Therapeutic potential of medicinal plant *Thespesia populnea* (L.) Soland ex Correa with special emphasis towards its antiproliferative activity against different cancer cell lines

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CERTIFICATE

This is to certify that the project entitled "Isolation and characterisation of anti-carcinogenic compounds from selected medicinal plants."Submitted by Megha K B, Research scholar, Department of Biotechnology, under the guidance of Dr. MV Joseph, Professor of Biotechnology, University of Calicut has been approved by the IAEC of the University of Calicut. The approval is given for performing the above mentioned research work in the approved animal house facility of the University with the registration number 426/2/CPCSEA.

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DECLARATION

I hereby declare that the work presented in the Thesis entitled "Therapeutic potential of medicinal plant *Thespesia populnea* (L.) Soland ex Correa with special emphasis towards its antiproliferative activity against different cancer cell lines" submitted to the University of Calicut, as partial fulfillment of PhD programme for a award of the degree of Doctor of Philosophy in Biotechnology is original research work carried out by me under the supervision of Dr. K K Elyas, professor, Department of Biotechnology, University of Calicut. This has not been submitted earlier either in part or in full for any degree or diploma of any university

Megha K B

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MEGHA K. B. “THERAPEUTIC POTENTIAL OF MEDICINAL PLANT THESPESIA POPULNEA (L.) SOLAND EX CORREA WITH SPECIAL EMPHASIS TOWARDS ITS ANTIPROLIFERATIVE ACTIVITY AGAINST DIFFERENT CANCER CELL LINES.” THESIS. DEPARTMENT OF BIOTECHNOLOGY, UNIVERSITY OF CALICUT, 2018.

CHAPTER 1

INTRODUCTION

Cancer is a multifaceted disease condition manifested by uncontrolled cell growth. The number of new cases as well as the number of individuals surviving with cancer is expanding continuously. The insidious nature of the disease as well as the challenges associated with its effective treatment has made this disease a leading cause of death in many countries [1]. Mortality rate due to cancer has fuelled to pursuit for effective anticancer agents to combat this disease. The WHO project that without immediate action, the global number of deaths from cancer will increase by nearly 80% by 2030. External factors such as tobacco, infectious organisms, chemicals and radiation and internal factors inherited mutations, hormones, immune conditions and mutations that occur from metabolism are mostly responsible for cancer. These causative factors may act together or in sequence to initiate or promote the development of cancer [2]. The most prevailing treatment now available is chemotherapy and radiotherapy although the strain and the future damage to the patient health is highly demanding to focus on the use of alternative treatments and

therapies against cancer [3]. Most anticancer agents display a narrow therapeutic window due to their lack of selectivity against cancer cells [4].

In the present scenario, compounds of natural origin play a very important role since many currently used drugs derive from or have been inspired by nature [5]. According to ayurvedic concept charaka and sushruta described cancer equivalent to “granthi” and “arbuda”. The synthetic drug creates adverse effect after treatment but natural therapies including plants and plant products are safe and effective against cancer. During 1950’s anti cancer agents like vincristine and vinblastine are developed from plant source. It is really significant that over 60% of currently used anticancer drugs are developed from one way or another from terrestrial and marine natural source [6]. The development of high-throughput screening techniques (HTS) has led to an increasingly high number of compounds that can be tested. The key areas in which natural products excel are their structural diversity and complexity [7]. Structure activity relationship studies and structural modification are helping in observing basis of compound characteristics and helps in modifying parent compound for creating an

improved and potent compound [8]. Plant based anticancer drugs currently use in clinical trials also provides a platform for design of novel and safe drugs through proper understanding of the complex synergistic interaction of various constituents of anticancer herbs [9].

Plants contain naturally occurring secondary metabolites/phytochemicals which are defined as bioactive non nutrient compounds which have been connected to reduction of major chronic disease risk [10, 11, 12]. Different studies revealed that phytochemicals can perform effectively as chemo preventive agent towards the human cancer by modulating cancer cell cycle, proliferation inhibition and initiation of apoptosis [13].

Cells in our body are frequently exposed to variety of oxidising agents. These factors may be present in air, water, food and are generated during metabolic activity within cells [14]. In the normal physiological condition there is an optimal balance between oxidants and antioxidants. Due to production of high amount of oxidants, which finally lead to oxidative stress that alters large biomolecules like lipid, protein and DNA resulting in cancer progression. In order to avoid or slow down oxidative stress caused by free radicals, adequate amount of antioxidants are needed. Antioxidants are those molecules that are involved in the scavenging of the reactive species causing oxidative stress. Oxidative stress and its detrimental effects can be prevented through the intake of naturally occurring antioxidants. It act as free radical scavengers and prevent oxidative reactions that lead to various disease condition. The majority of exogenous antioxidants are from the plants, the phytochemicals. In normal physiological process an organism could fight against the oxidative stress that occurs through various physiological processes. These include the enzyme catalase, superoxide dismutase, glutathione peroxidase and reduced glutathione which are endogenous antioxidants [15].

Thespesia populnea (L.) Soland ex Correa belongs to the family Malvaceae otherwise known as mallow family. It is mainly distributed in the coastal areas of Indian and Pacific oceans throughout Oceania. It has heart shaped leaves glossy green in color and has yellow hibiscus-type flower. *T. populnea* is small evergreen tree with average height 6-10m and they grow under full sunlight and tolerate drought conditions. The plant possesses many therapeutic potential including cancer. The bark, leaves, flowers

and fruits are useful in cutaneous infection such as scabies, psoriasis, eczema, ringworm and guinea worm [16]. A decoction of bark is commonly used for the treatment of skin and liver diseases. Oil of bark is mixed with vegetable oil and is useful in urethritis and gonorrhoea [17]. The bark, roots and fruits were used in dysentery, cholera and haemorrhoids. In the indigenous system of medicine, the paste of fruits, leaves and roots are applied externally for various skin diseases. The leaves are applied locally for their anti-inflammatory effects in swollen joints [18]. The alcoholic seed extract was reported for hypoglycaemic and anti-hyperglycemic [19], astringent, hepatoprotective [20] and antioxidant activity in rats [21]. The seed extract and its fractions were evaluated for analgesic and antipyretic activity in mice [22]. A polyherbal formulation containing *T. populnea* as one of the ingredient was shown useful remedy for Alzheimer's disease [23]. Gossypol was found to be the major component of *T. populnea*, producing antifertility effects in rats as well as in human beings [24, 25].

The present study is mainly focused on the leaves of medicinal plant *T. populnea* for its antioxidant and antiproliferative ability as it is the most preferred part of the plant for its metabolites. Preliminary phytochemical screening, antimicrobial and anti-inflammatory ability of the extracts were also evaluated. There is less scientific report available for the antiproliferative and apoptogenic ability of leaf extracts of *T. populnea*. An approach was also made to identify the compounds attributing the antiproliferative potential.

OBJECTIVES

1. **To identify the phytochemical constituents of leaf extracts of *T. populnea***
2. **Evaluation of antioxidant, antimicrobial and anti-inflammatory potentials of leaf extracts**
3. **Assessment of cytotoxicity of the leaf extracts against different cancer cell lines for its antiproliferative ability**
4. **Apoptogenic ability of the leaf extract against chronic myelogenous leukemia (K562) and colon cancer (HCT116) cell lines**
5. ***In vivo* study of leaf extract against Ehrlich Ascites Carcinoma in Swiss Albino Mice**
6. **Bioassay guided isolation and characterisation of anticarcinogenic compounds from leaf extract using chromatographic and spectroscopic techniques**

REVIEW OF LITERATURE

1.1 Cancer

Cancer is a severe metabolic syndrome, leading cause of mortality and morbidity worldwide and the number of cases are continuously rising [26, 27]. The disease ranks second in death cases after cardiovascular disorders in the developed nations [28, 29]. Cancer is responsible for one in eight deaths worldwide more than AIDS, tuberculosis and malaria together [30]. According to Globocan 2018, a project supported by WHO and International Agency for Research and Cancer shows that 18078 thousand new cases, 9555 thousand cancer death and 43841 thousand prevalent cases (within 5 years) in 2018 [31]. The most prevalent cancer affecting men were lung, prostrate, colorectal, stomach and liver. The cancers mostly seen in females were breast, colorectal, lung, cervical and thyroid. While checking the statistics in Indian population- lip/oral cavity, lung, stomach, colorectal and oesophageal cancers were commonly seen in men. In females, breast, cervix uteri, ovary, lip/oral cavity and colorectal were most prevalent cancers. In India, 1157 thousand new cases were reported and 784 thousand lives were lost due to cancer. There are 2258 thousand people surviving with cancer in 2018 [32]. Globally the number of cancer deaths is projected to increase from 7.1 million in 2002 to 11.5 million in 2030 [33].

The cancer is a phenomenon described by uncontrolled proliferation and dedifferentiation of normal cells. Cancer cells have some marked features i.e., they tune out the signals of proliferation and differentiation, they are capable to sustain proliferation, they overcome the apoptosis, and they have the power of invasion and angiogenesis. Sequential genetic alterations which produce genetic instabilities accumulate in the cell and a normal cell becomes a malignant cell [34]. These alterations include mutations in DNA repair genes, tumor suppressor genes, oncogenes and genes involve in cell growth and differentiation. External – radiations, smoking, pollution and infectious organisms and internal factors genetic mutations, immune conditions and hormones can cause cancer.

As various types of cancer exists in human lung, breast and colorectal cancer being the most common forms [35]. Several genes coordinate together for the growth and differentiation of a normal cell. In cancer, one or group of these genes get altered and

express aberrantly [36]. Modifications of epigenetic processes involved in cell growth and differentiation also lead to the development of a cancer.

1.2 Classification of cancer

Cancer classification is based on the site of origin, the cell grading and the extend of the disease. [37, 38].

1.2.1 Carcinoma- Cancer that begins in the skin or in the tissues that line or cover internal organs. They are of different subtypes a) adenocarcinoma b) basal cell carcinoma c) squamous cell carcinoma d) transitional cell carcinoma [37].

1.2.2 Sarcoma- Cancer that begins in the connective or supportive tissues such as muscle, cartilage, fat, bone and blood vessels [37].

1.2.3 Leukemia- Cancer that starts in blood forming tissue such as the bone marrow and causes abnormal cells to be produced and get into the blood. There are different types- acute myelocytic leukemia, chronic myelogenic leukemia, acute lymphatic leukemia and chronic lymphocytic leukemia [37].

1.2.4 Lymphoma and myeloma- Cancer of the lymphatic system (spleen, thymus, tonsils etc). Lymphoma is of mainly two types- Hodgkin's and Non- Hodgkin's lymphoma. Myeloma is the cancer originates in the plasma cells of bonemarrow [37].

1.2.5 Blastoma- Cancer that originates from embryonic tissue of organs [37].

1.3 Treatment for cancer

Treatment for cancer depends upon the stage and location of tumor. According to National Cancer Institute, USA the major treatment methods available against cancer are mentioned below;

In surgery, treatment procedure includes the surgical removal of the tumor from the boby. Radiation therapy uses high doses of radiation to eradicate cancer cells. chemotherapy type of treatment that uses drugs to kill cancer cells and shrink tumors. In immunotherapy, treatment method will make the patient's own immune system to fight against cancer. In targeted therapy the changes in the cancer cells are targeted by the drug such as the growth, division and metastasis. Hormone therapy slows or stops the growth of cancers like breast and prostate cancers. Stem cell transplant treatment

restores blood forming stem cells to treat cancers. Eg- Bone marrow transplantation [39].

1.4 Chemotherapy

In 1900s, the famous German chemist Paul Ehrlich coined the term chemotherapy and defined the use of chemicals to treat disease. Chemotherapy involves the use of low-molecular- weight drugs to selectively destroy tumor cells or atleast limit their proliferation [40]. The treatment method depends upon the stage and location of tumor. The major cancer drug screening program was started in 1937 under the leadership of Murray Shear at the National cancer Institute. They have screened over 3,000 compounds including natural products [41]. The use of nitrogen mustard for lymphomas spread rapidly throughout the US after the publication of the Lindskog in 1946 [42]. In 1948, Farber showed the antifolate activity of methotrexate in childhood leukemia. The antibiotic, actinomycin D had significant use in pediatric tumors in the 1950s and 1960s [43]. By 1951, Hitchings and Elion developed 2 drugs, for acute leukemia: 6-thioquanine and 6-mercaptopurine [44, 45]. The very first example of targeted therapy drug Fluoropyrimidine- 5- fluorouracil (5-FU) was developed by Charles Heidelberger. This agent was found to have a broad spectrum activity against a range of solid tumors and remains the leading drug for the treatment for colorectal cancer [46].

In the late 1960s combination chemotherapy was used against breast cancers and some encouraging results were found [47]. A combination of cyclophosphamide, methotrexate and 5-flurouracil are specifically designed for use as adjuvant chemotherapy [48, 49]. The advent of monoclonal antibody enhanced the effects of chemotherapy. In the mid 1990s monoclonal antibodies were introduced clinically and they seem to be work best. When they are used in conjuction with chemotherapy trastuzumab in breast cancer, cetuximab and bevacizumab in colorectal cancer and rituximab in non-Hodgkin’s lymphoma each are integral part of chemotherapy [46]. Chemotherapy has changed to the age of targeted therapy. The first and the best example of targeted therapy is the development of the Bcr-Abl tyrosine kinase inhibition imatinib for the treatment of chronic myelocytic leukemia [50, 53].

Chemotherapy involves the use of cytotoxic and cytostatic drugs and proved to be very effective when used in combination with other therapies [54]. Chemotherapeutic

drugs include alkylating agents, topoisomerase inhibitors, tubulin acting agents and anti-metabolites. Alkylating agents bind covalently with DNA, crosslink them and generate strand breaks. Drugs which are now used as alkylating agents are carboplatin, cisplatin, oxaliplatin, cyclophosphamide and melphalan which work by causing DNA damage. Doxorubicin and irinotecan are topoisomerase inhibitors and hinder DNA replication. Tubulin acting agents interrupt mitotic spindles and arrest mitosis. Paclitaxel, docetaxel, vinblastine and vincristine act on tubulins. DNA cleaving agents such as bleomycin interact with DNA and cause strand scission at the binding site [55].

Paclitaxel has been proved an effective anticancer drug against most of the cancer types. Antimetabolites stop nucleic acid synthesis and examples are methotrexate and 5-fluorouracil. Some other drugs with specific targets are also approved for the treatment. Bevacizumab inhibits vascular endothelial growth factor receptor and has been used to treat metastatic cancers [56]. Rituximab targets CD20 in lymphoma, imatinib targets Bcr/Abl, gefitinib acts on epithelial growth factor receptor bortezomib is approved as proteosome inhibitor [57].

There are many limitations to the available anticancer drugs. The existing anticancer drugs targets on rapidly dividing cells. Some of the normal cells in our body proliferate quickly like bone marrow cells, digestive tract cells and hair follicle cells. So these normal cells are also affected by the above mentioned drugs [61] and various kinds of toxicities occur as a result of chemotherapeutic treatments. E.g. - 5-Flurouracil, a common therapeutic agent is known for myelotoxicity [58], cardiotoxicity [59]. Another widely used chemo drug is doxorubicin causes cardiac toxicity [60-62], renal toxicity [63] and myelotoxicity [64]. Similarly bleomycin is known for its pulmonary toxicity [63-67] and also shows cutaneous toxicity [68].

Cyclophosphamide, drug used to treat many malignant conditions has also shown to have bladder toxicity in the form of hemorrhagic cystitis, immunosuppression, alopecia and at high doses cardiotoxicity [79]. Another is the resistance of cancer cells to the available drugs. Cancer cells undergo mutations and become resistant to the introduced drugs. Therefore an ideal cancer drug should be specifically being cytotoxic towards the cancer cells without affecting the normal cells. So there is a focus on using alternative treatments and therapies against cancer. Based on the

research findings, phytochemicals and derivatives present in plants are promising option for the improved and less toxic drugs for cancer therapy.

1.5 Plants as indispensable resources for bioactive phytochemicals

Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients [70]. They protect the plants from disease and damage and contribute to the plants color, aroma and flavour. The plant chemicals protect cells from environmental hazards such as pollution, stress and drought, UV exposure and pathogenic attack [71, 72]. Phytochemicals accumulate in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits or seeds. It is predicted that more than 5000 particular phytochemicals have been recognised in grains, fruits and vegetables but a large percentage are still unknown and must be identified before understanding their health benefits in whole foods [73,74].

1.5.1 Classification of phytochemicals

The exact classification of phytochemicals is more complex due to wide variety of them [75]. Phytochemicals mainly are classified as primary and secondary constituents. Primary constituents includes the common sugars, aminoacids, proteins, purines and pyrimidines of nucleic acids, chlorophylls etc. Secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plant steroids, cucumines, saponins, phenolics, flavonoids and glucosides [76].

1.5.1.1 Phenolics

Phenolic phytochemicals are the largest category of phytochemicals and they are of mostly three important groups of dietary phenolics- flavonoids, phenolic acids and tannins. Flavonoids are polyphenolic compounds that are ubiquitous in nature. More than 4,000 flavonoids have been recognised which occur mostly in vegetables, fruits and beverages like tea, coffee and fruit drinks [77]. The six membered ring condensed with the benzene ring is either –pyrone (flavones and flavonols) or its dihydroderivative (flavanones and flavan-3-ols). Flavonoids exert multiple biological properties including antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor activities but the best described property is their capacity to act as powerful antioxidants which protect the human body from free radicals and reactive oxygen

species [75]. Phenolic acids in general designate phenols that possess one carboxylic acid functional group. Naturally occurring phenolic acids contain two distinctive carbon frameworks: the hydroxycinnamic and hydroxybenzoic acids. Phenolic acids reported to varied biological activities as it increases bile secretion, reduces blood cholesterol and lipid levels and antimicrobial activities [76]. It's also reported for other biological properties like anti-ulcer, anti-inflammatory, antioxidant [77] and anti-depressant activities [78]. Tannins encompasses some diverse oligomers and polymers [79,80]. Tannins has the capacity to form reversible and irreversible complexes with proteins, polysaccharides (cellulose, hemicelluloses, pectin etc), alkaloids, nucleic acids and minerals [81, 82, 83]. Tannins are divided into four major groups: Gallotannis, ellagitannins, complex tannins and condensed tannins [84, 85, 86]. The tannin containing extracts are used as astringents, against diarrhoea, diuretic, against stomach and duodenal tumors [87], anti-inflammatory, antiseptic, antioxidant and haemostatic pharmaceuticals [88].

1.5.1.2 Alkaloids

Alkaloids are natural products containing heterocyclic nitrogen atoms and are basic in nature. They are seen in plants, bacteria and fungi. They are bitter in taste [75].The alkaloids are classified according to the heterocyclic ring system: Pyrrolidine alkaloids, pyridine alkaloids, pyrrolidine-pyridine alkaloids, pyridine-piperidine alkaloids, quinoline alkaloids, isoquinoline alkaloids. They are significant for the protecting and survival of plant by providing antibacterial and antifungal property, insects, herbivores (feeding deterrens) and also against other plants by means of allelopathically active chemicals [89]. They possess antihypersensitive, antimalarial and anticancer properties. Some alkaloids have stimulant property as caffeine and nicotine, morphine are used as the analgesic and quinine as the antimalarial drug [90].

1.5.1.3 Terpenoids

These are the class of natural compounds which are derived from five-carbon isoprene units. Most of the terpenoids have multi cyclic structures that differ from one another by their functional groups and basic carbon skeletons. Many of the terpenoids are commercially interesting because of their use as flavours and fragnances in food and cosmetics [91]. Terpenes are widespread in nature, mainly in plants as constituents of essential oils. Their building block is the hydrocarbon isoprene, CH_2 $=C(CH_3)$-

$CH=CH_2$. Terpene hydrocarbons therefore have molecular formula (C_5H_8) n and they are classified according to the number of isoprene units [92]; Hemiterpenoids, monoterpenoids, sesquiterpenes, Diterpenes, Triterpenes and tetraterpenoids. Terpenoids possess medicinal properties like anticarcinogenic, antimalarial, antiulcer, antimicrobial and diuretic activities. One of the sesquiterpenoid antimalarial drug is artimisinin and the diterpenoid anticancer drug is taxol [92, 93].

1.5.1.4 Saponins

Saponins are group of secondary metabolites that form stable foam in aqueous solutions. Chemically saponins include compounds that are glycosylated steroids, triterpenoids and steroid alkaloids. Saponins were found to possess membrane-permeabilising, immunostimulant, hypocholesterolaemic and anticarcinogenic ability. They are observed to have the potential to kill protozoans and molluscs, to be antioxidants, to impair the digestion of protein and the uptake of vitamins and minerals in the gut, to act as antifungal and antiviral property [94, 95, 96].

There is a considerable interest in the screening of plant and other natural product extracts in modern drug discovery programs, since structurally novel chemotypes with potent and selective biological activity may be obtained [97-100]. Bioactive phytochemicals possess diverse therapeutic functions (e.g., analgesic, anti-inflammatory, anti tumor and many more). These phytomedicines cover an important portion of our current pharmaceutics (table 1.1).

Table (1.1) Medicinal products derived from plants [61].

Drug	Pharmacological function
Aspirin	Analgesic, anti-inflammatory
Atropine	Pupil dilation
Bromelain	Anti-inflammatory
Colchicine	Anticancer
Digitoxin	cardiotonic
Ginkgolides	Brain disorders
Harpogoside	Rheumatism
Hyoscyamine	Anti-cholinergic
Morphine	Analgesic
Podophyllotoxin	Anticancer
Quinine	Antimalarial
Reserpine	Anti-hypertensive
Salicin	Analgesic
Silymarin	Antihepatotoxic
Sitosterol	Prostrate hyperplasia
Taxol	Anticancer
Vincristine and vinblastine	Anticancer

1.6 Cancer chemoprevention by phytochemicals

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years. The first records written on clay tablets in cuneiform, are from Mesopotamia and date from 2600 BCE. Egyptian medicine dates from about 2900 BCE, with best known Egyptian pharmaceutical record being the

Ebers Papyrus dating from 1500 BCE. The Chinese *Materia Medica* has been documented over the centuries with the first record (Wu Shi Er Bing Fang), containing 52 prescriptions, dating 1100 BCE and the documentation of the Indian Ayurvedic system dates from about 1000 BCE (Susruta and Charaka) [108].

Plants and their formulations are in medical uses since ancient times. Various herbal preparations with different philosophies and cultural origins are used by folk medicine practitioners to heal kinds of diseases [54]. Ayurveda, the ancient vedic literature of India, is the science of good health and well being [102]. It is the collection of traditional and cultural philosophies to cure the diseases. Plant derived natural products are nontoxic to normal cells and also better tolerated hence they gain attention of modern drug discovery. Estimated figures reveal that plant kingdom comprises atleast 250,000 species and only 10 percent have been investigated for pharmacological applications [54]. Phytochemicals and their derived metabolites present in root, leaf, flower, stem and bark perform several pharmacological functions in the body. It is being very long since plants are used for cancer treatment [101].

Cancer is currently the second leading cause of death worldwide. It is believed that the number of new cancer cases can be reduced and many cancer deaths can be prevented [103]. The primary aim is to completely prevent or atleast delay the onset of cancer through maintenance of healthy lifestyle, avoidance of exposure to toxicants / carcinogens, and dietary consumption of chemopreventive agents and drugs. The secondary aim depends on the early detection of cancer, thereby providing better management and treatment of these tumors and the secondary aim of cancer prevention involve reducing the risk of metastases, development of secondary tumors and recurrence, using preventive agents. Natural products as well as minerals and vitamins have been demonstrated to affect all three areas of cancer prevention [104, 105]. Natural products have garnered increasing attention in cancer chemotherapy because they are viewed as more biologically friendly and consequently more co-evolved with their target sites and less toxic to normal cells [106]. The National Cancer Institute (NCI) has screened approximately 35,000 plant species for potential anticancer activities. Among them, about 3,000 plant species have demonstrated reproducible anticancer activity [107].

1.6.1 Plant derived anticancer agents in clinical use

1.6.1.1 Vinca alkaloids

The vinca alkaloids: vinblastine (VLB) and vincristine (VCR) are isolated from the *Cantharanthus roseus* G. Don. comes under family Apocynaceae are the first agents introduced. The further extraction and fractionation leads to the isolation of two active alkaloids- vincristine and vinblastine. Now semi-synthetic analogues of vinca alkaloids are developed namely vinorelbine (VRLB) and vindesine (VDS). They are usually used alone or in combination to treat various type of cancer. VLB is used for treating lymphomas, breast cancer, leukemias, testicular cancer, Kaposi's sarcoma and lung cancer. VCR showed efficacy against leukemia, particularly for treating acute lymphocytic leukemia in childhood [108].

1.6.1.2 Podophyllotoxin

The species of podophyllaceae family such as *Podophyllum peltatum* Linn., *P. emodii* Wallich have were reported for a long history of therapeutical use, including the treatment of skin cancers and warts. *Podophyllum peltatum* were used by the Native Americans for the treatment of cancer. Several closely related podophyllotoxin- like lignans were introduced into clinical trials and was later dropped due to lack of efficacy and unacceptable toxicity [101]. The development of etoposide and teniposide are used as clinical agents. They act through inhibition of topoisomerase II, important enzyme involved in DNA replication.

1.6.1.3 Taxanes

The recent advancement in the development of naturally derived chemotherapeutic agent is the development of a class of molecules called taxanes. Paclitaxel otherwise known as Taxol® was first isolated from the bark of *Taxus brevifolia* Nutt. (Taxaceae). The various parts of *T. brevifolia* and other *Taxus* species (*T. canadensis* , *T. baccata*) are used in Ayurvedic medicine system reported to be used against cancer [108]. Paclitaxel, along with several precursors (the baccatins) occurs in the leaves of various *Taxus* species. The baccatins are semisynthetic conversion to paclitaxel and its analog docetaxel (Taxotere®) is major renewable natural source of its important class of drugs. Paclitaxel is used in the treatment for cancers including the breast, ovarian and non-small-cell lung cancer and also effective towards Kaposi sarcoma. It

showed most potent towards in the treatment of multiple sclerosis, psoriasis and rheumatoid arthritis. Docetaxel, primarily used in treatment of breast cancer is a semi synthetic derivative [101].

1.6.1.4 Campothecin derivatives

Campothecin, isolated from the Chinese ornamental tree *Campotheca acuminate* Decne (Nyssaceae) was advanced to clinical trials by NCI in the 1970s but later dropped due to its severe bladder toxicity [108]. Extensive research was conducted for the development of the semi-synthetic derivatives: topotecan (Hycamptin®), irinotecan (Camptostar®) and belotacan which are currently in clinical use. Topotecan is used for the treatment ovarian and small cell cancer while Irinotecan is used against colorectal cancers and belotecan is used for cervical carcinoma [101].

1.6.1.5 Homoharringtonine

Homoharringtone was originally isolated from the Chinese tree *Cephalotaxus harringtonia* var. *drupacea* (Cephalotaxaceae). Elliptinium was isolated from species of several genera of the Apocynaceae family including *Bleekeria vitensis*, a Fijian medicinal plant with reputed anticancer properties. A racemic mixture of harringtonine and homoharringtonine has been successfully in china for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia [101]. Purified homoharringtonine has shown efficacy against various leukemias, including some resistant to standard treatment. It is also reported to produce complete hematologic remission in patients with late chronic phase chronic myelogenous leukemia [108]. Elliptinium is marketed in France for the treatment of breast cancer [109].

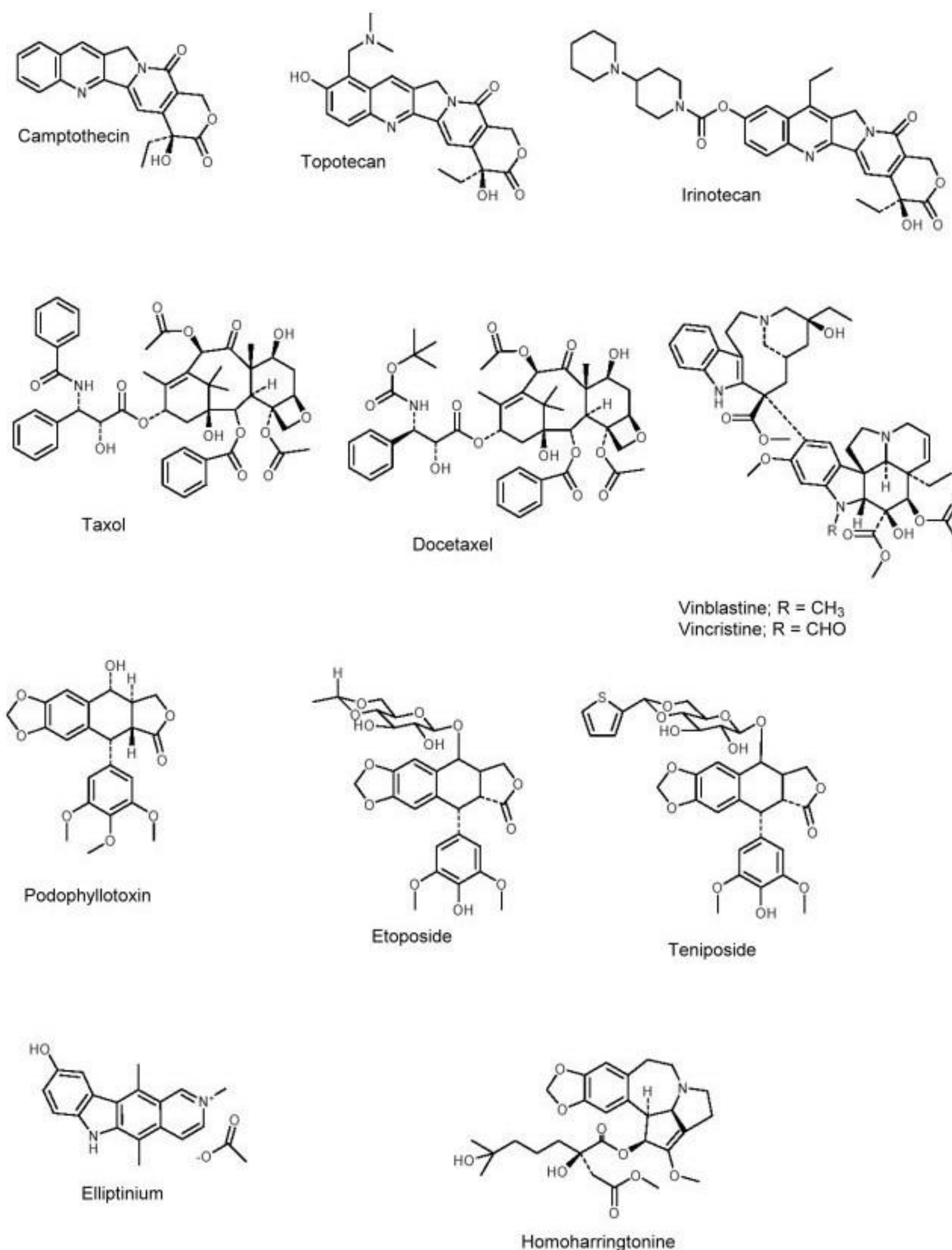


Figure (1.1) Plant derived anticancer agents in clinical use

1.7 Apoptosis and cancer treatment

Apoptosis is a mode of programmed cell death which is an active physiological process used to eliminate unnecessary cells. Induction of apoptosis in cancer cells is an important strategy for cancer treatment. It was described by Kerr *et al* in the 1970s and it remains as the most investigated processes in biological research [110].

Apoptosis is a highly selective process and involved in both physiological and pathological conditions [111, 112].

Table (1.2) Conditions involving apoptosis [95]

Physiological conditions

- During embryonic development the sculpting of tissues by programmed cell destruction
- Physiologic involution such as shedding of endometrium, regression of lactating breast
- Normal destruction of cells followed by replacement and proliferation in gut epithelium
- Involution of thymus in early age
- Clonal selection of autoreactive cells during the development of immune system

Pathological conditions

- Anticancer drug induced cell death in tumors
- Cytotoxic T cell induced cell death such as in immune rejection and graft versus host disease
- Progressive cell death and depletion of CD4+ cells in AIDS
- Some forms of virus induced cell death such as hepatitis B or C
- Cell death due to injurious agents like radiation, hypoxia and mild thermal injury
- Cell death in degenerative diseases such as Alzheimer's disease and Parkinson's disease
- Cell death that occurs in heart disease such as myocardial infarction

1.7.1 Morphological changes in apoptosis

For an apoptosis event to carry out usually several hours are required from the initiation of cell death to the final cellular fragmentation. However the time required depends upon the cell type, the stimulus and the apoptotic pathway [113]. Morphological changes occurring during apoptosis in the nucleus are chromatin condensation and nuclear fragmentation, which are accompanied by rounding up of the cell, reduction in the cellular volume (pyknosis) and retraction of pseudopodes [114]. Chromatin condensation starts at the periphery of the nuclear membrane, forming a crescent or ring like structure. The chromatin further condenses until it breaks up inside a cell with an intact membrane a feature referred as karyorrhexis [115]. The plasma membrane remains to be intact throughout the process. At the later stage of apoptosis some of the morphological changes include membrane blebbing, ultrastructural modification of cytoplasmic organelles and loss of membrane integrity are observed [114].

1.7.2 Biochemical changes in apoptosis

Mostly three main types of biochemical changes are observed in apoptosis-1) Activation of caspases 2) DNA and Protein breakdown 3) Membrane changes and recognition by phagocytic cells [116]. During the early stage of apoptosis, there is an expression of phosphatidylserine (PS) in the outer layers of the cell membrane, which has been flipped out from the inner layers. This allows the early recognition of the dying cells by macrophages, resulting in the phagocytosis without the release of proinflammatory cellular components [117].

After the externalisation of phosphatidylserine, it is then followed by a characteristic breakdown of DNA into large 50 to 300 kilobase pieces [118]. Later there is an internucleosomal cleavage of DNA into oligonucleosomes in multiples of 180 to 200 base pairs by endonucleases. This feature is characteristic of apoptosis and forms a typical DNA ladder in agarose gel electrophoresis [119]. Another important characteristic of apoptosis is the activation of group of enzymes belonging to the cysteine protease family named caspases. The ‘c’ in caspase refers to the cysteine protease while the ‘aspase’ denotes the enzyme’s unique property to cleave after aspartic acid residues [116]. Caspases and its cleaved products are monitored for the progression of the apoptotic activities.

1.7.3 Mechanisms of Apoptosis

The mechanisms of apoptosis are crucial and help in the understanding of the pathogenesis of conditions as a result of disordered apoptosis. This will help in the development of drugs that target certain apoptotic genes or pathways [120]. Caspases are central to the mechanisms of apoptosis as they are both the initiators and executioners. There are three pathways by which the caspases are activated. There are mainly three pathways by which caspases are activated. The two commonly described initiation pathways are the extrinsic (or death receptor) and intrinsic (or death receptor) pathways of apoptosis. Both pathways eventually lead to a common pathway or the execution phase of apoptosis [120]. The third less well-known initiation pathway is the intrinsic endoplasmic reticulum pathway [121].

1.7.3.1The extrinsic death receptor pathway

The extrinsic pathway begins when death ligands bind to a death receptor. The best known death receptors is the type 1 TNF receptor (TNFR1) and a related protein called Fas (CD95) and their ligands are called TNF and Fas ligand (FasL) respectively [117]. These death receptors have an intracellular death domain that recruits adapter proteins such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), as well as cysteine proteases like caspase 8 [122]. Binding of the death ligand to the death receptor results in the formation of a binding site for an adaptor protein and the whole ligand –receptor-adaptor protein complex is known as the death- inducing signalling complex (DISC) [121]. DISC then initiates the assembly and activation of pro-caspase 8. The activated form of the enzyme, caspase 8 is an initiator caspase, which initiates apoptosis by cleaving other downstream or executioner caspases [123].

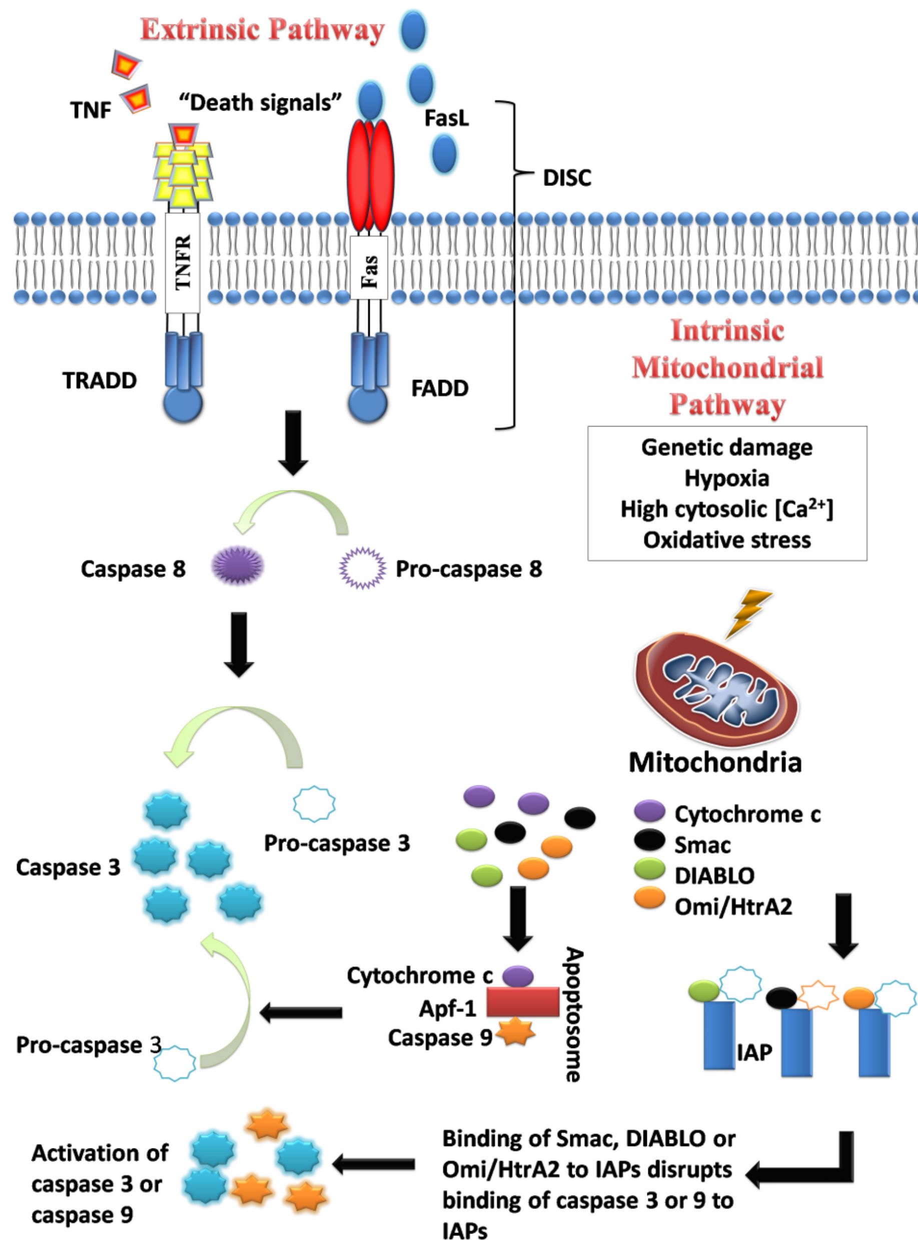


Figure (1.2) The extrinsic and intrinsic pathways of apoptosis [120]

1.7.3.2 The intrinsic mitochondrial pathway

The intrinsic pathway is initiated within the cell by the internal stimuli such as irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic Ca^{2+} and severe oxidative stress are some triggers of the initiation of the intrinsic mitochondrial pathway [123].

This pathway is the result of increased mitochondrial permeability and the release of pro-apoptotic molecules such as cytochrome-c into the cytoplasm [124]. This pathway is closely regulated by a group of proteins belonging to the Bcl-2 family. There are two main groups of Bcl-2 proteins, namely the pro-apoptotic proteins e.g. Bax, Bak,

Bad, Bcl-Xs, Bid, Bik, Bim and Hrk and the anti-apoptotic proteins e.g. Bcl-2, Bcl-X_L, Bcl-W, Bfl-1and Mcl-1[100]. The anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-c, the pro-apoptotic proteins act by promoting such release. It is the balance between the pro- and anti-apoptotic proteins that determines whether apoptosis would be initiated [125].

The other apoptotic factors that are released from the mitochondrial intermembrane space into the cytoplasm include apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP Binding protein with Low pI (DIALBO) and Omi/high temperature requirement protein A (HtrA2) [133]. The Cytochrome-c release activates caspase 3 through the formation of a complex known as apoptosome which is made up of cytochrome c, Apaf-1 and caspase 9 [126]. The Smac/DIABLO or Omi/HtrA2 promotes caspase activation by binding to inhibitor of apoptosis proteins (IAPs) which subsequently leads to disruption in the interaction of IAPs with caspase-3 or -9 [126, 127].

1.7.3.3 The common pathway

A series of caspases are involved in the execution phase of apoptosis. The upstream caspase for extrinsic pathway is caspase 8 while that of the intrinsic pathway is caspase 9. The extrinsic and the intrinsic pathway converge to caspase 3. Caspase 3 then cleaves the inhibitor of the caspase- activated deoxyribonuclease, which is responsible for nuclear apoptosis [128]. In addition to that the downstream caspases induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins and inhibitory subunits of endonucleases family. They also have an effect on the cytoskeleton, cell cycle and signalling pathways which together contribute to the typical morphological changes in apoptosis [128].

1.7.3.4 The intrinsic endoplasmic reticulum pathway

The intrinsic endoplasmic reticulum (ER) pathway is the less known third pathway. In this pathway, it is believed that the caspase 12 execute apoptosis and is mitochondria-independent in nature [129]. When the ER is injured by cellular stresses like hypoxia, free radicals or glucose starvation, there is unfolding of proteins and reduced protein synthesis in cell. There is the involvement of an adaptor protein known as TNF receptor associated factor 2 (TRAF2) dissociates from procaspase-12, resulting in the activation of the latter [121].

1.8 Apoptosis and carcinogenesis

Cancer is the result of succession of genetic changes during which a normal cell is transformed to a malignant cell with the evasion of cell death, which is one of the essential requirement in a cell to get transformed to a malignant cell [130]. In the early 1970s Kerr *et al* linked apoptosis to the elimination of potentially malignant cells, hyperplasia and tumor progression [130]. Generally the mechanisms by which evasion of apoptosis occurs can be broadly divided into: a) disrupted balance of pro-apoptotic and anti-apoptotic proteins b) reduced caspase function c) impaired death receptor signalling [120].

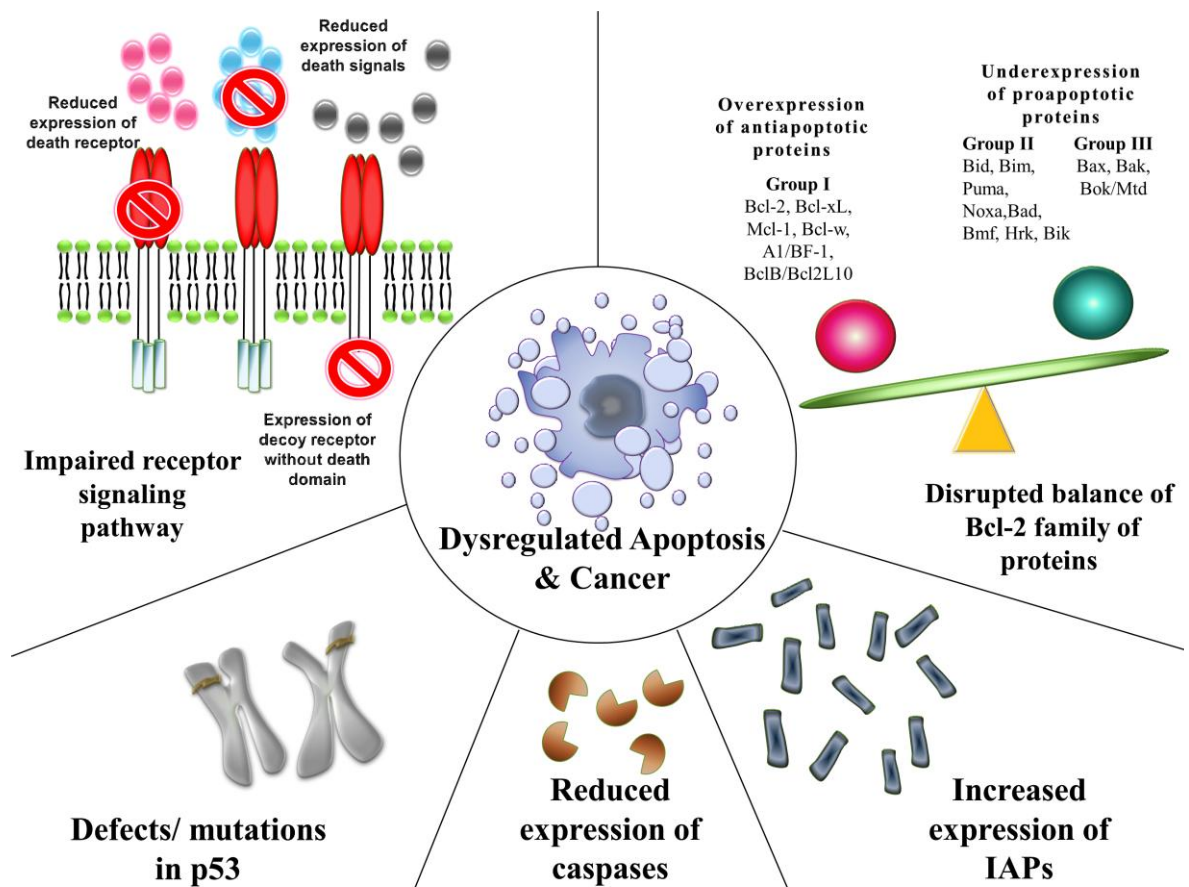


Figure (1.3): Mechanism contribution to evasion of apoptosis and progression of carcinogenesis [120].

1.8.1 Disrupted balance of pro-apoptotic and anti-apoptotic proteins

There are so many proteins reported to exert pro- or anti-apoptotic activity in the cell. It is not the quantity but rather the ratio of the pro- and anti-apoptotic proteins that plays an important role in the regulation of cell death. The over- or under-expression of certain genes found to contribute to carcinogenesis by reducing apoptosis in cancer cells [120].

1.8.2 The Bcl-2 family of proteins

The Bcl-2 family of proteins is comprised of pro-apoptotic and anti-apoptotic proteins that play an important role in the regulation of apoptosis, especially through the intrinsic pathway as they reside upstream of irreversible cellular damage and act mainly at the mitochondrial level [132]. The Bcl-2 members are located on the outer mitochondrial membrane. When there is disruption in the balance of anti-apoptotic and pro-apoptotic members of the Bcl-2 family, the result is dysregulated apoptosis in the affected cells [120]. This may be due to overexpression of one or more anti-apoptotic proteins or an underexpression of one or more pro-apoptotic proteins or a combination of both [120]. Raffo *et al* showed that the overexpression of Bcl-2 protected prostate cancer cells from apoptosis [133]. Fulda *et al* reported that the Bcl-2 overexpression led to inhibition of TRAIL- induced apoptosis in neuroblastoma, glioblastoma and breast carcinoma cells [134]. Overexpression of Bcl-xL has also been reported to confer a multi-drug resistance phenotype in tumor cells and prevent them from undergoing apoptosis [134].

1.8.3 p53

The p53 protein is also called as tumor protein 53 (or TP 53). It is one of the best known tumor suppressor proteins encoded by the tumor suppressor gene *TP53*. It is named after its molecular weight, i.e., 53 kDa [136]. It is a key player in cell cycle regulation, development, differentiation, gene amplification, DNA recombination, chromosomal segregation and cellular senescence [137] and is called the "guardian of the genome" [138]. Defects in the p53 tumor suppressor gene have been linked to more than 50% of human cancers [139]. Recently it is reported by Avery –Kieida *et al* reported that some target genes of p53 involved in apoptosis and cell cycle regulation are aberrantly expressed in melanoma cells, leading to abnormal activity of p53 and contributing to the proliferation of these cells [140].

1.8.4 Inhibitor of apoptosis proteins (IAPs)

The inhibitor of apoptosis proteins are a group of structurally and functionally similar proteins that regulate apoptosis, cytokinesis and signal transduction. IAPs are endogenous inhibitors of caspases and they can inhibit caspase activity by binding their conserved domains to the active sites of caspases or by keeping the caspases away from their substrates [141]. Dysregulated IAP expression has been reported in many cancers. For example, Lopes *et al* demonstrated abnormal expression was also responsible for resistance to chemotherapy [142].

1.8.5 Reduced caspase activity

The caspases can be broadly classified into two groups: 1) those related to caspase 1(e.g. caspase-1, -4, -5, -13, and -14) and are mainly involved in cytokine processing during inflammatory processes and 2) those that plays a central role in apoptosis (e.g. caspase -2, -3, -6, -7,-8, -9 and -10). The second group was further classified into a) initiator caspases (e.g. caspase-2, -8, -9 and -10) which are responsible for the initiation of the apoptotic pathway and b) effector caspases (caspase-3, -6 and -7) which are responsible for the cleavage of cellular components during apoptosis [143]. The low levels of caspases and the impairment in caspase function may lead to a decrease in apoptosis events and enhance carcinogenesis [120]. It was reported in one study that down regulation of caspase-9 was found to be frequent event in patients with stage II colorectal cancer and correlates with poor clinical outcome [144].

1.8.6 Impaired death receptor signalling

The death receptors and ligands of the death receptors are key players in the extrinsic pathway of apoptosis. The receptors possess a death domain and when triggered by a death signal, a number of molecules are attracted to the death domain, resulting in the activation of a signalling cascade [145]. However, death ligands can also bind to decoy death receptors that do not possess a death domain and latter fail to form signalling complexes and initiate the signalling cascade [145].

Several abnormalities in the death signalling pathways that can lead to evasion of the extrinsic pathway of apoptosis have been identified. These abnormalities include downregulation of the receptor or impairment of receptor function regardless of the mechanism or type of defects, as well as reduced level in the death signals. These changes contribute to impair signalling and thus lead to the reduction of apoptosis [120]. It has been reported that downregulation of receptor surface expression has been identified as a mechanism of acquired drug resistance. A reduced expression of CD95 was found to play a role in treatment- resistant leukaemia [146] or neuroblastoma [147] cells.

1.9 Therapeutic agents induced apoptosis in cancer cells

The success of each therapeutic approach depends mainly on the ability of the therapeutic agent to induce apoptosis either by targeting the overexpressed anti-apoptotic proteins or by stimulating the expression of the proapoptotic molecules. Many of the cancer treatments are challenged due to the resistance that the cancer cell attain and survive the treatment. Alterations in the expression levels or mutation of a chemotherapeutic drug target have an impact on apoptosis by such drugs.

1.9.1 5-Fluorouracil

The fluoropyrimidine 5-fluorouracil (5-FU) is widely used in cancer treatments including colorectal and breast cancer treatments [148, 149]. The drug targets the tumor suppressor p53 and subsequently triggers the cell cycle. 5-FU induces apoptosis in p53 dependant mechanism.

1.9.2 Antimicrotubule agents (AMA)

Microtubules are cytoskeletal fibres composed of tubulin subunits- α and β tubulin subunits and are important in maintaining cell shape, cell signalling, cell division and mitosis and in the transport of vesicles, mitochondria and other cellular components throughout the cell [150]. Taxanes, such as paclitaxel and docetaxel and vinca alkaloids, such as vinblastine and vincristine act by slowing or blocking of mitosis at the metaphase-anaphase boundary [151]. These compounds can block or slow mitosis and eventually cells die by apoptosis [152].

1.9.3 Cisplatin

The chemotherapeutic agent cisplatin and carboplatin interacts with DNA, RNA and protein. These compounds exert their cytotoxic effect mainly by the formation of DNA interstrand and intrastrand crosslinks.

1.10 Plant derived compounds exhibiting anti-apoptosis activity

Due to the increasing resistance to the present chemotherapy more research is focussing towards traditional therapies which are less toxic and not harmful to normal cells. Plant based or derived compounds are typically non-toxic to normal cells [153]. For more than 5000 years, plants have been utilized as medicines and therapies and many of the drugs used in modern medicine are directly or indirectly derived from plants [154].

Graviola is the fruit from *Annona muricata* that has been used in both alternative and traditional medicine for a variety of ailments [155], particularly for its anticancer properties. Graviola has been shown to inhibit BCL-2 proteins while increasing BAX

and promoting apoptosis [156]. The mechanism in which graviola uses is still unknown, however it is having potential anticancer property and nontoxic towards healthy cells [155].

There are many other plant-derived compounds that induce apoptosis in cancer cells and are mentioned in the table (1.3). These include black cohosh of *Actaea racemosa* [157], Juglone from *Juglans mandshurica* [158] and genistein [159]. Quercetin is found in apples and red onions, activates caspases which leads to the apoptotic response [159]. Green tea is thought to induce apoptosis in cancer cells, specifically by the compounds epigallocatechin-3-gallate [160]. Aloe-emodin, found in *Rheum palmatum*, also has caspase activation activity in cancer cells [160].

Table (1.3). Plant-derived compounds and their mechanism for inducing apoptosis [163]

Compound	Present in	Mechanism
Aloe-emodin	*Rheum palmatum*	Induces cytochrome c release [161]
Black cohosh	*Actaea racemosa*	Activates caspases [157]
Curcumin	Turmeric	Inhibits BCL-2 and activates BAX [162]
Epigallocatechin-3-gallate	Green tea component	Activates cell death receptors [163]
Genistein	Soybeans	Cell cycle arrest activation [164]
Graviola	*Annona muricata*	Inhibits BCL-2 and activates BAX [155]
Juglone	*Juglans mandshurica*	Increase caspase 9 cleavage [165]
Quercetin	Bark of many plants	Modulating cell cycle regulators to arrest cell cycle [166]

Targeting the apoptotic pathway is an intriguing approach to find new anticancer therapies as it is nonspecific to cancer types [155]. There are so many mutations found in both extrinsic and intrinsic pathways of cancer and making the cells to evade apoptosis which is found to be a characteristic of cancer. The ability to target and activate an apoptotic pathway would provide a more widespread cancer therapy. Predominantly most promising compounds to trigger apoptosis are plant derived compounds that are additionally nontoxic to healthy cells.

1.11 Role of antioxidants in cancer chemoprevention

The regulation of redox homeostasis is crucial for the maintenance of normal cellular growth, metabolism, and survival. Oxidative stress is defined as the imbalance between the production of reactive oxygen species (ROS) and the capability of the cell to elicit an effective antioxidant response. At lower concentrations, ROS are important signaling molecules involved in cellular proliferation, migration, and apoptosis [167, 168]. Several sources of ROS in cells and tissue have been identified, including mitochondrial electron transfer chain [169] and NADPH oxidase (NOX) enzymes [170]. At higher concentrations, these molecules could be useful against pathogens, resulting in increased leukocyte and platelet activation, and increased leukocyte recruitment [171]. ROS and oxidative stress have been implicated in a number of diseases such as fibrosis and cancer [172]. For cancer, there are several treatment approaches including, chemotherapy, surgery, radiation, immunotherapy and other novel targeted therapies. Cures can be achieved in some cases but resistance and recurrence are common. Chemotherapy and radiation also generate ROS, which, at high levels, are toxic to cancer cells. But sub-lethal ROS generated by these treatments were also reported to promote cancer invasion and metastasis [173]. ROS are thus considered one of the mediators of drug resistance and metastasis in cancer [173-175]. In recent years, antioxidants have drawn much attention as potential therapeutic interventions due to their ability to fight against oxidative stress and thereby interfering in the cancer development. The main function of antioxidants is to scavenge or neutralize free radical formation and to inhibit the deleterious downstream effects of ROS.

Consumption of fruits and herbal medicines in the diet is a convenient and effective method of administering phytochemicals in a cost-effective manner [176, 177]. Oxidative stress results from an imbalance in the production of reactive oxygen species (ROS) and the antioxidant capability of the cells [178]. ROS, such as, superoxide anion ($O2^-$), hydrogen peroxide (H2O2), and hydroxyl radicals (OH^-) are constantly produced in aerobic cells by incomplete reduction of molecular O2 to H2O during mitochondrial oxidative phosphorylation [178]. ROS are generated during a number of processes such as inflammation, infection, mechanical and chemical stresses, and exposure to UV rays and ionizing radiation. Basal levels of ROS act as signalling molecules to activate cell proliferation, survival, apoptosis, differentiation,

immune responses, motility, and stress-responsive pathways [178-180]. On the other hand, increased levels of ROS damage DNA, protein, and lipids which, if unrepaired, cause mutations and promote carcinogenesis [181]. However, excessive production of ROS results in extensive irreversible DNA damage, such as single or double-strand breaks, base modifications, and DNA cross links which ultimately leads to cell death [182-133]. Therefore, regulating cellular ROS is critical for maintaining cellular homeostasis.

ROS-induced oxidative stress plays a key role in cancer development and progression. Suppression of ROS using phytochemicals is crucial for cancer chemoprevention, however, at the same time, these have also been recognized as ROS-inducing agents in a wide variety of cancers. As compared to normal cells, cancer cells have heightened ROS levels [184]. However, high levels of ROS are also detrimental to cancer cells and so, they rely on a robust endogenous antioxidant system that attenuates oxidative stress in order to proliferate [185]. A number of phytochemicals have been shown to enhance the anticancer properties of chemotherapeutic agents by elevating ROS levels.

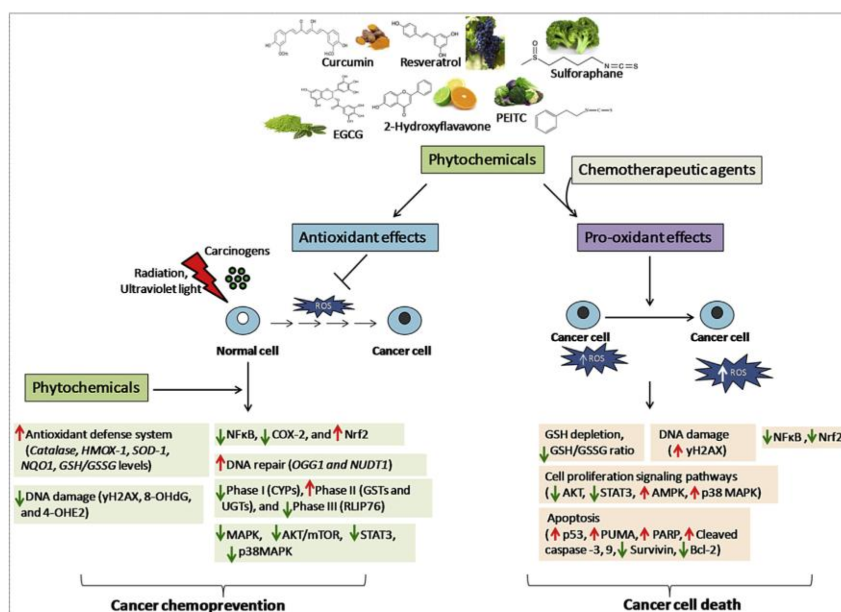


Figure (1.4): The antioxidant and pro-oxidant role of phytochemicals

1.12 Antioxidant potential of phytochemicals in cancer chemoprevention and treatment

Oxidative stress contributes to all phases of tumorigenesis either by a direct mechanism involving DNA damage or indirectly by modulating cell signal transduction. Therefore, reducing oxidative stress may play a critical role in chemoprevention. Diet-derived phytochemicals via their antioxidant property have shown promising chemopreventive effects in a wide variety of cancer types. In addition, these phytochemicals display minimal or no toxicity towards healthy tissue, thus making them ideal chemopreventive agents [186]. The roles of some dietary phytochemicals are mentioned below.

1.12.1 Curcumin

Curcumin, responsible for the yellow color of common Indian spice turmeric, is classified under bioactive compounds known as curcuminoids [187]. Curcumin, comprising of 2-5% of turmeric, has been shown to suppress ROS-induced tumorigenesis, and simultaneously protect normal tissues from ROS-mediated DNA damage [187]. The varied biologic properties of curcumin and lack of toxicity to healthy tissue, even when administered at doses as high as 8 g/d [188], makes it an attractive anticancer agent.

1.12.2 EGCG (Epigallocatechin gallate)

In several studies it has been suggested that consumption of green tea may reduce the risk of a variety of cancers. In addition, an inverse association between green tea consumption and DNA damage has been observed in several case-control studies. In chronic smokers consumption of green tea led to a significant reduction in smoking-induced micronuclei, an indicator of DNA damage, in peripheral white blood cells [189]. EGCG, a natural chemotherapeutic agent and a major active catechin in green tea, is known to possess antioxidant and anti-inflammatory properties [190]. EGCG has also demonstrated remarkable success in clinical trials for cancer prevention and cancer treatment. It has been clinically demonstrated that three out of four patients having low grade B cell malignancies showed partial response after oral ingestion of EGCG [191].

1.12.3 Resverastol

The cancer chemopreventive properties of resveratrol were first appreciated in 1997. Jang and colleagues found that resveratrol possess chemopreventive activity against all the three stages of carcinogenesis [192]. Resveratrol, a natural polyphenol found in blueberries, cranberries, nuts, red grapes, and wine, exerts anticancer effects via its antioxidant and anti-inflammatory properties. Epidemiologic study showed a 50% or greater reductions in breast cancer risk in women consuming grapes rich in resveratrol [193].

1.12.4 Phenethylisothiocyanate- PEITC

Cruciferous vegetables such as broccoli, brussels sprouts, cabbage, and cauliflower are rich in glucosinolates that can endogenously be converted into isothiocyanates such as phenethylisothiocyanate (PEITC) [194]. Phytochemicals such as PEITC and sulforaphane derived from consuming cruciferous vegetables such as broccoli have been studied for their potential chemotherapeutic and chemoprevention role in a variety of cancer types [195,196].

1.12.5 Sulforaphane

Sulforaphane, a potent antioxidant phytochemical from broccoli and broccoli sprouts has been shown to inhibit or retard tumor incidence and progression in carcinogenesis models of breast [197], colon [198], stomach [199] and lung [200].

1.12.6 Citrus flavonoids

Citrus fruits and peels are a rich source of flavonoids such as hesperedin, nobiletin, 2’-hydroxyflavanone (2HF), and quercetin which possess anticancer effects. Hesperidin has been shown to protect from carcinogen-induced hepatocarcinogenesis by activating Nrf2/ARE/HO-1 pathway and increasing GSH levels in liver tissues [201]. Nobiletin has been shown to inhibit inflammation induced colon carcinogenesis by increasing HO-1 and NQO1 levels [202]. The citrus flavonoid 2HF has been shown to exhibit strong anticancer effects in renal cancer by decreasing protein expression and activity of GSTs [203,204]. In addition, quercetin treatment inhibits Aurora kinase B, an inhibitor of p53 [205], and regressed lung tumors in mice xenografts model of lung cancer via p53-mediated ROS pathway [206]. Thus, citrus flavonoids exert potent regulatory effects on oxidative stress pathways both in cancer chemoprevention and treatment.

1.13 Process development for isolation and purification of anticancer phytochemicals

Therapeutic efficacy of any medicinal plant depends upon the quality and quantity of the active phyto-constituent, which vary with latitude, altitude, climate and season. Different parts of a plant may possess different level of therapeutical activity. Additive or synergistic effects of bioactive phyto-constituents might be responsible for the concerned pharmacological function rather than the purified one. These bioactive phytoconstituents can be developed in antitumor therapeutic entities but they demand intense effort. Several approaches can be used to purify these pharmacologically active phytochemicals. These include isolation and assay, combinatorial chemistry and bioassay-guided fractionation. Bioassay guided fractionation involves the separation of bioactive phytochemicals from a mixture of compounds using various analytic techniques based on biological activity testing.

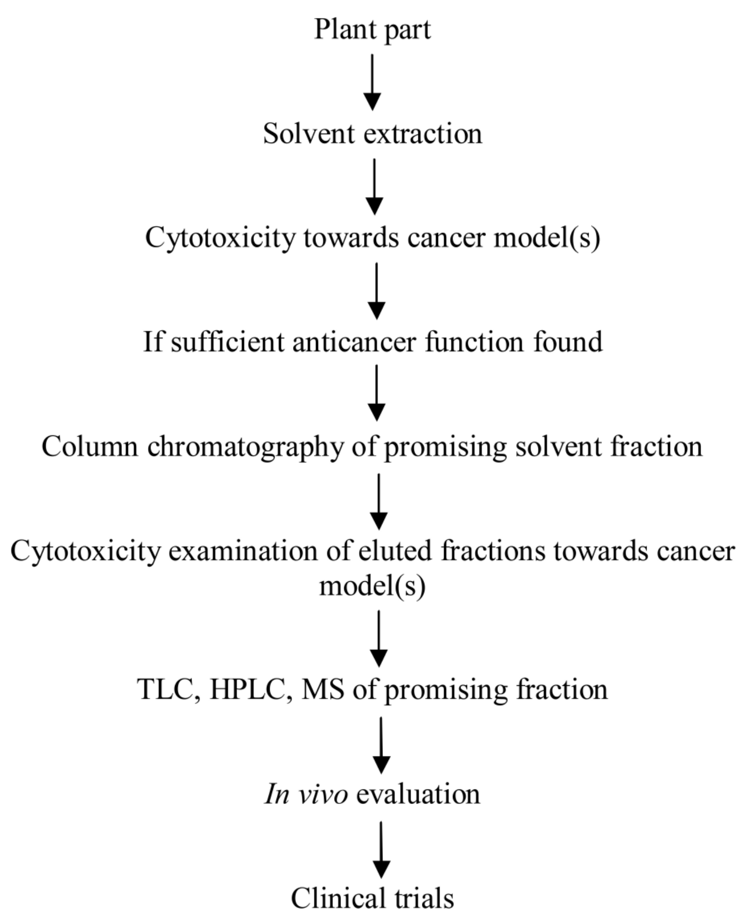


Figure (1.5): Development of the bioactive phytoconstituents into an anticancer therapeutic product

The process begins with the testing of natural extract with the confirmed bioactivity. The active extract is fractionated on suitable matrices, eluted fractions are tested for bioactivity and active fractions are examined by various analytic techniques, viz. thin layer chromatography (TLC), HPLC, FTIR, and Mass spectroscopy (MS). This approach can also be used to purify antimicrobial, antilipolytic and antioxidant compounds [34].

Solvents should be used in an increasing polarity order. Different TLC spraying reagents can be used for the detection of natural compounds. The procedures may be modified but purity and quantity of the bioactive molecule must be high as much as possible and this can be achieved by using good quality of solvents, experimental careful handling and efficiency of the procedure. After purification, molecule(s) must be examined *in vivo* for the anticancer effects. If a promising tumor killing is achieved by the molecule then other parameters i.e., safety and adverse effects, dose concentration, pharmacokinetics, drug interactions etc. must be explored for the drug molecule [34].

1.14 *Thespesia populnea* (L.) Soland ex Correa

T.populnea belongs to the family malvaceae or otherwise known as mallow family. It is a small evergreen tree averaging 6-10m in height, with a short, often crooked stem and a broad dense crown. It is commonly seen in the coastal areas of Indian and pacific oceans.

Figure (1.6): *Thespesia populnea* (L.) Soland ex Correa (Research specimen)

The plant is well known for its medicinal property as it is widely used in traditional medicine. Bark and fruit possess more curative properties. The plant is astringent, depurative, anti-inflammatory, antibacterial, antidiarrhoeal. It is also used for scabies, psoriasis, ringworm, diabetes, cough, asthma [207]. The fruits are employed in treating herpetic disease. A compound oil of bark is used in urithritis, gonorrhoea. The leaves are applied to inflamed and swollen joints [208].

The major chemical constituent present in *T.populnea* was gossypol. There are many other compounds isolated from the dichloromethane extract of wood and dark heartwood of *T.populnea*. Populene, Mansonones, 7-hydroxycadalene, 7-hydroxy-2,3,5,6-tetrahydro-3,6,9-trimethylnaphtol[1,8-b,c]pyran-4,8-dione and Thespesone from dark heartwood. Gossypol and 6, 6' methoxy gossypol was isolated from wood. Mansonones, gossypol, populene has been reported for their cytotoxicity. There are also some other compounds like kaempferol, quercetin, kaempferol 3-glucoside, rutin, nonacosane, lupenone, myricyl alcohol, lupeol, β-sitosterol and β-sitosterol-β-D-glucoside, 5,8 dihydroxy-7-methylflavone, 7 Hydroxy isoflavone, thespone, populneol and Thespesin reported in *T.populnea*. 3-O-methylthespesilactum is an anticancer molecule isolated from heartwood of *T. populnea* [269].

Even though the plant possesses many medicinal properties, it is not well studied for its antiproliferative and apoptosis inducing ability of this plant. So the plant was selected to know more about its cytotoxicity towards different cancer cell lines. Other properties such as antioxidant, antimicrobial and anti-inflammatory property were also checked in this study.

MEGHA K. B. “THERAPEUTIC POTENTIAL OF MEDICINAL PLANT
THESPESIA POPULNEA (L.) SOLAND EX CORREA WITH SPECIAL
EMPHASIS TOWARDS ITS ANTIPROLIFERATIVE ACTIVITY AGAINST
DIFFERENT CANCER CELL LINES.” THESIS. DEPARTMENT OF
BIOTECHNOLOGY, UNIVERSITY OF CALICUT, 2018.

CHAPTER 2
MATERIALS AND METHODS

2.1 Preparation of plant extracts

2.1.1 Collection of plant material

Fresh matured leaves of *T. populnea* were collected and identified and authenticated by Dr A. K. Pradeep, Assistant Professor, Department of Botany, University of Calicut, Kerala an angiosperm taxonomist. A voucher specimen with accession number-6934 was deposited in the herbarium at the same department.

2.1.2 Preparation of plant extracts

The fully matured leaves were collected, washed thoroughly and shade dried. The dried leaves were coarsely powdered. The 100g of leaf powder was subjected to extraction sequentially with organic solvents petroleum ether, chloroform, ethyl acetate and methanol of increasing polarity for 48h with continuous shaking. After each extraction, extracts were filtered through Whatmann No1 filter paper. The fitrate was concentrated and dried and dissolved in suitable solvents for further analysis. The residue was again extracted with the subsequent solvents.

2.2 Phytochemical analysis of powder and extracts

2.2.1 Fluorescence analysis and behaviour of leaf powder

The dried leaf powder were analysed under day light, short ultraviolet light, long ultraviolet light without any treatment and also after treatment with various organic and inorganic reagents like NaOH, HCl and H_2SO_4.The color of the fluorescence is noted [209].

The behaviour of *T. populnea* leaf powder was identified after subjected to treatment with different chemical reagents to detect the occurrence of phytoconstituents. Precipitate formation after the addition of picric acid confirms alkaloids. Reddish brown color formation after adding Con. H_2SO_4 confirms steroids. Tannins confirmed by the production of black precipitate on addition of Aq. $FeCl_3$. On adding iodine solution blue color confirms the presence of starch. Alkaloid presence confirmed by precipitate formation on adding Mayer’s reagent. Formation of yellow color on

addition of aqueous NaOH confirms flavonoids. On addition of Mg-HCl dark pink color confirms flavonoids. Dark brown precipitate on addition of Dragendroff's reagent detects alkaloids. Proteins/aminoacid was detected by the formation of pale white color with ninhydrin.

2.2.2 Qualitative phytochemical analysis of extracts

Sequentially prepared extracts of *T. populnea* were tested for the presence of bioactive compounds using standard methods [210, 211].

Proteins-

- **Biuret's test-** To 1 ml of hot aqueous extract 5-8 drops of 10% sodium hydroxide solution and followed by 1 or 2 drops of 3% copper sulphate solution were added and mixed. Formation of violet red color denotes the presence of proteins.
- **Ninhydrin test-** Crude extract when boiled with 2 ml of 0.2% solution of ninhydrin. Appearance of violet color confirms the presence of amino acids and proteins.

Carbohydrates-

- **Mohlish's test-** Crude extracts were mixed with 2 ml of Mohlish's reagent and shaken properly and immediately 2 ml of Con. H_2SO_4 was poured carefully along the sides of the test tube. Appearance of a violet ring at the interphase indicates the presence of carbohydrate.
- **Iodine test-** 1 ml of crude extracts was mixed with 2 ml of iodine solution. The presence of carbohydrate is indicated by dark blue or purple coloration.
- **Benedict's test-** Crude extracts when mixed with 2 ml of Benedict's reagent and boiled, a reddish brown precipitate formed when carbohydrates are present.

Alkaloids-

- **Dragendorff's reagent test-** To 1 ml of the extract, 2 ml of Dragendroff's reagent was added and mixed. To this 2 ml of dilute HCl was added. Formation of an orange color precipitate indicates the presence of alkaloids.
- **Mayer's test-** Few drops of Mayer's reagent was added to the 1 ml of extract. Formation of cream color precipitate indicates the presence of alkaloids.
- **Wagner's test-** To 1 ml of extract equal volume of Wagner's reagent added. Formation of reddish brown precipitate indicates the presence of alkaloids.

Glycosides-

- **Keller killiani test-** The extracts (1 ml) were dissolved in 1 ml of glacial acetic acid and cooled, after cooling 2-3 drops of ferric chloride was added. To this solution 2 ml of Con. H_2SO_4 was added carefully along the walls of the test tube. Appearance of reddish brown colored ring at the junction of two layers indicates the presence of glycosides.
- **Molish's test-** Described earlier. Formation of reddish purple colored ring at the junction of two layers indicates the presence of glycosides.
- **Con. H_2SO_4-** To 1 ml of test solution, 1 ml of Con. H_2SO_4 added and allowed to stand for 2 min. A reddish purple colored ring at the junction of two layers signifies the occurance of glycosides.

Tannins-

- **Ferric chloride test-** Few drops of ferric chloride solution was added to the test solution. Blackish precipitate indicates the presence of tannins.
- **Gelatin test-** The extracts were treated with few drops of gelatin solution. Formation of a white precipitate confirms the tannin.
- **Lead acetate test-** Basic lead acetate solution was added separately to 1 ml of test solution, bulky red precipitate confirms the presence of tannins.
- **Alkaline reagent test-** To the extracts (1 ml), a solution of NaOH was added. The presence of tannins can be confirmed by the yellow to red coloration.

Phenols-

- **Ellagic acid test-** The extracts were treated with few drops of 5% glacial acetic acid followed by 5% $NaNO_2$ solution. Formation of muddy brown color confirms the presence of phenols.
- **Phenol test-** To the extracts 1 ml of ferric chloride solution was added. The development of intense coloration indicates the presence of phenols.

Sterols-

- **Salkowski test-** To the test solutions 5ml of chloroform was added. To the above mixture, 1 ml of Con.H_2SO_4 was added carefully along the walls of the tube and mixed. The reddish color formation in the lower layer is the indication of steroids.

Terpenoids-

- To 1-2 ml of extracts 1% HCl was added and allowed to stand for 5-6 hours. Later, these extracts were treated with 1 ml of Trim-Hill reagent and heated in a boiling water bath for 5-10 min. Formation of bluish green color confirms the presence of terpenoids.

Flavonoids-

- **Shinoda test-** To the 1 ml of the test solution, add few fragments of magnesium ribbon. Then Con. HCl was added carefully along the sides of the test tube drop wise. Crimson red color indicates flavonoids.
- **Lead acetate test-** Basic solution of lead acetate was added separately to 1 ml of test solutions. Bulky reddish brown precipitate confirms the presence of flavonoids.
- **Alkaline reagent test-** The test solutions were treated with Sodium hydroxide solution. Flavonoid presence was obtained by a yellow to red color change.

Coumarins-

- The extracts were treated separately by covering with a piece of paper soaked in NaOH and heated. When the tubes yield a yellow fluorescence under UV light after treatment indicates the presence of coumarins.

Anthraquinones-

- 1 ml of extract was mixed with equal volumes of benzene and then about 1 ml of 10% ammonia solution was added. Formation of red color on addition of ammonia gives a clear evidence for anthraquinone.

Phlobatannins-

- 2-3ml of 10% HCl was added to 10 ml of extract in a boiling tube and the contents were boiled for 5-6 min. Formation of red color precipitate indicates the presence of phlobatannins.

Resins-

- To the extracts 2-3 ml of copper sulphate solution was added mixed well for about 2 min and then allowed to separate. Resins were indicated by green color precipitate.

Quinones-

- Extracts were treated separately with alcoholic KOH solution. Appearance of colors ranging from red to blue indicates the presence of quinones.

Fixed oils-

- Small quantity of extract was taken and pressed between two Whatman No.1 filter paper. The stain on the filter paper indicates the presence of fixed oils.

Saponins-

- 5 ml of each extract is taken in separate test tube and shaken vigorously to obtain a stable froth. Saponins are confirmed by a stable froth.

2.3 Antioxidant property of leaf extracts

2.3.1 DPPH radical scavenging assay

DPPH radical scavenging assay was performed to identify the antioxidant properties of *T. populnea* leaf extracts. 0.1 ml of the test extract was added with 1.9 ml of 0.1mM DPPH dissolved methanol. It was mixed thoroughly and kept for incubation for 30 minutes in the dark. After incubation the optical density of the extracts was taken at 517nm, methanol was kept as blank [212]. The percentage DPPH scavenged by the extracts were calculated using the equation-

% DPPH scavenging= [1-(sample/control)] X 100

All the extracts except PEE exhibited a potent DPPH radical scavenging ability. The IC_{50} value was calculated using ED50plusv1.0 software.

2.3.2 Nitric oxide radical scavenging inhibition assay

The extracts were subjected to evaluate the nitric oxide radical scavenging ability. For the reaction, 1ml extract is mixed equally with PBS followed by 4ml 10mM Sodium nitroprusside solution. Later, incubated for 150 minutes at room temperature. 0.5 ml of reaction mixture mixed with 1ml sulphanilic acid reagent (0.33% sulphanilic acid in 20% glacial acetic acid and kept for 5 minutes to diazotise. Later 1ml N-(1-naphthyl) ethylene-di-amine dihydrochloride was added and kept in diffused light for 30 minutes. Absorbance was taken at 540nm [213].

% Nitric oxide scavenging = [1-(sample/control)] X 100

CHFE exhibited nitric oxide radical scavenging ability and compared with the standard ascorbic acid.

2.3.3 Metal chelating ability of extracts

The chelating of ferrous ion by the extracts of *T.populnea* was estimated using the method mentioned below [214]. The different concentrations of the extract were mixed with 0.1 ml of 2 mM ferric chloride and 0.2 ml of 5mM ferrozine added to start the reaction. The reaction mixture incubated for 10 minutes in room temperature. The absorbance was measured spectrometrically at 562 nm. The EAE only exhibited metal chelation ability and was compared with standard EDTA.

2.3.4 Total reducing power assay

The reducing ability of the extracts was determined by the following method [216]. 2.5 ml of the test extract was mixed with 2.5 ml of 200mM sodium phosphate buffer. To this mixture 2.5 ml of 1% potassium ferricyanide was added, mixed and incubated for 50°C for 20 minutes. After incubation, 2.5 ml of 10% trichloroacetic acid was added to the reaction mixture and centrifuged for 3000 rpm for 10 minutes. From the upper layer 5ml was taken separately and mixed equally with deionised water. To this 1ml of 0.1% ferric chloride added and absorbance was measured at 700 nm against a blank. A higher absorbance indicates higher reducing ability. All the extracts except PEE exhibited the reducing power ability and ascorbic acid was used as standard.

2.3.5 Total antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of the leaf extracts of *T. populnea* was determined by phoshomolybdenum method [215] using ascorbic acid as standard. To 0.1 ml sample solution, 1 ml of reagent solution (0.6 M Sulphuric acid, 28 mM Sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 minutes. After cooling the absorbance of the mixture was measured at 695 nm. Total antioxidant capacity was not exhibited by PEE.

2.3.6 Total phenolic content of the extracts

The total phenolic content of the extracts was determined by Folin-Ciocalteu method [217]. About 0.1 ml of the extract was mixed with 0.5 ml of Folin-Ciocalteu reagent which was diluted in 1:10 ratio with distilled water and 1.5 ml sodium carbonate. The mixture was shaken thoroughly and made upto 10 ml using distilled water. The reaction mixture was incubated for 2 h. The absorbance was measured at 750 nm. Gallic acid was used as the standard. The total phenolic content was expressed as

gallic acid equivalent in mg/g of the extract. In CHFE, EAE and MOHE the phenolic content was estimated.

2.3.7 Total flavonoid content of the extracts

Total flavonoid content of the extracts was estimated using aluminium chloride colorimetric method [218]. The 0.5 ml of the extracts were mixed with 1.5 ml of 75% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. The reaction mixture was incubated for 30 minutes at room temperature. The absorbance was taken at 435 nm. The total flavonoid content of CHFE and EAE was expressed as the quercetin equivalent in mg/g of the extracts.

2.4 Antimicrobial potential of extracts.

2.4.1 Antimicrobial testing using microdilution method

The leaf extracts of *T. populnea* were tested for their antimicrobial activity against *Salmonella typhi* (MTCC 734), *Klebsiella pneumoniae* (MTCC 109), *Fusarium oxysporum* (MTCC 284), *Aspergillus niger* (MTCC 282) and *Candida albicans* (MTCC 227) using microdilution method.

2.4.1.1 Preparation of microbial culture

Using aseptic techniques a single pure colony was transferred into 10 ml nutrient broth for bacterial strains and potato dextrose broth for fungal strains capped and placed in incubator overnight at 37°C. After incubation, using aseptic preparation, turbidity of suspensions was calculated and adjusted using McFarland standards as a reference [219].

2.4.1.2 Preparation of microtiter plates

Microtitre plates were prepared under aseptic conditions. A sterile 96 well plate was labeled. A volume of 20µl, 40 µl, 60 µl, 80 µl, 100 µl of test material was pipetted into the wells. 100 µl of medium was added to each well. Finally, 100µl of microbial suspension was added to each well. Control dilutions of test material were also kept. A column with all solutions except the test compound was prepared as organism controls. Plate was wrapped loosely with cling film to ensure that organism did not become dehydrated. The plates were incubated at 37^{0} C for 24 hours and OD reading was taken (OD_{600}) after sufficient incubation.

Optical density of final test was obtained from subtracting the extract control OD from the test OD. The % of inhibition was calculated from the following equation;

% of inhibition= (Control- final Test)/Control $\times$100

Where control = organism control

2.5 Anti-inflammatory potential of extracts

2.5.1 BSA antidenaturation assay

The anti-inflammatoty activity of the leaf extracts was done using BSA antidenaturation assay [220]. To 0.2 ml of extract solution/standard prepared in isosaline, 1.8 ml of 1% BSA was added. This solution was incubated at 57°C for 10-15 min. After cooling the turbidity was measured at 660 nm. Diclofenac sodium was used as standard. The denaturation inhibition concentration-DIC_{50} values were calculated using the ED50 plus V1.0 software program. The CHFE exhibited anti-inflammatory activity.

2.6 Antiproliferative potential of extracts

2.6.1 Cell lines

The cell lines of various site of origin were used in the present study. The cell lines used in the present study are leukemic cell lines- Jurkat E6.1 (acute T cell leukemia), HL-60 (acute promyelocytic leukemia) and K-562 (chronic myelogenous leukemia), adenocarcinoma cell lines- PC-3 (prostate cancer), HeLa (cervical cancer) and MDA-MB-231 (Breast cancer), carcinoma cell lines- A549 (lung carcinoma) and HCT116 (colorectal carcinoma). Two cell lines of animal origin L929- Mouse fibroblast, Vero-Monkey kidney cells. The cell lines were obtained from National Centre for Cell Science, Pune, India. The entire cell lines were maintained in the recommended media supplemented with 10% heat inactivated FBS, gentamycin 40 µg/ml, streptomycin 100 µg/ml and penicillin 50 IU/ml and were grown at 37°C in a humidified atmosphere with 5% CO_2.

2.6.2 Sub-culturing the cell lines

The cells were maintained in 25 cm^2 flask. The suspension cells especially all leukemic cell lines were sub cultured without trypsinisation. The sub culturing ratio used was 1:2 to 1:4. The adherent cells were trypsinised before sub culturing. For trypsinisation, medium was completely removed and washed with PBS to remove the

traces of serum. Add 2 ml Trysin EDTA solution (0.25% trypsin in 0.53 mM EDTA) and incubated for 5 minutes for complete detachment of the attached cells. Complete growth media was added and incubated. All the leukemic cell lines were maintained in RPMI-1640. HeLa, HCT 116, MDA-MB-231 L929, Vero were maintained in DMEM, A549 in MEM and PC-3 in Hams F-12 nutrient mixture supplemented with 10% heat inactivated FBS, gentamycin 40 µg/ml, streptomycin 100 µg/ml and penicillin 50 IU/ml.

2.6.3 MTT assay

Cell proliferation was measured using 3-(4,5-dimethyl thiazol-2-yl)-2,5 di phenyl tetrazolium bromide (MTT) assay [221] which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product [222]. The method in brief, the cells were seeded in a 96 well cell culture plate with a density of 15000 cells/well and incubated overnight. After incubation, fresh medium containing varying concentration of extracts (10-100 µg/ml) was added to respective wells and incubated for 48 hours in a CO_2 incubator. At the end of incubation period medium was aspirated and replaced with new media containing MTT (5 mg/ml) and incubated for 3-4 hours. The formazan crystals thus formed were dissolved in DMSO and the absorbance was measured at 570 nm. The experiments were done triplicates and the IC_{50} values were calculated using the ED50 plus V1.0 software program. The antiproliferative properties of the extracts were performed and it was found that only CHFE exhibited the potential to inhibit the growth of cancer cell lines.

2.6.4 SRB assay

This is another assay used to check the antiproliferative ability of CHFE in PC-3, MDA-MB-231, A549 and HCT116. The initial steps in the SRB are similar to MTT assay with the desired concentration of treatment until 48h incubation. After incubation, the medium was completely removed and cells were fixed for 5 minutes at -20°C with methanol: acetic acid (95:5). Then the plates was washed thrice with distilled water and dried. Later, 100 µl 0.4% SRB in 1% acetic acid was added and incubated for 30 minutes at room temperature. After incubation, the plates were washed thrice with 1% acetic acid and the SRB dye was dissolved by adding 200 µl 10 mM Tris pH 10.5. The absorbance was taken at 450 nm and IC_{50} values were calculated using the ED50 plus V1.0 software program [223].

2.6.5 Acridine orange/Ethidium bromide double staining

The Acridine orange/Ethidium bromide staining was done in K562 and HCT116 cells. The cells were seeded at a concentration of $1X10^5$cells/ml and treated with different concentration of CHFE for 48h. In K562 (10, 25 and 50 μg/ml) and HCT 116 (10, 37 and 50 μg/ml) concentrations were used. The cells were harvested and washed with PBS and 10 μl of the acridine orange/ethidium bromide staining solution was added and incubated for 2 minutes. The cells were then observed under fluorescent microscope [224].

2.6.6 Nuclear Hoechst staining

The nuclear Hoechst staining was done in K562 and HCT116 cells. The cells were seeded at a concentration of $1X10^5$ cells/ml and treated with different concentration of CHFE for 48h. In K562 (10, 25 and 50 μg/ml) and HCT 116 (10, 37 and 50 μg/ml) concentrations were used. The cells were harvested and stained with Hoechst 33342 to a final concentration of 10 μM for 15 min in the dark at 37^oC and then examined using fluorescent microscope [225].

2.6.7 Scanning electron microscopic analysis

The SEM analysis was done to observe the morphological changes occurred in cells during apoptosis. In K562 and HCT116 cells treatment was done for 48h with different concentrations as mentioned earlier. The control and the treated cells after treatment was washed with ice cold PBS. Cells were then prefixed with 4% gluteraldehyde prepared in PBS and incubate for 2 minutes. Wash the prefixed cell pellet with ice cold PBS for 2 minutes. The cells were dehydrated by washing with acetone. The dried cell pellet was observed using SEM facility (Carl Zeiss Gemini 300 Germany using Smart SEM software).

2.6.8 DNA fragmentation

The cells were seeded at a concentration $1X10^6$ cells/ml and treated with different concentration of CHFE for 48h. In K562 (10, 25 and 50 μg/ml) and HCT 116 (10, 37 and 50 μg/ml) concentrations were used. The cells were harvested and centrifuged for 1000g for 5 minutes. The cell pellet was resuspended in lysis buffer containing 0.2% Triton X 100, 10mM Tris-HCl, 1mM EDTA with pH 8 and centrifuged for 15 minutes at 13,800g. Transfer the supernatant and treat with 15 μl RNase for 37^oC for

1h. Add 20µl of 20% SDS, 8 µl of proteinase K and 25 µl of 5M NaCl to the tube and incubate at 37°C for 30 minutes. After incubation 370 µl of phenol was added and centrifuged for 10 min at 13,000g. The upper layer of the liquid was added to equal volume of phenol/chloroform/isoamylalcohol (50:49:1) and centrifuged for 10 min at 13000g. The upper layer was transferred and equal volume of isopropanol was added and incubated for overnight at -20°C.The isolated DNA was suspended in TE buffer and separated over 1.2 % agarose gel and visualised under UV light using ethidium bromide [226].

2.6.9 Propidium iodide staining and cell cycle analysis

For staining the cells with Propidium iodide, the treated cells were fixed with methanol: acetone (1:1) at -20°C for 10 min. After fixation, washed with PBS and the cells were centrifuged. Propidium iodide stain was added and incubated at 37°C for 30 minutes. To perform cell cycle analysis both K562 and HCT116 cells were incubated with different concentration as mentioned earlier for 48h. After incubation the cells were washed with PBS and treated with 50 µl RNase (200 µg/ml) for 5 minutes followed by 400 µl propidium iodide stain (50 µg/ml) in PBS containing 0.1% Triton X and incubated for 30 minutes [227]. The cells were analysed using BD FACS ARIA II and results were analysed using BD FACS DIVA^{TM} software.

2.6.10 Annexin V-FITC staining and analysis

In K562 cells both Annexin V FITC staining and flow cytometric analysis were performed and in HCT 116 cells AnnexinV FITC staining alone was performed. After incubation with desired concentration of CHFE as mentioned earlier the cells were harvested and washed with ice cold PBS and then resuspended the cells in 1X binding buffer and then 5 µl of Annexin V FITC and PI were added according to the manufactures instruction (Alexa Fluor 488 Annexin V/Dead cell Apoptosis Kit, Life Technologies Corporation, USA). Gently vortex the cells and incubate for 15 minutes at room temperature in the dark. From this stage the cells were mounted in slides and subjected to fluorescence microscopy. For apoptosis analysis by flow cytometry again 400 µl of binding buffer was added and the cells were analysed using BD FACS ARIA II and results interpreted using BD FACS Diva software.

2.6.11 Determination of mitochondrial membrane potential

The K562 and HCT116 cells were treated with desired concentration of CHFE. After 48h the cells were harvested and washed with PBS and exposed to rhodamine 123 (10 µg/ml) for 30 minutes at 37°C. The cells were then washed with PBS and resuspended in 0.5 ml of PBS. It was then observed under fluorescent microscopy.

2.6.12 Western blot analysis

The K562 and HCT116 were treated with earlier mentioned concentration of CHFE. After incubation the cells were collected and washed with PBS. The cells were lysed using 50mM Tris-HCl pH 7.5, 100mM Nacl, 1mM DTT and PMSF. The cell lysate was then sonicated and incubated at -20°C. Equal quantities of proteins were loaded in 12% SDS-PAGE after boiling for 5 minutes at 95°C. After electrophoresis, the proteins were electrotransfered to PVDF and washed with TBST. The membrane was blocked for 1h at RT by 5% skimmed milk in TBST. The membrane was then washed thrice with TBST. The PARP, Caspase-9 and -3 primary antibodies (1:1000) (cell signalling technology, USA) and beta actin antibody (1:1000) (sigma Aldrich, USA) overnight at 4°C. In the next step, membrane was finally washed thrice and incubated with DAB (3, 3'-diaminobenzidinetetrahydrochloride) the bands will be developed within 3-5 minutes.

2.6.13 Colony inhibition assay

HCT116 cells were treated with 10, 37 and 50 µg/ml concentrations of CHFE and incubated for 48h. After incubation the media was changed regularly till 14-15 days. Finally the colonies were stained with Giemsa and counted [228]. The experiments were performed in triplicate.

2.6.14 Cell migration inhibition assay

HCT116 cells were seeded in 35 mm dish to form a confluent layer. The monolayer was made by scratching in a straight line [228]. To remove the debris formed it was washed with PBS. Fresh media and CHFE of concentration 10, 37 and 50 µg/ml was added and incubated for 24h. The migration of the cells towards the scratched region was monitored at different time intervals and phase contrast microscopic images were taken.

2.7 *In vivo* antitumor activity of leaf extract

Male swiss albino mice weighing 22-25g were used for the experiment. Before commencement of the experiment the mice were acclimatized to laboratory conditions for 7 days. All procedures described were reviewed, approved and study was conducted after obtaining ethical committee clearance from the Institutional Animal Ethics Committee of University of Calicut, Registration number 426/2/CPCSEA.

2.7.1 Acute toxicity study

Acute toxicity study of CHFE was conducted as per OECD guidelines 420 (fixed dose procedure) using Swiss mice. Each animal was administered test samples at a dose of 1000mg/kg by oral route. The animals were observed for any changes continuously for 1, 3, 6, 9h and for up to 24h. The animals were then kept for 15 days to observe daily cage side observations and for mortality [229].

2.7.2 Treatment Schedule to check the mean survival time

30 Swiss albino mice were used in the experiment, which were divided into five groups (n=6), they were fed with food and water *ad libitum*. All the animals in the group received EAC cells 1X10^6 cells intra peritoneally. Group I animals served as EAC control without any drugs. 24h after EAC transplantation, group II-IV received CHFE at a dose of 50, 100 and 200 mg/kg body weight CHFE in 0.1% carboxymethycellulose and group V received reference drug cyclophosphamide 25 mg/Kg body weight orally for continuous 10 days.

2.8 Bioassay guided isolation and characterisation of anticarcinogenic compounds

2.8.1 Thin layer chromatography

The thin layer chromatographic separation of CHFE was performed. The plates were prepared manually by using Silica Gel G for thin layer chromatography. The required amount of silica was mixed with distilled water to prepare a suspension. This was then uniformly distributed on the glass plate and kept for air dry. The plates were charged by keeping it under 110°C for 1h. Sample was prepared by dissolving it in chloroform. The sample was loaded 2 cm above its bottom. The plate was loaded with 5µl of the CHFE and then kept in glass chamber saturated with solvent combination petroleum ether: ethyl acetate (6:4). The mobile phase was allowed to move through

adsorbent phase up to 3/4th of the plate. The plates were then observed under visible, long and short ultra violet light.

2.8.2 High performance thin layer chromatography

The HPTLC profile of CHFE was also performed. The samples were spotted in the form of bands of width 5mm with Camag microlitre syringe on precoated silica gel plate 60F-254 using CAMAG Linomet 5 'Linomet 5_192444'.The mobile phase used was petroleum ether: ethyl acetate (6:4) for CHFE. Densitometric scanning was performed on Camag TLC scanner 'scanner_192930'. Scanning performed in 254 and 366 nm and operated by winCATS.

2.8.3 Thin layer chromatographic detection of compounds using different spraying reagents.

The thin layer chromatography was performed with CHFE as mentioned in 2.8.1 and different spraying reagents were used to detect the phytoconstituents in separated bands/regions. Anisaldehyde-sulfuric acid for terpenoids, Dragendorff reagent for alkaloids, Fast blue reagent for phenolics, potassium hydroxide to detect anthraquinones, Conc. H_2SO_4 for detecting cardiac glycosides and lignans, Vanillin-sulphuric acid for essential oil, terpenoids, steroids etc, Ethanolic aluminium chloride for detecting flavonoids, ammonia vapours for tetracyclins, iodine reagent to detect compounds having conjugated double bonds and vanillin phosphoric acid used to detect terpenoids, lignans etc.

2.8.4 FTIR analysis

The FTIR analysis of CHFE was performed to identify the functional groups present in the phytocompounds. The spectra were recorded using a Jasco 4100 FTIR spectrophotometer in the wavelengths ranging from 4000-400 cm^{-1}.

2.8.5 Column chromatography

The silica gel column chromatography was performed to isolate the phytoconstituents present in them. The silica gel column of mesh size 60-120 was used. The sample was loaded by mixing with silica gel on the top of the packed column with a height of 25 cm. The solvent system used was petroleum ether: ethyl acetate (100:0, 0:100). Fractions were eluted in the solvent mixture combinations like 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100. The eluted fractions were collected, dried and subjected MTT assay (2.6.3) to check the antiproliferative ability. The fraction which showed antiproliferative ability was further analysed by TLC (2.8.1) and GC-MS analysis.

2.8.6 GC-MS analysis

The fraction which showed antiproliferative property was subjected to GC-MS analysis. GC-MS analysis of the column chromatographic fraction was carried out in an Aglient gas chromatography 6850 Network GC system. It is fitted with a HP-5MS capillary column (30m X 0.25 mm), 0.25 μm film thickness and interfaced with an Agilent 5975C VLMSD with triple axis mass detector (Agilent technologies, USA).

2.8.7 Preparative HPLC analysis

For the isolation of active phytoconstituents present in CHFE preparative HPLC analysis with gradient method was performed. Preparative HPLC system Shimadzu LC 20 AP with column length 250 mm, column id 20 and particle size 10. The mobile phase used was methanol: water. Sample concentration used was 25mg/ml and 5 ml sample were loaded in a single run. The fractions collected were checked for its antiproliferative property by MTT assay. The active fraction was further analysed by TLC (2.8.1), HPLC and LC-MS analysis was performed.

2.8.8 HPLC analysis

The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with two Shimadzu LC-10 ATVP reciprocating pumps, a variable Shimadzu SPD-10 AVP UV-VIS detector and a Rheodyne Model 7725 injector with a loop size of 20 µl. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column, 4.6 mm i.d., particle size 5 µm, Luna 5µ C-18(2); phenomenex, Torrance, CA, USA) at 25°C. Running conditions included: injection volume, 20µl;

mobile phase, methanol: water (95: 5 v/v); flowrate, 1 ml min-1; and detection at 280 nm. Samples were filtered through an ultra membrane filter (pore size 0.22 nylon membrane) prior to injection in the sample loop. The PDA detector is used for recording.

2.8.9 LC-MS analysis

LC-ESI/MS analysis was conducted on Agilent 6520 accurate mass Q-TOF LC/MS coupled with Agilent LC 1200 equipped with Extend-C18 column of 1.8 µm, 2.1 x 50 mm. Gradient elution was performed with water/0.05% formic acid (solvent A) and Methanol (solvent B) at a constant flow rate of 0.8 ml/ min. The MS analysis was performed using ESI in the negative mode. The conditions for mass spectrometry were: drying gas (nitrogen) flow 5 L/min; nebulizer pressure 40 psig; drying gas temperature 325°C; capillary voltage - 3000 V; fragmentor volt 125V; Oct RF Vpp 750 V. The mass fragmentation was performed with varying collision energy 4 V/ 100 DA with an offset of 6V.

MEGHA K. B. “THERAPEUTIC POTENTIAL OF MEDICINAL PLANT THESPESIA POPULNEA (L.) SOLAND EX CORREA WITH SPECIAL EMPHASIS TOWARDS ITS ANTIPROLIFERATIVE ACTIVITY AGAINST DIFFERENT CANCER CELL LINES.” THESIS. DEPARTMENT OF BIOTECHNOLOGY, UNIVERSITY OF CALICUT, 2018.

CHAPTER 3

RESULTS

3.1 Phytochemical constituents present in *T.populnea* leaf

3.1.1 Fluorescence analysis of *T. populnea* leaf powder

Matured leaves of *T. populnea* were collected, shade dried and coarsely powdered for the analysis. Fluorescence characteristic of leaf powder of *T. populnea* were observed under visible, short and long ultra-violet light after treatment with various organic and inorganic reagents. The results obtained were shown in table 3.1.

Table 3.1: Fluorescence analysis of powdered leaf of *T. populnea*

Treatment	Colour observed		
	Day light	UV light short, 254 nm	UV light long, 365 nm
Leaf powder	Green	Green	Green
1N NaOH	Brown	Black	Brown
1NNaOH in methanol	Green	Green	Orange
1N HCl	Green	Green	Light Green
Powder + H_2SO_4	Brown	Black	Blue

3.1.2 Phytochemical constituents in *T. populnea* leaf powder

The occurrence of different phytoconstituents was detected after reacting the leaf powder with different chemical reagents. It showed the presence of alkaloids, terpenoids, glycosides, tannins, flavonoids and phenolics (Table 3.2).

Table 3.2: Phytochemical constituents in *T. populnea* leaf powder

Reagents	Color/Precipitate	constituents present
Picric acid	Precipitate formed	Alkaloids
Con. H_2SO_4	Reddish brown	Steroids/triterpenoids
Aq. $FeCl_3$	Black precipitate	Tannins
Iodine solution	No change	Starch absent
Mayers reagent	Precipitate formed	Alkaloids
Aq. NaOH	Yellow color	Flavonoids
Mg-HCl (Shinoda)	Dark pink color	Flavonoids
Dragendroff’s reagent	Dark brown precipitate	Alkaloids
Ninhydrin	Pale white precipitate	Proteins/amino acids

3.1.3 Dry weight of the solvent extracts and their appearances

T. populnea leaf powder was sequentially extracted using solvents like petroleum ether, chloroform, ethyl acetate and methanol based on their increasing polarity. The extracts were filtered and dried; their dry weight and appearances are depicted in table 3.3.

Table 3.3: Dry weight of the extracts and their appearances

Extract	Yield (%)	color	Consistency
Petroleum ether	1.790	Yellowish green	Greasy
Chloroform	3.140	Dark green	Sticky
Ethyl acetate	0.506	Brownish green	Sticky
Methanol	10.50	Brown	Syrupy

3.1.4 Qualitative phytochemical analysis of *T. populnea* leaf extracts

The extracts obtained are subjected for qualitative preliminary phytochemical analysis and revealed the presence of various types of phytoconstituents. The alkaloids, glycosides and tannins are commonly present in all the four solvent extracts. The presence of phenols, flavonoids, anthraquinones and quinines were observed in chloroform, ethyl acetate and methanol extracts. Terpenoids and resins presence was noted only in chloroform and ethyl acetate extracts. Fixed oil occurrence is seen in three extracts except for chloroform extract. The presence of coumarins in petroleum ether extract, sterols in chloroform, phlobatannins and saponins in methanol extracts were also noted (Table 3.4).

Table 3.4: Preliminary qualitative phytochemical analysis of *T. populnea* leaf extracts

Phytochemical test	PEE	CHFE	EAE	MOHE
Tests for Proteins				
Biuret's test	-	-	-	-
Ninhydrin test	-	-	-	-
Tests for carbohydrates				
Mohlish's test	+	+	+	+
Iodine test	-	-	-	-
Benedict's test	-	++	++	++
Tests for alkaloids				
Dragendorff's test	-	++	++	-
Mayer's test	++	-	-	+
Wagner's test	++	++	++	++
Tests for glycosides				
Keller killiani test	+	++	+	++
Mohlish's test	+	++	++	+
Con.H_2SO_4	-	++	++	++

Tests for tannins				
Ferric chloride test	-	++	++	+
Gelatin test	++	+	-	+
Lead acetate test	-	-	+	-
Alkaline reagent test	-	++	++	+
Tests for phenols				
Ellagic acid test	-	++	++	+
Phenol test	-	++	++	+
Tests for sterols				
Salkowski test	-	++	-	-
Tests for terpenoids	-	++	++	-
Tests for flavonoids				
Shinoda test	-	-	-	++
Lead acetate test	-	+	++	+
Alkaline reagent test	-	++	++	+
Tests for coumarins	++	-	-	-
Tests for anthraquinones	-	++	++	+
Tests for phlobatannins	-	-	-	+
Tests for resins	-	++	++	-
Tests for quinines	-	++	++	+
Tests for fixed oils	++	-	+	++
Tests for saponins	-	-	-	++

PEE- petroleum ether extract, CHFE- chloroform extract, EAE- ethyl acetate extract MOHE- methanol extract. Strongly present (++), Present (+), absent (-).

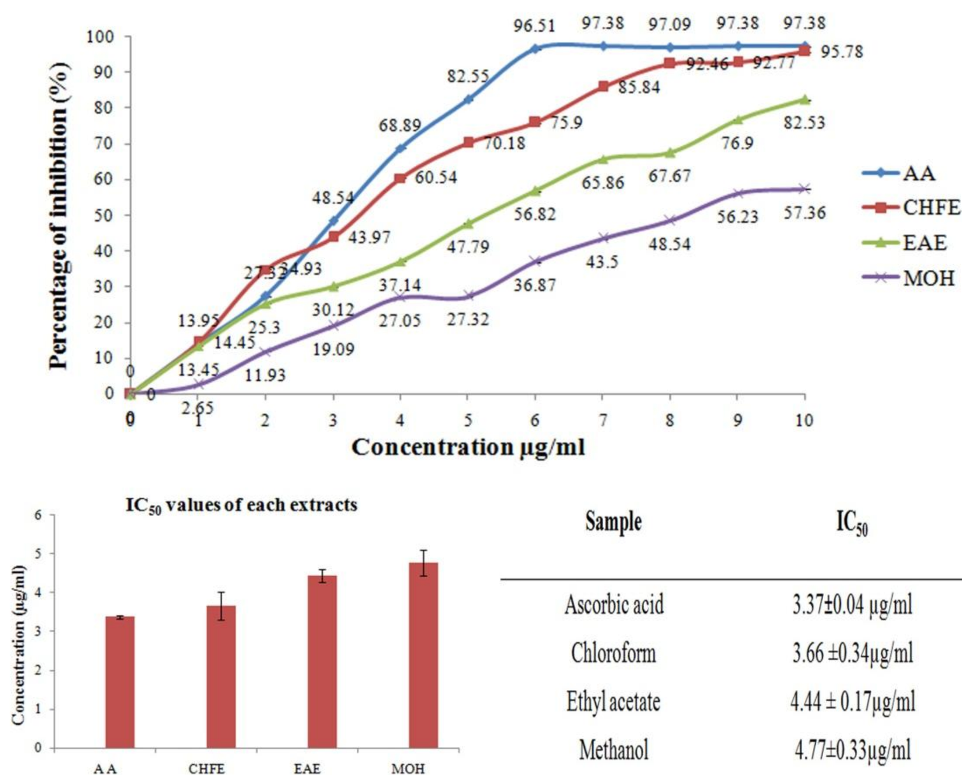
3.2 Evaluation of antioxidant, antimicrobial and anti-inflammatory potentials of leaf extracts

3.2.1 Antioxidant potentials of leaf extracts

Medicinal plants are commonly considered as the main source of secondary metabolites which constitute a rich diversity of compounds such as flavonoids, phenolic acids, lignins, stilbenes, terpenoids etc. These compounds differ in their structure thus leading to variation in having multiple biological effects including antioxidant activity. The leaf extracts of *T. populnea* was tested for its *in vitro* antioxidant activity using various methods. The petroleum ether extract lacks the antioxidant property and does not react to any of the tested methods.

3.2.1.1 DPPH radical scavenging property of leaf extracts

DPPH (2,2-diphenyl-1 picryl hydrazyl) is a dark colored crystalline powder composed of stable free radical molecule. DPPH assay method depends on the reduction of purple DPPH to a yellow colored diphenyl picryl hydrazine. The extracts exhibited potent radical scavenging activity in concentration dependent manner. The data were expressed as mean ± SD as shown in figure (3.1).



Sample	IC_{50}
Ascorbic acid	3.37±0.04 µg/ml
Chloroform	3.66 ±0.34µg/ml
Ethyl acetate	4.44 ± 0.17µg/ml
Methanol	4.77±0.33µg/ml

Figure 3.1: DPPH radical scavenging activity of the *T. populnea* extracts were determined. Ascorbic acid was used as an internal standard. AA-ascorbic acid, CHFE-chloroform extract, EAE- Ethyl acetate extract, MOH- methanol extract.

3.2.1.2 Nitric oxide radical inhibition assay

Nitric oxide was generated from sodium nitroprusside and measured using Griess reaction. Nitric oxides produced during their reduction with oxygen or superoxides are very reactive. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Nitrite ions react with Griess reagent and form a purple azo dye reflects the presence of scavengers in the test compound. Only the chloroform extract showed a good nitric oxide radical scavenging activity with an IC_{50} of 492.54±34.25 µg/ml and the scavenging ability was higher while comparing with that of the standard ascorbic acid i.e., 635.78±74.64 µg/ml. Data were represented as n=3, mean ±SD in figure(3.2).

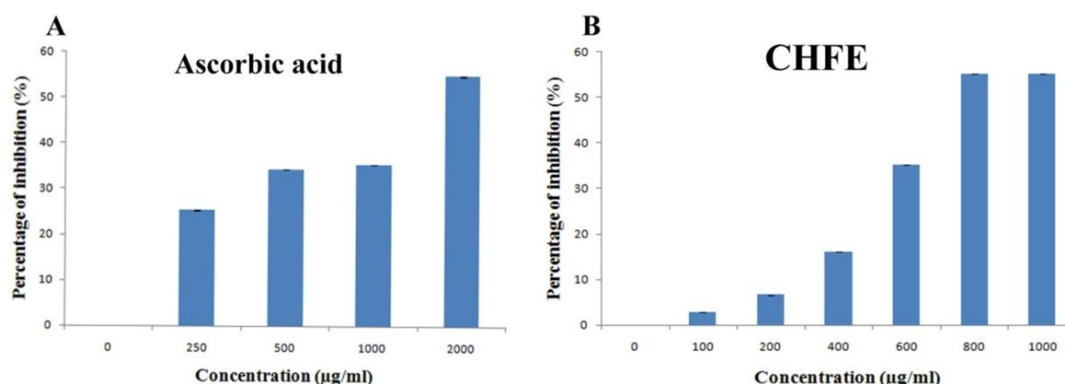


Figure 3.2: Graph representing the nitric oxide radical inhibiting ability of the (A) ascorbic acid and (B) chloroform extract. The IC_{50} concentration of the chloroform extract was found to be 492.548±34.25 µg/ml and that of ascorbic acid is 635.78±74.64 µg/ml.

3.2.1.3 Chelating ability on ferrous ions

Iron in nature can be found as either ferrous or ferric ion. Ferrous ion (Fe^{2+}) chelation may render important antioxidative effects by retarding metal-catalysed oxidation. Minimizing ferrous (Fe^{2+}) ions may afford protection against oxidative damage by inhibiting production of ROS (reactive oxygen species) and lipid peroxidation. Ferrozine can quantitatively form complexes with Fe^{2+} in this method. In the presence of chelating agents the complex formation is disrupted, resulting in a decrease in the red color of the complex. Chelation of ferrous ions retards metal catalyzed oxidation, which in turn provides protection against oxidative damage by inhibiting ROS production and lipid peroxidation. A lower absorbance of the reaction mixture

indicated a higher Fe^{2+} chelating ability. In this assay, the chelating effect was shown by ethyl acetate extract and exhibited an IC_{50} concentration of scavenging ability at 679.81±71 µg/ml and that of the standard EDTA is 1.27± 0.006 µg/ml (Figure 3.3).

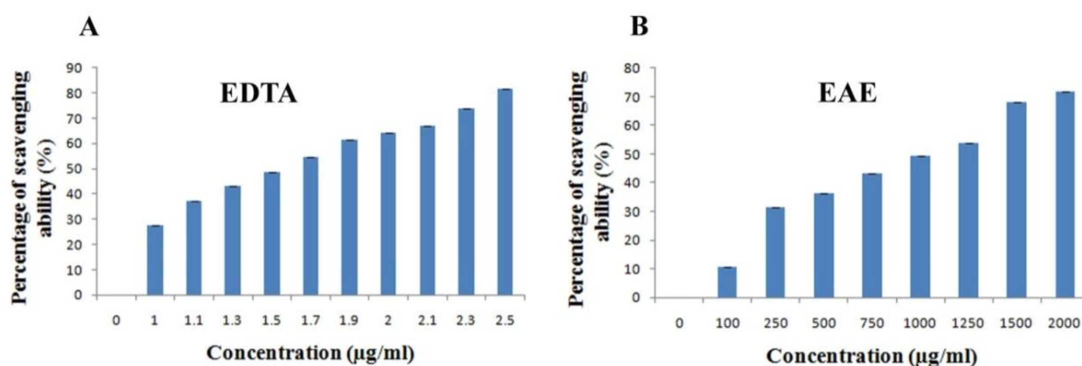


Figure 3.3: Graph represents the metal chelating ability of ethyl acetate extract of *T. populnea* and the standard ascorbic acid. Data were expressed as mean ± standard deviation.

3.2.1.4 Determination of total reducing ability

The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron. This assay is determined by the ability of extracts to reduce ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}). The yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of the test specimen. Higher value absorbance of the reaction mixture indicate greater reducing power. In the present study, the reducing power of the CHFE, EAE and MOHE was found to be excellent and steadily increases in direct proportion to the increasing concentrations of the extract (figure 3.4). The higher reducing value is an indicative of the hydrogen donating ability of the active species present in the extract.

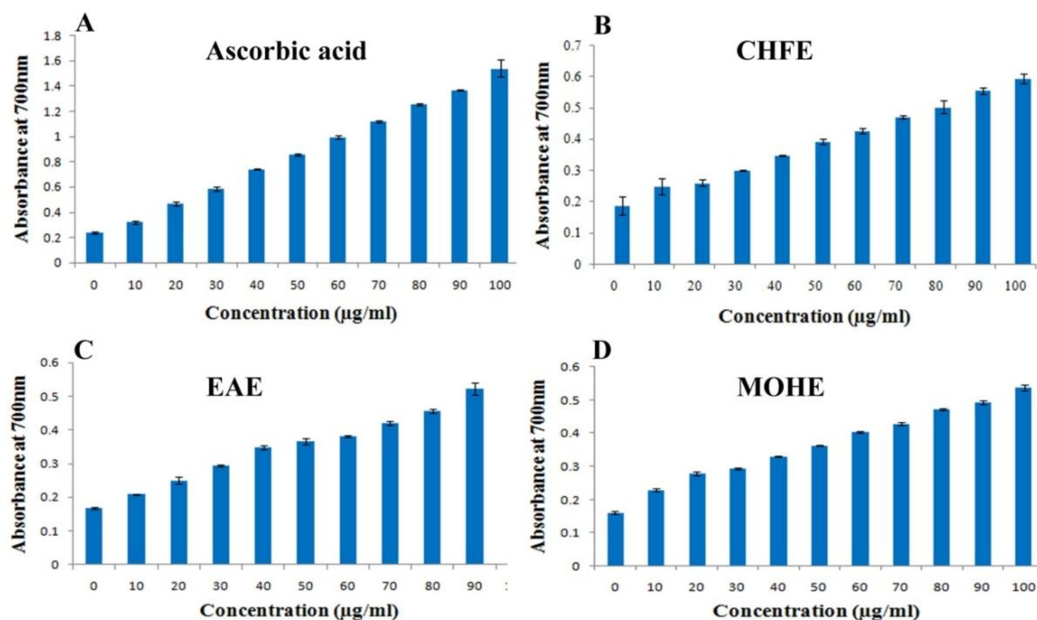


Figure 3.4: Total iron reducing ability of A-ascorbic acid, B-CHFE, C-EAE and D-MOHE of *T. populnea*. The values were average of three determinants. Data were expressed as mean ± standard deviation.

3.2.1.5 Total antioxidant capacity by phosphomolybdenum method

The result of total antioxidant capacity is shown in figure 3.5. The phosphomolybdenum method was used to evaluate total antioxidant capacity of the extracts. Mo(VI) is reduced to Mo(V) by the antioxidants in the sample with generation of a green phosphate/Mo(V) complex at acidic pH measured at 695 nm. TAC of the phosphomolybdenum model evaluates both water soluble and fat soluble antioxidant capacity. This method gives a combined measure of the antioxidant activity of the range of chemically diverse phenolics and flavonoid present in the leaf extracts of *T. populnea*.

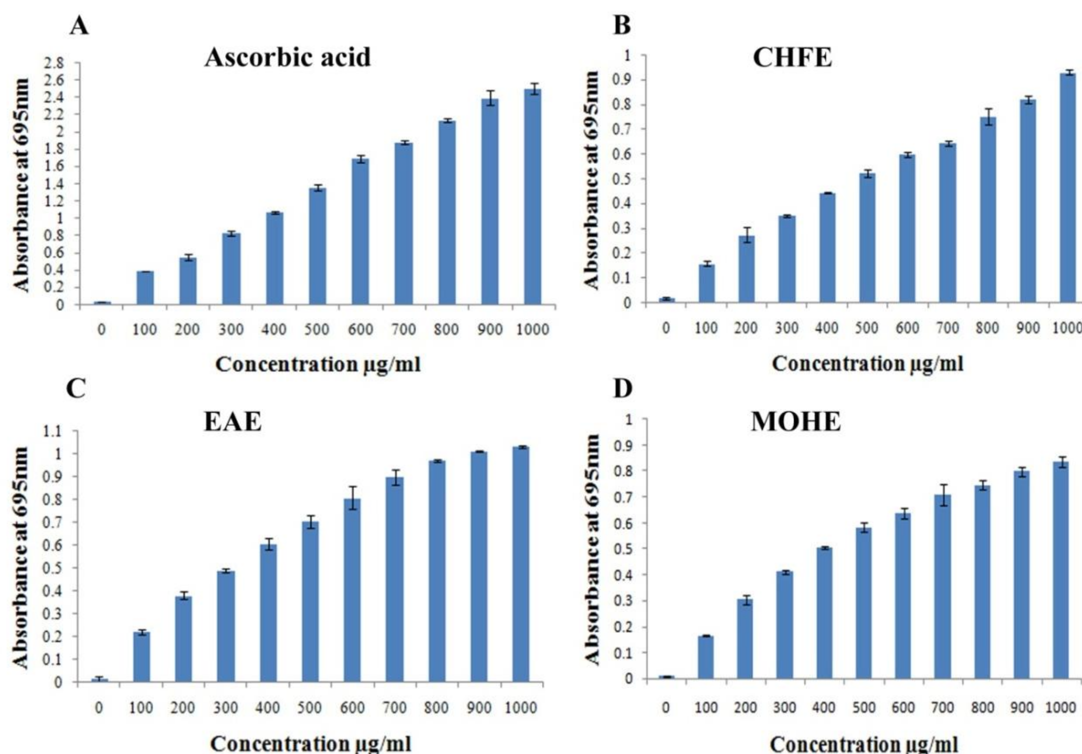


Figure 3.5: Graph representing the total antioxidant capacity of leaf extracts of *T. populnea* and with standard ascorbic acid; A-Ascorbic acid, B-CHFE, C-EAE, D-MOHE by using phosphomolybdenum method.

3.2.1.6 Total phenolic content of the extracts

The total phenolic content of the extracts were estimated using Folin–Ciocalteu (F–C) reagent and is very sensitive towards reducing compounds, polyphenols and thus produces a blue colour complex. Gallic acid was used as the standard. The total phenolic content of chloroform, ethyl acetate and methanol extracts were found to be 245±0.01, 254±0.01 and 152±0.01 mg gallic acid equivalent per gram of the extract respectively and is represented in figure 3.6.

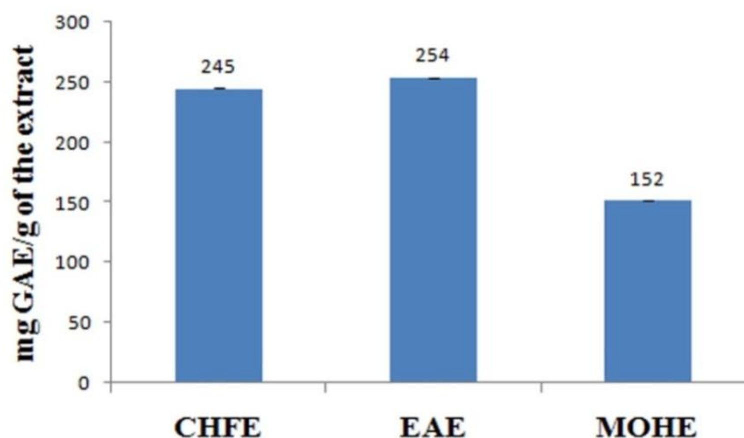


Figure 3.6: Graph representing the total phenolic content of the extracts expressed as mg gallic acid equivalent (GAE) per g of extract. The values were average of the determinants and data were expressed as mean ± standard deviation.

3.2.1.7 Total flavonoid content of the extracts

The flavonoid content of the extracts was estimated using aluminium chloride colorimetric method. The principle involved in this method is that $AlCl_3$ forms acid labile complexes with the ortho-dihydroxyl groups in any of the two rings of flavonoid. The results were expressed as quercetin equivalent in mg/g of extract. In chloroform and ethyl acetate extract the total flavonoid content was found to be 361±1.26 and 188±0.67 mg of quercetin equivalent/g of the extract respectively (figure 3.7).

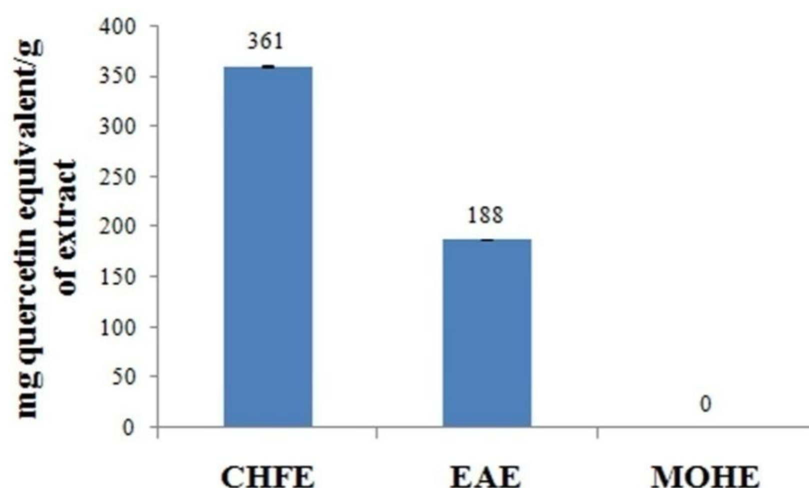


Figure 3.7: Graph representing the total flavonoid estimation. The values are expressed as mg quercetin equivalent/g of extract. The average values of three determinants were taken data were expressed as mean ± standard deviation.

3.2.2 Antimicrobial potentials of leaf extracts

The leaf extracts of *T. populnea* were tested for their antimicrobial activity against *Salmonella typhi* (MTCC 734), *Klebsiella pneumoniae* (MTCC 109), *Fusarium oxysporum* (MTCC 284), *Aspergillus niger* (MTCC 282) and *Candida albicans* (MTCC 227) using microdilution method. MIC value is recorded and is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested and is expressed in µg/ml. The anti microbial potential of the extracts were expressed as percentage of inhibition (%) and is shown in figure 3.8. It is clearly evident that the antimicrobial efficiency of the extracts responds to different microbial species in a dose dependent manner.

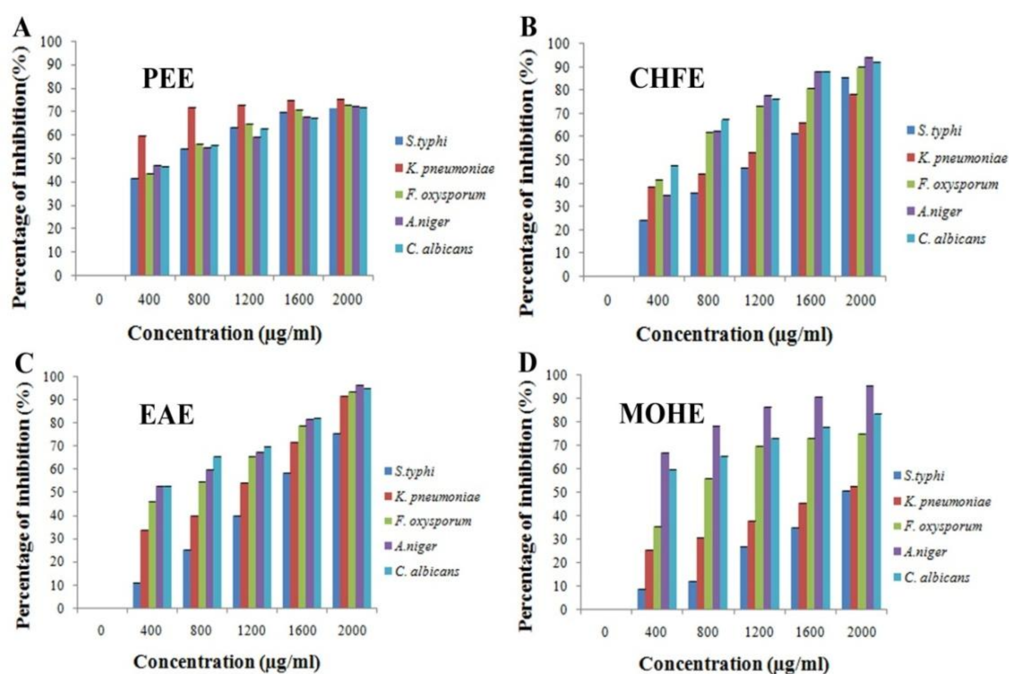


Figure 3.8: Graph showing the antimicrobial potentials of A-PEE, B-CHFE, C-EAE and D-MOHE on different microorganisms. The data were expressed as mean ± standard deviation of average values of three determinants.

Table 3.5 demonstrates the MIC_{50} and MIC_{90} values of the leaf extracts of *T.populnea* against gram negative bacteria- *S. typhi* and *K. pneumonia* and three fungal strains- *F. oxysporum*, *A. niger* and *C. albicans* which shows an effective antimicrobial property. It was found that PEE was showing more antimicrobial ability while comparing with other extracts.

Table 3.5: Minimum inhibitory concentrations for the leaf extracts of *T. populnea*

Extracts	MIC50 and MIC90 of leaf extracts (µg/ml)									
	S. typhi		*K. pneumoniae*		*F. oxysporum*		*A. niger*		*C. albicans*	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
PEE	554.3 ±0.78	1442.2 ±1.99	289.2 ±3.10	1286.11 ±3.12	537.27 ±2.30	1435.5 ±3.7	540.63 ±0.44	1484.56 ±1.91	515.41 ± 2.60	1457.28 ±2.47
CHFE	1008.42±0.78	1880.09±0.91	780.888±2.5	1685.88 ±2.29	686.212±2.21	1555.77±2.6	728.94 ±2.77	1546.61 ±3.1	624.84 ±1.82	1498.27 ±3.43
EAE	1068.83±1.06	1861.05 ±1.13	935.53 ±2.74	1811.68 ±2.78	764.816±0.36	1675.68±3.1	720.23 ±0.91	1657.27 ±2.77	668.69 ±1.48	1606.06 ±1.41
MOHE	1124.06±2.06	1943.03 ±2.25	754.208±4.79	1645.15 ±4.2	605.205±4.13	1441.1 ±1.2	445.961±0.94	1406.19 ±0.94	467.18 ±2.8	1444.52 ±1.8

3.2.3 BSA anti-denaturation assay

The denaturation of proteins is well documented cause of inflammation. As a part of the study, anti-inflammatory activity of the leaf extracts of *T. populnea* was checked, using BSA anti-denaturation assay with diclofenac sodium as standard. The CHFE only showed inhibition to BSA denaturation in a concentration dependant manner (100-1000 µg/ml). The denaturation inhibition concentration-DIC_{50} value was determined and was found to be 501.93±19.76 µg/ml for CHFE and 175.91±9.37 µg/ml for diclofenac sodium (figure 3.9).

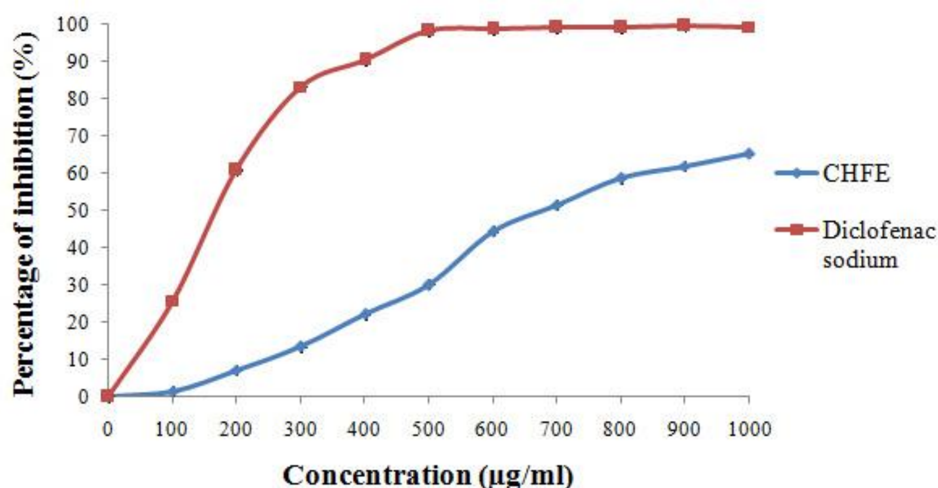


Figure 3.9: BSA- antidenaturation assay for checking the anti-inflammatory activity of CHFE and standard drug diclofenac sodium. The experiments were done in triplicates and the data were represented as mean±standard deviation.

3.3 Assessment of antiproliferative potential of leaf extracts against different cancer cell lines

To evaluate the antiproliferative effect of leaf extract of *T. populnea*, MTT and Sulforhodamine-B assays were performed.

3.3.1 MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

The viability of the cells were assessed by 3-(4,5- dimethyl thiazol-2-yl)-2,5 di phenyl tetrazolium bromide (MTT) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. The amount of formazan crystals produced is propotional to the number of viable cells. The antiproliferative potential of *T. populnea* leaf extracts were evaluated against various cancer cell lines. The cells were treated with different concentrations of the solvent extracts to identify their cytotoxic/antiproliferative action. Only the chloroform extract (CHFE) exhibited remarkable antiproliferative ability against various cancer cell lines. The cells were treated with various concentrations (10-100µg/ml) of CHFE for 48h and their IC_{50} values were identified.

After 48h treatment with chloroform extract, cell lines responded differently with almost different IC_{50} values. The leukemic cell lines Jurkat E6.1, HL-60 and K562 exhibited IC_{50} values of 35.72±0.95, 25.80±1.13 and 24.93±2.63 µg/ml respectively. Treatment in adenocarcinoma cell lines PC-3, HeLa and MDA-MB-231 exhibited an IC_{50} value of 60.79±1.84, 46.94±0.83 and 72.16±0.56 µg/ml. The two carcinoma cell lines included in the study were A549 and HCT116 responded after treatment with an IC_{50} value of 55.69±2.18 and 37.22±4.04 µg/ml respectively. Treatment of L929 cell line with chloroform extract exhibited an IC_{50} value of 60.88±1.45 µg/ml. Vero cell line after 48h treatment exhibited an IC_{50} value of 83.48±2.05 µg/ml. The results are summarised in the table 3.6. The graph showing the percentage of viability after treatment with CHFE for 48h treatment with different concentrations is represented in figure 3.10-3.13.

Table 3.6: MTT assay result: IC_{50} values of CHFE after 48h treatment on different cell lines

S.No	Cell line	Type of Cancer	IC_{50} value (µg/ml)
1	Jurkat E6.1	Acute T cell leukemia	35.72±0.95
2	PC-3	Grade IV, adenocarcinoma	60.79±1.84
3	HeLa	Adenocarcinoma	46.94±0.83
4	MDA-MB-231	Breast adenocarcinoma	72.16±0.56
5	HL- 60	Acute promyelocytic leukemia	25.80±1.13
6	A549	Lung carcinoma	55.69±2.18
7	K562	Chronic myelogenous leukemia	24.93±2.63
8	HCT116	Colorectal carcinoma	37.22±4.04
9	Vero	Monkey kidney normal cells	83.48±2.05
10	L929	Mouse fibroblast	60.88±1.45

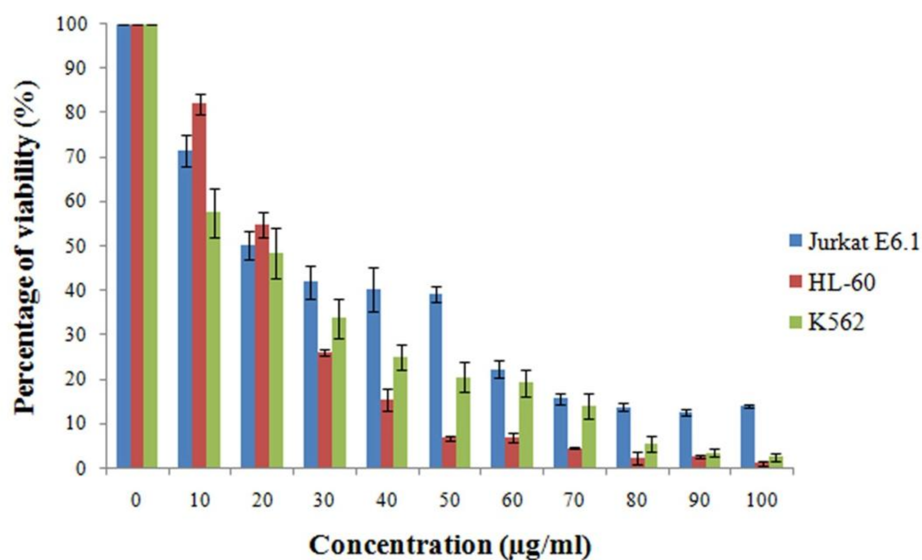


Figure 3.10: The antiproliferative property of *T. populnea* chloroform leaf extract on Jurkat E6.1, HL-60 and K562 cell lines. The cells were treated with different concentration from 0-100 µg/ml for 48 h. The IC_{50} concentration exhibited by CHFE

against Jurkat E6.1, HL-60 and K562 was found to be 35.72±0.95 µg/ml, 25.80±1.13 µg/ml and 24.93±2.63 µg/ml respectively.

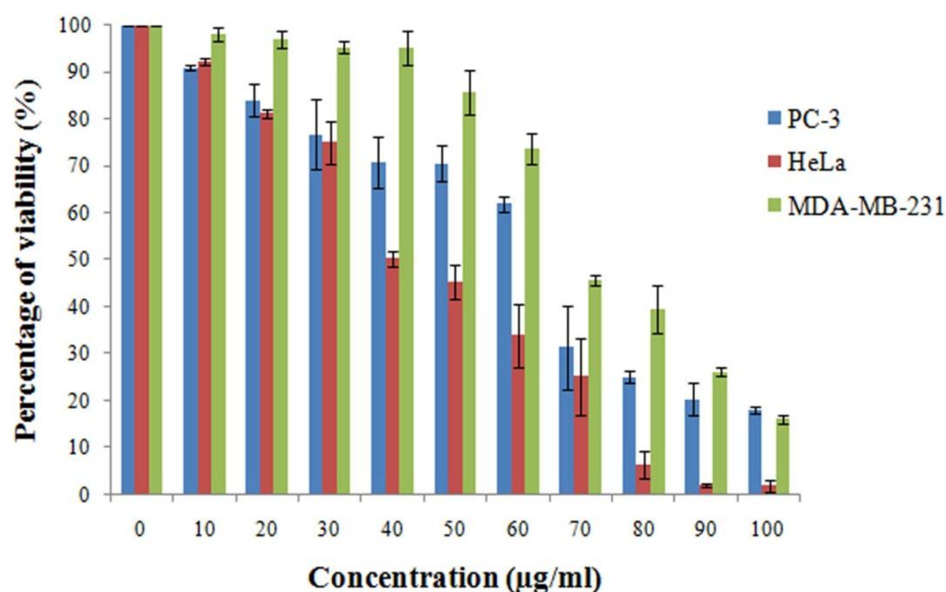


Figure 3.11: The antiproliferative property of *T. populnea* chloroform leaf extract on PC-3, HeLa and MDA-MB-231 cell lines. The cells were treated with different concentration from 0-100µg/ml for 48 h. The IC_{50} concentration exhibited by CHFE against PC-3, HeLa and MDA-MB-231 cell lines was found to be 60.79±1.84 µg/ml, 46.94±0.83 µg/ml and 72.16±0.56 µg/ml respectively.

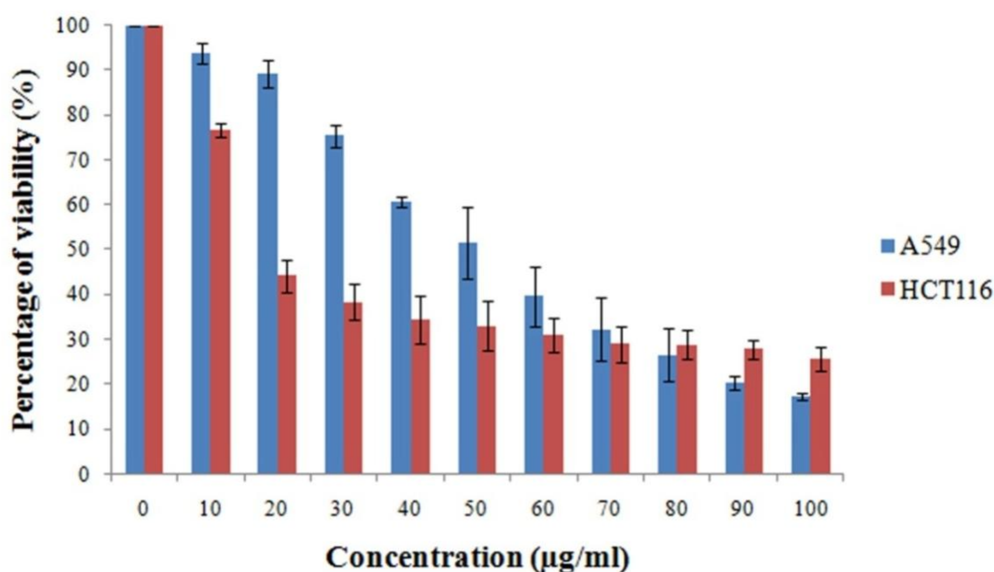


Figure 3.12: The antiproliferative property of *T. populnea* chloroform leaf extract on A549 and HCT116 cell lines. The cells were treated with different concentration from 0-100µg/ml for 48 h. The IC_{50} concentration exhibited by CHFE against A549 and

HCT116 cellines was found to be 55.69±2.18 µg/ml and 37.22±4.04 µg/ml respectively.

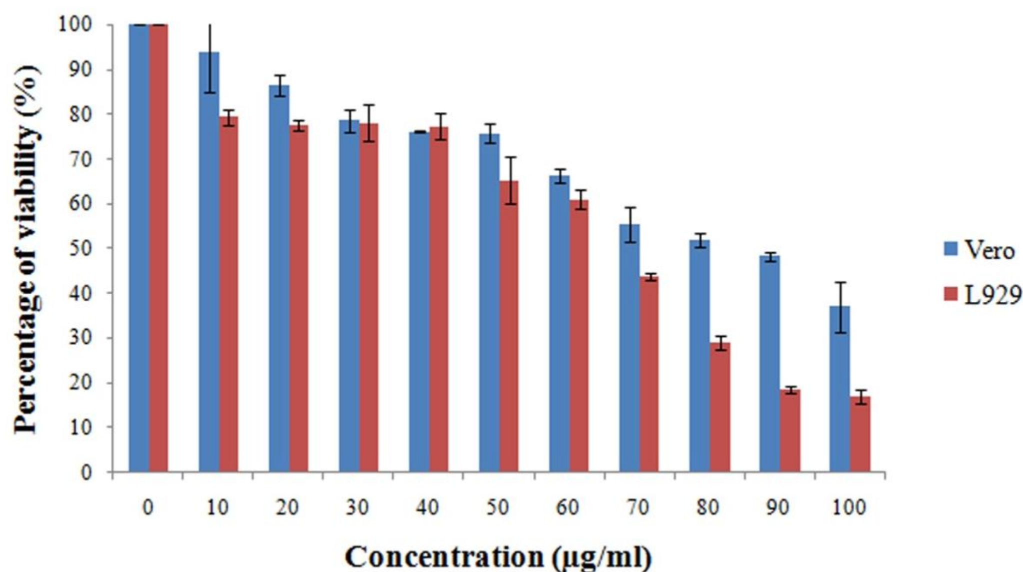


Figure 3.13: The antiproliferative property of *T. populnea* chloroform leaf extract on Vero and L929 cell lines. The cells were treated with different concentration from 0-100µg/ml for 48 h. The IC_{50} concentration exhibited by CHFE against Vero and L929 was found to be 83.48±2.05 µg/ml and 60.88±1.45 µg/ml respectively.

3.3.2 Sulforhodamine B assay

SRB assay relies on the uptake of the negatively charged pink aminoxanthine dye, sulphorhodamine B (SRB) by basic aminoacids in the cells. The greater the number of cells, the greater the amount of dye is taken up and after fixing, the cells are lysed, the released dye will give a more intense color and greater absorbance.

The SRB assay was conducted in addition to MTT assays to evaluate the antiproliferative effect of CHFE on cancer cell lines. The SRB assay revealed an encouraging antiproliferative effect for CHFE on PC-3, MDA-MB-231, A549 and HCT116. Their IC_{50} values are 50.16±0.92, 78.62±2.97, 142.51±13.2 and 37.81±3.74 µg/ml respectively. The graph representing the percentage of viable cells against increasing concentration of CHFE and their IC_{50} values are shown in figure 3.14 and 3.15.

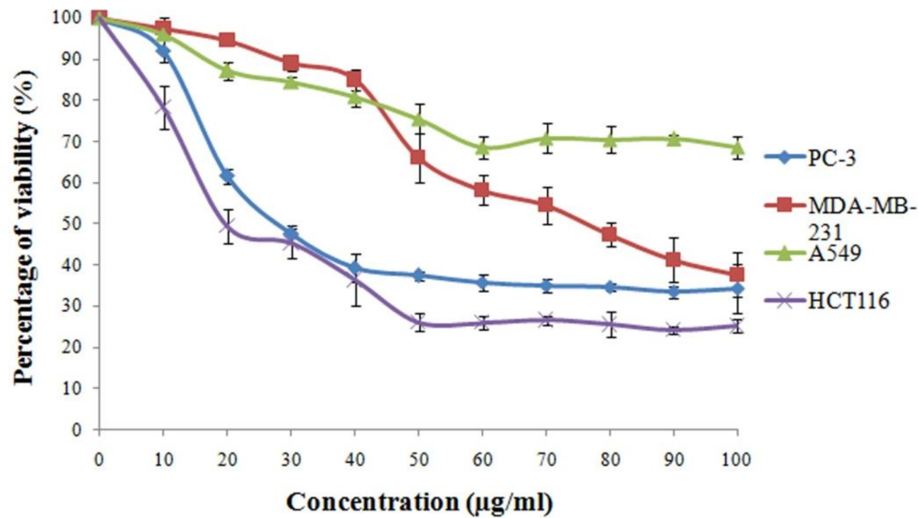


Figure 3.14: Graph showing the antiproliferative property of *T. populnea* chloroform leaf extract on PC-3, MDA-MB-231, A549 and HCT 116 cell lines using SRB assay. The cells were treated with different concentration from 0-100µg/ml for 48 h.

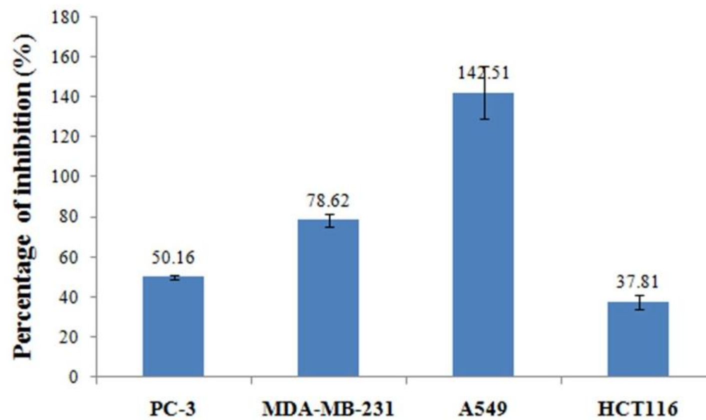


Figure 3.15: SRB assay result- IC_{50} values of CHFE after 48h treatment on different cell lines

3.4 Apoptogenic ability of the leaf extract against chronic myelogenous leukemia (K562) and colon carcinoma (HCT116) cell lines.

The antiproliferative potency of CHFE was tested in 10 different cell lines for 48h with different concentration (10-100μg/ml). Among the tested cell lines K562 and HCT116 cells were more sensitive towards CHFE exhibiting very low IC_{50} values. Therefore these cell lines were used for further analysis.

3.4.1 Antiproliferative and apoptogenic ability of the leaf extract against chronic myelogenous leukemia (K562) cell line

The antiproliferative and apoptogenic ability of chloroform leaf extract (CHFE) of *T. populnea* was evaluated against K562 cell line using various techniques.

3.4.1.1 Morphological changes occurred after treatment with CHFE at different time periods and concentrations in K562 cell line

The K562 cells were treated with different concentration of CHFE for 3, 6, 12, 24 and 48h. The morphological changes of the K562 cells were observed after treatment through phase contrast microscopy. It was observed that cells were shrunk into smaller size and their morphology got completely distorted as the treatment concentration increases while compared to the control cells and is shown in figure 3.16.

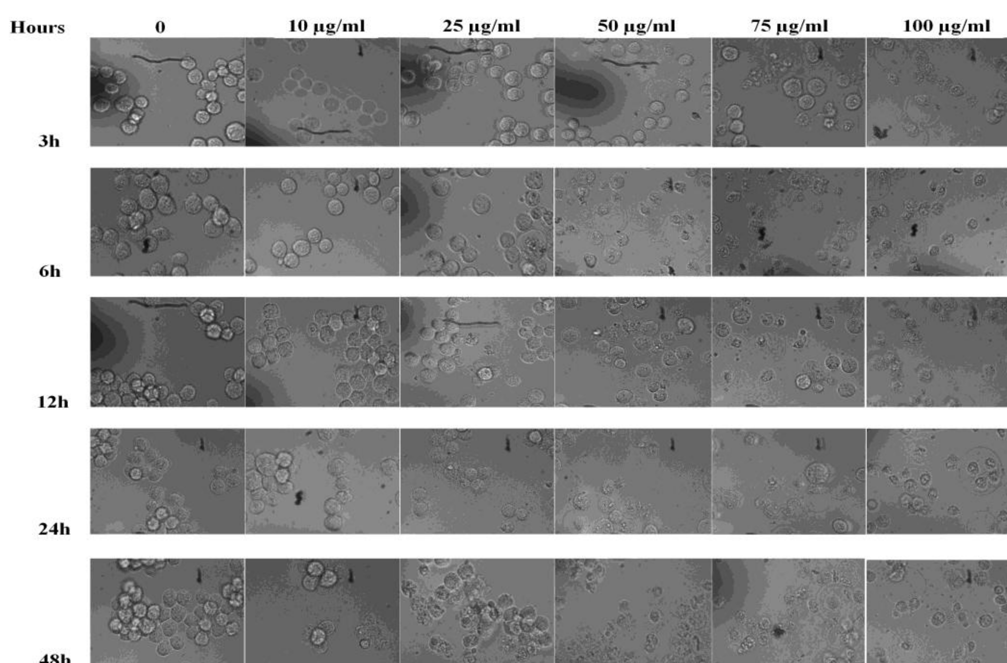


Figure 3.16: Phase contrast microscopic images of K562 cells under 400X magnification after A-3h, B-6h, C-12h, D-24h, E-48h of treatment using CHFE (10-100) μg/ml concentration.

3.4.1.2 MTT assay-

The antiproliferative abilty of CHFE were assessed using MTT assay after treating the K562 cells with different concentrations 10-100 µg/ml and at different time intervals. Their percentage of viablilty and IC_{50} concentration after different time intervals was calculated and shown in figure 3.17 and 3.18. The respone towards CHFE exhibited a dose dependent manner.The IC_{50} value decrease with increase in time and drug concentration. The IC_{50} value of CHFE against K562 at different time intervals were 51.09±2.34 µg/ml at 3 hour, 39.58±1.52 µg/ml at 6 hour, 35.18±1.53 µg/ml at 12 hour, 25.29±0.65 µg/ml and 24.93±2.63 µg/ml at 24 hour and 48 hour respectively.

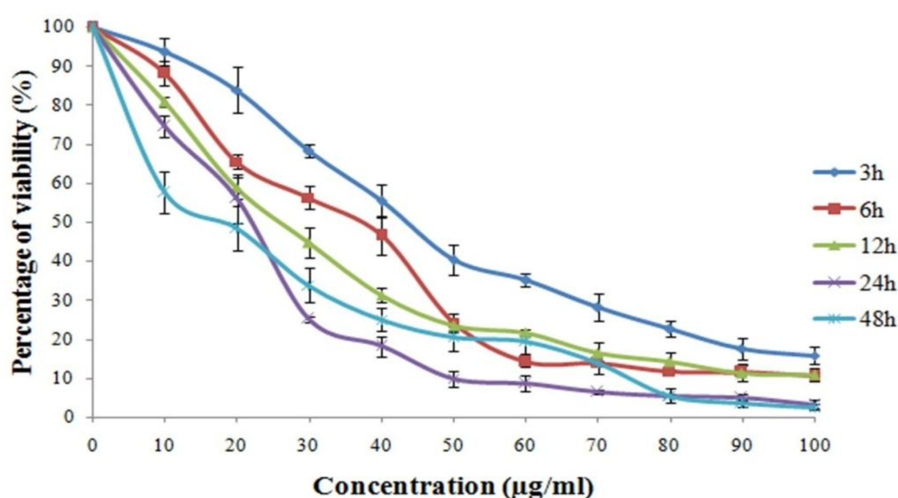


Figure 3.17: Graph representing the percentage of viability of K562 cells after treatment with different concentration (10-100 µg/ml) for different time periods (3h, 6h, 12h, 24h and 48h).

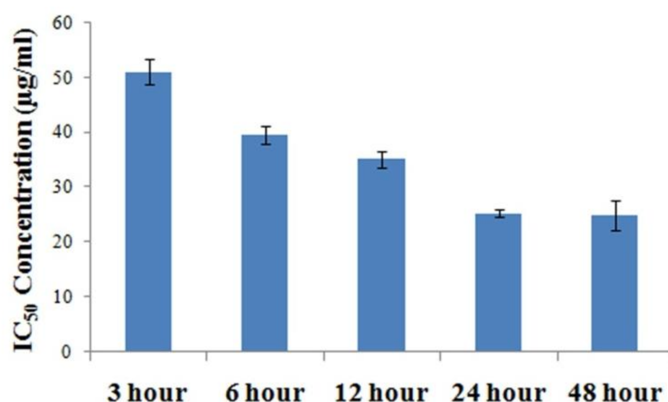


Figure 3.18: The results obtained shows the anti-proliferative potential of CHFE of *T. populnea* against K562 with an IC_{50} of 51.09±2.34 µg/ml at 3 hour, 39.58±1.52 µg/ml at 6 hour, 35.18±1.53 µg/ml at 12 hour, 25.29±0.65 µg/ml and 24.93±2.63 µg/ml at 24 hour and 48 hour respectively.

3.4.1.3 Acridine orange/Ethidium bromide double (AO/EB) staining

The CHFE of *T. populnea* treated K562 cells were subjected to AO/EB staining. AO/EB is used to visualise nuclear changes and apoptotic body formation that are characteristic of apoptosis. The treated cells were viewed under fluorescence microscope. AO is a vital dye and will stain both live and dead cells and EB will stain cells had lost membrane integrity. The control cells appeared green with intact nuclei and normal cell morphology. The low 10 µg/ml and IC_{50} value concentration 25 µg/ml showed typical early apoptotic cell features as they are stained green to orange with highly condensed or fragmented chromatin and apoptotic bodies. The late apoptotic and necrotic cells appeared to orange to red color with condensed and fragmented chromatin represented in figure 3.19.

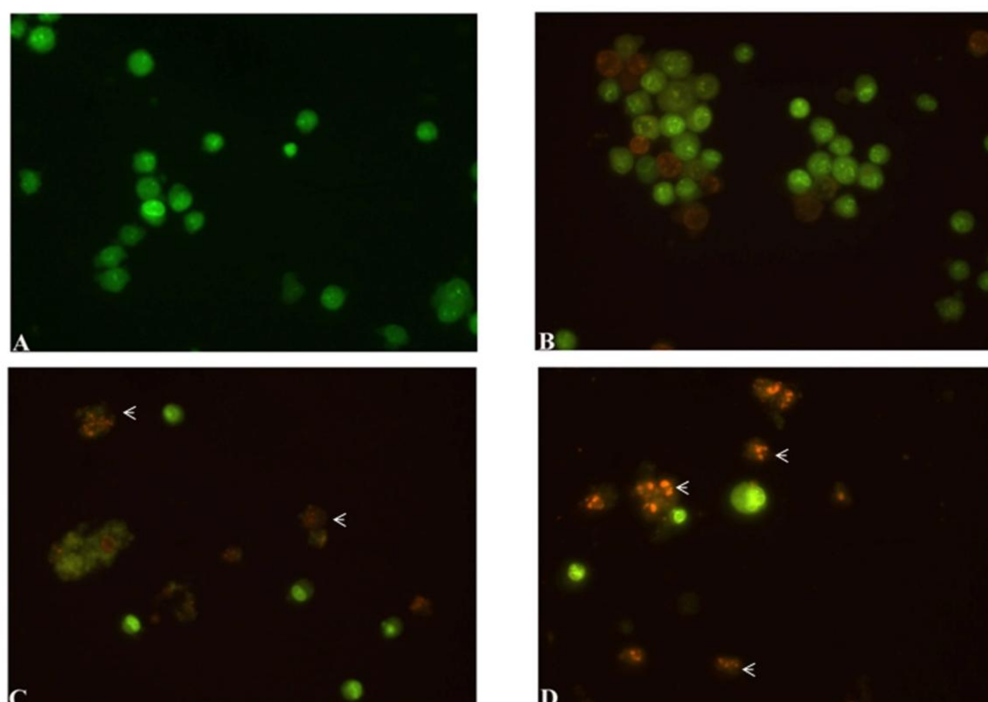


Figure 3.19: Acridine orange/ethidium bromide double staining (A) control group-No significant apoptosis is detected, (B) & (C) is the treated group of 10µg/ml and 25µg/ml of CHFE-Early stage apoptotic cells can be detected (D) 50µg/ml treated group-orange to red fluorescence due to late apoptotic and dead cells.

3.4.1.4 Nuclear Hoechst staining

Apoptosis induction is evaluated by morphological examination after treatment with CHFE in K562 under fluorescence microscopy after Hoechst (33342) staining as shown in figure 3.20. Control cells are uniformly stained by the dye. However cells treated with CHFE for 48h clearly exhibited significant morphological changes during apoptosis. Condensed chromatin and fragmented punctuate blue nuclear fluorescence were seen in a concentration dependant manner.

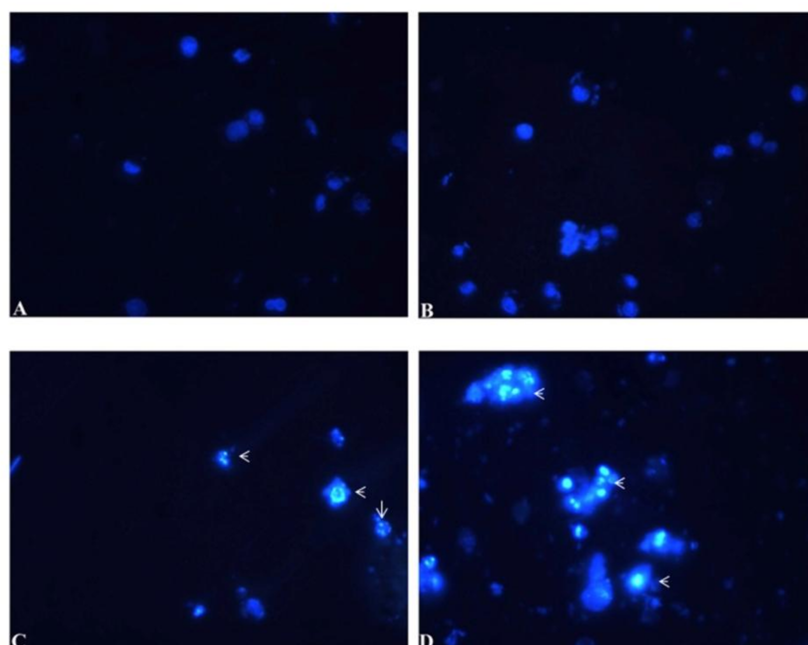


Figure 3.20: Nuclear Hoechst staining of CHFE treated K562 cells. (A)- control, (B)- 10 µg/ml arrows indicates apoptotic bodies of nuclear fragmentation observed in (C) 25 µg/ml and (D) 50 µg/ml concentration of CHFE after 48h of treatment.

3.4.1.5 Scanning electron microscopy

The characteristic morphological signs of apoptosis include cellular shrinkage, plasma membrane blebbing, nuclear condensation and fragmentation. The K562 cells were treated with CHFE concentration of 10, 25, 50 µg/ml for 48 h. After treatment, the cells were checked for its morphological changes under scanning electron microscopy. The control group were showing normal intact cell structure without any morphological alterations. The 10 and 25 µg/ml treated group shows the remarkable morphological changes including the plasma membrane blebbing. Whereas in the higher concentration treated group plasma membrane blebbing and cell shrinkage are clearly evident (figure 3.21).

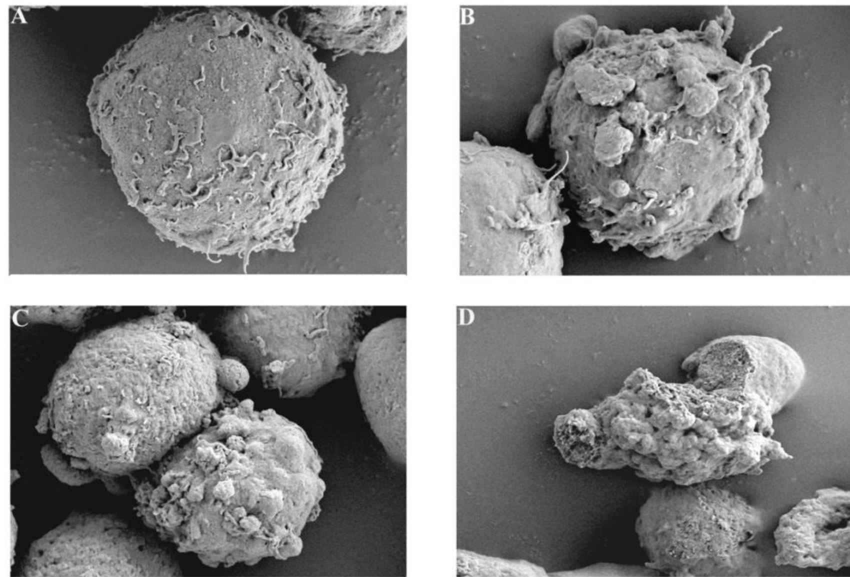


Figure 3.21: Scanning electron micrographs of K562 cells after 48h treatment with CHFE A-control, B-10 µg/ml, C-25 µg/ml, and D-50 µg/ml. Membrane blebbing and cell shrinkage are clearly visible after treatment with increase in drug concentration.

3.4.1.6 DNA fragmentation

In this study K562 cells were treated with CHFE of different concentration for 48h. The treated cells were analysed for DNA fragmentation pattern for the confirmation of apoptosis. The result showed a clear DNA fragmentation pattern visualised at IC_{50} concentration 25 µg/ml and higher concentration 50 µg/ml. The intensity of ladder increased with increase in concentration of drug treatment and is shown in figure 3.22.

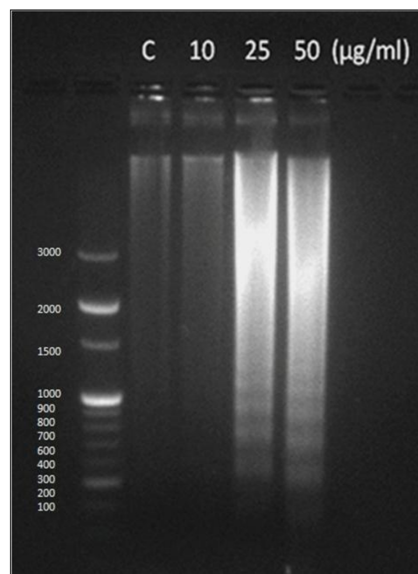


Figure 3.22: DNA fragmentation assay-formation of DNA ladder after treating K562 cells with CHFE (10, 25 and 50 µg/ml) for 48h and was analysed in 1.5% agarose gel electrophoresis.

3.4.1.7 Propidium iodide staining and Cell cycle analysis

Propidium iodide staining and cell cycle analysis of K562 cells were conducted after treating the cells with 10, 25 and 50 µg/ml concentration of CHFE for 48h. The morphological changes after staining with propidium iodide is shown in figure 3.23. It is clearly observed that the nuclear damage is more visible in IC_{50} and its higher concentration.

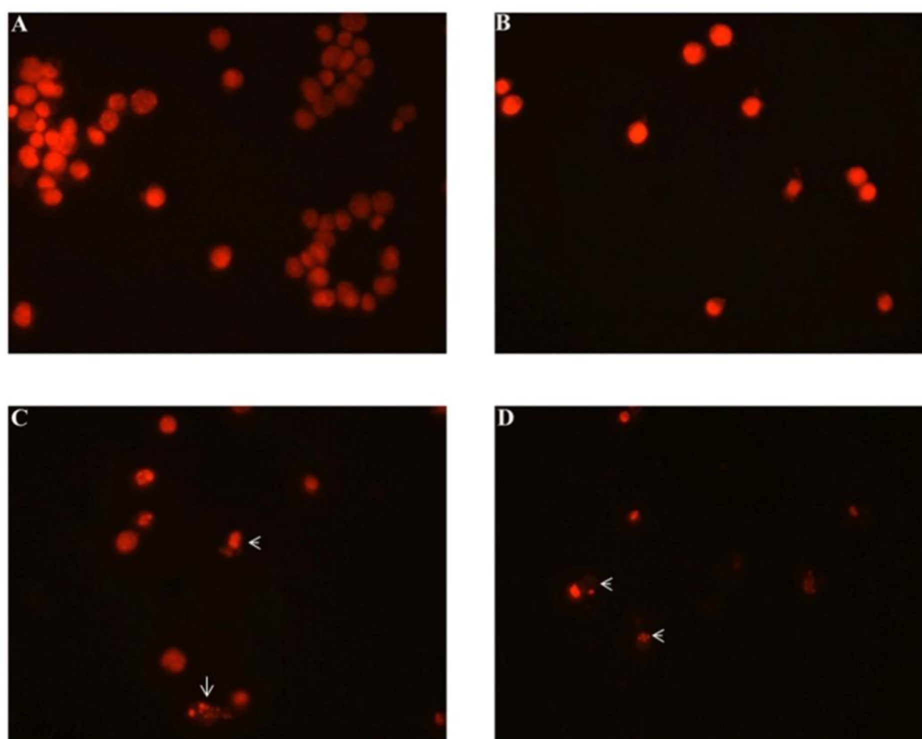


Figure 3.23: Propidium Iodide staining done after treatment with different concentration of CHFE on K562 cells after 48h. A-control, B-10 µg/ml, C-25 µg/ml, D-50 µg/ml, nuclear damage was clearly observed with increase in concentration of extract.

The DNA content of K562 cells after treatment is shown in figure 3.24. The cell cycle distribution of K562 cells treated with different concentration of CHFE as examined by flow cytometric analysis showed a dose dependent increase in the sub G_0 peak indicating induction of apoptosis. The DNA content histogram analysis obtained from PI stained cells showed an increase in the trend of cells in sub G_0 peak. The percentage of cells in sub G_0 phase is represented in figure 3.25. From the cell cycle analysis study, it is confirmed that the CHFE has the ability to induce apoptosis in a concentration dependent manner.

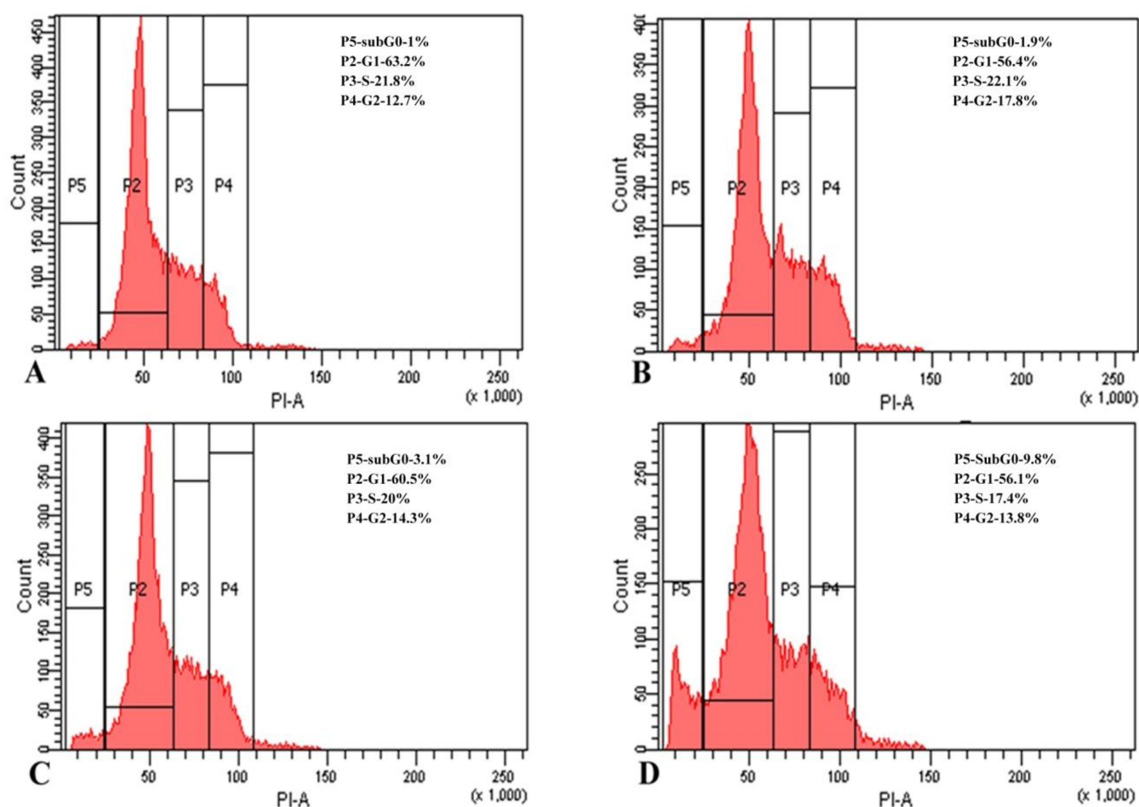


Figure 3.24: Histogram represents the cell cycle distribution of K562 cells after 48h treatment with CHFE A-control, B- 10 µg/ml, C-25 µg/ml, D-50 µg/ml. There is an increase in the Sub G_0 phase of the cell cycle.

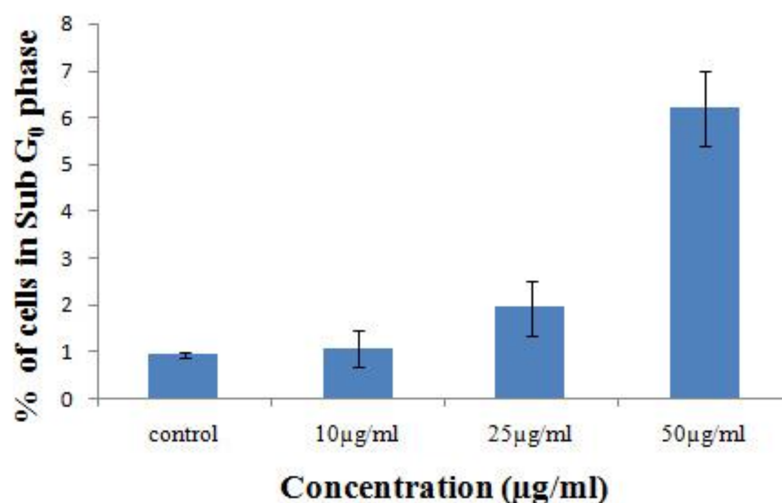


Figure 3.25: Graph showing an increase in Sub G_0 phase after treatment with different concentration (10 µg/ml, 25 µg/ml, and 50 µg/ml) of CHFE on K562 cells after 48h.

3.4.1.8 Analysis of Phosphatidylserine exposure using Annexin V- FITC

The indication of early stage of apoptosis was determined using Annexin V. In the non-apoptotic cell, most phosphatidylserine molecule are localised at the inner layer of plasma membrane. However, in this assay the traslocation of phosphatidylserine residues from the internal layer to outer face of the plasma membrane is an indicative for an early apoptotic induction.

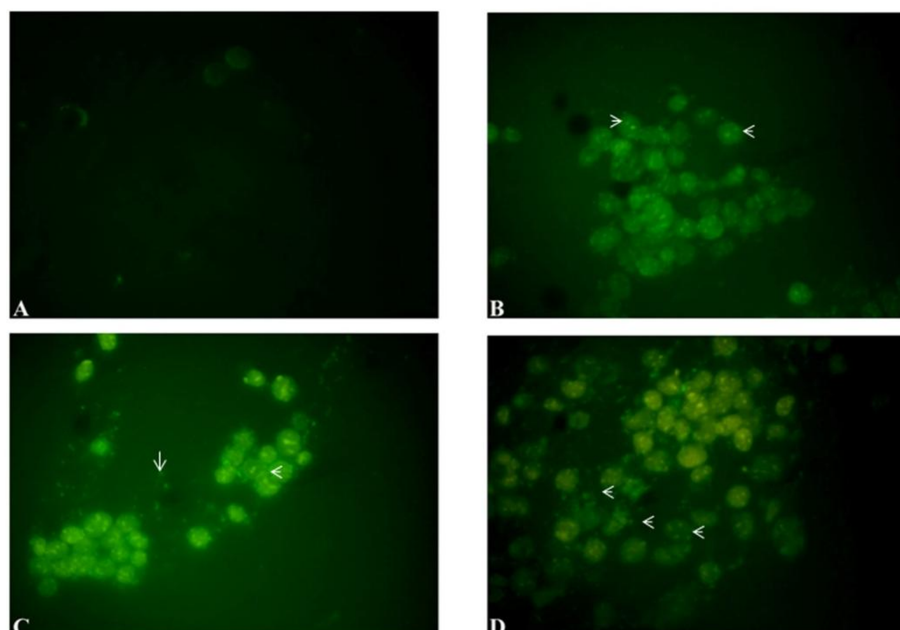


Figure 3.26: Fluorescent image of K562 cells treated with CHFE after 48h followed by Annexin V FITC and PI staining A-control cells, B-10 µg/ml, C-25 µg/ml and D-50 µg/ml

In this study, Annexin V FITC and PI staining flow cytometric analysis was performed for the determination of apoptosis. In the staining technique early and late apoptotic events was clearly visualised in the treated groups and is shown in figure 3.26. The externalisation of phosphatidylserine was triggered by the CHFE treatment in K562 cells in a dose dependent manner. Thus there was a drastic decrease in the percentage of viable cells in untreated group to the treated group and moreover there was a significant increase in the apoptotic cells in a dose dependent response. It was clearly observed in the histogram that there is an increase in the induction of apoptosis by CHFE in K562 treated cells and is illustrated in the figure 3.27. The percentage live, early and late apoptotic and necrotic cells are represented in figure 3.28.

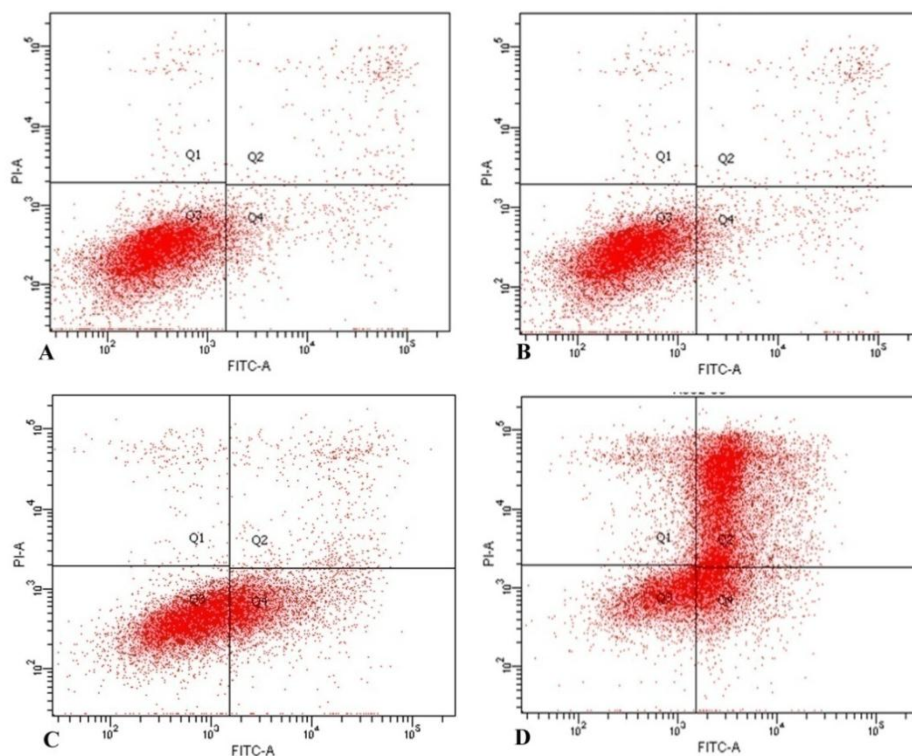


Figure 3.27: Histogram showing the analysis of PS exposure using Annexin V-FITC after 48h treatment with different concentration of CHFE A-control, B-10 µg/ml, C-25 µg/ml, and D-50 µg/ml.

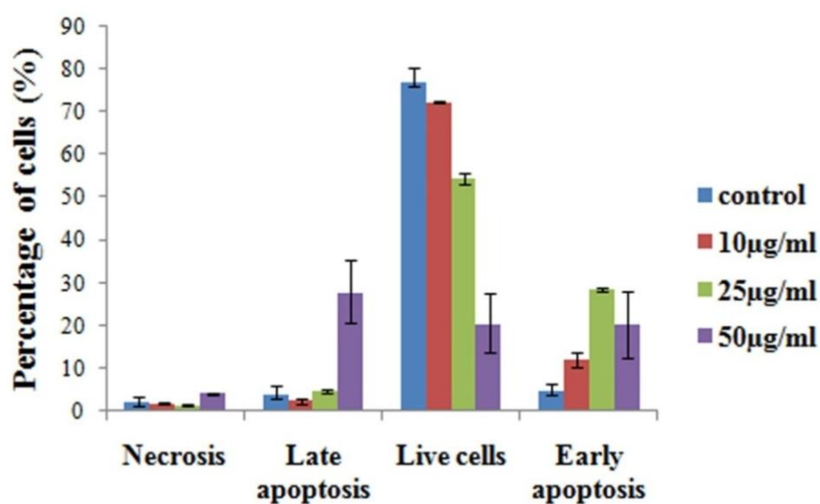


Figure 3.28: Graph represents the detection of increase in number of apoptotic cells as the concentration 10, 25 and 50 µg/ml of CHFE is increased after 48h of treatment.

3.4.1.9 Determination of mitochondrial membrane potential (ΔΨm)

Rhodamine 123 (R123) is a fluorescent cationic dye that binds to polarised mitochondrial membrane and accumulates as aggregates in the mitochondria of normal cells. For determining the mitochondrial membrane potential, K562 cells were treated with indicated concentration of CHFE 10, 25 and 50 µg/ml for 48h (figure 3.29). It was observed that there is an increase in the fluorescent intensity with increase in concentration and this corresponds to the decrease in the mitochondrial membrane potentiality. Mitochondrion is an integral part of apoptotic machinery and loss of mitochondrial membrane potential is a classical evidence for apoptosis.

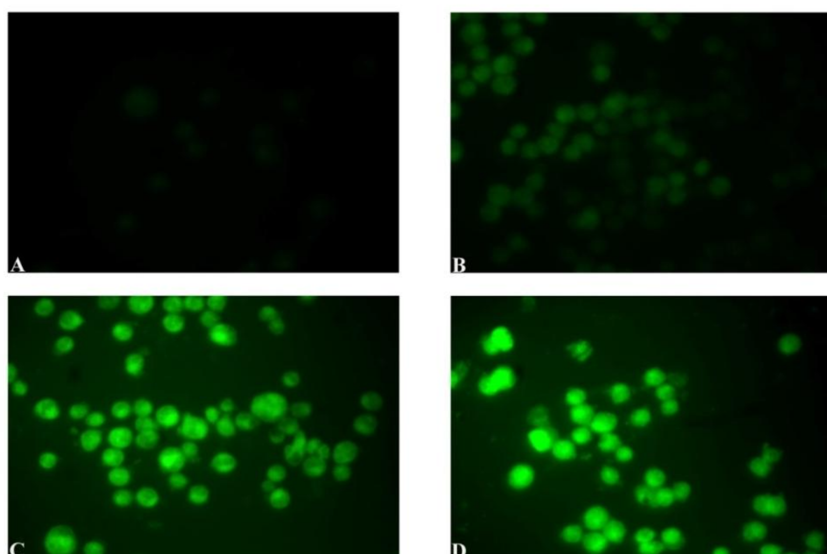


Figure 3.29: Effect of CHFE on mitochondrial membrane potential. K562 cells were treated with 10, 25 and 50µg/ml of CHFE for 48h and the cells were harvested and disruption of mitochondrial membrane potential was measured using fluorochome dye R123. A, B, C and D represents the fluorescent images of cells after treatment control, 10, 25 and 50µg/ml.

3.4.1.10 Western blot analysis

For elucidating the molecular mechanism of CHFE of *T. populnea*, the apoptotic signal transduction was investigated. Caspases plays an important role in initiation and execution of apoptosis. Caspases-8 and -9 are known as initiator caspases, whereas caspase-3 is considered as executioner caspase. Two distinct and well known initiator caspases, caspase-8 for death receptor mediated and caspase-9 for the mitochondria mediated pathways. The expression levels of Caspase-3, -9 and PARP, the substrate of caspase-3 were detected using western blotting analysis (figure 3.30). Proteolytic cleavage of mitochondria mediated apoptotic cell death initiator caspase-9

was observed in CHFE treated K562 cells. Cleaved caspase-3 and PARP was also observed in CHFE treated K562 cells.

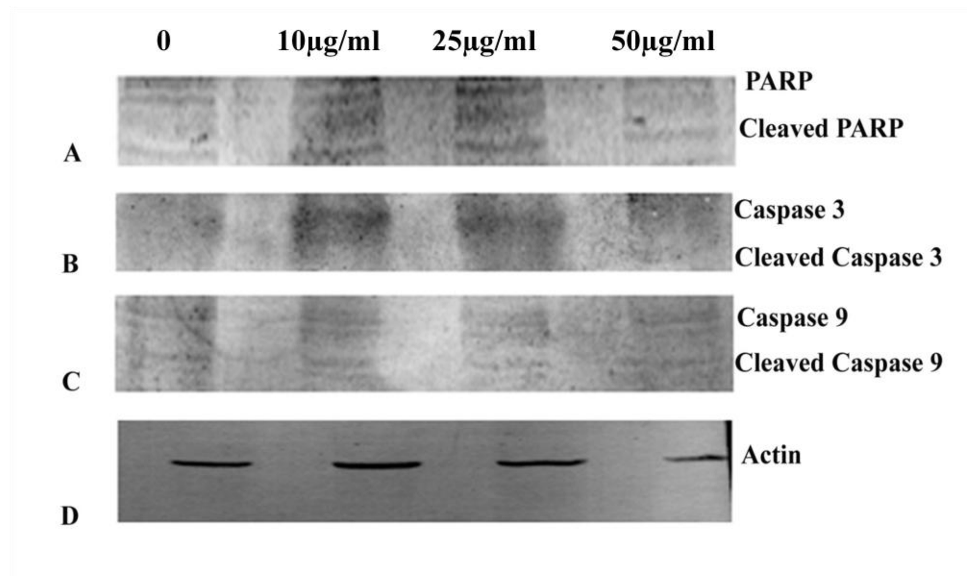


Figure 3.30: Total proteins from CHFE treated K562 cells were extracted and the levels of proteins were analysed by Western blotting. Cells were treated with 0 µg/ml, 10 µg/ml, 25 µg/ml and 50 µg/ml and checked for the expression of PARP, caspase 3 and Caspase 9. Actin was used as the internal control.

3.4.2 Antiproliferative and apoptogenic ability of the leaf extract against colorectal carcinoma HCT116 cell line

For the confirmation of antiproliferative and apoptogenic inducing ability of CHFE in HCT116 cells, different techniques were employed and are mentioned below.

3.4.2.1 Morphological changes occurred in HCT 116 cells after treatment with CHFE

The HCT116 cells were treated with 10, 37, 50 µg/ml concentrations of CHFE for 48h. The morphological changes occurred in HCT116 cells were observed after treatment through phase contrast microscope and observed that cells were shrunk into smaller round size and at higher concentration treated group it completely lost their anchorage capacity while compared to the control cells and is shown in figure 3.31.

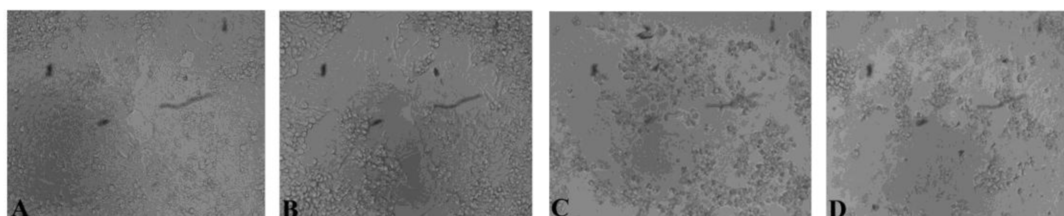


Figure 3.31: Phase contrast image of HCT116 cells after treatment with CHFE of different concentrations A-control, B-10 µg/ml, C-37 µg/ml and D-50 µg/ml for 48h. Cell morphology got altered after increase in concentration of CHFE.

3.4.2.2 Scanning electron microscopic images of HCT 116 after treatment with CHFE

The scanning electron microscopic images of HCT116 were analysed for the characteristic morphological signs of apoptosis. The cells were treated with 10, 37 and 50 µg/ml concentration of CHFE for 48h. It was observed that in the control group the cells was found to be intact with stable and exhibited normal morphology. In the treated groups, it was observed that the morphology of the cells was altered by showing the characteristic features of apoptosis like cell shrinkage and membrane blebbing formation (figure 3.32). It is clearly evident that the apoptosis inducing capability of CHFE responded in a concentration dependent manner.

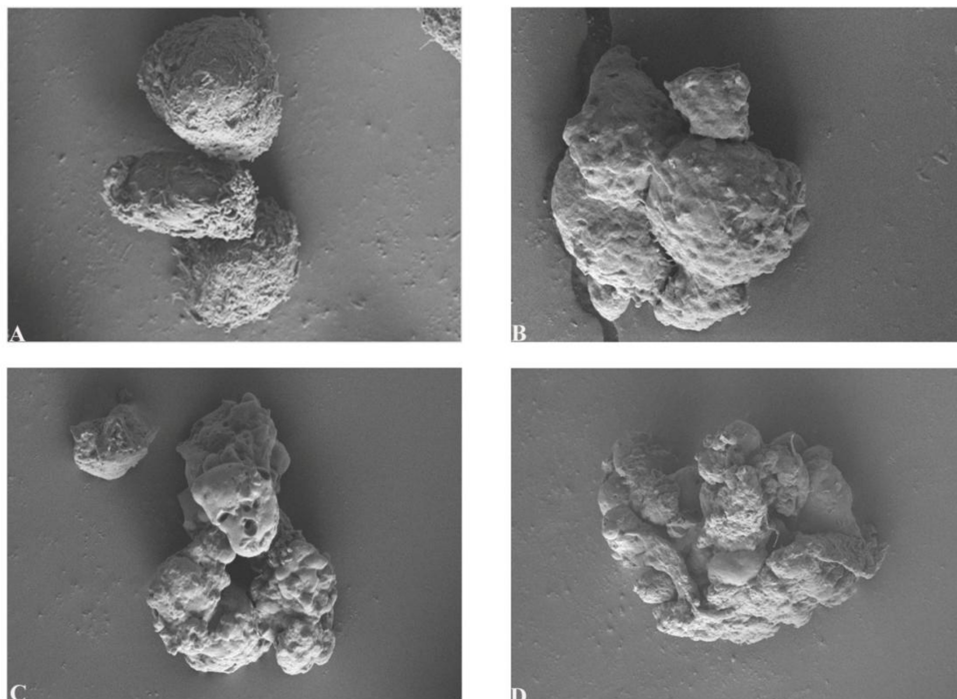


Figure 3.32: Scanning electron microscopic images of HCT116 cells after treatment with CHFE: A-control, B-10 µg/ml, C-37 µg/ml and D-50 µg/ml for 48h. The cell morphology was changed with increase in concentration of CHFE and also showed the characteristic apoptotic changes.

3.4.2.3 Assessment of antiproliferative potential of CHFE on HCT116 cell line using MTT and SRB assay

The antiproliferative ability of CHFE of *T.populnea* was checked against HCT116 cell line using MTT and SRB assay. The cells were treated with different concentration of CHFE varying from 10-100µg/ml for 48h. The percentage viability and their IC_{50} concentration were determined and shown in figure 3.33. In both the assays CHFE revealed encouraging antiproliferative ability against HCT 116 cells. In MTT assay the IC_{50} concentration was found to be 37.22±4.04 µg/ml and for SRB assay displayed a similar value of 37.81±3.74 µg/ml.

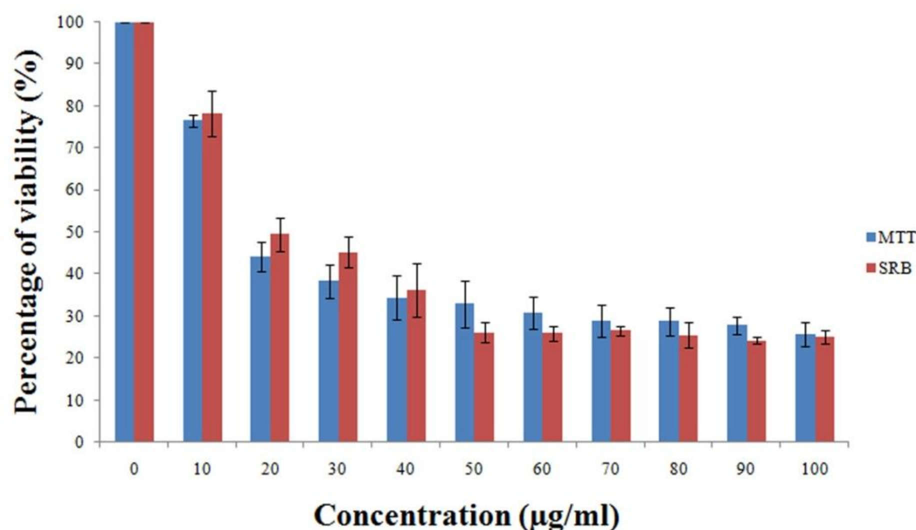


Figure 3.33: Graph representing the percentage of viability of CHFE against HCT116 cell using both MTT and SRB assay. The MTT assay showed an IC_{50} concentration of 37.22±4.04 µg/ml and 37.81±3.74 µg/ml for SRB assay

3.4.2.4 Acridine orange/Ethidium bromide double staining

Induction of apoptosis by CHFE was also assessed in HCT116 cells by double staining method using fluorescent microscopy. Acridine orange penetrates into the living cell and emits green fluorescence. Ethidium bromide stains the cells that lost membrane integrity. The untreated control group appeared green with intact nuclei and normal cell morphology. In the treated groups stained orange to red fluorescence showed the characteristic sign of apoptosis such as chromatin condensation, nuclear fragmentation and alteration in the size and shape of the cells as shown in figure 3.34.

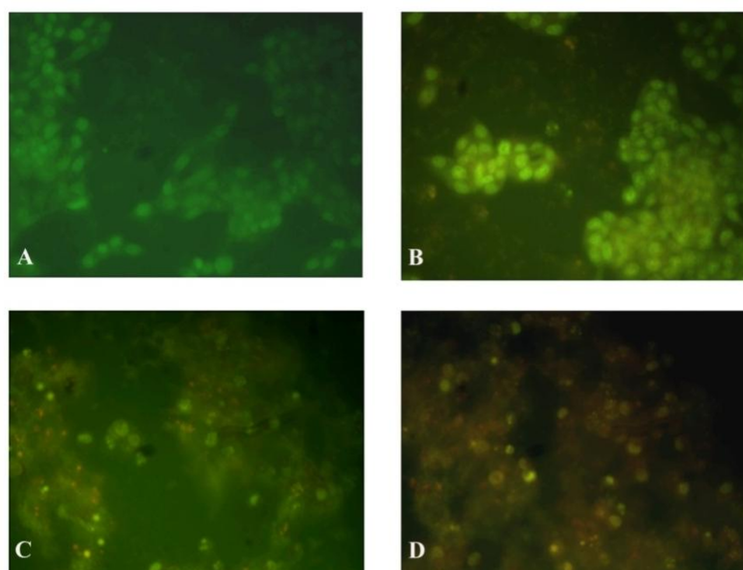


Figure 3.34: Acridine orange/ethidium bromide double staining (A) control group- No significant apoptotic changes detected, (B) & (C) is the treated group of 10 µg/ml and 37µg/ml of CHFE-early stage apoptotic cells detected (D) 50 µg/ml treated group orange to red fluorescence due to late apoptotic and dead cells.

3.4.2.5 Nuclear Hoechst staining

The nuclear Hoechst staining was performed on CHFE treated HCT116 cells. It is a membrane permeable nuclear blue fluorescent dye. The untreated cells displayed intact and round uniformly stained nuclei which was emitting lesser bright blue fluorescence. There is a concentration dependent morphological changes like loss of membrane integrity, cell shrinkage, nuclear fragmentation and condensation were observed in figure 3.35. Thus confirmed the apoptosis inducing ability of CHFE.

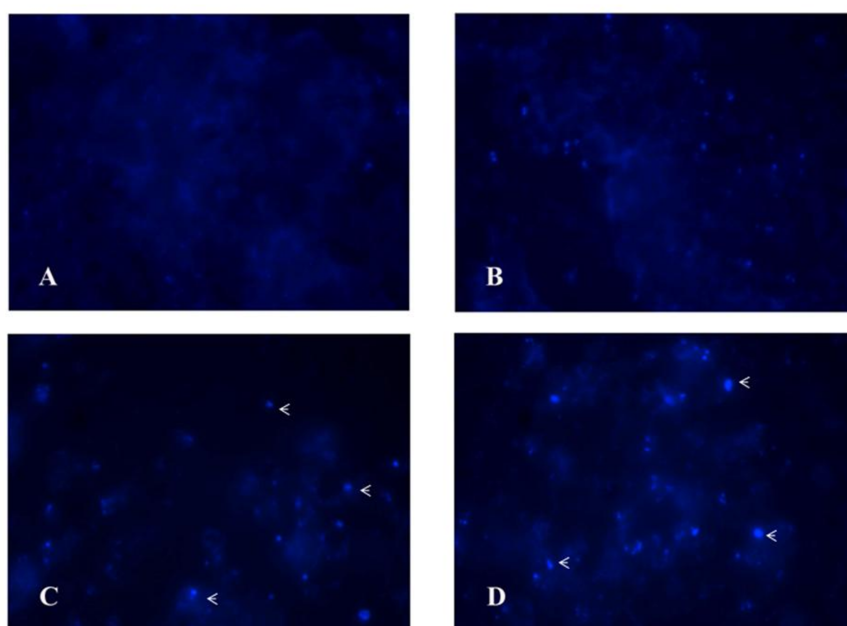


Figure 3.35: Nuclear Hoechst staining of CHFE treated HCT116 cells. A- control, B- 10 µg/ml arrows indicates apoptotic bodies of nuclear fragmentation observed in C -37 µg/ml and D-50 µg/ml concentration of CHFE after 48h of treatment.

3.4.2.6 Mitochondrial membrane potential

The loss of mitochondrial membrane potential is a classical evidence for apoptosis. The mitochondrial membrane integrity of HCT116 cells was evaluated by staining with Rhodamine 123 dye. For checking the mitochondrial membrane potential the cells was treated with CHFE of 10, 37 and 50 µg/ml concentration for 48 hours. It was observed that the fluorescence intensity was increased with increase in concentration

of the treatment and illustrated in figure 3.36. Thus confirmed the mitochondrial membrane potential integrity was lost with increasing dosage of treatment.

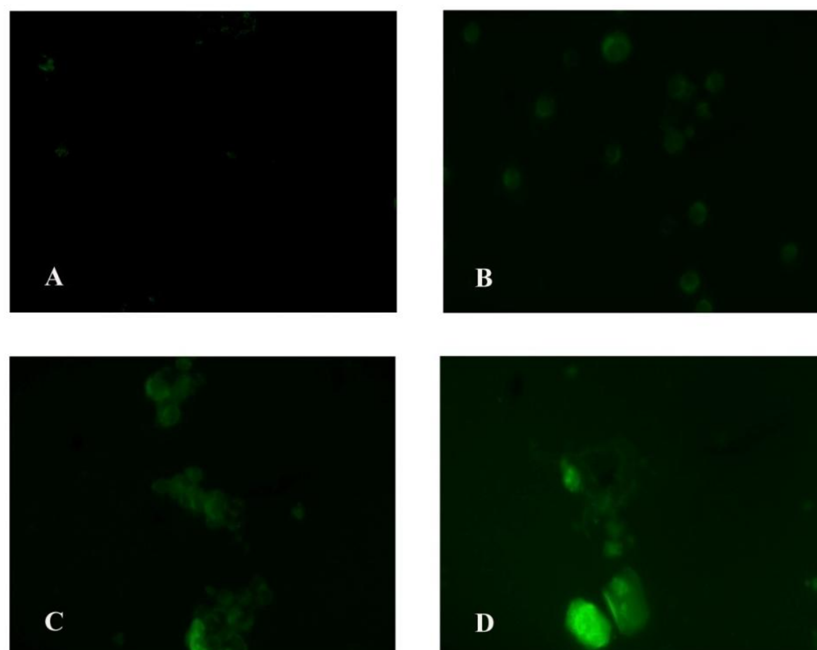


Figure 3.36: Fluorescent images of R123 stained HCT116 cells after 48h treatment with CHFE A-control, B-10μg/ml, C-37μg/ml and D-50μg/ml. The mitochondrial membrane potential was lost with increase in concentration of drug.

3.4.2.7 Propidium iodide staining and cell cycle analysis

The propidium iodide staining and cell cycle analysis of HCT116 cells was conducted after treating the cells with 10, 37 and 50 μg/ml concentration of CHFE for 48h. The morphological changes after staining with propidium iodide illustrated in figure 3.37. The nuclear damage was mostly observed in IC_{50} and its higher concentration. To elucidate the mechanism by which CHFE inhibits HCT116 cells, flow cytometric analysis was performed to determine the effects of cell cycle progression in cells treated with varying concentration of CHFE. The DNA content distribution after treatment is shown in figure 3.39. The cell cycle distribution of CHFE treated showed a dose dependent increase in the sub-G_0 peak indicating the apoptosis inducing ability. The percent of cells in different phase of cell cycle was illustrated in figure 3.38. Thus based on the percentage distribution of cells it is evident that the CHFE has the ability to induce apoptosis in a concentration dependent manner.

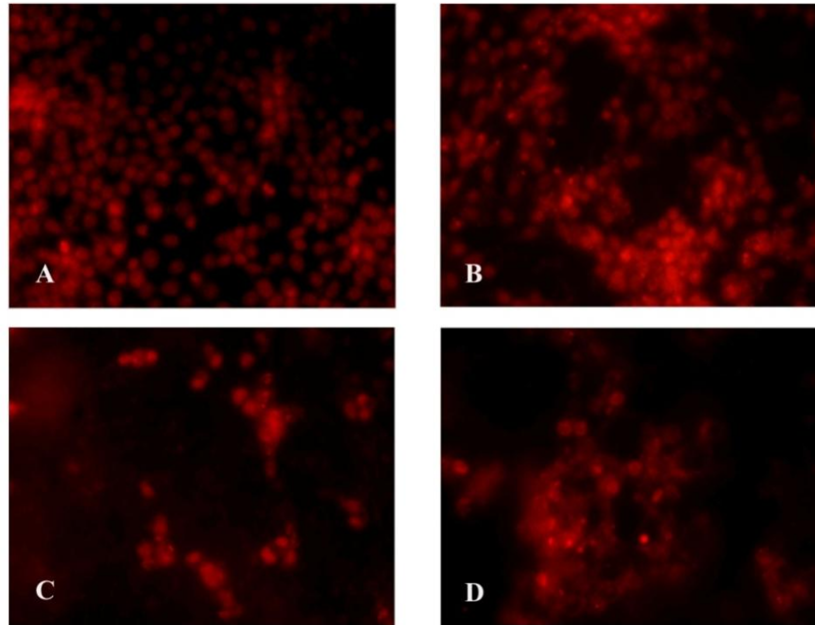


Figure 3.37: Propidium Iodide staining done after treatment with different concentration of CHFE on HCT116 cells after 48h. A-control, B-10 µg/ml, C-37 µg/ml, D-50 µg/ml, nuclear damage was clearly visualised with increase in concentration of extract.

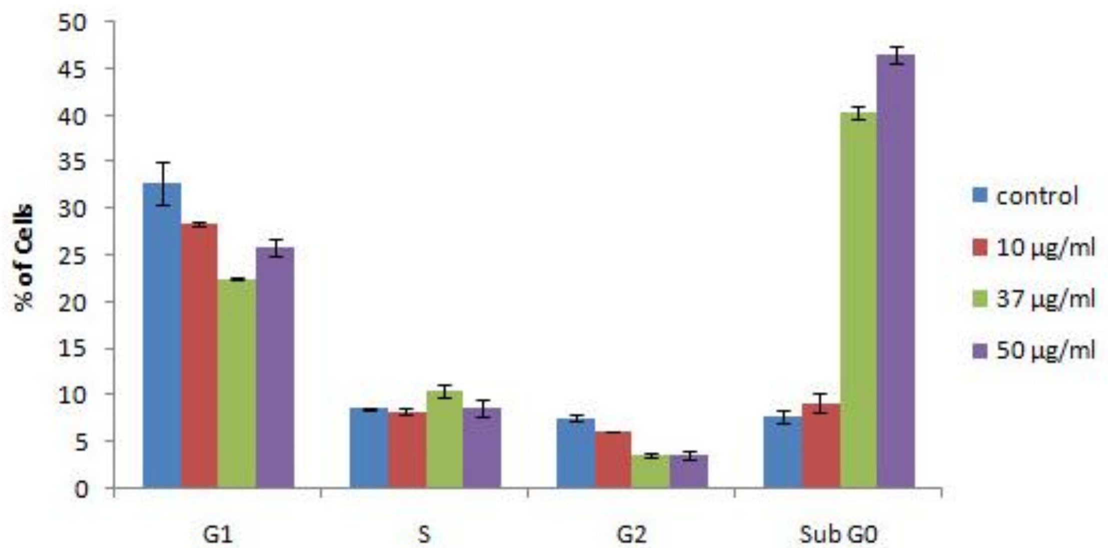


Figure 3.38: Graph representing percentage of cells in different phases of cell cycle after CHFE treatment in HCT116 cells. After treatment with CHFE observed a dose dependent increase of cells in sub G_0 phase.

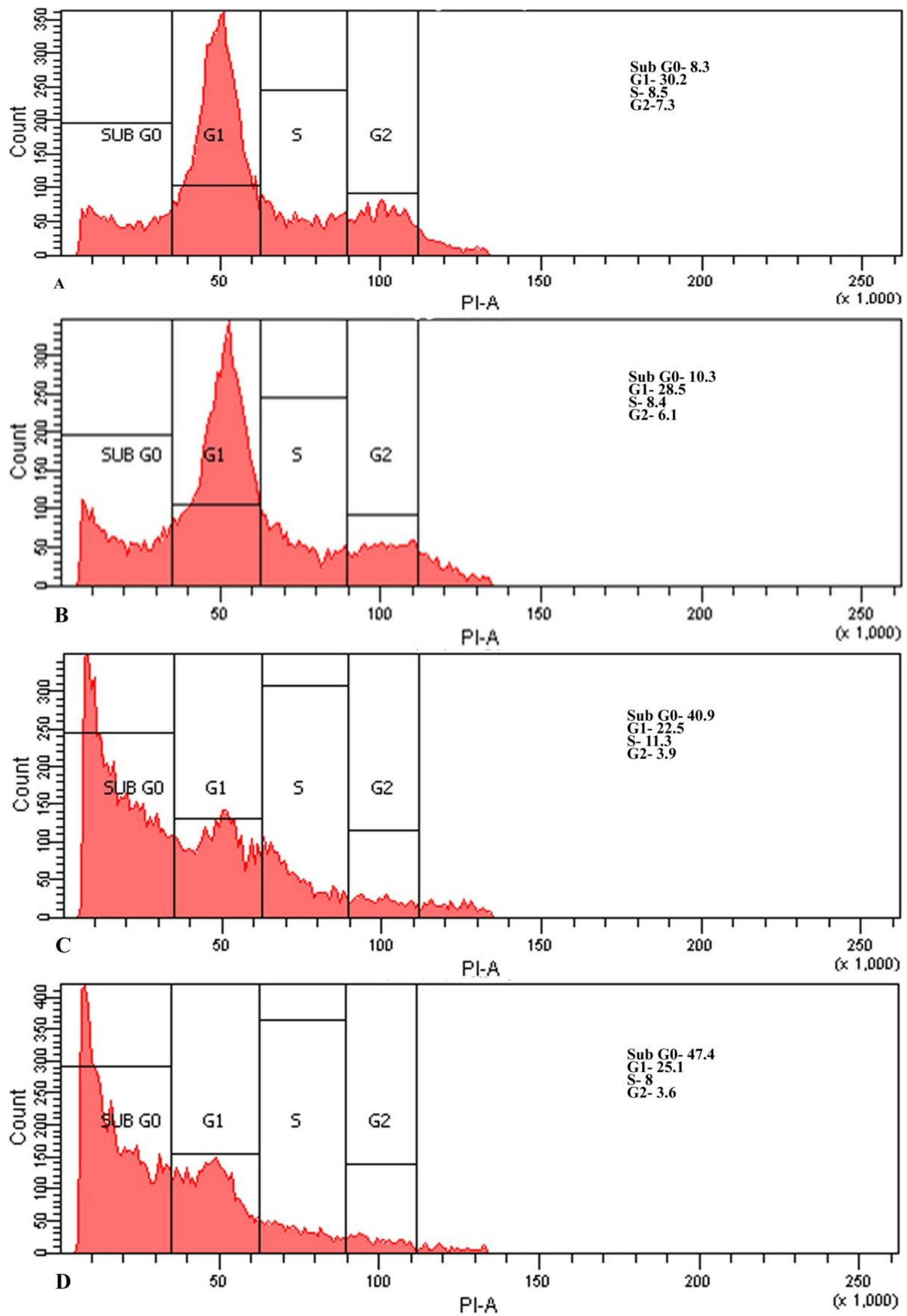


Figure 3.39: Histogram representing the increase in sub G_0 peak after treatment with CHFE A-control, B-10 µg/ml, C-37 µg/ml, D-50 µg/ml in HCT116 cells for 48h.

3.4.2.8 Annexin V- FITC fluorescent staining

Annexin V is a cellular protein, commonly used to detect apoptotic cells. It has the ability to bind to phosphatidylserine, a marker of apoptosis when it is in the outer leaflet of the plasma membrane. In normal condition the phosphatidyl serine are seen in the inner leaflets of plasma membrane, whereas in the case of apoptotic cells the phosphatidyl serine is tranlocated from the inner layer to the outer face of the plasma membrane. In this study after the treatment of HCT116 cells with different concentration of CHFE, early and later stages of apoptosis were observed at 37 and 50 µg/ml concentration shown in figure 3.40. The CHFE induced apoptosis in a dose-dependent manner in HCT116 cells.

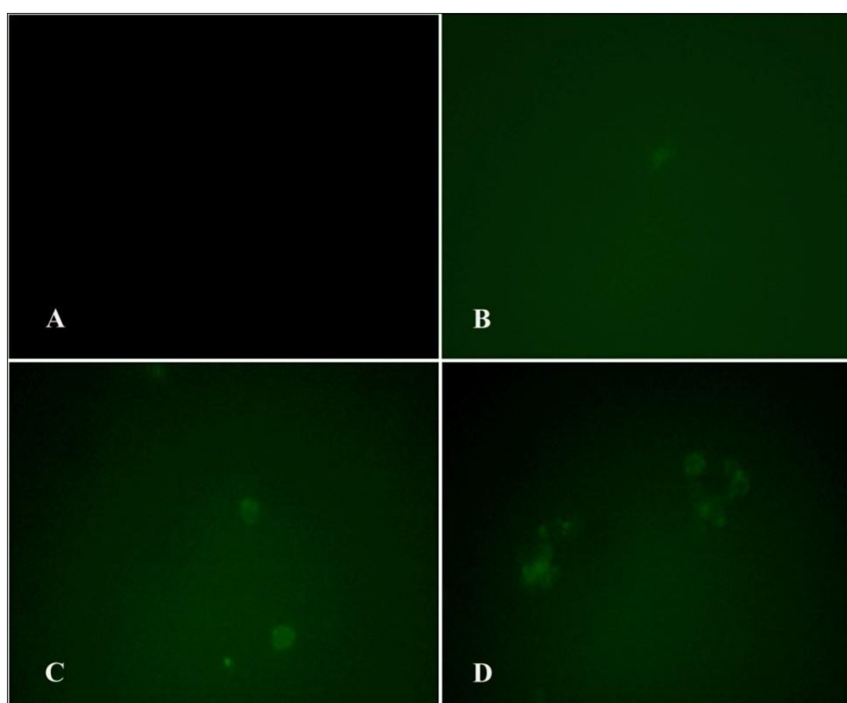


Figure 3.40: Annexin-V FITC fluorescent staining of HCT116 cells after treatment with CHFE. A-control, B-10 µg/ml, C-37 µg/ml and D-50 µg/ml.

3.4.2.9 DNA fragmentation assay

The DNA fragmentation an important characteristic feature of apoptotic cells. The study was conducted to investigate the cytotoxic ability of CHFE has the potential to induce apoptosis. For this study, the HCT116 cells were treated with 10, 37 and 50 µg/ml of CHFE for 48 hours. The ladder pattern was visualized in agarose gel as shown in figure 3.41. There was a clear ladder pattern exhibited at 37 and 50µg/ml

after CHFE treatment. Thus confirmed the apoptosis inducing ability of CHFE in HCT116 cells.

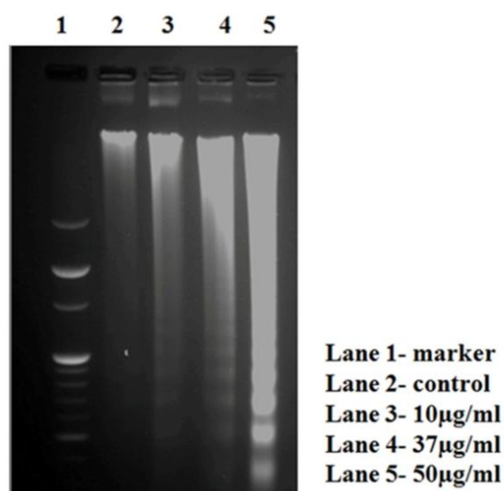


Figure 3.41: DNA fragmentation assay: Formation of DNA ladder after treating HCT116 cells with CHFE for 48h (0, 10, 37 and 50 µg/ml).

3.4.2.10 Colony inhibition assay

This assay analyse the capacity of a single cell to form a colony after treatment with cytotoxic compound. The *in vitro* potential of CHFE to inactivate the colony forming capacity of the HCT116 was investigated. The colony formation capacity of the cells was inhibited as the concentration of the CHFE is increased as represented in figure 3.42.

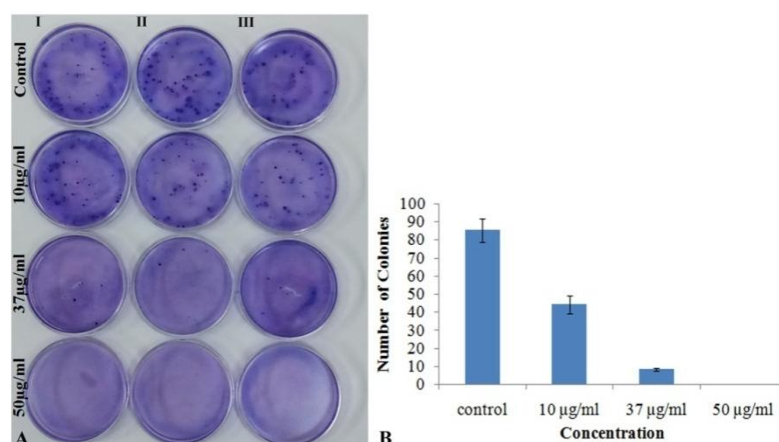


Figure 3.42: A- Image showing the colony inhibiting property of CHFE against HCT116 cells. B- Graph representing the decrease in number of colonies as the drug concentration is increased.

3.4.2.11 Cell migration inhibition assay

The effect of CHFE on the migration ability of HCT116 was analysed at 10, 37 and 50 µg/ml concentration using cell migration inhibition or scratch assay. This assay was performed by rendering a wound in a monolayer of cells and allowing the cells to respond by healing the wound in the presence of CHFE for 24h. After 12h and 24h the ability of the cells to migrate to clear the wound was captured under phase contrast microscope and shown in figure 3.43. The cells in the untreated group, the cells migrated towards the wound region after 12 and 24h. Thus the study clearly shows that the CHFE inhibited the migration of the cells and confirmed the anti-metastatic ability.

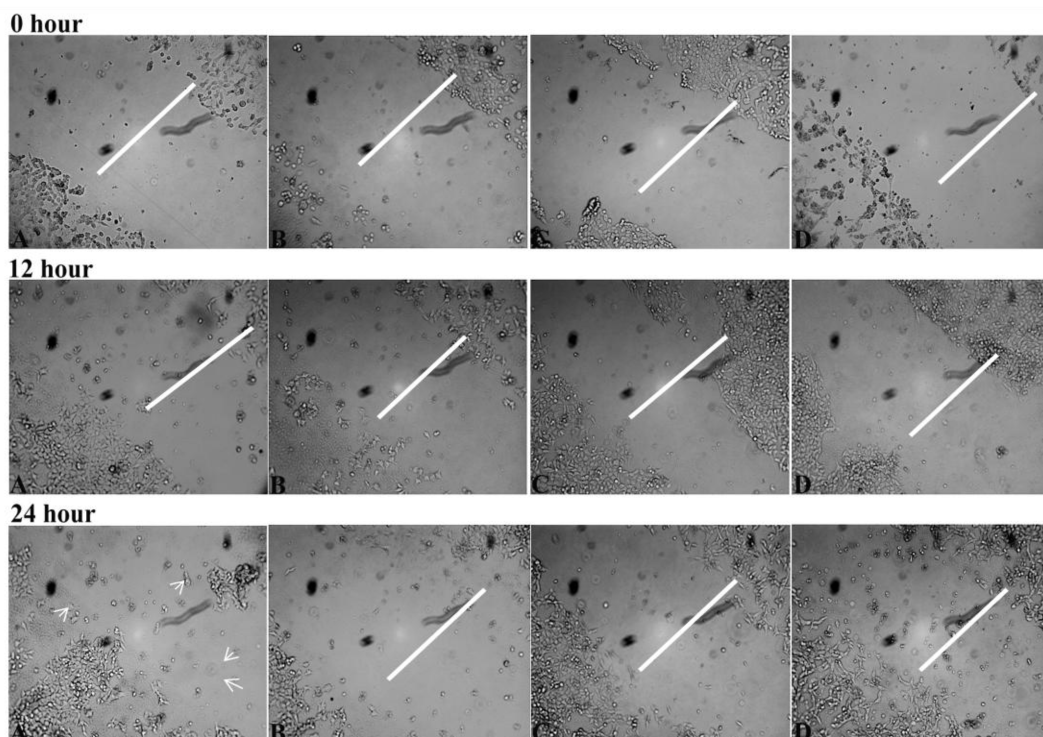


Figure 3.43: Phase contrast microscopic image of cell migration inhibition assay. The confluent HCT116 cells were scratched and CHFE of different concentration was added A-control B-37 and C-50 µg/ml observed at different time interval. After 24h the cells in the untreated group started migration towards the scratched area.

3.4.2.12 Western blotting

PARP cleavage is a characteristic feature of apoptosis. To confirm the apoptosis inducing ability of CHFE on HCT116 cells, western blot analysis of protein poly (ADP-ribose) polymerase (PARP) cleavage was performed. The result showed that 48

h treatment with 37 and 50 µg/ml of CHFE cleaved PARP in HCT 116 cells, which is a confirmation marker for apoptosis induction (figure 3.44).

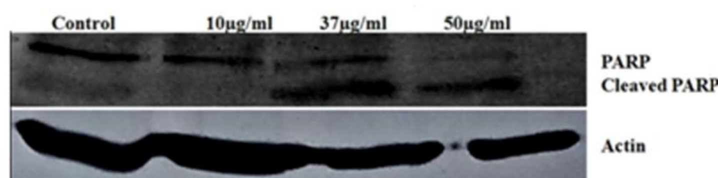


Figure 3.44: Western blot analysis: The result showing the development of PARP cleavage in HCT116 cells after treatment with CHFE at 37 and 50 µg/ml for 48h.

3.5 *In vivo* anti tumor activity of leaf extract against Ehrlich Ascites Carcinoma in Swiss Albino Mice

3.5.1 Acute toxicity study

Acute toxicity study (OECD 420) was carried out for chloroform leaf extract of *T.populnea* using 6 swiss albino mice. The animals were administered a single dose of 1g/Kg body weight of CHFE orally and observed closely for 1,3, 6, 9h and for upto 24h. There was no sign of toxicity and observed further for 15 days.

3.5.2 Mean survival time study

To check the mean survival time, 30 Swiss albino mice were used in the experiment, which were divided into five groups (n=6), they were fed with food and water *ad libitum*. All the animals in the group received EAC cells $1X10^6$ cells intra peritoneally. Group I animals served as EAC control without any drugs. 24h after EAC transplantation, group II-IV received CHFE at a dose of 50, 100 and 200 mg/kg body weight CHFE in 0.1% carboxy methycellulose and group V received reference drug cyclophosphamide 25 mg/Kg body weight orally for continuous 10 days.

The chloroform leaf extract at the doses of 100 and 200 mg/kg body weight when administered orally, increased the survival time of EAC bearing mice. CHFE considerably improved the mean survival time in tumour bearing mice while compared with the control group. No visible signs of toxicity were observed in any of the treated groups. The prolongation of life span is a reliable criterion for judging the efficacy of anticancer drugs.

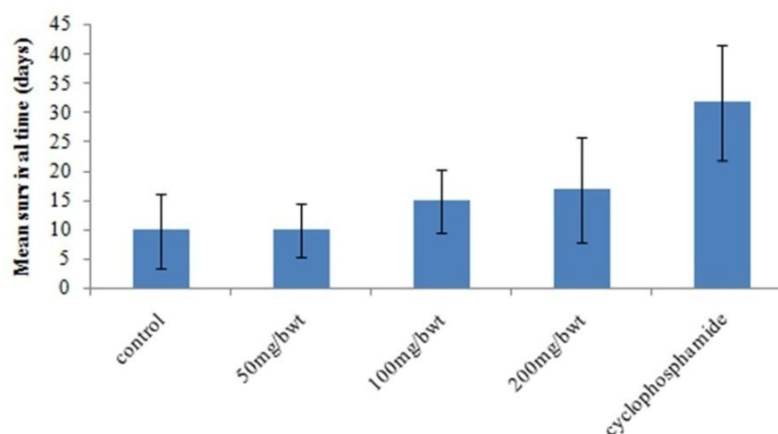


Figure 3.45: Graphical representation of mean survival time after treatment with CHFE for 10 day.

3.6 Bioassay guided isolation and characterisation of anticarcinogenic compounds

To isolate the active constituents compounds present in the chloroform leaf extract of *T. populnea* different methods were employed. Chromatographic and spectroscopic techniques are carried out for the isolation and characterisation of the bioactive compounds and checked for its antiproliferative ability by MTT assay. The techniques employed are thin layer chromatography, HPTLC, Column chromatography, HPLC, Preparative HPLC, GC-MS, FTIR and LC-MS analysis

3.6.1 Thin layer chromatographic separation of chloroform leaf extract of *T. populnea*

The CHFE showed a very prominent antiproliferative property against different cell lines. The biological activities exhibited by the extract may be due to the presence of various bioactive components present in them. Thin layer chromatography (TLC) is widely used for the rapid analysis of drugs and drug preparation. The time required for the demonstration of most of the characteristic constituents is very short. It gives a qualitative and also provides semi quantitative information on the major active constituents.

Chloroform extract of *T.populnea* was separated by TLC using Petroleum ether: Ethyl acetate solvents in the ratio 6:4 which showed maximum separation and illustrated in figure 3.46. Different compounds present in the extract were analysed using various spraying reagents.

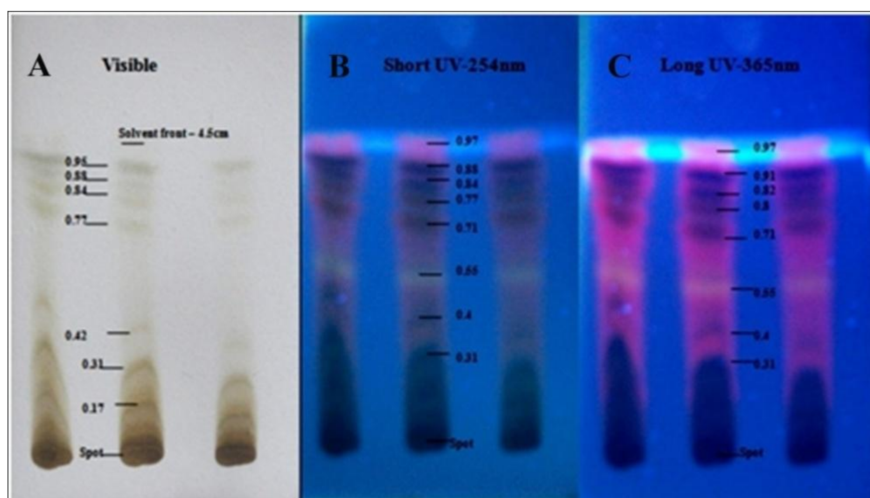


Figure 3.46: Thin layer chromatogram of chloroform leaf extracts of *T. populnea* observed under A-visible light, B-short UV 254nm and C-long UV 365nm after separating using petroleum ether: ethyl acetate (6:4)

3.6.2 High performance thin layer chromatography (HPTLC)

High performance thin layer chromatography is a modified version of thin layer chromatography. The separation of CHFE was done using high performance layer of pre-coated sorbent plates. The reduction in thickness of layer and the particle size results increased plate efficiency along with nature of separation. CHFE was separated using Petroleum ether: Ethyl acetate in the ratio 6:4, the parameter such as number of compounds and their corresponding peaks under UV 254 nm and 366 nm were illustrated. The HPTLC chromatograms and profiles showed in figure 3.47 and 3.48.

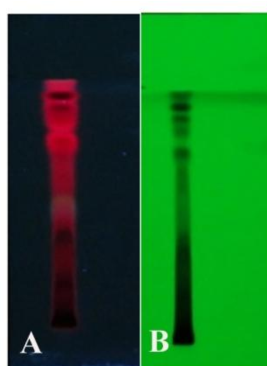
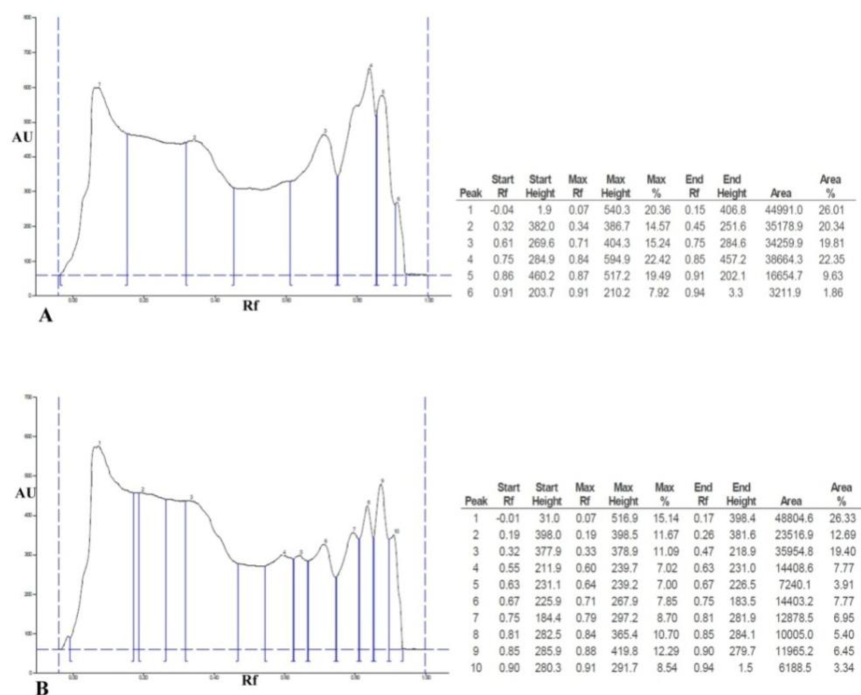


Figure 3.47: HPTLC chromatogram of chloroform leaf extract of *T. populnea* A-366nm B-254nm.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.04	1.9	0.07	540.3	20.36	0.15	406.8	44991.0	26.01
2	0.32	382.0	0.34	386.7	14.57	0.45	251.6	35178.9	20.34
3	0.61	269.6	0.71	404.3	15.24	0.75	284.6	34259.9	19.81
4	0.75	284.9	0.84	594.9	22.42	0.85	457.2	38664.3	22.35
5	0.86	460.2	0.87	517.2	19.49	0.91	202.1	16654.7	9.63
6	0.91	203.7	0.91	210.2	7.92	0.94	3.3	3211.9	1.86

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.01	31.0	0.07	516.9	15.14	0.17	398.4	48804.6	26.33
2	0.19	398.0	0.19	398.5	11.67	0.26	381.6	23516.9	12.69
3	0.32	377.9	0.33	378.9	11.09	0.47	218.9	35954.8	19.40
4	0.55	211.9	0.60	239.7	7.02	0.63	231.0	14408.6	7.77
5	0.63	231.1	0.64	239.2	7.00	0.67	226.5	7240.1	3.91
6	0.67	225.9	0.71	267.9	7.85	0.75	183.5	14403.2	7.77
7	0.75	184.4	0.79	297.2	8.70	0.81	281.9	12878.5	6.95
8	0.81	282.5	0.84	365.4	10.70	0.85	284.1	10005.0	5.40
9	0.85	285.9	0.88	419.8	12.29	0.90	279.7	11965.2	6.45
10	0.90	280.3	0.91	291.7	8.54	0.94	1.5	6188.5	3.34

Figure 3.48: HPTLC chromatogram of CHFE (A) - under 366nm and (B) - under 254nm

3.6.3 Thin layer chromatographic detection of compounds using different spraying reagents

After preliminary inspection in UV-254 and UV-365nm, each chromatogram is analysed for the presence of compounds by spraying with appropriate reagents for the detection of compounds. Most of the plant constituents react with the reagent and developed a colored reaction in visible and also under UV light as illustrated in figure 3.49 and 3.50 and table 3.7. The preliminary screening of the chloroform extract of *T.populnea* showed the presence of alkaloids, terpenoids, phenolics, flavonoids, anthraquinones, lignans to the specific spraying reagents.

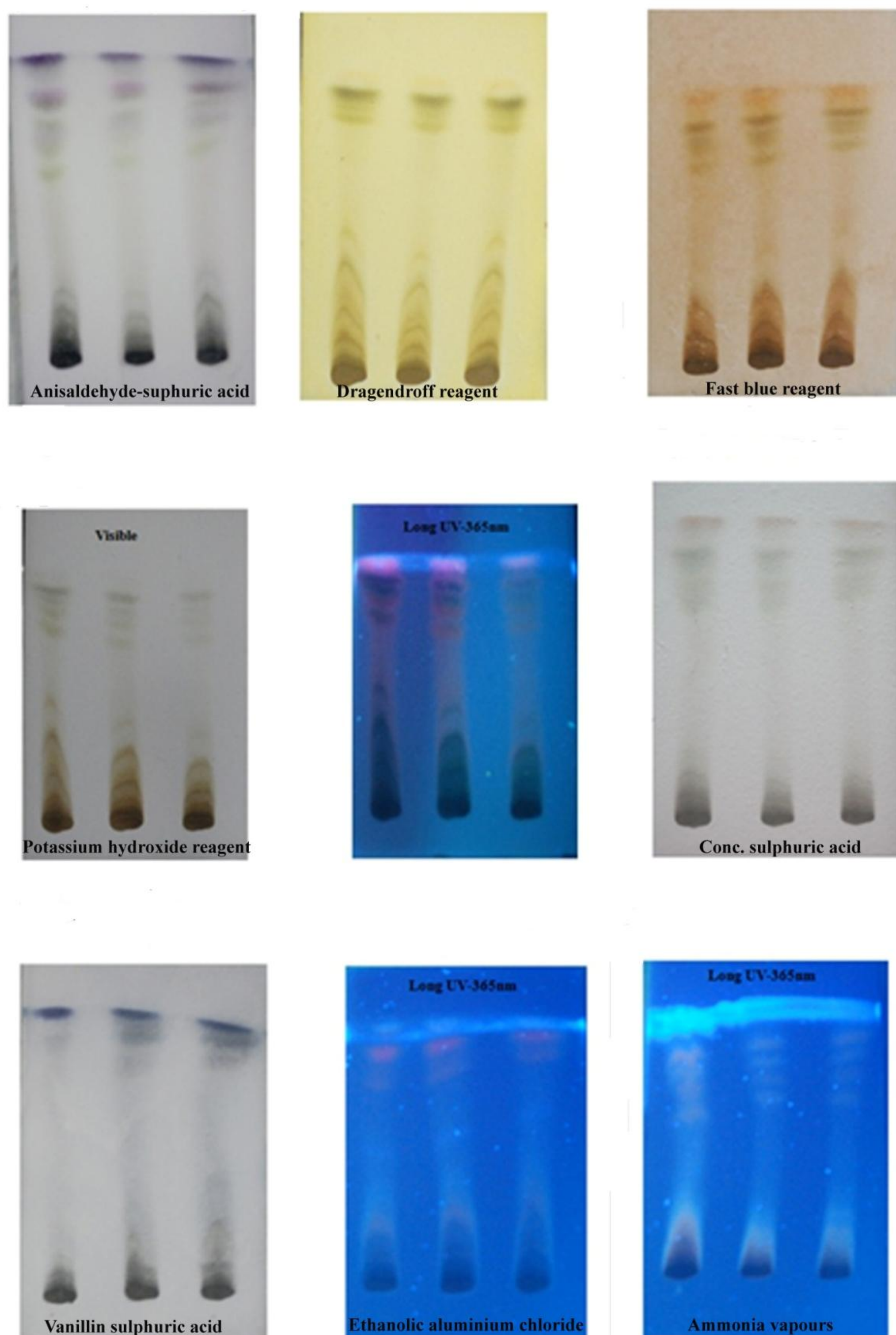


Figure 3.49: Preliminary screening of phytoconstituents using appropriate spraying reagent after developing TLC.

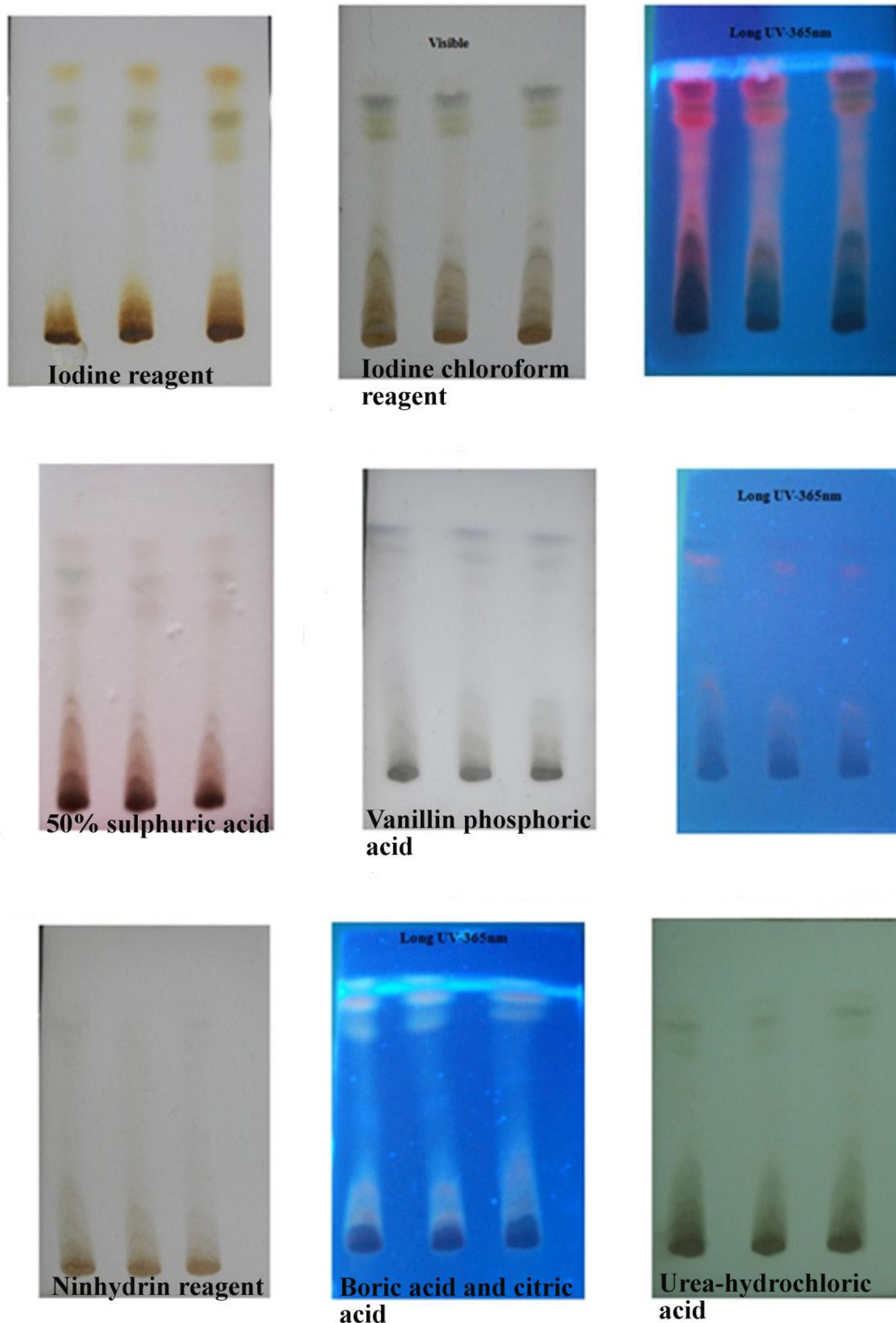


Figure 3.50: Preliminary screening of phytoconstituents using appropriate spraying reagent after developing TLC.

Table 3.7: Preliminary phytochemical screening of CHFE using various spraying reagents

S. No	Spraying reagent/ Test	Phytoconstituents	Detection in CHFE
1	Anisaldehyde sulphuric acid	Terpenoids, phenolic compounds	+
2	Dragendorff's	Alkaloids	+
3	Fast blue salt	Phenolic compounds	+
4	Iodine	Compounds having conjugated bonds	+
5	Potassium hydroxide	Anthraquinones	+
6	Con. H_2SO_4	Cardiac glycosides, lignanas	+
7	Vanillin Phosphoric acid	terpenoids	+
8	Vanillin Sulphuric acid	Essential oils (terpenoids)	+
9	Ethanolic aluminium chloride	Flavonoids	+
10	Ammonia vapours	Tetracyclins	-
11	Ninhydrin	Aminoacids	-
12	Boric acid and citric acid	Quinolines	-
13	Urea hydrochloric acid	Sugars	-
14	Iodine – chloroform	Ipecacuanha alkaloids	-

3.6.4 FT IR analysis

Fourier Transform infrared spectrophotometer–FTIR is the most powerful tool for identifying the types of chemical bonds that corresponds to the functional groups present in the compound. The FTIR spectrum of chloroform leaf extracts (CHFE) of *T. populnea* is shown in figure 3.51. The characteristic absorption band were exhibited at 2917 cm^{-1} for C-H stretching, 2341 cm^{-1} for C-H stretching, 1716 cm^{-1} for C=O carboxyl group and 1616 cm^{-1} for C=C group were exbibited in the CHFE.

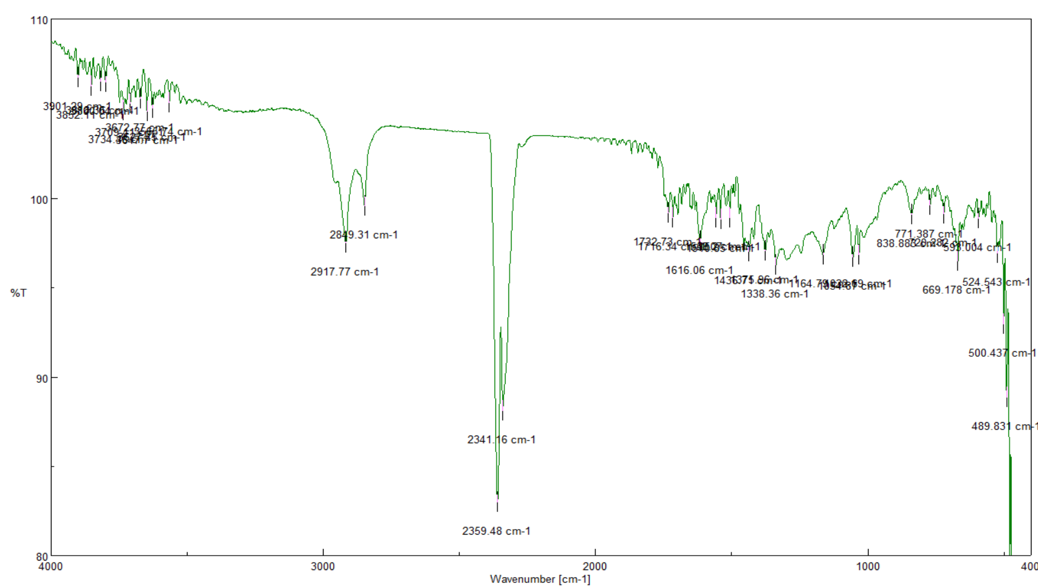


Figure 3.51: FT-IR spectrum of Chloroform leaf extract of *T. populnea*.

3.6.5 Column chromatography

To isolate the bioactive constituent present in the CHFE that contribute the antiproliferative property silica gel column chromatography was performed. The solvent combination that used for the separation of compounds was petroleum ether: ethyl acetate. The fractions were eluted accordingly using petroleum ether: ethyl acetate solvent mixture (100:0-0:100). The fractions were collected and checked for its cytotoxicity in K562 cells by MTT assay. The fraction collected in the combination 80:20 exhibited antiproliferative activity with an IC_{50} value of 69.36±2.15 μg/ml. The TLC chromatogram of active fraction showed in figure 3.52 and the graph representing the percentage of viability represented in Figure 3.53. The TLC profile of the active fraction showed the presence of variety of compounds. Further GC-MS analysis of this fraction was performed.

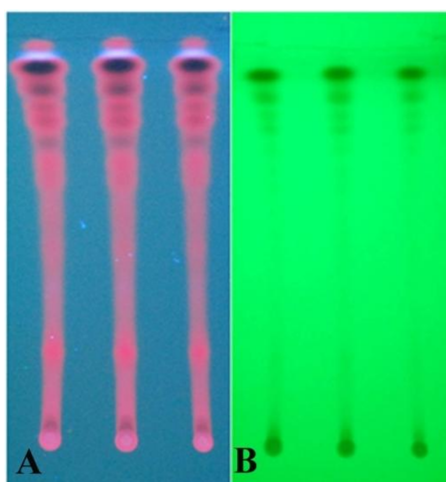


Figure 3.52: TLC chromatogram of column chromatographic fraction showing antiproliferative property A-under 365nm and B-254nm.

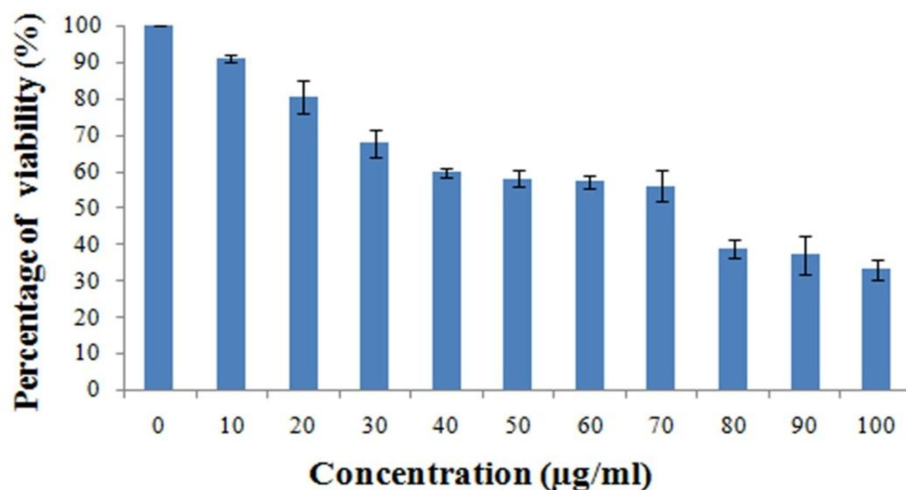


Figure 3.53: Graph representing the percentage of viability of column chromatographic fraction against K562 cell line and exhibited an IC_{50} value of 69.36±2.15µg/ml.

3.6.6 GC-MS analysis

The gas chromatograms of column chromatographic fraction of chloroform leaf extract of *T. populnea* which was showing antiproliferative activity confirmed the presence of various compounds with their retension time was shown in figure 3.54. The identification of the compounds was done through mass spectrometry attached with gas chromatography. The identified compounds, their retension time and peak area with more than 95% similarity was shown in table 3.8. The identification of the compounds is mainly based on the retension time and by comparison of the reported mass spectral fragmentation pattern in the literature and also based on MS library (NIST database NIST08 and 0.8L) by National Institute of standards and technology US). The compounds oleic acid and n-hexadecanoic acid was reported for antiproliferative property.

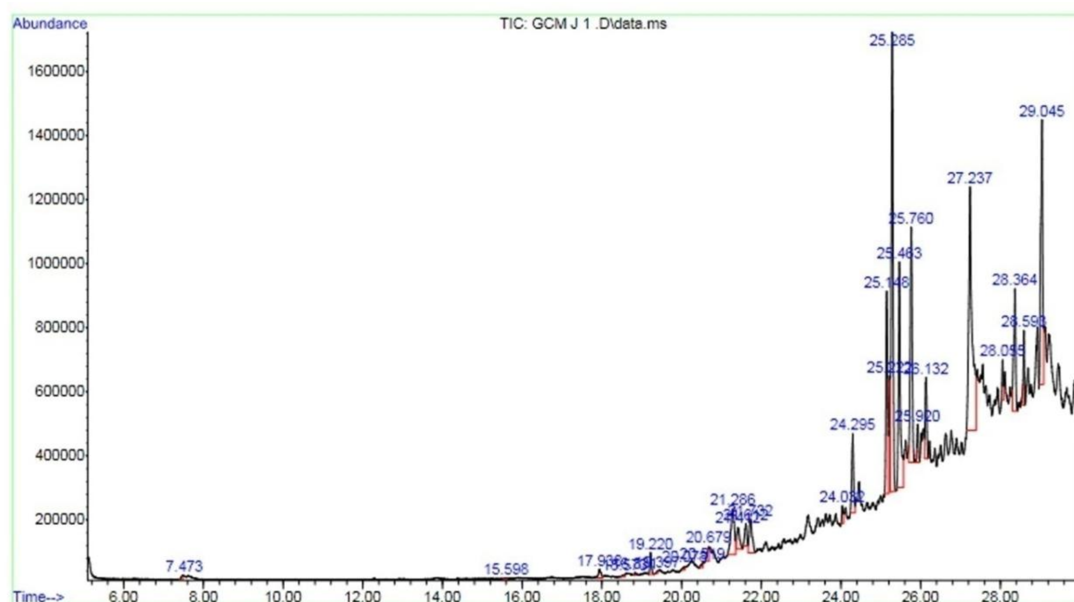


Figure 3.54: GC-MS chromatogram of column chromatographic fraction showing of CHFE.

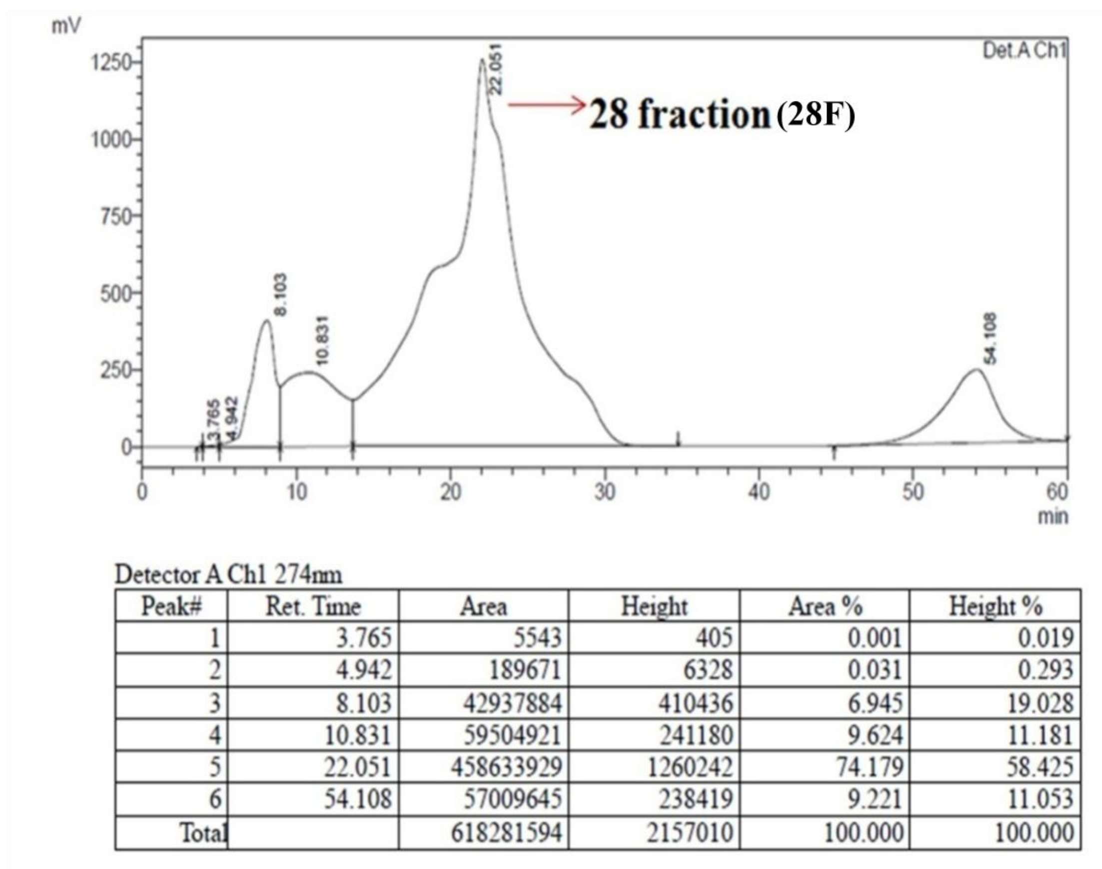
Table 3.8: Compounds identified in the choloform leaf extract of *T. populnea* by GC-MS analysis.

No	RT	Peak area (%)	Compound name	Mol weight (amu)
1	17.944	0.0767	Humulene	204.36
2	19.2258	0.259	2,4-Di-tert-butylphenol	206.32
3	20.7878	0.3481	2-Methyl-Z,Z-3,13-octadecadienol	280.45
4	21.2857	1.6681	9-octadecenal,(Z)-	266.469
5	24.038	0.2311	Linoelaidic acid	256.43
6	25.4684	4.2036	n-Hexadecanoic acid	268.438
7	26.5041	1.0383	Heptadecanolide	268.434
8	27.2365	9.4297	cis-Vaccenic acid	282.468
9	27.637	1.4622	Oleic acid	282.47
10	27.7286	1.6899	Trans-13-Octadecenoic acid	282.468
11	27.8774	0.3988	Cis-13-Octadecenoic acid	282.46
12	28.9302	2.8233	Squalene	410.718
13	29.222	3.9273	Cyclopropaneoctanal, 2-octyl-	280.49

3.6.7 Preparative HPLC

To isolate the active constituents attributing the cytotoxicity in chloroform leaf extract of *T. populnea*, preparative HPLC with gradient method was performed. The fractions were collected and checked for its antiproliferative ability. The peak which showed maximum intensity at retension time 22.051 detected at 274 nm exhibited cytotoxicity. It was named as 28F. Preparative HPLC chromatogram was shown in

figure 3.55. The 28F fraction was further analysed for its purity by analytical HPLC using 95% methanol as mobile phase. A prominent peak with maximum intensity at retension time 2.432 was detected at 274 nm illustrated in figure 3.56.



Detector A Ch1 274nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.765	5543	405	0.001	0.019
2	4.942	189671	6328	0.031	0.293
3	8.103	42937884	410436	6.945	19.028
4	10.831	59504921	241180	9.624	11.181
5	22.051	458633929	1260242	74.179	58.425
6	54.108	57009645	238419	9.221	11.053
Total		618281594	2157010	100.000	100.000

Figure 3.55: Preparative HPLC chromatogram of CHFE using Gradient method. The peak showed maximum intensity at retension time 22.051 detected at 274 nm, flow rate 8 ml/min.

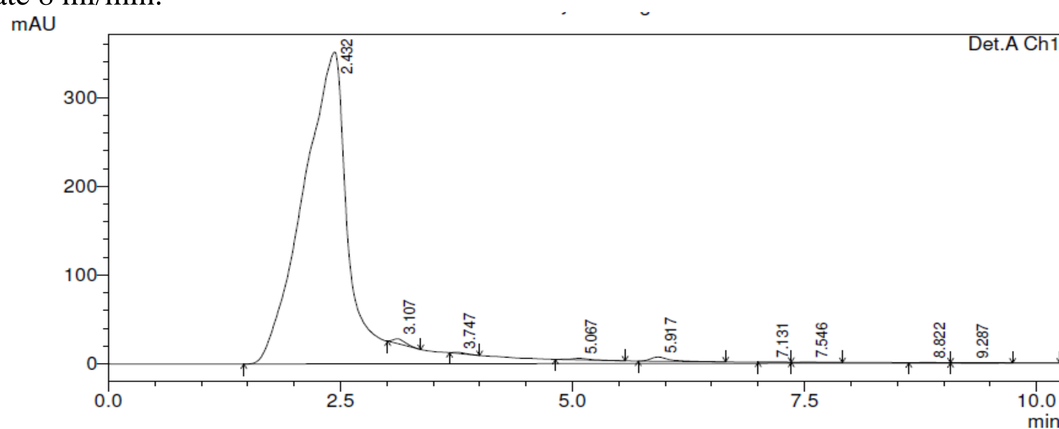


Figure 3.56: Analytical HPLC chromatogram of 28 fraction from preparative column by 95% methanol, 1ml/min flow rate. The peak intensity was maximum at 2.432 min detected at 274nm.

The antiproliferative ability of 28F was checked against K562 cell lines with different concentration ranging from 10-100 µg/ml for 48h. The 28F exhibited cytotoxicity in concentration dependent manner showed in figure 3.57. The IC_{50} concentration was found to be 64.26±3.24 µg/ml.

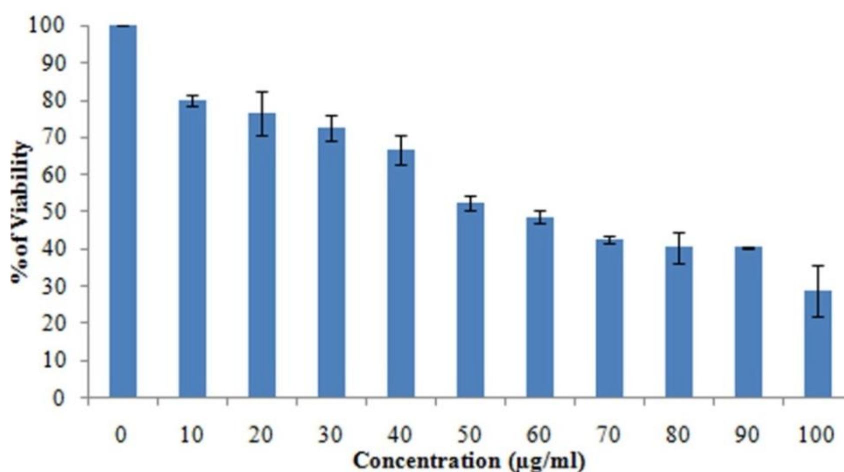


Figure 3.57: Graph representing the percentage of viability of 28F of preparative HPLC against K562 cell line showed an IC_{50} of 64.26±3.24 µg/ml.

The 28F of preparative HPLC was further separated using TLC. TLC separated 28F showed 4 separate regions namely 28F(1), 28F(2), 28F(3) and 28F(4) by using solvent combination of petroleum ether:ethyl acetate 6:2 shown in figure 3.58 A. Each separated region was checked for its antiproliferative potential, it was observed that only 28F(4) region exhibited a significant antiproliferative ability shown in figure 3.58 B.

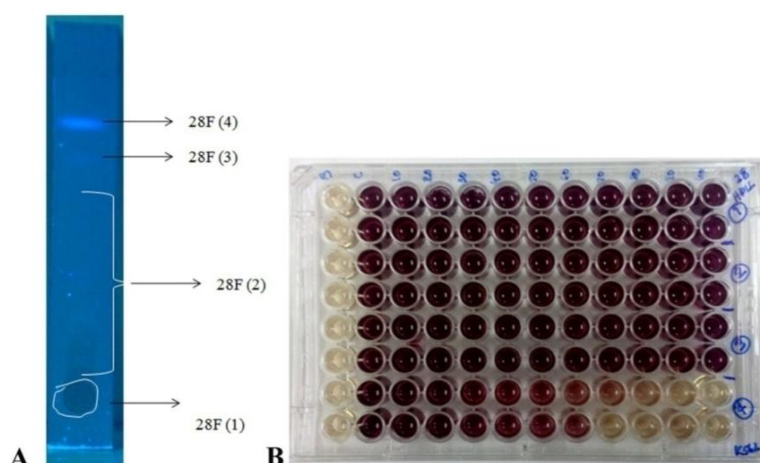
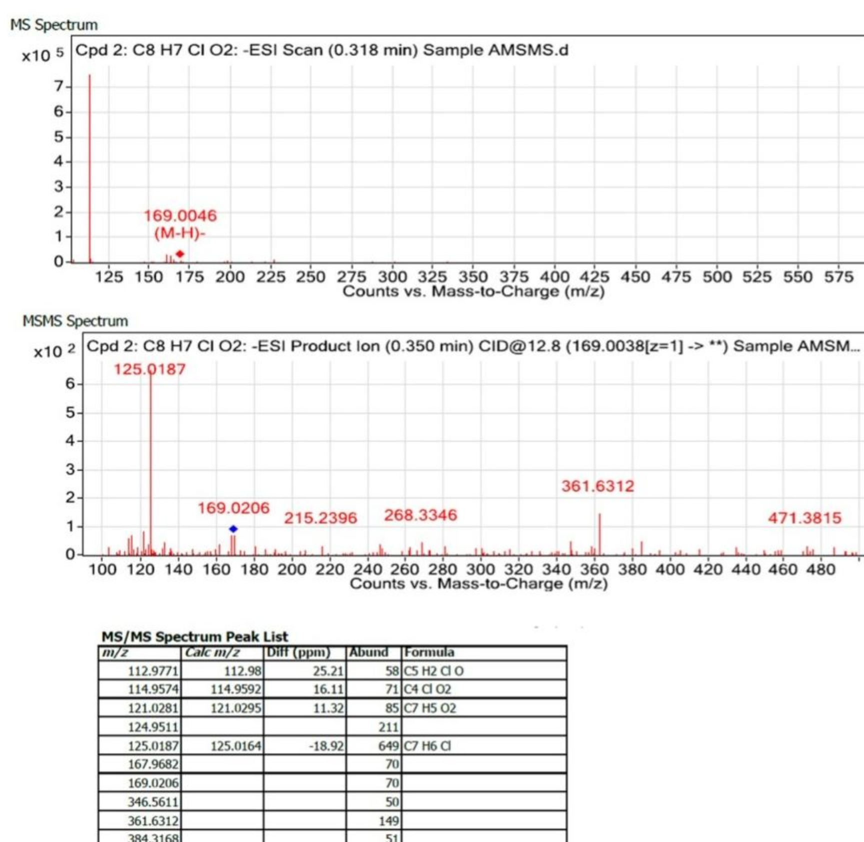


Figure 3.58: A- TLC profile of 28F and B- MTT picture of TLC isolated and separated regions of 28F

3.6.8 LC-MS analysis of TLC isolated 28 fraction (28F)

The TLC separated region of 28F, which exhibited the antiproliferative ability 28F(4) was further analysed for LC-MS to identify the molecular mass of the bioactive compound. The LC-MS spectrum is depicted in figure 3.59. The MS spectrum of 28F(4) showed similarity towards the MS spectrum of gallic acid or its derivative [293]. To evaluate the cytotoxic effect of gallic acid MTT assay was performed against K562 cells and compared with the antiproliferative effect of 28F(4) using concentration ranging from (10-100µg/ml) for 48h. The result was encouraging as it showed dose dependent response against K562 by both 28F (4) and gallic acid figure 3.60. The IC_{50} concentration was calculated and was found to be 38.47±1.033µg/ml for 28F(4) and for gallic acid found to be 32.8±1.2µg/ml.



MS/MS Spectrum Peak List

m/z	Calc m/z	Diff (ppm)	Abund	Formula
112.9771	112.98	25.21	58	C5 H2 Cl O
114.9574	114.9592	16.11	71	C4 Cl O2
121.0281	121.0295	11.32	85	C7 H5 O2
124.9511			211	
125.0187	125.0164	-18.92	649	C7 H6 Cl
167.9682			70	
169.0206			70	
346.5611			50	
361.6312			149	
384.3168			51	

Figure 3.59: LC-MS spectrum of 28F(4)

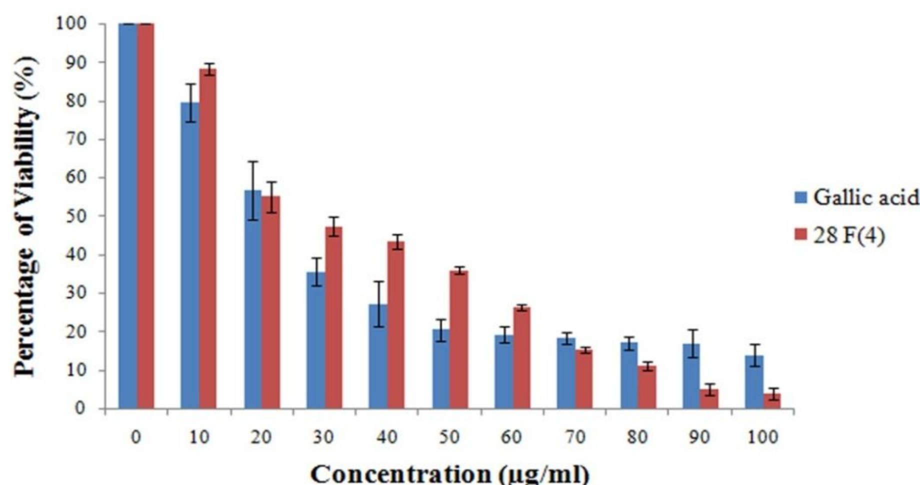


Figure 3.60: Graph representing the antiproliferative ability of gallic acid and 28F(4) against K562 cell line.

3.6.9 Combined effect of column chromatographic fraction and preparative HPLC fraction against K562 cell lines.

The bioassay guided isolation and characterisation of anticarcinogenic compounds from chloroform leaf extract was performed using chromatographic and spectroscopic techniques. It was observed that one fraction in the column chromatography exhibited antiproliferative ability against K562 cells and represented IC_{50} value of 69.36±2.15µg/ml. Similarly in the preparative HPLC isolated fraction 28F exhibited IC_{50} value 64.26±3.24 µg/ml. Both the fractions were mixed together and the antiproliferative ability was checked against K562 cells with different concentration ranging from 5-50 µg/ml for 48h. The result was so encouraging that the combined effect showed decrease in the IC_{50} value to 23.31±0.88 µg/ml which was comparable with the IC_{50} value of 25 µg/ml of CHFE. The graph representing the antiproliferative ability was shown in figure 3.61.

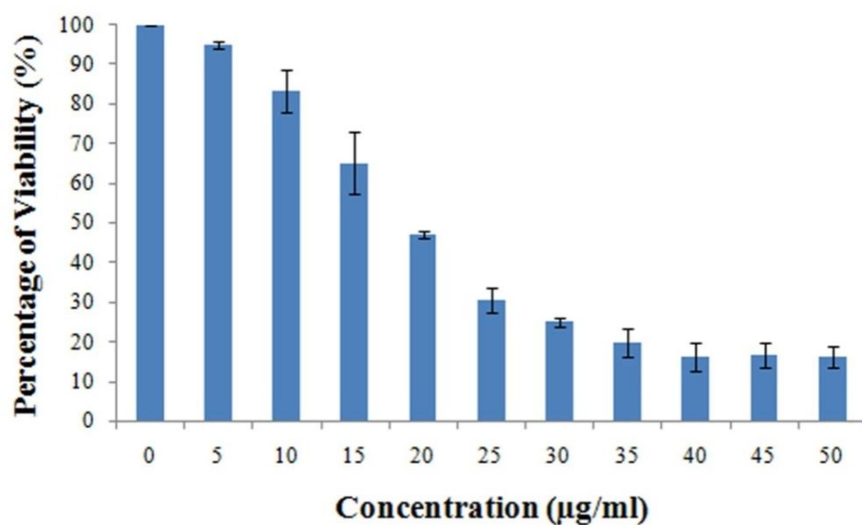


Figure 3.61: Graph representing the combined effect of column chromatographic fraction and preparative HPLC 28F exhibited IC_{50} value of 23.31±0.88 µg/ml.

Thus the study strongly supports the synergestic effect of the phytoconstituents towards its antiproliferative ability. The combined actions of the individual phytoconstituents contribute towards a better and enhanced cytotoxic activity.

MEGHA K. B. “THERAPEUTIC POTENTIAL OF MEDICINAL PLANT THESPESIA POPULNEA (L.) SOLAND EX CORREA WITH SPECIAL EMPHASIS TOWARDS ITS ANTIPROLIFERATIVE ACTIVITY AGAINST DIFFERENT CANCER CELL LINES.” THESIS. DEPARTMENT OF BIOTECHNOLOGY, UNIVERSITY OF CALICUT, 2018.

CHAPTER 4

DISCUSSION

Cancer is considered as multistage process leading to uncontrolled and abnormal growth of cells and becoming the leading cause of death worldwide. The unpredictable nature of the disease and lack of effective treatment methods made this disease distinct from other type of ailments. There are so many external and internal factors that contribute to promote and accelerate the disease progression. Plants have a long history in the use for cancer treatment due to their richness in the availability of variety of bioactive phytoconstituents owing diverse therapeutic potential. The search for the anticancer drugs started during 1950 and during this period plant derived anticancer compounds vinca alkaloids- vincristrine, vinblastine and podophyllotoxin were identified as anticancer agents. Many successful anticancer drugs currently in use or their analogues are plant derived and many more are under clinical trials. The search for finding novel and effective compounds of natural origin has put a major point of concern for research in the anticancer drug development. The World Health organisation report highlights the importance of traditional medicine as more than 80% of the world's population depending on it for their primary health requirements [230].

Thespesia populnea (L.) Soland ex Correa belongs to the family Malvaceae or commonly known as mallow. It is mainly distributed in the coastal regions of India and Pacific. This medicinal plant possesses different types of curative property and well known in traditional medicine. Majority of the plant parts like bark, leaves, flowers and fruits are useful in cutaneous infection such as scabies, psoriasis, eczema, ringworm and guineaworm [23]. The decoction of bark commonly used for the treating skin and liver diseases [24] and leaves are applied locally for their anti-inflammatory effects in swollen joints [25]. Gossypol, a major component in this plant reported to possess antifertility effects in rats as well as in human beings [31, 32]. The present study explored the presence of various types of phytochemicals, identified the therapeutic beneficial properties like antioxidant, antimicrobial and anti-inflammatory of the extracts were studied. The antiproliferatve and apoptogenic ability of the extract and its mechanism of action were reported. The isolation and characterisation of anticarcinogenic compounds were also studied.

The sample quality, purity and authentication are crucial measures for assessment of standards for the requirement of quality control of crude drug. Fluorescence analysis and behaviour of powdered drug with various chemicals/ reagents are rapid method to identify doubtful, adulterated or if the samples lack the physico-chemical properties [231]. The leaf powder of *T. populnea* is subjected for fluorescence analysis and their characteristic fluorescence is depicted in table 3.1. The phytochemical constituents present in leaf powder was also identified and showed the presence of alkaloids, terpenoids, glycosides, tannins, flavonoids and phenolics (table 3.2).

The extraction is the first and foremost crucial step in any kind of herbal preparation. There are so many methods for extraction and commonly used are soxhlation, maceration, decoction, percolation and sonication [232]. For the extraction of biologically active molecules various solvents like petroleum ether, chloroform, ethyl acetate, methanol, water etc or different combinations are commonly used [232]. For the study fresh leaves were collected, shade dried and coarsely powdered. The powdered leaves were sequentially extracted with increasing polarity of solvents. The dry weight of the extract, their appearances and yield percentage were noted (table 3.3). The methanol extract (MOHE) showed highest yield of 10.50% followed by chloroform extract (CHFE)-3.14%, petroleum ether extract (PEE)-1.790% and ethyl acetate extract (EAE)-0.51% respectively. The selection method used to isolate active components with highest yield and purity from natural sources mainly depends on the nature of the compounds and the raw materials used for the processing [233].

All the above mentioned extracts were subjected for qualitative phytochemical analysis. The presence of alkaloids, glycosides and tannins were commonly present in all extracts. Except in PEE, the presence of phenols, flavonoids, anthraquinone and quinines were observed. The presence of terpenoids and resins was noted only in CHFE and EAE. Fixed oil presence was detected in PEE, EAE and MOHE. Other constituents like coumarins in PEE, sterols in CHFE, phlobatannins and saponins in MOHE were also identified and represented in table 3.4. These bioactive constituents present in the extracts are known to exhibit medicinal as well as physiological activities [234]. Phenolic compounds are well known to possess biological properties such as anti-aging, anti-inflammatory, anti-atherosclerotic, cardiovascular protective and improve endothelial function as well as inhibition of angiogenesis and cell proliferation activities [235]. Medicinal plants rich in phenolic compounds are

reported to possess natural antioxidant activity [236, 237]. Tannins bind to proline rich protein and interfere with protein synthesis. Flavonoids possess antibacterial, effective antioxidant and anticancer activity [238]. Saponins are known to produce inhibitory effect on inflammation [239] and steroids are reported to have antibacterial properties [240]. Alkaloids have common biological properties like cytotoxicity, analgesic and antibacterial properties [238]. Glycoside has the ability to lower the blood pressure [241].

Free radicals are responsible for aging and cause various human diseases such as atherosclerosis, diabetes, cancer, hypertension, Alzheimer's disease, parkinsonism and cirrhosis [242]. Antioxidants deals with important role in the prevention and treatment of a variety of disease by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves [243]. The antioxidant activity of the leaf extracts of *T. populnea* was investigated against various *in vitro* models, since free radicals are of different chemical entities, it is essential to test the extract against many free radicals to prove its antioxidant activity. Free radicals are chemical species which contain one or more unpaired electrons. They are highly unstable and cause damage to other molecules by interacting electrons from them in order to attain stability. They are continuously formed inside one system and are highly reactive and potentially damaging transient chemical species. These unstable molecules/free radicals are produced in the human body as a part of chemical signaling, energy supply and immune function. Free radicals are regulated in one body by the endogenous antioxidant system but due to over production of free radicals by empower to environmental oxidant substances like pollution, cigarette, UV radiation etc or due to a failure in the antioxidant defence mechanism the risk increases for many diseases such as Alzheimer's disease, mild congestive impairment, Parkinson's disease, cardiovascular and liver disorders ulcerative colitis, inflammation and cancer [244].

Our body possesses defence mechanisms against oxidative stress which involves preventive and repair mechanisms. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPx) etc and non-enzymatic antioxidants such as carotenoids, ascorbic acid, phenolic compounds, flavonoids etc. These non enzymatic antioxidants act by one or more mechanisms like reducing activity, free radical scavenging potential, complexing of pro-oxidant metals

and quenching of singlet oxygen. It is better to reduce or prevent the risk of chronic diseases either by enhancing body's natural mechanism or by supplementing with proven antioxidants [245]. To analyse the antioxidant property of the extracts different methods were employed. The PEE lacks the antioxidant property and does not respond to any of the test methods. DPPH is a stable free radical and accepts electron or hydrogen radical to become a stable molecule [245]. This assay helps to identify the extracts having free radical scavenging ability. The extracts exhibited potent free radical scavenging ability in a concentration dependent manner (figure 3.1). The extracts CHFE, EAE and MOHE exhibited a promising DPPH free radical scavenging ability with IC_{50} values of 3.66±0.04 μg/ml, 4.44±0.17 μg/ml and 4.77±0.33 μg/ml respectively. The ascorbic acid exhibited IC_{50} value of 3.37±0.04 μg/ml which was comparable with CHFE. Nitric oxide being a potent pleiotrophic mediator in physiological processes and a diffusible free radical in the pathological conditions and reacts with superoxide anion and form a potentially cytotoxic molecule, the peroxy nitrite (ONOO-)'. Its protonated form, peroxynitrous acid (ONOOH) is a very strong oxidant and is responsible for oxidative damage of proteins [246]. Nitric oxide formed during their reduction with oxygen or with superoxide such as NO_2, N_2O_4, N_3O_4 is very reactive. These very reactive radicals have the capability to alter the structure and functions of many important cellular components. They are reported to be the reason for inflammation, cancer and other pathological conditions [247]. The CHFE of *T.populnea* are found to be an efficient scavenger of nitric oxide radicals in nitroprusside with an IC_{50} of 492.54±34.25 μg/ml. The CHFE showed a better activity in competing with oxygen to react with nitric oxide and inhibited the generation of anions than the standard ascorbic acid 635±74.64μg/ml (figure 3.2). The phytoconstituents present may have the capability to counteract the effect of nitric oxide formation and in turn can prevent the ill effects [248].

Iron is essential for life as it is required for oxygen transport, respiration and for the activity of many enzymes. Chelating agents inhibit lipid peroxidation by stabilising the transition metals [249]. Transition metals are considered as the catalysts for the initial formation of radicals. Chelating agents may stabilize transition metals in living systems and inhibit generation of radicals, consequently slows down free radical induced damage [249]. The chelating ability of the extracts was checked and it was

the EAE exhibited the property with an IC_{50} of 679.81±7.1 µg/ml and that of the standard is EDTA 1.27±0.006 µg/ml (figure 3.3). The chelation ability of the extract showed a dose dependent response. The iron (II) chelating activity of plant extracts is of great significance because the transition metal ion contributes to the oxidative damage in neurodegenerative disorders like Alzheimers and Parkinson's disease [250]. In the treatment of thalassemia and other anemias chelation therapy is the common practise of neutralising iron overload in the body. The current scenario suggests that the chelation therapy makes use of synthetic compounds which creates certain side effects [251]. Therefore chelation of metal ions by natural phytochemicals will be a breakthrough for therapeutics.

Fe (III) reduction is often considered as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action [252]. The reducing ability of a compound generally depends on the presence of reductones (antioxidants) which exert the antioxidant activity by breaking the free radical chain by donating hydrogen atom [253]. Figure 3.4 depicts the reductive capabilities of various leaf extracts of *T.populnea*. The extracts showed good reducing power ability in a dose dependent manner which was comparable with that of the standard except for petroleum ether extract. The reducing ability of the standard ascorbic acid was found to be higher than all the tested extracts. The active antioxidant principle present in the extracts of *T.populnea* caused the reduction of Fe^{3+}/ferricyanide complex to the ferrous form and thus proved the reducing power ability.

Total antioxidant capacity (TAC) assay by phosphomolybdenum method was based on the reduction of Mo(VI) to Mo(V) by the sample analyte and subsequent formation of a green phosphate/Mo(V) complex at acidic pH, usually detects antioxidants such as some phenolics, ascorbic acid, α-tocopherol and carotenoids [254]. The extracts except PEE showed high content of TAC with increase in concentration of extracts and was compared with standard ascorbic acid (figure 3.5). This could be due to the high content of total phenolics in these extracts. Due to the redox properties of phenolic compounds, they can play an important role in absorbing and neutralising free radicals, quenching singlet and triplet oxygen or decomposing peroxides [255]. Several reports have conclusively shown a strong connection between the antioxidant activity and amount of total phenolics and flavonoids present in them [256, 257]. It is also mentioned that the antioxidant capacity of the plant extracts is not limited to

phenolics or flavonoids may also due to the presence of other antioxidant constituents like carotenoids, vitamins etc [256, 257, 258]. The antioxidant activity of phenolic acids is generally governed by their chemical structures; the activity improves as the number of hydroxyl (OH) and methoxy groups increases, and the number of OH groups is more important. The phenolic compounds from plants are active antioxidants owing to their redox potential and particular chemical structure. They have a significant role in reducing the effects of free radicals, chelating transitional metals and quenching singlet and triplet oxygen [259]. The potential antioxidant activity of these phenolic compounds is demonstrated in this study due to their ability to scavenge the various radicals. So it is therefore necessary to determine the total amount of phenols present in the extracts. The contents of total phenols were estimated and expressed as gallic acid equivalents (figure 3.6). The presence of total phenolic content in the extracts can be represented in the increasing order as EAE>CHFE>MOHE. Phenolic compounds are known powerful chain breaking antioxidants (260). Phenolic acids are one of the main phenolic classes and can be obtained in the form of esters, glycosides or amides, but rarely in free form. Within this group, flavonoids are one of the most common phenolics widely distributed in plants [261]. In this study, total flavonoid content were estimated and expressed as quercetin equivalents. It was noted that chloroform extract showed the highest content of flavonoids 361 mg quercetin equivalent/g of extract followed by ethyl acetate extract 188 mg quercetin equivalent/g of extract (figure 3.7). Several reports have shown that there is a strong correlation between the antioxidant activity and amount of total phenolics and flavonoids [262, 263].

The efficacy of the current antimicrobial agents has been reduced due to the continuous development of resistant organisms to the commonly used antimicrobial agents. So the search for new drugs from plants is becoming a source to explore, therapeutically potent and effective antimicrobial agents. The screening of plant extracts and their products for antimicrobial activity has shown higher plants representing a potential source of novel antibiotic prototypes [264]. The selection of crude plant extracts for screening programs is potentially more successful in initial steps than the pure compounds [265]. Medicinal plants are an important source for the development of potential, new antibacterial drugs. Hence an attempt has been made to identify the antimicrobial activity of leaf extracts of *T.populnea* against five clinically

important microbes including both bacteria and fungi. The strains used for the study were *Salmonella typhi*, *Klebsiella pneumoniae*, *Fusarium oxysporum*, *Aspergillus niger* and *Candida albicans* using microdilution method. Broth dilution has been standardised by CLSI for testing bacteria that grow aerobically [266], yeast [267] and filamentous fungi [268]. The antimicrobial potential of the extracts were expressed as percentage of inhibition (%) and illustrated in figure 3.8. All the extracts of *T.populnea* exhibited antimicrobial ability. The MIC_{50} and MIC_{90} were calculated and shown in table 3.5. It was found that PEE was showing more antimicrobial potency than other extracts. Previous studies by earlier workers reported that the compounds isolated from the dark heartwood and wood of *T.populnea* showed antibacterial properties against *Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecalis*. None of the compounds were active against *Salmonella typhi*, *Salmonella sonei* and *Pseudomonas aeruginosa* [269]. In the present study, the leaf extracts exhibited a potent antimicrobial property. This may be due to the presence of secondary metabolites such as phenol [270], essential oil [271, 272], terpenoids [273, 274], alkaloids [275] and flavonoids [276] which was reported to have antimicrobial property.

Inflammation is a very common symptom of many chronic diseases. It is a very normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents [277]. Denaturation of protein is a well documented cause of inflammation and rheumatoid arthritis. Several anti-inflammatory drugs indomethacin, ibufenac, diclofenac sodium, salicylic acid and flufenamic acid [277] inhibit thermally induced protein denaturation. Non steroidal anti-inflammatory drugs commonly used for inflammatory conditions are associated with many types of unwanted side effects such as gastric irritation, ulcers etc [278]. Among several traditional claims, the usefulness of *Thespesia populnea* bark in inflammation and pain has been emphasised in many literatures [279,280]. The anti-inflammatory activity of the ethanolic extract of *T. populnea* bark has been established in both acute and chronic inflammation models [282]. The anti-inflammatory property of the extracts was done by using simple and viable protein denaturation bioassay method. The chloroform leaf extract exhibited the ability to inhibit protein denaturation in a concentration dependent manner with denaturation inhibition concentration (DIC_{50}) of 501.93±19.76 μg/ml and 175.91±9.37 μg/ml for diclofenac sodium (figure 3.9).

The antiproliferative effects of the leaf extracts were tested in 10 different cell lines i.e. Jurkat E6.1, HL-60, K-562, PC-3, HeLa, MDA-MB-231, A549, HCT116, L929 and Vero cell lines. The prominent antiproliferative activity was exhibited only by the chloroform leaf extract and the concentration used for the study was (10-100) µg/ml. The leukemic cell lines Jurkat E6.1, HL-60 and K-562 exhibited IC_{50} values of 35.72±0.95, 25.80±1.13 and 24.93±2.63 µg/ml respectively (figure 3.10). The adenocarcinoma cell lines PC3, HeLa and MDA-MB-231 exhibited an IC_{50} value of 60.79±1.84, 46.94±0.83 and 72.16±0.56 µg/ml respectively (figure 3.11). The two carcinoma cells used for study were A-549 and HCT 116 responded after treatment with an IC_{50} value of 55.69±2.18 and 37.22±4.04 µg/ml respectively (figure 3.12). The cell lines of animal origin were also used in the study. The L929 cell line with chloroform extract exhibited an IC_{50} value of 60.88±1.45 µg/ml and Vero cell line after 48h treatment exhibited an IC_{50} value of 83.48±2.05 µg/ml (figure 3.13). The IC_{50} values were shown in table 3.6. The IC_{50} concentration varies with different cell lines. In addition to MTT assay another antiproliferative assay SRB were also performed with PC-3, MDA-MB-231, A549 and HCT116. It estimates the total protein content present in the cells. The IC_{50} values were calculated was found to 50.16±0.92, 78.62±2.97, 142.51±13.2 and 37.81±3.74 µg/ml respectively (figure 3.14 and 3.15). Among the 10 different cell lines used to check the antiproliferative property, K-562 and HCT116 cells were more sensitive towards CHFE exhibiting low IC_{50} values. These cell lines were used for further analysis to check the apoptogenic ability of the CHFE. The morphological changes after treatment with different time periods was monitored in K562 cell lines. The treatment time period was 3, 6, 12, 24 and 48h. It was noticed that cells were shrunk into smaller size and their morphology got completely distorted as the treatment time and concentration increases (figure 3.16). The antiproliferative ability at different time periods and concentration by MTT assay were also performed. As the treatment dose and time increases it is observed that IC_{50} concentration decreased. The IC_{50} value of CHFE against K-562 at different time intervals were 51.09±2.34 µg/ml at 3 hour, 39.58±1.52 µg/ml at 6 hour, 35.18±1.53 µg/ml at 12 hour, 25.29±0.65 µg/ml and 24.93±2.63 µg/ml at 24 hour and 48 hour respectively (figure 3.17 and 3.18). The antiproliferative ability of CHFE against HCT 116 cells was also performed. It was observed that the cells morphology got altered and lost their anchorage capacity at higher concentration (figure 3.31) after treatment with 10, 37 and 50 µg/ml CHFE. The antiproliferative ability of CHFE was

checked against HCT116 cell line by MTT and SRB assay. In MTT assay the IC_{50} concentration was found to be 37.22±4.04 µg/ml and for SRB assay displayed a similar value of 37.81±3.74 µg/ml (figure 3.33). In K-562 cells the IC_{50} concentration after cisplatin treatment was found to be 0.36±0.11 µg/ml after 48h treatment and in the case of HCT 116 cells, after 24h treatment they exhibited an IC_{50} concentration of 4.2 µg/ml [295]. Cheng *et al* reported a study using different cell line showing a concentration dependent cell viability pattern for *B. scrozonerifolium* which was similar to that of CHFE in both K562 and HCT116 cells [283].

For the further studies the concentration used in K-562 cell lines were 10, 25 and 50 µg/ml and in HCT116 cells were 10, 37 and 50 µg/ml for 48h. The morphological changes during apoptosis induced by CHFE against K562 and HCT116 cells were performed using scanning electron microscopy. During the later stage of apoptosis morphological changes include membrane blebbing, ultrastructural modification of cytoplasmic organelles and loss of membrane integrity**.** Apoptosis involves the specific morphological and biochemical changes such as chromatin condensation, membrane blebbing, cell shrinkage, DNA fragmentation [284, 285]. In scanning electron microscopic evaluation after 48h treatment the K-562 control cells were intact with normal morphology, 10 and 25 µg/ml treated groups plasma membrane blebbing were observed and in the higher concentration treated group plasma membrane blebbing and cell shrinkage were prominent (figure 3.21) . Similar pattern of morphological changes were observed in HCT116 cells also (figure 3.32). For the assessment of apoptosis, acridine orange/ethidium bromide double staining method were employed at the IC_{50} concentration in both cell line exhibited typical early apoptotic cell features as they are stained green to orange with highly condensed or fragmented chromatin and apoptotic bodies. The late apoptotic and necrotic cells appeared to orange to red color with condensed and fragmented chromatin represented in figure 3.19 and 3.34. Nuclear Hoechst staining was also performed to evaluate apoptosis by morphological examination. As shown in figure 3.20 and 3.35, condensed chromatin and fragmented punctuate blue nuclear fluorescence observed in a concentration dependent manner. Propidium iodide staining and cell cycle analysis were carried out in both K-562 and HCT116 cells. The morphological changes after staining with propidium iodide is shown in figure 3.23 and 3.37. There is a general trend in the nuclear damage with increase in concentration of CHFE. The degree of

apoptosis was determined by cell cycle analysis by flow cytometry. There is evidence that the apoptotic death induced by chemopreventive or chemotherapeutic agents is closely connected to specific phase of cell cycle [286]. There is a dose dependent increase in the sub-G_0 peak which directly connects to the apoptotic inducing ability of CHFE.The cell cycle distribution is illustrated in figure 3.24 and 3.25 for K-562 cells and figure 3.38 and 3.39 for HCT116 cell. Phosphatidylserine (PS) externalisation during apoptosis is a specific phenomenon only observed in apoptotic cells. Annexin V was found to bind specifically to PS, located at the outer membrane leaflet of cells in the presence of calcium, while necrotic cells have damaged membranes and takes up PI. Annexin V-FITC and PI staining was done in K-562 and HCT116 cells after treatment observed that early and late stages of apoptosis was clearly visualised in the treated groups as shown in figure 3.26 and 3.40. The Annexin V-FITC flow cytometric assay showed there was a significant dose-dependent increase in early stage of apoptosis when K-562 cells exposure to CHFE for 48h as shown in figure 3.27 and 3.28. Evidences suggest that the disruptions of mitochondrial functions are crucial in apoptotic cell deaths [283]. Rhodamine 123 accumulates as aggregates in polarised mitochondrial membrane. It was observed that there was an increase in fluorescence due to decrease in mitochondrial membrane potential in both K-562 and HCT116 cells after treatment with CHFE for 48h (figure 3.29 and 3.36). DNA fragmentation assay was performed as it is most important phenomenon in apoptosis. The K-562 and HCT116 showed a well distinct ladder pattern formation at IC_{50} and higher concentration. The intensity of ladder increased with increase in concentration of drug treatment as shown in figure 3.22 and 3.41. For understanding the molecular mechanism of action induced by CHFE in K-562 and HCT116 western blot analysis was performed. Proteolytic cleavage of caspase -9 and -3 and PARP expression was observed in CHFE treated K-562 cells (figure 3.30). PARP cleavage was observed in HCT116 after 48h CHFE treatment (figure 3.44). It is very well known that caspases play a critical role in initiation and execution of apoptosis [287]. Metastasis is the major cause of death due to the movement of cancer cells to other regions of the body [288]. The metastasis inhibition capacity of CHFE was checked against HCT 116 using *in vitro* scratch assay or wound healing assay. It was observed that after 24h, the cells started migrating towards the scratched and proved that CHFE possess the ability to inhibit metastasis and shown in figure 3.43. The clonogenic assay helps to analyse the capacity of a single cell to form colonies after treatment

with cytotoxic drug. The result showed that increasing concentration of drug reduced the colony forming ability as mentioned in (figure 3.42). Therefore all the above mentioned studies support the *in vitro* antiproliferative and apoptogenic ability of CHFE.

To support the *in vitro* antiproliferative property of CHFE, *in vivo* study of leaf extract against Ehrlich Ascites carcinoma induced swiss albino mouse was conducted. Acute toxicity study (OECD 420) fixed dose procedure was carried out for chloroform leaf extract of *T. populnea*. It was observed that there was no sign of toxicity and noticeable behavioural changes in treated animals. Further study was carried out to check the mean survival rate of CHFE on tumor induced mice. The dose 100 and 200 mg/kg body weight drug administered group improved the mean survival time than tumor bearing mice (figure 3.45). The prolongation of life span is an important criterion for judging the efficacy of anticancer drugs [289].

The chloroform extract showed a very prominent antiproliferative property towards different cell lines. To isolate the active principle that contributes towards its anticarcinogenecity was checked using different methods. Chromatographic and spectroscopic techniques are employed for the isolation and characterisation of the bioactive molecules by MTT assay. The major techniques used were thin layer chromatography, HPTLC, column chromatography, HPLC, preparative HPLC, GC-MS, FTIR and LC-MS analysis. TLC was employed to separate the major compounds based on their polarity. The solvent system petroleum ether: ethyl acetate (6:4) showed maximium separation in both short and long ultraviolet light and illustrated in figure 3.46. Further HPTLC was also done to identify the maximum separated compounds in CHFE. The chromatogram shows a maximum separation (figure 3.47 and 3.48) and was similar to the preparative TLC pattern. Further qualitative preliminary phytochemical identification was done using various spraying reagents. The spraying reagents used are highly specific for the characteristic class of phytochemicals. The developed TLC plates showing the corresponding characteristic secondary metabolites are illustrated in figure 3.49 and 3.50. The phytoconstituents detected in the CHFE are tabulated in Table 3.7. The preliminary screening showed the presence of alkaloids, terpenoids, phenolics, flavonoids, anthraquinones, lignans to the specific spraying reagents. FTIR analysis of the CHFE was performed to identify the major functional groups present in them (figure 3.51). Identification of the

chemical nature of phytochemical compounds present in the medicinal plants will provide some information on the different functional groups responsible for their medicinal properties [290].

To isolate the bioactive compounds present in CHFE, column chromatography is used. Column chromatography is a common technique to isolate the compounds from the plant extracts. The stationary phase the silica gel was used and the mobile phase solvents of increasing polarity were employed. The mixture of Petroleum ether: ethyl acetate (100:0 and 0:100) was used as mobile phase. The fraction collected in the combination 80:20 showed prominent antiproliferatve ability against K-562 cells and showed in figure 3.53 and figure 3.52 shows the TLC profile of the active fraction. The active column chromatographic fraction exhibited an IC_{50} value of 69.36±2.15 µg/ml. For further characterisation of the active compounds present, GC-MS analysis were performed (figure 3.54 and table 3.8). In GC-MS analysis a variety of volatile compounds were identified. The compounds identified in the GC-MS spectrum showed the presence of oleic acid [291] and hexadeconoic acid [292] are reported to have antiproliferative ability. So the antiproliferative ability of the active column chromatographic fraction may be due to presence of this compound.

The next technique emoployed for the isolation of active compounds is preparative HPLC (figure 3.54). Fractions collected were checked for its antiproliferative ability. The fraction collected containing the major peak named as 28 F exhibited the antiproliferative activity against K562 cells. The 28F exhibited cytotoxicity in concentration dependent manner showed in figure 3.57. The IC_{50} concentration was found to be 64.26±3.24 µg/ml. The 28F fraction was further subjected to TLC and four separated regions were again checked for its antiproliferative ability (figure 3.58). It was found that the region 28F(4) showed prominent antiproliferative ability. 28F(4) was analysed for LC-MS to identify molecular mass of the active compound. The mass was found to be 169 and the MS spectrum of the 28F(4) showed (figure 3.59) similarity towards the MS spectrum of gallic acid or its derivative [293]. To evaluate the cytotoxic ability of gallic acid MTT assay were performed. The IC_{50} concentration was calculated and was found to be 38.47±1.033µg/ml for 28F(4) and for gallic acid found to be 32.8±1.2µg/ml (figure 3.60).

The bioassay guided isolation and characterisation of anticarcinogenic compounds from chloroform leaf extract resulted in the isolation of active compounds in two

fractions. The column chromatographic column fraction exhibited antiproliferative ability against K562 cells and represented IC_{50} value of 69.36±2.15 µg/ml. Similarly in the preparative HPLC isolated fraction 28F exhibited IC_{50} value 64.26±3.24 µg/ml. Both the fractions were mixed together and the antiproliferative ability was checked against K562 cells with different concentration ranging from 5-50 µg/ml for 48h. The result showed a decrease in the decrease in the IC_{50} value to 23.31±0.88 µg/ml which was comparable with the IC_{50} value of 25 µg/ml of CHFE. The graph representing the antiproliferative ability was shown in figure 3.61. Synergestic interactions are documented for constituents within a total extract of a sinlge herb as well as between different herbs. The concept that a whole or partial purified extract of a herb offers advantages over a single isolated ingredient [294]. Thus it is proved that the antiproliferative ability increases with the combined action of fractions rather than its isolated form.

The present study identified a variety of phytochemicals present in the leaf extracts of *T.populnea.* The extracts exhibited some biologically important activities like antioxidant, antimicrobial, anti-inflammatory and antiproliferative ability. The chloroform leaf exhibited antiproliferative and apoptogenic ability. The apoptogenic ability of the extract was well studied using different techniques and confirmed the ability of the extract to induce apoptosis by mitochondria mediated intrinsic pathway. Further work was carried out to isolate active compounds that contribute antiproliferative ability. But it was able to prove that the phytoconstituents present in the extract works synergistically to give better antiproliferative ability.

SUMMARY AND CONCLUSION

1. *Thespesia populnea* (L.) Soland ex Correa leaves were dried and sequentially extracted with increasing polarity of solvents. The four extracts petroleum ether (PEE), Chloroform (CHFE), Ethyl acetate (EAE) and Methanol (MOHE) were prepared. Before extract preparation, the leaf powder was subjected for its fluorescence analysis and phytochemical constituents were detected. It showed the presence of alkaloids, terpenoids, glycosides, tannins, flavonoids and phenolics.
2. All the four extracts were subjected for its qualitative phytochemical analysis and the alkaloids, glycosides and tannins are commonly present in all the four solvent extracts. The presence of phenols, flavonoids, anthraquinones and quinines were observed in chloroform, ethyl acetate and methanol extracts. Terpenoids and resins presence was noted only in chloroform and ethyl acetate extracts. Fixed oil occurrence is seen in three extracts except for chloroform extract. The presence of coumarins in petroleum ether extract, sterols in chloroform, phlobatannins and saponins in methanol extracts were also noted.
3. The extracts were studied for its antioxidant property. Except the PEE showed very prominent antioxidant ability for the tested methods. In DPPH radical scavenging ability test the extracts exhibited potent radical scavenging activity in concentration dependent manner. The CHFE showed a good nitric oxide radical scavenging activity with an IC_{50} of 492.54±34.25 µg/ml and the scavenging ability was higher while comparing with that of the standard ascorbic acid i.e., 635.78±74.64 µg/ml. The chelating effect was shown by EAE and exhibited an IC_{50} concentration of scavenging ability at 679.81±71 µg/ml. The reducing ability and total antioxidant capacity was exhibited by the extracts in a concentration dependent manner. The total phenolic content of CHFE, EAE and MOHE were found to be 245±0.01, 254±0.01 and 152±0.01 mg gallic acid equivalent per gram of the extract respectively. In CHFE and EAE the total flavonoid content was found to be 361±1.26 and 188±0.67 mg of quercetin equivalent/g of the extract respectively. Thus the extracts exhibited a significant antioxidant potential.
4. The antimicrobial capability of the extracts was tested against different microbial species like *Salmonella typhi* (MTCC 734), *Klebsiella pneumoniae*

(MTCC 109), *Fusarium oxysporum* (MTCC 284), *Aspergillus niger* (MTCC 282) and *Candida albicans* (MTCC 227) using microdilution method. The anti microbial potential of the extracts were expressed as percentage of inhibition (%). The MIC_{50} and MIC_{90} values of the leaf extracts were calculated and found that PEE showed a significant response against the microbial strains used for the study.

5. The anti-inflammatory ability of the extracts was tested using BSA anti-denaturation assay. The denaturation inhibition concentration-DIC_{50} value was determined and was found to be 501.93±19.76 µg/ml for CHFE and 175.91±9.37 µg/ml for diclofenac sodium.
6. The extracts were tested for its antiproliferative ability and were tested in 10 different types of cell lines. Their IC_{50} values were determined and were found to be 35.72±0.95, 25.80±1.13 and 24.93±2.63 µg/ml for Jurkat E6.1, HL-60 and K562 respectively. The PC-3, HeLa and MDA-MB-231 exhibited an IC_{50} value of 60.79±1.84, 46.94±0.83 and 72.16±0.56 µg/ml. A549 and HCT116 responded after treatment with an IC_{50} value of 55.69±2.18 and 37.22±4.04 µg/ml respectively. L929 and Vero cell line with chloroform extract exhibited an IC_{50} value of 60.88±1.45 µg/ml and 83.48±2.05 µg/ml. The SRB assay revealed a significant antiproliferative effect for CHFE on PC-3, MDA-MB-231, A549 and HCT116. Their IC_{50} values are 50.16±0.92, 78.62±2.97, 142.51±13.2 and 37.81±3.74 µg/ml respectively.
7. For the further studies K-562 and HCT116 cells were used as they were more responsive to the CHFE. The morphological changes after treatment with different time periods was monitored in K-562 cell lines. The treatment time period was 3, 6, 12, 24 and 48h. It was noticed that cells were shrunk into smaller size and their morphology got completely distorted as the treatment time and concentration increases. As the treatment dose and time increases it is observed that IC_{50} concentration decreased. The IC_{50} value of CHFE against K-562 at different time intervals were 51.09±2.34 µg/ml at 3 hour, 39.58±1.52 µg/ml at 6 hour, 35.18±1.53 µg/ml at 12 hour, 25.29±0.65 µg/ml and 24.93±2.63 µg/ml at 24 hour and 48 hour respectively.
8. The antiproliferative ability of CHFE against HCT 116 cells was also performed. It was observed that the cells morphology got altered and lost their anchorage capacity at higher concentration (10, 37, 50 µg/ml). The

antiproliferative ability of CHFE was checked against HCT116 cell line by MTT and SRB assay. In MTT assay the IC_{50} concentration was found to be 37.22±4.04 μg/ml and for SRB assay displayed a similar value of 37.81±3.74 μg/ml.

9. The morphological changes during apoptosis induced by CHFE against K-562 and HCT116 cells were performed using scanning electron microscopy. K-562 control cells were intact with normal morphology, 10 and 25 μg/ml treated groups plasma membrane blebbing were observed and in the higher concentration treated group plasma membrane blebbing and cell shrinkage were prominent. Similar pattern of morphological changes were observed in HCT116 cells also. For the assessment of apoptosis, acridine orange/ethidium bromide double staining method were employed at the IC_{50} concentration in both cell line exhibited typical early apoptotic cell features as they are stained green to orange with highly condensed or fragmented chromatin and apoptotic bodies. The late apoptotic and necrotic cells appeared to orange to red color with condensed and fragmented chromatin represented. Nuclear Hoechst staining was also performed to evaluate apoptosis by morphological examination. In both cells, condensed chromatin and fragmented punctuate blue nuclear fluorescence observed in a concentration dependent manner.
10. Propidium iodide staining and cell cycle analysis were carried out in both K-562 and HCT116 cells. The morphological changes after staining with propidium iodide showed a general trend in the nuclear damage with increase in concentration of CHFE. The degree of apoptosis was determined by cell cycle analysis by flow cytometry. There is a dose dependent increase in the sub-G_0 peak which directly connects to the apoptotic inducing ability of CHFE.
11. Phosphatidylserine (PS) externalisation during apoptosis is a specific phenomenon only observed in apoptotic cells. The Annexin V-FITC flow cytometric assay showed there was a significant dose-dependent increase in early stage of apoptosis when K-562 cells exposure to CHFE for 48h. Rhodamine 123 accumulates as aggregates in polarised mitochondrial membrane. It was observed that there was an increase in fluorescence due to decrease in mitochondrial membrane potential in both K-562 and HCT116 cells after treatment with CHFE for 48h.

12. DNA fragmentation assay was performed as it is most important phenomenon in apoptosis. The K-562 and HCT116 showed a well distinct ladder pattern formation at IC_{50} and higher concentration. Western blotting was performed and proteolytic cleavage of caspase -9 and -3 and PARP expression was observed in CHFE treated K-562 cells. PARP cleavage was observed in HCT116 after 48h CHFE treatment.
13. The metastasis inhibition capacity of CHFE was checked against HCT 116 using *in vitro* scratch assay. It was observed that after 24h, the cells started migrating towards the scratched and proved that CHFE possess the ability to inhibit metastasis. The clonogenic assay was also performed and the result showed that increasing concentration of drug reduced the colony forming ability.
14. *In vivo* study of leaf extract against Ehrlich Ascites carcinoma induced swiss albino mouse was conducted. Acute toxicity study (OECD 420) fixed dose procedure was carried out for chloroform leaf extract of *T. populnea*. It was observed that there was no sign of toxicity and behavioural changes after drug administration. The dose 100 and 200 mg/kg body weight drug administered group improved the mean survival time than tumor bearing mice.
15. Chromatographic and spectroscopic techniques are employed for the isolation and characterisation of the bioactive molecules by MTT assay. The major techniques used were thin layer chromatography, HPTLC, column chromatography, HPLC, preparative HPLC, GC-MS, FTIR and LC-MS analysis. TLC and HPTLC were employed to separate the major compounds based on their polarity. The solvent system petroleum ether: ethyl acetate (6:4) showed maximium separation in both short and long ultraviolet light. Further qualitative preliminary phytochemical identification was done using various spraying reagents. The preliminary screening showed the presence of alkaloids, terpenoids, phenolics, flavonoids, anthraquinones, lignans to the specific spraying reagents. FTIR analysis of the CHFE was performed to identify the major functional groups present in them.
16. To isolate the bioactive compounds present in CHFE, column chromatography were performed using the mixture of Petroleum ether: ethyl acetate (100:0 and 0:100) was used as mobile phase and silica gel mesh size 60-120 as stationary phase. The fraction collected in the combination 80:20 showed prominent

antiproliferative ability against K-562 cells. The active column chromatographic fraction exhibited an IC_{50} value of 69.36±2.15 μg/ml. For further characterisation of the active compounds present, GC-MS analysis were performed. In GC-MS analysis a variety of volatile compounds were identified. The compounds identified in the GC-MS spectrum showed the presence of oleic acid and hexadeconoic acid are reported to have antiproliferative ability.

17. The next technique emoployed for the isolation of active compounds is preparative HPLC. Fractions collected were checked for its antiproliferative ability. The fraction collected containing the major peak named as 28F exhibited the antiproliferative activity against K562 cells. The IC_{50} concentration was found to be 64.26±3.24 μg/ml. The 28F fraction was further subjected to TLC and four separated regions were again checked for its antiproliferative ability. It was found that the region 28F(4) showed prominent antiproliferative ability. 28F(4) was analysed for LC-MS to identify molecular mass of the active compound. The mass was found to be 169 and the MS spectrum of the 28F(4) showed similarity towards the MS spectrum of gallic acid or its derivative. To evaluate the cytotoxic ability of gallic acid MTT assay were performed. The IC_{50} concentration was calculated and was found to be 38.47±1.033 μg/ml for 28F(4) and for gallic acid found to be 32.8±1.2 μg/ml.
18. The bioassay guided isolation and characterisation of anticarcinogenic compounds from chloroform leaf extract resulted in the isolation of active compounds in two fractions. Both the fractions were mixed together and the antiproliferative ability was checked against K562 cells with different concentration ranging from 5-50 μg/ml for 48h. The result showed a decrease in the IC_{50} value to 23.31±0.88 μg/ml which was comparable with the IC_{50} value of 25 μg/ml of CHFE. The increase in the antiproliferative property after mixing the fractions proved that the antiproliferative capacity is getting enhanced by the combined action of fractions rather than its isolated form.

The present study identified a variety of phytochemicals in the leaf extracts of the *T. populnea*. The extracts exhibited a variety of biologically important activities like antioxidant, antimicrobial, anti-inflammatory and

antiproliferative ability. The apoptogenic ability of the extract was well studied using different techniques and confirmed the ability of the extract to induce apoptosis by mitochondria mediated intrinsic pathway. *In vivo* study supported the increasing survival of tumor bearing mice and doesn't show any symptoms of toxicity. Further experimentation was carried out to isolate the bioactive molecule contributing antiproliferative property. But it was able to prove that the phytoconstituents present in the extract works synergistically to give better antiproliferative ability. Thus the medicinal plant *T. populnea* is a promising medical aid in therapeuticals and for herbal formulations.

MEGHA K. B. “THERAPEUTIC POTENTIAL OF MEDICINAL PLANT THESPESIA POPULNEA (L.) SOLAND EX CORREA WITH SPECIAL EMPHASIS TOWARDS ITS ANTIPROLIFERATIVE ACTIVITY AGAINST DIFFERENT CANCER CELL LINES.” THESIS. DEPARTMENT OF BIOTECHNOLOGY, UNIVERSITY OF CALICUT, 2018.

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LIST OF PUBLICATIONS AND ABSTRACTS PRESENTED FROM THESIS

LIST OF PUBLICATIONS

1. Preliminary screening of phytochemicals, antiproliferative and anti-inflammatory properties of *Thespesia populnea* (L.) Soland Leaf extracts. **Megha K B,** Sanith Cheriyamundath, Joseph Madassery, Elyas KK. Vol 11 issue 5 May 2018 382-386.
2. Cytotoxic Effect of (*Z*)-Ethylidene-4,6-Dimethoxycoumaran-3-One solated from *Pogostemon quadrifolius* (Benth.) on PC-3and DU-145 prostate CancerCells.Sanith Cheriyamundath,Rahul Raghavan,Deepika Vinod, **K. B. Megha,**Asoke Banerji,Karel D.Klika,RobertOwen,Joseph Madassery December 2018, Volume 88, Issue 4, pp 1581–1588.
3. (Z)-ethylidene-4,6-dimethoxycoumaran-3-one induces apoptosis in chronic myelogenous leukemia cell line, Sanith Cheriyamundath, Rahul Raghavan, **Megha K B**, Joseph Madassery, Vol 9, issue 2, June 2015, Page 86-91. International journal of Biological Chemistry.
4. Ethylidene-4,6-dimethoxycoumaran-3-one: the $C_{2=}C_8$ double bond configuration, Karel D Klika, Sanith Cheriyamundath, Rahul Raghavan, **Megha K B**, Asoke Banerji, Robert Owen, Joseph Madassery, Vol 55, issue 48, November 2014, Page 6550-53. Tetrahedron letters.

DETAILS OF SEMINARS ATTENDED

- Oral presentation in three day International Conference on Phytomedicine-2018, organised by the Dept. of Botany, Bharathiar University, Coimbatore, during 29th to 31st August 2018.
- Poster presentation in three days seminar on "Recent advances in Molecular Biology and Biotechnology (RAMBB-2018)" held at Kannur University during 14-16 March 2018.
- Oral presentation in the International conference on "Emerging synergies in Agriculture, Food Processing Engineering and Biotechnology" organised by School of Agriculture and Biosciences, Karunya Institute of Technology and Sciences, Coimbatore from 21.02.2018-23.02.2018
- Poster presentation in UGC sponsored two day international seminar on 'Molecular Biology- an underpinning to life sciences' organised by the Post Graduate & Research Department of Zoology, Farook college, Kozhikode from 08 to 09 January 2018.
- Paper presentation in 104th Indian Science Congress held at S. V. University, Tirupati from January 3 to 7, 2017-09-22
- Participated in the National workshop on "Ethics and welfare concerns in Research for Human and Animal Health" at college of veterinary and Animal Sciences, Pookode, Wayanad, Kerala on 15th September 2017.
- Attended the workshop on "Advanced tools of Biostatistics" organised by the CEPCI Laboratory & Research Institute, Kollam during 26th-27th, June 2017 at Cashew Bhavan, Kollam.
- National workshop in Cheminformatics conducted by Department of Biotechnology, University of Calicut under the Bioinformatics Infrastructure Facility sponsored by DBT, Govt. Of India, from 6th-8th of December, 2016.
- Oral presentation in National Conference on "Indigenous medicinal plants and their therapeutic value" held at Karpagam University, Coimbatore on 18th and 19th August, 2016.
- Attended the one day workshop hosted by Interuniversity Centre for Plant Biotechnology, Calicut University on "Facilitating Innovation and Patent Filing organized by Inter University Centre for Intellectual Property Rights Studies (IUCIPRS), CUSAT on February 3, 2016.

- Participated in the 28th Kerala Science Congress, 28-30 January 2016, University of Calicut, Malappuram.
- Workshop/Training on “Bioinformatics Tools and Applications” on 18th and 19th February 2015, conducted by Department of Biotechnology, University of Calicut under the Bioinformatics Infrastructure Facility sponsored by DBT, Govt. Of India