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PRELIMINARY PHYTOCHEMICAL ANALYSIS AND CYTOTOXIC ACTIVITY OF FRUIT PERICARP EXTRACT OF *Terminalia catappa*

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF KERALA IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREEOF

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

SUBMITTED BY

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DECLARATION

I hereby declare that the dissertation entitled **"PRELIMINARY PHYTOCHEMICAL ANALYSIS AND CYTOTOXIC ACTIVITY OF FRIUT PERICARP EXTRACTS OF *Terminalia catappa*"** submitted to Sree Narayana College ,Kollam in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology ,is a bonafied record of original and independent work done by me under the guidance and supervision of **Dr. A. Jayakumaran Nair,** Head of the department of Biotechnology, Kariavattom Campus, University of Kerala,Thiruvananthapuram and **Dr. Supriya R,** Lecturer, Department of Biotechnology, University of Kerala, Kariavattom, Thiruvananthapuram. All sources of help received by me during the course of my study have been duly acknowledged.

Kariavattom **AYANA S Y**
AUG 2017

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AYANA S Y

ABBREVIATIONS

ALT	**-**	**Alanine amino Transferase**
CCl4	**-**	**Carbon tetra Chloride**
CHO-k1	**-**	**Chineese Hamster Ovary cells**
Cm	**-**	**Centi meter**
D-GalN	**-**	**D-Galactose Nitrosamine**
DMEM	**-**	**Dulbecco's Modified Eagle's Medium**
DMSO	**-**	**Dimethyl sulfoxide**
DPPH	**-**	**1,1-Diphenyl -2-picrylhydrazl**
EAC	**-**	**Ehrlich Ascites induced Carcinoma**
EDTA	**-**	**Ethylene Diamine Tetra Acetic acid**
FBS	**-**	**Fetal Bovine Serum**
HBV	**-**	**Hepatitis B Virus**
HCC	**-**	**Hepato Cellular Carcinoma**
HCV	**-**	**Hepatitis C Virus**
HeLa	**-**	**Henrietta Lacks**
HGPRT	**-**	**Hypoxanthine-Gaunine Phosphoribosyl Transferase**
HPB	**-**	**Hepatic-Pancreatico Biliary**
HPLC	**-**	**High Performance Liquid Chromatography**
HPTLC	**-**	**High Performance thin Layer Chromatography**
HPV	**-**	**Human papilloma Virus**
Hrs	**-**	**Hours**
IC50	**-**	**Concentration at which 50% inhibition occur**
IL-6	**-**	**Interleukin-6**
LLC	**-**	**Lewis Lung Carcinoma**

KOH - Potassium hydroxide

Mg - micro gram

Mg - milli gram

Ml	**-**	**milli litre**
MTT	**-**	**3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium Bromide**
NADPH	**-**	**Nicotinamide dinucleotide phosphate**
PBS	**-**	**Phosphate buffered saline**
Ppm	**-**	**Parts per million**
Rf	**-**	**Retardation factor**
Rpm	**-**	**Rotation per minute**
SD	**-**	**Standard deviation**
TLC	**-**	**Thin layer chromatography**
T.catappa	**-**	***Terminalia catappa***
UV	**-**	**Ultra violet**

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ABSTRACT

Preliminary phytochemical analysis and cytotoxic activity of fruit pericarp extract of *Terminalia catappa*

Terminalia catappa is a large spreading tree belonging to the family *Combretacea*, is distributed through out the tropical areas and hence the name 'tropical almond'. Different parts of this tree have been used in folklore medicines and research studies also exhibited various medicinal properties like antibacterial,anti-fungal , anti- inflammatory ,anti-tumor ,anti-oxidant,anti- diabetic and hepato protective activities. In this study, we focus on cytotoxic effects of pericarp extracts (chloroform, hexane, and methanol) of *Terminalia catappa* will be screened on A549(lung cancer cell line) and HeLa(cervical cancer cell line).The work include extraction of air dried sample extract with different solvents based on the polarity and determining the phytochemical analysis of all the extracts followed by determining cytotoxic/anti-proliferative activity using MTT assay. The active fraction was further selected and screened for the identification of major bioactive compounds responsible for the cytotoxicity of cancer cell lines. The study finds positive for using the selected plant as potential source for producing anti -cancer drugs.

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Chapter 1

INTRODUCTION

We know cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to another parts of the body. Over 100 types of cancer affects humans. In the developing world , nearly 20% of cancer are due to infection such as Hepatitis B ,Hepatitis C and Human Papiloma Virus (HPV).But about 5-10% of cancer due to the inherent genetic defect from a person's parent. Cancer can be detected by certain signs and symptoms or screening tests. It is then typically, further investigated by medical imaging and confirmed by biopsy. Cancer is often treated with some combination of radiation therapy, surgery , chemotherapy and targeted therapy.

There are number of researches which reports anti –cancer property in plants and its bioactive compounds. In case of chemotherapy ,it results in some ill effects, like multi drug resistance , excessive toxicity to normal cells and insufficient amount of drug reaching target cells,angiogenesis and metastasis. Many plants have been evaluated in clinical studies and are currently being investigated phytochemically to understand their tumourocidal actions against various cancers (Premalatha and Rajagopal;2005) .There are many *in vitro* studies carried out by scientists and researchers on different cancer cell lines and the cytotoxicity assay are one of the major tools to screen herbs as anti - cancer drugs. The plants exhibited strong anticancer, hepato- protective and several other activities .*Terminalia catappa* is one such tree underwent similar studies. It a large tropical tree inthe lead wood tree family – *Combretacea*. It grows mainly in the tropical regions of Asia

,Africa and Australia. The tree grows 35m tall, with an upright, symmetrical crown and horizontal branches. *T.catappa* has corky light edible fruit that are dispersed by water. The fruit is a drupe 5-7cm long and 3-5.5 cm broad, green at first then yellow and finally red

when ripe, and containing a single seed. *T.catappa* are claimed to have many therapeutic effects. It had reported that the antibacterial activity (Shahina et al;2007). The fruit is also helpful in the treatment of leprosy & headaches & leaves are specifically used in getting rid of intestinal parasite, treatment of eye problem (Kirtikar&Basu.,1991;Corner.,1997;Manjunath.,1976;).Fruit has demonstrated antidiabetic activity (Nagappa et al; 2003).

The chloroform extract and methanol extract of the bark & root displayed strong anti microbial activity (Pawar & Pal,2002). The various extracts of leaves and bark of *T catappa* have been reported to be anticancer, anti-HIV reverse transcripts and hepato- protective (Lin et al., 1997) as well as anti-inflammatory (Lin et al., 1999), anti-hepatitis (Chen et al., 2000) antidiabetic (Nagappa et al., 2003) and aphrodisiac (Ratnasooriya and Darmasuri, 2000). Aqueous, ethylacetate and hexane extracts of *T.catappa* wood and bark has anti fungal activity (Parimalagandhi et al;2015) . Fallen leaves are used to treatliver diseases in Taiwan (Wee, 1992) and is a potential herb in treating sickle cell disorders (Tan et al., 1991). The leaves have antioxidant as well as anticlastogenic properties (Masuda et al., 1999). Consumption of seed kernel is useful in the treatment of men with sexual dysfunctions- premature ejaculation (Ratnasooriya and Darmasuri,2000).Osmotically-induced hemolysis of human erythrocytes is inhibited bythis species of plant in a dose dependent manner (Chen et al., 2000).Dermatitis and hepatitis can be treated by, punicalagin and punicalin from leaves, since both have strong antioxidative activity (Lin et al., 1999). Consumption of this almond lowers low density lipoprotein (LDL) and cholesterol as well as reduce the risk of heart diseases. Such curing properties are attributed to the antioxidant activity of vitamin E and monounsaturated fats as well as the presence of phenolics in the stem, root, fruit, nuts and leaf parts of the almond plant (Subashinee et al., 2002).

This present study was set up with the objective of assessing the phytochemical analysis and cytotoxic activity of pericarp extract against various cancer cell lines other than the ones already studied.

1.1. AIM AND OBJECTIVE

Aim: Preliminary phytochemical analysis and cytotoxic activity of fruit pericarp of *Terminalia catappa*

The main objectives of this work are the following:

- To fractionate the compounds present in pericarp of *Terminalia catappa* fruit.
- Phytochemical analysis of various solvent extracts of pericarp extract.
- To check the *in vitro* cytotoxic activity of *T.catappa* using cancer cell lines – lung cancer and cervical cancer cell lines.
- Analysis of compounds present in the active fraction showing comparably highest cytotoxicity using thin layer chromatography.

Chapter 2

REVIEW OF LITERATURE

Terminalia catappa is a large tropical tree that belongs to the lead wood tree family *Combretacea* (Nous et al., 2008). The generic name derived from the Latin word "terminals", which referred to the position of leaves which are spirally clustered at the ends of its branches. *Terminalia* species are native from Africa and are now widely spread out in tropical and sub-tropical regions (Collins et al., 1992)

2.1. SCIENTIFIC CLASSIFICATION

Kingdom	*Plantae*
Division	*Magnoliophyte*
Class	*Magnoliopsida*
Order	*Myrtles*
Family	*Combretacea*
Genus	*Terminalia*
Species	*Terminaliacatappa*

2.2. COMMON NAMES

Indian almond, Tropical almond, Desi bad am, Malabar almond, Bengal almond, Umbrella tree, False kamani,almedra, nattuvadumai(Tamil), kottamba,ketapang et

2.3. BOTANICAL DESCRIPTION

Trunk

The tree is a tall deciduous and erect reaching to a height of 35-40m with horizontal branches.Its branches are characteristically arranged in tiers.

Leaves

Leaves are alternate, short-petioled, clustered at branch tips, usually obovate, but sometimes more or less elliptic, leathery and glossy. The leaves turn bright scarlet, dark red, dark purplish red or yellow before falling (because plants draws nutientsfrom leaves for recycling).There is a foliage change twice a year. Leaves of *T .catappa* have many pharmaceutical uses as well. These leaves show antiparasitic (Elizabeth, 2005) antibacterial (Elizabeth., 2005; Rajarajan et al., 2010; Kinoshita et al., 2007), antifungal (Elizabeth.,2005), antimetastic effects (Chen et al., 2007)etc.Dried leaves are used as water conditioner for aquarium since they have anti fungal and antibacterial properties.

Flowers

The tree is monoecious,with distinct male and female flowers. Located at the terminal end of branch. Male flowers,are present at apical part of branch and have stalk while female flowers are sessile and seen at the base.The flowers are small, greenish-white or creamy coloured with a barbate disk, five calyx lobbed , petals are absent .Flowering occurs up to 3 times a year.

Fruit

The fruit is sessile, ovoid, laterally compressed drupe. As the fruit matures,it changes its colour from green to bright red/purple and finally brown when dried .The kernel consist of two delicate and intricately entwined cotyledons enclosed in an inconspicuous cream coloured testa. Fruits are produced when tree is three years old and are edible in nature.

T

Fig 1: **Fruits of *T.catappa***

Seeds

Fruit contains a cream coloured seed, which encloses the nut. The rind of the fruit has a light corky tissue, that enables the fruit to float and be dispersed by sea currents.

Fig 2: **Seeds of *T.catappa***

Nuts

The kernel/nut are edible when raw or roasted.Its taste similar to standard almond. Contains high quality proteins and vital minerals (Eley.,1976). Nuts are good sources of edible oil and fats.

Bark

The bark is gray to dark gray-brown. The bark is used for treating many health problems like gastric ailments, bilious, diarrhea and dysentery.

Wood

The tree provides a red, good-quality, elastic, cross-grained timber that seasons well and works easily. Strong and pliable wood is used for the construction of buildings, boats, bridges, floors, boxes, planks, carts, wheelbarrows, barrels and water troughs. The trunk is a source of gum and black dye; it is used in leather preparation and as a base for ink.

2.3. PHYTOCHEMISTRY

T.catappa contains phytochemicals like alkaloids, saponins, tannins, terpinoids, phenols, steroids, flavonoids, glycosides and carbohydrates.The concentration of phytochemicalsis different in different parts of the plant. *T. catappa* leaf extracts exhibit biological activities, including antioxidant (punicalagin, punicalin, terfluvina A and B, chebulic acid, benzoic acid, cumaric, and its derivatives) (Chen and Li.,2006; Chyau et al., 2006; Kinoshitaet al., 2007), antidiabetic (β-carotene) (Anand et al., 2015), anticancer (punicalagin) (Naitik et al., 2012),antiviral(ellagic acid) (Tan et al., 1991),anti-inflammatory (triterpenic acids, especially ursolicacid and its derivatives) (Fan et al., 2004), antimicrobial (flavones and flavanols) (Kloucek et al., 2005; Nair and Chanda, 2008; Shinde et al., 2009)and hepato- protective activities (punicalagin, punicalin) (Kinoshita et al., 2007).

Glucose, pentosans, corilagin, carboxylic acid, carotene, ellagic acid, gallic acid, and tannin are present in fruits(Duke et al;2008). Fixed oil , olein and stearin are present in seeds.Glycoside, cardiac tannins, volatile oils, saponin, steroid, glycosides, and phenols are the major compounds in bark. Tannins present in the bark,fruit,leaves etc,can be used to dye materials like cotton and rattan to brown, orange or red colour(Rao etal.,1989).Both punicalagan and punicalin showed anti- inflammatory activity on carrageenan-induced hind paw edema in rats (Dukes.,2008)

2.4. PHARMACOLOGICAL ACTIVITIES

Antibacterial activity

The chloroform and methanolic extracts have proved antibacterial activity against Gram positive and negative bacteria. The chloroform extract showed efficient activity against *Staphylococcus. aureus* and *E. coli* while methanol extract has activity against *E. coli*(Taganna et al.,2011).It was also reported the antibacterial activity of *T.catappa* leaves and fruits against *Corynebacteria, Staphylococci, Enterococci, Escherichia, Salmonella* and *Shigella (*Shahina et al;2007*,).*Aqueous and ethanolic extracts of *T. catalpa* leaves also show activity against *S. typhi,E. coli, S. aureus* and *P. aeruginosa*. Anti bacterial activities of aqueous and ethanolic extracts of leaves and bark of *T. catalpa* (Neelavadhi et al.*,2013)*and aqueous ethyl acetate and hexane extracts of *T. catalpa* bark were also carried out against some pathogenic bacteria (Sangavi et al.,2015).

Antifungal activity

Many anti-fungal assays were done on various extracts of *T.catappa*. Out of this the methanol extract showed potential antifungal activity against *Pythium ultimum* and *Phytophthora parasitica (Goun et al.,2003).*It had also reported the anti-fungal activity of aqueous, ethyl acetate and hexane extracts's from wood and bark (Parimalgandhi et al.,2015).Out of the three extracts, hexane extract showed strong anti-fungal activity against all the selected fungal species. The activity is compared with a standard antibiotic Clotrimazole. It was reported that,leaf extracts of *T.catappa* can reduce fungal infections in the eggs of economically important food fish,tilapia (Reverter et al.,2014).

Anti-diabetic activity

Nagappa et al., (2003) investigated the antidiabetic ability of methanol, petroleum ether and aqueous extracts of tropical almond fruit on (FBS) fasting blood sugar levels and serum biochemical analysis in alloxan-induced diabetic rats. The three extracts of this fruit generated a significant antidiabetic activity at dosage levels of 1/5 of their lethal doses.

Antioxidant activity

Chukwuma (2015) evaluated the antioxidative properties of the tropical almond leaf using 1,1diphenyl-2-picrylhydrazyl (DPPH), total phenolic content and reducing power assays. The results showed that 80% methanolic leaf extracts exhibited the highest percentage 73.42% inhibition of free radical. This is followed by water and then ethanol extract at the rate of 95%. The three different solvent extracts from the tropical almond leaf exhibited its antioxidative action by scavenging DPPH radical as well as with a reasonable amount of phenols and its concomitant reducing activity. The study supports that the *T.catappa* leaf could be used as a natural source of managing and dealing with an oxidative stress-related health condition.

Biodiesel production

Adewuyi et al.,2011, produced biodiesel from the seed oil of tropical almond using a two-step reaction system. The first step involved the pretreatment process and this step involved using 2% sulphuric acid in methanol. The second step involved the transesterification reaction whereby KOH is used as the catalyst. The results showed a conversion that gave an ester content of above 97% with phosphorus content below 1 ppm in the biodiesel. The biodiesel generated from the oil of the tropical almond showed properties that are aligned with the recommended American standards (ASTM D 6751-07b) and European standards (EN 14214). Therefore these researchers support the use of crude *T. catappa* as raw feedstock for producing biodiesel.

Antiviral activity

HIV replication in infected H9 lymphocytes and purified HIV reverse transcriptase were inhibited by the punicalin and punicalagin (tannin) present in *T.catappa* (Liu et al.,1996) The chebulagic acid and punicalin blocked the binding of recombinant HIV coat protein gp120 (rgp120) to its normal cellular receptor, CD4(Liu et al.,1996).The fruit of *T. catappa* contains ellagic-acid which has anti-HIV activity (Chitmanat et al.,2005)

Anti-inflammatory activity

Lin et al(1999) evaluated the anti-inflammatory effects of punicalagin and punicalin in carrageenan-induced hind paw edema in rats. The punicalin and punicalagin ,potent tannins isolated from the leaves of *T.catappa*. Examining the anti-inflammatory effects, the edema rates that increased due

to the carrageenan administration ,showed a vast reduction after drug treatment (Punicalagin and punicalin) . The results showed that both punicalin and punicalagin possess anti- inflammatory properties, however, it is important to note that treatment with larger dosages of punicalin can possibly lead to some cell damages.

Antiparasitic activity

Dried leaves of Indian almond were grind and dissolved in water. A variety of concentrations of this solution were used to determine resulting activities against tilapia pathogens. The results indicated that *Trichodina*, fish ectoparasites, were eradicated

Hepato-protective activity

Liver injuries or damage of its cells may occur,by external and metabolic means .External causes include alcohol abuse ,HPB virus etc.Studies reveal that chloroform fraction of *T.catappa* leaves can block liver injury, by CCl4 induced over transcription of of IL-6 (Tang et al.,2003). CCl4 causes dramatic effects in liver,such as massive fatty change, necrosis, and broad infiltration of lymphocytes and Kupffer cells around the central vein and loss of cellular boundary in the liver. Treatment with chloroform extract of *T.catappa* prevent CCl4-induced disruption of liver intra mitochondrial Ca2+ overload ,suppression of Ca2+-ATPase activity(Tang et al.,2006).It was also reported extract of *T. catappa* leaves can protect liver from damage induced by D-galactose amine(D-GalN). This D-GalN protection is enabled by blocking the Alt enzyme activity.ALT enzyme is an index of cell membrane damage ,whose increase is promoted by D-GalN.Thus, the T.catappa extract promote liver protection(Tang et al.,2004). .

Hypocholesterolemic activity

Work on lipid profile in transplanted fibrosarcoma mice showed ,*T.catappa* has a lipid regulating activity That is *T.catappa* fraction can reversed the altered lipid levels to normal range in mice(Naitik et al.,2012).It is also reported that *T. catappa* fruit extract and fallen dry leaf decoction have hypocholesterolemic effects on rats(Ibegbulem et al.,2011).Thus ,the consumption of such decoction can regulate the cholesterol level in man as well.

Immunomodulatory activity

Immunomodulatory activity of flavonoid fraction of *T. catappa* leaves in Swiss albino mice reveals that it can be a good immunomodulator. This activity is checked on healthy albino mice,by administering the flavonoid fraction by intra-peritoneally . Administration of flavonoid fraction reveals a significant increase in neutrophil adhesion and phagocytic index (Saroja et al.,2013).

Wound healing activity

Another important and amazing fact is that,*T.catappa* can also be a potent wound healer as well.The wound healing property of this plant(bark) , was evaluated on excision wound models in Winster rats.The wound healing ability was measured by applying the ointment on the wounds and as a function of a specific time interval and percentage of wound healed within that interval.Wound healing activity is compared with that of control and Betadine ointment as standard drug. *T. catalpa* ointment showed 97% reduction in wound area when treated in animals. It was observed that ointment treated wounds , induce epithelization faster as compared to the control (Khan et al.,2014). Research on various medicicinal plants showed that, *T. catappa* exhibit to have wound healing effect similar to various herbs (Shalini et al.,2015).

Antitumor activity

Today the discoveries of new phytocompounds as cancer drugs is a boon to field of oncology. The harmful effects and toxicities induced by the use of chemotherapeutics can be overcomed by its use. Extracts of various plant parts of *T.catappa* show potent cytotoxicity /antitumour activities. These include various solvent extracts of bark, leaves, fruits etc..Reaserches are going on searching further potentialities of this plant in anti tumour activity.

It was reported that aqueous extract of *T. catappa* leaves and its major tannin component, punicalagin had effectively protected CHO-K1 (Chinese hamster ovary cells) cells against bleomycin-induced hgprt gene mutation (Chen et al.,2000) . In another study, it was revealed that the dose-dependent growth inhibition of both human hepatoma and normal liver cells,by the supercritical CO_2 extract of *T.catappa* (KO et al.,2003*). T.catappa* can also be used for preventing colon cancer. The new study reveals that, the hot water extract of *T. catalpa* had short-term chemopreventive action on biomarkers of colon carcinogenesis in F344 male rats and this

protective effect of *T.catappa* against colon carcinogenesis was believed to be,possible due to its antioxidant activity (Morioka et al.,2005).Recently a novel tannin component has isolated from *T. catappa* called Punicalagin .In a study on H-ras transformed NIH3T3 cells,it was reported that,water extract of *T.catappa* had suppressed its growth in a concentrationdependent manner. More over punicalagin present in the extract also inhibited H-rastransformed NIH 3T3 cell growth.(Chen et al.,2006). The anti-metastatic effect of *T.catappa* leaves were studied on highly metastatic human lung cancer cell-A549 and Lewis lung carcinoma (LLC) *invitro* and *invivo* respectively. The study shows that the water extract of *T.catappa*,decreased the level of specific endogenous proteolytic enzyme inhibitors in both A549 and LLC cells. Thus the study concludes that water extract of *T.catappa* is a potent agent for the prevention of lung cancer metastasis(Chu et al.,2007). In another study, concentration depended cytotoxic effect of methanolic extract of *T.catappa* leaves in EAC (Ehrlich Ascites induced carcinoma) cells were discovered(Saroja et al.,2011). EAC(Ehrlich Ascites induced Carcinoma) cells in mice was evaluated for the antitumour activity of flavonoid fraction of *T.catappa* leaves. (Saroja et al.,2013).By trypan blue exclusion and MTT assay,antitumour activity of *T.catappa* bark was evaluated against EAC cell lines, the result showed the extract had a potent antitumor activity (Venkatalakshmi et al.,2014).

As we are discussing about cytotoxicity/Anticancer activity in this current work, it is relevant to discuss some about cancer and its major types .

2.6. CANCER

Cancer is one of the malignant diseases, which is a leading cause of death in both developing and developed countries, worldwide. One in four deaths are due to lung cancer lung cancer and is the most common cancer in men while breast cancer is the most common cancer in women. An estimated 12.7 million people were diagnosed with cancer across the world in 2008, and 7 .6 million (around 13% of all death) people died from the cancer during the same year. Lung cancer, breast cancer, colorectal cancer and stomach cancer accounted for two fifth of the total cases of cancer diagnosed worldwide. In 1996 there were 10 million new cancer cases worldwide and six million deaths attributed to cancer. Globalization of unhealthy lifestyles, environmental risk factor, which include tobacco and alcohol use dietary factors, insufficient regular consumption of fruit and vegetable, overweight and obesity, physical inactivity ,environmental risks include exposure to ionizing and non ionizing radiation, chronic infection from Helicobacter pylori, Hepatitis B virus (HBV) Hepatitis C virus (HVB) and some type of Human papilloma virus(HPV).

Cancer is believed to be a curable disease . Chemoprevention of cancer can be possible by the use of natural, synthetic or biological substances that intervene in the early precancerous stages, therefore reverse and suppress the formation of tumour . Chemo preventive agents can be targeted for intervention at either the stage of initiation, promotion or progression of carcinogenesis (Wattenberg.,1990).The use of natural products as anticancer agents has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine. Many cancer patients use herbal medicine as alternatives including phytochemicals following the failure of standard cancer therapy (Eisenberg et al., 1998). Currently, several plant-derived compounds have been successfully employed in cancer treatment. Paclitaxel , camptothecin, combrestatin (Cirla & Mann, 2003), epipodophyllotox (Canel et al., 2000) and Vinca alkaloids (vinblastine, vincristine) (Johnson et al., 1963) are some examples of such phytochemicals used for cancer treatments.

Carcinogenesis is a multistep process, consisting of tumor initiation, promotion and progression. Cancer initiation can be blocked by activating protective mechanisms, either in the extracellular environment or intracellular environment by modifying transmembrane transport, modulating metabolism, blocking reactive oxygen and nitrogen species, maintaining DNA structure, modulating DNA metabolism and repair and controlling gene expression. Tumor promotion is the second stage of carcinogenesis and is followed by tumour progression. Both stages can be suppressed by inhibiting effects of genotoxic , favourin antioxidant and anti-inflammatory activity, inhibiting proteases and cell proliferation including cell differentiation, modulating apoptosis and signal transduction pathways. By affecting the hormonal status and the immune system in various ways,can also inhibit tumour progression.

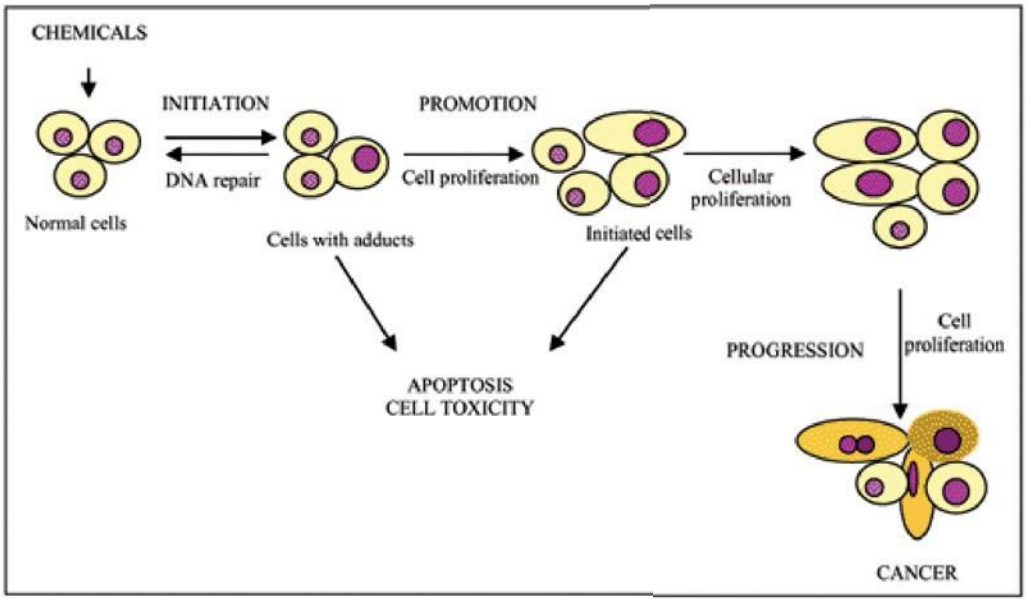

Figure 3: **Main step of multistep theory of cancer: intiation, promotion and progression**.

2.6.1. MODE OF ACTION OF PHYTOCHEMICALS FROM PLANTS

Apoptosis is a common phenomenon in multicellular organisms,which serves for programmed cell death. It is initiated when the cells get sensitized by abnormal stress such as DNA damage, or abnormal proliferation (Okada and Mak el al., 2014). It consists of a series of events which results in the cell death, by the action of different proteins of the caspase protein family. Once caspase cascade get activated, results in systematic cell death (Wang et al., 2005). Apoptosis activation, may be an extracellular (extrinsic pathway) or an intracellular (intrinsic pathway), depending on the mode of cellular abnormalities.

Recently many chemotherapeutic agents has been reported, which induces apoptosis. Cancer is associated with accumulation of neoplastic cells due to enhanced cell proliferation or negligible apoptotic activity or both. Currently it is commonly accepted that inhibition of apoptosis play an important role in the carcinogenic process. Many anti-apoptotic process leads to abnormal proliferation of cells, which leads to cancer. These facts enhance the need for a better molecule that arrest the cancer, either by blocking anti-apoptotic stimuli ,apoptosis or its activator. But most of the chemotherapeutics available today, that mean for inhibiting apoptosis, may itself damage

normal cells, limiting their clinical potential. These drawbacks can be compensated by the use of phytochemicals.

The commonly used plant-derived anti-cancer chemicals are vinca alkaloids, vinblastine and vincristine. Vinblastine and vincristine are isolated from Catharanthus roseus (Apocynaceae) Madagaskar periwinkle, these are used in the combination with other cancer chemotherapeutic drug for the treatment of a variety of cancers including leukemias , lymphomas, bladder cancer, advanced testicular cancer, breast cancer, lung cancer and kaposi's sarcoma (Mohammed shoch et al.,2006). It is used intravenously and works by inhibiting mitosis.

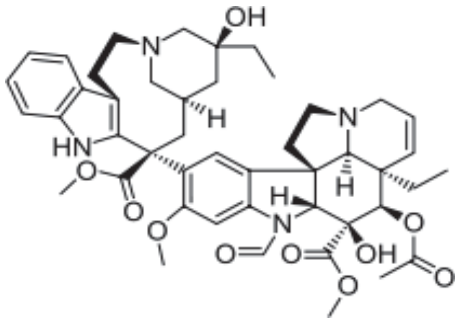

Figure 3: **Vincristine**

The discovery of Paclitaxel (Wani et al., 1971), from the bark of the Pacific Yew, Taxus brevifolia (Texaceae), is another trade mark discovery in the class of phytochemical drugs.Paclitaxel is a very potent anticancer agent. Various parts of Taxus brev,jolia and other Taxus species have been used for the treatment of some non-cancerous case . Paclitaxel is significantly active against ovarian cancer, advanced breast cancer, small and non-small lung cancer (Sharma Pet al., 2009).

Let’s discuss about some major cancers, their causes and symptoms.

2.6.1. CERVICAL CANCER

Cervical cancer kills 260,000 women annually, and nearly 85% of these deaths occur in developing nations, where it is the leading cause of cancer deaths in women. Disparities of health and poverty play a large role in this high mortality rate. Whereas routine Papanicolaou and human papillomavirus (HPV) testing has dramatically reduced cervical cancer deaths in Westernnations, without proper infrastructure, facilities, and medical training, the rates of cervical cancer in developing nations will remain high. Studies on HPV DNA testing and the low-technology method of "screen and treat" are promising. In addition, reducing the cost and increasing the availability of HPV vaccines in developing nations brings hope and promise to the next generation of women (Rev Obstet Gynecol., 2009).

One of the first things a biomedical researcher learns is that it's very hard to grow most human cells in the lab for an extended period. In fact, once removed from the human body, most cells will either die immediately or reproduce only a limited number of times. That's why it was so significant in 1951 that this barrier was overcome for the first time, using cancer cells taken from a 31 year old African American woman named Henrietta Lacks. The HeLa cell lines were perpetual, everlasting, death-defying, or whatever other word you want to use to describe immortal. This property meant that researchers would now have a handy stock of cells available for their experiments. It also meant that rather than each researcher using different cell types,labs all around the world could have access to cells of a common origin for their experiments. HeLa cells have been used to explore the complex processes involved in the growth, differentiation, and death of cells—processes that underlie a vast array of human diseases. HeLa cells have also served as the foundation for developing modern vaccines, including the polio vaccine; understanding viruses and other infectious agents; and devising new medical techniques, such as in vitro fertilization. The most unfortunate that Ms. Lacks did not receive the thanks she deserved from researchers during her lifetime. However, I'm glad that we now have a chance to thank the Lacks family for continuing to share her enduring legacy with the biomedical research community. Their generosity extends to the millions of people who have benefited, or will benefit in the future, from research using HeLa cells (Hudson K.L et al., 2013).

2.6.1.1. SIGN AND SYMPTOMS

In its early stages, cervical cancer typically does not cause symptoms. It may be detected by Pap screening and subsequent testing even before symptoms have developed. When symptoms do occur, one of the most common symptoms is abnormal vaginal bleeding. Unusually heavy menstrual bleeding and bleeding after menopause are also possible symptoms of cervical cancer. This can include bleeding: Between menstrual periods,bleeding after sexual intercourse or a pelvic exam,bleeding after douching (William C et al., 2015).

2.6.2.2.CAUSES

Human papillomavirus can cause cancer of the cervix, vagina, vulva, penis, and anus, as well as some head and neck cancers, anogenital warts, and recurrent respiratory papillomatosis. The World Health Organization (WHO) estimates that of the 500,000 new cases annually, 80% affect women between the ages of 15 and 45 years who live in developing nations. It predominantly impacts women living in Latin America and the Caribbean, sub-Saharan Africa, and Southeast Asia. Only 5% of women in these regions have been screened for cervical disease in the past 5 years. Because cervical cancer is caused by a sexually transmitted virus, the risk factors are the same as other sexually transmitted infections: early age at first sexual activity, multiple sexual partners, early age at first delivery, and increased number of pregnancies, smoking, immunosuppression and long- term oral contraceptive use. Preventive health measures should ensure education on safe sexual partners. The low status of women and their lack of empowerment play a significant role in the high rate of cervical cancer. These factors impose challenges on health providers who seek to educate, and on the women themselves, who may hesitate to seek adequate health care.

2.6.3. LUNG CANCER

The most common type is non-small cell lung cancer (NSCLC). NSCLC makes up about 80 to 85 percent of all cases. Thirty percent of these start in the cells that form the lining of the body's cavities and surfaces. This type usually forms in the outer part of the lungs (adenocarcinomas). Another 30 percent begins in cells that line the passages of the respiratory tract (squamous cell carcinoma). A rare subset of adenocarcinoma begins in the tiny air sacs in the lungs (alveoli). It's called adenocarcinoma in situ (AIS). This type isn't aggressive and may not invade surrounding tissue or need immediate treatment. Faster-growing types of NSCLC include large-cell carcinoma and large-cell neuroendocrine tumours. Small-cell lung cancer (SCLC) represents about 15 to 20 percent of lung cancers. SCLC grows and spreads faster than NSCLC. This also makes it more likely to respond to chemotherapy, but it's also less likely to be cured with treatment. Not all smokers get lung cancer, and not everyone who has lung cancer is a smoker. But there's no doubt that smoking is the biggest risk factor, causing 9 out of 10 lung cancers. In addition to cigarettes, cigar, and pipe smoking are also linked to lung cancer. The more you smoke and the longer you smoke, the bigger your risk of lung cancer. Breathing in other people's smoke increases the risk of lung cancer. According to the Canters for Disease Control and Prevention CDC), second hand smoke is responsible for about 7,300 lung cancer deaths each year in the United States.

Pulmonary research requires models that represent the physiology of alveolar epithelium but concerns with reproducibility, consistency and the technical and ethical challenges of using primary or stem cells has resulted in widespread use of continuous cancer or other immortalized cell lines. The A549 'alveolar' cell line has been available for over four decades but there is an inconsistent view as to its suitability as an appropriate model for primary alveolar type II (ATII) cells. Since most work with A549 cells involves short term culture of proliferating cells, we postulated that culture conditions that reduced proliferation of the cancer cells would promote a more differentiated ATII cell phenotype. We examined A549 cell growth in different media over long term culture and then used microarray analysis to investigate temporal regulation of pathways involved in cell cycle and ATII differentiation; we also made comparisons with gene expression in freshly isolated human ATII cells. Analyses indicated that long term culture in Ham's F12 resulted in substantial

modulation of cell cycle genes to result in a quiescent population of cells with significant up-regulation of autophagic, differentiation and lipidogenic pathways. There were also increased numbers of up and down-regulated genes shared with primary cells suggesting adoption of ATII characteristics and multilamellar body (MLB) development. Subsequent Oil Red-O staining and Transmission Electron Microscopy confirmed MLB expression in the differentiated A549 cells. This work defines a set of conditions for promoting ATII differentiation characteristics in A549 cells that may be advantageous for studies with this cell line (James Ross Cooper et al., 2016).

3.4.1 SIGN AND SYMPTOMS

Symptoms of non-small cell lung cancer and small cell lung cancer are basically the same. Tumours at the top of the lungs can affect facial nerves, leading to drooping of one eyelid, small pupil, or lack of perspiration on one side of the face. Together, these symptoms are called Horner syndrome. It can also cause shoulder pain. Tumours can press on the large vein that transports blood between the head, arms, and heart. This can cause swelling of the face, neck, upper chest, and arms.Early symptoms may include: Lingering or worsening cough,coughing up phlegm or blood,chest pain that worsens when you breathe deeply, laugh, or cough,hoarseness.

2.6.3.2.CAUSES

Anyone can get lung cancer, but 90 percent of lung cancer cases are the result of smoking. From the moment you inhale smoke into your lungs, it starts damaging your lung tissue. The lungs can repair the damage, but continued exposure to smoke makes it increasingly difficult for the lungs tokeep up the repair. Once cells are damaged, they begin to behave abnormally, increasing the likelihood of developing lung cancer. Small-cell lung cancer is almost always associated with heavysmoking. When you stop smoking, you lower your risk of lung cancer over time. Exposure to radon,a naturally existing radioactive gas, is the second leading cause, according to the American

Lung Association. Radon enters buildings through small cracks in the foundation. Smokers who are also exposed to radon have a very high risk of lung cancer. Breathing in other hazardous substances, especially over a long period of time, can also cause lung cancer. A type of lung cancer called mesothelioma is almost always caused by exposure to asbestos (Alberg et al., 2016).

2.7. BIOASSAYS

2.7.1. PHYTOCHEMICAL SCREENING

Phytoconstituents are the natural bioactive compounds found in plants,which have always been in great interest for scientists working on infectious diseases (Burkill.,2000; Roja et al., 2000;Sofowora 1982). It has been estimated that today about 25% of all prescribed medicines are substances derived from plants (Egwaikhide and Gimba 2007; Zheng and Wang 2001). A phytomedicine to be used for the treatment of diseases (Iwu, 1993).The medicinal value of these plants lies in the bioactive phytochemical constituents that produce definite physiological effects on human body. Phytochemical evaluation of plant is essential to study the pharmacological activities. It can be done by qualitative chemical analysis using specific reagents for specific constituents followed by confirmation with different chromatographic techniques, like TLC, HPTLC, HPLC, GC etc.

There are several standard methods used for the phytochemical screening of medicinal plants. They are described for alkaloids (Harbome, 1973), steroids (Trease and Evans, 1989), phenolics (Ayve and Sodipo, 2001), flavonoids (Malbry et al, 1970; Awe and Sodipo, 2001), saponins (Hungund & Pathak 1971; Sofowora, 1993), cardiac glycosides (Balbaa et al, 1981; Sofowora, 1993), tannins (Trease and Evans, 1978; Odebiyi and Sofowora, 1978). Methods for quantitative analysis of phytochemicals are also described for phenolics (Edeoga et al, 2005), flavonoids (Boham and KocipalAbyazan, 1974), alkaloids (Harbome, 1973), saponins (Obadoni and Ochuko, 2001) and glycosides (El-Olemy et al, 1994).

In this present work, chloroform ,hexane and methanol fractions of *T.catappa* extract were phytochemically analysed using standard procedures.

2.7.1.MTT ASSAY

In vitro assessment of cytotoxicity of any extract or material is done by MTT assay.

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation . The yellow tetrazolium salts MTT (3(4,5-dimethylthialthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced metabolically active cells , in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events leads to apoptosis or necrosis, the reduction in cell viability.The number of assay steps has been minimized as much as possible to expedite sample processing.The MTT reagents yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation .

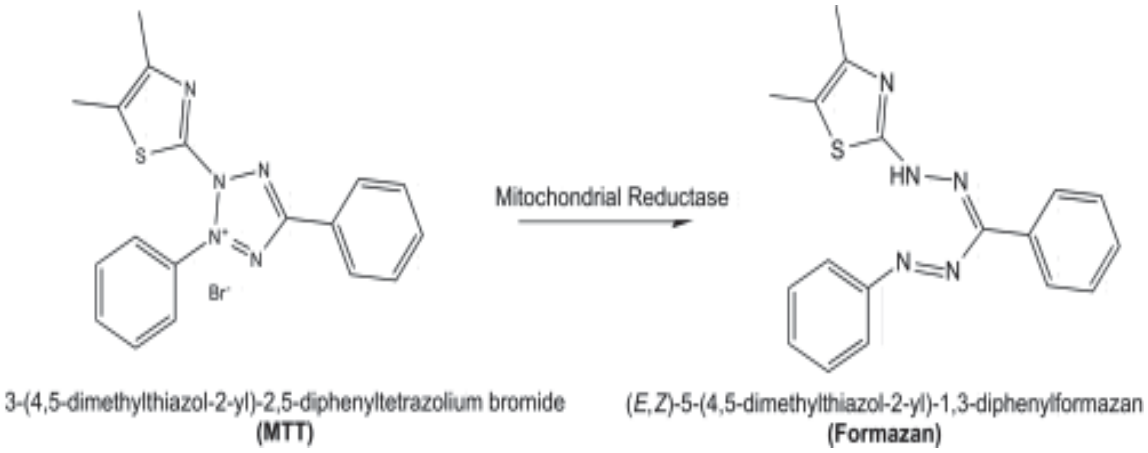

Figure 5 : MTT reaction

2.7.3.THIN LAYER CHROMATOGRAPHY

Thin Layer Chromatography (TLC) is performed for various extracts to confirm the presence of different phytoconstituents (Paulami Mandal and Tapan Ganguly, 2009). For carrying out TLC

an adsorbent/stationary phase and a mobile phase is needed.Stationary phase is generally ,a plate coated with silica gel (type 60),while the mobile phase is a single or a mixture of solvents. The extract is applied as a small spot or band at the origin of thin sorbent layer supported on a glass plastic metal plate. The mobile phase migrates through the stationary phase by capillary action. The separation of solutes takes place due to their differential adsorption (Lazarowych and Pekos.,1998) partition coefficient with respect to both mobile and stationary phases. The more strongly a given component of a mixture is adsorbed onto the stationary phase, the less time it will spend in the mobile phase and the more slowly it will migrate up the plate.

For example, the phenolic compounds of red clover (Trifolium pratense L.), the polar compounds, such as clovamide, have a strong affinity for a polar adsorbent like silica and remain near the origin (OR), while less polar compounds, such as the three isoflavones near the solvent front (SF), partition more readily into the solvents (which are less polar than silica unless water, acids, or bases are included) and migrate farther up the plate. TLC is widely used because it is relatively rapid and can be performed on different adsorbents (e.g. silica, starch, alumina), as well as providing good resolution and sensitivity.

Handmade plates are prepared by using techniques like, pouring, dipping or spraying.Now-a- days, readymade pre coated plates are also available. The plates need to be activated at 110 ^{0}C for 1 hr. This removes water / moisture loosely bound to silica gel surface. Plate markings were made with soft pencil. Glass capillaries were used to spot the sample for TLC applied sample volume 1-micro litre by using capillary at distance of 1 cm at 5 tracks. In the twin trough chamber with different solvent system Chloroform: Methanol (14:1) solvent system. After presaturation with mobile phase for 20 min for development were used. After the run plates are dried and sprayed freshly prepared reagents like anisaldehyde, dragendroff, iodine were used to detect the bands on the TLC plates. The movement of the active compound was expressed by its retention factor (Rf), values were calculated for different samples

$$Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent front}}$$

Phytochemical screening determination by some chemical tests and thin layer chromatographic study can be carried out by using various solvent system of varying polarity of hexane, chloroform, ethyl acetate, acetone and methanol extracts. Phytochemical screening reflects presence of alkaloids, glycosides, saponins, phenolic compounds, tannins, phytosterols, carbohydrates, proteins, amino acids, flavonoids, quinones and terpenoids shows different types of results in different solvents extracts. For eg., thin layer chromatographic studies of the Aerva lanata root extracts constituted different coloured phytochemical compounds with different Rf values. The chloroform and methanol extracts in the drug is carried out to establish the biomarker compound. The result proved that Aerva lanata root as a rich source of natural source of natural antioxidants, and provides evidence that solvent extract of Aerva lanata contains medicinally important bioactive compounds and this justifies the use of plant species as traditional medicine for treatment of various diseases (Rajendra Prasad Gujjeti and Estari Mamidala., 2013)

In this work, the cytotoxicity is checked in three extract fractions – Chloroform, Hexane and Methanol. By confirming cytotoxicity of each fraction by MTT assay, the active fraction is further analyzed by thin layer chromatography. TLC help in separation and identification of bioactive compounds.

Chapter 3

MATERIALS AND METHODS

3.1. MATERIALS

For studying cytotoxic analysis ,pure and analytical grade chemicals were purchased from E. Merck (India) Ltd., E. Merck ,Germany ;Hi-Media, Mumbai; Sigma Chemical Company ,USA; and used throughout the period of study.

3.2. PLANT MATERIALS

The plant material used in this study is *T.catappa* (pericarp of fruit) which were collected from Kariavattom Campus, University of Kerala. The sample were washed with distilled water and kept for drying .The dried samples were crushed and powdered using an electric blender.

3.3. EXTRACTION AND FRACTIONATION

3.3.1. EXTRACTION

Solvent Extract: The extraction method used to prepare crude solvent extract was hot continuous extraction using Soxhlet apparatus. Approximately 50g of sample was weighed, loaded in Soxhlet apparatus and extracted by adding 500ml of various solvents (hexane, chloroform, methanol and water) in a serial manner. After each extraction using a particular solvent the sample should be properly dried and weighed for next solvent extraction. The extracts were collected in a separate flask and condensed in vacuum dry evaporator. The crude extracts used in this study were stored at 4^0 C under sterile conditions until it is used.

3.3.2. FRACTIONATION

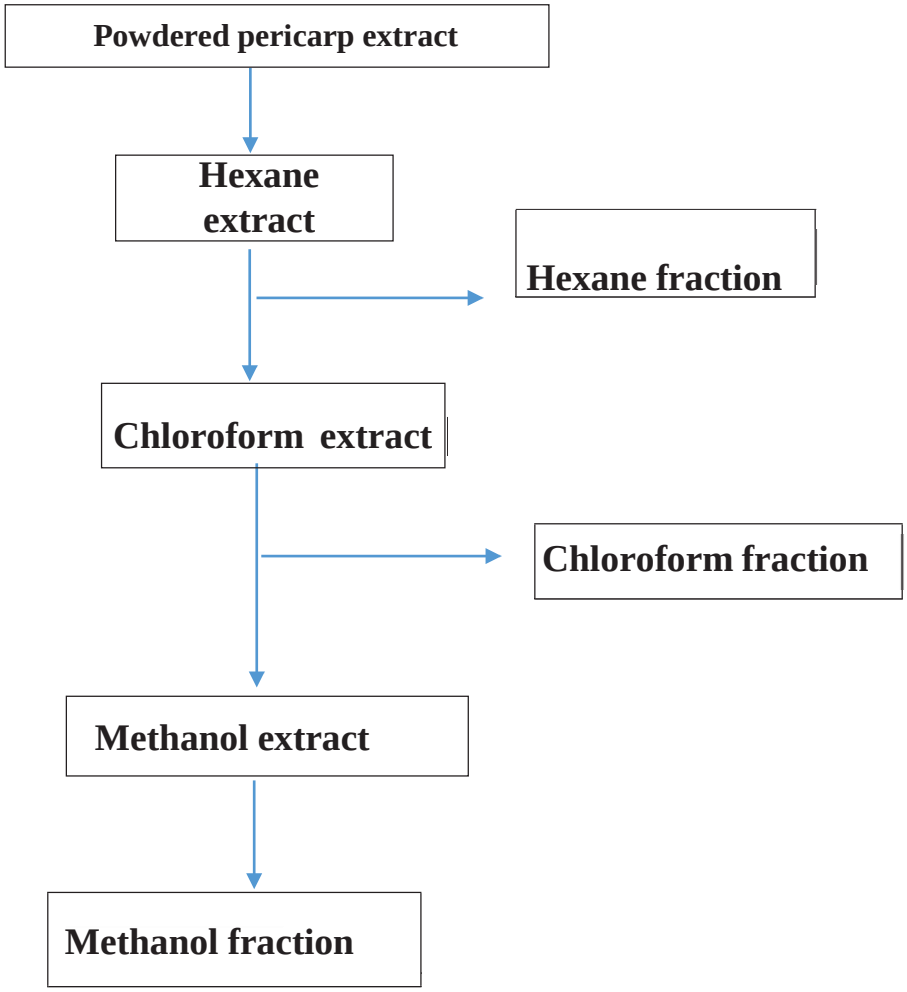

Figure 6 :Flow sheet of fractionation of crude extract

Crude extracts were partitioned with different organic solvents (hexane, chloroform,and methanol) in increasing order of polarity by using soxhlet apparatus. Residual aqueous layer was again separated in chloroform and methanol respectively. For all fractions, different volumes of solvent were used depending on its composition and solubility of components.

3.4. PLANT EXTRACT PREPARATION FOR SCREENING

Stock solutions containing plant extracts were prepared fresh on the day of the experiment. Dimethyl sulfoxide (DMSO)was used for dissolving the extracts to make a stock solution at 1mg/ml. Final test concentrations were obtained by diluting the stock solution with Dulbecco's minimum essential medium(DMEM)(Hi-media)supplemented with 10% fetal bovine serum(FBS) (Gibco).The final concentration of DMSO to which cell cultures were exposed never exceeds 0.2%(Nicoline Fri Tanih et al.,2013)

3.4 CELL LINE GROWTH AND MAINTENENCE

This study was conducted on two cancer cell lines, HeLa ,A549 and a non cancerous fibroblast cell line 3T3.All three cell lines were adherent type.

3.4.1. MEDIA PREPARATION

Before media preparation Fetal Bovine Serum (FBS)and Dulbecco's Modified Eagle Medium (DMEM)were incubated at 37^0C.Penicillin and streptomycin was used for preparation of antibiotic solution .For 1L of DMEM 10% FBS and 10% antibiotic was used .After syringe filtration this media was stored till further use at 2-8 ^{0}C.

3.4.2. DEFROSTING CELLS

Media for the cells ,DMEM was warmed upto 37^0 C at least one hour before use.The cryovial containing the frozen cells from -80 ^{0}C storage was removed and immediately placed into a 37 ^{0}C water bath.The cells (<1 minute)were thawed quickly by gently swirling the vial in the 37 ^{0}C water bath until there was a small bit of ice left in the vial.Pre-warmed complete growth medium was transferred drop wise into the centrifuge tubes containing the thawed cells.The cell suspension was centrifuged at approximately 200x g for 5 minutes.After the centrifugation ,the clarity of supernatant and visibility of a complete pellet was checked and the supernatant was decanted asceptically without disturbing the cell pellet.The cells were gently resuspended in complete growth medium and transferred into 25cm^2 culture vessel and diluted with 5ml of complete media (20%FBS). Flask was labelled with cell type, date, intials and passage number. Flask was incubated for overnight at 37 ^{0}C humidified incubator (New Brunswick Galaxy 48S) with 5% CO_2 for multiplication and adherence. After 24 hr, media was removed and fresh complete growth media (10%FBS) was added to the flask.

3.4.3. TRYPSINIZING AND SUBCULTURING CELLS FROM A MONOLAYER

Adherent cells are anchorage dependent and propagate as monolayer attached to the cell culture vessel. This attachment is essential for proliferation, many adherent cell culture will cease proliferating once they become confluent (i.e., when they completely cover the surface of culture vessel), and some will die if they are left in this confluent state for too long .To expose each cell to nutrients and environment provided, trypsinization was done when 80% confluence was reached. Media was removed and cells was washed thrice using 1ml PBS(Phosphate Buffer Saline) to partially remove the dead cells as they do not attach to the flask surface. 1X TrypsinEDTA (Appendix) was warmed before use in 1-3 min at 37 ^{0}C and then added to the culture flask. Flask was tapped softly to break the cell aggregates and then examine under phase contrast microscope (Leica DMIL Led).When the cells were completely detached from the flask,1ml of the prepared media was added to the flask. Media pipetted up and down many times to break up any cell lumps, and then collected to a centrifuge tubes. The cells were centrifuged for approximately 5 min at 200Xg, following which ,the media above the cell pellet formed was aspired and the cells were resuspended in 1ml complete media (10%FBS).The cells were counted *via* haemocytometer. The solution was divided into fresh flasks (1:3 split ratio was used). About 5ml DMEM was added to each flask and kept in an incubator at 37 ^{0}C and when it becomes confluent continue to passage as necessary.

3.4.4. CELL COUNTING

Cells were trypsinized in the normal way as mentioned above.DMEM was added and cells were centrifuged in a falcon tube for 1 min at 1000 rpm. Pellet was resuspended in 1ml of DMEM 10µ l of the cell suspension and 90 µl of PBS were added. Cells were pipetted up and down several times to mix the PBS thoroughly.10 µ l of suspension was added to prepare hemocytometer slide.
Cells were counted in each of 4 square and mean was calculated .Number of cells was calculated using the following equation:

Number of cells/ml = Mean of cells x 10^4 x 10 (dilution factor)

3.4.5.FREEZING CELLS

The process of storing cells is to freeze them. They were frozen when they were 80-100% confluent. Cells are passaged as described before. After dislodging the cells ,small volume (about 5ml)of media was added to stop the action of trypsin if still present. Cells were pelleted by centrifugation for 5 min at 2300 rpm .The pellet was suspended in a mixture of 90% FBS and 10%DMSO and shifted to cryovial tube.The tubes were placed at -80 ^{0}C to freeze overnight.

3.5. In Vitro CYTOTOXICITY ASSAY

The cytotoxicity of the extracts and fractions were determined using MTT assay as orginally described by Mossman in 1983.

3.6. PRINCIPLE

This is the colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)2,5,diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase .The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble ,colored (dark purple) formazan product .The cleavage and conversion of soluble yellow dye to the insoluble purple formazan product has been used to develop the assay system alternative to the conventional 3H-thymidine uptake and other assays for measurement of cell proliferation. Active mitochondrial dehydrogenase of living cells will cause this conversion .Dead cells do not cause this change. This reaction only takes place in living cells as only their mitochondria and mitochondrial enzymes are functional. Cytotoxicity of medicinal agents and other toxic materials can be found out by using this technique. Because this agents are involved in cell toxicity, deregulation of metallic process and hence decreased performance in the assay .Yellow MTT ,3(4,5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide ,a tetrazole ,get reduced to purple formazan in living cells by mitochondrial reductase (Mosmann,1983). DMSO is used to dissolve the insoluble purple formazan product. Then spectrophotometer is used to measure absorbance of this solution at 570 nm.

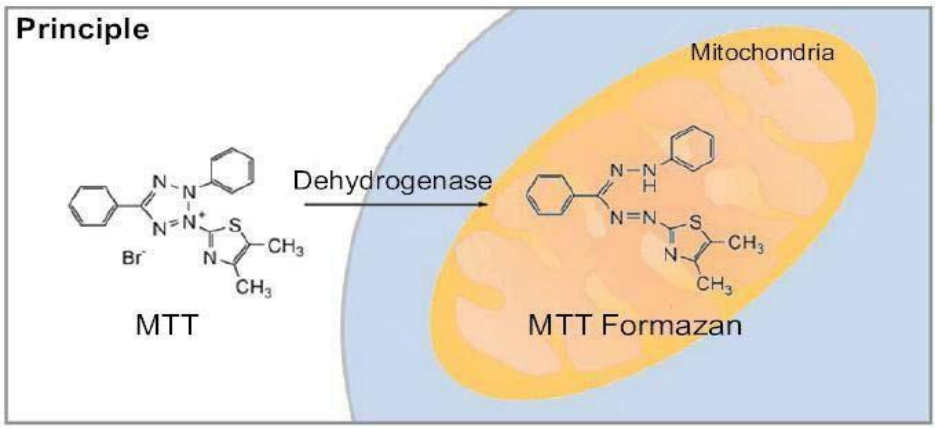

Fig 7: Principle of MTT assay

Principle:

MTT(3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (water soluble)

↓

Insoluble formazan

↓

Further solubilization, using **DMSO**

↓

Concentration determined by OD at **570 nm**

Fig 8: Principle and procedure of MTT assay

3.6.1. PREPARING MTT SOLUTION

MTT solution was prepared at concentration of 5 mg/ml in PBS(Phosphate buffered saline solution)as stock solution.This solution was diluted depending upon the concentration of cells needed to use.Stock solution was diluted to 1:10 PBS.

3.6.2. MTT ASSAY METHODOLOGY

To reduce interference with the dissolution of formazan crystals in the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), an improved solvent, alkaline dimethyl sulphoxide (DMSO),which could dissolve formazan crystals in approximately 10 minutes so as to give a stable spectrum by eliminating buffering effects of the residual medium was used. Briefly, cells were treated with different concentrations (62.5, 125, 250, 500 and 1000 µg/mL) of solvent and aqueous extracts of *T.catapa* in 96 – well plates and incubated for 24,48,and 72 h. After the incubation time, MTT dye (10 L,5mg/ml,HI-media) was added to the cells for 4h followed by incubation with DMSO,for 10 minutes.The colorimetric assay was measured at a absorbance of 570 nm using a microplate reader.The anti-proliferative potential of extracts was expressed as IC50 values.

% of proliferation = Absorbance of sample/Absorbance of control *100

% Cytotoxicity = 100 - % of Proliferation

3.6.3. STATISTICAL ANALYSIS

All the tests were performed as individual triplicate experiment. Results obtained from crude extracts and fraction activity were calculated for average % inhibition while data obtained from the results of activity of fractions was statistically analyzed.

3.7. IDENTIFICATION OF BIOACTIVE COMPOUND BY PHYTOCHEMICAL SCREENING

On each solvent type extract, test for alkaloids, Saponin, glycosides, phenols, Flavonoids, Tannins, Polyphenolic compounds, terpenoids ,alkaloids, steroids, carboxylic acid, coumarin carried out using standard Phytochemical methods. The tests performed for the presence of compounds are as:

3.7.1 Tannins:

Tannins are naturally occurring compounds that exist inside grape skins, seeds and stems. The scientific word for these compounds is polyphenols. Polyphenols release from the skins, seeds and stems.

- 0.5g of extract dissolved in 5 - 10ml of distilled water and was filtered.
- A few drops of a 5% FeCl3 solution were added to the filtrate.
- A blue, blueblack, green, or blue-green colour/precipitate is an indication of the presence of tannins (Trease and Evans, 2002).

3.7.1. Steroids:

A steroid is an organic compound with four rings arranged in a specific molecular configuration. Examples include the dietary lipid cholesterol, the sexhormones estradiol and testosterone and the anti-inflammatory drug dexamethasone.

- Extract dissolved in chloroform and equal volume of con. sulphuric acid added.
- Colour development from violet to blue or bluish-green indicate the presence of a steroidal ring i.e. a glycone portion of cardiac glycoside (Sofowora, 1993).

3.7.2. Saponins:

Saponins are glucosides with foaming characteristics. Saponins consist of a polycyclic aglycones attached to one or more sugar side chains. The aglycone part, which is also called sapogenin, is either steroid (C27) or a triterpene (C30). The foaming ability of saponins is caused by the combination of a hydrophobic (fat-soluble) sapogenin and a hydrophilic (water-soluble) sugar part. Saponins have a bitter taste. Some saponins are toxic and are known as sapotoxin.

- One gram of each portion was boiled with 5 ml of distilled water,filtered.
- To the filtrate, about 3 ml of distilled water was further added and shaken vigorously for about 5 minutes.
- Frothing which persisted on warming was taken as an indication of saponins(Sofowora,1993).

3.7.4. Flavonoids:

Flavonoids are a diverse group of phytonutrients (plant chemicals) found in almost all fruits and vegetables. Along with carotenoids, they are responsible for the vivid colors in fruits and vegetables. Flavonoids are the largest group of phytonutrients.

0.5mg of each portion was dissolved in ethanol, warmed and then filtered.
Three pieces of magnesium chips was then added to the filtrate followed by few drops of conc. HCl.
A pink,orange, or red to purple colouration indicates the presence of flavonoids (Trease and Evans,2002).

3.7.5. Alkaloids:

Alkaloids are in the class of naturally occurring organic nitrogen-containing bases. Thet have diverseand important physiological effects on plants, humans and other animals. Wellknown alkaloids include morphine, strychnine, quinine, ephedrine, and nicotine

- ***Mayer's test*** : To 3ml extract,few drops of Mayer's reagent added ,formation of buff coloured precipitate indicates presence of alkaloids(Sofowora,1993).

- ***Dragendroff test:*** To 3ml of extract add few drops of dragendroff reagent,brownish fluorescent precipitate indicate presence of alkaloids.

3.7.6. Glycosides:

- A small amount of alcoholic extract was dissolved in 1ml of water.
- A few drops of aqueous NaOH solution added.
- A yellow colour signify the presence of glycosides.

3.7.7. Coumarin:

- Few ml of extract mixed with few drops of methanol and alcoholic KOH.
- Formation of yellow color,which disappears by the addition of con.HCl.

3.7.8. Terpenoids:

- Crude extract (5mg) dissolved in 2ml chloroform ,then add acetic unhydride .
- Add con.sulphuric acid,results in the formation of reddish violet colour.
- Indicates presence of terpinoids.

3.7.9. Carboxylic acid :

- Sodium bicarbonate test: A small amount of extra was dissolved in water and to this, a pinch of sodium bicarbonate was added.
- A brisk effervescence confirms the presence of carboxylic acid.

3.7.10. Phenols: Ferric chloride test:

- 1 ml extracts was dissolved in distilled water and a few drops of dilute ferric chloride solution was added. The formation of a red, blue, green or purple colour indicates the presence of phenols.

3.8. SEPARATION OF PLANT COMPOUNDS BY THIN LAYER CHROMATOGRAPHY

Thin Layer Chromatography (TLC) studies were carried out for various extracts to confirm the presence of different phytoconstituents (Paulami Mandal and Tapan Ganguly, 2009). TLC is a mode of liquid chromatography in which plant part containing a mixture of any compounds is spotted into a TLC plate and an organic solvent is allowed to move up on the plate by capillary action ;potentially carrying with it the various components in the plant extract. The different

components of the plant extract are separated based on their affinities for stationary phases (the silica on the plate) and for mobile phase (the solvent that moving up the plate). Each separated component has same migration time but different migration distance. Compound with more efficiency with the silica (hydrophilic compounds) will not move any far, while compounds with high affinity for organic solvent (hydrophobic compounds) will move much further.

Hand-made plates are prepared by using techniques like, pouring, dipping or spraying. Now-a- days, readymade pre-coated plates are also available. The plates need to be activated at 110 ^{0}C for 1 hr. Glass capillaries were used to spot the sample for TLC applied sample volume 1-micro litre by using capillary at distance of 1 cm at 5 tracks. In the twin trough chamber with different solvent system Chloroform:Methanol(14:1) solvent system. After pre-saturation with mobile phase for 20 min for development were used. After the run plates are dried and sprayed freshly prepared reagents (anisaldehyde, dragendroff, iodine etc) were used to detect the bands on the TLC plates. The mobility of compound in a particular TLC system is the retension factor(Rf value).

$$\mathbf{Rf = \frac{Distance\ travelled\ by\ a\ compound}{Distance\ travelled\ by\ solvent\ front}}$$

3.8.1. DEVELOPMENT OF THIN LAYER CHROATOGRAPHIC PLATE

3.8.1.1.p-ANISALDEHYDE TEST - STAIN 1

p-Anisaldehyde stain 1 is an excellent multipurpose visualization method for examining TLC plates. It is sensitive to most functional groups, especially those which are strongly and weakly nucleophilic. It tends to be insensitive to alkenes, alkynes, and aromatic compounds unless other functional groups are present in the molecules which are being analyzed. It tends to stain the TLC plate itself, upon mild heating, to a light pink colour, while other functional groups tend to vary with respect to coloration. It is recommended that a record is kept of which functional group stains which colour for future reference, although these types of comparisons may be misleading when attempting to ascertain which functional groups are present in a molecule . The shelf-life of this stain tends to be quite long except when exposed to direct light or solvent is allowed to evaporate.

It is recommended that the stain be stored in a 100 ml wide mouth jar wrapped with aluminium foil to ensure a long life time.

Procedure

To 135 ml of absolute ethanol was added 5 ml of concentrated sulphuric acid, 1.5 ml of glacial acetic acid and 3.7 ml of p-anisaldehyde. The solution is then stirred vigorously to ensure homogeneity. The resulting staining solution is ideally stored in a 100 ml wide mouth jar covered with aluminium foil.

3.8.1.2. p-ANISALDEHYDE TEST - STAIN 2

p-Anisaldehyde Stain 2 is more specialised than compared to p-Anisaldehyde Stain 1. It is used for terpenes, cineoles, with anolides, acronycine, etc. As above, heating with a heat gun must be employed to effect visualization.

Procedure

The solution is prepared as follows; Anisaldehyde:HClO4:Acetone:Water (1:10:20:80)

3.8.1.3.DRAGENDORFF TEST

Dragendorff staining is used for the identification of phenols.

Procedure

Reagent Preparation: Solution A: 1.7 g basic bismuth nitrate in 100 ml water/acetic

3.8.1.4.IODINE CHAMBER TEST

The staining of a TLC plate with iodine vapour is among the oldest methods for the visualization of organic compounds. It is based upon the observation that iodine has a high affinity for both unsaturated and aromatic compounds.

Procedure

A chamber may be assembled as follows: To 100 ml wide mouth jar (with cap) is added a piece of filter paper and few crystals of iodine. Iodine has a high vapour pressure for a solid and the chamber will rapidly become saturated with iodine vapour. Insert your TLC plate and allow it to remain within the chamber until it develops a light brown colour over the entire plate. Commonly if

compound has an affinity for iodine, it will appear as a dark brown spot on a lighter brown background. Carefully remove the TLC plate at this point and gently circle the spots with a dull pencil. The iodine will not remain on the TLC plate for long periods of time so circling these spots is necessary if one wishes to refer to these TLC's at a later date.

Chapter 4

RESULTS AND DISCUSSION

Keeping focus on the objectives of this project the extracts of the selected plants (*T.catappa)* was subjected for testing its potential bioactivity. The present study investigated the identification of different phytochemicals followed by checking cytotoxic effects of extracts on different cancer cell lines (A549 and HeLa) *in vitro*, measuring cell viability compared to healthy 3T3 fibroblast cell line.

4.1. EXTRACTION AND FRACTIONATION

Selected plant material was subjected to extraction by hot extraction method and resulted in 20g of *T.catappa*. Crude extracts obtained from selected plants are fractionated with different organic solvents in increasing order of polarity to obtain three fractions of each plant extract. Yield obtained by fractionation is summarized in Table 1.

Table 1: Yield obtained by fractionation of crude extracts

Plant species	Yield obtained by fractionation of crude extract(ml)		
	Chloroform fraction	Hexane fraction	Methanol fraction
T.catappa	410	420	290

Crude extract of the plant and their fractions were subjected for analysis of phytochemical screening and cytotoxicity.

4.2. PHYTOCHEMICAL SCREENING

The first part of present study carried out in *T.catappa* revealed the presence of medicinal active constituents. The phytochemical active compounds of *T. catappa* were qualitatively analyzed in the fleshy pericarp in various solvent extracts using standard procedures. The results are presented in Table 2.

In these screening process, alkaloids, glycosides, saponins, phenolic compounds, tannins, glycosides, flavanoids, steroids, terpenoids and carboxylic acid shows different types of results in different solvents extracts. Among these phytochemical screening, phenolic compounds and steroids were present in all solvent extracts. Whereas tannins were present in all extracts except chloroform. Flavanoids, Coumarin and Saponins are present only in methanol extract . Glycosides and alkaloids are present in chloroform and methanol. Terpenes and carboxylic acids are absent in three extracts.

Table2: Phytochemical constitute of the fleshy pericarp extract of *T.catappa*

SL.No	Compound	Chloroform extract	Hexane Extract	Methanol extract
1	Coumarin	-	-	+
2	Saponins	-	-	+
3	Alkaloids	+	-	+
4	Phenols	+	+	+
5	Flavanoids	-	-	+
6	Tannins	-	+	+
7	Terpenes	-	-	-
8	Glycosides	+	-	+
9	Steroids	+	+	+
10	Carboxylic acid	-	-	-

After the phytochemical analysis of pericarp extract of *T.catappa* ,next we go for the analysis of its cytotoxic effects in different cancer cell lines – A549 and HeLa cell lines.

4.3. BIOLOGICAL ACTIVITY

In vitro Anticancer Activity

To test the activity of crude plant extracts and fractions against different cell lines, MTT assay was performed.

4.3.1. Effect of fractions of *T. catappa* on cell viability of A549 cell line

The time-course effects of different concentrations of the methanol, chloroform and hexane fractions of T.*catappa* on the viability of A549 cancer cell line for 24,48 and 72 hours are shown in tables. Healthy 3T3 fibroblast cell lines were used as control. The result shows that in A549, the Hexane fraction of *T.catappa* evoked a slight decrease in cell viability to untreated cells .An incubation time interval of 24 hours was employed in all the dose dependent experiments of this study.From the intial time course experiment.,it was established that comparably a slight increase in cell death occur after 48 hr of incubation.The result presented in the figures also shows a,comparably increased cytotoxic activity over the cells,than other two fractions(Chloroform and Methanol).The percentage cytotoxicity of Hexane fraction of *T.catappa* is 53.1052 at 48 hrs

MTT assay of Chloroform fraction on A549cell line							
Time(h)	**Concentration**	**1000**	**500**	**250**	**125**	**62.5**	**Control**
24	Mean +_SD	0.178 ±0.0655	0.1735 ±0.0509	0.1895 ±0.0259	0.18713 ±0.338	0.1910 ±0.0331	0.1955 ± 0.0281
	Viability(%)	91.9693	88.746	96.930	95.703	97.698	
	Cytotoxicity(%)	8.0307	11.254	3.069	4.296	2.3017	
48	Mean +_SD	1.469 ±0.3157	1.3087 ±0.024	1.0965 ±0.0620	1.1456 ±0.0122	1.0459 ±0.0545	1.5716 ± 0.0325
	Viability(%)	93.4716	83.271	69.769	72.893	66.492	
	Cytotoxicity(%)	6.6528	16.728	30.231	27.1061	33.508	
72	Mean +_SD	3.2298 ±0.0117	2.5251 ±0.5114	3.7098 ±0.1173	3.6476 ±0.0208	3.782 ±0.0285	3.8012 ± 0.0381
	Viability(%)	84.967	66.429	97.595	95.959	99.494	
	Cytotoxicity(%)	15.032	33.5709	2.4045	4.0408	0.505	

Table 3 : Effect of Chloroform extract at various time intervals on A549, show no cytotoxicity

MTT assay of Hexane fraction on A549							
Time(h)	**Concentration**	**1000**	**500**	**250**	**125**	**62.5**	**Control**
24	Mean ± SD	0.1611 ± 0.0373	0.1613 ± 0.2268	0.1611 ±0.0919	0.1711 ±0.0122	0.17201 ±0.0102	0.1955 ± 0.0281
	Viability(%)	82.404	82.506	82.42	87.519	87.984	
	Cytotoxicity(%)	22.686	21.742	17.580	12.481	12.0154	
48	Mean ± SD	1.4704 ±0.3471	1.0697 ±0.2607	0.7337 ±0.0989	0.8047 ±0.0306	1.01715 ±0.0993	1.5713 ± 0.0325
	Viability(%)	93.5607	68.0643	46.8948	51.2025	64.7206	
	Cytotoxicity(%)	6.439	31.9357	53.1052	48.797	35.279	
72	Mean ± SD	3.4432 ±0.3882	3.7190 ±0.120	3.3000 ±0.1959	2.8124 ±0.5501	3.6028 ±0.0254	3.8012 ± 0.0381
	Viability(%)	90.5819	97.8375	86.8146	73.9871	94.7805	
	Cytotoxicity(%)	9.4180	2.1624	13.1853	26.0128	5.2194	

Table 4 : Effect of hexane fraction on A549 shows, a slight cytotoxicity at 48 hr.

MTT assay of Methanol fraction on A549 cell line							
Time(h)	**Concentration**	**1000**	**500**	**250**	**125**	**62.5**	**Control**
24	Mean ± SD	0.1895 ±0.332	0.1799 ±0.0561	0.1897 ±0.014	0.1802 ±0.0147	0.1913 ±0.0264	0.1955 ±0.0281
	Viability(%)	96.930	92.0204	97.0332	92.1739	97.851	
	Cytotoxicity(%)	3.069	7.979	2.9667	7.826	2.149	
48	Mean ± SD	1.560 ±0.1044	1.44185 ±0.2031	1.3649 ±0.0281	1.30405 ±0.1169	1.2057 ±0.0262	1.5716 ±0.0325
	Viability(%)	99.261	91.740	86.8477	82.972	76.717	
	Cytotoxicity(%)	0.739	8.26	13.152	17.027	23.28	
72	Mean ± SD	3.4298 ±1.0759	3.733 ±0.084	3.4776 ±0.2131	3.5944 ±0.0504	3.6997 ±0.0161	3.8012 ±0.0381
	Viability(%)	90.2294	98.205	91.486	94.559	97.329	
	Cytotoxicity(%)	9.7705	1.794	8.513	5.4403	2.6702	

Table 5 : Effect of methanol fraction at various time interval on A549 shows no cytoxicity

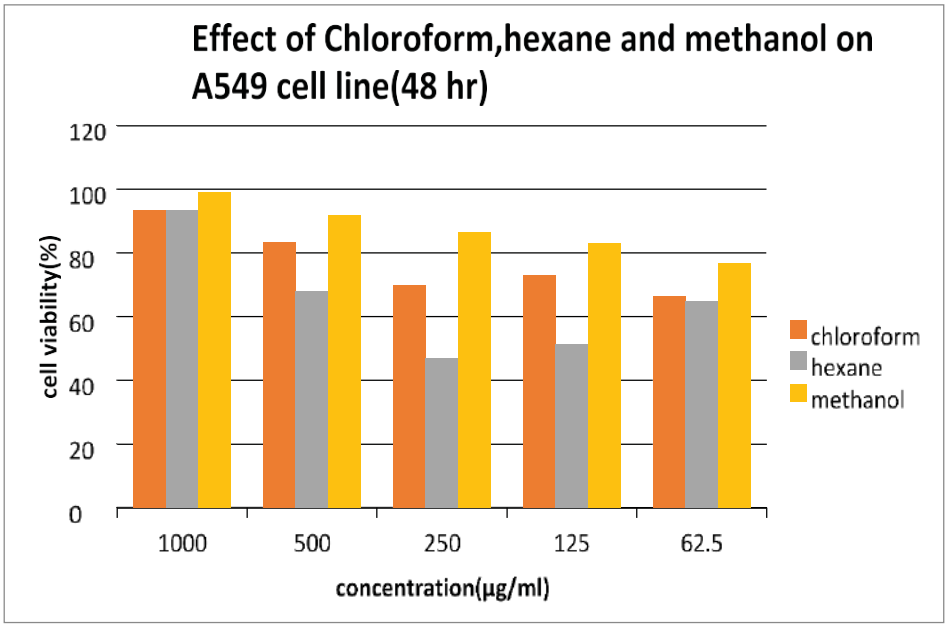

Fig 9: Percentage cell viability of A549 cell line when treated with different solvent fractions

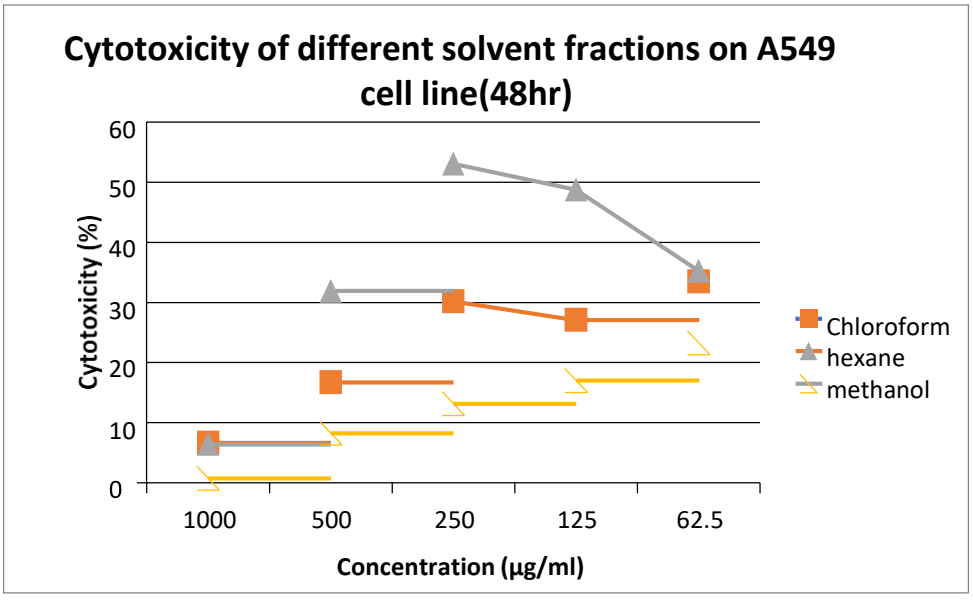

Fig 10 :Cytotoxic effect of Chloroform, Hexane & Methanol on A549 cell line at 48 hr

4.3.2. Effect of fractions of *T.catappa* on cell viability of HeLa cells

In the case of HeLa cell lines ,the result shows that there is no significant cell cytotoxicity by any fractions.The time course effect of different concentration of Chloroform, Hexane and Methanol fractions on HeLa cells at 24,48 and 72 Hours are shown in the tables.The result reveals ,there is no significant decrease in cell viability. Thus we can conclude that,the selected fractions of the plant extract have no cytotoxicity over HeLa cell lines.

MTT assay of Chloroform fraction on HeLa cell line							
Time(h)	**Concentration**	**1000**	**500**	**250**	**125**	**62.5**	**Control**
24	Mean ± SD	0.3621 ±0.0598	0.3552 ±0.0298	0.3554 ±0.0272	0.3712 ±0.0394	0.3728 ±0.0120	0.3754 ± 0.0425
	Viability(%)	96.457	97.282	94.672	98.881	99.3074	
	Cytotoxicity(%)	3.542	2.7171	5.327	1.1188	0.6926	
48	Mean ± SD	1.1904 ±0.2144	0.99305 ±0.099	1.2152 ±0.171	1.19165 ±0.1920	1.06905 ±0.268	1.5159 ± 0.0894
	Viability(%)	78.527	65.508	80.163	78.6100	92.238	
	Cytotoxicity(%)	21.473	34.491	19.836	21.3811	7.7610	
72	Mean ± SD	3.134 ±0.288	3.1548 ±1.309	3.2941 ±0.0486	2.932 ±1.5569	2.5921 ±0.1386	3.3861 ± 0.0693
	Viability(%)	92.554	93.169	97.283	86.589	76.551	
	Cytotoxicity(%)	7.445	6.830	2.717	13.410	23.448	

Table 6 : Effect of Chloroform extract on HeLa cell line at various time interval show no cytotoxicity

MTT assay of Hexane fraction on HeLa cells							
Time(h)	**Concentration**	**1000**	**500**	**250**	**125**	**62.5**	**Control**
24	Mean ± SD	0.3152 ±0.0171	0.2875 ±0.0188	0.2247 ±0.0547	0.3432 ±0.0278	0.3344 ±0.0702	0.3754 ± 0.0425
	Viability(%)	83.963	76.584	59.854	91.4224	89.078	
	Cytotoxicity(%)	16.0362	23.415	40.143	8.5775	10.92	
48	Mean ± SD	1.4988 ±0.127	1.4668 ±0.08195	1.1728 ±0.0243	1.2563 ±0.1536	1.3098 ±0.0441	1.5159 ± 0.0894
	Viability(%)	98.871	96.761	77.366	82.874	86.4074	
	Cytotoxicity(%)	1.1280	3.238	22.633	17.125	13.592	
72	Mean ± SD	3.1709 ±0.0501	3.3431 ±0.054	2.9729 ±0.7263	3.1553 ±0.0769	3.0087 ±0.068	3.3861 ± 0.0673
	Viability(%)	93.644	98.730	87.797	93.183	88.854	
	Cytotoxicity(%)	6.355	1.269	12.202	6.8161	11.145	

Table 7: Effect of hexane fraction on HeLa cell line on various time interval show no cytotoxicity

MTT assay of Methanol fraction on HeLa cell line cytotoxicity							
Time(h)	**Concentration**	**1000**	**500**	**250**	**125**	**62.5**	**Control**
24	Mean ± SD	0.337 ±0.0111	0.3492 ±0.349	0.3441 ±0.0678	0.3372 ±0.4824	0.3057 ±0.0915	0.3754 ±0.0425
	Viability(%)	89.7709	93.020	91.662	89.824	81.433	
	Cytotoxicity(%)	10.229	6.980	8.337	10.175	18.566	
48	Mean ± SD	1.4381 ±0.0888	1.3991 ±0.222	1.4321 ±0.0512	1.4173 ±0.0238	1.478 ±0.0201	1.5159 ±0.0894
	Viability(%)	94.867	92.295	94.471	93.495	97.499	
	Cytotoxicity(%)	5.1322	7.7049	5.5280	6.505	2.5001	
72	Mean ± SD	3.277 ±0.3920	2.547 ±0.6166	2.978 ±0.450	2.4632 ±0.4677	3.237 ±0.432	3.3861 ±0.0673
	Viability(%)	96.778	75.219	87.947	72.744	95.596	
	Cytotoxicity(%)	3.221	24.780	12.052	27.255	4.403	

Table 8: Effect of Methanol fraction on HeLa cells at various time interval shows no cytotoxicity.

4.3.3. Effect of fractions of *T.catappa* on the viability of 3t3 cells

The time course effect of different concentration of Chloroform, hexane and methanol fractions of *T.catappa* on the viability of 3t3 cell line for 24, 48 and 72 hours are shown in the figure.The result shows that , in 3t3 cell line, the hexane fraction does not evoke marked decrease in the cell viability(cell death). From these intial time course experiment ,it was established that cell death does not increase to its maximal level even after 72 hour of incubation. The incubation time interval of 24 hr was employed in all the dose dependent experiments of this study.The cytotoxicity of plant fractions over 3t3 cells was not significant and IC_{50} of any plant fraction was not found.

MTT Assay of hexane fraction of 3t3 cells							
Time(hr)	**Concentration**	**1000**	**500**	**250**	**125**	**62.5**	**Control**
24	Mean± SD	0.7427 ± 0.0263	0.6856 ± 0.1075	0.7361 ±0.0843	0.7145 ±0.0791	0.6819 ± 0.0314	0.7565 ± 0.0698
	Viability(%)	98.1258	90.6278	97.3033	94.4481	90.1387	
	Cytotoxicity(%)	1.8242	9.3721	2.69663	5.55189	90.1387	
48	Mean ± *SS DD*	1.0011 ± 0.0917	0.989 ±0.0254	1.1257 ±0.0115	1.1050 ±0.1184	09956 ±0.0237	1.1614 ±0.0784
	Viability(%)	86.1976	85.1558	96.9261	95.1437	85.7241	
	Cytotoxicity(%)	13.8023	14.8441	3.0738	4.8562	14.2758	
72	Mean± SD	0.2301 ±0.1931	0.2081 ±0.0130	0.2109 ±0.0123	0.2259 ±0.0251	0.2310 ±0.0315	0.2437 ±0.0424
	Viability(%)	94.4193	85.3918	86.5408	96.9593	94.7886	
	Cytotoxicity(%)	5.5806	14.6081	13.4591	7.3040	5.2113	

Table 9: The time course effect of crude hexane fractions on healthy 3t3 fibroblast

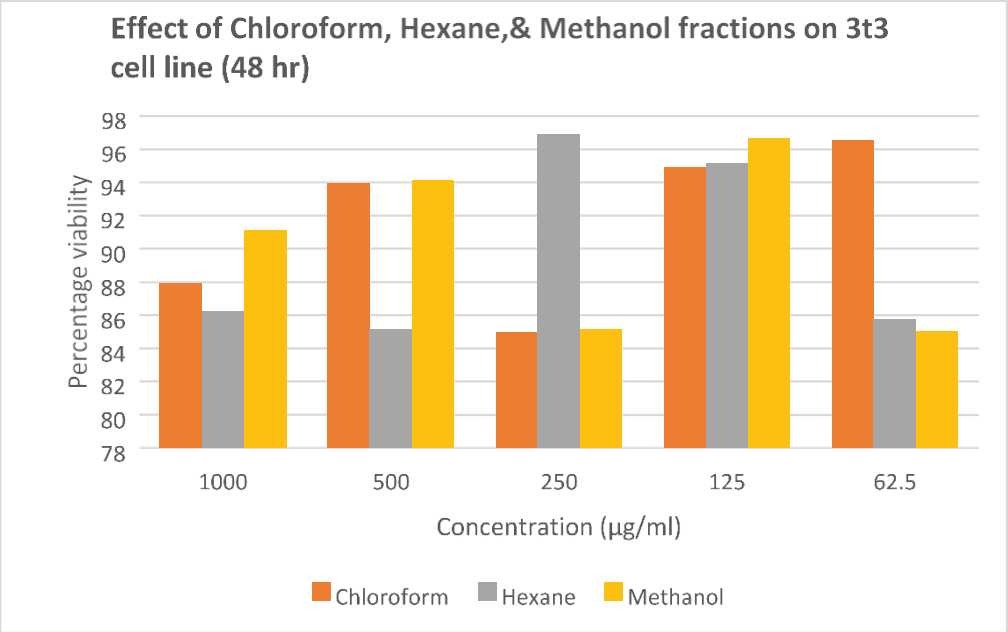

Fig 11:Percentage cell viability of different solvent fractions on 3t3 cell lines at hours 24 hours

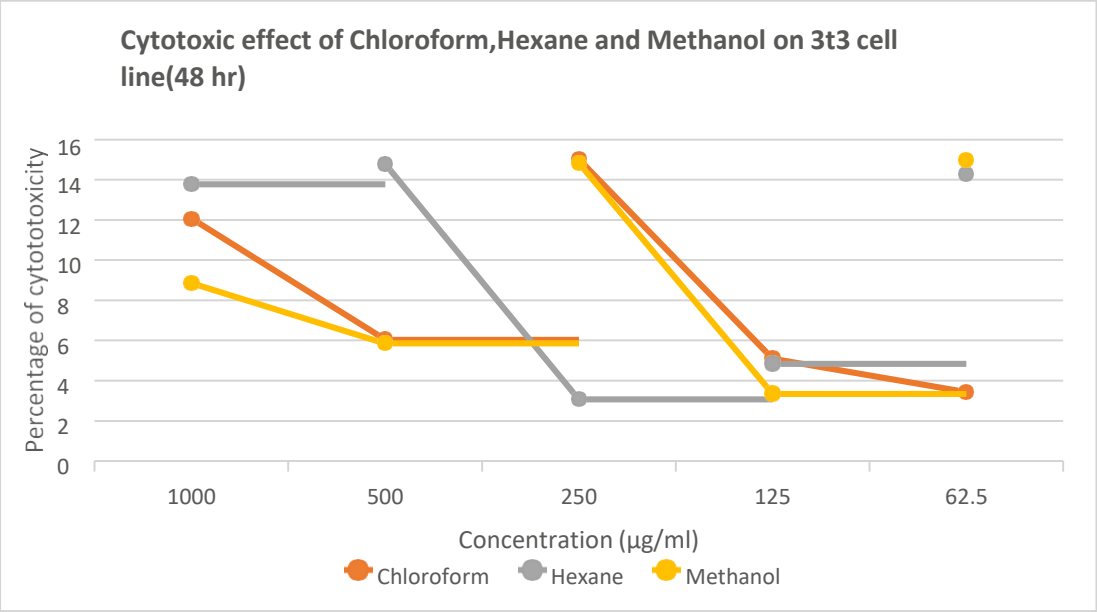

Fig12 : Effect of different solvent fractions on 3t3 cell lines

4.4 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography is the primary method for the separation of compounds and here, the hexane fraction have comparably higher cytotoxicity,it is taken for conducting TLC. Hence the bioactive compound can be analysed.

A large number of solvent systems were tried to achieve a good resolution. Finally, the solvent **Chloroform : Methanol** (15 : 1) was used. Thin layer chromatographic studies of the hexane extract of *T.catappa*, shows a several number of bands.Approximately 9-10 bands were clearly seen. Their Rf values are shown in the table.

$$Rf = \frac{\text{Distance Travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

TLC results indicate all the mobile phases are suitable to separate the compounds with good resolution. The Rf values are as follows;

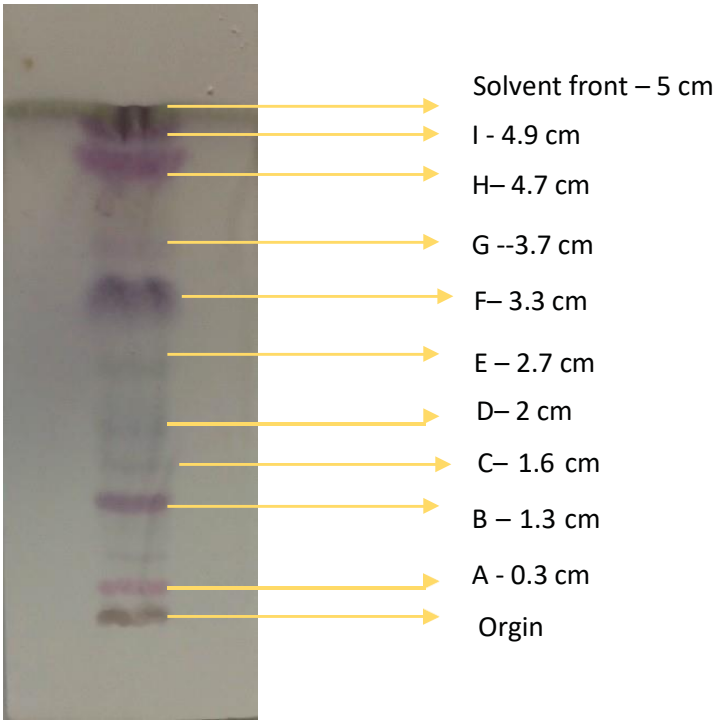

Fig 13: Thin layer chromatography –showing various bands of compounds in hexane extract

Table 10 : Rf values of separated compounds

Bands	Rf value
A	0.06
B	0.26
C	0.32
D	0.4
E	0.54
F	0.66
G	0.7
H	0.94
I	0.98

However the compound can be identified only when ran with suitable standards.

4.4.1. Iodine Chamber test

The TLC plate after the iodine chamber test showed three discrete bands which confirm the presence of organic volatile compounds in hexane extract of the pericarp

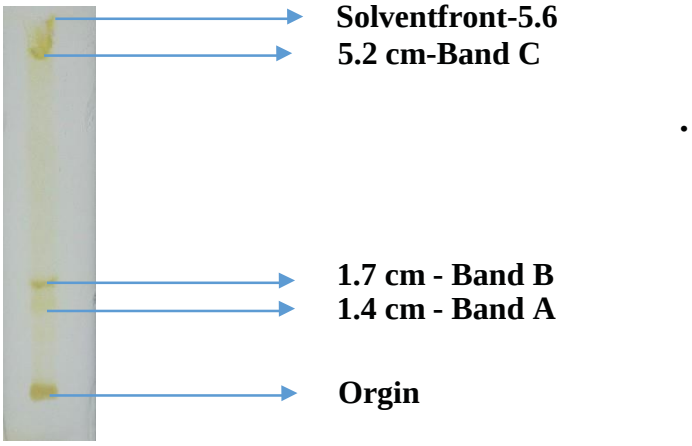

Figure 14: TLC Plate of hexane extract of pericarp of *T.catappa* after the iodine test

Bands	Rf value (cm)
Band A	0.25
Band B	0.30
Band C	0.92

Table 11:Rf value obtained from iodine chamber test

DISCUSSION

In the present study, qualitative phytochemical screening and ctotoxicity assay of the various extracts of pericarp of T.catappa was analysed.

a) Phytochemical screening

The phytochemical screening conducted in various solvent extracts (chloroform, hexane, and methanol) detects the presence of various secondary metabolites which may be responsible for the medicinal properties of the plant. The study revealed the presence of various bioactive components such as phenols, tannins, flavonoids, glycosides, saponins and alkaloids in the plant extracts among which phenols, tannins and steroids were most prominent. These secondary metabolites were reported to possess biological activities such as antidiabetic, antimicrobial, antioxidant, antiinflammatory, anticarcinogenic, hypoglycemic, activities etc [Negi et al.,2011].

Polyphenols are naturally occurred secondary metabolites present in plants that are generally used in defense mechanism against pathogens [Beckman C.H.,2000]. Polyphenols are responsible for the bitterness, color, flavor, odor and oxidative stability in food. Several studiesreveals that the long term consumption of diets rich in plant polyphenols which will offer prevention against the development of various cardiovascular diseases, neurodegenerative diseases, cancer, diabetes and osteoporosis [Graf et al.,2005; Arts I.C.W et al.,2005].

Flavonoids are water soluble polyphenolic compounds containing 15 carbon atoms. Based on the difference in the type of heterocycle involved, flavonoids may be classified into six subclasses such as flavonols, flavanones, flavones, anthocyanins, flavanols and isoflavones [Spencer,J.P ;et al.,1999]. The previous studies have reported that flavonoids revealed a wide range of biological activities such as anti-inflammatory, antioxidant, antiallergic, and antimicrobial [Kokate,K.C.,1997;Hossain,M.A et al.,2011]. Studies have shown that flavonoids prevent the oxidation of low-density lipoprotein and thereby prevent the development of atherosclerosis.

Tannins are polyphenolic compounds that are present in various parts of plants[Waterman,P.G et al.,1994]. Several studies confirmed that the tannins exhibit anti-oxidant, anti-microbial, and anti-inflammatory properties [Okwu,D.E et al.,2004]. The tannin can also be used asdrugs to heal the burning injury and on cuts to stop bleeding [King –Thom Chung et al.,2004].

b) Cytotoxicity analysis by MTT assay

The *in vitro* cytotoxicity assay of hexane extract of *T.catappa* showed a slight toxicity over the cell lines. As we are analyzing the phtochemical screening test, we can found that the phytoconstituents that predominently found in hexane extract are polyphenols and tannins.Studies shows that Polyphenols and tannins have antioxidant as well as anti-cancer activity.For example polyphenols (ellagic acid, gallocatechins,resveratrol) exerts anticancer effects through apoptosis induction (Heo et al.,2014;Wen et al.,2014).The study shows that ,ellagic acid isolated from *T.catappa* also have anti-oxidant activity(Toshiya et al.,1999). Recent studies shows that ,tannin component ,'punicalagin', present in *T.catappa* can protect CHO-k1 cells from induced gene mutation(Chen et al.,2000).Similarly ,antioxidant activity of tannin component punicallagin and punicalin from *T.catappa* were evaluated (Lin et al.,2001).

The hexane extract was selectively cytotoxic to the A549 cancer cells investigated in the present study and contained secondary metabolites, including phenols, tannins, steroids etc. The anti-cancerous activity of the extract can also be correlated due to the presence of these compounds which should be proved further.

It is reported that plant phenolics and tannins plays important role on cancer chemoprevention and chemotherapy, a major role by interacting between different types of genes and enzymes. Many mechanisms of action have been identified for these compounds which includes carcinogen inactivation, anti-proliferation, cell cycle arrest, induction of apoptosis, inhibition of angiogenesis, anti-oxidation, and reversal of multidrug resistance or a combination of these mechanisms (Yildrim and Kutlu, 2015; Chen et al., 2015).

In conclusion, the results of the present study provide confirmatory evidence for the presence of anticancer compounds in the fruit pericarp of *T.catapa*. Of the three solvent extracts identified to be cytotoxic (hexane, chloroform and methanol extracts), the hexane extract demonstrated a greater cytotoxicity in A549 cancer cell line and reduced cytotoxicity in normal 3T3 cells. Thus we can conclude that, the cytotoxicity of hexane fraction of *T.catappa* may be due to the presence of tannins and phenolic compounds.

Chapter- 5

SUMMARY AND CONCLUSION

India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants. The medicinal value of plants lies in some chemical substances , called phytochemicals that produce a definite physiological action on the human body. Phytochemicals are increasingly being used in the prevention and treatment of several human ailments including, cancer, heart diseases, diabetes, neuro degeneration and metabolic diseases (Manas KM et al.,2012). The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds. The use of herbs, rich in specific phytochemicals has been used to treat various types of cancer and this has been found to be effective with minimal or no side effects. Among the class of phytochemicals, polyphenols are known to possess chemo preventive activity (Zubair H et al.,2012).The actual compounds from the plant may not serve as the drug but leads to the development of potential novel agents. Natural agents are proving to be an important source of novel inhibitors & have the potential for development into selective anticancer agents.

In the present work, phytochemical screening and cytototoxic activity of different solvent fractions like chloroform, methanol, and hexane fractions of pericarp extract of *T. catappa* was studied. The solvent extraction was done by hot continuous process. The phytochemical screening reveals that the pericarp extract is rich in phenols, phytosterols and tannins. Cytotoxicity was measured using MTT Assay. Remarkable cytotoxicity was found to be exhibited by hexane extract of *T. catapa* against A549 cell line at 48 hr. Phenols, tannins, and phytosterols, the major phyto constituents identified in the extract which may be responsible for its cytotoxic potential. This studies concludes that, the extract would be used as a potent anti-cancer drug after further purification and evaluation

Chapter-6

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Chapter - 7

APPENDIX

- **PHOSPHATE BUFFERED SALINE**
  - Sodium chloride -8g
  - Potassium chloride -0.20g
  - Disodium hydrogen phosphate -1.44g
  - Potassium di hydrogen phosphate -0.24g
  - Triple distilled water -1000ml
  - Ph 7.2 with NaOH or HCL
- **PBS-EDTA solution**
  - EDTA -20g
  - PBS -100ml
- **Trypsin solution (0.25%)**
  - Trypsin -0.25g
  - PBS-EDTA -100mL
  - Sterilized by filtration
- **DMEM medium (1L plain medium)**
  - DMEM powder -13.48 (as per kit)
  - HEPES buffer -1.98g
  - $NaHCO_3$ -3.75g
  - Antibiotic Stored in fridge
- **10% DMEM**

  Prepare plain DMEM add 10ml FBS to 90ml medium and stored in fridge
- **Freezing mixture**
  - FBS -950µL
  - DMSO -50µL
- **MTT Assay (stoke for one plate)**
  - MTT -5g
  - PBS -1mL
- **FERRIC CHLORIDE**
  - Ferric (III) chloride -1%
  - Aqueous methanol -50%
- **NINHYDRIN**
  - Ninhydrin -1.5g
  - n-Butanol -100mL
  - Acetic acid -3mL

- **p- Anisaldehyde stain 1**
  - ❖ Absolute ethanol -135mL
  - ❖ Concentrated sulphuric acid -5mL
  - ❖ glacial acetic acid-3.7
  - ❖ p-anisaldehyde-3.7
- **p- Anisaldehyde stain 2**
  - ❖ Anisaldehyde -1mL
  - ❖ $HClO_4$ -10mL
  - ❖ Acetone -20mL
  - ❖ Water -80mL
- **DRAGENDORFF**

  Solution A
  - ❖ Bismuth nitrate -1.7g
  - ❖ Water -100mL

  Solution B
  - ❖ Potassium iodide -40g
  - ❖ Water -100mL
  - ❖ Water-phosphoric acid -50%