EVALUATION AND CHARACTERIZATION OF SELECTED PLANT SPECIES OF MIZORAM FOR THEIR CYTOTOXICITY AGAINST DIFFERENT CANCER CELL LINES

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

MALSAWMDAWNGLIANA

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DEPARTMENT OF HORTICULTURE AROMATIC AND MEDICINAL PLANTS
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SUBMITTED
IN PARTIAL FULFILLMENT OF THE REGUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN HORTICULTURE AROMATIC AND MEDICINAL PLANTS OF MIZORAM UNIVERSITY, AIZAWL

DECLARATION

I, **Malsawmdawngliana**, hereby declare that the subject matter of this thesis entitled "**Evaluation and characterization of selected plant species of Mizoram for their cytotoxicity against different cancer cell lines.**" is the record of the work done by me, that the contents of this thesis did not form basis of the award of any previous degree 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 for the degree of **Doctor of Philosophy** in Horticulture Aromatic and Medicinal Plants.

(MALSAWMDAWNGLIANA)

HEAD	SUPERVISOR
PROF. T.K. HAZARIKA	DR. AWADHESH KUMAR

CERTIFICATE

This is to certify that the thesis entitled **"Evaluation and characterization of selected plant species of Mizoram for their cytotoxicity against different cancer cell lines** submitted by **Mr. Malsawmdawngliana (**Ph.D. Regn. No.**MZU/Ph.D/1001 of 30.05.2017)**, in partial fulfillment of the requirements for the award of degree of Doctor of Philosophy in Horticulture Aromatic and Medicinal Plants of Mizoram University, Aizawl embodies the record of his original investigation under my supervision. He has duly registered and the thesis presented is worthy of being considered for the award of the Doctor of Philosophy (Ph. D.) Degree. The work has not been submitted previously for any degree to this or any other university.

(Dr. Awadhesh Kumar)

Supervisor

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Place: Aizawl.

Date: (**MALSAWMDAWNGLIANA**)

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LIST OF ABBREVIATIONS

°	Degree
Km	Kilometer
C	Celcius
Cm	Centimeter
WHO	World Health Organisation
%	Percentage
BC	Before Christ
TGF	Tumour Growth Factor
PDGE	Platelet Derived Growth Factor
ml	millilitres
µ	Microliters
g	gram
GAE	Gallic Acid Equavalent
pRb	retinoblastoma protein
DNA	Deoxyribonucleic acid
RNA	Ribonucleic Acid
PARP	Poly Adenosine Diphosphate-ribose Polymerase
mTOR	mammalian Target of Rapamycin
mg	Milligram
LDLR	Low-Density Lipoprotein Receptor
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
BHA	Butylated Hydroxyanisole
FRAP	Ferric-reducing antioxidant power
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
RSV	Respiratory Syncytial Virus
CPE	Cytopathic Effect
DW	Distilled water

Hrs	hours
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
$AlCl_3$	Allunimium chloride
NBT	Nitro blue tetrazolium
DMSO	Dimethyl sulfoxide
BCG	Bromocresol green solution
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
EDTA	Ethylene diamine tetra-acetic acid
UV-VIS	Ultraviolet–visible
CO_2	Carbon dioxide
3D	3 Dimentional
RCSB	Research Collaboratory for Structural Bioinformatics
PDB	Protein Data Bank
RAF	Rapidly Accelerated Fibrosarcoma
CDK2	Cyclin-dependent kinases
π	Pi
HIV	human immunodeficiency virus
ATP	Adenosine triphosphate

Chapter-1

INTRODUCTION

INTRODUCTION

1. Mizoram

Mizoram, a state in north-east India, is located between 21° 58′ and 24° 35′ north latitude and 92° 15′ and 93° 29′ east longitude. Mizoram's total area is 21,087 km2, with a total forest area of 15,825 km2. The elevation varies from 500 to 2157 metres. Summer temperatures range from 18 to 29°C, while winter temperatures range from 11 to 24°C. There is no snow in Mizoram, but frost can be seen in the eastern part of the state. Monsoon has a direct impact on Mizoram, with strong rains from May to September with an average annual rainfall of 245 cm (Lallianthanga 1990; Anonymous 1994)

Mizoram is a small state in India's north-east, bordering Myanmar and Bangladesh. It is part of the Indo-Burma biodiversity hotspots (Sharma et al., 2001). The state is rich in dense forest, which supports a diverse range of species and medicinal plants. Several rural tribal people in Mizoram still rely on plant-derived herbal medicines for their primary health care needs for the treatment of disorders like scorpion stings, insane instances, skin problems, and oxidative damage.

According to the World Health Organization, over three-quarters of the population in underdeveloped nations still rely on plant-based treatments as the basic necessity of primary health care in their conventional system. In light of such admiration, it looks feasible to establish a scientific foundation for the medicinal plants' potential application in second-hand conventional treatments to heal a variety of diseases for thousands of years. As a result, a thorough scientific investigation is required. (Rai and Lalramnghinglova 2010), WHO 2003.

1.1 Medicinal Plants

Plants have been utilised as medicine since the dawn of humanity, and many current medications are produced either directly or indirectly from plants (Newman and Cragg, 2014). Many modern medications for the treatment of various diseases, including cancer, are derived from natural sources (Mathieu et al., 2015). Since the 1940s, natural compounds have accounted for over 75% of the small antitumor chemicals reported (Newmann and Cragg, 2014).

Many chemotherapeutic medications were derived from natural sources, such as flowering plants, before they were chemically synthesised. Vinblastine and vincristine, two vinca alkaloids isolated from the Madagascar periwinkle *Catharanthus roseus*, are used in combination with other medications to treat a variety of diseases, including leukaemia, testicular cancer, and both Hodgkin and non-Hodgkin lymphomas (Moudi et al., 2013). Another anticancer compound, taxol, was discovered in the bark of the Pacific Yew, Taxus brevifolia (Rowinsky et al., 1995; Creemers et al., 1996; Bertino, 1997), and is used to treat endometrial cancer, cervical carcinoma, breast cancer, non-small-cell lung cancer, and bladder cancer (Rowinsky et al., 1995; Creemers et al., 1996; Bertin (Hajek et al., 2005; Khanna et al., 2015). The podophyllotoxins etoposide and teniposide, which have been identified from *Podophyllum peltatum* and *Podophyllum hexandrum*, are another family of cytotoxic medicine obtained from plants. In clinical practise, they are used alone or in conjunction with other chemotherapeutic medicines to treat testicular cancer, leukemias, and small cell lung cancer (Liu et al., 2016.). Camptothecin, a chemotherapy agent discovered from *Camptotheca acuminata* in 1966, was the other plant-derived chemotherapeutic medication; nonetheless, its insolubility was a big problem. Topotecan and irinotecan, which are utilised in the treatment of ovarian cancer, cervical cancer, small-cell lung cancer, and colorectal cancer, were synthesised to tackle this problem (Venditto and Simanek, 2010). The extreme toxicity of these contemporary synthetic chemicals, as well as the development of drug resistance, has resulted in therapeutic failure (DeVita and Chu, 2008; Housman et al., 2014).

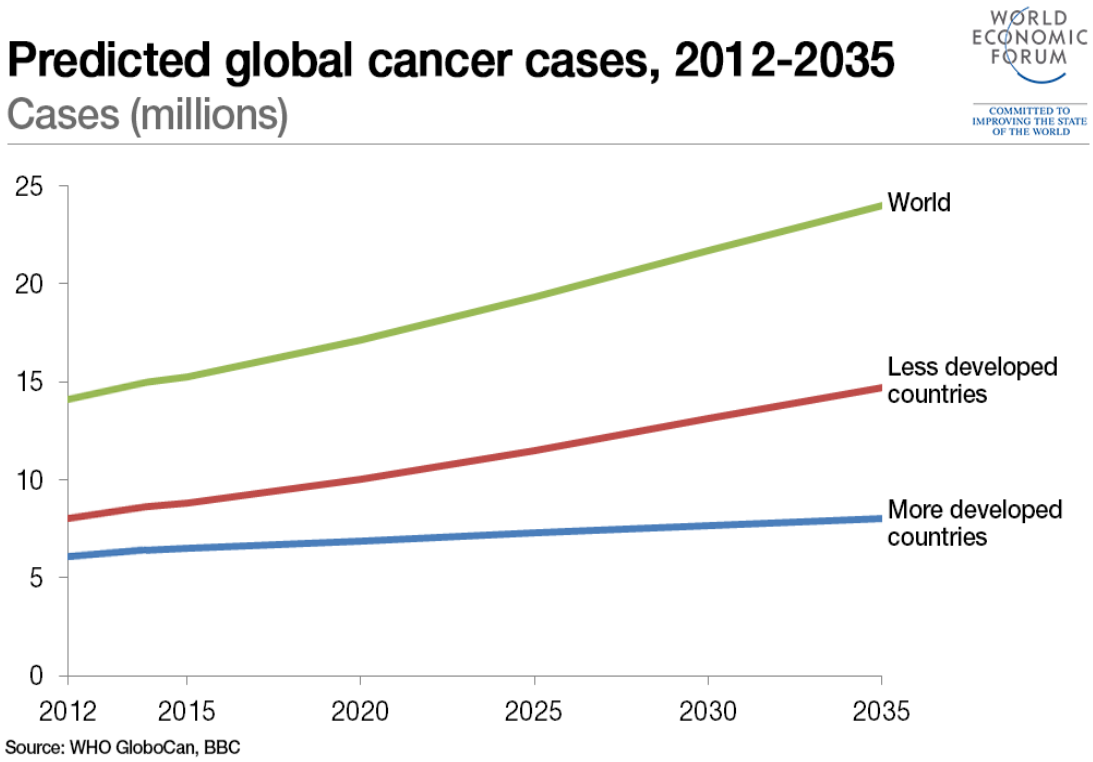
Cancer treatment is always a multimodality approach, combining surgery, radiation, and chemotherapy to cure the disease. Radiation and chemotherapy are

hazardous to normal cells, and their use as a treatment for cancer has been linked to the development of secondary cancers in survivors (Kumar, 2012; Pendelton et al., 2014; Murray et al., 2015). This suggests that plants may yet be able to provide novel compounds with cancer-curing properties. Plant secondary metabolites are thought to be a possible source for cancer treatment, fueling worldwide study on traditionally used medicinal plants (Demain and Vaishnav, 2011).

1.2 Cancer

Cancer is a category of disorders characterised by mutations or dynamic changes in a cell's genome that produce proteins that disrupt the cell's normal biological balance, resulting in uncontrolled cell proliferation (Bishop and Weinberg, 1996; Hejmadi, 2010; American Cancer Society, 2015). Though cell proliferation is required for embryogenesis, development, and correct function in a variety of adult tissues, it can also lead to cancer and death if its regulating mechanisms are absent or dysregulated (DeBerardinis et al., 2008). The cells start the active proliferative phase only after receiving mitogenic growth signals in normal conditions, and they cannot multiply without them. Cancer cells, on the other hand, are no longer dependent on these stimulatory signals and can multiply whether or not these signals are present (Hanahan and Weinberg, 2010). Cancer cells have the potential to manufacture their own growth factors that are similar to normal growth factors, making them self-sufficient (Fedi et al., 1997).

Even if there are over 100 different forms of cancer and subtypes from a single cause, the six hallmarks of cancer are six traits that most cancers share. Insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), boundless replicative capacity, prolonged angiogenesis, and tissue invasion and metastasis are all examples of self-sufficiency in growth signals (Hanahan and Weinberg, 2000). Later, the ability to reprogramme energy metabolism and elude immune destruction were added to the six cancer hallmarks (Sonnenschein and Soto, 2013).



1.3 History

Tumors were found in animals in prehistoric times, long before humans arrived on the scene, according to paleopathologic data. The Edwin Smith Papyrus, written around 3000 BC, contains the earliest recorded account of cancer, a breast carcinoma. The Ebers Papyrus, which dates from around 1500 BC, has the first mention of a soft-tissue tumour, a fatty tumour, as well as references to skin, uterus, stomach, and rectum tumours (Ebbell 1937). To treat cancer, the Sumerians, Chinese, Indians, Persians, and Hebrews of the same era employed herbal medicines including tea, fruit juices, figs, and cooked cabbage, but in advanced instances, they did not hesitate to use iron, copper, sulphur, and mercury solutions and pastes. For more than 3000 years, several of these mixtures were used externally and internally in varied concentrations (Wolff 1928; Castiglioni 1931). Although the Egyptians and Greeks used chemical agents sporadically before the Common Era (BC), Paracelsus (1493-1541) introduced mercury, lead, sulphur, iron, zinc, copper, arsenic, iodine, and potassium as internal treatments in the 16th century (Paracelsus 1562). In 1829, a French gynaecologist named Joseph Recamier (1774-1852) coined the term

metastasis. He was able to detect cancer blood vessel invasion with the naked eye by studying the growth and spread of malignancies. Cancers were first defined by Johannes Muller in 1838 as "special clusters of aberrant cells and stroma." Cancer, he said, is caused by the development of new cells in damaged organs that have the potential to be destructive and spread to other parts of the body through vascular invasion. He linked cancer to ageing and identified tumour necrosis (apoptosis) as a regressive symptom. He was able to tell the difference between epithelial and connective tissue cancers using microscopy. Sarcoma simplex (squamous carcinoma), carcinoma alveolare (adenocarcinoma), carcinoma fasciculatum (spindle cell carcinoma), carcinoma medullare (medullary carcinoma), and carcinoma melanodes were Muller's classifications for malignant epithelial tumours (malignant melanoma). Muller defined infiltrating fibrous tumours (desmoid tumour and fibrosarcoma), cystosarcoma of the breast, chondrosarcoma, and osteosarcoma of the bones as malignant connective tissue tumours. Virchow developed the terms "hyperplasia" and "metaplasia" in 1846, recognising that both are probable cancer precursors and that cancer cells differ significantly in size and shape from benign cells. Julius Cohnheim proposed the embryonal features in 1877. Moritz Wilhelm Hugo Ribbert, a Zurich pathologist, hypothesised twenty years later that mechanical irritation, such as chronic inflammation and trauma, can lead to the formation of cancer, particularly in epithelial and connective tissue cells (Cohnheim 1877; Ribbert 1904). For decades, Schistosoma haematobium was thought to be the sole microorganism that caused cancer (bladder) until Clonorchis sinensis, a causal factor for bile cancer, was discovered (Harrison 1889). Despite the fact that medicine had advanced significantly in certain ancient civilizations, progress in cancer therapy has been slow, and the disease is still feared today due to the persisting belief that it is an incurable disease (Diamandopoulus 1996; Kardinal 1979).

1.4 CHARACTERISTICS OF CANCER AND CANCER CELLS

In a normal state, the adult human body contains approximately 10^{15} cells, of which approximately 10 are formed, which divide and differentiate each day to replace the dead and worn-out cells. These cells will only enter the active proliferative phase after receiving mitogenic growth signals, and they will not

multiply without them. Cancer cells, on the other hand, have lost their requirement for these stimulatory signals and can proliferate whether or not these signals are present, producing their own growth factors that imitate regular growth factors, making them independent of normal growth factors (Fedi et al. 1997; Hanahan and Weinberg 2000). Despite the fact that there are over 100 different types of malignancies and tumours, and each organ has subtypes, human cancers have certain essential traits. Hanahan and Weinberg postulated the 'hallmarks of cancer,' a set of six characteristics that are shared by all malignancies (Hanahan and Weinberg, 2000). These capabilities included:

i. Growth signal self-sufficiency: Tumor cells have a lower reliance on exogenous growth stimulation because they generate their own growth signals by altering extracellular growth signals, transducers of those signals, and intracellular circuits that translate those signals into action, disrupting the tissue's normal homeostatic mechanism. Glioblastomas and sarcomas, for example, produce PDGF (platelet-derived growth factor) and TGF (tumour growth factor), respectively.

ii. Anti-growth signal sensitivity: Tumor cells can dodge antiproliferative signals by altering the retinoblastoma protein (pRb) pathway, which prevents cells from progressing through the G1 phase of the cell cycle, allowing them to proliferate and making them immune to antigrowth factors (Weinberg 1995).

iii. Evasion of apoptosis: Mutations in the p53 tumour suppressor gene, which is seen in more than half of all human malignancies, can make cancer cells resistant to apoptosis (Harris 1996).

iv. Limitless replicative potential: The three typical acquired capacities of cancer cells, such as growth signal autonomy, antigrowth signal insensitivity, and apoptosis resistance, can lead to a decoupling of a cell's growth programme from external signals. This is accomplished by increasing the expression of the telomerase enzyme.

v. Sustained angiogenesis: Because the development of new blood vessels is required for tumour cells to grow quickly, tumour cells appear to engage the angiogenic switch by altering the balance of angiogenesis inducers like VEGF and countervailing inhibitors like thrombospondin-1 (Hanahan and Folkman 1996).

vi. Tissue invasion and metastasis: During the progression of most human malignancies, tumour cells spread out and infect surrounding organs, where they may succeed in establishing new colonies through a process known as metastasis. Metastases are responsible for approximately 90% of all cancer deaths in humans (Sporn 1996). The lack of function of E-cadherin due to mutational inactivation, transcriptional repression, or proteolysis of the extracellular cadherin domain in the majority of epithelial malignancies is one of the reasons for this capability (Christofori and Semb 1999).

1.4.1 Treatment

Surgery, radiation, and systemic therapy are commonly used in cancer treatment. Low-risk patients with early-stage cancer can typically be cured with just surgery; however, many cases require combination treatment, which involves combining one or more treatment modalities to cure cancer. Hormonal therapy, targeted therapy, and chemotherapy are examples of systemic therapies. It is the only option if the disease has spread since cancer cells must be delivered through the bloodstream to multiple locations (Caley and Jones, 2012).

1.4.2 Surgery

Surgery is the earliest method of cancer treatment, and it is still employed to treat solid tumours in the modern world. If cancer is identified early enough, surgery can be utilised to cure it. When used alone, it is known to be the most effective and has a better success rate for cancer treatment than other forms of treatment (Harvey, 1974; Caley and Jones, 2012). Surgery, on the other hand, has its own set of constraints. Some cancer cells may be overlooked during surgery, and removing more of the surrounding tissues in order to eliminate all cancer cells can result in

organ loss or diminished function. Furthermore, it cannot be utilised to treat cancer that has already spread to other parts of the body.

1.4.3 Radiation

Radiation treatment is the use of radiation to eliminate cancer cells. Despite the fact that radiation is thought to cause cancer, research over the last century has shown that low amounts of ionising radiation can be beneficial. Evidence also revealed that complete or half-body low-dose irradiation could cure cancer or greatly slow its course, reducing cancer mortality without causing noticeable side effects. When cobalt-60 gamma radiation became available in the 1950s, it became widely used as a cancer treatment, followed by particle accelerators in the 1970s (Cuttler and Pollycove, 2003). It can also be given with a palliative intent, which means that the goal is to treat the symptoms of cancer rather than cure it. Radiation delivered prior to surgery to decrease the tumour is known as neoadjuvant therapy, and radiation given after surgery to eradicate the microscopic tumour cells is known as adjuvant therapy. By depositing energy in the form of ions, ionising radiation kills or causes genetic changes in the cells it passes through, creating single or double strand breaks in DNA (Lomax et al., 2013). This DNA damage affects not only tumour cells, but also normal cells close to or surrounding the tumour. This alters the genome's integrity, resulting in cancer recurrence. Cancer cells, on the other hand, are less effective than normal cells at repairing damage, resulting in differential cancer death (Begg et al., 2011). One of the most significant limitations of radiotherapy is that tumour cells developing in a low-oxygen environment, known as hypoxia, are 2 to 3 times more resistant to radiation damage than those growing in a normal oxygen environment (Harrison et al., 2002). In such cases, combined therapy is frequently used.

1.4.4 Chemotherapy

Chemotherapy is a cancer treatment that uses chemicals to kill cancer cells. Chemotherapy medicines have different mechanisms of action. Paul Ehrlich, the pioneer of chemotherapy, described the first alkylating agent in 1898. (Mann, 1999). He was also the first to demonstrate the efficacy of in vivo models for screening a

series of compounds for possible anti-disease action, a feat that has far-reaching implications for cancer medication development (DeVita and Chu, 2008). For the treatment of various forms of malignancies, there are over 100 distinct types of chemotherapeutic medicines that can be used alone or in combination. Cancer cells' DNA is directly damaged by alkylating chemicals, which prevents them from replicating. They can be used to treat a variety of cancers because they can kill cancer cells at any stage of the cell cycle. Alkylating agents include nitrogen mustards, nitrosoureas, alkyl sulfonates, triazines, and ethyleneamines (Colvin, 2003). Antimetabolites, unlike alkylating agents, function solely during the S-phase of the cell cycle and cause programmed cell death. They disrupt mitosis by stopping the production of DNA or RNA by blocking the enzymes required for DNA synthesis. They can also insert themselves into DNA because many of them have nucleotide-like structures. Anti-metabolites include anti-folates, fluoropyrimidines, deoxynucleoside analogues, and thiopurines, all of which are used in clinics to treat cancer (Lind, 2008; Parker, 2009). The drugs that can cause microtubule dysfunction are split into two categories: those that hinder microtubule assembly, such as vinca alkaloids, and those that block microtubule disassociation, such as taxanes. Despite their opposing mechanisms of action, both eventually cause apoptosis by inhibiting mitosis. Vincristine and vinblastine are vinca alkaloids recovered from Catharanthus roseus, whereas paclitaxel extracted from Taxus brevifolia is a taxanes (Lind, 2008; Yue et al., 2010; Liu et al., 2007). Cytotoxic antibiotics, on the other hand, stop cell division by intercalating into the DNA. The antibiotic category has three subgroups: doxorubicin, anthracycline, and bleomycin. They're used to treat a variety of cancers, including breast, ovarian, bladder, and lung cancers, as well as lymphomas and sarcomas (Chabner and Longo, 2001). Chemotherapy medications are delivered in treatment cycles, which are repeated at regular intervals. Each cycle kills a fraction of the cells, rather than a steady quantity. Because normal cells have a higher potential for repair than tumour cells, repeated cycles allow normal cells to mend and repopulate while tumour cells continue to decline in number (Caley and Jones, 2012). A tumour that is detectable has usually doubled over 30 times and has 108-109 cells (Price et al., 2008). As a result, even if no cells are discovered after

treatment, there are still a significant number of cells that can induce illness return (Caley and Jones, 2012).

Chemotherapy has its own set of restrictions because it does not target tumour cells precisely. Chemotherapeutic drugs harm normal dividing cells, particularly those in quickly regenerating tissues like bone marrow, gut mucosa, and hair follicles, by preventing cell division or inhibiting enzymes involved in DNA replication or metabolism (Wu et al., 2008). Furthermore, it can contribute to the creation of drug-resistant cells, and many tumor-killing medications can trigger mutations that cause normal cells to become cancerous (Aqeilan et al., 2009). Another issue with chemotherapy is that it has no effect on non-homogenous cancer stem cells, which means they cannot be eradicated even if all cancer cells die. As a result, these cancer stem cells have the potential to create cancer again (Cetin and Topcul, 2012). Combination chemotherapy is frequently used to achieve maximum cell destruction while minimising host harm and preventing resistance (Page and Takimoto, 2004).

1.4.5 Targeted therapy

Anticancer medications meant to interfere with a specific molecular target, usually a protein involved in tumour growth or progression, are referred to as targeted cancer therapy. Since it was discovered that many diseases are regulated by an abundance of proteins such as receptors and hormones, this has been a promising technique (Meiyanto et al., 2012). Monoclonal antibodies, antiangiogenic agents, hormones and hormone receptors, inhibitors of PARP, tyrosine kinase, proteasome, cyclin dependent kinases, Raf kinase, farnesyl transferase, matrix metalloproteinase, protein kinase, mTOR (mammalian target of rapamycin), glutathione-S-transferase, gluta (Topcul and Cetin, 2014).

1.4.6 Phytoceuticals

Plants have been a primary source of numerous medications, with 75 percent of contemporary chemotherapeutic treatments having their origins in plants or natural sources (Cragg and Newman, 2013; Harvey et al., 2015). Several

phytoceuticals have already been tested for anticancer properties. Anticancer activity has been discovered in various preclinical systems for *Alsotnia scholaris, Aegle marmelos, Aphanmixis polystychya, Solanum khasianum,* and *Tinospora cardiofolia* (Jagetia et al., 1998; Jagetia and Baliga, 2005; Jagetia et al., 2005, Jagetia and Venkatesha, 2012; Rosangkima and Jagetia, 2015). Anticancer action has also been reported in extracts from *Urtica membranacea*, *Artemesia monosperma*, and *Origanum dayi* post (Solowey et al., 2014). As a result, plants and natural products continue to be a major source of harmless compounds, including cancer therapy medicines, for screening and development.

Aims and objectives:

1. To select the most effective plant species against different cancer cell lines.
2. To characterize the active constituents and analyzed potentially cytotoxic plants species.
3. To identify the mechanism of action of the potent cytotoxic plants.

Chapter-2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Cancer is as old as recorded human history, but it remains one of the major causes of death worldwide, in both industrialised and developing countries (Torre et al., 2012). Every fourth citizen in developed countries is predicted to be cancer-stricken at some point in their lives, with around 400 new cases diagnosed for every 100,000 people diagnosed each year (Siegel et al., 2015). Despite significant progress and huge efforts to combat cancer, cancer mortality has not decreased significantly (Siegel et al., 2015). This shows that stronger and novel pharmacological agents are still needed to develop them as medications for human healthcare, including cancer cures. (Pan et al., 2012; Newman and Cragg, 2014). Plants have been utilised as medicine since the dawn of humanity, and many current medications are produced either directly or indirectly from plants (Newman and Cragg, 2014). Many modern medications for the treatment of various diseases, including cancer, are derived from natural sources (Mathieu et al., 2015). Since the 1940s, natural compounds have accounted for over 75% of the small anticancer chemicals reported (Newmann and Cragg, 2014).

Many chemotherapeutic medications were derived from natural sources, such as flowering plants, before they were chemically synthesised. Vinblastine and vincristine, two vinca alkaloids derived from the Madagascar periwinkle Catharanthus roseus, are used in combination with other medications to treat a variety of malignancies, including leukemias, testicular cancer, and both Hodgkin and non-Hodgkin lymphomas (Moudi et al., 2013). Another anticancer compound, taxol, was discovered in the bark of the Pacific Yew, Taxus brevifolia (Rowinsky et al., 1995; Creemers et al., 1996; Bertino, 1997), and is used to treat endometrial cancer, cervical carcinoma, breast cancer, non-small-cell lung cancer, and bladder cancer (Rowinsky et al., 1995; Creemers et al., 1996; Bertin (Hajek et al., 2005; Khanna et al., 2015). The podophyllotoxins etoposide and teniposide, which have been identified from *Podophyllum peltatum* and Podophyllum *hexandrum*, are another family of cytotoxic medicine obtained from plants. In clinical practise, they are used alone or in conjunction with other chemotherapeutic medicines to treat testicular cancer, leukemias, and small cell lung cancer (Liu et al., 2016.).

Camptothecin, a chemotherapy agent discovered from *Camptotheca acuminata* in 1966, was the other plant-derived chemotherapeutic medication; nonetheless, its insolubility was a big problem. Topotecan and irinotecan, which are utilised in the treatment of ovarian cancer, cervical cancer, small-cell lung cancer, and colorectal cancer, were synthesised to tackle this problem (Venditto and Simanek, 2010). The extreme toxicity of these contemporary synthetic chemicals, as well as the development of drug resistance, has resulted in therapeutic failure (DeVita and Chu, 2008; Housman et al., 2014).

2.1 *Clerodendrum infortunatum* Linn.

Clerodendrum belongs to the family Lamiaceae (Verbenaceae) and is commonly known as glory bower, bag flower, and bleeding-heart. It is native to tropical region of Africa and southern Asia (Harley et al,. 2004, Yuan et al,. 2010).
Clerodendrum species are employed in the field of traditional medicine for curing different ailment such as inflammation, toothache, cold, hypertension, asthma, furunculosis, rheumatism, anorexia, leprosy, dysentery, arthrophlogosis, mammitis and leucoderma in different region of the globe. (Shrisastava et al., 2007; Chethana et al., 2013; Baker et al., 1995; Hazekamp et al., 2001). It is well explored in scientific study relating to anticancer properties including human cell such as T47D (Breast), PC-3 (prostate), A549 (lung) and HCT-116 (colon) (Haris et al., 2016).

Clerodendrum were known composed of scutellarin, luteolin-7-3 glucuronide, apigen, stimasterol, D-glucose of sitosterol, queretonic acid, baicalin, oleonolic acid, serratagenic acid, D-mannitol and α-spinasterol in different parts such as leaf, bark and root.

In the past few decades, many researchers studied in the genus *Clerodendrum* for its high impact of medicinal efficacy. The leaf extract of *Clerodendrum serratum* Linn was reported for *in vivo* and *in vitro* studies of its anti-anthelmintic activity, hepatoprotective, anti-oxidant, anti-inflammatory, analgesic and antiasthmatic.
The species such as *C. philippinum, C. splendens and C. viscosum* were evaluated for antimicrobial, antioxidants, larvicidal and pupicidal activities. Moreover, *Clerodendrum colebrookianum* is common vegetable among local people and as

herbal medicine for treating hypertension, diabetes and colics in infants particularly in Mizoram (Poornima et al,. 2015; Kare et al,. 2019).

2.2 *Girardinia diversifolia* (Link) Friis

Girardinia diversifolia (Urticaceae) is a stout, erect, perennial herb, 25–200 cm tall, with a perennial rootstock. The aerial parts are armed with numerous slender stinging hairs and the leaves have 3–5 deep lobes, and are saw-toothed with bristles. The flowers are yellowish, clustered in a panicle; the male ones are white and borne in lower axillary panicles; the female ones are grouped in upper bristly axillary and terminal panicles. Flowers appear from July to September and fruits from September to November (Gurung et al 2012).

The plant is found on a clump with many stems, whose bark contains strong, smooth and light fibers, largely used for textiles in Nepal. *G. diversifolia* occurs abundantly in different parts of Nepal, notably in hill forests on moist and damp soil, at altitudes of 1200–3000 m; it is also found in northern India, Bhutan, Sri Lanka, eastward of central China, Myanmar, Malaysia, Indonesia and Africa. In Nepali, the plant is known as “allo” or “chalnesisno” as its leaf is broad and palm-like. In English it is commonly known as “Himalayan nettle” due to the presence of stinging hairs that cause irritation on the skin when touched.

G. diversifolia is largely used in traditional medicine for the treatment of several diseases such as gastric disorders, chest pain, rheumatism, tuberculosis, headache, joint aches, diabetes, asthma, gastritis, headache, joint pain, tuberculosis, gonorrhea and delivery problems. Other traditional uses are related to the treatment of bone fracture, internal injury and blood purification (Shrestha et al 2020, Subedee et al 2020).

Sharan et al., (2020) reported that the Phytochemical studies of *Girardinia diversifolia* showed that the roots and stems of *Girardinia diversifolia* contained saponins, tannins and cardiac glycosides but lacked alkaloids and anthracene glycosides. Thakur et al., (2020) found that phytochemicals like phenol (16.746±0.077) mg, flavonoid (8.033±0.105) mg/g, tannin (1.277±0.005) mg/g, terpenoid (1.381±0.044) mg/g, ascorbic acid (0.874±0.060) mg/g, tocopherol (10.419±0.465) µg/g and carotenoids (182.24±0.623) µg/g were present in

methanolic extract of *Gerardiana diversifolia* and it exhibit a high antioxidant and antibacterial activity.

Thakur et al., (2020) reported that *Girardinia diversifolia* shows a good antioxidant activity having an IC_{50} value of1.819µg/ml and 2.396 µg/ml by ABTS and DPPH respectively.

Study made by Sharan et al., (2020) observed that the *in vitro* test of total methanol extract showed a significant cytotoxic effect on BxPC3 and Huh7 cells. *G. diversifolia* extracts were able to reduce the expression of LDLR, and this may explain, in part, their cytotoxic effects on this cell line

2.3 *Mikania micrantha* Kunth

Mikania micrantha is an invasive weeds species belonging to the family of Asteraceae. It is a creepy plant with the soft stem, heart-shaped leaves, flowers contained many white to greenishwhite florets, and locally known as 'Selaput Tunggul'. The seed dissemination of this plant relies on the wind power and might successfully germinate within 8 days (Fengjian et al 2007).

Previous studies reported that *Mikania* genera are rich with hydroalcoholic acid, coumarin, steroids, diterpenes and sesquiterpenes (Lobitz, 1997; Sola Veneziani and De Oliveira, 1999; Bighetti, 2005). This group of plants genus were used as medicinal herbs for anti-spasmodic, antiulcerogenic, anti-rheumatic, fever, influenza and respiratory diseases (Bighetti, 2005; Perez-Amador, 2010).

The plant has been reported to possess anti-inflammatory activity, antibacterial activity, antistress activity, trypanocidal activity, antiviral activity, inhibitory effect against plant pathogens and antispasmodic effect (Jyothilakshmi 2015). *Mikania decora* had been reported with cytotoxic activity against various cancer cell lines (Aponte et al 2011, Matawali et al 2016).

Sumantri et al., 2020 reported that phytochemical screening of methanolic extract revealed the presence of alkaloids, flavonoids, reducing sugars, saponins, phenolic compounds and tannins, amino acids and proteins whereas petroleum ether extracts

revealed only saponins, chloroform extract revealed alkaloids and saponins and water extracts revealed saponins and amino acid and proteins presence.

As given by Sumantri et al., (2020) the total phenolic content of *Mikania micrantha* was 45.9 mg GAE/g extract. The IC_{50} values based on the DPPH for methanolic extract was 41.8 µg/ml. It showed lower than standard ascorbic acid (129.9 µg/ml) and BHA (153.1 µg/ml). Dev et al., (2015) reported the presence of alkaloids, flavonoids, tannins, Saponins, glycosides, terpenoids and steroids in *Mikania micrantha.* Ittiyavirah et al (2014) also reported that alkaloid, flavonoid, steroid, tannins and phenolics were found in *Mikania micrantha.*

The total phenolic of extract of *Mikania micrantha* leaf ranged from 13.19 ± 0.74 to 34.24 ± 1.24 mg gallic acid equivalent/g and total flavonoid ranged from 1.11 ± 0.11 to 20.63 ± 0.16 mg quercetin/g given by Dev et al., 2015

Dong et al 2017 studied *Mikania micrantha* and revealed that seven compounds isolated from the plant demonstrated good ABTS radical cation scavenging activity more potent than L-ascorbic acid, and four compounds exhibited more potent DPPH radical scavenging activity than L-ascorbic acid. Moreover, the ferric-reducing antioxidant power (FRAP) of four compounds were comparable to or even more potent than L-ascorbic acid. This study indicates that the invasive plant *M. micrantha* is rich in structurally diverse phenolic compounds with functional potential beneficial for human health and is worthy of further investigation.

Debaprotim et al (2014) reported that n-butanolic extract of *M. micrantha* reduce the volume of tumour and prolong the lifespan of EAC cancer mice. Matawali et al., (2016) reported that MTT assay of *Mikania micrantha* plant extracts also showed good cytotoxic activity against HL60 cell line. Dou et al. (2014) reported that the different concentrations of *Mikania micrantha* aqueous extract (50, 100, 200, 400 µg/mL) inhibited the proliferation of both K562 and HeLa cancer cells. The IC_{50} values of K562 and Hela at 48 h were 167.16 and 196.27 µg/mL and at 72 h 98.07 and 131.56 µg/mL respectively. The effects showed time-dose dependence. *Mikania micrantha* aqueous extract led to damages of organelles and induced

apoptosis. Saikia et al., (2020) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay showed that *Mikania micrantha* oil has excellent cytotoxicity against HeLa cervical cancer cell lines (IC_{50} 5.44 ± 1.33 μg/mL) and PA1 ovarian cancer cell lines (IC_{50} 10.57 ± 1.44 μg/mL).

2.4 *Senecio scandens* Buch.-Ham. ex D. Don

The genus *Senecio* (family Asteraceae) consists of more than 1000 species, 63 of which are found in China (Chen, 1999a, 1999b). *Senecio scandens* is one of the most popular species used as a Chinese medicinal medicinal plant and is distributed mainly in southwest China; its use and distribution is also found in Bhutan, India, Japan, Myanmar, Nepal, Philippines, Sri Lanka, and Thailand (Chen et al 1999).

This medicinal plant has been applied to treat the following maladies: carbuncles, furuncles, colds, fevers, pruritus, diarrhea, eczema, upper respiratory tract infection, pneumonia, acute tonsillitis, mumps, acute enteritis, bacterial dysentery, icteric hepatitis, cholecystitis, acute urinary tract infection, erysipelas, ringworm sore, trichomoniasis vaginalis, burns, and swollen red eyes (Roeder 2000). *Scenecio scandens* possesses significant anti-tumor effect on H22 tumor in vitro and in vivo, and that the mechanism of the anti-tumor effect is closely related to the induction of mitochondria-mediated apoptosis. (Dou et al 2017).

Wang et al., (2013) studied the phytochemical contents and have showed the presence of numerous valuable compounds, such as flavonoids, alkaloids, phenolic acids, terpenes, volatile oils, carotenoids, and trace elements.

Study made by Lu et al., (2010) reported the DPPH study of antioxidant activity of 70% alcohol extract of the whole plant of *Senecio scandens* achieved a value of 54.93% inhibition and therefore had a remarkably strong effect on the antioxidation. Liu et al., (2001) reported that the water extract of *Senecio scandens* showed strong antioxidative and free radical scavenging activities, as well as low side-effects.

He et al. (2010) reported that the total flavonoid extract from *Senecio scandens* showed antitumor and antiviral activity *in vitro*. The inhibitive effect of the

total flavonoid extract on human hepatocellular carcinoma cell line SMMC-7721, human gastric carcinoma cell line SGC-7901, and human breast carcinoma cell line MCF-7 studied by MTT assay showed the inhibitive effect on human respiratory syncytial virus (RSV) detected by cytopathic effect (CPE) and the IC_{50} of the total flavonoid solution on SMMC-7721, SGC-7901, and MCF-7 was 48.73, 61.32, and 31.26 μg/mL, respectively.

Yang et al., (2010) reported that The IC_{50} values for the lipid peroxidation were 0.980 and 0.950 mg/mL, and the IC_{50} of DNA damage were 0.690 and 0.140 mg/mL. These results suggested that the *Senecio scandens* polyphenol extract was an excellent natural antioxidant and free radical scavenger (Yang et al., 2010)

2. 5 *Paris polyphylla* Sm.

Paris polyphylla belongs to the family Melanthiaceae. The term Paris is derived from 'pars' which means symmetry and polyphylla means many leaves. It originates from Asia and mainly found in China, Indian subcontinent and in Himalayan regions (Danu et al 2015). In Indian Himalayan region, the *Paris polyphylla* is found distributed in Arunachal Pradesh, Assam, Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Manipur, Meghalaya, Mizoram, Nagaland and Sikkim (Paul et al 2015).

Paris polyphylla is a creeping rhizomatous forest plant with erect stem growing to 40 cm. It has 4-9 elliptic leaves occuring in a whorl. Flowers occur single at the end of branches. The flower has a ring of 4-6 green leaf-like perianth segments (which play the role of speals/petals), which are 5-10 cm long (Li et al 1998). The chemical constituents of the rhizome of these plants have been studied, and steroidal saponins are regarded as their main active ingredients. In recent years, steroidal saponins have attracted scientific attention for their structural diversity and significant bioactivities, including their antitumor, hemostatic, immunotropic, and analgesic properties, treatment of skin infection, sore throat, snakebite, convulsion, and traumatic bleeding, malarial fever, inflammation of the mammary gland, pimples, tuberculosis and asthma. The pharmacological activities of steroidal

saponins are reportedly associated with the sugar residues comprising the sugar chain and their sequences and with the structure of the aglycone moiety (Nguyen et al 2016; Zhao 2004; Trouillas et al., 2005; Zhang et al 2005; Li et al 2003; Zhang et al., 2007). Steroidal saponin comprises of diosgenin with their glycosyl derivatives is the major bioactive compounds reported from the rhizome of Paris polyphylla which accounts for 80% of the total phytoconstituents that possesses several therapeutic and pharmacological potential (Guan et al., 2018).

The IUCN and CAMP listed *P. polyphylla* Sm. as vulnerable medicinal plant (Anonymous 2001)

Rajsekhar et al. (2016) reported that flavonoids, carbohydrates, cardiac glycosides, terpenoids and sterols were present in water, methanol, ethanol, ethyl acetate and chloroform extract of *Paris polyphylla*. Alkaloids were present only in ethyl acetate and chloroform extracts. Saponins, phenols and tannins were present in all the extracts except ethyl acetate extract. Quinones were present in methanol, ethanol and chloroform extracts. Glycosides were present only in chloroform extract. These phytochemicals exhibit various medicinal and pharmacological activities.

Mayirnao et al. (2017) reported the methanolic rhizome extract was found to contain significant quantity of phenolic compounds 43.01±0.17mg/g GAE/g DW of sample. Flavonoids on the other hand also were quite significant in the sample containing 28±0.12 mg quercetin equivalent/g DW of the sample. It was observed that the methanol rhizome extract showed IC50 value of 1.09 mg/ml. The percentage scavenging effect on DPPH radical was increased with the increase in the concentration of the sample.

Lepcha et al. (2019) repoprted that the total phenolic contents of methanolic extracts of rhizomes of *P. polyphylla* from Tholung and *P. polyphylla* from Uttaray. The higher and lower altitude samples contained 16.29 ± 0.03 mg GAE/g DW and 5.96 ± 0.02 mg GAE/g DW respectively. Substantial quantity of flavonoids were present in both the samples from high and low altitudes with *P. polyphylla* from

Tholung and *P. polyphylla* from Uttaray showing total falvonoid content of 30.46 ± 0.06 mg QAE/g DW respectively

The effect of methanolic rhizome extract on the inhibition of cell growth was investigated by Lepcha et al. (2019) the result showed that the methanolic extract had an extremely high dose-dependent inhibition of HeLa cell growth reaching >90% inhibition at 100 µg/ml concentration within 72 hrs of treatment.

Sun et al. (2007) reported that *Paris polyphylla* showed a low IC_{50} on two liver carcinoma cell lines such as SMMC-7721 and HepG2 with IC_{50} value 12 µg/ mL and 10 µg/mL, respectively. The results indicated that *Paris polyphylla* may be a promising source for anticancer drug development.

P. polyphylla exerts anti-tumor effects against many tumors, including bladder cancer, pancreatic cancer, breast cancer, liver cancer, and lung cancer(Lee et al., 2005; Ong et al., 2008; Chen et al., 2014; Kong et al., 2010).

Watanabe et al., (2017) reported cancer cell lines IMR-32, NB-69 and LA-N-2 showed reduced cell viability in response to polyphyllin D treatment. No caspase-3/-7, -8,and -9 activity was observed in IMR-32 and LA-N-2 cells treated with polyphyllin D whereas activation of caspase-3/-7, and -8 activity was observed in NB-69 cells. When polyphyllin D and specific inhibitors of RIPK3 involved in necroptosis were added to IMR-32 and LA-N-2 cell lines, polyphyllin D-induced cell death was inhibited.

Wang et al. (2007) reported the identification of six compounds and their structures were recognised as diosgenin-3-O-alpha-L-arabinofuranosyl(1-->4) -beta-D-glycopyranoside (1), pennogenin-3-O-alpha-L-arabino-furanosyl(1-->4)-beta-D-glycopyranoside (2), isorhamn etin-3-O-beta-D-glycopyranoside (3), ethyl-alpha-D-fructofuranoside (4), pennogenin-3-O-alpha-L-rhamnopyranosyl (1-->4)-[alpha-L-rhamnopyranosyl (1-->2)]-beta-D-glycopyranoside (5) and pennogenin-3-O-alpha-L-rhamnopyranosyl(1-->4)-alpha-L-rhamnopyranosyl(1-->4)-[alpha-L-rhamnopyrano-syl (1-->2)]-beta-D-glycopyranoside (6).

Gupta et al. (2021) reported that the ethanolic extract of *Paris polyphylla* rhizome obtained through cold extraction method using 70% ethanol showed maximum diosgenin contain 17.90% quantified through GC-MS while similar compounds pennogenin (3.29%), 7β-Dehydrodiosgenin (1.90%), 7-Ketodiosgenin acetate (1.14%), and 7 β-hydroxydiosgenin (0.55%), and thus confirmed diosgenin as major and lead phytochemical. However, Diosgenin enriched *Paris polyphylla* rhizome extract obtained through both cold and repeated hot extraction with the same solvent (70% ethanol) showed diosgenin content of 60.29% which is significantly higher ($p < 0.001$) than the diosgenin content in ethanolic extract of *Paris polyphylla* rhizome.

Chapter-3

MATERIALS AND METHODS

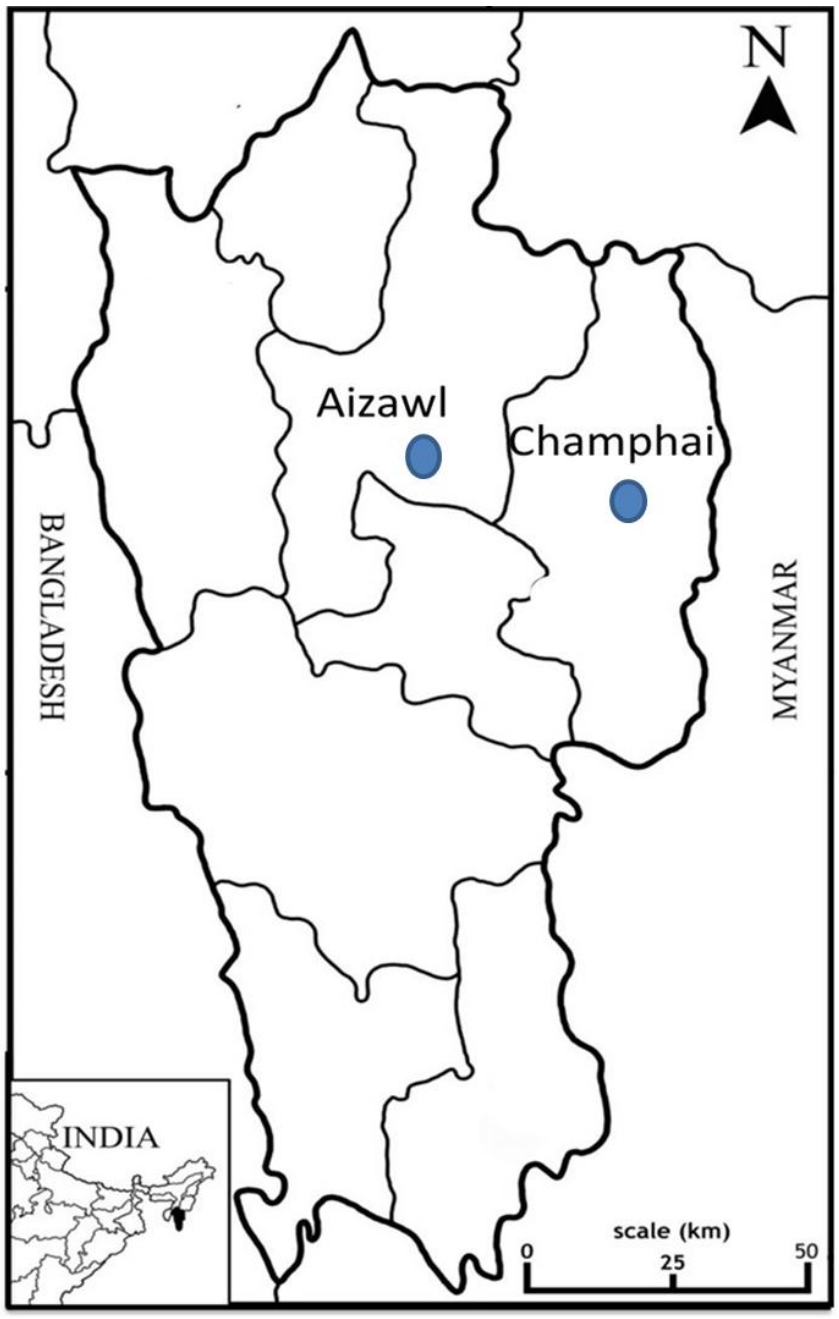
MATERIALS AND METHODS

3.1 Plant selection

Information regarding medicinal plants used against cancer were collected based on literature survey, traditional healers from Keifang and Champhai who have been practicing the used of ethno-medicinal plants for the treatment of different ailments including cancer and from cancer patient using herbal plants as ethno-medicine instead of chemotherapy or in combination of medicinal plants and chemotherapy.

3.1.1 Collection of Plant materials

The healthy leaves and rhizome of *Clerodendrum infortunatum* (leaves), *Mikania micrantha* (leaves), and *Girardinia diversifolia* (leaves), were collected from Aizawl, Mizoram. *Senecio scandens* (leaves), and *Paris polyphylla* (rhizome), were collected from Champhai District, Mizoram. The specimens were identified and authenticated by Prof. H. Lalramnghinglova, a known botanist as well as the Coordinator of Mizoram University Herbarium. The collected plants were properly submitted to the Mizoram University herbarium as well as departmental herbarium bearing the accession number *Clerodendrum infortunatum* (564), *Mikania micrantha* (101), *Girardinia diversifolia* (136), *Senecio scandens* (085), and *Paris polyphylla* (155).



MAP of Study Area

3.1.2 Extract Preparation

The collected plant samples were carefully sorted; washed thoroughly cleaned with water and then shade dried. The dried samples were grinded to finely powered using electric grinder. Sequential extraction was done by maceration process using petroleum ether, chloroform and ethanol. The crude extract was concentrated at 40°C,

using a rotary evaporator under low pressure. The extracts were dried and then stored in air-tight containers at 4°C until further use. Ethanol extract was selected for further studies.

MME- *Mikania mikrantha*

GDE- *Girardinia diversifolia*

SSE- *Senecio scandens*

CIE- *Clerodendrum infortunatum*

PPE- *Paris polyphylla*

3.2 Chemicals and reagents

All the chemicals and reagents Quercetin, Folin-Ciocalteu, Gallic Acid, Allunimium chloride ($AlCl_3$), Hydrochloric acid, Nitro blue tetrazolium (NBT), Ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Ethanol, Dimethyl sulfoxide (DMSO), Bromocresol green solution (BCG), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), trypsin and Ethylene diamine tetra-acetic acid (EDTA) used in this study were of analytical grade and obtained from Merck Company, Germany.

3.3 Preliminary phytochemical screening

3.3.1 Phenols

$FeCl_3$ tests: 1 mL of aqueous extraction solution was mixed with 1 mL of 2% FeCl3 solution. Development of a blue-green or black colour indicated the presence of phenols.

3.3.2 Flavonoids

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

3.3.3 Alkaloids

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

3.3.4 Cardiac glycosides (Keller-Killani test)

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

3.3.5 Saponins

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

3.3.6 Tannins

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

3.3.7 Terpenoids (Salkowski test)

The terpenoids in aqueous extract of *the plant extracts* were detected by mixing 5 ml of each extract with 2 ml of chloroform with the careful addition of 3 ml

concentrated H_2SO_4 so as to allow the formation of a layer. The formation of a reddish brown colour at the interface confirmed the presence of terpenoids.

3.3.8 Anthraquinones (Ammonium hydroxide test)

One drop of concentrated ammonium hydroxide was added to 10 mg of each extract, previously dissolved in isopropyl alcohol. After two minutes, formation of red color indicated the presence of anthraquinone.

3.3.9 Quinones (Sulfuric acid test)

One drop of concentrated sulfuric acid was added to 10 mg of each extract dissolved in isopropyl alcohol. Formation of red color indicated the presence of quinones.

3.4 Quantification of secondary metabolites

3.4.1 Alkaloids

For evaluation of the total alkaloid content, Bromocresol green method was used. A solution (BCG) was mixed with 1 mL of C. infortunatum extract and the mixture was agitated and complex formed was extracted with 5 mL of chloroform. Absorbance was taken at 415 nm against blank. The solutions were stable for 2 h. Atropine was used as positive control and extract was calculated against blank.

3.4.2 Phenols

The total phenols were determined by Folin-Ciocalteau reagent (McDonald et al., 2001). 500 µl of ethanolic extract of *the plants* was mixed with Folin-Ciocalteau solution (5ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4ml, 1M). The mixture was incubated for 15 min and the phenol content was determined at 756 nm using a UV/Visible double beam spectrophotometer. The phenol content was expressed in terms of gallic acid equivalent (mg/g of dry weight), which is a common reference compound.

3.4.3 Flavonoids

The aluminium chloride technique was used for flavonoids (Chang et al., 2002). Various concentrations of leaf extract 0.5 ml (1 mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It was then incubated at room temperature for 30 min. The amount of flavonoids was quantified through the optical density at 415 nm with a UV/Visible double beam spectrophotometer.

3.5 *In vitro* Antioxidant assays

3.5.1 DPPH radical scavenging activity

The test was carried out according to Leong and Shui (2002) with modification. To different concentrations of various extracts of the plant extract (0.5 ml each), 1 ml of methanol solution of 0.1 mM DPPH was added. After thorough mixing, the mixture was allowed to stand in the dark for 30 min and the absorbance was measured at 523 nm using UV-VIS double beam spectrophotometer (Epoch 2, Biotek, USA). Methanol was utilized for the baseline correction. The results have been compared with that of the control prepared as above without sample. Radical scavenging activity has been expressed as a percentage and calculated using the following formula:-

$$\% \text{ Scavenging} = \frac{(\text{A control O.D} - A \text{ sample O.D})}{\text{A control O.D}} \times 100$$

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

3.5.2 ABTS cations scavenging activity

ABTS scavenging activity of different extracts of the plant extracts was carried out as described earlier (Re et al., 1999). Briefly, 37.5 mg of potassium persulphate was dissolved in 1 ml of distilled water. 44 µl of this solution was added to 9.7 mg of ABTS dissolved in 2.5 ml of distilled water to prepare ABTS solution. The ABTS solution was allowed to stand in the dark at room temperature for 12-16 hours. The working solution was prepared by mixing 1 ml of ABTS solution with 88 ml of 50% ethanol. 25 µl of different concentrations (1- 5000 µg/ml) of the different

extracts (Chloroform, ethanol and aqueous) of *Oroxylum indicum* was mixed with 250 µl of the working ABTS solution and allowed to react for 4 minutes. The absorbance was then measured at 734 nm in a UV-VIS Softmax spectrophotometer. Trolox was used as the standard antioxidant and the activity was expressed as trolox equivalent. The percentage scavenging activity was calculated as follows:

$$\% \text{ Scavenging} = \frac{(\text{Control O.D} - \text{Sample O.D})}{\text{Control O.D}} X100$$

3.5.3 Superoxide anion scavenging activity

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

3.6 Cell Culture and maintenance

HeLa (Cervical cancer), AGS (Gastric cancer), A549 (Lung cancer), HT-29 (Colon cancer) and HepG2 (Liver cancer) cells were procured from the National Centre for Cell Science, Pune, India. The cells were routinely grown in 25 cm^2 culture flasks (HiMedia, Mumbai, India) containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 µg/ml gentamicin sulfate with loosened caps at 37°C in an atmosphere of 5% CO_2 in humidified air in a CO_2 incubator (Eppendorf AG, Hamburg, Germany).

3.6.1 Experimental Design for Cytotoxicity

The cytotoxic activity of plants extract were carried out by performing the experiments listed below.

3.6.1.1 Selection of most cytotoxic extract

This experiment was carried out to select the extract which provided the highest cytotoxic effect among GDE, SSE, MME, CIE and PPE by MTT assay, where HeLa, AGS, A549, HT-29 and HepG2 cell lines were treated with 5, 10, 20, 40, 80, or 100 µg/mL of GDE, SSE, MME, CIE and PPE or 1, 2, 4 or 8 µg/mL of DOX.

After MTT assays, their IC_{50} concentration was established and the remaining experiments were conducted using their IC_{50} concentrations, where the cell cultures were divided into the following groups: -

3.6.1.2 *MEM group*: The cells of this group were treated with 2 µL/mL of DMSO.

Treatment groups*:*

3.6.1.2.1 *GDE group*: 5, 10, 20, 40, 80, or 100 µg/mL of GDE were used.

3.6.1.2.2 *SSE group*: 5, 10, 20, 40, 80, or 100 µg/mL of SSE were used.

3.6.1.2.3 *MME group*: 5, 10, 20, 40, 80, or 100 µg/mL of MME were used.

3.6.1.2.4 *CIE group*: 5, 10, 20, 40, 80, or 100 µg/mL of CIE were used.

3.6.1.2.5 *PPE group*: 5, 10, 20, 40, 80, or 100 µg/mL of PPE were used.

3.6.1.2.6 DOX group: The cell cultures of this group were treated with or 1, 2, 4 or 8 µg/mL of doxorubicin and served as the positive control.

3.7 Cell cytotoxicity

The cytotoxic activity of the plants extract was determined by reduction of metabolically active cells through mitochondrial enzymes on HeLa, AGS, A549, HT-29 and HepG2 cell lines (Mossman, 1983). Approximately, 1×10^4 cells per well were seeded in 96-well plates. The cells were allowed to attach to the bottom of flask prior to experiment. After incubation for 16 h, the cells with 70–80% confluency were treated with the extracts at different concentrations and incubated for 72 h. Then, 10 µL of MTT (5mg/mL) solution was added to cells per well, and the plate was moved to a cell incubator for another 4 h. The proliferating cells were calculated using a Spectramax M2 Microplate Reader (Molecular Devices, USA.) at a wavelength of 550 nm. Relative viability was calculated taking wells with untreated cells as 100% control. The results are expressed as mean values (±SD) of three repeats.

3.7.1 Cysteine aspartic protease 9, 8 and 3 activities

The caspase 9, 8 and 3 are crucial in carrying out apoptosis and their activities were measured at 48 h in HeLa cells, A549 and HepG2 after *Paris polyphylla* ethanol extract treatment using ELISA kits following the instructions given by the manufacturer (Bioassay Technology Laboratory, China). Usually, 10^4 HeLa cells were seeded into 48 well plates and treated with IC_{50} concentration and grouped as described above.

3.8 HPLC chromatogram analysis

The presence of bioactive compounds were quantified using Shimadzu Instrument (Shimadzu Corp, Kyoto, Japan) coupled with a diode array detector (DAD, SPD N 20A) and C18 column (5 μM;4.6×250 mm). The analytical procedure was executed by applying the chromatographic conditions. The gradient system began with a concentration of 100% solvent A at 0.1 min, then progressively increased the concentration of solvent B to 35% in 25 min, 50% in 45 min, and eventually 100% in 65 min. The standard compounds quercetin and rutin were dissolved in sterile water and filtered by PVDF (0.45 μM) and a volume of 20 μL was eluted. The solvent system of HPLC grade H2O (solvent A) and H2O: C2H3N: CH3COOH (48:51:4 v/v) (solvent B) were employed as mobile phase. The flow rate was kept at 1 mL/min and 20 μL of the sample was injected for the analysis. The reference compounds such as Caffeic acid, Coumaric acid, Gallic acid, Naringenin, Quercetin and Rutin were analyzed discretely to obtain the retention periods.

3.8.1 Linearity

The acquisition of linearity was acquired by repetitive elution of various concentrations of standards (Caffeic acid, Coumaric acid, Gallic acid, Naringenin, Quercetin and Rutin). Analytical curves were obtained for every compound compared with their correlation in the peak area and the concentration using linearity.

3.9 Identification of compound using GC-MS analysis

The GC-MS analysis for *Paris polyphylla* was performed by using Clarus 690 Perkin-Elmer (Auto system XL) Gas Chromatograph equipped and coupled to a mass detector Turbo mass gold - Perkin Elmer Turbomass 5.1 spectrometer with an Elite - 1 (100% Dimethyl poly siloxane), 123.5m x 678µm of capillary column. The instrument was fixed an initial temperature of 40°C ramp 5°C/min to 115°C, hold 5 min, ramp 5°C/min to 140°C, hold 5 min, ramp 2°C/min to 210°C, hold 8 min, and maintained at this temperature for 3 min. At the end of this period the oven temperature was increased up to 250°C, at the rate of an increase of 5°C/min, and maintained for 9 min. Injection port temperature was ensured at 250°C and Helium flow rate at 1.5 ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 50-800 (m/z). The ion source temperature was maintained at 230°C and Interface temperature was at 240°C. The MS start time was 3 min, and end time was 75 min with solvent cut time was of 3 min. The spectrums obtained of volatile compounds detected the through GC-MS were compared and matched with NIST 17 online library Ver. 2.3.

3.10 Molecular Docking Analysis

3.10.1 Preparation of the Target 3 Dimensional structure and Initial Energy minimization:

After downloading the 3 Dimentional (3D) structures in from Protein Data Bank (RCSB-PDB) (https://www.rcsb.org/,). The Active site residues were searched using ligand interaction map provided in RCSB-PDB (Burley *et al.*, 2022). Only Chain A of the targets were used for the further binding affinity studies. After removal of the original ligands, other protein chains and water molecules from the

protein targets, the targets were treated for modelling any missing side chain residues. WHATIF Server (https://swift.cmbi.umcn.nl/servers/html/corall.html).

Table 1: Active sites for CDK2 and RAF

CDK2 (5A14)		**RAF (3Omv)**	
Lys 34	Val 65	ILE 355	LYS 375
Leu 56	-	PHE 475	GLU 393
Leu 125	-	CYS 424	VAL 363
Leu 144	-	ALA 373	-

The energy minimization methods utilised viz. steepest descent (20 Steps) and Conjugate Gradient (20 Steps). The Energy Computations were done in vacuo with the GROMOS96 43B1 parameters set, without reaction field. The computations were performed in Swiss-PDB Viewer v 4.10_PC (Guex et al., 1997.)

Table 2: Energy Minimization Steps using Swiss PDB Viewer Version 4.10_PC

PDB ID (CHAIN-A)	**Steepest descent 20 Steps (KJ/mol)**	**Conjugate gradient (20 Steps) (KJ/mol)**
3omv	-12272.841	-12681.288
5a14	-13068.831	-13601.903
The computations were made using Steepest descent and Conjugate Gradient using GROMOS96 force field.		

3.10.2 Preparation of Ligands 3 Dimension Structure:

The ligand 3D Structures were downloaded from PubChem (Kim et al., 2021). The structures were extracted in PDB format. The structures of all the ligands are optimized using MMFF94 forcefield before carrying out docking.

3.10.3 Binding Pose Analysis:

In binding pose analysis, stochastic Search method was employed by using Genetic Alogorthim and Monte Carlo (MC) algorithm. Stochastic methods allow

investigating the conformational space by random modification of the ligand conformation or a library of large set of ligands (Meng *et al.*, 2016). AutoDock Tool version 4.2.0 was employed for the Binding pocket analysis of each target against the selected set of ligands. The binding pockets were analysed using in terms of minimum binding energy of the docked conformation of the complexes.

Chapter-4

RESULTS

RESULTS

The results of phytochemicals of *Clerodendrum infortunatum, Mikania micrantha, Girardinia diversifolia, Senecio scandens* and *Paris polyphylla* have been shown in Tables 3.

4.1 Phytochemical Screening (Table 1)

4.1.1 Phenols

Development of a blue-green or black colour indicated the presence of phenols in all the extract.

4.1.2 Flavonoids

A yellow colour was observed indicating that all the plant extract contained flavonoids.

4.1.3 Alkaloids

The formation of a reddish brown precipitate on reaction with Dragendorff's reagent indicated the presence of alkaloids in all the extract.

4.1.4 Cardiac glycosides (Keller-Killani test)

The appearance of a brown ring at the interface indicates the presence of cardiac glycosides in the leaves extract of *Clerodendrum infortunatum,* whereas it was found absent in *Mikania micrantha, Girardinia diversifolia, Senecio scandens* and *Paris polyphylla.*

4.1.5 Saponins

Formation of emulsion indicated the presence of saponin in all the plant extract.

4.1.6 Tannins

A brownish colour was observed in *Mikania micrantha, Girardinia diversifolia, Senecio scandens* and *Paris polyphylla* but was found absent in *Clerodendrum infortunatum*

4.1.7 Terpenoids (Salkowski test)

The formation of a reddish brown colour at the interface confirmed the presence of terpenoids in all the plant extract.

4.1.8. Anthraquinones (Ammonium hydroxide test)

There was no formation of red colour indicating the absence anthraquinone in all the plant extracts.

4.1.9 Quinones (Sulfuric acid test)

No formation of red color was visualised which shows the absence of quinones in all the plant extracts.

4.2 The quantification of secondary metabolites

4.2.1 Phenols

The total phenol content of all the five plant extracts were evaluated using standard protocol as mentioned. Gallic acid was used as a standard. *Paris polyphylla* showed the highest content of phenol having 72.12 mg/g, *Girardinia diversifolia* contain 64.85mg/g, *Mikania micrantha* showed 64.57mg/g, closely followed by *Clerodendrum infortunatum* having 63.01mg/g and the least content of phenol was observed in *Senecio scandens* that is 61.44 mg/g.

4.2.2 Flavonoids

The total alkaloid content of all the five plant extracts were evaluated using standard protocol as mentioned. Quercetin was used as a standard. Flavonoid was highest in *Girardinia diversifolia* (57.79 mg/g) followed by *Clerodendrum infortunatum* (50.41 mg/g), *Mikania micrantha* (46.19 mg/g), *Senecio scandens* (41.55 mg/g) and *Paris polyphylla* (4.64 mg/g).

Table 3: Preliminary phytochemical screening of Medicinal plant of Mizoram.

Samples	Tannins	Saponins	Flavonoids	Alkaloids	Phenols	Cardiac glycosides	Quinone	Terpenoids	Anthroquinone
MME	+	+	+	+	+	-	-	+	-
SSE	+	+	+	+	+	-	-	+	-
GDE	+	+	+	+	+	-	-	+	-
CIE	-	+	+	+	+	+	-	+	-
PPE	+	+	+	+	+	-	-	+	-

Legend: + present, - absent

MME- *Mikania micrantha;* **SSE-** *Senecio scandens;* **GDE-** *Girardinia diversifolia;* **CIE-** *Clerodendrum infortunatum;* **PPE-** *Paris polyphylla*.

4.2.3 Alkaloids

The total alkaloid content of all the five plant extracts were evaluated using standard protocol as mentioned. Atropine was used as a standard. Alkaloid was highest in *Paris polyphylla* (4.64 mg/g) followed by *Girardinia diversifolia* (3.18 mg/g), *Mikania micrantha* (3.57 mg/g), *Clerodendrum infortunatum* (3.54 mg/g) and *Senecio scandens* (3.18 mg/g).

Table 4: Secondary metabolite quantification of medicinal plants extracts

Secondary Metabolites	MME	CIE	GDE	SSE	PPE
Phenol	64.57±0.59	63.01±0.94	64.85±0.64	61.44±0.12	72.12±1.05
Flavonoid	46.19±0.12	50.41±0.63	57.79±1.12	41.55±3.57	38.25±1.26
Alkaloid	3.57±0.99	3.54±0.75	3.80±0.67	3.18±0.51	4.64±1.45

The data present as Mean± SD, n=3.

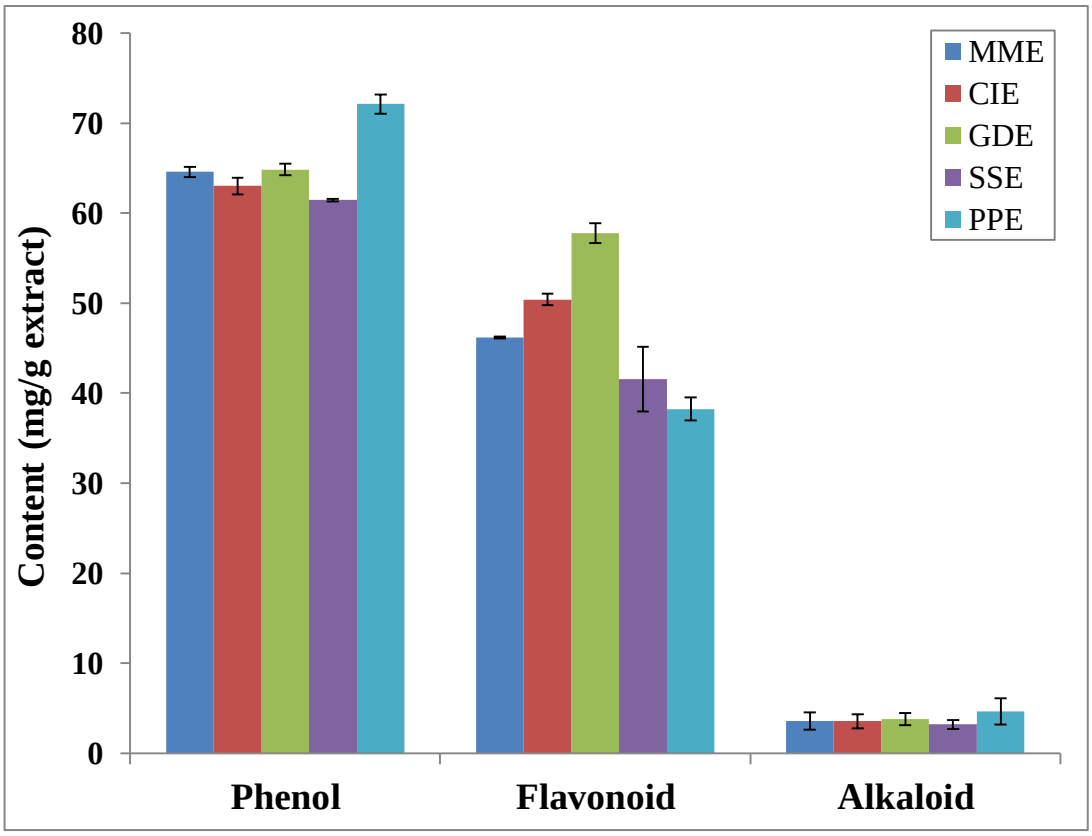


Figure 1: The secondary metabolite quantification of medicinal plants extracts. The data present as Mean± SD, n=3.

4.3.1 DPPH radical scavenging activity

Various plant extracts showed a concentration dependent, increase in the scavenging of DPPH radicals as indicated by the discolouration of DPPH which is purple in colour. Among the five plants, maximum scavenging was observed at a concentration of 80µg/ml in *Clerodendrum infortunatum* with the scavenging percentage of 86.41%. The IC_{50} of *Clerodendrum infortunatum* is 48.10 µg/ml.

Table 5a: The antioxidant potency of five medicinal plant of Mizoram using DPPH assays. The data present as Mean± SD, n=3.

Conc. (µg/ml)	AA	MME	CIE	GDE	SSE	PPE
5(0.5)	35.77±2.40	4.46±1.01	10.62±0.14	5.46±3.08	5.31±0.30	12.31±0.58
10(1)	64.921±4.16	14.15±2.15	15.03±0.41	14.39±0.52	20.39±0.52	22.35±0.49
20(2)	89.23±2.42	17.58±0.69	19.53±0.75	15.29±0.18	31.00±0.15	35.01±0.40
40(4)	92.03±0.45	25.06±0.38	27.67±0.36	16.66±2.21	43.37±1.29	47.34±2.10
80(8)	92.65±0.52	42.11±0.05	86.41±0.67	17.86±0.26	53.42±0.20	58.84±1.45
100(10)	93.10±0.02	49.83±0.34	83.86±2.18	27.04±0.20	61.86±1.67	66.81±2.64
IC50	6.70	119.2	48.10	>1000	61.15	58.78

The data present as Mean± SD, n=3.

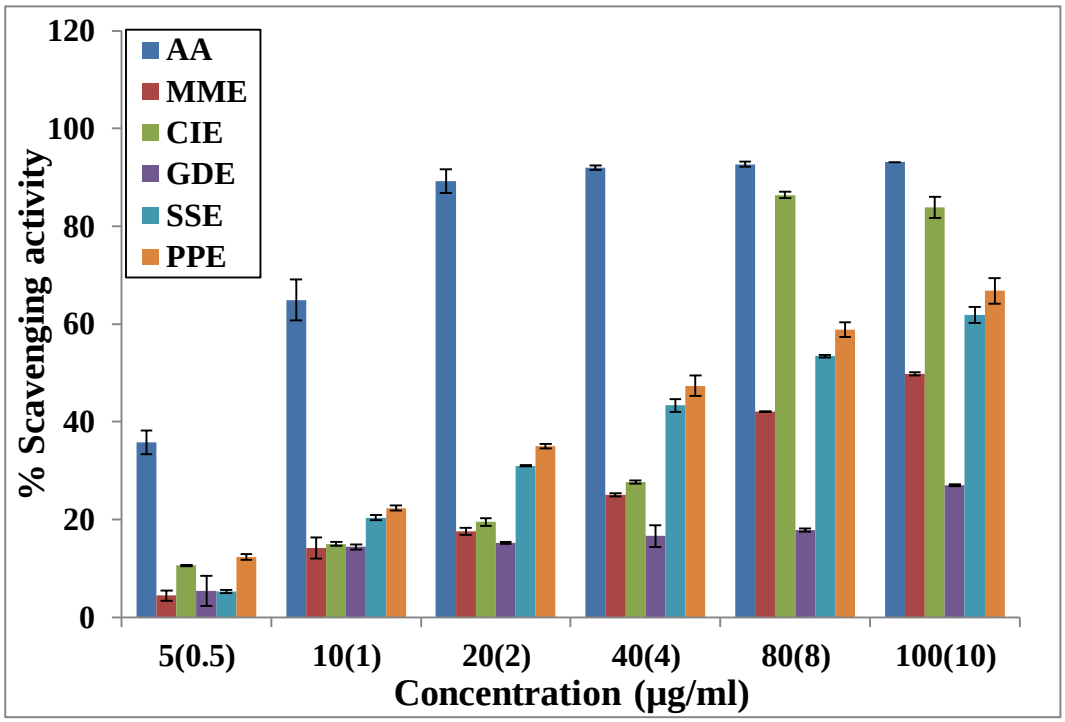


Figure 2a: The antioxidant potency of five medicinal plant of Mizoram using DPPH assays.

4.3.2 ABTS cation scavenging activity

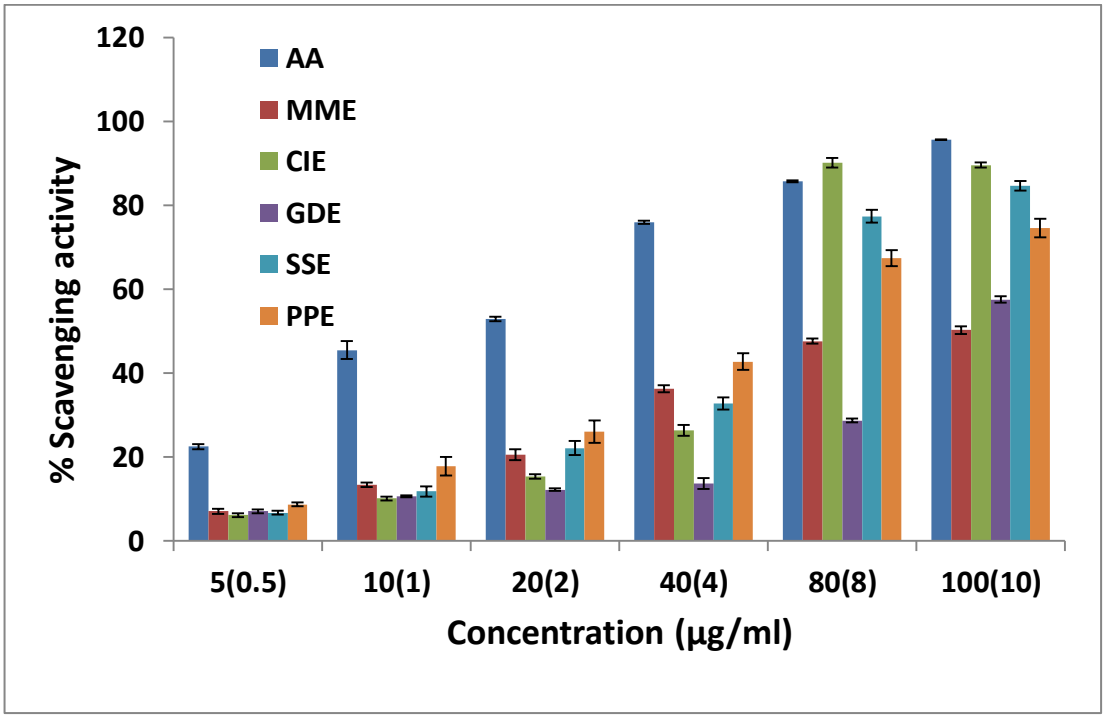
The different plant extracts showed a concentration dependent rise in the scavenging activity of the ABTS free radicals scavenging activity. A single concentration of *Clerodendrum infortunatum* extract 80 µg/ml showed the best scavenging percentage at 90.14%. The overall IC_{50} was lowest in *Paris polyphylla* with 45.1µg/ml.

Table 5b: The antioxidant potency of five medicinal plant of Mizoram using ABTS cation scavenging activity assays

Conc. (µg/ml)	AA	MME	CIE	GDE	SSE	PPE
5(0.5)	22.43±0.63	7.02±0.59	6.06±0.46	6.98±0.43	6.62±0.49	8.64±0.45
10(1)	45.43±2.18	13.28±0.50	10.04±0.44	10.58±0.21	11.72±1.20	17.78±2.20
20(2)	52.81±0.52	20.44±1.27	15.29±0.53	12.15±0.25	22.06±1.66	26.01±2.66

40(4)	75.93±0.41	36.21±0.84	26.26±1.28	13.61±1.26	32.66±1.43	42.66±1.96
80(8)	85.69±0.21	47.58±0.58	90.14±1.18	28.61±0.45	77.32±1.51	67.35±1.88
100(10)	95.57±0.03	50.22±0.93	89.55±0.59	57.47±0.78	84.57±1.18	74.53±2.18
IC50	13.75	91.3	50.1	108	46.6	45.1

The data present as Mean± SD, n=3.



The data present as Mean± SD, n=3.

Figure 2b: The antioxidant potency of five medicinal plant of Mizoram using ABTS cation scavenging activity assays

4.3.3 Superoxide anion scavenging activity

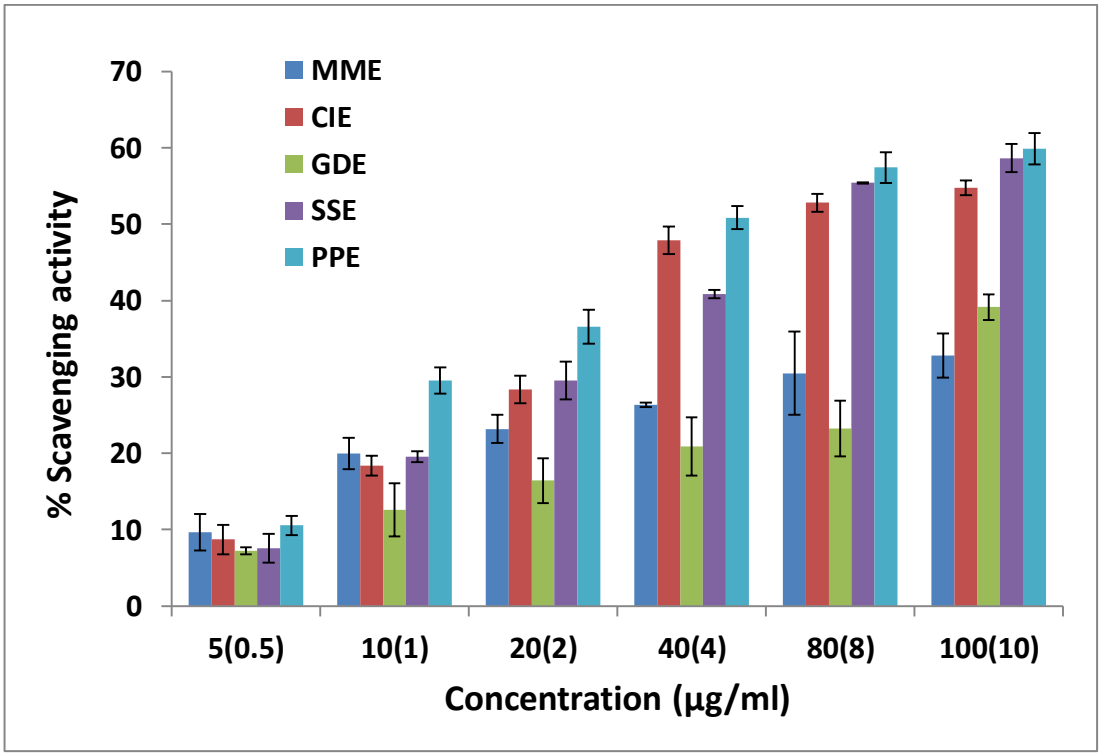
Clerodendrum infortunatum, Mikania micrantha, Girardinia diversifolia, Senecio scandens and *Paris polyphylla* showed a concentration dependent inhibition of superoxide radical generation up to a certain concentration. The gallic acid was used as a standard. *Paris polyphylla* at100 µg/ml gives the best scavenging

percentage at 59.89%. The IC_{50} for *Paris polyphylla* is the lowest with the value of 41.78 µg/ml.

Table 5c: The antioxidant potency of five medicinal plant of Mizoram using Superoxide anion scavenging activity assays

Conc.(µg/ml)	MME	CIE	GDE	SSE	PPE
5(0.5)	9.62±2.38	8.70±1.93	7.23±0.43	7.58±1.89	10.56±1.24
10(1)	19.98±2.05	18.36±1.31	12.58±3.49	19.52±0.70	29.52±1.70
20(2)	23.18±1.83	28.35±1.82	16.43±2.93	29.53±2.48	36.53±2.22
40(4)	26.32±0.27	47.84±1.79	20.88±3.81	40.84±0.57	50.84±1.52
80(8)	30.47±5.43	52.78±1.16	23.24±3.60	55.39±0.08	57.39±2.02
100(10)	32.79±2.87	54.74±0.97	39.13±1.68	58.64±1.84	59.89±2.05
IC50	>1000	64.98	324.8	62.38	41.78

The data present as Mean± SD, n=3.



The data present as Mean± SD, n=3

Figure 2c: The antioxidant potency of five medicinal plant of Mizoram using Superoxide anion scavenging activity assays

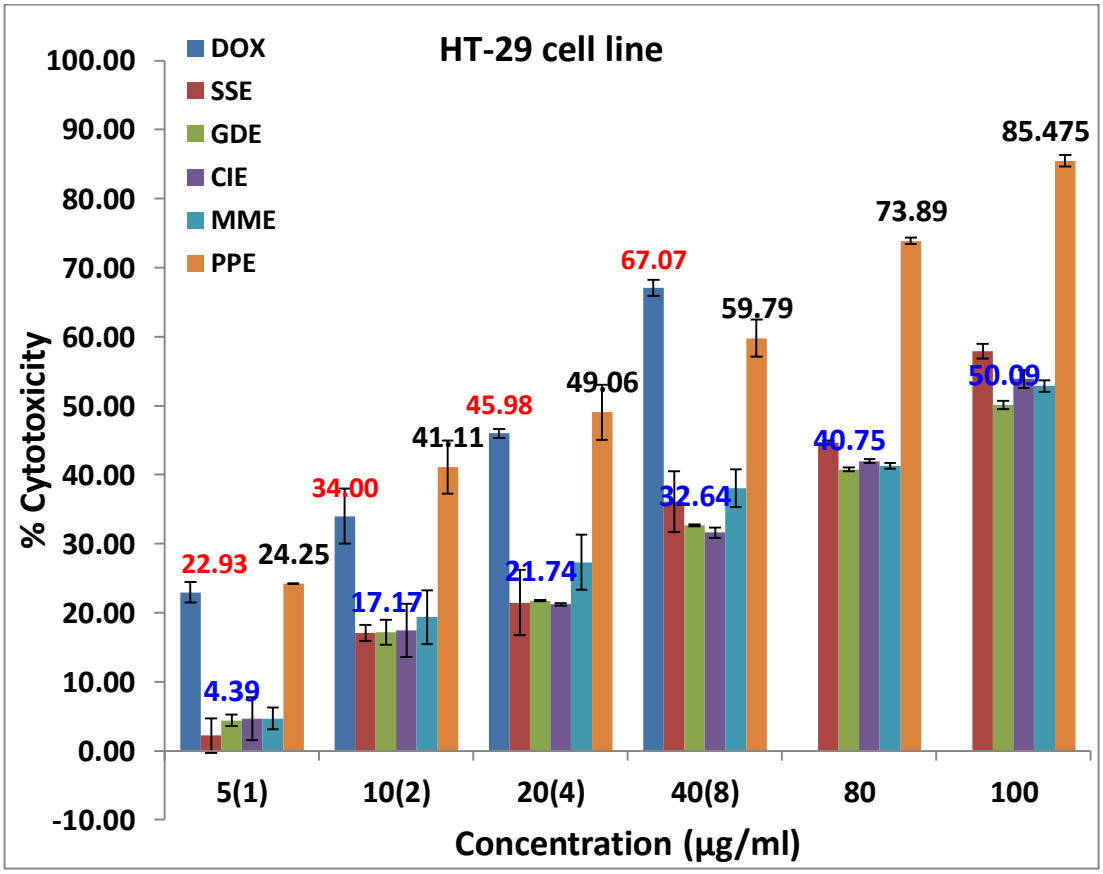
4.4 Cell cytotoxicity against HT-29(Colon) cancer cell lines

Treatment of HT-29 (Colon) cancer cells with *Clerodendrum infortunatum*, *Mikania micrantha, Girardinia diversifolia*, *Senecio scandens* and *Paris polyphylla* with different concentrations caused a concentration dependent increase in the cell cytotoxicity and the maximum cytotoxic effect was observed at the highest concentrations of *Paris polyphylla* (100µg/ml*)* having the cytotoxic effect of 85.47%. The lowest IC_{50} was obtained in *Paris polyphylla* with the concentration of 19.29µg/ml.

Table 6a: The cytotoxicity of medicinal plant extracts by MTT assay against HT-29 (colon) cancer cell line

Conc. (µg/ml)	DOX	SSE	GDE	CIE	MME	PPE
5(1)	22.93±1.48	02.18±2.49	4.39±0.85	4.60±3.10	4.67±1.57	24.24±0.03
10(2)	34.00±3.97	17.04±1.15	17.16±1.82	17.45±3.88	19.35±3.88	41.10±3.88
20(4)	45.97±0.68	21.470±4.70	21.74±0.10	21.21±0.19	27.31±3.99	49.06±3.99
40(8)	67.06±1.15	36.12±4.40	32.63±0.13	31.61±0.75	38.03±2.71	59.78±2.71
80	-	44.63±0.26	40.74±0.27	41.97±0.27	41.26±0.42	73.89±0.42
100	-	57.87±1.08	50.09±0.58	53.86±1.33	52.85±0.85	85.47±0.85
IC50	4.11	81.42	109.9	98.29	95.67	19.29

The data present as Mean± SD, n=3.



The data present as Mean± SD, n=3

Figure 3a: The cytotoxicity of medicinal plant extracts by MTT assay against HT-29 (colon) cancer cell line

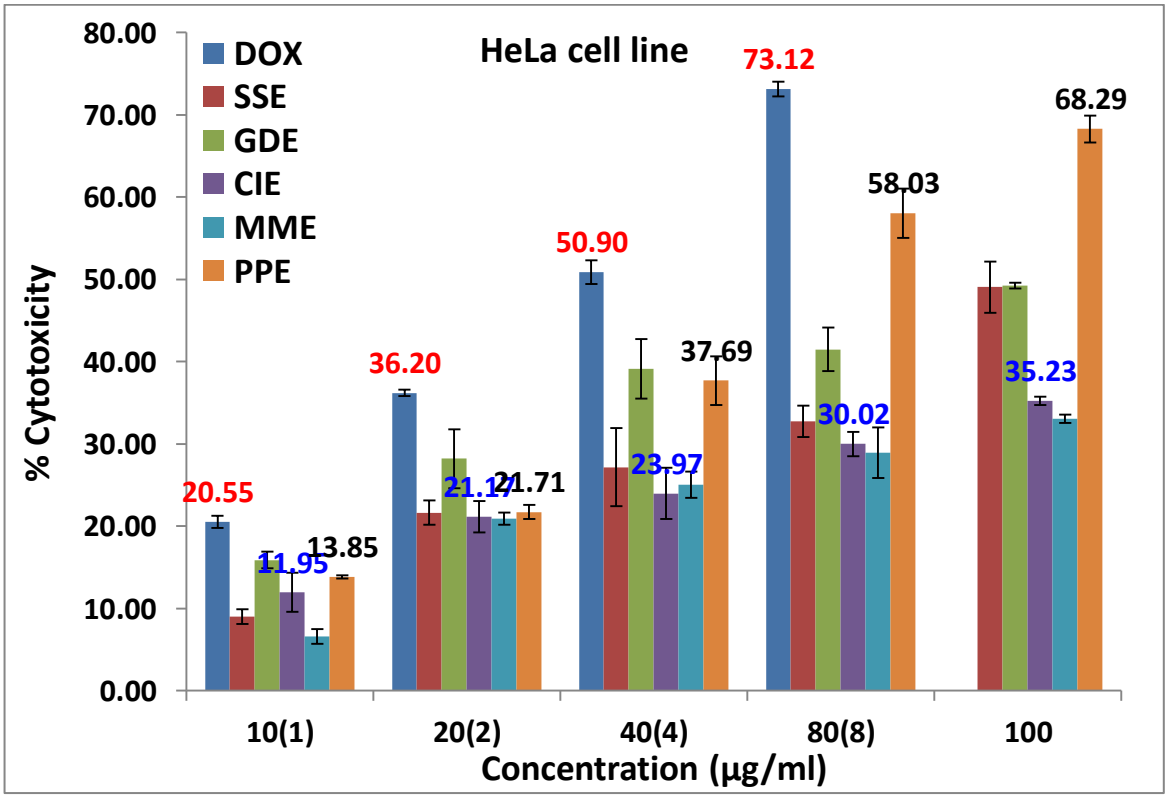
4.4.1 Cell cytotoxicity against HeLa (Cervical) cancer cell lines

The cell cytotoxicity against HeLa cell lines was in concentration dependent increase pattern. The maximum cytotoxicity was observed in 100 µg/ml concentration of *Paris polyphylla* having the cytotoxic effect of 68.29%. The IC_{50} of *Paris polyphylla* showed the lowest with 57.71µg/ml.

Table 6b: The cytotoxicity of medicinal plant extracts by MTT assay against HeLa (cervical) cancer cell line

Conc.(µg/ml)	Dox	SSE	GDE	CIE	MME	PPE
10(1)	20.54±0.75	9.01±0.92	15.89±0.98	11.95±2.38	6.60±0.87	13.84±0.17
20(2)	36.19±0.39	21.64±1.49	28.20±3.59	21.16±1.90	20.90±0.74	21.70±0.85
40(4)	50.89±1.43	27.154.74	39.12±3.59	23.96±3.11	25.03±1.60	37.69±2.99
80(8)	73.12±0.87	32.73±1.90	41.46±2.64	30.01±1.48	28.95±3.05	58.03±2.99
100		49.05±3.09	49.25±0.34	35.22±0.49	33.08±0.49	68.29±1.62
IC50	3.48	137.5	110.6	360.4	329.4	57.71

The data present as Mean± SD, n=3.



The data present as Mean± SD, n=3.

Figure 3b: The cytotoxicity of medicinal plant extracts by MTT assay against HeLa (cervical) cancer cell line

4.4.2 Cell cytotoxicity against AGS (Gastric) cancer cell lines

The cell cytotoxicity of *Clerodendrum infortunatum*, *Mikania micrantha*, *Girardinia diversifolia*, *Senecio scandens* and *Paris polyphylla* against AGS cell lines was also increase with the increase in concentration. The maximum cytotoxicity was observed in 100 µg/ml concentration of *Senecio scandens* having the cytotoxic effect of 83.02%. The IC_{50} of *Senecio scandens* showed the lowest with 25.19 µg/ml.

Table 6c: The cytotoxicity of medicinal plant extracts by MTT assay against AGS (gastric) cancer cell line

Conc.(µg/ml)	DOX	SSE	GDE	CIE	MME	PPE
5(1)	34.68±2.39	9.53±2.86	9.90±1.84	9.61±0.45	1.59±2.15	15.00±2.15
10(2)	47.28±0.21	21.10±1.09	22.99±5.20	17.17±0.87	14.44±2.98	27.85±2.98
20(4)	68.89±1.16	49.63±0.32	34.82±3.39	30.14±1.02	21.84±0.66	35.25±0.66
40(8)	86.70±1.60	64.40±2.54	44.16±0.02	39.14±3.59	28.91±0.50	55.73±0.50
80		75.95±3.31	54.95±0.37	51.60±0.35	30.81±0.60	71.04±0.60
100		83.02±0.97	66.43±4.94	58.53±1.21	38.38±0.63	78.61±0.63
IC50	1.94	25.19	50.88	67.97	220.1	31.13

The data present as Mean± SD, n=3.

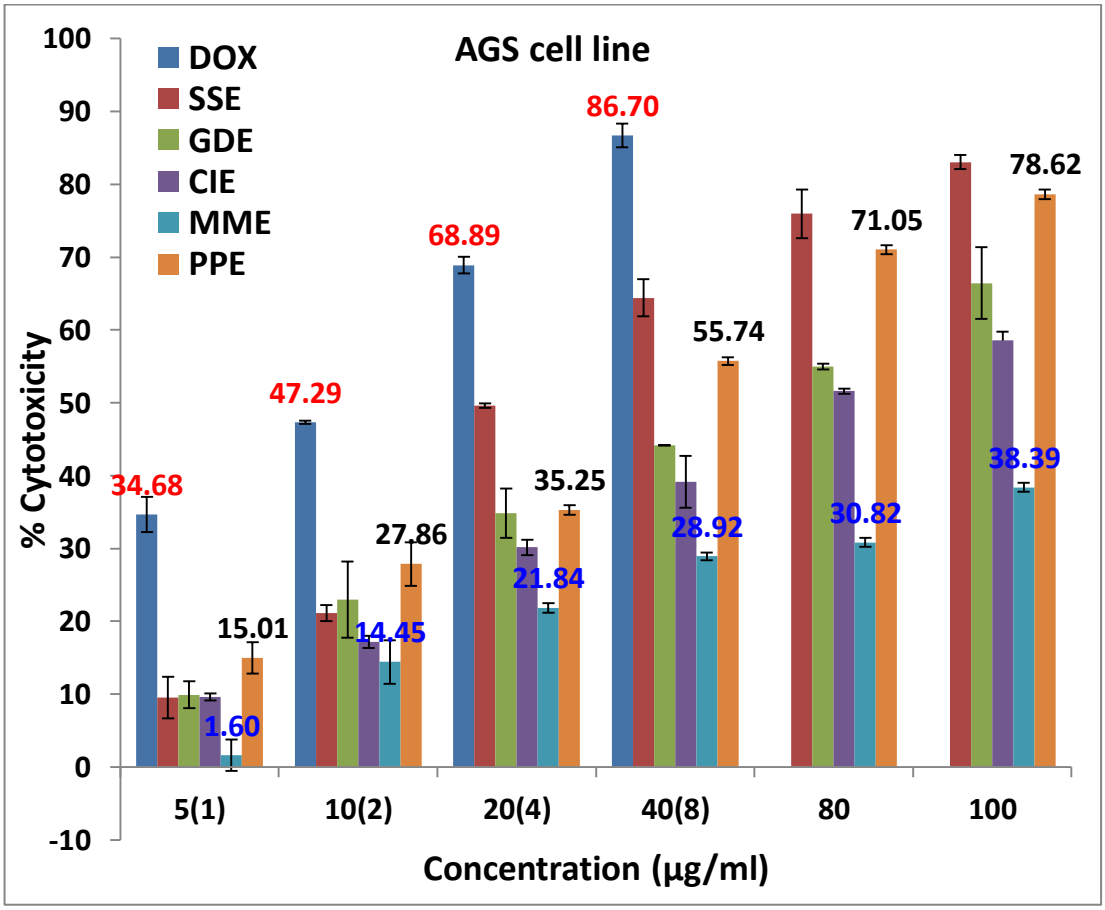


Figure 3c: The cytotoxicity of medicinal plant extracts by MTT assay against AGS (gastric) cancer cell line

4.4.3 Cell cytotoxicity against HepG2 (liver) cancer cell lines

The treatment of HepG2 cells with different plant in different concentrations resulted in a concentration dependent rise in its cytotoxic effects and a maximum cytotoxicity was recorded for the highest concentration (100µg/ml) of *Senecio scandens* having 80.98% cytotoxicity. *Paris polyphylla* exhibited overall lowest IC_{50} at 27.11 µg/ml.

Table 6d: The cytotoxicity of medicinal plant extracts by MTT assay against HepG2 (liver) cancer cell line

Conc. (µg/ml)	DOX	SSE	GDE	CIE	MME	PPE
10(1)	26.07±1.61	11.62±1.23	3.21±0.54	8.72±1.15	7.17±0.90	19.01±0.77
20(2)	35.11±2.36	43.57±0.36	26.99±3.80	21.74±1.16	12.45±0.74	44.58±0.29
40(4)	50.01±3.93	60.13±2.85	37.45±0.03	35.59±1.29	20.37±0.56	67.11±2.51
80(8)	80.72±1.24	73.06±3.72	48.03±1.70	48.79±0.39	22.50±0.68	73.01±1.64
100.00		80.98±1.09	55.63±0.23	55.80±1.83	30.98±0.71	77.94±0.58
IC50	3.16	30.72	77.7	79.19	387.3	27.11

The data present as Mean± SD, n=3.

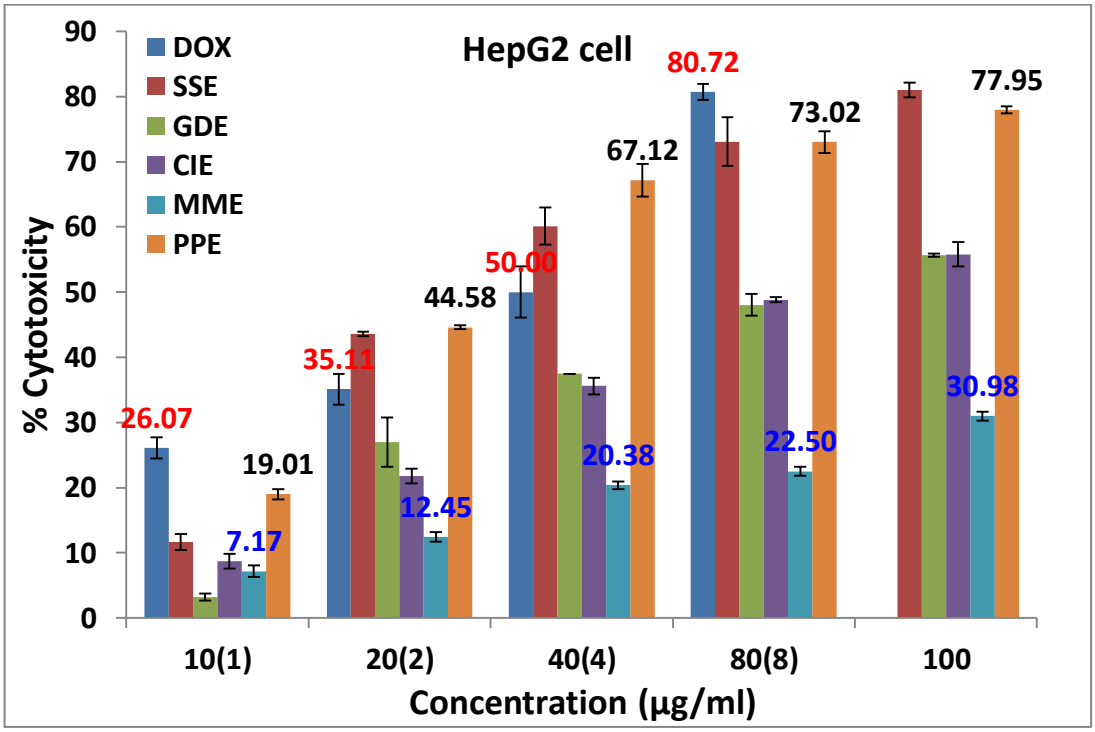


Figure 3d: The cytotoxicity of medicinal plant extracts by MTT assay against HepG2 (liver) cancer cell line

4.4.4 Cell cytotoxicity against A549 (Lung) cancer cell lines

The cell cytotoxicity of *Clerodendrum infortunatum*, *Mikania micrantha*, *Girardinia diversifolia*, *Senecio scandens* and *Paris polyphylla* against A549 cell lines was in concentration dependent increase pattern. The maximum cytotoxicity was observed in 100 µg/ml concentration of *Paris polyphylla* having the cytotoxic effect of 80.44%. The IC_{50} of *Paris polyphylla* showed the lowest with 20.49 µg/ml.

Table 6e: The cytotoxicity of medicinal plant extracts by MTT assay against A549 (Lung) cancer cell line

Conc. (µg/ml)	DOX	SSE	GDE	CIE	MME	PPE
10(1)	31.57±2.05	8.69±1.27	10.87±6.02	5.70±1.19	4.09±0.93	18.36±3.40
20(2)	41.07±1.05	41.70±0.37	24.57±3.93	19.15±1.19	9.54±0.77	56.80±2.04
40(4)	61.28±2.06	58.81±2.94	35.380.03	33.45±1.33	17.73±0.58	78.74±0.51
80(8)	85.08±0.26	63.63±2.74	46.31±1.76	47.10±0.41	19.93±0.70	79.42±0.17
100		80.35±1.13	54.16±0.24	54.34±1.89	28.69±0.74	80.44±0.85
IC50	2.37	34.8	85.73	84.44	359	20.49

The data present as Mean± SD, n=3.

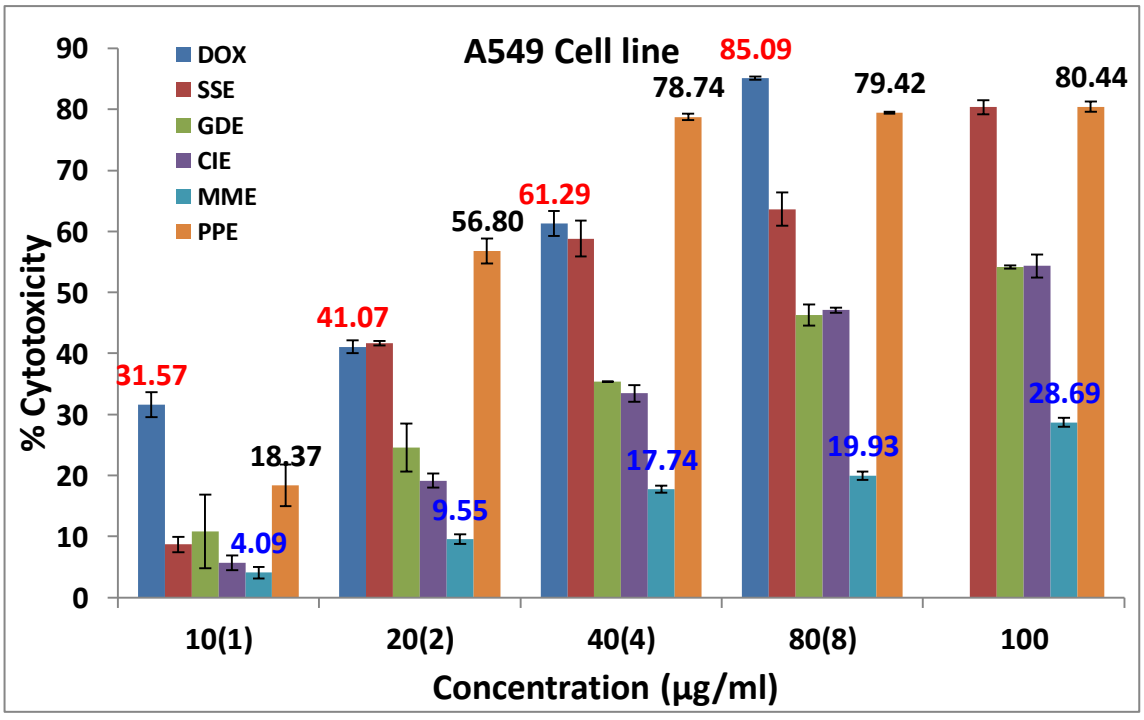


Figure 3e: The cytotoxicity of medicinal plant extracts by MTT assay against A549 (Lung) cancer cell line.

4.5 Caspase Activity

Since *Paris polyphylla* ethanolic extract exhibited overall best activity in phytochemical, antioxidant and anticancer study. Therefore, it was chosen for further analysis. The Caspase activity revealed that the *Paris polyphylla* ethanol extract induced apoptosis through intrinsic pathway in A549 cell lines by expression of initiator caspase 9 and executioner caspase 3. The *Paris polyphylla* induced both intrinsic and extrinsic apoptosis cell death in HeLa cancer cell. In HepG2 cell line, both initiator caspases (8 and 9) are expressed, meaning that cell death happened, but the executioner caspase 3 was not expressed, therefore we can imply that the kind of cell death may be necrosis.

Table 7: Caspase Activity

Caspases	Cell lines		
	HepG2	A549	HeLa
Casp-9	7.91	8.63	9.02
Casp-8	7.50	0.96	9.33
Casp-3	0.82	8.49	7.61

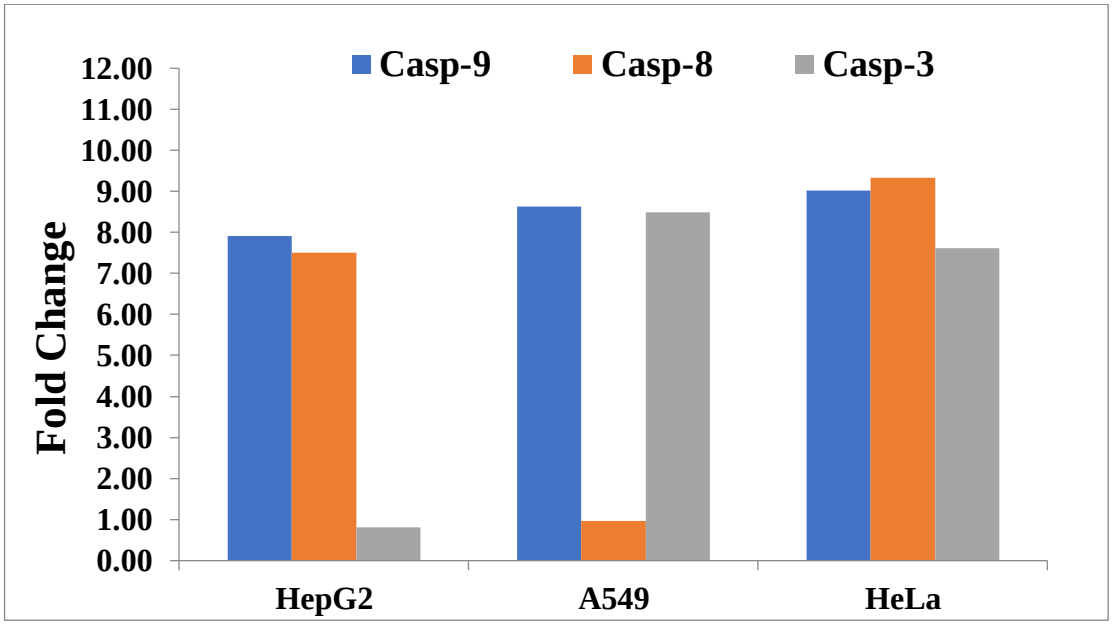
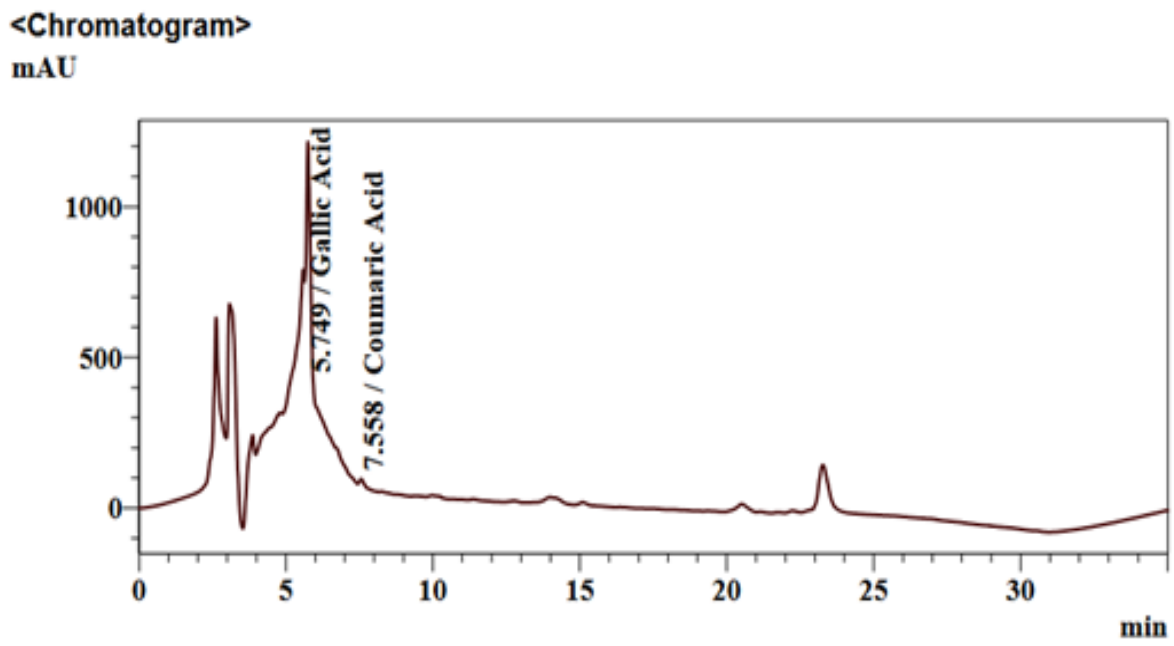


Figure 4: The caspase activity

4.6 High-performance liquid chromatography (HPLC)

The HPL study which was conducted using Caffeic acid, Coumaric acid, Gallic acid, Naringenin, Quercetin and Rutin confirmed the presence of Coumaric acid and Gallic acid in *Paris polyphylla* ethanolic extract.

<Chromatogram>



<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	5.749	3852816	534097	0.000	mg/L	M	Gallic Acid
2	7.558	181203	20842	0.000	mg/L	M	Coumaric Acid

Figure 5: The HPLC graph of *Paris polyphylla*

Table 8: The compounds identified using HPLC from *Paris polyphylla* and their medical uses

Name of Bio-active compounds	Uses
Gallic acid	Anti-oxidants, anti-inflammatory, anti-microbial
Coumaric acid	Anti-oxidants, anti-inflammatory, anti-diabetic, anti-ulcer and anti-cancer activity

4.7 Gas chromatography–mass spectrometry (GC-MS)

Table 9 : GC-MS profiling of the identified compound from the ethanol extract of *Paris polyphylla*

Peak	RT	Molecular weight	Molecular formula	Area %	Name of the compound
1	7.220	358.5	C20H38O5	2.46	Succinic acid, heptadecyl 4-methoxy-2-methylbutyl ester
2	7.998	170.30	C10H22N2	0.63	Iminobispropylamine
3	12.330	180.16	C6H12O6	20.34	D-Allose
4	13.319	180.1559	C6H12O6	6.87	D-Glucopyranose
5	15.163	121.139	C7H7NO	1.20	Benzamide
6	22.773	241.21	C9H14F3NO3	0.83	Sarcosine, N-(4-chlorobenzoyl)-, butyl ester
7	23.018	412.6	C27H40O3	1.66	7-Dehydrodiosgenin

Table 10 : Compounds identified from *Paris polyphylla* using GCMS and their medical uses

Name of the Bio-active compounds	Uses
Succinic acid	Antibiotic and curative agent
Iminobispropylamine	Antiseptics
D-Allose	Anti-oxidant, anti-inflamatory, anti-hypertensive
D-Glucopyranose	Hypglycemia
Benzamide	Anti-microbial, analgesics, anti-inflammatory, cardiovascular
Sarcosine, N-(4-chlorobenzoyl)-, butyl ester	Showed depressive symptoms in patient with schizophrenia
Diosgenin	Anti-oxidant, anti-inflammatory, anti-diabetic, anti-cancer

4.8 MOLECULAR DOCKING

Three dimensional structures of RAF (Rapidly Accelerated Fibrosarcoma) (PDB entry code 3OMV) and CDK2 (Cyclin-dependent kinases) (PDB entry code 5A14) were retrieved from the Protein Data Bank (PDB) http://www.pdb.org. The two compounds Diosgenin and Coumaric acid which were identified from HPLC and GCMS were used for docking. The 3D structures of both these compounds are obtained from Pubchem (https://pubchem.ncbi.nlm.nih.gov/).

4.8.1 Diosgenin and RAF

Diosgenin was subjected to molecular docking studies using the AutoDock Tool version 4.2.0. The target protein structures were docked with ligand which provided the least values of the binding energy. The Diosgenin bound with RAF receptor showed least binding energy of -7.97kcal/mol. This compound exactly fitted to the binding site of RAF receptor and interacted with six amino acids, Leusine-406, Alamine-373, Valine-363, Isoleusine-355, Tryptophan-423, Phenylalanine-475 (Fig.).

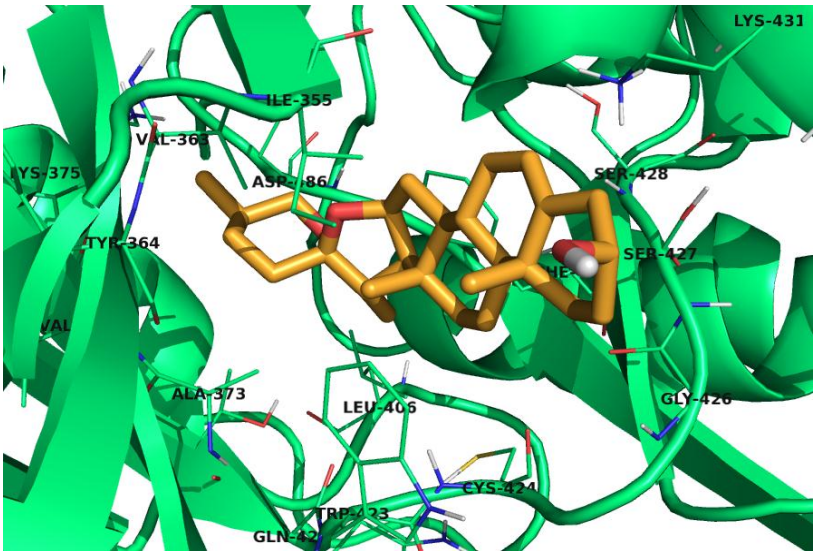


Figure 6: 3D image of how Diosgenin binds with RAF

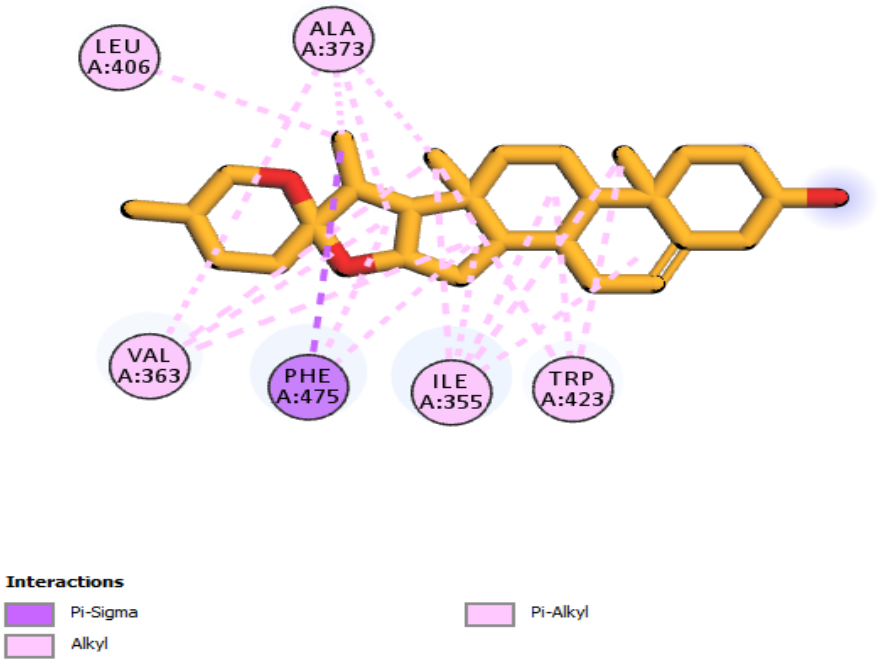


Figure 7: 2D image of how Diosgenin binds with RAF

Table 11a: The binding mode of Diosgenin and RAF

Amino Acids	Binding type	Distance (Å)
Phenylalanine-475	π–Sigma bond	3.59
Leusine-406	Alkyl bond	3.67
Alamine-373	Alkyl bond	4.14
Valine-363	Alkyl bond	3.81
Isoleusine-355	Alkyl bond	4.60
Tryptophan-423	π-Alkyl bond	3.66
Phenylalanine-475	π –Alkyl bond	3.41

4.8.2 Diosgenin and CDK2

Diosgenin bound with CDK2 receptor and showed good binding energy of-7.63 kcal/mol. This compound exactly fitted to the binding site of CDK2 receptor and interacted with five amino acids, Aspartic acid-86, Lysine-129, Alamine-149, Valine-164, Phenylalanine-146.

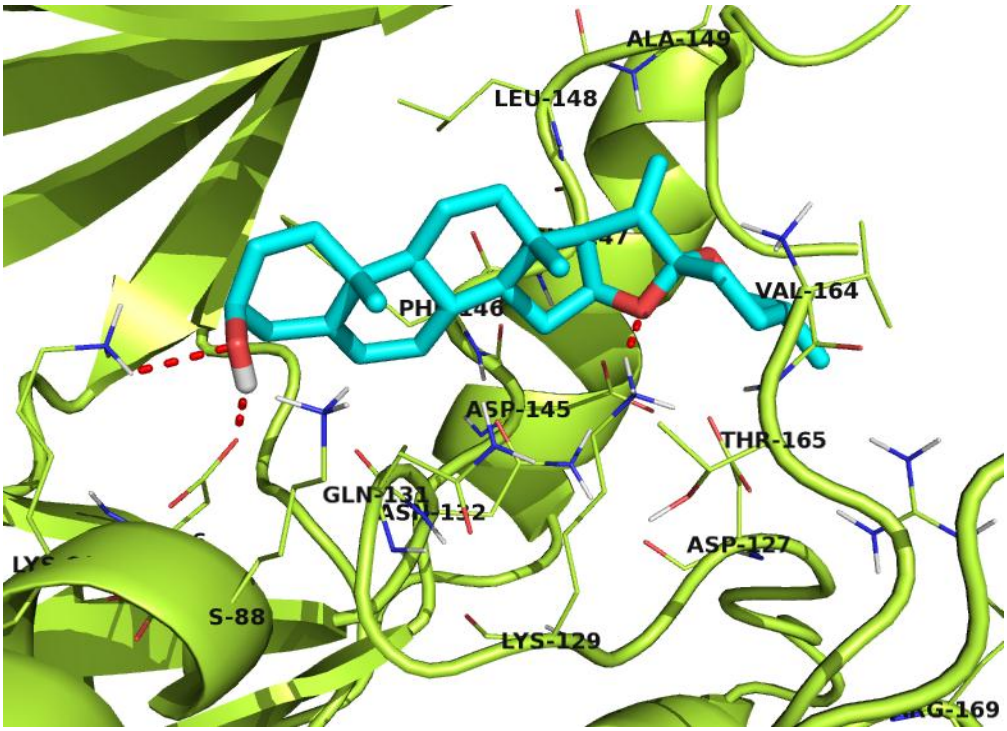


Figure 8: 3D image of how Diosgenin binds with CDK2

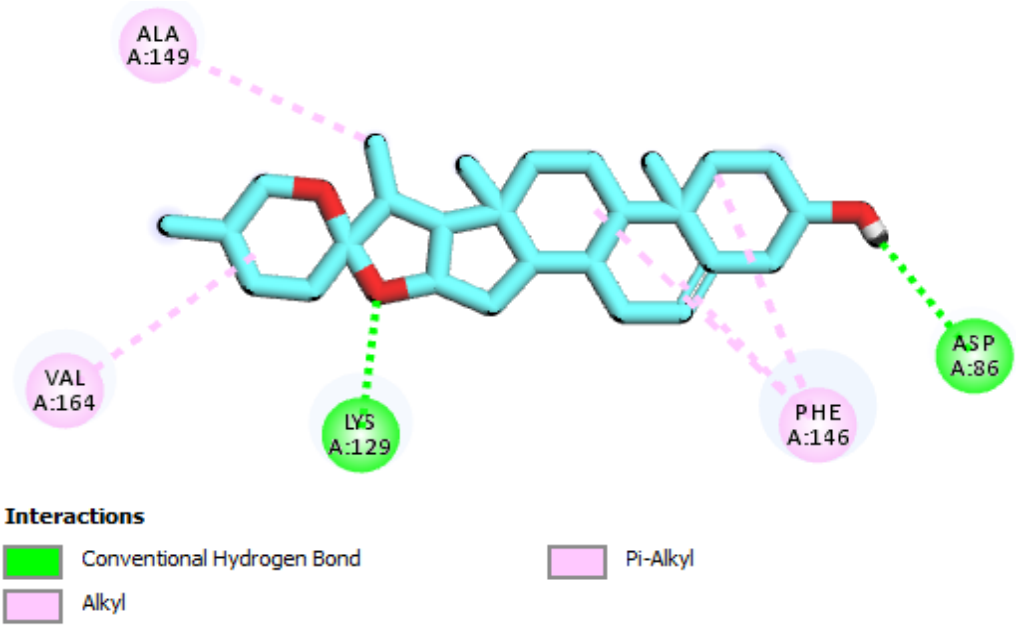


Figure 9: 2D image of how Diosgenin binds with CDK2

Table 11b: The binding mode of Diosgenin and CDK2

Amino Acids	Binding type	Distance (Å)
Aspartic acid-86	Hydrogen bond	2.08
Lysine-129	Hydrogen bond	1.77
Alamine-149	Alkyl Bond	3.61
Valine-164	Alkyl bond	4.24
Phenylalanine-146	π—Alkly bond	5.12
Phenylalanine-146	π—Alkly bond	4.22

4.8.3 Coumaric acid and RAF

Coumaric acid bound with RAF receptor showed binding energy of -6.44 kcal/mol. This compound exactly interacted with 6 amino acids, Glysine-426, Lysine-431, Glutamine-422, Cystine-424, Tryptophan-423, Alamine-373, (Fig.).

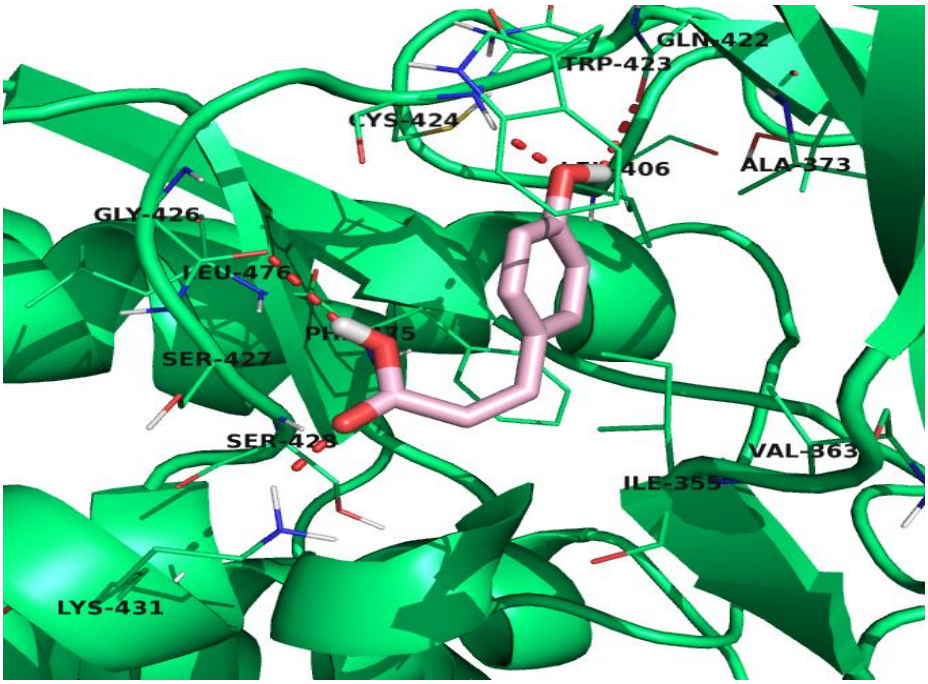


Figure 10: 3D image of how coumaric acid binds with RAF

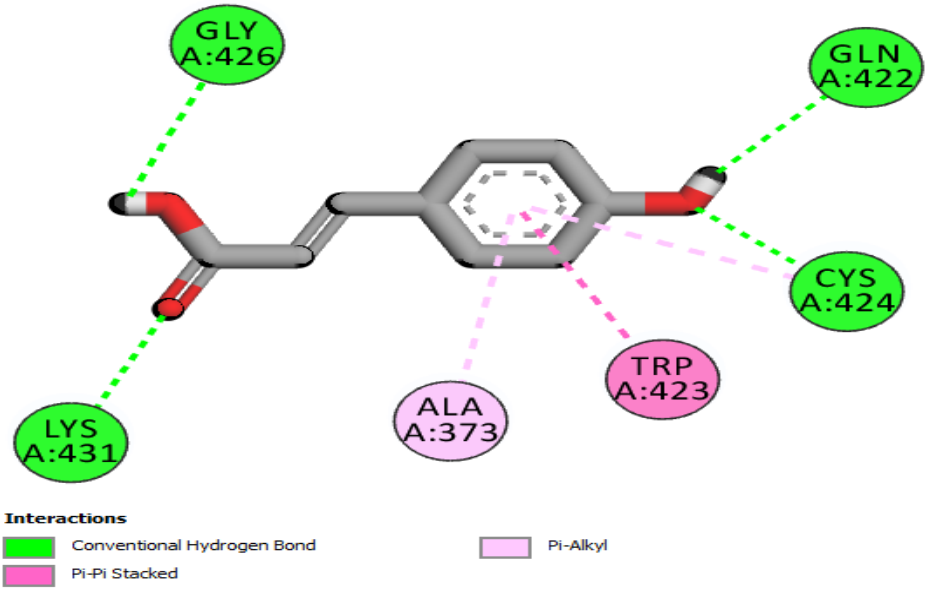


Figure 11 : 2D image of how coumaric acid binds with RAF

Table 11c: The binding mode of Coumeric acid and RAF

Amino Acids	Binding type	Distance (Å)
Glysine-426	Hydrogen bond	2.32
Lysine-431	Hydrogen bond	2.07
Glutamine-422	Hydrogen bond	2.22
Cystine-424	Hydrogen bond	1.98
Tryptophan-423	π—π bond	5.77
Alamine-373	π– Alkyl bond	4.30
Cystine-424	π– Alkyl bond	5.06

4.8.4 Coumaric acid and CDK2

The target protein structure (CDK2) was docked with Coumaric acid which provided values of the binding energy -4.0 kcal/mol. The binding profile of the Coumaric acid docking with CDK2 showed interaction with Histidine-125 and Cystine -118.

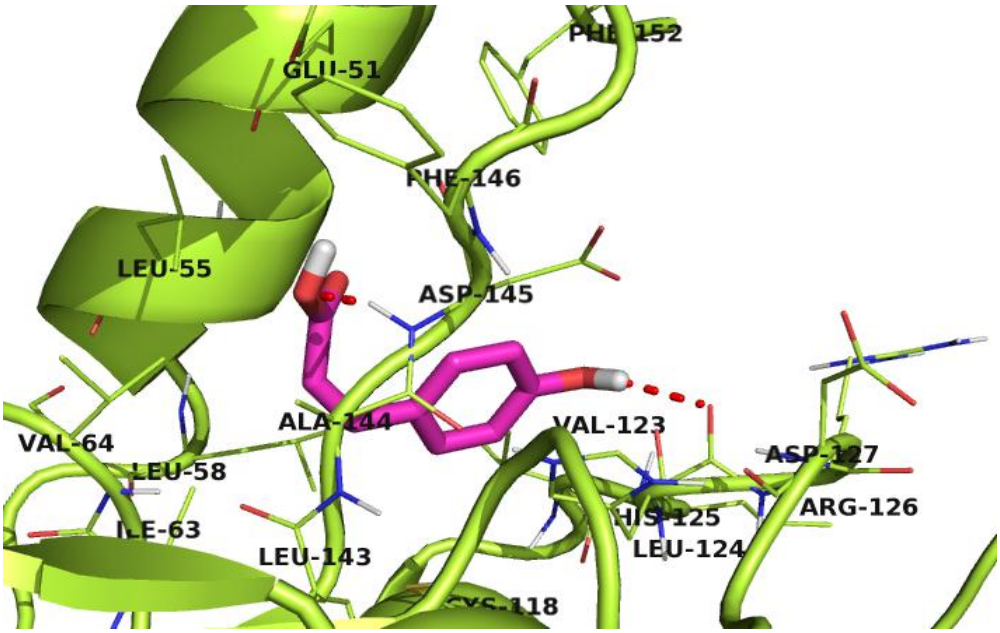


Figure 12 : 3D image of how coumaric acid binds with CDK2

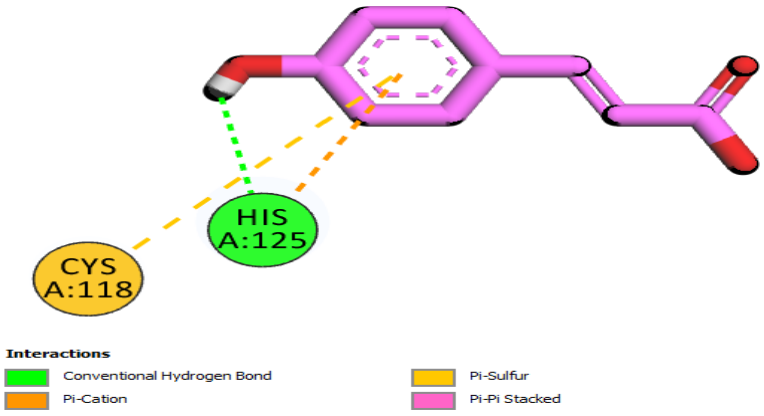


Figure 13: 2D image of how coumaric acid binds with CDK2

Table 11d: The binding mode of Coumeric acid and CDK2

Amino Acids	Binding type	Distance (Å)
Histidine-125	Hydrogen bond	2.12
Cystine -118	π—sulphur	5.35
Histidine-125	π—cation	3.75
Histidine-125	π—π	3.99

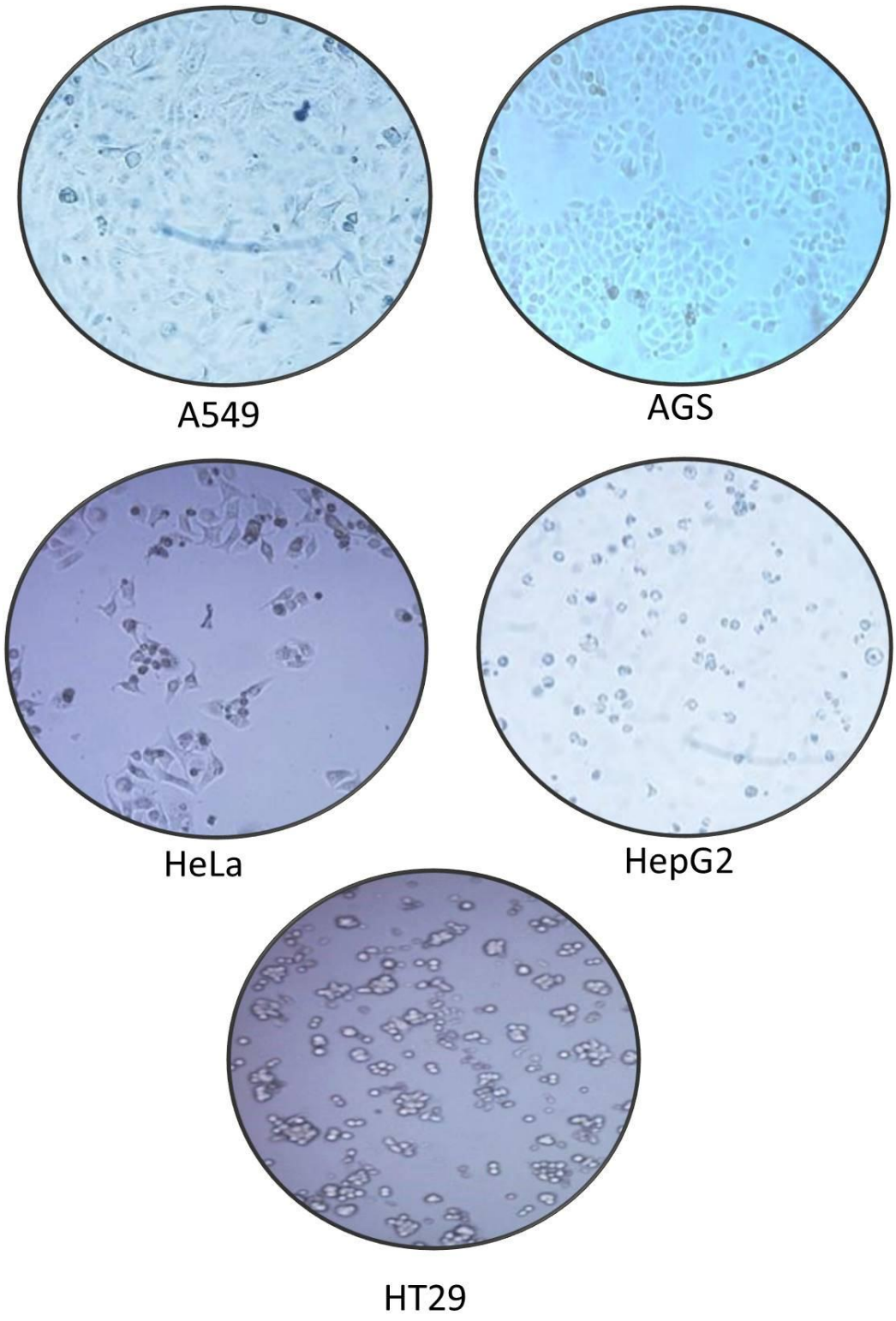


Plate 1- Cancer Cell lines

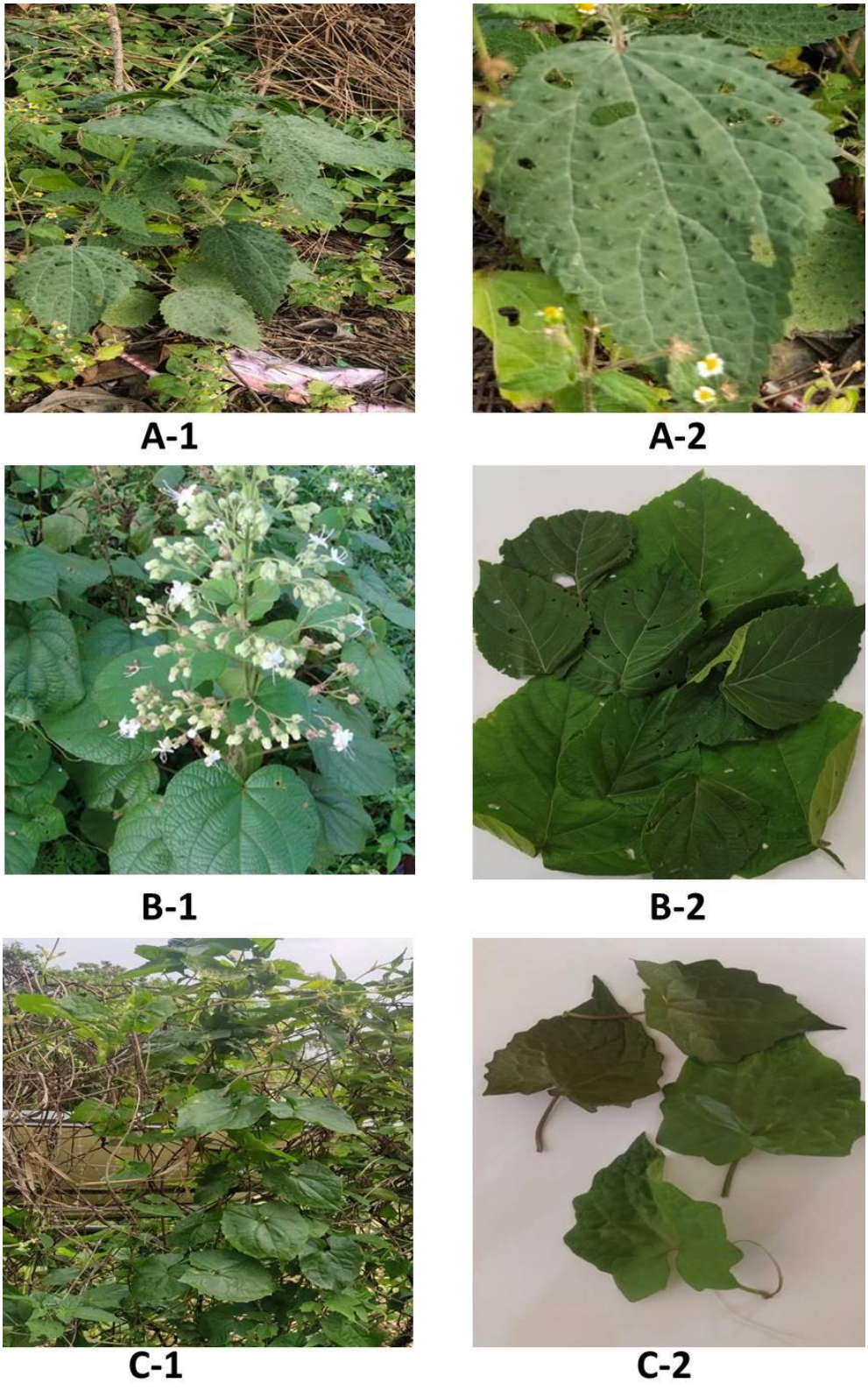


Plate 2- Colleted plants samples: A-1 &A-2-*G. diversifolia; B-1&B-2- C. infortunatum* and C-1&C-2- *M. micrantha*

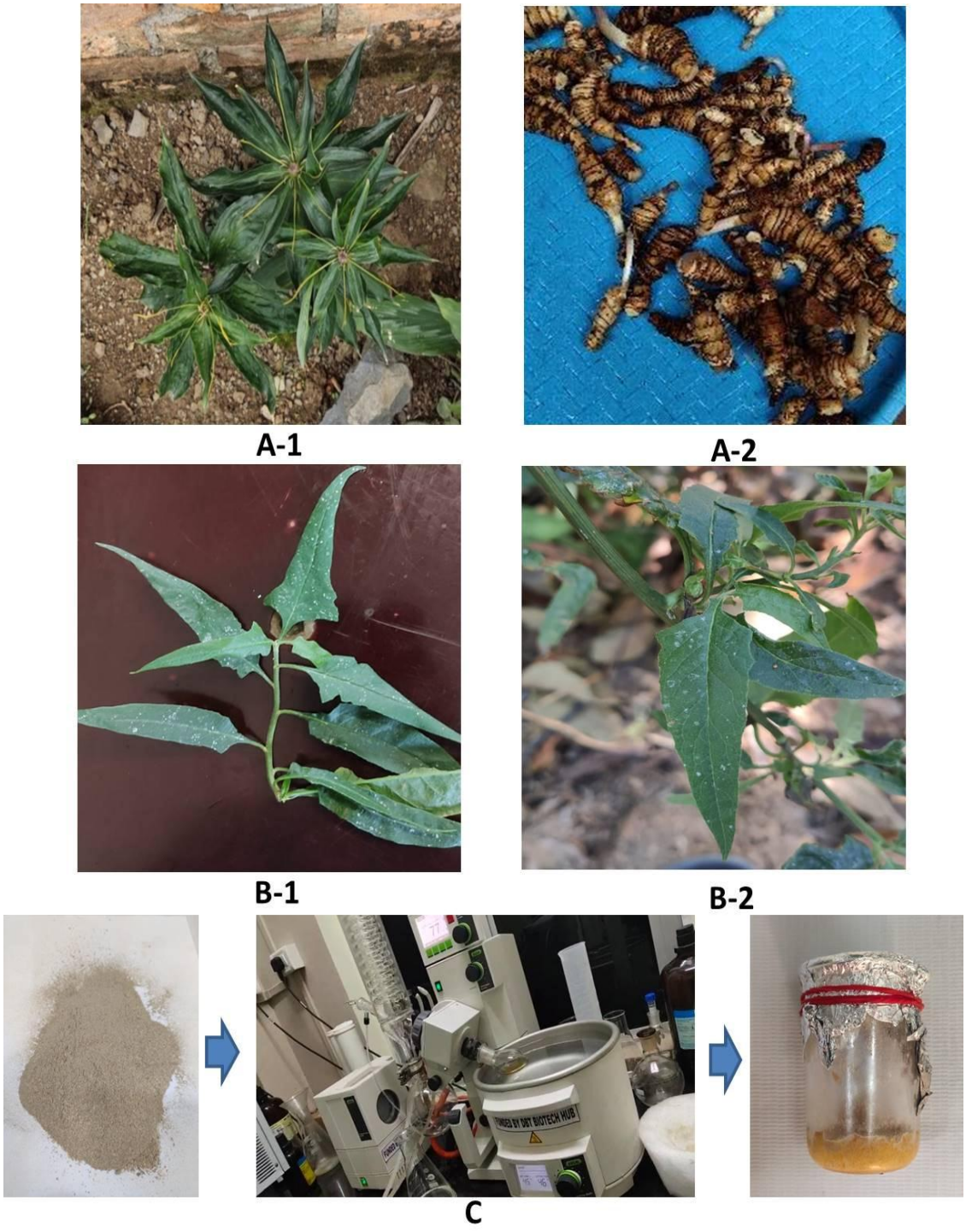


Plate 3- Colleted plants samples and laboratoty protocols: A-1 &A-2-*P. polyphylla;* B-1&B-2- *S. scandens* and C- preparation of extracts






Plate 4- Laboratoty protocols for antioxidants, anticancer activity and compound idendification by HPLC and GC-MS

Chapter-5

DISCUSSION

DISCUSSION

Alkaloids are a type of nitrogenous chemical produced by a variety of plants as secondary metabolites. Plants produce them for defence, harbivory, and protection against pathogenic organisms and dangerous insects (Kutchan, 1995). Plants are known to produce over 10,000 different alkaloids. Many of the alkaloids produced by plants are highly toxic to people and have been discovered to have remarkable physiological actions in humans, making them widely employed as medicines to treat a variety of human illnesses (Yang and Stöckigt, 2010). Hypertension, arrhythmia, malaria, cancer, cardiovascular problems, and HIV have all been linked to plant alkaloids. (Wink et al., 1998; Hagel and Facchini, 2013; Pan et al., 2013; Amoa Onguéné et al., 2013; Xing 2014; Chaves Valadão, 2015). All the ethanolic extract of the plants *Girardinia diversifolia, Mikania micrantha, Senecio scandens, Clerodendrum inforunatum* and *Paris polyphylla* showed the presence of alkaloids and this presence of alkaloids shows that the medicinal activities of this plant in humans may be in part due their alkaloid contents .

Flavonoids are found in all vascular plants, and there are around 10 different types of them (Harborne, 1998). There are around 8000 flavonoids found in various plant species. Along with anthocyanins, flavonoids are primarily responsible for the gorgeous colours of flowers (Iwashina, 2015). Many flavonoids act as copigments in the flower, contributing to colour diversity. Flavonoids are also important in plant stimulation, protection, flavouring, pigmentation, and plant-microbe communication (Ghasemzadeh and Ghasemzadeh, 2011). Plants and people both benefit from flavonoids as antioxidants. They also help plants grow by protecting them from stress and assisting in their development (Brunetti et al., 2016). Humans have been shown to benefit from flavonoids consumption in a variety of ways (Ivey et al., 2015). Flavonoids have been shown to have a wide range of functions in humans.

Antiallergic, anticancer, hepatoprotective, cardioprotective, anticatatactogenic, antiosteoporotic, antidiabetic, antibacterial, antiinflammatory, and antiviral properties are all present in them. (Hegarty et al., 2000; Cushnie and Lamb, 2005; Chahar et al., 2011; Kumar and Pandey, 2013, Tanaka, 2013). Similar to alkaloids all the plants *Girardinia diversifolia, Mikania micrantha, Senecio scandens, Clerodendrum inforunatum* and *Paris polyphylla* contained flavonoids and various medicinal properties of this plant may due to the presence of flavonoids and polyphenolic compounds in them.

Present study confirms the presence of Saponins in all the plants taken for this study. Saponins are a large group of glycosides that are distinguished from other glycosides by their surface active characteristics. They have foaming properties as well as detergent properties, which account for their soap-like behaviour in water and allow them to be employed as detergents (Oleszek, 2002; Chen et al., 2010; Couraud et al., 2014). They are allelopathic, which means they protect plants from insects and pathogens (Vincken et al., 2007). Saponins have antimicrobial, antimalarial, antiplasmodial, antiproliferative, antipsoriatic, antiallergic, antiatherosclerosis, antiatherosclerosis, antidiabetic, insecticidal, molluscicidal, anti-inflammatory, and anticancer properties. Takagi et al., 1980; Mert-Türk, 2006; Man et al., 2010; Dinda et al., 2010; Elekofehinti, 2015; Mroczek, 2015) have also been reported to be active against obesity. The presence of saponins in the plant may be responsible for some of the plant's medicinal properties.

Terpenoids, which is also found in all the ethanol extract of the five plants namely *Girardinia diversifolia, Mikania micrantha, Senecio scandens, Clerodendrum inforunatum and Paris polyphylla*. They are the primary constituents of the essential oils in many plants and they are synthesized for defence or as signals against indirect defence including herbivory and other enemies (Maatalah et al., 2012). Plant synthesize nearly 40,000 terpenoid molecules as secondary metabolites and they have diverse applications as industrial chemicals, flavouring agents,

pharmaceuticals, fragrance, pesticides and disinfectants (Cheng et al., 2007). Terpenoids have been shown to have a wide range of therapeutic properties, including antiviral, antibacterial, antimalarial, antiinflammatory, anticancer, and chemopreventive properties. They've been discovered to stop cholesterol from being made (Mahato and Sen, 1997; Wen et al., 2007; Bohlmann and Keeling, 2008; Sarala et al., 2011; Thoppil and Bishayee, 2011). These plants' therapeutic benefits could potentially be related to their terpenoid concentration.

The plants *Girardinia diversifolia, Mikania micrantha, Senecio scandens, and Paris polyphylla* shows the presence of tannins. Tannins are polyphenolic phytochemicals that are produced as secondary metabolites by a variety of plants (Khanbabaee and van Ree, 2001; Lu et al., 2012). Plants are protected against herbivores and insects by tannins, which reduce the availability of proteins or cause toxicity (Robbins et al., 1987; Frutos et al., 2004). Because of their ability to form complexes with protein, carbohydrates, and other macromolecules, they act as a barrier for pathogens and protect plants (Barbehenn and Constabel, 2011). Tannins have been shown to operate as both antioxidants and prooxidants in the presence of oxygen in vertebrates. (Robbins et al., 1987). Astringent, antibacterial, antiulcerogenic, antiviral, antitumor, antithrombogenic, and anti-inflammatory properties have been documented for tannins (Takechi and Tanaka, 1987; Banso and Adeyemo, 2007; Clinton, 2009; Ashok and Upadhyaya, 2012). These observations confirm the medicinal uses of these plants may be due to their tannins content.

Cardiotonic steroids are natural compounds with a steroid-like structure and an unsaturated lactone ring, commonly known as cardiac glycosides. They have cardiotonic action and frequently contain sugar moieties in their structure. (Schoner and colleagues, 2007). Digoxin, ouabain, bufalin, marinobufagenin, and telecinobufagin are some of the cardiac glycosides found in amphibians and mammals. In cardiology, certain cardiac glycosides are used to treat heart congestion and some forms of cardiac arrhythmias (Rahimtoola and Tak 1996). Some cardiac glycosides (e.g., digitoxin) have been shown to have powerful and selective

anticancer effects in vitro and ex vivo tests (Haux 1999, Sreenivasan 2006The cardiac medicines digitoxin and digoxin, as well as the semisynthetic cardiac glycoside UNBS1450 and two extracts from the Nerium oleander plant, have all entered clinical studies for cancer treatment. (Prassas and Diamandis 2008). Only the *Clerodendrum infortunatum* showed the presence of cardiac glycosides among all other plants.

Oxygen is one of the most important components for aerobic life, and its use by organisms has been linked to a cost in the form of harmful free radical production (Harman, 1956). The use of oxygen for energy production during respiratory pathways generates oxygen-derived free radicals, particularly superoxide and hydroxyl, which, if not managed carefully by the cells, cause oxidative stress (Cadenasa and Davies, 2000). Neutrophils, like other immune cells, use free radicals to fight infection. The creation of free radicals is kept under control to keep the individual healthy. Excess free radical production, on the other hand, is linked to autoimmune diseases, rheumatoid arthritis, cataracts, ageing, cardiovascular disorders, neurological diseases, and cancer (Valko et al., 2007). This suggests that fresh paradigms for neutralising excess free radical formation and protecting humans from the harmful effects of free radicals or free radical-induced oxidative stress are needed. Plants create a variety of biomolecules to defend themselves from insects, pests, and fungi, as well as other environmental challenges. These biomolecules could be extremely beneficial to human health because they are biocompatible and have no harmful effects at their maximum acceptable concentrations (Jagetia et al., 2003a; 2003b; Jagetia, 2007). Therefore the present study was under taken to evaluate the free radical scavenging activity of *Girardinia diversifolia, Mikania micrantha, Senecio scandens, Clerodendrum infortunatum* and *Paris polyphylla* which were used by the tribal people for treating several ailments.

Estimating DPPH scavenging is a straightforward way to investigate any pharmacological agent's antioxidant activity. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical that accepts an electron or hydrogen radical to form a stable diamagnetic molecule. It has long been used to estimate antioxidants' free radical scavenging properties. DPPH has a free electron and is violet in colour. When it

absorbs an electron from an antioxidant, it changes colour to yellow and is transformed to DPPH-H. (Goldschmidt and Renn, 1922). Previous studies suggest that the extracts of *Paris polyphylla, Clerodendrum infortunatum, Scenecio scandens, Grardinia diversifolia, Mikania micrantha* has shown to have antioxidant properties (Shen et al., 2014; Modi et al., 2010; Paciolla et al., 2011; Shrestha et al., 2020; Ishak et al., 2018)

Other plant extracts have been reported to scavenge DPPH radical earlier (Narayanaswamy and Balakrishnan, 2001; Baliga et al., 2003; Wong et al., 2006; Aparadh et al., 2012). Similarly, flavonoids like naringin, and mangiferin have also been reported to scavenge DPPH free radicals in a concentration dependent manner (Jagetia and Venkatesha, 2005).

The blue/green coloration of the ABTS•+ chromophore was obtained by reacting ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) with potassium persulfate. The addition of antioxidants to the pre-formed radical cation decreases ABTS, as seen by the blue/green colour deterioration. The % inhibition of the ABTS•+ enzyme was used to measure the extent of decolorization (Re et al., 1999). The trapping of the ABTS•+ radical cation by free radical scavengers is a standard approach for determining the total charge of antioxidants present in complex mixtures (Aliaga and Lissi, 1998).

Similarly, some plant flavonoids, such as naringin and mangiferin, have previously been shown to scavenge nitric oxide free radicals in a concentration-dependent manner (Jagetia et al., 2003a; 2012; Jagetia and Venkatesha, 2005).

The superoxide anion free radical is formed as a byproduct of incomplete oxygen metabolism during cellular respiration (Kirkinezosa and Morae, 2001). In the presence of metals, the superoxide anion produces H2O2, which produces hydroxyl free radicals (Turrens, 2003). As a result, neutralising the superoxide radical will stop the cascade of ROS production and protect the cells from oxidative stress. The plants *Paris polyphylla, Clerodendrum infortunatum, Scenecio scandens, Grardinia diversifolia, Mikania micrantha* have been found to inhibit the production of superoxide radical in a concentration dependent manner. Other plant extracts and some flavonoids have been reported to

scavenge the superoxide anion free radical earlier (Jagetia et al., 2003a; 2003b; Jagetia and Venkatesha, 2005; Jagetia et al., 2012).

The exact mechanism of free radical scavenging by the selected plants is not known. However, the free radical scavenging and antioxidant activity of *Girardinia diversifolia, Mikania micrantha, Senecio scandens, Clerodendrum infortunatum* and *Paris polyphylla* may be due to the presence of various phytochemicals like polyphenols and flavonoids, which may be able to donate or accept electron thus neutralizing their oxidative effects. Plants produce phenolic chemicals, particularly flavonoids, as secondary metabolites that aid pollination, protect against fungal infections, and give flowers appealing colours (Middleton and Chithan, 1993; Harborne and Baxter, 1999; Harborne and Williams, 2000). Because of their potential to scavenge free radicals and their antioxidant properties, these flavonoids have been found to have a beneficial effect on human health.

Chemotherapy was first used to treat metastatic cancer in 1956, and various synthetic chemical compounds have subsequently been effectively utilised to treat cancer, either alone or in combination with radiation and surgery. Normal toxicity, on the other hand, has been a serious worry because most chemotherapeutic medicines damage cellular DNA, and long-term use of these drugs has been linked to the development of second cancers in survivors (Pendelton et al., 2014). Before their actual chemical production, a large number of contemporary chemotherapy medicines were first obtained from plants (Cragg and Newman, 2014). The plant remains the most important source of novel medication discovery. Additionally, chemotherapeutic medications must not cause systemic toxicity and must be well tolerated by patients. Plant screening is a significant field for novel drug discovery. Therefore the present study was designed to evaluate the antineoplastic action of *Girardinia diversifolia, Mikania micrantha, Senecio scandens, Clerodendrum infortunatum* and *Paris polyphylla in vitro*.

The MTT assay is a rapid and standard technique to determine the cytotoxicity of any drug and the treatment of HeLa, AGS, A549, HT-29 and

HepG2 cells caused a concentration dependent rise in the cytotoxicity of ethanol extract of *Girardinia diversifolia, Mikania micrantha, Senecio scandens, Clerodendrum infortunatum* and *Paris polyphylla* due the reduction of MTT by mitochondrial succinate dehydrogenase by these cells. Because MTT can only be reduced in metabolically active cells, the degree of activity is a measure of the cells' viability. As a result, the more dead cells there are, the weaker the colour created. Previously, the MTT assay was used to assess cytotoxicity in a variety of cell lines (Mossmann, 1983).

Cell suicide, also known as apoptosis, is a carefully regulated process that happens in almost all living cells. Cell death is defined by cellular, morphological, and biochemical changes and is caused by the activation of a series of molecular events. Cell shrinkage, chromatin condensation, and nuclear fragmentation, membrane blebbing, caspase activation, and the development of membrane bound vesicles known as apoptotic bodies are all examples of these (Chen et al., 1999; Liu et al., 1996). Apoptosis can be triggered in two ways. The first, also known as the extrinsic or cytoplasmic pathway, is triggered by the Fas death receptor, which belongs to the TNF receptor superfamily (Zapata et al., 2001). The intrinsic or mitochondrial pathway, when activated, results in the release of cytochrome c from the mitochondria and the activation of the death signal (Hockenbery et al., 1990). Both paths eventually lead to the activation of a cascade of proteases known as caspases, which cleave regulatory and structural components, resulting in the cell's death. The activation of a group of proteases known as caspases is the key mechanism that leads to the execution of the death signal. Caspases aren't all engaged in apoptosis. Apoptosis, necrosis, and inflammation are all mediated by cysteine aspartic proteases' caspases. Caspases are controlled at the post-translational level, allowing them to be activated quickly. Caspases involved in apoptosis can be split into two functional groupings depending on their known or speculative involvement in the process: initiator caspases (caspase-2, -8, -9, and -10) and effector caspases (caspase-2, -8, -9, and -10). (caspase-3, -6, and -7). Caspases-3, -6, -7, -8, and -9 are the caspases that have been well described (Thornberry and Lazebnik, 1998; Mancini et al., 1998). Our study suggest that the

Paris polyphylla ethanol extract induced apoptosis through intrinsic pathway in A549 cell lines by expression of initiator caspase 9 and executioner caspase 3. The *Paris polyphylla* induced both intrinsic and extrinsic apoptosis cell death in HeLa cancer cell. In HepG2 cell line, both initiator caspases (8 and 9) are expressed, meaning that cell death happened, but the executioner caspase 3 was not expressed, therefore we can imply that the kind of cell death may be necrosis. Similar finding was observed by Lalrinzuali et al., 2021; Wong et al., 2013; Shoja et al., 2015 for different plants and different cell lines.

A-RAF, B-RAF, and C-RAF are three protein-serine/threonine kinases that were identified in 1983 and are related to retroviral oncogenes. Raf are serine/protein kinases that have been revealed as cellular components hijacked by transforming retroviruses. The human oncogene RAS can activate the three mammalian RAF proteins (A, B, and CRAF), which then have both kinase-dependent and kinase-independent tumor-promoting effects downstream. The MEK/ERK pathway, whose activation is linked to proliferation in a wide range of human malignancies, is primarily responsible for the kinase-dependent actions. Activating BRAF mutations were discovered in a fraction of human cancers almost a decade ago, and small-molecule RAF inhibitors have delivered exceptional response rates in melanoma patients in the last year. Raf thus qualifies as an excellent molecular target for anticancer therapy. (Maurer et al,. 2011)

A drug specifically targeting BRAFV600E (PLX4032/RG7204; Plexxikon/ Roche, Berkeley, CA, USA (Tsai et al., 2008; Joseph et al., 2010) has recently produced dramatic results, with response rates of 70–80 percent as a single agent in metastatic melanoma patients (Tsai et al., 2008; Joseph et al., 2010). (Bollag et al., 2010; Flaherty et al., 2010). Another ATP competitive BRAF inhibitor (GSK 2118436; GlaxoSmithKline, Brentford, UK (Kefford et al., 2010) produced similar results.

The protein kinase CDK2 belongs to the cyclin-dependent kinase family. It is crucial in the regulation of several processes in the eukaryotic cell division cycle. Evidence suggests that overexpression of CDK2 causes aberrant cell-cycle control, which is linked to cancer cell hyperproliferation. As a result, CDK2 was considered a possible therapeutic target for cancer treatment. We were able to comprehend the manner of CDK2 inhibition thanks to crystallography and the availability of the X-ray crystal structure of CDK2, which aided the development of a number of CDK2 inhibitors. Some CDK2 inhibitors have been tested in clinical trials to see if they may be used as anti-cancer drugs. (Chohan and colleagues, 2015)

Many pieces of data suggested that inhibiting CDKs could play an important role in cancer suppression. CDKs have become attractive targets for cancer therapy due to their frequent misregulation in malignant cells (Ahmed et al., 2011; Malumbres, 2009).

Our result shows that the compounds diosgenin and coumaric acid which were identified through HPLC and GCMS have good binding affinity with both RAF and CDK2 proteins which could result in the inhibition of these proteins expression and thus resulting in prevention of cancer. Similar to the present work, many bioactive compounds identified from the various organisms showed good docking scores against different types of cancer proteins. Ravi et al. (2017) described the compound gancidin - W derived from *Streptomyces paradoxus* (VITALK03) showed good binding energy -7.55 Kcal/mol against the targeted breast cancer protein. Shanta et al. (2018) screened five bioactive compounds derived from *Phyllanthus emblica* namely quercetin, kaempferol 3-bita-D-glucopyranoside, kaempferol, 1,1-diphenyl-2- picrylhydrazyl and isocorilagin against breast cancer protein estrogen receptor alpha (PDB id: 3ERT). Similarly, Paul et al. (2016) identified six bioactive compounds from *Hopea odorata,* screened against the breast cancer protein estrogen receptor alpha (PDB id: 3ERT). Further, Farhad et al. (2016) divulged the compounds stearic acid, carvacrol, palmitic acid

and vicenin from Ocimum sanctum screened against breast cancer protein estrogen receptor alpha (PDB id: 3ERT).

Chapter-6

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Plants have been the major source for several drugs and it is well known that 75% of the modern chemotherapeutic drugs have their origin in plants or natural products. Extracts from *Urtica membranacea, Artemesia monosperma,* and *Origanum dayi post* have also been reported to exert anticancer activity. *Artocarpus obtusus, Blumea balsamifera, Boerhaavia diffusa, Calotropis procera, Citrus maxima, Emblica officinalis, Moringa oleifera, Panax ginseng, Pfaffia paniculata, Rheum officinale, Saxifraga stolonifera, Vitex negundo, Withania somnifera,* and *Zingiber officinale* have also been found to possess anticancer activity under different conditions. Therefore plants and natural products still provide a major avenue for screening and developing of new nontoxic molecules including drugs for cancer treatment.

Samples of *Clerodendrum infortunatum* (leaves), *Mikania micrantha* (leaves), and *Girardinia diversifolia* (leaves), were collected from Aizawl, Mizoram. *Senecio scandens* (leaves), and *Paris polyphylla* (rhizome) were collected from Champhai, Mizoram was collected, cleaned properly, shade dried and powdered. extracted with ethanol and the liquid extracts were concentrated with rotary evaporator and stored at -70°C until further use. Preliminary phytochemical analysis showed the presence of Saponins, flavonoids, alkaloids, phenols and terpenoids present in all the plants.Tannins was present in all except *Clerodendrum infortunatum* and cardiac glycosides was absent in all except *Clerodendrum infortunatum.* Secondary metabolites like phenol, flavonoid and alkaloid were quantified and the amount of total phenols and flavonoids also increased with increasing concentration. *Paris polyphylla* showed highest content of phenol and alkaloid.

The *in vitro* antioxidant activity of *Clerodendrum infortunatum, Mikania micrantha, Girardinia diversifolia, Senecio scandens* and *Paris polyphylla* ethanolic extracts were analysed following DPPH, ABTS and superoxide free radical scavenging activity standard method. Free radicals are necessary evils as they are produced during normal metabolism of the body as well as a defense against the

pathogenic attack. However, excess of free radicals generation produce oxidative stress, inflammation and various diseases and any agent that can reduce the oxidative stress will be useful in treating oxidative stress related disorders. The various plant extracts inhibited the generation of DPPH, ABTS and Superoxide anion in a concentration dependent manner and this ability depended on the type of extract, the concentration. However, the IC_{50} scavenging activity for the free radicals was lowest for *Paris polyphylla* in ABTS and Superoxide. Therefore, the antioxidant activities of these plants may be due to the presence of good amount of secondary metabolites.

The cytotoxicity of the ethanol extract of *C. infortunatum, M. micrantha, G.diversifolia, S. scandens* and *P. polyphylla* on HT-29, AGS, HeLa, HepG2 and A549 cancer cells using MTT assay where the cytotoxicity was increased in a dose dependent manner. It also gives an account on the effect of different concentrations of the ethanol extract on the cytotoxicity. The ethanolic rhizome extract of *Paris polyphylla* showed the overall best result in cell cytotoxicity and was chosen for further result. The caspase activity assay revealed that apoptosis occurs in both A549 and HeLa cells following both intrinsic and extrinsic pathway and necrosis cell death happened in HepG2 cell since expression of caspase 3 was not seen.

Several compound including gallic acid, coumaric acid, diosgenin, benzamide and D-Allose were identified using HPLC and GC-MS.

The 3D image of RAF (Rapidly Accelerated Fibrosarcoma) – 3OMV and CDK2 (Cyclin-dependent kinases) - 5A14 were obtained from Protein Data Bank (PDB) and 3D image of diosgenin and coumaric acid were procured from Pubchem. These proteins which have huge involvement in cell division and the identified compounds were subjected to molecular docking using AutoDock Tool version 4.2.0. Diosgenin and RAF showed the binding affinity of -7.97 kcal/mol binded in the ATP binding site of RAF.

Since the plant *Paris polyphylla* showed a good activity in all the experiment undertaken, it can be a promising source of medicine in the future. To have a better understanding of this plants potency to cure cancer, animal model can be used.

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Curcuma aeruginosa Roxb. exhibits cytotoxicity in A-549 and HeLa cells by inducing apoptosis through caspase-dependent pathways

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ABSTRACT

The aim of the current study was to examine the efficacy of the leaf, stem and rhizome of *Curcuma aeruginosa* Roxb. for their phytochemical content, antioxidant and anti-cancer activities. The different parts of *C. aeruginosa* were subjected to sequential extraction to give three fractions viz., hexane, ethyl acetate and methanol extract. The cytotoxic effect and the mode of action against A-549 human lung adenocarcinoma and HeLa cell lines were examined. *C. aeruginosa* presented no significant toxic effect in normal human lung cells (L-132). The methanol extracts were found to be the most cytotoxic and further investigation was carried out to understand the effects. The methanol extracts induced DNA damage after 24 h with significant increase in tail DNA and tail moment when compared to untreated control. Up-regulation in the expression of the caspase − 8 and − 3 activity was observed after 48 h of treatment. The mechanism of cell death and apoptosis induced by the methanol extracts on A549 and HeLa cells were studied using fluorescent staining. Bioactive compounds detected from the HPLC revealed phenol and flavonoid compounds: Gallic acid, quercetin, caffeic acid, kaempferol, rutin, coumaric acid and naringenin. GC-MS results identified the presence of sesquiterpenoids: α-curcumene, curzerene curcumenol, curzerenone epicurzerenone, caryophyllene oxide and diterpenoid, andrographolide. These compounds are known for inducing apoptosis in human cancer cells through caspase - dependent pathways. Therefore, *C. aeruginosa* and its potential to induce apoptosis in cancer cells suggest that they have potential in medical applications.

1. Introduction

Curcuma aeruginosa Roxb. (Zingiberaceae) a rhizomatous herbaceous plant is also known as 'Pink and blue ginger' [1]. They are aromatic, deep blue or bluish black coloured cortex with pungent smell. It is known to be rich in ethno medicinal values with wide range of pharmaceutical properties. It is native to Myanmar [2]. In India, it grows in parts of the South-east and central regions and is used by the tribals to cure several ailments [3]. Due to the high therapeutic value and repeated harvest, the natural population has greatly declined, according to IUCN report, it is in the critically endangered category [4–6]. Curcuma plants are usually considered to be antibacterial, anti-viral, and anti-tumor [7]. C. aeruginosa has been identified to have various pharmacological activities like antimicrobial [8], antioxidant [9], anti-inflammatory, analgesic effect [10], anti-HIV-1 [11], anti-cancer [12], uterine relaxant effect [13], anti-androgenic and is traditionally used for treating gastrointestinal complications [14]. The rhizome contains several essential oils [15,16] and reported to possess pharmacological actions in treating various illnesses such as tumors, asthma and bronchitis [17]. It is vital to evaluate the developmental metabolic characteristics observed in these plants for better utilization and usage. Due to lack of systematic scientific proof on the molecular mechanism of herbs, it is often considered as only a substitute choice to conventional drugs [18].

Cancer is a primary cause of death worldwide, 10 million deaths was reported in 2020 [19]. As many as 139 thousand people in India had cancer in 2020. Lung and cervix were found to be among the major sites of the disease [20]. Cancer treatments are not entirely safe and the chemotherapeutic agents we utilize develop drug resistance overtime [21]. Since time immemorial, various parts of the plant such as leaves, roots, stem etc. are being used to a treat number of diseases and infections. The complications of allopathic medicines have made researchers turn interest towards plant-based phytochemicals in treating various diseases [22]. Due to the minimum toxic effects of herbal

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materials as compared to chemical drugs, they are considered a good option in cancer treatment [23]. It is greatly desired to acquire compounds that cause cancer cell death through apoptosis [24].

The literature survey showed that as far as our knowledge, no work has been done to compare the anti-cancer effect of the leaf, stem and rhizome of *C. aeruginosa*. Therefore, considering the significance of natural product-based antioxidants in treating several human disorders, the present study was focused towards characterizing the anticancer potential of *C. aeruginosa* in a comprehensive manner.

2. Materials and method

2.1. Collection of plant sample

The uninfected *Curcuma aeruginosa* Roxb. (Family: Zingiberaceae) was collected from Durtlang (23° 47' 0" North, 92° 44' 0" East), Mizoram, India during the month of May, 2021. The plant sample was authenticated by Prof. H. Lalramnghinglova, Dept. of HAMP, Mizoram University. The herbarium was deposited in the Institutional Herbarium, Mizoram University, Aizawl with voucher number bearing No.00397. The plant sample was washed with water, air dried and crushed into fine powder with electric grinder.

2.2. Preparation of extract

The powder sample was subjected for sequential cold extraction using solvents of increasing polarity namely hexane, ethyl acetate and methanol. The samples were extracted at 37 °C in continuous stirring till the solvent turn colourless. The extract samples were filtered and dried by rotary evaporator and stored at − 20 °C till further use.

The hexane, ethyl acetate and methanol extract from rhizome, stem and leaves were written as:

RH- Rhizome Hexane, SH- Stem Hexane, LH- Leaf Hexane, RE- Rhizome Ethyl acetate, SE- Stem Ethyl acetate, LE- Leaf Ethyl acetate, RM- Rhizome Methanol, SM- Stem Methanol and LM- Leaf Methanol.

2.3. Phytochemical analysis

The qualitative phytochemical analysis of the different extracts of *C. aeruginosa* was performed by standard procedures given by Trease and Evans [25], Harbone [26], Sofoware [27] to detect flavonoid, tannin, alkaloid, phenol, terpenoid, saponin, quinone, cardiac glycosides, anthraquinone in the plant.

The selective quantitative study was carried out for phenol [28], flavonoid [29] and alkaloid content [30] and results were expressed as gallic acid, quercetin and atropine equivalents respectively.

2.4. Antioxidant activity free radical scavenging activity

2.4.1. DPPH scavenging assay

The activity was performed by a method described by Leong and Shui [31]. 1 ml solution of 0.1 mM DPPH was added to the various concentrations of the different extracts. Absorbance was measured at 523 nm after an incubation time of 30 min.

2.4.2. ABTS scavenging assay

Determined according to the method of Re et al. [32] with minor modifications. To 1 ml of distilled water, 37.5 mg of potassium persulfate was added. 44 μl from this solution and 9.7 mg of ABTS were mixed in 2.5 ml of distilled water. For 16 h, the solution was kept at room temperature in dark condition. Absorbance was measured at 734 nm.

2.4.3. Superoxide anion scavenging assay

The activity was determined by following Hyland et al [33] with slight modifications.

0.6 ml of the different extracts and 0.2 ml of NBT were added. To this, 2 ml of alkaline DMSO was added to make a final volume of 2.8 ml.

DMSO was used as blank. The absorbance was measured at 560 nm.

2.4.4. Phosphomolybdenum assay

The total antioxidant capacity was determined according to the method of Prieto et al. [34]. An aliquot of 0.1 ml of each extract was added to 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Incubation was done for 90 mins in a water bath at 95 °C. The samples were cooled to room temperature. Absorbance was measured at 765 nm against a blank.

2.4.5. Hydroxyl radical scavenging assay

The activity was measured by following Smirnoff and Cumbes [35]. 1.0 ml of 1.5 mM $FeSO_4$ was added to 0.7 ml of 6 mM hydrogen peroxide. To this, 0.3 ml of 20 mM sodium salicylate and different concentrations of the extract was added together to make up a reaction mixture of 3 ml. Followed by incubation at 37 °C for one hour.

2.5. Selection of most cytotoxic extract

MTT assay was carried out for selecting the extract with the highest cytotoxic effect among the 9 samples. Based on the IC_{50} values, the methanol extract of *C. aeruginosa* showed the best cytotoxic effect overall and the remaining experiments were carried out using the methanol extract from rhizome, stem and leaves and the cell cultures were divided into the following groups:

MEM group: The cells of this group were untreated and serve as negative control.

Dox group: The cells were treated with IC_{50} concentration of doxorubicin and used as positive control.

Treated group: Cells were treated with IC_{50} concentration of the following extracts.

1. RM group – Rhizome methanol
2. SM group – Stem methanol
3. LM group – Leaf methanol

2.6. MTT assay

In vitro anticancer activity of different parts of *C. aeruginosa* against A-549 and HeLa cell line and their toxicity in normal cell line (L-132) was studied using MTT assay described by Mosmann [36]. Briefly, 10^4 cells were seeded in 96 well plates containing 100 μl minimum essential medium (MEM) in each microwell. The cells were kept at 37°C in a CO_2 incubator with an atmosphere of 5% CO_2 in 95% humidified air and were allowed to attach for 24 h. Different concentrations (5, 10, 20, 40, 80 & 100 μg/ml) of the samples or doxorubicin were added into the well of the microplates and incubated in the CO_2 incubator. After 72 h of cell plating, 20 μl of MTT was added and the microplates were incubated for another 2 h. The media were removed and the insoluble purple formazan formed was dissolved with DMSO and incubated once again for 4 h. Then, the absorbancy reading was analysed at 560 nm and the cytotoxicity was calculated.

Cytotoxicity (%) = Control-Treatment/Control X 100.

2.7. Comet Assay

It was performed using modified version of Olive & Banáth [37].The A-549 and HeLa cells were seeded separately in 6-well plates for 24 h at 37°C in 5% CO_2 to allow cell adherence. After adherence, cells were treated with the IC_{50} concentration of the methanol extracts (RM, SM and LM) for 24 h. Firstly, the slides were coated with 1% agarose solution. The cells were trypsinised and centrifuged. The supernatant was discarded, the cells were mixed with the low melting agar (0.7%) and then mounted on the slide with cover slip and were allowed to solidify. Lysis buffer (1%SDS, 0.5 M NaCl, 0.1 M NA2EDTA, 0.01 M Tris HCl,

Table 1
Phytochemical analysis of different extract of *C. aeruginosa* Roxb.

	Qualitative phytochemical analysis using standard protocol									
Part used	**Solvent**	**Phenols**	**Flavonoids**	**Alkaloids**	**Saponins**	**Quinones**	**Cardiac glycosides**	**Terpenoids**	**Tannins**	**Anthraquinones**
Leaf	Methanol	+	+	+	+	–	+	–	+	–
Stem		+	+	+	–	–	+	–	+	–
Rhizome		+	+	+	+	+	+	+	+	–
Leaf	Ethyl acetate	+	+	+	–	+	+	–	–	–
Stem		+	+	+	–	+	+	+	+	–
Rhizome		+	+	+	+	+	+	+	+	–
Leaf	Hexane	+	+	–	+	–	+	+	+	–
Stem		+	+	–	+	+	+	+	+	–
Rhizome		+	+	–	+	+	+	+	+	–

	Quantitative phytochemical analysis (mg/g of dry weight)			
		Phenols	**Flavonoids**	**Alkaloids**
Leaf		7.66 ± 0.05	7.57 ± 0.022	3.19 ± 0.52
Stem	Methanol	1.67 ± 0.24	0.96 ± 0.15	3.24 ± 0.48
Rhizome		3.67 ± 0.32	3.92 ± 0.08	3.28 ± 0.15
Leaf		4.37 ± 0.06	3.23 ± 0.42	3.05 ± 0.03
Stem	Ethyl acetate	1.97 ± 0.02	1.04 ± 0.13	0.28 ± 0.04
Rhizome		5.19 ± 0.32	2.3 ± 0.05	1.12 ± 0.27
Leaf		2.29 ± 0.34	0.88 ± 0.35	NA
Stem	Hexane	0.57 ± 0.42	0.40 ± 0.03	NA
Rhizome		2.26 ± 0.02	1.04 ± 0.08	NA

pH10), 1%Triton X and 10% DMSO was added after removal of cover slip and was allowed to stand overnight. The slides were taken out and run with electrophoresis buffer (100 mM Na2 EDTA, I M NaOH) for 50 mins at 70 mA. Then, the slides were taken out and kept in neutralized buffer (IM Tris HCl pH7.5). Finally, the slides were stained with EtBr and observe under Fluorescence Microscope (EVOS® FL Cell Imaging System, Thermo Fisher Scientific). Image capture and analysis were done with Open Comet v1.3.1 and Graph Prism 7.

2.8. Apoptotic morphological changes by Acridine orange (AO)/Ethidium bromide (Et-Br) (2:1) staining

The morphological alterations induced by the extracts in HeLa, A-549 and L-132 cells were studied using AO/Et-Br staining. Two DNA-binding dyes AO and Et-Br were used for the morphological detection of apoptotic and necrotic cells. The different cell lines were seeded separately in 6- well plates for 24 h at 37^{O}C in 5% CO_2 to allow cell adherence. The cells were treated with the IC_{50} concentration of the treated group for 24 h after adherence. Then, the cells (1×10^5 cells/ml) were washed with ice-cold 1X PBS (ph 7.4). Cells were then fixed in 4% formaldehyde in 1X PBS at room temperature for 15 mins. The dye was incubated with the cells at room temperature in dark for 10 min. Fluorescence microscope (EVOS® FL Cell Imaging System, Thermo Fisher Scientific) was used for observing the stained cells and the apoptotic and necrotic index was calculated.

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

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

2.9. Measurement of Caspase-3 and Caspase-8 enzyme activation

Caspase-3 and Caspase-8 activities were measured using ELISA kits according to the manufacturer's instructions (Bioassay Technology Laboratory, China). Briefly A-549 and HeLa cells were kept in a T25 flask for 24 h prior to the experiment. Cells were exposed to the IC_{50} concentrations of the treated group for 48 h to induce apoptosis. 10 μg/ml doxorubicin was taken as positive control. Fold increase in Caspase-3 and 8 activities were examined by direct comparisons to the level of untreated controls.

2.10. Identification of bioactive compounds using GC/MS analysis

The GC/MS analysis was done using a Clarus 690 Perkin/Elmer (Autosystem XL) Gas Chromatograph mass detector Turbo mass gold Perkin Elmer Turbomass 5.1 spectrometer and an Elite 1 (100% Dimethyl poly siloxane) capillary column measuring 123.5 m x 678 m. The instrument was fixed at a temperature of 40 °C ramp 5 °C/min to 115 °C, hold 5 min, ramp 5 °C/min to 140 °C, hold 5 min, ramp 2 °C/min to 210 °C, hold 8 min, and maintained for 3 min. After this, the oven temperature was allowed to rise up to 250 °C, at the ratio of 5 °C/mins for a duration of 9 mins. The injection port temperature was maintained at 250 °C, while the flow rate of Helium was kept at 1.5 ml/min. 70 eV was set as the ionisation voltage. The samples were injected in a 10:1 split mode. The mass spectral scan range was set to 500–800 (*m/z*). The ion source was ensured at 230 °C, while the interface was kept at 240 °C. The start time of the MS was 3 min, 75 min was the end time and the solvent cut time was 3 min. These compounds were matched with PubChem Compound (NCBI) and NIST 17 online library Ver. 2.3.

2.11. HPLC chromatogram analysis

The secondary metabolites were identified by Shimadzu Instrument (Shimadzu Corp, Kyoto, Japan) using a diode array detector (DAD, SPD N 20A) and C18 column (5 μm; 4.6×250 mm). The analytical procedure was done by applying the chromatographic conditions. The gradient system initiated with a concentration of 100% solvent A at 0.1 min, then progressively increased the concentration of solvent B to 35% in 25 min, 50% in 45 min, and eventually 100% in 65 min. The standard compounds were dissolved in sterile water and filtered by PVDF (0.45 μm) and a volume of 20 μl was eluted. The solvent system of HPLC grade H_2O (solvent A) and H_2O: C_2H_3N: CH_3COOH (48:51:4 v/v) (solvent B) were employed as mobile phase. The flow rate was kept at 1 ml/min and 20 μl of the sample was injected for the analysis. The reference compounds such as kaempferol, gallic acid, quercetin, caffeic acid, coumaric acid, naringenin and rutin were analyzed discretely to obtain the retention periods.

3. Statistical analysis

The statistical analysis was carried out using Microsoft excel 2010, Washington, USA. The IC50 was calculated using Graph Pad Prism 7 software, California, USA. Experimental data were analyzed by one-way ANOVA followed by Tukey's post-hoc test. A P value of < 0.05 was

Table 2
Antioxidant activity (IC_{50} values (μg/ml) of the different extract of *C. aeruginosa* Roxb. Values expressed are mean ± standard deviation (SD) of triplicate measurements.

Solvent	DPPH			ABTS+			O_2-			Hydroxyl radical			Phosphomolybdenum		
Methanol	Leaf	Stem	Rhizome	Leaf	Stem	Rhizome	Leaf	Stem	Rhizome	Leaf	Stem	Rhizome	Leaf	Stem	Rhizome
	31.81 ± 0.38	18.89 ± 0.23	12.22 ± 0.11	24.69 ± 2.67	37.56 ± 0.88	12.87 ± 1.16	128 ± 0.3	91.86 ± 0.61	35.92 ± 0.3	7.73 ± 0.5	5.30 ± 0.1	5.32 ± 0.09	10.25 ± 0.06	7.11 ± 0.02	3.97 ± 0.01
Ethyl acetate	20.91 ± 0.72	81.92 ± 0.40	64.27 ± 2.08	66.71 ± 2.82	57.4 ± 2.18	13.42 ± 0.72	142 ± 3.5	103.4 ± 0.6	54.23 ± 1.35	14.42 ± 0.28	6.74 ± 0.9	3.42 ± 0.09	8.85 ± 0.15	9.51 ± 0.03	4.90 ± 0.02
Hexane	157.5 ± 0.14	144.3 ± 0.1	33.715 ± 0.63	109.8 ± 0.28	147.8 ± 2.65	34.69 ± 2.3	167.5 ± 3.04	163.46 ± 4.03	98 ± 1.4	9.35 ± 0.017	8.89 ± 0.03	5.85 ± 0.04	52.55 ± 0.63	27.68 ± 1.06	17.90 ± 0.42

Table 3
Cytotoxic activity (IC_{50} values (μg/ml) of *C. aeruginosa* Roxb extracts against human cancer and normal cell lines. Values expressed are mean ± standard deviation (SD) of triplicate measurements.

Solvent	A-549			Hela			L-132		
Methanol	Leaf	Stem	Rhizome	Leaf	Stem	Rhizome	Leaf	Stem	Rhizome
	35.71 ± 0.39	34.40 ± 2.5	15.42 ± 1.5	57.09 ± 0.22	33.69 ± 0.54	25.40 ± 0.13	86.09 ± 2.96	102.7 ± 3.6	113 ± 0.56
Ethyl acetate	162.6 ± 1.27	109.75 ± 1.48	36.93 ± 0.69	154.5 ± 0.07	104.8 ± 0.07	61.24 ± 0.03	407.5 ± 0.1	398.05 ± 0.2	197.1 ± 1.7
Hexane	116.45 ± 0.49	96.7 ± 1.10	65.06 ± 0.94	138.85 ± 1.6	93.21 ± 0.53	43.65 ± 0.52	522.95 ± 3.8	387.6 ± 1.8	250.15 ± 1.6
Dox	3.65 ± 0.05			4.54 ± 0.39			3.464 ± 0.8		

Table 4
Effect of the methanol extract of *C. aeruginosa* on the induction of apoptosis and expression of caspase-3 and − 8 activity in HeLa and A-549 cells. The data represent Mean ± SD, n = 5. The one-way ANOVA was performed to test the significant level between the control and treatment groups at a significant level of * <0.5, @<0.01 and # < 0.001.

	HeLa cell						A-549 cell				
Treatment	**Apoptosis (%)**	**Necrosis (%)**	**Live (%)**	Caspase (Fold change/increase)		**Treatment**	**Apoptosis (%)**	**Necrosis (%)**	**Live (%)**	Caspase (Fold change/increase)	
	Mean±SD	Mean±SD	Mean±SD	C8	C3		Mean±SD	Mean±SD	Mean ±SD	C8	C3
Control	2.03 ± 0.70	1.63 ± 1.22	96.34 ± 1.22	0.00	0.00	**Control**	2.51 ± 0.95	1.50 ± 0.08	95.99 ± 1.01	0.00	0.00
DOX	76.63 ± 2.31#	14.76 ± 1.34	8.62 ± 1.34	4.01	2.25	**DOX**	79.40 ± 2.06#	13.35 ± 1.24	7.24 ± 1.24	11.14	10.48
RM	70.91 ± 1.97@	9.40 ± 0.27	19.69 ± 0.27	1.75	1.85	**RM**	75.42 ± 2.09#	8.19 ± 1.74	16.40 ± 1.07	2.27	2.61
SM	63.80 ± 2.49@	8.03 ± 2.27	28.18 ± 2.27	1.29	1.58	**SM**	66.96 ± 1.22#	9.19 ± 2.04	23.85 ± 0.98	2.16	2.54
LM	59.56 ± 2.34@	10.73 ± 1.02	29.71 ± 1.02	1.50	1.52	**LM**	62.80 ± 1.70#	10.29 ± 0.56	26.91 ± 1.31	1.93	1.73

L-132 cell			
Treatment	**Apoptosis (%)**	**Necrosis (%)**	**Live (%)**
	Mean±SD		Mean±SD
Control	2.77 ± 0.67	1.14 ± 1.31	94.82 ± 1.55
DOX	67.62 ± 1.01	13.25 ± 1.27	17.67 ± 0.68
RM	15.62 ± 1.82	6.76 ± 1.59	78.71 ± 1.56
SM	16.37 ± 0.65	7.89 ± 1.75	75.66 ± 2.24
LM	18.38 ± 1.22	10.99 ± 1.35	69.85 ± 1.38

considered statistically significant. The results are expressed as mean ± standard deviation of the mean (SED).

4. Result

4.1. Phytochemical analysis

The qualitative phytochemical analysis of the leaf, stem and rhizome of *C. aeruginosa* revealed a broad variety of phytochemicals. The key phytochemical components: phenols, flavonoid, terpenoids, alkaloids, cardiac glycosides, saponin, quinones and tannins were present in the extracts (Table 1). Alkaloids was found to be absent in the hexane extracts of *C. aeruginosa* and anthraquinone was absent in all the extracts.

The quantification of phytochemicals of the various plant parts of *C. aeruginosa* revealed that LM contained the highest phenol (7.66 ± 0.05 mg GAE/g of dry weight) followed by RE (5.19 ± 0.32 mg GAE/g of dry weight). The highest flavonoid content was found in LM (7.57 ± 0.02 mg quercetin equivalent/g of dry weight) followed by RM (3.92 ± 0.08 mg quercetin equivalent /g of dry weight). The alkaloid content was the highest in RM (3.28 ± 0.15 mg atropine equivalents/g of dry weight) (Table 1).

4.2. Antioxidant activities

To validate the antioxidant activity of *C. aeruginosa* extracts, phosphomolybdenum assay and different in-vitro assays were conducted. Free radical scavenging activity was assessed using DPPH and ABTS+ , superoxide and hydroxyl assays (Table 2).

Based on the IC_{50} values, RM showed the highest ability to scavenge DPPH. The observed order of IC_{50} value was as follows: RM (12.22 ±

Table 5(a)
GC-MS profiling of the identified compound from the rhizome methanol (RM) extract of *Curcurma aeruginosa*.

S.no	Retention Time	CAS	Compound name	Molecular formula	Molecular weight	Peak area (%)
1	7.315	20085-85–2	Epicurzerenone	$C_{15}H_{18}O_2$	230	1.03
2	9.926	17910-09-7	Curzerene	$C_{15}H_{20}O$	216	34
3	12.397	1139-30–6	Caryophyllene oxide	$C_{15}H_{24}O$	220	.34
4	14.038	1877-77–6	Benzenemethanol, 3-amino	C_7H_9ON	123	.62
5	14.573	101628-22–2	Valerenol	$C_{15}H_{24}O$	220	0.33
6	20.295	644-30–4	α- Curcumene	$C_{15}H_{22}$	202	5.31
7	17.369	20493-56–5	Curzerenone	$C_{15}H_{18}O_2$	230	30.18
8	18.009	19431-84–6	Curcumenol	$C_{15}H_{22}O_2$	234	2.20
9	19.210	900151-99-7	Cycloisolongifolene	$C_{15}H_{24}$	204	30.97
10	19.770	118-55-8	Phenyl salicylate	$C_{13}H_{10}O_3$	214	12.27
11	20.295	60-33-3	9,12-Octadecadienoic acid (z,z)-	$C_{18}H_{32}0_2$	280	9.42
12	21.541	2198-92-7	Verrucarol	$C_{15}H_{22}O_4$	266	.49
13	22.086	5508-58-7	Andrographolide	$C_{20}H_{30}O_5$	350	4.40
14	23.367	900150-40-2	Tricyclo[8.6.0.0(2,9)]hexadeca-3,15-diene, cis-2,9-anti-9,10-cis-1,10-	$C_{16}H_{24}$	216	2.01

Table 5(b)
GC-MS profiling of the identified compound from the stem methanol (SM) extract of *Curcuma aeruginosa*.

S. no	Retention time	CAS	Compound name	Molecular formula	Molecular weight	Peak area (%)
1	14.633	506–24–1	9-octadecynoic acid	$C_{18}H_{32}O_2$	230	1.85
2	14.943	900294–14–4	Benzo[*b*]perhydroazonine-2,7-dione, 9-methyl	$C_{13}H_{15}O_2N$	217	.30
3	15.338	900293–99–0	Naphthalene-2-sulfonamide, n-(2-methoxyphenyl)-	$C_{17}H_{15}O_3NS$	313	.41
4	15.659	900382–90–8	Carbonic acid, prop-1-en-2-yl tridecyl ester	$C_{17}H_{32}O_3$	284	.26
5	16.284	900382–90–6	Carbonic acid, prop-1-en-2-yl undecyl ester	$C_{15}H_{28}O_3$	256	14.92
6	16.729	900336–45–3	Methyl10,11-octadecadienoate	$C_{19}H_{34}O_2$	294	1.64
7	17.349	35237–63–9	10-dodecenol	$C_{12}H_{24}O$	184	1,64
8	17.999	900269–47–3	Pyrimidine-2,4(1 h,3 h)-dione, 5-(2-methoxyphenylaminosulfonyl)− 6-methy	$C_{12}H_{13}O_5N_3S$	311	2.45
9	19.035	1877–77–6	Benzenemethanol, 3-amino	C_7H_9ON	123	27.61
10	19.655	6627–60–7	Pyridine, 2-aminomethyl-6-methyl	$C_7H_{10}N_2$	122	6.31
11	20.676	1877–77–6	Benzenemethanol, 3-amino	C_7H_9ON	123	30.72
12	21.371	21226–32–4	Benzenesulfon-o-anisidide	$C_{13}H_{13}O_3NS$	263	.17
13	21.761	123–06–8	Propanedinitrile, (ethoxymethylene)-	$C_6H_6ON_2$	122	.51
14	22.221	6295–94–9	Benzenesulfonamide, N-(2-methoxyphenyl)− 4-methyl	$C_{14}H_{15}O_3NS$	277	.75
15	23.512	529–63–5	4(5 h)-benzofuranone, 6,7-dihydro-3,6-Dimethyl-, (R)	$C_{10}H_{12}O_2$	164	.22
16	25.092	900277–36–5	Pyrazol-5-ol, 4-(2-methoxyphenylazo)− 3-methyl	$C_{11}H_{12}O_2N_4$	232	1.85
17	31.360	900399–09–9	Cholest-20(22)-en-3-one, 4,5-epoxy-11-hydroxy	$C_{27}H_{42}O_3$	414	.68
18	33.871	113261–27–1	1 H-1,2,3-triazol-1-amine, n-[(4-methoxyphenyl)methylene]− 4,5-dimethyl	$C_{12}H1_4ON_4$	230	1.02
19	34.736	900336–46–2	Methyl 13,14-octadecadienoate	$C_{19}H_{34}O_2$	294	1.56
20	36.937	103–70–8	Formamide, n-phenyl-	C_7H_7ON	121	5.11

Table 5c
GC-MS profiling of the identified compound from the leaf methanol (LM) extract of *Curcurma aeruginosa*.

S.no	Retention Time	CAS	Compound Name	Molecular Formula	Molecular Weight	Peak Area (%)
1	9.936	900149–54–6	Bicyclo[5.1.0]octan-2-one,4,6 diisopropylidene-8,8-dimethyl	$C_{16}H_{24}O$	232	2.61
2	14.943	330569–99–8	[1,2,4]triazolo[1,5-*a*]pyrimidin-7-ol, 2-amino-5-methyl-6-nitro-	$C_6H_6O_3N_6$	210	.92
3	16.174	900263–78–5	Pyrazole, 3-methyl-5-(3,5-dimethylpyrazol-1-yl)− 4-nitro-	$C_9H_{11}O_2N_5$	221	23.65
4	16.719	2541–75–5	8-heptadecanol	$C_{17}H_{36}O$	256	4.77
5	17.364	5405–58–3	Hexane, 1,1′-[ethylidenebis(oxy)]bis-	$C_{14}H_{30}O_2$	230	5.42
6	18.705	5405–58–3	Hexane, 1,1′-[ethylidenebis(oxy)]bis	$C_{14}H_{30}O_2$	230	23.98
7	19.750	57–11–4	Octadecanoic acid	$C_{18}H_{36}O_2$	284	9.29
8	20.285	629–96–9	1-eicosanol	$C_{20}H_{42}O$	298	15.44
9	21.746	56438–07–4	1,1-dodecanediol, diacetate	$C_{16}H_{30}O_4$	286	2.12
10	24.522	765–14–0	Vinyl lauryl ether	$C_{14}H_{28}O$	212	2.09
11	25.387	1454–84–8	N-nonadecanol-1	$C_{19}H_{40}O$	284	1.69
12	36.372	506–24–1	9-octadecynoic acid	$C_{18}H_{32}O_2$	280	4.56
13	39.933	2566–97–4	9,12-octadecadienoic acid, methyl ester, (e,e)-	$C_{19}H_{34}O_2$	294	3.46

0.11)> SM (18.89 ± 0.23)> LE (20.91 ± 0.72)> LM (31.81 ± 0.38) > RH (33.71 ± 0.63)>RE (64.27 ± 2.08)> SE(81.92 ± 0.4)> SH (144.3 ± 0.1) > LH (157.5 ± 0.14) with respect to ascorbic acid (1.071 ± 0.016 μg/ml).

$ABTS^+$ cations scavenging of extracts exhibited maximum activity in RM having IC_{50} value of 12.87 ± 1.16 μg/ml followed by RE with IC_{50} value of 13.42 ± 0.72 μg/ml.

The extracts' radical scavenging activity were effective in the order: RM (12.87 ± 1.16)> RE (13.42 ± 0.72)> LM (24.69 ± 2.67)> RH (34.69 ± 2.3)> SM (37.56 ± 0.88)> SE (57.4 ± 2.18)> LE (66.71 ± 2.82) > LH (109.8 ± 0.28)> SH (147.8 ± 2.65) and ascorbic acid was used as standards at the concentration 1–25 μg/ml with an IC_{50} of 0.013 ± 0.006 μg/ml.

RM displayed the best superoxide radical activity (Table 2) with an IC_{50} value of 35.92 ± 0.3 μg/ml. The decreasing order of scavenging activity of the extracts were found to be: RM (35.92 ± 0.3)> RE (54.23

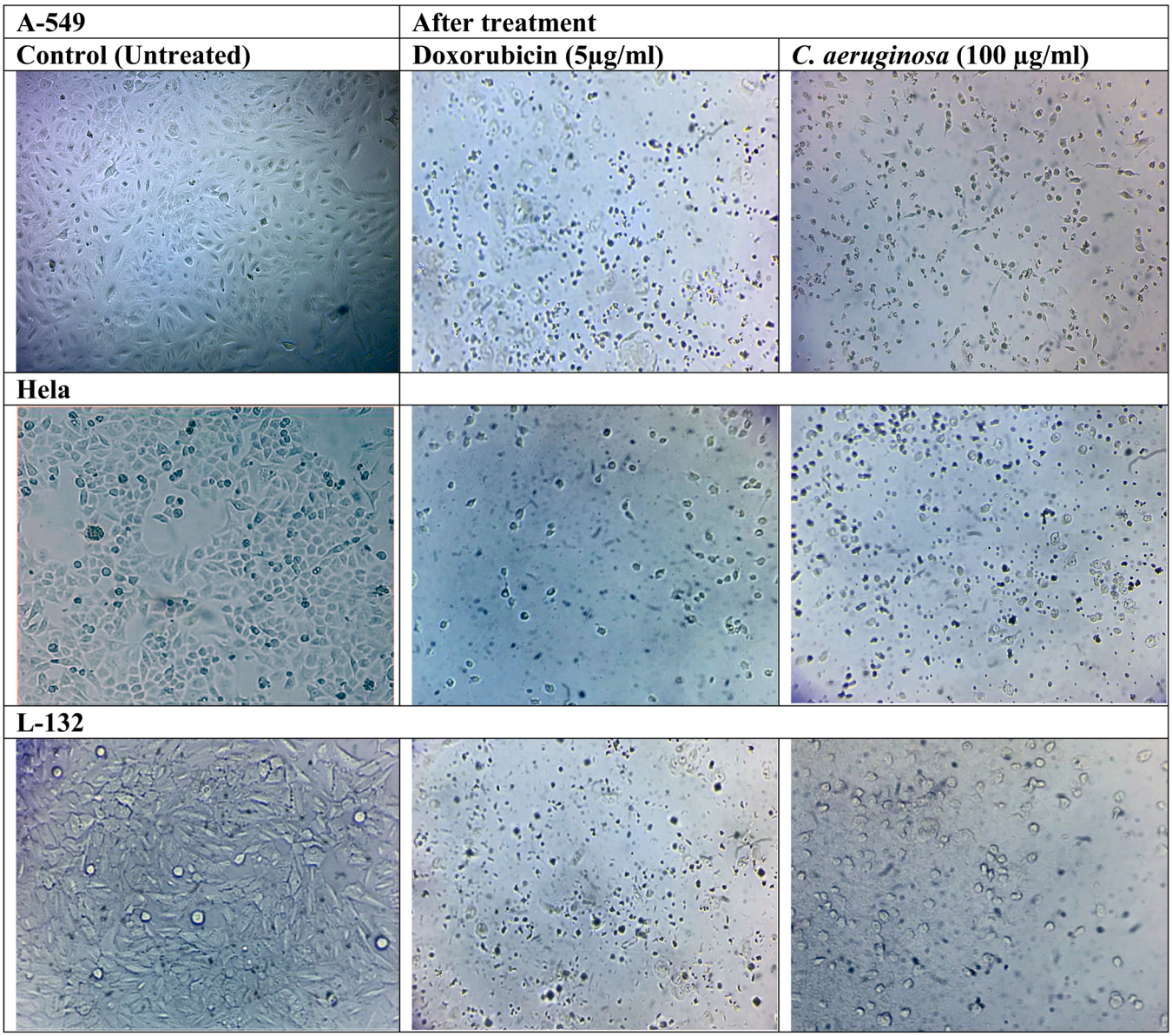


Fig. 1. The cytotoxicity effect of C. aeruginosa Roxb. extract against A-549 (lung cancer cell) and Hela (Cervical cell).

± 1.34)>SM (91.86 ± 0.61)> RH (98 ± 1.4)> SE (103.4 ± 0.6)> LM (128 ± 0.3)> LE (142 ± 3.5)> SH (163.46 ± 4.03)> LH (167.5 ± 3.04) with respect to Gallic acid (8.51 ± 0.31 µg/ml).

The best IC_{50} value for hydroxyl scavenging activity was exhibited by RE (3.42 ± 0.09 µg/ml).

The scavenging activity were effective in the order: RE (3.42 ± 0.09)> SM (5.30 ± 0.1)> RM (5.32 ± 0.09)> RH (5.85 ± 0.04)> SE (6.74 ± 0.9)> LM (7.73 ± 0.5)> SH (8.89 ± 0.03)> LH (9.35 ± 0.01)> LE (14.42 ± 0.28) with respect to ascorbic acid (0.85 ± 0.018 µg/ml).

The total antioxidant capacity of the extracts was evaluated using phosphomolybdate method.

RM showed the most promising IC_{50} value (4.903 µg/ml).The detected order of IC_{50} value: RM (3.97 ± 0.01)> RE (4.90 ± 0.02)> SM (7.11 ± 0.02)> LE (8.85 ± 0.15)> SE (9.51 ± 0.03)> LM (10.25 ± 0.06)> RH (17.90 ± 0.42)> SH (27.68 ± 1.06)> LH(52.55 ± 0.63) with respect to ascorbic acid (0.85 ± 0.018 µg/ml).

4.3. Cytotoxicity

Table 3 shows the results of the screening of the different extracts of *C. aeruginosa*. IC_{50} values were calculated for these extracts against two human cancer cell lines, namely, A549 and Hela and a normal cell: L-132.

The cytotoxicity revealed that the different extracts of *C. aeruginosa* was capable of killing A-549 and HeLa cells extracts but insignificant toxicity against normal (L-132) cell.

The RM (rhizome methanol) extract of *C. aeruginosa* exhibited no significant cytotoxicity against the L-132 cell line achieving an IC_{50} value of 113.6 ± 0.56 µg/ml. On the contrary, RM, exhibited significant activity against the A-549 and Hela cells with an IC_{50} value of 15.42 ± 1.5 µg/ml and 25.40 ± 0.13 µg/ml respectively (Table 3). Doxorubicin at the concentration 0.5, 1, 2, 4 and 8 µg/ml was used as positive control and the IC_{50} value was found to be between 2 and 4 µg/ml for A-549, Hela and L-132 cells (Table 3).

4.4. Comet assay

The level of DNA damage was examined using the alkaline comet assay. The methanol extracts of *C. aeruginosa* induced DNA damage in A-549 and HeLa cells with prominent increased tail length and tail

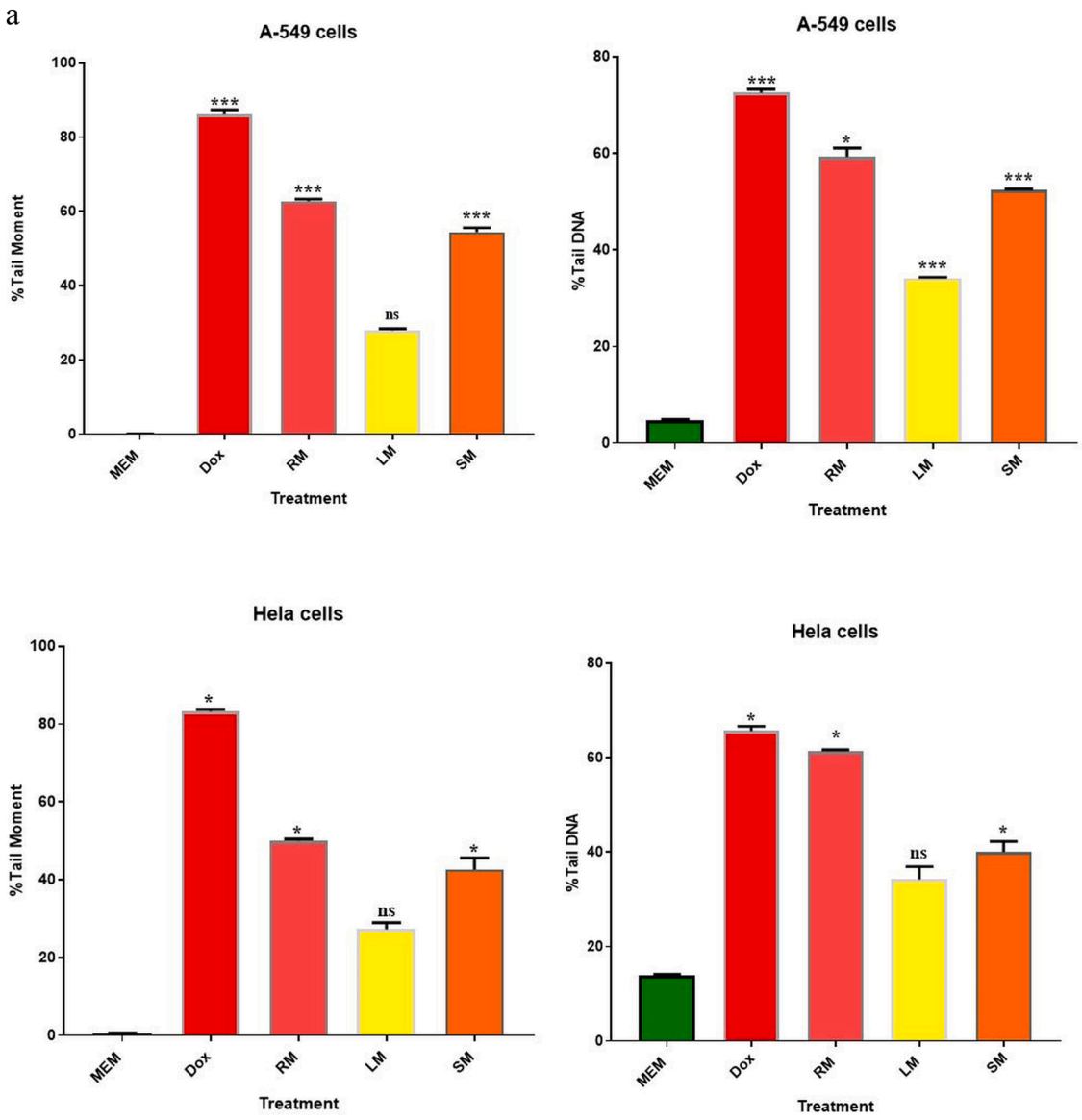


Fig. 2a. The extent of DNA damage expressed in terms of Tail DNA (%) and Tail moment (%) in A-549 and HeLa cells. The data represent Mean ± SD, n = 5. The one-way ANOVA was performed to test the significant level between the control and treatment groups at a significant level of * <0.5, * *< 0.01 and * ** <0.001.

moment in treated group when compared to untreated control (Fig. 2 a).

Among the extracts, RM showed significant increased tail DNA (61.43 ± 0.24%) and tail moment (50.06 ± 0.45%) when compared to untreated control (13.86 ± 0.6% tail DNA and 0.47% ± 0.14% tail moment) in Hela cells (Fig 2b). Treatment with RM against A549 cells also showed induction of DNA damage with increased tail DNA (59.30 ± 1.77%) and tail moment (62.80 ± 0.55%). The untreated control in A-549 showed 4.74 ± 0.17% tail DNA and 0.11 ± 0.03% tail moment. SM induced greater DNA damage with longer tail DNA (52.41 ± 0.19%) and tail moment (54.51 ± 1.13%) when compared with LM (34.17 ± 0.17% tail DNA and 27.95 ± 0.44% tail moment). Doxorubicin was used as positive control.

4.5. Activation of apoptotic cell death by the methanol extracts of C. aeruginosa

AO/EB staining method was used for analyzing the apoptotic effect in A-549 and HeLa cells. Treatment with the IC_{50} concentration of the methanol extract of *C. aeruginosa* against the A-549 and HeLa cells revealed induction of apoptotic and necrotic cells for 48 h with an increased rate of cell death when compared to the L-132 cells (Table 4). The apoptosis induction was similar to doxorubicin, which was significantly higher than the untreated control. Among the extracts, RM was found to exhibit highest apoptotic cells in HeLa cells with an apoptotic index of 70.91 ± 1.97%.The necrotic cell index was the highest in LM with 10.73 ± 1.02%. A-549 cells treated with 15.42 ± 1.5 μg/ml of RM increased the apoptotic cells by 75.42 ± 2.09% and necrotic cells by 8.19 ± 1.74% in comparison to the control. Normal cell line (L-132) was included for comparison against the extracts activity and found that apoptotic index and necrotic index was insignificant as compared to A-549 and HeLa cells.

To investigate the cleaved effector caspase in the apoptotic pathway, the activation of caspase-8 and caspase-3 were measured by the colorimetric assay. The result showed that there was an increment in caspase-3 and caspase-8 activities in comparison to untreated cells in both HeLa and A-549 cells. Treatment of A-549 cells with RM resulted in the up-regulation of caspase-8 and caspase-3 activities by 2.27-fold and 2.61-fold respectively. The cleavage of caspase-3 and caspase-8 activity induced by RM showed a fold change of 1.85 and 1.75 respectively in HeLa cells (Table 4). RM exhibited the highest increment in caspase-8

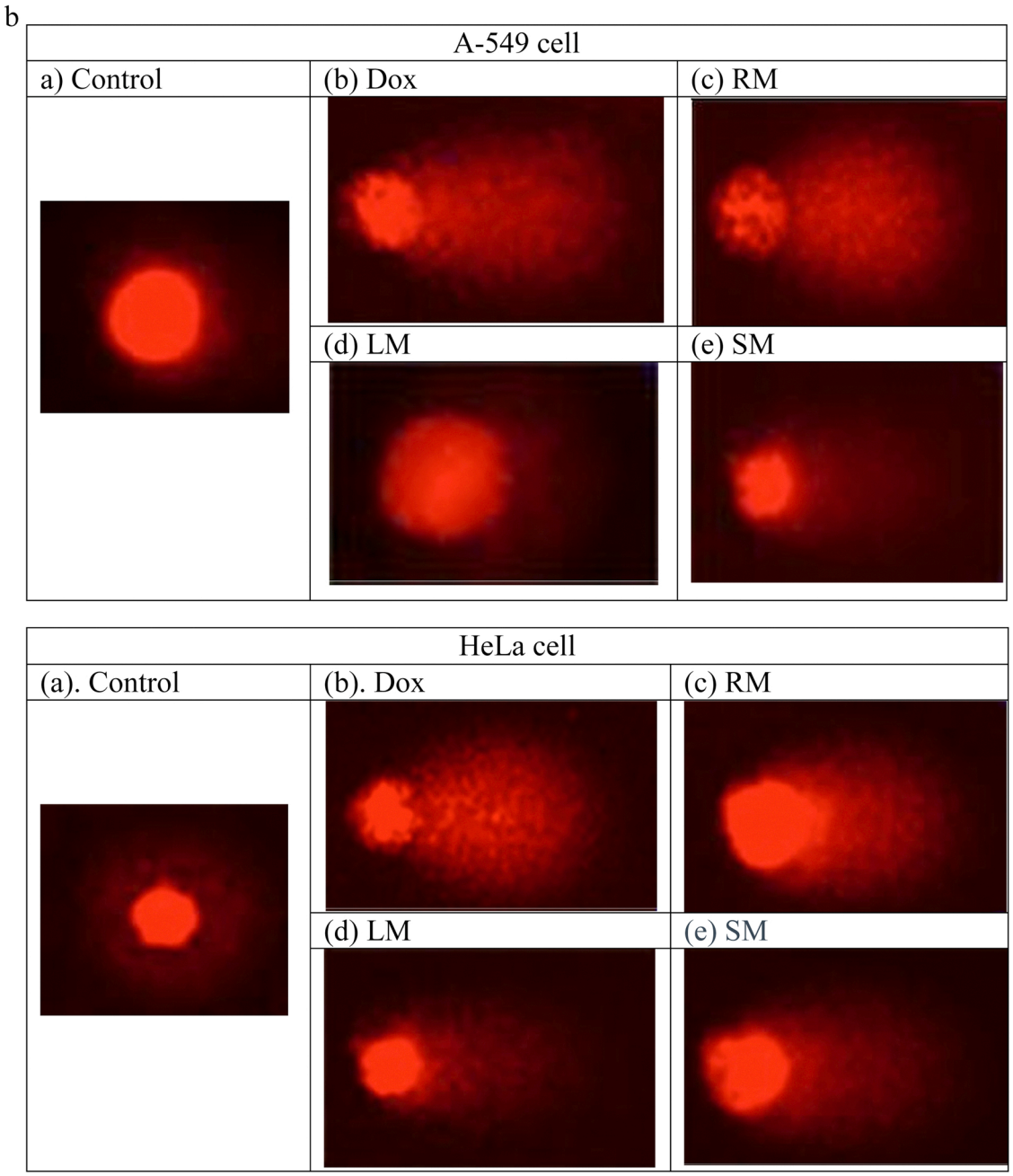


Fig. 2(b). Fluorescence Comet images observed in A549 and HeLa cells.

and caspase-3 activities in A-549 and HeLa cells.

4.6. Identification of compound using HPLC analysis

The HPLC analysis of the methanol extract of *C. aeruginosa* recorded the presence of important bioactive phenol and flavonoid compounds like gallic acid (5.669), caffeic acid (6.819, quercetin, (8.637) and kaempferol (22.59) in RM (Fig. 3a). SM contain gallic acid (5.689), caffeic acid (6.817) and quercetin (8.651) (Fig. 3b). LM showed the presence of gallic acid (5.691), rutin (6.796), coumaric Acid (7.973), naringenin (11.002) (Fig. 3c).

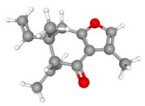
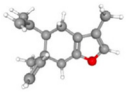
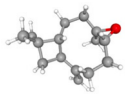
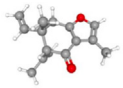
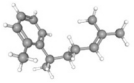
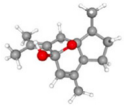
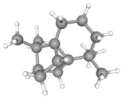
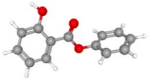
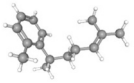
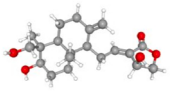
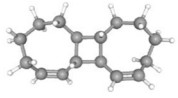
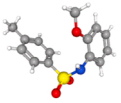
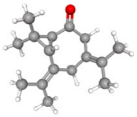
4.7. Identification of compound using GC-MS analysis

The GC–MS analysis of the methanol extracts of *C. aeruginosa* recorded a total of 47 peaks that were identified by peak retention time and peak area (%) to that of the known compounds described by the PubChem Compound (NCBI) and NIST library. Results revealed that 14 compounds were identified in RM (Table 5a). The GC–MS analysis of SM and LM recorded 20 and 13 peaks respectively (Tables 5b and 5c). Overall, the 15 phytocompounds with known biological activity identified in the methanol extracts of *C. aeruginosa* are presented in Table 5d.

5. Discussion

Our result showed the phytochemical and antioxidant activity of *C. aeruginosa* Roxb relating to in vitro anticancer activity against A-549 and HeLa cells. The phytochemical content of *C. aeruginosa* Roxb. showed the presence of many important bio-active compounds (Table 1). The results of this study revealed that the extracts exhibited potent antioxidant activity. The methanol and ethyl acetate extracts exhibited greater potency of free radical scavenging activity compared

Table 5d
Biological activity of phytochemical constituents identified in the methanol extracts of *C. aeruginosa* using gas chromatography-mass spectrometry. (Picture source: Pubchem (NCBI).

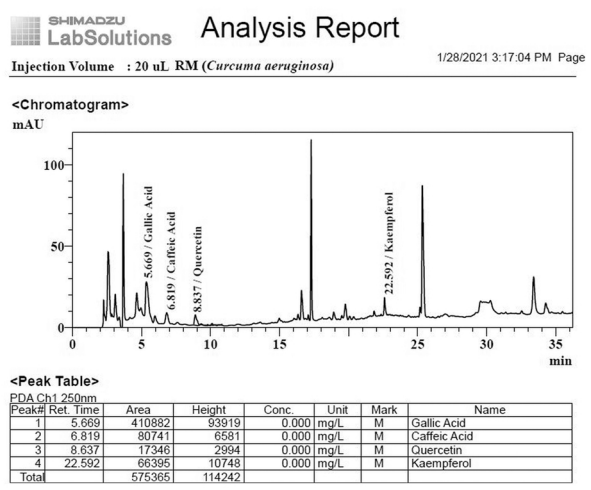
S. no	Compound name	Chemical structure	Biological activity	Reference
1	Epicurzerenone		Anti-cancer	Cui et al. [75]
2	Curzerene		Anti-cancer	Wang et al. [73]
3	Caryophyllene oxide		Anti-cancer	Delgado et al. [66]
4	Curzerenone		Anti-cancer	Rahman et al. [64]
5	α- curcumene		Anti-cancer	Shin et al. [65]
7	Curcumenol		Anti-cancer	Hamdi et al. [70], Lee et al. [71], Han et al. [72]
8	Cycloisolongifolene		Antimicrobial	Zhao et al. [76]
9	Phenyl salicylate		Antiseptic; mild analgesic;	Sneader [77]
10	9,12-octadecadienoic acid (z,z)-		Anti-cancer	Yong et al. [74]
11	Andrographolide		Anti-cancer	Zhou et al. [67], Ku et al. [68], Banerjee et al 201669
12	Tricyclo[8.6.0.0(2,9)]hexadeca-3,15-diene, cis-2,9-anti-9,10-cis-1,10-		Antimicrobial, Anti-inflammatory, Anticancer,	Pakkirisamy et al. [78]
13	Benzo[*b*]perhydroazonine-2,7-dione, 9-methyl		Anti-microbial activity	Anjali et al. [79]
14	Benzenesulfonamide, n-(2-methoxyphenyl)− 4-methyl		Anti-microbial activity	Ahmad and Farrukh [80]
15	Bicyclo[5.1.0]octan-2-one, 4,6-diisopropylidene-8,8-dimethyl		Anti-microbial	Yang et al. [81]

to the hexane extracts (Table 2). The efficacy of antioxidant activity is significantly associated with total phenolic content and total flavonoid content [38]. The stable radical scavenging and cytotoxic activities are directly associated with phytochemical compounds present [39].

MTT assay is extensively applied in the evaluation of cytotoxic drug therapy serving as an analytical tool for chemotherapy [40].The cytotoxicity results in our study revealed that the different extracts of *C. aeruginosa* was capable of killing A-549 and HeLa cells but exhibit insignificant toxicity against normal cells (L-132) cells (Table 3 and Fig. 1). The most valuable substance are those that can selectively kill cancer cells while exerting no significant damage to normal cells [41]. The extracts in this study are not as efficient as doxorubicin in killing cancer cells, however, they have lower cytotoxicity against the normal cells. Thus, these results support the traditional use of this medicinal plant in treating lungs and cervical cancer. According to the NCI, a plant extract with incubation between 48 and 72 h is considered to possess active cytotoxic effect with IC50 value $\leq$ 25 μg/ml [42,43]. In this respect, the methanol extracts- RM, SM and LM were selected for carrying out the remaining experiments. Previous studies have showed the antitumor activity of SCF extract of *C. aeruginosa* against HeLa cell line in a dose dependent manner [44].

The present results are in agreement with earlier findings where the anticancer activity of *C. aeruginosa* was assessed using two different cell lines, HT-29 and MCF-7 cancer cells, by using the MTT assay [45]. Other

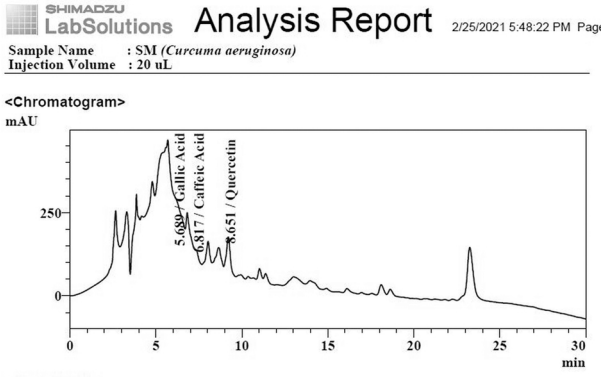
a



<Peak Table>
PDA Ch1 250nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	5.669	410882	93919	0.000	mg/L	M	Gallic Acid
2	6.819	80741	6581	0.000	mg/L	M	Caffeic Acid
3	8.637	17346	2994	0.000	mg/L	M	Quercetin
4	22.592	66395	10748	0.000	mg/L	M	Kaempferol
Total		575365	114242				

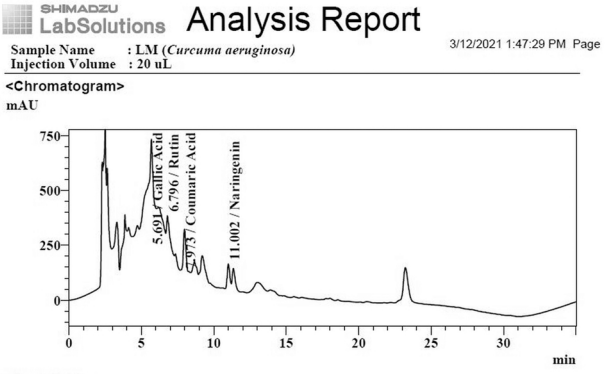
b



<Peak Table>
PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	5.689	5983864	167933	0.000	mg/L	M	Gallic Acid
2	6.817	694952	62286	0.000	mg/L	M	Caffeic Acid
3	8.651	817427	48956	0.000	mg/L	M	Quercetin
Total		7496244	279175				

c



<Peak Table>
PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	5.691	3757659	284053	0.000	mg/L	M	Gallic Acid
2	6.796	1057031	96165	0.000	mg/L	M	Rutin
3	7.973	2154556	189156	0.000	mg/L	M	Coumaric Acid
4	11.002	1044378	98006	0.000	mg/L	M	Naringenin
Total		8013624	667379				

Fig. 3. (a) Detection of bioactive compound of the rhizome methanol (RM) extract of *Curcuma aeruginosa* by HPLC method. Fig. 3b Detection of bioactive compound of the stem methanol (SM) extract of *Curcuma aeruginosa* by HPLC method. Fig. 3c. Detection of bioactive compound of the leaf methanol (LM) extract of *Curcuma aeruginosa* by HPLC method.

species of curcuma namely *Curcuma longa* have been reported to show cytotoxic activities against A549 cells [46] and HeLa cells [47]. *Curcuma amada* was also found to inhibit the growth of large human lung carcinoma (NCI-H460) and small human lung carcinoma (A-549) [48].

Evaluation of the level of DNA damage was studied by using the Alkaline Comet assay. The comet assay is a used for quantifying DNA strand breaks in eukaryotic cells [49]. To assess the extract induced DNA damage, the damage index was used in the visual score and the tail DNA % and tail moment % were used as indexes in the software analysis. The damage index of treatments was calculated (Fig. 2a). There was a major reduction of migration in the untreated cells when compared to the treated group and Doxorubicin (Fig. 2b).

Cell death happens in cancers following cytotoxic drug therapy and apoptosis is the most popular underlying mechanism of the anticancer effects of various anticancer drugs, including natural compounds [50]. Our interest was to know whether the methanol extracts of *C. aeruginosa* induces cytotoxicity by triggering apoptosis in A-549 and HeLa cells. The AO/EB staining is most reliable method to detect apoptosis [51]. Observation under the fluorescence microscope revealed induction of apoptotic and necrotic cells for 48 h in the A-549 and HeLa cells with an increased rate of cell death when compared to the L-132 cells (Table 4). The nuclei of the viable cells exhibited organized structure showing green fluorescence. The nuclei of cells with early apoptotic phase were characterized with fragmented yellow chromatin. The necrotic cells were visible by orange chromatin in round nuclei were categorized as necrotic cells [52]. Cytotoxic agents may induce apoptosis by initiating death signaling pathways in susceptible target cells [53]. We examined the cancer cells for apoptosis and whether the cytotoxicity was due to apoptosis, the apoptotic pathway activated by anticancer compounds. The caspases are associated with apoptosis. Apoptosis is a process of eliminating damaged cells and is executed by a family of caspases [54].

The methanol extracts of *C.aeruginosa* up-regulated the caspase-8 and caspase − 3 activities (Table 4), thereby exerting cytotoxic effect by triggering apoptosis. This is in agreement with the morphology of DNA fragmentation and apoptosis induced by the extracts in A-549 and HeLa cells. In another study, the ethanol extract of rhizome of *C. zedoaria* was found to induce apoptosis by activation of caspases on human esophageal carcinoma cells [55]. The bulb methanol extract of *A. atroviolaceum* was reported to induce apoptosis triggering the activation of caspase-8 and caspase-3 in MCF7 and MDA-MB-231 cells [56]. Likewise, the ethanol stem bark extract of *Oroxylum indicum* was reported to do the same in HeLa cells [57].

The most cytotoxic fractions, the methanol extracts were analyzed by GC-MS and HPLC to identify the active compounds. Detection of bioactive compounds was done to identify the compounds that establish their pharmacological properties. Zingiberaceae families have phytochemicals such as terpenoids, flavonoids, phenylpropanoids and sesquiterpenes which possess anti-tumor activities [58,59]. Genotypes and environmental factors can affect the bioactive contents and composition of medicinal plants [60]. Geographic location, environmental differences and variability of genes affects the polyphenol, flavonoids and curcuminoids present in *C. aeruginosa* accessions [61].

GC-MS results reveal the presence of various compounds which were earlier detected in previous works [62,63]. Biological activities of phytochemical constituents detected in *C. aeruginosa* are given in Table 5d. Seven sesquiterpenoids, curcumenol, curzerenone, epicurzerenone, α-curcumene, curzerene, caryophyllene oxide, 9,12-Octadecadienoic acid (Z,Z) and a diterpenoid, andrographolide detected in the rhizome methanol extract (RM) (Table 5(a)) have cancer protective properties.

Curzerenone, α-curcumene, caryophyllene oxide and andrographolide are reported to inhibit the growth of human cancer cells, MCF-7, Ca Ski, HCT-116, SiHa, PC-3 and MDA-MB-231 and induce apoptosis through the activation of caspase-signalling pathway [64–70]. Curcumenol, curzerene, 9,12-Octadecadienoic acid (Z,Z) and epicurzerenone have been reported to induce apoptotic cell death in MCF-7, A549, AGS,

SPC-A1 MG-63 and L-02 cells [71–81]. The HPLC analysis showed that the methanol extract of the different parts of *C. aeruginosa* contain seven active substances: gallic acid, kaempferol, quercetin, caffeic acid, coumaric acid, naringin, rutin (Fig. 3a-3c). These compounds are recognized as apoptosis inducers in human lung and cervical cancer cells evidenced by their ability to induce the activation of caspase-involved apoptotic pathway [82–101].

Therefore, the apoptosis-inducing effect of *C. aeruginosa* on A549 and HeLa cells came from these naturally active phenol and flavonoid compounds. The GC-MS result revealed that the rhizome of *C.aeruginosa* possess naturally occurring sesquiterpenoids and diterpenoid, that can induce apoptosis in cancer cells, thereby exhibiting greater anticancer potential than the other parts examined as evident in this study.

6. Conclusion

Our result showed the phytochemical and antioxidant activity of *C. aeruginosa* Roxb. relating to in vitro anticancer activity against A-549 and HeLa cells. The screening of the *C. aeruginosa* extracts showed that RM has significant cytotoxicity against lung adenocarcinoma (A-549) cells and human cervical cancer (HeLa) with low IC_{50} value of 15.42 $\pm$ 1.5 μg/ml and 25.40 $\pm$ 0.13 μg/ml respectively showing increased DNA fragmentation, apoptotic and necrotic indices. Our results indicate that the methanol extracts of *C. aeruginosa* are capable of inducing apoptosis in A-549 and HeLa cells by up-regulating the activities of caspase-8 (initiator) and caspase-3 (executioner) which is reported for the first time in this communication. Overall study shows that the anticancer activity of the rhizome was better than other parts examined. The phenol, flavonoid and terpenoid compounds identified in the present study might be responsible for the anticancer activities of the plant. From these findings, the plant *C. aeruginosa* can be further exploited to develop a potential lead compound in the search for natural compounds particularly for lung and cervical cancer chemotherapy.

CRediT authorship contribution statement

Alex Zohmachhuana: Conceptualization, Methodology, Writing – original draft. **M. Vabeiryureilai:** Writing – review & editing. **Malsawmdawngliana**: Formal analysis, **N. Senthil Kumar:** Supervision, Resources, **K. Lalrinzuali:** Software, Analyzed the data. **F. Lalnunmawia:** Supervision.

Conflict of interest statement

None declared.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113039.

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Antioxidant efficacy and cytotoxicity of ethanol extract of *Clerodendrum infortunatum* against different cell lines

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Clerodendrum infortunatum belongs to the Lamiaceae family and is a perennial shrub. It is widely known for their important medicinal values among the Mizo tribe. In the present study, the preliminary phytochemical screening, quantification of phenols, flavonoids and alkaloids, antioxidant activities by DPPH, O^{2-} and ABTS assays and cytotoxicityby MTT assay against AGS (gastric cancer), HeLa (cervical) and HT-29 (colon) cell lines compared with normal cell line (Chang liver) were performed. Furthermore, the GC-MS profiling was also conducted. The results imply the presence of saponin, alkaloid, cardiac glycoside, phenol and flavonoid. The quantification shows that phenol content (64.35 mg/ g) was highest followed by flavonoid (61.93 mg/ g) and alkaloid (13.33 mg/ g). Its scavenging efficiency against DPPH with IC_{50} value was 47.99, against O^{2-} with IC_{50} was108 µg/mL and against ABTS cations with IC_{50} was 50.05 µg/mL, respectively. The ethanol extract exhibited a maximum cytotoxicity against HeLa with IC_{50} value of 53.55 µg/mL, AGS with IC_{50} value 82.44 µg/mL and HT-29 with IC_{50} value of 142.2 µg/mL. However, the extract showed comparatively less toxicity against normal cell lines. Moreover, 14 active compounds were confirmed in the GC-MS analysis of the extract. HPLC study also infers the occurrence of the flavonoids rutin and quercetin. Therefore, the results of *C. infortunatum* ethanolic extract clearly specified that it has a very high antioxidant activity as well as cytotoxic properties; which proved that this ethnomedicinal plant can be used as an alternative agent to treat a variety of illnesses.

Keywords: Antioxidant, Cell lines, *Clerodendrum infortunatum*, GC-MS, Mizoram

The use of plant based drug is slowly rising in popularity since the past decade in both the developing and developed countries. These herbal drugs are more readily available for consumers as compared to the synthetic drug since they are easily accessible in many shops not only in drug stores. It is believed that herbal medicine is used by 80% of the world's population[1-3]. The people of Mizoram rely largely on herbal drugs which have been inherited for generations traditionally. It is through nature, especially the plants that the human race obtained many drugs or chemicals for the treatment of different ailments. Many drugs which play a crucial part in modern medical science are prepared from plants[4]. Since cancer has become a significant concern in public health worldwide, vinblastine and vincristine were among the earliest cancer treatments to enter clinical trials which were extracted from the plant, *Catharanthus roseus*[5]. They are frequently used in amalgamation with other chemotherapeutic medications to treat a variety of cancers[6]. The pursuit for newer anti-cancer drugs perseveres due to the numerous adverse effects of the currently used drugs especially the synthetic ones besides the high chance of tumour recurrence[7]. Besides antineoplastic and other activities, medicinal plants become more important sources for antioxidant compound[8]. The presence of compounds like phenolic and flavonoids possesses high antioxidants activities that can inhibit the free radicals to prevent several harmful ailments[9-11].

Clerodendrum pertains to the category family Lamiaceae and is also known as glory bower, bag flower, and bleeding-heart[12,13]. It is indigenous to tropical regions of Africa and southern Asia[14]. *Clerodendrum* are often known to have pesticidal properties[15], and various *Clerodendrum* species are employed in the discipline of traditional medicine for curing different ailment such as inflammation, toothache, cold, hypertension, asthma, furunculosis, rheumatism, anorexia, leprosy, dysentery, arthrophlogosis, mammitis and leucoderma in many parts of the world[16-19]. It is well explored in scientific study relating to anticancer properties including human cells

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such as T47D (Breast), PC-3 (prostate), A549 (lung) and HCT-116 (colon)[20,21]. *Clerodendrum* were known to constitute of scutellarin, luteolin-7-3 glucuronide, apigen, stimasterol, D-glucose of sitosterol, queretonic acid, baicalin, oleonolic acid, serratagenic acid, D-mannitol and α-spinasterol in various parts such as leaf, bark and root[22]. In the past few decades, many researchers studied the genus *Clerodendrum* for its high impact of medicinal efficacy. *Clerodendrum serratum* Linn. leaf extract was reported for *in vivo* and *in vitro* studies of its anti-anthelmintic activity, hepatoprotective, anti-oxidant, anti-inflammatory, analgesic and antiasthmatic[23,24]. The species such as *C. philippinum, C. splendens* and *C. viscosum* were evaluated for antimicrobial, antioxidants, larvicidal and pupicidal activities[25-27].

Moreover, *Clerodendrum colebrookianum* is a common vegetable among local people and as herbal medicine for treating hypertension, diabetes and colics in infants particularly in Mizoram. However, *C. infortunatum* is not considered edible but used as medicinal purposes including treatment for scabies, as antidandruff agent, some skin diseases[28]. *C. infortunatum* is not yet explored scientifically in this particular region though it is used in various medicinal purposes by the local people. Therefore, the purpose of this study is to assess the antioxidant property and cytotoxicity of ethanolic extract of *C. infortunatum in vitro*.

Materials and methods

Chemicals and reagents

All the chemicals and reagents Quercetin, Folin-Ciocalteu, Gallic Acid, Aluminium chloride ($AlCl_3$), Hydrochloric acid, Nitro blue tetrazolium(NBT), Ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Ethanol, Dimethyl sulfoxide (DMSO), Bromocresol green solution (BCG), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), trypsin and Ethylene diamine tetra acetic acid (EDTA) used in this study are analytical grade and obtained from Merck Company, Germany.

Collection of Plant materials

The *Clerodendrum infortunatum* L. was collected from Tanhril Village, Aizawl, Mizoram. The specimen was assigned an accession number 564 after the proper identification was made by Prof. H. Lalramnghinglova, a known botanist as well as the Coordinator of Mizoram University Herbarium (it is an authorized herbarium in the university/state). Further, the specimen as a herbarium was submitted in this Mizoram University Herbarium for future reference.

Preparation of extracts

The freshly cleansed *C. infortunatum* leaves were air-dried for 72 h at room temperature before being ground in a grinder. The powder (250 g) was extracted for 24 h with 500 mL ethanol in a glass conical flask using a shaker at 25°C and filtered. Using a rotary evaporator under low pressure, the crude extract was concentrated at 40°C. The extract was preserved after drying and stored at 4°C in airtight containers until needed.

Preliminary phytochemical screening

The phytochemicals screening of the plants extract was done using standard protocols[29-31].

Determination of the total phenolic, flavonoids and alkaloids content

Total Phenol content

Folin-Ciocalteau reagent was used to figure out the total phenols[32]. 500 µL of ethanolic extract of *C. infortunatum* leaves was mixed with Folin-Ciocalteau solution (5 mL, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 mL, 1 M). A UV/Visible double beam spectrophotometer at 756 nm was used to find out the phenol concentration after 15 min of incubation. Gallic acid equivalent was used for the calculation of the phenol content (mg/g dry weight), which is a widely used standard.

Total flavonoids content

Flavonoids quantification was done following the aluminium chloride method[33]. 0.5 mL (1 mg/mL) of *C. infortunatum* extract was combined with 1.5 mL methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL distilled water in various concentrations. It was then kept at room temperature for 30 min. The quantity of flavonoids was estimated by measuring the optical density at 415 nm using a UV/Visible spectrophotometer.

Total Alkaloids content

For evaluation of the total alkaloid content, Bromocresol green method[34] was used. A solution (BCG) was mixed with 1 mL of *C. infortunatum* extract and the mixture was agitated and complex formed was extracted with 5 mL of chloroform. Absorbance was taken at 415 nm against blank. The solutions were stable for 2 h. Atropine was used as positive control and extract was calculated against blank.

Antioxidants Activities

DPPH scavenging activity

The DPPH free radical scavenging activity was measured using the Leong and Shui method[35], with slight modifications. In 1 mL solution of 0.1 mM DPPH, 0.5 mL of each concentration of the *C. infortunatum* extract was added. The absorbance was measured after it was thoroughly mixed and incubated for 30 min (523 nm). The OD of the plant extract and the control were compared. The result was expressed as a percentage and was calculated using the following formula:

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

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

ABTS cation scavenging assay

The *C. infortunatum* extract ABTS cation scavenging activity was assessed using the decolorization method[36], in which the plant extract transformed the green colour of an unstable ABTS solution to colourless. The formula used to compute the percent suppression of absorbance at 734 nm is:

$$\text{ABTS scavenging effect } (\%) = \frac{(AB - AA)}{AB} \times 100$$

where, AB is absorbance of ABTS radical + methanol; AA is absorbance of ABTS radical + sample extract/standard. Trolox was used as positive standard substance.

Superoxide anion scavenging assay

This Superoxide anion scavenging activity was determined with slight modifications[37]. To the mixture, 0.2 mL of NBT (1 mg/mL of solution in DMSO), 0.6 mL *C. infortunatum* extract, 2 mL of alkaline DMSO (1 mL DMSO containing 5 mM NaOH in 0.1 mL H_2O) were added in a final volume of 2.8 mL. The ascorbic acid was used as a positive control.

Cell lines and Culture medium

HeLa (cervical cancer), HT-29 (colon cancer), AGS (gastric cancer) cell lines were obtained from NCCS, Pune, India. Cell lines were grown at 37°C in DMEM with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL), and amphotericin B (5 µg/mL) in a humidified environment of 5% CO_2 until 85% confluency. Trypsin solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS) was used to separate the cells.

Cytotoxicity

The cytotoxic activity of the plant extract was determined by reduction of metabolically active cells through mitochondrial enzymes[38] on HeLa, HT-29 and AGS cell lines. Approximately, 1×10^4 cells per well were seeded in 96-well plates. The cells were allowed to attach to the bottom of flask prior to the experiment. After 16 h incubation period, cells with 70-80% confluency were treated with *C. infortunatum* extracts at various doses and incubated for 72 h. The cells were then given 10 µL of MTT (5 mg/mL) solution and the plate was placed to a cell incubator for another 4 h. At a wavelength of 550 nm, the proliferating cells were determined using a Spectramax M2 Microplate Reader (Molecular Devices, USA). The untreated wells were taken for measuring the relative viability, where cells were found 100% viable. The results are expressed as mean values (±SD) of three repeats.

Identification of bioactive compound using GC-MS analysis

The GC-MS analysis was carried out using a Clarus 690 Perkin/Elmer (Auto system XL) Gas Chromatograph with a mass detector Turbo mass gold Perkin Elmer Turbomass 5.1 spectrometer and an Elite 1 (100% Dimethyl poly siloxane) capillary column measuring 123.5 M × 678 M. The instrument was fixed at an initial temperature of 40°C ramp 5°C/min to 115°C, hold 5 min, ramp 5°C/min to 140°C, hold 5 min, ramp 2°C/min to 210°C, hold 8 min, and maintained at this temperature for 3 min. At the end of this period, the oven temperature was increased up to 250°C, at the rate of 5°C/min, and maintained for 9 min. The injection port temperature was maintained at 250°C, while the flow rate of Helium was kept at 1.5 mL/min. 70 eV was used as the ionisation voltage. The samples were injected in a 10:1 split mode. The scan range for mass spectral scanning was set to 500-800 (m/z). The temperature of the ion source was ensured at 230°C, while the temperature of the interface was kept at 240°C. The MS start time was 3 min, and end time was 75 min with solvent cut time of 3 min. The spectrums obtained of volatile compounds detected the through GC-MS were compared and matched with NIST 17 online library Ver. 2.3[39].

HPLC chromatogram analysis

The presence of bioactive compounds were quantified using Shimadzu Instrument (Shimadzu Corp, Kyoto, Japan) coupled with a diode array detector (DAD, SPD N 20A) and C18 column (5 µM;

4.6×250 mm). The analytical procedure was executed by applying the chromatographic conditions[40]. The gradient system began with a concentration of 100% solvent A at 0.1 min, then progressively increased the concentration of solvent B to 35% in 25 min, 50% in 45 min, and eventually 100% in 65 min. The standard compounds quercetin and rutin were dissolved in sterile water and filtered by PVDF (0.45 µM) and a volume of 20 µL was eluted. The solvent system of HPLC grade H_2O (solvent A) and H_2O: C_2H_3N: CH_3COOH (48:51:4 *v/v*) (solvent B) were employed as mobile phase. The flow rate was kept at 1 mL/min and 20 µL of the sample was injected for the analysis. The reference compounds such as quercetin and rutin were analyzed discretely to obtain the retention periods.

Table 1 — Preliminary phytochemical screening of ethanol extract of *Clerodendrum infortunatum* leaves

Phytochemicals	Test result
Saponins	+
Tannins	-
Cardiac Glycosides	+
Flavonoids	+
Alkaloids	+
Phenols	+

Linearity

The acquisition of linearity was acquired by repetitive elution of various concentrations of standards (quercetin and rutin). Analytical curves were obtained for every compound compared with their correlation in the peak area and the concentration using linearity.

Statistical analysis

The statistical analysis was carried out using Microsoft excel 2010, Washington, USA. The IC_{50} was calculated using GraphPad Prism 7 software, California, USA. The results are expressed as mean ± standard error of the mean (SEM).

Results

Preliminary phytochemical screening

According to the findings, the ethanol leaf extract of *C. infortunatum* showed the presence of saponin, alkaloid, cardiac glycosides, phenol and flavonoid but tannin was found to be absent (Table 1).

Determination of the total phenolic, flavonoids and alkaloids content

The *C. inforunatum* contain high phenol 64.35 mg/g extract followed by flavonoid 61.93 mg/g extract and alkaloid 13.33 mg/g extract expressed as gallicacid, quercetin and atropine equivalents, respectively (Fig. 1).

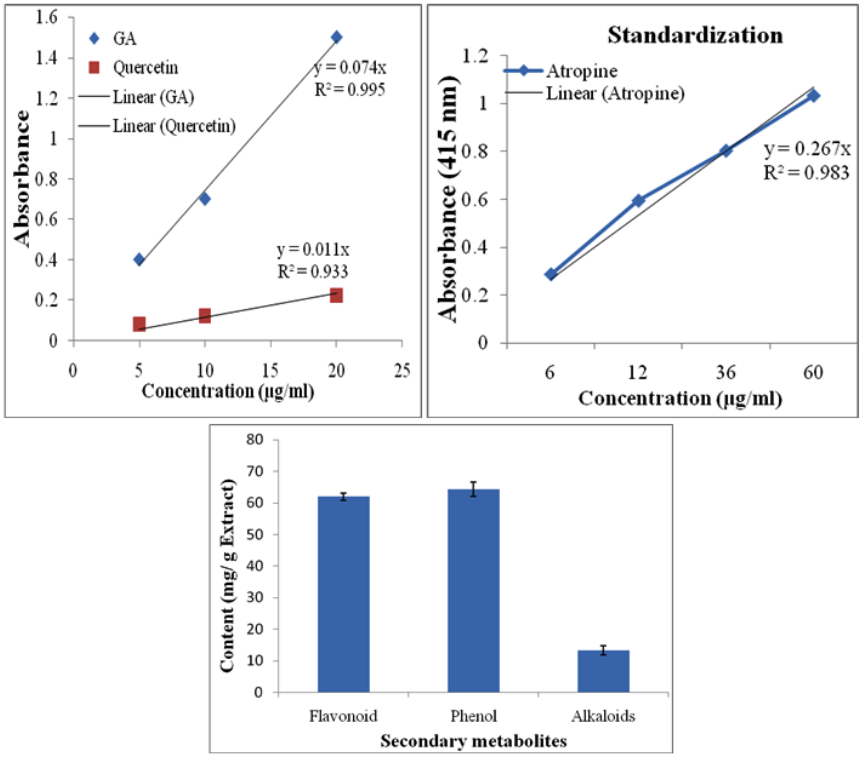

Fig. 1 — The calibration and quantification of secondary metabolites content of *C. infortunatum.* (A & B) Standardization of phenol and flavonoids; and (C) Quantification of secondary metabolites. The data present as mean ± SEM, n=3

Antioxidants Activities (DPPH scavenging activity, ABTS cation scavenging assay, Superoxide anion scavenging assay)

The antioxidant activity was assessed by the scavenging ability of DPPH, ABTS and Superoxide free radicals. The percentage inhibition of ABTS at 80 µg/mL sample gives the highest inhibition (90.14%) with an IC_{50} value of 50.05 followed by DPPH at 80 µg/mL inhibition 86.90% with IC_{50} of 47.99 and superoxide at 100 µg/mL sample shows 54.02% inhibition with IC_{50} of 83.57, respectively, (Fig. 2).

Cytotoxicity

The cytotoxicity of *C. inforunatum* extract was carried out on different cancer cell-lines such as HeLa, AGS and HT-29 along with chang liver (normal cell line). The result exhibited concentration dependant toxicity where the IC_{50} against HeLa is 52.48 followed by AGS (82.44) and HT-29 (142.2). However, the normal cell line (chang liver) showed less toxicity against plant extract except HT-29 where inhibitory concentration was found to be increased (Fig. 3).

Identification of compound using GC-MS analysis

The GC-MS analysis of the *C. inforunatum* showed the presence of 14 active compounds. The identified compounds were as follows, 9,12,15-octadecatrienoic acid(Z,Z,Z)- (3.864%), Ethyl 13-Methyl-Tetradecanoate (3.668 %), neophytadiene (3.372%), tridecanoic acid,12-methyl-Methyl ester (2.588%), methyl 11-Mrthyl-Dodecanoate (1.546%), Phenol,4-(-Methylpropyl) (1.422%), Bis (1,2,2-Trimethylpropyl) Methylphodhonate (1.285%), Benzofuran,2,3-Dihydro (0.909%), Phosphinothioic fluoride (0.883%), Imidazole,4-fluro- (0.604%), Oleic acid (0.595%), 3,7,11,15-Tetramethyl-2-Hexadecen-1-ol (0.456%), 4-cyclopentene-1,3-dione (0.441 %), Cyclobutanethiol (0.373%) (Table 2 & Fig. 4).

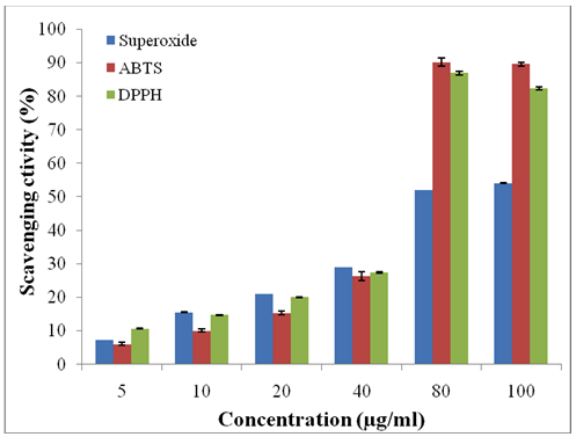

Fig. 2 — The free radical scavenging activity of *Clerodendrum infortunatum* extract. The data measured as percentage inhibition and represent as Mean± SEM, n=3

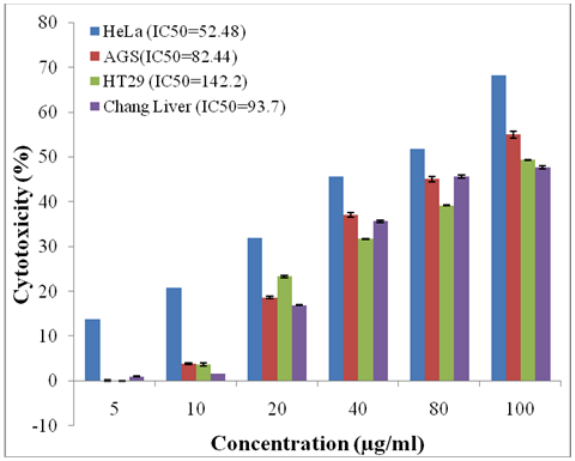

Fig. 3 — The cytotoxicity of *Clerodendrum infortunatum* extract against AGS, HeLa, HT-29 and normal cell line. The data measured as percentage inhibition and represent as Mean± SEM, n=3

Table 2 — GC-MS profiling of the identified compound from the ethanol extract of *Clerodendrum infortunatum* leaves

Peak	RT	Molecular weight	Molecular formula	Area %	Name of the compound
1	7.129	86.07	$C_3H_3FN_2$	0.604	Imidazole,4-fluro-
2	7.939	88.17	C_4H_8S	0.373	Cyclobutanethiol
3	11.171	96.08	$C_5H_4O_2$	0.441	4-cyclopentene-1,3-dione
4	18.492	306.21	$C_{10}H_9F_6PS$	0.883	Phosphinothioic fluoride
5	20.454	-	-	1.233	Unknown
6	23.909	120.15	C_8H_8O	0.909	Benzofuran,2,3-Dihydro
7	24.521	264.34	$C_{13}H_{29}O_3P$	1.285	Bis(1,2,2-Trimethylpropyl) Methylphosphonate
8	27.676	225.28	$C_{12}H_{19}NO_3$	1.422	Phenol,4-(-Methylpropyl)
9	55.124	278.5	$C_{20}H_{38}$	1.557	Neophytadiene
10	57.360	296.5	$C_{20}H_{40}O$	0.456	3,7,11,15-Tetramethyl-2-Hexadecen-1-ol
11	59.980	228.37	$C_{14}H_{28}O_2$	1.546	Methyl 11-Methyl-Dodecanoate
12	62.745	282.5	$C_{18}H_{34}O_2$	0.595	Oleic acid
13	63.373	270.5	$C_{17}H_{34}O_2$	3.668	Ethyl 13-Methyl-Tetradecanoate
14	68.468	298.9	$C_{18}H_{31}ClO$	0.467	9,12-octadecadienoylchloride, (Z,Z)-
15	69.823	242.4	$C_{15}H_{30}O_2$	2.588	Tridecanoic acid,12-methyl-Methyl ester

Table 3 — HPLC method validation of the standards

Standards	RT	R^2	Formula (Y=m(X)+C)	LOD (µg/L)	LOQ (µg/L)	Accuracy (%)	Peak purity index
Rutin	6.603	0.99	Y = 2283X - 915	0.73	0.3	100.3	0.78091
Quercetin	8.470	0.99	Y = 3569X - 147	0.24	0.15	103.1	0.8385

Linear calibrations curves were not forced to zero; RT-Retention time; R^2- Coefficient correlation; LOD-Limit of detection; LOQ-Limit of quantification

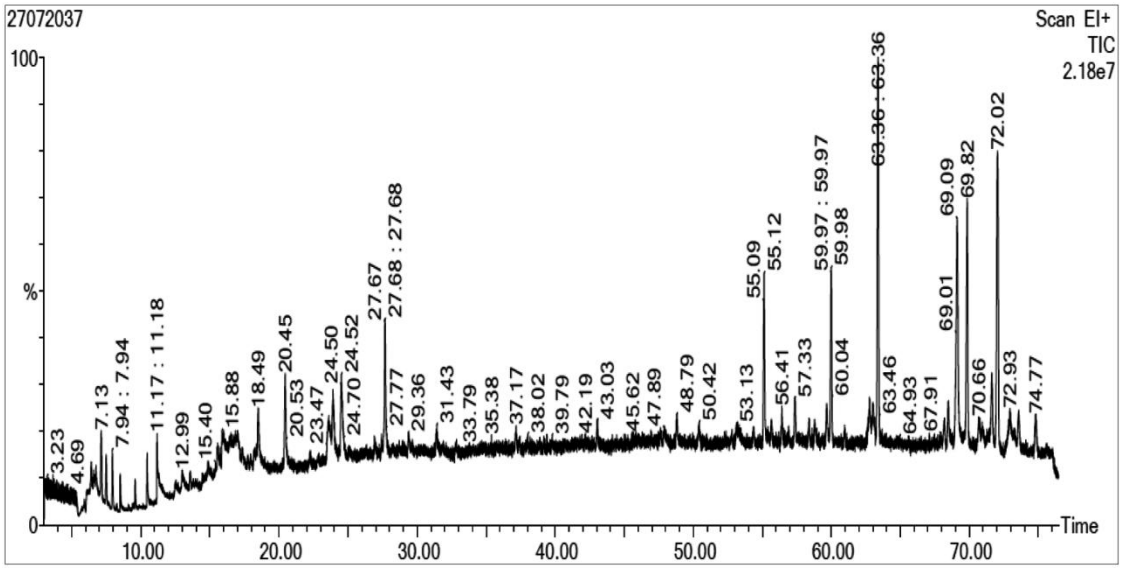

Fig.4 — GC-MS chromatogram of the ethanol extract of *Clerodendrum infortunatum*

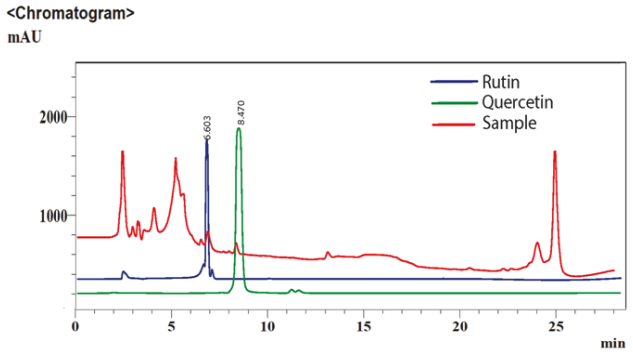

Fig. 5 — Detection of bioactive compound of *Clerodendrum infortunatum* extract by HPLC method

The HPLC analysis of *C. inforunatum* revealed the presence of rutin at the retention time of 6.603 and quercetin at the retention time of 8.470 (Table 3 & Fig. 5).

Discussion

Herbal medication is still practiced in many developing countries and is believed to be the cheapest and safest medication and promotes healthier living as well[41]. This could be the reason for the boom in sales of herbal medicines which constitutes a significant share of the global drug market[42,43]. Therefore, it was decided to investigate the phytochemical constituents of *C. infortunatum* plant which is known to have many uses customarily for various medications. It is also a well-known ethno medicinal plant amongst the Mizo tribe of Mizoram state. The phytochemical analysis revealed that various secondary metabolites such as saponin, flavonoid, phenol, alkaloid and cardiac glycosides were present (Table 1). Previous research reported that phenolics, steroids, di- and triterpenes, flavonoids, volatile oils are present in this genus[44]. It is reported that plant phytochemical contents ensured the medicinal properties such as anti-cancer, anti-inflammatory, antioxidant activities[45,46].

The present study also showed that phenolics and flavonoids content were comparatively high whereas alkaloid is found to be lesser as compared to the other secondary metabolites (Fig. 1). The phenols possess high antioxidant capacity and are an important dietary additive of plants. It has been reported that the scavenging effect of the DPPH free radical significantly elevated with the increase in concentration of the sample and standard to a certain degree and hence are strongly

reliant on the extract concentration[47-49]. Phenolics have been a centre of attraction as potential agents to prevent and treat many oxidative stress-related diseases[50-52]. The flavonoids have a major contribution for the attractive colours of flowers with anthocyanins[53,54]. The antioxidant activity depends mostly on the secondary metabolites found in the plants. It expresses the amount of antioxidant needed to neutralize the unstable free radicals in the system[55]. The assessment of the ability to scavenge DPPH is a very useful method to estimate the antioxidant activity[56]. It is a violet coloured compound and has an unpaired electron in the outermost shell which turns yellow after accepting electron from any antioxidant[57]. The high antioxidant properties might be due to the presence of high flavonoid and phenol present in the leaf extract of *C. infortunatum* (Fig. 2).

The toxicity studies of *C. infortunatum* against HeLa, AGS and HT-29 cells exhibited an increase in cytotoxicity with the rise in concentration (Fig.3). It was reported that methanolic extract of *C. serratum* leaves reduced tumour development in 7,12-dimethylbenz[α]anthracene (DMBA) induced skin carcinogenicity in testis, liver and kidney of mice[58-59]. Previous report stated that the methanolic extract of *Clerodendrum serratum* illustrates good *in vivo* activity using Dalton's Lymphoma Ascites[60]. Other plants containing flavonoids such as *Oroxylum indicum*, *Tragopogon porrifolius*, *Trigonella foenum-graecum*, *Cassia acutifolia* and *Rhazya stricta* have also been shown to have antioxidant and anticancer activities[61-63].

The gas chromatography-mass spectrometry analyses indicated the occurrence of 14 bioactive volatile compounds. The gas chromatographic study reported the presence of bioactive compounds such as hispidulin-glucuronide, eupafolin, scutellarin, 2-acetoxyclerodendrin B and hispidulin 7-O-glucuronide from *C. infortunatum*[64]. These chemicals, however, were not found in present experiment; it may be because of geographical as well as ecological and plant growth factors[42]. The detection of neophytadiene (diterpene) in our study (Fig. 4) which is histamine release inhibitor is a noble compound with the activities of antioxidants, analgesic, anti-inflammatory and antimicrobial[65,66]. Further, the presence of oleic acid, octadecatrienoic acid, Phenol,4-(-methylpropyl) were already reported and discussed in the previous studies of *C. infortunatum* leaves extract[67]. The present study indicated that benzofuran, 2,3-dihydro is compound of dihydro derivative of benzofuran which were used as anticancer agents and inhibitors of NF-κB[68].

In previous report, Waliullah *et al*. reported that the ethanol leaf extract of *C. infortunatum* has shown noteworthy inhibitory potency against the fungal and bacterial strain by testing out the MIC and its zone of inhibition[69]. Similarly, Saha *et al.* also testified that the chloroform extract of *C. infortunatum* has high content of phenol and flavonoids and it is effective against *Phomopsis vexans* and the antifungal activity was also proven by Kharkwal *et al*[70,71]. In addition to that, there were also studies that *C. infortunatum* possessed a substantial antitumor activity against DLA cells *in vitro* as well as EAC cell lines *in vivo*[72,73]. The presence of rutin and quercetin detected using HPLC (Fig. 5) which has been shown to have anti-cancer activity both *in vitro* and *in vivo*[74,75]. Rutin is able to curb the expression of Bax and Bcl-2 which eventually triggers different caspase enzymes leading to apoptosis in colon cancer cells[76] and can also reduce AP-1 and NF-κB transactivation significantly[77]. Quercetin has the capacity to induce DNA interaction, cell cycle arrest as well as activating apoptosis in different cancer cells[78,79]. Hence, rutin and quercetin along with benzofuran identified using HPLC and GC-MS could be the reason for the anti-cancer activity observed in our study.

Conclusion

Clerodendrum infortunatum contain various phytochemicals and exhibits significant anti-oxidant activity leading to cell cytotoxicity against HeLa, HT-29 and AGS, respectively. Therefore, disseminating the awareness about the significance of *C. infortunatun* is required which is a known ethnomedicinal plant in traditional system of Mizo tribe for its unique medicinal values especially in rural areas. Parallely, the conservation measures also needs to be taken into consideration for this plant. Moreover, the mechanism of action might be essential to study at molecular level for dosage formulation in future. Our study revealed that the anti-tumor activity of *C. infortunatum* could be possibly due to the flavonoids rutin and quercetin as well as benzofuran. Besides, our study also validates the plant's traditional use to treat a variety of diseases by the Mizo ethnic tribe. Hence, the *C. infortunatum* could be a viable candidate for more research as anti-cancer agent.

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Conflict of interest

All authors declare no conflict of interest.

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The assessment of the free radical scavenging activity and flavonoid contents of selected medicinal plants of Mizoram

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Abstract

Herbal medicine has proven to be one of the most well-known fields of traditional medicine worldwide. Extracts from different traditional medicinal plants have been screened to discover the source of therapeutic effects and nature has been a source of medicinal agent for thousands of years. Antioxidants protect the body from the harmful damage produced by free radical induced oxidative stress. The present study is the assessment of the total flavonoid contents and antioxidant potential of medicinal plants. Different parts of the plant were used namely the stem bark of *Schima wallichi* (DC) Korth., *Milletia pachycarpa* Benth., the leaves of *Eleagnus caudata* Schlecht, *Dysoxylum gobara* Buch.-Ham and the fruit of *Castanopsis indica* (Roxb.)A.DC. The chloroform and ethanol extracts of all these plants showed free radical scavenging activity in a concentration dependent manner. *S. wallichi* showed the maximum scavenging activity followed by *E. caudata*, *M. pachycarpa*, *C. indica* and *D. gobara* accordingly. The chloroform and ethanol extracts both showed an increase in the flavonoid content in a concentration dependent manner. The amount of total flavonoids was higher in the ethanol extracts than that of chloroform extracts. The ethanol extracts also has greater flavonoid content and possess higher antioxidant activity when compared to the chloroform extracts. Our study concluded that *S. wallichi* showed highest ABTS scavenging activity among all the five plants. The antioxidant activity was not directly proportional to the total flavonoid contents of a plant species. The amount of total flavonoids was found to be lower in *S. wallichi*, which shows that other secondary compound like alkaloids, phenols, etc may have contributed to this effect.

Key words: ABTS; Total flavonoids; Medicinal plants.

Introduction

In spite of the recent domination of the synthetic chemistry as a method to discover and produce drugs, the potential of bioactive plants or their extracts to provide new and novel products for disease treatment and prevention is still enormous (1). For thousands of years nature has been the major source of medicine (2). Bioactive plants and their extracts have the potential to provide novel products for prevention and treatment of diseases. Due to its effectiveness in treating various diseases, minor side effects and cheaper cost, medicinal plants are highly popular among developing countries (3). Medicinal plants are indispensible to the global economy. Many secondary metabolites are commercially sold and are crucial to many pharmaceutical companies (4).

In India, the traditional knowledge of medicinal plants used by indigenous people are well documented and has been passed on the next generation for many years (5). Phyto-chemicals are natural chemical compounds found in plants. Plants produce these chemicals as a mode of defense mechanism for their protection, but these phytochemicals also provide protection

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for humans from diseases according to recent studies (6). Flavonoids are ubiquitous in nature and occurs as glycones, glycosides and methylated derivatives in vascular plants. Flavonoids are polyphenolic compounds (7). Due to their extensive biological and pharmacological properties, flavonoids have been widely studied. Flavonoids are potent antioxidants that can protect human beings from free radicals and reactive oxygen species. Their antioxidant capacity and scavenging activities depends upon their molecular structure, mainly on the position of hydroxyl groups in its chemical structure (8). Flavonoids contain many substances that protect biological systems from the toxic effects of oxidative processes (9). Free radicals have harmful effects on human beings and is related to toxicity and causing diseases like diabetes, chronic renal failure, cancer, mellitus, atherosclerosis, immune dysfunction and aging (10). Food sources like fruits and vegetables contain many free radical scavenging antioxidants (11). Free radicals have a damaging effect on cells and cause various degenerative disorders when they are produced excessively (12).

Antioxidants can intervene the production of free radicals during the main steps of the free radical mediated oxidative processes, viz., initiation, propagation and termination (13). Most living organisms have an effective defense system to protect themselves against the oxidative stress induced by Reactive Oxygen Species (ROS) (14). Due to the certain health benefits, less toxicity, cheap price and accessibility, antioxidants from plant sources are getting popular (15). Therefore, this study targets to investigate antioxidant potential of different medicinal plants including *Schima wallichi*, *Milletia pachycarpa*, *Eleagnus caudata*, *Castanopsis indica* and *Dysoxylum gobara in vitro* by evaluating ABTS scavenging activity.

Medicinal uses:

***Schima wallichii* (DC) Korth:** The leaves and the stem bark are traditionally used for its medicinal properties. The bark is used as an antiseptic for wounds. It is also used as a vermicide, and treating gonorrhea (16), decoction of bark is effective against head lice and reduces fever. The bark juice of Chilauni is used in animals as a liver fluke disinfesting agent (17). People of Western Mizoram use the fruit juice of Chilauni for treating snakebite (18). *Schima wallichii* has anti-cancer activities, and have the ability to induce apoptotic mechanisms (19).

***Elaeagnus caudata* Schlecht:** The fruit is taken as a health tonic (20). The extract of the fruit or stem bark is mixed with *Piper longum* and is taken for 2-3 weeks on a daily basis to cure jaundice and other liver troubles (21). The root decoction is taken to expel the retained placenta, ease labor and as a treatment after child birth. The leaf infusion is used for strengthening the function of uterus after child birth (22).

***Milletia pachycarpa* Benth:** *M.pachycarpa* is used in Chinese traditional medicine for the preparation of 'Jixueteng' that induce the growth of red blood cells (23). The compounds isolated from *M. pachycarpa* has been reported to be cytotoxic and induce apoptosis in HeLa cells (24) and also show cytotoxic effect in Brine shrimp assay (25) with anti-inflammatory activity (26). In India and China, it is used traditionally in treating cancer and infertility. It is also used as a pesticide and as a blood tonic (27). The bark paste is also used in treating diseases like skin infections and itches (28).

***Castanopsis indica* (Roxb.) A. DC:** *C. indica* is traditionally used for treating stomach disorders, chest pain, skin diseases, headache and diarrhea (29). The leaf decoction is used to treat stomach disorder and skin diseases (30). The seeds are consumed raw in Nepal (31) and Mizoram (32). The resin is given to treat diarrhea and the leaf paste is applied for headache. The bark paste is also applied on the chest to control chestpain (33).

***Dysoxylum gobara* Buch.-Ham:** The leaf and bud decoction is used to treat diarrhea and dysentery (34-36). The tender leaves and flowers are cooked

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and eaten as a vegetable. The decoction of leaves is used as a remedy for food poisoning, diarrhea and dysentery (37).

Materials and Methods

Chemicals: Ascorbic acid, trolox and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), aluminium chloride, chloroform, ethanol, potassium acetate, quercetin, potassium persulfate.

Collection of Plant Materials: The healthy stem bark of *Milletia pachycarpa* Benth., *Schima wallichi* (DC) Korth., the leaves of *Eleagnus caudata* Schlecht., *Dysoxylum gobara* Buch.-Ham. and the fruit of *Castanopsis indica* (Roxb.) A.DC. were collected during the dry season from different parts of Mizoram. The plant specimens were identified by Prof. Lalramnghinglova, Department of HAMP, Mizoram University, Aizawl. The herbarium specimens are deposited at the Department of Zoology, Mizoram University. The stem bark, leaves or fruits were examined visually for infection, washed thoroughly with clean water and allowed to shade dry at RT in the dark, clean and hygienic conditions. The dried plant material was powdered using an electrical grinder at room temperature and sequentially extracted with chloroform and ethanol using a Soxhlet apparatus. The liquid extracts were filtered and dried using rotary vacuum evaporator and stored at-70^{o} C until further use.

Total flavonoid content: The total flavonoid content was determined by $AlCl_3$ method (38). The ethanol and chloroform extracts of different concentrations were mixed with 1.5 ml of methanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. Then it was incubated at RT for 30min. The absorbance was measured at 415 nm with double beam UV spectrophotometer). The calibration curve was prepared by preparing Quercetin solution at different concentrations.

Antioxidant capacity using ABTS scavenging assay: The ABTS cation scavenging activity was determined for the different extracts using a minor modification of Re R et al (39). 37.5 mg of potassium persulfate was taken and dissolved in 1 ml of distilled water. 44 ml was taken from this solution and dissolved in 2.5 ml of distilled water with 9.7 mg of ABTS. The absorbance was measured at 734 nm after the solution was kept in dark condition at RT for 16 hours. The results have been represented as ascorbic acid equivalent. The scavenging activity was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{\text{(A control OD-Atest OD)}}{\text{(A control OD)}} \times 100$$

Results and Discussion

Phytochemicals found in plants possesses significant benefits to human health such as ascorbic acid, carotenoids and phenolic compounds (40). Phytochemical compounds that naturally possess anticarcinogenic (41-42) and other beneficial properties are referred to as chemo-preventers and this protective action is due to their antioxidant activity and their capacity to scavenge free radicals (43).

The total flavonoid contents of chloroform extracts of *Milletia pachycarpa*, *Schima wallichi*, *Eleagnus caudata*, *Castanopsis indica* and *Dysoxylum gobara* showed a concentration dependent rise up to 2500 μg/ml (Figure.1). The highest total flavonoid was present in *E. caudata* (130.36±2.15 mg/g) followed by *D. gobara* (94.62±1.58 mg/g), *M. pachycarpa* (51.27±2.14 mg/g), *C. indica* (48.61±3.40 mg/g) and *S. wallichi* (26.82±1.25 mg/g). The least total flavonoid content was detected in *S. wallichi.*

The presence of total flavonoid contents in ethanol extracts of all five plants also showed an increase in flavonoid content with an increase in concentration manner. The highest flavonoid content were also found at 2500 μg/ml for all the extracts with the highest value present in *Dysoxylum gobara* (75.73±0.98 mg/g), followed by *E. caudata* (53.43±3.27 mg/g), *C. indica* (38.04±23 mg/g), *S. wallichi* (32.06±2.29 mg/g). The lowest content was found in *M. pachycarpa* (19.43±0.71 mg/g). The order of the flavonoid

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content observed was as follows: ECC (130.36±2.15) > DGC (94.62±1.58) > DGE (75.73±0.98) > ECE (53.43±3.27) > MPC (51.27±2.14) > CIC (48.61±3.40) > CIE (38.04±23) > SWE (32.06±2.29) > SWC (26.82±1.25) > MPE (19.43±0.71) at the concentration 2500 µg/ml. The present study revealed high content of both phenols and flavonoids (Figure 1), both flavonoids and flavones are widely distributed secondary metabolites with antioxidant and antiradical properties (44).

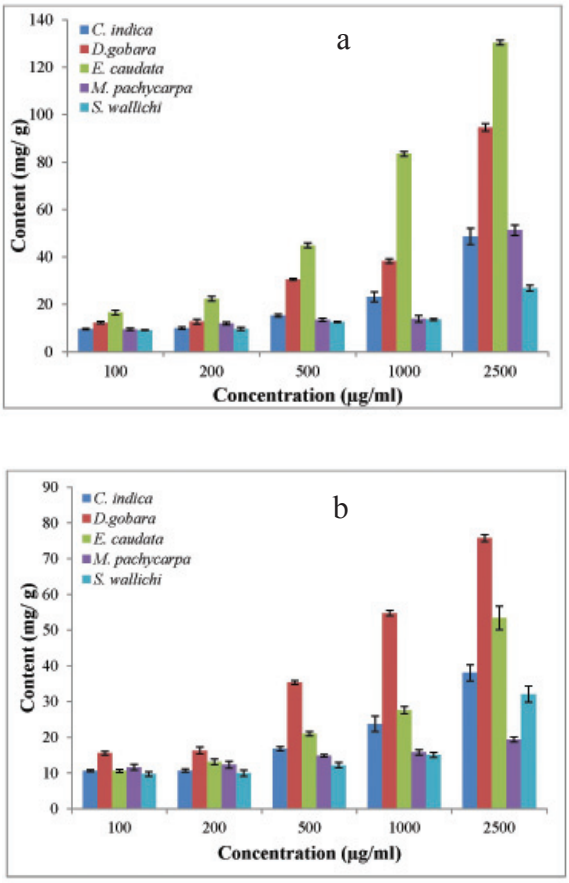

Fig. 1: The total flavonoid content of various medicinal plant of Mizoram extract with chloroform (a) and ethyl alcohol (b) as Quercetin equivalent. The values represented as Mean+ SEM, n=3.

The chloroform extracts of the five plants showed a concentration dependent rise up to 5000 µg/ml in the ABTS scavenging activity. The maximum ABTS inhibition for chloroform extract was found in *S. wallichi* (78.45±0.95 µg/ml) followed by *C. indica* (65.78±0.76 µg/ml), *M. pachycarpa* (63.44±2.58 µg/ml), *E. caudata* (63.28±2.44 µg/ml) and *D. gobra* (22.93±0.53 µg/ml). The ethanol extract of all the plants also showed the highest ABTS scavenging at 5000 µg/ml. The ethyl alcohol extract of *M. pachycarpa* showed the highest activity (89.02±1.79 µg/ml), followed by, *S. wallichi* (88.84±2.46 µg/ml), *E. caudata* (88.60±0.94 µg/ml), and *C. indica* (79.90±1.65 µg/ml) and *D. gobara* (78.67±2.21 µg/ml).

The order of scavenging activity was observed as follows: MPE (89.02±1.79%) > SWE (88.84±2.46%) > ECE (88.60±0.94%) > CIE (79.90±1.65%) > DGE (78.67±2.21) > SWC (78.45±0.95%) > CIC (65.78±0.76%) > MPC (63.44±2.58%) >ECC (63.28±2.44%) > DGC (22.93±0.53%) at the concentration 5000 µg/ml.

The analysis of ABTS scavenging in respect of TROLOX equivalent was similar as maximum scavenging activity was observed at concentration of 5000 ìg/ml for chloroform extracts and ethyl alcohol extracts of all the five plants.

Correlation analysis of total flavonoid content and scavenging activity of certain medicinal plants of Mizoram, extracted with chloroform and ethyl alcohol shows that an increase in the flavonoid content causes an increase in the scavenging activity. The chloroform extracts of *M. pachycarpa*, *S. wallichi*, *E. caudata*, *C. indica* and *D. gobara* did inhibit the generation of ABTS free radicals in concentration dependent manner and this activity was highest at the concentration of 5000 µg/ml with EC50 values of 2140 µg/ml, 475.9 µg/ml, 74.18 µg/ml, 2295 µg/ml respectively, however *D. gobra* did not show insignificant effective concentration compared to the others, the maximum effect was observed for 5000 ìg/ml with 22.93±0.53% of scavenging activity.

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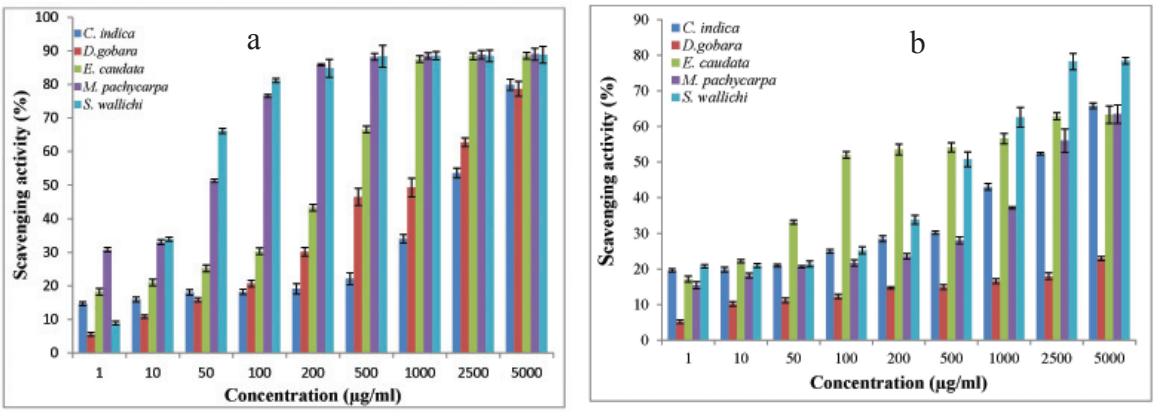

Fig. 2: Scavenging activity of various medicinal plant of Mizoram extracted with chloroform (a) ethyl alcohol (b). The values represented as Mean+ SEM, n=3.

Table 1(a) : Correlation of total flavonoid and scavenging activity certain medicinal plants of Mizoram, extracted with chloroform.

Conc.	*C. indica*		*D.gobara*		*E. caudata*		*M. pachycarpa*		*S. wallichi*	
(µg/ml)	TF	SA	TF	SA	TF	SA	TF	SA	TF	SA
100	9.51	24.94	12.35	12.26	16.6	51.97	9.47	21.65	9.14	25.22
200	9.97	28.49	12.68	14.68	22.35	53.51	11.9	23.55	9.67	33.81
500	15.26	30.19	30.51	14.95	44.83	54.14	13.49	28.04	12.55	50.76
1000	23.18	43.02	38.2	16.58	83.5	56.6	13.97	37.15	13.61	62.57
2500	48.61	52.36	94.62	17.99	130.37	62.89	51.28	56.02	26.83	78.24
Correlation (R- value)			**0.95**	**0.85**	**0.98**	**0.94**	**0.88**			

Table 1(b) : Correlation of total flavonoid and scavenging activity certain medicinal plants of Mizoram, extracted with ethyl alcohol.

Conc (µg/ml)	*C. indica*		*D.gobara*		*E. caudata*		*M. pachycarpa*		*S. wallichi*	
	TF	SA	TF	SA	TF	SA	TF	SA	TF	SA
100	10.62	18.17	15.57	20.68	10.6	30.29	11.64	76.6	9.76	81.21
200	10.68	19.12	16.31	30.11	13.15	43.34	12.33	85.87	9.99	84.84
500	16.79	22.12	35.36	46.5	21.05	66.55	14.85	88.23	12.21	88.41
1000	23.76	34.05	54.76	49.29	27.58	87.55	15.77	88.53	15.05	88.55
2500	38.04	53.6	75.73	62.77	53.43	88.38	19.44	88.84	32.06	88.56
Correlation (R- value)		**0.99**		**0.95**		**0.82**		**0.71**		**0.55**

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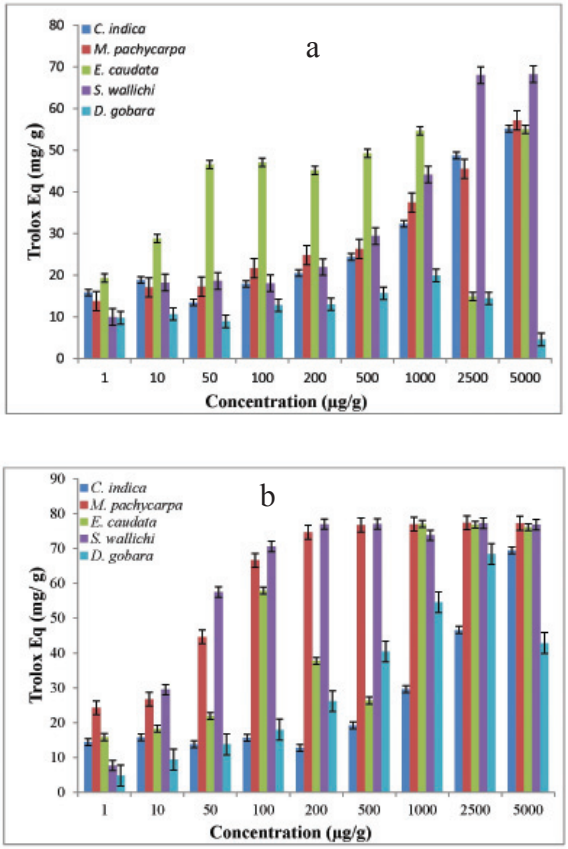

Fig. 3: The trolox equivalent of various medicinal plant of Mizoram extracted with chloroform (a) ethyl alcohol (b). The values represented as Mean+ SEM, n=3.

The ethyl alcohol extracts of *C. indica* and *D. gobra* also showed maximum inhibition at the concentration of 5000 µg/ml, with EC50 value of 2524 µg/ml and 908 µg/ml, respectively.

The maximum ABTS inhibition for ethyl alcohol extract of *E caudata* was at 200 µg/ml, which remained almost same at 1000 µg/ml, that remained almost similar up to a concentration of 5000 µg/ml with an EC50 value of 239 µg/ml. The maximum scavenging was observed at 200 µg/ml for *M. pachycarpa* with an EC50 value of 1.27 µg/ml, whereas this concentration was 100 µg/ml for *S. wallichi* with EC50 value of 0.060 µg/ml, both remained almost similar up to a concentration of 5000 µg/ml. The comparison between activity and content show positive correlation and the order of positive correlation were as follows: CIE (r = 0.99) > ECC (r = 0.975) > CIC (r = 0.951) > DGC (r = 0.950) > MPC (r = 0.93) > SWC (r = 0.88) > DGC (r = 0.85) > ECE(r = 0.82) > MPE (r = 0.71) > SWE (r = 0.55).

The free radical scavenging activity of plants extract against ABTS cations revealed significant reduction with less concentration (Figure 2). The active metabolites present in the plants are responsible for the antioxidant activity and the natural phenol and flavonoid contents are present in fruit, leaves, flower and the seeds of plants (45). The TROLOX equivalent activity showed the plant extract possesses high efficacy of scavenging properties (Figure 3). The correlation analysis of both concentration and activity revealed that the activity increased with increase in concentration (Table. 1). The natural antioxidants maybe useful as they may have fewer side effects or no side effects, because of their biologic origin. *E. caudata* scavenged the ABTS free radicals in a concentration dependent manner indicating its antioxidant potential. The other species of *Eleagnus* including *E. angustifolia* have been reported to scavenge ABTS radical (46).

S. wallichi inhibit the generation of ABTS free radicals in a concentration dependent manner and this activity was highest for this plant (Figure. 2). The present study correspond with the previous reported by (47, 17, 48). The resulting antioxidant effect observed was almost akin to the present study. The chloroform and ethanol extracts of *M. pachycarpa* did inhibit the generation of DPPH free radicals in concentration dependent manner. The ethyl alcohol extract of *M. pachycarpa* showed the highest activity.

M. pachycarpa has been known to scavenge free radicals (49-50) and the results are also in accordance with the findings in this study.

C. indica scavenged the ABTS free radicals in a concentration dependent manner indicating

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its antioxidant potential. The *C. indica* has been reported to scavenge superoxide, hydroxyl and ferric free radicals earlier and this activity was attributed to the presence of phenolic compunds (51).

The other species of Castanopsis including *Castanopsis cuspidate* have been reported to have antioxidant property (52).

The antioxidant activity of *D. gobara* was lesser than the other plants and has the least antioxidant activity among all the five plants. *D. gobara* has been reported to possess antioxidant activity in DPPH assays earlier (50) and the results are also in accordance with the findings in the present study. The other species of *Dysoxylum* namely *Dysoxylum cauliflorum* have been reported to possesses antioxidant activity in both the DPPH scavenging and FRAP assay.

Conclusion

The present study of medicinal plants including *M. pachycarpa, S. wallichi, E. caudata, C. indica* and *D. gobara* revealed high content of bioactive compound and potential antioxidant efficiency, which might be useful resources for future ethno-medicine. However, the mechanism of action of secondary metabolites required further investigation in future.

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Abstract

The aim of this study is to evaluate the *in vitro* anti-inflammatory effect of *Mikania micrantha* (L.) Kunth. leaf extract on RAW 264.7 murine leukemia cell line. The qualitative phytochemical analysis of the different extracts of the leaves of *M. micrantha* revealed the presence of carbohydrates, flavonoids, quinones, terpenoids, phenols, and coumarins, whereas the quantification revealed that the methanol extract contained the highest phenol content (259.88 mg catechol equivalent/g dried sample) as well as flavonoid content (156.55 mg quercetin equivalent/g of dried sample). The different extracts were tested for antioxidant activity using a DPPH scavenging assay. The antioxidant capacity of ethyl acetate extract at 100 µg concentration showed the highest DPPH scavenging ability with an IC50 value of 40.34 µg/ml in comparison with the standard (39.92 µg/ml). *Allium cepa* assay and MTT assay were performed to assess the cytotoxicity effects. The fresh leaf extract increased the incidence of anomalous mitosis. Cytotoxicity study showed that ethyl acetate extracts exhibit the highest cytotoxicity with an IC50 value of 47.68 µg/ml. Reverse-transcription polymerase chain reaction analysis exhibited the suppression of the RAGE gene. This is the first report on the effect of the anti-inflammatory activity of *M. micrantha* leaf extract on RAW 264.7 murine leukemia cell line. This study concluded that *M. micrantha* possesses antioxidant property and limiting RAGE gene expression suggests anti-inflammatory properties.

Keyword: Anti-inflammatory Antioxidant Cytotoxicity Mitosis Phytochemical.

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DEGREE: Ph. D.

DEPARTMENT: HORTICULTURE AROMATIC AND MEDICINAL PLANTS

TITLE OF THESIS: EVALUATION AND CHARACTERIZATION OF SELECTED PLANT SPECIES OF MIZORAM FOR THEIR CYTOTOXICITY AGAINST DIFFERENT CANCER CELL LINES

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Head
Department of Horticulture Aromatic and Medicinal Plants

ABSTRACT

EVALUATION AND CHARACTERIZATION OF SELECTED PLANT SPECIES OF MIZORAM FOR THEIR CYTOTOXICITY AGAINST DIFFERENT CANCER CELL LINES

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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DEPARTMENT OF HORTICULTURE AROMATIC AND MEDICINAL PLANTS
SCHOOL OF EARTH SCIENCES AND NATIURAL RESOURCES MANAGEMENT

APRIL, 2022

EVALUATION AND CHARACTERIZATION OF SELECTED PLANT SPECIES OF MIZORAM FOR THEIR CYTOTOXICITY AGAINST DIFFERENT CANCER CELL LINES

BY

MALSAWMDAWNGLIANA

DEPARTMENT OF HORTICULTURE AROMATIC AND MEDICINAL PLANTS

NAME OF THE SUPERVISOR: DR. AWADHESH KUMAR

SUBMITTED
IN PARTIAL FULFILLMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN DEPARTMENT OF HORTICULTURE AROMATIC AND MEDICINAL PLANTS OF MIZORAM UNIVERSITY, AIZAWL

Abstract

Plants have been the major source for several drugs and it is well known that 75% of the modern chemotherapeutic drugs have their origin in plants or natural products. Extracts from *Urtica membranacea, Artemesia monosperma,* and *Origanum dayi post* have also been reported to exert anticancer activity. *Artocarpus obtusus, Blumea balsamifera, Boerhaavia diffusa, Calotropis procera, Citrus maxima, Emblica officinalis, Moringa oleifera, Panax ginseng, Pfaffia paniculata, Rheum officinale, Saxifraga stolonifera, Vitex negundo, Withania somnifera,* and *Zingiber officinale* have also been found to possess anticancer activity under different conditions. Therefore plants and natural products still provide a major avenue for screening and developing of new nontoxic molecules including drugs for cancer treatment.

Samples of *Clerodendrum infortunatum* (leaves), *Mikania micrantha* (leaves), and *Girardinia diversifolia* (leaves), were collected from Aizawl, Mizoram. *Senecio scandens* (leaves), and *Paris polyphylla* (rhizome) were collected from Champhai, Mizoram was collected, cleaned properly, shade dried and powdered. extracted with ethanol and the liquid extracts were concentrated with rotary evaporator and stored at -70°C until further use. Preliminary phytochemical analysis showed the presence of Saponins, flavonoids, alkaloids, phenols and terpenoids present in all the plants.Tannins was present in all except *Clerodendrum infortunatum* and cardiac glycosides was absent in all except *Clerodendrum infortunatum.* Secondary metabolites like phenol, flavonoid and alkaloid was quantified and the amount of total phenols and flavonoids also increased with increasing concentration . *Paris polyphylla* showed highest content of phenol and alkaloid.

The *in vitro* antioxidant activity of *Clerodendrum infortunatum, Mikania micrantha, Girardinia diversifolia, Senecio scandens* and *Paris polyphylla* ethanolic

extracts were analysed following DPPH, ABTS and superoxide free radical scavenging activity standard method. Free radicals are necessary evils as they are produced during normal metabolism of the body as well as a defense against the pathogenic attack. However, excess of free radicals generation produce oxidative stress, inflammation and various diseases and any agent that can reduce the oxidative stress will be useful in treating oxidative stress related disorders. The various plant extracts inhibited the generation of DPPH, ABTS and Superoxide anion in a concentration dependent manner and this ability depended on the type of extract and the concentration. Therefore, the antioxidant activities of these plants may be due to the presence of good amount of secondary metabolites. However, the IC_{50} scavenging activity for the free radicals was lowest for *Paris polyphylla* in ABTS and Superoxide.

The cytotoxicity of the ethanol extract of *C. infortunatum, M. micrantha, G.diversifolia, S. scandens* and *P. polyphylla* on HT-29, AGS, HeLa, HepG2 and A549 cancer cells was estimated using MTT assay, where the cytotoxicity was increased in a dose dependent manner. It also gives an account on the effect of different concentrations of the ethanol extract on the cytotoxicity. The ethanolic rhizome extract of *Paris polyphylla* showed the overall best result in cell cytotoxicity and was chosen for further result. The caspase activity assay revealed that apoptosis occurs in both A549 and HeLa cells following both intrinsic and extrinsic pathway and necrosis cell death happened in HepG2 cell since expression of caspase 3 was not seen.

Several compound including gallic acid, coumaric acid, diosgenin, benzamide and D-Allose were identified using HPLC and GC-MS.

The 3D image of RAF (Rapidly Accelerated Fibrosarcoma) – 3OMV and CDK2 (Cyclin-dependent kinases)- 5A14 were obtained from Protein Data Bank (PDB) and 3D image of diosgenin and coumaric acid were procured from Pubchem. These proteins which have huge involvement in cell division and the identified compounds were

subjected to molecular docking using AutoDock Tool version 4.2.0. Diosgenin and RAF showed the binding affinity of -7.97 kcal/mol binded in the ATP binding site of RAF.

Since the plant *Paris polyphylla* showed a good activity in all the experiment undertaken, it can be a promising source of medicine in the future. To have a better understanding of this plants potency to cure cancer, animal model can be used.