SCREENING FOR PHYTOCHEMICALS AND CYTOTOXICITY OF SELECTED ETHNO-MEDICINAL PLANTS FROM MIZORAM, NORTHEAST INDIA

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

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IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN BOTANY OF MIZORAM UNIVERSITY, AIZAWL.

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CERTIFICATE

This is to certify that this study **"Screening for phytochemicals and cytotoxicity of selected ethno-medicinal plants from Mizoram, Northeast India."** submitted by Alex Zohmachhuana (MZU/Ph.D/1012 of 26.05.2017) in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Botany is a record of bonafide work carried out by him under my supervision and guidance.

Place: Aizawl

Date: 19th May, 2022 **(Prof. F. LALNUNMAWIA)**

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DECLARATION BY THE CANDIDATE

MIZORAM UIVERSITY

May, 2022

I, **Alex Zohmachhuana**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do 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 by me.

This is being submitted to the Mizoram University for degree of Doctor of Philosophy in Botany department.

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Alex Zohmachhuana

Preface

Medicinal plants have been extensively studied due to their antioxidant effects and other positive health benefits such as prevention of cardiovascular diseases, atherosclerosis, inflammation, or reducing the risk of cancer. These plants have been used for the treatment of diverse ailments since ancient times, but their anti-cancer properties have not been well studied. Globally, the most common types of cancer encountered include lung, cervical, colorectal, stomach, prostrate and skin cancers. Plants belonging to the family Zingiberaceae are commonly used medicinal plants with known antioxidant activity and are recognized widely in uses of food, medicine and traditional knowledge. While modern medicine is the primary form of healthcare in many countries, there remains a tradition of using local medicinal plants for health promotion and the treatment of diseases. Medicinal plants are generally considered to be safe and contain biologically active constituents that have beneficial physiologic effects, some plants are safe in modest amounts but they may show some adverse effects from their usage or become toxic at higher doses. Medicinal plants are usually consumed only in small quantities, so their dietary contribution is relatively small and insufficient to show medicinal effects. However, if eaten regularly, medicinal plants in the form of some preparations, herbs and spices could provide useful amounts of beneficial bioactives, including both ubiquitous and less common phytochemicals. Because of this reason, researchers stress that people should not self-medicate without consulting qualified practitioners. Whole medicinal plants, parts of these plants, and their preparations are usually complex mixtures of numerous active compounds with health effects that are concentration dependent. Biologically active substances in plants could often cooperate to show synergism, and therefore, the advantage of their usage is that minor constituents can also contribute to the overall quality. The medicinal plants'

research is presently intensively focused also on the identification of naturally occurring anticarcinogens, which were found in certain plants.

The interest in these natural medicines is mainly due to the fact that diseases such as cancer are still difficult to cure. As a result, there is a great scientific effort to delay the process of carcinogenesis and to reduce the morbidity and mortality of cancer. In addition, the usage of potent biologically active components of medicinal plants as chemopreventive agents seems to be very promising. There has been research into various anticancer effects of medicinal plants, through such mechanisms as protecting DNA from free radical-induced structural damage, encouraging the self-destruction of aberrant cells (apoptosis), and inhibiting tumor growth. Because cancer is a multifactorial disease, there are many ways in which plant anticarcinogens are able to exert a protective effect. Phenol compounds belong to constituents that may inhibit carcinogenesis by affecting the molecular events in the initiation, promotion, and progression stages. Flavonoids of the flavone, flavonol, flavanone, and isoflavone classes possess antiproliferative effects in different cancer cell lines including colon, prostate, leukemia, liver, stomach, cervix, pancreas, and breast cancer cell lines. The capability of these bioactive compounds for growth inhibition and induction of apoptosis is well documented. In addition, the bioavailability of the dietary polyphenols is discussed extensively to determine the biological activity. Understanding the bioavailability of polyphenols is also important in extrapolating results from studies in cell lines to animal models and humans. Several commonly used aromatic plants have been identified by the National Cancer Institute as possessing cancer-preventive properties. Since the amounts of medicinal plants consumed are usually small, individual plants do not make a significant contribution to the diet. When used as remedies or drug constituents, the bioactive components of medicinal plants will be more concentrated.

The objective of the present study is to investigate the phytochemical and antioxidant activity of *Costus pictus*, *Curcuma aeruginosa* and *Alpinia galanga* relating to *in vitro* anticancer activity against cells against four human cancer cell lines namely,

A-549 (human lung), HeLa (human cervical), AGS (human gastric), HT-29 (human colon) cancer cell lines and a normal cell: L-132 cells. The different parts of the plants were subjected to sequential extraction and the efficacy of the leaf, stem and rhizome of the selected plants was examined for their phytochemical content, antioxidant and anti-cancer activities.

The qualitative and quantitative phytochemical analysis of the selected plants was done using standard protocols. The identification of the phytochemical composition including the bioactive compounds of these plants, is one of the necessary steps that must be taken to explain their medicinal properties. It is required to identify the active components responsible for the anti-cancer effects. As every plant has its own metabolite fingerprinting, profiling by some platform technology is crucial to have a holistic overview of all metabolites present in the extracts. The present study investigated the phytochemical constituents of *the selected plants* by using two different techniques of HPLC and GC-MS analysis. The information is essential to proceed with the significant use of herbal plant as the source of health-related products such as functional foods or pharmaceuticals to prevent or treat certain diseases. Phytochemicals from plants are reported to be effective against cancer cells because they have many molecular targets. It is worth mentioning that antioxidant activities and antitumor or anticancer properties of plant extracts are always reported concomitantly in several plants (Kathiriya *et al*., 2010), and some studies demonstrated that there is a positive linear relationship between antioxidant activity and anticancer effect of plant extracts (Li *et al*., 2007).

The antioxidant activity of the different extracts was conducted using phosphomolybdenum assay and different *in-vitro* assays: DPPH and $ABTS^{+}$, superoxide and hydroxyl assays. MTT assay was carried out for selecting the extract with the highest cytotoxic effect. The different extracts show cytotoxicity towards the cancer cells with insignificant toxicity against normal cells (L-132) cells, which supports the traditional use of the plants in treating cancer. Further investigation was carried out to

understand the effects. The mechanism of cell death and apoptosis induced by the plants on A549 and HeLa cells were studied using fluorescence staining. The level of DNA damage was examined using the alkaline comet assay and induction of DNA damage after 24 hours with significant increase in tail DNA and tail moment was observed when compared to untreated control. Induction of the apoptotic morphological alterations was studied using Acridine orange (AO)/Ethidium bromide (Et-Br) staining, revealing that the different extracts are capable of inducing apoptotic and necrotic cells for 48 hours with an increased rate of cell death when compared to the L-132 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. Up-regulation in the expression of caspase-8 and caspase-3 activities in both HeLa and A-549 cells after 48 hours of treatment was observed, which might have triggered cell death through apoptosis. The present study investigated the phytochemical constituents of the selected plants by using two different techniques of HPLC and GC-MS analysis. The information is essential to proceed with the significant use of herbal plant as the source of health-related products such as functional foods or pharmaceuticals to prevent or treat certain diseases. The chromatographic techniques revealed the presence of mainly phenol and flavonoid compounds like gallic acid, kaempferol, quercetin, caffeic acid, coumaric acid, catechin, naringin and rutin including vitamins and several terpenoid compounds. These compounds are known for inducing apoptosis in different human cancer cells through caspase-dependent pathways. The present findings contributes to the scientific evidence for the use of traditional herbs for cancer treatment, and pave the way for the development of new therapeutic agents, possibly in the development of a potential lead natural compound for cancer chemotherapy.

The literature survey showed that as far as my knowledge, no work has been done to compare the anti-cancer effect of the leaf, stem and rhizome of *Costus pictus*, *Curcuma aeruginosa and Alpinia galanga*.

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ABBREVIATIONS

CPRH	*Costus pictus* Rhizome Hexane
CPRE	*Costus pictus* Rhizome Ethyl acetate
CPRM	*Costus pictus* Rhizome Methanol
CPSH	*Costus pictus* Rhizome Hexane
CPSE	*Costus pictus* Stem Ethyl acetate
CPSM	*Costus pictus* Stem Methanol
CPLH	*Costus pictus* Leaf Hexane
CPLE	*Costus pictus* Leaf Ethyl acetate
CPLM	*Costus pictus* Leaf Methanol
CURH	*Curcuma aeruginosa* Rhizome Hexane
CURE	*Curcuma aeruginosa* Rhizome Ethyl acetate
CURM	*Curcuma aeruginosa* Rhizome Methanol
CUSH	*Curcuma aeruginosa* Stem Hexane
CUSE	*Curcuma aeruginosa* Stem Ethyl acetate
CUSM	*Curcuma aeruginosa* Stem Methanol
CULH	*Curcuma aeruginosa* Leaf Hexane
CULE	*Curcuma aeruginosa* Leaf Ethyl acetate
CULM	*Curcuma aeruginosa* Leaf Methanol
APRH	*Alpinia galanga* Rhizome Hexane
APRE	*Alpinia galanga* Rhizome Ethyl acetate
APRM	*Alpinia galanga* Rhizome Methanol
APSH	*Alpinia galanga* Stem Hexane
APSE	*Alpinia galanga* Stem Ethyl acetate
APSM	*Alpinia galanga* Stem Methanol
APLH	*Alpinia galanga* Leaf Hexane
APLE	*Alpinia galanga* Leaf Ethyl acetate

APLM	*Alpinia galanga* Leaf Methanol
%	Percentage
±	More or less
^{o}C	Degree Celsius
µg	Microgram
Edn.	Edition
mg	Milligram
i.e	*Id est* (that is)
et al.,	*et alii:* and others
e.g.	Example
IC_{50}	Half-maximal inhibitory concentration
w/v	Weight in volume
HPLC	High Performance Liquid Chromatography
GC-MS	Gas Chromatography- Mass Spectrometry
WHO	World Health Organization
EDTA	Ethylenediamine tetraacetic acid
µl	Microliter
µg/ml	Microgram per milliliter
TCM	Traditional Chinese Medicine
TIM	Traditional Indian Medicine
AM	Alternate medicine
NCEs	New Chemical Entities
IARC	International Agency for Research on Cancer
PBCR	Population Based Cancer Registries
Dox	Doxorubicin

Chapter 1
Introduction

1.1. Medicinal Plants

1.1.1. General

A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Robinson and Zhang, 2011). This description makes it possible to distinguish between medicinal plants whose therapeutic properties and constituents have been established scientifically, and plants that are regarded as medicinal but which have not yet been subjected to a thorough scientific study. A number of plants have been used in traditional medicine for many years. Some do seem to work although there may not be sufficient scientific data to confirm their efficacy. Such plants should qualify as medicinal plants. The term 'crude drugs of natural or biological origin' is used by pharmacists and pharmacologists to describe whole plants or parts of plants which have medicinal properties. There are several factors that influence how people select plants for medicinal purposes: tradition, efficacy, abundance, accessibility, doctrine of signatures, as well as taxonomic affiliation (Phumthum *et al*., 2018; Bennett and Husby, 2008). Interestingly, some plant families comprise higher proportion of medicinally useful species than expected from the null model of a linear relationship between species diversity and number of medicinally useful species. Traditional use of plants for medicines is not random but determined, to a certain degree, by taxonomic affiliation (Moerman, 1996; Bennett and Husby, 2008).

Medicinal plants have many characteristics when used as a treatment:

1. **Synergic medicine**- Each plant has many compounds that may interact simultaneously leading to either complement or damage the functions of each other, or neutralize their possible negative effects.
2. **Support of official medicine**- The ingredients of plants can be used along with chemical products to achieve the desired outcome.
3. **Preventive medicine**-Some components of plants have proved to be effective in preventing or reducing the risk of certain disease (e.g. flu), and this can help in reduce the burden and cost of using chemical remedies (Hassan, 2012).

Medicinal plants and their products are used worldwide for thousands of years due to their health effects (anti-inflammatory, antioxidant, antibacterial, digestive, antispasmodic, carminative, diuretic, hypolipidemic, sedative, enhancing the function of the immune system as well as anticancer, antitumor activity, etc.) and a key role in preventing various diseases such as cardiovascular diseases, gastrointestinal disorders, inflammatory diseases, and cancer initiation (Yuan *et al*., 2016; Sofowora *et al*., 2013).

1.1.2. Ethno-botanical Studies on Medicinal Plants Used in Disease Prevention

Two remaining living traditions, the traditional Chinese medicine (TCM) and Ayurveda, the traditional Indian medicine (TIM) have provided most of the current knowledge related to medicinal plants (Patwardhan *et al*., 2005). In order that a comprehensive compilation of medicinal plants that can be used in disease prevention is obtained, collation of original data from the traditional custodians of such knowledge is essential (Tan *et al*., 2010). This is especially so in the case of African Traditional Medicine (ATM) where information is passed on from generation to generation orally about the plants used. Unlike in Chinese Traditional Medicine (CTM) and the Indian systems of medicine (Ayurveda, Unani and Sithda) where the information is available in books (and now online), a lot of the information on African traditional medicine is yet to be documented. Efforts are, however, being made by WHO-AFRO to augment the

various isolated databases on medicinal plants through the provision of guidelines for documentation of herbal recipes (WHO/AFRO, 2012).

1.1.3. Importance of medicinal plants

The use of plants in order to achieve a medicinal purpose is called alternative medicine (AM). AM has been used almost in all cultures particularly Asian and western culture. Medicinal plants represent an alternative treatment to various diseases, and their use is increasingly prevalent throughout the world. Due to the large number of plant species with medicinal properties, some sciences have emerged such as medicinal herbalism, which has allowed the wide development of traditional medicine in some countries. The traditional uses attributed to each medicinal plant depend on where the plant is consumed and the social groups that use them (Singh, 2015; Mohammed *et al*., 2019).

Medicinal plants have been recommended as an efficacious adjuvant for the treatment and prevention of diseases since ancient times. It is estimated that 70–95% of the population in developing countries continues to use traditional medicines. Knowledge of the biodiversity and traditional medical uses of these herbal medicines has contributed to the development of almost 70% of the conventional drugs currently in use. At least 700 natural products, natural products-derived or natural products-inspired New Chemical Entities (NCEs) were approved between 1981 and 2019, aside from natural product mimics (Newman and Cragg, 2020). Natural products have, therefore, been an invaluable source of drug leads for many years as a result of their chemical diversity (Ogbourne and Parsons, 2014). In addition, these molecules also present diverse industrial uses, including applications as insecticides, in cosmetics, and for pharmaceutical purposes (Newman and Cragg, 2020). Up to date, there are more than hundred thousand of plants globally that are either undiscovered, or their medical activities are not investigated and analyzed yet. It is predicted that plants are going to play an essential role in the medical field, especially in the treatment of critical disease such as cancer, so their medical efficacy should be tested in the present and future

studies (Mohammed *et al*., 2019). Active compounds produced during secondary metabolism are usually responsible for the biological properties of plant species used throughout the globe for various purposes, including treatment of infectious diseases (Singh, 2015)

1.1.4. The strategy of using antioxidant activity of medicinal plants in prevention of diseases

Oxidative stress, caused by reactive oxygen species, plays an important role in many chronic and degenerative diseases, such as atherosclerosis, ischemic heart disease, cancer, diabetes mellitus, neurodegenerative diseases and ageing (Azizova, 2002). The antioxidants prohibit the oxidative damage to various macromolecules like nucleic acids, proteins, and lipids and scavenge free radicals generated from biochemical reactions (Kampa and Castanas, 2008). A reaction of these free radicals with macromolecules has been reported to stimulate apoptosis that may cause various physiological, cardiovascular, and neurological disorders (Granda and Pascual, 2018). Various types of phytochemicals such as phenolic acids, ascorbic acid, tocopherols, and bioflavonoids having antioxidant characteristics have been used to treat many diseases (Floyd and Hensley, 2002). Numerous epidemiological studies have shown an inverse relationship between the intake of natural antioxidants from plant products and the incidence of some diseases because dietary plant antioxidants are capable of removing free radicals. The antioxidant activity of medicinal plants depends on each plant (variety, environmental conditions, harvesting methods, postharvest treatment, and processing), and composition and concentration of present antioxidants. For appropriate determination of antioxidant capacity, the extraction technique, its conditions, solvent used, and particular assay methodology are important. Extracts from natural plant materials are mixtures of many components. Plant materials, such as medicinal plants (herbs, spices), could be promising sources of effective antioxidants. The body's non-enzymatic antioxidant defense system includes some antioxidants, such as vitamin C, vitamin E, vitamin K and glutathione. Some synthetic antioxidants, widely used in food industry to protect food

from oxidation and spoiling, are harmful because of their potential toxicity and carcinogenicity (Botterweck *et al*., 2000). However, natural antioxidants in fruits and vegetables are inversely related with the risk of the chronic diseases mentioned above (Leifert and Abeywardena, 2008).

Natural antioxidants, therefore, provide alternative strategy to prevention as well as treatment of these diseases. Phenolic compounds because of their oxidative activity are potential agents for preventing and treating many oxidative stress-related diseases. The antioxidant activity of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelator (Kratchanova *et al*., 2010). Some medicinal plants possess more potent antioxidant activity than common dietary plants (Cai *et al*., 2004). Therefore, their extract, if not toxic, can serve as food additive and also be used for disease prevention (Liu, 2003; Liu *et al*., 2008).

1.2. Secondary metabolites extracted from plants used as anticancer agents

Overtime researchers detected that plants are found to be enriched with natural compounds called secondary metabolites, these metabolites characterized by several points make them effective antitumor agents. These compounds can be classified into three main groups which are terpenoids (polymeric isoprene derivatives and biosynthesized from acetate via the mevalonic acid pathway), phenolics (biosynthesized from shikimate pathways, containing one or more hydroxylated aromatic rings), and the extremely diverse alkaloids (non-protein nitrogen-containing compounds), biosynthesized from amino acids such as tyrosine, with a long history in medication. Yearly several new metabolites are extracted from plants, but only a limited number of them have been used to synthesize new potent anticancer agents. (Seca *et al*., 2018)
Discovery of plant-derived substances has evolved during the last 200 years due to the variety of experience and expertise needed in order to identify such compound. Initially, a plant is identified by a botanist or ethnobotanist, ethnopharmacologist, or plant ecologist. Next, plant extracts followed by biological screening assays are performed by

a phytochemist to identify the potential therapeutic activity followed by isolation of the active compound. Finally, molecular biology studies are required to reveal the mode of action and relevant molecular targets (Balunas and Kinghorn, 2005). The expertise to select the right plants, methods of drug concoction and their specific use has been first transferred orally from one generation to the next until set down (Samuelsson, 2004). This empirical knowledge comes from the plant defense system, which generates numerous compounds with diverse molecular structures, far superior to those derived from synthetic products, so there is great interest in the elucidation of new active principles (Pradeepa *et al*., 2014).

Recent reports released by the World Health Organization (WHO) showed that although many advanced countries have considered traditional herbal treatment as an official treatment for cancer, only 5–15% of these herbs have been investigated to detect their bioactive compounds, i.e., anticancer compounds (Ahmad *et al*., 2016; Shabani, 2016). Despite a lot of development, the current therapeutic options still face a lot of challenges. The most important challenges include the absence of precise therapy and low drug buildup inside the cancer cells (Han *et al*., 2017). Since cancer develops as a result of deregulation of single and or multiple cellular mechanism(s) involved in, such as, cell division, and apoptosis that are required by normal growth and proliferation of healthy cells. Identifying the difference(s) in regulatory mechanism at play in cancer cell responsible for transformation, and specifically targeting that mechanism is the major task for drug development and candidate screening (Wiman and Zhivotovsky, 2017; Matsuoka and Yashiro, 2018).

1.3. Cancer

1.3.1. General

Cancer is one of the principal causes of mortality and morbidity around the globe and the number of cases are constantly increasing estimated to be 21 million by 2030 (Siegel *et al*., 2016). Cancer does not represent only one disease but it is a group involving about 100 diseases. It is characterized by two things: Firstly there is no control for the growth of cancer cells, and secondly it is the ability of the cancer cells to metastasize and migrate from the original site to different parts of the body. There are two types of tumors which are malignant and benign. Cancer can attack any person, and its occurrence increases as the age of the individual increases too (Dewitt, 2002; Markman, 2002). Cancer is a major public health burden in both developed and developing countries. These cells are born due to imbalance in the body and by correcting this imbalance, the cancer may be treated. There are many problems associated with cancer diseases either solid or hematological cancer such as nausea, vomiting, diarrhea, constipation, hypercalcemia, pain, loss of appetite, anemia, fatigue, cachexia, leucopenia, neutropenia, and thrombocytopenia. Hence due to these reasons, cancer is considered as one of the major diseases that will affect the quality of life (Sitamvaram 2005; Stephens, 2005). Carcinogenesis involves many complex biological processes, including activation of oncogenes and epigenetic mutations, inactivation of tumour suppressor genes, obstruction of DNA repair mechanisms, and destruction of apoptosis mechanisms, which forces cells to undergo uncontrolled cell division (Melo *et al*., 2018). The treatment process of cancer still has a destructive effect on normal cells and tissues of the body (Cragg and Pezzuto, 2016). In addition, there are still no effective drugs to fully eradicate cancer and the long-term use of certain drugs can lead to the development of drug resistance in tumour cells (Chen and Song, 2019).

1.3.2. Prevalence of cancer globally

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020 (Ferlay *et al*., 2020). Over the years, several population-based epidemiological and experimental animal model systems and clinical reports emphasized a major rise in the prevalence of the disease leading up to 70% of cancer deaths globally (Siegel *et al*., 2020). Despite the tremendous advances made to fight cancer, recent studies revealed that the mortality rate of cancer worldwide is increasing every year (Zhang *et al*., 2020). It is also estimated that every fourth citizen is cancer-stricken sometimes during his/her life and approximately 400 new incidents of cancer emerge for every 100,000 individuals diagnosed annually in the developed countries (Ferlay *et al*., 2019). As per the reports of World Health Organization (WHO), 8.97 million mortalities per year are caused due to cancer, thus making it the second major cause of mortality after ischemic heart disease (Mattiuzzi and Lippi, 2019). Cancer, the second deadliest disease worldwide, had imposed a severe burden on public health with an estimated 192 thousand cancer incidences and 61 thousand cancer mortalities in 2021 (Siegel *et al*., 2022). The latest statistics of the International Agency for Research on Cancer (IARC) in 2020 estimate that the number of new cancer cases in the world is 19.29 million, among which the number of new lung cancer cases is 2.20 million, accounting for about 11.4 % of new cancer cases. The most frequent ones were breast (2.26 million) and lung (2.20 million). Colorectal cancer was ranked as the third most prevalent cancer with a total of 1.93 million cases followed by prostate cancer (1.41 million cases), stomach cancer (1.08 million cases), liver (0.90 million cases), and cervical cancer (0.60 million cases) (Gonzalez-Valdivieso *et al*., 2021).

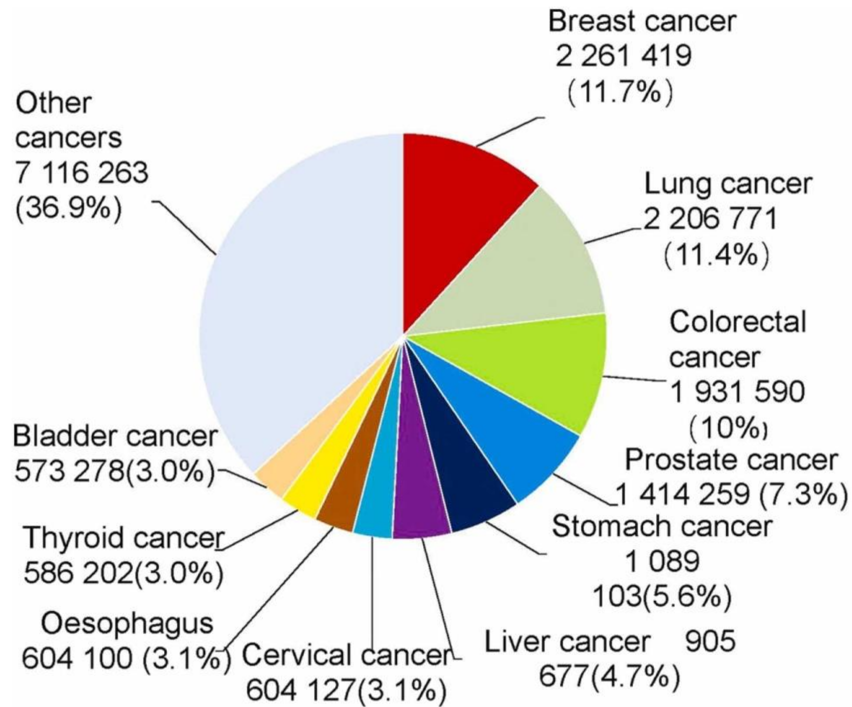

Fig.1. International Agency for Research on Cancer (IARC) issued global cancer 2020 statistics (Globocan, 2020).

1.3.3. Prevalence of cancer in India

As many as 139 thousand people in India had cancer in 2020 (Mathur *et al*., 2020). India exhibits heterogeneity in cancer. According to Population Based Cancer Registries, Lung (9 PBCRs), mouth (9 PBCRs), esophagus (5 PBCRs), stomach (4 PBCRs), and nasopharynx (1 PBCR) cancers were the most common cancers in men. Lung cancer was the leading site in metropolitan cities and the southern region, whereas mouth cancer was the leading site in the West and Central regions. Lung cancer and oral/mouth cancer were the most common cancers among males in the Indian

subcontinent (Bray *et al*., 2018). Cancer of the breast (19 PBCRs) and cervix uteri (7 PBCRs) were the most common cancers in women (Chaturvedi *et al*., 2014). Presently, breast cancer and cervix uteri are the leading sites of cancer among women in India, posing an important public health problem that needs important input from various health and other agencies to tackle (Takiar, 2018).

Northeast India is the cancer capital of the country as the highest cancer incidence rate was observed in the North- East region (6 PBCRs for males and 4 PBCRs for females) than other areas in the country and the leading sites of cancer in the region were nasopharynx, hypopharynx, esophagus, stomach, liver, gallbladder, larynx, lung, breast, and cervix uteri. A substantial proportion of patients with cancer from the North-East region are traveling outside the North-East for treatment and cancer care (Ngaihte *et al*., 2019). Local cultural factors and lifestyle choices may have contributed to the heterogeneity in cancer incidence pattern and differences in India.

Mizoram's Aizawl district has reported the highest age-adjusted cancer incidence rate among males in the country for five years from 2012. According to the National Cancer Registry Programme (NCRP) 2012-2016 report, Aizawl district has recorded the highest age-adjusted incidence rates among males with 269.4 cancer cases per one lakh population. The report, released by the Indian Council of Medical Research (IMCR) and National Centre for Disease Informatics and Research (NCDIR), stated that in Asia, Aizawl district has the highest incidence rate of lung cancer in females and also the highest incident rate of stomach cancer in males when compared with Non-Asian countries, the study also showed. Aizawl district also topped in the incidence of colon cancer in the country among males and females.

1.3.4. Current approach in cancer

Currently, the therapeutic approaches of cancers are mainly composed of surgical resection, chemotherapy, radiotherapy, target therapy and immunotherapy (Siegel *et al*., 2022). Today, solid tumors are surgically removed and patients receive adjuvant radiation treatment and chemotherapy that cause severe sides effects and dramatically reduce quality of life. In addition, the toxicity of some treatments restricts their use and effectiveness. Certain types of cancer such as breast cancer, can be treated using biological drugs (Herceptin), however the cost of these drugs is very high and their effectiveness is limited in most cases to certain kinds of tumors. In many cases, the tumor develops resistance to a particular drug and the patient is transferred to a different drug. In addition, many patients are treated with a combination of several drugs (DeSantis *et al*., 2014). A large number of efforts have been made to minimize the harmful side effects of drugs during the process of cancer therapy like preventing the side effects on the nearby cells and tissues, increasing drug accumulation and efficacy in the lesion, developing novel drug delivery and targeting systems (Vinogradov and Wei, 2012).

Comparing chemotherapy with other types of treatments, it still remains potentially high risk with many side effects which are difficult to manage. The chemotherapy used required the involvement of various clinical professionals during its various stages of administration, and enormous patient health care is needed to overcome its side effects. Treatment methods depend upon the cancer type, stage and location (Rizzo and Cloos, 2002). One of the most critical problems associated with cancer treatment is chemotherapy resistance, that’s why researchers trying their best to prevent or reduce incidence of resistance by detecting new anticancer agents as an alternate (Ahmad *et al*., 2016).

Chemotherapeutic agents involve cytostatic and cytotoxic drugs which have shown promising results alone or in combination with other cancer therapies. These chemotherapeutic agents involve topoisomerase inhibitors [e.g. irinotecan (side effects include: neutropenia, sensory neuropathy, and diarrhoea) and doxorubicin (side effects include cardiotoxicity), alkylating agents e.g. oxaliplatin, melphalan, carboplatin, cisplatin and cyclophosphamide (side effects include: nephrotoxicity, gastrointestinal toxicity, cardiovascular toxicity, pulmonary and hematologictoxicity), microtubules acting agent e.g. vincristine, vinblastine, docetaxel and paclitaxel etc] (Weaver, 2014; Caruso *et al*., 2000). The above mentioned drugs are highly effective against a wide range of cancers, but these drugs are also having some limitations (side effects, expensive, very complex, not eco-friendly and toxic). There are cells in our body which multiply rapidly under normal physiological conditions like hair follicle cells, bone marrow cells and digestive tract cells etc., These present anticancer drugs also target these rapidly dividing normal cells which is a big challenge thus, harmful side effects arise. Due to these side effects there is decreased blood production, GIT inflammation, hair loss, immunosuppression, heart diseases and nervous disorders may arise. Another limitation is that these cancer cells resist to these drugs as they go through mutations. e.g., Drug resistant genes (ABCA4 and ABCA12) were over-expressed in human MCF-7 breast cancer cells respectively when docetaxel was applied. However, when phytochemical curcumin was applied in association with docetaxel down regulation of drug resistance genes was observed (Aung *et al*., 2017). Thus, treating cancer cells by employing mono-target chemical agent is not an effective method. Therefore, based on extensive research findings, phytochemicals and their derived analogues possess most promising option for the better and less toxic cancer treatment (Singh *et al*., 2016).

1.3.5. Current cancer therapy via phytochemicals: a novel approach

Various types of chemotherapies fail due to adverse reactions, drug resistance, and target specificity of some types of drugs. There is now emerging interest in developing drugs that overcome the problems stated above by using natural compounds, which may affect multiple targets with reduced side effects and which are effective against several cancer types. Natural compounds from plants offer a great opportunity for discovery of novel therapeutic candidates for the treatment of cancer (Newman *et al*., 2003).

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 (Yan-Wei *et al*., 2009). Medicinal plants serve as nature's gift to humans to help them pursue better health. Plants and their bioactive compounds are in medicinal practices since ancient times. Several medicinal plant species and their phytochemicals inhibit the progression and development of cancer (Aung *et al*., 2017). Researchers mentioned that herbal-based medicines are found to be one of the best choices for treating and/or preventing incidence of cancer. This is mainly because of the varieties of active substances that plants contained which work against many types of cancers in several mechanisms. These compounds can be extracted and can be used alone or in combination with other anticancer treatments. In comparison with synthetic drugs, these natural compounds from plant sources (phytoextracts) commonly employed in traditional medicine are found to be naturally available, cheaper, longer usage duration, greater potential in targeting diseases and easy to administered orally and they are found to be rich of various biologically active chemotypes (Seca and Pinto, 2018; Lichota and Gwozdinski, 2018). The therapeutic value of a medicinal plant is attributed to the presence of specific phytochemical compounds such as flavonoids, phenylpropanoids and terpenoids present in them, which exert critical biological functions in plants, such as protection against the

action of herbivores and pathogens, and contribute to their color, taste, and flavor (Simoh, 2014).

Phytochemicals and their derived analogues are present in different parts of the plant, *e.g.,* flower, flower stigmas, pericarp, sprouts, fruits, seeds, roots, rhizomes, stem, leaf, embryo, bark and perform several pharmacological functions. Several plant products such as alkaloids, flavonoids, lignans, saponins, terpenes, taxanes, vitamins, minerals, glycosides, gums, oils, biomolecules and other primary and secondary metabolites play significant roles in either inhibiting cancer cell activating proteins, enzymes and signaling pathways [Cdc2, CDK2 and CDK4 kinases, topoisomerase enzyme, cycloxigenase and COX-2 (Cycloxigenase), Bcl-2, cytokines, PI3K, Akt, MAPK/ERK, MMP, TNK, mechanistic target of rapamycin (mTOR) or by activating DNA repair mechanism (p21, p27, p51, p53 genes and their protein products), Bax, Bid, Bak proteins, stimulating the formation of protective enzymes (Caspase-3, 7, 8, 9, 10, 12)], inducing antioxidant action, thus showing strong anticancer effects in terms of their efficacy on the above mentioned proteins, enzymes and signaling pathways (Thakore *et al*., 2012; Tariq *et al*., 2017).

Bioactive natural products are molecules perfected by evolution, which, based on their physico-chemical properties, are much more likely to become potential drug candidates than synthetic compounds produced by combinatorial chemical methods (Larsson *et al*., 2005). A majority of those who cannot afford expensive drugs depend on herbal medicines, an alternative to Western medicine that has been used for centuries, based on the ability of plants or their extracts to treat diseases and promote health and well-being (Ishola *et al*., 2016). It is believed that the crude extracts from medicinal plants are more biologically active than the isolated compounds due to their synergistic effects (Jana and Shekhawat, 2010). Due to its extensive biological and pharmacological properties, bio active compounds have been widely studied. They are potent antioxidants that can protect human beings from free radicals and reactive oxygen species. Free radicals have harmful effects on human beings and are related to toxicity and causing

diseases like diabetes, chronic renal failure, cancer, mellitus, atherosclerosis, immune dysfunction and aging (Das *et al*., 2014). Certain medicinal plants have been shown to contain bioactive compounds with medicinal properties, such as antioxidant, anti-inflammatory, antibacterial, antimutagenic, antidiabetic and anticarcinogenic activities. However, only a small percentage has been sufficiently evaluated to date for their potential as therapeutic agents (Close *et al*., 2016).

1.4. Cytotoxocity

Cytotoxicity is defined as the toxicity caused due to the action of chemotherapeutic agents on living cells. The method for determination of cytotoxicity and cell viability involves dyes, such as trypan blue, alamar Blue, neutral red, and coomassie Blue. The method differentiates the various cells in terms of colors. The cells are differentiated based on the ratio of the color uptake of both living and dead cells. The other methods for assaying cytotoxicity include tritium-labeled thymidine uptake assay, the MTT method, WST assay, and dehydrogenase-based assay (Li *et al*., 2012). Cytotoxicity is one of the most important methods for biological evaluation as the ability to accurately measure cytotoxicity can prove to be a very valuable tool in identifying compounds that might pose certain health risks in humans The cytotoxicity test is one of the most important indicators of the biological evaluation system in vitro to observe the cell growth, reproduction and morphological effects by chemicals, and with the progress of modern cell biology; experimental methods to evaluate cytotoxicity are also continuously being developed and improved (Li *et al*., 2015). Cytotoxicity studies are a useful initial step in determining the potential toxicity of a test substance, including plant extracts or biologically active compounds isolated from plants. Minimal to no toxicity is essential for the successful development of a pharmaceutical or cosmetic preparation and in this regard, cellular toxicity studies play a crucial role. Various bioassays and a number of different cell lines have been used to assess cytotoxicity of chemicals. The ability to measure early indicators of toxicity is an essential part of drug discovery. *In vitro* cytotoxicity assays involving tissue specific cell cultures are considered as valuable

predictors of human drug toxicity. Measuring cell cytotoxicity also proves to be quite indispensable in the process of developing therapeutic anti-cancer drugs. By determining the cytotoxicity levels of cancer cells, anti-cancer medications can hinder the proliferation of target.

Additionally, understanding the mechanisms involved in cytotoxicity can likewise give researchers a more in-depth knowledge on the biological processes (both normal and abnormal) governing cell growth, cell proliferation, and death. The mitochondrial dehydrogenase performance measurement, also known as the 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-tetrazolium bromide (methyl thiazolyl tetrazolium; MTT) assay, is a rapid assessment of cell proliferation and cytotoxicity colorimetric assay to measure cell metabolism or function used The main principle is as follows: Mitochondrial dehydrogenase in the cytochrome *b* and *c* sites of the living cells can cleave the tetrazole ring, and the yellow, water-soluble MTT is reduced to produce a purple crystalline formazan. This substance is soluble in dimethyl sulfoxide and other organic solvents, but is insoluble in water. The amount of crystals formed has a positive correlation to the number of cells and their activity, and measuring the absorbance (optical density) colorimetric value reflects the number of surviving cells and metabolic activity (Riss *et al*., 2013; Marshal *et al*., 1995; Berridge *et al*., 1996).

1.5. Treatment of Cancer through Targeting Apoptosis

Apoptosis is a self-destructive programmed sequence of signal transduction events that destroys cells that become a threat, or are no longer necessary to the organism (Alberts *et al*., 2002). When apoptosis is defective in one of the main apoptotic pathways, it increases the likelihood of the cell becoming cancerous. Various well-established treatments have been designed to destroy cancer cells through apoptosis. It is highly desirable to have compounds that can cause cancer cell death via apoptosis. Apoptosis eliminates malignant or cancer cells without damaging normal cells and surrounding tissues (Ashkenazi, 2008). Apoptosis is triggered through two well-characterized pathways in mammalian cells: extrinsic and intrinsic apoptosis. Extrinsic

apoptosis occurs when cell death is triggered by binding of extracellular stress ligands to transmembrane receptors such as death receptors CD95 (APO-1/Fas) and tumor necrosis factor (receptor1) (Wajant *et al*., 2002). The extrinsic apoptosis pathway is initiated through the binding of ligand (Fas-associated death domain) to death receptors that contain an intracellular death domain (death-inducing signaling complexes (Millimouno *et al*., 2014; Fulda, 2010). In contrast, intrinsic apoptosis occurs in the mitochondria through heterogeneous signaling cascades dependent or independent of caspases (Galluzzi *et al*., 2012) mediated by molecules released from the mitochondria (e.g., Bcl-2 protein family). The intrinsic pathway is activated by physical or chemical stimulations, such as hypoxia, growth factor deprivation, cell detachment, or stress signals**.** Resistance to treatments that target apoptotic cell death is indicative of treatment failure. Anti-apoptotic mutations during cancer progression reduce chemotherapy-induced apoptosis in spontaneous murine tumors (Schmitt *et al*., 1999) and produce multi-drug resistance (Dive and Hickman, 1991).

Apoptosis is characterized by chromatin condensation and DNA fragmentation, and it is mediated by caspases. Caspases are cysteine-dependent aspartate-specific proteases and are regulated at a post-translational level which ensures that they can be rapidly activated. Both pathways cause the activation of the initiator caspases, which then activate effector caspases. They are first synthesized or expressed in cells as inactive proenzyme which consists of a prodomain, a small subunit, and a large subunit forms that require oligomerization and/or cleavage for activation. Many apoptotic signals are mediated to cell death machinery through p53 with other proteins such as TNF, Fas, and TRAIL receptors that are highly specific physiologic mediators of the extrinsic signaling pathway of apoptosis. Mitochondria are involved in a variety of key events, such as release of caspases activators, changes in electron transport, loss of mitochondrial membrane potential (MMP), and participation of both pro-and antiapoptotic Bcl-2 family proteins (Zhou *et al*., 2016). This breakthrough finding may have important implication for targeted cancer therapy and modern application of natural compounds.

Thus, understanding how to induce cell cytotoxicity via chemosensitization is as important as how to trigger apoptosis in cancer cells with chemotherapies. It has been reported that natural compounds with anti-tumor activities are able to not only kill cancer cells but also restore drug sensitivity (Tian and Pan, 1997; Choi *et al*., 1998). This suggests that natural compounds can have therapeutic effects in cancer chemo-radiotherapy.

1.6. Future Perspectives

Plants provide a broad spectrum of sources for modern anticancer drugs. Various preclinical findings and results of several *in vitro* and *in vivo* studies convincingly argue for potent role of natural compounds in the prevention and treatment of many types of cancer. Many reports on mechanism of actions of the promising compounds target multiple signaling pathways, which vary widely depending on cancer origin (Aung *et al*., 2017). According to the literature, the major molecular targets that have been characterized are the key challenge for researchers and scientists to use this information for effective cancer prevention in populations with different cancer risks. Moreover, low potency and poor bioavailability of natural compounds pose further challenges to scientists and researchers. The future, full with convergence of chemoprevention and chemotherapy drug development will open new avenues for natural compounds in reducing the public health impact of major cancers. However, additional preclinical studies and clinical trials are certainly yet required to elucidate the full spectrum of cytotoxic activities of the selected natural compounds either alone or in synergistic combination with other small molecules to further validate the usefulness of these agents as potent anticancer agents.

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 selected medicinal plants in a comprehensive manner.

1.7. Objectives

The present study was designed to achieve the following objectives:

1. To carry out the qualitative and quantitative study of various phytochemicals present in *Curcuma aeruginosa*, *Costus pictus*, and *Alpinia galanga*, and identification of bioactive compounds using HPLC and GC/MS analysis.
2. To determine the antioxidant activity of the extracts using different assays: DPPH, ABTS+, Superoxide, Hydroxyl and Phosphomolybdenum.
3. To examine the cytotoxic potential of the extracts against A-549, Hela, AGS, HT-29 and L-132 cell line using MTT Assay.
4. To assess the level of DNA damage induced by the extracts in A-549 and Hela cells using Comet assay.
5. To evaluate the apoptotic morphological alterations and measurement of Caspase-8 and Caspase-3 enzyme activation induced by the extracts in A-549, HeLa and L-132 cells.

Chapter 2
Review of Literature

2.1. *Curcuma aeruginosa* Roxb.

Curcuma aeruginosa Roxb. (Zingiberaceae) is a rich ethno medicinal plant with wide range of medicinal properties. *C. aeruginosa* is a rhizomatous perennial herbaceous plant, fresh rhizomes 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 (Srivastava *et al*., 2006). In India, it grows in parts of the South-east and central regions. *C. aeruginosa* is also an ethno-medicinal plant in Indonesia, Malaysia, Thailand, Northern Australia, and Papua New Guinea (Sulfianti *et al*., 2019). In Indonesia, *C. aeruginosa* is known as Temu Ireng (Choudhury *et al*., 2013; George and Britto, 2015), and in English, it is known as pink and blue ginger (Sulfianti *et al*., 2019), *temu hitam* in Malaysia, and *waan-maha-mek* in Thailand. 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 (Khan *et al* 2005; Behar *et al*., 2014; Kumar *et al*., 1998).

C. aeruginosa has been identified to have various pharmacological activities like antimicrobial (Akarchariya *et al*., 2017), antioxidant (Nurcholis *et al*., 2012), anti-inflammatory, analgesic effect (Reanmongkol *et al*., 2006), anti HIV-1 (Otake *et al*., 1995), anti-cancer (Jantan *et al*., 2005) and uterine relaxant effect (Thaina *et al*., 2009). The rhizome contains several essential oils (Dung *et al*., 1995; Jarikasem *et al*., 2005) and reported to possess pharmacological actions in treating various illnesses such as tumors, asthma and bronchitis (Choudhury *et al*., 2013). According to the study conducted by Nurcholis *et al*., (2015), the phytochemical screening of the ethanol extract of *C.aeruginosa* rhizome revealed the presence of tannins and triterpenoids. Thomas and

Jose, (2014) and Anu *et al*. (2020) reported that the methanol extracts of *C.aeruginosa* rhizome demonstrated the presence of alkaloids, phenol, flavonoids, terpenoids, tannins, glycosides and saponin. Pintatum *et al*. (2020) investigated the antioxidant activity of the rhizome of *C.aeruginosa* and the study revealed good scavenging effects of the ethyl acetate extracts on DPPH and ABTS radical. A study done by Atun *et al*. (2016) showed that the n-hexane and chloroform fraction of *C. aeroginosa* exhibited cytotoxic activity against MCF-7 and Ca-ski cells and the extract and fraction of *C. aeroginosa* are not toxic (LC50> 500 µg/mL) against Vero cell lines.

2.1.1 Ethno medicinal uses

The plant is gathered from the wild for use in traditional medicine and as a food. It is often grown in Malaysia as a medicinal plant and is also sometimes cultivated as an ornamental. In India, it is used by the tribals to cure several ailments (Rajkumari and Sanatombi, 2017). The rhizome of *C. aeruginosa* was traditionally used to treat gastrointestinal problems such as diarrhea, fungal infections (Suphrom *et al*., 2013), amebic dysentery, stomach ache, ulcer, indigestion, sprains, bruises, and cosmetic (Pandey and Chowdhury, 2003). Traditionally, its rhizomes have been used medically to treat colic, obesity and rheumatism, asthma and coughs, scurvy and mental derangement (Perry, 1980).

2.2. *Costus pictus* D.Don

Earlier it is mentioned that all the species of Costus were placed under the family Zingiberaceae. Later with the inclusion of many new floras, Costaceae were further classified as Costoideae, a subfamily within Zingiberaceae. Followed with the addition of genera like Dimerocostus, Monocostus and Tapeinochilus into the genus Costus, a new family of Costaceae was created from the family of Zingiberaceae which described the reasons for considering Costaceae as a separate family. The uniqueness in the leaf and root anatomy of the Costus species supported its leap from a genus to family. Costaceae was well distinguished from the other families within Zingiberales due to its

distinctive monistichous spiral phyllotaxy. *Costus pictus* is a rhizomatous perennial herb, commonly known as 'Spiral flag' is a newly introduced plant in India from South and Central America. It is distributed throughout the tropical zones of the world. In India, it had been seen in sub-Himalayan range, some regions of central India and across the Western Ghats (Selvakumarasamy *et al*., 2021).

This is a recent introduction to India from America as an herbal cure for diabetes and hence commonly called as 'insulin plant' (Jose and Reddy, 2010). It is widely grown in gardens as ornamental plant in South India and also run wild in many places (Benny, 2004). This is grown in fertile and moist soils preferably in shade. Highly humid tropical climate and temperatures around 13 °C favours its growth.

Devi and Urooj (2010) reported that *C. pictus* revealed a broad spectrum of phytochemical compounds such as alkaloids, glycosides, tannins, phenols, steroids, terpenoids and flavonoids. It was revealed in another study that the methanol extract was found to contain the highest number of phytochemicals such as carbohydrates, triterpenoids, proteins, alkaloids, tannins, saponins, and flavonoids (Jothivel *et al*., 2007). According to Shankarappa *et al*. (2011), the sequential screening for phytochemicals of *C. pictus* leaves revealed that it is rich in protein, iron, and antioxidant components such as ascorbic acid, α-tocopherol, β-carotene, terpinoids, steroids, and flavonoids.

Jayasri *et al*. (2009) reported that the leaves and rhizomes of *C. pictus* showed good antioxidant activity of about 89.5% and 90.0% when compared with standard BHT (Butylated Hydroxy Toulene) (85%) at a concentration of 400 µg/ml. Results obtained revealed that methanol extracts of both leaves and rhizomes of *C. pictus* possess higher antioxidant activity when compared with other extracts. Majumdar and Parihar, (2012) reported the antioxidant activity of the leaves of *C. pictus*. Methanol extracts showed highest antioxidant activity. He also reported antioxidant activity in stems and flowers as well. Sathuvan *et al*. (2012) also reported that among the extracts tested, chloroform extract of *C. pictus* stem possessed high antioxidant activity.

According to the study conducted by Nadumane *et al*. (2011), the ethanol extract of leaves of *C. pictus* was found to have anti-proliferative and anti-cancer potential in *in-vitro* mammalian fibrosarcoma (HT-1080) cells. The ethanol extract was found to be anti-proliferative and cytotoxic even at lower concentrations on fibrosarcoma cells whereas no cytotoxic effects were seen on normal lymphocytes. All the extracts of stem had potent anti-cancer properties against HT 29 and A549 cells (Sathuvan *et al*., 2012). Prejeena *et al*. (2017), evaluated the anticancer effects of the *C. pictus* leaves against breast cancer cell lines (MCF-7). Ethanolic, aqueous, and petroleum ether leaf extracts reduced the growth rate, thereby decreasing the survival of MCF-7 cells. Due to the presence of significant biological attributes, *C. pictus* emerged as a vital medicinal plant of substantial therapeutic importance.

2.2.1. Ethno medicinal uses

It is used as a traditional medicine for it's diuretic, carminative and antiseptic properties. The leaves served as a good source for the treatment of diabetes. The rhizomes are used for a wide range of ailment. In Southeast Asia, it was used to control diarrhoea, headache and vomiting. The Japanese utilized it for the treatment of syphilis disease. In India, it is used to control pneumonia and rheumatism (Jose and Reddy, 2010). It is used in India to control diabetes, and it is known that diabetic people eat one leaf daily to keep their blood glucose low (Devi and Urooj, 2010). Leaves of *C. pictus* were one among the plants known to be effectively used for treating diabetes by the tribal people of Kolli hills of Namakkal district, Tamilnadu (Elavarasi and Saravanan, 2012). In Mexican folk medicine, the aerial part of *C. pictus* D. Don is used as an infusion in the treatment of renal disorders (Camargo *et al*., 2006).

2.3. *Alpinia galanga* (L.) Willd

This plant is commonly known as Greater galangal in English and Kulanjan in Hindi, Ai-chal in Mizo. The related species are *A. officinarum* and *A. calcarata*, which are known as lesser galangal. All the three species have more or less similar properties and uses and hence in trade practically no distinction is made among them It grows in open, sunny places, forests and brushwood. It is commonly cultivated in the mid and low country in Sri Lanka (Arambewela *et al*., 2006). *A. galanga* is cultivated in all South-East Asian countries, India, Bangladesh, China and Surinam. In India, it shows exuberant growth along the eastern Himalayas and in southwest India and is cultivated throughout the Western Ghats (Ravindran and Balachandran, 2006). It is a rhizomatous, perennial herb, and commonly found in Indonesia, India, China, and Arabic gulf areas, Malaysia, Egypt and Sri Lanka. Galangal is a native of Indonesia though the exact origin is not known.

According to the phytochemical investigations conducted by Ajay and Vijaykumar (2015), *A. galanga* is rich in a variety of phenolic compounds and essential oils and presented many biological activities, including anti-inflammatory, anti-tumor, anti-viral, anti-microbial, anti-oxidant, anti-allergic, and gastro-protective activities. Extracts of *A. galanga* showed significant anti-oxidative behavior in many in vitro assays. Zaeoung *et al*. (2005) reported significant free radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals in methanol and water extracts and volatile oils. The ethanolic galangal extract containing the highest concentrations of total phenolic compounds showed the highest DPPH free radical scavenging ability, as well as the highest oxygen radical absorbance capacity compared with the water extract and the essential oil. According to a study conducted by Chan *et al*. (2008), *A. galanga* leaf extract exhibited outstanding ferrous ion-chelating value which was more than 20 times higher than that of rhizomes.

In another study, the antioxidant activity of methanol extract of *A. galanga* was determined by using three complementary in vitro assays: Inhibition of DPPH and reducing power assay. From these results, it could be inferred that *A. galanga* possesses innate antioxidant properties (Das and Santhy, 2015). A comparing study found that *A. galanga* showed better free radical scavenging activity against DPPH and ABTS than *A. calcarata*, a substitute of *A. galanga* in Indian materia medica, due to high contents of phenolic compounds, flavonoids, and polyphenolic bioflavonoids (Nampoothiri *et al*., 2015). Methanol extracts of *Alpinia galanga* has been evaluated for total phenolic content (TPC) and antioxidant activities using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), reducing power (RP), ferrous ion chelating as well as β-carotene bleaching assays. *A.galanga* leaves and flowers showed highest chelating and β-carotene bleaching abilities. So the leaves of the plant may serve as potential dietary source of natural antioxidant (Wong *et al*., 2009). Samarghandian *et al*. (2014) investigated the potential of *A. galanga* rhizomes to induce cytotoxic and apoptotic effects in the cultured human breast carcinoma cell line, (MCF-7) in comparison with the non-malignant (MRC-5) cells cultured in DMEM medium, the percentage of apoptotic cells was determined. It was found that *A. galanga* induced apoptosis in MCF-7 cells, as determined by flow cytometry.

Another study showed that the crude aqueous extract of galangal rhizomes displayed a wide range of cytotoxicity in lung adenocarcinoma cell (A549), normal fibroblast cell (CRL2522), normal epithelial cells derived from breast (MCF-12A), mammary carcinoma cells [CRL2321 and CRL2335 (p53-inactive)], and p53-mutated fibroblast line (2800T) (Muangnoi *et al*., 2007). According to study done by Suja and Chinnaswamy (2008), the ethanol extract of *A. galanga* was shown to inhibit the cell growth of prostate cancer cell lines (PC-3 cell). Hadjzadeh *et al*. (2014) also reported that the aqueous extract of *A. galanga* showed anti-proliferative effect on human gastric tumor (AGS) and L929 cell lines.

2.3.1. Ethno medicinal uses

Alpinia galanga (L.) Willd is effectively used as a therapeutic treatment for various diseases (Rao *et al*., 2010). It can be used to treat stomach pain, back pain, rheumatism, asthma, diabetes, heart disease, disorders of the liver, kidney disease, and to increase the appetite (Rajpal and Kohli, 2009). Galangal rhizome can also be used as a substitute for antibiotics, disinfectants, and food seasonings (Ram and Rastogi, 2006). The rhizome is generally used as a spice. It is also a good source of essential oil. The flowers and young shoots are also used as a vegetable or as a spice (Arambewela and Wijesinghe, 2006). *A. galanga* is a well-known traditional Chinese medicine and have been widely used as a remedy for gastrointestinal diseases, such as stomachache, dyspepsia, and gastrofrigid vomiting. Investigations have shown *A. galanga* and *A. officinarum* have many biological activities, including effectiveness as anti-inflammatory, anti-tumor, anti-viral, anti-microbial, anti-oxidant, anti-allergic, and gastro-protective agents. Both the rhizomes and seeds of *A. galanga* are used as spices and medicine, and for this reason the composition of rhizomes and seeds have been studied by researchers (Ajay and Vijaykumar, 2015). *A. galanga* is an important medicinal plant in different traditional systems of medicine to treat several diseases, including microbial infections, inflammations, rheumatic pains, chest pain, and dyspepsia, fever, burning of the liver, kidney disease, tumours, diabetes and even HIV (Ramesh *et al*., 2011). The plant has an active role in the treatment of eczema, bronchitis, coryza, mobile, pityriasis versicolor, otitis internal, gastritis, ulcers, and cholera. The seed is used for emaciation and to clean the mouth. It stimulates the digestive power, appetite and acts as a purgative. The rhizome is generally used as a spice. It is also a good source of essential oil.

2.4. Medicinal plants used to prevent cancer

Medicinal plants are traditionally used in folk medicine as natural healing remedies with therapeutic effects such as prevention of cardiovascular diseases, inflammation disorders, or reducing the risk of cancer. In addition, pharmacological industry utilizes medicinal plants due to the presence of active chemical substances as agents for drug synthesis. Yasukawa, (2012) has reviewed the chemopreventive activity of natural sources, foods, supplements, crude drugs and Kampo medicines (traditional Japanese herbal prescriptions). In that review, he observed that cancer chemoprevention is currently one of the most urgent projects in public health. Cancer chemoprevention is defined as the use of specific natural and synthetic chemical agents to reverse or suppress carcinogenesis and prevent the development of invasive cancers. Recently, dietary non-nutrient compounds have demonstrated important effects as chemo-preventive agents, and considerable work on the cancer chemopreventive effects of such compounds in animal models has been undertaken. Epidemiological surveys have shown that the majority of human cancers are related to two factors, namely, diet and smoking (Banning, 2005; Hirayama, 1984). However, in the general population, daily consumption of certain foods has also been shown to have anticancer effects. This highlights the importance of environmental factors such as diet in cancer chemoprevention (Banning, 2005).

Therefore, to prevent cancer, it is essential to find plants that contain effective compounds (anti-tumour promoters) that delay, inhibit or block tumour promotion, which is a reversible and long-term process (Yasukawa, 2012). It is estimated that 67% of drugs used in chemotherapy are derived from natural products (Wangkheirakpam, 2018). This applies to the discoveries of active compounds such as vincristine (Raviña, 2011), taxol (Fischer *et al*., 2010), and artemisinin (Tu, 2011). Moreover, medicinal plants also offer an opportunity for rural dwellers to generate a cash income (EL-Hilaly *et al*., 2003).

2.5. Plant-derived anti-cancer agents in clinical use

Natural compounds with potent anti-cancer activities are widely available from different plant tissues. Naturally occurring compounds target tumor cells by regulating cell death pathways such as extrinsic and intrinsic apoptosis pathways and autophagic pathways. A collection of plant-derived natural anti-cancer compounds can be found at Naturally Occurring Plant-based Anti-cancer Compound-Activity-Target Database (NPACT, http://crdd.osdd.net/raghava/npact/) where approximately 1980 experimentally validated compound-target interactions are documented (Mangal *et al*., 2013). Probably the most well-known plant-derived anti-cancer drug is Paclitaxel (Taxol®). The cytotoxic activity of this taxane dipertene found in extracts from the bark of *Taxus brevifolia* Nutt. (Western yew) was first reported by Wani *et al*. (1971).

Detailed information about these medicinal plants, family, part used and specific type of anticancer phytochemical and their mechanism of action against particular type of cancer is important. Furthermore, the generalized model of carcinogenesis, anti-cancer mechanism of body and natural phytochemicals against cancer.

1. **Vinca alkaloids**

They are one of oldest class of agent used to treat cancer and are the second-most commonly used agents in the clinic. They were first developed in the 1950s by Canadian scientists Robert Noble and Charles Beer. These alkaloids were isolated from *Catharanthus roseus* (Apocynaceae) and were given to patients with breast cancer, Hodgkin's lymphoma, leukaemia, testicular cancer and lung cancer (Singh *et al*., 2013). Vinblastine, vincristine and vinorelbine are approved for use in the USA and vinflunine was approved in 2008 in Europe.

2. **Campothecin derivatives**

Campothecin (family of topoisomerase I poisons) is another class of plant derived clinically-active chemotherapeutic agents possesses strong anticancer potential inhibiting topoisomerase I in a large number of cancers. (Kim *et al*., 2015). It was first isolated from *Camptotheca acuminata* (Nyssaceae). The isolate of *C. acuminata* has been the only agent out of 1000 different plant extracts screened out for anticancer activity which have shown efficacy and the active constituents isolated has been identified as camptothecin. Extensive research is performed by several research organizations for effective camptothecin derivatives like topotecan (hycamtin) and irinotecan, where irinotecan is used to treat colorectal cancer while topotecan is used to treat ovarian and lung cancer (Cragg and Kingston, 2005).

3. **Triterpenoid acids**

Triterpenoid acids are also naturally occurring phytomolecules with anticancer properties. Moreover, these potent anticancer agents have shown strong anticancer results in both *in-vitro* and *in-vivo* against leukemia, pancreatic and breast cancer. Other anticancer agents like, CDDO (2-cyano-3, 12-dioxoolean1, 9-dien-28-oic acid) and its methyl ester are active against ovarian cancer (Cragg and Newman, 2005). Betulinic acid is another triterpenoid isolated from *Ziziphus* mauritiana, *Ziziphusrugosa* and *Ziziphus oenoplia* and *Betula* Sp. (Betulaceae) and is cytotoxic against a wide range of cancer including human melanoma (Prakash *et al*., 2013).

4. **Phenols**

Numerous pure polyphenolic compounds and natural polyphenolic extracts possess antioxidant, anticancer and anti-inflammatory activities (Romier *et al*., 2009). Moreover, polyphenolic compounds in plant have biological activities such as carcinogenic inactivation, antiproliferation, cell cycle arrest, induction of apoptopsis, and inhibition of angiogenesis. In addition, polyphenolic compounds have antioxidant activity that can prevent damage from reactive oxygen species (Hoelz *et al*., 2010).

5. **Flavones/Flavonoids**

Flavonoids are also plant-specific secondary metabolites widely present in fruits and vegetables that are consumed daily with around 8 000 diverse compounds and play a vital role for the treatment of large number of cancer (Ververidis *et al*., 2007). Research has shown that flavonoids inhibit cell proliferation and angiogenesis, cause cell cycle arrest, induce cell apoptosis, and reverse multi-drug resistance and/or a combination of the aforementioned mechanisms (Chahar *et al*., 2011). A flavonoid, quercetin, is abundant in daily-consumed foods such as onions (*Allium cepa*) with a wide range of anecdotally reported health benefits that include anti-oxidant, anti-inflammatory, and anti-cancer activities in vitro and is effective against various cancer cells (Sak, 2014). Flavopiridol is a plant-derived semisynthetic flavone that inhibit cyclin-dependent kinase with anticancer activity against esophageal and gastric cancers (Rathkopf *et al*., 2009). To date, meta-analyses have primarily focused on the functions of dietary flavonoids including inhibition of DNA topoisomerase I, and cycloxigenase and are used against breast (Morimoto *et al*., 2014), lung (Tang *et al*., 2009), stomach and colorectal cancer (Woo and Kim, 2013).

6. **β-elemene**

β-elemene a sesquiterpene, is a promising anticancer agent with a wide range of its effect against drug-resistant tumors (Liu *et al*., 2011). β-elemene is major component of traditional Chinese medicine and inhibit different forms of cancer, induces apoptosis and cell death in human gastric cancer (Jiang *et al*., 2017; Jiang *et al*., 2016).

7. **Vitamin E from plant oil**

Vitamin E has been reported as anti-tumor agent and represents a group of compounds consisting of both tocotrienols and tocopherols. It is fat-soluble anti-oxidant present in sunflower oil, germ oil, safflower oils and wheat. It has been researched that both tocopherols and tocotrienols exhibit antitumor properties like proapoptotic, anti-proliferative effects in both either *in-vitro* and *in-vivo* studies (Wada, 2012).

7. **Chalcone**

Chalcone is also a naturally occurring anticancer flavonoid in fruits and vegetables. It is responsible for activation of different caspases (caspase-8, 9, 12 enzymes), upregulate the of pro-apoptotic proteins expression (Bid, Bax, and Bak proteins), decreases anti-apoptotic Bcl-2 gene expression and have been used for the treatment of the treatment of colon, lung, breast, liver and prostate cancer (Das and Manna, 2016).

8. **Kaempferol**

Kaempferol is also a naturally occurring anticancer agent and possess significant antitumor potential on a large number of cancer cells e.g. colorectal cancer and HT-29 cancer cells by activating the expression of caspase-3 enzyme, *p53* gene and its products (Lee *et al*., 2014).

Chapter 3
Methodology

3.1. Collection of plant sample

The uninfected *Curcuma aeruginosa* Roxb., *Costus pictus* D.Don were collected from Durtlang (23° 47' 0" North, 92° 44' 0" East), Mizoram and *Alpinia galanga* L. (Willd) was collected from Bairabi; 24.1853° N, 92.5371° E.

The plant samples were identified by Prof. H. Lalramnghinglova, Department of Environmental Science and Dr. Khomdram Sandhyarani Devi, Department of Botany**,** Mizoram University, Aizawl. The herbarium was deposited in the Institutional Herbarium, Mizoram University with reference to Botanical Survey of India, Eastern Regional Centre.

3.2. Details of the plants used in this study

3.2.1. Curcuma aeruginosa

Kingdom- Plantae

Phylum- Tracheophyta

Class- Liliopsida

Order- Zingiberales

Family- Zingiberaceae

Genus- Curcuma

Species- *C. aeruginosa*

3.2.2. Botanical description

Curcuma aeruginosa is a perennial rhizomatous herb producing unbranched leafy stems up to 200 cm tall from a large underground rhizome that can be 16cm long and 3cm wide (Fayaz, 2011). Blue in the centre, merging towards grey, the blue colour is highly variable with age of the rhizome, strongly aromatic; sessile tubers branched, condensed; roots fleshy; root tubers many, ovate-oblong. Plants, 70-100 cm tall, pseudostem 30-35 cm tall, sheaths green. Leaves distichous, 30-40 x 10-12 cm, oblong-lanceolate, tip acute, base acuminate, glabrous purple or reddish-brown patch along the sides of the distal half of the mid rib on upper side only, fading at maturity, groove of the midrib green; petiole as long as lamina. Inflorescence lateral, 25-30 cm long, peduncle 12-18 cm; spike 12-15 x 5 cm; coma bracts large, pink to violet, lower ones streaked green. Lobes. The inflorescence develops from the rhizome, usually before the leaves are produced Fertile bracts 18-20, 4.5-5 x 4.4-5 cm lower half fused, tip rounded, green with a pink tip, subtends a cincinnus of 8-10 flowers; bracteoles 3.5 x 2.5 cm, white with a median light green patch. Flowers 4.5-5 cm, equal to or slightly shorter than the bracts. Calyx 1 cm, truncate, 3-lobed at apex, split on one side, Corolla tube 3-3.3 cm long, pink, lobes unequal; dorsal lobes 1.5 x 1.2 cm, concave, hooded; lateral lobes 1.5 x 1 cm, tip rounded, pink. Labellum 1.5-1.7 x 1.8 cm tip emarginated, yellow with a deep yellow median band. Lateral staminodes 1.5 x 1 cm, yellow. Anther 7 mm, without crest, spurred at base, spurs 3mm long, divergent. Epigynous glands two, 5 mm long, linear, yellowish green. Ovary 5 mm, trilocular, with many ovules; style long, filiform; stigma bilipped, slightly exserted above the anther. Flowering and fruiting is seen during the month of April – May.

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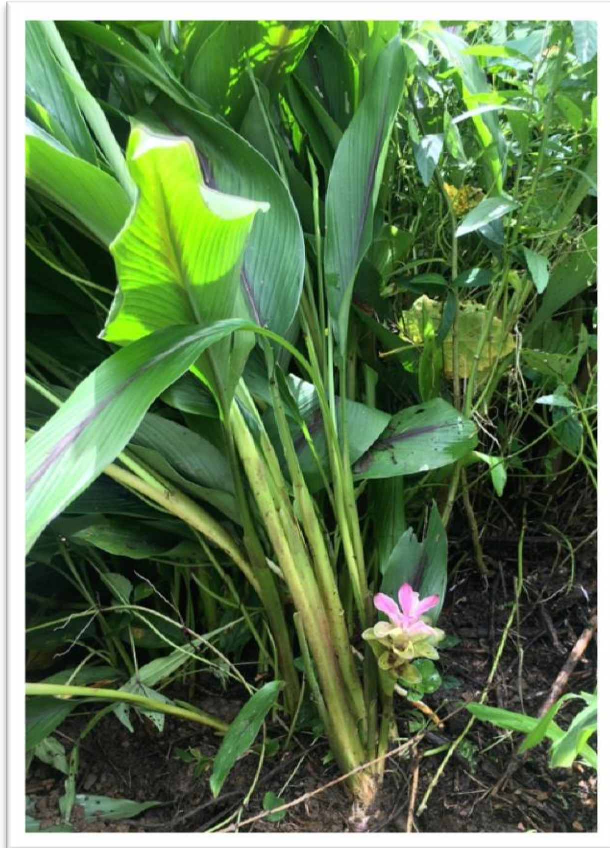
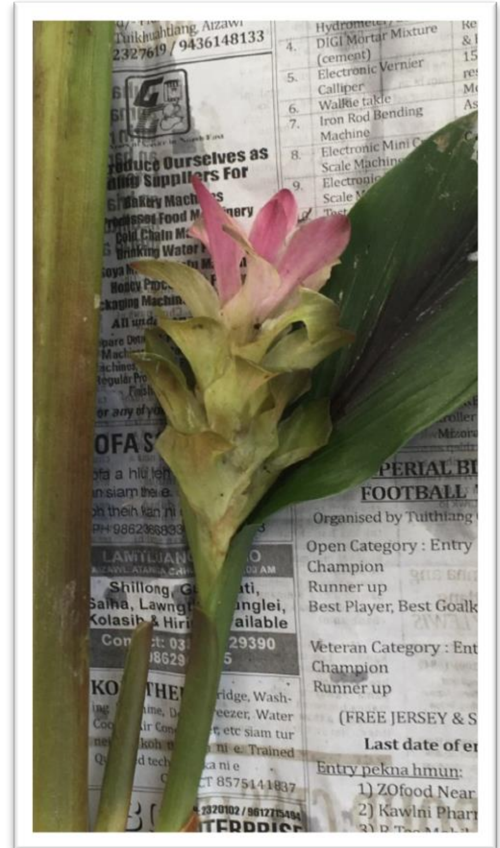


Curcuma aeruginosa



Inflorescence

Photo plate 1. Habit and inflorescence of *Curcuma aeruginosa*.

3.2.3. *Costus pictus*

Kingdom- Plantae

Phylum- Tracheophyta

Class- Liliopsida

Order- Zingiberales

Family- Costaceae

Genus- Costus

Species- *C. pictus*

3.2.4. Botanical description

Costus pictus is a perennial, upright, spreading plant reaching about two feet tall, with the tallest stems falling over and lying on the ground. The leaves show a simple, spiral arrangement and is characterised by an oblong-lanceolate appearance. The average length of a leaf varies between 10 to 25 cm and has a width of about 2.5 to 6 cm. Internode length was around 2–5 cm. The rhizomes are the parts from which new shoots arise. Rhizomes are thick, horizontal underground part which continues to increase upto 3 feet width in a span of two years under desirable growth conditions. *Costus pictus* reproduced vegetatively mainly by rhizomes, however, stem cuttings could also aid in reproduction. Seed germination played a minimal role due to its low germination percentage. Yellow colored flowers with red stripes are seen with green color bracts. Flowers occurred only in late summer. Flowering generally commenced from July and continued till September. Later as the flowers fade away, cone shaped bracts prevailed. Fruits remained inconspicuous. Seeds are minute and black colored with white fleshy aril.

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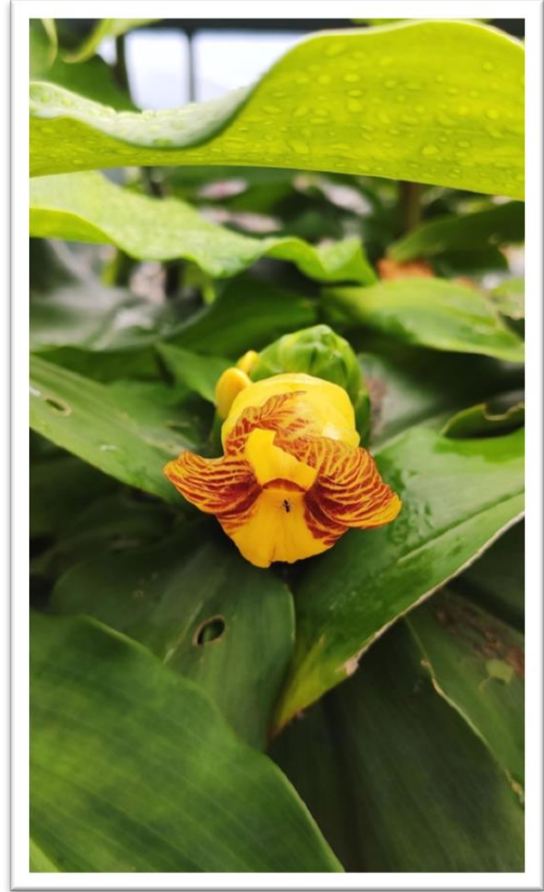


Costus pictus **Inflorescence**

Photo plate 2. Habit and inflorescence of *Costus pictus*.

3.2.5. *Alpinia galanga*

Kingdom- Plantae

Phylum- Tracheophyta

Class- Liliopsida

Order- Zingiberales

Family- Zingiberaceae

Genus- Alpinia

Species- *A. galanga*

3.2.6. Botanical description

The plant is a perennial herb, successfully cultivated only on sandy loam soils and in humid tropical climate with adequate irrigation provisions,. It can be grown in open sunny areas with sufficient rainfall. It grows up to a height about 5 feet. Leaves are oblong-lanceolate, tuberous root, slightly aromatic. The rhizome is from 3.5-7.5 cm in length, and seldom more than 2 cm thick. The leaves are long, oblong-lanceolate, acute, glabrous, ligules are short and rounded. Flowers are greenish white in colour about 3 cm long, and occur in dense panicles, bracteate, bracts are ovate lanceolate. Tubular calyx, Corolla lobes oblong, claw green, blade white, striated with red, rather more than 1 cm long, broadly elliptic, shortly 2-lobed at the apex, with a pair of subulate glands at the base of the apex, with a pair of subulate glands at the base of the claw. Fruit the size of the small cherry, orange red. Flowering occurs in May and June, while fruiting occurs in August and September. Fruits are orange-red in colour.

Accession no: MZUEVS29520, B.S.I (E.R.C):133416

Alpinia galanga

Inflorescence

Photo plate 3. Habit and inflorescence of *Alpinia galanga*.

3.3. Preparation of extract

The plant samples were washed with water, air dried and crushed into fine powder with electric grinder. The powder sample was subjected for sequential hot and 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 of *C. pictus* were written as:

CPRH- *Costus pictus* Rhizome Hexane, CPSH- *Costus pictus* Stem Hexane, CPLH- *Costus pictus* Leaf Hexane, CPRE- *Costus pictus* Rhizome Ethyl acetate, CPSE- *Costus pictus* Stem Ethyl acetate, CPLE- *Costus pictus* Leaf Ethyl acetate, CPRM- *Costus pictus* Rhizome Methanol, CPSM- *Costus pictus* Stem Methanol and CPLM- *Costus pictus* Leaf Methanol.

The hexane, ethyl acetate and methanol extract from rhizome, stem and leaves of *C. aeruginosa* were written as:

CURH- *Curcuma aeruginosa* Rhizome Hexane, CUSH- *Curcuma aeruginosa* Stem Hexane, CULH- *Curcuma aeruginosa* Leaf Hexane, CURE- *Curcuma aeruginosa* Rhizome Ethyl acetate, CUSE- *Curcuma aeruginosa* Stem Ethyl acetate, CULE- *Curcuma aeruginosa* Leaf Ethyl acetate, CURM- *Curcuma aeruginosa* Rhizome Methanol, CUSM- *Curcuma aeruginosa* Stem Methanol and CULM- *Curcuma aeruginosa* Leaf Methanol.

The hexane, ethyl acetate and methanol extract from rhizome, stem and leaves of *A.galanga* were written as:

APRH- *Alpinia galanga* Rhizome Hexane, APSH- *Alpinia galanga* Stem Hexane, APLH- *Alpinia galanga* Leaf Hexane, APRE- *Alpinia galanga* Rhizome Ethyl acetate,

APSE- *Alpinia galanga* Stem Ethyl acetate, APLE- *Alpinia galanga* Leaf Ethyl acetate, APRM- *Alpinia galanga* Rhizome Methanol, APSM- *Alpinia galanga* Stem Methanol and APLM- *Alpinia galanga* Leaf Methanol.

3.4. Phytochemical analysis

The qualitative phytochemical analysis of the extracts were performed by standard procedures given by Trease and Evans (1989), Harbone (1998), Sofowara (1993) to detect flavonoid, tannin, alkaloid, phenol, terpenoid, saponin, quinone, cardiac glycosides, anthraquinone in the plant sample such as leaf, stem and rhizome.

1. Test for Tannins

To 1 ml of plant extracts, 2 ml of 0.7 M Sodium carbonate and few drops of Folins reagent were added. Formation of dark blue or greenish black indicates the presence of tannin.

2. Test for Saponins

To 2 ml of plant extracts, 2 ml of distilled water was added and mixed in a graduated cylinder for 15 minutes. Formation of 1cm layer of foam indicates the presence of saponins.

3. Test for Flavonoids

To 2 ml of plant extracts, 1 ml of 2 N Sodium hydroxide was added. Presence of yellow indicates the presence of flavonoids.

4. Test for Alkaloids

The presence of alkaloids in the aqueous extracts of different plants was determined by mixing 1 ml of each plant extract with Dragendorff's reagent. The formation of reddish brown precipitate indicated the presence of alkaloids.

5. Test for Quinones

To 1 ml of plant extracts, 1 ml of concentrated Sulphuric acid was added. Formation of red colour indicates the presence of quinones.

6. Test for Cardiac glycosides

To 1 ml of plant extracts, 2 ml of Glacial acetic acid and few drops of 5% Ferric chloride were added. This was under layered with 1 ml of concentrated Sulphuric acid. Formation of brown ring at the interface indicates the presence of cardiac glycosides.

7. Test for Terpenoids

To 1 ml of plant extracts, 2 ml of Chloroform was added and then concentrated Sulphuric acid was added carefully. Formation of red brown colour at the interface indicates the presence of terpenoids.

8. Test for Phenol

To 1 ml of plant extracts, few drops of 15% sodium carbonate and a few drops of folins reagent was added. Formation of blue colour indicates presence of phenol

9. Test for Anthraquinones

To 1 ml of plants extracts few drops of 10% Ammonia solution was added, appearance of pink colour precipitate indicates the presence of anthraquinones.

3.5. Quantitative Phytochemical Analysis

3.5.1. Quantification of Phenols

The total phenolic content was determined by using Folin-Ciocalteu reagent following a slightly modified method of Ainsworth and Gillespie (2007). Gallic acid was used as a reference standard for plotting calibration curve. A volume of 0.5 ml of the plant extract (10 mg/ml) was mixed with 5 ml of the Folin-Ciocalteu reagent and were neutralized with 4 ml of sodium carbonate (NA_2CO_3) solution (7.5 %, w/v). The reaction mixture was incubated at room temperature for 30 min with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 765 nm. The total phenolic contents were determined from the linear equation of a standard curve prepared with gallic acid. The content of total phenolic compounds expressed as mg/g gallic acid equivalent (GAE) of dry extract.

3.5.2. Quantification of Flavanoids

Total flavonoid content was estimated by Aluminium chloride colorimetric method (Chang *et al*., 2002). Standard quercetin solutions of various concentrations were used to make the calibration curve. 10 mg of quercetin was dissolved in methanol and then diluted to 1, 2, 5, 10, 20, 40 µg/ml using methanol. Stock solution of extracts was prepared by dissolving 10 mg of the each extract in 1ml methanol for the 27 samples. Aluminium chloride and 1M potassium acetate were prepared using distilled water. The assay was determined using 0.5 ml of each extract stock solution and each dilution of standard quercetin taken separately in test tubes. To each test tube 1 ml methanol, 0.1 ml aluminium chloride solution, 0.1ml potassium acetate solution and 2.8 ml distilled water were added and mixed well. Sample blank for all the dilution of standard quercetin were prepared in similar manner by replacing aluminium chloride solution with distilled water. All the prepared solutions were filtered through Whatmann filter paper if necessary before measuring their absorbance. Absorbance was Taken at 415 nm against the suitable blank.

3.5.3. Quantification of Alkaloids

Alkaloid quantification was done following the Bromocresol green method proposed by Luyang *et al*. (2014). The extract samples were dissolved in 2 N of Hydrochloric acid (HCl) and then filtered. Phosphate buffer solution (pH 4.7) was prepared by adjusting the pH of 2 M sodium phosphate (71.6 g Na_2HPO_4 in 1 L distilled water) to 4.7 (4.5 to 4.9) with 0.2 M citric acid (42.02 g citric acid in 1 L distilled water. Bromocresol green solution (BCG) (10-4 M) was prepared by heating at 50-60 degrees Celsius, 10-15 min of 69.8 mg bromocresol green with 3 ml of 2 N NaOH and 5 ml distilled water until completely dissolved and the solution was diluted to 1000 ml with distilled water. 1 ml of extract was transferred to separating funnel and add 5 ml of phosphate buffer pH 4.7 and 5 ml of bromocresol green (BCG) solution. The mixture was shaken and complex formed was extracted with 5 ml of chloroform. Chloroform layer was collected in 10 ml of volumetric flask and make the volume up to mark with chloroform. Absorbance was taken at 415 nm against blank .The solutions were stable for 2 hours. The total alkaloids were determined by the regression equation.

3.6. 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 123.5m x 678m. 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.

3.7. 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 x 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 minute, then progressively increased the concentration of solvent B to 35% in 25 minutes, 50% in 45 minutes, and eventually 100 % in 65 minutes. 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.

3.8. Antioxidant activities

To validate the antioxidant activity of the different 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).

3.8.1. DPPH free radical scavenging assay

The free-radical scavenging activity was measured by a method described by Leong and Shui (2000). 1ml methanol solution of 0.1 mM DPPH was added to the different concentration of the methanol extract. The absorbance was measured at 523 nm after incubation time of 30 minutes.

3.8.2. ABTS free radical scavenging assay

ABTS scavenging activity of the different extracts were determined using a minor modification of Re *et al*. (1999). Briefly, 37.5 mg of potassium persulfate was dissolved in 1 ml of distilled water. A total of 44 µl of this solution was added to 9.7 mg of ABTS dissolved in 2.5 ml of distilled water so as to prepare ABTS solution. The ABTS solution was allowed to stand in the dark for about 15 h at room temperature. The working solution was prepared by mixing 1 ml of ABTS solution with 88 ml of 50% ethanol. A total of 50 µl of the extracts were mixed with 100 µl of ABTS working solution and allowed to stand for 4 min. The absorbance was read at 734 nm. The results have been expressed as ascorbic acid equivalent which was used as a standard.

3.8.3. Superoxide anion scavenging assay

This activity was measured using a method given by (Hyland *et al*., 1983) with slight modifications. 0.2 ml of NBT (1 mg/ml of solution in DMSO), 0.6 ml different extracts, 2 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml H2O) were added in a final volume of 2.8 ml. The blank consisted of pure DMSO instead of alkaline DMSO. The results have been expressed as ascorbic acid equivalent which was used as a standard. The absorbance was measured at 560 nm.

3.8.4. Hydroxyl radical scavenging assay

The activity was measured by following Smirnoff and Cumbes (1989). 1.0 ml of 1.5 mM FeSO4 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.

3.8.5. Phosphomolybdenum assay

The total antioxidant capacity was determined according to the method of Prieto *et al*. (1999). 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

3.9. Selection of most cytotoxic extract

MTT assay was carried out for selecting the extract with the highest cytotoxic effect among the 27 samples. Based on the IC_{50} values, *C. aeruginosa* showed the best cytotoxic effect overall and the remaining experiments were carried out using the methanol extract from rhizome, stem and leaves. The hexane leaf extract of *A. galanga* also showed promising cytotoxic activity, so it was included in the proceeding experiments. The cell cultures were divided into the following groups:

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

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

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

1. CURM group – *Curcuma aeruginosa* Rhizome methanol
2. CUSM group – *Curcuma aeruginosa* Stem methanol
3. CULM group – *Curcuma aeruginosa* Leaf methanol
4. APLH group – *Alpinia galanga* Leaf hexane

3.10. Cell lines and Culture medium

The cells were procured from The National Centre for Cell Science, Pune, India – 4 human cancer cell lines, namely, A-549, HeLa, AGS, HT-29 and a normal cell: L-132. Stock cells were cultured in MEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5 % CO2 at 37°C until confluent. The cells were dissociated with trypsin solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks, and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., India).

3.11. MTT assay

In vitro anticancer activity of the extracts against A-549, HeLa, AGS, HT-29 cell line and their toxicity in normal cell line (L-132) was studied using MTT assay described by Mosmann, (1983). 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 37ºC in a CO_2 incubator with an atmosphere of 5 % CO_2 in 95 % humidified air and were allowed to attach for 24 hours. 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 hours of cell plating, 20 µl of MTT was added and the microplates were incubated for another 2 hours. The media were removed and the insoluble purple formazan formed was dissolved with DMSO and incubated once again for 4 hours. Then, the absorbancy reading was performed using a Spectramax M2 Microplate Reader (Molecular Diagnostic, Inc.) at a wavelength of 570 nm and the cytotoxicity was calculated

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

3.12. Comet Assay

It was performed using modified version of Olive and Banáth (2006). The A-549 and HeLa cells were seeded separately in 6-well plates for 24 hours at 37°C in 5% CO2 to allow cell adherence. After adherence, cells were treated with the IC_{50} concentration of the treated groups (CURM, CUSM, CULM and APLH) for 24 hours. 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, 0.5M NaCl, 0.1 M NA_2EDTA, 0.01 M Tris HCl, 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 Na_2 EDTA, I M NaOH) for 50 mins at 70 mA. Then, the slides were taken out and kept in neutralized buffer (I M 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.

3.13. 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 hours at 37°C in 5% CO_2 to allow cell adherence. The cells were treated with the IC_{50} concentration of the treated group for 24 hours after adherence. Then, the cells ($1x10^5$ 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 minutes. Fluorescence microscope (EVOS® FL Cell Imaging System, Thermo Fisher Scientific) was used for observing the stained cells.

The apoptotic and necrotic index was calculated.

$$\text{Apoptotic index (\%)} = \frac{\text{Number of apoptotic cells scored}}{\text{Total number of cells counted}} \text{ X } 100$$

$$\text{Necrotic index (\%)} = \frac{\text{Number of necrotic cells scored}}{\text{Total number of cells counted}} \text{ X } 100$$

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

Caspase-8 and Caspase-3 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 hours prior to the experiment. Cells were exposed to the IC_{50} concentrations of the treated group for 48 hours to induce apoptosis. 10 µg/ml doxorubicin was taken as positive control. Fold increase in Caspase-8 and -3 activities were examined by direct comparisons to the level of untreated controls.

3.15. Statistical analysis

The statistical analysis was carried out using Microsoft excel 2010, Washington, USA. The IC_{50} 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 considered statistically significant. The results are expressed as mean ± standard error of the mean (SEM).

Chapter 4

Results

4.1. Phytochemical analysis

4.1.1. Qualitative phytochemical analysis

The qualitative phytochemical analysis of the different extracts 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 and anthraquinone was absent in all the extracts.

4.1.2. Quantitative phytochemical analysis

The quantification of phytochemicals of the various plant parts of *C. pictus* revealed that CPLM contained the highest phenol (7.62 ± 0.35 mg GAE/g of dry weight) followed by CPLE (4.65 ± 0.65 mg GAE/g of dry weight). The highest flavonoid content was found in CPLM (4.37 ± 0.23 mg quercetin equivalent/g of dry weight) followed by CPLH (2.87 ± 0.74 mg quercetin equivalent /g of dry weight). The alkaloid content was the highest in CPLM (1.05 ± 0.35 mg atropine equivalents/g of dry weight) (Table 2).

The quantification of phytochemicals of the various plant parts of *C. aeruginosa* revealed that CULM contained the highest phenol (7.66 ± 0.05 mg GAE/g of dry weight) followed by CURE (5.19 ± 0.32 mg GAE/g of dry weight). The highest flavonoid content was found in CULM (7.57 ± 0.02 mg quercetin equivalent/g of dry weight) followed by CURM (3.92 ± 0.08 mg quercetin equivalent /g of dry weight). The alkaloid content was the highest in CULM (1.52 ± 0.77 mg atropine equivalents/g of dry weight) (Table 2).

Table1. Qualitative phytochemical analysis of the different extracts of *C. pictus, C. aeruginosa* and *A. galanga*.

Sample	**Phenol**	**Flavonoid**	**Alkaloid**	**Saponin**	**Quinone**
CPRH	+	+	-	+	-
CPRE	+	+	+	+	-
CPRM	+	+	+	+	-
CPSH	+	+	-	+	-
CPSE	+	+	+	-	-
CPSM	+	+	+	-	-
CPLH	+	+	-	+	+
CPLE	+	+	+	-	+
CPLM	+	+	+	+	+
CURH	+	+	-	+	+
CURE	+	+	+	+	+
CURM	+	+	+	+	+
CUSH	+	+	-	+	+
CUSE	+	+	+	-	+
CUSM	+	+	+	-	-
CULH	+	+	-	+	-
CULE	+	+	+	-	+
CULM	+	+	+	+	-
APRH	+	+	-	-	+
APRE	+	+	+	-	+
APRM	+	+	+	+	+
APSH	+	+	-	-	-
APSE	+	+	+	-	-
APSM	+	+	+	+	+
APLH	+	+	-	+	-
APLE	+	+	+	+	-
APLM	+	+	+	+	+

Sample	Cardiac glycosides	Terpenoid	Tannin	Anthraquinone
CPRH	+	+	-	-
CPRE	+	+	-	-
CPRM	+	+	-	-
CPSH	+	+	-	-
CPSE	+	+	-	-
CPSM	+	+	-	-
CPLH	+	+	-	-
CPLE	+	+	-	-
CPLM	+	+	-	-
CURH	+	+	+	-
CURE	+	+	+	-
CURM	+	+	+	-
CUSH	+	+	+	-
CUSE	+	+	+	-
CUSM	+	-	+	-
CULH	+	+	+	-
CULE	+	-	+	-
CULM	+	-	+	-
APRH	-	+	-	-
APRE	-	+	-	-
APRM	-	+	+	-
APSH	-	+	+	-
APSE	-	+	+	-
APSM	-	+	+	-
APLH	-	+	+	-
APLE	-	+	+	-
APLM	-	+	+	-

The quantification of phytochemicals of the various plant parts of *A. galanga* revealed that APRM contained the highest phenol (3.98 ± 0.83 mg GAE/g of dry weight) followed by APLM (3.77 ± 0.33 mg GAE/g of dry weight). The highest flavonoid content was found in APRM (3.02 ± 0.05 mg quercetin equivalent/g of dry weight) followed by APLM (2.80±0.40 mg quercetin equivalent /g of dry weight). The alkaloid content was the highest in APSE (0.90 ± 0.05 mg atropine equivalents/g of dry weight) (Table 2).

4.2. 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 CURM (Table 3a and Figure 2a). The GC–MS analysis of CUSM and CULM recorded 20 and 13 peaks respectively (Table 3b and Figure 2b, Table 3c and Figure 2c). Overall, the 15 phytocompounds with known biological activity identified in the methanol extracts of *C. aeruginosa* are presented in Table 4a.

The GC–MS analysis of APLH recorded 18 peaks (Table 3d and Figure 2d). The 11 compounds with known biological activities was identified in APLH (Table 4b).

Table 2. Quantitative phytochemical analysis of different extract of *C. pictus, C. aeruginosa* and *A. galanga.*

S.no	Sample	Phenol	Flavonoid	Alkaloid
1	CPRH	1.80 ± 0.05	1.47 ± 0.22	-
2	CPRE	1.89 ± 0.23	1.30 ± 0.55	0.87 ± 0.38
3	CPRM	4.25 ± 0.52	2.63 ± 0.85	0.97 ± 0.22
4	CPSH	0.9 ± 0.82	0.81 ± 0.53	-
5	CPSE	1.35 ± 0.30	1.02 ± 0.64	0.38 ± 0.83
6	CPSM	3.92 ± 0.28	1.39 ± 0.52	0.72 ± 0.32
7	CPLH	3.19 ± 0.43	2.87 ± 0.74	-
8	CPLE	4.65 ± 0.65	2.23 ± 0.32	0.89 ± 0.52
9	CPLM	7.62 ± 0.35	4.37 ± 0.23	1.05 ± 0.35
10	CURH	2.26 ± 0.02	1.04 ± 0.08	-
11	CURE	5.19 ± 0.32	2.3 ± 0.05	0.87 ± 0.62
12	CURM	3.67 ± 0.32	3.92 ± 0.08	0.79 ± 0.25
13	CUSH	0.57 ± 0.42	0.30 ± 0.03	-
14	CUSE	1.97 ± 0.02	1.04 ± 0.13	0.28 ± 0.28
15	CUSM	1.67 ± 0.24	0.96 ± 0.15	0.88 ± 0.53
16	CULH	2.29 ± 0.34	0.88 ± 0.35	-
17	CULE	4.37 ± 0.06	3.23 ± 0.42	1.39 ± 0.23
18	CULM	3.66 ± 0.05	3.57 ± 0.02	1.52 ± 0.77
19	APRH	2.25 ± 0.34	1.56 ± 0.50	-
20	APRE	2.28 ± 0.55	2.07 ± 0.83	0.39 ± 0.52
21	APRM	3.98 ± 0.83	3.02 ± 0.05	0.67 ± 0.63
22	APSH	1.07 ± 0.52	0.78 ± 0.03	
23	APSE	1.01 ± 0.8	0.65 ± 0.02	0.90 ± 0.05
24	APSM	1.20 ± 1.2	1.065 ± 0.82	0.83 ± 0.21
25	APLH	2.20 ± 0.77	1.64 ± 0.32	-
26	APLE	3.36 ± 0.35	2.46 ± 0.82	0.45 ± 0.83
27	APLM	3.77 ± 0.33	2.80 ± 0.40	0.67 ± 0.22

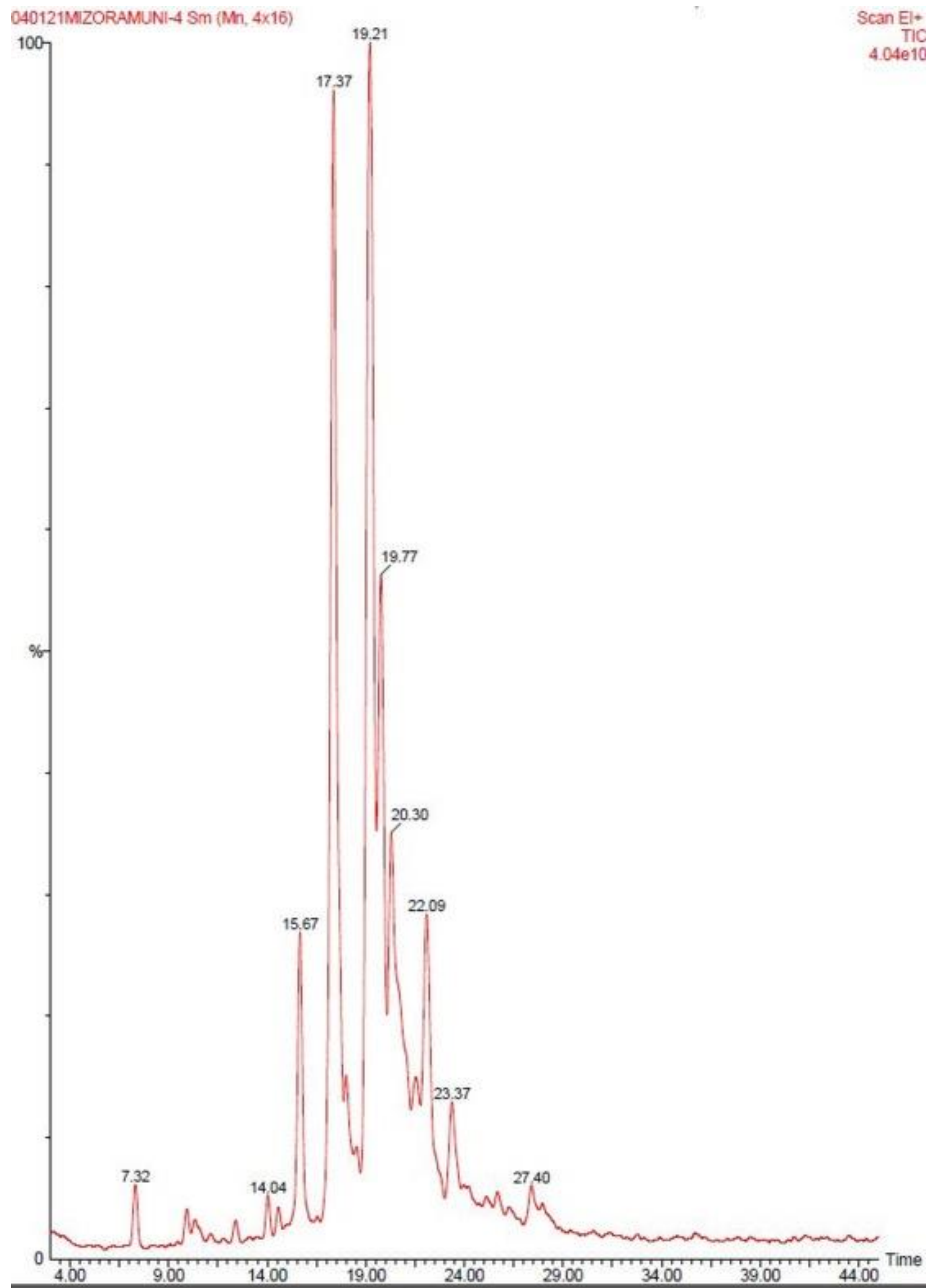


Figure 2a. GC-MS chromatogram of CURM

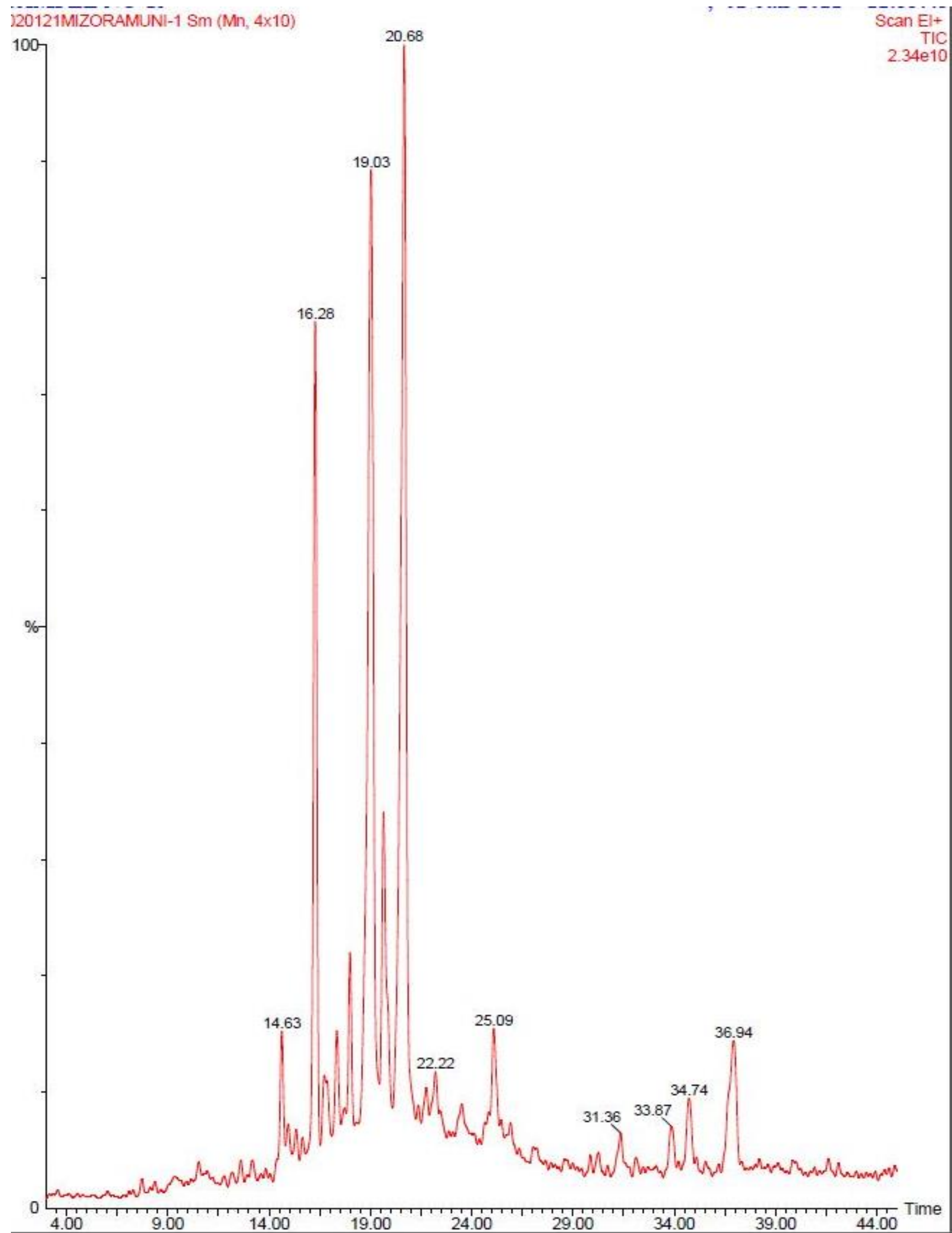


Figure 2b. GC-MS chromatogram of CUSM

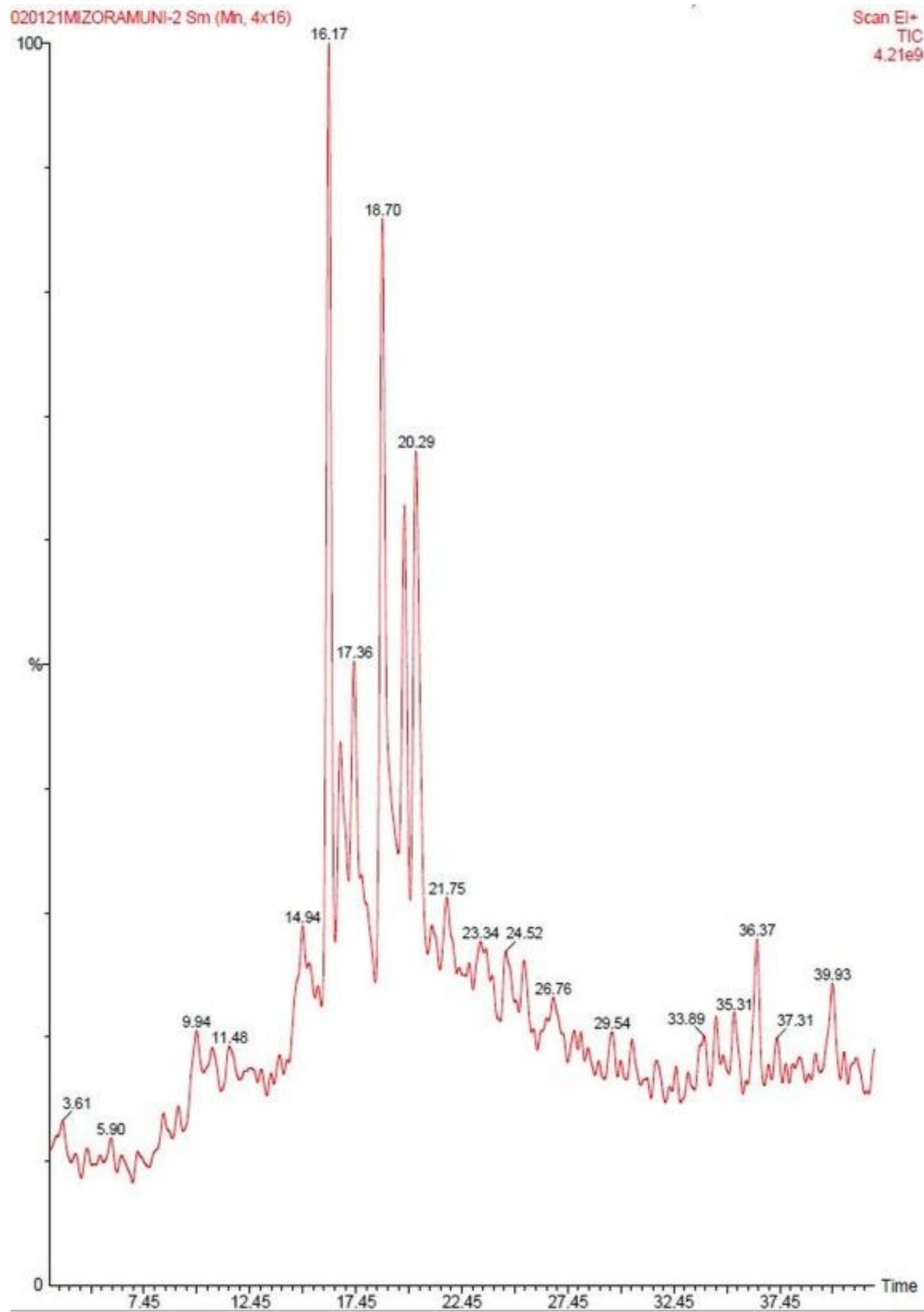


Figure 2c. GC-MS chromatogram of CULM

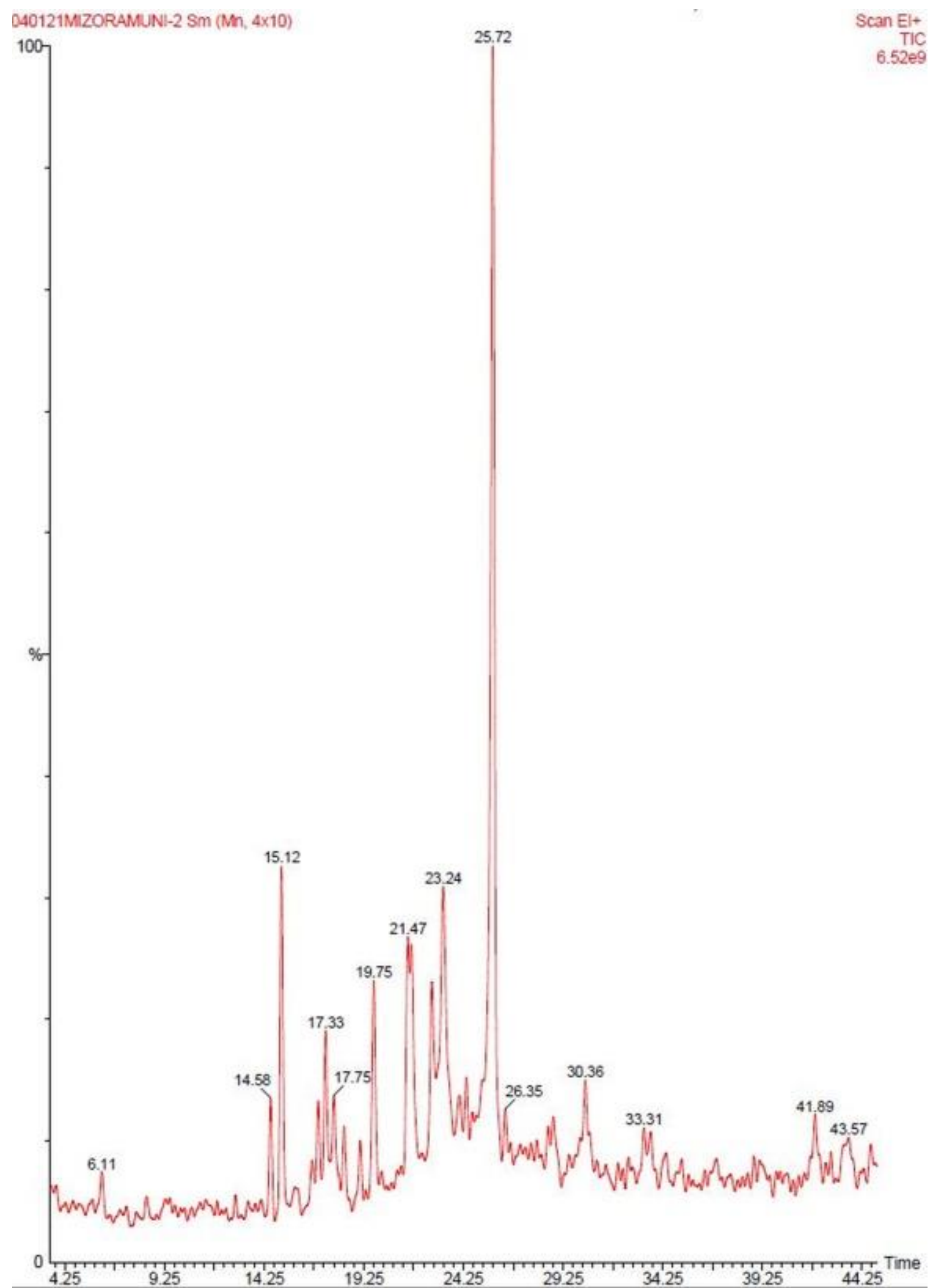


Figure 2d. GC-MS chromatogram of APLH

Table 3a. GC-MS profiling of the identified compound from CURM

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	0.34
4	14.038	1877-77-6	Benzenemethanol, 3-amino	C_7H_9ON	123	0.62
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	0.49
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 3b. GC-MS profiling of the identified compound from CUSM.

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	0.30
3	15.338	900293-99-0	Naphthalene-2-sulfonamide, n-(2-methoxyphenyl)-	$C_{17}H_{15}O_3NS$	313	0.41
4	15.659	900382-90-8	Carbonic acid, prop-1-en-2-yl tridecyl ester	$C_{17}H_{32}O_3$	284	0.26
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(1h,3h)-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	0.17
13	21.761	123-06-8	Propanedinitrile, (ethoxymethylene)-	$C_6H_6ON_2$	122	0.51
14	22.221	6295-94-9	Benzenesulfonamide, N-(2-methoxyphenyl)-4-methyl	$C_{14}H_{15}O_3NS$	277	0.75
15	23.512	529-63-5	4(5h)-benzofuranone, 6,7-dihydro-3,6-Dimethyl-, (R)	$C_{10}H_{12}O_2$	164	0.22
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	0.68
18	33.871	113261-27-1	1H-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 3c. GC-MS profiling of the identified compound from CULM

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	0.92
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

Table 3d. GC-MS profiling of the identified compound from APLH

S.no	Retention Time	CAS	Compound name	Molecular formula	Molecular weight	Peak area (%)
1	14.578	463-40-1	9,12,15-octadecatrienoic acid, (z,z,z)-	$C_{18}H_{30}O_2$	278	2.92
2	15.118	3243-36-5	Ambrial	$C_{16}H_{26}O$	234	8.68
3	16.659	78046-17-0	Tricyclo[6.3.3.0]tetradec-4-ene,10,13-dioxo	$C_{14}H_{18}O_2$	218	0.71
4	16.969	2407-61-6	N-acrylonitrylaziridine	$C_5H_6N_2$	94	1.63
5	17.334	138-86-3	Limonene	$C_{10}H_{16}$	136	3.23
6	17.749	506-24-1	9- octadecynoic acid	$C_{18}H_{32}O_2$	280	1.60
7	18.265	900295-24-5	Furan-2(3h)-one, 4,5-dihydro-5-(benzimidazolthioacethyl)-3-butyl-5-met	$C_{18}H_{22}O_3N_2S$	346	1.62
8	19.075	7616-22-0	Gamma.-tocopherol	$C_{28}H_{48}O_2$	416	1.23
9	19.750	916113-36-0	(R)-6-methoxy-2,8-dimethyl-2-((4r,8r)-4,8,12-trimethyltridecyl)chroma	$C_{28}H_{48}O_2$	416	6.24
10	20.140	148-03-8	Beta- tocopherol	$C_{28}H_{48}O_2$	416	0.32
11	21.466	6053-49-2	Cyclopentaneundecanoic acid	$C_{16}H_{30}O_2$	254	12.90
12	22.666	33672-51-4	Disulfide, 1-methylethyl propyl	$C_6H_{14}S_2$	150	6.39
13	23.237	52910-25-5	4H-1,3,2-benzodioxaborin, 2-(pentyloxy)-	$C_{12}H_{17}O_{3B}$	220	13.95
14	24.042	110823-68-2	β-elemene	$C_{15}H_{24}$	204	1.10
15	24.392	91509-06-7	Cyclopropane, hexylidene	C_9H_{16}	124	1.17
16	25.723	77899-11-7	(Z)6,(z)9-pentadecadien-1-ol	$C_{15}H_{28}O$	224	34.11
17	30.360	74744-50-6	5-pentadecen-7-yne, (z)	$C_{15}H_{26}$	206	1.27
18	41.889	85721-25-1	Oxirane, (7-octenyl-)	$C_{10}H_{18}O$	154	0.91

Table 4a. Biological activity of phytochemical constituents identified in the methanol extracts of *C. aeruginosa* using gas chromatography-mass spectrometry.

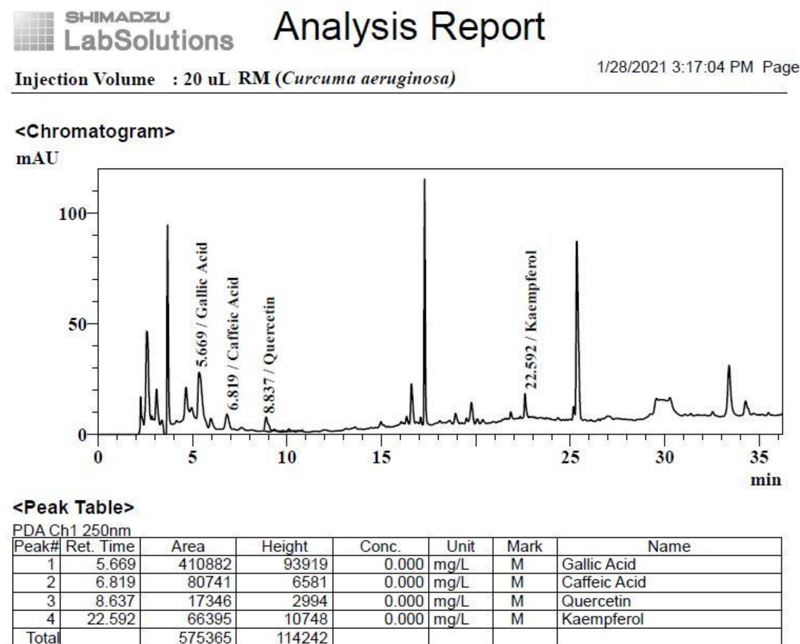
S.no	Compound name	Biological activity	Reference
1	Epicurzerenone	Anti-cancer	Cui *et al*., 2019
2	Curzerene	Anti-cancer	Wang *et al*., 2017
3	Caryophyllene oxide	Anti-cancer	Delgado *et al*., 2021
4	Curzerenone	Anti-cancer	Rahman *et al*., 2013
5	α- curcumene	Anti-cancer	Shin *et al*., 2013
7	Curcumenol	Anti-cancer	Hamdi *et al.,* 2014; Lee *et al*., 2018; Han *et al*., 2019
8	Cycloisolongifolene	Antimicrobial	Zhao *et al*., 2010
9	Phenyl salicylate	Antiseptic; mild analgesic;	Sneader, 2005
10	9,12-octadecadienoic acid (z,z)-	Anti-cancer	Yong *et al*., 2012
11	Andrographolide	Anti-cancer	Zhou *et al*., 2008; Ku *et al*., 2011; Banerjee *et al*., 2016
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*., 2017
13	Benzo[b]perhydroazonine-2,7-dione, 9-methyl	Anti-microbial activity	Anjali *et al*., 2013
14	Benzenesulfonamide, n-(2-methoxyphenyl)-4-methyl	Anti-microbial activity	Ahmad and Farrukh 2012
15	Bicyclo[5.1.0]octan-2-one, 4,6-diisopropylidene-8,8-dimethyl	Anti-microbial	Yang *et al*., 2015

Table 4b. Biological activity of phytochemical constituents identified in APLH using gas chromatography-mass spectrometry.

S.no	Compound name	Biological activity	Reference
1	9,12,15-octadecatrienoic acid, (z,z,z)	Hypocholesterolemic, hepatoprotective, antiandrogenic, 5-alpha reductase inhibitor, anticoronary,	Dr. Dukes Phytochemical and Ethnobotanical Databases
2	Ambrial	Anti-microbial	M. Singh *et al*., 1999
4	Tricyclo[6.3.3.0]tetradec-4-ene,10,13-dioxo	Anti-bacterial	Ololade *et al*., 2017
5	N-Acrylonitrylaziridine	Antitumor, antioxidant, neurostimulant.	Dr. Dukes Phytochemical and Ethnobotanical Databases
6	Limonene	Anti-cancer	Xiao *et al*., 2018; Chaudhary *et al*., 2012; Ye *et al*., 2020; Jie *et al*., 2016; Jia *et al*., 2013; Miller *et al*., 2013
7	Gamma.-tocopherol	Anti-cancer	Jiang *et al*., 2004; Rezaei *et al*., 2014; Pédeboscq *et al*., 2012; Shah *et al*., 2003
8	β-Tocopherol	Anti-spasmodic	Lanuzza *et al*., 2017
9	Disulfide, dipropyl	The treatment of infectious viral diseases including corona virus	Mehmood *et al*., 2021
10	β-elemene	Anti-cancer	Liu *et al*., 2017; Gong *et al*., 2015; Liu *et al*., 2015; Li *et al*., 2014; Wang *et al*., 2018
11	(Z)6,(Z)9-pentadecadien-1-o	Anti-bacterial	Dr. Dukes Phytochemical and Ethnobotanical Databases

4.3. Identification of compound using HPLC analysis

The HPLC analysis of the treated groups 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 CURM (Figure 3a.). CUSM contain gallic acid (5.689), caffeic acid (6.817) and quercetin (8.651) (Figure 3b). CULM showed the presence of gallic acid (5.691), rutin (6.796), coumaric acid (7.973), naringenin (11.002) (Figure 3c). APLH contain gallic acid (5.649), catechin (5.886), rutin (6.862) and quercetin (8.562) (Figure 3d).



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				

Figure 3a. Detection of bioactive compound of CURM by HPLC method.

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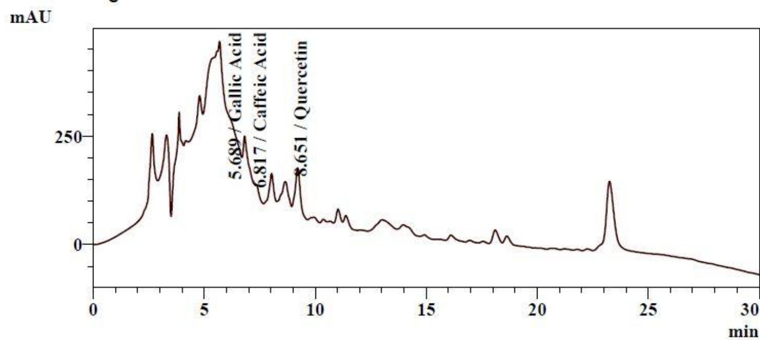
Sample Name : **SM** ***(Curcuma aeruginosa)***
Injection Volume : **20 uL**

<Chromatogram>



<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				

Figure 3b: Detection of bioactive compound of CUSM by HPLC method.

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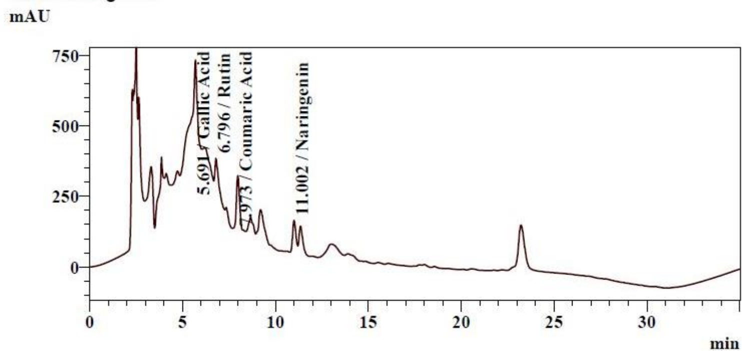
LabSolutions **Analysis Report** 3/12/2021 1:47:29 PM Pag

Sample Name : **LM** **(*Curcuma aeruginosa*)**
Injection Volume : **20 uL**

<Chromatogram>

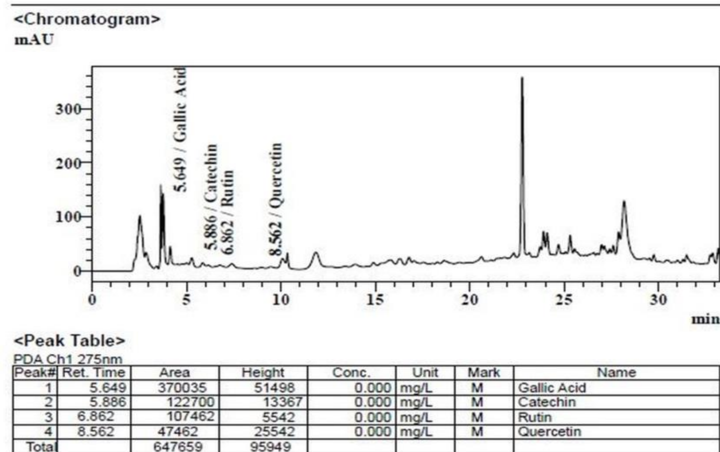


<Peak Table>

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				

Figure 3c. Detection of bioactive compound of CULM by HPLC method.



Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	5.649	370035	51498	0.000	mg/L	M	Gallic Acid
2	5.886	122700	13367	0.000	mg/L	M	Catechin
3	6.862	107462	5542	0.000	mg/L	M	Rutin
4	8.562	47462	25542	0.000	mg/L	M	Quercetin
Total		647659	95949				

Figure 3d. Detection of bioactive compound of APLH by HPLC method.

4.4. Antioxidant activities

To validate the antioxidant activity of the different 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 5 and Figure 4-8).

4.4.1. DPPH free radical scavenging activity

The DPPH scavenging activity of extracts of *C. pictus* showed rise in activity with an increase in concentration manner (5-100 µg/ml) as shown in Figure 4a. The scavenging activity wwas effective in the order: CPSM (9.06 ± 0.07)> CPLM (20.89 ± 0.3)> CPRE (24.61 ± 0.04)> CPRH (44.33 ± 0.14)> CPRM (56.66 ± 0.7)> CPSH (90.17 ± 1.5)> CPLH (114.7 ± 0.02)> CPSE (115 ± 0.02)> CPLE (174.2 ± 0.28) with respect to ascorbic acid (1.071 ± 0.016µg/ml) (Table 5).

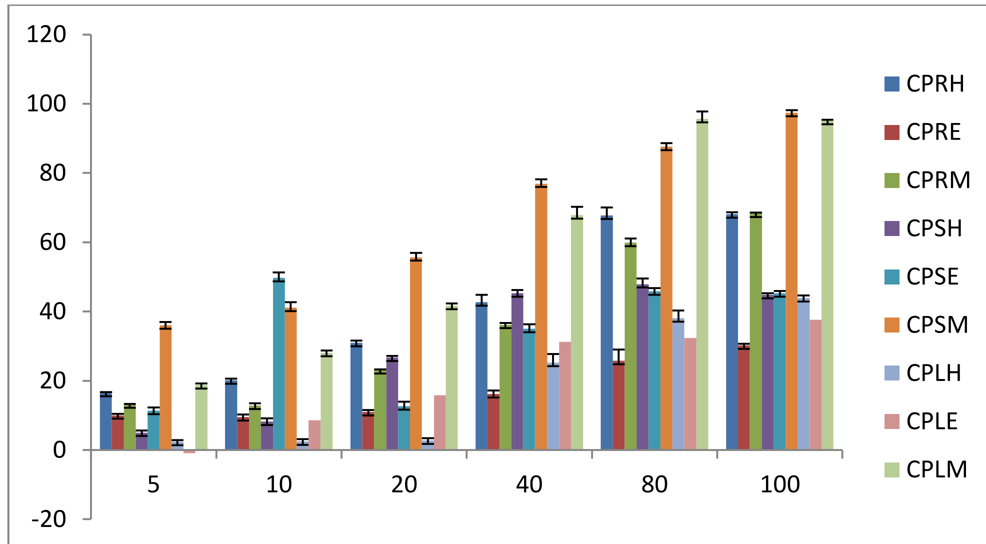


Figure 4a. The DPPH free radical scavenging activity of the different extracts of *C. pictus*. The data shown as Mean ± SEM, N=3**.**

The DPPH scavenging activity of extracts of *C. aeruginosa* showed rise in activity with an increase in concentration manner (5-100 µg/ml) as shown in Figure 4b. Based on the IC_{50} values, CURM showed the highest ability to scavenge DPPH. The observed order of IC_{50} value was as follows: CURM (12.22 ± 0.11)> CUSM (18.89 ± 0.23)> CULE (20.91 ± 0.72)> CULM (31.81 ± 0.38) > CURH (33.71 ± 0.63)> CURE (64.27 ± 2.08)> CUSE (81.92 ± 0.4)> CUSH (144.3 ± 0.1) > CULH (157.5 ± 0.14) with respect to ascorbic acid (1.071 ± 0.016µg/ml) (Table 5).

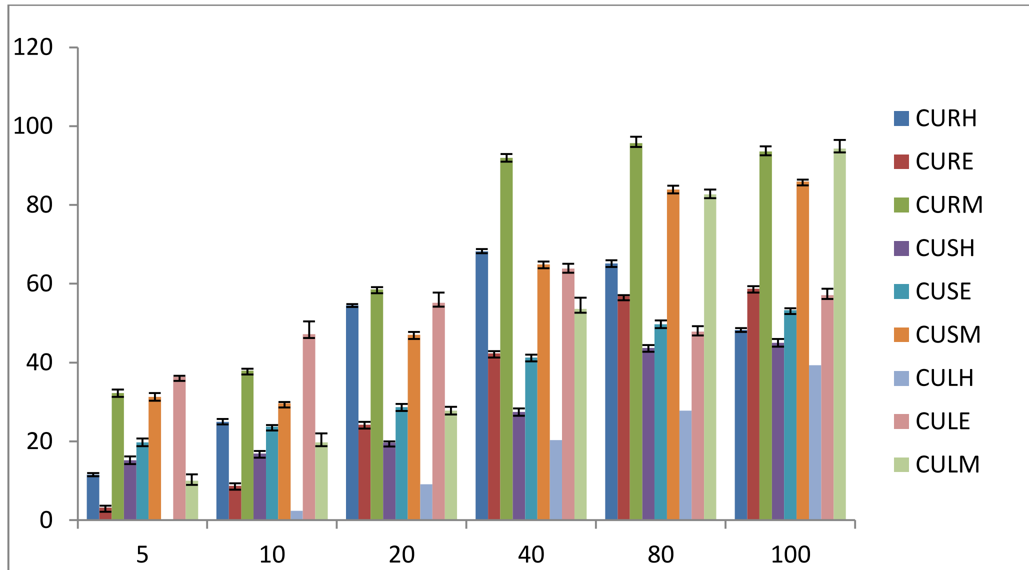


Figure 4b. The DPPH free radical scavenging activity of the different extracts of *C. aeruginosa*. The data shown as Mean ± SEM, N=3**.**

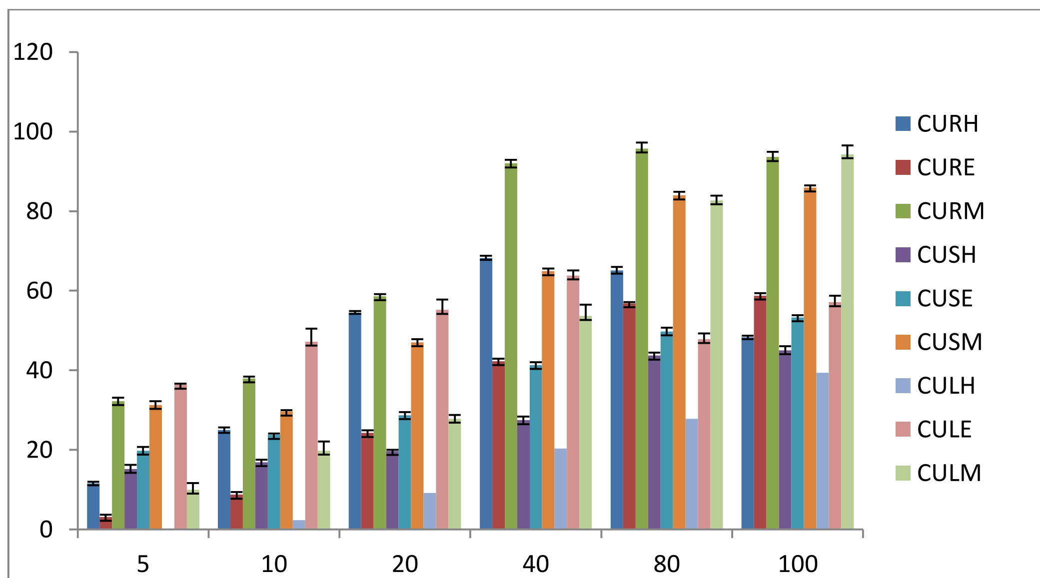


Figure 4b. The DPPH free radical scavenging activity of the different extracts of *C. aeruginosa*. The data shown as Mean ± SEM, N=3**.**

The DPPH scavenging activity of extracts of *A. galanga* showed rise in activity with an increase in concentration manner (5-100 µg/ml) as shown in Figure 4c. The scavenging activity were effective in the order: APLE (6.44 ± 0.07)> APSH (7.81 ± 0.3)> APLH (10.19 ± 0.04)> APRM (25.51 ± 0.14)> APRH (33.37 ± 0.7)> APSM (34.21 ± 1.5)> APLM (41.13 ± 0.02)> APRE (45.19 ± 0.02)> APSE (55.02 ± 0.28) with respect to ascorbic acid (1.071 ± 0.016µg/ml) (Table 5).

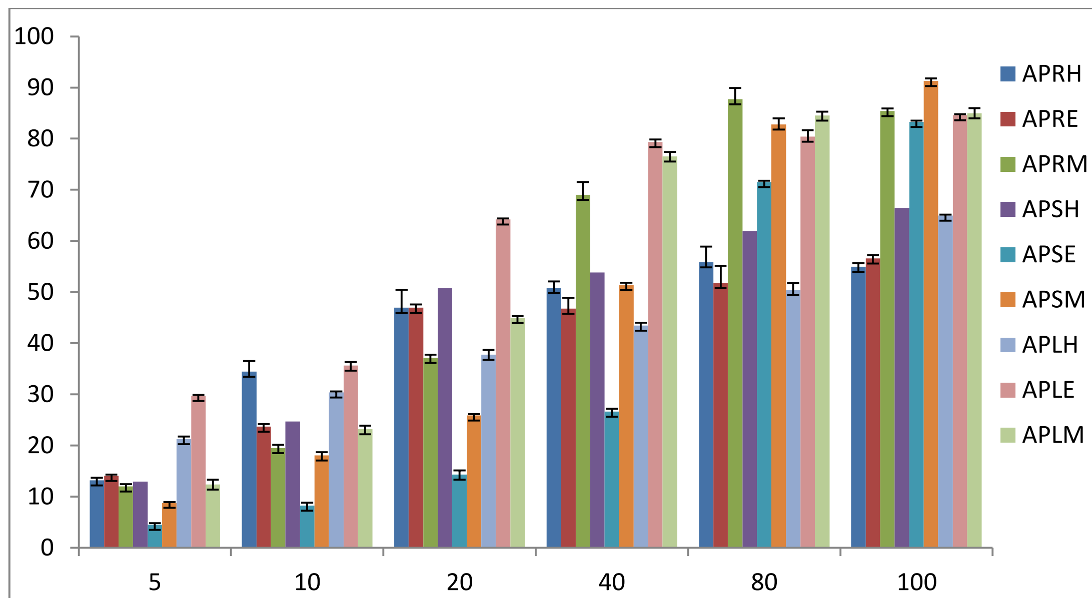


Figure 4c. The DPPH free radical scavenging activity of the different extracts of *A. galanga*. The data shown as Mean ± SEM, N=3**.**

4.4.2. ABTS^{+} cations scavenging activity

The ABTS^{+} cations scavenging of the extracts of *C. pictus* exhibited rise in activity with an increase in concentration manner (5-100 µg/ml) as shown in Figure 5a. The scavenging activity was effective in the order: CPLE (5.23 ± 0.12)> CPSH (15.45 ± 0.7)> CPLM (15.81 ± 0.23)> CPRH (28.07 ± 0.12)> CPLH (30.07 ± 0.5)> CPRM (44.11 ± 0.8)> CPSM (67.37 ± 0.05)> CPSE (79.14 ± 0.15)> CPRE (93.02 ± 0.14) with respect to ascorbic acid (1.31-± 0.006 µg/ml) (Table 5).

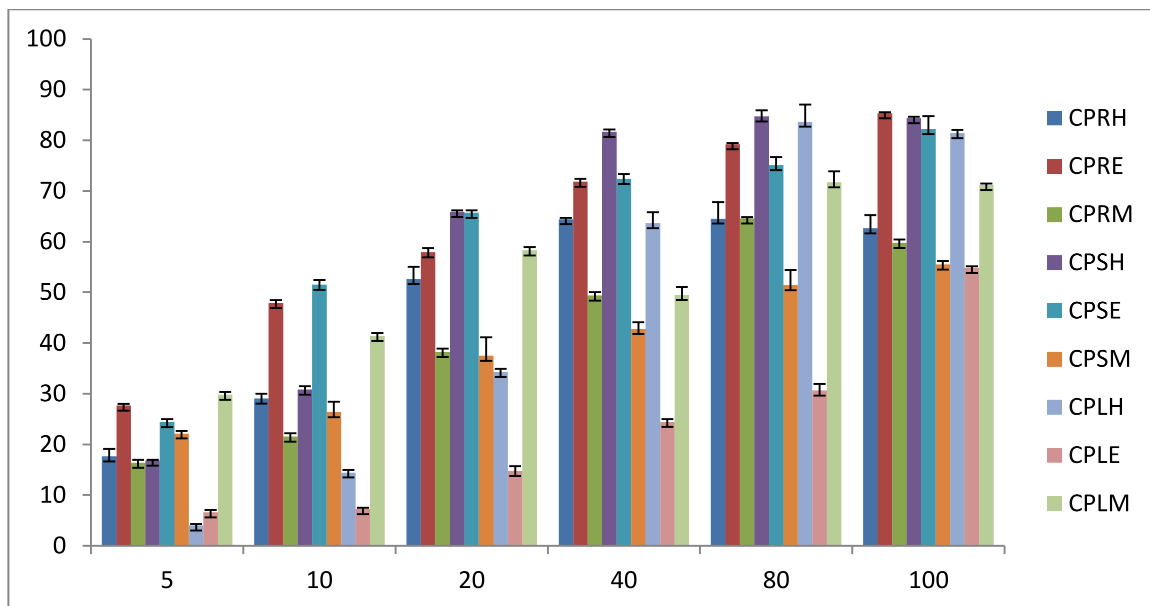


Figure 5a. The ABTS cation scavenging activity of the different extracts of *C. pictus*. The data shown as Mean ± SEM, N=3.

The $ABTS^+$ cations scavenging of the extracts of *C. aeruginosa* exhibited rise in activity with an increase in concentration manner (5-100 µg/ml) as shown in Figure 5b. The extracts' radical scavenging activity were effective in the order: CURM (12.87 ± 1.16)> CURE (13.42 ± 0.72)> CULM (24.69 ± 2.67)> CURH (34.69 ± 2.3)> CUSM (37.56 ± 0.88)> CUSE (57.4 ± 2.18)> CULE (66.71 ± 2.82) > CULH (109.8 ± 0.28)> CUSH (147.8 ± 2.65) and ascorbic acid was used as standards at the concentration 1–25 µg/ml with an IC_{50} of 1.31 ± 0.006 µg/ml) (Table 5).

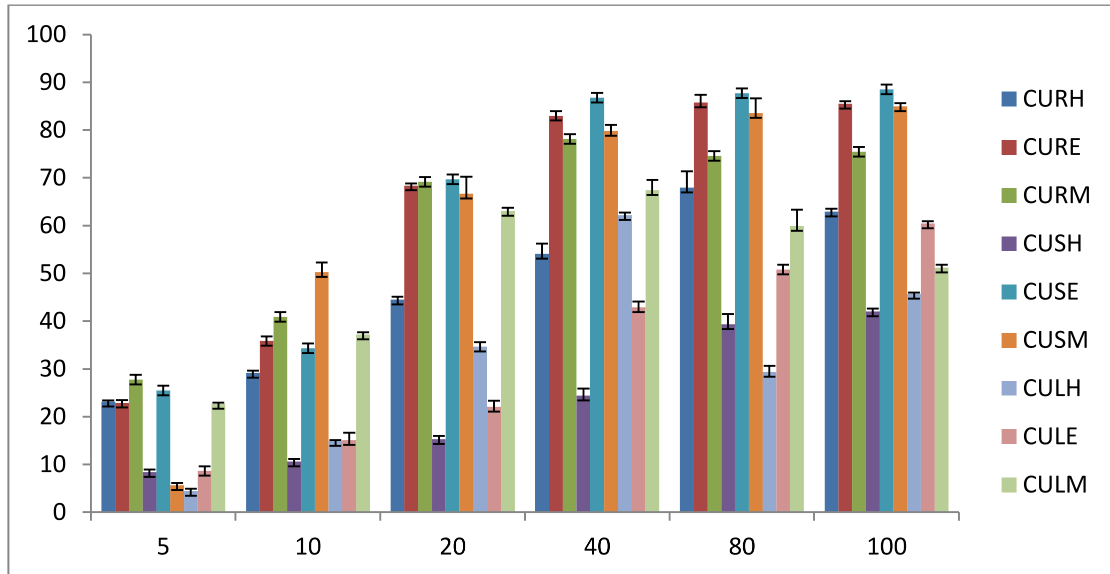


Figure 5b. The ABTS cation scavenging activity of the different extracts of *C. aeruginosa*. The data shown as Mean ± SEM, N=3.

The ABTS$^+$ cations scavenging of the extracts of *A. galanga* exhibited rise in activity with an increase in concentration manner (5-100 µg/ml) as shown in Figure 5c. The scavenging activity was effective in the order: APSH (1.18 ± 0.7)> APLE (5.15 ± 0.5) > APRM (14.74 ± 0.12)> APLH (20.66 ± 0.23)> APLM (25.36 ± 0.77)> APRH (28.07 ± 0.12> APSE (37.23 ± 0.8)> APSM (67.37 ± 0.05)> APRE (78.92 ± 0.15) with respect to ascorbic acid (1.31 ± 0.006 µg/ml) (Table 5).

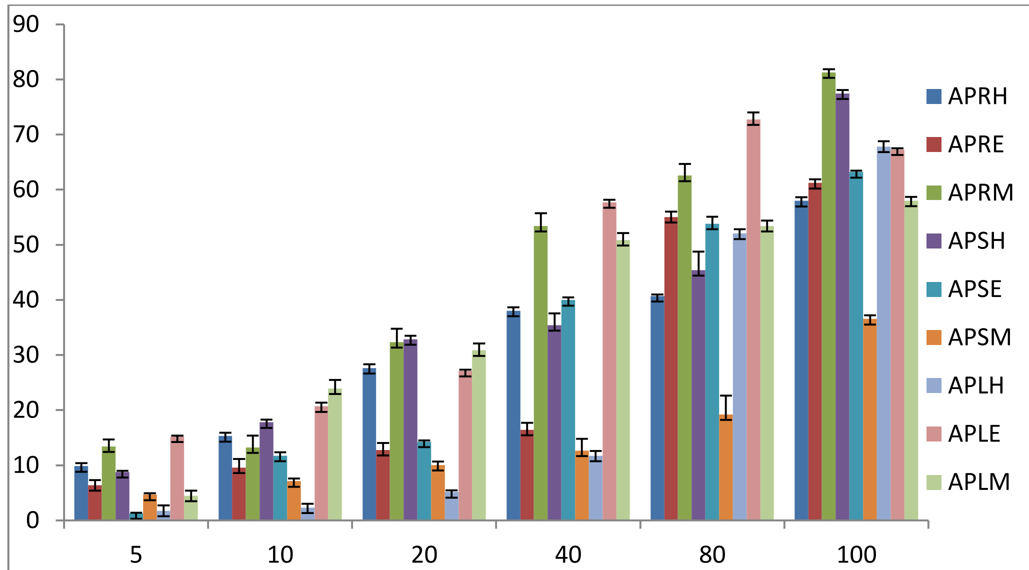


Figure 5c. The ABTS cation scavenging activity of the different extracts of *A. galanga*. The data shown as Mean ± SEM, N=3.

4.4.3. Superoxide anion scavenging assay

The superoxide scavenging activity of extracts of *C. pictus* exhibited increase in activity with a rise in concentration manner (5-100 µg/ml) as shown in Figure 6a. The decreasing order of scavenging activity of the extracts was found to be: CPLM (70.06 ± 0.7)> CPLE (86.43 ± 0.32)>CPLH (90.05 ± 0.73)> CPSE (90.86 ± 0.8)> CPRH (121.5 ± 0.5)> CPSM (134.9 ± 1.2)> CPRM (149.1 ± 0.5)> CPRE (160.7 ± 3.02)> CPSH (176.1 ± 3.04) with respect to Gallic acid (8.51 ± 0.31 µg/ml) (Table 5).

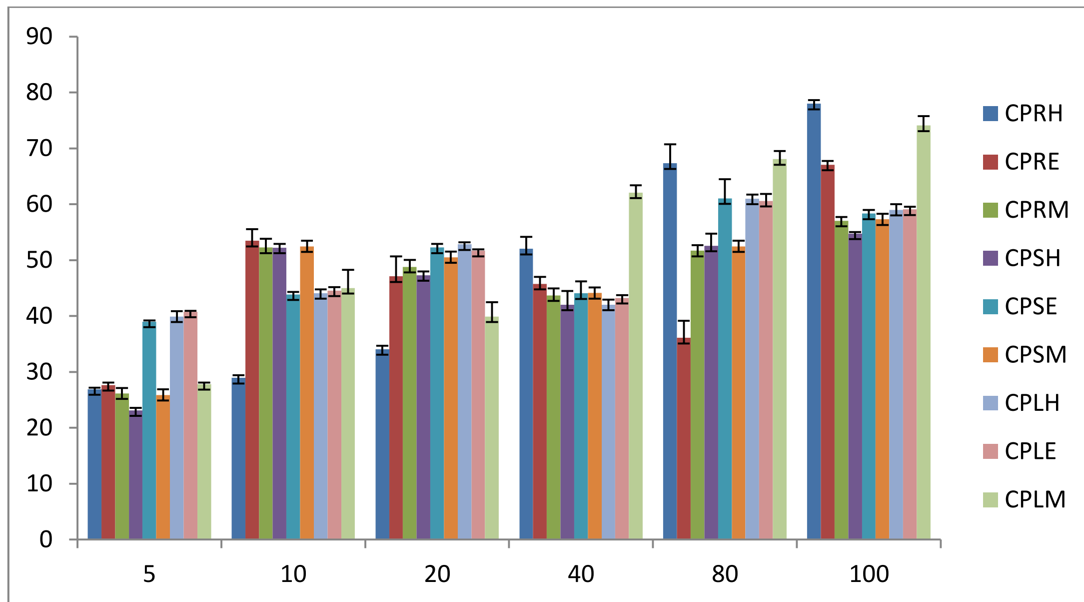


Figure 6a. The superoxide (O_2^-) scavenging activity of the different extracts of *C. pictus*. The data shown as Mean ± SEM, N=3.

The superoxide scavenging activity of extracts of *C. aeruginosa* exhibited increase in activity with a rise in concentration manner (5-100 µg/ml) as shown in Figure 6b. CURM 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 was found to be: CURM (35.92 ± 0.3)> CURE (54.23 ± 1.34)>SM (91.86 ± 0.61)> CURH (98 ± 1.4)> CUSE (103.4 ± 0.6)> CULM (128 ± 0.3)> CULE (142 ± 3.5)> CUSH (163.46 ± 4.03)> CULH (167.5 ± 3.04) with respect to Gallic acid (8.51 ± 0.31 µg/ml) (Table 5).

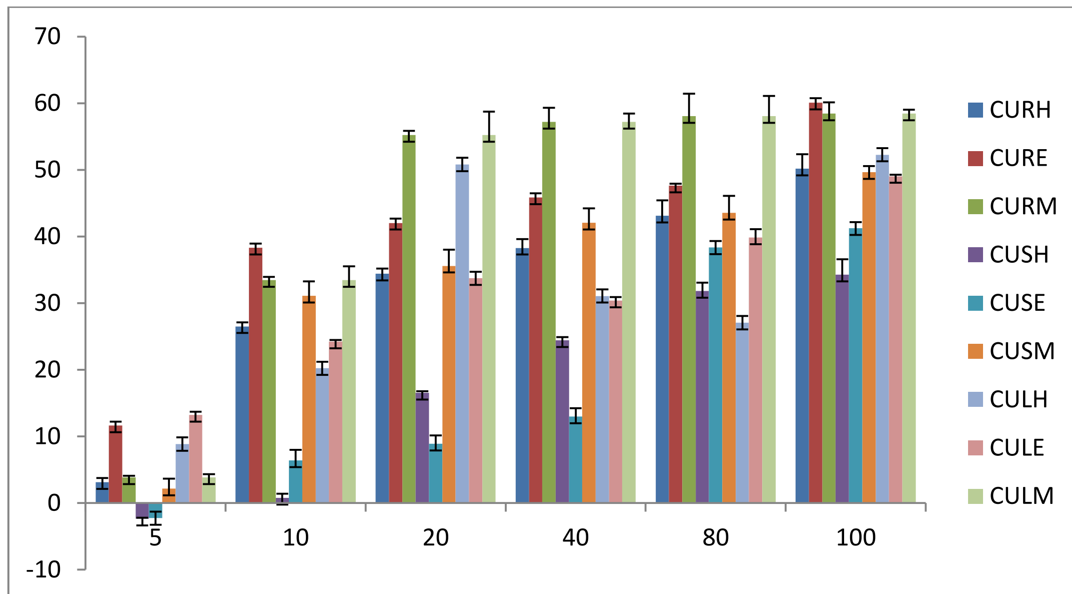


Figure 6b. The superoxide anion (O_2^-) scavenging activity of the different extracts of *C. aeruginosa* Roxb. The data shown as Mean ± SEM, N=3.

The superoxide scavenging activity of extracts of *A. galanga* exhibited increase in activity with a rise in concentration manner (5-100 µg/ml) as shown in Figure 6c. The decreasing order of scavenging activity of the extracts was found to be: APSM (24.39 ± 1.7)> APLM (47.96 ± 0.28)>APSE (57.37 ± 0.57)> APLE (59.58 ± 1.7)> APLH (75.43 ± 0.7)> APRM (95.28 ± 1.5)> APRE (161.8 ± 0.8)> APRH (179.1 ± 1.05)> APSH (183.1 ± 0.72) with respect to Gallic acid (8.51 ± 0.31 µg/ml) (Table 5).

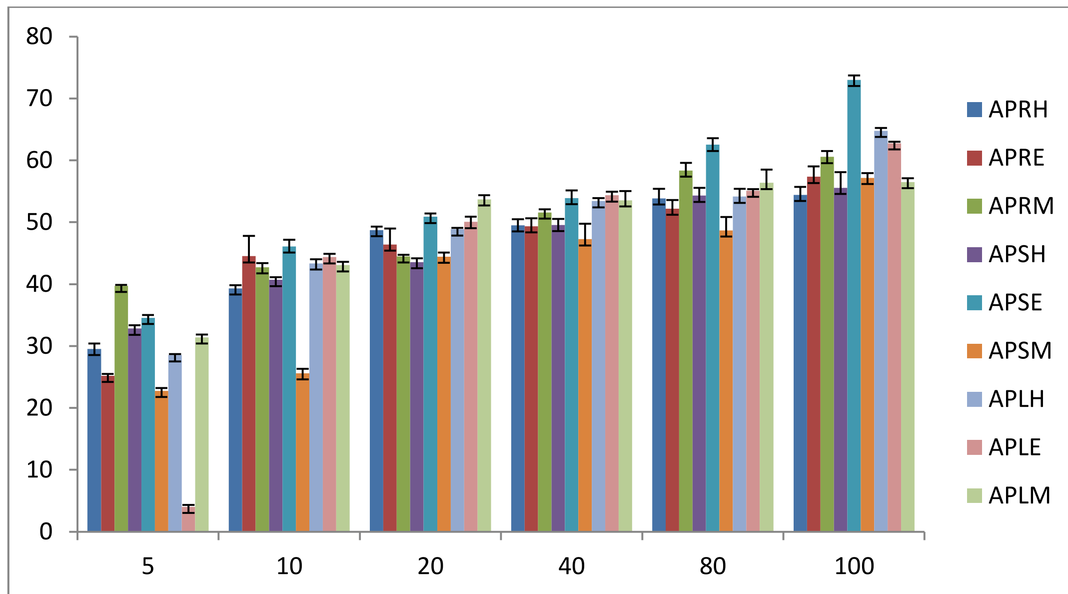


Figure 6c. The superoxide anion (O_2^-) scavenging activity of the different extracts of *A. galanga*. The data shown as Mean ± SEM, N=3.

4.4.4. Hydroxyl radical scavenging assay

The hydroxyl scavenging activity of extracts of *C. pictus* showed rise in activity with an increase in concentration manner (5-100 µg/ml) as shown in Figure 7a. The scavenging activity was effective in the order: CPSH (1.55 ± 0.05)> CPSM (2.35 ± 0.8)>CPSE (4.2 ± 0.7)> CPLM (4.25 ± 1.07)> CPLH (4.62 ± 0.52)> CPRH (5.06 ± 1.2)> CPRE (6.34 ± 0.8)> CPLE (9.184 ± 0.05)> CPRM (9.72 ± 0.07) with respect to ascorbic acid (0.85 ± 0.018 µg/ml) (Table 5).

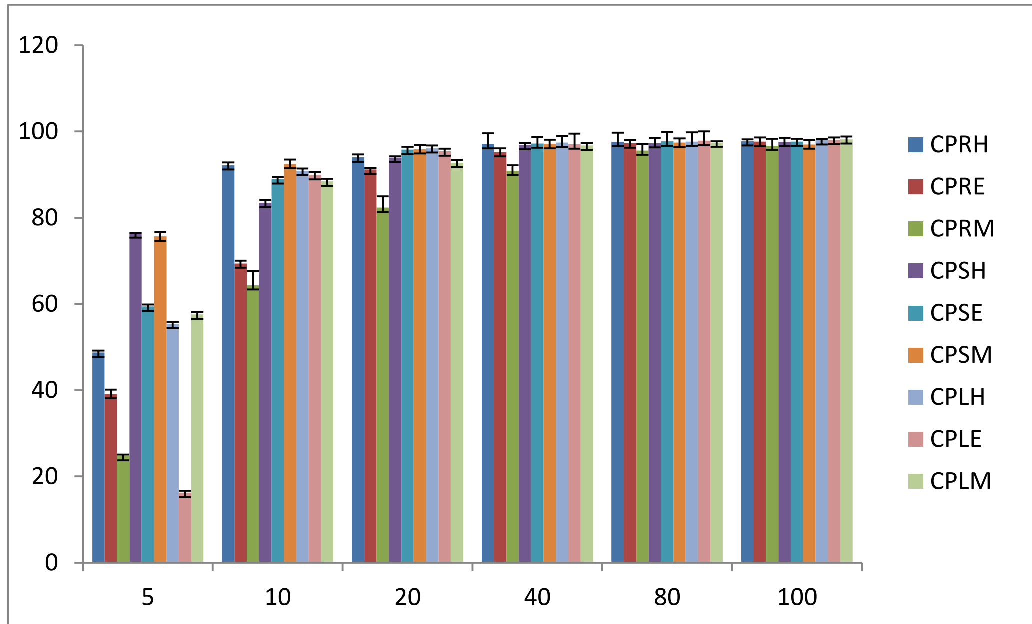


Figure 7a. The hydroxyl radical scavenging activity of the different extracts of *C. pictus*. The data shown as Mean ± SEM, N=3.

The hydroxyl scavenging activity of extracts of *C. aeruginosa* showed rise in activity with an increase in concentration manner (5-100 µg/ml) as shown in Figure 7b. The best IC_{50} value for hydroxyl scavenging activity was exhibited by CURE (3.42 ± 0.09 µg/ml). The scavenging activity was effective in the order: CURE (3.42 ± 0.09)> CUSM (5.30 ± 0.1)> CURM (5.32 ± 0.09)> CURH (5.85 ± 0.04)> CUSE (6.74 ± 0.9)> CULM (7.73 ± 0.5)> CUSH (8.89 ± 0.03)> CULH (9.35 ± 0.01)> CULE (14.42 ± 0.28) with respect to ascorbic acid (0.85 ± 0.018 µg/ml) (Table 5).

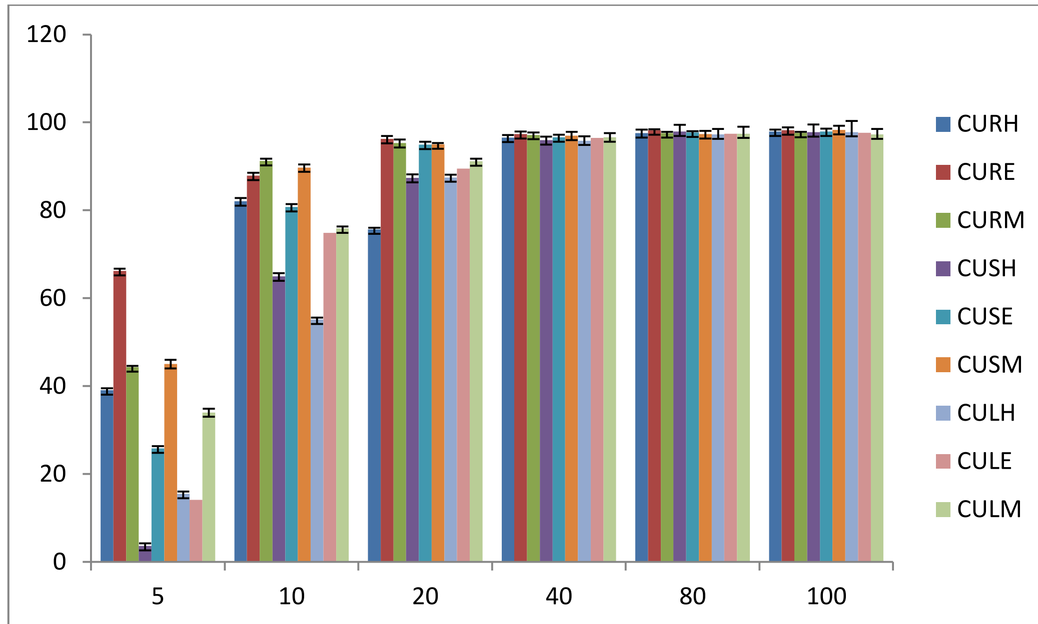


Figure 7b. The hydroxyl radical scavenging activity of the different extracts of *C. aeruginosa* The data shown as Mean ± SEM, N=3.

The hydroxyl scavenging activity of extracts of *A. galanga* showed rise in activity with an increase in concentration manner (5-100 μg/ml) as shown in Figure 7c. The scavenging activity was effective in the order: APSE (1.81 ± 0.07)> APLH (2.36 ± 0.5)>APSM (2.53 ± 0.3)> APSH (3.00 ± 0.05)> APLE (3.57 ± 1.02)> APRE (3.65 ± 0.40)> APRH (4.06 ± 0.8)> APLM (4.13 ± 0.87)> APRM (5.06 ± 0.03) with respect to ascorbic acid (0.85 ± 0.018 μg/ml) (Table 5).

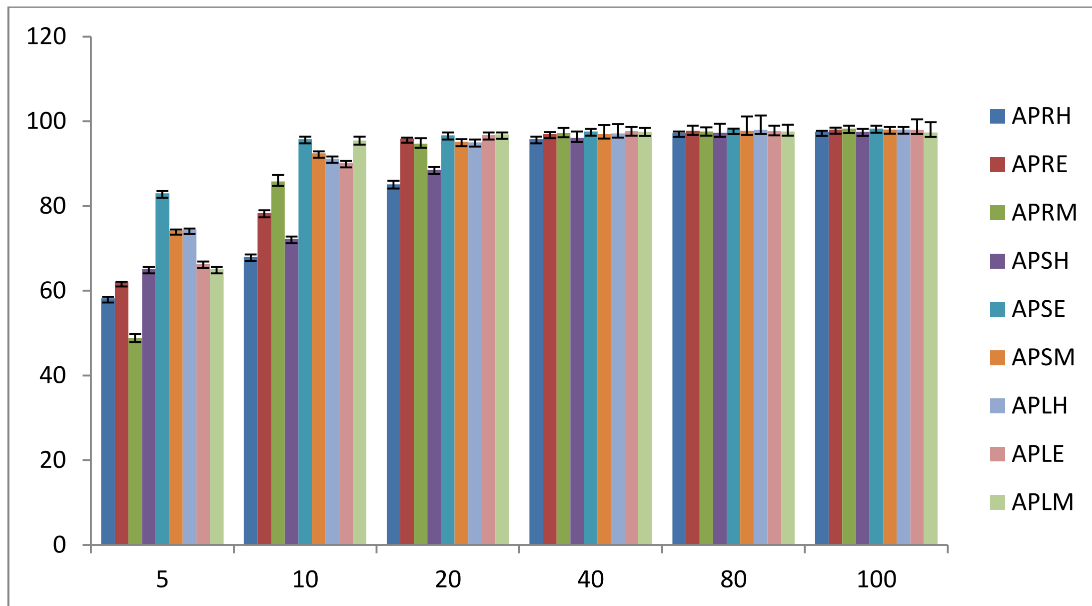


Figure 7c. The hydroxyl radical scavenging activity of the different extracts of *A. galanga*. The data shown as Mean ± SEM, N=3.

4.4.5. Phosphomolybdenum assay

The total antioxidant capacity of the extracts was evaluated using phosphomolybdate method. The total antioxidant capacity of the extracts of *C. pictus* exhibited increase in activity with a rise in concentration manner (5-100 µg/ml) as shown in Figure 8a. The detected order of IC_{50} value: CPRM (1.36 ± 0.05)> CPLH (23.42 ± 0.07)> CPRH (43.98 ± 0.05)> CPLM (44.56 ± 0.28)> CPSE (68.19 ± 0.15)> CPLE (70.93 ± 0.08)> CPRE (77.83 ± 0.55)> CPSM (82.6 ± 0.20)> CPSH (117.1 ± 0.07) with respect to ascorbic acid (0.85 ± 0.018µg/ml) (Table 5).

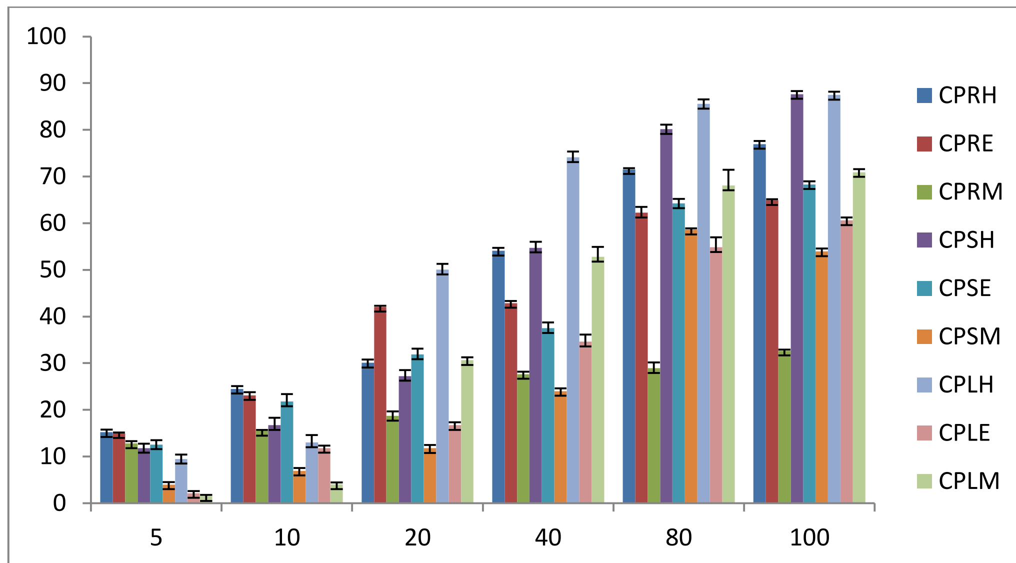


Figure 8a. The antioxidant capacity of the different extracts of *C. pictus* by phosphomolybdenum assay. The data shown as Mean ± SEM, N=3.

The total antioxidant capacity of the extracts of *C. aeruginosa* exhibited increase in activity with a rise in concentration manner (5-100 µg/ml) as shown in Figure 8b. The detected order of IC_{50} value: CURM (3.97 ± 0.01)> CURE (4.90 ± 0.02)> CUSM (7.11 ± 0.02)> CULE (8.85 ± 0.15)> CUSE (9.51 ± 0.03)> CULM (10.25 ± 0.06)> CURH (17.90 ± 0.42)> CUSH (27.68 ± 1.06)> CULH (52.55 ± 0.63) with respect to ascorbic acid (0.85 ± 0.018µg/ml) (Table 5).

The total antioxidant capacity of the extracts of *A. galanga* exhibited increase in activity with a rise in concentration manner (5-100 µg/ml) as shown in Figure 8c. The detected order of IC_{50} value: APLH (3.93 ± 0.05)> APSM (25.02 ± 0.07)> APSH (28.77 ± 0.05)> APRE (30.3 ± 0.28)> APLM (34.19 ± 0.15)> APLE (35.67 ± 0.08)> APRH (41.89 ± 0.55)> APRM (49.76 ± 0.20)> APSE (68.24 ± 0.07) with respect to ascorbic acid (0.85 ± 0.018µg/ml) (Table 5).

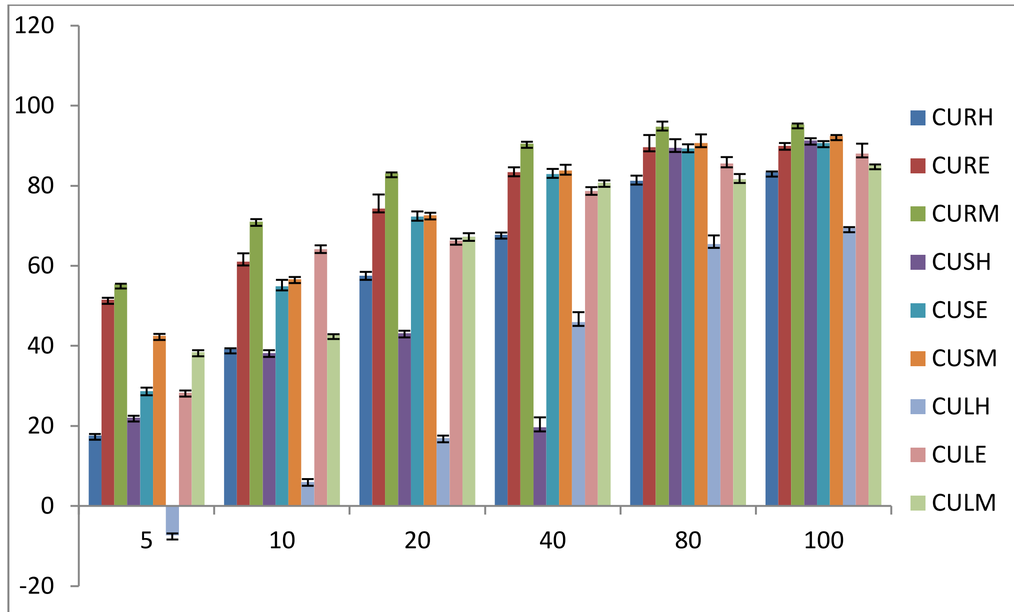


Figure 8b. The antioxidant capacity of the different extract of *C. aeruginosa* by phosphomolybdenum assay. The data shown as Mean ± SEM, N=3.

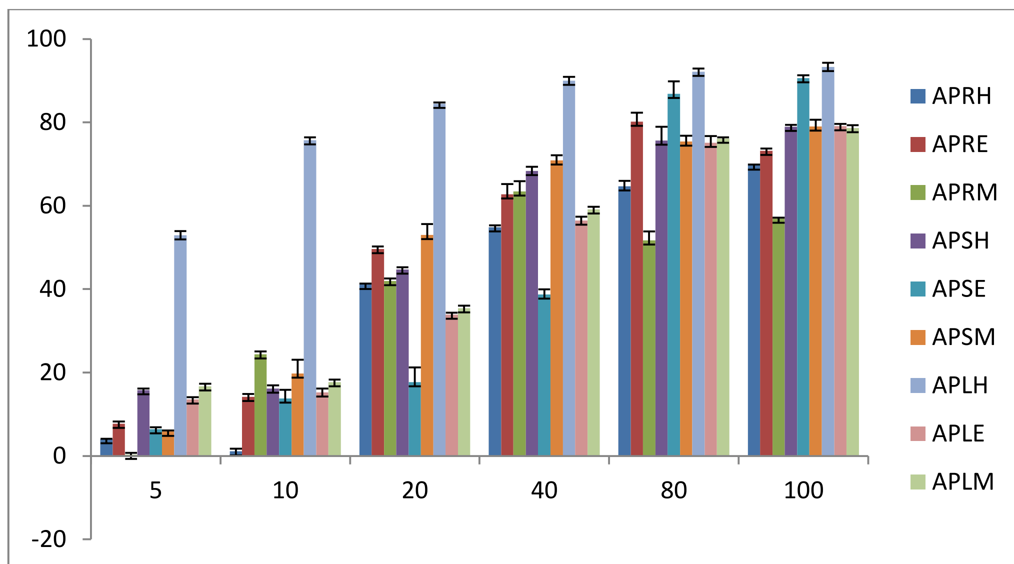


Figure 8c. The antioxidant capacity of the different extracts of *A. galanga* by phosphomolybdenum assay. The data shown as Mean ± SEM, N=3.

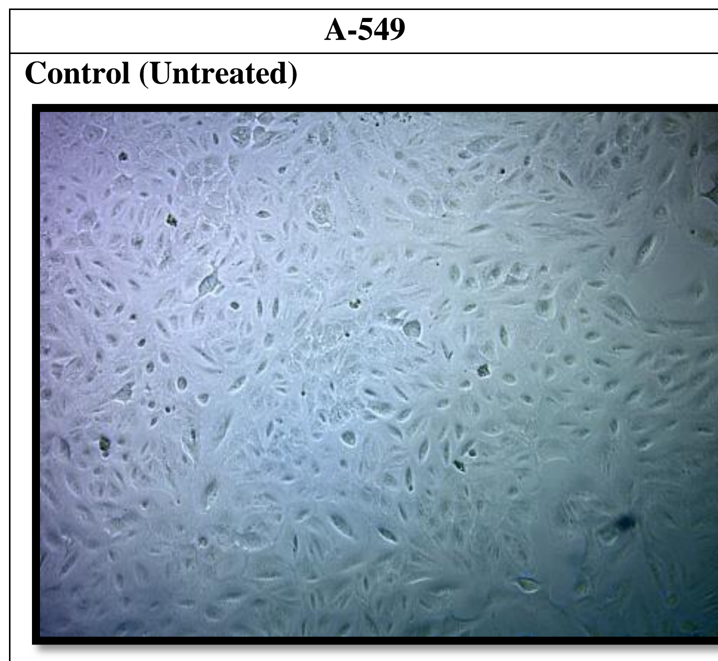
Table 5. Antioxidant activity (IC_{50} values (µg/ml) of the different extract of *C. pictus*, *C. aeruginosa* and *A. galanga*. Values expressed are mean ± standard deviation (SEM) of triplicate measurements.

Sample	DPPH	ABTS+	Superoxide anion (O2-)	Hydroxyl radical	Phosphomolybdenum
CPRH	44.33 ± 0.14	28.07 ± 0.12	121.5 ± 0.5	5.06 ± 1.2	43.98 ± 0.05
CPRE	24.61 ± 0.04	93.02 ± 0.14	160.7 ± 3.02	6.34 ± 0.8	77.83 ± 0.55
CPRM	56.66 ± 0.7	44.11 ± 0.8	149.1 ± 0.5	9.72 ± 0.07	1.36 ± 0.05
CPSH	90.17 ± 1.5	15.45 ± 0.7	176.1 ± 3.04	1.55 ± 0.05	117.1 ± 0.07
CPSE	115 ± 0.02	79.14 ± 0.15	90.86 ± 0.8	4.2 ± 0.7	68.19 ± 0.15
CPSM	9.06 ± 0.07	67.37 ± 0.05	134.9 ± 1.2	2.35 ± 0.8	82.6 ± 0.20
CPLH	114.7 ± 0.02	30.07 ± 0.5	90.05 ± 0.73	4.62 ± 0.52	23.42 ± 0.07
CPLE	174.2 ± 0.28	5.23 ± 0.12	86.43 ± 0.32	9.18 ± 0.05	70.93 ± 0.08
CPLM	20.89 ± 0.3	15.81 ± 0.23	70.06 ± 0.7	4.25 ± 1.07	44.56 ± 0.28
CURH	33.71 ± 0.63	34.69 ± 2.3	98 ± 1.4	5.85 ± 0.04	17.90 ± 0.42
CURE	64.27 ± 2.08	13.42 ± 0.72	54.23 ± 1.34	3.42 ± 0.09	4.90 ± 0.02
CURM	12.22 ± 0.11	12.87 ± 1.16	35.92 ± 0.3	5.32 ± 0.09	3.97 ± 0.01
CUSH	144.3 ± 0.1	147.8 ± 2.65	163.46 ± 4.03	8.89 ± 0.03	27.68 ± 1.06
CUSE	81.92 ± 0.4	57.4 ± 2.18	103.4 ± 0.6	6.74 ± 0.9	9.51 ± 0.03
CUSM	18.89 ± 0.23	37.56 ± 0.88	91.86 ± 0.61	5.30 ± 0.1	7.11 ± 0.02
CULH	157.5 ± 0.14	109.8 ± 0.28	167.5 ± 3.04	9.35 ± 0.01	52.55 ± 0.63
CULE	20.91 ± 0.72	66.71 ± 2.82	142 ± 3.5	14.42 ± 0.28	8.85 ± 0.15
CULM	31.81 ± 0.38	24.69 ± 2.67	128 ± 0.3	7.73 ± 0.5	10.25 ± 0.06
APRH	33.37 ± 0.7	28.07 ± 0.12	179.1 ± 1.05	4.06 ± 0.8	41.89 ± 0.55
APRE	45.19 ± 0.02	78.92 ± 0.15	161.8 ± 0.8	3.65 ± 0.40	30.3 ± 0.28
APRM	25.51 ± 0.14	14.74 ± 0.12	95.28 ± 1.5	5.06 ± 0.03	49.76 ± 0.20
APSH	7.81 ± 0.3	1.18 ± 0.7	183.1 ± 0.72	3.00 ± 0.05	28.77 ± 0.05
APSE	55.02 ± 0.28	37.23 ± 0.8	57.37 ± 0.57	1.81 ± 0.07	68.24 ± 0.07
APSM	34.21 ± 1.5	20.66 ± 0.23	24.39 ± 1.7	2.53 ± 0.3	25.02 ± 0.07
APLH	10.19 ± 0.04	20.66 ± 0.23	75.43 ± 0.7	2.36 ± 0.5	3.93 ± 0.05
APLE	6.44 ± 0.07	5.15 ± 0.5	59.58 ± 1.7	3.57 ± 1.02	35.67 ± 0.08
APLM	41.13 ± 0.02	25.36 ± 0.77	47.96 ± 0.28	4.13 ± 0.87	34.19 ± 0.15

4.5. Cytotoxicity

Table.6 shows the results of the screening of the different extracts of *C. aeruginosa, C. pictus and A. galanga*. IC_{50} values were calculated for these extracts against four human cancer cell lines, namely, A549, Hela, AGS, HT-29 and a normal cell: L-132.

The cytotoxicity revealed that the different extracts were capable of killing the cancer cells but insignificant toxicity against normal (L-132) cell (Table 6 and Figure 9a- Figure 9e). CURM 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, CURM, exhibited significant activity against A-549, AGS and Hela cells with an IC_{50} value of 15.42 ± 1.5 µg/ml, 14.02 ± 0.57 µg/ml and 25.40 ± 0.13 µg/ml respectively, which is in agreement to NCI, USA cutoff of ≤ 25 µg/mL for crude plant extracts (Geran et al 1972, Lee & Houghton 2005). Overall, the different extracts of *C. aeruginisa* showed the best cytotoxic activity against the cancer cells. Therefore, the methanol extracts of the different parts of *C. aeruginisa* were selected for further investigations. APLH also showed significant cytotoxic activity against A-549, AGS, HT29 cells with an IC_{50} value of 13.2 ± 0.8 µg/ml, 15.87 ± 1.24 µg/ml and 20.46 ± 0.72 µg/ml respectively, then was included for the remaining experiments. 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 2-4 µg/ml for A-549, Hela, AGS, HT-29 and L-132 cells (Table 6).

A-549
Control (Untreated)


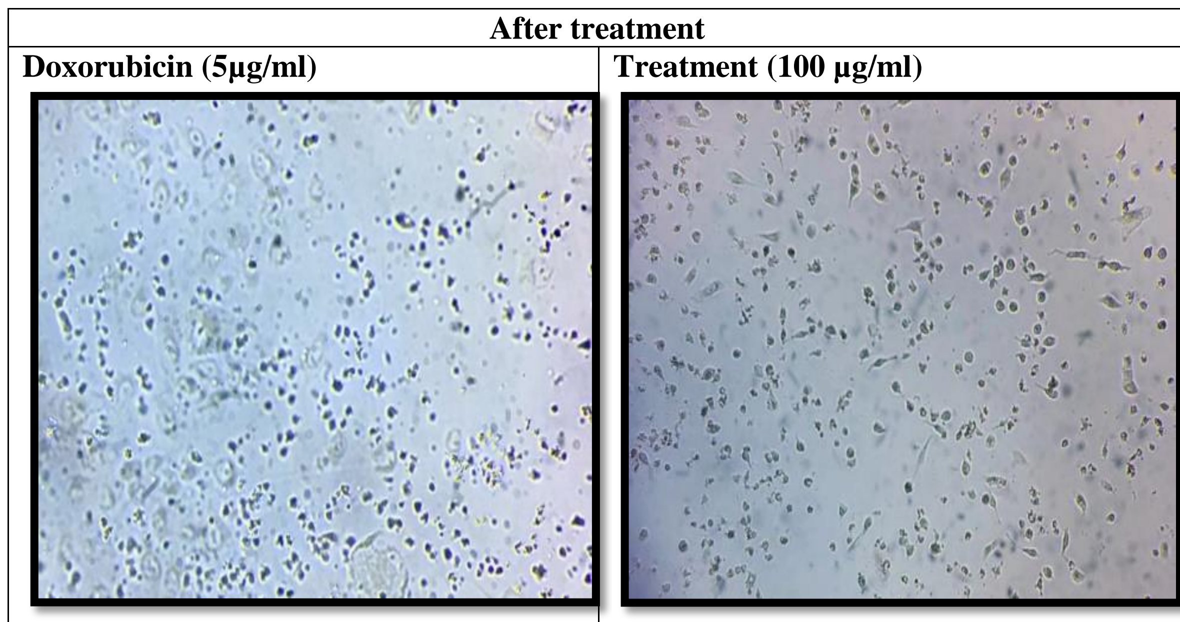
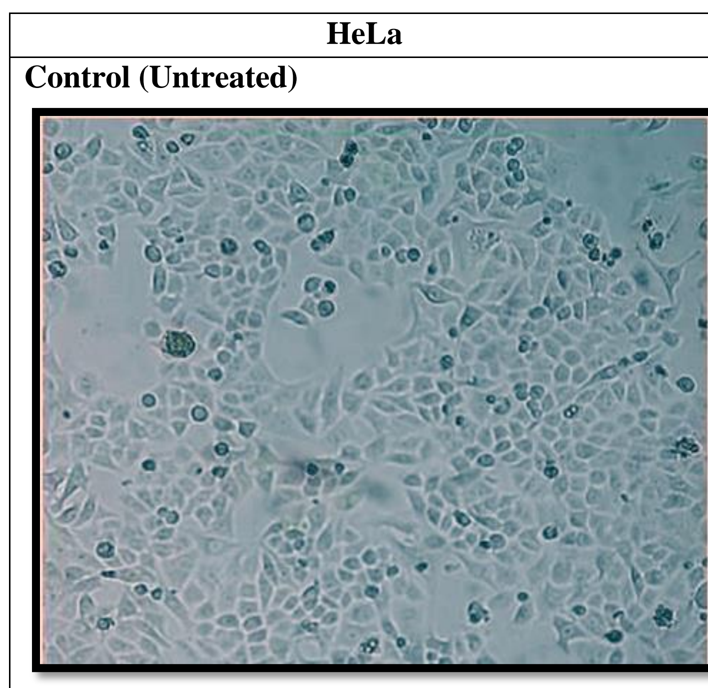
After treatment	
Doxorubicin (5µg/ml)	Treatment (100 µg/ml)
	

Figure 9a: The cytotoxicity effect of the different extracts against A-549 cells.



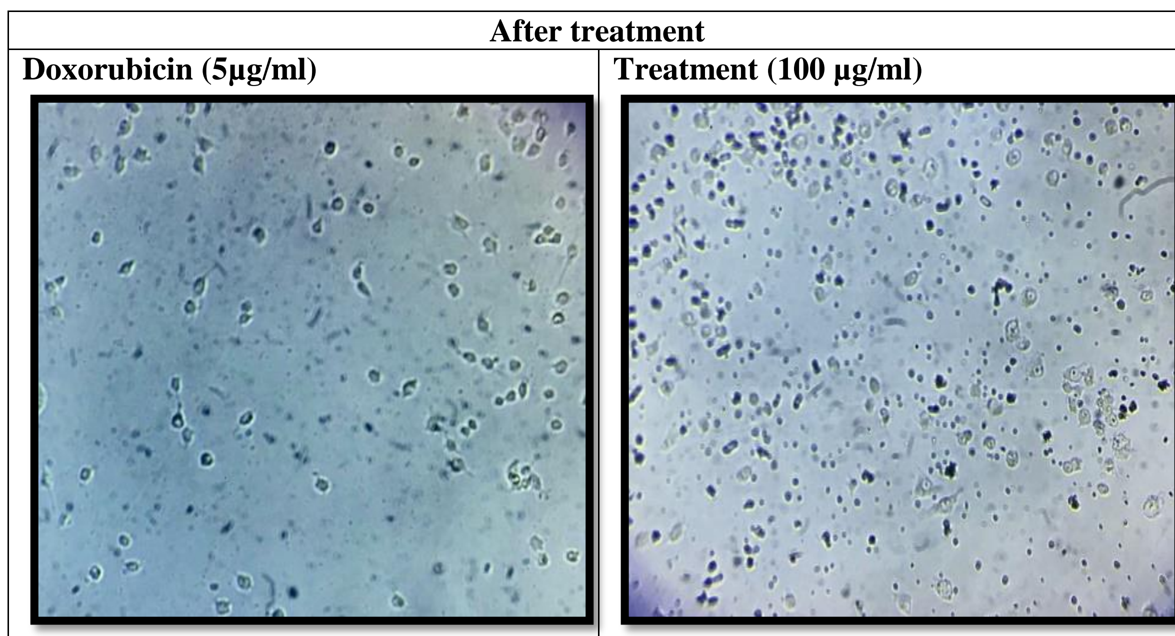
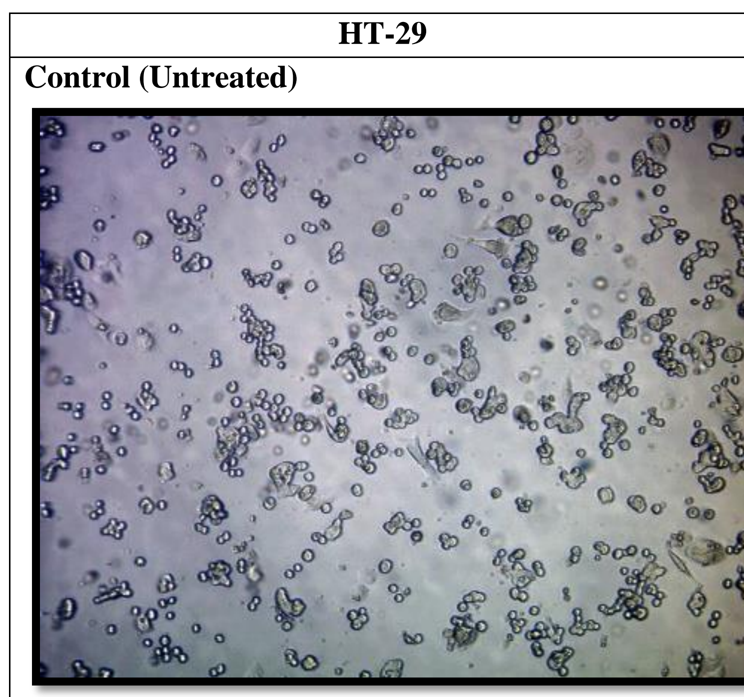


Figure 9b: The cytotoxicity effect of the different extracts against HeLa cells.



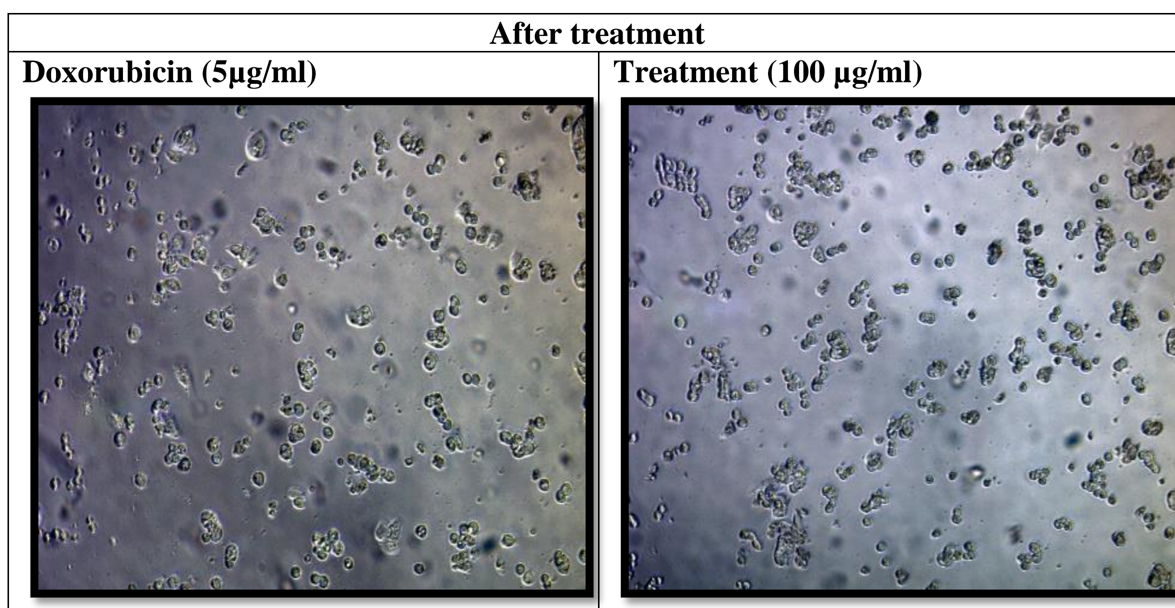
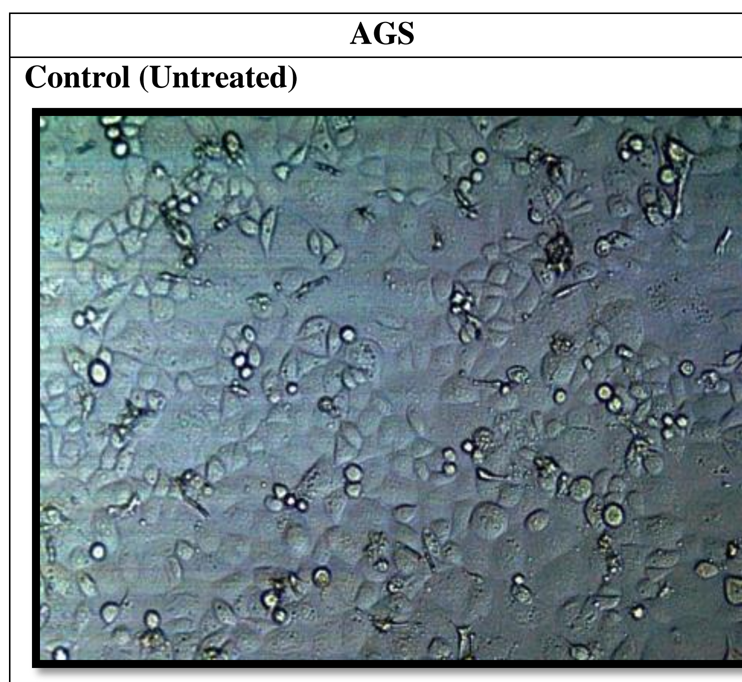


Figure 9c: The cytotoxicity effect of the different extracts against HT-29 cells.



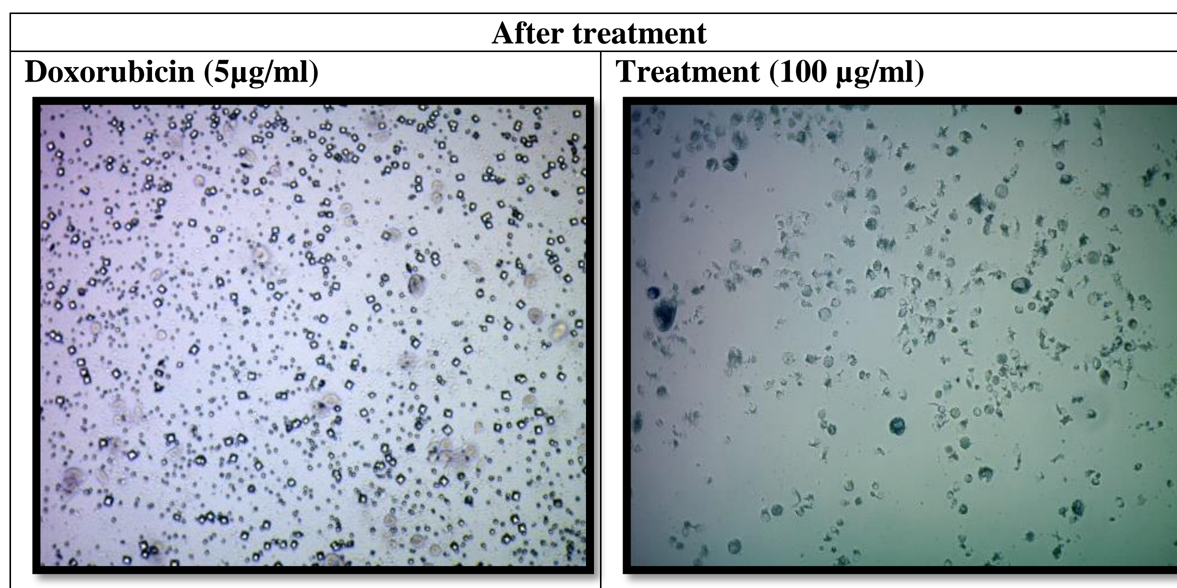
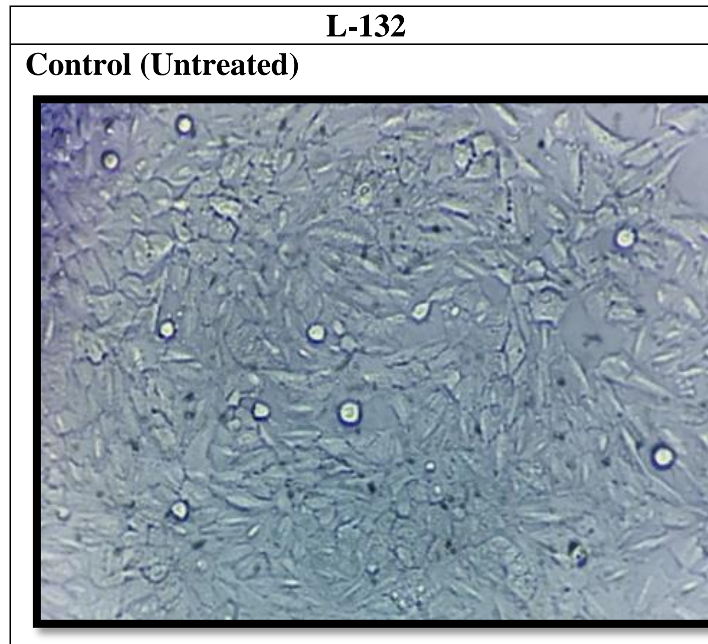


Figure 9d: The cytotoxicity effect of the different extracts against AGS cells.



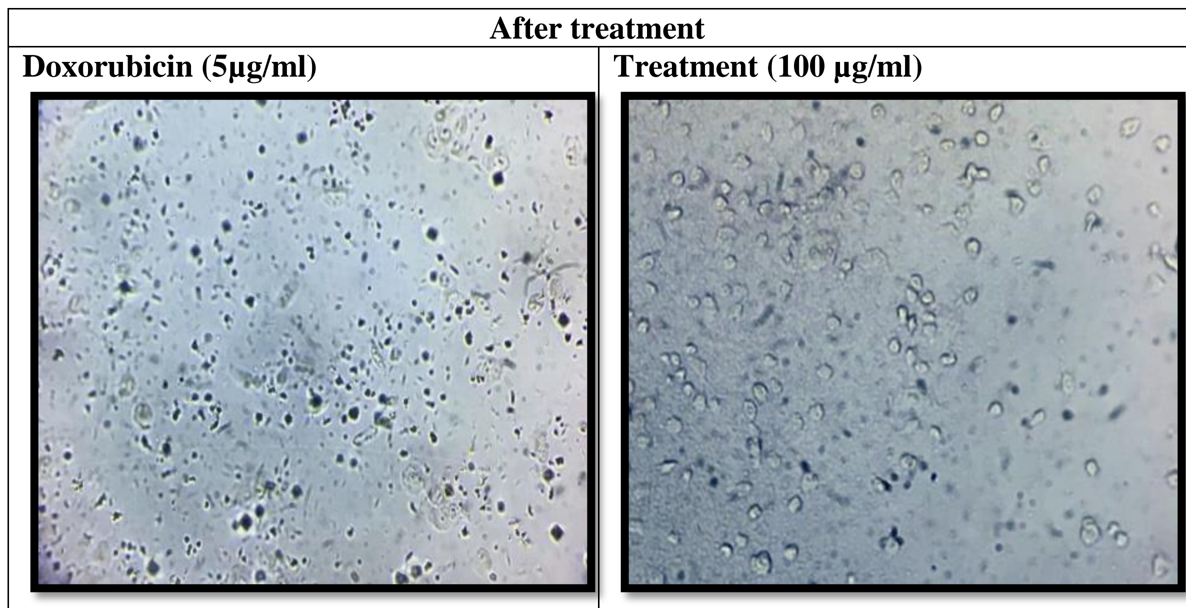


Figure 9e: The cytotoxicity effect of the different extracts against L-132 cells.

Table 6. Cytotoxic activity (IC_{50} values (μg/ml) of different extracts against human cancer and normal cell lines.

	A549	**AGS**	**HT29**	**HELA**	**L-132**
CPRH	143.6	73.3	26.09	68.31	178
CPRE	158.8	123.5	115.5	97.39	109.2
CPRM	34.22	56.63	65.59	>180	150.6
CPSH	116.8	128.2	69.6	26.51	143
CPSE	25.09	92.96	25.28	35.65	82.62
CPSM	68.78	144	57.2	69.55	117.6
CPLH	105.6	148	105	>180	170.4
CPLE	169.9	95.13	118.6	>180	102.8
CPLM	71.97	90.2	73.36	105	99.55
CURH	65.06	142.5	66.11	43.65	>180
CURE	36.93	25.2	24.33	61.24	>180
CURM	15.42	14.02	31	25.4	113
CUSH	96.70	163.7	105.7	93.21	>180
CUSE	109.75	138.5	69.65	104.8	>180
CUSM	34.4	148	89.11	33.69	102.7
CULH	116.45	165.3	178.8	138.85	>180
CULE	162.6	97.88	143.3	154.5	>180
CULM	35.71	21.55	53.35	57.09	86.09
APRH	48.66	115.3	67.63	75.9	133.6
APRE	65.73	101.8	70.46	35.3	>180
APRM	65.38	140.7	33.44	32.2	124.3
APSH	36.59	74.88	43.23	41.6	94.75
APSE	79.75	132.3	>180	73.7	142
APSM	38.86	>180	168.1	>180	148
APLH	13.2	15.87	20.46	45.83	81.76
APLE	134	39.31	124.6	>180	>180
APLM	164.5	81.52	150	77.76	82.28
DOX	2.038	2.552	3.464	3.65	3.448

4.6. Comet assay

The level of DNA damage was examined using the alkaline comet assay. The treated groups induced DNA damage in A-549 and HeLa cells with prominent increased tail length and tail moment in treated group when compared to untreated control (Figure.10a and Figure 10b). Doxorubicin was used as positive control.

Treatment with APLH against A549 cells showed induction of DNA damage with increased tail DNA (64 ± 0.90 %) and tail moment (63.24 ± 0.77 %). Treatment with CURM also showed DNA damage with increased tail DNA (59.30 ± 1.77 %) and tail moment (62.80 ± 0.55 %) (Figure 11a). The untreated control in A-549 showed 4.74 ± 0.17 % tail DNA and 0.11 ± 0.03 % tail moment. CUSM induced greater DNA damage with longer tail DNA (52.41 ± 0.19 %) and tail moment (54.51 ± 1.13 %) when compared with CULM (34.17 ± 0.17 % tail DNA and 27.95 ± 0.44 % tail moment).

Among the extracts, APLH showed significant increased tail DNA (65.36 ± 0.26%) and tail moment (63.24 ± 0.77 %) when compared to untreated control (13.86 ± 0.6% tail DNA and 0.47 % ± 0.14 % tail moment) followed by CURM with significant increased tail DNA (61.43± 0.24 %) and tail moment (50.06 ± 0.45 %) in Hela cells (Figure 11). CUSM induced greater DNA damage with longer tail DNA (40.04 ± 2.23 %) and tail moment (42.69 ± 2.9 %) when compared with CULM (34.2 ± 2.73 % tail DNA and 27.29 ± 1.70 % tail moment). Doxorubicin showed significant increased tail DNA (65.62 ± 1.01%) which is almost similar to the tail DNA induced by APLH. Tail moment induced by Doxorubicin was higher (83.28 ± 0.57 %) than all the treated groups (Figure 11b).

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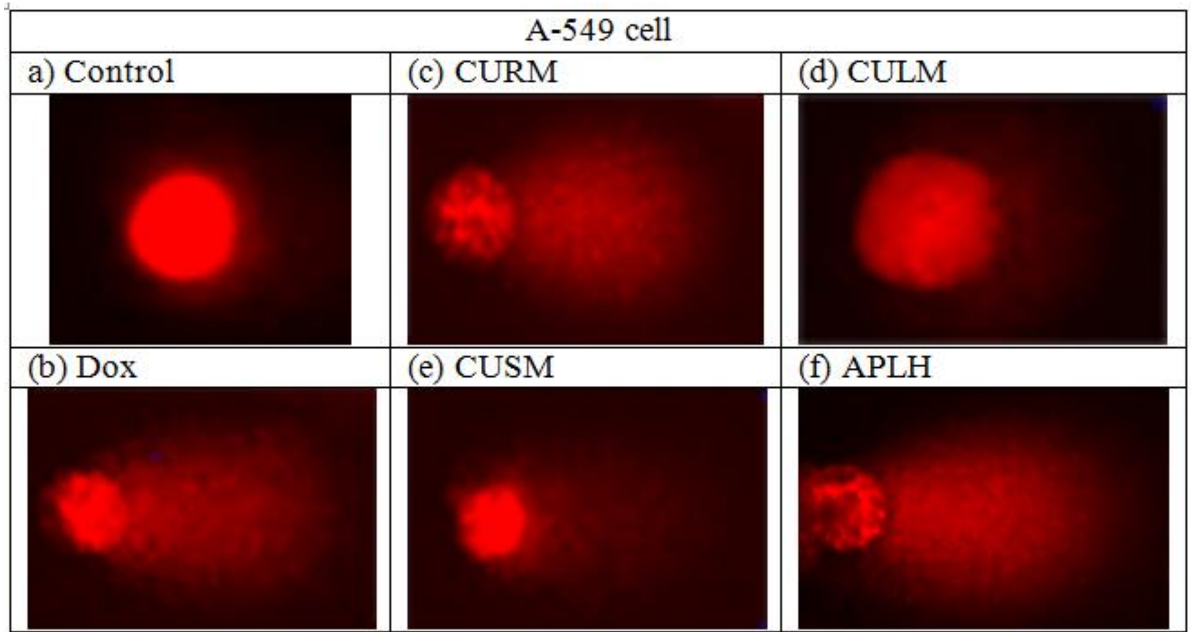


Figure 10a. Fluorescence Comet images observed in A-549 cells.

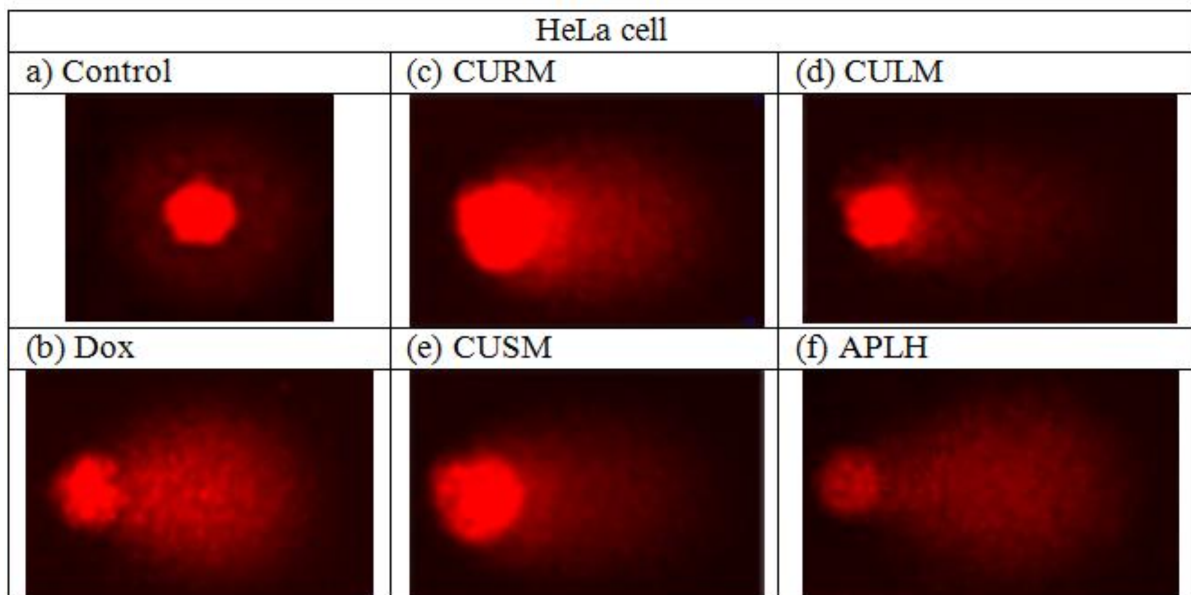
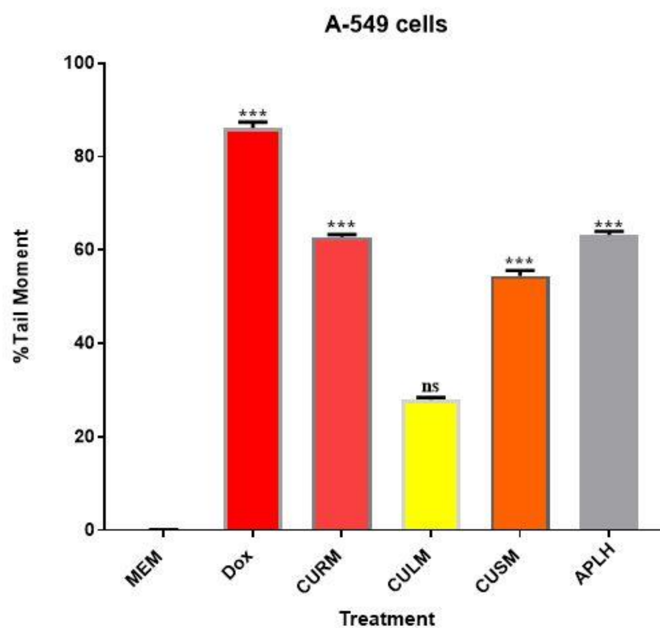


Figure 10b. Fluorescence Comet images observed in HeLa cells.



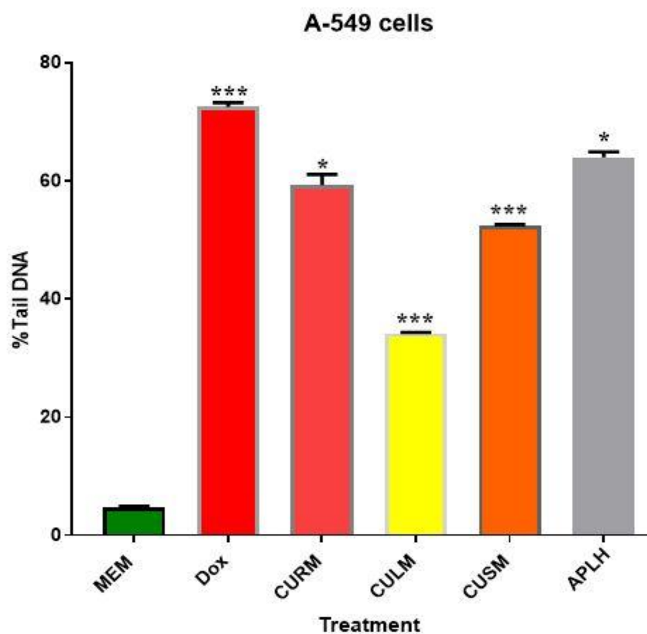
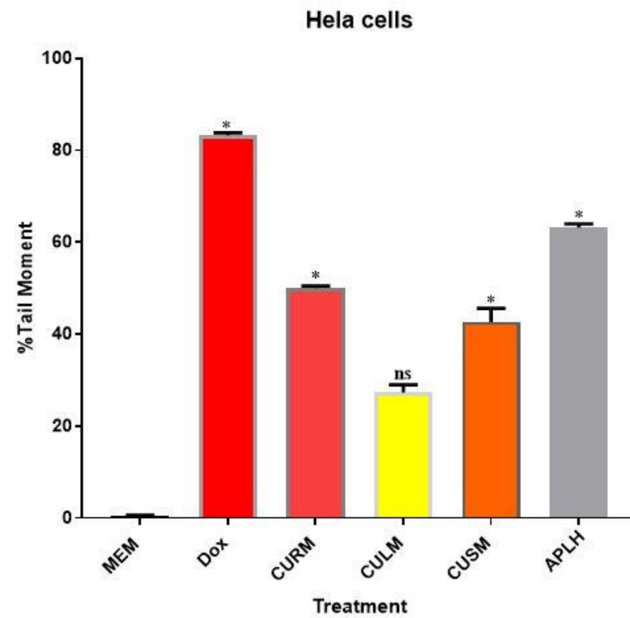


Figure 11a. The extent of DNA damage expressed in terms of Tail DNA (%) and Tail moment (%) in A-549 cells. The data represent Mean ± SEM, N=3. The one-way ANOVA was performed to test the significant level between the control and treatment groups at a significant level of *p<0.5, **p<0.01 and ***p<0.001.



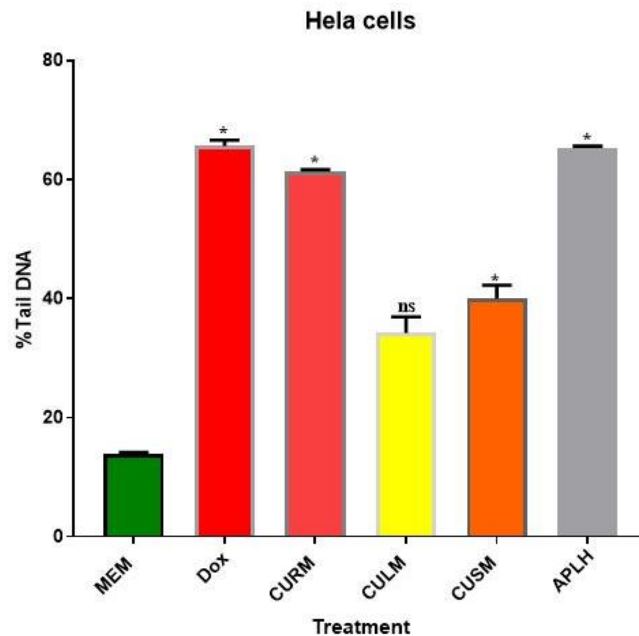


Figure 11b. The extent of DNA damage expressed in terms of Tail DNA (%) and Tail moment (%) in HeLa cells. The data represent Mean ± SEM, N=3. The one-way ANOVA was performed to test the significant level between the control and treatment groups at a significant level of *p<0.5, **p<0.01 and ***p<0.001.

4.7. Activation of Apoptotic Cell Death by the treated groups

AO/EB staining method was used for analyzing the apoptotic effect in A-549 and HeLa cells. Treatment with the treated groups against the A-549 and HeLa cells revealed induction of apoptotic and necrotic cells for 48 hours with an increased rate of cell death when compared to the L-132 cells (Figure 12). The apoptosis induction was similar to doxorubicin (76.63 ± 2.31), which was significantly higher than the untreated control (2.03 ± 0.70%).

A-549 cells treated with 15.42 ± 1.5 µg/ml of CURM increased the apoptotic cells by 75.42 ± 2.09 % and necrotic cells by 8.19 ± 1.74 % in comparison to the control. APLH was also found to exhibit apoptotic cells and necrotic cells with an apoptotic index of 70.03 ± 1.23% and necrotic index of 3.50 ± 1.44 % followed by CUSM with an apoptotic index and necrotic index of 66.96 ± 1.22 % and 9.19 ± 2.04 % respectively. Lowest apoptotic index of 62.80 ± 1.70 was displayed by CULM and the necrotic cell index was the highest in CULM among the treated groups with 10.29 ± 0.56 %. (Table 7a and Figure 12a)

Among the extracts, APLH was found to exhibit highest apoptotic cells in HeLa cells with an apoptotic index of 72.57 ± 1.75 % and necrotic index of 5.83 ± 1.05 % followed by CURM with an apoptotic index and and necrotic index of 70.91 ± 1.97 % and 9.40 ± 0.27 % respectively. CUSM also showed apoptotic index of 63.80 ± 2.49 % and necrotic index of 8.03 ± 2.2 7%. Lowest apoptotic index of 59.56 ± 2.34 % was displayed by CULM and the necrotic cell index was the highest in CULM among the treated groups with 10.73 ± 1.02 %. (Table 7b and Figure 12b)

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 (Table 7c and Figure 12c).

Table 7a. Effect of the treated groups on the induction of apoptosis in A-549 cells. The data represent Mean ± SEM, N=5.

A-549 cell			
Treatment	**Apoptosis (%)** Mean ± SEM	**Necrosis (%)** Mean ± SEM	**Live (%)** Mean ± SEM
Control	2.51 ± 0.95	1.50 ± 0.08	95.99 ± 1.01
DOX	79.40 ± 2.06#	13.35 ± 1.24	7.24 ± 1.24
CURM	75.42 ± 2.09#	8.19 ± 1.74	16.40 ± 1.07
CUSM	66.96 ± 1.22#	9.19 ± 2.04	23.85 ± 0.98
CULM	62.80 ± 1.70#	10.2 9± 0.56	26.91 ± 1.31
APLH	70.03 ± 1.23#	3.50 ± 1.44	10.97 ± 1.44

The one-way ANOVA was performed to test the significant level between the control and treatment groups at a significant level of *p<0.5, @p<0.01 and #p<0.001.

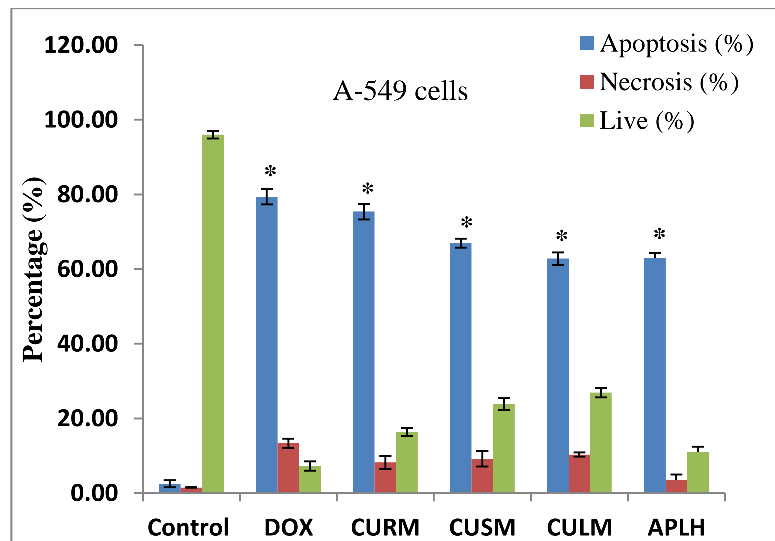


Figure 12a. Effect of the treated group on the induction of apoptosis in A-549 cells. The data represent Mean ± SEM, N=5. The one-way ANOVA was performed to test the significant level between the control and treatment groups at a significant level of *p<0.5, **p<0.01 and ***p<0.001.

Table 7b. Effect of the treated groups on the induction of apoptosis in HeLa cells. The data represent Mean ± SEM, N=5.

HeLa cell			
Treatment	**Apoptosis (%)** Mean ± SEM	**Necrosis (%)** Mean ± SEM	**Live (%)** Mean ± SEM
Control	2.03 ± 0.70	1.63 ± 1.22	96.34 ± 1.22
DOX	76.63 ± 2.31#	14.76 ± 1.34	8.62 ± 1.34
CURM	70.91 ± 1.97@	9.40 ± 0.27	19.69 ± 0.27
CUSM	63.80 ± 2.49@	8.03 ± 2.27	28.18 ± 2.27
CULM	59.56 ± 2.34@	10.73 ± 1.02	29.71 ± 1.02
APLH	72.57 ± 1.75@	5.83 ± 1.05	16.05 ± 0.57

The one-way ANOVA was performed to test the significant level between the control and treatment groups at a significant level of *p<0.5, @p<0.01 and #p<0.001.

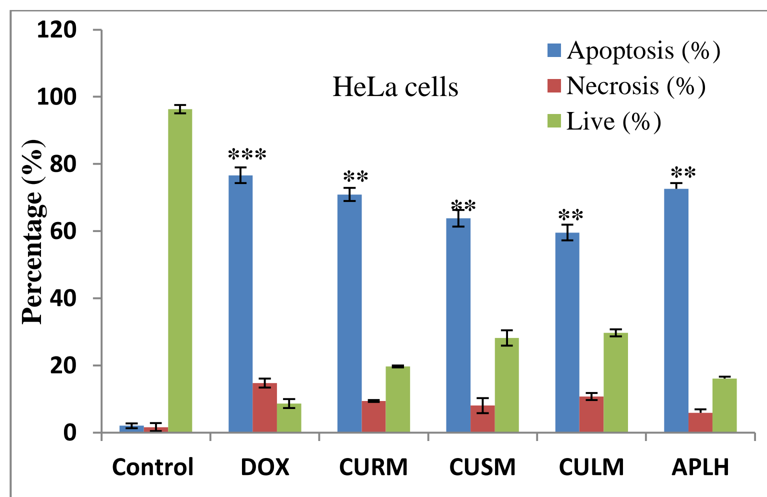


Figure 12b. Effect of the treated group on the induction of apoptosis in HeLa cells. The data represent Mean ± SEM, N=5. The one-way ANOVA was performed to test the significant level between the control and treatment groups at a significant level of *p<0.5, **p<0.01 and ***p<0.001.

Table 7c. Effect of the treated group on the induction of apoptosis in L-132 cells. The data represent Mean ± SEM, N=5.

L-132 cell			
Treatment	**Apoptosis (%)** Mean ± SEM	**Necrosis (%)** Mean ± SEM	**Live (%)** Mean ± SEM
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
CURM	15.62 ± 1.82	6.76 ± 1.59	78.71 ± 1.56
CUSM	16.37 ± 0.65	7.89 ± 1.75	75..66 ± 2.24
CULM	18.38 ± 1.22	10.99 ± 1.35	69.85 ± 1.38
APLH	14.25 ± 0.86	8.32 ± 0.87	72.54 ± 0.87

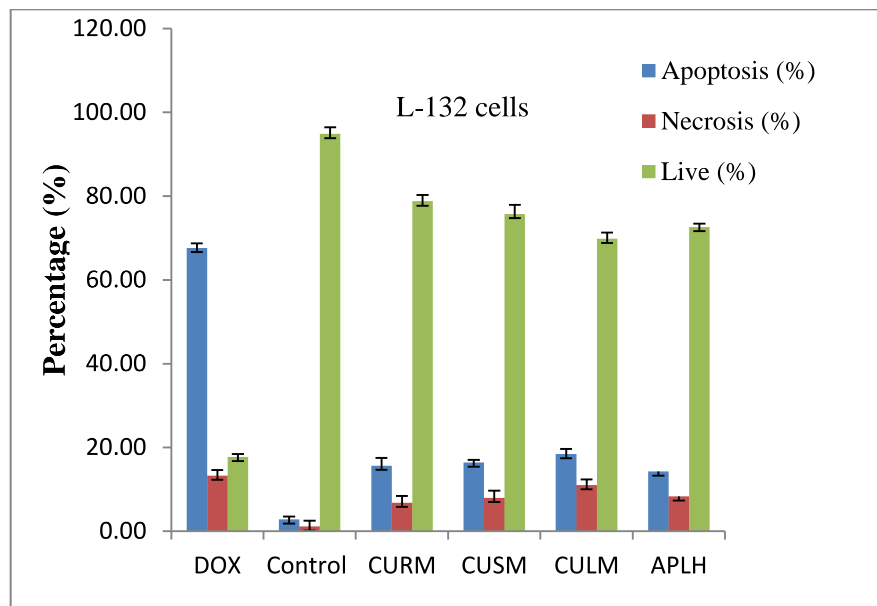


Figure 12c. Effect of the treated group on the induction of apoptosis in L-132 cells. The data represent Mean ± SEM, N=5.

4.8. Effect of the treated group on the expression of caspase-8 and -3 activity

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 APLH resulted in the up-regulation of caspase-8 and caspase-3 activities by 4.57-fold and 5.79-fold respectively followed by CURM with a fold change of 2.27 and 2.61 respectively (Table 8 and Figure 13a).

The cleavage of caspase-3 and caspase-8 activity induced by APLH showed a fold change of 2.25 and 2.24 respectively in HeLa cells followed by CURM with 1.85-fold and 1.75-fold increase respectively. APLH exhibited the highest increment in caspase-8 and caspase-3 activities in A-549 and HeLa cells (Table 8 and Figure 13b).

Table 8. Effect of the treated group on the expression of caspase-8 and -3 activity in HeLa cells. The data represent Mean ± SEM, N=3.

	A-549		**HeLa**	
Treatment	Fold change		Fold change	
	Caspase-8	Caspase-3	Caspase-8	Caspase-3
Control	0.00	0.00	0.00	0.00
DOX	8.95	7.75	4.02	2.25
CURM	2.27	2.61	1.75	1.85
CUSM	2.16	2.54	1.29	1.58
CULM	1.93	1.73	1.50	1.52
APLH	4.57	5.78	2.24	2.25

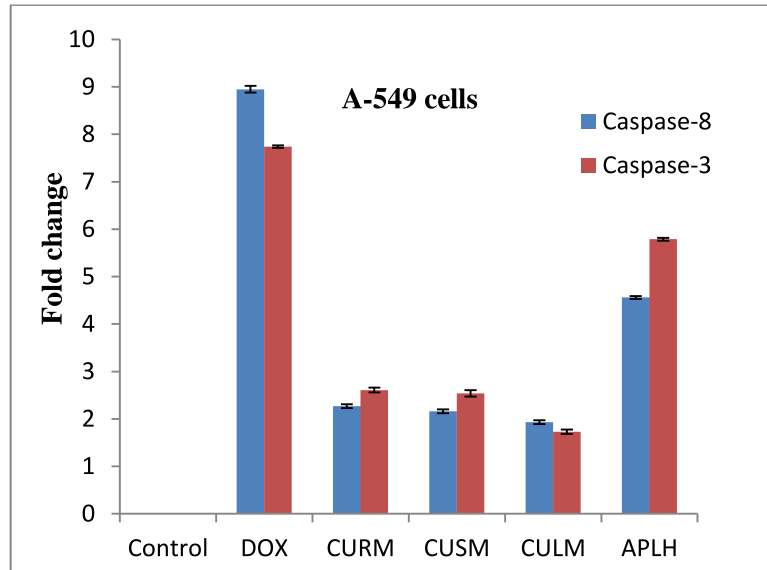


Figure 13a. Effect of the treated group on the expression of caspase-8 and -3 activity in A-549 cells. The data represent Mean ± SEM, N=3.

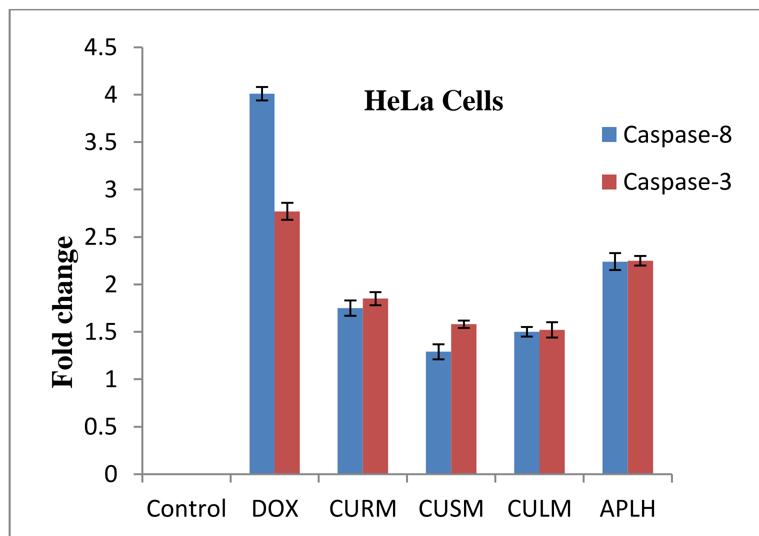


Figure 13b. Effect of the treated group on the expression of caspase-8 and -3 activity in HeLa cells. The data represent Mean ± SEM, N=3.

Chapter 5
Discussion

The anticancer characteristics of plants have been recognized for centuries and are still being actively researched and some have shown promising results. The phytochemical analysis of the different extracts revealed the presence of phenols, flavonoid, terpenoids, alkaloids, tannins cardiac glycosides, saponins and quinones (Table 1 and Table 2). The present results are in agreement with earlier findings where the methanol extracts of *C.aeruginosa* rhizome demonstrated the presence of alkaloids, phenol, flavonoids, terpenoids, tannins, glycosides and saponin (Thomas and Jose, 2014; Anu *et al*., 2020). Jothivel *et al*. (2007) also reported that *Costus pictus* revealed a broad spectrum of phytochemicals such as carbohydrates, triterpenoids, proteins, alkaloids, tannins, saponins, and flavonoids. *A. galanga*, when screened for the phytochemical constituents revealed the presence of a rich variety of phenolic compounds and essential oils (Ajay and Vijaykumar, 2015). The efficacy of antioxidant activity is significantly associated with total phenolic content and total flavonoid content (Sarepoua *et al*., 2013). In many research studies concerning determination of antioxidant activity, the correlation between antioxidant capacity results and phenolics concentration was observed (Katalinic *et al*., 2006). The results also depend on the chemical nature and structure of the phenolic compounds present in the extracts. In many assays, extracts with higher total phenolic contents were noticeable in antioxidant activity (Dorman *et al*., 2003). However, in some cases, the content of major antioxidants (phenol compounds) was rather low, a synergy can occur between them, and therefore, the other minor plant constituents might significantly influence the differences in their overall antioxidant activity (Kulisic *et al*., 2004). The antioxidant properties of medicinal plants depend on the plant and its characteristics such as growing conditions and processing, further concentration and composition of present antioxidants (phenolic compounds such

as phenolic acids, flavonoids, terpenes, carotenoids, and vitamins). In addition, extraction technique and determination method of antioxidant capacity are important for evaluation of medicinal plants quality. The results of this study revealed that the different extracts exhibited potent antioxidant activity (Table 5 and Figure 4-8). The present results are in akin to the studies of Pintatum *et al*. (2020), Jayasri *et al*. (2009), Majumdar and Parihar (2012), Sathuvan *et al*. (2012), Zaeoung *et al*. (2005) and Das and Santhy (2015) who reported that the different extracts display significant antioxidative behavior in many in vitro assays.

The stable radical scavenging and cytotoxic activities are directly associated with phytochemical compounds present (Nurcholis *et al*., 2017). MTT assay is extensively applied in the evaluation of cytotoxic drug therapy serving as an analytical tool for chemotherapy (Jiao *et al*., 1992). The cytotoxicity results in our study revealed that the different extracts were capable of killing the cancer cells but exhibit insignificant toxicity against normal cells (L-132) cells (Table 6 and Figure 9). The most valuable substance are those that can selectively kill cancer cells while exerting no significant damage to normal cells (Malek *et al*., 2009).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 IC_{50} value ≤25 µg/ml (Geran *et al*., 1972; Lee and Houghton, 2005). In this respect, based on the IC_{50} values, *C. aeruginosa* showed the best cytotoxic effect overall and the remaining experiments were carried out using the methanol extract from rhizome, stem and leaves. The hexane leaf extract of *A. galanga* also showed promising cytotoxic activity and it was included in the proceeding experiments. Previous studies have showed the antitumor activity of SCF extract of *C. aeruginosa* against HeLa cell line in a dose dependent manner (Dessy and Sivakumar, 2018). 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

(Chandra *et al*., 2003). Other species of curcuma namely Curcuma longa have been reported to show cytotoxic activities against A549 cells (Shah *et al*., 2015) and HeLa cells (Shukla *et al*., 2016). *Curcuma amada* was also found to inhibit the growth of large human lung carcinoma (NCI-H460) and small human lung carcinoma (A-549) (Muthukumar *et al*., 2012). The ethanol extract of leaves of *C. pictus* demonstrate anti-cancer potential in *in-vitro* mammalian fibrosarcoma (HT-1080) cells (Nadumane *et al*., 2011). All the extracts of stem of *C. pictus* have been reported to be have potent anti-cancer properties against HT 29 and A549 cells (Sathuvan *et al*., 2012). The anticancer effects of the *C. pictus* leaves against breast cancer cell lines (MCF-7) was also reported by Prejeena *et al*. (2017). Muangnoi *et al*. (2007) evaluated the crude aqueous extract of galangal rhizomes. His findings showed that that the extract possess a wide range of cytotoxicity in lung adenocarcinoma cell (A549), normal fibroblast cell (CRL2522), normal epithelial cells derived from breast (MCF-12A) and mammary carcinoma cells. The aqueous extract of *A. galanga* have antiproliferative effect on human gastric tumor (AGS) and L929 cell lines. (Hadjzadeh *et al*., 2014). A study conducted by Suja and Chinnaswamy (2008), revealed that *A. galanga* can inhibit the cell growth of prostate cancer cell lines (PC-3 cell).

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 (Collins, 2004). 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 (Figure 10-11). There was a major reduction of migration in the untreated cells when compared to the treated group and Doxorubicin. 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 (Shi, 2004). Our interest was to know whether the treated group induces cytotoxicity by triggering apoptosis in A-549 and HeLa cells. The AO/EB staining is most reliable method to detect apoptosis (Liu *et al*., 2015). Observation under the fluorescence microscope revealed induction of

apoptotic and necrotic cells for 48 hours in the A-549 and HeLa cells with an increased rate of cell death when compared to the L-132 cells (Figure 12a-c). 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 (Kasibhatla, 2006). Cytotoxic agents may induce apoptosis by initiating death signaling pathways in susceptible target cells (Fisher, 1994). 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 (Liao *et al*., 2015). The treated groups up-regulated the caspase-8 and caspase -3 activities (Figure 13a-b), 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 (Hadisaputri *et al*., 2015). The rhizome of *A. galanga* is also reported to induce apoptosis in MCF-7 cells according to the work done by Samarghandian *et al*. (2014) 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 (Khazaei *et al*., 2017). Likewise, the ethanol stem bark extract of *Oroxylum indicum* was reported to do the same in HeLa cells (Lalrinzuali *et al*., 2021).

The treated groups 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 (Lakshmi *et al*., 2011; Lai *et al*., 2004). Genotypes and environmental factors can affect the bioactive contents and composition of medicinal plants (Batubara *et al*., 2020). Geographic location, environmental differences and variability of genes affects the polyphenol, flavonoids and curcuminoids present in *C. aeruginosa* accessions

(Nurcholis *et al*., 2016). GC-MS results reveal the presence of various compounds which were earlier detected in previous works (Dessy *et al*., 2019).

Nurcholis, (2021) reported that the ethanolic extract of *C. aeruginosa* accessions is mainly composed of sesquiterpenes (19.86 -43.72%) and monoterpenes (6.3 -25.86%). The isocurcumenol was found to be the most dominant metabolite in ethanol extract of *C. aeruginosa* rhizome. The major phytochemical constituents found in leaf extracts of *A. galanga* were benzenepropanal (37.35±0.5%) and 3-phenyl-2-butanone (20.49±0.6%) (Singh *et al*., 2020).

Biological activities of phytochemical constituents detected in the methanol extracts of *C. aeruginosa* are given in Table 4a. Seven sesquiterpenoids, curcumenol, curzerenone, epicurzerenone, α-curcumene, curzerene, caryophyllene oxide, 9,12-Octadecadienoic acid (Z,Z) and a diterpenoid, andrographolide detected in CURM (Table 3a) 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 (Rahman *et al*., 2013; Banerjee *et al*., 2016; Delgado *et al*., 2021; Zhou *et al*., 2008; Hamdi *et al*., 2014). 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 (Jung *et al*., 2018; Wang *et al*., 2017; Cui *et al*., 2019; Zhao *et al*., 2010; Sneader, 2005).

GC-MS profiling of the identified compound from APLH identified11 compounds with known biological activities (Table 4b). D-limonene, β-elemene, Gamma-Tocopherol detected in APLH have antiproliferative activity by inducing apoptosis in cancer cells. D-limonene is one of the most common terpenes in nature and has well-established chemopreventive activity against many types of cancer. Ye *et al*. (2020) reported that limonene might be used as a potent anticancer agent against human bladder cancer, inducing significant apoptosis with an increase in the expression of caspase-3. The induction of apoptosis by D-limonene in human colon cancer cells and lung cancer was

also reported by Jia *et al*. (2013), Zhao *et al*. (2012) and Yu *et al*. (2018). β-elemene induces caspase-dependent apoptosis in human glioma (U87, U251), human brain (A172, CCF-STTG1 and U-87MG), human NSCLC (H460), gastric (SGC7901) and prostate (DU145) cells (Li *et al*., 2014; Li *et al*., 2013; Zhang *et al*., 2012; Wang *et al*., 2005; Liu *et al*., 2015; Li *et al*., 2010). Gamma-Tocopherol is also reported to induce cell death in human prostate cancer (PC-3, LNCaP), lung cancer (A549), colon (HT- 29) and cervical (Hela) cells by inducing apoptosis through involvement of caspase-independent pathways (Jiang *et al*., 2004; Rezaei *et al*., 2014; Pédeboscq *et al*., 2012).

The HPLC analysis showed that the treated group contain seven active substances: gallic acid, kaempferol, quercetin, caffeic acid, coumaric acid, naringin, rutin (Figure 3a-3d). 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 (You *et al*., 2010; Maurya *et al*., 2010; Li *et al*., 2020; Nguyen *et al*., 2003; Zheng *et al*., 2012; Priyadarsini *et al*., 2010; Sundaram *et al*., 2019; Chang *et al*., 2010; Lin *et al*., 2012; Shi *et al*., 2021; Kilinc *et al*., 2019; Rodríguez *et al*., 2021; Lu *et al*., 2020; Zeng *et al*., 2014; Khan *et al*., 2021; Hoai *et al*., 2020).

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

Chapter 6
Conclusion

Medicinal plants have various advantages over chemical products, because plant derived compounds are more tolerated and non-toxic to the normal human cells. Already available conventional therapies for the treatment of cancer are radiotherapy and chemotherapy and they have possesses various side effects like neurological, cardiac, renal and pulmonary toxicity, which seriously affects the health of the person. Therefore, an alternative method is required to develop less toxic and more potent anticancer drug. Phytochemicals from ethno-botanically important traditional plants from have gained scientific attention for their use as agents in cancer treatment and prevention.

The present study showed the phytochemical and antioxidant activity of *Curcuma aeruginosa*, *Costus pictus*, and *Alpinia galanga* relating to *in vitro* anticancer activity against cells against four human cancer cell lines namely, A-549 (human lung), HeLa (human cervical), AGS (human gastric), HT29 (human colon) cancer cell lines and a normal cell: L-132 cells. The qualitative phytochemical analysis of the different extracts revealed the presence of key phytochemicals: phenols, flavonoid, terpenoids, alkaloids, cardiac glycosides, saponin, quinones and tannins. The quantitative phytochemical analysis indicated that the different extracts possess high phenol and flavonoid content when compared to alkaloid which was lower in content. Validation of the antioxidant activity of the different extracts was conducted using phosphomolybdenum assay and different in-vitro assays: DPPH and ABTS+, superoxide and hydroxyl assays. The results revealed that the different extracts exhibited potent antioxidant activity.

MTT assay was carried out for selecting the extract with the highest cytotoxic effect. Among the 27 extracts, CURM and APLH showed great cytotoxicity towards the cancer cells and insignificant toxicity against normal cells (L-132) cells, CURM, exhibited significant activity against human lung adenocarcinoma (A-549), human cervical cancer

(HeLa) and human gastric cancer (AGS) cells with an IC_{50} value of 15.42±1.5 µg/ml, 14.02±0.57 µg/ml and 25.40±0.13 µg/ml respectively. It was further observed that APLH also showed significant cytotoxic activity against against human lung adenocarcinoma (A-549), human gastric cancer (AGS), and human colon cancer (HT29) cells with an IC50 value of 13.2 ±0.8 µg/ml, 15.87±1.24 µg/ml and 20.46±0.72 µg/ml respectively, thereby showing increased DNA fragmentation, apoptotic and necrotic indices. AO/ Et-Br staining revealed that APLH and CURM exhibited highest apoptotic cells in HeLa and A-549 cells respectively. The treated group upregulated caspase-8 and caspase -3 activities in A-549 and HeLa cells thereby exerting cytotoxic effect by triggering apoptosis. Our results indicate that CURM, CUSM, CULM and APLH 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 the present study. Among the treated group, APLH exhibited the highest increment in caspase-8 and caspase-3 activities. The chromatographic techniques- HPLC and GC-MS revealed the presence of phenol, flavonoid compounds like gallic acid, kaempferol, quercetin, caffeic acid, coumaric acid, naringin, rutin and diferent terpenoid compounds that contributes to the anticancer activities of the plant.

Therefore, the apoptosis-inducing effect of *C. aeruginosa* and *A. galanga* on A549 and HeLa cells came from these naturally active phenol and flavonoid compounds. The GC-MS result revealed that CURM and the APLH possess naturally occurring terpenoids and vitamins, that can induce apoptosis in cancer cells, thereby exhibiting greater anticancer potential than the other parts examined as evident in this study. From these findings, it is inferred that the plant *C. aeruginosa* and *A. galanga* can be use to develop a potential lead compound for cancer chemotherapy.

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BIO-DATA

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Educational Qualification

Sl. No.	Examination Passed	Subject	Year	Board / University	Division	Percentage of Marks
1.	HSLC	Regular	2008	MBSE	First Division	64%,
2.	SSCE	Science	2010	CBSE	First Division	74 %,
3.	B.SC	Botany	2014	Madras Christian College (University of Madras)	First Division	74%,
4.	M.SC	Botany	2016	Madras Christian College (University of Madras)	First Division (Distinction)	77.47%,

Ph.D registration No and Date : MZU/Ph.D./1012 of 26.05.2017

Department : Botany

Title of research : Screening for phytochemicals and cytotoxicity of selected ethno-medicinal plants from Mizoram, Northeast India.

Supervisor : Prof. F. Lalnunmawia

PUBLICATIONS

1. Alex Zohmachhuana, Malsawmdawngliana, F. Lalnunmawia*, Vabeiryureilai Mathipi, K. Lalrinzuali and N. Senthil Kumar. 2022. *Curcuma aeruginosa* Roxb. exhibits cytotoxicity in A-549 and HeLa cells by inducing apoptosis through caspase-dependent pathways. *Biomedicine and Pharmacotherapy*. 150: 113039. https://doi.org/10.1016/j.biopha.2022.113039
2. Alex Zohmachhuana, Malsawmdawngliana, K. Lalrinzuali, F. Lalnunmawia,M. Vabeiryureilai, N. Senthil Kumar. 2021. The Assessment of the Free Radical Scavenging Activity and Flavonoid Contents of Selected Medicinal Plants of Mizoram. *Current Trends in Biotechnology and Pharmacy*.15 (1) 34-43. DOI:10.5530/ctbp.2021.1.4
3. Alex Zohmachhuana, Malsawmdawngliana Tlaisun, Vabeiryureilai Mathipi, Lalrinzuali Khawlhring, Joyce Sudandara Priya. 2022. Suppression of the RAGE gene expression in RAW 264.7 murine leukemia cell line by ethyl acetate extract of *Mikania micrantha* (L.) Kunth. *Journal of Applied Biology and Biotechnology*. 10(5) 107-114. DOI: 10.7324/JABB.2022.100513
4. Malsawmdawngliana, Alex Zohmachhuana, M Vabeiryureilai, Nurpen Meitei Thangjam, K Lalrinzuali,N Senthil Kumar & Awadhesh Kumar. (2021). Antioxidant efficacy and cytotoxicity of ethanol extract of *Clerodendrum infortunatum* against different cell lines. *Indian Journal of Biochemistry & Biophysics*. 58: 572-581.
5. J.C. Angel Lalrindiki, Alex Zohmachhuana and F. Lalnunmawia. 2020. Phytochemical Screening and Allelopathic Effects of *Ageratum conyzoides* L. Science and Technology Journal .8 (2).

PAPERS PRESENTED

1. Presented a paper on "Phytochemical analysis and anti-cancer activity of the methanol extracts of *Curcuma aeruginosa* Roxb." at the International Conference on Biodiversity, Environment and Human Health: Innovations and Emerging Trends (BEHIET & The 12th Annual Convention of Association of Biotechnology and Pharmacy (ABAP).
2. Presented a paper on "Evaluation of phytochemicals, antioxidant and anti-cancer activity of *Alpinia galanga* (L.) Willd." at the National conference on Microbes in Health, Agriculture and Enivironment.
3. Presented a paper on "The Assessment of the Free Radical scavenging Activity and Flavonoid contents of selected medicinal plants of Mizoram." at the International Seminar on Recent Advances in Science and Technology (ISRAST).
4. Poster presentation on the "Evaluation of phytochemicals, antioxidant and anti-inflammatory activity of *Mikania micrantha* (L.) Kunth." at the International Virtual Conference on Natural Products and Synthetic Biology (ICNSB2020).

SEMINAR AND WORKSHOP ATTENDED

1. Participate in the Hands-On Training Workshop on " Stem Cell Biology.'' organized by Advanced Centre for Treatment, Research and Education in Cancer, DBT Biotechnology/Bioinformatics Training Centre, Mumbai, held during 13-18t^{h} February, 2017.
2. Participate in the workshop on "Statistical and Computing methods for Life Science Data Analysis" organized by Department of Botany, Mizoram University and Biological Anthropological Unit, Indian Statistical Institute, Kolkata held during 5-10th March, 2018.
3. Participated in the ATMA Mizoram State Level Workshop-cum-Exhibition with the theme "Research-Extension-Farmer Linkage'' from 31st January to 1st February, 2019 at Synod Conference Centre, Aizawl.
4. Participated at the exhibition in Mizoram Science Congress held at Mizoram University during 13th-14th October, 2016.
5. Participated in the national workshop on ''A brief introduction Bioinformatics and Systems Biology held during 13th-14th December, 2018 organised by Bioinformatics Infrastructure Facility (BIF), Department of Biotechnology, Mizoram University.
6. Participated in the workshop on "Cancer Epidemiology": Mizoram, 2016 (International) held duringn29th-30th November organized by Department of Biotechnology, Mizoram University and State Biotech Hub Facility, Department of Biotechnology (DBT), New Delhi.
7. Participated in the workshop on "National Level Workshop on Biostatistics and Bioinformatics held during 1st-7th September, 2016, organised by Department of Biotechnology, Mizoram University and Bioinformatics Infrastructure Facility, Department of Biotechnology (DBT), New Delhi.

PARTICULARS OF THE CANDIDATE

Name	: Alex Zohmachhuana
Degree	: Ph.D
Department	: Botany
Title of thesis	:Screening for phytochemicals and cytotoxicity of selected ethno-medicinal plants from Mizoram, Northeast India.
Date of Admission	: 17.08.2016
Approval of research proposal	
1. DRC	: 24.04.2017
2. BOS	: 01.05.2017
3. SCHOOL BOARD	: 26.05.2017
MZU REGISTRATION No.	: 1600794 of 2016
Ph.D REGISTRATION No. & DATE	: MZU/Ph.D/1012 of 26.05.2017

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Head

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ABSTRACT

SCREENING FOR PHYTOCHEMICALS AND CYTOTOXICITY OF SELECTED ETHNO-MEDICINAL PLANTS FROM MIZORAM, NORTHEAST INDIA

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

ALEX ZOHMACHHUANA

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May, 2022

ABSTRACT

SCREENING FOR PHYTOCHEMICALS AND CYTOTOXICITY OF SELECTED ETHNO-MEDICINAL PLANTS FROM MIZORAM, NORTHEAST INDIA

BY

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DEPARTMENT OF BOTANY

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MIZORAM UNIVERSITY

SUBMITTED

IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN BOTANY OF MIZORAM UNIVERSITY, AIZAWL

Abstract

The aim of the current study entitled "Screening for phytochemicals and cytotoxicity of selected ethno-medicinal plants from Mizoram, Northeast India" was to examine the efficacy of the leaf, stem and rhizome of *Costus pictus, Curcuma aeruginosa* and *Alpinia galanga* for their phytochemical content, antioxidant and anti-cancer activities. The different parts of the plants were subjected to sequential extraction to give three fractions viz., hexane, ethyl acetate and methanol extract.

The qualitative phytochemical analysis of the selected plants indicated the presence of phenols, flavonoid, terpenoids, alkaloids, tannins cardiac glycosides, saponins and quinones. The quantification of phytochemicals of the various plant parts of *C. aeruginosa* revealed that *C. aeruginosa* leaf methanol extract (CULM) contained the highest phenol (7.66 ± 0.05 mg GAE/g of dry weight), flavonoid (7.57 ± 0.02 mg quercetin equivalent/g of dry weight) and alkaloid content (1.52 ± 0.77 mg atropine equivalents/g of dry weight). The quantitative phytochemical analysis of *C. pictus* revealed that *C. pictus* leaf methanol extract (CPLM) contained the highest phenol (7.62 ± 0.35 mg GAE/g of dry weight) and flavonoid content (4.37 ± 0.23 mg quercetin equivalent/g of dry weight). The alkaloid content was the highest in *C.pictus* leaf methanol extract (CPLM) (1.05 ± 0.35 mg atropine equivalents/g of dry weight). The quantification of phytochemicals of the various plant parts of *A. galanga* revealed that *A. galanga* rhizome methanol extract (APRM) contained the highest phenol (3.98 ± 0.83 mg GAE/g of dry weight) and flavonoid content (3.02 ± 0.05 mg quercetin equivalent/g of dry weight). The alkaloid content was the highest in *A. galanga* stem ethyl acetate extract (APSE) (0.90 ± 0.05 mg atropine equivalents/g of dry weight)

To validate the antioxidant activity of the different extracts, phosphomolybdenum assay and different *in-vitro* assays were conducted. Free radical scavenging activity was assessed using DPPH and $ABTS^+$, superoxide and hydroxyl assays. The scavenging activity of the different extracts showed rise in activity with an increase in concentration manner. In *C. aeruginosa*, among the different extracts, *C. aeruginosa* rhizome methanol extract (CURM) showed the highest ability to scavenge DPPH, $ABTS^+$ cations, superoxide radicals and reduce Mo (VI) to Mo (V) using phosphomolybdate method, with an IC_{50} value of 12.22 ± 0.11 µg/ml, 12.87 ± 1.16 µg/ml, 35.92 ± 0.3 µg/ml and 3.97 ± 0.01 µg/ml respectively. The best IC_{50} value for hydroxyl scavenging activity was exhibited by *C. aeruginosa* rhizome ethyl acetate (CURE) (3.42 ± 0.09 µg/ml).

In *C.pictus*, the best IC_{50} value for DPPH scavenging activity was exhibited by *C.pictus* stem methanol extract (CPSM) (9.06 ± 0.07). The $ABTS^+$ cations scavenging of extracts exhibited maximum activity in *C. pictus* leaf ethyl acetate extract (CPLE) (5.23 ± 0.12 µg/ml. *C.pictus* leaf methanol extract (CPLM) displayed the best superoxide radical activity with an IC_{50} value of (70.06 ± 0.7 µg/ml). The best IC_{50} value for hydroxyl scavenging activity was exhibited by *C. pictus* stem hexane extract (CPSH) (1.55±0.5 µg/ml). The total antioxidant capacity of the extracts was evaluated using phosphomolybdate method and *C. pictus* rhizome methanol extract (CPRM) (1.36 ± 0.05 µg/ml) showed the most promising IC_{50} value. The DPPH scavenging activity of extracts of *A. galanga* showed that *A. galanga* leaf ethyl acetate extract (APLE) (6.44 ± 0.07 µg/ml) has the highest ability to scavenge DPPH radical. The $ABTS^+$ cations scavenging of the extracts revealed that *A. galanga* stem hexane extract (APSH) (1.18 ± 0.7 µg/ml) exhibited maximum activity. *A. galanga* stem methanol extract (APSM) (24.39 ± 1.7 µg/ml) was found to have the highest superoxide scavenging activity. *A. galanga* stem ethyl acetate extract (APSE) displayed the best superoxide radical activity with an IC_{50} value of 1.81 ± 0.07 µg/ml. The results of the phosphomolybdenum assay showed *A. galanga* leaf hexane (APLH) (3.93 ± 0.05 µg/ml) has significant antioxidant activity.

Lung, cervical, gastric and colon cancer were found to be the leading sites of cancer in India, particularly North- East India. Thus, the cytotoxic effect and the mode of action were examined against four human cancer cell lines namely, A-549 (human lung), HeLa (human cervical), AGS (human gastric), HT-29 (human colon) cancer cell lines and a normal cell: L-132 cells. The cytotoxicity results revealed low cell survival rate on MTT assay compared to non- cancerous (L132) cells. MTT assay was carried out for selecting the extract with the highest cytotoxic effect among the 27 samples. Based on the IC_{50} values, *C. aeruginosa* showed the best cytotoxic effect overall and the remaining experiments were carried out using the methanol extract from rhizome, stem and leaves - CURM, CUSM and CULM respectively. *A. galanga* leaf hexane extract (APLH) also showed promising cytotoxic activity, so it was included in the proceeding experiments and were labeled as treated groups. Among the 27 extracts, *C. aeruginisa* rhizome methanol extract (CURM) and *A. galanga* leaf hexane extract (APLH) were found to be the most cytotoxic showing significant cytotoxicity towards the cancer cells with insignificant toxicity against normal cells (L-132) cells, thus supporting the traditional use of this medicinal plant in treating cancer. Further investigation was carried out to understand the effects.

The mechanism of cell death and apoptosis induced by CURM, CUSM, CULM and APLH (treated group) on A549 and HeLa cells were studied using fluorescence staining. The level of DNA damage was examined using the alkaline comet assay. The treated groups induced DNA damage after 24 hours with significant increase in tail DNA and tail moment when compared to untreated control. Among the extracts, APLH showed significant increased tail DNA (65.36 ± 0.26 %) and tail moment (63.24 ± 0.77 %) when compared to untreated control (13.86 ± 0.6 % tail DNA and 0.47 % ± 0.14 % tail moment) in HeLa cells. Treatment with APLH against A-549 cells showed induction of DNA damage with increased tail DNA (64 ± 0.90 %) and tail moment (63.24 ± 0.77 %). The untreated control in A-549 showed 4.74 ± 0.17 % tail DNA and 0.11 ± 0.03 % tail moment. The apoptotic morphological alterations induced by the extracts in A-549, HeLa and L-132 cells were studied using Acridine orange (AO) / Ethidium bromide

(Et-Br) staining. Treatment with the treated groups against the A-549 and HeLa cells revealed induction of apoptotic and necrotic cells for 48 hours with an increased rate of cell death when compared to the L-132 cells.

Among the treated group, APLH was found to exhibit highest apoptotic cells in HeLa cells with an apoptotic index of 72.57 ± 1.75 % and lowest necrotic index of 5.83 ± 1.05 %. A-549 cells treated with 15.42 ± 1.5 µg/ml of CURM 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 treated groups up-regulated the caspase-8 and caspase -3 activities in both HeLa and A-549 cells after 48 hours of treatment, inducing cytotoxic effect and triggering apoptosis. APLH exhibited the highest increment in caspase-8 and caspase-3 activities in A-549 and HeLa cells. This is in agreement with the morphology of DNA fragmentation and apoptosis induced by the treated group in A-549 and HeLa cells.

The extracts were fractionated by GC–MS (Gas Chromatography-Mass Spectrometry) and HPLC (High-Performance Liquid Chromatography) analysis for their phytochemical content. The chromatographic techniques revealed the presence of many bioactive compounds. The GC–MS analysis of the methanol extracts of *C. aeruginosa* namely, CURM, CUSM and CULM recorded a total of 47 peaks. Seven sesquiterpenoids, curcumenol, curzerenone, epicurzerenone, α-curcumene, curzerene, caryophyllene oxide, 9,12-Octadecadienoic acid (Z,Z) and a diterpenoid, andrographolide detected in CURM are reported to have cancer protective properties inhibiting the growth of human cancer cells. The GC–MS analysis of APLH recorded 18 peaks. Limonene, β-elemene, Gamma-Tocopherol detected in APLH have antiproliferative activity by inducing apoptosis in cancer cells. The GC-MS result revealed that CURM and the APLH possess naturally occurring terpenoids and vitamins,

that can induce apoptosis in cancer cells, thereby exhibiting greater anticancer potential than the other parts examined as evident in this study.

The HPLC analysis of the treated groups recorded the presence of important bioactive phenol and flavonoid compounds: Gallic acid, quercetin, caffeic acid, catechin, kaempferol, rutin, coumaric acid and naringenin. These compounds are known for inducing apoptosis in human cancer cells through caspase-dependent pathways. Therefore, *C. aeruginosa* and *A. galanga* and its potential to induce apoptosis in cancer cells suggest that they have potential in medical applications and can be further use to develop a potential lead compound in the search for natural compounds particularly for lung and cervical cancer chemotherapy.