ANTIPROLIFERATIVE ACTIVITY OF *CELASTRUS PANICULATUS* WILLD.

BHAGYA UNNI
(2018-09-008)

B. Sc. – M. Sc. (INTEGRATED) BIOTECHNOLOGY

DEPARTMENT OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM – 695 522

KERALA, INDIA

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by

BHAGYA UNNI

(2018-09-008)

THESIS

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KERALA, INDIA

2023

DECLARATION

I hereby declare that the thesis entitled "**Antiproliferative activity of *Celastrus paniculatus* Willd.**" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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LIST OF ABBREVIATIONS

%	Percentage
^{o}C	Degree Celsius
$^{\Delta}$	Delta
µg	Microgram
µl	Microlitre
mL	Milli litre
5-FU	5 Flurouracil
Anti- Anti	Antibiotic and Antimycotic
BAX	Bcl-2-like protein 4
BCL-2	B-cell lymphoma/leukemia-2
CASPASE	Cysteine-dependent aspartate-directed proteases gene
CO_2	Carbon dioxide
cm	centimetre
CPMLC	Cold-extracted *Celastrus paniculatus* Methanolic leaf extract
CPMLS	Soxhlet extracted *Celastrus paniculatus* Methanolic leaf extract
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
et al.	*et alia*
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal Bovine serum
g	gram
h	Hour
hTERT	Human telomerase reverse transcriptase
IC_{50}	Half maximal inhibitory concentration

kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	milligram
min	Minute
ml	Millilitre
mM	millimolar
MTT	3- (4,5- dimethyl thiazol-2-yl)- 2,5- diphenyl tetrazolium bromide
nm	Nanometre
PARP1	Poly (ADP-Ribose) Polymerase 1 gene
PBS	Phosphate Buffered Saline
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
rpm	rotations per minute
sec	seconds
T_a	Annealing Temperature

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Introduction

1. INTRODUCTION

Cancer is a generic term for a broad spectrum of diseases characterized by uncontrolled and aggressive proliferation of cells. It is one of the leading causes of morbidity and mortality and a growing burden on the finance and healthcare systems of developing and developed nations around the world. Despite advances in cancer treatment, drug resistance, high cost, and associated undesirable side effects are downsides of currently available chemotherapeutic drugs used to treat cancer (Senapati *et al*., 2018). Hence, there is a constant need to find affordable and novel anticancer medicines with little or no detrimental effects.

Natural Products (NPs) are a valuable source of compounds with significant structural diversity and numerous bioactivities that may be developed directly or utilized as stepping stones for developing novel drugs (Atanasov *et al*., 2021). Plants are a rich source of numerous phytochemicals with pleiotropic function and are promising anticancer drug candidates. Paclitaxel from *Taxus brevifolia*, Vinca alkaloids from *Catharanthus roseus*, and β-lapachone from *Tabebuia avellanedae* are some of the promising plant-derived anticancer agents (Asati, 2022).

Two of the major "Hallmarks of cancer" that dictate the conversion of normal cells into cancerous cells are evasion of apoptosis and limitless replicative potential. Apoptosis or programmed cell death, is evolutionarily conserved and crucial in maintaining appropriate cell numbers in the body. Genes involved in apoptosis can be proapoptotic (*CAS 3*, *CAS 9*, *BAX*) or antiapoptotic (*BCL2*, *PARP1*). Apoptotic signalling components are often deregulated in cancer and hence its regulation is a promising treatment strategy (Pistritto *et al*., 2016). Human chromosome termini consist of repetitive DNA sequences called telomeres. Most eukaryotes stabilize the chromosome termini using telomerase enzyme, which compensates for the loss of telomeres due to DNA end replication problem (Bonnell *et al*., 2021). The telomerase reverse transcriptase (*hTERT*) gene maintains telomeres; induces the proliferation of cancer cells and is associated with malignant changes. Understanding the molecular mechanisms responsible for the evasion of apoptosis and telomere elongation in cancer cells provides a rational approach to combating cancer. Drug combinations that target various molecular changes or cancer hallmarks may be required to

produce long-term survival effects. This is likely to be the most challenging but intriguing precision cancer therapy in the future.

Celastraceae (staff-vine or bitter-sweet) family of the order celastrales is home to numerous medicinally important plants. *Celastrus paniculatus* Willd. is an important medicinal plant of the *Celastrus* genus, extensively utilized in Ayurveda and Unani medicinal systems. It is an unarmed climbing herb native to the Indian subcontinent, which can grow in various climatic conditions, mainly in tropical and subtropical forests. The plant possesses multiple pharmacological effects including nootropic and neuroprotective effects and is called "The Elixir of Life" due to its exclusive and miraculous health benefits (Maurya *et al*., 2021).

There are only a few preliminary studies regarding the cytotoxicity of *Celastrus paniculatus*. Beta-dihydroagarofuranoid sesquiterpenes, an essential constituent of *C. paniculatus*, have been shown to inhibit the viability of MCF-7 breast cancer cells and to induce apoptosis and autophagy with an IC_{50} value of 17±1μM (Weng *et al*., 2013). Cuprous oxide nanoparticles (CuO NPs) phytofabricated from the aerial extract of *Celastrus paniculatus* Willd. is found to hold good anticancer potential with a half-maximum inhibitory concentration (IC_{50}) value of 107.56 μg/mL and 208.57 μg/mL against MCF-7 and HT-29 cell lines, respectively (Giridasappa *et al.*, 2021).

Hence, the study aims to identify the true potential of *C. paniculatus* Willd. as a promising anticancer agent and possible mechanisms involved in inhibiting or killing cancer cells via induction of apoptosis and suppression of telomerase. The antiproliferative potential of methanolic leaf extract of *C. paniculatus* Willd. against breast and colon cancers, two of the most prevalent forms of cancer will be investigated using MCF-7 breast cancer and HCT-116 colon cancer cell lines. The induction of reproductive death of cancer cells by the extract which implies its effect on the long-term proliferation of cancer cells is assessed. Further, the expression of key apoptotic genes *CAS3, CAS9, PARP1*, *BAX, BCL2*, and telomerase gene *hTERT* is analysed for a better understanding of the molecular mechanism of cytotoxic activity of *C. paniculatus* leaf extract on cancer cells which can give a base for further in-depth investigations.

Review of Literature

2. REVIEW OF LITERATURE

Cancer has afflicted humanity since prehistoric times. According to the WHO, "cancer" (Latin word meaning 'crab'), is a generic term for a group of diseases characterized by the creation of abnormal cells from normal cells which grow beyond their boundaries, spread, and eventually invade other parts of the body through metastasis (WHO, 2022). The words "cancer" and "tumour" are often used interchangeably. However, the distinction between the two terms is important in cancer biology. Tumours are masses of unregulated and continually proliferating cells that can be either benign or malignant. On the other hand, malignant tumours that eventually spread throughout the body through circulatory or lymphatic systems are cancerous. In short, not all tumours are cancerous. Benign tumours are harmless but can turn cancerous and life-threatening if left untreated.

2.1 TYPES OF CANCER

Cancer is highly heterogeneous and can affect nearly all tissues in the body. Cancer can be classified according to the tissue of origin or its primary location in the body. Some cancers are even mixed types. Classification of cancer is represented in Figure 1 given below.

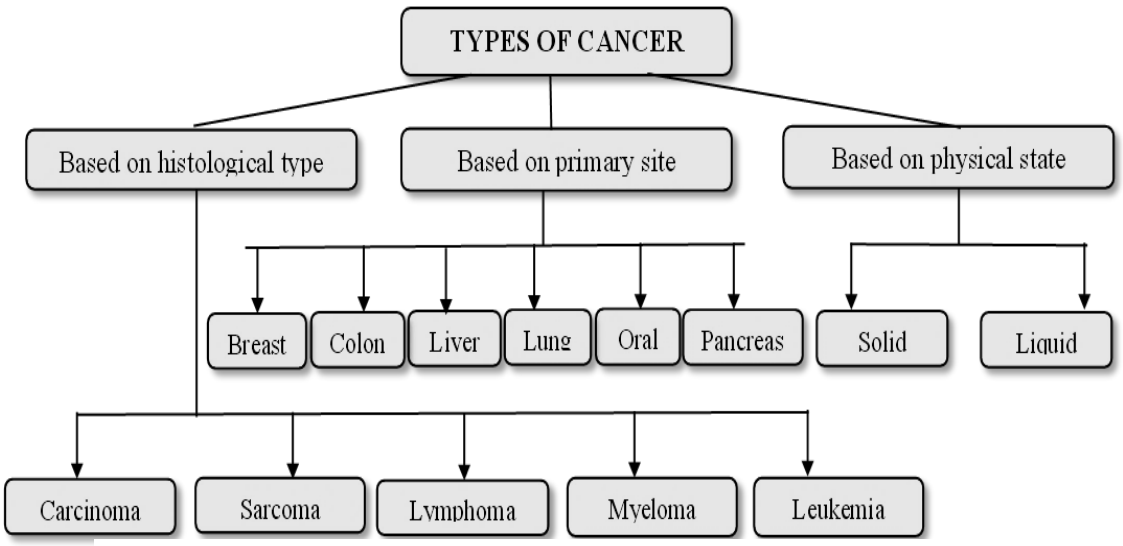

Fig. 1: Classification of cancer

a. Five broad categories of cancer according to their tissue of origin are as follows (Cancer Research UK, 2021):

1. Carcinoma:

   Carcinoma is a cancer of epithelial tissues that cover and line all the organs and body cavities. They are the most common type of cancer (80-90%). Carcinomas are of two major subtypes – adenocarcinoma which originates in an organ or gland and squamous cell carcinoma which originates in squamous epithelium.

2. Sarcoma:

   Sarcoma is a cancer of connective tissues which are the supporting tissues of the body. Connective tissues include muscles, bones, cartilage, and fat. A characteristic feature of sarcomas is that they can resemble the tissue they grow. They are less common than other types of cancers, less than 1% are diagnosed every year. They include Osteosarcoma (bone), Chondrosarcoma (cartilage), Leiomyosarcoma (smooth muscle), Rhabdomyosarcoma (skeletal muscle), Liposarcoma (adipose tissue), Glioma and others.

3. Lymphoma

   Lymphoma is a cancer of the lymphatic system which originates in lymphatic glands, lymphatic organs, and lymph nodes that act as a filter to resist body infections. They are solid tumours that can be of two major subtypes: Hodgkin lymphoma and Non-Hodgkin lymphoma diagnostically differentiated with the absence of Reed-Sternberg cells in the latter.

4. Leukemia

   Leukemia is the cancer of white blood cells resulting from the overproduction of immature leukocytes. Immature leukocytes are hence unable to perform their primary function of protecting the body from infections. It is also called blood cancer or liquid cancer as it usually does not form solid tumors. They include Acute myelocytic leukemia (AML), Chronic myelocytic leukemia (CML), Acute lymphocytic leukemia (ALL), Chronic lymphocytic leukemia (CLL) and erythremia.

5. Myeloma

   Like Lymphoma, Myeloma is also a malignancy of the lymphatic system. It is also named multiple myeloma and originates in plasma cells which are a type of white blood cells that produce antibodies. Cancerous plasma cells do not make antibodies appropriately to fight infection. They are also a less common type of cancer.

a. Based on the primary location, cancer can be classified as breast cancer, colon cancer, liver cancer, lung cancer, pancreatic cancer, cervical cancer, testicular cancer, prostate cancer, oropharyngeal cancer, and others based on their site of occurrence. Both breast cancer (2,26 million cases) and colon cancer (1.93 million cases) are invasive cancer types being among the top 5 most common cancers by incidence and mortality in 2022 (WHO, 2022).

b. Based on the physical state of cancer, they can be of two types.
   1. Solid cancers (organ cancers) are cancers that form masses or multiple cell masses and do not contain cysts or liquid areas. Carcinoma, sarcoma, and lymphoma are solid tumours and are not present enough in body fluids.

   2. Liquid cancers often referred to as blood malignancies such as leukemia create masses that travel through the bloodstream.

2.2 EPIDEMIOLOGY OF CANCER

Cancer has become a pressing health issue, causing a financial burden on healthcare systems worldwide. Recent global estimates reveal that cancer is the second leading cause of mortality after heart diseases. According to WHO, Australia, followed by New Zealand, Ireland, and the United States, have the highest cancer rates per 100,000 people (IARC, n.d.). The pace of decline in cancer mortality has gradually increased from about 1% per year in the 1990s to 1.5% in the 2000s and to 2% per year from 2015 to 2020 (Siegel *et al*., 2023). The figures for the cost of cancer care underscore the significant economic troubles associated with diagnosis and treatment and the urgent need to escalate cancer research to provide better cancer health care.

Cancer is a menace to India, like other nations around the world. India is a vast country with diverse people and lifestyles, resulting in a heterogeneous distribution of disease across

the country. India's highest number of cancer cases was in Kerala, followed by Mizoram, Haryana, Delhi, and Karnataka, and the lowest rate was in Bihar in 2016 (Dhillon *et al.*, 2018). A systematic study and data collection performed across India under the National Cancer Registry Programme revealed over 1.3 million anticipated cancer patients in 2020. The five leading sites are the breast, lung, mouth, cervix, uterus, and tongue (Mathur *et al.*, 2020). Current cancer prevalence in India shows that lung, mouth, oesophagus, stomach, and nasopharynx cancers are more common in men, whereas breast and uterine cervix cancers are more common in women. The expected rise in the total number of new cancer cases in males is from 0.589 million in 2011 to 0.934 million by 2026, while the number is from 0.603 million to 0.935 million in females during the same period (D'Souza *et al*., 2013).

2.3 MOLECULAR BASIS OF CANCER

Cell division is a tightly regulated process controlled by several genes. This includes genes whose protein products stimulate cell division and growth which in their normal counterparts are termed as proto-oncogenes and mutated forms are known as oncogenes. Exposure to chemical, environmental, or viral carcinogens activates mutated oncogenes, causing cell changes and the production of proteins that are either incorrectly expressed within normal cells or inappropriately expressed in other tissues, which promotes cellular proliferation and ultimately results in the development of cancer.

The other group of genes includes those which can directly or indirectly cause cell death and are known as tumour suppressor genes. Inactivation of tumour suppressor genes is one of the common mechanisms that contribute to tumorigenesis (Wang *et al*., 2018). The gain of function mutations in protooncogenes and loss of function mutations in tumour suppressor genes lead to the development of cancer. Several key oncogene and tumour suppressor signalling networks responsible for cancer progression are listed in Table 1 below (Kontomanolis *et al*., 2020).

Table 1: Proto-oncogenes and tumour suppressor genes with their respective functions

Proto-oncogenes	Function	Tumor suppressor genes	Function
MYC	Transcription factor in the induction of apoptosis	*RB*	Prevent cell division
ABL	Tyrosine kinase in cell division and differentiation	*TP53*	Controls cell proliferation and growth
RAS	G-protein in cellular signal transduction	*JAK*	Promote cell division
BCL-2	Inhibition of apoptosis	*APC*	WNT signaling pathway regulator
HER2	Protein receptors in cell division	*PTEN*	Prevent cell division

2.4 HALLMARKS OF CANCER

Douglas Hanahan and Robert Weinberg (2000) described six crucial and distinctive functional capabilities that collectively enable malignant growth known as "Hallmarks of Cancer" which provide a comprehensive framework and unravel the complexity of cancer and are as given in Table 2.

Table 2: Hallmarks of cancer and their characteristic features (Lazebnik, 2010)

Hallmark	**Characteristic Features**
Acquired Capability: Self-Sufficiency in Growth Signals	Cancer cells produce many growth factors like PDGF (platelet-derived growth factor) and TGFα (Tumor Growth Factor alpha) and thus overcome the requirement of external growth factors like the Epidermal Growth Factor (EGF). The reduction in dependence on exogenous growth signals breaches the anticancer defense mechanism of normal cells that maintain tissue

	homeostasis through a tightly regulated cell cycle.
Acquired Capability: Insensitivity to Antigrowth Signals	Cancer cells evade antiproliferative signals that maintain tissue homeostasis by balancing cell proliferation. They suppress tumor suppressor genes and lack contact inhibition which helps the cancer cells to grow and divide independent of their surroundings.
Acquired Capability: Evading Apoptosis	Apoptosis or programmed cell death is a cell suicide cascade that is a major barrier to cancer formation and progress. Hence cancer cells evade apoptosis by activating various anti-apoptotic genes and suppressing pro-apoptotic genes necessary for maintaining normal cell populations.
Acquired Capability: Limitless Replicative Potential	Cancer cells achieve replicative immortality surpassing Hayflick's limit (60-70 population doublings) of mammalian cells by maintaining telomeric ends. Telomeres shorten after each cell division due to the end replication problem and it shortens to such an extent that it marks the start of cellular senescence at Hayflick's limit. Telomerase reverse transcriptase *hTERT* is responsible for replicative immortality in cancer cells by maintaining telomere ends.
Acquired Capability: Sustained Angiogenesis	Angiogenesis or formation of vascular framework from pre-existing vessels is necessary for the growth and spread of cancer as it ensures an adequate supply of nutrients and oxygen. Vascular endodermal Growth Factor (*VEGF*), fibroblast growth factor (*FGF*), and

	platelet-derived growth factor (*PDGF*) play an important role in angiogenesis in cancer cells.
Acquired Capability: Tissue Invasion and Metastasis	Cancer cells develop the ability to migrate and invade neighbouring tissues from their primary site and establish secondary tumours through a process called metastasis. It involves extensive changes to cell-cell and cell-matrix interactions It is responsible for malignant growths and tumour dissemination around the body and responsible for 90 % of death in cancer patients.

2.4.1 Apoptosis: A Distinct Mode Of Programmed Cell Death

Cell death or cell suicide, a natural process of cell ceasing is fundamental to remove unwanted cells essential to maintain tissue homeostasis. It can take place either by programmed cell death (apoptosis and non-apoptosis including autophagy) or necrosis. Apoptosis (Greek "Dropping off") is a sophisticated way of programmed cell death (PCD) with wide-ranging implications in tissue kinetics (Kerr *et al.*, 1972). It is an evolutionarily conserved mechanism and morphological changes associated with apoptosis are similar across cell types and species. It is essential for normal cellular turnover, to remove damaged cells that can interfere with normal functions. The necrosis or accidental cell death is caused by pathological stimuli. It is accompanied by rounding-up of the cell, retraction of pseudopodia, reduction of cellular volume (pyknosis), condensation of the chromatin, fragmentation of the nucleus (karyorrhexis) with little or no ultrastructural modification of cytoplasmic organelles and plasma membrane blebbing (Kroemer *et al*., 2005). There are two main pathways of apoptosis based on how apoptosis is induced and both these pathways converge at the later stage as represented in Figure 2.

a. Intrinsic Pathway/ Mitochondrial Pathway

It is mediated by mitochondria in response to internal signals including oncogenes, direct DNA damage and hypoxia. This occurs via permeabilization of the outer mitochondrial membrane by proapoptotic members of the Bcl family of proteins (Ola *et al.*, 2011).

Mitochondrial outer membrane permeabilization (MOMP) is often considered the "point of no return" for apoptosis. Hence, proteins normally found in the space between the inner and outer mitochondrial membranes are released, including cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G (Saelens *et al.*, 2004). Then, an apoptosome complex made up of Apaf-1, caspase 9 and cytochrome c is formed, which leads to a caspase cascade. As part of the cascade, caspase 9 is activated first, which further activates caspase 3 and caspase 7, eventually leading to nuclear fragmentation and cell death. Tumour suppressor gene p53 is critical in the intrinsic apoptosis pathway where p53 activates the pro-apoptotic Bcl family members and represses anti-apoptotic Bcl proteins. (Wei *et al.,* 2021). Bcl2, Bcl XL, and Mcl-1 are anti-apoptotic proteins whose role is to prevent the release of cytochrome c and maintain mitochondrial integrity, whereas Bax, Bak, Bad, and Bok are pro-apoptotic proteins of the Bcl2 family whose role is to allow the release of cytochrome c (Edlich, 2018).

b. Extrinsic pathway/ Receptor Pathway

It is triggered by ligand molecules or external stimuli and is mediated by the binding of ligands to extracellular death receptors such as Fas receptors, DR4/DR5, Tumor Necrosis Factor Receptors (TNF-R), and TNF-Related Apoptosis-Inducing Ligand Receptors (TRAIL-R) present at the surface of different cells (Kashyap et al., 2021). TNF superfamily comprises death receptor ligands such as CD95 ligand (CD95L), TNFα, lymphotoxin-a (the latter two bind to TNFRI), TRAIL and TWEAK, a ligand for DR3. Adapter proteins are recruited and a complex of ligand-receptor-adaptor leads to the formation of the death-inducing signalling complex (DISC). This leads to the activation of caspase 8 which further crosslinks with the execution phase of the intrinsic pathway with the activation off caspase 3 finally causing cell death.

2.4.1.1 Genes involved in apoptosis

Caspases: Caspases, a family of aspartic acid-specific proteases play a key role in apoptosis. There are both initiator and effector caspases based on the phase of apoptosis they act. Caspase 8 and 9 are initiator caspases, while Caspase 3, Caspase 6, Caspase 7, and Caspase 10, plays a key role in the execution phase of apoptosis and are effector or executioner caspase.

Bcl2 family of proteins: The Bcl2 family of proteins can either promote or suppress apoptosis. Antiapoptotic Bcl2 is the founding member of the group. Other anti-apoptotic proteins of the family are Bcl2, Bcl-XL, Mcl-1, Bfl-1, Bcl-W, and Bcl2 L10, Bcl2 and Bcl-xL in the mitochondria limit the release of apoptotic mediators such as cytochrome c and apoptosis-inducing factors. The pro-apoptotic Bcl2 family members can also be differentiated into BH3-only proteins (only the BH3 region is homologous to Bcl2) and effector proteins with several BH domains (BH1, BH2, and BH3). Bim, Bad, Bid, Noxa, Puma, Bmf, Hrk, and Bik are BH3-only proteins (Roufayel *et al*., 2022). Multi-BH-domain effector proteins include Bax, Bak, and Bok.

PARP: Poly (ADP-ribose) polymerase (PARP), a nuclear protein involved in DNA repair, is one of the known substrates of caspases. It catalyzes the poly (ADP-ribosyl)ation of a variety of nuclear proteins**.** Hence, PARP cleavage is considered to be a critical event of apoptosis. PARP inactivation has been hypothesised to prevent NAD (a PARP substrate) and ATP depletion, both of which are thought to be essential for following steps in apoptosis (Boulares *et al*., 1999).

Dysregulation of apoptosis results in various pathological conditions including cancer and autoimmune diseases. The anticancer drugs that cause apoptosis include Bcl2 antagonists, Smac/Diablo mimics, Herceptin, Gleevec and Iressa, Rituximab, and the p53-MDM2 complex (Gerl *et al*., 2005). There have been continued efforts in the search for new plant-based anticancer drugs targeting various aspects of apoptosis. *Moringa oleifera* crude aqueous leaf extract is reported to result in cell-cycle arrest and induce apoptosis in cancerous human liver hepatocellular carcinoma (HepG2) (Tiloke *et al*., 2019). Another study revealed that ethanolic extract of *Cyperus rotundus* rhizomes showed higher antiproliferative activity than methanolic extract and resulted in the induction of apoptosis by mitochondrial membrane depolarization, the upregulation of death receptor 4 (DR4), DR5 and pro-apoptotic Bax, as well as downregulation of anti-apoptotic surviving, Bid expression and activated caspase 8 and 9, and Bcl2 (Hemanth Kumar *et al*., 2014).

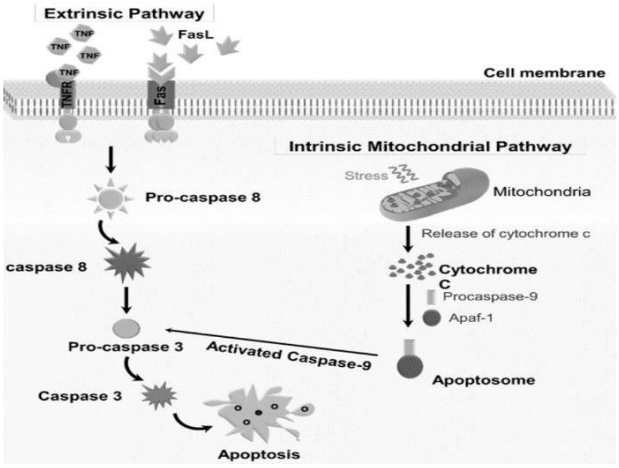

Fig. 2: Schematic representation of apoptotic pathways (Bhosale *et al*., 2020)

2.4.2 Telomerase And Their Role In Cancer Development

Telomeres are arrays of non-coding highly repetitive DNA sequences that cap the ends of chromosomes and maintain genomic integrity protecting from end-to-end fusions. They progressively shorten during each cell division due to the DNA end replication problem and when the chromosome shortening reaches a critical length, the cells stop dividing, further triggering chromosome senescence and is correlated to the cell attaining Hayflick limit (Olovnikov, 1996). Human telomeres consist of a highly conserved guanine-rich repetitive sequence 5'-$(TTAGGG)_n$-3' ranging from 5-15 kb followed by a terminal 3′ G-rich single-stranded overhang (150-200bp) in somatic cells (Moyzis *et al*., 1988). The T loop structure is a characteristic feature of telomeres in which the 3' single-stranded overhang folds back and invades the homologous duplex-repeat array forming a displacement (D) loop of TTAGGG repeats (Greider, 1999). T loop structure protects the telomeric ends from inappropriate DNA Damage response pathways. The structure of the telomere is given in Figure 3 below.

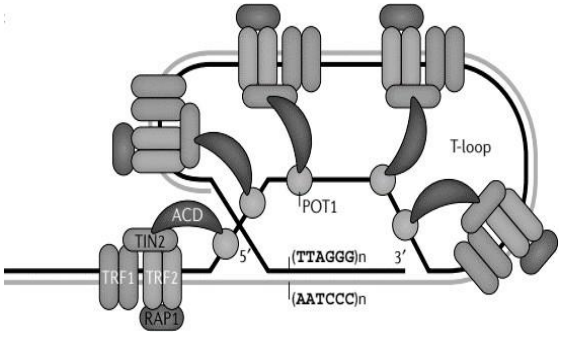

Fig. 3: Structure of telomere (Shay and Wright, 2019)

Telomerase is a ribonucleoprotein that maintains telomere length by adding telomeric repeats to the chromosomal ends. It consists of human telomerase reverse transcriptase (hTERT) which forms the catalytic core and non-coding human telomerase RNA (hTR) which serves as a template for adding new sequences onto the ends of chromosomes (Roake and Artandi, 2020). Six specialised proteins called shelterin proteins make up a shelterin complex that associates with telomere to provide for the capping functions as described in Table 3 below (Turner *et al*., 2019).

Table 3: Shelterin proteins and their functions

Shelterin Proteins	Functions
Telomere repeat binding factor 1 (TERF1/TRF1)	Homodimer; Bind telomeric dsDNA
Telomere repeat binding factor 2 (TERF2 or TRF2)	Homodimer; Bind telomeric dsDNA
TERF1 interacting nuclear factor 2 (TINF2 or TIN2)	2 connects TRF1 and TRF2 dimers
Protection of Telomeres 1 (POT1)	caps the telomeric 3′ tail
Shelterin complex subunit and telomerase recruitment factor (ACD or TPP1)	caps the telomeric 3′ tail
TERF2 interacting protein (TERF2IP also known as RAP1)	accessory subunit of TRF2; Protect critically short telomeres

Cancer cells generally have an average telomere length of about 5 kb when compared to the telomere length of normal cells which is about 12 kb and germ cells' average telomere length of 15-20 kb. Maintenance of telomere length is required for the proliferation of cancer cells. Expression of telomerase is highly regulated and restricted to certain cell types like gametes and diminished in somatic cells after birth (Hiyama and Hiyama, 2007). Telomerase activity was first identified by G. B. Morin in the HeLa breast cancer cell line (Morin, 1989). Telomerase activity has been detected in approximately 85% of cancers while 15% maintain telomere length using an ALT (Alternative Lengthening of Telomeres) mechanism. Expression of *hTR* and *hTERT* is upregulated in almost all human malignant tumours making it a useful marker for cancer diagnosis and a prognostic marker of cancer including breast, colon, stomach, and neuroblastoma (Hiyama and Hiyama, 2002).

Telomerase is a relatively selective cancer target as normal cells express little or no telomerase as compared to cancer cells. Many therapeutic vaccines targeting telomerase protein TERT are currently under trial in leukemia, breast, renal, prostate, lung, skin, and pancreatic cancer (Harley, 2008). Telomestatin, tamoxifen, 3'azido-3'deoxythymidine, and pyridinium-ceramides are anticancer agents that are designed to target and interfere with telomerase activity (Ruparel *et al*., 2011).

Telomerase inhibitors derived from natural sources are an appealing cancer therapeutic option. Plant secondary metabolites such as polyphenols, alkaloids, triterpenoids, xanthones and sesquiterpenes show anticancer potential by targeting telomerase and conferring anti-proliferative properties (Ganesan and Xu, 2017). Curcumin, a yellow-coloured dietary pigment from *Curcuma longa* L. at a concentration of 100mM inhibited 93.4% telomerase activity in MCF-7 breast cancer cells compared to untreated cells (Ramachandran *et al*., 2002). *Melissa officinalis* hydroalcoholic extract shows potent antiproliferative activity by downregulating *BCL2*, *HER2*, *VEGF-A* and telomerase gene *hTERT* in a study on human breast, lung and prostate cancer cells (Jahanban-Esfahlan *et al*., 2017).

2.5 CURRENT TREATMENT STRATEGIES

Standard treatment regimens and the response of cancers to these treatment strategies are largely dependent on cancer type, stage, and patient's performance status. Current common cancer treatments include chemotherapy, surgery, radiotherapy, targeted therapy, and immunotherapy as shown in Figure 4.

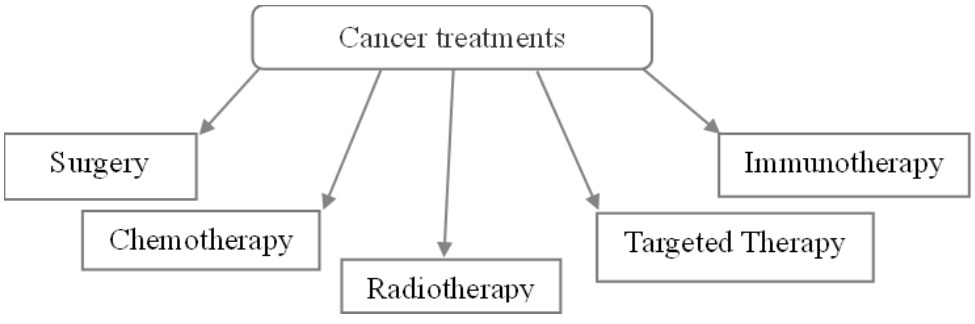

Fig. 4: Common cancer treatment options

Surgery is one of the oldest of cancer treatment which works best for solid tumours restricted to one area. Surgery involves the removal of tumour tissue by specialised surgeons and can be open or minimally invasive. Surgical complications include discomfort, haemorrhage, impairment to other organs, and a slow return to normal bodily functions (De Boniface *et al.,* 2022).

Chemotherapy refers to intravenous, intrathecal, subcutaneous injection or oral administration of drugs depending on the type and stage of cancer mostly being intravenous due to 100% absorption rate (Amjad, 2023). It can be used solely or in combination with other treatments. It is associated with serious side effects depending on the type of chemotherapeutic agent administered and individualised patient characteristics. It can be adjuvant or adjunct chemotherapy (in addition to the primary course of treatment) or neoadjuvant (prior to the primary course of treatment) chemotherapy. Common side effects can be acute or prolonged and range from fatigue, mouth sores, nausea, and hair loss to hypersensitivity and neurotoxicity.

Radiation therapy or radiotherapy uses high doses of radiation to kill cancer cells and prevent them from spreading to other parts of the body. Radiation therapy is often used to shrink the tumour before surgery. Early cancers including skin cancers, prostate, lung and cervix carcinomas and lymphomas can be solely with radiotherapy (Baskar *et al*., 2012).

Immunotherapy is gaining attention in cancer treatment as it effectively uses the immune system to find and kill cancer cells (Esfahani *et al*., 2020). It is a kind of biological therapy which makes use of monoclonal antibodies, vaccines, cytokines and CAR T-Cell therapy. It is not widely used as other treatment strategies.

Targeted therapy has revolutionized the field of oncology and is the foundation of precision medicine in which drugs target specific genes that help in tumour development and progression (Padma, 2015). It is less toxic to normal cells than chemotherapeutic drugs, nevertheless, high cost and potential side effects are drawbacks of targeted therapy.

2.5.1 Chemotherapeutic Drugs And Mechanism Of Action

Chemotherapeutic drugs used against cancer can be classified in several ways according to the mechanism of action exhibited by them diagrammatically represented in Figure 5 (Amjad, 2023).

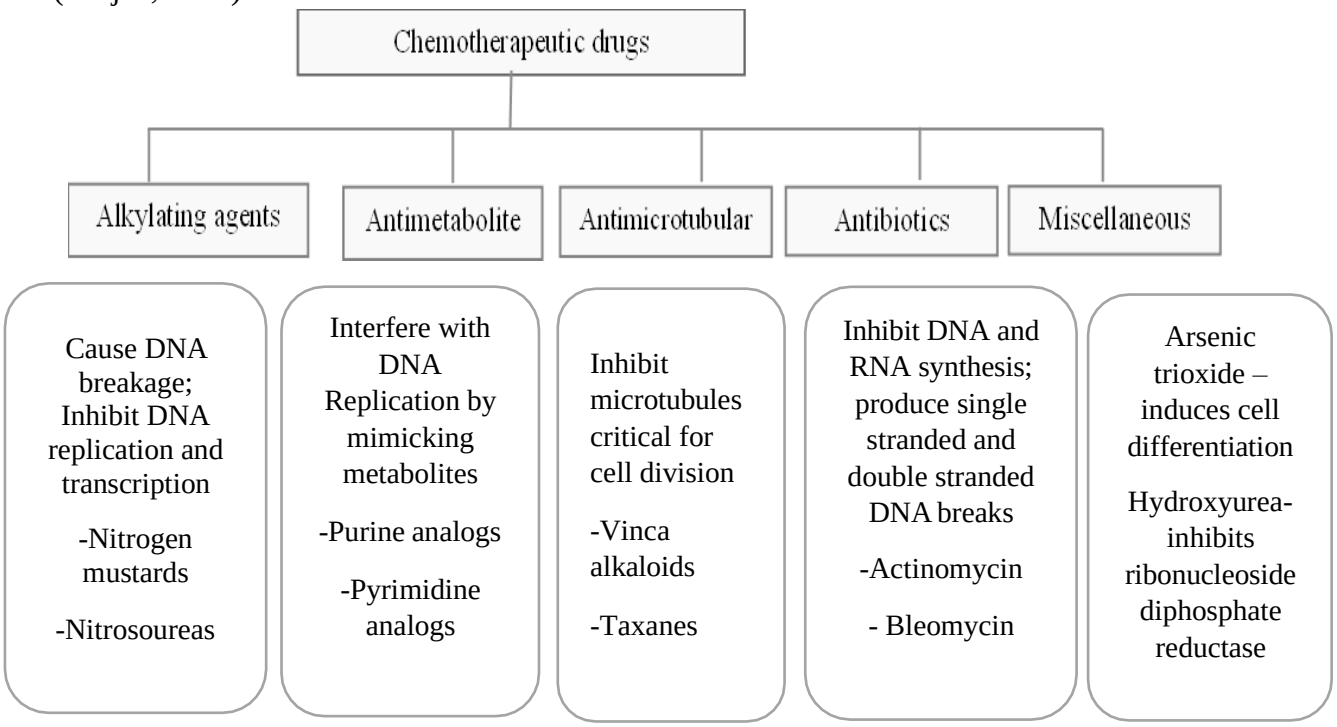

Fig. 5: Major classification of Chemotherapeutic drugs with examples

2.5.2 Natural products in cancer therapy

There are numerous potential adverse effects of existing drugs which increase patient morbidity including nausea, weight loss, chemical imbalances in the body, persistent joint pains, and life-threatening complications. Cancer survivors are also at acceptable risk of cancer recurrence, which affects the quality of life of cancer patients. Hence, there have been increasing efforts to develop a reliable low-cost therapeutic intervention with reduced side effects and toxicity without compromising therapeutic efficacy.

According to WHO, more than 80% of the world's population relies on herbal remedies to meet their primary healthcare needs (Akerele, 1993). Various phytochemicals with pleiotropic effects have demonstrated antitumor activities in various preclinical and clinical studies. Vinblastine, vincristine, taxol, elliptinium, etoposide, colchicinamide, 10-hydroxycamptothecin, and curcumol are among the indispensable phytochemicals with antitumour activity (Singh *et al*., 2016).

Research attempts demonstrated that many plant extracts have an essential function in the management of several malignancies, including breast, stomach, oral, colon, lung, hepatic, cervical, and blood cancer cell lines through *in vitro* studies. Furthermore, *in vivo,* studies on the anticancer properties of these plants yielded brimming results in combating cancer in animal models. Lang *et al.* (2019) reported that *Artemisia annua* extract showed antiproliferative potential on breast, pancreas, prostate and non-small cell lung cancer cell lines as compared to normal mammary epithelial cells, lymphocytes, and PBMC cells. Also, the extract was responsible for apoptosis-mediated cell death *in vitro* and *in vivo*. The quest for novel anticancer plants is currently receiving focus in anticancer research. Motadi and coworkers (2020) identified and evaluated the molecular the mechanism of cytotoxic activity of *Tulbaghia violacea* and it was revealed that methanol, hexane, and butanol extracts exhibited cytotoxic activity in breast and cervical cancer cells with methanolic extract being the most cytotoxic and apoptosis was induced in a p53 dependent pathway.

Although these phytochemicals are present in different plant parts as a complex mixture with a variety of properties, their relative quantity depends on a variety of variables, including the environment, culture, timing of collection, extraction methods, etc. Since crude extracts include different amounts of pharmacologically active ingredients, it is crucial to identify the bioactive chemicals and determine their ideal concentrations for maximal therapeutic efficacy.

2.5.2.1 Celastrus paniculatus Willd. -"Elixir of life"

Members of the Celastraceae family have a plethora of bioactive constituents that have been used since antiquity for treating various ailments. *Celastrus paniculatus* Willd. (Black oil plant or Intellect plant) is an important medicinal plant of Celastraceae with versatile ethnopharmaceutical properties widely used in the Unani medicare system.

The morphological features include: - large deciduous twining, unarmed shrubs, stem with a height of 10 m and up to 23 cm in diameter with pale brown branches- cracked, exfoliating in small scales; branchlets pubescent or glabrous, with prominent elliptic lenticels; axillary buds ca. 1-2 mm, triangular. Leaves simple, alternate, spiral; stipules lancinate; petioles ca. 6-10 by 2-6 cm. mm long; lamina ca. 5-17 x 2-10 cm, obovate-orbicular, elliptic to ovate-oblong, rounded or acute at base, bluntish or shortly acuminate at apex, crenate-serrulate along margins, glabrous, pubescent beneath, coriaceous; secondary nerves 4-8 pairs.

Inflorescences in terminal drooping panicles ca. 15 cm long, pubescent; Flowers ca. 3-4 mm across, pale greenish; male flowers: ca. 2 mm long, sepals 5-lobed; lobes semi-orbicular, ciliate; petals ca. 3 mm long, oblong to obovate-oblong; stamens ca. 3 mm long; anthers ovoid; ovary sterile, columnar; disc cupular; lobes obscure; female flowers: sepals, petals, and disc as in the male flowers; stamens sterile; ovary globose; style columnar; stigma 3-lobed. Capsule ca. 1-1.3 cm in diam., bright yellow, depressed, globose, 3-valved. Seeds 3-6, ca. 3-5 x 2-4 mm, brownish, smooth, arillate (*Celastrus Paniculatus | Species*, n.d.).

2.5.2.1.1 Scientific classification

Table 4: Scientific classification of *Celastrus paniculatus*

Kingdom	Plantae
Sub-Kingdom	Tracheobionta
Super-Division	Spermatophyta
Phylum	Tracheophyta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Celastrales
Family	Celastraceae
Genus	Celastrus
Species	Paniculatus

2.5.2.1.2 Distribution

Celastrus paniculatus Willd. is native to the Indian continent, but is known to grow wildly across Asia as well as many of the Pacific islands. The plant is primarily utilized to produce seed oil and plant seeds are taken indiscriminately from both wild and cultivated sources. As a result, the plant is very vulnerable and noted as critically endangered in the Western and Eastern Ghats.

2.5.2.1.3 Secondary metabolites and medicinal properties

Phytochemical investigations have shown that *C. paniculatus* is a rich source of a diverse array of metabolites such as the presence of monoterpenes, sesqueterpene esters, diterpenoids, triterpenoids, alkaloids, fatty acids, steroids (β-sitosterol, carpesterol

benzoate), flavonoids (paniculatin), benzoic acid, and vitamin C responsible for its various pharmacological activities (Nagpal *et al*., 2022). *Celastrus paniculatus* Willd. shows various biological effects with health benefits including antioxidant properties responsible for cognitive enhancing effects (Bhanumathy *et al.,* 2010), hypolipidaemic activity (Choudhary and Soni, 2021), wound healing (Harish *et al*., 2008), analgesic (Ma *et al*., 2014), and anti-inflammatory activity (Parimala *et al*., 2009).

There are only few reports on the cytotoxicity of *C. paniculatus* Willd. plant extract on cancer cells. Beta-dihydroagarofuranoid sesquiterpenes isolated from *C. paniculatus* have been demonstrated to reduce the survival of MCF-7 breast cancer cell lines and cause apoptosis and autophagy with a half maximum inhibitory concentration of 17 ±1μM (Weng *et al*., 2013). CuO NPs synthesized from the aerial extract of *Celastrus paniculatus* Willd. have been found to have potent anticancer potential, with half maximum inhibitory concentration (IC_{50}) values of 107.56 μg/ml and 208.57 μg/ml against breast (MCF-7) and colon (HT-29) cancer cells respectively (Giridassappa *et al*., 2021).

2.6 METHODS USED IN THE STUDY

The first step in identifying prospective medication candidates is *in vitro* experiments. Several *in vitro* assays/techniques have been developed to investigate cytotoxic potential and molecular mechanisms of cytotoxicity. The particular research question to be examined mostly influences the choice of an *in vitro* assay or procedure.

2.6.1 Phytoextraction: Soxhlet extraction technique and Cold extraction method

The initial step in preparing phytomedicine from medicinal plants is phytoextraction. Ethnopharmacological knowledge is used to identify plant species, parts, and extract types for targeted phytomedicinal validation. There are numerous approaches for isolating, purifying, and characterising phytomedicinal substances. Soxhlet extraction, a conventional extraction method is still considered as the reference method as it is an efficient and continuous extraction technique. Franz Ritter Von Soxhlet, a German scientist, was the one who first proposed the Soxhlet extractor (Jensen, 2007). One cycle of the Soxhlet extraction method involves extraction following the evaporation of the solvent and this cycle is repeated many times to maximise the yield of the compound. The minimum time taken for a regular Soxhlet extraction is around 8 hours. This technique cannot be used for thermolabile compounds as prolonged heating causes degradation of compounds. Generally, polar solvents are used for extraction based on their increasing

polarity such as petroleum ether, hexane, toluene, benzene, dichloromethane, isopropanol, methanol and ethanol (Brusotti *et al*., 2014). The cold extraction or cold maceration method is another widely used method for phytoextraction. The dried plant part is dissolved in respective solvent and mixed by shaking for one to seven days and the extract is obtained after filtering. Cold extraction technique unlike Soxhlet extraction does not employ a heat source and is maintained at low or room temperatures. Soxhlet extraction is an effective and continuous extraction technique, which is less time-consuming and solvent-consuming but is not suitable for the extraction of thermolabile compounds.

2.6.2 3-(4,5-Dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide MTT ASSAY

MTT assay is a well-known, rapid, precise, and quantitative colourimetric detection assay for mammalian cell survival and proliferation. MTT assay relies on the principle that mitochondrial activity is constant for most viable cells; hence, an increase or decrease in viable cells has a linear correlation with mitochondrial activity (Mosmann, 1983). This is based on the reduction of the light-coloured tetrazolium salt 3-(4,5-Dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple-blue formazan crystals by mitochondrial enzyme which are then solubilized and their absorbance quantified using a microplate absorbance reader. The MTT assay is appropriate for determining the drug sensitivity of both primary cells and established cell lines (Van Meerloo *et al.*, 2011).

2.6.3 Clonogenic assay

The clonogenic assay, also known as the colony formation assay, first introduced by Puck and Marcus in 1956 is used in the assessment of the ability of single mammalian cells plated in culture dishes with an appropriate medium to form colonies (Puck and Marcus, 1956). A colony is defined to have at least 50 cells in it. It is the method of choice to determine the reproductive death of cells after treatment with ionizing radiation, but can also be used to determine the effectiveness of other cytotoxic agents (Franken *et al*., 2006). Cells are seeded in appropriate dilutions, treated with the cytotoxic agent or ionizing radiation source, and then incubated for 1-3 weeks to allow divisions. The cells are then fixed with methanol, stained with crystal violet and the colonies are counted under the stereo microscope.

2.6.4 Real-time PCR

In molecular biology investigations, measuring gene expression is crucial, and real-time PCR or q PCR enables the accurate, sensitive, and high-throughput quantification of nucleic

acids. Either intercalator dyes or probes are used in real-time PCR. The probe-based method is accurate yet expensive and uses a probe with fluorescent reporter dye attached to a quencher dye. It works on the fluorescence resonance energy transfer (FRET) principle, and the fluorescence is only emitted when the probe is cleaved during amplification (Cardullo *et al.,* 1988). Intercalator dye-based qPCR uses SYBR green, a non-sequence-specific double-stranded DNA dye in the reaction mixture, which binds to newly synthesized double-stranded DNA (but does not bind to single-stranded DNA) and gives fluorescence (Morrison *et al*., 1998). The threshold cycle (CT), the PCR cycle at which the fluorescence signal crosses the threshold, is the quantitative endpoint of real-time PCR. The lower the CT value, the greater the amount of amplicon and this relationship between fluorescence and the amount of amplified product allows for sensitive and precise quantification of target molecules across a wide dynamic range (Schmittgen and Livak, 2008). The target gene can then be relatively quantified to the expression of a housekeeping gene, e.g., *GAPDH or β-ACTIN*.

Materials and Methods

1. MATERIALS AND METHODS

The study entitled **"**Antiproliferative activity of *Celastrus paniculatus* Willd." was conducted at the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram from July 2022 - to May 2023. The methodology adopted for the study is provided in this chapter.

MATERIALS

Dulbecco's Modified Eagle Medium (DMEM), powder, low glucose (Thermofisher Scientific), Fetal Bovine Serum, qualified, Brazil (Thermofisher Scientific), Antibiotic-Antimycotic (100X) (Thermofisher Scientific), Trypsin-EDTA (0.25%), phenol red (Thermofisher Scientific), Tissue Culture Flask sterile (25 cm^2) (Tarson), 6 well plates (Tarson), 96 well plate (Tarson), Cryovial (Genaxy**),** Dimethyl sulfoxide (DMSO) (HiMedia), MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (HiMedia), 5-FU (HiMedia), Isopropanol (Sigma-Aldrich), Ethanol (Sigma-Aldrich), **,** Methanol (Merck), Sodium dodecyl sulfate (SDS) (Sigma-Aldrich), Ethylenediamine tetraacetic acid (EDTA) (Sigma-Aldrich), Sodium acetate (Sigma-Aldrich), Sodium chloride (NaCl) (Sigma-Aldrich), Crystal violet (HiMedia), Agarose (G-Biosciences), Tris Base (G-Biosciences), Nuclease free water (Origin), , 5 X RNA gel loading dye (HiMedia), 100 bp and 1 kb ladder (Origin), RNase Out (G-Biosciences), TRIzolTM Reagent (Thermofisher scientific), Verso cDNA Synthesis Kit (Thermofisher scientific), 2X AB HS SYBR Green qPCR Mix (G-Biosciences), primer sets purchased from G- bioscience, Trivandrum, Kerala.

3.1 CULTURING AND MAINTENANCE OF CELL LINES

3.1.1 Cell Line Information

Two cancer cell lines HCT-116, and MCF-7, and a normal cell line HEK-293 were used in the study.

HCT-116

HCT-116 is a highly aggressive and fast-growing human colorectal carcinoma cell line characterized by epithelial morphology. The cells are adherent in nature and grow as monolayers.

MCF-7

MCF-7 human breast adenocarcinoma cell line, positive for oestrogen and progesterone receptors, forms a monolayer of adherent cells with mammary epithelium-like morphology and the presence of dome-shaped structures due to fluid accumulation between monolayer and vessel surface.

HEK-293

HEK-293 - human kidney (embryonic) cell line is a robust, fast-growing, and widely used low-maintenance normal cell line in cancer research with epithelial morphology and characterized by a monolayer of polygonal-shaped cells.

The above cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune. The purchased cell lines were sub-cultured and maintained appropriately in DMEM (Gibco) supplemented with 10% Foetal Bovine Serum (Gibco) and 1 % anti-anti (Antibiotic-Antimycotic) (Gibco) solution. T-25 culture flasks (TARSON) with cells were incubated in a CO_2 incubator (PANASONIC) at a temperature of 37 ° C and 5% carbon dioxide.

3.1.2 Subculturing of Cell Lines

Subculturing or passaging was done to maintain the cells at optimal density and further the propagation and expansion of the cell line on reaching 80% confluency or log phase. Initially, the used media was removed from the culture flask and HCT-116, MCF-7, and HEK-293 cells were washed in phosphate-buffered saline (PBS) free of Mg^{2+} and Ca^{2+} inside the biosafety cabinet to eliminate any unwanted dead cells. The monolayer was then covered with digestive enzymes and chelating agents (trypsin and EDTA) and incubated at 37°C in a CO_2 incubator to dislodge cells from the vessel surface. The extent of dissociation was observed under the inverted microscope and ensured that all cells were detached by gently tapping the sides of the culture vessel. The primary culture flask was rinsed with a complete medium containing an enzyme inhibitor (FBS) to prevent enzymatic digestion and cell dissociation, and the dissociated cells were collected in a sterile falcon tube. Cells are collected by centrifugation at 300g for 10 minutes prior to transfer to new flasks. The supernatant was discarded and the undisturbed cell pellet was gently mixed by resuspending in the new medium and transferred to culture flasks with complete DMEM (10% FBS). The reserve cells were cryopreserved using a freezing mixture containing cryoprotective agents DMSO (dimethyl sulfoxide) and FBS ((95:5 ratio of FBS and DMSO) in cryovials

for future use. Cells were revived at requisite times using prewarmed DMEM containing 20% FBS and used.

3.2 PREPARATION OF PLANT EXTRACT

Fresh leaves of *Celastrus paniculatus* Willd. were collected from the Regional Ayurveda Research Institute Campus, Poojappura, Thiruvananthapuram, Kerala-695012. Leaves were washed well with water, cut into small pieces, midribs removed, shade-dried and finely powdered using a mixer grinder. Methanolic leaf extract of *Celastrus paniculatus* was then prepared using Soxhlet extraction and cold extraction techniques.

3.2.1 Cold Extraction

The cold extract was prepared by soaking 5 g dry leaf powder in 250 mL of methanol and keeping it overnight in an orbital shaker. The extract was then collected into clean glassware by filtering through Whatman filter paper, methanol evaporated, dry extract collected, weighed and then redissolved in DMSO to yield a final concentration of 10 mg/ml and stored at -20^0C. Hereafter, cold extracted crude methanolic leaf extract of *Celastrus paniculatus* Willd. is denoted as CPMLC.

3.2.2 Soxhlet Extraction

Soxhlet extraction technique was adopted to prepare the methanolic leaf extract of *Celastrus paniculatus* Willd at high-temperature conditions. 5g of powdered leaf sample is loaded onto a disposable thimble made of Whatman filter paper and placed in the thimble chamber of Soxhlet apparatus (Borosil). 200 mL of methanol was taken in a round bottom flask and the Soxhlet extractor was assembled properly. Methanol was heated at 60^0C which evaporates and moves up to the condenser of the apparatus. The condensate drips and falls to the thimble chamber and continues to wet the thimble, followed by siphoning of phytoconstituents enriched methanol through the siphon tube into the bottom flask after a certain period under the influence of gravity. The vaporization of methanol from the bottom flask, filling of condensed methanol into the Soxhlet chamber and siphoning of extract-enriched methanol into the bottom flask was continued for several cycles for 10 hours. The phytoextract-loaded methanol was then allowed to evaporate and the dry residue was weighed, redissolved in DMSO to yield a final concentration of 10 mg/ml and stored at -20^0C. Hereafter, the crude methanolic leaf extract of *Celastrus paniculatus* Willd. prepared using the Soxhlet extraction technique is denoted as CPMLS.

3.3 DETERMINATION OF CYTOTOXICITY

3.3.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

MTT assay, a colourimetric assay based on the reduction of a yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to purple formazan crystals by viable cells was used to determine the cytotoxic effect of plant extract on different cell lines (Mosmann, 1983). MCF-7, HCT-116, and HEK-293 cells were seeded at a density of 5000 cells per well of 96-well plates leaving all the outer wells of the plate. The seeded 96-well plate was treated with different concentrations (0-120 µg/mL) of CPMLC and CPMLS along with DMSO (Vehicle control) and 5-Flurouracil (5-FU) as negative control and incubated for 72 hours. After the incubation period, the plate was observed for any morphological changes and the media was discarded followed by a PBS wash (100 µL/well). 100µL of working standard of MTT dye (1 mg/ml in PBS) was prepared from 10mg/ml stock solution (HiMedia) and was added under dark conditions. The plate was sealed with tin foil and incubated for 2 hours. Thereafter, MTT dye was discarded and 100 µL of lysis buffer (comprising DMSO and isopropanol in a 1:1 ratio) was added into each well and kept for 5 minutes for completely solubilizing formazan crystals formed. Absorbance was measured at 595 nm using a microplate reader (BIO-RAD). The cell viability percentage and inhibition percentage were calculated using the formulas given below:

Cell Viability % = (OD treatment / OD control) *100

Inhibition % = 100 - Cell Viability %

The half-maximum inhibitory concentration (IC_{50} value) of the CPMLC and CPMLS for each cell line was calculated by plotting the inhibition % on the Y-axis and different concentrations of the extract on the X-axis.

3.4 IDENTIFICATION OF REPRODUCTIVE DEATH OF CANCER CELLS

A clonogenic assay was carried out to assess the differences in the reproductive death of cancer cells upon the treatment with *C. paniculatus* methanolic leaf extract.

MCF-7 and HCT-116 cell lines were seeded at a low density of 1000 cells per well and allowed to adhere in 6-well plates. Each well was treated with IC_{50}, IC_{25}, and IC_{10} values of CPMLS extract along with control, vehicle control (DMSO) and positive control (IC_{50}

of 5-Fluro uracil) and incubated for one week to allow for 6-7 cell divisions and colony formation. After incubation, the media was gently removed from the wells and rinsed with PBS followed by fixing the colonies with methanol (500µL/well) of the 6-well plate. The colonies were stained using 500 µL/well of 1% crystal violet. After staining, excess crystal violet was washed off and the plates were dried at room temperature and the colonies were counted. The survival fraction was calculated based on plating efficiency as mentioned below and the cell survival curve was plotted.

Plating Efficiency (PE) = (Number of colonies formed after treatment *100%) / Number of cell seeded

Survival Fraction = No of colonies formed / Number of cells seeded *PE

3.5 EXPRESSION ANALYSIS OF APOPTOTIC GENES (*CAS3, CAS9, PARP, BCL2)*

RNA isolation, quantification, and cDNA conversion followed by quantitative Real-time PCR were used to analyze the expression of key apoptotic genes including *CASPASE3, CASPASE9, BAX, PARP* and *BCL2.*

3.5.1 RNA Isolation

5 lakh cells per well were seeded in six-well plates and kept for cell adherence. The cells were treated with IC_{50} values of CPMLS extract, vehicle control (DMSO), and IC_{50} of positive control/drug (5-FU) in addition to control, and incubated for 72 hours. All experiments were carried out with three biological replicates. RNA was isolated in three biological replicates of all samples using the modified TRIzol method (Jogalekar and Serrano, 2019).

RNA isolation was carried out in a sterile, RNase-free zone using a ribonuclease inhibitor (RNase out) in the workbench, gloves, and pipettes used. Media was discarded from the wells followed by a PBS wash. Cells were lysed by adding 1mL of TRIzol (Thermo Scientific) followed by gentle mixing. Cells were transferred to fresh RNase-free microfuge tubes. 200µL of chloroform (per mL of TRIzol used) was added to each tube. The tubes were vortexed vigorously for 5 sec and left at RT for 5 min. The tubes were centrifuged at 12000g for 15 mins at 4°C. The upper aqueous phase alone was transferred to a new tube without disturbing the interphase. RNA was precipitated by adding 500µL of chilled isopropanol per 1mL of TRIzol followed by overnight incubation at -20°C. The

next day, RNA was pelleted by centrifuging at 14000g for 10 min (4 °C). The supernatant was removed completely and the pellet was washed twice with 1ml 75% ethanol; and centrifuged at 14000 g for 5 min at 4 °C. Ethanol was decanted and the pellet was air-dried and resuspended in 20µL of nuclease-free water and stored at -80^{0}C.

3.5.2 Quantification of RNA

RNA was quantified using a Nanodrop 1000 spectrophotometer (ThermoScientific). The A_{260} and A_{280} values were recorded. The quality and quantity of the isolated RNA were obtained.

Purity of RNA = A_{260} / A_{280} (ratio should be 2 for pure RNA)

Quantity of RNA (ng/µL) = A_{260} × 40 × Dilution factor

3.5.3 cDNA synthesis

Reverse transcription of RNA samples was performed using a Verso cDNA synthesis kit (ThermoScientific). The reaction mix and the thermal profile for cDNA synthesis are shown in Tables 5 and 6 respectively. cDNA was diluted in a 1:4 ratio in nuclease-free water and stored at -20^{0}C until use.

Table 5: Reaction mix used for cDNA synthesis

Reagent	**Volume(µl)**
5X cDNA synthesis buffer	4
dNTP Mix	2
RNA Primer	1
RT Enhancer	1
Verso Enzyme Mix	1
Template (RNA)	1-5
Water, nuclease-free	To make up to 20
Total volume	20

Table 6. Thermal profile of cDNA synthesis

	Temperature	**Time**	**Number of cycles**
cDNA synthesis	42 °C	30 min	1 cycle
Inactivation	95 °C	2 min	1 cycle

3.5.4 Relative expression of apoptotic genes

Gradient PCR using the CFX96 Real-Time system (BIO-RAD) was carried out in a temperature range of 55°C to 63 °C for *PARP1, BCL-2, BAX, CASPASE 3, CASPASE 9* and the reference gene *β-ACTIN* used in the study. The melt curve was analysed for each gene at each temperature and the optimum annealing temperatures were finalised. The expression of the apoptotic genes was analysed using real-time PCR. The cDNA from untreated control, CPMLS extract treated cells (IC_{50}), vehicle control treated cells, and 5 FU (IC_{50}) treated cells were amplified and the C_t values were obtained using CFX96 Real-Time system (BIO-RAD) and 2XAB HS SYBR Green qPCR mix (Gbioscience). The reaction mix and the thermal profile of cDNA are shown in Tables 7 and 8 respectively.

Table 7. Reaction mix used for Real-Time PCR

Reagent	**Volume(µl)**
SYBR Green qPCR mix	5
Forward Primer	1
Reverse Primer	1
Template (cDNA)	1
Water, nuclease-free	2
Total volume	10

Table 8. Thermal profile of Real-Time PCR

Stage	**Temperature**	**Time**	**Repeat**
Initial denaturation	95°C	5 min	1
Denaturation	95 °C	45 s	34x
Annealing	Specific to genes	30s	
Extension	72	45s	
Final extension	72	1 min	1

The threshold cycle (C_t) was noted and the level of gene expression was determined using the comparative Ct technique. To determine the product specificity, melt curve analysis was carried out. The relative gene expression level of control and treated cells were represented in terms of relative fold change calculated as follows:

$\Delta C_t = C_t$ (target gene) – C_t (reference gene)

$\Delta\Delta C_t = \Delta C_t$ (sample) – ΔC_t (control)

Relative fold change = $2^{-\Delta\Delta Ct}$

3.6 DETERMINATION OF TELOMERASE EXPRESSION (*hTERT*)

Quantitative real-time PCR was performed to analyze the expression of telomerase *hTERT* using the c DNA synthesized as mentioned in 3.5.4.

3.6.1 Expression of Telomerase Gene

The melt curve was analysed and optimum annealing temperature was found. The cDNA from untreated control, CPMLS extract treated cells (IC_{50}), vehicle control, and 5 FU (IC_{50}) treated cells were amplified and the cycle threshold ($C_{t)}$ values were obtained using CFX96 Real-Time system and 2XAB HS SYBR Green qPCR mix (Gbioscience) as mentioned before. The reaction mix and the thermal profile of cDNA are shown in Tables 5 and 6 in 3.5.3. Melt curve analysis was done and the relative level of gene expression was determined using the delta-delta C_t method. The relative gene expression levels of control and treated cells were represented in terms of relative fold change using the formula mentioned above in 3.5.4.

Results

2. RESULTS

The results of the study entitled "Antiproliferative activity of *Celastrus paniculatus* Willd." carried out at the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani during 2022 - 2023 are presented in this chapter.

4.1 CULTURING AND MAINTENANCE OF CELL LINES

MCF-7 and HCT-116 cancer cell lines and normal cell line HEK-293 were cultured and maintained in DMEM without contamination at appropriate culture conditions (5% CO_2 at 37^0C). The morphology of cancer and normal cell lines observed under the inverted microscope is given in Table 9.

Table 9: Morphology of cancer and normal cell lines

NAME OF CELL LINE	HISTOLOGY	MORPHOLOGY
MCF-7	Human breast adenocarcinoma	Dome-shaped monolayer structures with epithelial morphology
HCT-116	Human colorectal carcinoma	A monolayer of adherent cells forming larger spheres with epithelial morphology
HEK-293	Human embryonic kidney tissue	Polygonal-shaped cells with epithelial morphology

Microscopic view of MCF-7 breast cancer cell line, HCT-116 colon cancer cell line, and HEK-293 cells are shown in Plate 1.

4.2 PREPARATION OF PLANT EXTRACT

C. paniculatus Willd. leaves collected from State Medicinal Plants Board Kerala, Pujappura, Thiruvananthapuram were shade-dried and finely powdered as shown in Plate 2. Cold extraction and Soxhlet extraction technique was carried out to prepare *Celastrus paniculatus* methanolic leaf extract as shown in Plate 3 and Plate 4 respectively. 5g of finely powdered *C. paniculatus* Willd. leaves yielded 432 mg of crude *C. paniculatus* methanolic extract (CPMLC) through cold extraction and 672mg of crude *C. paniculatus*

methanolic leaf extract (CPMLS) through the Soxhlet extraction technique respectively which were dissolved in DMSO and stored at -20^0C.

4.3 DETERMINATION OF CYTOTOXICITY

MTT assay was carried out in all three cell lines: HCT-116, MCF-7, and HEK-293 using CPMLC and CPMLS extract at a dose ranging from 0-120ug after 72 hours of incubation. Both CPMLC and CPMLS extract showed a dose-dependent cytotoxicity. The vehicle control DMSO showed very negligible cytotoxicity in both cancer and normal cell lines while positive control chemotherapeutic drug 5-Flurouracil (IC_{50}) showed nearing 50% inhibition in both MCF-7 and HCT-116 cells while negligible cytotoxic cytotoxicity to normal cell line HEK-293.

In HCT-116 colon cancer cells after 72 hours, CPMLS extract exhibited a dose-dependent reduction in cell viability ranging from 6.70% to 60.85%, while CPMLC extract showed a dose-dependent reduction in cell viability ranging from 5.39% to 56.19% as shown in Table 10. After 72 hours, CPMLC extract showed a dose-dependent reduction in cell viability in HCT-116 colon cancer cells ranging from 6.70% to 60.85 % and in MCF-7 breast cancer cells ranging from 15.45% to 81.89% as given in Table 11. After 72 hours, CPMLC extract showed a dose-dependent reduction in cell viability in MCF-7 breast cancer cells ranging from 12.51 to 77.03%, whereas CPMLS revealed a dose-dependent reduction in cell viability in MCF-7 breast cancer cells ranging from 15.45 to 81.89%. Dose-response curves of MCF-7, HCT-116, and HEK-293 cells treated with different concentrations of CPMLC are shown in Figures 6, 7, and 8 respectively. From the dose-response curves, the half-maximum inhibitory concentration or IC_{50} value of CPMLC extract was determined to be 72.13µg and 102.07µg on HCT-116 and MCF-7 cells respectively. Dose-response curves of MCF-7, HCT-116, and HEK-293 cells treated with different concentrations of CPMLS are shown in Figures 9, 10, and 11 respectively. From the dose-response curves, the half-maximum inhibitory concentration or IC_{50} value of CPMLS extract was determined to be 69.55µg and 95.58µg on HCT-116 and MCF-7 cells respectively.

Soxhlet-extracted methanolic leaf extract of *C. paniculatus* (CPMLS) exhibited a comparable but slightly higher cytotoxic effect on cancer cells compared to cold-extracted methanolic leaf extract (CPMLC) as given in Table 13 and it was used for further studies. Further, the extract did not show a significant cytotoxic effect on the HEK-293 normal cells

at the obtained IC_{50} values for both cancer cell lines. MCF-7 and HCT-116 cancer cells exposed to different doses of CPMLS extract showed extensive cell death as evidenced by decreased cell quantity, cell shrinkage, rounding off, and detachment from the culture vessels (Plate 5 and Plate 6). Similar time and dose treatment of the extract did not show observable morphological changes on normal cell line HEK-293 (Plate 7).

Table 10: Inhibition of viability of MCF-7 cells after 72 hours of incubation with different concentrations of the extract

CPMLC		***CPMLS***	
Concentration (µg)	Inhibition %	Concentration (µg)	Inhibition %
T1 (0)	0.00	T1 (0)	0.00
T2 (20)	5.39	T2 (20)	6.70
T3 (40)	13.69	T3 (40)	18.49
T4 (60)	22.35	T4 (60)	24.97
T5 (80)	30.53	T5 (80)	32.54
T6 (100)	40.75	T6 (100)	51.63
T7 (120)	56.19	T7 (120)	60.85
Vehicle Control (DMSO)	4.39	Vehicle Control (DMSO)	3.76
Negative Control (5-FU)	50.80	Negative Control (5-FU)	49.19

Table 11: Inhibition of viability of HCT-116 cells after 72 hours of incubation with different concentrations of the extract

CPMLC		***CPMLS***	
Concentration (µg)	Inhibition %	Concentration (µg)	Inhibition %
T1 (0)	0.00	T1 (0)	0.00
T2 (20)	15.45	T2 (20)	12.51
T3 (40)	32.32	T3 (40)	36.47
T4 (60)	42.75	T4 (60)	46.15
T5 (80)	63.34	T5 (80)	52.70
T6 (100)	68.50	T6 (100)	70.14
T7 (120)	81.89	T7 (120)	77.03
Vehicle Control (DMSO)	4.50	Vehicle Control (DMSO)	3.27
Negative Control (5-FU)	51.30	Negative Control (5-FU)	52.17

Table 12: Inhibition of viability of HEK-293 cells after 72 hours of incubation with different concentrations of the extract			
CPMLC		***CPMLS***	
Concentration (µg)	Inhibition %	Concentration (µg)	Inhibition %
T1 (0)	0.00	T1 (0)	0.00
T2 (20)	2.38	T2 (20)	8.16
T3 (40)	4.90	T3 (40)	11.22
T4 (60)	6.03	T4 (60)	11.56
T5 (80)	12.89	T5 (80)	17.35
T6 (100)	21.93	T6 (100)	19.73
Vehicle Control (DMSO)	1.70	Vehicle Control (DMSO)	1.88
Negative Control (5-FU)	3.40	Negative Control (5-FU)	3.17

Table 13: Comparison of IC_{50} values of CPMLC and CPMLS on breast and colon cancer cells

Cell line	IC_{50} value of CPMLC (µg)	IC_{50} value of CPMLS (µg)
MCF-7	102.07µg	95.58µg
HCT-116	72.13µg	69.56µg

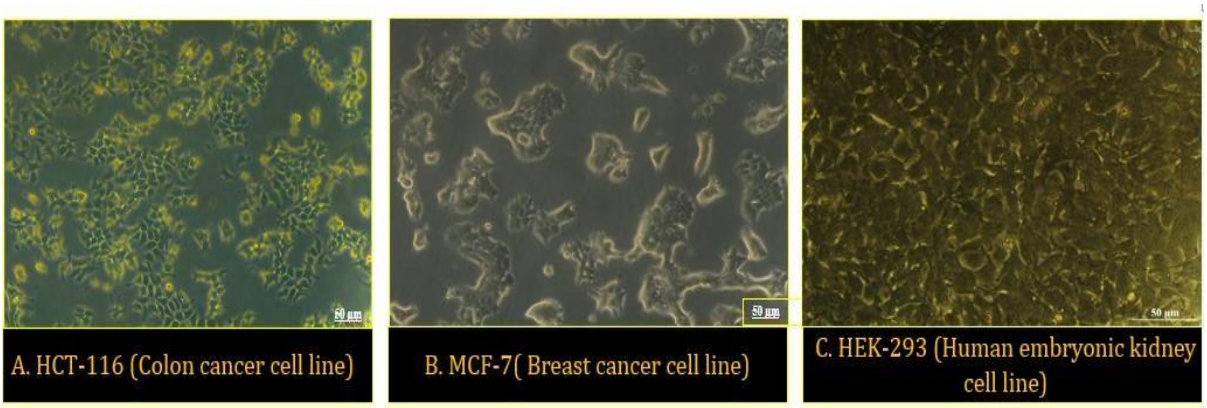

Plate 1: Microscopic view of the cell lines under 10x magnification A. HCT-116 (Colon cancer cell line) B. MCF-7 (Breast cancer cell line) C. HEK-293 (Normal cell line)

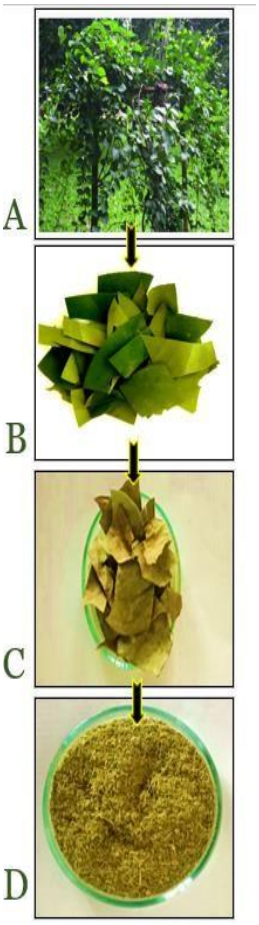

Plate 2: Preparation of *Celastrus paniculatus* powdered plant sample A: *C. paniculatus* Willd. plant B: Leaf sample of *C. paniculatus* C: Dried leaf sample of *C. paniculatus* D: Powdered leaf sample

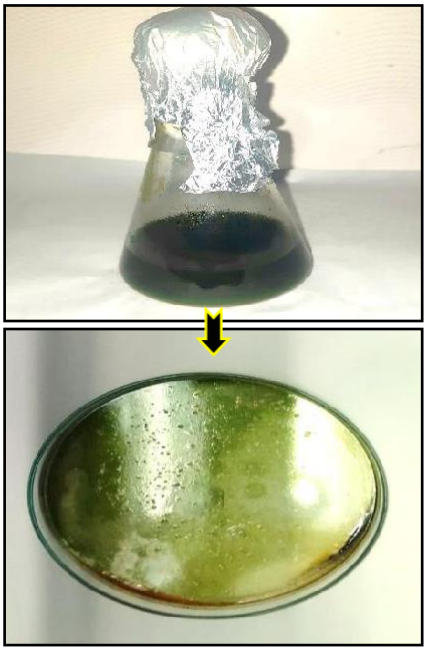

Plate 3: Methanolic extraction of *Celastrus paniculatus* leaves by cold extraction

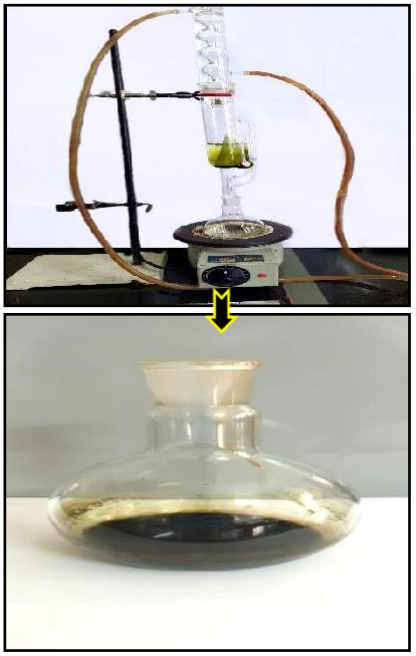

Plate 4: Methanolic extraction of *Celastrus paniculatus* leaves by Soxhlet extraction

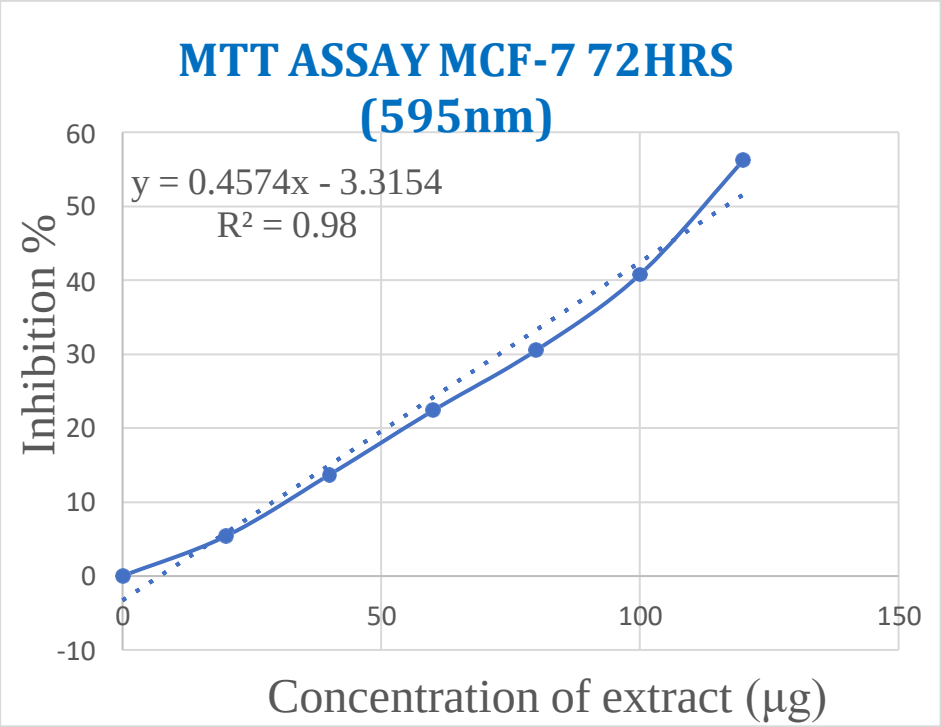

Fig. 6: Cytotoxic effect of *Celastrus paniculatus* methanolic extract prepared through cold extraction technique on MCF-7 cell line

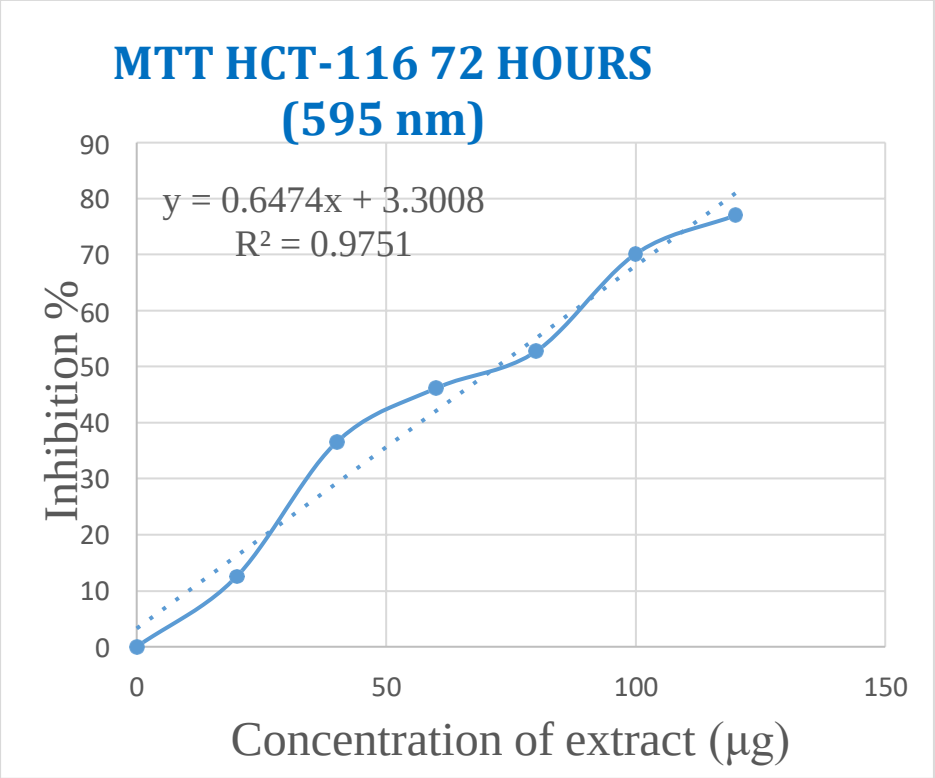

Fig. 7: Cytotoxic effect of *Celastrus paniculatus* methanolic extract prepared through cold extraction technique on HCT-116 cell line

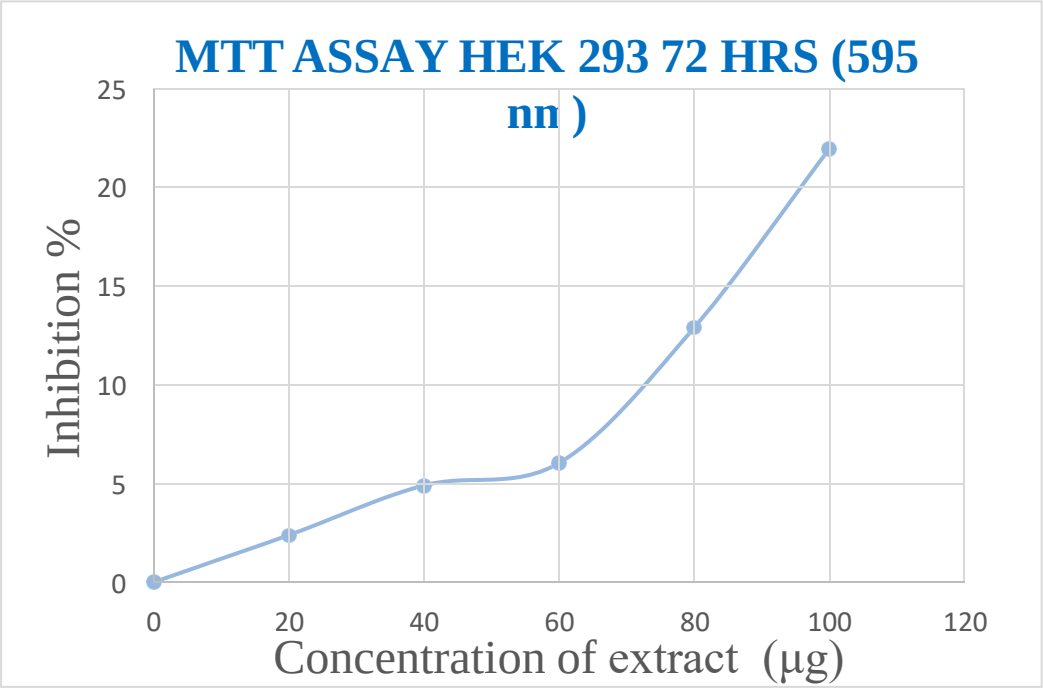

Fig. 8: Cytotoxic effect of *Celastrus paniculatus* methanolic extract prepared through cold extraction technique on HEK-293 cell line

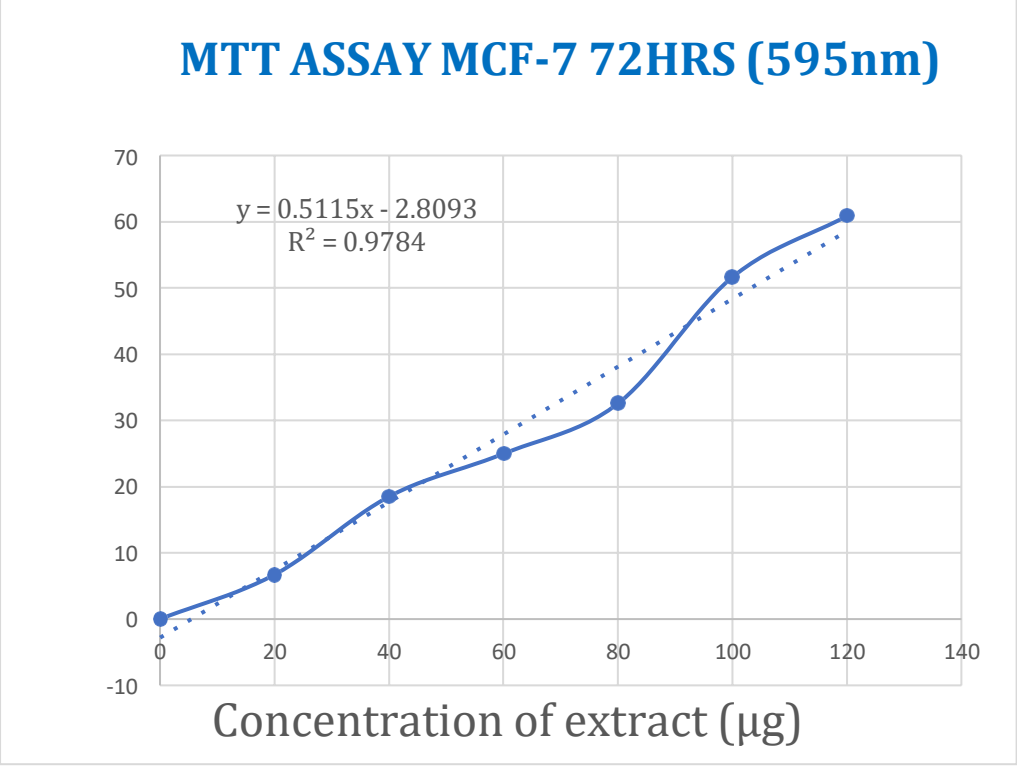

Fig. 9: Cytotoxic effect of *Celastrus paniculatus* methanolic extract prepared through Soxhlet extraction technique on MCF-7cell line

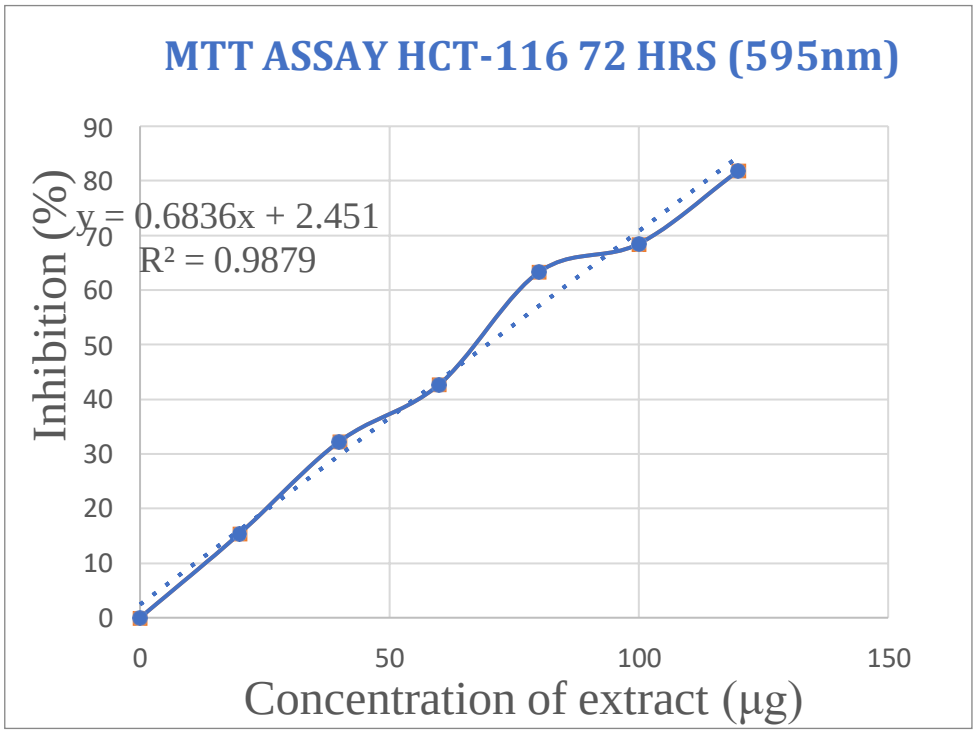

Fig. 10: Cytotoxic effect of *Celastrus paniculatus* methanolic extract prepared through Soxhlet extraction technique on HCT-116 cell line

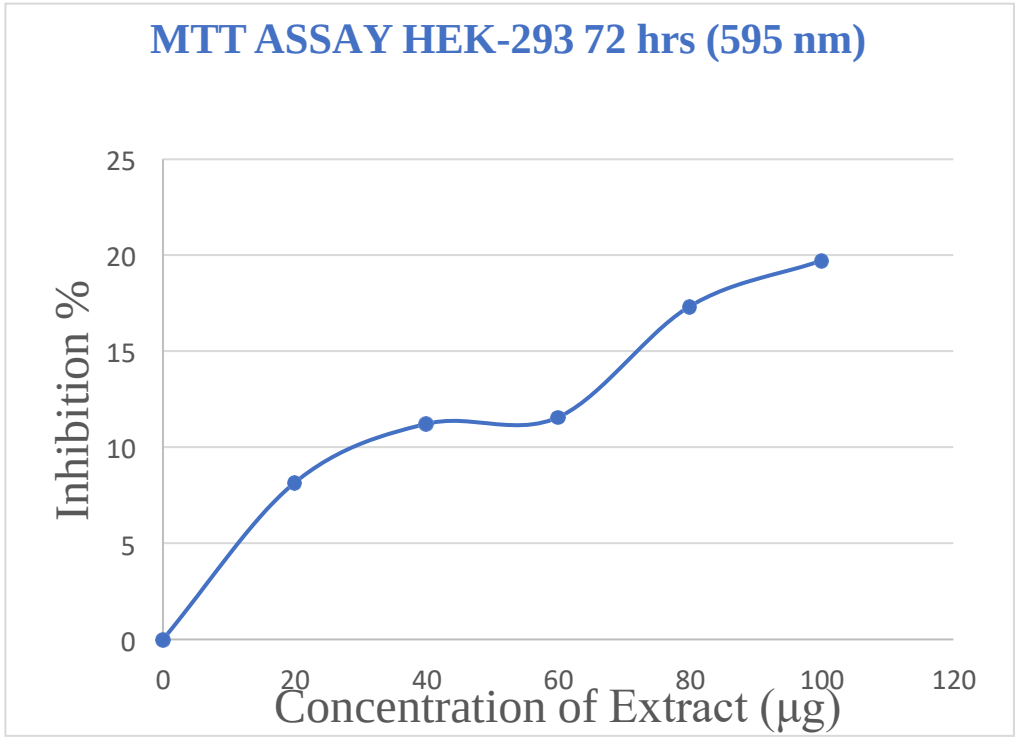

Fig. 11: Cytotoxic effect of *Celastrus paniculatus* methanolic extract prepared through Soxhlet extraction technique on HEK-293 cell line

4.1 IDENTIFICATION OF REPRODUCTIVE DEATH OF CANCER CELL LINES

Clonogenic assay performed to assess the reproductive death of cancer cells treated with the crude methanolic leaf extract of *C. paniculatus* showed that CPMLS inhibited colony formation in MCF-7 and HCT-116 cell lines (Plate 8, 9). The survival fraction MCF-7 and HCT-116 cells were calculated and plotted as shown in Figure 12 and Figure 13 respectively. CPMLS extract at its IC_{50} concentration inhibited colony formation by 69 % in MCF-7 cells and by 41% in HCT-116 cells when compared to control.

Table 14: Survival fraction by MCF-7 cells upon treatment with CPMLC

Treatments	Number of colonies	Survival Fraction
Control	555	1.00
Vehicle control (DMSO)	467	0.84
IC_{10}	449	0.81
IC_{25}	395	0.71
IC_{50}	169	0.31
Positive control (5-FU)	47	0.09

Table 15: Survival fraction by HCT-116 cells upon treatment with CPMLS

Treatments	Number of colonies	Survival Fraction
Control	305	1.00
Vehicle control (DMSO)	252	0.83
IC_{10}	242	0.79
IC_{25}	224	0.73
IC_{50}	170	0.56
Positive control (5-FU)	54	0.18

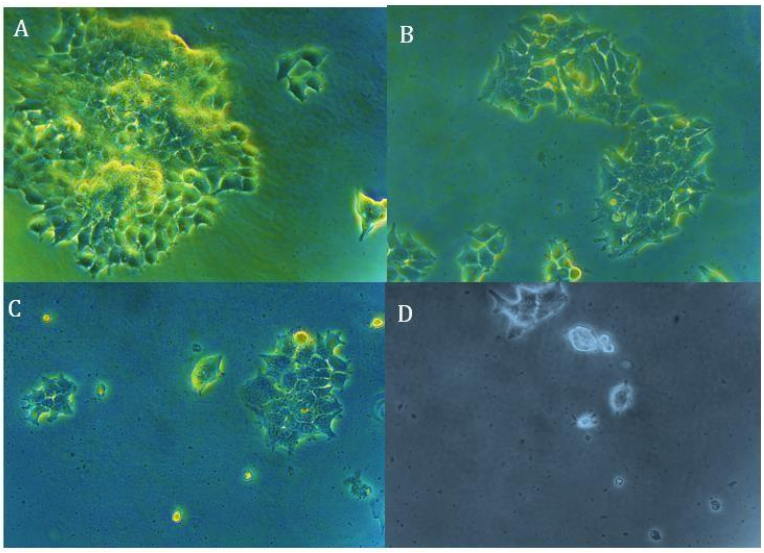

Plate 5: Morphological changes of MCF-7 cells treated with methanolic leaf extract of *C. paniculatus* A. Control B. Vehicle Control C. IC_{50} D. Positive Control

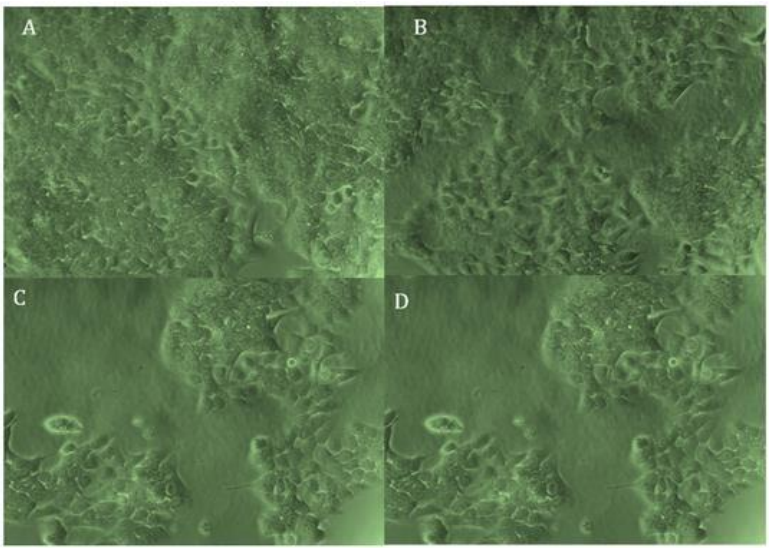

Plate 6: Morphological changes of HCT-116 cells treated with methanolic leaf extract of *C. paniculatus* A. Control B. Vehicle Control C. IC_{50} D. Positive Control

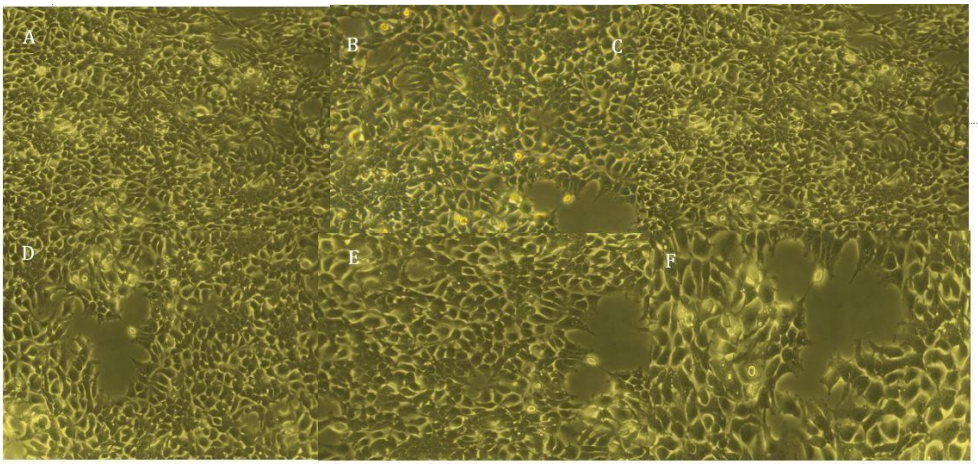

Plate 7: Morphological changes of HEK-293 cells treated with methanolic leaf extract of *C. paniculatus* A. Control B. 20µg C.40µg C. 60µg D.80µg E. 100µg

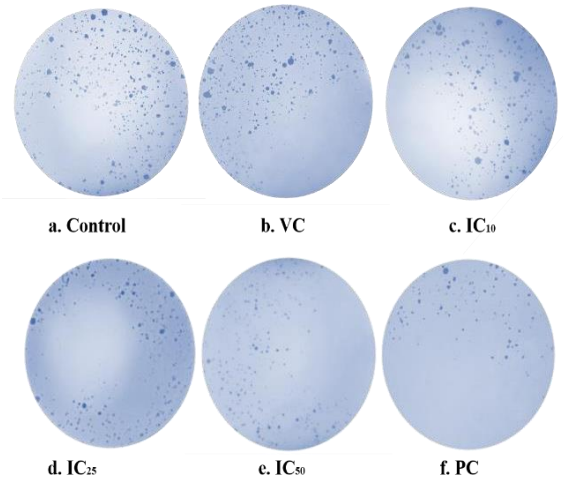

Plate 8: Colony formation by MCF-7 under the following treatments a. Control b. Vehicle Control (VC) c. IC_{10} d. IC_{25} e. IC_{50} f. Positive Control (PC)

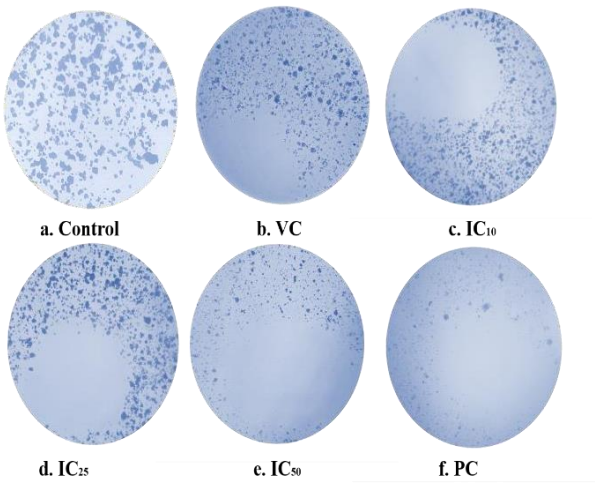

Plate 9: Colony formation by HCT-116 under the following treatments a. Control b. Vehicle Control (VC) c. IC_{10} d. IC_{25} e. IC_{50} f. Positive Control (PC)

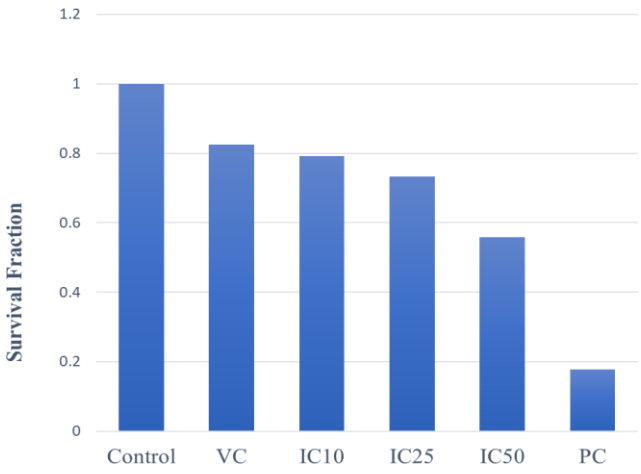

Fig. 12: Percentage reduction in the number of colonies in MCF-7 cells after the treatment with methanolic leaf extract of *C. paniculatus*

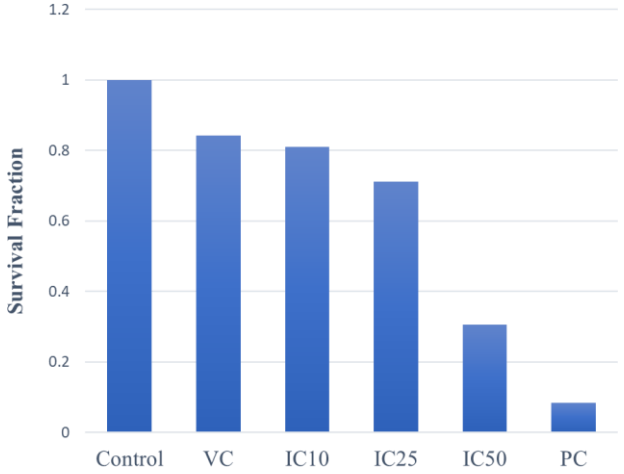

Fig. 13: Percentage reduction in the number of colonies in HCT-116 cells after the treatment with methanolic leaf extract of *C. paniculatus*

5.1 EXPRESSION ANALYSIS OF KEY APOPTOTIC GENES (*CAS3*, *CAS9*, *PARP1*, *BCL2* AND *BAX*)

5.1.1 RNA Isolation

RNA was isolated from both control and treated cells (each in three biological replicates) of MCF-7 and HCT-116 breast cancer cells using the Trizol method. The isolated RNA from MCF-7 and HCT-116 were visualised as two intact bands (28S and 18S) without degradation in 1.8 % agarose gel as shown in Plates 10 and 11 respectively. All the isolated RNA samples from MCF-7 and HCT-116 cells were found to be of good quality and quantity using a Nanodrop 1000 spectrophotometer (Table 16, 17).

Table 16: Quality and quantity of RNA isolated from HCT-116 cell line

SAMPLE NAME	A_{260}	A_{280}	Purity A_{260}/A_{280}	Concentration (ng/µl)
C_1	15.81	7.91	2.0	632.3
C_2	9.86	5.01	1.97	394.5
C_3	13.05	6.46	2.02	521.8
IC_1	9.39	4.74	1.98	375.7
IC_2	10.01	5.11	1.96	405.2
IC_3	9.58	4.81	1.99	383.3
VC1	4.45	2.27	1.96	178.0
VC_2	5.94	3.00	1.98	237.4
VC_3	9.53	4.79	1.99	381.2
5-FU_1	19.56	9.98	1.96	795.0
5-FU_2	6.95	3.56	1.95	277.8
5-FU_3	19.54	10.02	1.95	794.5

Table 17: Quality and quantity of RNA isolated from HCT-116 cell line

SAMPLE NAME	A_{260}	A_{280}	Purity A_{260}/A_{280}	Concentration (ng/µl)
C_1	11.13	6.02	1.85	445.34
C_2	9.61	5.17	1.86	384.58
C_3	17.91	9.58	1.87	716.20
IC_1	6.79	3.59	1.89	271.44
IC_2	7.62	4.05	1.88	304.72
IC_3	9.31	4.85	1.92	372.50
VC_1	10.26	5.43	1.89	410.25
VC_2	11.46	6.10	1.88	458.32
VC_3	10.56	5.71	1.85	422.76
5-FU_1	10.61	5.58	1.90	424.53
5-FU_2	9.87	5.09	1.94	394.76
5-FU_3	8.27	4.31	1.92	330.64

5.1.2 Optimisation Of Annealing Temperatures Of Primers Of Key Apoptotic Genes

A total of six sets of primers (reconstituted to a working stock of 10 pM in nuclease-free water) were used for studying the differential expression of apoptotic genes including β-actin as the reference gene. The primer sequence and melt curve of key apoptotic genes and reference gene *β-ACTIN* are given in Table 18. The optimized annealing temperature of *β-ACTIN*, *PARP1,* and *CAS 9* was found to be 60.1 °C, *BAX* at 62.4 °C, *BCL2* at 56.6 °C, and *CAS 3* at 55.5 °C and melt curve of key apoptotic genes and reference gene *β-ACTIN* is given in Figure 14.

Table 18: Primer sequence of apoptotic genes

Target gene	**Orientation**	**Primer sequence (5' – 3')**	**T_a**	**Reference**
CAS 3	F R	TTAATAAAGGTATCCATGGAGAACACT TTAGTGATAAAAATAGAGTTCTTTTGTGAG	55.5˚C	(Pozo-Guisado *et al.*,2005)
CAS 9	F R	TTCCCAGGTTTTGTTTCCTG CCTTTCACCGAAACAGCATT	60.1˚C	Zhao *et al.*,2018
BCL2	F R	ATCGCCCTGTGGATGACTGAGT GCCAGGAGAAATCAAACAGAGGC	56.6˚C	(Jiang *et al.*,2020)
BAX	F R	TCAGGATGCGTCCACCAAGAAG TGTGTCCACGGCGGCAATCATC	62.4˚C	(Qiu *et al.*,2018)
PARP1	F R	CGGAGTCTTCGGATAAGCTCT TTTCCATCAAACATGGGCGAC	60.1˚C	(Romero-Moreno *et al.*,2019)
β-ACTIN	F R	GACCTCTATGCCAACACAGT AGTACTTGCGCTCAGGAGGA	60.1˚C	(Shen *et al.*, 2019)

5.1.3 Relative expression of apoptotic genes

The average cycle threshold values (C_t) obtained during real-time PCR of key apoptotic genes in MCF-7 and HCT-116 cell lines are given in Table 19 and the graphical representation of the same is given in Figure 15 and Figure 16 respectively.

The fold change of each gene expression was determined using the comparative C_t ($2^{-\Delta\Delta}C_t$) method. Assessment of apoptotic marker gene expression (*BAX/BCL2* ratio) was found to be greater than 1 (Table 20 and Figure 17) in both HCT-116 (3.18 fold) and MCF-7 cells (28.75 fold), indicative of apoptosis on cancer cells upon treatment with plant extract. Table 21 represents the relative fold change obtained for the key apoptotic genes used in the study. Proapoptotic genes *BAX, CAS3, and CAS9* were found upregulated in

HCT-116 (*BAX* 2.9 fold, *CAS3* 1.02 fold, *CAS9* 1.65 fold) and MCF-7 (*BAX* 2.3 fold, *CAS3* 1.6 fold, *CAS9* 1.13) cells when treated with the crude methanolic leaf extract compared to the untreated control. Antiapoptotic *BCL2* and *PARP1* genes were downregulated in both HCT-116 (*PARP1* 0.44 and *BCL2* 0.91fold) and MCF-7 (*PARP1* 0.38 and *BCL2* 0.08 fold) cells compared to the control. Figures 19 and 20 represent the relative fold change of apoptotic genes in HCT-116 and MCF-7 cell lines respectively.

Table 19: Average C_t values of apoptotic genes in MCF-7 and HCT-116 cell line

Cell lines	**Treatments**	**Ct average**				
		PARP 1	***BAX***	***BCL2***	***CAS 3***	***CAS 9***
MCF-7	**Control**	27.41	18.38	25.38	29.68	26.40
	Vehicle control	25.29	17.91	23.14	24.58	27.63
	IC_{50}	24.85	17.90	26.15	24.12	26.20
	Drug	27.33	17.55	24.30	25.09	25.69
HCT-116	**Control**	31.07	26.56	27.48	23.64	31.64
	Vehicle control	29.86	27.52	25.01	24.46	27.98
	IC_{50}	29.52	27.06	27.10	24.30	29.97
	Drug	31.22	27.35	26.29	26.04	27.45

Table 20: *BAX / BCL2* ratio of MCF-7 and HCT-116 cancer cell line

Cell lines	**Treatments**	***BAX/BCL2 Ratio***		
		BAX	***BCL2***	***BAX/BCL2***
MCF-7	Control	1.00	1.00	1.00
	Vehicle control (DMSO)	0.23	0.75	0.30
	IC_{50}	2.30	0.08	28.75
	Positive control (5-FU)	7.41	0.067	110.60
HCT-116	Control	1.00	1.00	1.00
	Vehicle control (DMSO)	0.30	0.97	0.31
	IC_{50}	2.90	0.91	3.18
	Positive Control (5-FU)	4.20	0.53	7.92

Table 21: Relative fold change in gene expression of apoptotic genes in MCF-7 and HCT-116 cell lines

Cell lines	**Treatments**	**Relative fold change**				
		Antiapoptotic genes		**Proapoptotic genes**		
		PARP1	***BCL2***	***BAX***	***CAS 3***	***CAS 9***
MCF-7	Control	1.00	1.00	1.00	1.00	1.00
	Vehicle control	0.74	0.75	0.23	0.07	0.11
	IC_{50}	0.38	0.08	2.3	1.60	1.13
	Drug	0.28	0.07	7.41	3.35	6.84
HCT-116	Control	1.00	1.00	1.00	1.00	1.00
	Vehicle control	0.74	0.97	0.3	0.143	0.65
	IC_{50}	0.44	0.91	2.90	1.02	1.65
	Drug	0.56	0.53	4.20	7.10	9.70

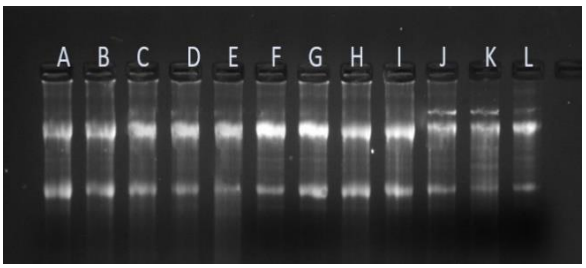

Plate 10: Gel Profile of RNA isolated from HCT-116 cancer cells A-C: Control D-F: IC_{50} G-I: Vehicle Control J-L: Drug

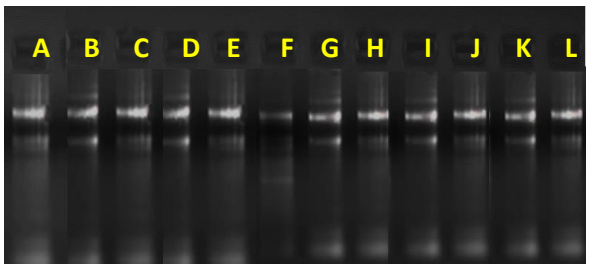

Plate 11: Gel Profile of RNA isolated from MCF-7 cancer cells A-C: Control D-F: IC_{50} G-I: Vehicle Control J-L: Drug

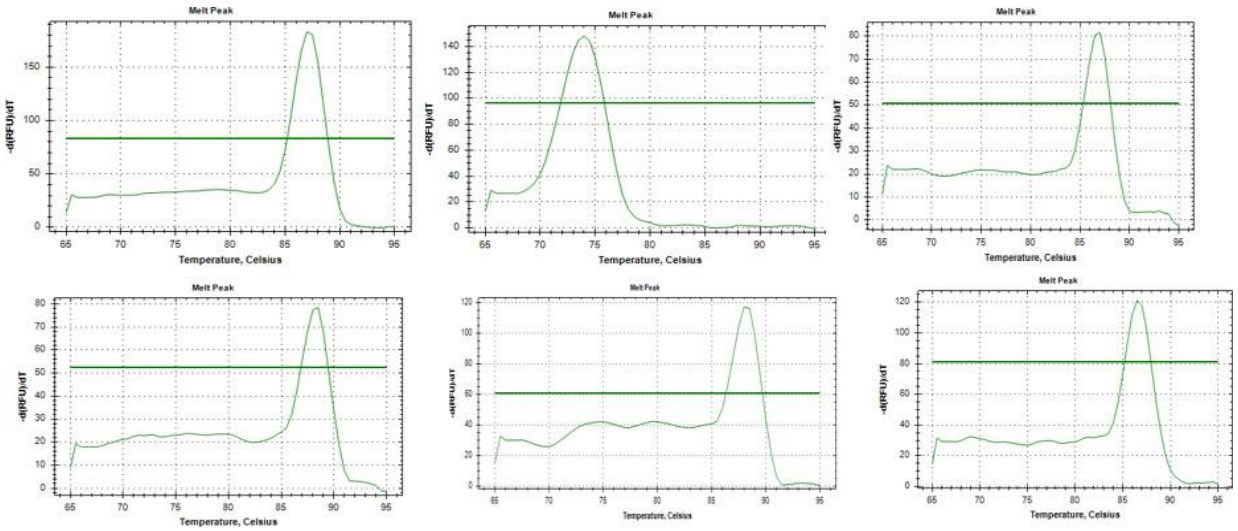

Fig.14: Melt curve analysis: (A) *β-ACTIN* at 60.1˚C; (B) *CAS 3* at 55.5˚C; (C) *CAS* 9 at 60.1˚C; (D) *PARP 1* at 60.1˚C;(E) *BCL2* at 56.6˚ C; (F) *BAX* at 62.4˚C

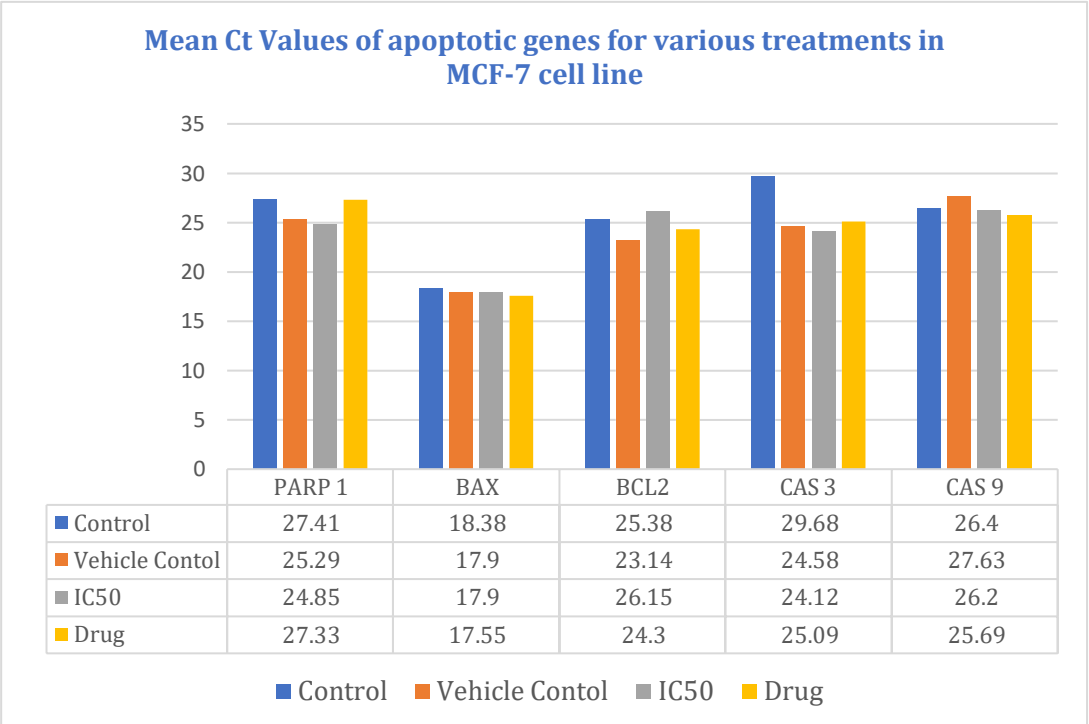

Fig. 15: Mean Ct values of apoptotic genes in MCF-7

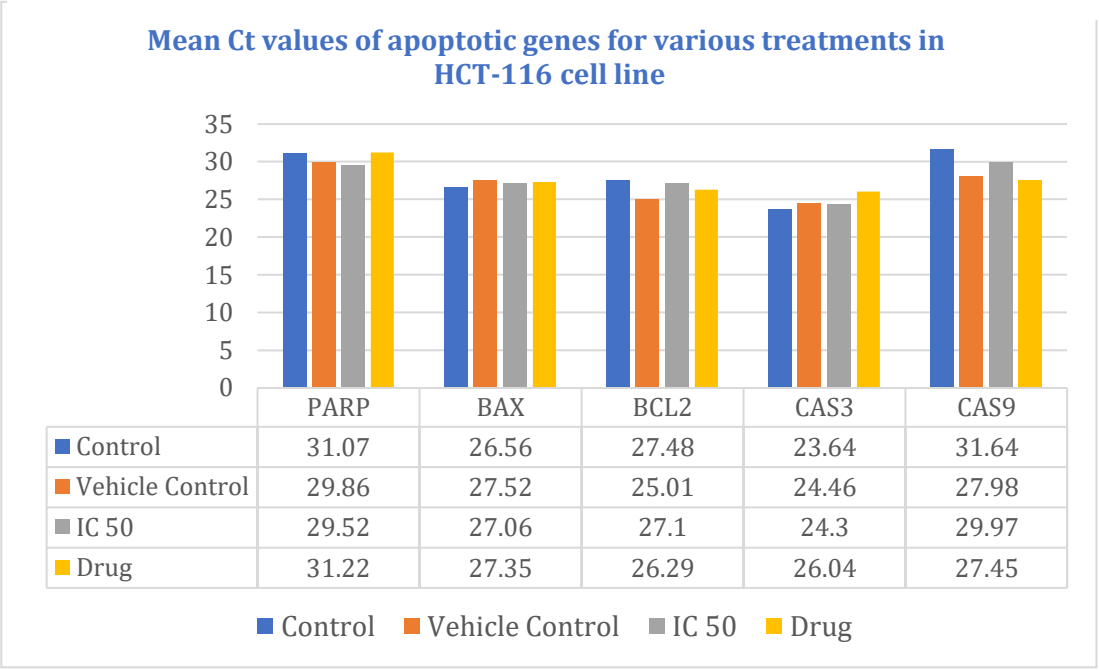

Fig. 16: Mean Ct values of apoptotic genes in HCT-116

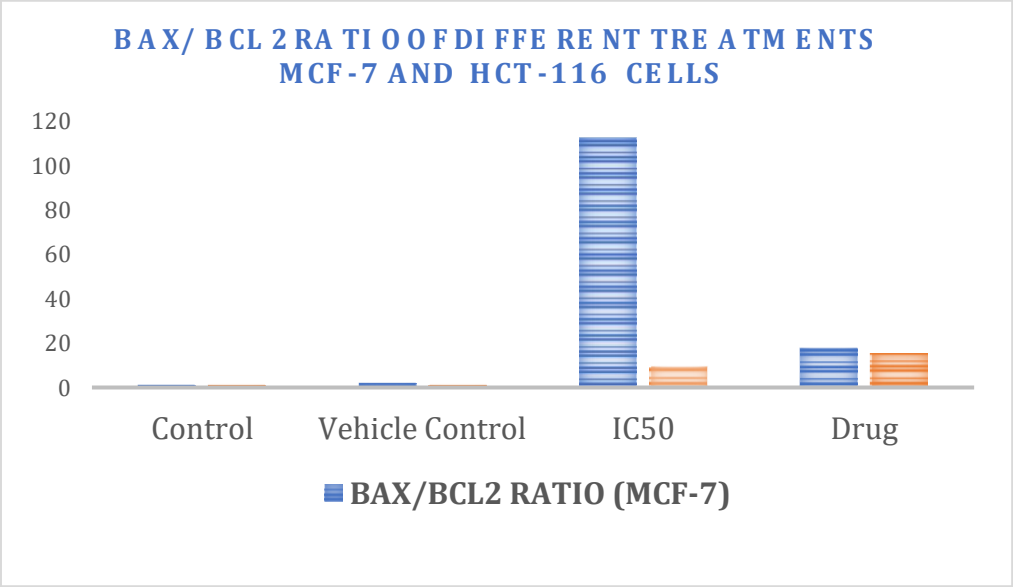

Fig. 17: *BAX/BCL2* ratio in MCF-7 and HCT-116 cell lines

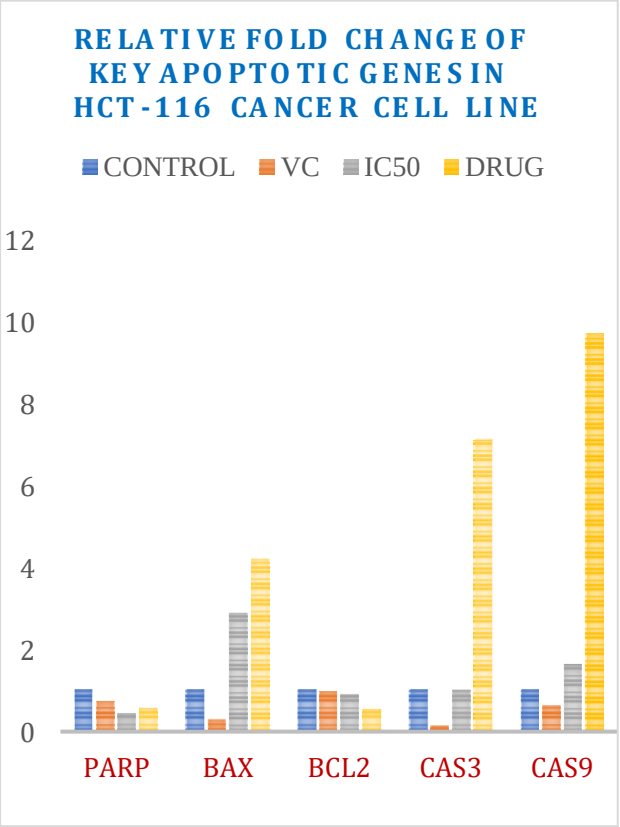

Fig. 18: Relative fold change in apoptotic gene expression after various treatments in HCT-116 cell line

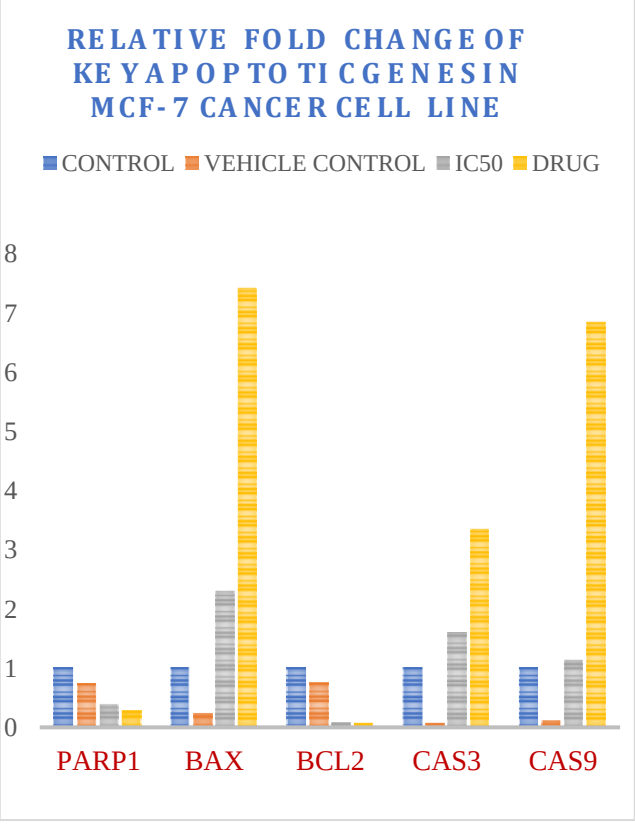

Fig. 19: Relative fold change in apoptotic gene expression after various treatments in MCF-7 cell line

4.6 DETERMINATION OF TELOMERASE EXPRESSION (*hTERT*)

The determination of telomerase expression was performed using real-time PCR.

4.6.1 Isolation and Quantification Of RNA

RNA samples isolated from both cancer cell lines and quantified using a Nanodrop Spectrophotometer were found to be of good quantity and purity as mentioned in 4.5.1. The primer sequence and melt curve of the telomerase gene are given in Table 22 and Figure 21 respectively.

Table 22: Primer sequence of telomerase gene

Target gene	Orientation	Primer Sequence (5' – 3')	T_a	Reference
hTERT	F R	CGGAAGAGTGTCTGGAGCAA GGATGAAGCGGAGTCTGGA	51.5 °C	(Liu *et al*., 2013)

4.6.2 Relative Expression of Telomerase Gene

The optimized annealing temperature for the *hTERT* gene was found to be 51.5^0C and the melt curve is given in Figure 14. The average C_t values obtained during real-time PCR of telomeres gene *hTERT* in MCF-7 and HCT-116 cell lines are given in Table 23 and the graphical representation of the same is given in Figure 22. The relative fold change of telomerase gene *hTERT* was calculated using the $2^{-\Delta\Delta}C_t$ method and is represented in Table 24. The telomerase gene was found to be downregulated in both MCF-7 (0.45 fold) and HCT-116 (0.61 fold) cell lines upon treatment with CPMLS as compared to the untreated control indicative of suppression of telomerase activity in breast and colon cancer cells upon treatment with CPMLS extract. Figure 23 shows the graphical representation of the relative fold change of *the hTERT* gene in the MCF-7 and HCT-116 cell lines.

Table 23: Average C_t values of *hTERT* in MCF-7 and HCT-116 cell line

Cell lines	Treatments	Ct average
MCF-7	Control	29.97
	Vehicle control	27.64
	IC_{50}	30.61
	Positive Control (Drug)	30.04
HCT-116	Control	30.3
	Vehicle control	30.4
	IC_{50}	31.7
	Positive Control (Drug)	31.8

Table 24: Relative fold change in gene expression of telomerase gene in MCF-7 and HCT-116 cell lines

Cell lines	Treatments	Relative fold change
		hTERT
MCF-7	Control	1.00
	Vehicle control (DMSO)	0.88
	IC_{50}	0.45
	Positive Control (Drug)	0.22
HCT-116	Control	1.00
	Vehicle control (DMSO)	0.89
	IC_{50}	0.61
	Positive Control (Drug)	0.44

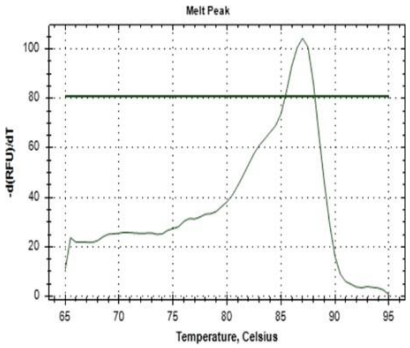

Fig. 20: Melt curve analysis: *hTERT* at 51.5 ˚C

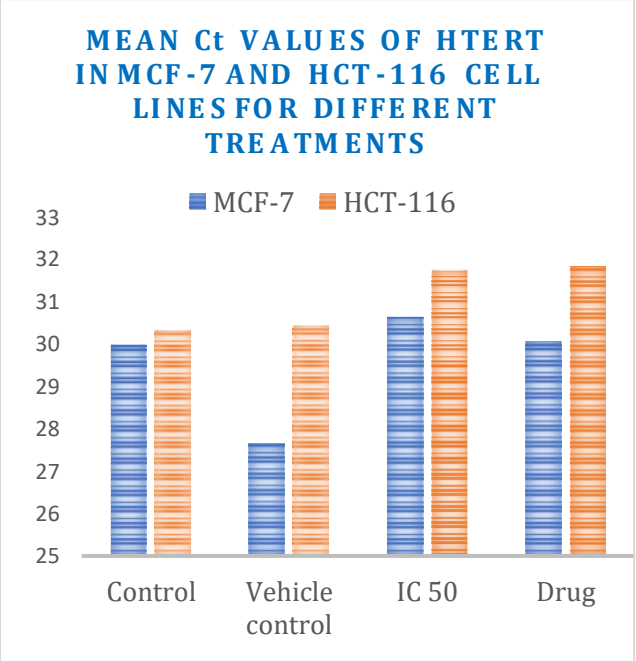

Fig. 21: Mean C_t values of telomerase gene in MCF-7 and HCT-116 cell lines

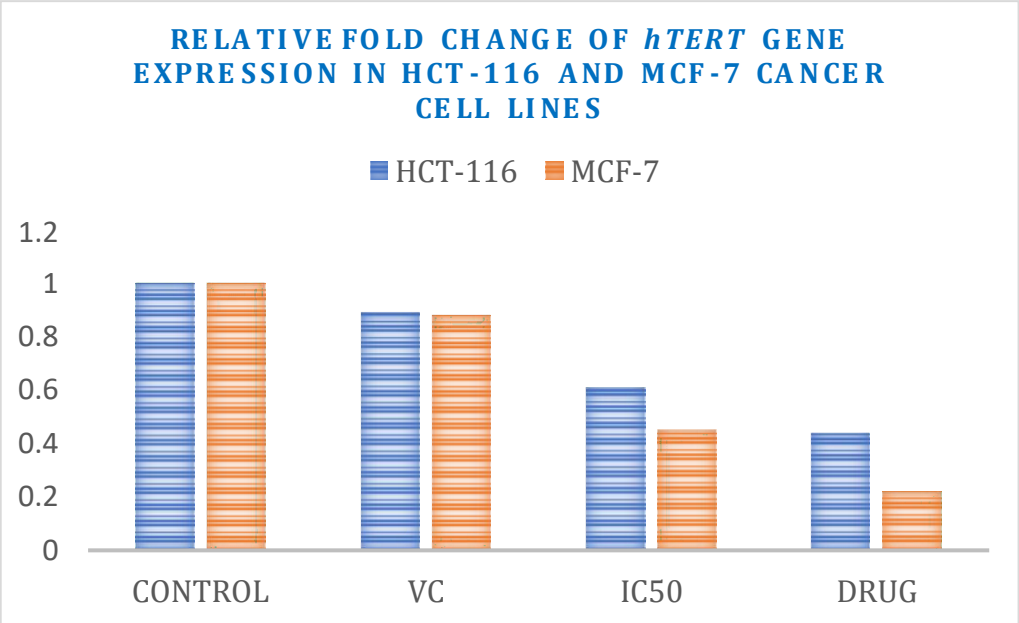

Fig. 22: Relative fold change of telomerase gene in MCF-7 and HCT-116 cell lines

Discussion

5. DISCUSSION

The ever-increasing demand for novel and clinically effective anticancer agents has shifted attention to natural compounds as a source of anticancer drugs. Due to their safety and efficacy over currently used clinical drugs, plant-derived anticancer medications have an advantage over conventional cancer treatment modalities. Plants are a source of a myriad of bioactive compounds with valuable potential for combating various diseases including cancer (Desai *et al*., 2008). A few phytochemicals have been clinically approved for cancer treatments and numerous phytochemicals have shown anticancer properties in preclinical and clinical stages but advanced research is necessary to develop them into effective drugs (Garcia-Oliveira *et al*., 2021).

Celastus paniculatus Willd. (Black oil plant) is a medicinally relevant climbing shrub of the Celastraceae family found in the Indian subcontinent. The plant is known for its analgesic, anti-inflammatory, and antiarthritic activity in addition to its cognition-enhancing activity (Aleem, 2021). There has been accumulating evidence of the bioactive compounds from the Celastraceae family with reported anticancer activities (Abdollahi *et al*., 2015). Kupchan and others (1972) reported that ethanolic extract of *Tripterygium wilfordii* Hook F. displayed significant anticancer potential against two leukemias in vivo and against nasopharynx (KB) carcinoma cells *in vitro*. The antimetastatic activity of triptolide from *Tripterygium wilfordii* was characterized by Johnson and coworkers (2011) revealing the suppression of various cytokine receptors, along with inhibition of COX-2 and VEGF in colon cancer cells. Celastrol, a bioactive compound found in certain Celastraceae family species has been reported to regulate hypoxia-inducible factor-1α (HIF-1α) and thus a potent inhibitor of hypoxia-induced angiogenic and metastatic activity (Hwang *et al.*, 2011). Another study reported that pristimerin, a triterpenoid quinone-methide induced G1 cell cycle arrest and caused apoptosis of cells as well as autophagy via activation of ROS/ASK1/JNK pathway on breast cancer cells (Zhao *et al*., 2019).

There are a few preliminary reports on the cytotoxic potential of *C. paniculatus* and the molecular mechanism behind the anticancer potential was not much studied and established. The present study is the first report that demonstrates the antiproliferative potential of *C. paniculatus* methanolic leaf extract in breast and colon cancer, two of the most common cancers and elucidates its mode of action through induction of apoptosis and suppression of expression of telomerase.

Celastrus paniculatus Willd. leaves collected from Thiruvananthapuram were shade-dried finely powdered and extracted with methanol by cold and hot extraction methods. When choosing an extraction process for the preparation of plant extract, it is also important to take into account the extract yield and retention of the properties of the bioactive compounds (Estrella Aspé, 2011). Among the two different extraction techniques; Soxhlet extraction and cold extraction Soxhlet extraction gave a higher yield and cytotoxic activity. Methanol was selected as the solvent for extract because it gives a higher yield and can effectively extract both highly polar and moderately polar compounds (Abubakar and Haque, 2020). Cold extraction at room temperature yielded only 432mg of methanolic leaf extract (CPMLC) while the Soxhlet extraction yielded 672mg of methanolic leaf extract (CPMLS). According to a study reported by Shirsath and coworkers (2017), Soxhlet extraction had a greater extraction yield of Curcumin from *Curcuma amada* as compared to cold extraction. The greater extraction yield obtained through the Soxhlet extraction techniques also implies a thorough extraction procedure as compared to cold maceration and active thermostable phytocompounds being present in the extract as Soxhlet extraction causes degradation of thermolabile phytocompounds present while boiling (Zhang *et al*., 2018).

In cancer research, *in vitro*, cytotoxicity assays are the gold standard for determining the preliminary cytotoxic effect of both synthetic and natural extracts. The IC_{50} value is a useful and informative measure of a drug's efficacy as it refers to the concentration of the drug which inhibits the cell viability by half *in vitro*. Generally, a drug with a lower IC_{50} value is regarded as more potent (Aykul and Martinez-Hackert, 2016). MTT assay was performed and the IC_{50} of the CPMLC were found to be 72.13µg and 102.07µg on the MCF-7 breast cancer cell line and the HCT-116 colon cancer cell line, respectively, while the IC_{50} of the CPMLS were found to be 69.55µg and 95.58µg on the same cell lines. The low IC_{50} values obtained for the colon cancer cell line indicate that both CPMLC and CPMLS extracts are more cytotoxic to colon cancer than breast cancer. Comparison of IC_{50} values of CPMLC and CPMLS extracts on MCF-7 and HCT-116 cancer cells reveal that CPMLS shows a comparable but lower IC_{50} and hence higher cytotoxicity as compared to CPMLC on both types of cancer cells. As a result, CPMLS extract was utilized to perform subsequent assays in the study.

The results obtained are in accordance with previous preliminary cytotoxicity analyses report that cuprous oxide nanoparticles synthesized from the aerial extract of *C. paniculatus* induced cell death and IC_{50} value - 97.39 µg/mL and 205.11µg/mL in MCF-7 and HT-29 cell lines respectively. AgNPs synthesized from *C. paniculatus* aerial extract triggered cell cycle arrest in MCF-7 and HT-29 cell lines a dose-dependent relationship with IC_{50} values of 207.19 ± 0.64 and 221 ± 0.57 µg/mL, respectively (Giridasaapa *et al*., 2021). The lethal effect of newly identified β-dihydroagarofuranoid sesquiterpenes extracted from the whole plant (IC_{50} value of 17 ±1µM), stimulating apoptotic death in breast cancer via targeting cell cycle progression, and induction of autophagy are all indicative of a pleiotropic mechanism of action (Weng *et al*., 2013).

The MTT assay used to test the biosafety of the plant extract in a normal cell line model revealed no potential harmful effect in HEK-293 normal cells at the same dosage, indicating that the extract has minimal effect on the growth of healthy cells as compared to cancer cells. The cytotoxicity of the extract may be attributed to vehicle control in the study, DMSO, showed low cytotoxicity in all cell lines, indicating that the cytotoxic impact is related to the plant extract rather than DMSO. 5-Fluorouracil (5-FU), a widely used chemotherapeutic medication when used as a positive control in its IC_{50} concentration as expected showed around 50% cell viability in both cancer cell lines and high cell viability in normal cell lines. Compared to 5-FU, the cytotoxic effect of both CPMLC and CPMLS is quite low as expected in the case of direct solvent extracts containing a compendium of phytoconstituents of diverse activity.

Cancer cells possess the trait of forming colonies which determine their tumor-initiating capabilities. At its IC_{50} concentration, CPMLS showed a severe 69% reduction in colony formation in MCF-7 breast cancer cells and a 41% reduction in HCT-116 colon cancer cells following one week of incubation to allow for colony formation. A lower reduction rate in the colony formation in the HCT-116 cell line can be attributed to the highly aggressive and invasive nature of the cell line to form colonies compared to the comparatively slow-growing and non-aggressive MCF-7 cell line when incubated for the same period (Rajput *et al*., 2008). This is the first report on *C. paniculatus* methanolic leaf extract inhibiting reproductive ability in breast and colon cancer cells. Hence, the results of *in vitro* acute cytotoxicity and long-term cytotoxicity assays further support the anticancer potential of *C. paniculatus* methanolic leaf extract as assessed through MTT assay.

Evading apoptosis is one of the major hallmarks of cancer and they circumvent the apoptotic machinery, adopting various strategies including upregulating proapoptotic genes and downregulating antiapoptotic genes. Apoptosis can be triggered by intrinsic factors or external death signals. Expression analyses of the genes involved in the apoptotic pathway were also carried out in breast and colon cell lines treated with CPMLS extract to analyze the predisposition of HCT-116 and MCF-7 cells to apoptosis upon treatment with CPMLS. Expression of genes regulating the intrinsic pathway of apoptosis namely *CAS3* (executioner caspase), *CAS9* (initiator caspase), *PARP1* (a DNA damage repair protein), *BAX*, and *BCL2* (BCL2 family of apoptotic regulators) was studied using real-time PCR by obtaining relative fold change with respect to housekeeping gene β-actin. *CAS3*, *CAS9*, and *BAX* are proapoptotic genes that promote apoptosis, whereas *BCL2* and *PARP1* aid in avoiding apoptosis and are antiapoptotic genes. Upon the treatment with CPMLS extract, the expression of proapoptotic *BAX* was upregulated (2.30 and 2.90 fold) while antiapoptotic *BCL2* (0.08 and 0.91fold) was significantly downregulated in both breast and colon cancer cell lines indicating active apoptosis happening in cancer cells. *BAX* and *BCL2* are antagonistic in function that promote and inhibit apoptosis respectively. A high *BAX/BCL2* ratio is a characteristic of drug-sensitive cells in cancer studies and is recognized as an apoptotic marker and used to assess the induction of apoptosis upon drug treatment in cancer cells. *BAX/BCL2* ratio greater than 1 is an indicator of apoptosis (Raisova *et al.*, 2001).

Analyzing the relative fold change of gene expression, it was found that *BAX/BCL2* ratio was greater than 1; 28.75 in MCF-7 cells and 3.10 in colon cancer cells. The higher *BAX/BCL2* ratio in breast cancer cells indicates apoptosis in breast cancer cells as compared to colon cancer cells. A similar report on the expression analysis of BAX and BCL2 in HCT-116 colon cancer cells by *Artemisia absinthium* methanolic extract triggers apoptosis as confirmed by Caspase 3 activation and western blot analysis (Nazeri *et al*., 2020). Another study revealed that *Ricinis communis* fruit extract altered BAX/BCL2 expression in MCF-7 cells prompting cytotoxicity via apoptosis (Majumder *et al*., 2019).

In the present study, chemotherapeutic 5-FU, utilized as a positive control, exhibits a substantially greater *BAX/BCL2* ratio compared to the value obtained for CPMLC extract,

demonstrating active apoptosis by the drug treatment in both cell lines. This is consistent with the widespread use of 5-FU in the management of colorectal and breast malignancies (Longley *et al*., 2003). On the other hand, the vehicle control used in the study, DMSO shows insignificant changes in relative fold change of gene expression confirming that the changes in gene expression are primarily attributable to treatment with the extract and not to DMSO.

Both the caspases used in the study, *CAS3* and *CAS9* were found to be upregulated in both breast and colon cancer cells upon treatment with CPMLS extract. In comparison to the control group, CPMLS-treated MCF-7 cells upregulated the expression of *CAS3* and *CAS9*, by 1.60 and 1.13 folds, respectively while CPMLS-treated HCT-116 cells upregulated the expression of *CAS3* and *CAS9*, by 1.02 and 1.65 folds, respectively. *CAS3* and *CAS9* expression are indicative of activation of the intrinsic apoptotic pathway (Brentnall *et al*., 2013). Cas3/9-mediated apoptosis has been reported in gastric cancer cells by baicalin, a natural flavonoid from the root of *Scutelleria baicalensis* (Hongwei *et al*., 2017). 5-FU treated cells showed a relatively higher fold of expression of *CAS3* (3.35 and 6.84 fold) and *CAS9* (7.10 and 9.70 fold) in breast and colon cancer cells when compared to untreated control. The results of the change in gene expression of apoptotic genes shown by 5-FU are consistent with previous reports on the cell lines (Shi *et al*., 2019).

Poly (ADP-ribose) polymerase 1 (PARP1) plays an indirect role in apoptosis by regulating apoptosis while protecting cells from DNA damage. *PARP1* was downregulated by 0.38 and 0.44 folds in MCF-7 and HCT-116, respectively. There are reports on leukemia cells respond to curcumin by going through a caspase-3-dependent mechanism that is mediated through PARP1 cleavage making it a relevant measure in determining apoptosis (Mishra *et al*., 2016).

PARP1 is one of the well-known substrates of *CASPASE 3 and 7* and *PARP* cleavage is considered to be one of the major characteristic features of apoptosis (*Mashimo et al., 2021*). On analyzing the expression of *CAS 3* it can be found that a greater expression of CAS3 (1.60 fold) in MCF-7 cells also showed a greater reduction in fold change (0.38 fold) in the downregulation of *PARP1* in MCF-7 cells compared to HCT -116 cells substantiating PARP cleavage by CAS3.

The overexpression of proapoptotic genes (*CAS3, CASP9,* and *BAX*) and the downregulation of anti-apoptotic genes (*PARP1* and *BCL2*) in both cell lines used strongly indicate that gene expression affects apoptotic action. On analyzing closely, proapoptotic effector *CAS3* showed a greater upregulation, and antiapoptotic *PARP1* and *BCL2* showed greater downregulation in MCF-7 Cells than HCT-116 cells suggesting more apoptosis occurring in MCF-7 cells upon treatment with CPMLS. On the other hand, proapoptotic *BAX* and initiator *CAS9* were more upregulated in HCT-116 cells. Apoptosis is multifaceted and the balance between death and survival is met by several caspases and BCL2 family proteins that determine the ultimate fate of the cell (Opferman and Kothari, 2018). Hence, these findings are inadequate to establish an exact pathway of apoptosis; further study is required to gain a greater understanding of the cytotoxic potential of *C. paniculatus* leaf extract.

Telomeres gradually shorten in normal cells leading to replicative senescence after successive cell divisions in normal cells (Jiang *et al*., 2007). Most cancer cells maintain telomeres by ribonucleoprotein telomerase enzyme. The telomerase's catalytic component with reverse transcriptase activity is known as *hTERT*. The study determined the expression of the telomerase gene *hTERT* using real-time PCR. According to expression profiling outcomes, *hTERT* was downregulated by 0.45 and 0.61fold in MCF-7 and HCT-116 cells treated with CPMLS extract. This is indicative of suppression of telomerase activity is more in MCF-7 cells compared to HCT-116 upon treatment with the extract. Downregulation of telomerase in breast cancer cells has been found to induce apoptosis-mediated cell death in breast cancer cells (Rubiś *et al.,* 2013). There are reports that downregulation of telomerase expression enhances apoptosis which correlates with the results obtained in this study (Nakajima *et al.,* 2003). In other words, a greater downregulation of telomerase *hTERT* can be correlated with enhanced apoptosis occurring in MCF-7 cells upon treatment with the plant extract.

To conclude, CPMLS is a promising cytotoxic agent against breast and colon cancer and its probable mode of action is through regulating apoptosis and suppressing telomerase based on the results of the *in vitro* experiments and expression analysis of key apoptotic and telomerase genes. Further research is needed to gain a better understanding of the apoptotic and antitelomerase mechanisms of the methanolic leaf extract of *C. paniculatus*.

Summary

5. SUMMARY

The study entitled "Antiproliferative activity of *Celastrus paniculatus* Willd." was undertaken in the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2022- 2023.

Cancer remains a significant global health concern, accounting for a high mortality rate. The drawbacks of current treatment options including limited efficacy, side effects, and high cost have opened doors to the hunt for new safer, and more effective anticancer drugs. Among natural sources, plants provide anticancer compounds with clinical significance including well-known taxanes and vinca alkaloids.

Celastrus paniculatus Willd. (Black oil plant or Jyotishmathi), an important medicinal plant of the Celastraceae family native to the Indian subcontinent is widely known for its pharmacological activities. However, there are limited studies on its anticancer and cytotoxic effects. In this study, the cytotoxic potential of *C. paniculatus* leaves and their mode of action on breast and colon cancer cells were investigated by performing preliminary *in vitro* assays followed by gene expression analyses of apoptotic and telomerase genes.

The leaf samples were obtained from the State Medicinal Plants Board Kerala, Thiruvananthapuram, dried, powdered, and extracted in methanol. *C. paniculatus* crude methanolic leaf extract was prepared using the cold and Soxhlet extraction techniques. In the current study, HCT-116 and MCF-7 cancer cell lines and HEK-293 normal cell lines were maintained in DMEM with 10% FBS. Soxhlet-extracted *C. paniculatus* methanolic leaf extract (CPMLS) gave a higher yield (672mg/5g) as compared to cold-extracted *C. paniculatus* methanolic leaf extract (432mg/5g).

MTT cell viability assay was performed on cancer and normal cell lines to determine the cytotoxicity of plant extract. CPMLC and CPMLS show a dose-dependent reduction in cell viability % with negligible cytotoxicity in normal cell lines. CPMLS showed a slightly more cytotoxic effect compared to CPMLC on both HCT-116 and MCF-7 cancer cell lines with a more potent cytotoxic effect on HCT-116 colon cancer cells. Hence, CPMLS extract

was used further in the study. Clonogenic assay was performed using IC_{10}, IC_{25}, and IC_{50} concentrations of the CPMLS to determine the colony-forming ability of cancer cells. The determination of survival fraction revealed a considerable percentage reduction in the total number of colonies formed after one week of incubation in both breast as well as colon cancer cell lines with a severe reduction in MCF-7 compared to the HCT-116 cell line.

The expression profiles of key apoptotic and telomerase genes were performed using Real-time PCR with *β-ACTIN* as an endogenous control to elucidate the mechanism of action of CPMLS on breast and colon cancer cells. RNA samples were isolated in biological replicates from control and treated samples and quantified to ensure purity and quantity. cDNA was synthesized and real-time PCR was performed. Mean C_t values were noted and relative fold change of genes were computed using the comparative Delta C_t method. Relative fold change of proapoptotic genes *BAX, CAS3*, and *CAS9* was found to be upregulated while antiapoptotic *PARP1* and *BCL2* genes were downregulated on CPMLS extract-treated samples compared to untreated control. *BAX/BCL2* ratio was found to be greater than 1 indicating the chance of apoptosis happening in CPMLS-treated samples. Telomerase gene *hTERT* was downregulated in CPMLS-treated breast and colon cancer cell lines compared to untreated control indicative of suppression of telomerase activity upon the treatment with CPMLS.

The findings of the particular study indicate the antiproliferative and cytotoxic activities of *C. paniculatus* methanolic leaf extract are mediated through the induction of apoptosis and suppression of telomerase expression in breast and colon cancer cells which require validation through further molecular investigations.

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Appendices

7. APPENDICES

APPENDIX I

Reagents required for maintenance of cell lines

1. Dulbecco's Modified Eagle Medium (DMEM)

- DMEM powder
- HEPES 1.95/L
- Sodium bicarbonate 3.76/L
- 1X antibiotic and antimycotic solution (100µL/L)
- Double distilled water 1L

2. Phosphate-buffered saline [PBS (pH 7.4)]

- NaCl 8g/L
- KCl 0.20g/L
- Na2HPO4 1.44g/L
- KH2PO4 0.24g/L

APPENDIX II

Reagents required for MTT assay

1. MTT stock

- MTT salt, 5 mg in 1 ml PBS.

2. MTT Lysis buffer

- Isopropanol and DMSO (1:1 ratio)

3. Plant extract for treatment

- Crude methanolic leaf extract of CPMLC and CPMLS 10mg in 1 mL 100% ethanol

APPENDIX III

Reagents required for Clonogenic assay

1. 1% crystal violet

- Crystal violet - 0.1g in 10mL methanol

APPENDIX IV

Reagents used for Agarose gel electrophoresis

1. ***Gel tray soaking buffer***
   - NaOH 0.1 M
   - EDTA 100 mM
2. ***TBE buffer (5X) (pH: 8)***
   - Tris base 54 g
   - Boric acid 27.5 g
   - 0.5 M EDTA 20 mL
3. ***Gel loading dye (6X)***
   - Bromophenol Blue 0.2 g
   - 50 % glycerol 6 mL
   - Milipore water 4 mL

ANTIPROLIFERATIVE ACTIVITY OF *CELASTRUS PANICULATUS* WILLD.

by

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Abstract of the thesis

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Abstract

8. ABSTRACT

The study entitled “Antiproliferative activity of *Celastrus paniculatus* Willd.” was conducted in the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2022-2023. *Celastrus paniculatus* Willd. (Black oil plant) is an important medicinal plant belonging to the family *Celastraceae.* Preliminary studies were conducted on the antiproliferative activity of *C. paniculatus* Willd. in breast cancer cells. However, the mode of action of antiproliferative activity of *Celastrus paniculatus* Willd. leaf extract is yet to be investigated. Hence, the objective of the study was to evaluate the cytotoxic potential of *Celastrus paniculatus* Willd. leaf extract on cancer cells and to elucidate its action on induction of apoptosis and suppression of telomerase in cancer cells.

C. paniculatus leaves were collected, dried, powdered, and extracted with methanol using cold extraction and Soxhlet extraction techniques. 5g finely powdered leaf samples yielded 432mg and 672mg crude extract through cold extraction and Soxhlet extraction, respectively. The cytotoxic effect of the leaf extract was determined by MTT assay in the human breast cancer cell line (MCF-7), human colon cancer cell line (HCT-116), and normal cell line HEK-293. The extract showed a dose-dependent reduction in cell survival percentage, and the *C. paniculatus* leaf extract prepared through the Soxhlet extraction technique was more cytotoxic than the cold extract. IC_{50} values of Soxhlet extracted methanolic extract of *C.paniculatus* were 69.55µg and 95.58µg for colon and breast cancer, respectively, while it was non-toxic to normal cells in the above dosage. The leaf extract also inhibited the colony-forming ability of both breast and colon cancer cells as determined by clonogenic assay.

Expression profiling of the apoptotic genes *BAX, BCL2, CAS 3, CAS 9,* and *PARP1* showed upregulation of *CAS 3, BAX,* and *CAS 9* in the CPMLS-treated cells, while the anti-apoptotic genes *BCL2* and *PARP1* were downregulated. The assessment of apoptotic marker expression (*BAX/BCL2* ratio) was found to be greater than one, which confirms that active apoptosis is happening in cancer cells upon treatment with *C. paniculatus* methanolic leaf extract. The downregulation of telomerase *hTERT* in treated cells as revealed by expression analysis indicates the suppression of telomerase activity in breast and colon cancer cells by the *C. paniculatus* leaf extract.

The findings of the study suggest that the methanolic leaf extract of *C. paniculatus* is a promising candidate for developing cancer therapeutics after further in-depth investigations.

സംഗ്രഹം

തിരുവനന്തപുരം വെള്ളായണി കാർഷിക കോളേജിലെ മോളിക്യുലർ ബയോളജി ആൻഡ് ബയോടെക്നോളജി വകുപ്പിൽ 2022 നും 2023 നും ഇടയിലാണ് 'സെലസ്ട്രസ് പാനിക്കുലേറ്റസ് വൈൽഡിന്റെ ആന്റിപ്രോലിഫറേറ്റീവ് ആക്റ്റിവിറ്റി' എന്ന പേരിൽ പഠനം നടത്തി. *Celastrus paniculatus* Willd. (ബ്ലാക്ക് ഓയിൽ പ്ലാന്റ്), സെലാസ്ട്രേസിയ സസ്യ കുടുംബത്തിലെ ഔഷധ ഗുണമുള്ള ഒരു കുറ്റിച്ചെടിയാണ്. സ്തനാർബുദ കോശങ്ങളിൽ സി. പാനിക്കുലേറ്റസ് വൈൽഡിന്റെ ആന്റിപ്രൊലിഫെറേറ്റീവ് പ്രവർത്തനത്തെക്കുറിച്ച് പ്രാഥമിക പഠനങ്ങൾ നടത്തിയിട്ടുണ്ട്. എന്നിരുന്നാലും, സെലാസ്ട്രസ് പാനിക്കുലേറ്റസ് വൈൽഡ് ഇലയുടെ സത്തിന്റെ ആന്റിപ്രോലിഫറേറ്റീവ് ശേഷിയുടെ പ്രവർത്തന സംവിധാനം ഇനിയും അന്വേഷിക്കേണ്ടിയിരിക്കുന്നു. അതിനാൽ, കാൻസർ കോശങ്ങളിലെ സെലാസ്ട്രസ് പാനിക്കുലേറ്റസ് വൈൽഡ് ലീഫ് എക്സ്ട്രാക്റ്റിന്റെ സൈറ്റോടോക്സിക് സാധ്യതകൾ വിലയിരുത്തുകയും, കാൻസർ കോശങ്ങളിലെ അപ്പോപ്റ്റോസിസിന് കാരണമാകുന്നതിലും ടീലോമറേസ് ജീനിനെ നിയന്ത്രിക്കുന്നതിനും ഉള്ള അതിന്റെ പ്രവർത്തനം വ്യക്തമാക്കുകയും ചെയ്യുക എന്നതായിരുന്നു പഠനത്തിന്റെ ലക്ഷ്യം.

സി. പാനിക്കുലേറ്റസ് ഇലകൾ ശേഖരിച്ച്, ഉണക്കി പൊടിച്ച്, മെഥനോൾ ലായകത്തിൽ കോൾഡ് എക്സ്ട്രാക്ഷൻ, സോക്സ്ലെറ്റ് എക്സ്ട്രാക്ഷൻ ടെക്നിക് എന്നിവ ഉപയോഗിച്ച് വേർതിരിച്ചെടുത്തു. 5 ഗ്രാം നന്നായി പൊടിച്ച ഇലയുടെ സാമ്പിളുകളിൽ നിന്ന് 432mg, 672mg അസംസ്കൃത സത്ത്, കോൾഡ് എക്സ്ട്രാക്ഷൻ, സോക്സ്ലെറ്റ് എക്സ്ട്രാക്ഷൻ എന്നിവയിലൂടെ യഥാക്രമം ലഭിച്ചു. ഹ്യൂമൻ സ്തനാർബുദ സെൽ ലൈൻ (MCF-7), ഹ്യൂമൻ കോളൻ കാൻസർ സെൽ ലൈൻ (HCT-116), നോർമൽ സെൽ ലൈൻ HEK-293 എന്നിവയിൽ MTT പരിശോധന നടത്തി ഇല സത്തിന്റെ സൈറ്റോടോക്സിക് സ്വഭാവം വിലയിരുത്തി. എക്സ്ട്രാക്റ്റ് നൽകുമ്പോൾ, കോശങ്ങൾ അതിജീവനത്തിന്റെ ശതമാനത്തിൽ ഡോസ്-ആശ്രിത കുറവ് കാണിച്ചു, സോക്ഷ്ലെറ്റ് എക്സ്ട്രാക്ഷൻ ടെക്നിക് വഴി തയ്യാറാക്കിയ സി. പാനിക്കുലേറ്റസ് ഇല സത്തിൽ കോൾഡ് എക്സ്ട്രാക്ഷൻ ടെക്നിക് വഴി തയ്യാറാക്കിയ ഇല സത്തിനേക്കാൾ കൂടുതൽ സൈറ്റോടോക്സിക് സ്വഭാവം ഉണ്ടായിരുന്നു. വൻകുടൽ അർബുദത്തിന്റെയും സ്തനാർബുദത്തിന്റെയും കോശങ്ങൾക്ക് യഥാക്രമം 69.55µg, 95.58µg എന്നിവയായിരുന്നു സി. പാനിക്കുലേറ്റസിന്റെ സോക്ഷ്ലെറ്റ് എക്സ്ട്രാക്റ്റഡ് മെഥനോളിക് എക്സ്ട്രാക്റ്റിന്റെ IC_{50} മൂല്യങ്ങൾ, അതേസമയം മുകളിൽ പറഞ്ഞ അളവിൽ സാധാരണ കോശങ്ങളിൽ ഇത് ശക്തമായ സൈറ്റോടോക്സിക് സ്വഭാവം കാണിച്ചില്ല. ക്ലോനോജെനിക് പരിശോധനയിലൂടെ, സ്തന, വൻകുടൽ കാൻസർ കോശങ്ങളുടെ കോളനി രൂപീകരണ ശേഷിയെ ഇല സത്തിന് തടയാൻ കഴിയുമെന്ന് കണ്ടെത്തി.
BAX, *BCL2*, *CASPASE 3*, *CASPASE 9*, *PARP1* എന്നീ അപ്പോപ്ടോട്ടിക് ജീനുകളുടെ എക്സ്പ്രഷൻ പ്രൊഫൈലിങ്ങിലൂടെ, എക്സ്ട്രാക്റ്റ്-ട്രീറ്റ് ചെയ്ത കോശങ്ങളിൽ പ്രോ-അപ്പോപ്റ്റോട്ടിക് ജീനുകളായ *BAX*, *CASPASE 3*, *CASPASE 9* എന്നിവയുടെ ജീൻ എക്സ്പ്രഷൻ വർധിക്കുന്നതായി കണ്ടെത്തി. പക്ഷേ ആന്റി-അപ്പോപ്റ്റോട്ടിക് ജീൻ *BCL2, PARP1* എന്നിവ നിയന്ത്രിത ജീൻ എക്സ്പ്രഷൻ കാണിച്ചു. അപ്പോപ്റ്റോട്ടിക് മാർക്കർ എക്സ്പ്രെഷന്റെ (*BAX/BCL2* അനുപാതം) ഒന്നിൽ കൂടുതലാണെന്ന്

കണ്ടെത്തിയതിലൂടെ സി. പാനിക്കുലേറ്റസ് മെത്തനോളിക് ഇല സത്ത് നൽകുമ്പോൾ കാൻസർ കോശങ്ങളിൽ സജീവമായ അപ്പോപ്റ്റോസിസ് സംഭവിക്കുന്നുവെന്ന് സ്ഥിരീകരിച്ചു. കൂടാതെ, കാൻസർ കോശങ്ങളിൽ ടീലോമറേസ് ജീൻ *hTERT*ന്റെ എക്സ്പ്രഷൻ കുറയുന്നതായി കണ്ടെത്തി.

കൂടുതൽ വിശദമായ പഠനത്തിന് ശേഷം, സി. പാനിക്കുലേറ്റസിന്റെ മെത്തനോളിക് ഇല സത്ത് ഉപയോഗിച്ച് പുതിയ കാൻസർ ചികിത്സ വികസിപ്പിക്കാമെന്ന് പഠനം സൂചിപ്പിക്കുന്നു.