"SCREENING OF MEDICINAL PLANTS USED IN TRADITIONAL INDIAN MEDICINE FOR *IN VITRO* CYTOTOXICITY AND ANTI-PROLIFERATIVE ACTIVITY"

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For the award of the degree of Doctor of Philosophy

In the Faculty of Pharmacy

By

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(Registration No: KLEU/Ph.D./2015-16/DO1215009)

Under the Guidance of

Prof. (Dr.) Kalpana S Patil ***M.Pharm, PhD***

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"The only limit to the height of your achievements is the reach of your dreams and your willingness to work for them."-Michelle Obama.

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Date: ***Rodrigues Jeswiny Leena***

Place: Belagavi

LIST OF ABBREVIATIONS USED

ASN	-	Asparagine
BSL	-	Brine Shrimp Lethality
CA IX	-	Carbonic Anhydrase IX inhibitor
CDK	-	cyclin-dependent kinases
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
DTH	-	Delayed type hypersensitivity
EGFR	-	Epidermal growth factor recepter
FDA	-	Food and Drug Administration
HA	-	Humoral antibody
IARC	-	International Agency for Research on Cancer
IL	-	Interleukins
LC_{50}	-	Lethal Concentration 50%
mL	-	Milli litre
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NET	-	Neuroendocrine Tumors
RNA	-	Ribonucleic acid
TGF	-	Transforming Growth Factor
TNF	-	Tumor Necrosis Factor
WBC	-	White Blood Cell
WHO	-	World Health Organisation

ABSTRACT

Background: Cancer is a major health burden globally. In recent years, a lot of research has been focussed on the synthesis of potential anticancer drugs from alternative sources due to side effects associated with current chemotherapeutic drugs. The use of plant based products in medicine, including cancer therapy, has been prominent since long. It has been evidenced through the successful development of anticancer drugs like vinca alkaloids (vincristine, vinblastin) from *Vinca rosea.* With the use of simple yet effective screening models like the BSL bioassay it is possible to screen numerous extracts/fractions for their cytotoxicity. The integration of molecular docking and *in vitro* studies can help cancer drug discovery with good consistency of the results between the two approaches.

Objectives: To identify potential cytotoxic constituents from plants from Indian system of medicine through bioactivity guided fractionation.

Methodology: The extracts and fractions were screened using the BSL bioassay for their bioactivity. The active fractions were further studied using the MTT assay against a panel of cell lines- MCF-7, HT-29, A-549, HepG2 and L6. The most cytotoxic fraction was then subjected to cell cycle analysis and assay for apoptosis. Molecular docking of phytoconstituents from cytotoxic fractions was conducted against Caspase -3,-7 and -9 for their binding affinity.

Results: Fraction 3 from *C.hirsutus* and *D.glaucescens* displayed promising cytoxicities against A-549 cell line. Purified Fraction 3 of C.hirsutus also demonstrated cell cycle inhibition and induction of apoptosis in A-549 cell line. Trilobine and Cocsoline showed significant binding affinity towards Caspase-3, -7 and -9 in molecular docking studies.

Conclusion: The study revealed cytotoxic alkaloid rich fractions of *C.hirsutus* and *D.glaucescens* among the 5 selected plants. The alkaloid rich fractions also inhibited A-549 cell line and induced apoptosis. Furthermore, trilobine and cocsoline were identified as potential lead molecules from *C.hirsutus*.

Keywords:

Alkaloids; Apoptosis; Bisbenzylisoquinoline; Brine shrimp; Caspase; Cell cycle; Cytotoxicity; Docking; Flowcytometry; Menispermaceae

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1.INTRODUCTION

1.1. Background

Cancer is a complex disease characterized by the uncontrolled and abnormal growth of cells which can begin anywhere in the body. Human cells multiply by cell division and when they age or get damaged these cells are eliminated and are replenished by new cells. When there is any disturbance in this process, damaged cells grow abnormally and form lumps of tissue called tumour. Such cancerous tumours may spread/invade adjacent tissues and migrate to other areas in the body. This process is called Metastasis. Metastasis is one of the major reasons of mortality in cancer.[1]

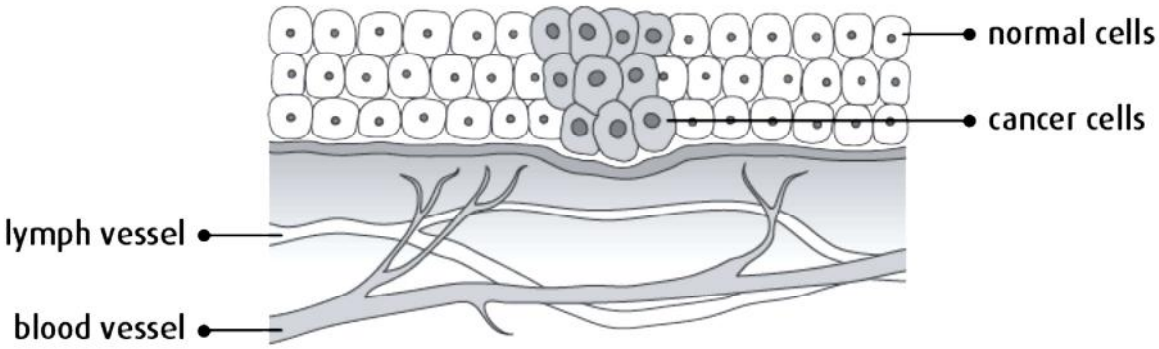


Figure 1. How cancer cells spread [2]

Hallmarks of cancer cells [3]

- Cancer cells are capable of growing without any growth signals, while normal cells divide only after receiving such signals.
- Cancer cells can ignore apoptotic signals which would otherwise direct the abnormal or damaged cell towards apoptosis (programmed cell death).
- Cancer cells can induce angiogenesis. The tumour cells are enriched with oxygen and other nutrient by these blood vessels.

- Our body's defence mechanism eliminates damaged or abnormal cells. Cancer cells capable of avoiding immune destruction or trick it to survive and grow.
- Cancer cells need more energy to multiply at such high rates and hence they utilise other sources of nutrients and thus follow abnormal pathways (deregulation of cellular energetics).
- Cancer cells have an increased tendency of genome alteration and mutations during cell division.

Recently research on cancer has been focussed on these differences to develop treatments which can target the abnormal behaviour of cancer cells.

Causes of cancer

There are a number of risk factors of cancer and some are preventable. Generally, the progression of pre-cancerous lesion to a malignant tumour occurs due to changes in genes which control cell functions especially cell growth and division through a multi-stage process. These genetic changes can be inherited or occur on account of aberration during cell division or due to environmental factors (carcinogens). Environmental factors (tobacco, infections, radiation, lifestyle, environmental pollutants) constitute around 90-95% of cases of cancer. Other causes of cancer cannot be prevented. Age is the most significant unpreventable risk factor of cancer.[4,5]

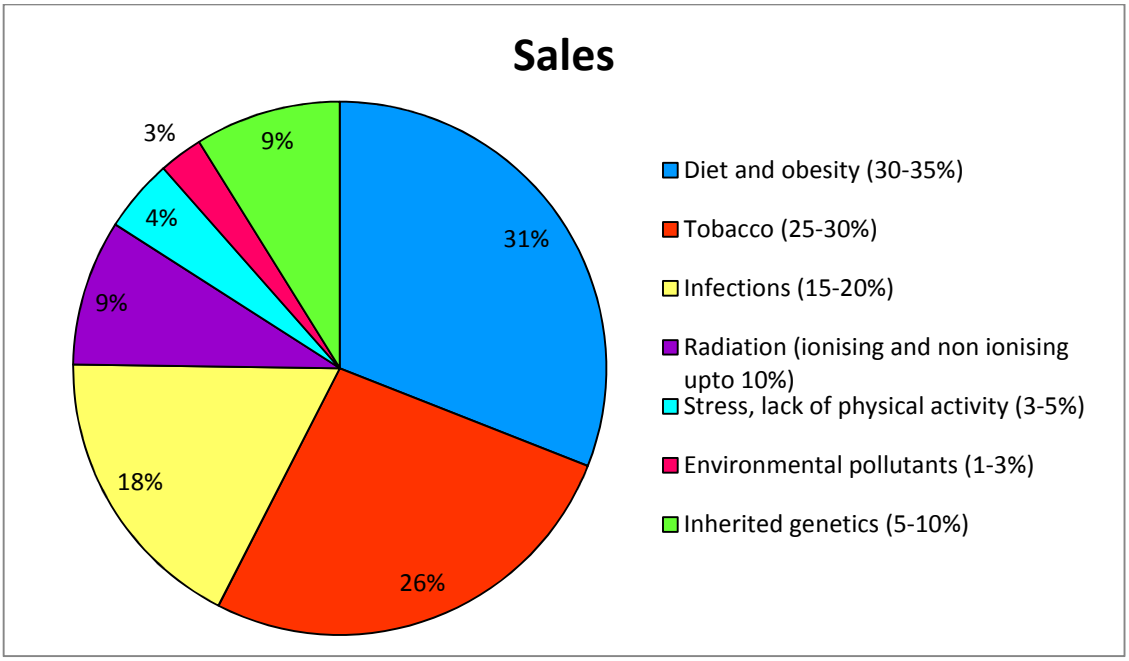


Figure 2. Risk factors in cancer[4]

Proto-oncogenes : any alteration or over-activity of these genes result in their transformation to oncogenes (cancer-causing genes) and thereby allow cells to survive when they should not.[6]

Tumour suppressor genes: these are also involved in cell growth and division and any alteration cause cells to multiply uncontrollably.[7]

DNA repair genes: these are involved in repairing damaged DNA. Alteration in these and their chromosomes cause duplication and deletions of chromosomal parts. These mutations cause cells to turn cancerous.[8]

In these past few years, there has been remarkable advance in our knowledge about the pathogenesis and molecular changes resulting in cancer. Cancer therapy targeting gene mutations in cancer is now trending.

Types of Cancer

1. **Carcinomas:** cancers that begin in the tissue or skin of glands and internal organs. E.g. Lung cancer, colorectal cancer, prostate cancer etc.[9]
2. **Sarcomas:** cancer of connective tissues which support the body. E.g. Bone cancer, cancers of blood and lymph vessels or cartilage. [9]
3. **Leukemia's:** blood cancer. E.g. acute/chronic myeloid leukemia, acute/chronic lymphocytic leukemia.9
4. **Lymphomas:** cancer which starts in the lymphatic system involved in fighting infection. E.g. Hodgkin lymphoma and Non-Hodgkin lymphoma.[9]
5. **Melanoma:** cancer originating from melanocytes (pigment-producing cells). E.g. uveal and Nodular melanomas. [10]
6. **Miscellaneous tumors:**
    i. **Germ Cell Tumors:** tumors that are developed in gonads (testes and ovaries) which can be either malignant or benign.[11]
    ii. **Neuroendocrine Tumors** (NETs)**:** these tumors are derived from endocrine cells which secrete hormones s a response to signals from the nervous system. These are rare and can occur anywhere in the body. E.g. Pancreatic neuroendocrine tumors, Adrenal cancer, Merkel cell carcinoma. [12]

Cancer Data and Statistics

WHO has stated cancer as the second highest cause of deaths worldwide. According to world cancer report, the incidence is going to increase at an alarming rate globally. Cancer accounted for almost 10 million deaths in 2020 worldwide.[13] In India, the projected incidence of cancer in 2020 was 679,421 (94.1 per 100,000) in males and 712,758 (103.6 per 100,000) among females.[14] The common cancers were:

- Breast cancer (178 361 new cases).
- Lung cancer (72 510 new cases).
- Stomach cancer (60 222 new cases).
- Liver cancer (34 743 new cases).
- Colon and rectal cancers (31 646 new cases).[15]

The International Agency for Research on Cancer (IARC), estimates that by 2040, the global cases and deaths may increase to 27.5 million and 16.3 million respectively owing to growing population and aging.[16]

Cancer therapy

There are a number of treatment options in cancer depending upon the type and stage of the disease, a single or combination of treatments is opted.

- Surgery
- Radiation therapy
- Chemotherapy
- Immunotherapy

Classification of anticancer drugs[17,18]:

1. Alkylating Agents (Altretamine, Bendamustine, Busulfan, Carmustine)
2. Taxanes (Docetaxel, Paclitaxel, Cabazitaxel)
3. Platinum Complexes (Carboplatin, Cisplatin, Oxaliplatin)
4. Antibiotics (Bleomycin, Doxorubicin, Mitoxantrone, Valrubicin)
5. Anti-metabolites (Methotrexate, Azathioprine, Fluorouracil)
6. Vinca Alkaloids (Vinblastine, Vincristine, Vinorelbine)
7. Topoisomerase Inhibitors (Etoposide, Irinotecan, Topotecan).
8. Hormonal Agents

i. Antiandrogens (Abiraterone, Apalutamide, Bicalutamide)

ii. Antiestrogens (Anastrozole, Tamoxifen)

iii. Analogues of Gonadotropin-Releasing Hormone (Degarelix, Histrelin)

iv. Peptide-hormone analogues: Pasireotide, Lanreotide.

9. Protein Kinase Inhibitors (Abemaciclib, Vemurafenib, Zanubrutinib)

10. Monoclonal Antibodies (Pertuzumab, Tositumomab, Alemtuzumab)

The treatment by chemotherapy can also affect the normal cells, tissue, and organs apart from cancerous cells. Therefore there are number of undesirable effects associated with chemotherapy, which sometimes is life threatening. Some common side effects include anemia, thrombocytopenia, infection, neutropenia, lymphedema, nausea, vomiting, peripheral neuropathy, bone marrow suppression, severe pain.[19]

Adverse/toxic effects and even mortality during chemotherapy has been increasing in cancer patients, hence in the past few decades there has been growing emphasis on finding newer drugs from alternative sources.[19]

Plants as a reservoir of anticancer drug discovery

The use of plants as medicine in various forms dates back to 2600 BC.[20] The Indian traditional systems of medicine: Ayurveda, Siddha and Unani used many medicinal herbs and spices (e.g turmeric) as therapy since ancient times including cancer. The term 'cancer' in ancient literature however, is undefined and often referenced to conditions like hard swellings, calluses, warts, corns, ulcers, abscesses, polyps, etc.[21]

The concept of cancer therapy and prevention from naturally derived compounds has gained popularity in recent years. Vinblastine and Vincristine were among the first natural agents to move forward into clinical use after isolation from plant *Catharanthus roseus*

species (Apocynaceae).[22] Drugs like Paclitaxel, podophyllotoxins and campthothecin from *Taxus brevifolia Phodophyllum peltatum,* and *Camptotheca acuminata* respectively are some of the other chemotherapeutic agents that followed and are widely being used in clinical therapy.[23] Hence the search is on for potential molecules from natural sources in anticancer drug development.

More than 60% of therapeutic agents being used clinically today have been obtained through natural sources, especially plants. India being one of the 12 "major diversities" centres serves as a large reservoir of plant genetic diversity with a greater likelihood to provide novel biomolecules for cancer.[24]

Plant derived cytotoxic agents exert their effect through multiple mechanisms and thereby inhibiting different stages of the cancer cell growth.[25] The structural diversity of phytoconstituents (e.g., flavonoids, alkaloids, terpenes, lignans, saponins and other secondary metabolites) is responsible for the selective inhibition of proliferation and cancer induction.[26] Natural anticancer agents may be cytotoxic on cellular level by inhibiting the cell division and cell proliferation. They may also inhibit certain immunosuppressant proteins produced by cancer cells and act as immunomodulators. Further, they could delay the process of carcinogensesis and act as chemopreventive agents.[27]

Bioactivity guided approach

The bioactivity guided identification of cytotoxic compounds from plants is a highly sorted technique in natural product research helpful in identifying potential lead molecules and it is essential to search rational methods, which screens large number of plant sources with minimum cost, time and still provides with reliable information.[28]

Mc Laughlin and Rogers were first to propose bench top bioassay techniques as a useful way to indicate a pharmacological activities of botanicals.[29] The Brine Shrimp Lethality (BSL) Bioassay developed by Meyer et al., is a rapid, convenient and reliable method in the detection and isolation of constituents with variable pharmacological effects. The bioassay depends on the principle that "Pharmacology is nothing but toxicology at low doses and molecules which show toxicity in simple zoological organisms such as *Artemia salina* (Leach) may possess certain biological activity in higher animals. This bioassay can identify a broad range of bioactivities of chemicals as well as their structural diversity.[30]

In recent years, advanced genomics and computer assisted *de novo* drug design have significantly facilitated the lead compounds' identification for drug development.[31] Molecular docking gives information about the atomic interaction between drugs and targets. Identification of numerous molecular targets has made high throughput screening of compounds possible which now sets the base for anti-cancer drug discovery. Molecular docking coupled with *in vitro* studies can help achieve identification of leads and speed up cancer drug discovery process while maintaining consistency of results between these two methods.[32]

1.2 Review of literature

1.2.1 The Cell Cycle

The cell cycle is a phenomenon whereby a cell duplicates and divides. The components regulating the cell cycle play an important role in arresting or inhibiting cell division. Since cancer is caused by disturbance in the cell cycle, its regulation or control is an important area in anti cancer research. The cell cycle produces two exact duplicates of the parent cell. There is a continuous growth phase which causes increase in cell mass partitions resulting in two daughter cells. There are special proteins and checkpoint systems which ensure proper cell cycle sequence. It is classified into two broad phases:

1. Mitosis (M) phase
2. Interphase

i. G_1(first gap)

ii. S (synthesis)

iii. G_2 (second gap)

iv. G_0 [39]

M Phase

This phase comprises of mitosis and cytokinesis. The daughter cells are formed when the chromosomes and cytoplasm divide, each of which receives organelles exact copy of the parent cell and a complement of genetic material.[40]

Interphase

This is the phase which includes growth of cells and replication of their DNA. There are 4 stages in this phase.[40]

G_1 Phase

In this phase, the cell is preparing to divide. This is also the longest phase of the cycle and also the most variable. At cytokinesis, the cells are half their original size before mitosis and the cells increase to the optimal size by the end of this stage. During this stage, there is a suppression of many processes of the cell cycle so that the cells do not initiate another round of proliferation by a system called 'Restriction point'. This occurs if there is a poor supply of nutrients or if the cell receives anti-proliferative stimuli and the cells delay their processes or the cycle enter G_0 phase. Upon exposure to positive stimuli the cells may overcome this restriction point and triggers the process of a new cycle of replication and division. Cancer cells have faulty restriction point which is responsible for the unchecked growth and division even if there are suitable signals.[40]

G_0 Phase

Cells which form cells that perform specialised functions and are unable to divide further belong in this phase. Here the cells are highly motile and involved in protein synthesis and secretion. Here the cells are not dormant and this phase may not be permanent. In special cases, exposure to appropriate signals can cause G_0 cells to re-enter the cell cycle accompanying changes in gene expression and protein stability. This phase if not regulated can lead to cancer due to the uncontrolled growth of cells.[40]

S Phase

This is the stage where DNA is replicated. The replication of DNA occurs only once. A diploid somatic cell consisting of 2N complement of DNA obtains 4N complement of DNA at the end of this phase. This is a significant stage of the cell cycle because the replication of genomic information from the nucleus of the cell occurs at this time. The

cohesion between sister-chromatids occurs in the S phase which is responsible for proper segregation of chromosomes during mitosis. The S-phase checkpoint is mediated by protein kinases in response to DNA damage and disturbances in replication. This checkpoint preserves the integrity of DNA replication and maintains the genome integrity.[40]

G_2 Phase

This is the shortest phase which is occurs soon after the S phase and just before mitosis. Any errors in the chromosomes are repaired and the cell prepares to enter the next cycle. The G_2 phase checkpoint prevents cells with damaged DNA from undergoing mitosis.[40]

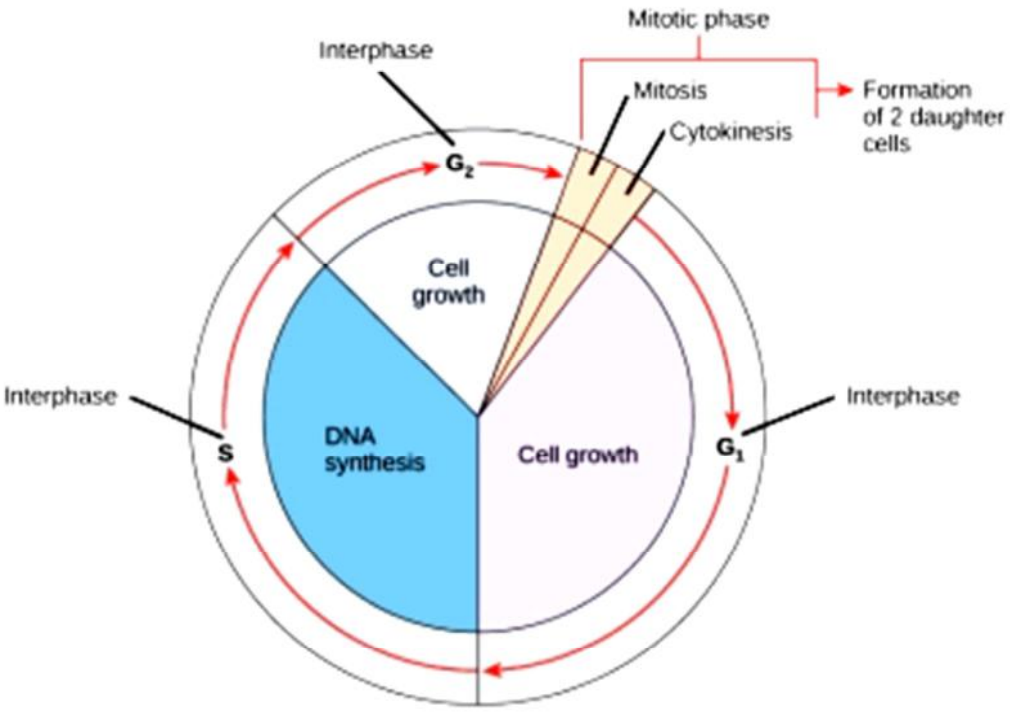


Figure 3. Phases of the cell cycle

1.2.2 Apoptosis

Apoptosis is the programmed cell death and is characterized by morphological and energy-dependent biochemical changes. It finds significance in regulation of the immunity, cell turn over, embryonic development etc. Disturbance in apoptosis is a key factor in various types of conditions including cancers.

Apoptosis takes place as a homeostasis mechanism that maintains cell population, during development and ageing of cells. It may also act as a defence mechanism when cells are damaged or exposed to harmful agents. Fas or TNF receptors expressed on certain cells may initiate apoptosis by protein cross-linking and ligand binding. It is a complex process, synchronized and energy-dependent. Some type of cysteine proteases (caspases) are activated which cascades events ultimately resulting in the cell death.[41]

1.2.3 Morphological changes in apoptosis

In the early stages of apoptosis, there is rounding of the cell, condensation of chromatin to dense mass and the cytoplasm shrinks. This condensation of chromatin (pyknosis) is a characteristic feature of apoptosis. Blebbing of membrane occurs (karyorrhexis) and transform of cell fragments into apoptotic bodies. These apoptotic bodies are made up of tightly packed cell organelles enclosed within the plasma membrane. These are then phagocytosed by macrophages which are degraded within phagolysosomes.[42]

Necrosis is the alternative to apoptosis and is considered to be a lethal process where cell follows an energy-independent mode of death. Necrosis is characterised by major morphological changes like cell swelling, condensed, swollen or ruptured mitochondria, distended endoplasmic reticulum, cytoplasmic blebs, cytoplasmic vacuoles, disrupted

organelle membranes, swollen and ruptured lysosomes, disaggregation and detachment of ribosomes which results in disruption of the cell membrane.[43]

1.2.4 Natural products as anticancer agents

Plant-derived anticancer agents act by inhibiting the cancer cell growth at various stages. Tubulin binding agents (Vinca alkaloids, Taxanes) causes a disruption in the alignment of daughter chromosomes and attachment to mitotic spindle by interfering with the microtubule function. This leads to mitotic arrest at the metaphase and consequent apoptosis.[44, 45]

The human immune system is capable of destroying cells of established cancers, (antitumor immunity). The immune system may detect oncogene proteins such as HER-2/neu, mutated *ras* and *p53* on the cell surface. Cytokines (IL-2) and interferones are majorly involved in the regulation of immune cells. There are many natural compounds which can stimulate or support the immune system. Immunosuppressive agents and cytokines like PGE2, TGF-β 4(Transforming growth factor) and IL-10 are produced by cancer cells which protects them from immune attack.[46]

Irinotecan, an anticancer agent which was approved by US FDA in 1996 is an alkaloid which inhibits DNA unwinding by topoisomerase-1 inhibition.[47] Flavopiridol derived from an alkaloid Rohitukine obtained from Amoora rohituka and Dysoxylum acutangulum is a cyclic dependent kinase (CDK) inhibitor.[48] A naphthaquinone compound, β-lapachone, isolated from the bark of Tabebuia sp is known to induce p53 dependent apoptosis and inhibit protein synthesis.[49]

Kupchan et.al (1973) first reported that Bisbenzylisoquinoline alkaloids possess antitumor properties.[50] Kuroda *et al.*, (1976) investigated a number of bisbenzylisoquinoline

alkaloids (tetrandrine, cepharanthin, barbarin, oxycanthin, epistephanine, dauricine, stebisimine) for anticancer potential in HeLa cella lines and animals.[51]

Su-Mi Yoo *et. al*, (2002) reported that Tetrandrine, a bisbenzylisoquinoline alkaloid derived from the root of *Stephania tetrandra* could inhibit cell growth. Apoptosis was also caused in HepG2.[52] Thavamani *et. al*, (2013) reported cytotoxicity of plants from Menispermaceae family against HeLa cell lines. Bisbenzylisoquinoline alkaloids being the major phytoconstituents may have been responsible for the cytotoxic activity.[53]

Campbell et.al., (2000) reported bioactive alkaloid 1-o-acetylnorpluvine from *Brunsviga radulosa* which showed cytotoxic activity in BL6 mouse melanoma cells *in vitro*.[54]

1.2.5 BSL bioassay as a pre-screening model

McLaughlin et.al did a comparative analysis of simple bench-top bioassays including the BSL Bioassay and various human tumor cell lines and it was found that the BSL bioassay was better or just as as accurate as the *in vitro* studies on solid tumor cell lines. This bioassay is well accepted and numerous reports demonstrate the success of the BSL bioassay in evaluating natural products for cytotoxicity.[29]

Gerwick et.al., (1994) used the BSL bioassay to isolate a unique metabolite Curacin A, from the marine cyanobacterium *Lyngbya majuscula* which was found to be responsible for the mammalian cell antiproliferative activity in the Chinese Hamster Aux B1 cell line.[55]

Ajaiyeoba et.al. (2006), evaluated 20 plants from the regions of Nigeria for cytotoxicity using the BSL bioassay out of which, the methanol extracts of 2 plant barks *Morinda lucida* and *Lippia multiflora* displayed cytotoxic effect. The results provided an

insight into the cytotoxic nature of the extracts and a basis for their selection of further fractionation and evaluation.[56]

Tan et.al. (2008), studied the extracts and fractions of the marine cyanobacterium, *Lyngbya majuscule*, wherein the BSL bioassay was used for the evaluation of toxicity. The brine shrimp bioassay active fractions were further purified and two new secondary metabolites, Besarhanamides A and B were isolated. Besarhanamides A was found to show moderate toxicity in this bioassay.[57]

Zhang et.al (2015), carried out a bioassay guided fractionation of a fungus from *Rhizophora stylosa* using the BSL bioassay as a screening method. The brine shrimp lethal EtOAc extract which was further fractionated, led to the discovery of new alkaloids 18-hydroxydecaturin B and penioxamide A. Both the new compounds showed potent cytotoxicity in the BSL Bioassay.[58]

Alves et al have screened 60 Brazilian medicinal plants for the Brine shrimp toxicity for detecting bioactive molecules. Plants were selected based on the ethnobotanical information. Six species have shown the LC_{50} values $\leq$ 100 ppm, of which dicholoromethane–methanol extracts of *Leonurus sibiricus* L (Lamiaceae) aerial parts and *Xylopia aromatica* (Lam) Mart (Annonaceae) bark were reported as the most bioactive having LC_{50} =12 ppm and =18 ppm, respectively.[59]

Olila et al., isolated a cytotoxic sesquiterpine muzigadial, from *Warburgia ugandensis* which was found to be highly lethal to brine shrimps and possess trypanocidal activity against a drug-resistant trypanosome strain IL 3338 and drug-sensitive IL1180 trypanosome strains.[60]

Karchesy et.al., (2016) screened 211 methanol extracts from plants of Pacific Northwest cytotoxic activity using the BSL assay. Following studies of the cytotoxic plants in

this bioassay confirmed its significance in identifying potential insecticidal and fungicidal leads.[61]

1.2.6 Molecular docking in cancer research

Molecular docking is recently being used modern drug discovery as a computational tool. It is used to quantify binding affinity between ligand and protein based on structure. Molecular docking has been successfully used to identify potential protease inhibitors and provide an insight in the mechanism of their binding to protease enzyme.[62]

Zhang et al. reported a structural component of peptide aldehydes (MG_{132}) which is a selective and potent protease inhibitor using molecular docking.[63]

Santoro et al. reported cationic and anionic porphyrins as potential protease inhibitors. The results showed the ability of cationic porphyrins to inhibit protease at three catalytic sites of the protease.[64]

Amresh et al. carried out molecular docking and reported five potential Carbonic anhydrase inhibitors (CA IX inhibitor) and identifying this class of enzymes as a potential anticancer drug target. Docking simulations help identify residues at CA IX active site by which interactions take place.[65]

The epidermal growth factor recepter (EGFR), a family of kinases involved in the physiological and metabolic processes is another promising target in anticancer research. García-Godoy et al. studied the interactions of EFGR inhibitors using molecular docking with wild-type and mutant EGFR. The results showed Met793 residue interaction with ligand present at the EGFR active site.[66]

Oliva et al. reported the inhibition of cytochrome c oxidase (CcO) by Chlorpromazine using molecular docking. This study provided evidence of the antiproliferative activity of Chlorpromazine against colon and brain tumors.[67]

Atidel et al. evaluated campthothecin-like moleculas with DNA topoisomerase 1 (Top1) using molecular docking tool. The results indicated the highest binding to the cavity by Arg364 residue suggesting that camptothecin similars as potential leads for the development of novel anticancer drugs.[68]

The interaction of 1,2,4-Oxadiazoles derivatives with caspase-3 enzyme has been reported which showed interaction with asparagine 273 (ASN 273) of the protein suggested activation of apoptosis as a promising strategy in anticancer drug development.[69]

1.2.7 Plant profile

I. *Cocculus hirsutus*[70]

Figure 4. *C.hirsutus* plant species

Scientific Classification

Kingdom	: Plantae
Phylum	: Spermatophyta
Subphylum	: Angiospermae
Class	: Dicotyledonae
Order	: Ranunculales
Family	: Menispermaceae
Genus	: *Cocculus*
Species	: *Cocculus hirsutus (L.) Diels*

Vernacular Name(s)[70,71]

English: Broom-Creeper, Ink-Berry.

Ayurvedic: Chhilihinta, Paataala garuda, Mahaamuulaa, Dirghavalli, Jalajamani.

Siddha/Tamil: Kattukodi

Distribution

Belgaum, Chikmagalur, Coorg, Hassan, Mysore, N.Kanara, Shimoga all districts of Maharashtra, Palakkad.[70]

Plant description

Climbing shrub; Leaves- velvety, ovate, apex obtuse, 5-7 x 3-4 cm, base cordate. Flowers: drupe to 8 mm, globose, purple. Female inflorescence grouped axillary fascicles. Six sepals obovate, arranged in two rows. Six oblanceolate, hairy petals, and three carpels. Male inflorescence- axillary branched raceme; six sepals, obovate; six obovate bifid petals, base auricle; six stamens, free.[70]

Traditional uses

Decoction of leaves was used for eczema, leucorrhoea and gonorrhoea. Roots were used in syphilitic cachexia, chronic rheumatism and gout. It is used as an antimicrobial and in treatment of wounds, burns and ulcers.[71,72]

Phytoconstituents

Phenolic compounds, flavonoids, bis-benzyl isoquinoline alkaloids – hirsutine, shaheenine, cohirsinine, trilobine.[71,72]

Pharmacological action

- Potent antimicrobial activities of petroleum ether and ethanolic extracts have been displayed against microorganisms *Staphylococcus aureus*, *Pseudomonas aeruginosa Escherichia* coli, *Pseudomonas aureus*, and *Salmonella typhi*.[73]
- Aqueous extract of *C.hirsutus* aerial parts has been reported for its diuretic and laxative effects.[74]
- The ethanolic and aqueous extract have Immunomodulatory effect by reportedly increasing carbon clearance, delayed type hypersensitivity (DTH), humoral antibody (HA) level, WBC count and reducing myelosuppression in rats.[75]
- Ethanolic extracts have shown Antiinflammatory and analgesic properties in albino rats.[76]
- Extracts of aerial parts have shown antioxidant and cytotoxic effect against MCF-7 (human breast cancer cell line).[77]

II. Diploclisia glaucescens[71,78]

Figure 5. *D.glaucescens* plant species

Scientific Classification

Kingdom	:	Plantae
Phylum	:	Tracheophyta
Subphylum	:	Angiospermae
Order	:	Ranunculales
Family	:	Menispermaceae
Genus	:	*Diploclisia*
Species	:	*Diploclisia glaucescens*

Vernacular Name(s)[71,78]:

English: Glaucous *Diploclisia*

Local name: Erumathirankodi

Marathi: Vatan vali

Tamil: Kottaiyachachi

Kannada: Bootha kannu

Distribution

Western & Eastern Ghats of India.[78]

Plant description

Woody climber. Leaves- broadly ovate to orbicular glaucous below, base truncate, apex obtuse, upto 8 cm. Petiole: 1.5-3 cm long. Unisexual flowers, droopy, bright yellow. Six sepals 3 mm long, obovate, two whorls; six petals, apex obtuse, incurved margins, upto 2 mm long, concave, emarginated.[78]

Traditional uses

Externally applied to relieve sprains. Herpes, pruritus, scabies, rheumatic arthritis, urethritis, cholecysititis, snake bites, leaves were used as anti inflammatory, in Gonorrhoea and Syphilis.[71,79]

Phytoconstituents

Pentacyclic triterpenoids (serjanic acid and phytolaccagenic acid), 3- O-beta- D-glucopyranosylphytolaccagenic acid, Ginnol, sitosterol, alkaloids (trilobine, stepharine), magnoflorine.[79,80]

Pharmacological action

- Various extracts of leaves have displayed Antimicrobial activity against different bacterial strains.[78]
- Methanolic extract of leaves have shown hypoglycemic effect in mice.[81]
- The saponins from stem of *Diploclisia glaucescens* have been reported to have molluscicidal activity.[82]

III. *Hyptis sauveolens* [71,83]

Figure 6. *H.sauveolens* plant species

Scientific Classification

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Asteridae
Order	:	Lamiales
Family	:	Lamiaceae
Genus	:	*Hyptis*
Species	:	*Hyptis sauveolens (L.) Poit.*

Vernacular Name(s)[71]

English – Pignut, Bush Mint

Hindi- Vilaiti Tulsi

Malayalam-Nattapoochedi

Marathi- Darp Tulas, Jungli Tulas

Others- Bhunsuri

Distribution

Chikmagalur, Mysore, Coorg, Shimoga, Hassan, all districts of Maharashtra, Kerala, Tamil Nadu.[71]

Plant description

Shrubs, 1.5 m height; stem thinly hairy,obtuse. Leaves acute, ovate, glabrate, hispid under, petiole- 5 cm long. Blue inflorescence, clusters; calyx- 8 mm, 10-ribbed, tubular, teeth spinulose upto 4 mm long, glandular hairy; corolla- 5 mm long, short glabrous lobes. Compressed nutlets, ridges on dorsal area, 4 x 2.5 mm, pubescent, mucilaginous when wet, dark brown.[71]

Traditional uses

As carminative, stimulant, sudorific and lactogogue Leaf extracts cure swellings, abscesses and haemorrhoids. Decoction of roots was used to purify the blood and uterine infections. Plant was used to relieve pain and as decongestant. Roots were used chewed to relieve stomach cramps and decoction used as an appetizer.[83]

Phytoconstituents

Phenolic compounds methyl rosmarinate and rosamarinic acid. Oleanoic acid, 3β-hydroxyl lup-20(29)-en-27-oic acid urs-12-en-3β-ol-27-oic acid, 1,19adihydroxy-urs-2(3), 3β-hydroxy lup-12-en-28-oic acid. Flavonoid, saponins, essential oils, terpenoids and sterols β-sitosterol, ursolic acid. Diterpenes: suaveolic acid, methyl suaveolate, suaveolol, alkaloids.[84]

Pharmacological action

- Extracts of *Hyptis* species have been reported to decrease inflammation or fever and wound healing activity in rats.[85]
- Aqueous extract of leaves have shown antinociceptive effects in chemical and thermal models using mice.[86]
- Essential oils of *Hyptis sauveolens* have reported antioxidant activity by increasing levels of catalase and superoxide dismutase in granuloma tissue.[87]
- Aqueous and ethanolic extract have shown antiulcer activity and hexane extract has been reported to have gastroprotective effect in animal models.[88]
- Dehydroabietinol from Hyptis sauveolens is reported to inhibit chloroquine-sensitive and chloroquineresistant strains of Plasmodium falciparum in erythrocytes.[89]
- The alcoholic extract of *H. sauveolens* is reported to have immunomodulatory and antioxidant potential.[90]
- Various leaf extracts have been reported to have broadspectrum antibacterial against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*. Also reported for its antifungal effect against *Helminthosporium oryzae*, *Aspergillus niger*, *Fusarium oxysporum.*[91]

IV. *Tiliacora acuminata*[70]

Figure 7. *T.acuminata* plant species

Scientific Classification

Kingdom	:	Plantae
Phylum	:	Tracheophyta
Subphylum	:	Angiospermae
Order	:	Ranunculales
Family	:	Menispermaceae
Genus	:	*Tiliacora*
Species	:	*acuminata (Lam.) Hook. F. & Thoms*

Vernacular Name(s)[70]

Synonym-*T.racemosa Coleber., Menispermum acuminatum Lamk*

Malayalam- Vallikanjiram

Folk- Teliakora

Siddha/Tamil - Kodaparuavalli.

Distribution

Kozhikkode, Thrissur, Wayanad, Kottayam, Malappuram, Palakkad, Alappuzha, Kollam, Pathanamthitta, Thiruvananthapuram, Kannur.[70]

Plant description

Climbing shrubs. Stems striate, sparsely puberulous or glabrous. Leaf around 8-14 x 3.5- 8 cm, ovate, alternate, truncate, cordate, apex acuminate, rarely acute base, glabrous, upto 5 nerves at base, petioles glabrous 1.5-3 cm in length,. Flowers axillary panicle, ~10 cm. Male and female flowers two to seven at apex, yellow; six sepals in 2 rows; broad elliptical, glabrous; six petals, obovate, smooth; six stamens, cylindrical. Eight to twelve carpels, glabrous, on stalked gynophore. Fruits-10-15 x 6-7 mm, oblong to obovoid, smooth, endocarp reticulate, red color.[70]

Traditional uses

Externally used for skin diseases, roots are rubbed between stones and dissolved in water as a drink to cure venomous snake-bites.[92]

Phytoconstituents

Alkaloids, anthraquinones, catechins, coumarins, flavonoids, phenols, quinones, saponins, steroids.[80]

Pharmacological action

- Ethanolic leaf extract has been reported for its antinociceptive activity using mice model and antidiarrhoeal activities such as castor oil and magnesium sulfate induced diarrhea in rats antioxidant activity.[93]
- Cytotoxic effect of root extracts of *T.acuminata* has been reported against HeLa cell line, leukaemia cell lines HL-60 and K-562 and breast cancer (MCF-7).[94]
- Extracts are reported to inhibition of broad range of gram positive, gram negative bacteria and fungi.[95]

V. *Pachygone ovata*[70]

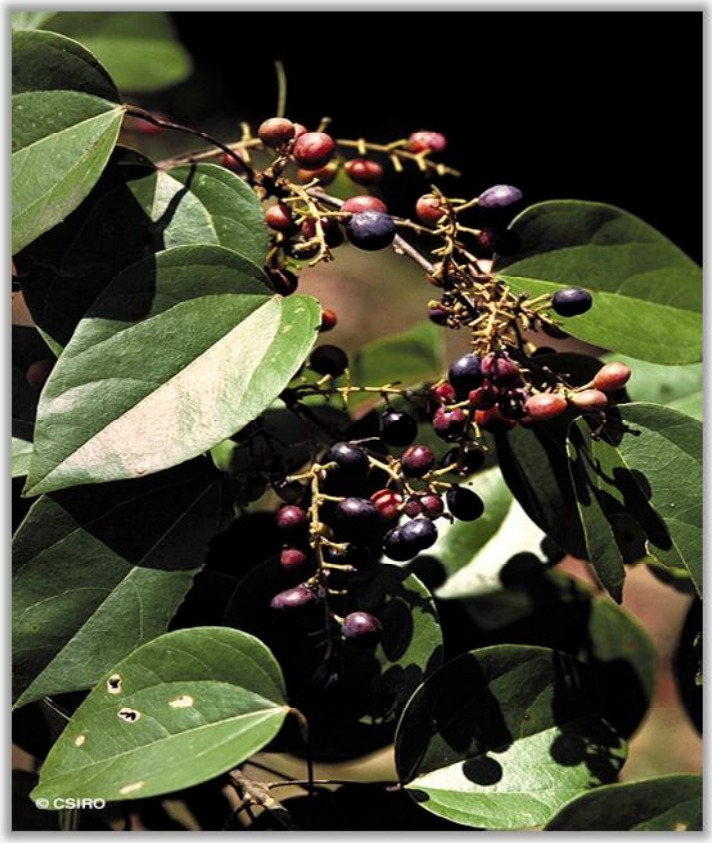


Figure 8. *P.ovata* plant species

Scientific Classification

Kingdom	: Plantae
Phylum	: Tracheophyta
Subphylum	: Angiospermae
Order	: Ranunculales
Family	: Menispermaceae
Genus	: *Pachygone*
Species	: *Pachygone ovata (Poir) J.D. Hook & Thompsons*

Vernacular Name(s)[70]

Synonym - *Cissampelos ovata (Poir)*

Folk- Kadukoddi

Malayalam- Katukodyvally

Others – Javanakodi

Distribution

Idukki, Palakkad, Mysore.[70]

Plant description

Climbing shrub; leaf size 3-7 x 2-3.5 cm, elliptical, three to five nerves, hairy petioles. Male flowers axillary white, pubescent, external sepals linear, small, internal sepals ovoidal; petals white, clinging to the filaments. Stamens filaments incurved, free. Female flowers with three carpels, six staminodes. Fruits 7 x 6 mm, smooth endocarp.[70]

Traditional uses

Snake bites**,** paste of leaves were externally applied to cuts and boils, painful swellings.[96]

Phytoconstituents

Alkaloids (N-methylcrotsparine, reticuline, reticuline N-oxide, quercitol, liriodenine, trilobine, coclaurine), flavonoids.[97]

Pharmacological action

- Various stem and leaf extracts of *Pachygone ovata have shown* antioxidant activity.[96]
- Methanol extracts have been reported for anti-inflammatory and antinociceptive effects in carrageenan-induced paw edema in rats and formalin test respectively.[98]

1.3 Justification of the study

The incidence of cancer is on the rise and is expected to continue growing in the following years to come. According to WHO, Cancer is said to be the second most common non communicable disease causing mortality worldwide.[13]

There are a number of medicinal plants in Indian system of Medicine which are traditionally known to be useful in treatment of cancer. However the scientific validation for their use in clinical therapy is still a lingering question.[33] These medicinal plants are rich in active phytochemical constituents that need to be isolated and experimentally evaluated for their cytotoxic and anti-proliferative activities. However the average time and cost involved in the evaluation of a single plant for cytotoxicity and isolation of bioactive compounds is high and the outcomes of the study may be unpredictable with numerous leads discarded in the process, which may further result in waste of the time and money spent.[34]

By the use of economical and consistent prescreening models such as the Brine Shrimp Lethality Bioassay, a number of medicinal plants can be precisely screened for cytotoxic properties in a relatively shorter time.[30] Therefore, in this study, the BSL bioassay is selected for the activity-guided identification of cytotoxic and antiproliferative agents from plants.

The medicinal plants from traditional Indian Medicine selected for the study are *Anarmita cocculus* (Menispermaceae), *Cocculus hirsutus* (Menispermaceae), *Diploclisia glaucescens* (Menispermaceae), *Pachygone ovata* (Menispermaceae), *Tiliacora accuminata* (Menispermaceae) and *Hyptis sauveleons* (Lamiaceae). These plants are reported to be traditionally used in treatment of conditions like ulcers, wounds, inflammations etc or as toxins. These plants are also reported to contain major phytochemical constituents such as alkaloids, which are known to possess antitumor properties. However, the bioactive

compounds responsible for the cytotoxic activity have yet not been identified from these plants. Thus there is a need to screen these plants for their potential anticancer properties and isolate the compound that may be accountable for this property.[35,36,37]

The identification of newer proteins with significant roles in regulation of tumour cell cycle progression and the use of these proteins as targets for high throughput screening helps to isolate molecules from plant sources and thus, proving to be an important reservoir of novel inhibitors of these key proteins, which could be developed into selective anti-cancer agents.[38]

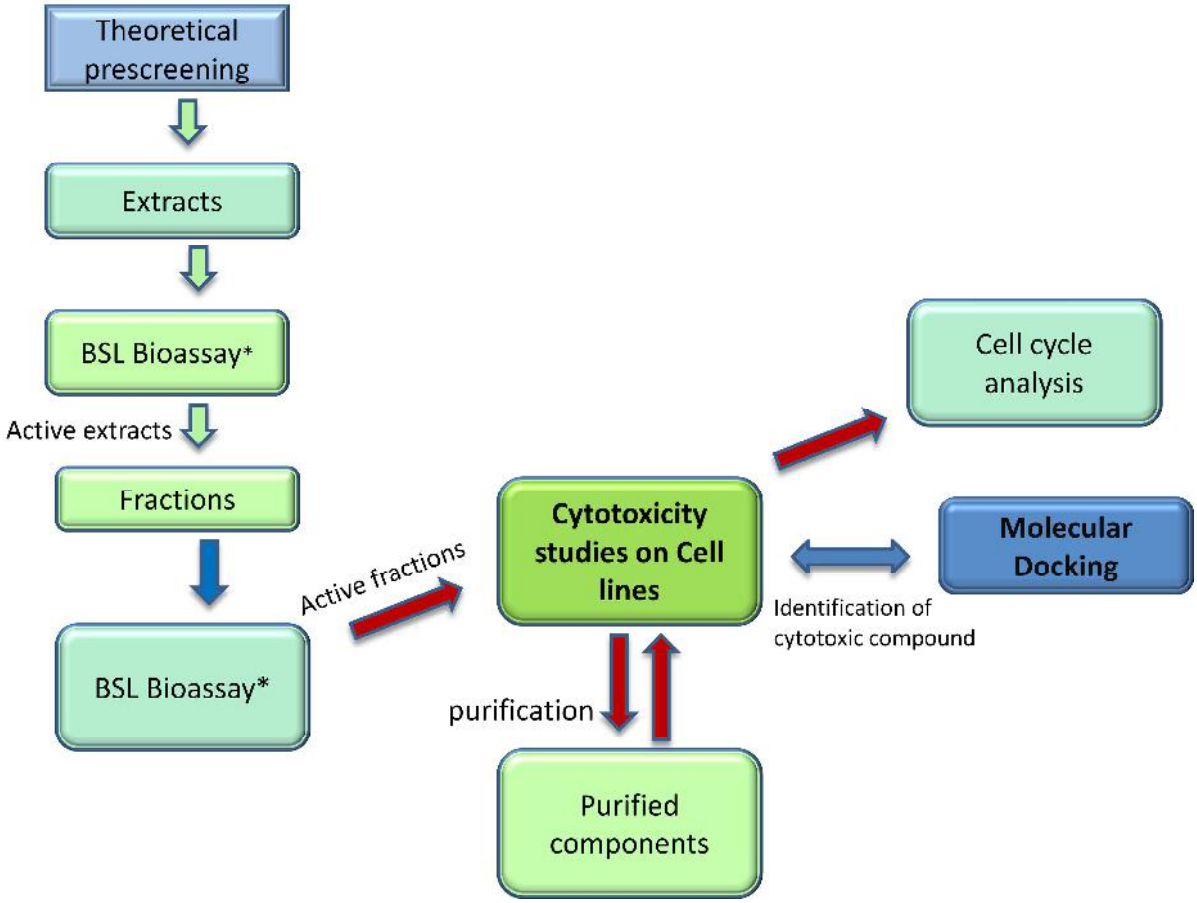
Thus, this research study is planned with the approach as to find potential lead molecule that will possibly be useful in the development of new anti-cancer drugs

1.4. Objectives

1. To identify lead molecules from medicinal plants of Traditional Indian System of Medicine through bioactivity guided fractionation.
2. To evaluate the *in vitro* cytotoxicity and anti-proliferative activity of the purified fractions using cell lines.

2. MATERIAL AND METHODS

Plan of work



2.2 Collection and authentication of plant material

The whole plant material of *C.hirsutus* was collected from wild regions of Belagavi and Jamboti, Western Ghats, Karnataka. Whole plants of *D. glaucescens* and *H. sauveolens* were collected from the wild from regions of Keri, Western Ghats, Goa. The authentication of plant materials of *C. hirsutus*, *D. glaucescens* and *H. sauveolens* was carried out by Dr. Harsha Hegde, Scientist 'D' at NITM-ICMR, Belagavi and herbaria were deposited with authentication numbers RMRC-1348, RMRC-1274 and RMRC-1349, respectively. The collection and authentication of whole plants of *P. ovata* and *T. accuminata* from the wild regions of Eastern Ghats, Andhra Pradesh, was done by Dr. K. Madhava Chetty, Botanist at Sri. Venkateswara University, Tirupati, Andhra Pradesh and herbaria deposited with voucher numbers 0948 and 0827, respectively. Plant material was washed under running water, dried, pulverised to coarse powder for further use.

2.3 Extraction and fractionation

The dried powdered plant was first subjected to cold maceration for 24 hours using 70% v/v ethanol. Following filtration, the dried marc was subjected to Soxhlet extraction using ethanol. The filtrates of both extraction methods were combined and final extract was obtained using a rotary evaporator (IKA-RV Digital). This extract was further subjected to fractionation via liquid-liquid partitioning to produce methanol fraction (F1), petroleum ether fraction (F2), dichloromethane fraction (F3) and aqueous fraction (F4).[99] The generic scheme of extraction and fraction has been shown in figure 3

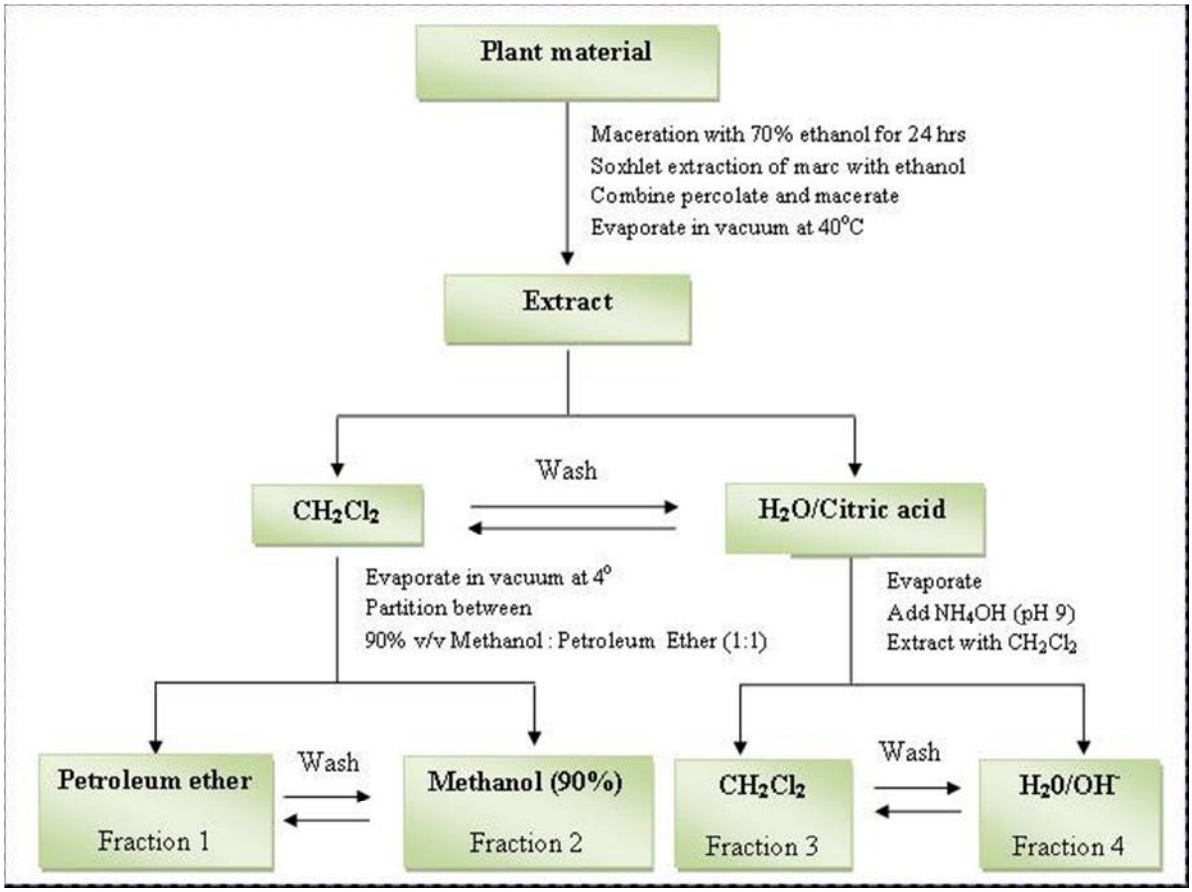


Figure 9. Extraction and fractionation scheme

2.4 Phytochemical investigations of extracts/fractions

Various tests were carried out on the extracts/fractions to detect the presence of major phytochemical classes of constituents i.e tannins, saponins, triterpenoids, steroids, alkaloids, glycosides, carbohydrates, fats and waxes.[100]

2.5 BSL Bioassay

Artemia salina Leach. eggs (Seamonk international Artemia cyst 003) were used for the assay. The bioassay method was carried out according to previous reports. Stock solutions (5000µg/ml) of test were prepared using 1% DMSO and serially diluted in geometric progression to get concentrations ranging 10-1000 µg/ml using natural sea water. Ten shrimps were exposed to 5ml of each test solution and number of surviving shrimps was

noted after 24 hours. Control tubes were maintained adding the same volume of distilled water. This assay was conducted in triplicates for all concentrations. Percentage mortality was calculated using the following formula and LC_{50} values were calculated by probit analysis.[101]

$$Percentage\ mortality\ (\%) = \frac{Total\ nauplii - Alive\ nauplii}{Total\ nauplii}\ X\ 100$$

2.6 Cell culture

Solid tumor cell lines HT-29 (Human, colon cancer), A-549 (Human, small cell lung carcinoma), HepG-2 (Human, hepatic cancer), MCF-7 (Human, breast cancer) and L-6 (Rat, normal skeletal muscle) cell lines were purchased from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were sub-cultured in 25 cm^2 culture flasks (Tarsons India Pvt. Ltd) using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% inactivated FBS, streptomycin (100 mg/ml), amphotericin B (5 mg/ml) and penicillin (100 IU/ml), incubated at 37°C in a 5% CO_2 incubator until confluent. Cells were detached by tripsinization using trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS).[102]

2.7 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay for evaluation of cytotoxicity

The active fractions from BSL assay were evaluated for cytotoxicity using MTT assay. Briefly, $1x10^4$cells were seeded per well in 96-well microtiter plates which were incubated for 24 hrs. Serial dilutions (10-1000 µl) of fractions were prepared and 100µl of each dilution was added to the wells. Plates were further incubated for 48 hrs in CO2 incubator at 37°C. 10µl of MTT (5mg/ml in PBS) was put in each well and incubated for

further 4 hrs. Supernatant was removed and DMSO was added into each well, following which, absorbance was quantified using a UVmicroplate reader at wavelength of 540 nm.[102]

2.8 Molecular docking

Docking of phytoconstituents with Caspase-3,-7 and -9 was conducted as previously described by Khanal *et al.* The selected ligand molecules i.e. coclaurine, cocsoline, cohirsinine, cohirsitinine, hirsutine, isotrilobine, shaheenine and trilobine were recovered from pubchem database. Files were converted using discovery studio 2017 and mmff94 force field was used to minimise and conjugate gradient method as optimization algorithm. The pose scoring minimum binding energy was selected as a ligand molecule for docking. Caspase -3,-7 and -9 (PDB ID: 4GHF) obtained from RCSB database was used as query sequence template for accession no: P11386.3 for homology modelling. Missing amino acid was added with the help of Modeller 9.10. This retrieved protein contained water molecules and other heteroatoms which were removed with Discovery Studio 2017; this avoids interference and docking was performed in autodock4.0 under Lamarckian GA 4.2. After docking, minimum pose score binding energy was selected to visualize the interaction between ligand-protein in Discovery Studio 2017.[103]

2.9 Cell cycle analysis

This assay was performed by the Propidium iodide staining method. 0.5-1x 10^5 cells were plated in 6 well- plates and kept in incubation for 24 hrs. 10 ug of C.F3, D.F3 and Paclitaxel were added to these wells and incubated for 6, 24, 30 and 48 hours. Control A549 cells were also maintained. Cells were harvested at the mentioned time points, fixed in 70% alcohol and stored at 4 ^oC for 30 minutes. After decanting the alcohol, cell were washed with 0.2% FBS-PBS and re-suspended in PBS containing PI (20ug/ml) and RNAse (100 ug/ml)

and incubated at room temperature for 30 min. Cells were analysed using flow cytometry (BD Accuri, photomultiplier tube 2).[104]

2.10 Apoptosis assay

The assay was carried out according to the procedure mentioned in the product manual of Annexin V-FITC Apoptosis Detection kit (Sigma-Aldrich). $1x10^5$ cells of A-549 were seeded in 6-well plates for 24 hrs incubation. 10ug/ml of C.F3, D.F3 and Paclitaxel were added to the cells and incubated for 6, 30 and 48 hours. Control cells without any treatment were also maintained. The cells were harvested at the mentioned time points and washed 2-3 times with DPBS and were re-suspended in 1xBinding buffer. Cell suspension (500µL) was taken in eppendorf tubes and Annexin V FITC Conjugate (5 µL) and Propidium iodide (10 µL) were added. These tubes were maintained at room temperature for exactly 10 minutes in dark and fluorescence was immediately measured with a flow cytometer (BD Accuri, photomultiplier tube 2).

2.11 Statistical analysis

All data were expressed as MEan±SEM using Graph Pad Prism ver 5.0. All assays were performed in triplicates. The IC_{50} values were calculated using a linear regression curve and lethal concentration (LC_{50}) was calculated probit analysis. The binding affinity of compounds was represented as binding energy.

3. RESULTS

3.1 Preliminary phytochemical screening

The extracts of *C.hirsutus, D,glaucescens, H.sauveolens, P.ovata* and *T.acuminata* were found to contain steroids, tri terpenoids, tannins, alkaloids, lipids, flavonoids and phenols.

3.2 BSL Bioassay for evaluation of bioactivity

The results of the toxicities of extracts and various fractions of *C.hirsutus, D,glaucescens, H.sauveolens, P.ovata* and *T.acuminata* against brine shrimps were found to be concentration-dependent. The percentage mortality of extracts and their various fractions at different concentrations have been depicted in figures 2,3,4,5 and 6 A total of 20 fractions were screened for their bioactivity using the BSL bioassay and 6 fractions: C3 (LC_{50}=34.48 ±2.405), D3 (LC_{50}=21.67 ± 2.53), P3 (LC_{50}=30.47±1.66), T3 (LC_{50}=45.57 ±1.67), H3 (LC_{50}=41.26 ± 1.237), H1 (LC_{50}= 56.178 ± 2.940) showed the most promising activity. The LC_{50} values of various extracts/fractions have been displayed in table 1.

Table 1. Effect of various extracts and fractions on brine shrimps

Name of the plant	Extract/Fraction	LC_{50} µg/ ml
C.hirsutus	Extract	59.71 ± 2.42
	Fraction 1	887.63 ± 2.346
	Fraction 2	108.54 ± 1.926
	Fraction 3	34.48 ± 2.405
	Fraction 4	119.401 ± 3.123

D. glaucescens	Extract	39.14 ± 1.97
	Fraction 1	85.59 ± 3.33
	Fraction 2	188.02 ± 1.62
	Fraction 3	21.67 ± 2.53
	Fraction 4	151.99 ± 1.33
P. ovata	Extract	58.411 ± 3.33
	Fraction 1	261.57 ± 2.14
	Fraction 2	163.92 ±3.73
	Fraction 3	30.47 ± 1.66
	Fraction 4	429.94 ± 3.27
H. suaveolens	Extract	63.043 ± 2.86
	Fraction 1	56.178 ± 2.940
	Fraction 2	150.993 ± 3.33
	Fraction 3	41.26 ± 1.237
	Fraction 4	284.89 ± 1.47
T. acuminata	Extract	51.299 ± 1.96
	Fraction 1	83.28 ± 0.66
	Fraction 2	332.7 ± 2.83
	Fraction 3	45.57 ± 1.67
	Fraction 4	395.22 ± 1.74

N=3, Values expressed as Mean±S.E.M

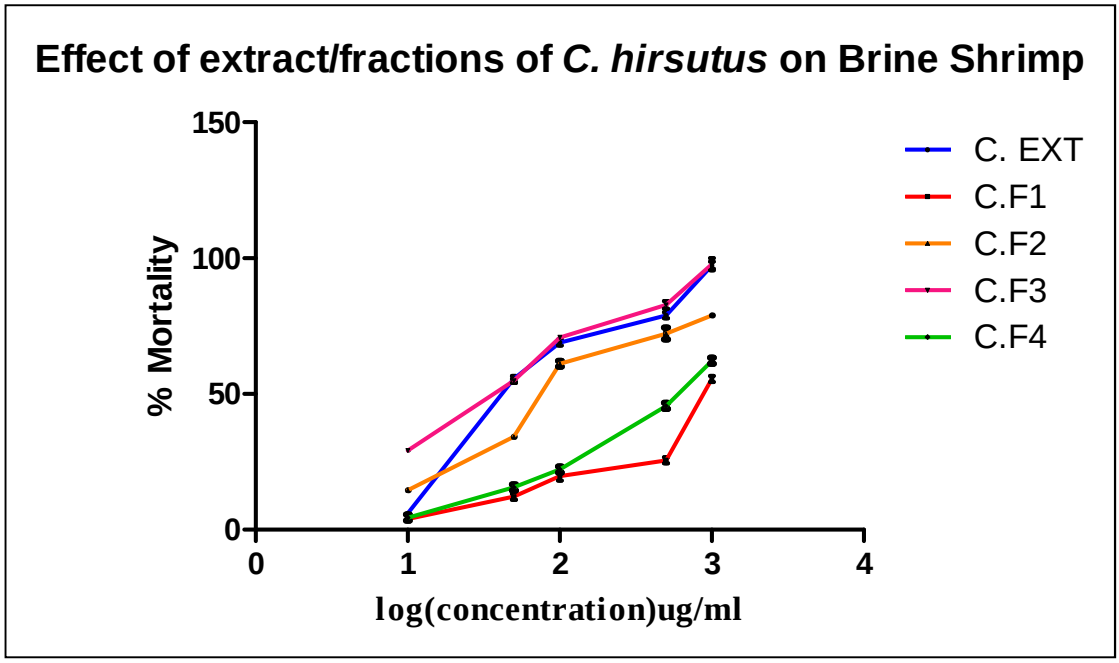


Figure 10 Effect of Extracts/fractions of *C.hirsutus* on brine shrimps

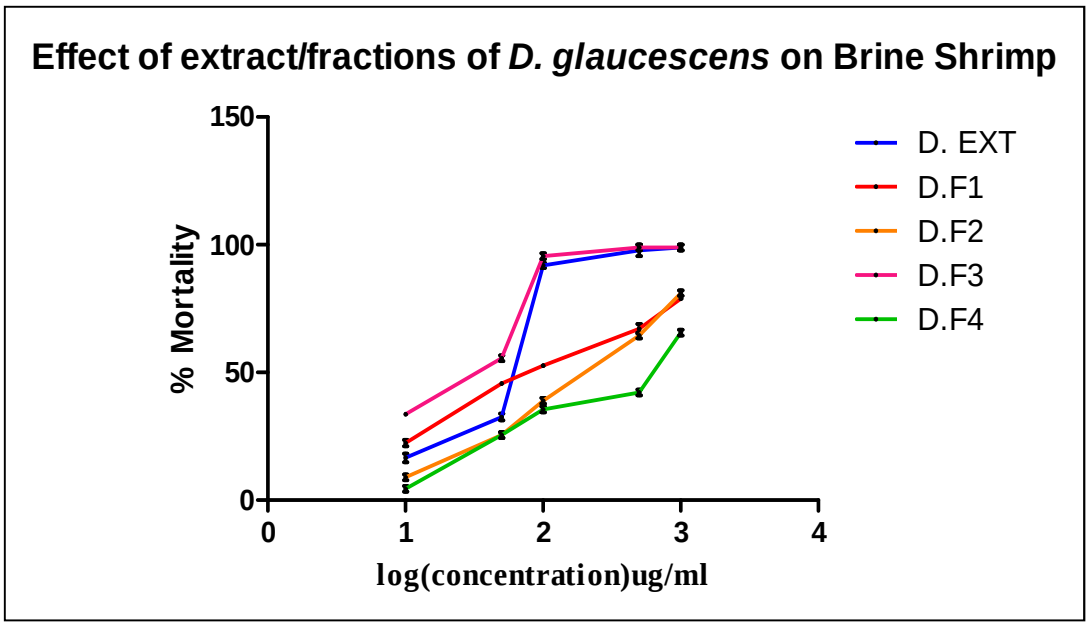


Figure 11. Effect of Extracts/fractions of *D.glaucescens* on brine shrimps

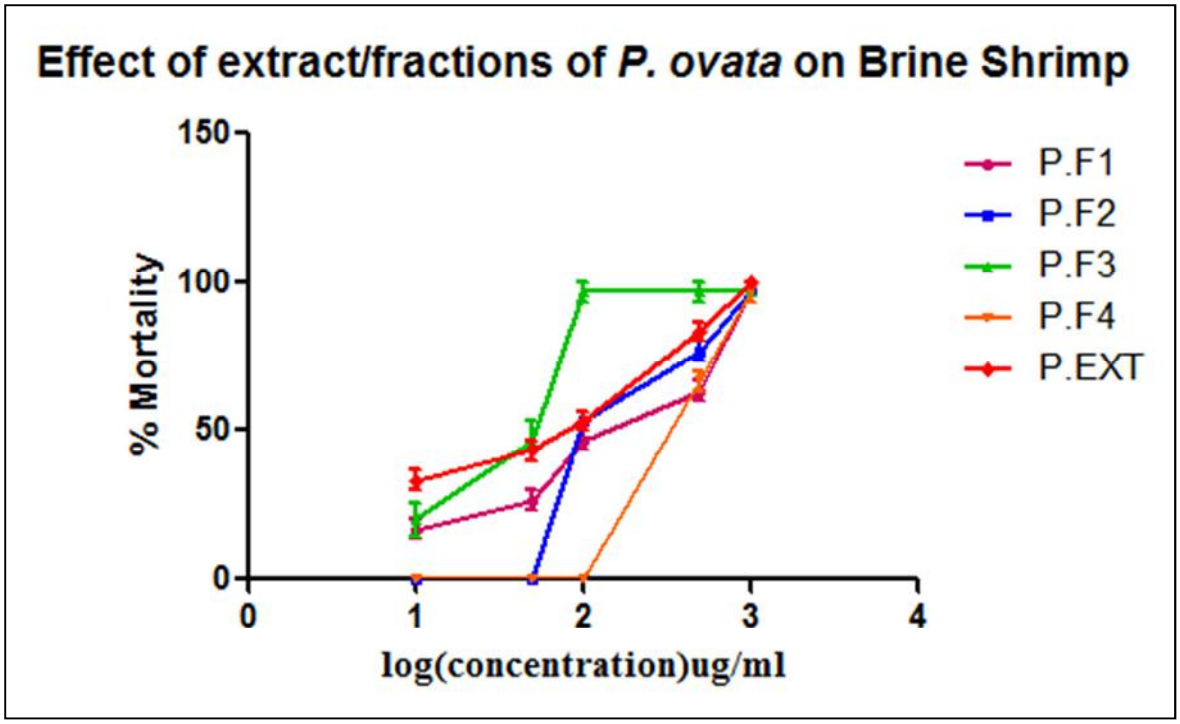


Figure 12. Effect of Extracts/fractions of *P.ovata* on brine shrimps

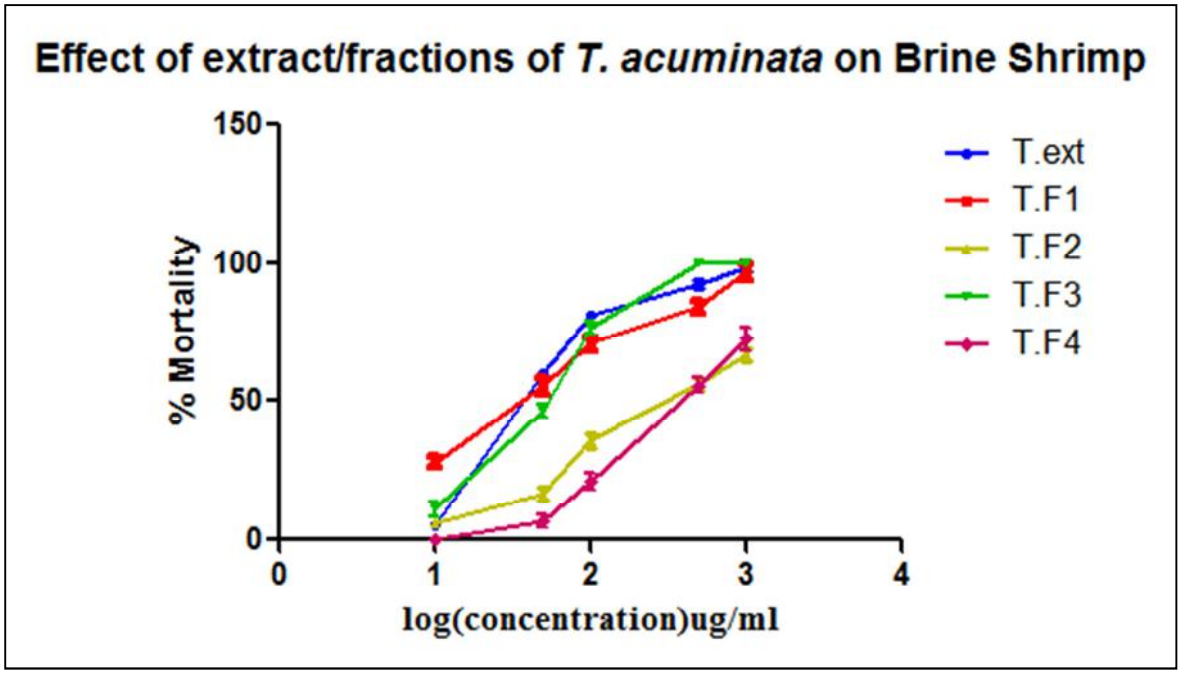


Figure 13. Effect of Extracts/fractions of *T.acuminata* on brine shrimps

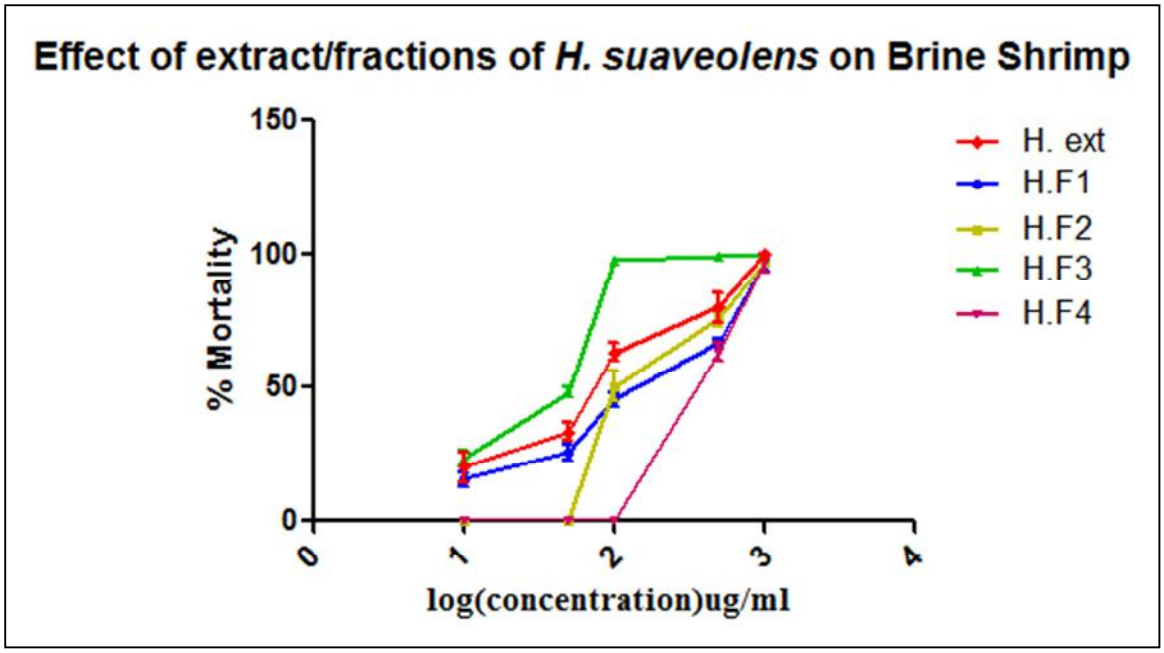


Figure14. Effect of Extracts/fractions of *H. sauveolens* on brine shrimps

3.3 MTT Assay for evaluation of cytotoxicity

The results of the MTT assay showed C3 and D3 to be more cytotoxic among the 6 fractions, with IC_{50} = 49.16 ± 4.970 and IC_{50} = 47.16 ± 0.7146, respectively against A-549 cell line. H3 did not show any noteworthy cytotoxicity in any of the cell lines. Only H1 was found to be cytotoxic towards MCF-7 cell line compared to the other fractions with IC_{50}=86.76 ± 5.210. The percentage inhibition was found to be concentration dependant and depicted in figures 15,16,17,18 and 19. The fractions were found to be biocompatible with normal cell line with IC_{50} >1000. The IC_{50} of the various fractions on a panel of cell lines have been shown in table 2.

Table 2. Effect of various fractions on cell lines

Sr. No	Sample	Cell lines IC_{50} µg/ml (mean ±SD)				
		A549	MCF-7	HT-29	HepG2	L-6
1	Fraction 3 *(C. hirsutus)*	47.16 ± 0.7146	217.17 ± 7.232	86.56 ± 9.953	37.2 ± 2.860	>1000
2	Fraction 3 *(D. glaucesens)*	49.16 ± 4.970	531.6 ± 13.75	45.08 ± 0.4950	96.76 ± 8.183	>1000
3	Fraction 3 *(P. ovata)*	84.76 ± 1.526	336.1 ± 25.02	91.12 ± 4.117	625.1 ± 22.72	>1000
4	Fraction 3 *(T. acuminata)*	127±6.44	>1000	79.52±9.690	185.1± 5.496	>1000
5	Fraction 3 *(H. suaveolens)*	398.1 ± 31.40	>1000	109.16 ± 4.801	>1000	>1000
6	Fraction 1 *(H. suaveolens)*	259.7 ± 19.14	86.76 ± 5.210	53.61 ±2.452	53.99 ± 130.5	>1000

N=3, Values expressed as Mean ±S.E.M.

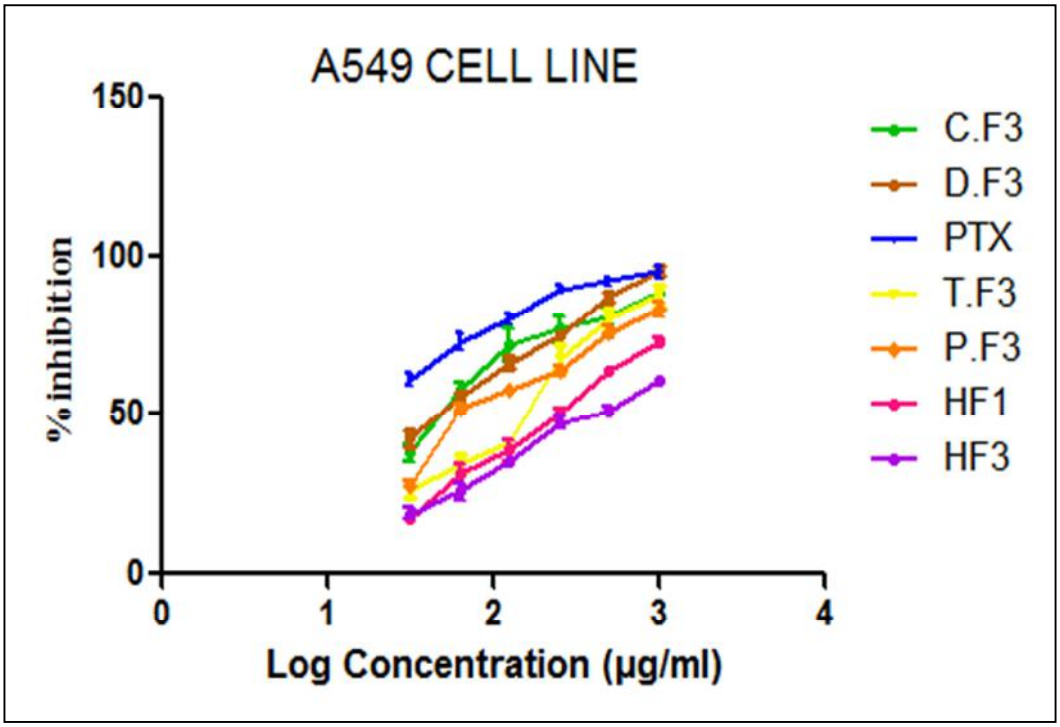


Figure 15. Percentage inhibition of various fractions on A-549 cell line

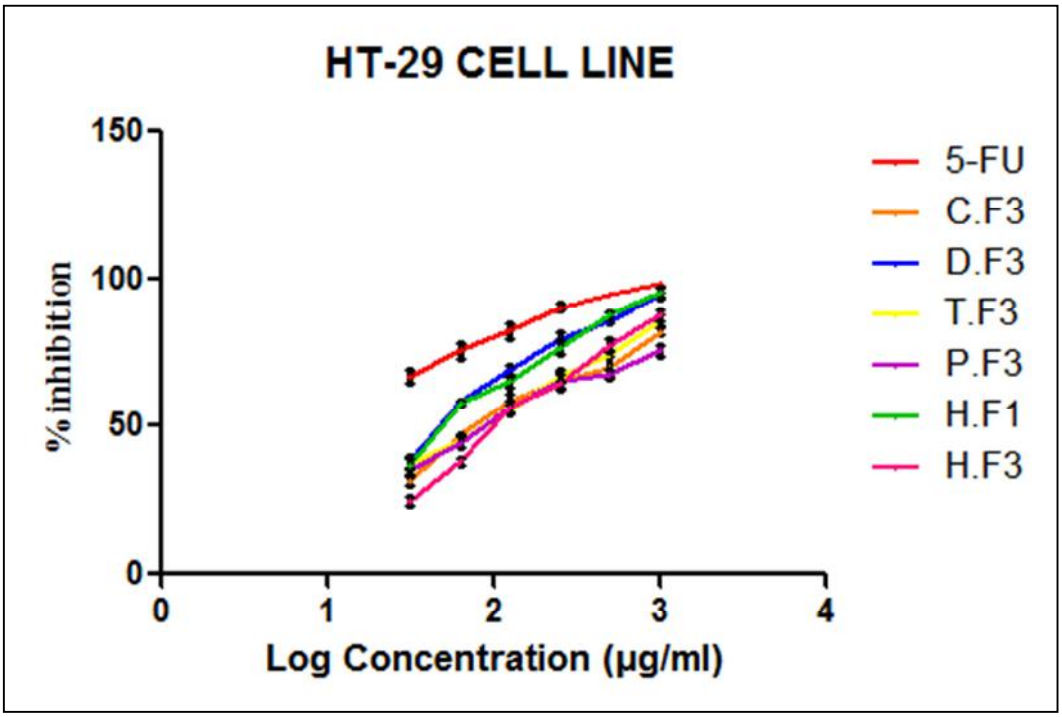


Figure 16. Percentage inhibition of various fractions on HT-29 cell line

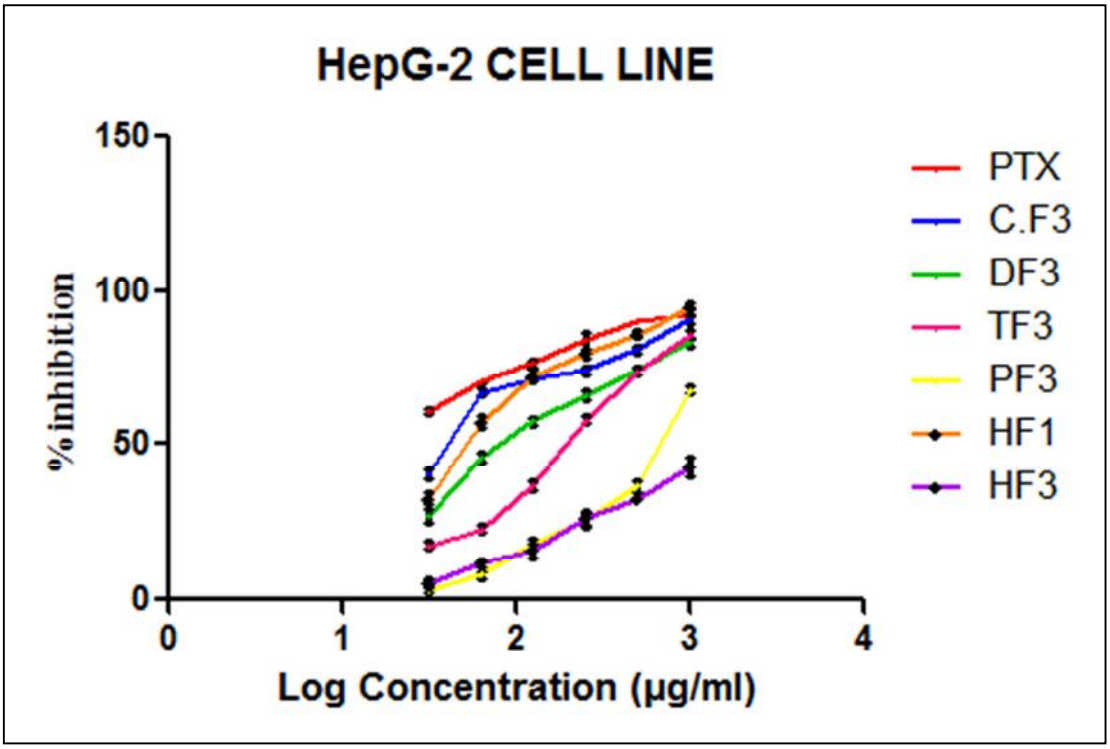


Figure 17. Percentage inhibition of various fractions on HepG-2 cell line

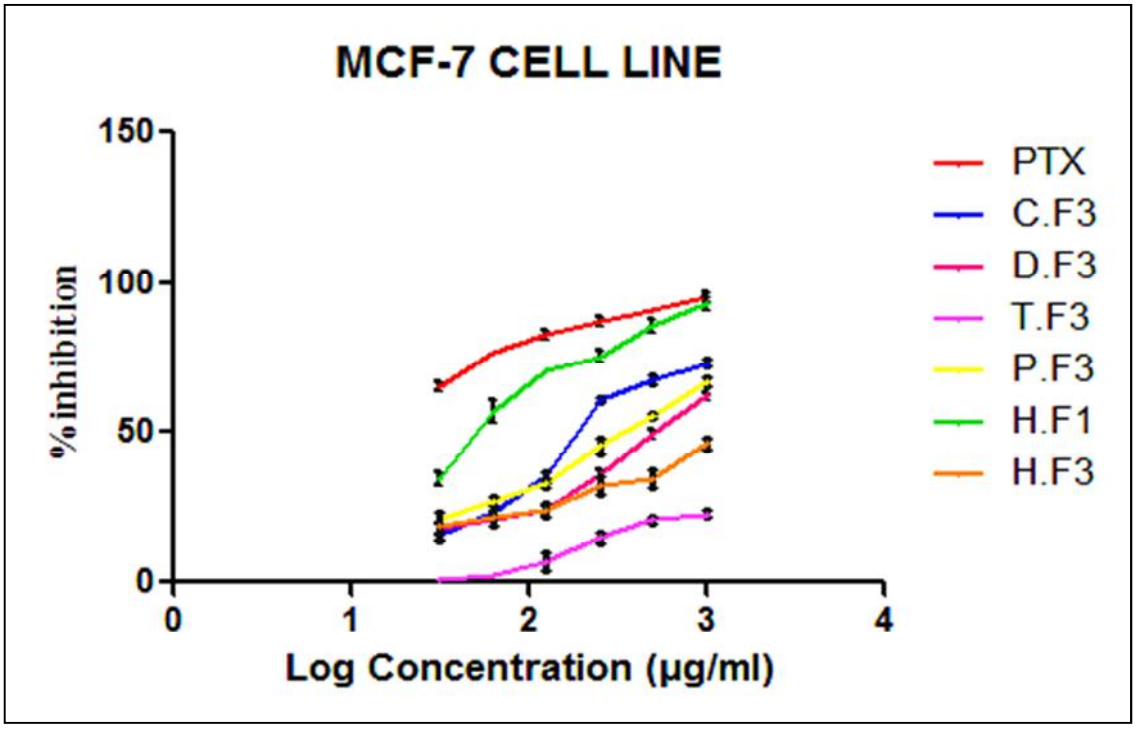


Figure 18. Percentage inhibition of various fractions on MCF-7 cell line

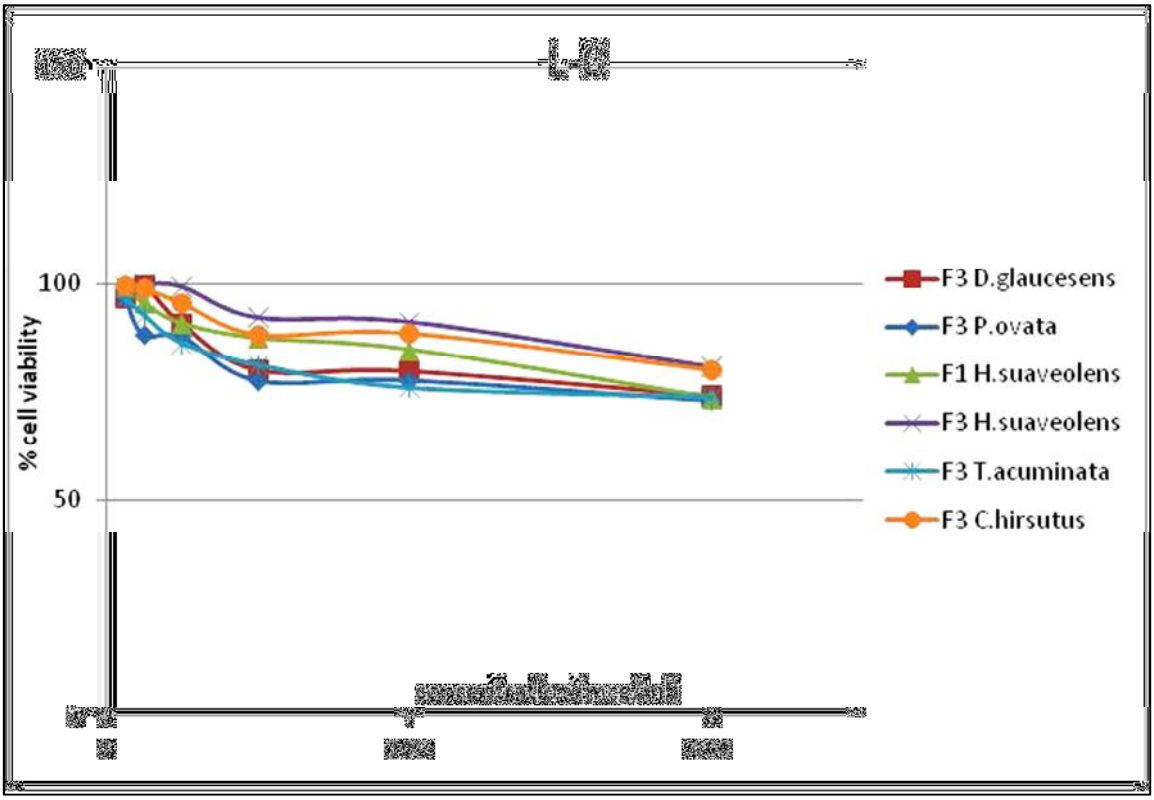


Figure 19. Percentage inhibition of various fractions on L6 cell line

3.4 Cell cycle analysis

Treatment of cells with C3 and D3 significantly inhibited cell growth in A549 cells at the selected time intervals in the study compared to untreated control cells. There was a noticeable shift in the population of cells in the treatment groups, indicated by the absence of the G2/M peak.(figure 20) Both the fractions, C3 and D3 exhibited a time-dependent effect on the cell cycle and causing significant G2/M arrest of A-549 cells. The representative PI plots at 24 hr interval have been shown in figure 21

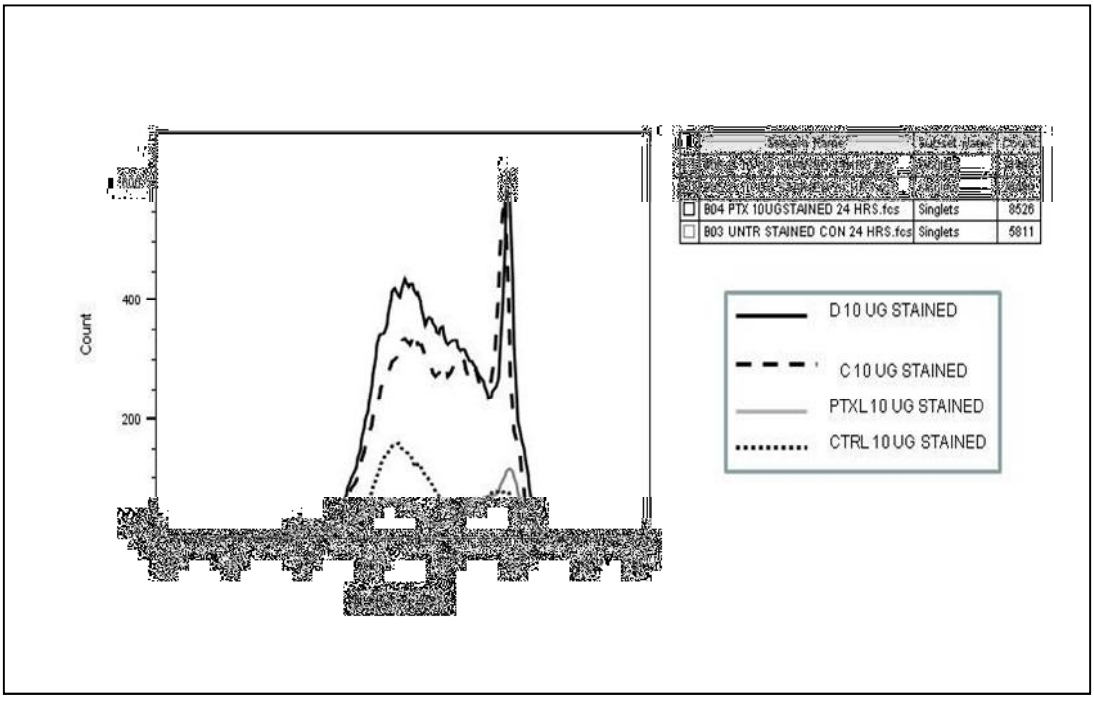
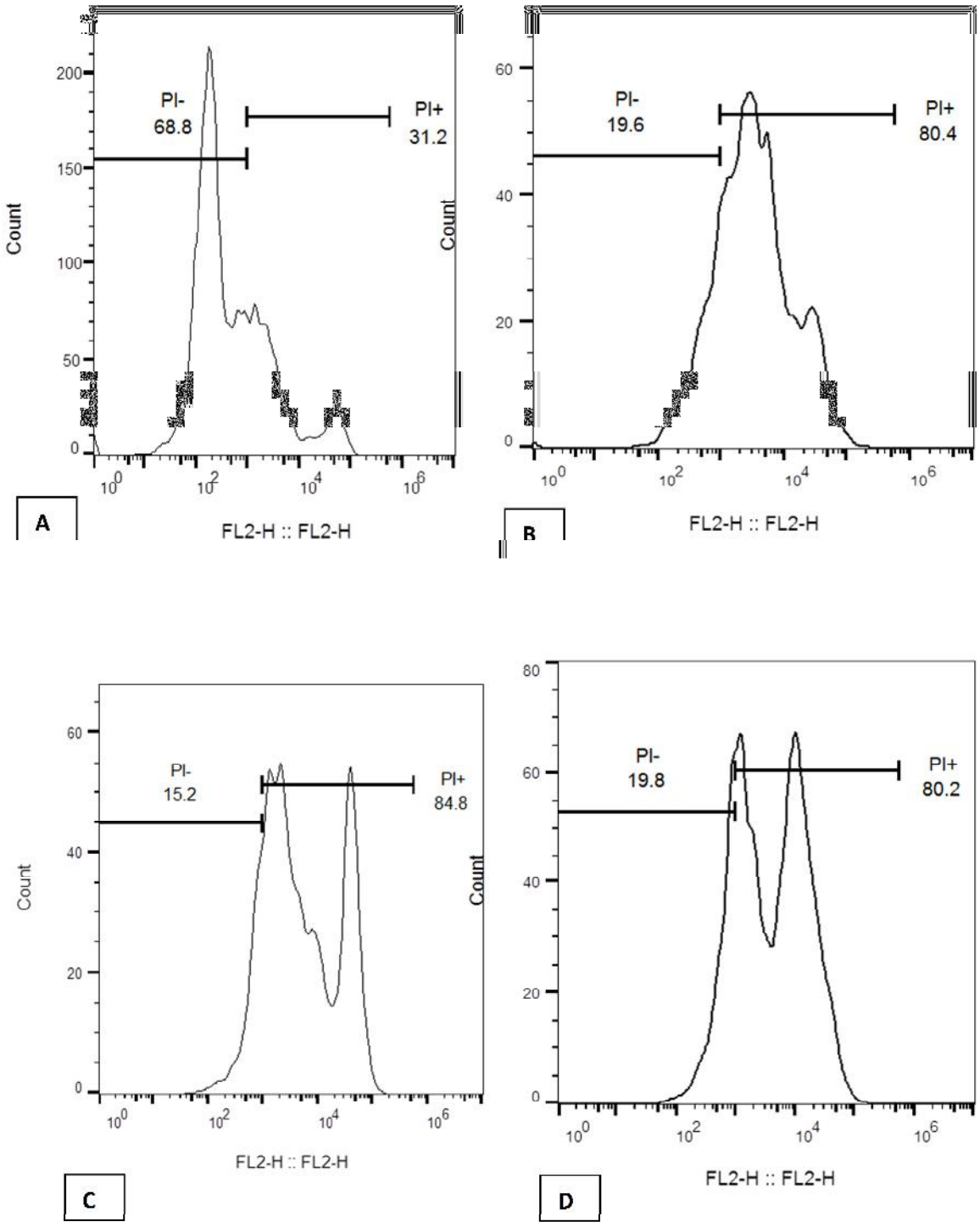


Figure 20. Cell cycle analysis after 48 hrs



A-Untreated A-549 cells, B- Paclitaxel (10mcg) treated A-549 cells, C- Fraction C3 (10mcg) treated A-549 cells, D- Fraction D3 (10mcg) treated A-549 cells

3.5 Apoptosis Assay

Apoptotic, early apoptotic, necrotic and viable cells were distinguished by the double staining method using Annexin-FITC and PI. Viable cells showed both PI and Annexin V negative staining. Early-apoptotic cells show PI positive and Annexin V negative. Apoptotic cells show negative PI staining and positive Annexin V staining. Necrotic cells show positive staining with both PI and Annexin V. Apoptosis was clearly observed upon examination of these molecular markers of apoptosis, seen after 6, 30 and 48 hours of exposure of both fractions C3 and D3 in A-549 compared to untreated control cells. A higher percentage of cells were seen in the early-apoptosis phase after 6 hours of treatment of both fractions. The percentage of cells in the apoptotic phase gradually increased over the time intervals. The viable, early apoptotic, apoptotic and necrotic cells have been shown in figure 15.

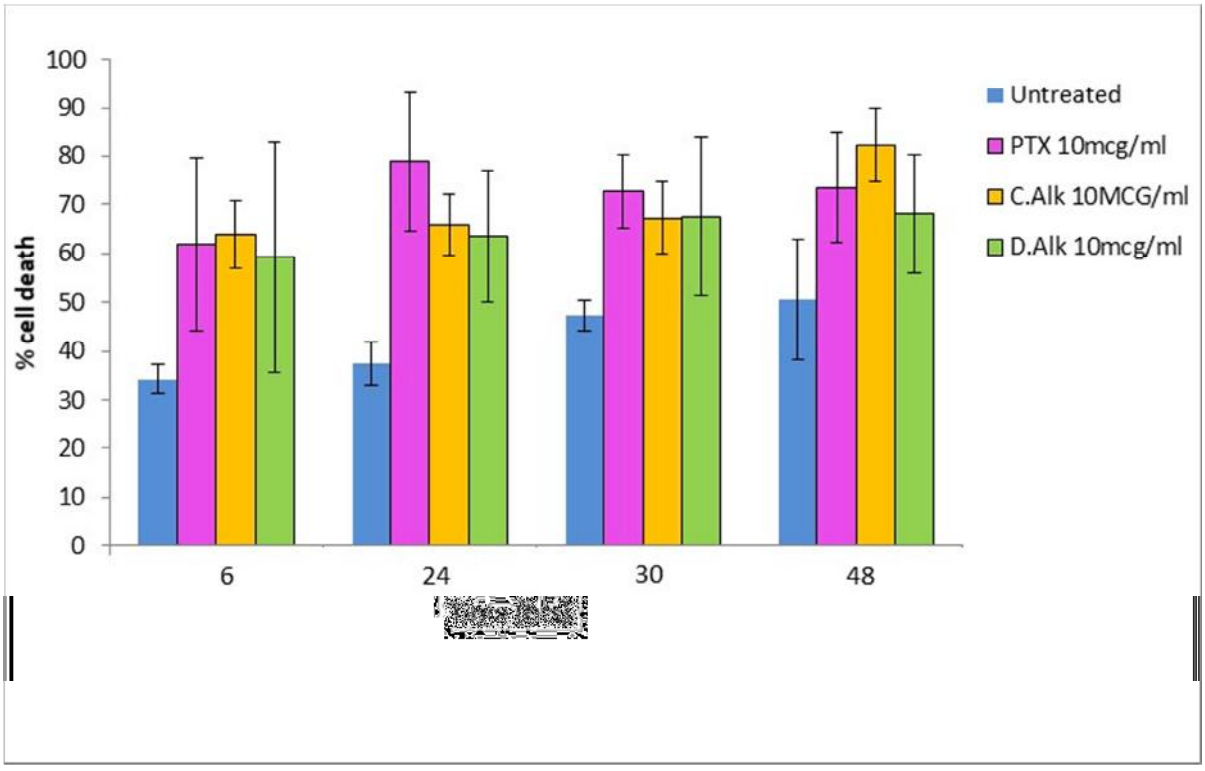
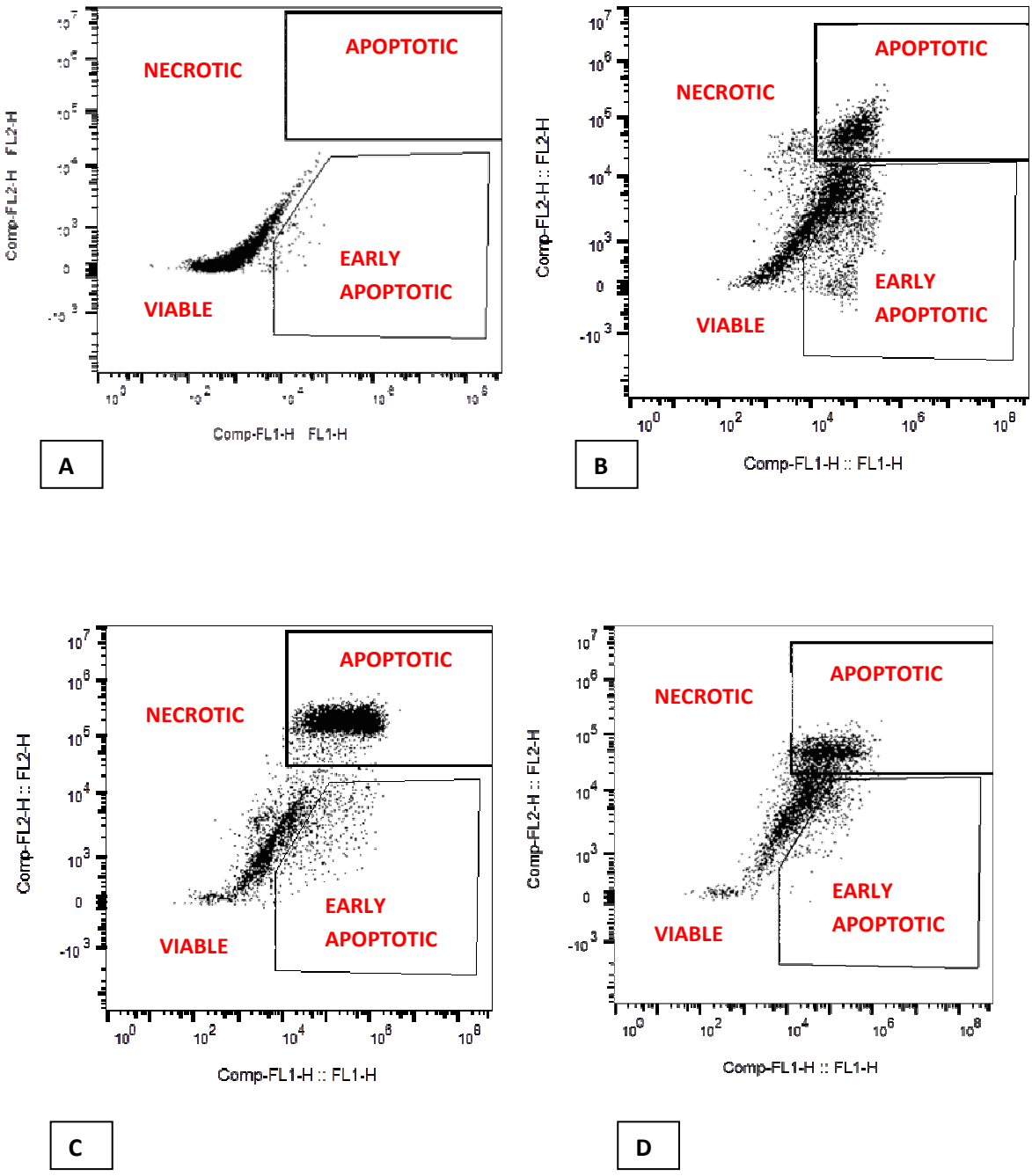


Figure 22. Percentage of cell death at different time intervals



A-Untreated A-549 cells, B- Paclitaxel (10mcg) treated A-549 cells, C- Fraction C3 (10mcg) treated A-549 cells, D- Fraction D3 (10mcg) treated A-549 cells

Figure 23. Representative images of cells in Viable, Early Apoptosis, Apoptosis and Necrosis phases after 24 hrs

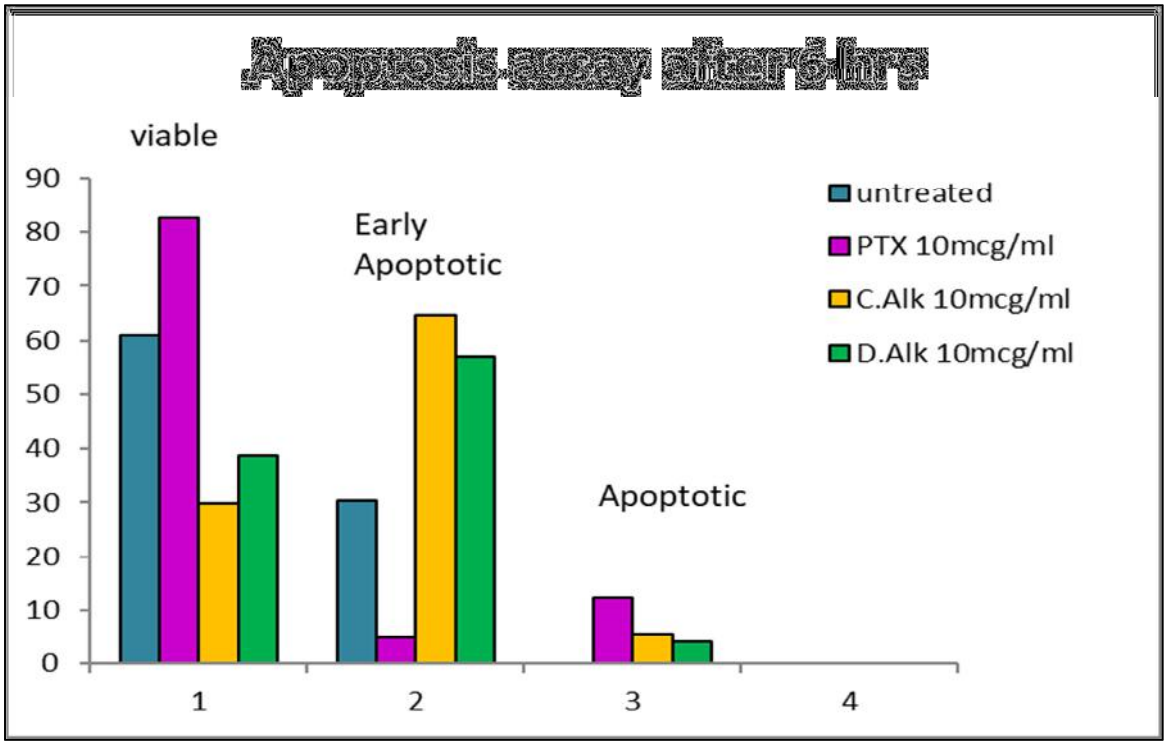


Figure 24. Apoptosis assay of C3 and D3 after 6 hrs

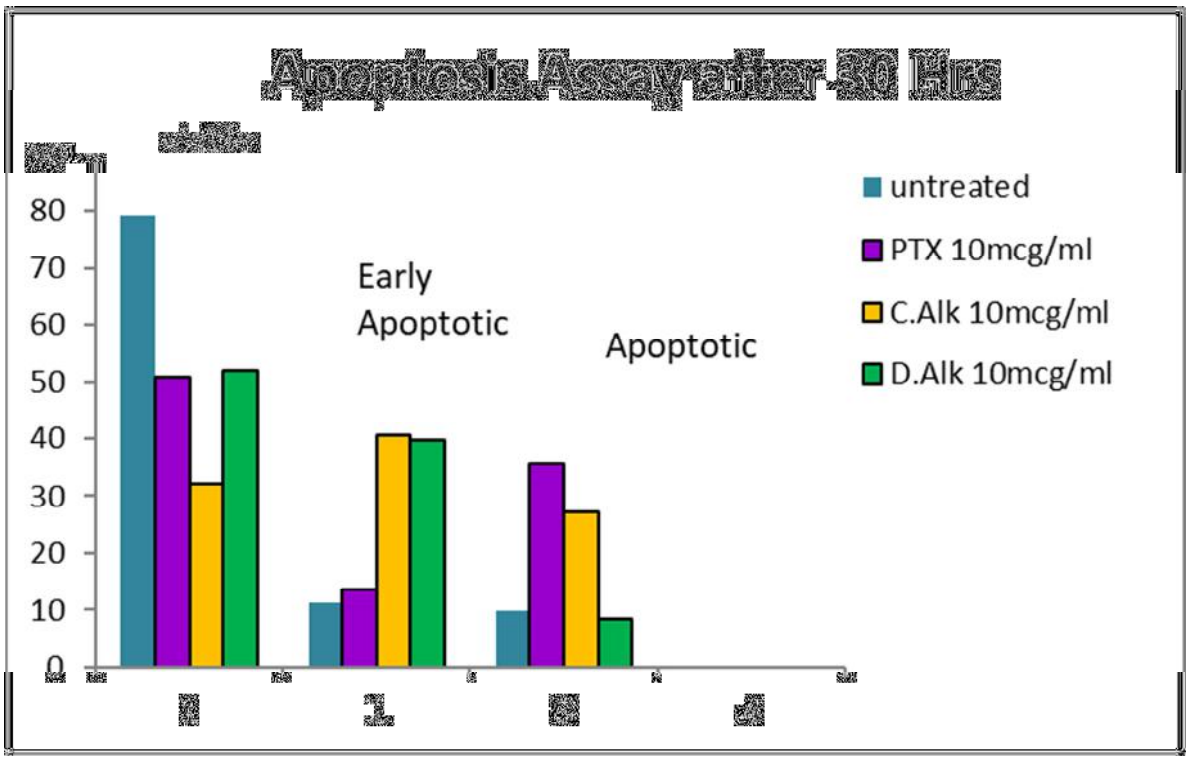


Figure 25. Apoptosis assay of C3 and D3 after 30 hrs

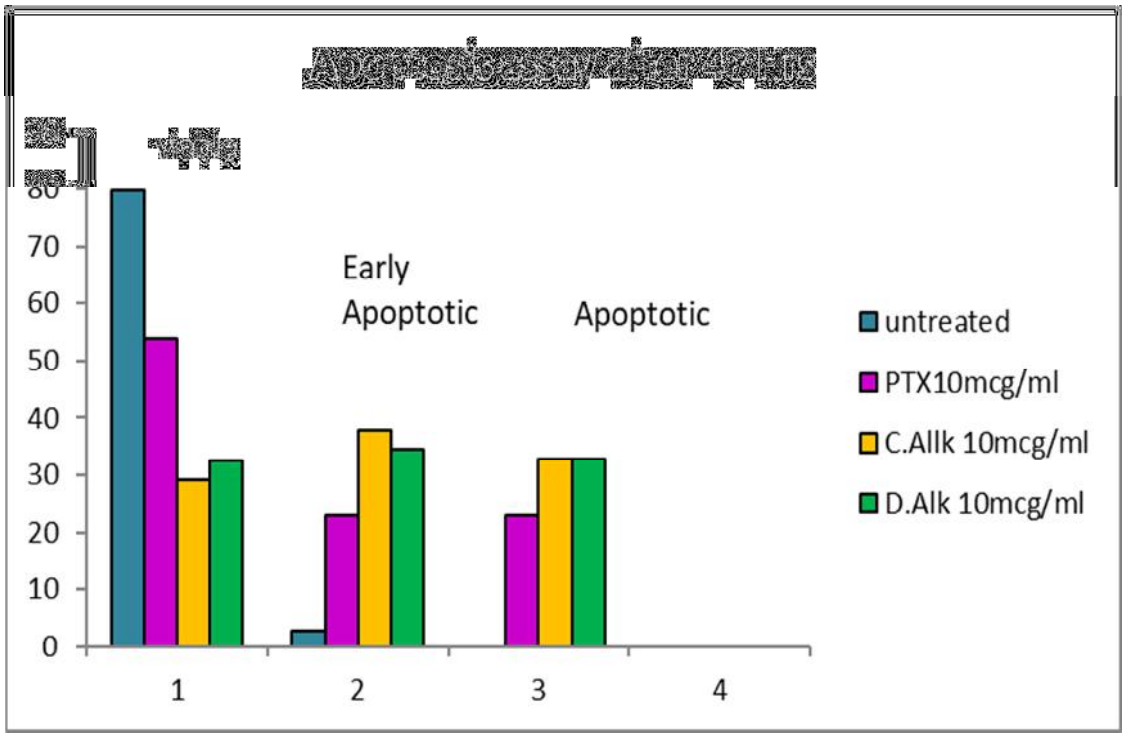


Figure 26. Apoptosis assay of C3 and D3 after 48 hrs

3.6 Molecular docking

Among the eight bioactives, trilobine was found to have highest binding affinity (-8.5 kcal/mol) with caspase-3 interacting with THR140. Highest number of hydrogen interaction was achieved by shaheenine with three amino acids i.e. ASP68, ARG75 and ASN89 of caspase-3 though it was found to have the binding energy (-6.1 kcal/mol) (table 3)

Similarly, cocsoline showed the most binding affinity (-8.8 kcal/mol) with caspase-7 with one H bond interaction with Asn148 (table 5). Shaheenine showed three H bond interactions with ASN91, GLU95, ASN112 of caspase-7 even though it showed lower binding affinity (-6.8 kcal/mol) compared to cocsoline. The binding affinities of Trilobine with Caspase-3 and cocsoline with Caspase-7 and -9 have been shown in figures 19,20 and 21, respectively.

Likewise, cocsoline showed highest binding affinity (-9.1kcal/mol) with caspase-9 via one H bond interaction with CYS403 (table 5). Hirsutine showed two H bond interactions with THR337 of caspase-9 even though it showed lower binding affinity (-6.7 kcal/mol) compared to cocsoline.

Table 3. Molecular docking of bioactives with caspase-3

Ligand	Binding energy (kcal/mol) with caspase 3	NHB with caspase 3	HBR with caspase 3
Coclaurine	-6.1	-	-
Cocsoline	-8.3	1	THR140
Cohirsinine	-6	1	THR140
Cohirsitinine	-5.9	2	GLU124, GLY125
Hirsutine	-6.2	1	ARG75
Isotrilobine	-7.9	-	-
Shaheenine	-6.1	3	ASP68, ARG75, ASN89
Trilobine	-8.5	1	THR140

Table 4. Molecular docking of bioactives with caspase 7

Ligand	Binding Affinity with caspase 7	NHB with caspase 7	HBR with caspase 7
Coclaurine	-6.1	1	ARG187
Cocsoline	-8.8	1	ASN148
Cohirsinine	-6.3	1	PHE166
Cohirsitinine	-6.3	1	ASN61
Hirsutine	-5.6	1	THR163
Isotrilobine	-8.3	1	ARG187
Shaheenine	-6.8	3	ASN91, GLU95, ASN112
Trilobine	-8.7	-	-

Table 5. Molecular docking of bioactives with caspase 9

Ligand	Binding Affinity with caspase 9	NHB with caspase 9	HBR with caspase 9
Coclaurine	-6.6	-	-
Cocsoline	-9.1	1	CYS403
Cohirsinine	-7.4	1	THE337
Cohirsitinine	-7.3	1	ASN265
Hirsutine	-6.7	2	THR337
Isotrilobine	-8.7	-	-
Shaheenine	-7.3	1	THR337
Trilobine	-8.8	1	PRO338

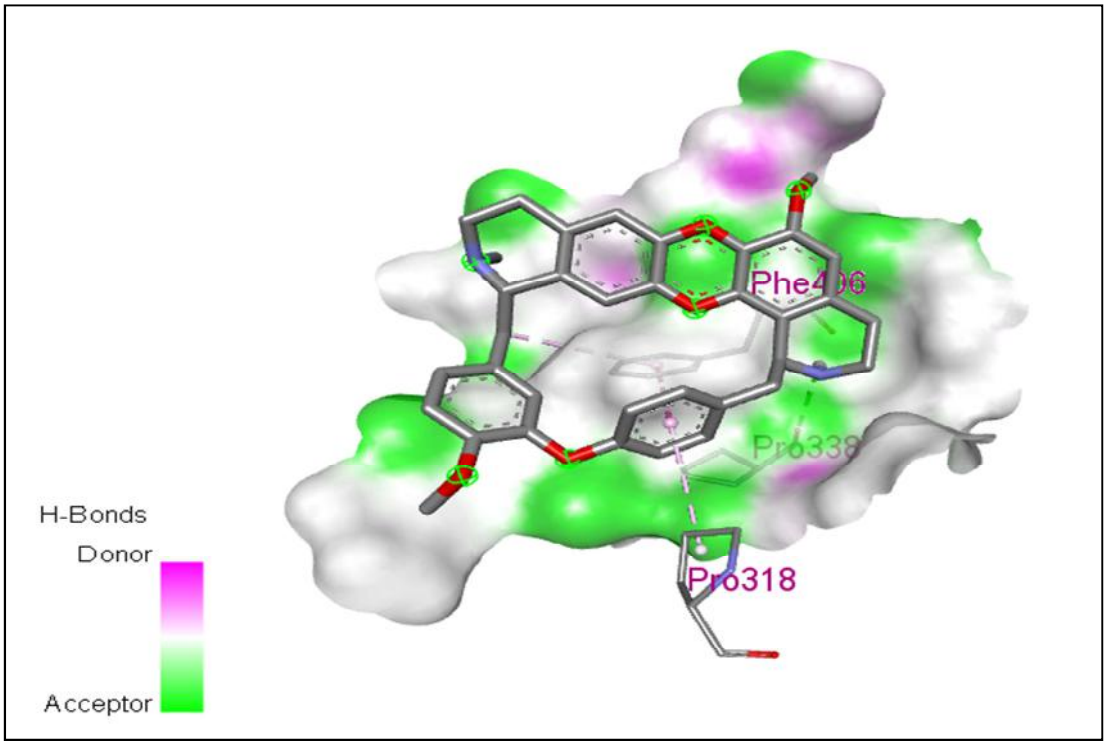


Figure 27. Interaction of trilobine with caspase 3

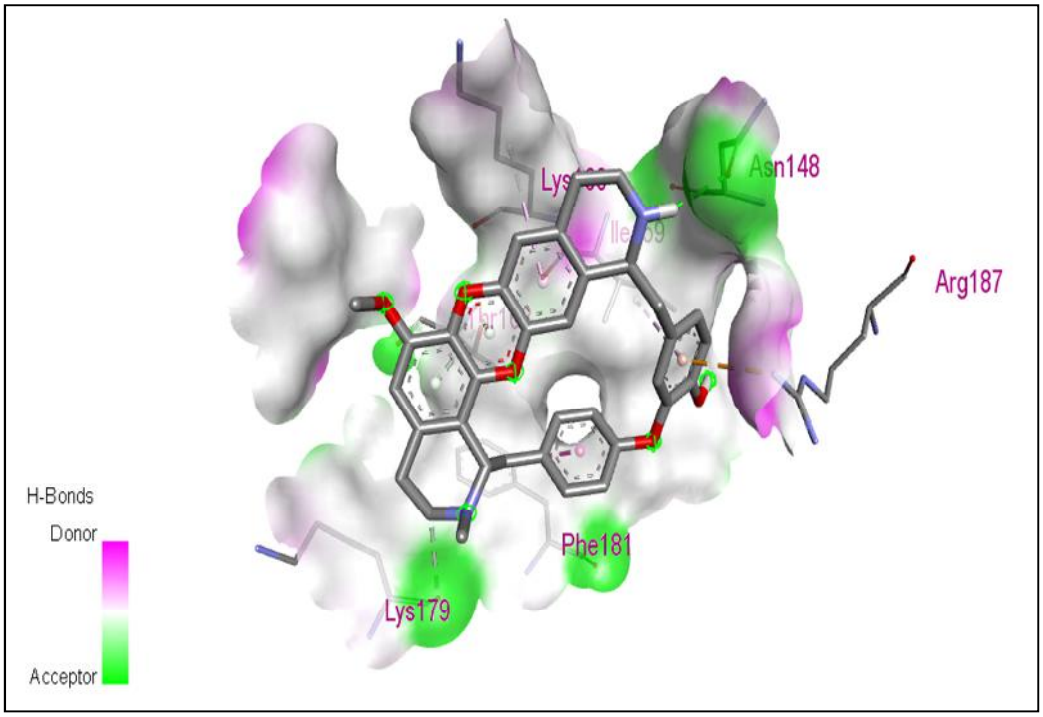


Figure 28. Interaction of COCSOLINE with caspase 7

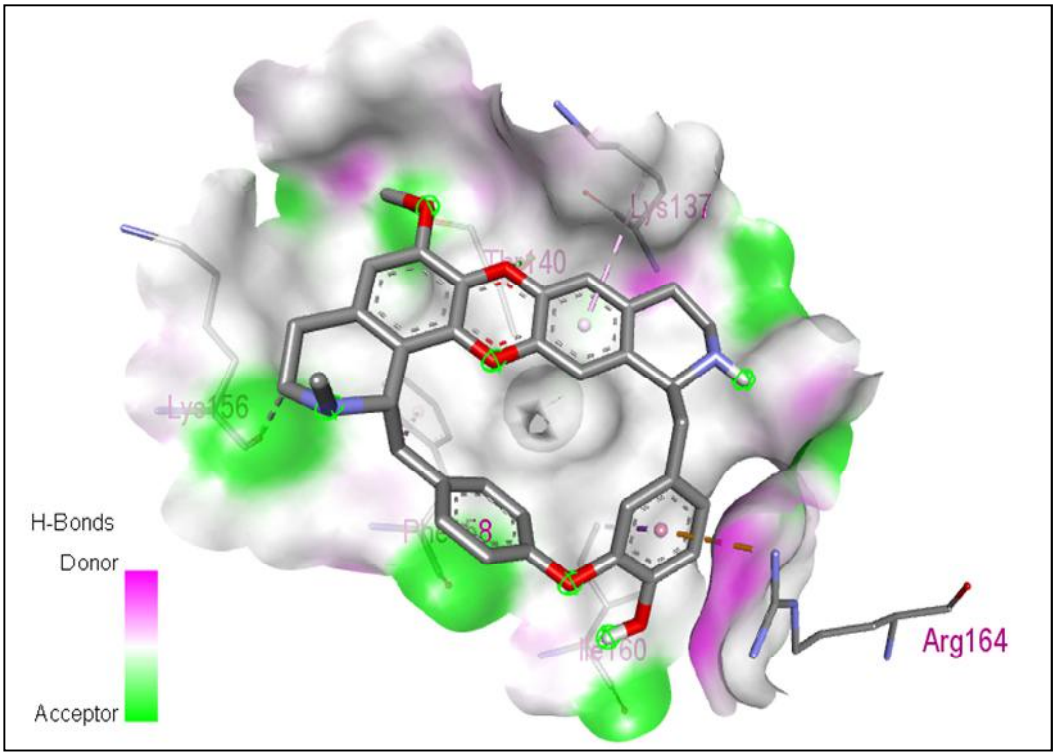


Figure 29. Interaction of COCSOLINE with caspase 9

4. DISCUSSION

In the present study, the initial screening of extracts of 5 plants: *C.hirsutus*, *D.glaucescens*, *H.sauveolens*, *T.acuminata* and *P.ovata* were done using the BSL bioassay. A pharmacological activity (bioactivity) of a phytoconstituent can be implied by its lethality towards lower organisms such as the brine shrimp larvae.[30] All 5 plant extracts were found to be bioactive in the BSL Bioassay. Hence 4 fractions of each plant extract were further evaluated for their bioactivities using BSL assay. Our results showed 6 out of 20 fractions with promising bioactivities. Compounds displaying LC_{50} <100 are often considered cytotoxic.[105] Thus in our study; the BSL bioassay effectively detected the potential toxicity or bioactivity of fractions C3, D3, H3, H1, T3 and P3. A positive correlation has been previously reported with the BSL bioassay to detect anticancer compounds in plant extracts. BSL bioassay and Human solid tumour cell lines have been found to have excellent correlation. Hence the 6 bioactive fractions from this assay were further taken forward for evaluation of *in vitro* cytotoxicity using cell lines.

The MTT assay was carried out against cell lines (A-549, MCF-7, HT-29 and HepG-2) to evaluate the cytotoxic effect of the 6 bioactive fractions from BSL bioassay. This assay measures the metabolic activity based on the reduction of the MTT dye which. 2 Fractions i.e. C3 and D3 were found to be more cytotoxic compared to the other fractions, displaying significant cytotoxic effect in 3 cell lines A-549, HT-29 and HepG-2. Fractions C3 (IC50 = 49.16 ± 4.970) and D3 (IC_{50} = 47.16 ± 0.7146) showed comparable cytotoxicities against A-549 cell line. H.F3 did not show any noteworthy cytotoxicity in any of the cell lines. On the other hand, H.F1 was the only fraction to display cytotoxicity towards the MCF-7 cell line.

Upon phytochemical screening, C3 and D3 fractions of *C. hirsutus* and *D.glaucescens* were found to be rich in alkaloids. Belonging to the Menispermaceae family, *C. hirsutus* and *D.glaucescens* are reported to contain isoquinooline and Bisbenzyl isoquinoline alkaloids.*C. hirsutus* is reported to contain bisbenzyl isoquinoline type alkaloids like cohirsitine shaheenine, trilobine, cohirsinine, hirsutine, cocsoline, cohirsine, haiderine and jamtinine.[80] *D.glaucescens* is reported to contain magnoflorine and stepharine.[106] A number of bisbenzyl isoquinoline alkaloids have shown antitumor effect in preclinical studies. Reports also suggest that the antitumor action may not only be directly on the tumors, but may also act via immunological mechanism of the hosts against tumors.[107] Kupchan et al., had also reported a correlation between the antitumor activity of bisbenzyl isoquinoline alkaloids and their chemical structures.[51] The extracts of leaves and rhizomes of *C. hirsutus* have previously shown cytotoxicity against UACC62 (melanoma) and TK10 (renal) cancer cell lines.[108] *C. hirsutus* leaf extract is also reported to possess positive modulator effects on the immune system in mice.[109] The extracts of *D.glaucescens* have previously shown cell cycle inhibition in mouse cell line ts-FT210.[110]

The cell cycle checkpoints are important in regulating the systematic flow of cell cycle events. In this study, we carried out cell cycle analysis to get insights into the possible mechanism of cytotoxicity of fractions C3 and D3. The results showed that treating cells with C3 and D3 fractions significantly inhibited cell growth in A549 cells at the various time intervals in the study. There was a noticeable shift in the population of cells in the treatment groups, indicated by the absence of the G2/M peak. This could be due to the strong cytotoxic activity of C3 and D3 which fragmented the DNA to such small fragments that the binding to PI was not possible and hence not detected. The cyclin/CDK complex closely regulates the cell cycle progression. So the level of cyclin expression is essential in progression of cell cycle, especially in G2/M and G1/S.[111] Previous studies of anticancer compounds on A-549

cell line have shown down regulation of cyclin D1 and increased p21 expression during G0/G1 or G2/M cell cycle arrests.[112] Bisbenzyl isoquinoline alkaloids have previously displayed decrease in expressions of cyclin D3, CDK4, E2F-1, phosphorylation of cdc2, c-Myc and cyclin D1 which could be responsible for the molecular mode of action of cell cycle arrest.[113] The cell cycle inhibitory effect of C3 and D3 could be possibly due to one or more of these molecular mechanisms.

Since cell cycle arrest often leads to Apoptosis, the ability of C3 and D3 to cause apoptosis in A-549 was carried out with Annexin V FITC apoptosis kit. The translocation of phosphatidylserine (PS) in the plasma membrane from the inner layer to the outer surface is a key feature in the Early-apoptosis phase. Quantification of Annexin V FITC binding to this PS in the external layer makes it easier to differentiate apoptotic cells and other cell populations. In our study, we examined the molecular markers of apoptosis. Cells in the apoptosis phase were observed as positive staining of both Annexin V FITC and PI using flowcytometry.[114] There was clear evidence of apoptosis after 6, 30 and 48 hours displayed by both fractions. Tetrandrine, a Bisbenzylisoquinoline alkaloid is reported to inhibit the growth of various types of cancer cells and reverse multidrug resistance by inhibiting P-glycoprotein activity.[113] Other bisbenzylisoquinoline alkaloids have reportedly inhibited the G0/G1 phase of hepatocellular carcinoma (SMMC7721) cells. They also reportedly decreased mitochondrial membrane potential and induced caspase-3 and caspase-9 activation.[115] Bisbenzylisoquinoline alkaloids have also shown apoptosis previously in human lung carcinoma, bladder cancer, colorectal cancer, gall bladder carcinoma, and prostate cancer cells.[116] Thus cell cycle inhibition and or consequent apoptosis may be the probable mechanisms of the cytotoxic effect of C3 and D3.

In this study, C3 from *C.hirsutus* showed a comparatively better potential of inducing apoptosis than D3. Since the progression of apoptosis is systematically regulated by a series of signal cascades, the binding affinity of some previously reported alkaloids from *C.hirsutus* with caspase -3,-7 and -9 was investigated through *in silico* molecular docking. The caspase-cascade system has significant function in the induction, transduction and amplification of intracellular apoptotic signals. Activated Caspase-9 has a role in cleaving Caspase-3, -6 and -7 and also initiates the caspase-cascade. Caspase-3 is vital in processes involving in dissembling the cell apparatus and development of apoptotic bodies. Caspase-7, on the other hand, is responsible for ROS production and promotes cell detachment during apoptosis.[117] The protein and ligand interaction occurs via a hydrogen bond and pi-interaction.[118]

Our results showed Trilobine with the most binding affinity with caspase -3 and Cocsoline showed the most promising binding affinity to caspase-7 and -9. The binding affinity implies that Trilobine and Cocsoline could fit into the binding cavity/pocket of their respective targeted proteins. Bisbenzyl isoquinoline alkaloids have shown interaction with caspase 3 in previous studies.[119] Although there are not many reports on the cytotoxic effect of Cocsoline, however there are reports suggestive of its strong antimalarial, antibacterial, anti fungal activities.[120] Trilobine has previously demonstrated multidrug-resistance-reversing activity in human breast cancer cells.[121] Thus the study finds Trilobine and Cocsoline to be promising molecules as seen by their binding potential with Caspase-3,-7 and -9.

5. SUMMARY

Cancer originates by genetic alterations which make cancer cells attain certain common properties that include infinite proliferation, self-sufficient growth signals, angiogenesis, invasion and metastasis and resistance to apoptotic and anti-growth signals. According to WHO, cancer is the second largest reason for deaths all over and is expected to continue in the upcoming years. Chemotherapy is the use of drugs to kill or inhibit the cancer cells. However, owing to the toxicities associated with current chemotherapeutic agents, it is necessary to find agents for cancer therapy from alternative sources. Plants have been a major provider of medicine to mankind since ancient times and still continue to hold importance in certain regions of the world. Indian system of medicine consists of thousands of medicinally active plants which could be explored for their cytotoxic potential as leads in anticancer research. With the use of rapid and reliable screening models such as the BSL bioassay, a number of plant extracts can be screened for their cytotoxicities. In the present study, 5 plants from Indian system of medicine- *C.hirsutus, D.glaucescens*, *H.sauveolens*, *T.acuminata* and *P.ovata* were screened so as to identify potential lead constituents. Six bioactive fractions from the BSL bioassay were further evaluated for their cytotoxicity against a panel of cancer cell lines. The most promising fraction i.e alkaloid rich fractions of *C.hirsutus* and *D.glaucescens* were further subjected to cell cycle analysis to get an insight into the mechanism of cell growth inhibition. Since cell cycle inhibition leads to apoptosis, these fractions were studied for their potential to induce apoptosis. The results showed the possible inhibition at the G2/M phase of cell cycle and apoptosis induction. The cytotoxic effect of alkaloid rich fractions could be attributed to one or both of these mechanisms. Further, molecular docking of alkaloids from C.hirsutus revealed most promising binding of Trilobine with caspase-3 and Cocsoline with caspase-7 and -9.

In conclusion, this study highlights alkaloids from *C.hirsutus* as a promising source of anticancer agents. The study also predicts trilobine and cocsoline as potential leads which may be useful in development of anticancer agents.

6. CONCLUSION

The present study carried out screening of 5 medicinal plants from the Indian traditional system of medicine for cytotoxicity and antiproliferative activity. The results revealed fractions rich in alkaloids from *C.hirsutus* and *D.glaucescens* to be cytotoxic agents against A-549 cell line.

The BSL Bioassay was successful in detecting potentially cytotoxic fractions containing alkaloids and thus, our approach of using the brine shrimp mortality assay as a guide for fractionation of the 5 plant extracts was productive, and supports the suitability of this assay as a pre-screening model for cytotoxicity.

Alkaloid rich fraction C.F3 from *C.hirsutus*, showed the most promising cytotoxicity against cell lines. Inhibition of cell cycle and/or apoptosis was revealed as the possible mechanisms of their cytotoxicity. The results also confirmed the apoptotic potential of D.F3 from *D.glaucescens*, Thus the alkaloid rich fractions of *C.hirsutus* and *D.glaucescens* may have importance as potential anticancer agents.

Furthermore, molecular docking predicted promising interaction of alkaloids from *C.hirsutus* with caspase 3,7 and 9. Trilobine and Cocsoline alkaloids were identified as potential lead molecules from *C.hirsutus*, which may be useful in anti cancer research.

In conclusion, this study revealed cytotoxic alkaloids from *C.hirsutus* which may be a promising group of phytoconstituents as a resource of newer anticancer agents. Further studies may be needed to establish the clinical value of these findings.

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8-ANNEXURES

PLANT AUTHENTICATION CERTIFICATES

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AUTHENTICATION CERTIFICATE

I hereby certify that the following plant species for pharmacognostical / pharmaceutical / pharmacological / phytochemical investigation research work is identified and their botanical name and family name is given.

Botanical Name	Voucher number	Family
Tiliacora acuminata (Lam.) Hook.f. &Thom	0948	Menispermaceae

Authenticated by

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AUTHENTICATION CERTIFICATE

I hereby certify that the following plant species for pharmacognostical / pharmaceutical / pharmacological / phytochemical investigation research work is identified and their botanical name and family name is given.

Botanical Name	Voucher number	Family
Pochygone ovata Miers. ex Hook.f. &Thom	0827	Menispermaceae

Authenticated by

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AUTHENTICATION

This is to authenticate that the plant brought by Ms. Jeswiny Rodrigues, Ph.D. Scholar, KLE University, Belagavi is identified as ***Diploclisia glaucescens*** **(Bl.) Diels.** belonging to family **Menispermaceae**.

Harsha Hegde
Scientist 'D'

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Date: 18-08-2016

AUTHENTICATION

This is to authenticate that the plants brought by Ms. Jeswiny Rodrigues, Ph.D. Scholar, KLE University, Belagavi are identified as ***Cocculus hirsutus* (L.) Diels. (Menispermaceae)** and ***Hyptis sauveolens* (L.) Poit. (Lamiaceae)**. The herbarium specimens of the same have been deposited in our herbaria with accession numbers RMRC-1348 and RMRC-1349 respectively.

Harsha Hegde
Scientist 'D'

RESEARCH PUBLICATIONS

1. Rodrigues J, Hullatti K, Jalalpure SS, Khanal P. In-vitro Cytotoxicity and in silico Molecular Docking of Alkaloids from *Tiliacora acuminata*. Indian J of Pharmaceutical Education and Research. 2020;54(2s):s295-s300. DOI: 10.5530/ijper.54.2s.86

2. Rodrigues J, Hullatti KK, Khanal P. *In silico* and *in vitro* cytotoxicity profile of hydroalcoholic extract/fraction(s) of *Pachygone ovata*. J Appl Pharm Sci, 2020; 10(05): 135–141. DOI: 10.7324/JAPS.2020.10518

Original Article

In-vitro Cytotoxicity and *in silico* Molecular Docking of Alkaloids from *Tiliacora acuminata*

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ABSTRACT

Background: The present study aimed to identify the cytotoxicity of *Tiliacora acuminata* extract/fraction(s) plant via bioactivity guided approach and predict the binding affinity with Topoisomerase II. **Materials and Methods:** Extract and fractions were screened using brine shrimp lethality bioassay and the potent fraction was further evaluated for its *in vitro* cytotoxicity using five different cell lines i.e. HT-29, HepG2, MCF-7 and A-549. The binding affinity of individual phytoconstituent from the potent fraction with Topoisomerase II was further predicted using autodock4. **Results:** The fraction containing alkaloids showed the highest cytotoxicity against the HT-29 cell line. Molecular docking study identified coclaurine as a potent alkaloid phytoconstituent to bind with topoisomerase II. **Conclusion:** The study revealed the potential cytotoxic alkaloids from *T. acuminata* which may find use in the development of cytotoxic or chemopreventive agents.

Key words: Alkaloids, Brine shrimp, Docking, Menispermaceae, MTT, Topoisomerase II.

INTRODUCTION

Cancer has emerged as a severe health issue worldwide, increasing at an alarming rate and it was responsible for 8.8 million deaths in 2015.[1] The primary abnormality that drives the development of cancer is the continuous unregulated proliferation of cancer cells, which invade normal tissues and eventually spread throughout the body; due to mutations leading to genetic alterations and abnormal proliferation of single-cell.[2]

Primarily, the current cancer therapy includes surgery, radiation and chemotherapy. Due to toxicity and contraindications associated with current drugs and limited selectivity towards cancer cells, the search for newer agents for cancer management and treatment is of great interest.[3] This necessitates the design and development of novel anticancer drugs or combinations in cancer biology. Targeted cancer therapy has come to attain great attention with a number of molecular targets being identified; opens door to potential treatment options for cancer pharmacotherapy. Topoisomerase II, a] catalyst in the cleavage and rejoining of double-stranded DNA is one such molecular target for the development of novel anticancer agents.[4]

Traditional medicine around the globe has utilized natural products as therapeutic agents since ancient times. The Indian traditional system of medicine itself offers a number of plants useful in the treatment of different conditions associated with cancer such as hard swellings, abscesses and inflammation.[5] The evaluation of their therapeutic efficacy may be useful in modern drug development. The chemotherapeutic properties of many phytochemicals have been well established in recent times. Currently, more than 60% of drugs used in therapy have been derived from natural sources.[6] The phytochemicals present in plants possess structural diversity that makes them a unique source in drug development. With the use of simple yet reliable

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pre-screening models such as the brine shrimp lethality assay, medicinal plants can be effectively screened for their cytotoxic potentials.[7]

Tiliacora acuminata (Lam.) belonging to the family Menispermaceae is a large woody climber. It is mentioned in Ayurvedic medicine as Krishnavetra which alleviates many ailments and cures cancerous diseases.[8,9] It is also traditionally used as an antidote to treat snake bites.[10] The root of *T. acuminata* contains a number of bisbenzylisoquinoline alkaloids and the cytotoxic activity of the root extract has been reported previously.[11] However; investigations on the active compounds are still in progress. Hence the present study aims to evaluate *T. acuminata* for *in vitro* cytotoxicity and *in silico* molecular docking.

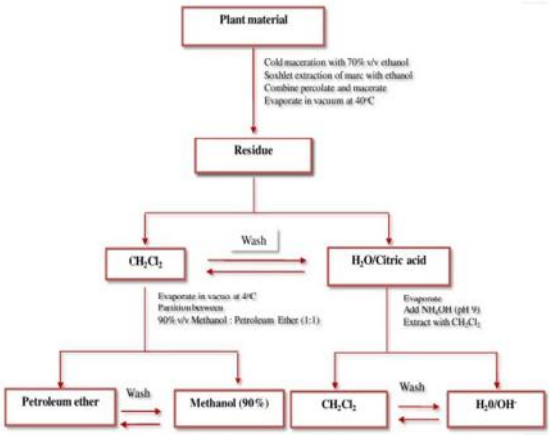

Figure 1: Scheme of extraction and fraction of *T. acuminata*.

MATERIALS AND METHODS

Collection of plant material

The whole plant of *T. acuminata* was collected from the wild regions of Tirupati, Eastern Ghats, Andhra Pradesh, authenticated by Botanist, Dr. K. Madhava Chetty at Sri. Venkateswara University, Tirupati, Andhra Pradesh and herbarium of the same was deposited with voucher number 0948.

Preparation of extracts and fractions

The extraction and fractionation were carried out according to the generic scheme described by Cos *et al.*[12] with minor modifications. The coarsely powdered material was subjected to maceration using 70% ethanol for 24 hrs. After filtration, the marc was dried and subjected to soxhlet extraction using ethanol as solvent. The macerate and percolate were then combined and concentrated using a rotary evaporator (IKA-RV Digital) to obtain the final extract. Fractionations of ethanol extract yielded methanol, petroleum ether, dichloromethane and aqueous fractions (Figure 1).

Brine shrimp Lethality (BSL) Bioassay

The brine shrimp (*Artemia salina* Leach.) eggs (Seamonk international Artemia cyst 003) were used for the assay. The bioassay was carried out according to the method described by McLaughlin *et al.*[13] with modifications. Stock solutions (5000µg/ml) of extracts/fractions were prepared in 1% DMSO and serial dilutions in geometric progression were made using seawater. Ten shrimps were exposed to 5ml of each test solution. Control tubes contained equal volumes of distilled water. The assay was carried out in triplicate for each concentration. At the end of 24 hr, the number of survivors was counted and LC_{50} values were calculated by probit analysis.

Cell culture

The cell lines A-549 (Human, small cell lung carcinoma), MCF-7 (Human, breast cancer), HepG-2 (Human, hepatic cancer), HT-29 (Human, colon cancer) and L-6 (Rat, normal skeletal muscle) were procured from National Centre for Cell Sciences, Pune, India. Stock cells were cultured and grown in 25 cm^2 culture flasks (Tarsons India Pvt. Ltd, Kolkata, India) using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were detached with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS).

MTT Assay for cytotoxicity

The bioactive fractions from brine shrimp bioassay were screened for cytotoxicity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[14] Briefly, cell monolayers were trypsinized, washed with culture medium and plated in 96-well microtiter plates and incubated for 24 hr. Each dilution of fraction 3 was added to the wells and plates were incubated for 48 hr in a humidified incubator at 37°C with 5% CO_2. After incubation, 10µl of MTT (5mg/ml in PBS) was added to each well and incubated again for 4 hr. The absorbance was measured using a microplate reader at a wavelength of 540 nm.

Molecular docking

Docking of phytoconstituents with topoisomerase II was performed as previously explained by Khanal *et*

al.[15] Briefly, the selected ligand molecules i.e. coclaurine, magnoflorine and stepholidine were retrieved from the PubChem database in. sdf format and converted into.pdb using discovery studio 2017; minimized using mmff94 force field and conjugate gradients as an optimization algorithm. After minimization, the pose scoring the minimum binding energy was chosen as a ligand molecule for docking. Topoisomerase II (PDB ID: 4GHF) was retrieved from the RCSB database; used as a template for query sequence for accession number: P11388.3 for homology modeling by adding the missing amino acid using Modeller 9.10. The retrieved protein molecule contained the water molecules and other heteroatoms; removed using Discovery Studio 2017; avoids docking interference and saved in .pdb format and docking was carried by using autodock4.0 under Lamarckian GA 4.2. After docking the pose scoring minimum binding energy was chosen to visualize the ligand-protein interaction using Discovery Studio 2017.

Statistical analysis

Data were expressed as Mean ± SD using Graph Pad Prism version 5.0. All experiments were performed in triplicates. IC_{50} was calculated using a linear regression curve and lethal concentration (LC_{50}) was calculated probit analysis. The binding affinity of compounds was represented as binding energy.

RESULTS AND DISCUSSION

BSL bioassay of extract and fractions

The extract of *T. acuminata* showed promising toxicity towards brine shrimps with LC_{50} of 51.299± 3.16 µg/ml. Fractions 3 showed the most cytotoxicity with LC_{50} of 45.57 ± 2.14 µg/ml; summarized in Table 1. The other fractions showed moderate to no activity in the BSL bioassay.

MTT Assay of the fraction

The fraction 3 showed the highest cytotoxicity against HT-29 (IC_{50}= 79.52±1.21 µg/ml) compared to the other cell lines A-549 (127±6.44µg/ml) and Hep G-2 (185.1± 8.16 µg/ml). Percentage cell growth inhibition was observed in a concentration-dependent manner and summarised in Figure 2. Fractions did not show cytotoxicity towards the MCF-7 cell line. The fraction was also found to be non-cytotoxic to normal cells displaying more than 70% cell viability at all the concentrations in the L6 normal cell line.

Molecular docking

Three phytoconstituents caclaurine, magnoflorine and stepholidine; previously reported in *T. acuminata* were studied by molecular docking for their binding affinity with topoisomerase II. Coclaurine was predicted for the highest binding affinity with topoisomerase II with a maximum number of H-bond interactions.

Stepholidine was found to show the least binding affinity with topoisomerase II. The binding energy, inhibitory coefficient and H-bond interactions of individual phytoconstituents with topoisomerase II are summarized in Table 2. The interaction of individual compounds with topoisomerase II is shown in Figure 3.

Table 1: Lethal concentration (LC_{50}) of extract/fractions of *T. acuminata* in BSL Bioassay.

Extract / Fractions	LC_{50} (ppm)
T. acuminata hydroalcoholic extract	51.299 ±1.13
Fraction 1	83.28±1.35
Fraction 2	332.7±2.26
Fraction 3	45.57±1.56
Fraction 4	395.22±1.19

Values are expressed as Mean ± SEM

Table 2: Binding energy and inhibition constant of compounds with Topoisomerase II.

Phytoconstituents	Topoisomerase II (PDB: 4GHF)		
	BE (kcal/mol)	IC_{50}(µM)	Interacting Hydrogen Bond Residues
Coclaurine	-5.29	133.53	ASP 479, ASP 446, ARG 450, GLU 454, LYS 480
Magnoflorine	-5.21	150.75	LEU 473, GLY 474,
Stepholidine	-4.48	520.33	SER 709

BE: Binding Energy, IC_{50}: Inhibitory Concentration 50

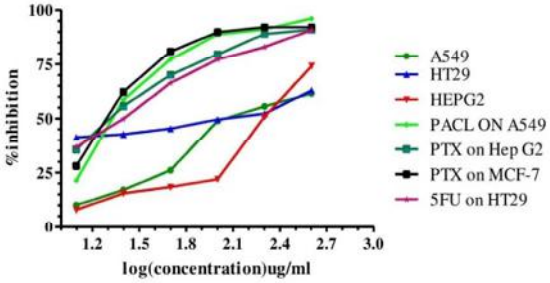

Figure 2: Percentage inhibition of cell growth of *T. acuminata* fraction 3 on various cell lines. Values are expressed as Mean±SEM (*n*=3).

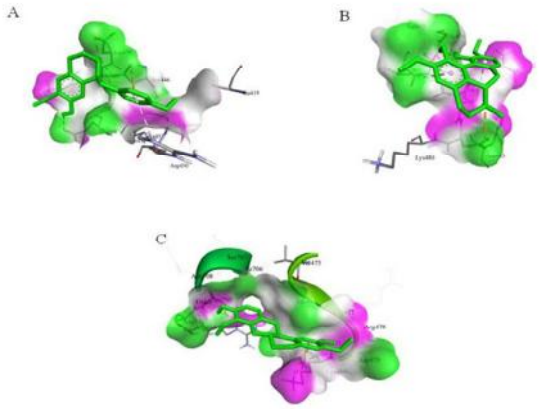

Figure 3: Interaction of (a) Caclaurine, (b) Magnoflorine, (c) Stepholidine.

The present study aimed to identify potential phytoconstituents from *T. acuminata* with special reference to cytotoxicity using *in vitro* and *in silico* approach. To deal with this approach, BSL bioassay and MTT cytotoxicity assay were performed which identified alkaloid fraction as a promising group of phytoconstituents. Initially, BSL bioassay was performed to pre-screen the fractions to narrow down the active fraction from *T. acuminata* extract. This bioassay has been successful in the identification of many bioactive components from natural sources including cytotoxic constituents.[16,17] Compounds with LC_{50} lower than 100 µg/ml may be considered as cytotoxic.[18] The lethality to brine shrimps has been frequently reported as a reliable pre-screening assay to the existing cytotoxicity assays.[19] Thus, rapid screening of extracts and fractions for potential cytotoxicity can be obtained by this assay.

In the present study, a fraction rich in alkaloids showed the highest toxicity to brine shrimps. The methanolic fraction 1, petroleum ether fraction 2 and aqueous fraction 4 did not show promising cytotoxicity in the BSL bioassay. Hence alkaloidal fraction of *T. acuminata* was taken assessed for *in vitro* cytotoxicity studies using a panel of cell lines. The ability of the fraction to inhibit cell growth was determined by reduction in the MTT dye to soluble formazan crystals by metabolically active mitochondrial enzymes.[20] The alkaloidal fraction showed the cytotoxicity in HEPG2 and HT29, however, the highest cytotoxic potency was towards the HT-29 cell line. Diphenylbisbenzylisoquinoline alkaloids have been identified as the principal components in *T. acuminata*.[21] The cytotoxic activity of *T. racemosa* has been previously reported in acute myeloblastic leukemia (HL-60), chronic myeloblastic leukemia (K-562) and cervical epithelial carcinoma (HeLa) tumor cell lines.[22] Tiliarine has previously shown *in vitro* inhibition of human melanoma cells.[23]

Targeting topoisomerase II is a well-accepted approach in cancer chemotherapy by inhibiting the enzyme-mediated DNA damage. Topoisomerase II enzyme is of vital use in cell processes namely, replication, transcription, chromosome separation and segregation since it acts as a catalyst in the cleavage and rejoining of double-stranded DNA. Topoisomerase II is reported to be highly overexpressed in proliferating cancer cells and hence makes it a potential target for new anticancer drugs.[24] Further, previous docking studies also report the interaction of multiple phytoconstituents with various protein molecules.[15,25] Hence, we investigated the binding affinity of reported alkaloids from *T. acuminata* with topoisomerase II. Nine different alkaloids i.e. coclaurine, coreximine, isoboldine, liridenine, magnoflorine, norjuziphine, nortrilobine, stepholidine and trilobine were investigated to predict the binding affinity and possible phytoconstituent-topoisomerase II interaction using *in silico* molecular docking. Interestingly, we identified coclaurine to have the highest binding affinity by interacting with ASP 479, ASP 446, ARG 450, GLU 454, LYS 480 amino acid residues (Table 2). The present study is also supported by the anti-cancer activity of coclaurine on various cell line studies.[26] Further, previous studies suggest that a lead molecule can target multiple proteins and regulate the multiple pathways in a particular disease[27,28] which is the future scope of the study to evaluate the role of hit molecules in cancer treatment. Further, a single compound can modulate multiple protein/pathway(s)[29] which can be further evaluated for Coclaurine via gene set enrichment analysis with special reference to the pathways involved in cancer pathogenesis.

CONCLUSION

The present study utilized bioactivity guided screening of hydroalcoholic extract/fraction(s) of *Tiliacora acuminata* which identified fraction rich in alkaloids to be a potent agent against multiple cell lines. Additionally, coclaurine was predicted to have the highest binding affinity with Topoisomerase II which needs to be further confirmed via *in vitro* enzyme assay. Additionally, we suggest performing a coclaurine-modulated gene set enrichment analysis with special reference to protein/pathway(s) involved in cancer pathogenesis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

BSL: Brine Shrimp Lethality, **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **PDB:** Protein Data Bank, **RCSB:** Research Collaboratory for Structural Bioinformatics, **IC_{50}:** Inhibitory concentration 50, **LC_{50}:** Lethal concentration 50.

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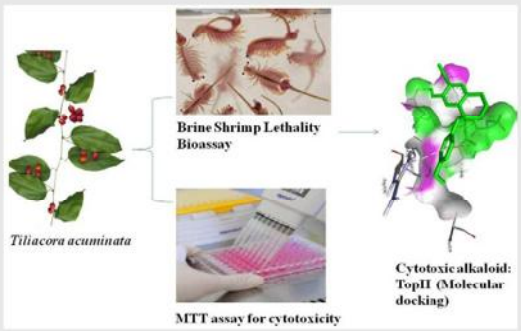
PICTORIAL ABSTRACT



About Authors

Jeswiny Rodrigues is a research scholar at KAHER Belagavi. Her interest is to screen multiple natural source-based compounds in multiple screening model. Her research area is cytotoxicity profiling of phytoconstituents.

Dr. Kirankumar Hullatti is presently working as a Professor at KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research (KAHER), Belagavi. His areas of research interests include isolation/ characterization of cytotoxic principles from medicinal plants and their pharmacological screening for various biological activities and standardization of Ayurvedic drugs; He has 18 years of Teaching and 5 years of Industrial experience. He has received Best paper award by IDMA in 2017 and also received funding from Dept. of Science and Technology, GoK, India for project related to quality assessment of raw materials used in Ayurveda.

SUMMARY

Cancer is one of the leading causes of death all over the world. The shortcomings in current drugs used in chemotherapy necessitate the discovery and development of newer drugs. Plants have long been associated with treatment in various forms and even today are recognized as important reservoirs for molecular leads. The current study evaluated the cytotoxic potential of *T. acuminata* using the bioactivity guided approach. The BSL the bioassay was used as a pre-screening model to rule out the least/inactive fractions. According to this assay, the alkaloid fraction showed the highest cytotoxicity and hence was further evaluated using a panel of human cancer cell lines. The alkaloid fraction showed the most cytotoxicity against the HT-29 cell line. Thus, nine alkaloids previously reported from *T. acuminata* were evaluated for their binding affinity with topoisomerase II enzyme using molecular docking. This enzyme plays a major role in vital cell processes. Caclaurine was found to show the highest binding affinity with topoisomerase II. Thus, the study revealed the presence of cytotoxic constituents from *T. acuminata* which may further be evaluated thoroughly. The study forms the base for the development of potential anticancer molecules from *T. acuminata*.

Dr. Sunil S. Jalalpure is presently working as a Principal and Professor at KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research (KAHER), Belagavi. His areas of research interests include isolation/ characterization of active principles from medicinal plants and their pharmacological screening for various biological activities and training the research students in Pharmacognosy, Phytochemistry and Biotechnological aspects with modern tools and techniques. He is recently involved in nanoparticle drug delivery system of herbal actives and green nanotechnology.

Pukar Khanal has been awarded the gold medal twice for his academic performance. His area of interest covers network pharmacology, ADMET profiling of lead hits from a natural source and their pharmacological evaluation, gene set enrichment analysis, prediction and assessment of protein-protein network interaction, *in silico* molecular docking, protein modeling and utilizing Danio rerio as a preliminary animal model. Further, he interests to utilize regression models for the evaluation of PKPD profiles and data correlation with wet-lab protocols.

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In silico and *in vitro* cytotoxicity profile of hydroalcoholic extract/ fraction(s) of *Pachygone ovata*

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Bisbenzylisoquinoline, brine shrimp, Menispermaceae, MTT, topoisomerase.

ABSTRACT

Medicinal plants have been used in the past for the treatment of diseases and continue to be an important reservoir for the development of new drugs. With the increasing burden of cancer globally, there is a need to find newer anticancer agents. The process of identification and evaluation of cytotoxic molecules from plants can be achieved conveniently by using simple yet reliable screening models and combining with *in silico* techniques. *Pachygone ovata*, least explored plant from Menispermaceae family, is known to be rich in alkaloids. This study aimed to identify the cytotoxic constituents from *Pachygone ovata* through bioactivity-guided fractionation using Brine shrimp lethality bioassay as a screening model. The active fraction in this assay was evaluated for its *in vitro* cytotoxic activity on human tumor cell lines. Some reported alkaloids were studied for their binding affinities with topoisomerase II by molecular docking. The study revealed the cytotoxic constituents from *P. ovata*. The study also revealed alkaloids with higher binding affinity with topoisomerase II, and the scope for further use leads to the development of new drugs.

INTRODUCTION

In the past, medicinal herbs were used to treat even the most complex conditions related to cancer such as inflammation, swellings, growths, and warts. Over 2,000 plant species of medicinal value have been recorded and are used for therapeutic purpose in different forms (Biljana, 2012). Cancer begins as a result of altered cell function, due to genetic and epigenetic changes within the cell leading to genetic instability (Anna, 2019). At present, cancer is the second largest cause of death worldwide. According to the WHO, cancer accounted for an estimated 9.6 million deaths in 2018 and continues to grow globally (WHO, 2020). The complication in treatment arises due to the uncontrolled proliferation of cells and invasion into other tissues, and thus, ineffective treatment and toxic side effects associated with chemotherapy necessitate the discovery of alternative treatment options (Chidambaram *et al*., 2011).

Medicinal plants still prove to be an important resource in the development of new drugs. Currently, a number of drugs derived from plants have been approved for clinical use, including cancer therapy (Cragg and Newman, 2005). Extensive research findings suggest that phytochemicals and their derived analogs are reported to inhibit the progression of cancerous cells through various mechanisms and have the most promising alternative for the treatment of cancer (Ana *et al*., 2018). The Indian system of medicine offers a number of plants that possess cytotoxic activities (Petrovska, 2012). *Pachygone ovata* (Poir.) Miers ex Hook. f. Et Thoms belonging to the family Menispermaceae is a deciduous woody shrub that can climb up to 15 m or more. The dried fruits of *P. ovata* have been used traditionally to repel insects and as fish poison (Shirin *et al*., 2014). It is one of the least explored plants, which is rich in active phytoconstituents, especially alkaloids. Different benzyl-isoquinoline-derived alkaloids have been reported from *P. ovata* stems, leaves, and roots (El-Kawi *et al*., 1984). Alkaloids have been well known for their inhibitory action on a wide range of tumors through different mechanisms (Lu *et al*., 2012).

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The brine shrimp lethality (BSL) assay is reported to be an effective screening model for potential cytotoxic constituents and has led to the identification of many cytotoxic compounds through bioactivity-guided approach (Meyer *et al*., 2005). Computational tools, in recent years, are being employed to study the modes of interaction, bioavailability, and toxicities of the possible lead compound with the target protein (Rosales-Hernandez *et al*., 2009). The inhibition of topoisomerase enzymes is one of the many modes, through which drugs may exert their effects on cancer cells. Important cellular functions such as replication, recombination, transcription, and DNA repair are governed by the activities of topoisomerase I and II (Kumar *et al*., 2013). The inhibition of such enzymes has been the target in anticancer drug research since recent years (Sivakumar *et al*., 2010). The use of simple and reliable screening models combined with modern *in silico* techniques can make the process of identification of bioactive constituents from plants effective and convenient (Khanal *et al*. 2019a, 2019b). Thus, this study was carried out to identify the potential cytotoxic constituents from *P. ovata* through the bioactivity-guided approach and molecular docking, which could be promising in the development of new drugs. The phytoconstituents used for docking i.e., coreximine, isoboldine, liriodenine, norjuziphine, pachygonine, reticuline, nortrilobine, and trilobine including standard etoposide are shown in Figure 1.

MATERIALS AND METHODS

Collection, authentication, extraction, and fractionation of plant

The wild-grown whole plant of *P. ovata* was collected in the month of July–September, from areas of Tirupati, Eastern Ghats, Andhra Pradesh, authenticated at Sri Venkateswara University, Tirupati, Andhra Pradesh, and the herbarium of the same was deposited with voucher number 0827 for future reference. The dried plant material was extracted by cold maceration using 70% v/v ethanol for 24 hours. After filtration, the marc was dried and further extracted by Soxhlet extraction. The macerate and percolate were then combined and concentrated using a rotary evaporator (IKA-RV Digital) to obtain the final extract. The fractionation of extract was carried as explained by Cos et al. (2006) to obtain methanol, petroleum ether, dichloromethane, and aqueous fractions.

Cell culture

MCF-7 (human, breast cancer), HT-29 (human, colon cancer), A-549 (human, small-cell lung carcinoma), HepG-2 (human, hepatic cancer), and L-6 (rat, normal skeletal muscle) cell lines were purchased from National Centre for Cell Science, Pune, India. The cells were cultured in 25 cm^2 culture flasks with Dulbecco's modified eagle medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 mg/ml), and amphotericin B (5 mg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution 0.2% trypsin, 0.02% Ethylenediaminetetraacetic acid (EDTA) and 0.05% glucose in Phosphate-buffered saline (PBS)).

Evaluation of cytotoxicity

BSL bioassay

The brine shrimp (*Artemia salina* Leach.) eggs were purchased from a local vendor. The procedure was carried out according to the procedure reported (Mc Laughlin and Roggers,

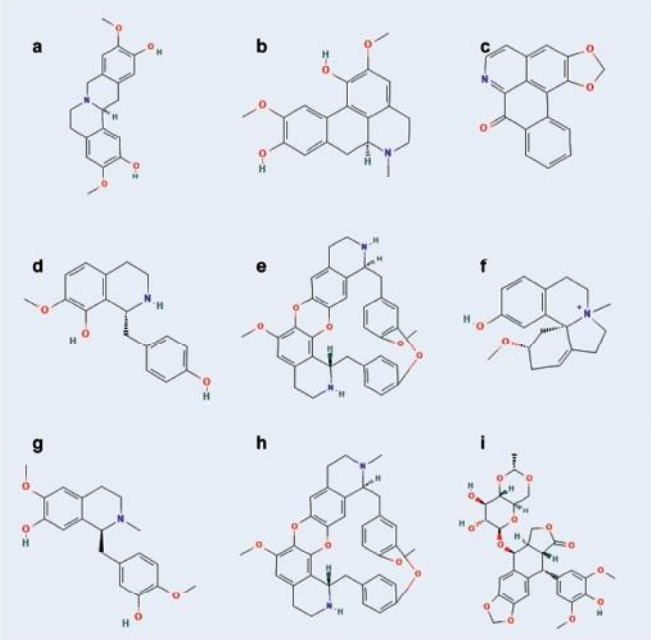


Figure 1. Structure of (a) coreximine, (b) isoboldine, (c) liriodenine, (d)norjuziphine, (e) nortrilobine, (f) pachygonine, (g) reticuline, (h) trilobine and (i) etoposide.

1998) with modifications. Briefly, the stock solution of extracts/ fractions was prepared in 1% DMSO and serially diluted using sea water to obtain solutions of 10, 50, 100, 500, and 1,000 µg/ml. Ten nauplii were added to 5 ml of each test solution. Control tubes contained equal volumes of distilled water. The assay was carried out in triplicate for each concentration. The tubes were kept under illumination, and the number of survivors was counted after 24 hours and the percentage of mortality was calculated. LC_{50} values were calculated by Probit analysis using SPSS-10.0.5 software (Armonk, NY).

3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay for cytotoxicity

The active fractions from BSL bioassay were screened for cytotoxicity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Lombardi *et al.*, 2017). Cells were collected by trypsinization, and the cell count was adjusted to 1.0×10^5 cells/ ml per well using DMEM medium containing 10% FBS. After 24 hours of incubation at 37°C in 5% CO_2 atmosphere, 100 µl of the various concentrations of the sample were added to the wells and incubated. After 48 hours, 20 µl of MTT was added to each well and incubated for 4 hours. The supernatant was discarded, and DMSO was added to each well. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition and IC_{50} values were calculated.

$$\text{Percentage growth inhibition (\%)} = \left[\frac{\text{Absorbance of test}}{\text{Absorbance of control}}\right] \times 100$$

where A (control)= Absorbance of control, A (sample) = Absorbance of sample

Molecular docking

The selected ligand molecules, i.e., coreximine, isoboldine, norjuziphine, nortrilobine, trilobine, liriodenine, pachygonine, and reticuline were retrieved from the PubChem database (.sdf format), converted into .pdb using Discovery Studio 2017 and minimized using mmff 94 force field and conjugate gradients as an optimization algorithm. After minimization, the pose with the least energy was chosen for docking. Topoisomerase II (PDB: 4GHF) was retrieved from the RCSB database and used as a template for query sequence for Accession number: P11388.3 for the homology modeling by adding the missing amino acid using Modeller 9.10. The protein molecule was made free from heteromolecules using Discovery Studio 2017 to avoid docking interference and saved in .pdb format, and the docking was carried by using AutoDock 4.0 under Lamarckian GA 4.2. The protein was viewed in Ramachandran plot to assess the distributed amino acid residues using Procheck (https://servicesn.mbi.ucla.edu/ PROCHECK/). After docking, the pose scoring minimum binding energy was chosen to visualize the ligand–protein interaction using Discovery Studio 2017. All the docking results were compared with the known topoisomerase II inhibitor, i.e., etoposide.

Statistical analysis

Data were expressed as mean ± SD using GraphPad Prism version 5.0. The IC50 was calculated using a linear regression model.

RESULTS

BSL bioassay

The hydroalcoholic extract of *P. ovata* displayed high toxicity toward shrimp nauplii with LC_{50}: 58.411 ± 1.33 µg/ ml. The fractions showed toxicity toward shrimp nauplii in a concentration-dependent manner and are shown in Figure 2. Fraction 3 showed the highest percentage mortality of nauplii and LC_{50}: 30.47 ± 1.66 µg/ml. Fraction 4 showed the least percentage mortality in this assay. The LC_{50} of hydroalcoholic extract/ fraction is shown in Table 1

MTT assay

Alkaloid-rich Fraction 3 of *P. ovata* displayed a notable cytotoxicity in the cell lines. The cytotoxicity increased with increasing concentration and is shown in Figure 3. The

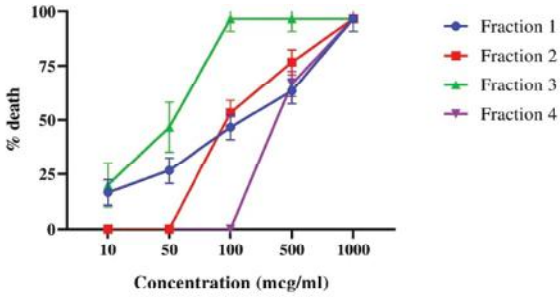


Figure 2. Brine shrimp mortality by various fractions of *P. ovata*.

Table 1. Evaluation of *P. ovata* extract and fractions in the BSL bioassay

Test agent	LC_{50} (µg/ml)
P. ovata alcoholic extract	58.411 ± 3.33
Fraction 1	261.57 ± 2.14
Fraction 2	163.92 ± 3.73
Fraction 3	30.47 ± 1.66
Fraction 4	429.94 ± 3.27

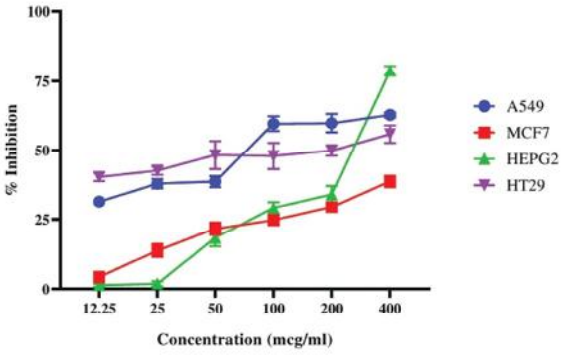


Figure 3. Cell growth inhibition of Fraction 3 of *P. ovata* in different cell lines.

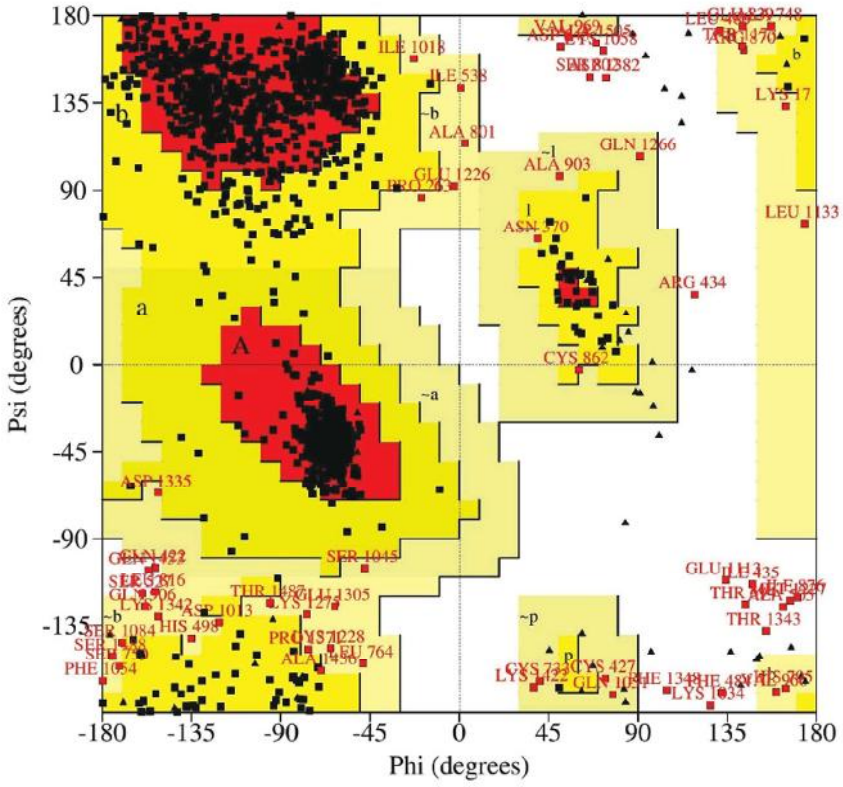
most cytotoxicity was observed towards the A-549 cell line compared to the others displaying IC_{50}= 84.76 ± 1.47µg/ml. The least cytotoxicity was noted in the MCF-7 cell line, whereas no cytotoxicity was observed in the normal cell line L6. Table 2 shows the IC_{50} of a fraction rich in alkaloids in multiple cell lines.

Molecular docking

In the homology modeled protein, the residues in most favored, allowed, and generously allowed regions were 81.5%, 14.2%, and 2.7%, respectively (Figure 4). The docking study predicted trilobine to possess the highest binding affinity (−11.2

Table 2. Evaluation of cytotoxicity using the MTT assay.

Test agent	IC_{50} (µg/ml)				
	A-549	MCF-7	HT-29	HepG2	L-6
Fraction rich in alkaloids	84.76±1.47	333.7±4.23	91.12±2.17	625.10±2.73	>1000
Paclitaxel	7.43±0.33	10.52±	12.67±1.29	15.81±2.26	323±2.73



Plot statistics

Residues in most favoured regions [A,B,L]	1118	81.5%
Residues in additional allowed regions [a,b,l,p]	195	14.2%
Residues in generously allowed regions [~a,~b,~l,~p]	37	2.7%
Residues in disallowed regions	21	1.5%
Number of non-glycine and non-proline residues	1371	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	84	
Number of proline residues	74	
Total number of residues	1531	

Figure 4. Ramachandran plot of homology modeled topoisomerase II.

kcal/mol) compared to others. However, no hydrogen bond interactions were found with the amino acids of topoisomerase II. Although reticuline was predicted to have binding energy −7.2 kcal/mol, it was found to be interactive with two amino acids, i.e., GLU1494 and MET1500. Table 3 shows the binding affinity of each ligand molecule with topoisomerase II. Figure 5 shows the interaction of liriodenine, pachygonine, norjuziphine, trilobine, nortrilobin, coreximine, isoboldine, reticuline, and etoposide with topoisomerase II.

DISCUSSION

The BSL bioassay, a general test for screening bioactive compounds, was used in this study to identify the potential cytotoxic constituents in *P. ovata*. According to several reports, the BSL test predicts cytotoxicity and has been successfully utilized in the bioassay-guided fractionation of active cytotoxic and antitumor agents (Arullappan *et al*., 2015; Zhanga *et al*., 2015). The reports suggest a significant correlation between the brine shrimp assay and *in vitro* inhibition of human tumor cell lines (Anderson *et al*., 1991).

Table 3. Binding affinity of ligand with Topoisomerase II.

Ligand	Binding affinity (kcal/mol)	Number of hydrogen bonds	Hydrogen bond residues
Liriodenine	−9.3	–	–
Norjuziphine	−7.6	1	MET1500
Pachygonine	−8.4	1	LYS512
Reticuline	−7.2	2	GLU1494, MET1500
Trilobine	−11.2	–	–
Coreximine	−4.58	2	GLY 474, LYS480
Isoboldine	−4.94	1	TYR 481
Nortrilobine	−4.42	–	–
Etoposide[a]	−9.2	3	HIS605, ASN508, GLN517

[a]Known molecule as topoisomerase inhibitor.

In this study, the fraction rich in alkaloids showed the highest mortality of the brine shrimp nauplii compared to the other three fractions, LC_{50} = 30.47 ± 1.66 µg/ml, which is considered as cytotoxic as explained by Pimentel Montanher *et al*. (2002). Hence, the alkaloid fraction was further evaluated for its *in vitro* cytotoxicity using multiple human tumor cell lines using the MTT assay. Although there have not been notable reports regarding the cytotoxicity of *P. ovata,* there are, however, studies reporting the antioxidant (Amalarasi and Jothi, 2019) and anti-inflammatory (Marahel and Sharanaiah, 2016) activities of *P. ovata* extracts, both of which have a close association with the pathogenesis of cancer (Yoshikawa and Naito, 2002; Rayburn *et al*., 2009). In accordance, a noteworthy cytotoxic effect of the alkaloid rich fraction was observed in this study. The highest cytotoxicity (IC_{50} = 84.76 ± 1.47 µg/ml) was noted against A-549 (human lung cancer) cell line. Moreover, worth noticing was that the fraction was relatively nontoxic toward the L-6 cell line indicating biocompatibility with the normal cell line. The preliminary screening of hydroalcoholic extract/fraction (s) in BSL bioassay was found to be effective in identifying potentially toxic fractions containing alkaloids, which further displayed significant cytotoxicity *in vitro*, and thus confirming its productive use in our study.

P. ovata, which is a member of the Menispermaceae family, is reported to constitute benzylisoquinoline alkaloids (El-Kawi *et al*., 1984). This study also identifies alkaloids from

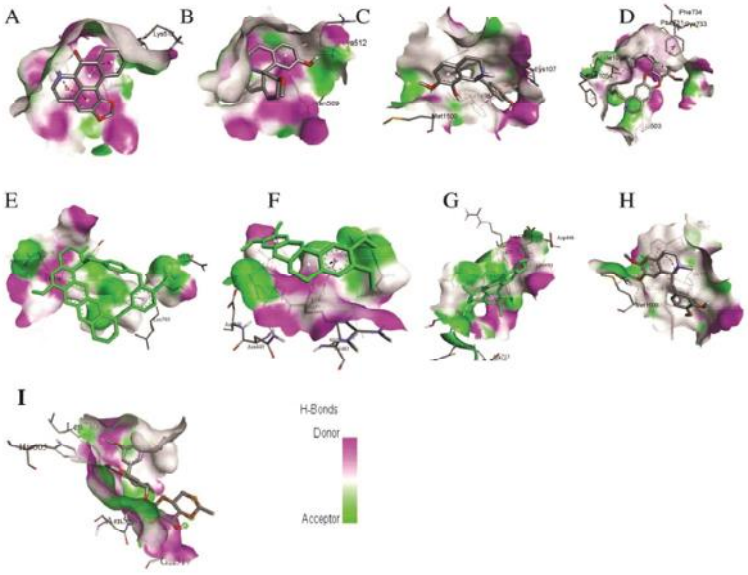


Figure 5. Interaction of (a) liriodenine, (b) pachygonine, (c) norjuziphine, (d) trilobine, (e) nortrilobin, (f) coreximine, (g) isoboldine, (h) reticuline, and (i) etoposide with topoisomerase II.

P. ovata which could be a relatively promising group of natural products as a source of new anticancer agents. Hence, *in silico* docking studies were carried out on previously reported alkaloids (liriodenine, trilobine, pachygonine, coreximine, reticuline, isoboldine, norjuziphine, and nortrilobine) against Topoisomerase II. Topoisomerase II is an enzyme involved in DNA replication. Topoisomerase II is radically upregulated in cancer cells due to rapid cell division and growth (Nainwal *et al*., 2014). Topo II is a potential target in the designing of newer anticancer agents (Heck *et al*., 1986). In this study, alkaloids from *P. ovata* were found to interact with topoisomerase II, suggesting their involvement in cancer management.

It was reported that the stability of the ligand–protein complex depends on the binding energy as well as hydrogen bond interactions. In this study, although trilobine was found to have the highest binding affinity with topoisomerase II, the complex may not be stable since it could not form any hydrogen bond interactions with any amino acid of protein molecules. Although reticuline was predicted to have binding energy −7.2 kcal/mol, it was found to be interactive with a protein molecule. Reticuline has previously shown *in vitro* cytotoxic activity in human tumor cell lines such as P-388, KB16, and A549 (Chen *et al*., 1997; Suresh *et al*., 2012), demonstrating its future scope in cancer research.

Further, cancer is a polygenic condition, in which multiple proteins are involved in its pathogenesis (Bredberg, 2011). The fraction rich in alkaloids could modulate the multiple proteins and pathways which can be accessed through the gene-set enrichment analysis (Khanal and Patil, 2019; Khanal and Patil, 2020) and network pharmacology (Khanal *et al*., 2019c) which is also a future scope of the present study.

CONCLUSION

This study revealed potential cytotoxic alkaloids from *P. ovata*. Further, the docking study predicted the binding ability of alkaloids from *P. ovata* with topoisomerase II; however, further investigations need to be carried out to validate the findings and the use of alkaloids from *P. ovata* leads to cancer treatment.

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CONFLICT OF INTEREST

Authors declare that they do not have any competing interests

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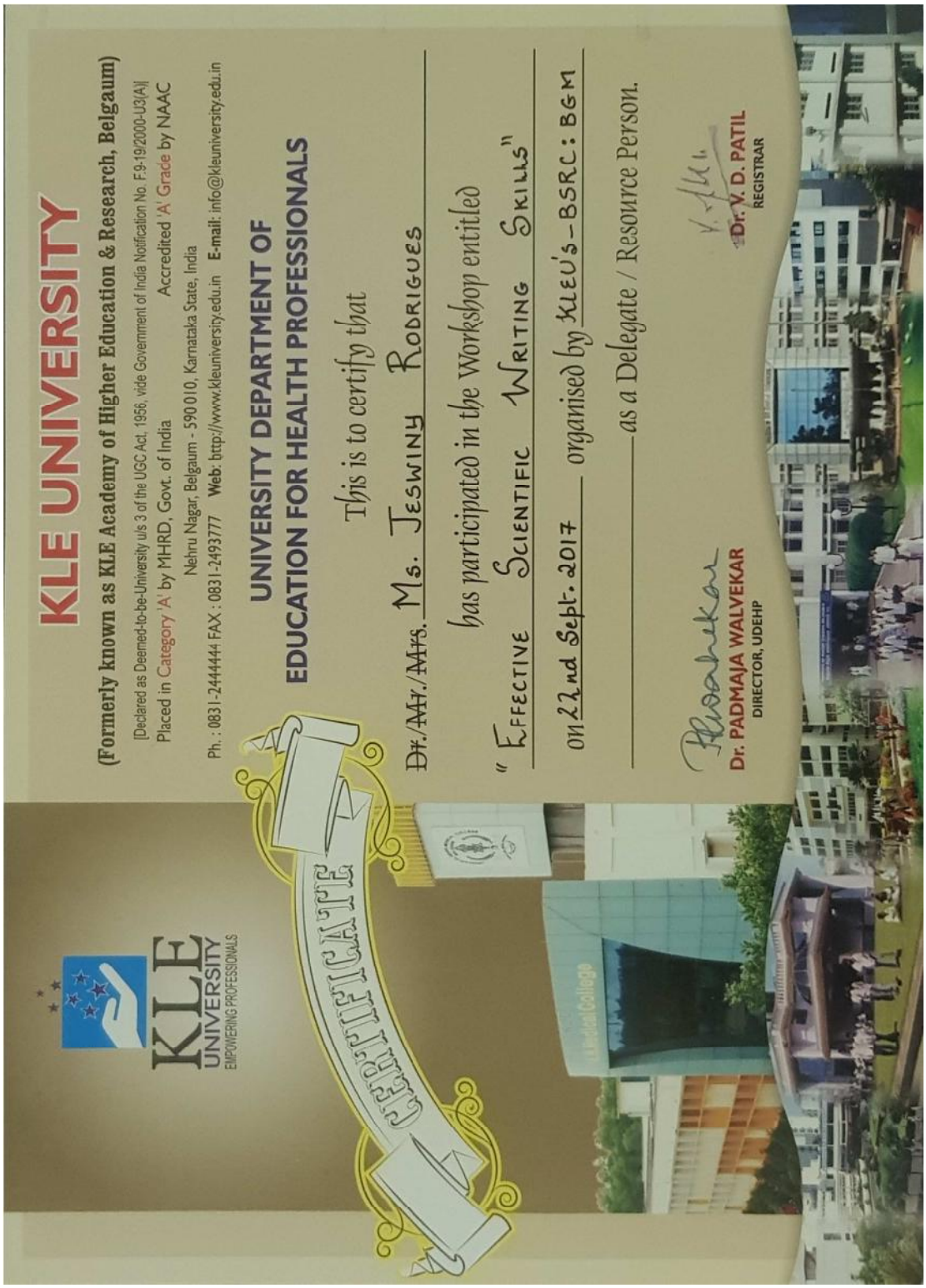

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&

INDIAN PHARMACOLOGICAL SOCIETY, LOCAL BRANCH, BELAGAVI

This is to certify that Ms. Jeswiny Rodrigues

has participated as a delegate in **Pharmacology CME 2018** on **"Current Trends in Pharmacological Research"** held on 06/04/2018 at the Department of Pharmacology, J.N.M.C. Belagavi.

Dr. (Mrs) N.S. Mahantashetti
Principal
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Dr. A.P Hogade
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Dr. Urmila Kagal
Organizing Secretary