. This can be achieved by upregulating the expression of telomerase enzyme.

v. **Sustained angiogenesis:** The formation of new blood vessel is a prerequisite for the rapid clonal expansion of tumor cells, so the tumor cells appear to activate the angiogenic switch by changing the balance of angiogenesis inducers such as VEGF and countervailing inhibitors such as thrombospondin-1 (Hanahan and Folkman 1996).

vi. **Tissue invasion and metastasis:** At the time of development of most of human cancers the tumor cells move out and invade adjacent tissues where they may succeed in finding new colonies by a process known as metastasis. About 90% of human cancer deaths are caused by metastases (Sporn 1996). One of the reasons for this capability is the loss of function of E-cadherin due to mutational inactivation, transcriptional repression or proteolysis of the extracellular cadherin domain in majority of epithelial cancers (Christofori and Semb 1999).

Chapter 1

CAUSES AND MECHANISM OF CANCER

In humans, carcinogenesis is a multifactorial and complex mechanism. It can be divided into four stages: tumor initiation, tumor promotion, malignant conversion, and tumor progression. Tumor initiation is an irreversible change caused by chemical carcinogens by forming DNA- adducts resulting in the activation of proto-oncogenes, inactivation of tumor suppressor genes and genomic instability. Tumor promotion occurs when the initiated cells undergo clonal expansion which will undergo further genetic changes and increased malignancy. Tumor promoters are not carcinogenic alone but they can act as mediators for tumor initiator and they can increase the latency period of tumor formation as well as increase the number of tumors in tissues. Examples of tumor promoters include dioxin, benzoyl peroxide, dichlorodiphenyltrichloroethane (DDT), phenobarbital, cigarette-smoke condensate,
Ultraviolet light, etc. Malignant conversion occurs with further genetic changes which can be due to infidelity during DNA synthesis leading to the transformation of a preneoplastic cell into the malignant phenotype. Tumor progression is the transformation of a malignant tumour with the accumulation of further mutations and selection of mutated cells and subclones. The mutations affect additional oncogenes and tumour suppressor genes. Metastasis may also occur where tumor cells are able to secrete proteases that allow invasion beyond the immediate primary tumor location (Schulz 2005).

The initiation and progression of cancer depends on exogenous factors (chemical, physical, or biological carcinogens) as well as endogenous factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These factors can act together or in a sequence, by different mechanisms at different stages of tumor development resulting in abnormal cell behavior and excessive proliferation resulting in metastasis. The

lifestyle, environment and age have a profound effect in the development of cancer. In 1950, the attendees of an international symposium sponsored by the World Health Organization learned that people who migrated to other countries, developed types of cancer common to their adopted countries, rather than their homelands. This implied that most cancers were caused by exposures in the environment, such as smoking, alcohol consumption and exposure to various carcinogens rather than inherited genetic factors. Genetic predisposition increases the chances of earlier development of cancer in less than 10% of cases such as childhood leukemias, retinal cancers etc. (Higginson 1992; Hejmadi 2010).

Chemical carcinogens

Chemical carcinogens can be grouped into organic and inorganic compounds. Inorganic compounds comprised of nickel, cadmium, or arsenic which could be encountered in the workplace or present as contaminants in water whereas organic compounds comprised of aliphatic compounds, like nitrosamines, which occur in smoked and pickled foods, or trichloro-ethylene, which is used for cleaning. Aromatic compounds like benzopyrenes and arylamines are generated from natural sources by burning of coal and fuels, and are among the many carcinogens in tobacco smoke. Nitrosamines are thought to contribute to stomach cancer whereas arylamines cause bladder cancer, in particular. Natural compounds produced by plants and mold such as Aflatoxin B1 is implicated as a carcinogen in liver cancer. Medical drugs used in cytostatic tumor therapy like cyclophosphamide, nitrogen mustards, and platinum compounds are also carcinogenic. Various hormones and hormone-like compounds from natural and pharmaceutical sources also influence the development of cancers in specific tissues e.g. in the breast and prostate. Reactive oxygen species produced at increased rates during certain

physiological processes such as immune defense and inflammation can also be mutagenic (Schulz 2005).

Physical carcinogens

Depending on its' dose and absorption any electromagnetic radiation can act as a carcinogen. Ultraviolet radiation (UVR) can be divided into three wavelength ranges based on their differences in photochemistry and biological importance such as UVA (320 to 400 nm), UVB (290 to 320 nm) and UVC (240 to 290 nm). Among them, UVB is mainly responsible for skin cancer through direct photochemical damage to DNA as it overlaps the upper end of the DNA and proteins' absorption spectra whereas UVA is photocarcinogenic and involved in photoaging but is weakly absorbed by the DNA and proteins. The relevant chromophores may therefore involve reactive oxygen species (ROS), which secondarily cause damage to DNA. Hence, although UVA and UVB light constitute a minute portion of the emitted solar wavelengths, they are primarily responsible for the Sun's pathologic effects. γ-radiation from natural, industrial, and iatrogenic sources (e.g., used in X-ray diagnostics) can penetrate the body and become carcinogenic to the extent to which it is absorbed, damaging DNA and cells by direct absorption but also indirectly by generating reactive oxygen species. Radioactive β-radiation and specifically α-radiation is most dangerous when nuclides are ingested or incorporated, eg. cesium, uranium, and plutonium. The effect of radioactive isotopes depends also on their distribution in the body. For instance, radioactive iodine is accumulated in the thyroid gland and therefore causes specifically thyroid cancers, whereas radioactive cesium isotopes tend to become enriched in the urinary bladder (Devita 2001; Schulz 2005).

Biological carcinogens

Biological carcinogens include certain bacteria and viruses like human papilloma virus HPV16 and HPV18 that causecervical and other genital cancers which can also influence the development of cancers of the skin and of the head and neck. Herpes simplex virus (HHV8) is involved in Kaposi sarcoma, hepatitis B virus in liver cancers, and Epstein Barr virus in lymphomas. HIV facilitate the development of cancers mostly by interfering with the immune system, but HTLV1 (human T-cell leukemia virus) causes a rare leukemia by direct growth stimulation of T-cells. Bacteria such as *Helicobacter pylori* causes stomach cancer through infection. Parasites like *Schistosoma trematodes* are also known to cause urinary bladder cancer (Schulz 2005).

Endogenous carcinogens

Endogenous factors alone may result in development of cancer or they can act along with exogenous agents as cancer modulators. The detoxification mechanism might not be able to remove the carcinogenic compounds such as nitrosamines, aromatic amines, quinones, reactive aldehydes and ROS generated during normal metabolism leading to the formation of cancer. However, inefficient DNA repair mechanism, genetic and even epigenetic errors during fetal development, ageing and chronic inflammation could all lead to the development of cancer at some point of life. Prolonged hormonal stimulation of a particular target organ can also lead to cancer such as breast and other reproductive organs which is mainly controlled by mutation of genetic sequence that encoded protein. (Kufe *et al*. 2003; Schulz 2005)

Chapter 1

CLASSIFICATION OF CANCER

Cancers are classified based on their degree of malignancy and histological subtype which is a prerequisite for better prognosis and appropriate treatment. The various classifications or characterizations include:-

Staging:

Cancer can be classified depending on the extension of tumor. Investigation by visual inspection, palpation and various imaging techniques (ultrasound, X-rays, scintigraphy, computer tomography, magnetic resonance, and positron emission tomography) prior to surgery to detect changes in tissue shape density, changes in metabolism and blood flow is called clinical stage and denoted by 'c' whereas inspection of the tumor site and histopathological investigation of the specimen post surgery is called pathological stage and denoted by 'p'.

The most widely used systematic staging system is the TNM classification where the extent of the primary tumor is normally described by T1-T4, where increasing numbers describe larger and/or more invasive tumors. The system varies for different tumor sites. The presence of cancer cells in lymph nodes is denoted by N0, N1, and in some cancers also N2, with N0 meaning none detected. The presence of metastases is indicated by M0 meaning none detected, M1, or in some cancers also M2. After surgery, to know whether all of the local tumor growth has been removedR value is used. R stands for resection margin, so R0 means that the tumor seems to be wholly contained within the removed specimen. In all categories, the affix 'x' is used for 'not determined/unknown'.

Grading: To further estimate the degree of malignancy grading system is being used. The most prevalent system is G grading, which usually ranks from G0 to G4.The designation G0 typically denotes normal differentiation and no cellular atypia, as would be found in a benign

tumor. At the other end, G4 would be assigned to cancers with a cellular morphology completely different from the normal tissue and pronounced atypia of the cells and nuclei. The grades G1-G3 are called well-differentiated, moderately and poorly differentiated tumors.

Histological classification: Histological typing of tumors is performed by evaluating their morphology. Routine procedures use a variety of specific stains developed over centuries in anatomy and pathology to highlight particular cell types as well as extracellular structures like basement membranes, fibers or mucous. Increasingly, tumor classification by histopathological investigation is being improved by specific molecular markers. Immunohistochemical staining with antibodies directed against specific antigens of the presumed tissue of origin, e.g. cytokeratins, or tumor-specific antigens, eg. carcinoembryonic antigen, is often performed. For leukemias, analysis of subtypes can be determined by antibody staining followed by flow cytometry (Schulz 2005; Webber *et al*. 2014; American Cancer Society, 2015).

CANCER EPIDEMIOLOGY

Cancer is one of the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases in 2012 (Farley *et al.* 2012) and it is the second leading cause of death globally, and was responsible for 8.8 million deaths in 2015. In 2017, 1,688,780 new cancer cases and 600,920 cancer deaths are projected to occur in the United States (Siegel *et al*. 2017). Globally, nearly 1 in 6 deaths is due to cancer and the number of new cases is expected to rise by about 70% over the next 2 decades. Approximately 70% of deaths from cancer occur in low- and middle-income countries. Around one third of deaths from cancer are due to the 5 leading behavioral and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco and alcohol use. Tobacco use is the most important risk factor for cancer and is responsible for approximately 22% of cancer deaths (GBD 2015). Cancer causing

infections, such as hepatitis and human papilloma virus (HPV), are responsible for up to 25% of cancer cases in low- and middle-income countries (Plummer *et al*. 2012). Late-stage presentation and inaccessible diagnosis and treatment are common. In 2015, only 35% of low-income countries reported having pathology services generally available in the public sector whereas more than 90% of high-income countries reported treatment available services. The economic impact of cancer is significant and is increasing; in 2010 the total annual economic cost of cancer was estimated at approximately US$ 1.16 trillion (Stewart 2014).

TREATMENT

For the treatment of cancer a different range of therapies are available. Surgery, irradiation or drugs can be employed, or a combination of these. The choice of therapy depends on the type of cancer; surgery or radiation treatment is chosen for localized cancers. In contrast, leukemias, lymphomas, and metastatic or locally advanced carcinomas and soft tissue cancers require drug chemotherapy, which is in some cases supplemented by radiotherapy or surgery of primary cancers or metastases. Conversely, adjuvant treatment can be given where surgery is followed by chemotherapy or irradiation to attack residual local tumor or metastases or neo-adjuvant treatment can be given where chemotherapy can be applied before surgery to shrink the tumor mass and facilitate its complete resection. The standard chemotherapy regimen for a cancer is usually designated as ‘first-line’, if it fails, ‘second-line’ therapy can be attempted (Schulz 2005).

Surgery

Surgery is the oldest treatment for cancer and forms the mainstay of treatment of solid tumors till today. It is most effective in the treatment of localized primary tumor and associated regional lymphatic. This is accomplished by en bloc surgical procedures that attempt to

encompass gross and microscopic tumor in all contiguous and adjacent anatomic locations. Intuitively, it appears logical that surgery should have little role in disease management once a neoplasm has spread from the primary location to a distant site. However, advances in surgical techniques and a better understanding of the patterns of spread of individual cancers have allowed successful resections for an increased number of patients (Kufe *et al*. 2003).

Radiotherapy

Radiotherapy refers to the treatment of benign and malignant tumor with ionizing radiation (IR). With the discovery of X-rays by Wilhelm Conrad Röntgen from Germany in 1895 and radium by Marie Curie, the clinical usefulness of radiation as a cancer therapy has been established (Baskar 2012). Along with surgery and chemotherapy, radiation therapy or radiotherapy gains an important modality for the treatment of cancer because of its highly cost effective single modality treatment and accounts only about 5% of the total cost of cancer care (Ringborg 2003). With the advancement in imaging techniques, computerized treatment planning systems, radiation treatment machines (with improved X-ray production and treatment delivery) as well as improved understanding of the radiobiology of radiation therapy, there has been a great progress in this field (Bernier 2004). Radiotherapy can be delivered primarily with high-energy photons (γ-rays and X rays) and charged particles (electrons) and other therapeutic modalities include neutrons (Vynckier 1998) and protons (Miller 1995). The radiation randomly affects the molecules of the cell where the main target is the deoxyribonucleic acid (DNA) which can result in single- and double-strand breaks (DSBs) in the sugar-phosphate backbone of the DNA molecule (Dizdaroglu, 1992; Lomax *et al*., 2013), damage caused to the cellular and nuclear membranes and other organelles may also play an important role. Cross-links between DNA strands and chromosomal proteins also occur. Depending on the type of radiation the

mechanism of DNA damage also differs. For example, X and γ radiation are indirectly ionizing where DNA damage is caused by the short-lived, hydroxyl free radicals produced primarily by the ionization of water components of the cell (Ward 1988; Desouky *et al*., 2015), however protons and other heavy particles are directly ionizing and damage DNA directly (Phillips 1997). Radiation damages both normal cells as well as cancer cells, so the main goal of radiation therapy is to maximize the radiation dose to abnormal cancer cells while minimizing exposure to normal cells. Since, normal cells usually repair themselves at a faster rate and retain its normal function status than the cancer cells which are generally not as efficient as normal cells in repairing the radiation damage resulting in the differential cancer cell killing (Begg 2011).

Chemotherapy

Chemotherapy is any drug which is used to treat any disease. But the word is commonly used in cancer therapy. The term 'chemotherapy' was coined by Paul Ehrlich while he was working on the treatment of infectious diseases by using antibiotics. In the early 1900s, George Clowes at Rosewell Park Memorial Institute used this idea to induce tumor model in rodents to screen the potential anticancer drugs. Alkylating agents, the first class of chemotherapeutic drugs to be used in the clinical setting were a product of the secret gas program of the United States in both world wars where the military seamen were exposed to mustard gas in World War II which led to the observation that alkylating agents caused marrow and lymphoid hypoplasia (Alexander 1944; Hersh 1968). This observation led to the direct application of such agents in humans with hematologic neoplasms, including Hodgkin's disease and lymphocytic lymphomas, at the Yale Cancer Center in 1943 (Marchall 1964; DeVita and Chu, 2008). During the same year, the significant proliferative effect of folic acid against leukemic cell growth in children with lymphoblastic leukemia had also been reported by Sidney Farber and the antifolates like

aminopterin (the predecessor of methotrexate) was found to kill tumor cells by blocking DNA replication (Farber, 1948). This led to the discovery of many drugs capable of blocking different functions of cell growth and replication. This was the beginning of the chemotherapy research. The use of methotrexate in 1956 treated metastatic cancer for the first time after the discovery of DNA. There are more than 100 different types of chemotherapeutic drugs for the treatment of different types of cancers, which are used either alone or in combination (Colvin 2003).

The chemotherapeutic drugs can be classified according to their mechanism of action such as the alkylating agents, antimetabolites, plant alkaloids, antitumor antibiotics, hormonal agents and targeted cancer therapies. The alkylating agents form covalent bonds with amino, carboxyl, sulfhydryl, and phosphate groups in biologically important molecules such as DNA, RNA or proteins which impaired their function. In DNA the electron-rich nitrogen at the 7 position of guanine is particularly susceptible to alkylation. Alkylating agents depend on cell proliferation for activity by killing a fixed percentage of cells in a given dose, but are not cell-cycle phase–specific. The alkylating agents are classified according to their chemical structures and mechanisms of covalent bonding; this drug class includes the nitrogen mustards used against cancer of the hematopoietic system, nitrosoureas used against a variety of brain tumors and platinum complexes like cisplatin is used against testicular, ovarian, bladder cancers etc. (Colvin, 1990; Pazdur *et al*. 2007).

Antimetabolites are structural analogs of the naturally occurring metabolites involved in DNA and RNA syntheses. Antimetabolites exert their cytotoxic activity either by competing with normal metabolites for the catalytic or regulatory site of a key enzyme or by substituting for a metabolite that is normally incorporated into DNA and RNA. Because of this mechanism of action, antimetabolites are most active when cells are in the S phase and have little effect on cells

in the G_0 phase. Consequently, these drugs are most effective against tumors that have a high growth fraction. The antimetabolites can be divided into folate analogs, purine analogs, adenosine analogs, pyrimidine analogs, and substituted ureas. These drugs are most effective against tumors that have a high growth fraction (Devita et al. 2001; Pazdur *et al*. 2007).

Plant alkaloids inhibit tubulin polymerization thereby disrupt the assembly of microtubules which result in mitotic arrest at the metaphase stage and eventually leading to the death of cells. They include vinca alkaloids (vincristine,vinblastin) derived from the periwinkle plant *Vinca rosea*, taxanes including paclitaxel and docetaxel (Taxotere) which are semisynthetic derivatives of extracted precursors from the needles of yew plants. The other class of plant based drugs that inhibit topoisomerases include epipodophyllotoxins such as etoposide, a semisynthetic epipodophyllotoxin extracted from the root of *Podophyllum peltatum* (mandrake), and camptothecin derived from the Chinese ornamental tree *Camptotheca acuminata* and its semisynthetic analogs including irinotecan (CPT-11 [Camptosar]) and topotecan (Hycamtin). Plant alkaloids are effective against different types of cancers (Pazdur *et al*. 2007).

Antitumor antibiotics intercalate DNA and inhibit topoisomerases I and II resulting in spontaneous oxidation and formation of free oxygen radicals that cause strand breakage and finally cell death. They include bleomycin and anthracycline which is produced by the *Streptomyces verticillus* and *Streptomyces percetus* var *caesius* (Umezawa *et al.*, 1966; Arcamone *et al*., 1969).

Hormonal agents such as estrogen inhibitors, androgen inhibitors, gonadotropin-releasing hormone agonists, aromatase inhibitors and glucocorticoids bind to their respective hormone receptors thereby suppressing their action. Estrogen inhibitor such as tamoxifen could bind to the estrogen receptors of breast tumor inducing competitive inhibition and suppresses the

production of insulin-like growth factor 1(IGF 1) and transforming growth factor alpha (TGF-α) (Kufe *et al*. 2003).

Targeted cancer therapies interfere with specific proteins involved in tumorigenesis. There are three main types of targeted cancer therapies; 1) monoclonal antibodies, 2) small molecule inhibitors and 3) immunotoxins (Baudino 2015). Monoclonal antibodies deregulate the functions of the cancer cell by the disruption of protein function and possible downstream signaling, antibody-dependent cytotoxicity and complement dependent cytotoxicity. Avastin (bevacizumab, Genentech) is a monoclonal antibody that targets VEGF by inhibiting VEGF signaling. Small molecule inhibitors competitively bind to the active or inactive ATP binding site of a tyrosine kinase. They are used to target proteins that have become either unregulated or upregulated during cancer progression, such as BCR-ABL. Gleevec (imatinib mesylate, Novartis) is a small molecule inhibitor targeted against the BCR-ABL tyrosine kinase domain, which is used in the treatment of several different cancers, including Philadelphia chromosome positive chronic myeloid leukemia (Ph+ CML), and c-Kit positive gastrointestinal stromal tumors (de Jong, 1997; Deininger, 2003). Immunotoxins are a new class of drugs that involves modifying monoclonal antibodies or growth factors which binds to a cell surface protein and becomes internalized via clathrin-coated pits and elicit apoptosis of the targeted cell (Alewine, 2015). Ontak (denileukin diftitox, Ligand Pharmaceuticals) is an interleukin-2 (IL-2)/DT fusion protein produced by creating recombinant DNA (rDNA) that fuses together a DT gene that has a truncated binding region and an IL-2 gene is used to treat patients with cutaneous T-cell lymphomas (CTCLs) (Foss, 2005). Activated T-cells highly express high affinity IL-2R on their cell membrane and thus, IL-2R becomes a potential target in T-cell lymphomas. The bacterial toxin, DT, results in ADP-ribosylation of elongation factor-2 (EF-2), which causes EF-2 to

become inactive, preventing protein synthesis (Carroll, 1987). DT is extremely potent and only one molecule needs to enter the cytosol of a cell to cause an apoptotic response (Kreitman, 2006).

However, cancer therapy have their own limitations as they also damage the normal dividing cells especially the rapidly regenerating tissues, such as hair follicles and can also lead to drug resistance as well induction second malignancies, (Wu *et al*. 2008; Morton et al., 2014) necessitate the need to utilize alternative concepts or approaches to treat the cancer.

AIM OF THE STUDY

Helicia nilagirica Bedd. (Family: Proteaceae) locally known as Pasaltakaza is a medium-sized tree, which grows up to 12 meters high. It is widely distributed in Sri Lanka, southern India, Burma (Myanmar), Indochina, Japan, Taiwan, and Thailand. It is also found scattered in lowland to montane rain forests, up to 2,000-3,350 m altitude. Some species prefer habitats along streams but other species are found on hilltops or ridges (Khamyong et al., 2004). This tree has been used as a folk medicine since time immemorial in Mizoram, India by the Mizo tribe. Its decoction prepared by boiling the leaves or bark is used to treat various stomach ailments including peptic ulcers, indigestion, mouth ulcer, urinary tract infection and gynaecological disorders. It is also used in scabies and other skin diseases (Sawmliana, 2003). The fruits of *H. nilagirica* have been used as a medicine to cure cough and cold in Sikkim (Chauhan, 2001). The scientific evaluation regarding its medicinal or other properties is scarce Except that, it has been shown to possess anti-inflammatory activity in rat cotton pellet granuloma model (Lalawmpuii *et al.,* 2014). This indicates the need to systematically evaluate its anticancer properties. The main objective of present investigation is to evaluate the anticancer activity of *Helicia nilagirica* extracts *in vivo* and *in vitro* by carrying out the following investigations:

Chapter 1

1. Activity guided fractionation of different extracts of *Helicia nilagirica*.
2. Evaluation of anticancer activity of the various extracts in different cultured neoplastic cell lines and in tumor bearing mice.
3. Study of the mechanism of action of active extract.

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CHAPTER 2

PHYTOCHEMICAL PROFILING OF *HELICIA NILAGIRICA* (BEDD.)

Chapter 2

Abstract

The mature non-infected stem bark of Helicia nilagirica was collected, dried and powdered and subjected to sequential extraction with increasing polarity using petroleum ether, chloroform, ethanol and distilled water. The different extracts were cooled and evaporated with rotary evaporator and kept at -80^{o}C for further use. Phytochemical analysis was done on all the three extracts in which the presence of flavonoids,tannins,terpenoids,cardiac glycosides were found in the chloroform and ethanol extracts whereas in the aqueous extract saponin, tannins and cardiac glycosides were present. The TLC profile also showed the presence of different phytochemicals as indicated by different Rf values revealed by the different extracts for the various solvent systems used.

1. INTRODUCTION

Medicinal plants have been used as the main traditional herbal medicine amongst rural dwellers worldwide since antiquity to date (Doughari, 2012). The earliest written evidence of use of plants as medicine has been found around 5000 years before on a Sumerian clay slab from Nagpur which comprised of 12 recipes for drug preparation comprising over 125 plants including poppy, henbane and mandrake. In 2500 BC the Chinese emperor has written a book for 365 drugs some of which have been used nowadays such as *Rhei rhisoma*, camphor, *Theae folium, Podophyllum*, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra (Bottcher 1965; Wiart 2006) .Over the years plants have been used as the main source of medicine especially in the developing countries and more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (WHO, 2010)

Phytochemicals are natural bioactive chemical compounds found in plants, protect plants from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack (Ali et al, 2006). These compounds known as secondary plant metabolites include, organic substances like alkaloids, carotenoids, glycosides, terpenoids, steroids, tannins, flavonoids,

vitamins, mucilages, minerals, organic acids etc (Bravo, 1998; Brown et al., 1999; Gosslau and Chen, 2004; Heber, 2004). These secondary metabolites are of great health benefits to humans. Some of the beneficial roles of phytochemicals are low toxicity, low cost, easy availability and they have an extensive range of therapeutic activities such as antioxidant, antimicrobial, hypoglycemic, antidiabetic, antimalarial, anticholinergic, antileprosy, and antineoplastic These phytochemicals also help in the modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism (Andre et al, 2010; Negi et al, 2011). Even with a remarkable progress in synthetic drugs, therapies using medicinal plants make a major contribution to the pharmaceutical industry because they are safe, easily available, cost effective, and there are synergistic effects of other biologically active ingredients and the presence of beneficial minerals. (Farnsworth and Soejarto, 1991, Jagetia, 2017).

Helicia nilagirica Bedd. (Family: Proteaceae) is locally known as Pasaltakaza, is a medium-sized tree, which grows up to a height of 12 meters. It is widely distributed in Sri Lanka, southern India, Burma (Myanmar), Indochina, Japan, Taiwan, and Thailand. It is also found scattered in lowland to montane rain forests, up to 2,000 -3,350 m altitude. Some species are found in habitats along the streams whereas other species are found on hilltops or ridges (Khamyong et al., 2004). This tree has been used as folk medicine since time immemorial in Mizoram, India by the Mizo people. Its decoction prepared by boiling the leaves or bark is used to treat various stomach ailments including peptic ulcers, indigestion, mouth ulcer, urinary tract infection and gynaecological disorders, It is also used in scabies and other skin diseases (Sawmliana 2003). The fruits of *H. nilagirica* have been used as a medicine to cure cough and

cold in Sikkim (Chauhan 2001). The ethnomedicinal use of *Helicia nilagirica* stimulated us to investigate its phytochemical composition.

2. MATERIALS AND METHODS

2.1.Chemicals and reagents

Potassium iodide, bismuth nitrate, sulphuric acid, ferric chloride, hydrochloric acid, aluminium chloride, ammonium hydroxide, glacial acetic acid, chloroform, ethanol, methanol, n-butanol, ethyl acetate, sodium chloride, sulphuric acid, olive oil, and Whatman filter paper were procured from Sd fine Chemical Ltd., Mumbai, India. The TLC plates were commercially procured from Merck India, Mumbai.

2.2. Collection and extraction

The mature non-infected stem bark of *Helicia nilagirica* Bedd. (Family: Proteaceae) was collected from Sialsuk, Aizawl District of Mizoram during the dry season and the plant was authenticated by the Botanical Survey of India, Shillong. The cleaned and non-infected bark was spread into stainless steel trays and allowed to dry in the shade at room temperature in dark in clean and hygienic conditions to avoid entry of insects, animals, fungus, and extraneous terrestrial materials. The exhaust and free air circulation was allowed. The dried bark was powdered in a grinder at room temperature. A sample of 100 g of the powder was extracted sequentially with chloroform, ethanol and water in a Soxhlet apparatus (Suffness and Dorous, 1979). The extract was then concentrated to dryness under reduced pressure and stored at -80 until further use.

2.3.Phytochemical screening

The different extracts of *Helicia nilagirica* were analyzed for the presence of various phytochemicals using standard procedures as described below.

2.3.1. Alkaloids

The presence of alkaloids was determined by mixing 0.1g of the extract with 0.5 ml of Mayer's reagent and Draggendorff's reagent. The formation of a creamy (Mayer's reagent) or reddish brown precipitate (Draggendorff's reagent) indicated the presence of alkaloids (Harborne, 1998; Doughari, 2012).

2.3.2. Tannins

About 0.5 g of dried powdered samples was boiled in 20 ml of water in a test tube and filtered. A few drops of 0.1% ferric chloride was added to the filtrate. The formation of brownish green or a blue-black colour indicated the presence of tannins (Harborne, 1998; Doughari, 2012).

2.3.3. Phlobatannins

The aqueous extract of *Helicia nilagirica* was boiled with 1% aqueous hydrochloric acid and deposition of a red precipitate indicated the presence of phlobatannins (Harborne, 1998; Doughari, 2012).

2.3.4. Saponins

About 2 g of the powdered sample was boiled with 20 ml of distilled water in a water bath for 10 minutes and filtered while hot and cooled before conducting the following tests:

***Frothing*:** 3 ml of filtrate was diluted up to 10 ml with distilled water and shaken vigorously for 2 minutes. The formation of a fairly stable froth indicated the presence of saponins in the filtrate.

Emulsification: 3 drops of olive oil was added to the solution obtained by diluting 3 ml filtrate to 10 ml distilled water and shaken vigorously for a few minutes. The formation of a fairly stable emulsion indicated the presence of saponins (Trease and Evans 1989; Harborne, 1998; Doughari, 2012).

2.3.5. Flavonoids

Three different methods were used to test the presence of flavonoids in all the extracts (Sofowara, 1993; Harborne, 1998; Doughari, 2012). Five ml of dilute ammonia solution was added to a portion of the aqueous filtrate of each plant extract followed by the addition of a concentrated H_2SO_4. Appearance of a yellow colour (disappeared on standing) in each extract indicated the presence of flavonoids.

A few drops of 1% aluminum solution was added to a portion of each filtrate. A yellow colour indicated the presence of flavonoids.

A portion of the plant powder was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colour indicated the presence of flavonoids.

2.3.6. Terpenoids

Salkowski test: Five ml of each extract was mixed with 2 ml of chloroform, with a careful overlaying of 3 ml concentrated sulphuric acid. The formation of a reddish brown precipitate at the interface indicated the presence of terpenoids (Harborne, 1998).

2.3.7. Cardiac glycosides (Keller-Killani test)

To determine the presence of cardiac glycosides, 5 ml of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution and underlay with 1ml of concentrated sulphuric acid. The appearance of brown ring at the interface indicated the presence of deoxysugar, which is a characteristic of cardenolides (Harborne, 1998; Doughari, 2012).

2.3.8. Carbohydrates

Benedict 's test: The filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

2.4.Quantitative determination of the phytochemicals

2.4.1. Determination of Saponins

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

2.4.2. Determination of Flavonoids

Ten g of the bark powder of *Helicia nilagirica* was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed (Bohm and Kocipai-Abyazan, 1994).

2.5. Determination of moisture content

Determination of the amount of volatile matter (i.e., water drying off from the drug) in the *Helicia nilagirica* is a measure of loss after drying of substances appearing to contain water

as the only volatile constituent. The powdered bark of *Helicia nilagirica* 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/W x 100

Where Pw = Pre weighed sample

Fw = Final weight of the dried sample

W = Total weight of the sample

2.6.Ash values

The ash values including total and acid insoluble ash were determined to estimate the total amount of the inorganic salts present in the drug. The ash contents remained after ignition of plant material was determined by two different methods to measure total and acid insoluble ash contents.

2.6.1. Total ash

The method measures the total amount of material remaining after ignition including both 'physiological ash', derived from the plant tissue itself, and 'non-physiological ash' which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface.

Procedure: Two grams of ground air-dried material of *Helicia nilagirica* was accurately weighed in a previously ignited and tared crucible. The material was spread as an even layer and ignited by gradually increasing the temperature up to 500-600˚C until it became white, indicating the absence of carbon. The crucible was cooled and weighed. The percentage of total ash content was calculated according to the following formula. Total ash content = Pw-Fw /W x 100

Where Pw = Pre weighed crucible

Fw = Final weight of the crucible containing ash

W = Total weight of powdered plant material

2.7. Extractive values

These are used to determine the amount of the matter which is soluble in the solvents used including alcohol and water. The percentage of alcohol and water-soluble extractives were calculated and used as standards.

2.7.1. Determination of alcohol-soluble extractive

Five grams of air dried coarsely powdered material was macerated in 100 ml of alcohol in a closed conical flask for twenty four hours, with frequent shaking during first six hours and allowed to stand for next eighteen hours thereafter it was filtered rapidly with caution to avoid loss of solvent. The 25 ml of the filtrate was evaporated to dryness at 105˚C in a tared flat-bottomed shallow dish and weighed. The percentage of alcohol-soluble extractive was calculated with reference to the air-dried material.

2.7.2. Determination of water-soluble extractive

Five grams of coarsely powdered air dried material was macerated in 100 ml of chloroform-water (0.1%) in a closed flask for 24 h, shaken frequently until six hours and allowed to stand for another eighteen hours. Thereafter it was filtered rapidly, with precautions to avoid loss of solvent by evaporation. The 25 ml of the filtrate was evaporated to dryness at 105˚C in a tared flat-bottomed shallow dish and weighed. The percentage of water soluble extractive was calculated with reference to the air dried material. All the tests were done in triplicate.

2.8.TLC Analysis:

TLC is a simple and rapid technique that is able to determine the number of components present in solution and helps in finding a suitable solvent for separating the components by column chromatography as well as for monitoring reactions' progress. The chloroform and ethanol extracts were spotted on to a number of TLC plates (Merck India, Mumbai) in 1 mm diameter above the bottom of the plates and placed into different mobile phases. The extracts

were allowed to move on the adsorbent (Stationary) phase according to the solvent system used. Several combinations of solvents of increasing polarity were evaluated as mobile phase for TLC run to determine the number of compounds present in different extracts of *Helicia nilagirica*. The different solvent systems were used as mobile phase for TLC, which consisted of chloroform: methanol (9:1, 8:2), pure chloroform, chloroform: ethyl acetate (1:1) and methanol: hydrochloric acid (9:1) solvent combinations. The resultant spots were observed under visible and ultra-violet light at 254 nm and 365 nm. The measure of the distance of a compound travelled is considered as the retention factor (R_f) value which was calculated using the following formula:-

R_f = Distance travelled by solute/Distance travelled by solvent

3. RESULTS

The results of phytochemical analyses and TLC profiling of *Helicia nilagirica* are presented in Table 1-6 .

Phytochemical analysis

The preliminary phytochemical screenings of different extracts of *Helicia nilagirica* showed the presence of tannins, phytosterols, saponins, phlobatannins, cardiac glycosides, flavonoids, phenol and terpenoids as chemical entities, whereas alkaloids were completely absent (Table 1).

Quantitative determination of phytochemicals

The quantitative determination of the chemical constituents showed that *Helicia nilagirica* contained 9.26% and 0.26% flavonoids and saponins, respectively (Table 2).

Determination of moisture content

The drying of 500 g of *Helicia nilagirica* bark yielded 296 g of dried bark, and this reduction in weight was due to 40.6% loss in its water contents The analysis of dried bark of *Helicia nilagirica* showed presence of 25.63% moisture (Table 3).

Determination of total ash content

The ash content of the crude bark powder was found to be 3.24% (Table 4).

Determination of extractive values

The *Helicia nilagirica* bark was found to contain 3.4% ethanol-soluble and 8% water-soluble extractives (Table 4).

Extract yield

The extraction of *Helicia nilagirica* stem bark yielded 2%, 4% and 6% in chloroform, ethanol and water extracts (Table 5).

TLC Analysis

The evaluation of chloroform and ethanol extracts of *Helicia nilagirica* showed the presence of different components as indicated by a varying number of spots and colours on a TLC plates using UV visualization method (Table 6).

Discussion

Plants synthesize several phytochemicals and have played an important role in the development of new therapeutic agents. The preliminary qualitative phytochemical analysis of the bark of *Helicia nilagirica* revealed the presence of phenol, flavonoid, tannins, saponins, cardiac glycosides and carbohydrate. These phytochemicals synthesized by plants are essential for the growth, pathogen attack, pollination, defence and other activities of plants (Reymond *et al.*, 2000; Hermsmeier *et al.*, 2001) however, at the same time these phytochemicals are of great

use for humans as a source of drugs and other healthcare agents (Shantabi *et al.*, 2014; Lalrinzuali *et al*., 2015).

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure which are ubiquitously present in plants. Approximately, more than 6000 varieties of flavonoids have been identified (Ferrer *et al*., 2008). They can be divided into a variety of classes such as flavones (e.g., apigenin, and luteolin), flavonols (e.g., quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others (Middleton 1998). They are the hydroxylated phenolic substances synthesized by plants in response to microbial infection (Dixon *et al*. 1983). Flavonols are the most abundant flavonoids in foods and they are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes (Yao *et al*. 2004). Flavonoids have been consumed by humans since the advent of human life on earth, that is, for about 4 million years. They have extensive biological properties that promote human health and help reduce the risk of diseases and they are known to possess antioxidant, hepatoprotective, antibacterial, anti-inflammatory, anticancer and antiviral properties (Ferry *et al*. 1996; Manthey 2000; Li *et al*. 2000; Kelly *et al*. 2002; Kumar *et al*. 2013; Mishra *et al*. 2013).

Tannins are polyphenols which occur widely in vascular plants particularly associated with woody tissues. They are water soluble and have molecular weights ranging between 500 and 3000 Daltons. Based on the chemical structures, tannins are divided into two groups: hydrolysable, and condensed. The hydrolysable tannins consist of gallic acid esters, and ellagic acid glycosides (Simões *et al*. 2003). They have an amazing astringent properties which is mainly related to their drug applications. They are known to be antimicrobial, antifungal, anthelminthic, antiviral, antiulcer and hasten the healing of wounds and inflamed mucous

membranes, (Khennouf *et al*. 2003; Li *et al*. 2011; Theisen 2014; Williams *et al*. 2014) They exert internal anti-diarrheal and antiseptic effects by waterproofing the outer layers of more exposed mucous membranes. Tannins are also haemostatic, and can serve as an antidote in poisoning cases (Albuquerque *et al*. 2005). In the process of healing wounds, burns and inflammations, tannins help by forming a protective layer (tannin-protein/tannin-polysaccharide complex), over injured epithelial tissues permitting the healing process below to occur naturally. Studies show that many tannins act as radical scavengers, intercepting active free radicals (Simões *et al*. 2003) various degenerative diseases such as cancer, multiple sclerosis, atherosclerosis and aging process itself are associated with high concentrations of intercellular free radicals.

Terpenoids are synthesized from five carbon isoprene units mainly isopentenyl pyrophosphate and its isomer dimethylallyl pyrophosphate by the enzyme terpene synthases. They are classified according to whether they contain two (C_{10}), three (C_{I5}), four (C_{20}), six (C_{30}) or eight (C_{40}) isoprene units. They range from the essential oil components, the volatile mono- and sesquiterpenes (C_{10} and C_{15}) through the less volatile diterpenes (C_{20}) to the involatile triterpenoids and sterols (C_{30}) and carotenoid pigments (C_{40}). Each of these various classes played a significant role in plant growth, metabolism or ecology (Harborne 1998). Approximately 40,000 terpenes have been identified and the majority of possible functions of these molecules are unknown (Goto *et al*., 2010). Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer, and they are also known to possess antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, anti-malarial, antihyperglycemic, antiinflammatory and immunomodulatory properties (Mujoo *et al*. 2001; Wagner *et al*. 2003; Salminen *et al* 2008; Rabi *et al*. 2009; Grace *et al*. 2013). Terpenoids

can also be used as protective substances in storing agriculture products as they are known to have insecticidal properties as well (Sultana *et al*. 2008).

Saponins are naturally occurring structurally and functionally diverse phytochemicals that are widely distributed among seventy families of plants. They are glycosides of both triterpenes and sterols (Hostettmann and Marston 1995). Due to the presence of both the hydrophobic aglycone backbone and hydrophilic sugar molecules the saponins are highly amphipathic and possess foaming and emulsifying properties. They play an important role in plant ecology and they are also exploited for a wide range of commercial applications in the food, cosmetic and pharmaceutical sectors (Güçlü-Ustündağ & Mazza, 2007; San Martín & Briones, 1999) These molecules are potent membrane permeabilizing agents, immunostimulatory, hypocholesterolemic, anti-carcinogenic, anti-inflammatory, anti-microbial, anti-protozoan, molluscicidal and have anti-oxidant properties (Francis *et al*. 2002; Sparg *et al*. 2004) . Saponins also act as antitumor by inhibiting tumor cell growth by apoptosis (Lee *et al*., 2011).

Cardiac glycosides are composed of two structural features: The sugar (glycoside) and the non-sugar (aglycone-steroid) moieties and they act on the contractile action of the cardiac muscle. These compounds have long been used for the treatment of cardiac arrhythmias and congestive heart failure due to their capability to increase the contractile force (Liu *et al*. 2000). Digitalis is the most commonly used cardiac glycoside and it is able to directly inhibits the proliferation of androgen dependent and androgen independent prostate cancer cell lines by initiating apoptosis and increasing intracellular Ca2+ .Cardiac glycosides have been reported to inhibit the four genes that are over expressed in prostate cancer cells including the inhibitors of apoptosis inhibitor and transcription factors (Newman *et al*. 2008). Cardiac glycosides have been

reported as active anticancer agents (Kepp *et al*., 2012; Calderón-Montaño *et al*., 2014). They are also reported to have antiviral properties against human cytomegalovirus. (Kapoor *et al*. 2012).

The *Helicia nilagirica* has been found to contain various phytochemicals like cardiac glycosides, flavonoids, saponins, terpenes, and tannins. Their presence has been confirmed by various analytical methods. The presence of these phytochemicals shows that it may act as promising anticancer agent.

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Table 1. Results of the Phytochemical analysis of *Helicia nilagirica*

Tests	Chloroform extract	Ethanol extract	Water extract
Alkaloids	-	-	-
Phenols	+	+	-
Flavonoids	+	+	-
Tannins	+	+	+
Terpenoids	+	-	-
Diterpenes	+	-	-
Saponin	-	-	+
Cardiac glycoside	+	+	+
Carbohydrate	-	-	+

Table 2. Quantitative determination of the chemical constituent of *Helicia nilagirica.*

Plant	Flavonoid			Saponin		
Helicia nilagirica	Quantity	Output	%	Quantity	Output	%
	10g	0.9256g	9.256	20g	0.051g	0.26

Table 3. Percentage of loss on drying fresh bark of *Helicia nilagirica*

Weight before drying (kg)	Weight after drying (kg)	Loss after drying (%)	Moisture content (%)
0.5	0.296	40.6	25.63

Table 4. Physicochemical parameters of dried bark powder of *Helicia nilagirica*

Total ash (%)	Ethanol- soluble extract (%)	Water- soluble extract(%)
3.24	3.4	8

Table 5. Yield of various extracts of *Helicia nilagirica.*

Dried powder	Chloroform extract (%)	Ethanol extract (%)	Water extract (%)
100g	2	4	6

Table 6. TLC profile of the different extracts of *Helicia nilagirica* on pre-coated aluminium TLC plates.

Extract	Solvent	Day light	R_f value	UV 254 nm	R_f value	UV 365 nm	R_f value
Chloroform	$CHCl_3$:CH_3OH 9:1	Streak	-	Two spots	0.92, 0.53	5 spots (1 red,1 blue, 3 yellowish)	0.92, 0.86, 0.57, 0.5 & 0.42
Ethanol		Streak	-	3 spots	0.57, 0.28 & 0.09	Not clear	-
Aqueous		Not visible	-	Not visible	-	Not visible	-
Chloroform	$CHCl_3$:CH_3OH-8:2	One spot	0.94	1 spot	0.94	3 spots (1 red, 2 bluish)	0.94, 0.88 & 0.84
Ethanol		Streak	-	3 spots	0.90, 0.82 & 0.5	1 spot (bluish)	0.38
Aqueous		Not visible	-	Not visible	-	Not visible	-
Chloroform	$CHCl_3$	Not visible	-	1 spot	0.94	3 spots (1 bluish, 2 red)	0.94, 0.09 & 0.05)
Ethanol		Not visible	-	Not clear	-	1 spot (bluish)	0.11
Aqueous		Not visible	-	Not visible	-	Not visible	-
Chloroform	$CHCl_3$:$C_4H_8O_2$-1:1	One spot	0.90	3 spots	0.90, 0.69 &0.48	4 spots (1 reddish, 3 bluish)	0.90, 0.86 & 0.84
Ethanol		Not visible	-	3 spots	0.69, 0.42 & 0.23	Not visible	-
Aqueous		Not visible	-	Not visible	-	Not visible	-

Chloroform	CH_3OH:HCl (9:1)	One spot	0.86	1 spot	0.84	2 spots (1 reddish, 1 bluish)	0.94 & 0.79
Ethanol		Two spots	0.82 0.43	1 spot	0.84	Not clear	-
Aqueous		Not visible	-	Not visible		Not visible	-

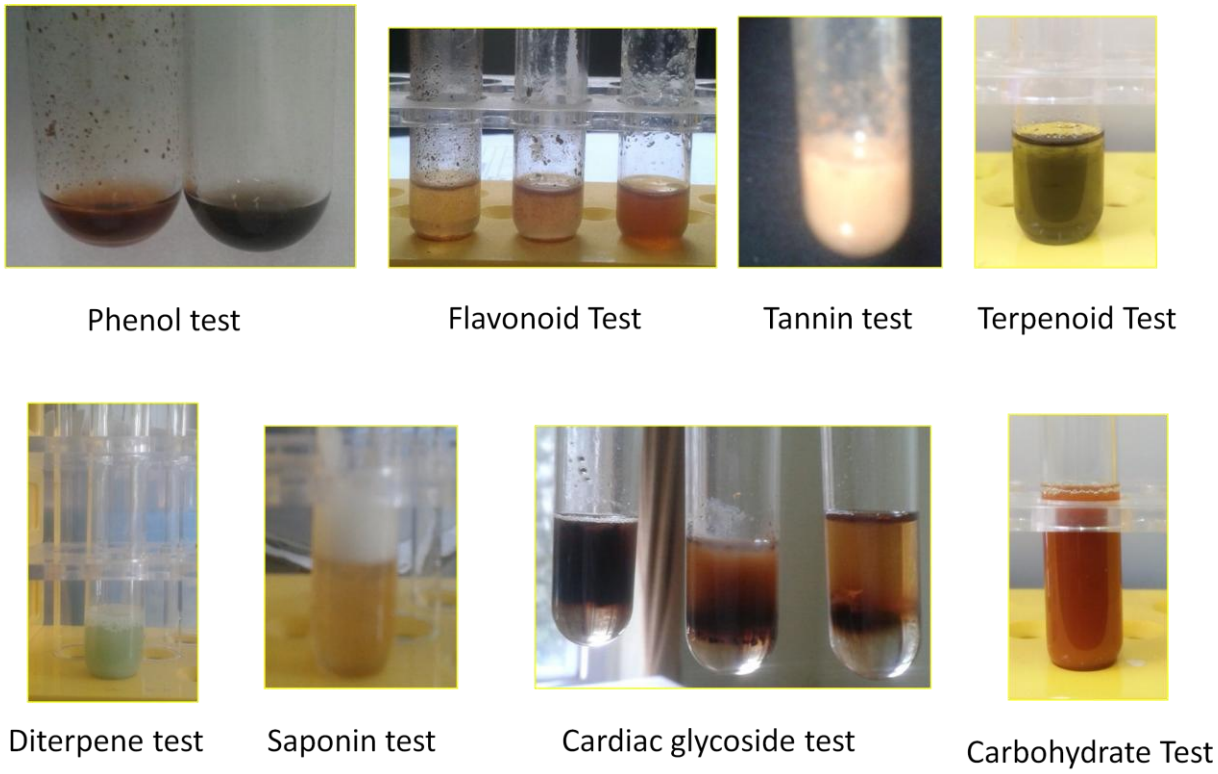

Figure 1: Phytochemical screening of *H.nilagirica* extracts.

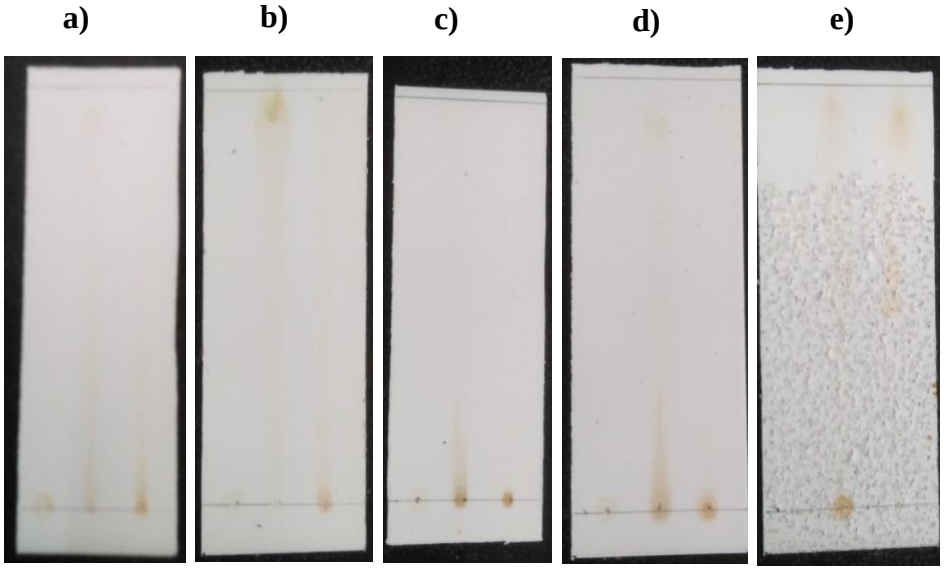

Figure 2: TLC profile of different extracts of *H.nilagirica* using different solvent systems observed under normal light to detect phytochemicals present in the extracts (aqueous, chloroform and ethanol).

a) $CHCl_3$:CH_3OH (9:1) **b)** $CHCl_3$:CH_3OH (8:2) **c)** $CHCl_3$ **d)** $CHCl_3$:$C_4H_8O_2$ (1:1) **e)** CH_3OH: HCl (9:1)

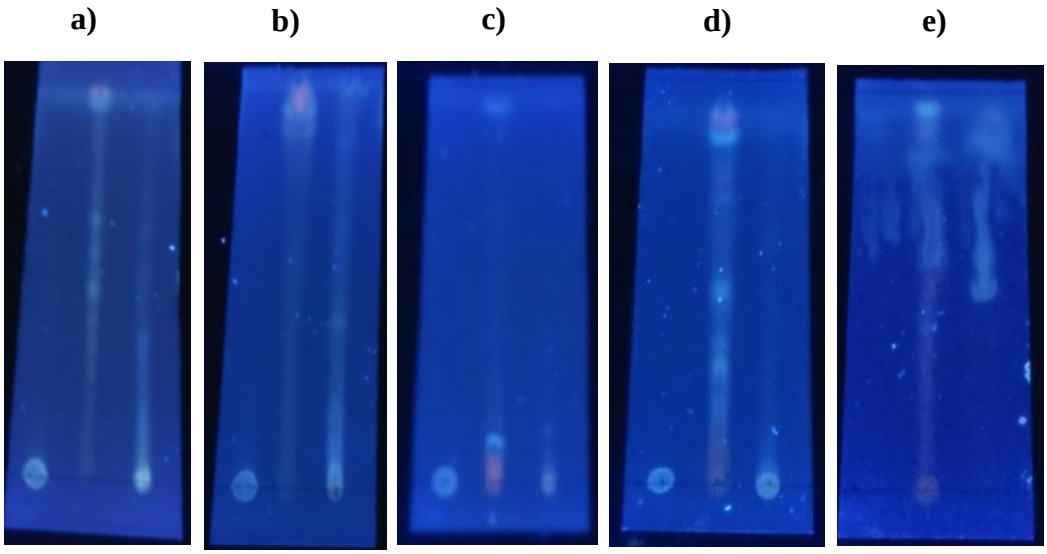

Figure 3: TLC profile of *H.nilagirica* on different solvent systems observed under UV 365 nm to detect phytochemicals present in the extracts (aqueous, chloroform and ethanol).
a) $CHCl_3$:CH_3OH (9:1) **b)** $CHCl_3$:CH_3OH (8:2) **c)** $CHCl_3$ **d)** $CHCl_3$:$C_4H_8O_2$ (1:1) **e)** CH_3OH: HCl (9:1).

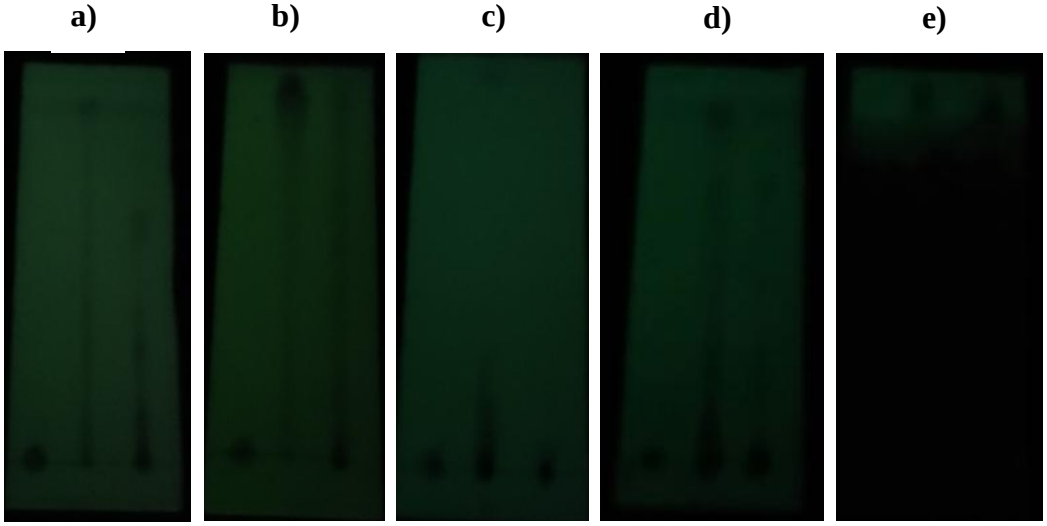

Figure 4: TLC profile of *H.nilagirica* on different solvent systems observed under UV 254 nm to detect phytochemicals present in the extracts (aqueous, chloroform and ethanol).
a) $CHCl_3$:CH_3OH (9:1) **b)** $CHCl_3$:CH_3OH (8:2) **c)** $CHCl_3$ **d)** $CHCl_3$:$C_4H_8O_2$ (1:1) **e)** CH_3OH: HCl (9:1).

CHAPTER 3

DETERMINATION OF FREE RADICAL SCAVENGING ACTIVITY OF *HELICIA NILAGIRICA IN VITRO*

Chapter 3

Abstract

Free radicals are produced during respiration and also to defend against pathogenic attack, however, their excess has been implicated in various chronic diseases. Excess free radicals may be neutralized by inbuilt mechanisms of the cells, supplementation with antioxidants or antioxidants present in diet. Unfortunately, available synthetic antioxidants produce adverse side effects indicating the need for safe natural antioxidants effects. Therefore, the present study was carried out to evaluate the antioxidant activity of Helicia nilagirica. The non-infected stem bark of Helicia nilagirica was collected, shade dried, powdered and sequentially extracted with petroleum ether, chloroform, ethanol and water using a Soxhlet apparatus. The ability of chloroform, ethanol and water extracts to scavenge various free radicals including DPPH, superoxide anion, hydroxyl, nitric oxide, ABTS and Fe^{3+} was studied. All the three extracts of H. nilagirica showed a concentration dependent rise in the free radical scavenging activity. The total flavonoid and phenolic contents also showed a concentration dependent increase in their quantity. Our findings suggest that H. nilagirica could be a good source of antioxidant as it has the ability to scavenge different free radicals. The presence of flavonoids and polyphenols may be responsible for the neutralization of various free radicals.

Chapter 3

1. INTRODUCTION

The transition of organisms from anaerobic to aerobic mode has led to an increased efficiency however; it has come with a price the organisms have to pay in the form of production of oxidative stress. The oxygen is a strong oxidizing agent and its use for the production of chemical energy in the form of ATP (adenosine triphosphate) in the mitochondria is associated with the production of free radicals or reactive oxygen species. During this process, free radicals are generated in the form of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) (Halliwell, 2011). In addition, the environmental pollutants including cigarette smoke, automobile exhaust, pesticides and electromagnetic radiation also trigger the generation of free radicals (Boora *et al*., 2014), which the endogenous antioxidant system may not be able to handle apprpriately. The ROS include $O_2^{\bullet-}$ (superoxide anion), $^{\bullet}OH$ (hydroxyl radical), H_2O_2 (hydrogen peroxide) and $^{1/2}O_2$ (singlet oxygen) . The free radicals are essential for various biological processes at low or moderate levels as they are required for nerve conduction, fighting against infection and execute immune responses; however their high concentrations result in the production of oxidative stress, a deleterious process leading to pathogenesis (Sies, 2017). The increased oxidative stress is indicated in the development of chronic and degenerative diseases including arthritis, aging, autoimmune disorders, cancer, cardiovascular and neurodegenerative diseases and many more (Valko *et al*., 2007).

The human body has several inbuilt mechanisms to counterbalance the increased oxidative stress during regular physiological functions by producing antioxidants. In certain cases external supplementation of natural antioxidants may be needed to keep the oxidative stress to minimum. Indeed, a broader definition of antioxidant was suggested by Halliwell *et al*. in 1995 as "any substance that when present at low concentrations, compared to those of an oxidizable substrate

significantly delays or prevents oxidation of that substrate". Later oxidative stress was redefined as ''any substance that delays, prevents or removes oxidative damage to a target molecule'' (Halliwell, 2007). The synthetic phenolic antioxidants include butylated hydroxyl anisole (BHA), butylated hydroxy toluene (BHT), and tertiary butylated hydroquinone, which are frequently used to combat oxidative stress (Carocho and Fereira, 2013). However, some physical properties of BHT and BHA such as high volatility and instability at elevated temperature, and carcinogenic nature of some the synthetic antioxidants dissuade their use by the consumers. The consumers always prefer to use natural products rather than synthetic chemicals, due their longstanding use and non-toxic nature. The preference for natural products against the synthetic chemicals has spurn interest to test the antioxidant activity of natural products.

Helicia nilagirica Bedd. (Family: Proteaceae) locally known as Pasaltakaza is a medium-sized tree, which grows up to 12 meters high. It is widely distributed in Sri Lanka, southern India, Burma (Myanmar), Indochina, Japan, Taiwan, and Thailand. It is also found scattered in lowland to montane rain forests, up to 2,000-3,350 m altitude. Some species prefer habitats along streams but other species are found on hilltops or ridges (Khamyong *et al*., 2004). This tree has been used as a folk medicine since time immemorial in Mizoram, India by the Mizo tribe. Its decoction prepared by boiling the leaves or bark is used to treat various stomach ailments including peptic ulcers, indigestion, mouth ulcer, urinary tract infection and gynaecological disorders. It is also used in scabies and other skin diseases (Sawmliana, 2003). The fruits of *H. nilagirica* have been used as a medicine to cure cough and cold in Sikkim (Chauhan, 2001). The scientific evaluation regarding its medicinal or other properties is scarce. However, it has been recently reported to possess anti-inflammatory activity in rat cotton pellet granuloma model (Lalawmpuii *et al*., 2014). This indicates

the need to systematically evaluate its medicinal properties. Therefore, the present study was carried out to evaluate the antioxidant activity of *Helicia nilagirica.*

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

All the chemicals used were of analytical grade and Milli Q water was used for the entire analysis. 1,1-Diphenyl-2-picrythydrazyl (DPPH), dimethyl sulfoxide (DMSO), ascorbic acid, nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), β-nicotinamide adenine dinucleotide (NADH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ethylenediamine tetraacetic 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 extract

Helicia nilagirica was identified and authenticated by Botanical Survey of India, Shillong. The non-infected stem bark of *Helicia nilagirica* was collected from Sialsuk, Aizawl District of Mizoram, India during the dry season. The bark was cleaned chopped into small pieces, spread into the stainless steel trays and allowed to shade dry at room temperature in dark, clean and hygienic conditions to avoid entry of insects, animals, fungus, and other extraneous terrestrial materials. The dried bark was powdered in an electrical grinder at room temperature. A sample of 100 g of bark powder was sequentially extracted in chloroform, ethanol and water using a Soxhlet apparatus until

the solvents became colourless. The extracts were evaporated to dryness under reduced pressure and stored at -80 until further use.

3. ESTIMATION OF FREE RADICAL SCAVENGING *IN VITRO*

3.1. DPPH free radical scavenging assay

The assay is based on the reduction of the DPPH free radical by an antioxidant into the 2, 2-diphenyl-1-picryl hydrazine. The DPPH free radical scavenging activity of various extracts of *Helicia nilagirica* (HNE) was determined as described earlier (Leong and Shiu, 2002). Briefly, various concentrations of HNE were dissolved in 40 µl of appropriate solvents and added to 3 ml of methanol solution of 0.1 mM DPPH and allowed to stand for 30 min at room temperature. The standard consisted of an equal amount of DPPH whereas an equal amount of methanol was taken as a blank. After 30 min, absorbance was recorded at 515 nm in Eppendorf UV/VIS Spectrophotometer (Eppendorf, Germany). Radical scavenging activity has been expressed as a percentage, which 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.

3.2. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of *Helicia nilagirica* was assayed according to the method of Klein *et al*. (1981). Briefly, 1 ml of different concentrations of HNE was transferred into a test tube and evaporated to dryness, and 1 ml of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml 0.018% EDTA, 1 ml DMSO (0.85%, *V/V*, in 0.1 mol L-1 phosphate buffer, pH 7.4) and 0.5 ml 0.22% ascorbic acid were added. The tubes were capped tightly and heated in a water bath at 80-90°C for 15 min. The reaction was terminated by adding 1

ml of ice-cold TCA (17.5% *w/v*). Three ml of Nash reagent (75.0 g ammonium acetate, 3 ml glacial acetic acid and 2 ml acetyl acetone were mixed and water was added to make up the volume up to 1 L) was added to each tube, which were left at room temperature for 15 min for colour development. The intensity of the yellow colour formed was measured at 412 nm using a UV/VIS Spectrophotometer against a blank. The antioxidant capacity of HNE based on its ability to decrease hydroxyl radicals generated in a Fenton reaction system was expressed as % inhibition.

3.3. Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of *Helicia nilagirica* extract was based on the method described earlier (Liu *et al*., 1997). Superoxide radicals were generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of NBT. Briefly, various concentrations of HNE were mixed with 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 µM), and 1 mL NADH (78 µM) solution. The reaction was initiated by adding 1 mL of 10 µM PMS to the mixture. The reaction mixture was incubated at 25 ^{o}C for 5 min, and the absorbance was measured against the blank at 560 nm in a spectrophotometer. L-Ascorbic acid was used as a control. Decrease in the absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula.

Inhibition (%) = $(A_o-A_1) /A_o x100$

Where A_o is the absorbance of the control, and A_1 is the absorbance of *Helicia nilagirica* extract.

3.4.ABTS scavenging activity

ABTS scavenging activity of different extracts of HNE was carried out as described earlier (Re *et al*., 1999). Briefly, 37.5 mg of potassium persulphate was dissolved in 1 ml of distilled water.

44 µl of this solution was added to 9.7 mg of ABTS dissolved in 2.5 ml of distilled water. The ABTS solution was allowed to stand in the dark at room temperature for 12-16 hours. The working solution was prepared by mixing 1 ml of ABTS solution with 88 ml of 50% ethanol. Usually, 25 µl of different concentrations of the various HNEs were mixed with 250 µl of ABTS working solution and allowed to react for 4 minutes. The absorbance was then read against the blank at 734 nm in a UV-VIS spectrophotometer. L-Ascorbic acid was used as the standard antioxidant. The percentage scavenging activity was calculated as follows:

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

3.5. Nitric oxide scavenging activity

The nitric oxide scavenging activity of HNE was estimated by Griess reaction as described earlier with minor modifications (Marcocci *et al*., 1994). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Marcocci *et al*., 1994). Briefly, the reaction mixture containing 1 ml of 5 mM sodium nitroprusside was incubated with different concentrations of HNE and incubated at 25°C for 150 min. 1 ml sample from the above was aspirated and diluted with 1 ml of Griess reagent and the absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and subsequent coupling with NED was read at 546 nm in a UV/VIS Spectrophotometer. The blank consisted of PBS and equal ratio of sodium nitroprusside and Griess reagent treated in an identical manner except the test sample (HNE). The antioxidant capacity of HNE has been expressed as % inhibition

3.6. Reducing power

The reducing power of the extracts was determined as described earlier (Oyaizu, 1986). Various extracts of *H. nilagirica* in distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50ºC for 20 min and subsequently 2.5 ml of trichloroacetic acid (10%) was added to this mixture, which was centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was collected and mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 1%). The absorbance was measured at 700 nm with a UV-VIS spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power.

3.*7. Determination of Total phenolic contents*

The total phenolic contents of the HNE were determined as described earlier (Singleton and Rossi, 1965). 500 µl of different concentrations of HNE were mixed with 1000 µl of 1:10 Folin-Ciocalteau's reagent and incubated for 5 min at room temperature followed by the addition of 900 µl of saturated 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 HRE have been expressed as gallic acid equivalents mg/100 g of the HNE.

3.8. Total Flavonoids Determination

The total flavonoids were determined by colorimetric method described by Chang et al. (2002). Briefly, 1 ml of various extracts of HNE were individually mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum 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 calibration curve was plotted using

different concentrations of quercetin in methanol. The presence of flavonoids in HNE has been expressed as mg quercetin equivalent /100 g of HNE.

4. STATISTICAL ANALYSIS

The statistical analyses between the treatments were performed using Origin Pro 8. All the results are expressed as mean ± standard error of mean (S.E.M).

5. RESULTS

The results of free radical scavenging activity are shown in Figure 1-5, whereas reducing power, total phenol and flavonoid contents in Figure 6 – 8.

5.1. DPPH radical scavenging activity

Analysis of DPPH scavenging activity of various extracts of *Helicia nilagirica* showed a concentration dependent increase in the scavenging of DPPH radicals (Figure 1) as indicated by the discolouration of DPPH from purple to colourless. The ethanol extract of *H. nilagirica* was most effective in scavenging of DPPH radical, where a maximum inhibition was recorded at a concentration of 160 µg/ml and plateaued thereafter (Figure 1). The chloroform, extract was less effective in scavenging of DPPH radical and a maximum activity was observed at 420 µg/ml. The aqueous extract was least effective than the other two extracts where a maximum scavenging could be observed only at 500 µg/ml (Figure 1). The IC50 was 270.85 µg/ml, 63.91 µg/ml and 362.32 µg/ml for chloroform, ethanol and aqueous HNE, respectively when compared to 13.34 µg/ml for the ascorbic acid, which has been used as a positive control.

5.2. Hydroxyl Radical Scavenging activity

Different extracts of *Helicia nilagirica* inhibited the generation of hydroxyl radical in a concentration dependent manner (Figure 2). The aqueous extract showed a maximum inhibitory effect up to 140 µg/ml and a decline thereafter up to 180 µg/ml, the last concentration of this extract

was evaluated (Figure 2). The chloroform and ethanol extracts of *H. nilagirica* showed a maximum inhibition of OH radical generation at 200 µg/ml, which plateaued thereafter (Figure 2). The IC50 for chloroform, ethanol and aqueous extracts were 75.40 µg/ml, 31.93 µg/ml and 33.19 µg/ml, respectively when compared with an IC50 value of 23.75 µg/ml for ascorbic acid.

5.3. Superoxide anion scavenging activity

The chloroform, ethanol and water extracts of *Helicia nilagirica* showed a concentration dependent rise in the superoxide radical scavenging activity (Figure 3). The aqueous extract was most effective as the maximum scavenging of $O_2^{\bullet-}$ was reported at 120 µg/ml, which remained almost unaltered up to a concentration of 300 µg/ml (Figure 3). The chloroform extract revealed the highest scavenging activity at 200 µg/ml, whereas maximum scavenging of $O_2^{\bullet-}$ was observed at 300 µg/ml for the ethanol HNE (Figure 3). The IC50 concentrations were also calculated and found to be 75.40 µg/ml, 40 µg/ml and 33.19 µg/ml for chloroform, ethanol and aqueous extracts, respectively. The IC50 value for ascorbic acid was 23.75 µg/ml.

5.4.ABTS scavenging activity

The determination of antioxidant activities of aqueous, ethanol and chloroform extracts of *Helicia nilagirica* showed a concentration dependent elevation in the scavenging of ABTS radical (Figure 4). The chloroform extract was found to be most efficient as the maximum scavenging activity was observed at 320 µg/ml, followed by ethanol extract, where the greatest scavenging effect was observed at 340 µg/ml (Figure 4). The aqueous extract was less effective as it required a higher concentration of 560 µg/ml to inhibit the generation of ABTS free radical to its maximum extent (Figure 4). The IC50 was found to be 156.85 µg/ml, 154.79 µg/ml and 430.51 µg/ml for chloroform, ethanol and aqueous extracts, respectively. The ascorbic acid had an IC50 concentration of 13.79 µg/ml.

5.5.Nitric oxide scavenging activity

Nitric oxide radical scavenging activity increased in a concentration dependent manner for all the extracts of *Helicia nilagirica* (Figure 5). HNE was very effective in suppressing the generation of NO radical as it inhibited the generation of NO radical at lower concentrations. The chloroform and ethanol extracts inhibited the NO generation to a greatest extent at 120 µg/ml whereas the concentration for maximum NO scavenging effect was seen at 140 µg/ml for the aqueous extract (Figure 5). The IC50 for chloroform, ethanol and aqueous extracts were found to be 57.13 µg/ml, 47.63 µg/ml and 103.51 µg/ml, respectively when compared to ascorbic acid, where the IC50 was 5 µg/ml.

5.6.Reducing power

The evaluation of reducing power of *Helicia nilagirica* extracts showed that different extract increased reducing power in a concentration dependent manner up to 5000 µg/ml for the chloroform, ethanol and aqueous extracts, respectively (Figure 6). However, the reducing power assay showed a gradual rise in the reducing power of all HNEs up to a concentration of 2000 µg/ml with an abrupt rise thereafter (Figure 6). The maximum reducing power for all extract was observed for 5000 mg/100 g gallic acid equivalent.

5.7.Total phenolic contents

The presence of phenolic compounds in the extract was estimated as total phenol contents. The total phenolic contents increased in a concentration dependent manner for chloroform, ethanol and aqueous extracts (Figure 7). The ethanol extract showed the highest phenolic contents of 1078 mg/100 g gallic acid equivalent of the extract (Figure 7). The chloroform and aqueous extract showed 531 and 588 mg/100 g gallic acid equivalent, respectively (Figure 7).

5.8.Total flavonoid contents

The total flavonoid contents in chloroform, ethanol and aqueous extract of *Helicia nilagirica* increased in a concentration dependent manner up to 10000 µg/ml for chloroform and aqueous extracts, whereas maximum flavonoid contents were recorded for 7000 µg/ml for ethanol extract equivalent to quercetin (Figure 8). The flavonoid contents for chloroform, ethanol and aqueous extracts at 7000 µg were 96, 685 and 326 mg/100 g quercetin equivalent, respectively (Figure 8).

DISCUSSION

Free radicals are mainly generated by various metabolic pathways; however, excess generation of free radicals leads to oxidative stress. The other main reason of increased oxidative stress is the failure of the cell's endogenous systems to cope with the excess generation of free radicals. The oxidative stress is a cause of several diseases including diabetes, autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular disorders, neurodegenerative diseases and cancer (Valko *et al*., 2007). Therefore there is always a need to alleviate the oxidative stress to inhibit several pathological conditions in humans. Several synthetic antioxidants or vitamin mixtures are available in the market however, they have adverse side effects. The natural products may be especially helpful in reducing oxidative stress. The benefit of natural products is that they are biological in origin and have lesser known side effects and they have been used by humans since the advent of human civilization. Therefore, the present study was undertaken to determine the free radical scavenging activity of various extracts of *Helicia nilagirica* in vitro.

The assay of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging is a widely used method to effectively evaluate antioxidant activities of different pharmacological agents. DPPH is a stable free radical, where the antioxidant compounds in the extract neutralize the free radical by

transferring either electrons or hydrogen atoms to DPPH, thereby changing the colour from purple to the yellow coloured stable diamagnetic molecule diphenylpicrylhydrazine causing a rapid decrease in the optical density at 517 nm indicating the scavenging capability of the extract (Goldschmidt and Renn, 1922). The various extracts of *Helicia nilagirica* stem bark inhibited the DPPH radical formation in a concentration dependent manner. The DPPH scavenging activity of methanol extract has also been observed in this plant earlier (Lalawmpuii *et al*., 2014). Similarly, different extract of other plants have shown the ability to scavenge DPPH free radical earlier (Baliga *et al*., 2003; Jagetia *et al*., 2003a; Wong *et al*., 2006; Aparadh *et al*., 2012; Jagetia *et al*., 2012; Shantabi *et al*., 2014; Lalrinzuali *et al*., 2015).

The use of oxygen by aerobic organisms is deleterious to cell as it is a strong oxidizing agent and it produces free radical during cellular respiration in the mitochondria. The coupling of oxygen during electron transport chain result in the production of super oxide radical, which if not neutralized is converted into less reactive H_2O_2, which is a highly toxic and strong oxidizing agent, and it produces a highly reactive hydroxyl free radical, especially in the presence of iron by Haber Weiss and/or Fenton reaction (Valko *et al*., 2007; Lushchak 2014). The highly reactive hydroxyl radical if not neutralized reacts with various important macromolecules including proteins and nucleic acids leading to severe detrimental effects in the form of bond breakage, DNA base and sugar damages, single or double strand breaks (Kohen and Nyska, 2002; Dizdaroglu and Jaruga, 2012). The macromolecular damage leads to several pathologic conditions listed earlier. The $^{\bullet}OH$ radical reacts with lipids leading to their peroxidation, which subsequently reacts with other macromolecules inflicting various kinds of damages and pathologies. The agents that can neutralize the $^{\bullet}OH$ radicals will be useful in arresting the various ROS-induced pathologies. Different extract of *Helicia nilagirica* scavenged $^{\bullet}OH$ radicals in a concentration dependant manner. A similar effect

has been reported earlier with methanol extract of this plant (Lalawmpuii *et al*., 2014). *Agele marmelos, Croton caudatus, Oroxylum indicum, Syzygium cumini, Zingiber officinale, Alstonia scholaris and Curcuma longa* have been reported to inhibit $^{\bullet}OH$ radical generation in vitro earlier (Jagetia *et al*., 2003a, b; Shantabi *et al*., 2014; Lalrinzuali *et al*., 2015).

Superoxide anion radicals ($O_2^{\bullet-}$) are generated by four-electron reduction of molecular oxygen into water. These radicals are also formed in aerobic cells due to electron leakage from the electron transport chain. Superoxide radicals ($O_2^{\bullet-}$) are also formed by activated phagocytes such as monocytes, macrophages, eosinophils and neutrophils, which are used as a defence to kill bacteria by phagocytes. The superoxide anion produces H_2O_2, which in turn generates hydroxyl free radicals in the presence of metals (Halliwell, 2007; Lussshchak, 2014). The different extracts from the stem bark of *Helicia nilagirica* inhibited the formation of superoxide radical in a concentration dependant manner. Several plants and plant formulations have also been reported to scavenge $O_2^{\bullet-}$ in a concentration dependent manner [Jagetia *et al*., 2003a, b; Shantabi *et al*., 2014; Lalrinzuali *et al*., 2015).

The ABTS (2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) is a stable free radical, which is generated by reacting a strong oxidizing agent (e.g., potassium permanganate or potassium persulfate) with the ABTS salt. Reduction of blue-green ABTS radical colored solution by hydrogen-donating antioxidant is measured by its characteristic long wave (734nm) absorption spectrum (Miller and Rice-Evans, 1997). The extent of decolorization is expressed as the percentage inhibition of the $ABTS^{\bullet+}$ (Re *et al*., 1999). This trapping of ABTS derived radical cation ($ABTS^{\bullet+}$) by free radical scavengers is a commonly employed method to evaluate the total charge of antioxidants present in complex mixtures. The various extracts of *Helicia nilagirica* scavenged the ABTS radical in a concentration dependant manner which shows that these extracts are able to

act as free radical scavengers and have antioxidant activity. A similar effect has been observed earlier with different plant extracts (Jagetia et al., 2003a; b).

The nitric oxide radical ($NO^{\bullet}$) is a diffusible free radical that plays many role as an effector molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities. However the elevation of $NO^{\bullet}$ produces several pathological conditions including cancer. Under aerobic conditions, the $NO^{\bullet}$ molecule is very unstable and reacts with the oxygen to produce intermediates such as NO_2, N_2O_4, N_3O_4, the stable products nitrate and nitrite and peroxynitrite when reacted with superoxide (Carr *et al.*, 2000). These progenitors are highly genotoxic. The chloroform, ethanol and aqueous extracts of *Helicia nilagirica* stem bark have inhibited the production $NO^{\bullet}$ formation and in turn may be of considerable interest in preventing the negative effects of excess $NO^{\bullet}$ generation in vivo. Several other plant extracts have been reported to inhibit the $NO^{\bullet}$ generation earlier (Jagetia *et al.*, 2004; Shantabi *et al.*, 2014; Lalrinzuali *et al.*, 2015).

Reducing power is another parameter that indicates the antioxidant activity of any pharmacophore and it has been widely used to evaluate the antioxidant activity earlier (Oktay *et al.*, 2003). The electron donor compounds possessing reducing power are able to reduce the oxidized intermediates of lipid peroxidation and thus can act as primary and secondary antioxidants. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound. The presence of reducers used in this method converts Fe3+/ferricyanide complex to ferrous form by donating an electron. Different extracts of *Helicia nilagirica* stem bark showed antioxidant activity by increasing reducing power. Our earlier studies on *Croton caudatus* and *Oroxylum indicum* have shown a similar effect (Shantabi *et al.*, 2014; Lalrinzuali *et al.*, 2015).

Two compounds have been isolated from the leaves of *Helicia nilagirica* where compound 1 was elucidated as 1-O-3-D-glucopyranosyl-(2S, 3S, 4R, 8Z)-2-[(2'R)-2'-hydroxylignocenoyl-amino]-8-octadecene-1, 3, 4-triol and compound 2 was an analogue of compound 1 (Wu et al., 2004). From the seeds five compounds have also been isolated from the dichloromethane and n-butanol extracts, identified as p-hydroxybenzaldehyde, p-hydroxybenzoic acid, gallic acid, helicide, 4-formylpymyl-O-beta-D-glucopyranoside (Liu et al., 2005). The presence of these compounds may have contributed to HNE's free radical scavenging and antioxidant activities The exact mechanism of radical scavenging of free radicals by *Helicia nilagirica* is not clearly understood. The presence of various phytochemicals including flavonoids and polyphenols may have been responsible for the neutralization of various free radicals.

CONCLUSIONS

Our study clearly demonstrated that chloroform, ethanol and aqueous extracts of *Helicia nilagirica* were able to scavenge the DPPH, OH, superoxide, nitric oxide and ABTS free radicals in a concentration dependent manner, which substantiates its folklore use for human healthcare. The free radical scavenging ability of *Helicia nilagirica* may be due to the presence of flavonoid and other polyphenolic phytochemicals in its stem bark.

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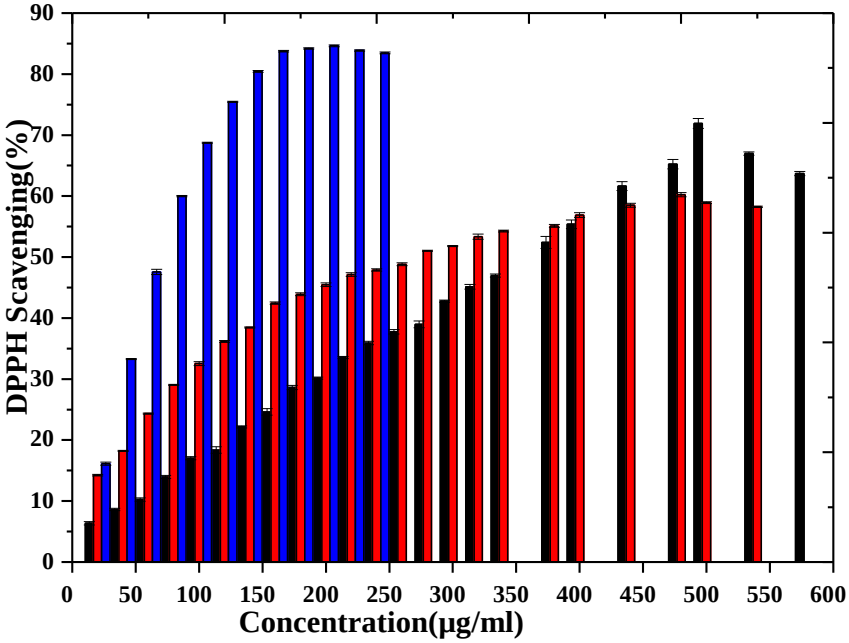


Figure 1: Effect of different *Helicia nilagirica* extracts on the DPPH free radical scavenging activity.
Black bars: Aqueous Extract, Red bars: Chloroform Extract and Blue bars: Ethanol Extract

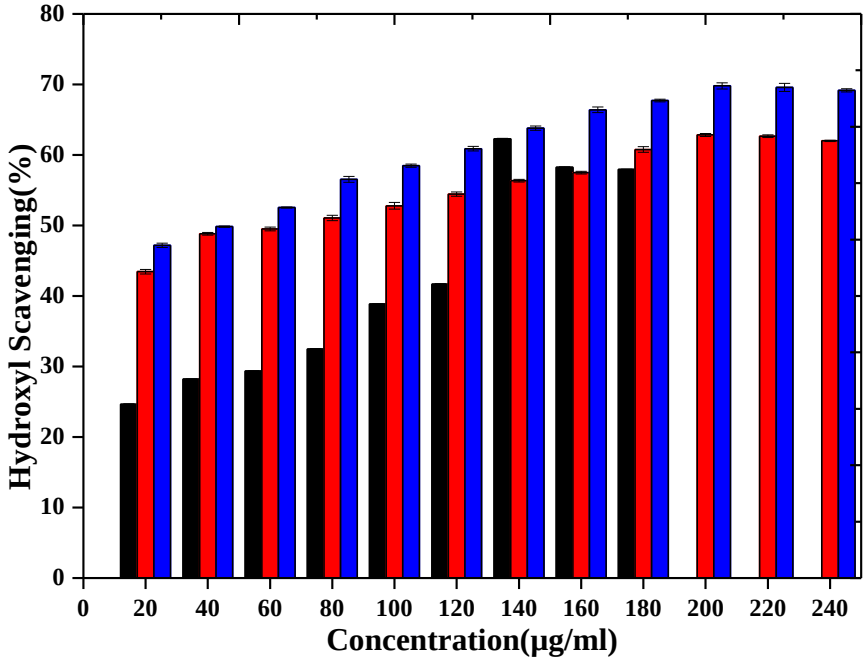


Figure 2: Effect of different *Helicia nilagirica* extracts on the hydroxyl free radical scavenging activity
Black bars: Aqueous Extract, Red bars: Chloroform Extract and Blue bars: Ethanol Extract

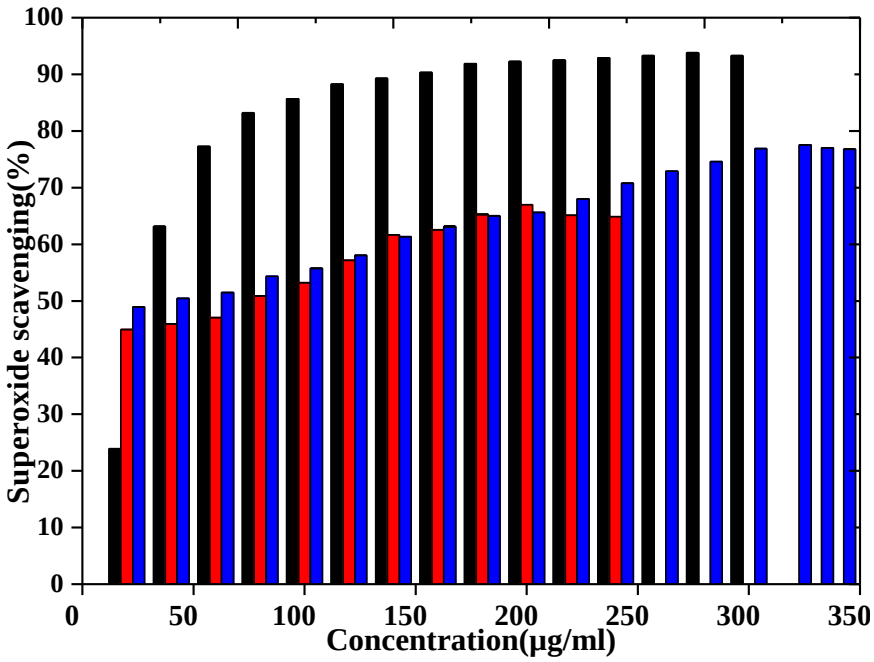


Figure 3: Effect of different *Helicia nilagirica* extracts on the superoxide anion free radical scavenging activity.
Black bars: Aqueous Extract, Red bars: Chloroform Extract and Blue bars: Ethanol Extract

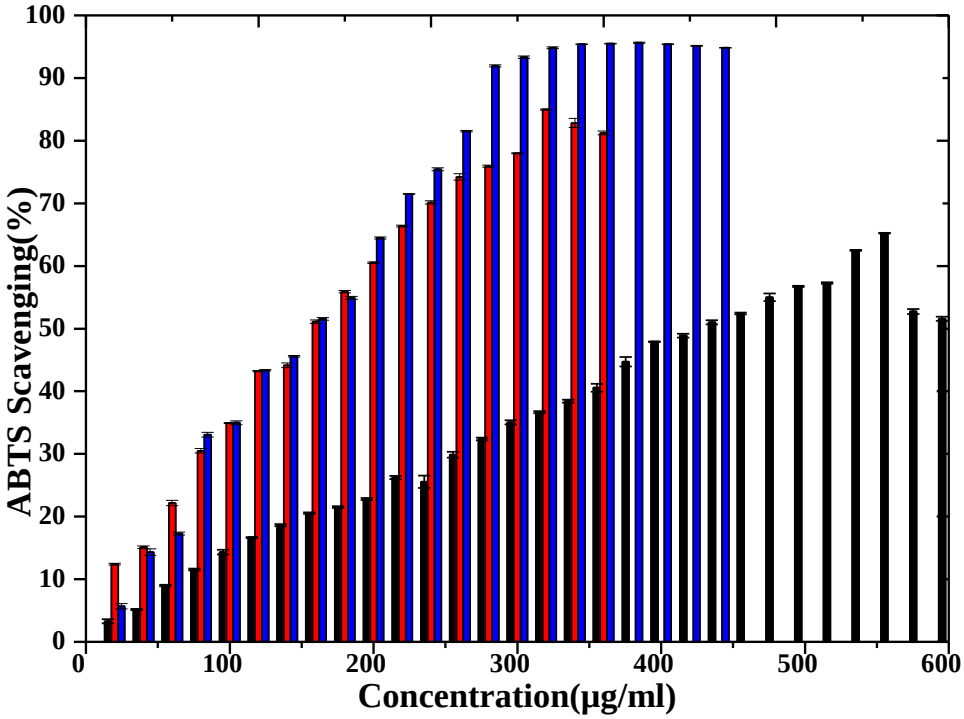


Figure 4: Effect of different *Helicia nilagirica* extracts on the ABTS cation free radical scavenging activity.
Black bars: Aqueous Extract, Red bars: Chloroform Extract and Blue bars: Ethanol Extract

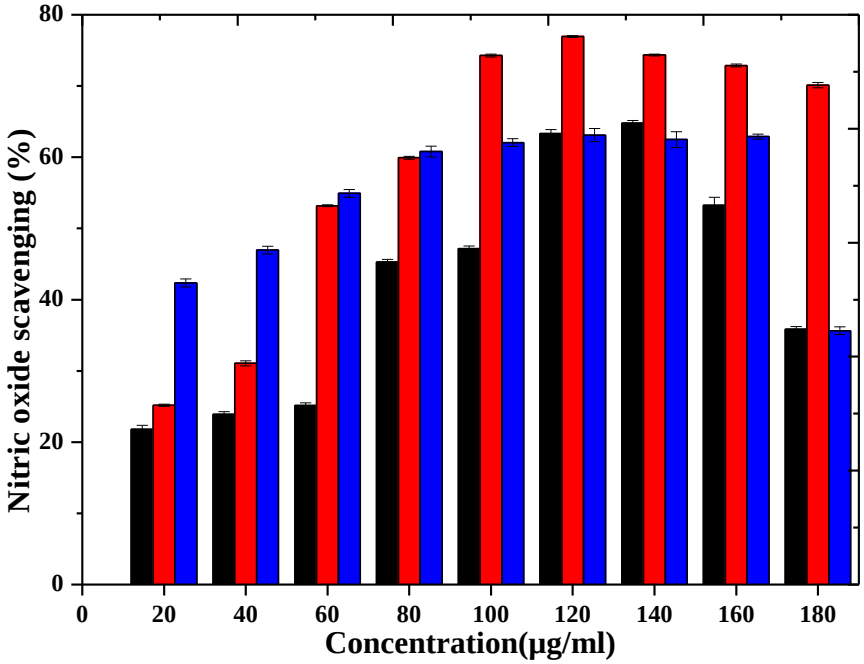


Figure 5: Effect of different *Helicia nilagirica* extracts on the nitric oxide free radical scavenging activity.
Black bars: Aqueous Extract, Red bars: Chloroform Extract and Blue bars: Ethanol Extract

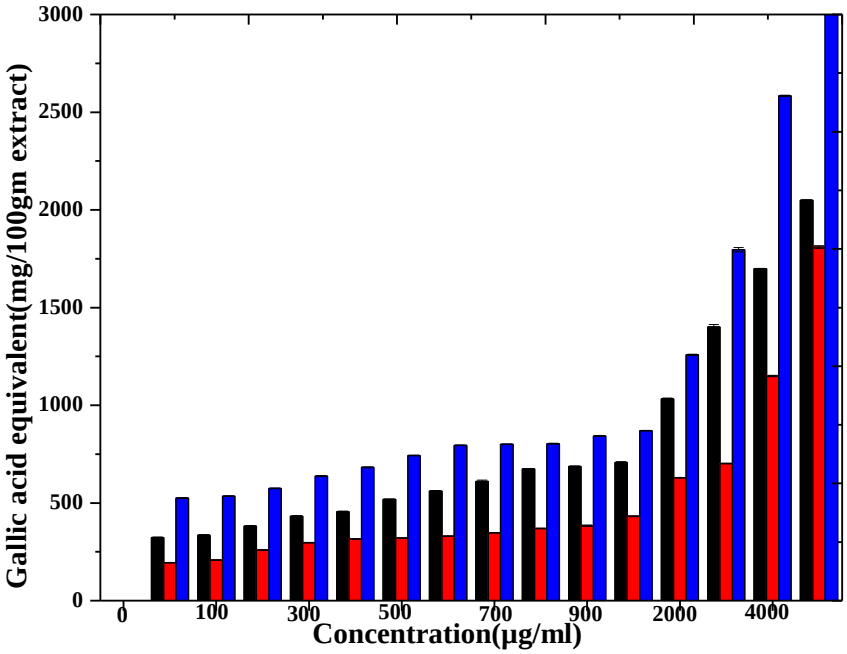


Figure 6: Total reducing power of different *Helicia nilagirica* extracts
Black bars: Aqueous Extract, Red bars: Chloroform Extract and Blue bars: Ethanol Extract

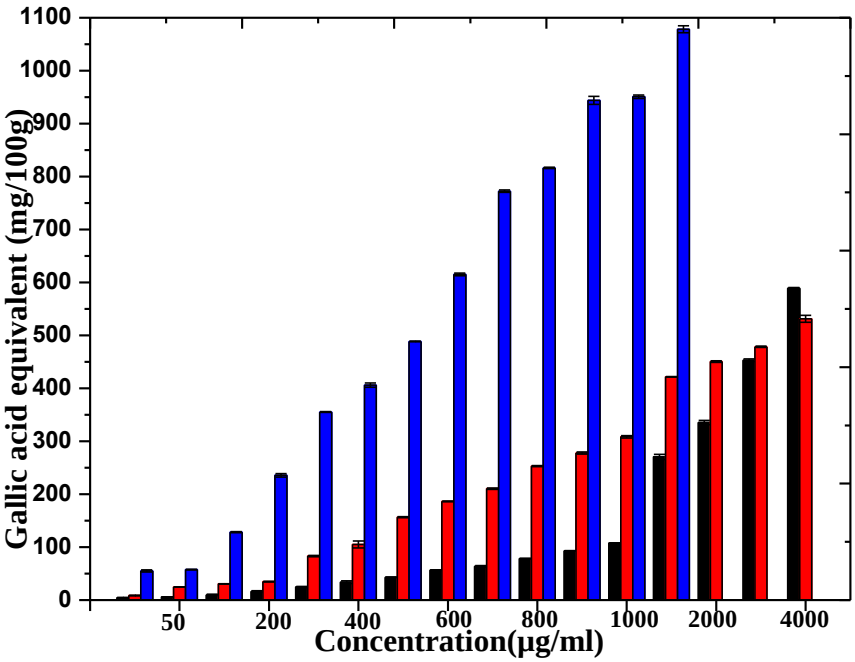


Figure 7: Total phenol content of different *Helicia nilagirica* extracts.
Black bars: Aqueous Extract, Red bars: Chloroform Extract and Blue bars: Ethanol Extract

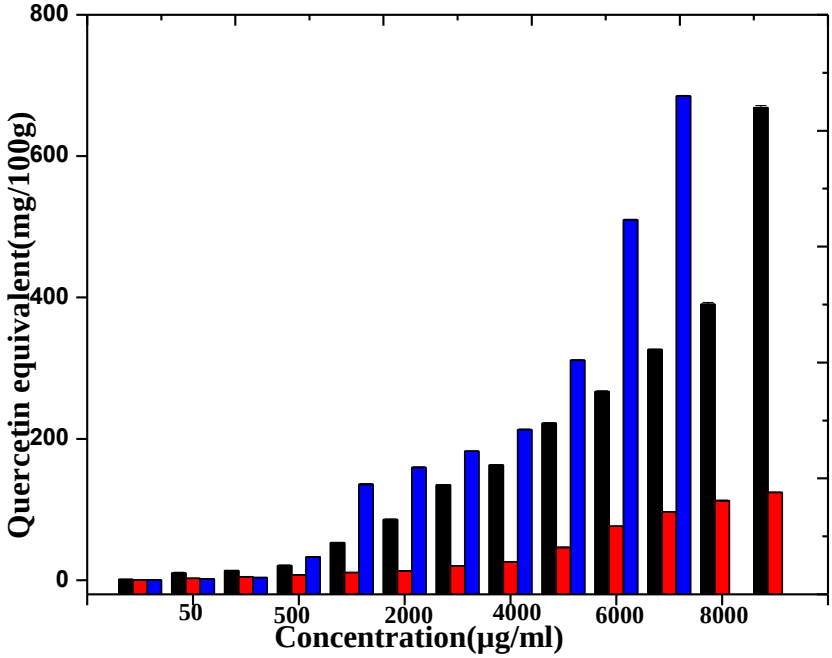
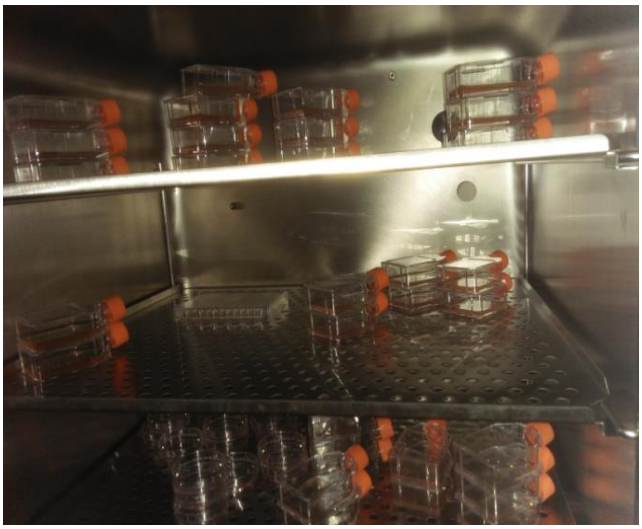


Figure 8: Total flavonoid content of different *Helicia nilagirica* extracts.
Black bars: Aqueous Extract, Red bars: Chloroform Extract and Blue bars: Ethanol Extract

CHAPTER 4

EVALUATION OF THE ANTINEOPLASTIC ACTIVITY OF *HELICIA NILAGIRICA* (BEDD.) IN PRECLINICAL MODELS IN VITRO



Chapter 4

Abstract

The cancer is a second largest killer disease and despite numerous advances made in the treatment strategies, the complete cure of cancer remains elusive. Therefore, the present study was undertaken to investigate the anticancer potential of aqueous extract of Helicia nilagirica (HNA) in vitro by MTT and clonogenic assays, where V79 and HeLa cells were treated with the different concentrations of aqueous extract of Helicia nilagirica. The treatment of V79 and HeLa cells with HNA resulted in a concentration dependent increase in cytotoxicity, which was maximum at the highest concentration of 400 µg/ml HNA in both the cell lines. The results of MTT assay were further confirmed by clonogenic assay, which also showed a concentration dependent decrease in the clonogenicity of HeLa cells.. To understand the mechanism of action the effect of HNA on glutathione (GSH) concentration, activities of glutathione-s-transferase (GST), catalase and superoxide dismutase (SOD) were studied at different post HNA treatment times. The exposure of HeLa cells to different concentrations of HNA at different post- treatment time alleviated the GSH content and also reduced the activities of antioxidant GST,CAT and SOD in a concentration and time dependent manner, except GST which was lowest and 6 h post-treatment and then marginally elevated at 12 h post-treatment.. The present study indicates that HNA exerted the cytotoxic effect on HeLa cells and recued the cell survival and this effect of HNA may be due to the alleviated level of the GSH, GST, catalase and SOD.

Chapter 4

1. INTRODUCTION

Cancer a multistage disease has been the second leading cause of death worldwide. The number of cancer cases has been predicted to rise by almost 70% in 2020 (WHO, 2017) indicating the need to find new paradigms to treat or prevent the occurrence of cancer. The chemotherapy is an established mode of treatment of several neoplasia and it is the only treatment when a patient presents with metastasis (Harrington and Smith, 2009). Almost all the modern chemotherapeutic treatments available today are associated with several adverse side effects due to limitation in site specificity, causing strain to the patient/s (Ochwang'I *et al*., 2014). This indicates the need to focus on the use of alternative treatments and therapies against cancer, which are non-toxic or possess negligible side effects.

Plants have formed the major source of several modern chemotherapeutic drugs until their chemical synthesis began and they will continue to play a major role to treat cancer (Kinghorn *et al.*, 2016). Plant-derived drugs have gained interest for anticancer treatment as they are natural and readily available, readily administered orally as part of patient's dietary intake (Cornblatt *et al*., 2007; Amin *et al*., 2009). Since they are naturally derived compounds from plants they are generally more tolerated and non-toxic to normal human cells (Jagetia, 2007; Unnati *et al*., 2013; Jagetia and Baliga, 2016).

The National Cancer Institute collected about 35,000 plant samples from 20 different countries, and has screened around 114,000 extracts for anticancer activity. 60% of the commercially available anticancer drugs are derived from natural sources. The anticancer agents, vinblastine and vincristine from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apo-cynaceae), were the first agents to advance into clinical use for the treatment of cancer. Vinblastine and vincristine are used in combination with other cancer chemotherapy drugs, for

the treatment of various kinds of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers (Moudi *et al*., 2013). The isolation of paclitaxel from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (*Taxaceae*) was clinically introduced to the US market in the early 1990s. Paclitaxel is active against a number of cancer types such as ovarian cancer, advanced breast cancer, small and non-small cell lung cancer, while *Taxus baccata* was reported to be used in India as a medicine for the treatment of cancer (Ahmed *et al*., 2013). The Camptothecin isolated from the Chinese ornamental tree, *Camptotheca acuminate* Decne (*Nyssaceae*), derivatives of camptothecin, Topotecan and irinotecan, are used for the treatment of ovarian and small cell lung cancers, and colon cancers, respectively (Venditto and. Simanek, 2010). However, induction of various adverse side effects including myelosuppression, gastrointestinal, hair follicle damage, reproductive and nephrotoxicities by these drugs has been the major stumbling block which necessitates the need to identify effective newer biomolecules to kill cancerous cells and spare normal cells with very low or negligible toxicity.

Helicia nilagirica Bedd. (Family: Proteaceae) locally known as Pasaltakaza is a tree, that grows up to a height of 12 meters and it grows in southern India, Indochina, Sri Lanka, , Burma (Myanmar), Japan, Taiwan, and Thailand. The *Helicia nilagirica grows*along streams some species are found on hilltops or ridges (Khamyong et al., 2004). Traditionally, *Helicia nilagirica* has been used as folk medicine in Mizoram, India by the Mizos since time immemorial . The decoction of leaves or bark of *Helicia nilagirica* is used to cure mouth ulcers, indigestion, stomach ailments, peptic ulcers, urinary tract infection gynaecological disorders and scabies and other skin diseases (Sawmliana 2003). In Sikkim the fruits of *H. nilagirica* are used to treat cough and cold (Chauhan 2001). A recent study has indicated that methnol extract of this plant possessed anti-inflammatory activity in rat cotton pellet granuloma model (Lalawmpuii *et al.*

2014). The systematic study on the anticancer properties of *Helicia nilagirica is lacking, which* indicates a need to evaluate its anticancer potential. Therefore, the present study was carried out to evaluate the anticancer activity of *Helicia nilagirica 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), reduced glutathione (GSH), ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), crystal violet were obtained from Sigma Chemical Co. (Bangalore, India). Sodium bicarbonate (Na_2CO_3), potassium chloride (KCl) and 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), hydrochloric acid (HCl), n-butanol, Tris buffer (Tris (hydroxymethyl) aminomethane and ammonium oxalate were requisitioned 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 (DOX), was obtained from Getwell Pharmaceuticals, Gurgaon, India.

2.2.Preparation of the extract

The identification and authentication of *Helicia nilagirica* Bedd. (Family: Protaeceae) was done by Botanical Survey of India, Shillong. The non-infected stem bark of *Helicia nilagirica* was collected from Sialsuk, Aizawl District of Mizoram, India during the dry season. The stem bark was peeled of the tree, cleaned chopped into small pieces, spread into the stainless steel trays and allowed to shade dry at room temperature in the dark, in the clean and hygienic

conditions free from insects, animals, fungus, and other extraneous terrestrial materials. The dried tree stem bark was powdered in an electrical grinder at room temperature. A sample of 100 g of bark powder was sequentially extracted with petroleum ether, chloroform, ethanol and water using a Soxhlet apparatus. The extracts were evaporated to dryness under reduced pressure and stored at -80 until further use. Only the aqueous extract of *Helicia nilagirica* (HNA) was used for evaluation of the anticancer activity.

2.3.Dissolution of drug/s

The doxorubicin was freshly dissolved in MEM and the aqueous extract of *Helicia nilagirica* was dissolved in MEM, filtered and sterilized immediately before use.

2.4.Cell line and Culture

HeLa S3, 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, 1% L-glutamine and 50 µg/ml gentamicin sulfate 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

Usually a fixed number of cells were inoculated into the desired culture vessels and they were divided into the different groups depending on the experimental protocol:

2.5.1. Determination of Cytotoxicity

2.5.1.1 MTT assay

MEM group: The cells of this group served as negative control group.

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

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

The cytotoxic effects of different concentrations of aqueous extract of *Helicia nilagirica* 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 37 ºC in a CO_2 incubator in an atmosphere of 5% CO_2 in 95 % humidified air. The cells were allowed to attach for 24 hours. The cells in microplates were exposed to different concentrations of HNA or doxorubicin and incubated in the CO_2 incubator for next 48 hours. Thereafter, 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 microplate reader (Spectramax M2). The cytotoxicity was calculated using the formula Control-Treatment/Control X 100. The IC50 was also determined.

2.5.2. Determination of optimum exposure time for cytotoxicity

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

2.5.3. The Determination of anticancer activity

Another experiment was setup to evaluate the anticancer activity of HNA, where grouping and other conditions were similar to that described in the experimental design section. Usually 10^6 exponentially growing HeLa cells were seeded into several culture flasks and the

cells were allowed to attach for 24 h. The cells were exposed to 5, 10 or 20 µg/ml DOX or 20, 300 or 400 µg/ml of HNA.

After 2 hours of drug/s treatment the media were removed and the flasks were washed twice with sterile PBS, subjected to trypsin EDTA treatment and the following studies were conducted.

2.5.4. Clonogenic Assay

Usually 200 HeLa cells were inoculated into several individual petridishes containing 5 ml MEM and allowed to grow for another 11 days. The resultant colonies of cells were stained with 1 % crystal violet in methanol and scored. The plating efficiency (PE) of the cells was determined and surviving fraction (SF) calculated according to Puck and Marcus (1955).

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

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

2.6.Biochemical assays

A separate experiment was performed to estimate the effect of HNA on the activities of various antioxidant enzymes in HeLa cells, where grouping and other conditions were essential similar to that described for anticancer activity except that the cell cultures were terminated at 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. The cells were pelleted and weighed and 5% homogenate of the cells was prepared in PBS (pH 7.4) using sonicator (PCI Analytics Pvt. Ltd., Mumbai, India).

2.6.1. Total proteins

The proteins were estimated by standard procedure of Bradford (1976).

2.6.2. Glutathione estimation

Glutathione was estimated as described earlier (Moron *et al*., 1979). Briefly, 1.8 ml of 0.2 M Na_2HPO_4 was mixed with 40 µl 10 mM DTNB and 160 µl of cell homogenate. The mixture was incubated for 2 minutes at room temperature and the absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India).

2.6.3. Glutathione - S – transferase estimation

Glutathione-s-transferase activity was estimated by the method of Habig *et al*., (1974). Briefly, 0.5 ml of 0.1 M phosphate buffer pH 6.5, 0.1 ml of 20 mM 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 read at 340 nm at 1 min intervals for 6 minutes in UV-VIS Biospectrophotometer. The GST activity was estimated using the following formula:-

GST activity = Absorbance of sample – Absorbance of blank × 1000/9.6 × Vol of sample

2.6.4. Catalase estimation

Catalase was assayed according to the technique of 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 initiated 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.

2.6.5. Superoxide dismutase estimation

SOD activity was estimated as described by Fried (1975). Briefly, 100 µl of cell homogenate was mixed with 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitroblue tetrazolium, and 200 µl of 780 µM NADH and 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 ANALYSES

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

4. RESULTS

The results are expressed in table 1-7 and figure 1-7 as mean ± standard error of the mean.

Determination of Cytotoxicity

The treatment of HeLa or V79 cells with different concentrations of HNA resulted in a concentration dependent rise in its cytotoxic effects (Figure 1)and a maximum cytotoxicity was recorded for the highest concentration of HNA (Table 1). The positive control doxorubicin also showed a similar pattern (Figure 1). The IC50 was also calculated and found to be 306.71µg/ml for HeLa and 300.64 µg/ml HNA for V79 cells, respectively.

Determination of optimum exposure time for cytotoxicity

The optimum exposure time for cytotoxicity of HNA against the two cell lines was determined using MTT assay at 2, 4, and 6 hours. The highest percent of cytotoxicity was observed at 4 h for HeLa and 2 h post treatment time for V79 cells, respectively (Figure 2). The

difference among various treatment times was statistically not significant (Table 2) Therefore further experiments were setup using 2 h HNA treatment time.

Clonogenic Assay

The clonogenicity of HeLa cells declines in a concentration dependent manner after treatment with different concentrations of HNA and the cell survival reached a nadir at a concentration of 400 µg/ml (Figure 3). The IC50 of HNA was also calculated and found to be 208.69 µg/ml.

Glutathione

The glutathione content of HeLa cells treated with different concentrations of HNA declined in a concentration dependent manner at all the post treatment times (Figure 4) and this decline was statistically significant when compared with the untreated control group Table 4). A maximum of 2.7 fold reduction in the glutathione content was observed at 12h post treatment at a concentration of 400 µg/ml (Table 4). The doxorubicin treatment also showed a pattern similar to HNA treatment (Figure 4).

Glutathione-s-transferase

Treatment of HeLa cells with different concentration of HNA showed a concentration dependent reduction in the GST activity at all the post treatment times (Figure 5). The maximum reduction (4.09 fold) was found at a concentration of 400 µg/ml after 6h treatment time (Table 5). The DOX treated group also reduced the enzyme activity in a concentration dependent manner. The reduction at all concentrations was found to be statistically significant ($p<0.05$).

Catalase

The activity of catalase in the HeLa cells treated with different concentrations of HNA showed a concentration dependent decrease at all post treatment times (Figure 6). A maximum of 2.87 fold decrease in catalase activity was observed at 400 µg/ml, at 12 h post treatment when compared with the non-drug treated control group. The catalase activity declined significantly in the HeLa cells treated with different concentrations of HNA or doxorubicin (Table 6).

Superoxide dismutase

The treatment of HeLa cells with different concentrations of HNA or DOX caused a significant but concentration dependent attrition in the SOD activity at all post treatment times (Figure 7 and Table 7). A maximum of 7.11 fold and 7.59 fold decrease in the SOD activity was observed for 400 µg/ml and 20 µg/ml of HNA and DOX, respectively, at 12h, post treatment (Table 7).

DISCUSSION

Chemotherapy has been a major treatment modality to treat various malignant cancers, either alone or in combination with radiation or surgery. It has also been used as a palliative treatment where the complete cure of cancer has not been affected (Morgan *et al*., 2004; Roeland). The active principles in chemotherapy have been derived from plants such as *Catharanthus roseus, Podophyllum peltatum, P. emodii, Taxus brevifolia, Ochrosia elliptica* and *Campototheca acuminate* (Kinghorn and Balandrin 1993). However, most of the modern chemotherapeutic agents have limitations in terms of toxicity, lack of tumor selection, ineffective against drug resistant cancers, expensive and teratogenic (Mellor and Callaghan 2008; Valko and McLeod 2009). Moreover, the patients who survive chemotherapy have shown the development of second malignancies associated with chemotherapeutic treatment (Morton *et al*., 2014).

Therefore, screening for non-toxic, cheaper, higher efficacy and better selectivity cancer drug/s, which are devoid of all the side effects of modern molecules is needed. The use of plants for treating various ailments have been practiced by humans since time immemorial and there is an unending quest in finding new and improved chemotherapeutic drugs till today. Since herbal products have been traditionally accepted and known to have lesser or no adverse effects, it is imperative to search new molecules from the plants. This has been the impetus to determine the anticancer activity of the stem bark of *Helicia nilagirica in vitro.*

Cell culture has provided a fast, efficient and economical way of cytotoxicity screening, elucidation of mode of action of drugs in a controlled and systematic manner with high resolution in a short period of time. The present study was also carried out using HeLa, a cervical cancer cells and non-cancerous V79, a Chinese hamster lung cells to estimate the cytotoxic effects of HNA by employing MTT assay. This assay is a rapid and a standard technique to test the cytotoxicity of drugs where metabolically active cells increase the formation of formazan crystals by mitochondrial succinate dehydrogenase and the level of enzyme activity is a measure of the viability of the cells and more intense color indicates more viable cells (Mossmann, 1983). The treatment of HeLa and V79 cells with HNA reduced the cell survival indicated by a concentration dependent rise in the cytotoxic effect. There seems to be no reports on the cytotoxicity of HNA and this is probably the first report where HNA has been found to be cytotoxic. However, other plants such as *Aphanamixis polystachya* ,*Tinospora cordifolia, Alstonia scholaris, Consolida orientalis, Ferula assafoetida, Coronill avaria* and *P. pellucidum* extract have been reported to induce cytotoxicity in HeLa cells in vitro (Jagetia *et al.,* 1994; Jagetia and Rao., 2006;Jagetia and Baliga, 2005; Widowati *et al.,* 2013; Jagetia and Venkatesha, 2016). Similarly, *Arctium lappa, Artemisia absinthium, Calendula officinalis, Centaurea,*

Cyanus, Tanacetum vulgare and *Tragopogon pratensis* have been reported to be cytotoxic to J-45.01 human acute T leukemia cells (Wegiera *et al.,* 2012). The cytotoxicity of HNA was further confirmed by performing clonogenic assay on the HeLa cell line. The IC50 was calculated to be 208.69 µg/ml.

The clonogenic assay is the gold standard to test the reproductive integrity of cells and HNA treatment has been found to reduce the Clonogenic potential of HeLa cells in a concentration dependent fashion. Likewise *Aphanamixis polystachya* , *Tinospora cordifolia,* and *Alstonia scholaris* have been reported to retard the clonogenicity of HeLa cells (Jagetia *et al.,* 1994; Jagetia and Rao., 2006; Jagetia and Venkatesha, 2016). Similarly, a natural product berberine has been found to reduce the clonogenic potential of HeLa cells in a concentration dependent manner (Jagetia and Rao, 2017).

Glutathione (γ -glutamylcysteinyl glycine) is a tripeptide synthesized in most cells and it is formed by the ATP dependent condensation of glutamic acid and cysteine, catalyzed by γ-glutamylcysteinyl synthetase. Glycine is then added by glutathione synthetase to form GSH. The presence of sulfhydryl (SH) group of the cysteinyl moiety is a powerful reducing agent and a strong nucleophile that is able to react with cellular toxicants directly or via the catalysis of the glutathione S-transferase family of enzymes. It is also a co-factor for several metabolic enzymes and is involved in intracellular transport, functions as an antioxidant and radioprotectant and facilitates protein folding and degradation (Halliwell and Gutteridge 1999; Gamcsik *et al*., 2012; Lu, 2013). In cancer cells the rise in GSH beyond normal level is an indication of chemotherapy resistance whereas low level of GSH has been reported to enhance oxidative stress, and subsequently cause cell death and apoptosis of the tumor cells. The loss of essential sulfhydryl groups lead to an alteration in the calcium homeostasis that eventually results in the loss of cell

viability which is indispensible for chemotherapy to be effective (Mayer *et al*., 1987; Neal *et al*., 2003; Ramsay and Dilda, 2014). Treatment of the HeLa cells with different concentration of HNA showed a concentration dependent reduction of GSH content which showed the effectiveness of HNA against neoplastic cells.

Glutathione-S-transferase isoenzymes are ubiquitous which catalyze the conjugation of reduced glutathione (GSH) with compounds that contain an electrophilic centre through the formation of a thioether bond between the sulphur atom of GSH and the substrate (Chasseud 1979; Mannervik 1985; Laborde, 2010). A number of GST isoenzymes also exhibit GSH-dependent catalytic activities such as reduction of organic hydroperoxides, isomerisation of various unsaturated compounds and also several non-catalytic functions such as sequestering of carcinogens, modulation of signal transduction pathways etc. (Ketterer *et al*. 1990; Jakoby and Habig 1980; Cho *et al*. 2001). The over expression of GST in cancer cells are common and can induce chemoresistance and resistance to apoptosis which makes it a promising target for research on the GST inhibitors to sensitize tumor cells (McIlwain *et al*., 2006; Zeng *et al*., 2014). The decline in GST activity after treatment with the HNA could be an indication that this extract act as an inhibitor compound for GST and thereby killing the cancer cells.

Catalases catalyze the conversion of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) in the presence of iron or manganese as a cofactor (Kodydková *et al*., 2014). It is localized in peroxisomes in eukaryotic cells. Suppression of catalase has been reported to induce the increase production of H_2O_2 which block TNF-induced NF-κB activation and sensitizes cells to apoptosis (Yang *et al*., 2011). The HNA also reduces the activity of catalase which may have contributed to its anticarcinogenic activity.

Superoxide dismutases (SOD) are the enzymes that catalyze the dismutation of superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and elemental oxygen. They consist of three isoforms in mammals: the cytoplasmic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3), all of which require catalytic metal (Cu or Mn) for their activation. The mechanism of dismutation of O_2^- to H_2O_2 by SOD involves alternate reduction and reoxidation of a redox active transition metal including copper (Cu) and manganese (Mn) at the active site of the enzyme (Abreu, 2010). The role of SOD level on tumor invasiveness is controversial as far as different studies are concerned on different tumors. In one study MnSOD overexpression protected HeLa cervical carcinoma cells from growth suppression under the condition of serum deprivation, which was suggested to be related to changes in the intracellular oxidative processes of these cells (Palazzotti 1999). When human prostate carcinoma cells were transfected with the cDNA for MnSOD, the clones overexpressing MnSOD grew more slowly under basal cell culture conditions in vitro than control cells (Li 1998). In one model recombinant CuZnSOD increased colon carcinoma liver metastasis in mouse models (Nonaka 1993), whereas in another mouse model recombinant CuZnSOD reduced fibrosarcoma pulmonary metastasis (Yoshizaki 1994). Therefore, modulation of the oxidant/antioxidant balance toward a more reduced state is likely to have a controlling influence limiting the survival and invasion of most cancer cells. The HNA also alleviated the activity of SOD which caused change in the cellular oxidant/antioxidant balance thereby increasing tumor cell kill in the present study.

The exact mechanism of action of cell killing by HNA is not clearly understood however, the present study clearly indicates that HNA has been able to reduce the activities of GST, catalase and SOD which are involved in the failure of chemotherapy. This reduced activity may

have played a major role in the cell killing by HNA in the present study. The GSH is another molecule which in indicated in the development of chemo resistance and it reduction by HNA would have made cells for sensitive to its cytotoxic effect and its decline may have also stimulated cell death by apoptosis. Although the effect of HNA at molecular level has not been studied. It is plausible that HNA may have blocked the transcription of NF-κB, COX-II, Nrf2 and some cell cycle proteins that help cancer cell division and ensure higher survival (Sobolewski *et al*., 2010; Lu and Stark, 2015; Choi and Kwak, 2016) and their inhibition may have led to the effective cell killing by HNA. The suppression of these proteins have been found to enhance cancer cell killing (Xu *et al*., 2014; Pozdeyev *et al*., 2015; Menegon *et al*., 2016). The HNA may have also stimulated apoptotic pathway by upregulating p53 and Bax proteins and bring effective cell killing.

CONCLUSIONS

The exact mechanism underlying the antineoplastic activity of the HNA is unknown, however cytotoxic effect against HeLa cells was observed with MTT and clonogenic assays. The alleviation in the level of glutathione, GST, catalase and SOD might have increased the level of oxidative stress, leading to DNA damage and cell death. The HNA may also have suppressed the transcription of Nrf2, NF-κB and COX-II genes at molecular level and up regulated p53 and Bax leading to effective cell kill in the present study.

Chapter 4

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Table 1: Effect of different concentrations on the cytotoxic effects of aqueous extract of *Helicia nilagirica* (HNA) in various cell lines by conventional MTT assay. The results were determined as percentage (%) cytotoxicity and expressed as Mean ± SEM.

Concentration (µg/ml)	Cytotoxicity in different cell lines (% ± SEM)			
	V79		HeLa	
	Doxorubicin	HNA	Doxorubicin	HNA
0	100	100	100	100
5	78.34±0.21*	--	53.95±0.90*	--
10	81.32±0.21*	--	59.24±0.17*	--
20	82.31±0.21*	--	71.55±0.22*	--
12.5	--	19.33±0.64*	--	12.49±1.76*
25	--	22.14±0.47*	--	24.50±1.42*
50	--	24.46±0.21*	--	28.52±0.82*
100	--	27.02±0.45*	--	29.13±1.80*
200	--	37.27±0.28*	--	41.05±1.61*
300	--	49.58±0.56*	--	49.28±2.02*
400	--	64.13±0.21*	--	60.00±2.54*
	IC50	300.64 µg/ml	**IC50**	306.71µg/ml

Standard error of the mean (SEM).

n=5, p<0.05.

Table 2: Effect of different exposure times on the cytotoxic effect of aqueous extract of *Helicia nilagirica* (HNA) and doxorubicin in various cell lines evaluated by MTT assay at different post treatment times.

Dose (µg/ml)	Cytotoxicity (% ± SEM)					
	Post treatment time (h)					
	V79			HeLa		
	2	4	6	2	4	6
0	100	100	100	100	100	100
Doxorubicin 5	75.22±0.51*	72.96±0.26*	66.73±0.12*	66.48±0.47*	65.20±0.48*	67.67±1.30*
Doxorubicin10	76.54±0.13*	73.61±0.29*	70.40±0.06*	70.83±0.78*	71.76±0.48*	73.53±0.60*
Doxorubicin20	78.97±0.21*	74.35±0.37*	75.04±0.31*	76.81±0.47*	77.23±0.65*	76.54±0.93*
HNA 200	66.58±0.14*	51.18±0.18*	59.21±0.16*	64.31±0.95*	65.20±0.79*	64.65±0.73*
HNA300	73.59±0.18*	54.37±0.18*	62.44±0.16*	66.48±0.65*	70.30±1.19*	65.82±0.76*
HNA400	77.45±0.22*	67.72±0.22*	71.83±0.50*	68.47±0.31*	71.76±0.96*	72.69±0.73*

The data are expressed as Mean ± SEM, n=5, p<0.05.

Table 3: Effect of different concentrations of the aqueous extract of *Helicia nilagirica* (HNA) and doxorubucin (DOX) treatment on the survival of HeLa cells. The result are expressed as Mean ± SEM.

Treatment	Surviving fraction
MEM	1.01±0.004
Dox 5 µg/ml	0.53±0.014*
Dox 10 µg/ml	0.31±-0.004*
Dox 20 µg/ml	0.26±0.010*
HNA 200 µg/ml	0.71±0.009*
HNA300 µg/ml	0.48±0.007*
HNA400 µg/ml	0.41±0.002*

**P<0.05 when treatment groups are compared to control group (MEM).*

No symbol= no significance. Standard error of the mean (SEM).

n=3.

Table 4: Alterations in the Glutathione contents of HeLa cells induced by different concentrations of *Helicia nilagirica* (HNA) and doxorubicin. The results were determined as µmol/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20
2	2.64±0.20	1.56±0.02*	1.44±0.01*	1.41±0.03*	1.10±0.003*	0.93±0.09*	0.87±0.03*
6	2.53±0.24	1.69±0.04*	1.41±0.009*	0.99±0.01*	1.01±0.003*	0.93±0.003*	0.78±-0.02*
12	2.33±0.06	1.01±0.01*	0.94±0.04*	0.88±0.04*	0.73±0.006*	0.59±0.01*	0.57±0.008*

**p<0.05 when treatment groups are compared with concurrent control (MEM) group.*

Standard error of the mean (SEM), **n=5.**

No symbol= no significant difference.

Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

Table 5: Alterations in the GST activity of HeLa cells treated with different concentrations of *Helicia nilagirica* (HNA) and doxorubicin. The results were determined as unit/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20
2	0.10±0.003	0.08±0.001*	0.05±0.002*	0.04±0.001*	0.08±0.001*	0.05±0.001*	0.02±0.001*
6	0.10±0.001	0.08±0.002*	0.04±0.003*	0.02±0.003*	0.07±0.001*	0.03±0.001*	0.02±0.003*
12	0.10±0.007	0.09±0.003	0.06±0.002*	0.04±0.007*	0.09±0.002	0.05±0.006*	0.04±0.003*

**p<0.05 when treatment groups are compared with concurrent control (MEM) group.*

Standard error of the mean (SEM), ***, n=5.***

No symbol= no significant difference.

Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

Table 6: Alterations in the catalase activity of HeLa cells treated with different concentrations of *Helicia nilagirica* (HNA) and doxorubicin (DOX). The results were determined as Unit/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20
2	7.64±0.19	6.11±0.50*	5.16±0.33*	4.58±0.33*	6.19±0.23	5.35±0.19*	3.68±0.16*
6	7.26±0.50	5.35±0.50*	3.82±0.38*	2.86±0.31*	4.37±0.18*	4.01±0.33*	2.58±0.16*
12	6.59±0.16	5.02±0.43*	2.77±0.34*	2.29±0.33*	4.09±0.11*	2.69±0.20*	1.91±0.38*

**p<0.05 when treatment groups are compared with concurrent control (MEM) group.*

Standard error of the mean (SEM), ***, n=5.***

No symbol= no significant difference.

Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

Table 7: Alterations in the SOD activity of HeLa cells treated with different concentrations of *Helicia nilagirica* extract (HNA) and doxorubicin (DOX). The results were determined as Unit/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20
2	3.03±0.12	2.87±0.13	2.39±0.06*	1.54±0.07*	2.63±0.13	2.11±0.10*	1.47±0.10*
6	2.84±0.12	1.82±0.09*	1.34±0.06*	1.04±0.18*	1.45±0.06*	1.16±0.10*	0.79±0.06*
12	2.52±0.07	0.61±0.02*	0.36±0.02*	0.35±0.003*	0.54±0.006*	0.40±0.002*	0.33±0.006*

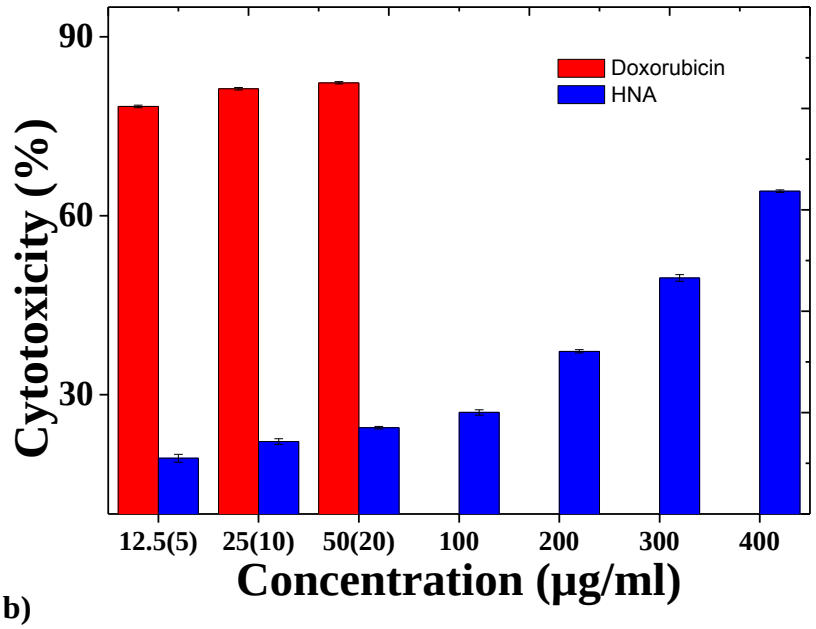
**p<0.05 when treatment groups are compared with concurrent control (MEM) group.*

Standard error of the mean (SEM), ***, n=5.***

No symbol= no significant difference.

Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

a)



b)

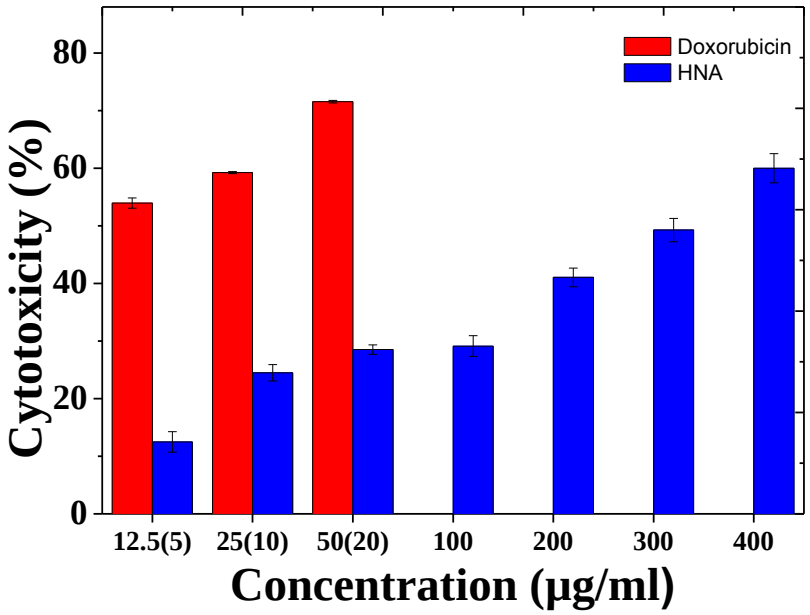
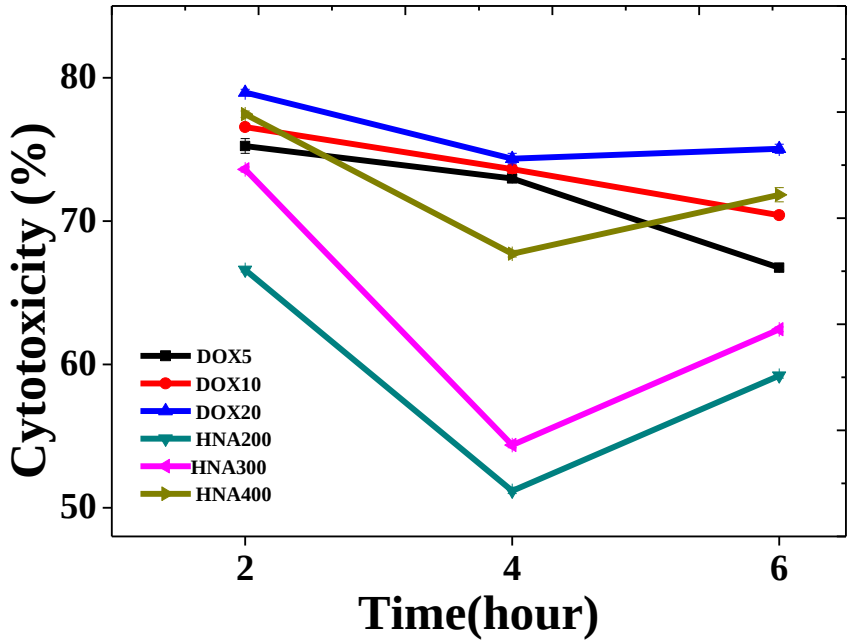


Fig. 1: Cytotoxic effect of different concentrations of aqueous extract of *Helicia nilagirica* in V79 (a) and HeLa (b) cell lines by conventional MTT assay. The results were determined as percentage (%) cytotoxicity and expressed as Mean ± SEM, n=5, $p<0.05$.

a)



b)

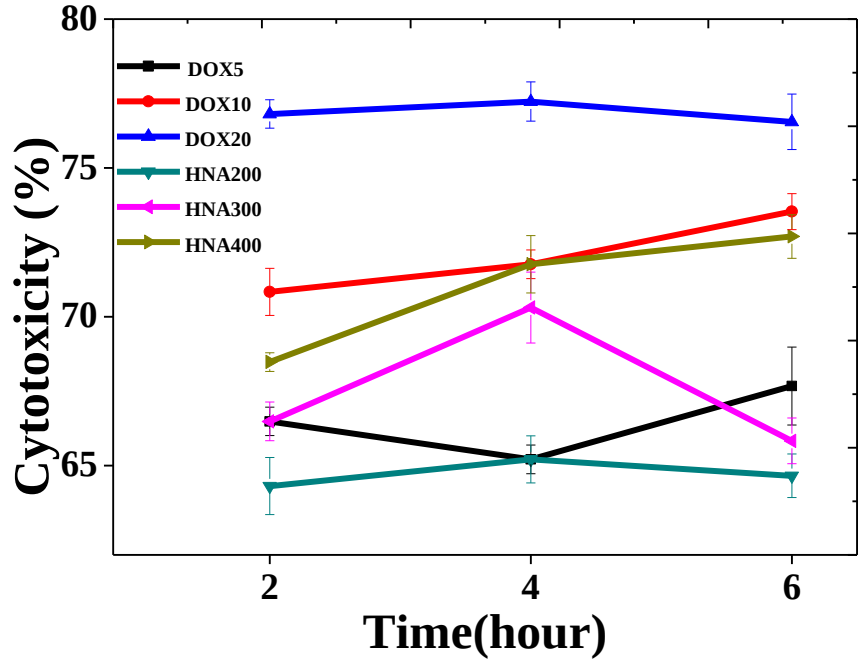


Fig 2: Cytotoxic effect at different exposure time of the aqueous extract of *Helicia nilagirica* and DOX in V79 (a) and HeLa (b) cell lines by MTT assay. The data are expressed as Mean ± SEM, n=5, p<0.05.

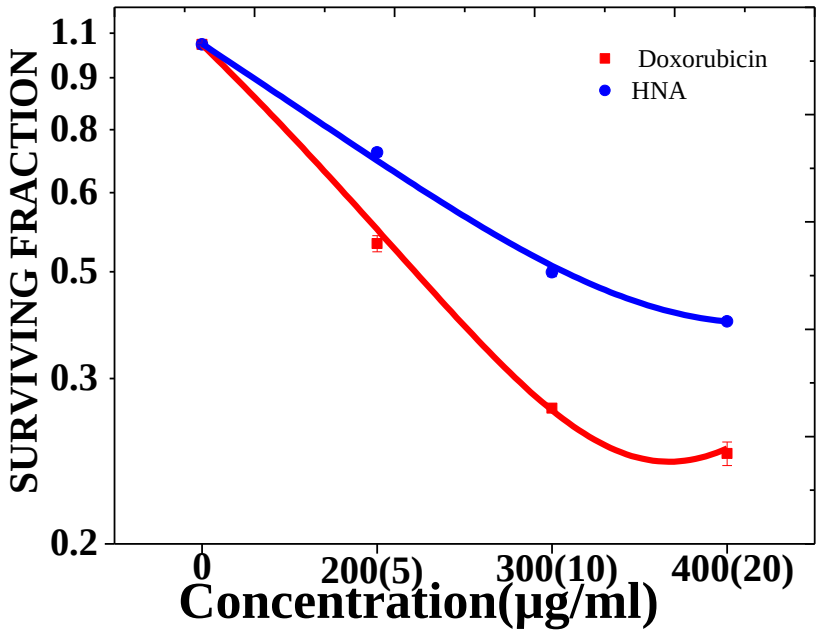


Fig 3: Effect of different concentrations of the aqueous extract of *Helicia nilagirica* (HNA) and Doxorubucin (DOX) treatment on the survival of HeLa cells.

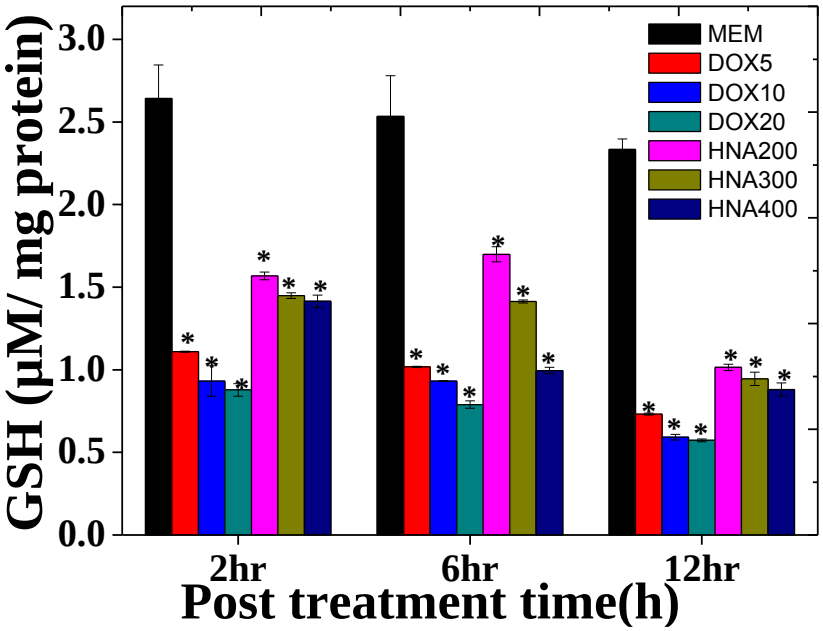


Fig 4: Alteration in the GSH content of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA- Aqueous extract of *Helicia nilagirica* (treatment), DOX- Doxorubicin (Positive Control).

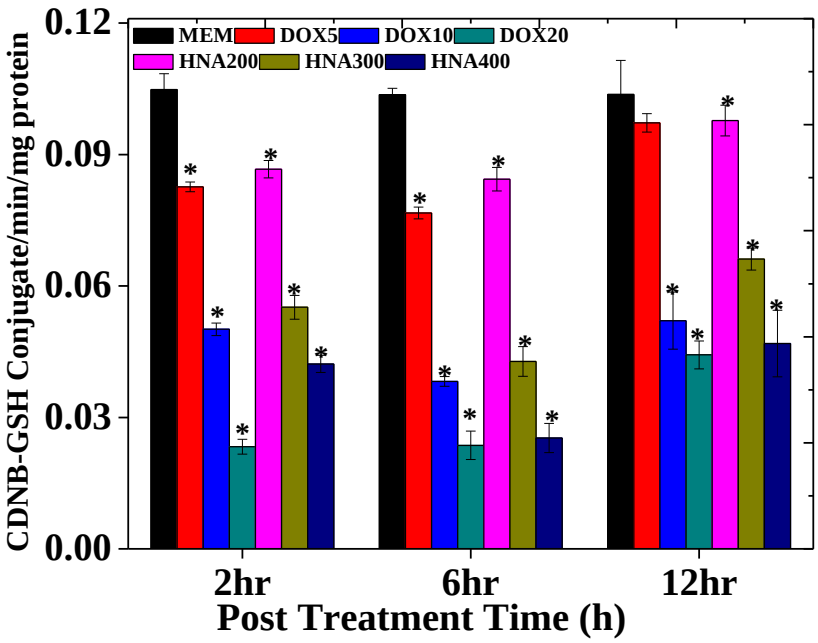


Fig 5: Alteration in the GST activity of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA- Aqueous extract of *Helicia nilagirica* (treatment), DOX- Doxorubicin (Positive Control).

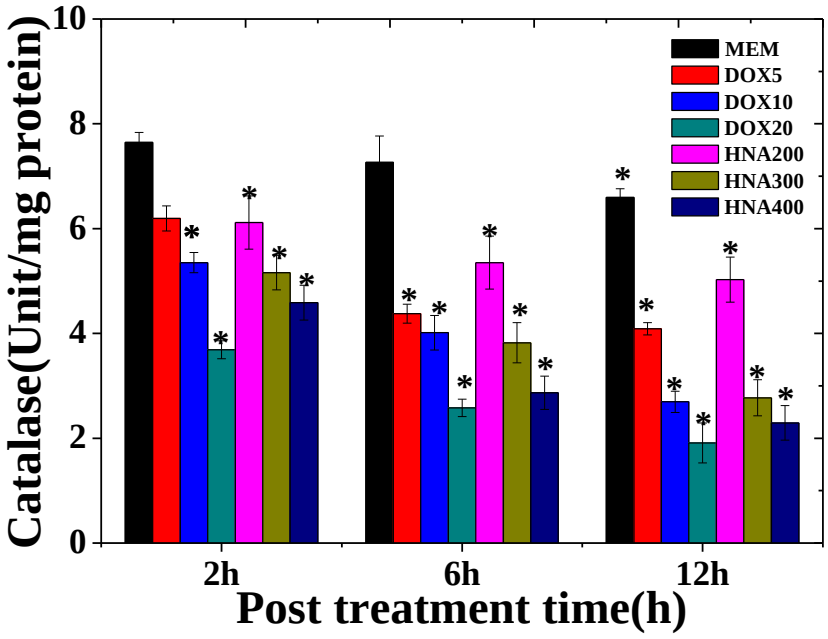


Fig 6: Alteration in the catalase activity of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA- Aqueous extract of *Helicia nilagirica* (treatment), DOX- Doxorubicin (Positive Control).

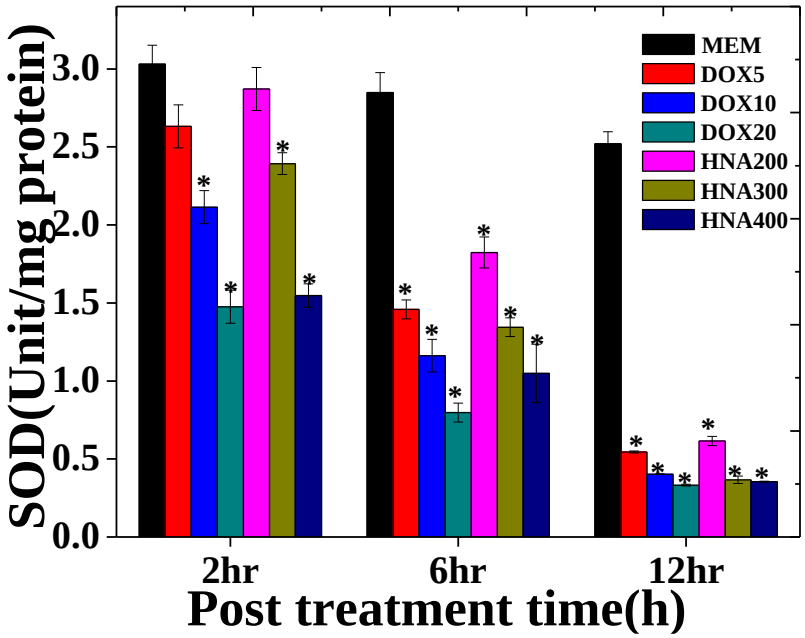


Fig 7: Alteration in the SOD activity of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA- Aqueous extract of *Helicia nilagirica* (treatment), DOX- Doxorubicin (Positive Control).

CHAPTER 5

INVESTIGATION OF THE ANTICANCER ACTIVITY OF *HELICIA NILAGIRICA* (BEDD.) IN THE SWISS ALBINO MICE TRANSPLANTED WITH DALTON'S LYMPHOMA TUMOR CELLS

Chapter 5

Abstract

The cancer is a dreaded disease and the definite cure, especially for solid tumors is not available in the advanced stages. Moreover, the modern chemotherapeutic regimens induce adverse toxic effect in the patients undergoing chemotherapy indicating the need to search newer treatment modalities which are less toxic and can cure cancer. The toxic effects of different extracts of Helicia nilagirica were studied by determining the acute toxicity in normal Swiss albino mice, where mice were injected with different doses of the chloroform, ethanol and aqueous extracts of Helicia nilagirica intraperitoneally. The LD50 was found to be 2 g/kg b. wt. for chloroform and 0.75g/kg b. wt. for aqueous extract, whereas the ethanol extract was nontoxic up to a dose of 2g/kg b. wt. The estimation of anticancer activity of Helicia nilagirica in the Dalton's lymphoma tumor bearing mice showed the administration of 50-175mg/kg b. wt. aqueous extract of Helicia nilagirica resulted in a dose dependent increase in the tumor free survival and the highest survival of 16.6 % was observed in the mice receiving 175mg/kg b. wt. aqueous extract, where the animals were able to survive beyond 120 days. The AST of 55 days and MST of 86 days were reported for this dose. The administration of different doses of ethanol extract of Helicia nilagirica also elevated the tumor free survival however only up to 38 days for 175 mg/kg b. wt. and no tumor free survivors were observed thereafter. The dose of 175 mg/kg. b. wt. aqueous extract was chosen for further investigation as maximum tumor free survivors were observed for this dose. The analysis of micronuclei frequency showed a time dependent elevation up to 24h and a decline thereafter. The apoptosis index also increased with assay time up to 36 h post treatment time. The determination of glutathione, glutathione-s-transferase, catalase, superoxide dismutase declined with increasing assay time up to 8 h post treatment, whereas the lipid peroxidation showed a time dependent rise until 24 h post treatment. The liver and kidney function test did not show any significant alteration. Our study demonstrates that Helicia nilagirica is non-toxic and increase the tumor free survival by inducing DNA damage and reducing the antioxidant levels in the tumor cells.

Chapter 5

1. INTRODUCTION

Cancer is a multifactorial disease which gives rise to the formation of rapidly dividing abnormal cells that grow beyond their usual boundaries, and invade adjoining parts of the body by spreading into other organs through the process of metastases. It is the second leading cause of mortality worldwide. The cancer incidence has been increasing at an alarming rate and with modern diagnostic techniques more cancer cases are coming to light than ever before. In India usually 700,000 cancer cases are diagnosed annually and the figure is suspected to increase by five folds in the year 2025 (IARC, 2014). It is expected that 1,688,780 new cancer cases are expected to be diagnosed and 600,920 patients of cancer will die in the year 2017 in the United States (Siegel et al., 2017). The global figure of cancer occurrence in the 2017 will still be higher. The last century has seen major advances in the understanding of the process of cancer induction and also in the treatment modalities. Despite this fact, the morality rates in patients suffering from cancer have not been significantly improved than it was is 1950, especially for the solid neoplasia (Siegel *et al*., 2017). This indicates that new treatment strategies need to be devised if the mortality rates have to be brought down in the cancer patients. Further, the current cancer therapies have shown severe side effects such as immunosuppression, organ failure and infectious diseases which cause the death of patient after recovery from cancer (Barh 2008) due to the adverse effect of modern chemotherapy molecules on normal cells, since these molecules are non-specific and target of cancer as well as normal cells equally. The use of plants by human beings for the treatment of various disorders and healthcare is as old as the human civilization. There are evidences from the written record on Sumerian clay slabs dating back to about 5000 years (Sumner 2000). Plants and natural products have provided a major source of new drugs and they have been the backbone of several chemotherapeutic drugs that are in common use to treat

cancer (Newman and Cragg, 2016; Kinghorn *et al*., 2016). The fact is that most modern chemotherapeutic drugs have been isolated from plants/natural products before their chemical syntheses began. The earliest plant derived chemotherapeutic drugs are vinca alkaloids, which have been isolated from *Cathranthus roseus.* The vinca alkaloids have been reported to be active against leukemias, lymphomas, Kaposi's sarcoma, testicular tumors, lung and breast cancers either alone or in combination with other cancer chemotherapy regimens (Duflos *et al*., 2002; Jordan, 2002). Similarly, many other modern chemotherapeutic agents have been isolated from plants to treat cancer in clinical situation successfully (Erba *et al*., 1983; O'Dwyer *et al*., 1985; Rowinsky, 1997; Zasadil *et al*., 2014; Kamal *et al*., 2015). Recently, scientists and medical professionals have shown increasing interest in this field as they recognize the true health benefits of herbal medicine, which has been playing an important role in the human healthcare system since time immemorial.

Helicia nilagirica Bedd belongs to plant family Proteaceae, and it is known as Pasaltakaza locally. *Helicia nilagirica* grows in lowlands to montane rain forests, up to an altitude of 2,000 -3,350 m and also along streams and hilltops or ridges (Khamyong et al., 2004). Mizo tribe has found its medicinal utility and it is used by Mizos to treat gynaecological disease, stomach ailments, peptic ulcers, indigestion, ulcers of mouth, and infection of the urinary tract. They also use it to treat scabies and some of the skin diseases (Sawmliana 2003). The *H. nilagirica* fruits are used in Sikkim to provide relief in cure cough and cold (Chauhan 2001). There has been a recent report regarding the anti-inflammatory activity of *H. nilagirica* (Lalawmpuii et al., 2014). However, the systematic scientific evaluation of medicinal properties of *Helicia nilagirica* is unavailable. Therefore, the present study was carried out to obtain an

insight into the antineoplastic activity of *Helicia nilagirica* in the Swiss albino mice transplanted with Dalton's lymphoma.

2. MATERIALS AND METHODS

2.1. Chemicals

Doxorubicin was requisitioned from Getwell Pharmaceuticals, Gurgaon, India. Dimethyl sulphoxide (DMSO), -chloro-2,4-dinitrobenzene (CDNB), 5, 5'dithio 2-nitrobenzoic acid (DTNB), ethylenediamine tetra-acetic acid (EDTA), reduced glutathione (GSH), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), 1, ethidium bromide, acridine orange, sodium pyruvate, thiobarbituric acid (TBA), and cytochalasin B were obtained from Sigma Aldrich Chemical Co. (Kolkata, India). Potassium chloride (KCl), hydrogen peroxide (H_2O_2) and trichloroacetic acid (TCA), were procured from SD Fine-Chem Ltd., Mumbai, India., Disodium biphosphate (Na_2HPO_4), hydrochloric acid (HCl), sodium chloride (NaCl), sodium hydroxide (NaOH), sulphuric acid (H_2SO_4), n-butanol, Tris (hydroxymethyl) aminomethane (Tris buffer) and ammonium oxalate were supplied by Merck India Limited, Mumbai. Coomassie brilliant blue, reduced nicotinamide adenine dinucleotide (NADH) was purchased from HiMedia, Mumbai, India.

2.2. Collection and Preparation of the Extract

The non-infected stem bark of *Helicia nilagirica* Bedd. (Family: Protaeceae) was collected from Sialsuk, Aizawl District of Mizoram, India during the dry season and it was identified and authenticated by Botanical Survey of India, Shillong. The cleaned bark was chopped into small pieces, and shade dried at room temperature in the dark and ground to powder at room temperature using an electrical grinder. The dried powder was weighed and 100 g of bark powder was sequentially extracted in petroleum ether, chloroform, ethanol and water in

a Soxhlet apparatus. All the extracts were collected and dried by evaporating their liquid contents under reduced pressure. The dried extracts were stored at -80 until further use.

2.3. Preparation of Drug and mode of administration

The ethanol and aqueous extracts of *Helicia nilagirica* were used for further studies. The chloroform extract (HNC) was dissolved in sterile physiological saline (SPS) and 1% CMC, ethanol (HNE) extract was dissolved in 5% ethanol in SPS whereas doxorubicin and aqueous extract (HNA) were dissolved in SPS alone. The animals were administered intraperitoneally according to their body weight.

2.4. Animal care handling

The animal care and handling were carried out according to the guidelines of the INSA (Indian National Science Academy, New Delhi, India) and the World Health Organization, Geneva, Switzerland and. Swiss albino mice were purchased from Pasteur Institute, Shillong and were bred before use in a controlled environment of temperature (24-25ºC), 50% humidity and light and dark (12 h each) cycle. Usually 5-6 animals were housed in a sterile polypropylene cage containing paddy husk (procured locally) as bedding material. Six to eight weeks old male and female Swiss albino mice weighing 20-30 g were used for the experimentation. The animals were maintained on commercially available food pellets and water ad libitum. All animal experiments were carried out according to NIH and Indian National Science Academy, New Delhi, India guidelines. The Institutional Animal Ethics Committee of Mizoram University, approved the entire study vide letter no. MZUIAEC141513, Aizawl, India.

2.5. Acute Toxicity Test

The acute toxicity study of all extracts was performed as per Organization for Economic Co-operation and Development (OECD) guidelines. The mice were selected by random sampling

technique (n=10) per dose. The animals were fasted for 18 hours (both food and water withdrawn) prior to intraperitoneal injection of different extracts of *Helicia nilagirica*. The control group received the vehicle used for dissolving the extracts. The animals were constantly monitored up to 14 days after drug treatment. If mortality was observed in 2-3 animals, then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxicity. The LD_{50} for each extract was calculated using probit analysis.

2.6. Tumor Model

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

2.7. Experimental

The anticancer activity of *Helicia nilagirica* was assessed in the Dalton's lymphoma tumor bearing mice, which were divided into the following groups:

2.7.1. Negative Control groups: This group received 0.01 ml/kg body weight of respective vehicle used for dissolving the extract/s. Each extract had its own control group. ***2.7.2. DOX groups:*** This group of animals was injected with 0.5 mg/kg body weight of doxorubicin, a standard anticancer drug and served as positive control.

2.7.3. HNE groups: This group of animals received 50, 75, 100, 125,150 or 175 mg/kg body weight of the ethanol extract of *Helicia nilagirica* (HNE).

2.7.4. HNA groups: The animals were injected with 50, 75, 100, 125,150 or 175 mg/kg body weight of the aqueous extract of *Helicia nilagirica* (HNA).

The tumor bearing animals were given the above treatments once daily 1 day after tumorization of mice that was followed up to subsequent 9 days (Geran *et al*., 1972). 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). Each group consisted of ten animals for each extract dose. The deaths, if any, of the tumor bearing mice were recorded daily and the survival was determined. The tumor response was assessed by calculating median survival time (MST) and average survival time (AST). The MST and AST were calculated from the animals dying within 120 days and those surviving beyond 120 days were excluded from the study (Geran *et al*., 1972). The increase in median life span (% IMLS), increase in average life span (% IALS) and the increase in life span (%ILS) was also calculated using the formulae:

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

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

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

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

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

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

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

2.8. Micronucleus Assay

The ability of HNA to induce DNA damage in Dalton's lymphoma cells was studied by performing a separate experiment, where 1 x 10^6 Dalton's lymphoma cells were transplanted into 5 – 8 weeks old mice and allowed to grow the tumor for 9 days. Thereafter, these animals were given a single treatment of 175 mg/kg body weight of HNA or 0.5 mg/kg body weight

doxorubicin intraperitoneally. One hour after the drug/s administration each of the tumorized mouse was injected with 150 µg of cytochalasin B so as to suppress cytokinesis in proliferating tumor cells. The mice were euthanized at 12, 24 and 48 h post-drug administration and the tumor cells were collected in individual tubes. The tumor cells were washed with ammonium chloride to lyse erythrocytes and centrifuged at 1000 rpm. The micronuclei were prepared according to the modified method of Fenech and Morley, (1985). In brief, cells were washed with sterile PBS and pelleted by centrifugation at 1000 rpm for 5 min. The cell pellet was disturbed and subjected to mild hypotonic treatment (0.75% ammonium oxalate) at 37°C, centrifuged once again and the resultant cell pellet was allowed to fix in Carnoy's fixative 3:1 (Methanol: Acetic acid) overnight. The cells were centrifuged and the resultant pellet was resuspended in a small volume of fixative. The cells were spread on to pre cleaned coded slides to avoid observer's bias. The cells were stained with 0.025% acridine orange 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 binucleated cells with well-preserved cytoplasm were scored for each post-treatment time in each group. The frequency of binucleated cell bearing micronuclei (MNBNC) was determined. The micronucleated cells were scored following the criteria of Kirsch-Volders *et al*., (2003) and Fenech *et al*., (2003).

2.9. Apoptosis Assay

Another experiment was conducted to investigate the ability of *Helicia nilagirica* to induce apoptosis in Dalton's lymphoma cells, where grouping and other conditions were exactly similar to that described for micronucleus assay except that tumor bearing mice were euthanized

at 2, 12, 24 and 36 h post drug treatment. The apoptosis assay was carried out according to Sahni *et al*., 1993. 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 preclean coded slides and stained with freshly prepared ethidium bromide and acridine orange (1:1) (Sigma Aldrich Chemical Co. Bangalore, India) dye mixture 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 was counted for each slide and a total of 5000 cells were counted for each group. The apoptotic index was calculated as follows:

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

2.10. Biochemical Assays

A separate experiment was performed to study the various biochemical changes in the HNA treated Dalton's lymphoma cells in vivo. The grouping and other conditions was essentially similar to that described for apoptosis assay. The animals were euthanized after nine days of drug/s treatment at an interval of 2, 4, 6, 8, 12 and 24 h post HNA treatment. Both the treated and untreated Dalton's lymphoma cells were aspirated under sterile conditions, washed with ammonium chloride followed by sterile phosphate buffer saline and pelleted. The cell pellets were weighed and 5% homogenate was prepared in cold, sterile PBS (pH 7.4), which was used for the estimation of various glutathione (GSH), glutathione-s-transferase (GST), catalase, superoxide dismutase (SOD) and lipid peroxidation (LOO).

2.10.1. Total proteins

The proteins were estimated by standard procedure of Bradford (1976).

2.10.2. Estimation of Glutathione

Glutathione concentration was estimated as described earlier (Moron et al., 1979). Glutathione was measured by its reaction with DTNB (Ellman's reaction) to give a compound that absorbs light at 412 nm. In brief, to 1.8 ml of 0.2 M Na_2HPO_4, 40 µl of 10 mM DTNB and 160 µl of cell homogenate were added, mixed and allowed to stand for 2 minutes. The absorbance was read against the blank at 412 nm in a Systronic UV-VIS double beam spectrophotometer (Systronics India Ltd., Ahmedabad, India).

2.10.3. Estimation of Glutathione - S – Transferase

Glutathione-s-transferase was determined by the method of Habig et al., (1974). Usually, 0.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.1 ml of 20 mM CDNB, and 8.8 ml distilled water were incubated at 37°C for 10 min followed by the addition of 0.5 ml of 20 mM GSH and 0.1 ml of cell homogenate. The absorbance was read at 340 nm with a UV-VIS double beam spectrophotometer at 1 min intervals for 6 minutes. The GST activity was estimated using the following formula:-

GST activity = Absorbance of sample – Absorbance of blank × 1000/9.6 × Vol of sample

2.10.4. Catalase Assay

Catalase was assayed according to technique described by Aebi (1984). In a 3 ml cuvette, 20 µl of cell homogenates was mixed with 1.98 ml of 50 mM phosphate buffer (pH 7.0). The reaction (maintained at 20ºC) was started by the addition of 1 ml of 30 mM H_2O_2. The decrease in absorbance was monitored at 240 nm for 60 seconds.

2.10.5. Superoxide Dismutase Assay

The activity of SOD was estimated by the Fried et al., (1975). Briefly, 100 µl of cell homogenate, 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitroblue tetrazolium,

200 µl of 780 µM NADH were mixed and incubated for 90 seconds at 30°C. The reaction was terminated by adding 1000 µl of acetic acid followed by the addition of 4 ml n-butanol. The absorbance was recorded at 560 nm using UV/VIS double beam spectrophotometer. The percent inhibition was calculated by measuring the absorbance of blank without SOD enzyme. The SOD activity was calculated using the formula (Blank-Sample)/Blank X 100.

2.10.6. Lipid Peroxidation Assay

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

2.11. Determination of Liver and Kidney function

2.11.1. Collection of serum

A separate experiment was conducted to estimate the toxicity of the HNA in the liver and kidney of the tumorized mice. The tumor bearing mice were injected with 175 mg/kg b. wt. of aqueous extract one day after transplantation of Dalton's lymphoma until nine subsequent days. After the treatment period was over, the blood was collected at different time intervals from the retro orbital sinuses using a capillary tube after anesthetizing the mice with ketamine. The

collected blood was allowed to stand for 30 min at 4ºC so as to separate the serum. The samples were centrifuged at 1000 rpm for 5 min and the serum was collected for the estimation of AST, ALT, creatinine and uric acid at 2, 4, 6, 8,12 and 24 hours after the last drug administration. Commercially available kits were used to measure AST, ALT, creatinine and uric acid. The aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphate (ALP), were measured with the help of commercial available Respons kits using a Respons 910 autoanalyzer (Diagnostic Systems GmbH¸ Holzheim, Germany).

3. Statistical Analyses

The statistical analyses were carried out using Origin Pro 8 SRO v8.0724 (B724), Northampton, MA, USA. The significance for survival analysis was determined by Kaplan Meier test and student's't' test was applied for biochemical studies and followed by Tukey's post -hoc tests for multiple comparisons, wherever necessary. The Wilcoxson's signed rank test was utilized for micronucleus and apoptosis assays. The results were confirmed by repetition of the experiments. Test of homogeneity was applied to determine any statistical differences between the repeat experiments. Since no significant differences were observed the data of all experiments were combined and expressed as mean ± standard error of the mean (SEM). A p value of < 0.05 was considered statistically significant.

4. **Results**

The results are expressed as the mean ± standard error of the mean (SEM), wherever required and are presented as Tables 1-12 and Figures 1-11.

Chapter 5

4.1. **Acute toxicity**

The acute toxicity of *Helicia nilagirica* was determined by intraperitoneal administration of its different solvent extracts. The highest toxicity was found for the aqueous extract with an LD_{50} of 750 mg/kg body weight followed by chloroform extract having an LD_{50} of 2000 mg/kg body weight. However, the ethanol extract was non-toxic up to a dose of 2000 mg /kg body weight (Table1).

4.2. Anticancer activity

The DLA bearing mice gained weight continuously due to tumor cell proliferation and growth and there was no sign of tumour regression in the negative control group. Treatment of DLA mice with 50, 75, 100, 125, 150 and 175 mg/kg body weight of aqueous extract of *Helicia nilagirica* exhibited only slight elevation in the body weight with elapse of time when compared with negative control group (Figure 1). The comparison of *Helicia nilagirica* extract treated groups with negative control revealed a significant reduction in the body weight due to alleviated cell proliferation (Table 2). The administration of ethanol extract also reduced the body weight in a dose dependent manner when compared to untreated control (Figure 1). However, the degree of weight reduction was lesser than HNA (Table 2).

Dalton's lymphoma transplanted in the peritoneum of mice developed rapidly with no signs of regression and all the untreated tumorized mice died within 20 days post tumor inoculation (Table 3; Figure 2). The AST and MST for this group were 20.5 and 20.67 days, respectively (Table3; Figure 3).

The treatment of tumor bearing mice with different doses of 50, 75,100,125, 150 and 175 mg/kg body weight of the ethanol extract resulted in a dose dependent rise in the survival and maximum number of survivors (20%) was observed at 175 mg/kg body until 40 days. The

administration of 175 mg/kg body weight HNE resulted in an AST of 32 days leading to an increase of 12 days, whereas MST of 44.5 days and increase by almost 24 days, when compared to negative control (Figure 2). The IMLS of 117.07% and an IALS of 54.88% was recorded from 175 mg/kg HNE (Table 4; Figure 3).

The treatment of tumor bearing mice with different doses of 50, 75,100,125, 150 and 175 mg/kg body weight of the aqueous extract resulted in a dose dependent rise in the survival and a maximum number of survivors (16.6%) was observed at 175 mg/kg body until 120 days and 50% of tumor free survivors were observed up to 40 days post HNA treatment (Table 3). The administration of 175 mg/kg body weight HNA resulted in an AST of 55 days, MST of 86 days, IMLS of 319.51% and an IALS of 166.21%, respectively (Table 4; Figure 3). This dose was considered as an optimum dose and remaining experiments were conducted using this dose of HNA.

4.3. Micronucleus assay

The treatment of tumor bearing mice with 175 mg/kg body weight of HNA as well as treatment with 0.5 mg/kg body weight of doxorubicin showed a significant increase in the frequency of one and two micronuclei in the binucleated cells scored at different time intervals (Figure 4). The frequencies of both cells with one and two micronuclei increased with assay time up to 24 h post treatment and declined thereafter (Figure 4). The frequency of micronuclei was greatest at 24 h post treatment ($p<0.05$) (Table 5).

4.4. Apoptosis assay

Treatment of DLA mice with HNA of DOX increased the apoptotic and necrotic index in a time dependent manner and maximum number of tumor cells died due to apoptosis or necrosis

by 36 h (Figure 5). The apoptotic and necrotic index showed a significant ($p<0.05$) elevation when compared to negative control in the HNA and DOX treated groups (Table 6).

4.5. Biochemical assays

4.5.1. Glutathione

The tumor bearing mice treated with 175 mg/kg body weight of HNA and 0.5 mg/kg body weight of doxorubicin showed a decrease in glutathione contents in a time dependent manner up to 24h post drug treatment (Figure 6). The maximum decline was found at 8 h post treatment for both HNA and DOX groups where the decreases were 2.10 fold for HNA and 2.8 fold for DOX respectively. Thereafter the GSH concentration started rising however, it did not reach to negative control level even at 24 h post treatment (Figure 6). The alleviation of glutathione contents in all the treatment groups was significant ($p<0.05$) when compared with the concurrent control group (Table 7).

4.5.2. Glutathione - S – Transferase (GST)

The admistration of HNA or DOX resulted in a time dependent decline of GST activity and a maximum decline in the GST activity was recorded at 4 h post drug treatment for HNA (2.39 fold) and 8 h for DOX (2.46 fold) (Figure 7). Thereafter there has been a marginal elevation in the GST activity however, it did not reach to negative control level (Table 8). The decline in the GST activity was significant ($p< 0.05$) at all the post treatment times except 2 h when compared with the concurrent negative control group (Table 8).

4.5.3. Catalase (CAT) activity

The catalase activity in the HNA and DOX treated groups declined with the increasing assay time interval (Figure 8) and the greatest reduction was found at 24h post drug treatment, where this decrease was 1.95 fold for HNA and 2.17 fold for DOX respectively (Table 8). All the

treatment groups showed a significant ($p<0.05$) decline when compared with the negative concurrent control group (Table 9).

4.5.4. Superoxide dismutase (SOD) activity

The administration of HNA (175mg/kg body weight) and DOX (0.5mg/kg body weight) resulted in a significant ($p<0.05$) decline in SOD activity in all the treatment groups (Figure 9). The greatest reduction in SOD activity was observed at 2 h for HNA (2.2 fold) and (2.5 fold) for DOX respectively (Table 10). The SOD activity increased thereafter and a second phase of decline was noticed at 8 h post treatment for both HNA and DOX groups where it was 1.8 and 2.4 folds, respectively (Table 10).

4.5.5. Lipid peroxidation

Treatment of DLA mice with HNA and DOX resulted in a continuous rise in the lipid peroxidation up to 24 h post treatment (Figure 10). The LOO increased significantly ($p<0.05$) at all post treatment assay times (Table 11). The increase in lipid peroxidation was 1.36 fold for HNA and 1.83 fold for DOX at 24 h post treatment time (Table 11).

4.5.6. Liver and kidney function test

The serum level of mice treated with HNA (175mg/kg body weight) and DOX (0.5mg/kg body weight) did not show significant change in the level of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) except a marginal rise at 8 h for HNA which was found to be within the normal range. The uric acid and creatinine level was also determined to test their toxic effect in the kidney, the result did not show any significant alteration except at 12 h post treatment for uric acid test which was also found to be within the normal range (Table 12; Figure 11). The toxic profile for both the liver and kidney showed that there was no adverse side effect associated with the dose of HNA being used for the study.

Chapter 5

Discussion

The main therapeutic treatment of cancer includes surgery, chemotherapy and irradiation and their optimum outcomes are limited due to their insensitivity to select tumor cells over normal cells, triggering of systemic toxicity and the development of drug resistance by tumor cells (Xu and McLeod, 2001; Housman et al., 2014). Therefore, there is a need to focus on a safe and effective therapeutic treatment that can cure cancer without adverse side effects and avoid the development of drug resistance. Herbal medicines have been used all over the world from ancient times as an alternative medicine and they are still used in developing countries to treat and prevent various diseases including cancer. The main advantage of plant derived products over synthetic drugs is the possession of properties which are optimized by evolution for serving different biological functions (e.g., binding to specific target proteins or other biomolecules) (Appendino *et al*., 2010; Hunter, 2008). The inclusion of herbal medicine as an adjuvant therapy in cancer treatment regimen may be able to reduce the toxicity of the drugs against normal cells while increasing the action of drugs by killing the neoplastic cells (Tannock, 1996). Therefore, the present study was undertaken to investigate the anticancer activity of the aqueous extract of the stem bark of *Helicia nilagirica*.

The acute toxicity studies after intraperitoneal administration did not show any toxic effect for ethanol extract up to 2 g/kg b. wt., whereas chloroform and ethanol extracts of *Helicia nilagirica* revealed toxicity and the . LD50 was found to be 2g/kg and 0.750g/kg body weight, for chloroform and aqueous extracts, respectively. The toxic effects of *Helicia nilagirica* has not been studied preclinically. However, other plant extract like *Alstonia scholaris, Nigella sativa* also did not exhibit toxicity up to 1000 mg after intraperitoneal administration in earlier studies (Baliga *et al*., 2004; Jagetia and Ravikiran, 2014).

The induction of tumor employing DLA showed that there was continuous growth of tumor cells in the absence of the extracts as confirmed by the gain in body weight and all the tumor bearing animals of control group died within 21 days showing an average survival time (AST) of 20.66 and median survival time (MST) of 20.5, whereas administration of the extracts inhibited tumor cell proliferation and growth as indicated by reduction in body weights and increased survival. Although the ethanol extract increased the tumor free survival the aqueous extract proved superior where 16.7% tumor free survivor could be observed at the end of 120 days. The 175mg/kg body weight aqueous extract increased the AST by 35 and MST by 66 days when compared to 20 days of negative control. Earlier various plant extracts such as *Alstonia scholaris, Aphnamixis polystachya, Dillenia pentagyna,* and *Tinospora cordifolia* have been reported to kill tumor cells and increase the tumor free survivors in different preclinical models effective against this type of tumor earlier (Jagetia and Baliga, 2004, 2016; Jagetia and Rao, 2006; Rosangkima et al., 2008; Jagetia and Venkatesha, 2012).

The induction of DNA damage is one of the important mechanisms to bring cell killing and the efficacy of HNA to bring out the DNA damage was assayed by studying micronuclei in the tumor cells. Administration of 175 mg/kg b. wt of HNA resulted in the increase in micronuclei frequency indicating that the cell killing effect of HNA was due to its ability to trigger damage to cellular genome, which is expressed as micronuclei after a cell division. The studies regarding the induction of DNA damage by HNA are lacking. However, *Tinospora cordifolia,* and *Aphnamixis polystachya* have been reported to kill tumor cells by inducing micronuclei formation (Jagetia et al., 1998; Jagetia and Venkatesha, 2016). Similar molecular DNA damage has been found to be responsible for tumor cell killing in earlier studies (Jagetia and Rao, 2015a,b) The formation of micronuclei as well as nucleoplasmic bridges (NPB) could

be used as a biomarker for the presence of DNA damage, chromosome rearrangement and genotoxic agents (Fenech, 2002; Thomas *et al*., 2003). Several studies have shown that the cells bearing micronuclei are dying cells (Jagetia and Adiga, 2000, Jagteia and Aruna, 1999; Jagetia and Adiga, 1995, 1998, 1999; Jagetia and Venkatesh, 2015; Jagetia and Venkatesha, 2016). The presence of micronuclei in the tumor cell being tested could be an indication of DNA damage caused by the *H. nilagirica* extract.

The induction of apoptosis in neoplastic cells is needed to reduce their proliferation through activation of p53 pathway or other apoptotic pathways (Lowe and Lin, 2000). The treatment of DLA mice with the HNA (175mg/kg b. wt.) induced apoptosis in a time dependent manner leading to the formation of cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation. The apoptosis induction as an important cell death mechanism has been reported (Aghbali, 2014). The aqueous extract of *Origanum dayi* and *Ochradenus baccatus* have been reported to induce apoptosis in HepG2 cells earlier (Bhatia et al., 2015). *Etlingera elatior*, *Rosa damascene* and *Rafflesia kerrii* extracts have been reported to induce apoptosis in epidermoid carcinoma A431 cells in an earlier study (Thuncharoen *et al*., 2013). Similarly the methanol extract of *Holarrhena floribunda* has been reported to trigger apoptosis in breast (MCF-7), colorectal (HT-29), and cervical (HeLa) cancer cells relative to normal KMST-6 fibroblasts (Badmus *et al*., 2015). Glutathione (GSH) a tripeptide (L-γ-glutamyl-L-cysteinylglycine) acts as an antioxidant molecule either directly by interacting with reactive oxygen/nitrogen species (ROS and RNS, resp.) and electrophiles or by operating as a cofactor for various enzymes and metabolic processes. The glutathione has been found to be elevated in majority of the human tumors (Gamcsik *et al*., 2012). Similarly the DLA tumor showed an increased GSH concentration. The elevated levels of GSH in tumors are the cause of

chemotherapy resistance and failure of tumor therapy (Traverso *et al*., 2013; Ramsay and Dilda, 2014). This indicates that reduced glutathione levels will make tumors more amenable to therapy. The administration of HNA led to a decline in the glutathione levels of the tumor cells when compared with the control group. This reduction could indicate increased oxidative stress leading to higher tumor cell kill in the present study The treatment of DLA tumor with the HNA reduced the activities of GST, catalase and SOD significantly in a time dependent manner which might also contributed to their effective tumor cell kill by increasing oxidative stress leading to the breakdown of cells, as the main function of these enzymes lies in their ability to the detoxification of foreign compounds such as superoxide anion which is dismutated by superoxide dismutases (SODs) to H_2O_2 that is catalyzed to H_2O by catalase, peroxiredoxins (Prxs), or glutathione peroxidases (GPx) (Sherrat and Hayes, 2001; Fukai and Fukai, 2011). The aberrant expression of GSTs in tumors has been linked to therapy resistance (McIlwain *et al*., 2006) and reduction in the activity of GST by HNA may have contributed to increased tumor cell kill in the present study. Similarly, the SOD enzymes are also found to be elevated in tumors (Khan *et al*., 2010) and the alleviated levels of SOD may have also been responsible for tumor cell death and increased tumor free survivors in the HNA treated group. The observation of increased lipid peroxidation level in the treated group when compared to the untreated control group also confirmed the increased oxidative stress in the cell and the depletion of antioxidant scavenger system leading to the oxidative degradation of lipid layer and increasing the cell death. The chemotherapy drugs have been reported to act by increasing oxidative stress and also products of lipid peroxidation leading to the effective cell killing (Barrera, 2012)

The administration of aqueous HNA (175mg/kg b. wt.) for nine consecutive days was found to be safe as it did not alter the level of aspartate aminotransferase (AST), alanine

aminotransferase (ALT), creatinine and uric acid significantly even though there was a slight elevation in ALT and uric acid level but these changes were within the normal range (Reitman and Frankel, 1957; Fossati and Prencipe, 1980).

The exact mechanism by which the aqueous extract of *H.nilagirica* stem bark exerted its cytotoxic effect on DLA is unknown. It is plausible that the HNA may have utilized multiple putative mechanisms to bring effective tumor cell kill and increase tumor free survivors in the present study. The HNA may have increased oxidative stress that may have killed tumor cells more effectively. This is supported by the observation that HNA increase the lipid peroxidation in the tumor cells and reduced antioxidants like GSH and activities of GST, catalase and SOD enzymes significantly. The DNA damage is one of the important events in cell kill and HNA induced DNA damage in the form of micronuclei may have played a crucial role in the tumor cell killing and increased tumor free survivors. The other important event in cell killing is HNA induced apoptotic cell death as it induced apoptosis in the tumor cells in a time dependent manner. Although no attempt has been made to investigate the molecular mechanisms however, the results of apoptosis studies indicate that HNA may have utilized molecular mechanism to induce cell killing of DLA cells. The HNA may have suppressed the transcription of NF-κB, COX-II and Nrf2 that are overexpressed in the tumors (Sobolewski et al., 2010; Lu and Stark, 2015; Choi and Kwak, 2016) thereby bringing effective cell kill in the present study. The HNA might be able to suppress the expression of Bcl_{xL}, survivin, IAP, and c-FLIP, or upregulate the levels P53 and Bax leading to the increase mortality of cancer cells.

Conclusions

The present study clearly demonstrates that HNA was more cytotoxic and increased the tumor free survivors beyond 120 days. The cell killing effect of HNA may be due to the reduction in

GSH and activities of GST, catalase and SOD accompanied by increased lipid peroxidation. The increased micornuclei formation and apoptosis also played a major role in the cell killing which may be due to reduced antioxidant status. The HNA may have also inhibited the transcriptional activation of NF-κB, COX-II, Nrf2, Bcl_{xL}, survivin, IAP, and c-FLIP and upregulation of p53 and Bax proteins.

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Table 1: Acute toxxcity of different extracts of *Helicia nilagirica* in Swiss albino mice after intraperitoneal administration. The LD50 was determined using probit analysis.

Extract	Dose (mg/kg body weight)	Survival (%)	LD_{50} (mg/kg)
Chloroform	500	100	2000
	1000	100	
	1500	75	
	2000	50	
Ethanol	500	100	>2000
	1000	100	
	1500	100	
	2000	100	
Aqueous	500	100	750
	1000	0	
	1500	0	
	2000	0	

n=10.

Table 2: Change in the body weight of Dalton's lymphoma bearing Swiss albino mice administered with ethanol or aqueous extract of *Helicia nilagirica*. The data are expressed as Mean ± standard error of the mean (SEM).

Treatment	Dose (mg/kg b.wt.)	Post tumor transplanted time (Days)						
		0	**3**	**9**	**15**	**21**	**27**	**30**
SPS	**0**	29.52±0.93	29.41±0.93	31.53±1.23	37.39±0.67	--	--	--
Ethanol	**50**	27.42±1.05	27.78±1.46	29.12±2.31	29.78±2.85	32.91±2.46	--	--
	75	28.33±0.83	26.42±0.39	29.4±0.53	34.09±0.91	35.74±2.26	--	--
	100	27.07±0.82	27.35±0.95	28.08±1.08	27.58±1.89	27.44±2.63	26.43±0	--
	125	24±0.89	25.23±0.61	27.53±1.59	30.83±1.02	35.62±0.84	34.44±0.24	--
	150	23.52±0.77	24.23±0.27	25.22±0.80	26.58±0.88	31.1±1.19	34.76±2.11	34.28±1.77
	175	23.47±0.66	23.73±0.55	26.8±0.80	29.52±1.40	31.99±1.81	33.96±0.79	34.46±1.15
Aqueous	**50**	25.16±1.38	24.98±0.77	27.77±1.38	33.65±1.53	37.1±3.7	--	--
	75	25.41±1.19	25.11±1.33	24.28±0.79	23.88±1.61	28.4±3.69	24	24.8
	100	23.78±1.82	24.41±2.04	24.91±3.06	26.72±2.74	27.02±2.82	27.55±1.84	27.6±2.70
	125	29.68±0.92	28.74±0.73	29.28±1.28	30.41±1.60	31.24±2.89	38.25±9.15	37.5±8.8
	150	30.48±1.76	29.21±1.45	28.65±2.14	28.68±1.62	29.66±1.19	31.5±1.22	31.8±1.21
	175	29.67±0.64	28.84±0.81	29.04±0.72	28.77±0.84	30.58±1.10	32.3±1.21	31.2±0.95

n=10.

Table 3: Alteratiuon in the Survival of Dalton's lymphomas ascites bearing mice treated with various doses of *Helicia nilagirica* intraperitoneally.

Post tumor transplant time (day)	Survival (%)												
	SPS (Control)	Ethanol extract						Aqueous extract					
	Dose (mg/kg)												
	0	50	75	100	125	150	175	50	75	100	125	150	175
0	100	100	100	100	100	100	100	100	100	100	100	100	100
20	0	50	80	100	100	100	100	83.33	100	100	100	100	100
24	0	0	20	40	60	100	100	16.66	50	66.66	71.4	83.33	100
28	0	0	0	20	20	50	80	0	16.66	66.66	28.5	66.66	71.42
30	0	0	0	0	0	30	60	0	0	50	28.5	50	60
34	0	0	0	0	0	0	20	0	0	16.66	28.5	50	60
38	0	0	0	0	0	0	20	0	0	16.66	28.5	33.33	50
40	0	0	0	0	0	0	0	0	0	16.66	28.5	33.33	50
44	0	0	0	0	0	0	0	0	0	0	28.5	33.33	32.85
48	0	0	0	0	0	0	0	0	0	0	28.5	33.33	32.85
50	0	0	0	0	0	0	0	0	0	0	16.66	33.33	32.85
54	0	0	0	0	0	0	0	0	0	0	0	16.66	28.57
58	0	0	0	0	0	0	0	0	0	0	0	16.66	28.57
60	0	0	0	0	0	0	0	0	0	0	0	0	16.66

n=10.

Table 4: Alteration in the survival time of Dalton's lymphoma ascites bearing mice treated with different extracts of *Helicia nilagirica*.

Treatment	Dose (mg/kg body weight)	MST	AST	IMLS (%)	IALS (%)
SPS (Control)	0	20.5	20.66	0	0
Ethanol extract	50	24.5*	20.67*	19.51*	0.03*
	75	33*	22*	60.97*	6.48*
	100	38*	25.2*	85.36*	21.97*
	125	38.5*	26*	87.80*	25.84*
	150	44.5*	29*	117.07*	40.36*
	175	44.5*	32*	117.07*	54.88*
Aqueous extract	50	28.5*	21.33*	39.02*	3.25*
	75	32*	24.16*	56.09*	16.97*
	100	80*	42.5*	290.24*	105.71*
	125	79*	38*	285.36*	83.93*
	150	75*	55.33*	265.85*	167.82*
	175	86*	55*	319.51*	166.21*

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

n=10

MST:Mean survival time

AST:Average survival time

IMLS:Increase in mean life span

IALS:increase in average life span

Table 5: Frequency of micronuclei in Dalton's lymphoma ascites bearing mice treated with 175 mg/kg b.wt. aqueous extract of *Helicia nilagirica* (HNA) or 0.5 mg/kg b. wt. doxorubucin (DOX) at different post treatment time.

Post treatment time (hour)	Frequency of micronuclei (Mean ± SEM)								
	SPS			HNA			DOX		
	One MN	Two MN	Total	One MN	Two MN	Total	One MN	Two MN	Total
12	8.2±0.64	2.6±0.45	10.8	66.6±1.48*	14.2±0.85*	80.8	71.1±0.9*	22.5±0.5*	93.6
24	11.5±0.5	3.3±0.61	13.8	83.8±1.45*	20.7±0.80*	104.5	91.7±1.4*3	24.6±1.01*	116.3
48	11.8±0.61	2.6±0.45	14.4	75.8±1.35*	20.1±0.82*	92.1	76.4±1.55*	24.3±1.14*	95.7

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

Table 6: Induction of apoptosis and necrosis in Dalton's lymphoma ascites bearing mice treated with 175 mg/kg.b.wt. aqueous extract of *Helicia nilagirica* (HNA) or 0.5 mg/kg b.wt. doxorubucin (DOX) at different post treatment time.

Post treatment time (hour)	Apoptosis & necrosis (% ±SEM)					
	SPS		HNA		DOX	
	Apoptotic	Necrotic	Apoptotic	Necrotic	Apoptotic	Necrotic
2	1.76±0.09	1.38±0.08	3.62±0.13*	2.68±0.13*	5.96±0.15*	4.48±0.13*
12	2.56±0.16	1.66±0.09	9.62±0.15*	7.5±0.15*	13.72±0.14*	10.48±0.16*
24	3.48±0.13	2.26±0.09	11.7±0.1*	10.44±0.15*	16.02±0.12*	11.34±0.09*
36	4.36±0.12	3.22±0.08	12.28±0.10*	9.64±0.12*	16.9±0.07*	11.4±0.13*

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

Table7: Alteration in the glutathione (GSH) contents of mice bearing Dalton's lymphoma treated with 175 mg/kg.b.wt. aqueous *Helicia nilagirica* extract (HNA) or 0.5 mg/kg b.wt. doxorubicin (DOX).

Post Treatment Time (h)	Glutathione (GSH) (µm/mg protein), Mean ± SEM		
	SPS	HNA	DOX
2	5.09±0.15	2.92±0.29*	2.39±0.09*
4	5.18±0.17	3.86±0.37*	2.36±0.18*
6	5.25±0.14	3.67±0.08*	2.74±0.14*
8	5.09±0.07	2.41±0.07*	2.23±0.09*
12	5.66±0.07	2.94±0.08*	2.34±0.05*
24	5.84±0.05	3.33±0.09*	2.58±0.06*

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

Table 8: Alteration in the glutathione-S-transferase (GST) activity of Mice bearing Dalton's lymphoma treated with 175 mg/kg.b.wt. aqueous *Helicia nilagirica* extract (HNA) or 0.5 mg/kg b.wt. doxorubicin (DOX).

Post Treatment Time (h)	Glutathione-S-transferase (GST) (U/mg protein), Mean ± SEM		
	SPS	HNA	DOX
2	0.054±0.001	0.041±005	0.041±011
4	0.076±0.008	0.032±004*	0.033±007*
6	0.070±0.012	0.035±003*	0.033±003*
8	0.058±0.004	0.033±007*	0.023±002*
12	0.072±0.007	0.043±003*	0.035±001*
24	0.065±0.008	0.038±004*	0.027±002*

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

Table 9: Alteration in the catalase activity of mice bearing Dalton's lymphoma treated with 175 mg/kg.b.wt. aqueous *Helicia nilagirica* extract (HNA) or 0.5 mg/kg b.wt. doxorubicin (DOX).

Post Treatment Time (h)	Catalase (CAT) (U/mg protein), Mean ± SEM		
	SPS	HNA	DOX
2	24.49±0.49	19.57±1.12*	15.49±0.31*
4	22.61±1.26	19.05±0.37*	17.09±0.28*
6	23.47±0.40	16.47±0.37*	13.73±1.72*
8	23.51±0.63	14.32±1.65*	13.81±0.84*
12	23.99±0.56	14.49±0.40*	11.18±1.28*
24	22.92±1.80	11.74±0.51*	10.55±0.39*

p<0.05 when treatment are compared with concurrent control (SPS) group.

No symbol= no significance.

n=10.

Table 10: Alteration in the superoxide dismutase (SOD) activity in mice bearing Dalton's lymphoma treated with 175 mg/kg.b.wt. aqueous *Helicia nilagirica* extract (HNA) or 0.5 mg/kg b.wt. doxorubicin (DOX).

Post Treatment Time (h)	Superoxide dismutase (SOD) (U/mg protein), Mean ± SEM		
	SPS	HNA	DOX
2	1.91±0.006	0.87±0.07*	0.76±0.05*
4	1.93±0.002	1.35±0.03*	0.88±0.04*
6	1.94±0.001	1.50±0.01*	1.18±0.14*
8	1.92±0.009	1.08±0.02*	0.79±0.08*
12	1.93±0.002	1.19±0.04*	1.01±0.05*
24	1.94±0.003	1.11±0.06*	0.58±0.06*

p<0.05 when treatment are compared with concurrent control (SPS) group.

No symbol= no significance.

n=10

Table 11: Alterations in the lipid peroxidation level in mice bearing Dalton's lymphoma treated with 175 mg/kg.b.wt. aqueous *Helicia nilagirica* extract (HNA) or 0.5 mg/kg b.wt. doxorubicin (DOX).

Post Treatment Time (h)	Lipid peroxidation (MDA) Mean ± SEM, (nmol /mg protein)		
	SPS	HNA	DOX
2	3.87±0.05	4.16±0.01*	5.06±0.02*
4	3.55±0.02	4.23±0.03*	5.53±0.02*
6	3.69±0.08	4.45±0.06*	5.82±0.14*
8	3.99±0.03	4.65±0.02*	6.47±0.62*
12	3.91±0.06	4.97±0.01*	6.51±0.10*
24	3.83±0.06	5.22±0.02*	7.03±0.02*

p<0.05 when treatment are compared with concurrent control (SPS) group.

No symbol= no significance.

n=10

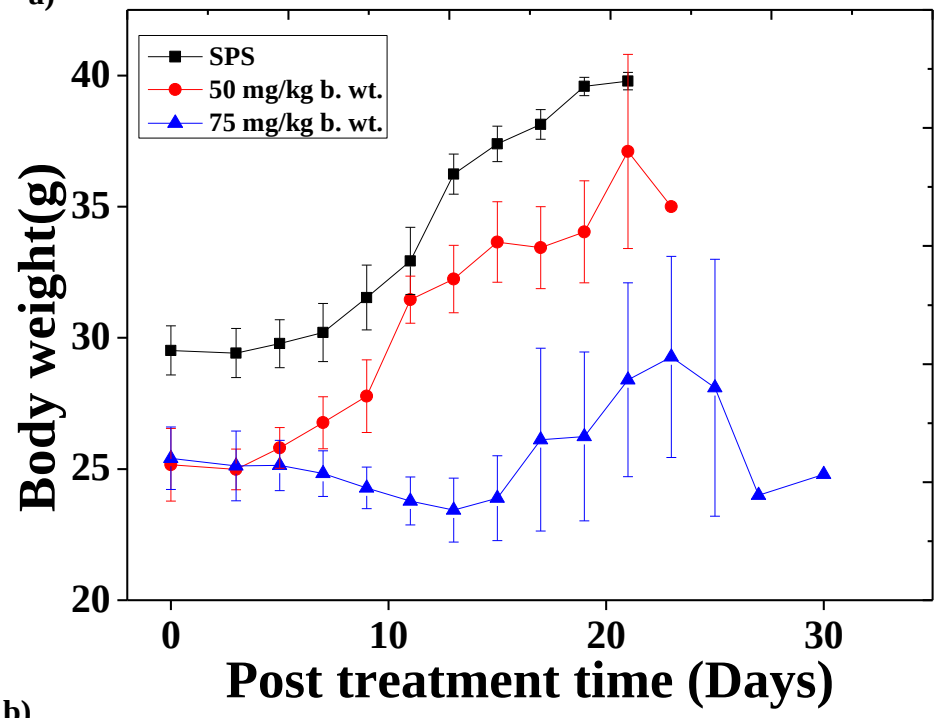
Table 12: Effect of 175 mg/kg.b.wt. aqueous *Helicia nilagirica* extract (HNA) or 0.5 mg/kg b.wt. doxorubicin (DOX) on the liver and kidney function tests of Dalton's lymphoma bearing mice.

Treatment	Dose (mg/kg b.wt.)	Post treatment time (h)	Liver function test		Kidney function test	
			AST (U/L)	ALT (U/L)	Creatinine (mg/dl)	Uric acid (mg/dl)
Control	0	2	120.43±0.29	19.01±0.32	0.4±0.05	2.75±0.02
		4	121.26±0.46	19.36±0.10	0.5±0.05	2.93±0.08
		6	128.44±0.63	19.16±0.16	0.4±0.05	3.01±0.06
		8	127.77±1.42	18.13±0.63	0.5±0.05	3.09±0.06
		12	133.80±0.47	19.27±0.33	0.53±0.08	2.86±0.09
		24	135.91±0.49	19.37±0.64	0.6±0.05	2.23±0.06
HNA	175	2	120.31±0.64	20.51±0.45	0.6±0.05	3.08±0.008
		4	122.54±1.05	21.76±0.24	0.61±0.02	3.25±0.01
		6	131.07±0.36	20.41±0.50	0.54±0.02	3.13±0.07
		8	128.73±3.33	20.51±0.38*	0.63±0.08	3.26±0.01
		12	134.50±0.33	20.25±0.22	0.69±0.05	4.05±0.02*
		24	134.82±0.16	21.69±0.72	0.6±0.05	2.94±0.27
Dox	0.5	2	121.78±0.22	20.02±0.24	0.53±0.08	3.15±0.01
		4	125.71±0.59	20.38±0.87*	0.46±0.03	3.28±0.01
		6	131.22±0.46	20.62±0.14	0.46±0.0.03	3.58±0.20
		8	131.21±1.36	21.14±0.26*	0.57±0.01	3.30±0.01
		12	135.58±0.32	21.12±0.11	0.6±0.05	3.16±0.01*
		24	135.36±0.85	21.45±0.11	0.8±0.05	3.2±0.10*

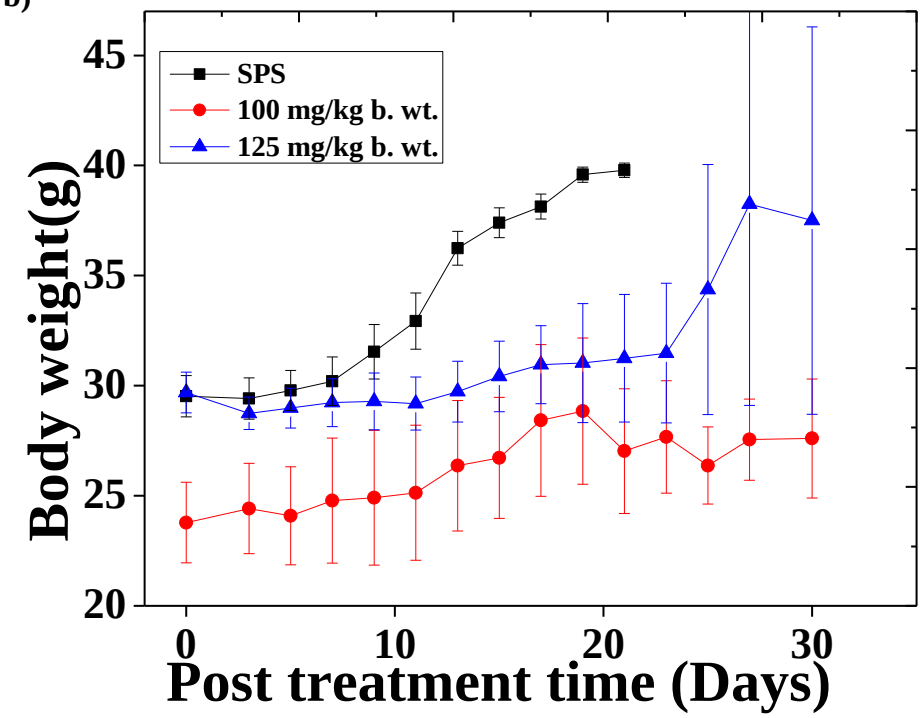
**p<0.05, when treatment groups are compared to spontaneous control group.*

No symbol= no significance. n=10.

a)


SPS
50 mg/kg b. wt.
75 mg/kg b. wt.
Body weight(g)
Post treatment time (Days)
20
25
30
35
40
0
10
20
30

b)


SPS
100 mg/kg b. wt.
125 mg/kg b. wt.
Body weight(g)
Post treatment time (Days)
20
25
30
35
40
45
0
10
20
30

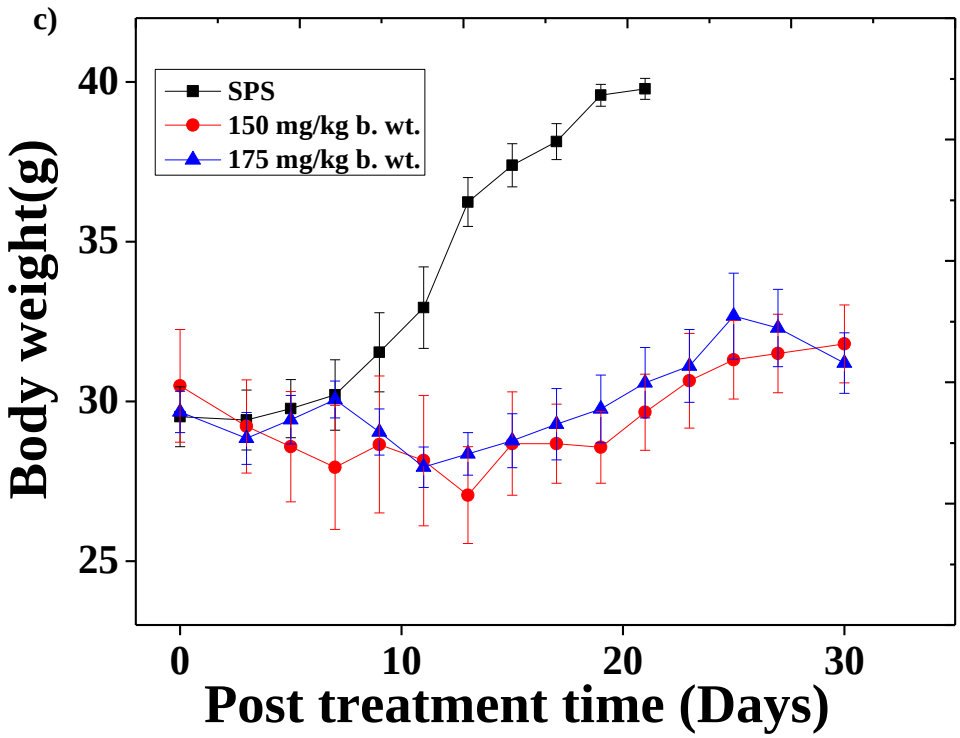
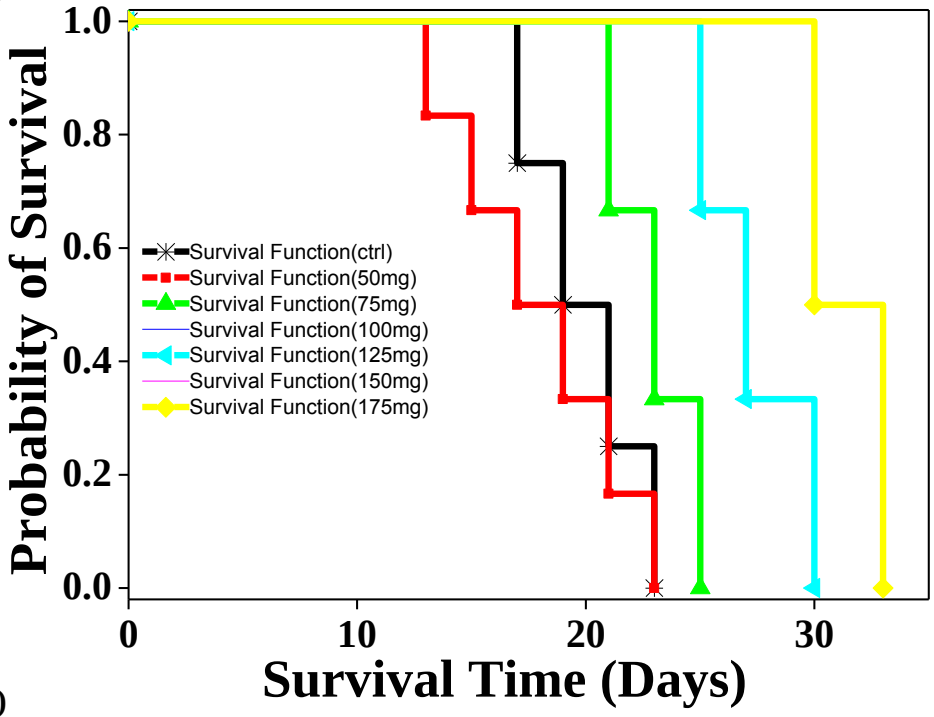


Fig 1: Change in body weight of Dalton's lymphoma bearing Swiss albino mice after treatment with different concentrations of the aqueous extract of *Helicia nilagirica.* The data are expressed as Mean ±SEM , n=10.

a)



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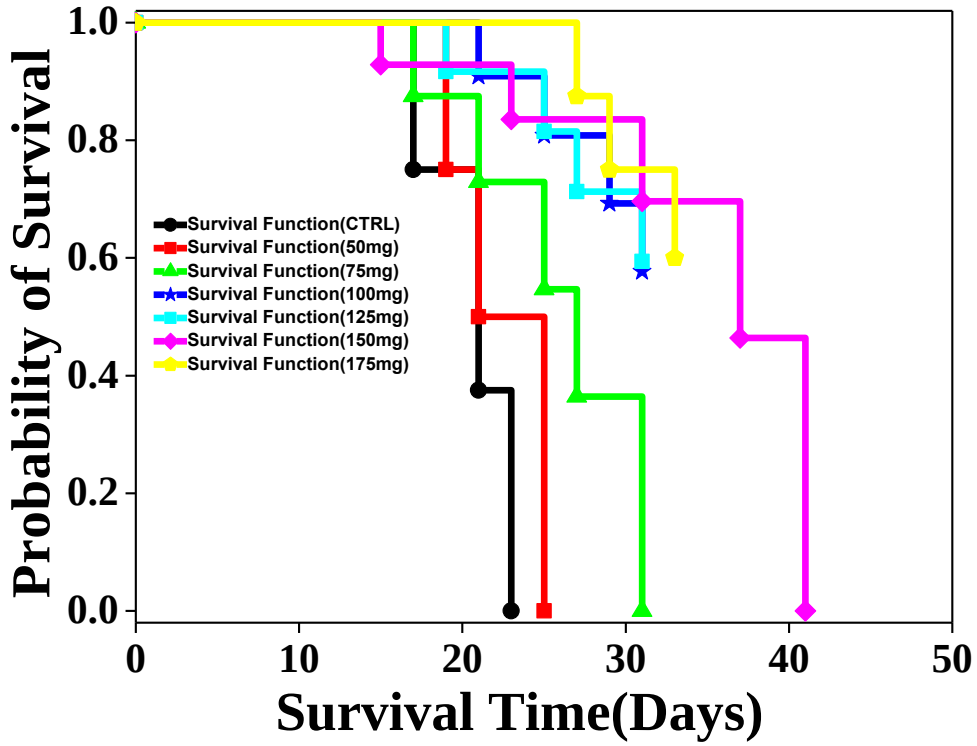

Fig 2: Effect on the survivality of Dalton's lymphoma ascites bearing mice after 9 days consecutive treatment with different doses of a) ethanol and b) aqueous extracts of *Helicia nilagirica in vivo*. The result indicates survival function , n=10.

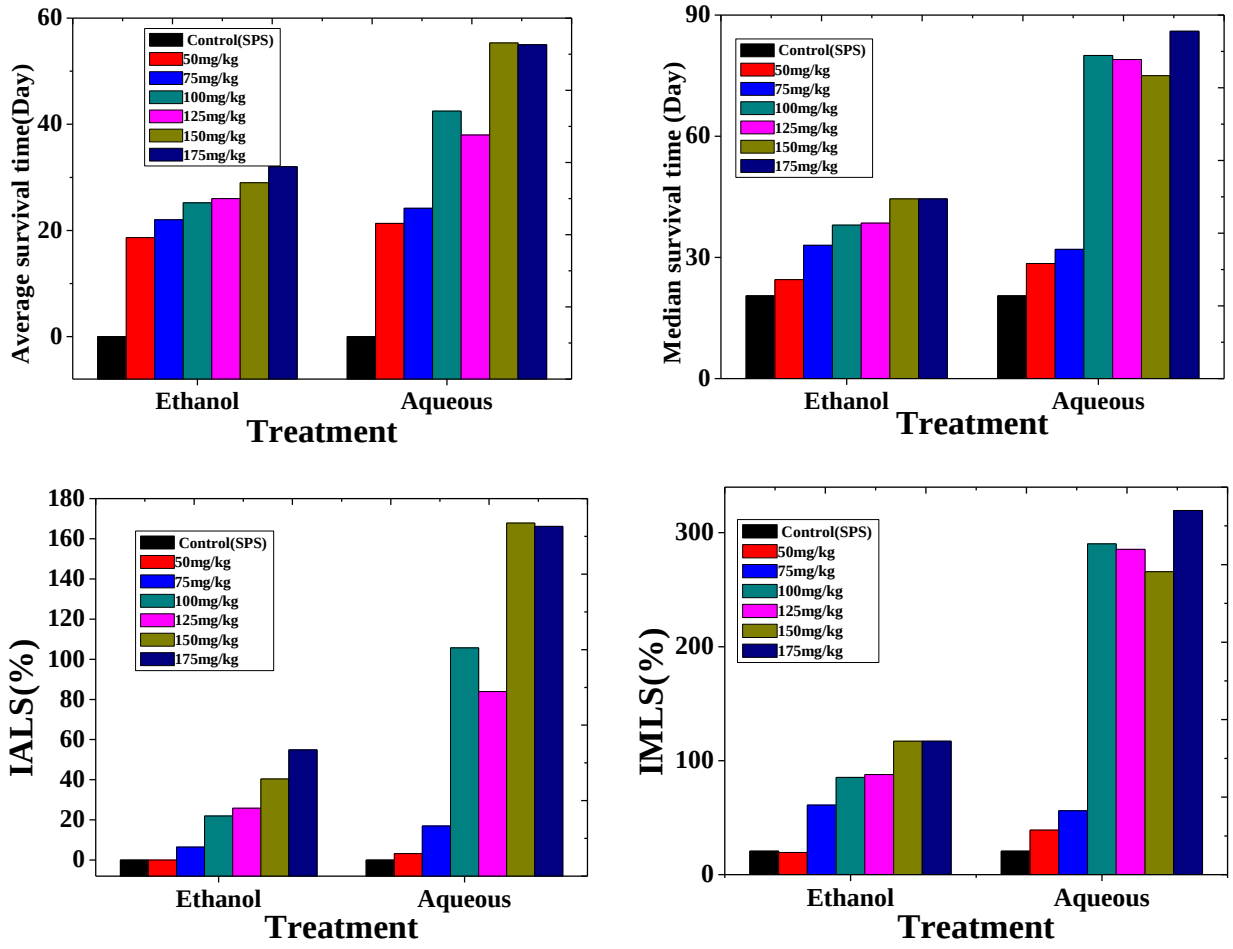

Fig 3: Effect of extract of *Helicia nilagirica* in Dalton's lymphoma ascites bearing mice on the tumor response assessment based on average survival time (AST), median survival time (MST), mean life span (% IMLS) and increase in average life span (% IALS) .*p<0.05, when treatment groups are compared to spontaneous control group.

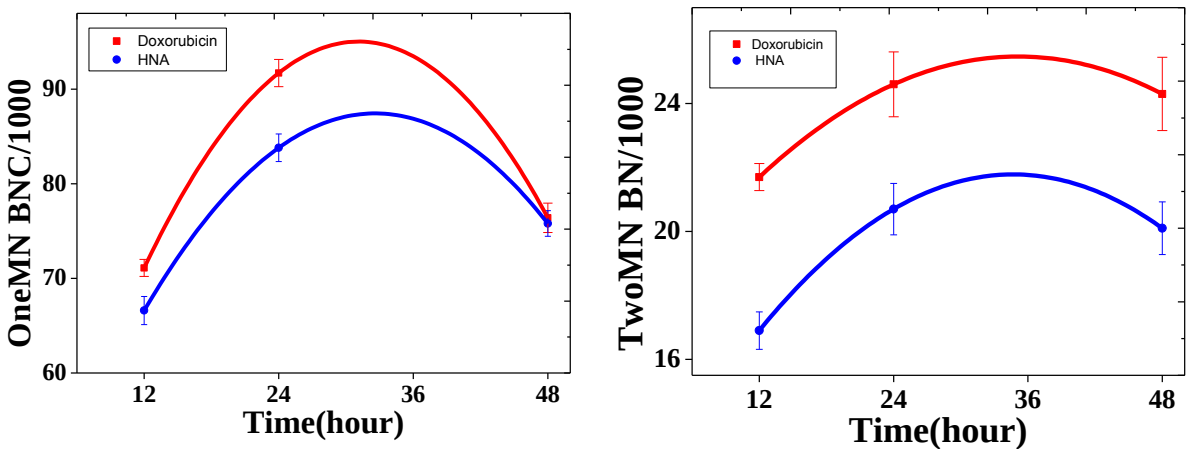

Fig 4: Induction of micronuclei in Dalton's lymphoma ascites bearing mice treated with 175mg/kg.b.wt. aqueous extract of *Helicia nilagirica* (HNA) and 0.5mg/kg.b.wt. doxorubucin (DOX) at different post treatment time. One and two micronuclei in binucleated cells. The results were determined as frequency of Micronuclei/1000 cells and expressed as the Mean ± SEM, n=10.

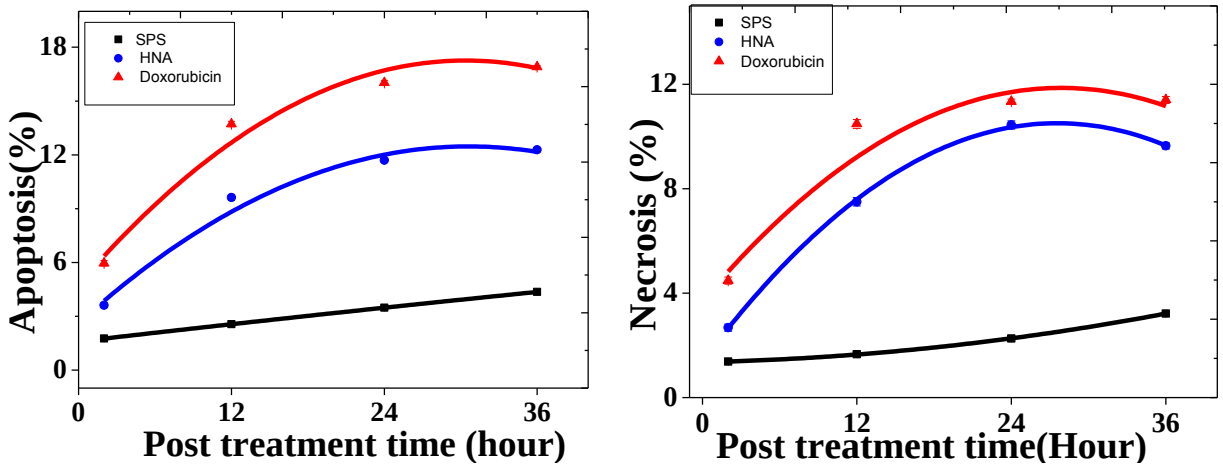

Fig 5: Induction of apoptosis and necrosis in Dalton's lymphoma ascites bearing mice treated with 175 mg/kg.b.wt. aqueous extract of *Helicia nilagirica* (HNA) and 0.5mg/kg.b.wt. doxorubucin (DOX) at different post treatment time. The results determined as percent index and expressed as the Mean ± SEM, n=10.

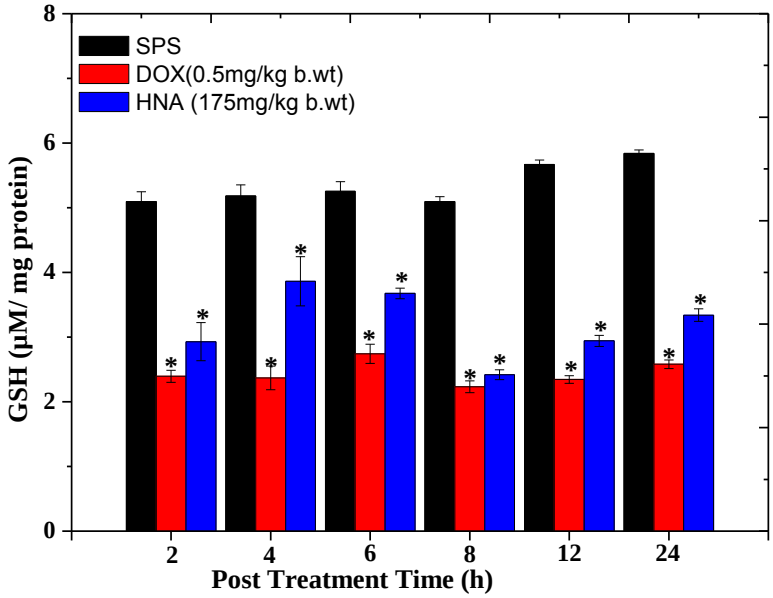

Fig 6: Alteration in the glutathione content of mice bearing Dalton's lymphoma ascites treated with 175 mg/ kg. body weight of *Helicia nilagirica* extract (HNA) and 0.5mg /kg b.wt. doxorubicin (DOX). The results expressed as the Mean ± SEM, n=10. **p<0.05 when treatment are compared with concurrent control (SPS) group. No symbol= no significance.*

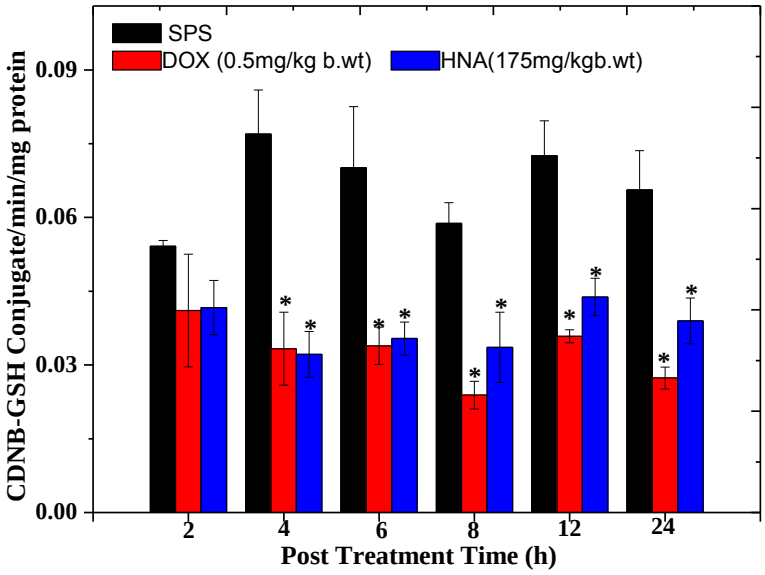

Fig 7: Alteration in the glutathione –s – transferase content of mice bearing Dalton's lymphoma ascites treated with 175 mg/ kg. body weight of *Helicia nilagirica* extract (HNA) and 0.5mg /kg b.wt. doxorubicin (DOX). The results expressed as the Mean ± SEM, n=10. **p<0.05 when treatment are compared with concurrent control (SPS) group. No symbol= no significance.*

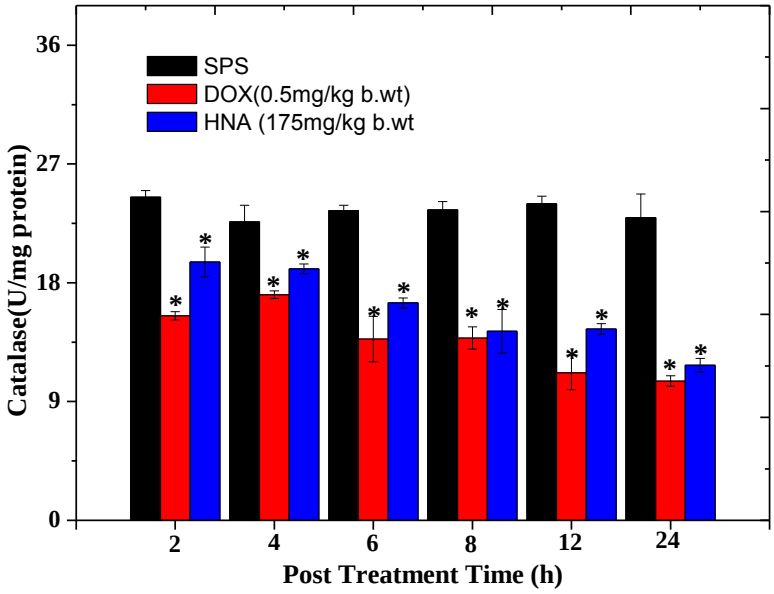

Fig 8: Alteration in the catalase activity of mice bearing Dalton's lymphoma ascites treated with 175 mg/ kg. body weight of *Helicia nilagirica* extract (HNA) and 0.5mg /kg b.wt. doxorubicin (DOX). The results expressed as the Mean ± SEM, n=10. **p<0.05 when treatment are compared with concurrent control (SPS) group. No symbol= no significance.*

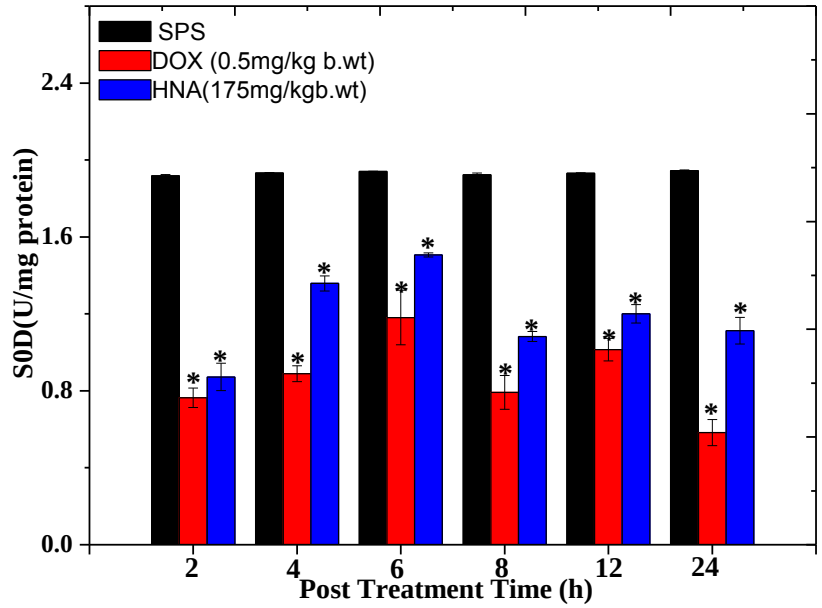

Fig 9: Alteration in the superoxide dismutase activity of mice bearing Dalton's lymphoma ascites treated with 175 mg/ kg. body weight of *Helicia nilagirica* extract (HNA) and 0.5mg /kg b.wt. doxorubicin (DOX). The results expressed as the Mean ± SEM, n=10. **p<0.05 when treatment are compared with concurrent control (SPS) group. No symbol= no significance.*

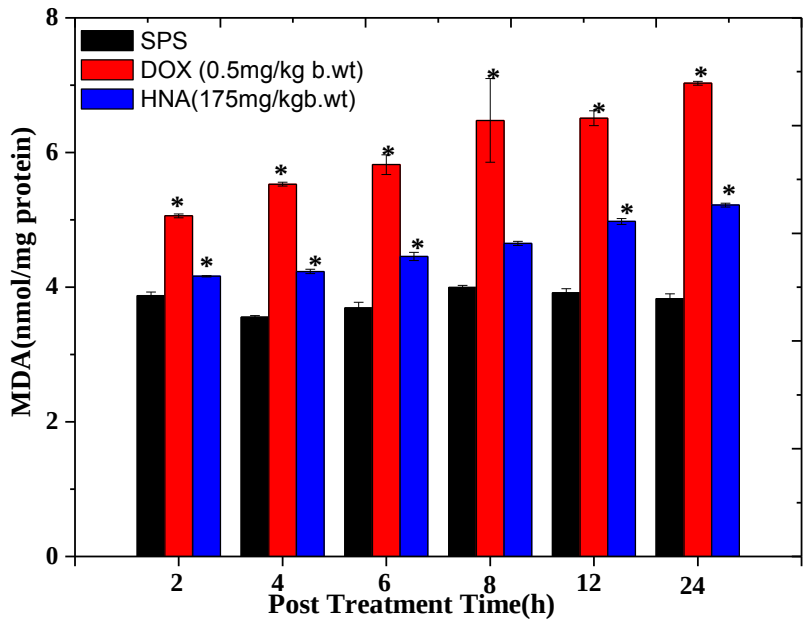

Fig 10: Alterations in the lipid peroxidation (LOO) level in the Dalton's lymphoma ascites bearing mice treated with 175 mg/ kg. body weight of *Helicia nilagirica* extract (HNA) and 0.5mg/kg b.wt. Doxorubicin (DOX). The results present the Mean ± SEM, n=10.

p<0.05 when treatment are compared with concurrent control (SPS) group.

No symbol= no significance.

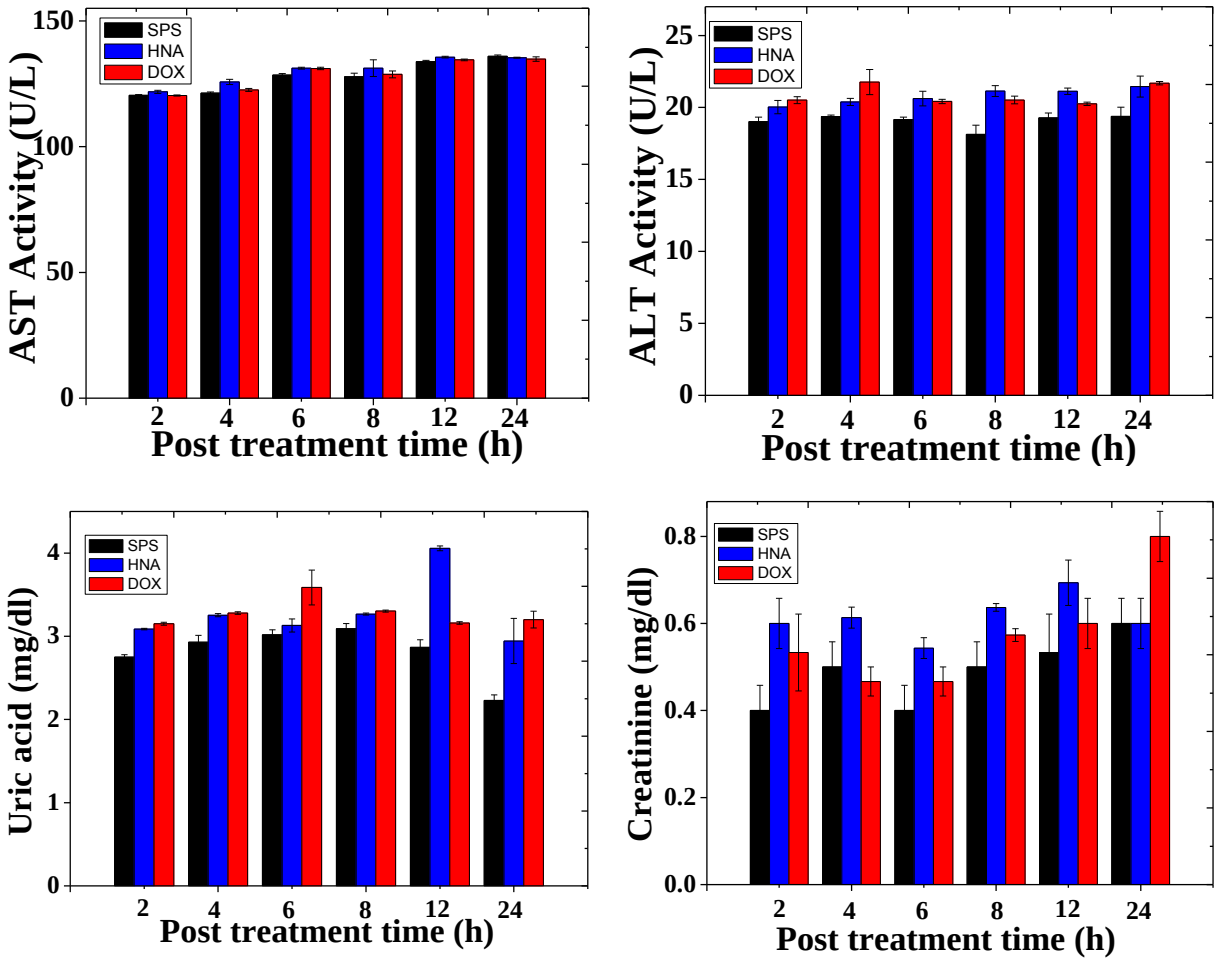

Fig 11: Effect on the liver and kidney function on Dalton's lymphoma ascites bearing mice when treated with 175 mg/kg.b.wt of aqueous extract of Helicia nilagirica (HNA) and 0.5 mg/kg.b.wt of DOX. The results present the Mean ± SEM, n=10. P< 0.05.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Cancer can be defined as a multifactorial disease in which a group of abnormal cells grow uncontrollably defying all the normal rules and regulatory mechanisms of cell growth and division or it can be due to changes or mutation into the cell genome which disrupt the cellular balance leading to their uncontrolled division and subsequently form cancer (Hejmadi, 2010). Paleopathologic findings have indicated that tumors already existed in animals in prehistoric times, long before men evolved on the Earth. The earliest written description of cancer, a breast cancer, was found in the Edwin Smith Papyrus that was written approximately 3000 BC. A set of six characteristic properties of cancers has been proposed by Hanahan and Weinberg that are called as the 'hallmarks of cancer' such as self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

Cancer is one of the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases in 2012 (Farley *et al.* 2012) and it is the second leading cause of death globally, and was responsible for 8.8 million deaths in 2015. In 2017, 1,688,780 new cancer cases and 600,920 cancer deaths are projected to occur in the United States (Siegel *et al*. 2017). Tobacco use is the most important risk factor for cancer and is responsible for approximately 22% of cancer deaths worldwide (GBD 2015). Cancer causing infections, such as hepatitis and human papilloma virus (HPV), are responsible for up to 25% of cancer cases in low- and middle-income countries (Plummer *et al.* 2012).

Cancer can be treated by a different range of therapies. Surgery, irradiation or drugs can be employed, or in combination of all these is also commonly used to treat cancer. The choice of therapy depends on the type of cancer; surgery or radiation treatment is chosen for localized

cancers. Surgery is the oldest treatment for cancer and forms the mainstay of treatment of solid tumors till today. It is most effective in the treatment of localized primary tumor and associated regional lymphatic. The cancer treatment received fillip when Wilhelm Conrad Röntgen discovery of X-rays in Germany in 1895 followed by the discovery of radioactivity by Henri Becquerel in 1896. The discovery of X-rays led to the birth of a new branch of cancer treatment the Radiotherapy. The radiation randomly affects the molecules of the cell where the main target is the deoxyribonucleic acid (DNA) which can result in single- and double-strand breaks (DSBs) in the sugar-phosphate backbone of the DNA molecule (Dizdaroglu, 1992; Lomax *et al*., 2013)., The damage caused to the cellular and nuclear membranes and other organelles by ionizing radiation plays an important role in cell death. The term Chemotherapy was coined by Paul Ehrlich while he was working on the treatment of infectious diseases by using antibiotics. There are more than 100 different types of chemotherapeutic drugs for the treatment of different types of cancers, which are used either alone or in combination (Colvin 2003). The chemotherapeutic drugs can be classified according to their mechanism of action such as the alkylating agents, antimetabolites, plant alkaloids, antitumor antibiotics, hormonal agents and targeted cancer therapies.

However, cancer therapy have their own limitations as they also damage the normal dividing cells especially the rapidly regenerating tissues, such as hair follicles and can also lead to drug resistance as well induction second malignancies, (Wu *et al*. 2008; Morton et al., 2014). This indicates that new utilize alternative concepts or approaches are required to treat the cancer.

AIM OF THE STUDY

Helicia nilagirica Bedd. (Family: Proteaceae) locally known as Pasaltakaza is a medium-sized tree, which grows up to 12 meters high. It is widely distributed in Sri Lanka, southern

India, Burma (Myanmar), Indochina, Japan, Taiwan, and Thailand. It is also found scattered in lowland to montane rain forests, up to 2,000-3,350 m altitude. Some species prefer habitats along streams but other species are found on hilltops or ridges (Khamyong et al., 2004). This tree has been used as a folk medicine since time immemorial in Mizoram, India by the Mizo tribe. Its decoction prepared by boiling the leaves or bark is used to treat various stomach ailments including peptic ulcers, indigestion, mouth ulcer, urinary tract infection and gynaecological disorders. It is also used in scabies and other skin diseases (Sawmliana, 2003). The fruits of *H. nilagirica* have been used as a medicine to cure cough and cold in Sikkim (Chauhan, 2001). The scientific evaluation regarding its medicinal or other properties is scarce Except that, it has been shown to possess anti-inflammatory activity in rat cotton pellet granuloma model (Lalawmpuii et al. 2014). This indicates the need to systematically evaluate its anticancer properties. The main objective of present investigation is to evaluate the anticancer activity of *Helicia nilagirica* extracts *in vivo* and *in vitro.*

CHAPTER 1

Chapter 1 gives an introduction on the subject of cancer, history, characteristics, epidemiology, causes and different treatment modalities of cancer. It also gives a brief description about the aims and objectives and scope of the thesis.

CHAPTER 2

This chapter gives an account on the phytochemical and physicochemical characteristics of *Helicia nilagirica*. Firstly, the mature non-infected stem bark was collected, cleaned, shade dried and powdered. Then it was subjected to extraction with different solvent with increasing polarity using petroleum ether, chloroform, ethanol and distilled water in a Soxhlet apparatus. The different extracts were dried and kept at -80^{o}C for further use. The phytochemical analysis

showed the presence of flavonoids, tannins, terpenoids, cardiac glycosides in the chloroform and ethanol extracts whereas saponin, tannins and cardiac glycosides were present in the aqueous extract. The TLC study also showed the presence of different phytochemicals as indicated by the different Rf values in different solvent systems.

CHAPTER 3

Chapter 3 gives an account of free radical scavenging activity of the chloroform, ethanol and aqueous extracts of *H. nilagirica*. The free radicals which are produced during the normal respiration could give rise to chronic diseases if produced in excess so it is necessary to control their level to maintain the normal homeostasis of the body. The scavenging activity of the extracts was evaluated against DPPH, superoxide anion, hydroxyl, nitric oxide and ABTS radicals. The total reducing power, total phenols and flavonoids were also estimated. The *H. nilagirica* extracts were able to scavenge all the free radicals effectively in a concentration dependent manner. The total reducing power, total phenol and total flavonoid contents also increased with increasing concentration of the extracts. These findings indicate that *H. nilagirica* could be a good source of antioxidant as it has the ability to scavenge different free radicals. The presence of flavonoids and polyphenols may be responsible for the neutralization of various free radicals.

CHAPTER 4

In this chapter the cytotoxicity of aqueous extract of *H. nilagirica* was evaluated on HeLa and V79 cell lines. MTT assay was done on HeLa and V79 cell lines to study the cytotoxicity and the optimum exposure time where the cytotoxicity increased with increasing concentration of the extract. The employment of clonogenic assay on HeLa cells confirmed the cytotoxicity of the extract by decreasing the clonogenic potential of the cancer cells with increasing

concentration. The level of GSH, GST, catalase and SOD also decreased with increasing concentration of the extract and increasing assay time. This alleviation could indicate an increase in oxidative stress leading to decline in the cancer cell survival.

CHAPTER 5

Chapter 5 gives an account on the acute toxicity of the three extracts which were administered intraperitoneally. The ethanol extract was found to be non-toxic up to 2g/kg b .wt. whereas the LD50 of the chloroform and aqueous extracts were found to be 2g/kg b .wt. and 0.75g/kg b. wt. , respectively. The anticancer activity of the extracts was evaluated and aqueous extract possessed the highest anticancer activity as shown by highest tumor free survivors. Treatment of DLA mice with 175 mg/kg b. wt. led to an increase in the tumor free survivors of 16.6 % beyond 120 days with an AST of 55 days and MST of 86 days. This dose was chosen as an optimum dose for further investigations. The study of micronucleus and apoptosis by 175mg/kg .b.wt of HNA showed a time dependent increase in micronuclei formation up to 24 h post treatment, whereas apoptotic index elevated up to 36 h post treatment. The level of GSH, GST, catalase and SOD declined significantly with increasing post treatment times, whereas the level of lipid peroxidation increased with increasing post treatment time. Toxicology studies on the level of AST, ALT, creatinine and uric acid showed no significant changes which indicated that the extract did not cause any severe damage to liver or kidney. The cytotoxicity towards the cancer cells i.e. Dalton's lymphoma could be due to the decline in the GSH, GST, catalase and SOD level and elevation of lipid peroxidation and DNA damage.

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BOOK CHAPTER

Analysis of phytochemical constituents and assessment of the antioxidant activity of selected plants of Mizoram. Proceedings of The Mizoram Science Congress 2016. Allied Publishers, New Delhi, India. ISBN: 978-93-85926-49-5.

SEMINARS PRESENTED

1. Presented a research paper in the" International Conference on Radiation biology" held on Nov 11-13, 2014 at NASC Complex, Dev Prakash Shastri Marg, Pusa, New Delhi, India.

2. Presented a research paper in the "Mizoram Science Congress 2016" held on 13th-14th Oct at Mizoram University, Aizawl.

3. Oral presentation in the National Seminar on "Biodiversity, Conservation and Utilization of Natural Resources with reference to Northeast India" held on 30th-31st March 2017, organized by Deparment of Botany at Mizoram University, Aizawl.

SEMINARS PARTICIPATED

1. Participated in "Five Days Workshop on Instrumentation in Science" from 6th-10th Aug 2012 held at Mizoram University sponsored by UGC.

2. Attended "One Day Seminar on Animal Welfare and Ethics" on 6th June 2013 at Hotel Regency, Aizawl organized by Department of Pharmacy, RIPANS in association with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

3. Participated in the "International Conference cum Exhibition on Drugs Discovery and Development from Natural Resources" organized by the Department of Pharmacy, RIPANS under the sponsorship of the Ministry of Health and Family Welfare, Govt. of India on 5th-6th Feb. 2014 at Art and culture Auditorium, Berawtlang, Aizawl.

4. Participated in the National conference on "Advances in Cancer Genomics" held on 3th -31st May 2014 organized by Mizoram State Cancer Institute and Department of Biotechnology

Mizoram University sponsored by Department of Biotechnology, New Delhi coordinated by Indian Institute of Technology, Guwahati.

5. Participated in the workshop on "Exploring the Cancer genomics" held during 22nd-27th Feb. 2016 organized by Department of Biotechnology, Mizoram University sponsored by State Biotech- Hub facility, Department of Biotechnology (DBT), New Delhi.

6. Attended workshop on "Mechanisms of Adaptation in the Temporal Environment" on 23rd May 2017 at Mizoram University organized by Department of Zoology, Mizoram University, Aizawl.

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EVALUATION OF THE CYTOTOXIC EFFECTS OF HELICIA NILAGIRICA BEDD IN VITRO

Zoology

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ABSTRACT

The cancer is a second largest killer disease and despite numerous advances made in the treatment strategies, the complete cure of cancer remains elusive. Therefore, the present study was undertaken to investigate the anticancer potential of aqueous extract of *Helicia nilagirica* (HNA) in vitro by MTT and clonogenic assays, where V79 and HeLa cells were treated with the different concentrations of aqueous extract of *Helicia nilagirica.* The treatment of V79 and HeLa cells with HNA resulted in a concentration dependent increase in cytotoxicity, which was maximum at the highest concentration of 400 µg/ml HNA in both the cell lines. The results of MTT assay were further confirmed by clonogenic assay, which also showed a concentration dependent decrease in the clonogenicity of HeLa cells.. To understand the mechanism of action the effect of HNA on glutathione (GSH) concentration, activities of glutathione-s-transferase (GST), catalase and superoxide dismutase (SOD) were studied at different post HNA treatment times. The exposure of HeLa cells to different concentrations of HNA at different post- treatment time alleviated the GSH content and also reduced the activities of antioxidant GST,CAT and SOD in a concentration and time dependent manner, except GST which was lowest and 6 h post-treatment and then marginally elevated at 12 h post-treatment.. The present study indicates that HNA exerted the cytotoxic effect on HeLa cells and recued the cell survival and this effect of HNA may be due to the alleviated level of the GSH, GST, catalase and SOD.

KEYWORDS

HeLa, MTT, clonogenic, glutathione, catalase

INTRODUCTION

Cancer a multistage disease has been the second leading cause of death worldwide. The number of cancer cases has been predicted to rise by almost 70% in 2020 (WHO, 2017) indicating the need to find new paradigms to treat or prevent the occurrence of cancer. The chemotherapy is an established mode of treatment of several neoplasia and it is the only treatment when a patient presents with metastasis (Harrington and Smith, 2009). Almost all the modern chemotherapeutic treatments available today are associated with several adverse side effects due to limitation in site specificity, causing strain to the patient/s (Ochwang'I *et al*., 2014). This indicates the need to focus on the use of alternative treatments and therapies against cancer, which are non-toxic or possess negligible side effects.

Plants have formed the major source of several modern chemotherapeutic drugs until their chemical synthesis began and they will continue to play a major role to treat cancer (Kinghorn *et al.*, 2016). Plant-derived drugs have gained interest for anticancer treatment as they are natural and readily available, readily administered orally as part of patient's dietary intake (Cornblatt *et al*., 2007; Amin *et al*., 2009). Since they are naturally derived compounds from plants they are generally more tolerated and non-toxic to normal human cells (Jagetia, 2007; Unnati *et al*., 2013; Jagetia and Baliga, 2016).

The National Cancer Institute collected about 35,000 plant samples from 20 different countries, and has screened around 114,000 extracts for anticancer activity. 60% of the commercially available anticancer drugs are derived from natural sources. The anticancer agents, vinblastine and vincristine from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apo-cynaceae), were the first agents to advance into clinical use for the treatment of cancer. Vinblastine and vincristine are used in combination with other cancer chemotherapy drugs, for the treatment of various kinds of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers (Moudi *et al*., 2013). The isolation of paclitaxel from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (*Taxaceae*) was clinically introduced to the US market in the early 1990s. Paclitaxel is active against a number of cancer types such as ovarian cancer, advanced breast cancer, small and non-small cell lung cancer, while *Taxus baccata* was reported to be used in India as a medicine for the treatment of cancer (Ahmed *et al*., 2013). The Camptothecin isolated from the Chinese ornamental tree, *Camptotheca acuminate* Decne (*Nyssaceae*), derivatives of camptothecin, Topotecan and irinotecan, are used for the treatment of ovarian and small cell lung cancers, and colon cancers, respectively (Venditto and. Simanek, 2010). However, induction of various adverse side effects including myelosuppression, gastrointestinal, hair follicle damage, reproductive and nephrotoxicities by these drugs has been the major stumbling block which necessitates the need to identify effective newer biomolecules to kill cancerous cells and spare normal cells with very low or negligible toxicity.

Helicia nilagirica Bedd. (Family: Proteaceae) locally known as Pasaltakaza is a tree, that grows up to a height of 12 meters and it grows in southern India, Indochina, Sri Lanka, , Burma (Myanmar), Japan, Taiwan, and Thailand. The *Helicia nilagirica grows*along streams some species are found on hilltops or ridges (Khamyong et al., 2004). Traditionally, *Helicia nilagirica* has been used as folk medicine in Mizoram, India by the Mizos since time immemorial . The decoction of leaves or bark of *Helicia nilagirica* is used to cure mouth ulcers, indigestion, stomach ailments, peptic ulcers, urinary tract infection gynaecological disorders and scabies and other skin diseases (Sawmliana 2003). In Sikkim the fruits of *H. nilagirica* are used to treat cough and cold (Chauhan 2001). A recent study has indicated that methnol extract of this plant possessed anti-inflammatory activity in rat cotton pellet granuloma model (Lalawmpuii *et al.* 2014). The systematic study on the anticancer properties of *Helicia nilagirica is lacking, which* indicates a need to evaluate its anticancer potential. Therefore, the present study was carried out to evaluate the anticancer activity of *Helicia nilagirica 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), reduced glutathione (GSH), ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), crystal violet were obtained from Sigma Chemical Co. (Bangalore, India). Sodium bicarbonate (Na_2CO_3), potassium chloride (KCl) and 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), hydrochloric acid (HCl), n-butanol, Tris buffer (Tris (hydroxymethyl) aminomethane and ammonium oxalate were requisitioned 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 (DOX), was obtained from Getwell Pharmaceuticals, Gurgaon, India.

Preparation of the extract

The identification and authentication of *Helicia nilagirica* Bedd. (Family: Protaeceae) was done by Botanical Survey of India, Shillong. The non-infected stem bark of *Helicia nilagirica* was collected from Sialsuk, Aizawl District of Mizoram, India during the dry season. The stem bark was peeled of the tree, cleaned chopped into small pieces, spread into the stainless steel trays and allowed to shade dry at room temperature in the dark, in the clean and hygienic conditions free from insects, animals, fungus, and other extraneous terrestrial materials. The dried tree stem bark was powdered in an electrical grinder at room temperature. A sample of 100 g of bark powder was sequentially extracted with petroleum ether, chloroform, ethanol and water using a Soxhlet apparatus. The extracts were evaporated to dryness under reduced pressure and stored at -80 until further use. Only the aqueous extract of *Helicia nilagirica* (HNA) was used for evaluation of the anticancer activity.

Dissolution of drug/s

The doxorubicin was freshly dissolved in MEM and the aqueous extract of *Helicia nilagirica* was dissolved in MEM, filtered and sterilized immediately before use.

Cell line and Culture

HeLa S3, 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, 1% L-glutamine and 50 µg/ml gentamicin sulfate at 37°C in an atmosphere of 5% CO_2 in humidified air in a CO_2 incubator (Eppendorf AG, Hamburg, Germany).

Experimental Design

Usually a fixed number of cells were inoculated into the desired culture vessels and they were divided into the different groups depending on the experimental protocol:

MEM group: The cells of this group served as negative control group.
HNA group: This group of cells was treated with different concentrations of HNA.

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

The cytotoxic effects of different concentrations of aqueous extract of *Helicia nilagirica* was studied by MTT assay in HeLa, and V79 cells as described by Mosmann (1983). Usually 10^3 cells were seeded into 96 well plates in 100 µl MEM. The cells were incubated at 37 ºC in a CO_2 incubator in an atmosphere of 5% CO_2 in 95 % humidified air. The cells were allowed to attach for 24 hours. The cells in microplates were exposed to different concentrations of HNA or doxorubicin and incubated in the CO_2 incubator for next 48 hours. Thereafter, 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 microplate reader (Spectramax M2). The cytotoxicity was calculated using the formula Control-Treatment/Control X 100. The IC50 was also determined.

Determination of optimum exposure time for cytotoxicity

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

The Determination of anticancer activity

Another experiment was setup to evaluate the anticancer activity of HNA, where grouping and other conditions were similar to that described in the experimental design section. Usually 10^6 exponentially growing HeLa cells were seeded into several culture flasks and the cells were allowed to attach for 24 h. The cells were exposed to 5, 10 or 20 µg/ml DOX or 20, 300 or 400 µg/ml of HNA.
After 2 hours of drug/s treatment the media were removed and the flasks were washed twice with sterile PBS, subjected to trypsin EDTA treatment and the following studies were conducted.

Clonogenic Assay

Usually 200 HeLa cells were inoculated into several individual petridishes containing 5 ml MEM and allowed to grow for another 11 days. The resultant colonies of cells were stained with 1 % crystal violet in methanol and scored. The plating efficiency (PE) of the cells was determined and surviving fraction (SF) calculated according to Puck and Marcus (1955).

PE = (Number of colonies counted x 100)/(Number of cells seeded)
SF = (Number of colonies counted)/(Number of cells seeded) x (mean plating efficiency).

Biochemical assays

A separate experiment was performed to estimate the effect of HNA on the activities of various antioxidant enzymes in HeLa cells, where grouping and other conditions were essential similar to that described for anticancer activity except that the cell cultures were terminated at 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. The cells were pelleted and weighed and 5% homogenate of the cells was prepared in PBS (pH 7.4) using sonicator (PCI Analytics Pvt. Ltd., Mumbai, India).

Total proteins

The proteins were estimated by standard procedure of Bradford (1976).

Glutathione estimation

Glutathione was estimated as described earlier (Moron *et al*., 1979). Briefly, 1.8 ml of 0.2 M Na_2HPO_4 was mixed with 40 µl 10 mM DTNB and 160 µl of cell homogenate. The mixture was incubated for 2 minutes at room temperature and the absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India).
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Glutathione - S – transferase estimation

Glutathione-s-transferase activity was estimated by the method of Habig *et al*., (1974). Briefly, 0.5 ml of 0.1 M phosphate buffer pH 6.5, 0.1 ml of 20 mM 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 read at 340 nm at 1 min intervals for 6 minutes in UV-VIS Biospectrophotometer. The GST activity was estimated using the following formula:-
GST activity = Absorbance of sample – Absorbance of blank_× 1000/9.6 × Vol of sample

Catalase estimation

Catalase was assayed according to the technique of 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 initiated 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 by Fried (1975). Briefly, 100 µl of cell homogenate was mixed with 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitroblue tetrazolium, and 200 µl of 780 µM NADH and 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.

STATISTICALANALYSES

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 table 1-4 and figure 1-7 as mean ± standard error of the mean.

Determination of Cytotoxicity

The treatment of HeLa or V79 cells with different concentrations of HNA resulted in a concentration dependent rise in its cytotoxic effects (Figure 1)and a maximum cytotoxicity was recorded for the highest concentration of HNA. The positive control doxorubicin also showed a similar pattern (Figure 1). The IC50 was also calculated and found to be 306.71µg/ml for HeLa and 300.64 µg/ml HNA for V79 cells, respectively.

Determination of optimum exposure time for cytotoxicity

The optimum exposure time for cytotoxicity of HNA against the two cell lines was determined using MTT assay at 2, 4, and 6 hours. The highest percent of cytotoxicity was observed at 4 h for HeLa and 2 h post treatment time for V79 cells, respectively (Figure 2). The difference among various treatment times was statistically not significant. Therefore further experiments were setup using 2 h HNA treatment time.

Clonogenic Assay

The clonogenicity of HeLa cells declines in a concentration dependent manner after treatment with different concentrations of HNA and the cell survival reached a nadir at a concentration of 400 µg/ml (Figure 3). The IC50 of HNA was also calculated and found to be 208.69 µg/ml.

Glutathione

The glutathione content of HeLa cells treated with different concentrations of HNA declined in a concentration dependent manner at all the post treatment times (Figure 4) and this decline was statistically significant when compared with the untreated control group (Table 1). A maximum of 2.7 fold reduction in the glutathione content was observed at 6h post treatment at a concentration of 400 µg/ml (Table 1). The doxorubicin treatment also showed a pattern similar to HNA treatment (Figure 4).

Glutathione-s-transferase

Treatment of HeLa cells with different concentration of HNA showed a concentration dependent reduction in the GST activity at all the post treatment times (Figure 5). The maximum reduction (4.09 fold) was found at a concentration of 400 µg/ml after 4h treatment time (Table 2). The DOX treated group also reduced the enzyme activity in a concentration dependent manner. The reduction at all concentrations was found to be statistically significant ($p<0.05$).

Catalase

The activity of catalase in the HeLa cells treated with different concentrations of HNA showed a concentration dependent decrease at all post treatment times (Figure 6). A maximum of 2.87 fold decrease in catalase activity was observed at 400 µg/ml, at 6 h post treatment when compared with the non-drug treated control group. The catalase activity declined significantly in the HeLa cells treated with different concentrations of HNA or doxorubicin (Table 3).

Superoxide dismutase

The treatment of HeLa cells with different concentrations of HNA or DOX caused a significant but concentration dependent attrition in the SOD activity at all post treatment times (Figure 7 and Table 4). A maximum of 7.11 fold and 7.59 fold decrease in the SOD activity was observed for 400 µg/ml and 20 µg/ml of HNA and DOX, respectively, at 6h, post treatment (Table 4).

DISCUSSION

Chemotherapy has been a major treatment modality to treat various malignant cancers, either alone or in combination with radiation or surgery. It has also been used as a palliative treatment where the complete cure of cancer has not been affected (Morgan *et al*., 2004; Roeland). The active principles in chemotherapy have been derived from plants such as *Catharanthus roseus, Podophyllum peltatum, P. emodii, Taxus brevifolia, Ochrosia elliptica* and *Campototheca acuminate* (Kinghorn and Balandrin 1993). However, most of the modern chemotherapeutic agents have limitations in terms of toxicity, lack of tumor selection, ineffective against drug resistant cancers, expensive and teratogenic (Mellor and Callaghan 2008; Valko and McLeod 2009). Moreover, the patients who survive chemotherapy have shown the development second malignancies associated with chemotherapeutic treatment (Morton *et al*., 2014). Therefore, screening for non-toxic, cheaper, higher efficacy and better selectivity cancer drug/s, which are devoid of all the side effects of modern molecules is needed. The use of plants for treating various ailments have been practiced by humans since time immemorial and there is an unending quest in finding new and improved chemotherapeutic drugs till today. Since herbal products have been traditionally accepted and known to have lesser or no adverse effects, it is imperative to search new molecules from the plants. This has been the impetus to determine the anticancer activity of the stem bark of *Helicia nilagirica in vitro.*

Cell culture has provided a fast, efficient and economical way of cytotoxicity screening, elucidation of mode of action of drugs in a controlled and systematic manner with high resolution in a short period of time. The present study was also carried out using HeLa, a cervical cancer cells and non-cancerous V79, a Chinese hamster lung cells to estimate the cytotoxic effects of HNA by employing MTT assay. This assay is a rapid and a standard technique to test the cytotoxicity of drugs where metabolically active cells increase the formation of formazan crystals by mitochondrial succinate dehydrogenase and the level of enzyme activity is a measure of the viability of the cells and more intense color indicates more viable cells (Mossmann, 1983). The treatment of HeLa and V79 cells with HNA reduced the cell survival indicated by a concentration dependent rise in the cytotoxic effect. There seems to be no reports on the cytotoxicity of HNA and this is probably the first report where HNA has been found to be cytotoxic. However, other plants such as *Aphanamixis polystachya* ,*Tinospora cordifolia*, *Alstonia scholaris, Consolida orientalis*, *Ferula assafoetida*, *Coronill avaria* and *P. pellucidum* extract have been reported to induce cytotoxicity in HeLa cells in vitro (Jagetia *et al.,* 1994; Jagetia and Rao., 2006;Jagetia and Baliga, 2005; Widowati *et al.,* 2013; Jagetia and Venkatesha, 2016). Similarly, *Arctium lappa, Artemisia absinthium, Calendula officinalis, Centaurea, Cyanus, Tanacetum vulgare* and *Tragopogon pratensis* have been reported to be cytotoxic to J-45.01 human acute T leukemia cells (Wegiera *et al.,* 2012). The cytotoxicity of HNA was further confirmed by performing clonogenic assay on the HeLa cell line. The IC50 was calculated to be 208.69 µg/ml.

The clonogenic assay is the gold standard to test the reproductive integrity of cells and HNA treatment has been found to reduce the Clonogenic potential of HeLa cells in a concentration dependent fashion. Likewise *Aphanamixis polystachya , Tinospora cordifolia*, and *Alstonia scholaris* have been reported to retard the clonogenicity of HeLa cells (Jagetia *et al.,* 1994; Jagetia and Rao., 2006; Jagetia and Venkatesha, 2016). Similarly, a natural product berberine has been found to reduce the clonogenic potential of HeLa cells in a concentration dependent manner (Jagetia and Rao, 2017).

Glutathione (γ -glutamylcysteinyl glycine) is a tripeptide synthesized in most cells and it is formed by the ATP dependent condensation of glutamic acid and cysteine, catalyzed by γ-glutamylcysteinyl synthetase. Glycine is then added by glutathione synthetase to form GSH. The presence of sulfhydryl (SH) group of the cysteinyl moiety is a powerful reducing agent and a strong nucleophile that is able to react with cellular toxicants directly or via the catalysis of the glutathione S-transferase family of enzymes. It is also a co-factor for several metabolic enzymes and is involved in intracellular transport, functions as an antioxidant and radioprotectant and facilitates protein folding and degradation (Halliwell and Gutteridge 1999; Gamcsik *et al*., 2012; Lu, 2013). In cancer cells the rise in GSH beyond normal level is an indication of chemotherapy resistance whereas low level of GSH has been reported to enhance oxidative stress, and subsequently cause cell death and apoptosis of the tumor cells. The loss of essential sulfhydryl groups lead to an alteration in the calcium homeostasis that eventually results in the loss of cell viability which is indispensible for chemotherapy to be effective (Mayer *et al*., 1987; Neal *et al*., 2003; Ramsay and Dilda, 2014). Treatment of the HeLa cells with different concentration of HNA showed a concentration dependent reduction of GSH content which showed the effectiveness of HNA against neoplastic cells.

Glutathione-S-transferase isoenzymes are ubiquitous which catalyze the conjugation of reduced glutathione (GSH) with compounds that contain an electrophilic centre through the formation of a thioether bond between the sulphur atom of GSH and the substrate (Chasseud 1979; Mannervik 1985; Laborde, 2010). A number of GST isoenzymes also exhibit GSH-dependent catalytic activities such as reduction of organic hydroperoxides, isomerisation of various unsaturated compounds and also several non-catalytic functions such as sequestering of carcinogens, modulation of signal transduction

pathways etc. (Ketterer *et al*. 1990; Jakoby and Habig 1980; Cho *et al*. 2001). The over expression of GST in cancer cells are common and can induce chemoresistance and resistance to apoptosis which makes it a promising target for research on the GST inhibitors to sensitize tumor cells (McIlwain *et al*., 2006; Zeng *et al*., 2014). The decline in GST activity after treatment with the HNA could be an indication that this extract act as an inhibitor compound for GST and thereby killing the cancer cells.

Catalases catalyze the conversion of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) in the presence of iron or manganese as a cofactor (Kodydková *et al*., 2014). It is localized in peroxisomes in eukaryotic cells. Suppression of catalase has been reported to induce the increase production of H_2O_2 which block TNF-induced NF-κB activation and sensitizes cells to apoptosis (Yang *et al*., 2011). The HNA also reduces the activity of catalase which may have contributed to its anticarcinogenic activity.

Superoxide dismutases (SOD) are the enzymes that catalyze the dismutation of superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and elemental oxygen. They consist of three isoforms in mammals: the cytoplasmic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3), all of which require catalytic metal (Cu or Mn) for their activation. The mechanism of dismutation of O_2^- to H_2O_2 by SOD involves alternate reduction and reoxidation of a redox active transition metal including copper (Cu) and manganese (Mn) at the active site of the enzyme (Abreu, 2010). The role of SOD level on tumor invasiveness is controversial as far as different studies are concerned on different tumors. In one study MnSOD overexpression protected HeLa cervical carcinoma cells from growth suppression under the condition of serum deprivation, which was suggested to be related to changes in the intracellular oxidative processes of these cells (Palazzotti 1999). When human prostate carcinoma cells were transfected with the cDNA for MnSOD, the clones overexpressing MnSOD grew more slowly under basal cell culture conditions in vitro than control cells (Li 1998). In one model recombinant CuZnSOD increased colon carcinoma liver metastasis in mouse models (Nonaka 1993), whereas in another mouse model recombinant CuZnSOD reduced fibrosarcoma pulmonary metastasis (Yoshizaki 1994). Therefore, modulation of the oxidant/antioxidant balance toward a more reduced state is likely to have a controlling influence limiting the survival and invasion of most cancer cells. The HNA also alleviated the activity of SOD which caused change in the cellular oxidant/antioxidant balance thereby increasing tumor cell kill in the present study.

The exact mechanism of action of cell killing by HNA is not clearly understood however, the present study clearly indicates that HNA has been able to reduce the activities of GST, catalase and SOD which are involved in the failure of chemotherapy. This reduced activity may have played a major role in the cell killing by HNA in the present study. The GSH is another molecule which in indicated in the development of chemo resistance and it reduction by HNA would have made cells for sensitive to its cytotoxic effect and its decline may have also stimulated cell death by apoptosis. Although the effect of HNA at molecular level has not been studied. It is plausible that HNA may have blocked the transcription of NF-κB, COX-II, Nrf2 and some cell cycle proteins that help cancer cell division and ensure higher survival (Sobolewski *et al*., 2010; Lu and Stark, 2015; Choi and Kwak, 2016) and their inhibition may have led to the effective cell killing by HNA. The suppression of these proteins have been found to enhance cancer cell killing (Xu *et al*., 2014; Pozdeyev *et al*., 2015; Menegon *et al*., 2016). The HNA may have also stimulated apoptotic pathway by upregulating p53 and Bax proteins bring effective cell killing.

CONCLUSIONS

The exact mechanism underlying the antineoplastic activity of the HNA is unknown, however cytotoxic effect against HeLa cells was observed with MTT and clonogenic assays. The alleviation in the level of glutathione, GST, catalase and SOD might have increased the level of oxidative stress, leading to DNA damage and cell death. The HNA may also have suppressed the transcription of Nrf2, NF-κB and COX-II genes at molecular level and up regulated p53 and Bax leading to effective cell kill in the present study.

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Table 1: Alterations in the Glutathione contents of HeLa cells induced by different concentrations of *Helicia nilagirica* (HNA) and doxorubicin. The results were determined as µmol/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20
2	2.64±0.20	1.56±0.02*	1.44±0.01*	1.41±0.03*	1.10±0.003*	0.93±0.09*	0.87±0.03*
6	2.53±0.24	1.69±0.04*	1.41±0.009*	0.99±0.01*	1.01±0.003*	0.93±0.003*	0.78±-0.02*
12	2.33±0.06	1.01±0.01*	0.94±0.04*	0.88±0.04*	0.73±0.006*	0.59±0.01*	0.57±0.008*

*p<0.05 when treatment groups are compared with concurrent control (MEM) group.
Standard error of the mean (SEM), **n=5.**
No symbol= no significant difference.
Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

Table 2: Alterations in the GST activity of HeLa cells treated with different concentrations of *Helicia nilagirica* (HNA) and doxorubicin. The results were determined as unit/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20
2	0.10±0.003	0.08±0.001*	0.05±0.002*	0.04±0.001*	0.08±0.001*	0.05±0.001*	0.02±0.001*
6	0.10±0.001	0.08±0.002*	0.04±0.003*	0.02±0.003*	0.07±0.001*	0.03±0.001*	0.02±0.003*
12	0.10±0.007	0.09±0.003	0.06±0.002*	0.04±0.007*	0.09±0.002	0.05±0.006*	0.04±0.003*

*p<0.05 when treatment groups are compared with concurrent control (MEM) group.
Standard error of the mean (SEM), , **n=5.**
No symbol= no significant difference.
Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

Table 3: Alterations in the catalase activity of HeLa cells treated with different concentrations of *Helicia nilagirica* (HNA) and doxorubicin (DOX). The results were determined as Unit/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20
2	7.64±0.19	6.11±0.50*	5.16±0.33*	4.58±0.33*	6.19±0.23	5.35±0.19*	3.68±0.16*
6	7.26±0.50	5.35±0.50*	3.82±0.38*	2.86±0.31*	4.37±0.18*	4.01±0.33*	2.58±0.16*
12	6.59±0.16	5.02±0.43*	2.77±0.34*	2.29±0.33*	4.09±0.11*	2.69±0.20*	1.91±0.38*

*p<0.05 when treatment groups are compared with concurrent control (MEM) group.
Standard error of the mean (SEM), **, n=5.**
No symbol= no significant difference.
Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

Table 4: Alterations in the SOD activity of HeLa cells treated with different concentrations of *Helicia nilagirica* extract (HNA) and doxorubicin (DOX). The results were determined as Unit/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20

2	3.03±0.12	2.87±0.13	2.39±0.06*	1.54±0.07*	2.63±0.13	2.11±0.10*	1.47±0.10*
6	2.84±0.12	1.82±0.09*	1.34±0.06*	1.04±0.18*	1.45±0.06*	1.16±0.10*	0.79±0.06*
12	2.52±0.07	0.61±0.02*	0.36±0.02*	0.35±0.003*	0.54±0.006*	0.40±0.002*	0.33±0.006*

*p<0.05 when treatment groups are compared with concurrent control (MEM) group.
Standard error of the mean (SEM), , **n=5.**
No symbol= no significant difference.
Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

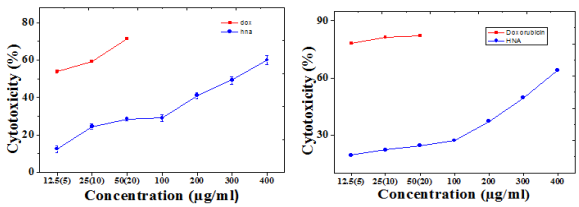

Fig. 1: Cytotoxic effect of different concentrations of aqueous extract of *Helicia nilagirica* in HeLa and V79 cell lines by conventional MTT assay. The results were determined as percentage (%) cytotoxicity and expressed as Mean ± SEM, n=5, p<0.05.

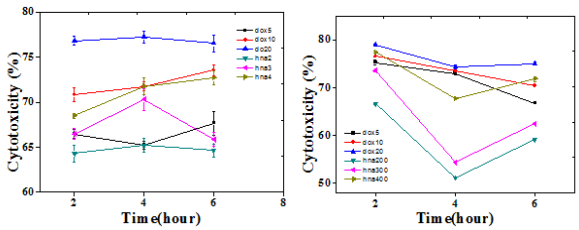

Fig 2: Cytotoxic effect at different exposure time of the aqueous extract of *Helicia nilagirica* and DOX in HeLa and V79 cell lines by MTT assay. The data are expressed as Mean ± SEM, n=5, p<0.05.

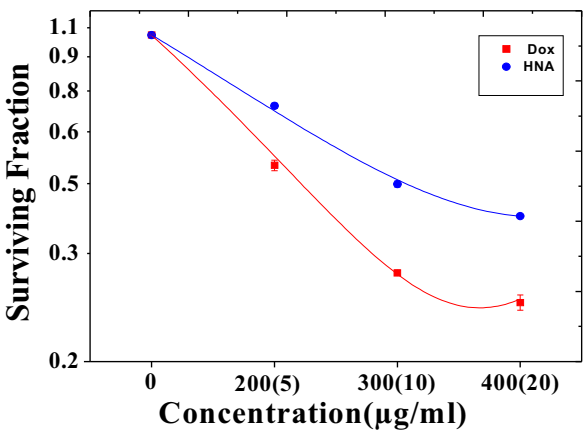

Fig 3: Effect of different concentrations of the aqueous extract of *Helicia nilagirica* (HNA) and Doxorubucin (DOX) treatment on the survival of HeLa cells.

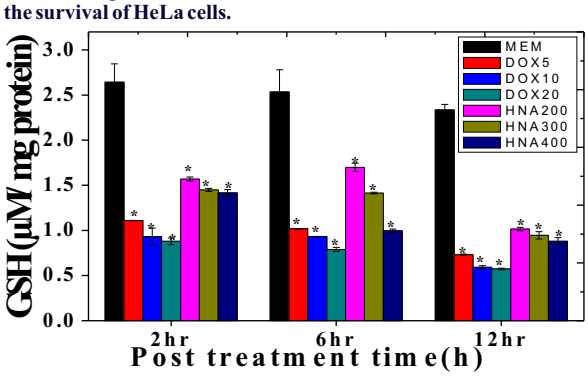

Fig 4: Alteration in the GSH content of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA-Aqueous extract of Helicia nilagirica (treatment), DOX- Doxorubicin (Positive Control).

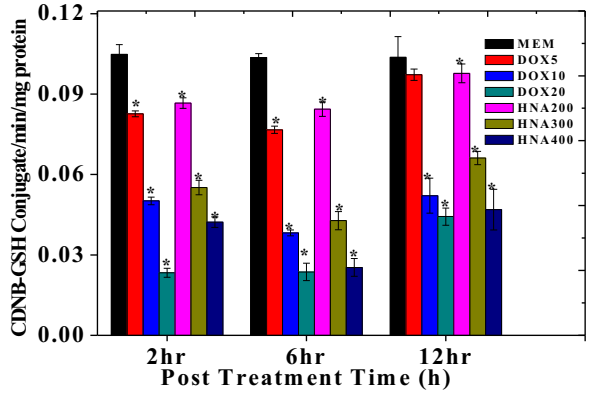

Fig 5: Alteration in the GST activity of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA-Aqueous extract of Helicia nilagirica (treatment), DOX- Doxorubicin (Positive Control).

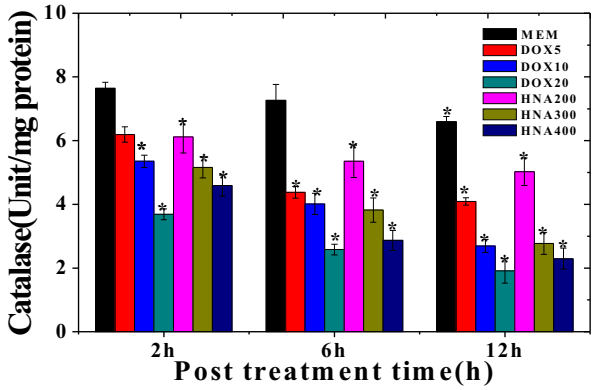

Fig 6: Alteration in the catalase activity of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA-Aqueous extract of Helicia nilagirica (treatment), DOX- Doxorubicin (Positive Control).

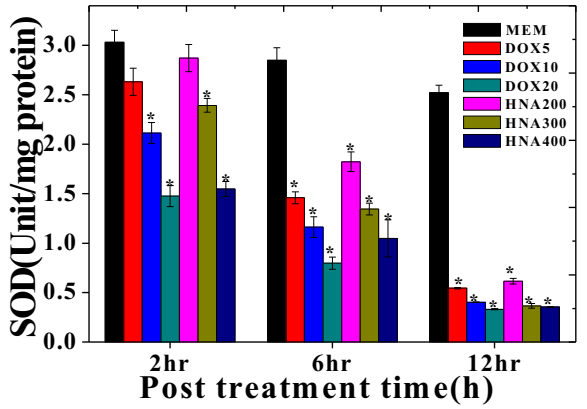

Fig 7: Alteration in the catalase activity of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA-Aqueous extract of Helicia nilagirica (treatment), DOX- Doxorubicin (Positive Control).

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Research Article

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In vitro effect of tuibur (tobacco brew) on the viability of human blood lymphocytes

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The use of tobacco and its products are known to cause many illnesses including cancer. A smokeless tobacco locally manufactured called *tuibur* (tobacco brew) has been consumed by the Mizos from a very long time. In this experiment we aim to determine the cytotoxicity of *tuibur* by an *in vitro* study on *tuibur*-treated human peripheral blood lymphocytes. We have found that 24 h treatment of human lymphocytes with two grades of commercial *tuibur* and nicotine showed a concentration dependent decrease in cell viability. We, therefore, concluded that as the *in vitro* use of *tuibur* has an adverse effect on cell survival, its consumption might have potential side effects on the health of the users.

Key words: Cell viability, lymphocytes, tobacco, tuibur.

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Introduction

Tobacco is linked with many diseases and has been known to contain more than eight thousand chemicals, out of which roughly 68 are probable carcinogens.[1-3] Some of the common toxic chemicals include benzo[a]pyrene (B[a]P), N′-nitrosonornicotine (NNN), N′-nitrosoanatabine (NAT), N′-nitrosoanabasine (NAB), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosodimethylamine (NDMA), nitrite, cadmium, lead, arsenic, nickel, chromium, etc.[4,5] The consumption of both smoking and smokeless tobacco is popular throughout the world and its detrimental effect could be observed from many medical records. Besides its deleterious consequence upon the pulmonary system, it has been linked with many forms of cancer. In fact, many studies suggested that almost all known cancer could be linked to tobacco use.[6,7]

It would be safe to say that every nation throughout the globe has tobacco users in its population.[8] The form of tobacco used may vary considerably. Some prefer smoking tobacco while others prefer smokeless tobacco, or both. But, it may be acceptable to say that more than half of the tobacco users used it in the form of smoking tobacco.[9] The Mizo tribes living in the northeastern part of India use both smoke and smokeless tobacco.[10] A form of smokeless tobacco locally called *tuibur* (tobacco brew) is used popularly and is commercially available in the

local market, generally in two grades, which largely depend on the amount of tobacco used in its production. The method of practice is the users of *tuibur* put the product in the mouth for roughly 5-10 minutes which is then spitted out. The duration is determined when the alkalinity of the *tuibur* is depleted.[11]

In this experiment, we aimed to determine the effect of two grades of commercial *tuibur* on the viability of *tuibur*-treated human peripheral blood lymphocytes *in vitro*.

Materials and Methods

Chemicals

A small quantity of two grades of commercial *tuibur*, labelled as *tuibur*-A (special grade) and *tuibur*-B (ordinary grade), produced in a local industry were purchased from the market. Although there is no standard protocol, the manufacturers graded the *tuibur* depending on the quantity of tobacco used in its production. Pure nicotine (Cayman Chemical Company) and trypan blue (Sigma) were purchased from local supplier. RPMI-1640 media (HiMedia) was obtained from local supplier and prepared in the laboratory using standard protocol.

Lymphocyte culture and treatment

Lymphocyte culture were performed using the protocol described by Jagetia *et al.* [12] Briefly, peripheral blood lymphocytes were collected by venipuncture in a heparinized vacutainer from a 27-year-old healthy male volunteer who has no known history of tobacco consumption. The collected blood was allowed to stand for roughly half an hour and the upper translucent layer containing lymphocytes was taken for culture. Approximately two million lymphocytes were cultured in different test tubes containing 2 ml RPMI-1640 culture media without the addition of any growth factor.

The tubes were separated into four groups (I, II, III & IV) and different volumes of *tuibur*-A and *tuibur*-B were added to group I & II (2.5, 5, 10, 20, 40, and 50 µl/ml) respectively. To group III, 2.5, 10, 20, 40, and 50 µg/ml of nicotine was added and this served as positive control. Group IV or blank acted as negative control and did not contain any chemical other than the cells and the media. All cultures were performed in triplicate. These tubes were incubated at 37°C for 24 h. After 24 h, the survival of the cells was checked by modified trypan blue exclusion assay.[13] The number of living and dead cells were counted in a hemocytometer and the mean percentage of surviving cells was taken as viability.

Statistical analysis

All statistical analysis were performed using Microsoft Excel 2013 and OriginPro-8. Correla-

Table 1 | Mean percentage of viable human peripheral blood lymphocytes for blank and treatment with different concentration of *tuibur*-A, *tuibur*-B and nicotine.

Concentration (µl/ml or µg/ml)	Mean % of viable cells±SEM			
	Tuibur-A	*Tuibur*-B	Nicotine	Blank
0	-	-	-	100.00±0.00
2.5	100.00±0.00	100.00±0.00	98.25±0.06	-
5	96.39±0.58	95.06±0.40	96.55±0.25	-
10	90.34±1.86	90.35±0.97	92.32±0.59	-
20	86.41±0.62	87.03±0.29	91.1±0.23	-
30	79.43±2.22	85.65±1.20	87.9±0.50	-
40	71.07±1.97	79.15±0.58	85.57±0.14	-
50	65.57±0.62	76.14±1.11	82.71±2.31	-

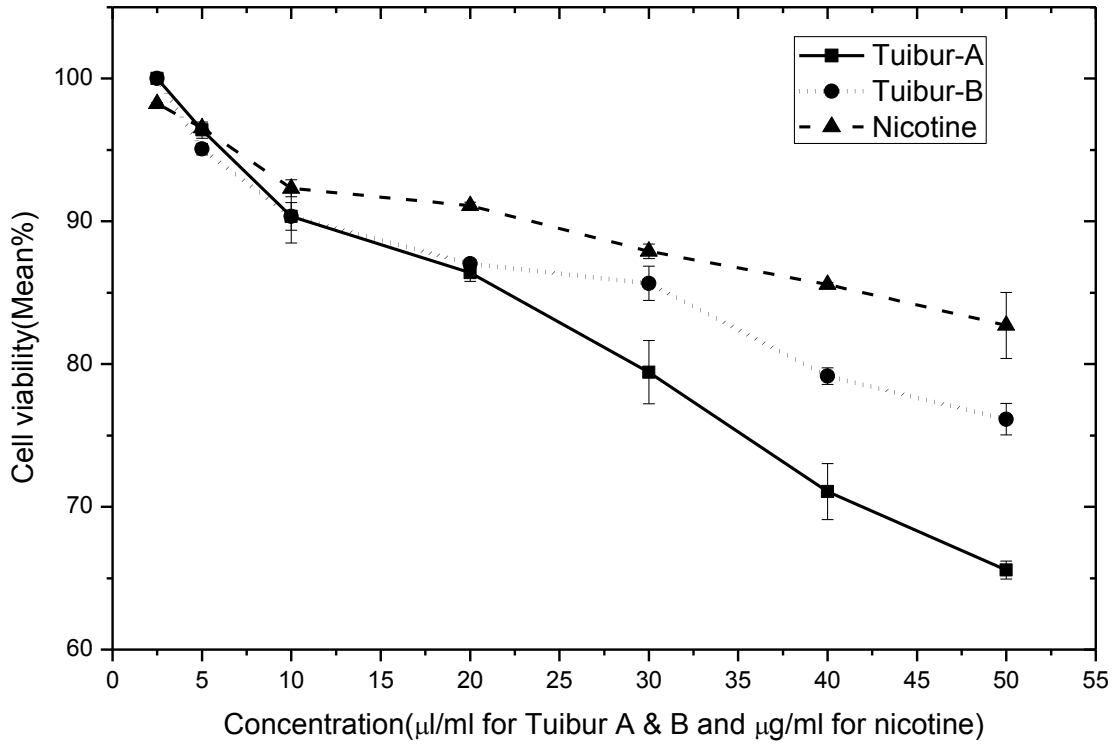

Figure 1 | Graph showing mean percentage of viable human peripheral blood lymphocytes treated with different concentration of *tuibur*-A, *tuibur*-B and nicotine.

tion coefficient was performed to determine relationship between different treatment concentrations and viability within a group. Student's *t*-test was employed to determine significant difference between the treatment groups.

Table 2 | Student's *t*-test between different treatment groups at 95% confidence interval.

Student's t-test between	p-value at 95% CI	Inference
Tuibur-A & *Tuibur*-B	0.60	No significant difference
Tuibur-A & Nicotine	0.27	No significant difference
Tuibur-B & Nicotine	0.45	No significant difference
Control & *Tuibur*-A	≤0.00	Significant difference
Control & *Tuibur*-B	≤0.00	Significant difference
Control & Nicotine	≤0.00	Significant difference

Results

The pH of *tuibur*-A and *tuibur*-B were found to be 9.81 and 10.09 respectively. Table 1 and Figure 1 showed the mean percentage of viable cells for the different treatment groups. The negative control showed 100% viability while *tuibur*-A, *tuibur*-B and nicotine showed a concentration dependent viability. Lymphocytes treated with a maximum concentration of 50 µl/mlof *tuibur*-A and *tuibur*-B showed 65.57% and 76.14% viability respectively while a minimum concentration of 2.5 µl/ml of both the two *tuibur* grades resulted in 100% viability in both the groups. A maximum concentration of 50 µg/ml and a minimum concentration of 2.5 µg/ml of nicotine showed 82.71% and 98.25% viability respectively. A strong negative correlation was observed between cell viability and concentration of *tuibur*-A (-0.994), *tuibur*-B (-0.969) and

nicotine (-0.979). This means higher the concentration of the chemicals, lower the viability and vice versa.

Statistical analysis by t-test at 95% CI (Table 2) between mean percentage of viable cells for blank and *tuibur*-A, blank and *tuibur*-B, blank and nicotine showed a significant difference (p-value≤0.00). However, comparison of *tuibur*-A and *tuibur*-B (p-value=0.60), *tuibur*-A and nicotine (p-value=0.27), *tuibur*-B and nicotine (p-value=0.45) showed that there is no significant difference in mean percentage of viable cells between these groups.

Discussion

Tobacco is known to contain enormous amount of different chemicals, many of which have been reported to have carcinogenic and cytotoxic properties.[1,14] Most studies, if not all, reported the use of tobacco in any form only have negative impact on the physiological well-being of the users. There have been only a handful of literatures on the scientific investigation of *tuibur*. A preliminary report on the chemical composition of *tuibur* showed the presence of polyaromatic hydrocarbons and carbonyl compounds in the tar phase.[11]

An epidemiological study among the Mizos showed that *tuibur* users have a higher risk of developing gastric cancer and the combine use and frequency of smoking, betel, *tuibur* and *sahdah* were reported to have a significant influence on the risk of gastric cancer.[10] Phukan *et al.*[15] have also reported *tuibur* use as a risk factor for gastric cancer. Besides gastric cancer patients in Mizoram, *tuibur* consumers were found to have a variety of mtDNA D-loop region mutations and polymorphisms.[16] Individuals with Arg/Pro genotype,GSTM1 null genotype and GSTT1 non-null genotype were also suggested to have a higher risk of gastric cancer if they have habits of using *tuibur* and smoking tobacco.[17,18]

The damaging effect of tobacco may be attributed to its vast array of chemical compositions. Heavy metals like cadmium and lead present in tobacco have also been found to cause glomerular dysfunction. Many of these effects may be because of nicotine's ability to affect certain antioxidant enzymes like lipid peroxidase, superoxide dismutase, catalase, glutathione-s-transferase, glutathione reductase, etc.[3] Cytological studies have reported nicotine to inhibited cell proliferation and decreased protein synthesis in a dose dependent manner in cultured periodontal ligament fibroblast,[19] while it was also reported to stimulate endothelial cell DNA synthesis and proliferation at concentrations lower than $<10^{-8}$ M. The cytotoxicity of nicotine was reported to be at a higher concentration, i.e. $>10^{-6}$.[20]

Onion bulbs treated with *tuibur* showed a reduced root growth, reduced mitotic index, formation of micronuclei, lagging chromosomes, and c-mitosis.[21] A study on seven smokeless tobacco aqueous extracts showed a concentration-dependent effects on the growth and viability of oral bacteria cultured under anaerobic conditions.[22] These effects may be a result of increase superoxide anion production, lipid peroxidation, DNA fragmentation and DNA ladders caused by the use of chewing tobaccos.[23]

Our result showed concentration dependent cell viability for the *tuibur* and nicotine treatment groups while the untreated negative control group showed 100% viability. We are uncertain as to what chemical(s) in the tobacco brew would cause the cells to die. But from the nicotine treatment group, we may be able to say, although carefully, that the nicotine might contributed significantly in this result. However, one study suggested other biologically active compounds like NNN, NNK, etc., other than nicotine present in tobacco leave extract to be the source of cytotoxicity.[24]

Another probable factor for the decrease in viability of the *tuibur* treatment groups would be the change in pH of the culture media. As we have shown in our result, the pH of both the two grades of *tuibur* are alkaline in nature, a slight rise in pH of the culture media was observed after the addition of both the *tuibur* (data not shown). This change in pH may be a factor that leads to decrease cell viability. In conclusion, our

result showed that 24 h treatment of human lymphocytes with *tuibur* and nicotine may have an adverse effect on their survival and hence these chemicals might have cytotoxic properties. Therefore, the consumption of *tuibur* might have potential side effects on the health of the users.

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The Phytochemical and Thin Layer Chromatograhy Profile of Ethnomedcinal Plant *Helicia Nilagirica* (Bedd)

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Research Article

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Abstract

The plants have provided valuable medicines in the form of secondary metabolites synthesized by them for various purposes. The present study deals with the phytochemical profiling of *Helicia nilagrica* using various phytochemical procedures and thin layer chromatography. The mature non-infected stem bark of *Helicia nilagirica* was collected, dried, powdered and subjected to sequential extraction with increasing polarity using petroleum ether, chloroform, ethanol and distilled water. The different extracts were cooled and evaporated to dryness with rotary evaporator. The phytochemical analyses were carried out on chloroform, ethanol and aqueous extracts. The chloroform extract revealed the presence of flavonoids, tannins, terpenoids, cardiac glycosides, whereas alkaloids, saponins and carbohydrates were completely absent. Similarly, the ethanol extract contained flavonoids, tannins, phenols and cardiac glycosides. The aqueous extract showed the presence of saponins, tannins, cardiac glycosides and carbohydrates. The TLC profile also showed the presence of different phytochemicals in the different extracts as indicated by different Rf values using various solvent systems.

Keywords: *Helicia nilagirica*; Phytochemical; Flavonoid; Thin layer chromatography

Introduction

Medicinal plants have been used as the main traditional herbal medicinal system amongst rural dwellers worldwide since antiquity and they are still in use for healthcare. The earliest written evidence of use of plants as medicine has been found around 5000 years before on a Sumerian clay slab from Nagpur which comprised of 12 recipes for drug preparation consisting over 125 plants including poppy, henbane and mandrake. The Rig Veda, the ancient knowledge book of Hindus dating between 3500 B.C. to1800 B.C. mentions several plant-based drugs for human healthcare and is the earliest systematic record in this regard. In 2500 BC the Chinese emperor has written a book for 365 drugs some of which have been used even today include *Rhei rhisoma*, camphor, *Theae folium, Podophyllum*, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra [1,2]. Over the years plants have been used as the main source of medicine especially in the developing countries and more than 80% of the world's population relies on traditional medicine for their primary healthcare needs [3].

Phytochemicals are natural bioactive chemical compounds produced by various plants to protect themselves from environmental hazards such as pollution,

stress, drought, UV exposure and pathogenic attack [4]. These compounds are known as secondary plant metabolites and include organic substances like alkaloids, carotenoids, glycosides, terpenoids, steroids, tannins, flavonoids, saponins, vitamins, mucilages, minerals, organic acids etc. [5-8]. These secondary metabolites are of great health benefits to humans. Some of the beneficial roles of phytochemicals include low toxicity, low cost, easy availability with an extensive range of therapeutic activities such as antioxidant, antimicrobial, hypoglycemic, antidiabetic, antimalarial, anticholinergic, antileprotic and antineoplastic. These phytochemicals also help in the modulation of detoxification of enzymes, stimulation of the immune system, decrease in platelet aggregation and modulation of hormone metabolism [9,10]. Even with a remarkable progress made in synthetic drugs in modern medicine, therapies using medicinal plants still make a major contribution to the pharmaceutical industry because of their safety, easy availability, and cost effectiveness. They also have synergistic effect with other biologically active ingredients due to the presence of beneficial minerals [11,12].

Helicia nilagirica Bedd. (Family:Proteaceae) is locally known as Pasaltakaza, is a medium-sized tree, which grows up to a height of 12 meters. It is widely distributed in Sri Lanka, southern India, Burma (Myanmar), Indochina, Japan, Taiwan, and Thailand. It is also found scattered in lowland to montane rain forests, up to an altitude of 500 - 3,350 meters. Some species are found in habitats along the streams whereas other species are found on hilltops or ridges [13]. It has been used as folk medicine since time immemorial in Mizoram, India by the Mizo people. The decoction prepared by boiling the leaves or bark is used to treat various stomach ailments including peptic ulcers, indigestion, mouth ulcer, urinary tract infection and gynecological disorders. The stem bark juice is applied to reduce muscular swelling and treat cuts and wounds. The stem bark is also used in scabies and other skin diseases [14]. *H. nilagirica* has been shown to possess anti-inflammatory and antioxidant properties recently [15,16]. *H. nilagirica* has been found to be cytotoxic in cultured HeLa cells indicating its anticancer potential in treating neoplastic disorders [17]. The fruits of *H. nilagirica* have been used as a medicine to cure cough and cold in Sikkim [18]. *Helicia nilagirica* has been used in traditional medicine by the people of Mizoram and other states for healthcare and the information has been collected by meeting the elders who practice ethnomedicine. There are only very few reports about phytochemical analysis of *Helicia nilagirica* and these studies were done on methanol extract and not all parameters were evaluated [14,19]. The ethnomedicinal use and scanty information on the phytochemical analysis of *Helicia nilagirica* stimulated us to investigate its phytochemical composition.

Materials and Methods

Chemicals and reagents

Potassium iodide, bismuth nitrate, sulphuric acid, ferric chloride, hydrochloric acid, aluminium chloride, ammonium hydroxide, glacial acetic acid, chloroform, ethanol, methanol, n-butanol, ethyl acetate, sodium chloride, sulphuric acid, olive oil, and Whatman filter paper were procured from Sd fine Chemical Ltd., Mumbai, India. The TLC plates were commercially procured from Merck India, Mumbai.

Collection and extraction

The mature and non-infected stem bark of *Helicia nilagirica* Bedd. (Family: Proteaceae) locally known as Pasaltakaza was collected from Sialsuk, Aizawl District of Mizoram, India during the dry season. Identity of *Helicia nilagirica* Bedd was authenticated by the Botanical Survey of India, Shillong. The cleaned and non-infected bark was spread into stainless steel trays and allowed to dry in the shade at room temperature in dark in clean and hygienic conditions to avoid entry of insects, animals, fungus, and extraneous terrestrial materials. The exhaust and free air circulation was allowed. The dried bark was powdered in a grinder at room temperature. A sample of 100 g of stem bark powder was extracted sequentially in chloroform, ethanol and water in a Soxhlet apparatus. Each extract was concentrated to dryness under reduced pressure and stored at -80°c until further use.

Phytochemical Screening

The different extracts of *Helicia nilagirica* were analyzed for the presence of various phytochemicals using standard procedures as described below.

Alkaloids

The presence of alkaloids was determined by mixing 0.1g of the extract with 0.5 ml of Mayer's reagent and Dragendorff's reagent. The formation of a creamy (Mayer's reagent) or reddish-brown precipitate (Dragendorff's reagent) indicated the presence of alkaloids [20,21].

Tannins

About 0.5 g of dried powdered samples was boiled in 20 ml of water in a test tube and filtered. A few drops of 0.1% ferric chloride was added to the filtrate. The formation of

brownish green or a blue-black colour indicated the presence of tannins [20,21].

Phlobatannins

The aqueous extract of *Helicia nilagirica* was boiled with 1% aqueous hydrochloric acid and deposition of a red precipitate indicated the presence of phlobatannins [20,21].

Saponins

About 2 g of the powdered sample was boiled with 20 ml of distilled water in a water bath for 10 minutes and filtered while hot and cooled before conducting the following tests:

Frothing: 3 ml of filtrate was diluted up to 10 ml with distilled water and shaken vigorously for 2 minutes. The formation of a fairly stable froth indicated the presence of saponins in the filtrate.

Emulsification: 3 drops of olive oil was added to the solution obtained by diluting 3 ml filtrate to 10 ml distilled water and shaken vigorously for a few minutes. The formation of a fairly stable emulsion indicated the presence of saponins [20,21].

Flavonoids

Three different methods were used to test the presence of flavonoids in all the extracts [20,21,22]. 5 ml of dilute ammonia solution was added to a portion of the aqueous filtrate of each sample followed by the addition of concentrated H_2SO_4. Appearance of a yellow colour (disappeared on standing) in each extract indicated the presence of flavonoids.

a) A few drops of 1% aluminum solution were added to a portion of each filtrate. A yellow colour indicated the presence of flavonoids.

b) A portion of the sample was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colour indicated the presence of flavonoids.

Terpenoids

The presence of terpenoids was detected as follows:

Salkowski test: Five ml of each extract was mixed with 2 ml of chloroform, with a careful overlaying of 3 ml concentrated sulphuric acid. The formation of a reddish-brown precipitate at the interface indicated the presence of terpenoids [23].

Cardiac glycosides (Keller-Killani test)

The cardiac glycosides were determined by adding 5 ml of each extract in 2 ml of glacial acetic acid containing one drop of ferric chloride solution that was underlayed with 1ml of concentrated sulphuric acid. The appearance of brown ring at the interface indicated the presence of deoxysugar, which is a characteristic of cardenolides [20,21].

Carbohydrates

The presence of carbohydrates in each extract of *Helicia nilagirica* was detected by the Benedict's test, where the filtrates of the extracts were treated with Benedict's reagent and heated gently. The appearance of orange red precipitate indicated the presence of reducing sugars.

Quantitative Determination of The Phytochemicals

The quantitative determination of saponins and flavonois was also carried out as described below.

Determination of Saponins

20 g of *Helicia nilagirica* powder was weighed in 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 in a water bath at 90 °C. The concentrate was transferred into a 250 ml separating funnel with the addition of 20 ml of diethyl ether 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 [24].

Determination of Flavonoids

Ten g of the bark powder of *Helicia nilagirica* was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed [25].

Determination of Moisture Content

Determination of the amount of volatile matter (i.e., water drying off from the drug) in the *Helicia nilagirica* is a measure of loss after drying of substances appearing to contain water as the only volatile constituent. The powdered bark of *Helicia nilagirica* 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/W x 100

Where Pw = Preweighed sample

Fw = Final weight of the dried sample

W = Total weight of the sample

Ash values

The ash values including total and acid insoluble ash were determined to estimate the total amount of the inorganic salts present in the drug. The ash contents remained after ignition of plant material was determined by two different methods to measure total and acid insoluble ash contents.

Total Ash Contents

The method measures the total amount of material remaining after ignition including both 'physiological ash', derived from the plant tissue itself, and 'non-physiological ash' which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface.

Procedure: Two grams of ground air-dried material of *Helicia nilagirica* was accurately weighed in a previously ignited and tared crucible. The material was spread as an even layer and ignited by gradually increasing the temperature up to 500-600˚C until it became white, indicating the absence of carbon. The crucible was cooled and weighed. The percentage of total ash content was calculated according to the following formula. Total ash content = Pw-Fw /W x 100

Where Pw = Preweighed crucible

Fw = Final weight of the crucible containing ash

W = Total weight of powdered plant material

Extractive Values

These are used to determine the amount of the matter which is soluble in the solvents used including alcohol and water. The percentage of alcohol and water-soluble extractives were calculated and used as standards.

Determination of Alcohol-Soluble Extractive

Procedure: Five grams of air dried coarsely powdered material was macerated in 100 ml of alcohol in a closed conical flask for twenty-four hours, with frequent shaking during first six hours and allowed to stand for next eighteen hours thereafter it was filtered rapidly with caution to avoid loss of solvent. The 25 ml of the filtrate was evaporated to dryness at 105˚C in a tared flat-bottomed shallow dish and weighed. The percentage of alcohol-soluble extractive was calculated with reference to the air-dried material.

Determination of Water-Soluble Extractive

Procedure: Five grams of coarsely powdered air-dried material was macerated in 100 ml of chloroform-water (0.1%) in a closed flask for 24 h, shaken frequently until six hours and allowed to stand for another eighteen hours. Thereafter it was filtered rapidly, with precautions to avoid loss of solvent by evaporation. The 25 ml of the filtrate was evaporated to dryness at 105˚C in a tared flat-bottomed shallow dish and weighed. The percentage of water soluble extractive was calculated with reference to the air-dried material. All the tests were done in triplicate.

TLC Analysis

TLC is a simple and rapid technique that is able to determine the number of components present in solution and helps in finding a suitable solvent for separating the components by column chromatography as well as for monitoring reactions' progress. The chloroform ethanol and aqueous extracts of *H. nilagirica* were spotted on to a number of TLC plates (Merck India, Mumbai) in 1 mm diameter above the bottom of the plates and placed into different mobile phases. The extracts were allowed to move on the adsorbent (Stationary) phase according to the solvent system used. Several combinations of solvents of increasing polarity were evaluated as mobile phase for TLC run to determine the number of compounds present in different extracts of *Helicia nilagirica*. The different solvent systems were used as mobile phase for TLC, which consisted of chloroform: methanol (9:1, 8:2), pure chloroform, chloroform: ethyl acetate (1:1) and methanol: hydrochloric acid (9:1) solvent combinations. The resultant spots were observed under visible and ultra-violet light at 254 nm and 365 nm. The measure of the distance of a compound travelled is considered as the

retention factor (F_{ry}) value which was calculated using the following formula: -

F_{ry}= Distance travelled by solute/Distance travelled by solvent

Results

The results of phytochemical analyses and TLC profiling of *Helicia nilagirica* are presented in Table 1-6 and Figures 1-3.

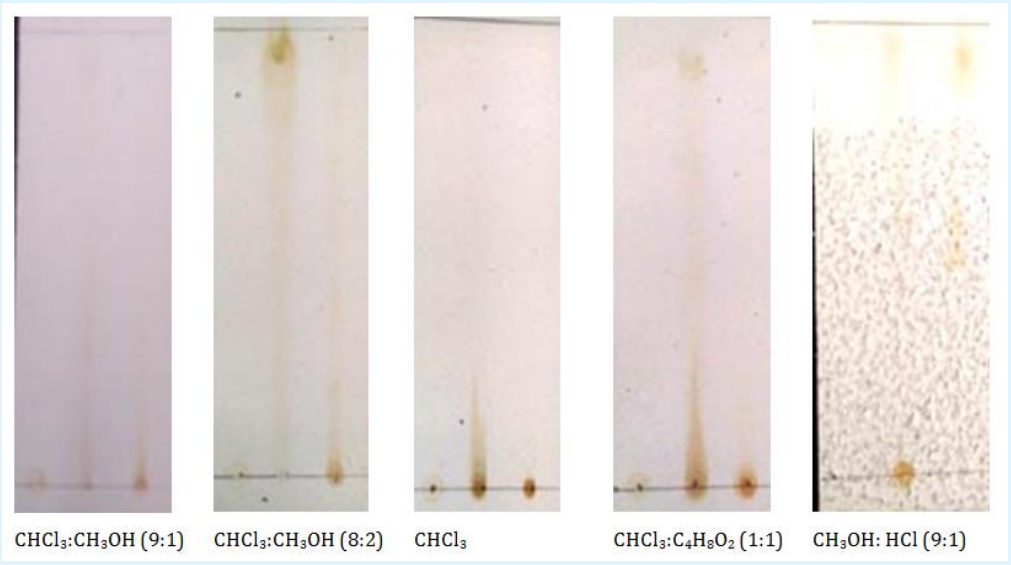

Figure 1: TLC profile of different extracts of *H. nilagirica* in different solvent systems observed under normal light to detect various phytochemicals present in the extracts. (aqueous, chloroform and ethanol). Lanes in each TLC plate: Left- aqueous; middle- chloroform and right-ethanol.

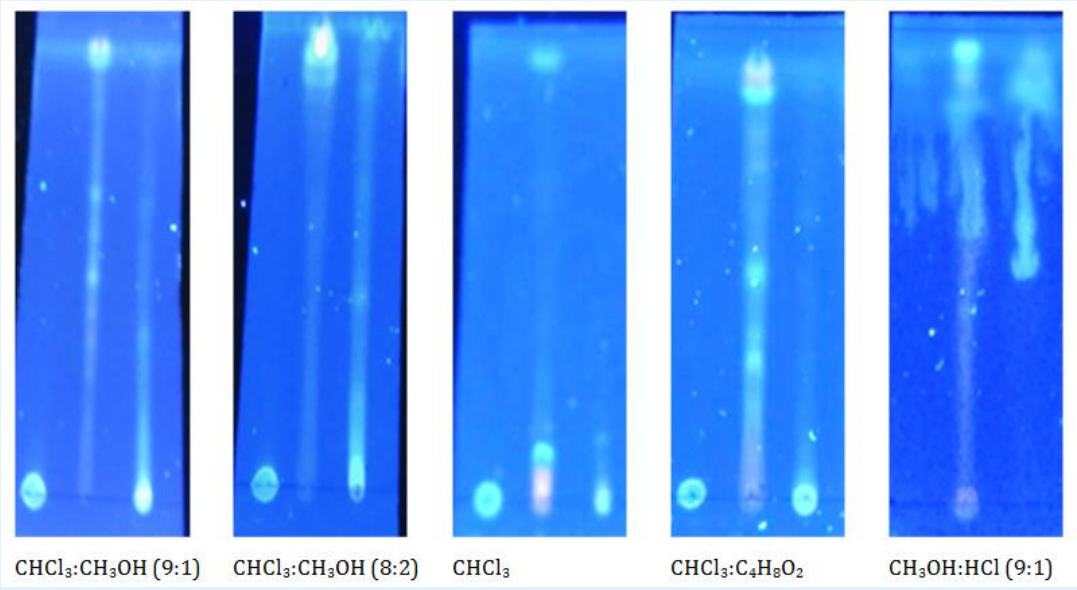

Figure 2: TLC profile of *H. nilagirica* with different solvent systems observed under UV 365 nm to detect phytochemicals present in the different extracts. Lanes in each TLC plate: Left- aqueous; middle- chloroform and right-ethanol.

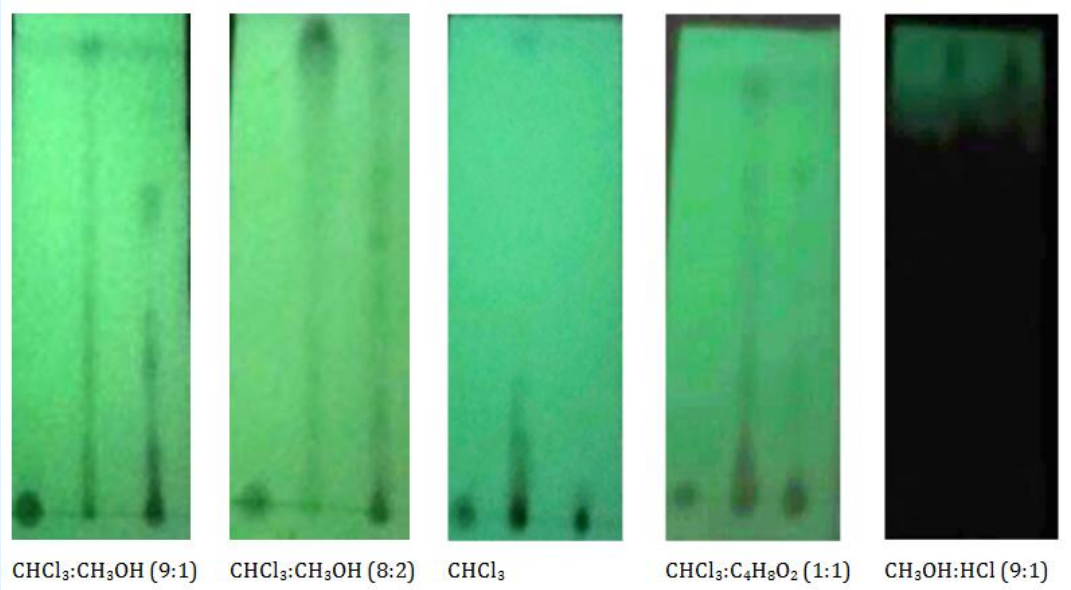

Figure 3: TLC profile of *H. nilagirica* on different solvent systems observed under UV 254 nm to detect phytochemicals present in the extracts. Lanes in each TLC plate: Left- aqueous; middle- chloroform and right-ethanol.

Phytochemical analysis

The phytochemical screenings of chloroform extract of *Helicia nilagirica* showed the presence of flavonoids, tannins, terpenoids, diterpenes, cardiac glycosides, whereas the alkaloids, saponins and carbohydrates were conspicuous by their absence (Table 1). The phytochemical analysis of ethanol stem bark extract of *Helicia nilagirica* revealed that it contained only flavonoids, phenols, tannins and cardiac glycosides. The other phytochemicals like, alkaloids, saponins, terpenoids and carbohydrates. flavonoids, phenol and terpenoids were not detected (Table 1). In aqueous extract tannins, saponins, cardiac glycosides and carbohydrates were detected, whereas all other phytochemicals could not be detected and the phlobatannins were absent in all extracts (Table 1).

Tests	Chloroform extract	Ethanol extract	Water extract
Alkaloids	-	-	-
Phenols	+	+	-
Flavonoids	+	+	-
Tannins	+	+	+
Terpenoids	+	-	-
Phlobatannins	-	-	-
Diterpenes	+	-	-
Saponins	-	-	+
Cardiac glycosides	+	+	+
Carbohydrates	-	-	+

Table 1: Results of the Phytochemical analysis of *Helicia nilagirica*.

Quantitative determination of phytochemicals

The quantitative determination of the chemical constituents showed that *Helicia nilagirica* contained 9.26% and 0.26% flavonoids and saponins, respectively (Table 2).

Plant	Flavonoid			Saponin		
Helicia nilagirica	Quantity	Output	%	Quantity	Output	%
	10g	0.9256g	9.256	20g	0.051g	0.26

Table 2: Quantitative determination of the chemical constituent of *Helicia nilagirica*.

Determination of moisture content

The drying of 500 g of *Helicia nilagirica* bark yielded 296 g of dried bark, and this reduction in weight was due to 40.6% loss in its water contents. The analysis of dried bark of Helicia *nilagirica* showed presence of 25.63% moisture (Table 3).

Weight before drying (kg)	Weight after drying (kg)	Loss after drying (%)	Moisture content (%)
0.5	0.296	40.6	25.63

Table 3: Weight loss on drying fresh bark of *Helicia nilagirica*.

Determination of total ash content

The ash content of the crude bark powder was found to be 3.24% (Table 4).

Total ash (%)	Ethanol- soluble extractive (%)	Water- soluble extractive (%)
3.24	3.4	8

Table 4: Physicochemical parameters of dried bark powder of *Helicia nilagirica*.

Determination of extractive values

The *Helicia nilagirica* bark was found to contain 3.4% ethanol-soluble and 8% water-soluble extractives (Table 4).

Extract yield: The extraction of *Helicia nilagirica* stem bark yielded 2%, 4% and 6% chloroform, ethanol and water extracts (Table 5).

Dried powder	Chloroform extract (%)	Ethanol extract (%)	Water extract (%)
100g	2	4	6

Table 5: Yield of various extracts of *Helicia nilagirica.*

TLC Analysis

The evaluation of chloroform and ethanol extracts of *Helicia nilagirica* showed the presence of different components as indicated by a varying number of spots and colours on a TLC plates using UV visualization method (Table 6).

Extract	Solvent	Day light	R_f value	UV 254 nm	R_f value	UV 365 nm	R_f value
Chloroform	$CHCl_3$:CH_3OH 9:1	Streak	-	Two spots	0.92, 0.53	5 spots (1 red,1 blue, 3 yellowish)	0.92, 0.86, 0.57, 0.5 & 0.42
Ethanol		Streak	-	3 spots	0.57, 0.28 & 0.09	Not clear	-
Aqueous		Not visible	-	Not visible	-	Not visible	-
Chloroform	$CHCl_3$:CH_3OH-8:2	One spot	0.94	1 spot	0.94	3 spots (1 red, 2 bluish)	0.94, 0.88 & 0.84
Ethanol		Streak	-	3 spots	0.90, 0.82 & 0.5	1 spot (bluish)	0.38
Aqueous		Not visible	-	Not visible	-	Not visible	-
Chloroform	$CHCl_3$	Not visible	-	1 spot	0.94	3 spots (1 bluish, 2 red)	0.94, 0.09 & 0.05)
Ethanol		Not visible	-	Not clear	-	1 spot (bluish)	0.11
Aqueous		Not visible	-	Not visible	-	Not visible	-
Chloroform	$CHCl_3$:$C_4H_8O_2$-1:1	One spot	0.9	3 spots	0.90, 0.69 & 0.48	4 spots (1 reddish, 3 bluish)	0.90, 0.86 & 0.84
Ethanol		Not visible	-	3 spots	0.69, 0.42 & 0.23	Not visible	-
Aqueous		Not visible	-	Not visible	-	Not visible	-

Chloroform	CH_3OH:HCl (9:1)	One spot	0.86	1 spot	0.84	2 spots (1 reddish, 1 bluish)	0.94 & 0.79
Ethanol		Two spots	0.82 0.43	1 spot	0.84	Not clear	-
Aqueous		Not visible	-	Not visible		Not visible	-

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

Discussion

Plants synthesize several phytochemicals, which have played an important role in the development of new therapeutic agents. The preliminary qualitative phytochemical analysis of the bark of *Helicia nilagirica* revealed the presence of phenol, flavonoid, tannins, saponins, cardiac glycosides and carbohydrates. These phytochemicals synthesized by plants are essential for the growth, pathogen attack, pollination, defence and other activities of plants [26,27]. However, at the same time these phytochemicals are of great use for humans as a source of drugs and other healthcare agents [28-30].

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure which are ubiquitously present in plants. Approximately, more than 10000 flavonoids have been identified [31,32]. They can be divided into a variety of classes such as flavones (e.g., apigenin, and luteolin), flavanols (e.g., quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others [33,34]. They are the hydroxylated phenolic substances synthesized by plants in response to microbial infection [35]. Flavanols are the most abundant flavonoids in foods and they are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes [36]. Flavonoids have been consumed by humans since the advent of human life on earth. They have extensive biological properties that promote human health and help to reduce the risk of diseases and they are known to possess antioxidant, hepatoprotective, antibacterial, anti-inflammatory, anticancer and antiviral properties [37-41]. The *Helicia niglagirica* have shown the presence of flavonoids like other plants. These results are in conformation with earlier studies, where the presence of flavonoids has been reported in the methanol extract of *H. nilagirica* [15]. The flavonoids have also been detected in *H. nilagirica* recently [19]. Earlier, flavonoids were detected in *Croton caudatus* and *Oxylium indicum* and several other plants from our laboratory [28,29].

The presence of tannins in the *Helicia nilagirica* is in conformation with other studies where various plants have been reported to contain tannins [28,29]. Tannins are polyphenols which occur widely in vascular plants particularly associated with woody tissues. They are water soluble and have molecular weights ranging between 500 and 3000 Daltons. Based on the chemical structures, tannins are divided into two groups: hydrolysable, and condensed. The hydrolysable tannins consist of gallic acid esters, and ellagic acid glycosides [41]. They possess an amazing astringent property, which is mainly related to their drug applications. They are known to be antimicrobial, antifungal, anthelmintic, antiviral, antiulcer. They are known to hasten the healing of wounds and alleviate inflammation in mucous membranes [42-44]. They exert internal anti-diarrheal and antiseptic effects by waterproofing the outer layers of more exposed mucous membranes. Tannins are also haemostatic, and can serve as an antidote in poisoning cases [45]. In the process of healing wounds, burns and inflammations, tannins help by forming a protective layer (tannin-protein/tannin-polysaccharide complex), over injured epithelial tissues permitting the healing process below to occur naturally. Studies show that many tannins act as radical scavengers, intercepting active free radicals [41]. Various degenerative diseases such cancer, multiple sclerosis, atherosclerosis and aging process itself are associated with high concentrations of intercellular free radicals and tannins are useful in these conditions.

Terpenoids are synthesized from five carbon isoprene units mainly isopentenyl pyrophosphate and its isomerdimethylallyl pyrophosphate by the enzyme terpene synthases. They are classified according to whether they contain two (C_{10}), three (C_{I5}), four (C_{20}), six (C_{30}) or eight (C_{40}) isoprene units. They range from the essential oil components, the volatile mono-and sesquiterpenes (C_{10} and C_{15}) through the less volatile diterpenes (C_{20}) to the involatile triterpenoids and sterols (C_{30}) and carotenoid pigments (C_{40}). Each of these various classes play a significant role in plant growth, metabolism or ecology [20]. Approximately 40,000 terpenes have been identified and the possible functions of majority of these molecules are not known [46]. Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer. They possess antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, anti-malarial, antihyperglycemic, anti-

inflammatory and immunomodulatory properties [47-51]. Terpenoids can also be used as protective substances in storing agriculture products as they are known to have insecticidal properties as well [52].

Saponins are naturally occurring structurally and functionally diverse phytochemicals that are widely distributed among seventy families of plants. They are glycosides of both triterpenes and sterols [53]. Due to the presence of both the hydrophobic aglycone backbone and hydrophilic sugar molecules the saponins are highly amphipathic and possess foaming and emulsifying properties. They play an important role in plant ecology and they are also exploited for a wide range of commercial applications in the food, cosmetic and pharmaceutical sectors [54,55]. These molecules are potent membrane permeabilizing agents with immunostimulatory, hypocholesterolemic, anti-carcinogenic, anti-inflammatory, anti-microbial, anti-protozoan, molluscicidal and have anti-oxidant properties [56,57]. Saponins also act as antitumor agents by inhibiting tumor cell growth and inducing apoptosis [42]. Terpenoids and saponins were detected in *Helicia nilagirica* like other plants. An earlier study has also reported the presence of saponins in *Helicia nilagirica* [19]. Likewise, several other plants have been found to synthesize terpenoids and saponins [28,29].

Cardiac glycosides composed of two structural features: The sugar (glycoside) and the non-sugar (aglycone-steroid) moieties and they act on the contractile action of the cardiacmuscle. These compounds have long been used for the treatment of cardiac arrhythmias and congestive heart failure due to their capability to increase the contractile force [58]. Digitalis is the most commonly used cardiac glycoside, which directly inhibits the proliferation of androgen dependent and androgen independent prostate cancer cell lines by initiating apoptosis and increasing intracellular Ca^{2+}. Cardiac glycosides have been reported to inhibit the four genes that are over expressed in prostate cancer cells including the inhibitors of apoptosis and transcription factors [59]. Cardiac glycosides have been reported to act as active anticancer agents [60,61]. They are also reported to have antiviral properties against human cytomegalovirus [62]. The *Helicia nilagirica* has been found to contain cardiac glycosides. Similarly, the glycosides have been detected in the methanol extract of stem bark of *H. nilagirica* [15]. The other plants have been reported to contain the cardiac glycosides [28,29]. The presence of these phytochemicals may have been responsible for its anticancer activity in our earlier study [17].

Conclusions

The phytochemical analyses have revealed the presence of different phytochemicals including, phenols, flavonoids, tannins, terpenoids, saponins, cardiac glycosides in the different extracts. However, alkaloids and phlobatannins were not present in this plant. The presence of various phytochemicals affirms its ethnomedicinal use in Mizoram.

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Conflict of interest statement

The authors do not have any Conflict of interest statement to declare.

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