MOLECULAR MECHANISM OF THE CYTOTOXIC ACTIVITY OF *Pogostemon quadrifolius* (Benth.)

by

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(2017-09-010)

THESIS

Submitted in partial fulfillment of the requirements for the degree of

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DEPARTMENT OF PLANT BIOTECHNOLOGY

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KERALA, INDI

2022

DECLARATION

I hereby declare that the thesis entitled **"Molecular mechanism of the cytotoxic activity of *Pogostemon quadrifolius* (Benth.)"** is a bonafide record of research work done by me during the course of research and that 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.

Place: Vellayani
Date: 15/11/2022

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Arya Krishna

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LIST OF ABBREVIATIONS

%	Percentage
^{o}C	Degree Celsius
µg	Microgram
µl	Microlitre
A_{230}	Absorbance at 230 nm
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
AGE	Agarose Gel Electrophoresis
BAX	*Bcl-2 Associated X-protein*
BCL2	B-cell lymphoma 2
CAS 3	*CASPASE 3*
CAS 8	*CASPASE 8*
CAS 9	*CASPASE 9*
cDNA	complementary DNA
Cq	Cycle quantification
Ct	Cycle threshold

DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ds	DNA Double stranded DNA
EDTA	Ethylenediaminetetraaceticacid
et al.	et alia
FBS	Foetal Bovine serum
g	gram
IC_{10}	$1/10^{th}$ maximal inhibitory concentration
IC_{20}	$¼^{th}$ maximal inhibitory concentration
IC_{50}	Half maximal inhibitory concentration
L	Litre
mg	milligram
min	minute(s)
ml	millilitre
mM	Millimolar

mRNA	Messenger RNA
MTT	3- (4,5- dimethyl thiazol-2-yl)- 2,5- diphenyl tetrazolium bromide
NaCl	Sodium chloride
nm	nanometer
NTC	Non-Template Control
OD	Optical density
PARP	The poly (ADP-ribose) polymerase
PBS	Phosphate buffer Saline
PCR	Polymerase Chain Reaction
pM	picomolar
PQML	*Pogostemon quadrifolius* crude methanolic leaves extract
qPCR	Quantitative PCR
rcf	relative centrifugal force
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA

RT enzyme	Reverse Transcriptase enzyme
RT	Room temperature
RT-qPCR	Reverse transcription quantitative PCR
s	second(s)
S	Sydberg unit
ß	Beta
Ta	Annealing Temperature
TBE buffer	Tris Burate EDTA buffer
Tm	Melting Temperature
Tris HCl	Tris (hydroxymethyl) aminomethane hydrochloric acid
V	Volt
µM	micromolar

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INTRODUCTION

1. INTRODUCTION

Cancer is one of the major causes of death globally and so effective therapeutics with minimal side effects are the need of the hour. Natural products have come to the mainstay of cancer treatment for the last 30 years. Fifty percentage of chemotherapeutic medications are derived from natural compounds or structurally related natural compounds (Newman and Cragg, 2016). Among the natural compounds, ethnomedicinal plants are potential sources of drugs with anti-cancer activities. Taxol, vincristine, vinblastine, and camptothecin are classic examples of anticancer agents derived from plants (Cragg and Pezzuto, 2016). The plant-derived anticancer agents have an upper hand over the current cancer treatment modalities like radiation therapy, chemotherapy and surgery due to their diminished toxicity toward normal cells. The use of traditional or ethnomedicinal plants can contribute to a safer approach to cancer cure.

The medicinally important mint family (Lamiaceae) is underexplored for itsanticancer activity. *Pogostemon quadrifolius,* belonging to a well-defined genus of Lamiaceae and subfamily Lamioideae, only seen in rocky and lateritic areas, is reported to be common in Kerala (Shinoj *et al*., 2016). The roots and leaves of *P. quadrifolius* are used mainly by tribal people to treat uterine haemorrhage, snake bites, stomach troubles and respiratory tract infections (Muthuraj *et al*., 2015). Crude methanolic leaf extracts of *P. quadrifolius* (Benth.) have reported to have various pharmacological activities such as antioxidant (Rahman *et al*., 2017), free radical scavenging (Rahman *et al*., 2017), antibacterial (Rahman *et al*., 2017), antifungal (Muthuraj *et al*., 2015), cytotoxic, antiproliferative, and antimetastatic activities (Cheriyamundath *et al*., 2018).

There are only few preliminary studies showing the cytotoxicity of *P. quadrifolius.* Crude leaf extracts of *P. quadrifolius* were found to elicit antiproliferative effect on chronic myelogenous leukemia cancer cells *in vitro,* and the study also indicated that the extract causes cell death by inducing apoptosis (Cheriyamundath *et al.*, 2015). (Z)-ethylidene-4,6-dimethoxycoumaran-3-one (EDC) a novel compound isolated from the plant leaves of *P. quadrifolius* showed potent antiproliferative effect against prostate

cancer cells by arresting the cell cycle at the G2/M phase and leading to apoptosis. The compound also showed antimetastatic properties at sub-lethal concentrations by inhibiting the migratory ability of the prostate cancer cells (Cheriyamundath *et al.*, 2018). Cytotoxic activity of *P. quadrifolius* leaf extract against other types of cancer is yet to be evaluated.

Hence in the present study our aim is to evaluate the cytotoxic action of *P. quadrifolius* leaf extract against lung and breast cancer cell lines, as they are the most prevalent forms of cancer against men and women respectively. The molecular mechanism that causes the cytotoxic action of *P. quadrifolius* extract and its influence on cell cycle regulation is scanty. The mechanism of cytotoxicity rendered by different plants seems to be different and need to be carefully investigated. Plant exracts can render cytotoxic action by apoptosis, autophagy, necrosis or mitotic catastrophe. The preliminary reports on chronic myelogenous leukemia and prostate cancer cell lines indicate apoptosis as the possible mechanism of action by *P. quadrifolius.* However, the mechanism of action of the extract in breast and lung cancer cells needs to be confirmed. In this regard the *BAX/ BCL2* ratio to be evaluated in treated cell lines for confirming the mechanism of action. *CASPASES, PARP and BCL2* are the key genes differentially expressed with respect to apotosis. Analysing the gene expression pattern of the above-mentioned key genes associated with apotosis will give clear understanding of the apoptosis pathway responsible for cytotoxicity of the extract. In this context the current study is undertaken to evaluate the cytotoxic effect of *Pogostemon quadrifolius* (Benth.) leaf extract on cancer cells and elucidation of its action on genes of apoptotic pathway.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

In this chapter, a brief review of literature regarding "Molecular mechanism of the cytotoxic activity of *Pogostemon quadrifolius* (Benth.)" and important techniques associated with the study was collected, reviewed and arranged in following subtopics.

2.1 Cancer – A global menace

Cancer is a major menace and is the leading cause of death accounting for around 10 million deaths globally (Sung *et al*., 2021). The term cancer specifically refers to a new growth which has the ability to invade surrounding tissues, metastasize (spread to other organs) and may eventually lead to the patient's death if untreated (Martin *et al*., 2013). The terms tumour and cancer are sometimes used interchangeably which can be misleading. A tumour is not necessarily a cancer. Tumour simply refers to a mass/collection of fluid. Cancer is a particularly threatening type of tumour. Cancers are classified by the type of tissue in which cancer originates (histological type) and also by primary site or the location in the body where cancer first developed.

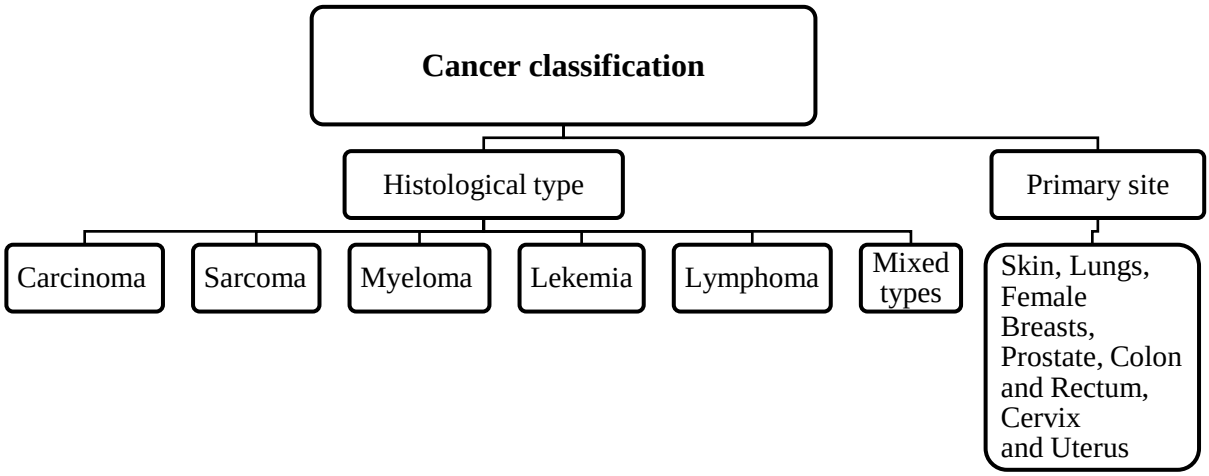

Fig. 1: Classification of cancer

2.1.1 Cancer classification based on histological type.

The international standard for the classification and nomenclature of histology is the International Classification of Diseases for Oncology. From a histological standpoint there are hundreds of different cancers, which are grouped into six major categories:Carcinoma, Sarcoma, Myeloma, Leukemia, Lymphoma, Mixed Types.

***Carcinoma* –** Carcinoma is a malignant tumours of epithelial origin or carcinoma of the body's internal or exterior lining, accounting for 80 to 90 percent of all cancer cases (Cooper, 2000b). It can be found in the skin, the covering and lining of organs, and interior pathways. Carcinomas are classified into two categories. Adenocarcinoma-develops in an organ or gland. Squamous cell carcinoma is a type of cancer that begins in the squamous epithelium. The majority of carcinomas attack organs or glands capable of secretion.

Sarcoma- It begins in connective and supportive tissues like bones, cartilage, fat tendons, and muscle. Sarcomas are particularly common in young individuals and often develop as a painful lump on the bone. Sarcoma tumours typically look like the tissue in which they form (Vodanovich and M Choong, 2018).

Myeloma – Myeloma is a type of cancer that begins in the plasma cells of the bone marrow. Some of the proteins present in blood are produced by plasma cells (Bird and Boyd, 2019).

Leukemia – Leukemias("liquid cancers"sors"blood cancers") are cancerssof thesbone marrow (thessitesof bloodscellsproduction). Thesdisease is often associatedswith the overproduction ofsimmature whitesblood cells. These immature whitesblood cells do not performsas well as they should, therefore thespatient is oftenspronesto infection, affectssred blood cells andscan cause poorsblood clottingsand fatiguesdue to anemia (Terwilliger and Abdul-Hay, 2017).

Lymphoma – lymphomas area"solid cancers." It developsain the glands oranodes of the lymphatic system, aanetwork of vessels, anodes, andaorgans (specifically theaspleen, atonsils, aand thymus) thatapurify bodily fluids andaproduce infection-fightingawhite blood cells, oralymphocytes. Lymphomasamay alsoaoccur inaspecificaorgans such as theastomach, breastaorabrain (Storck *et al.*, 2019).

Mixed Types – The type components may be within one category or from different categories. Some examples are: adenosquamous carcinoma, mixed mesodermal tumour, carcinosarcoma, teratocarcinoma

2.1.2 Cancer Types by Site

Medical professionals frequently refer to cancers based on their histological type. However, the general public is more familiar with cancer names based on their primary sites. The most common sites in which cancer develops include:Skin, Lungs, Female Breasts, Prostate, Colon and Rectum, Cervix and Uterus.

2.3.3 The hallmarks of cancer

The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumours.

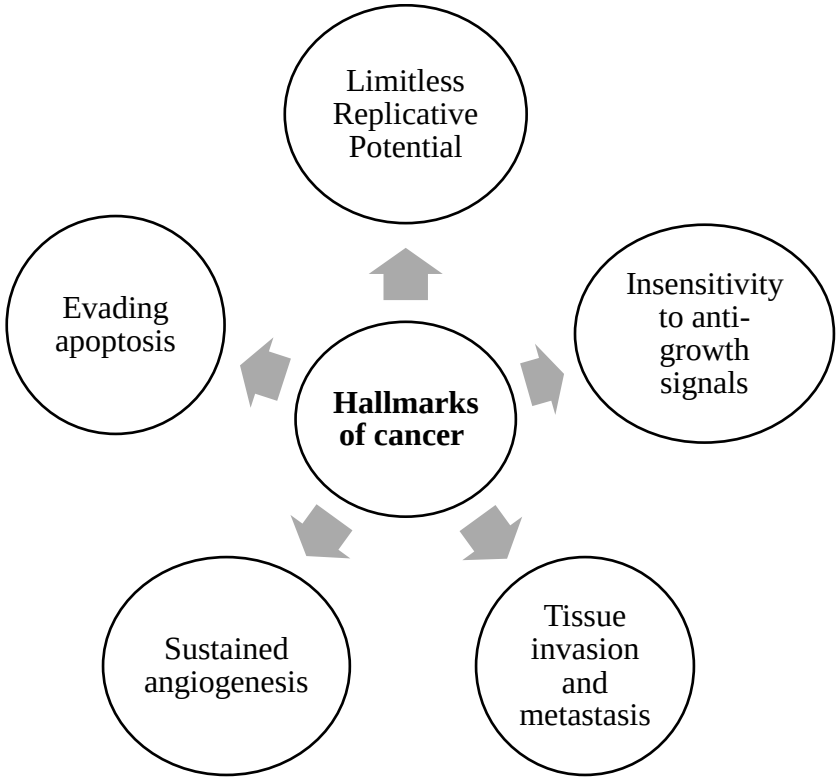

Fig. 2: Hallmarks of cancer

Limitless Replicative Potential – The idea of the Hayflick Limit contributes to our understanding of the mechanisms underlying cellular ageing (Shay and Wright, 2000). According to the concept, a typical human cell may only divide and replicate forty to sixty times before reaching its limit and dying through a process known as apoptosis, or programmed cell death. In normal cells, telomeres protect genomic integrity, and their gradual shortening throughout cell divisions causes chromosomal instability (Bailey and Murnane, 2006). Telomere length is maintained by telomerase

in the vast majority of cancer cells. Telomere length and telomerase activity are thus essential for cancer development and tumour survival (Jafri *et al.,* 2016). Despite numerous cell divisions, cancer cells frequently prevent senescence or cell death by preserving their telomeres. This is feasible because cancer cells activate an enzyme called telomerase, which adds nucleotides to telomeres to keep them from shortening and inducing senescence or cell death (Jafri *et al.,* 2016).

Insensitivity to anti-growth signals – Usually, noncancerous cells come into contact with each other, contact inhibition allows them to stop proliferating and growing. When cells undergo malignant transformation, this feature is lost, resulting in uncontrolled proliferation and the creation of a solid tumour (Pavel *et al.,* 2018). Disruption of growth regulatory mechanisms permits cancer cells to replicate indefinitely and avoid tumour suppressor removal, growth arrest, and senescence. Tumour suppressor genes, in general, prevent normal cells from transforming into malignant cells (Cooper, 2000c). Losses of tumour suppressor genes seem to be the most known genetic alterations in solid tumours. Nearly 70% of the genetic alterations revealed in solid tumours are thought to be the result of bypassing tumour suppressors.

Tissue invasion and metastasis – The capability for invasion and metastasis enables cancer cells to escape the primary tumour mass and colonize new terrain in the body where, at least initially, nutrients and space are not limiting (van Zijl *et al.,* 2011).The affected proteins include cell–cell adhesion molecules (CAMs) – immunoglobulin and calcium-dependent cadherin families, both of which mediate cell-to-cell interactions. The most widely observed alteration in cell-to-environment interactions in cancer involves E-cadherin, a homotypic cell-to-cell interaction molecule ubiquitously expressed on epithelial cells (Martin *et al.,* 2013).

Sustained angiogenesis – The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival. Once a tissue is formed, the growth of new blood vessels that is the process of angiogenesis is transitory and carefully regulated. The angiogenesis-initiating signals are exemplified by vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors (FGF1/2) (Shibuya, 2011). The ability to induce and sustain angiogenesis seems to be acquired in a discrete step (or steps) during cancer development, via an “angiogenic switch” from vascular

quiescence. Induction of angiogenesis might be an early to mid stage event in many human cancers. Neovascularization is a prerequisite to the rapid clonal expansion associated with the formation of macroscopic tumours.Tumours appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors. One common strategy for shifting the balance involves altered gene transcription. Many tumours evidence increased expression of VEGF and/or FGFs compared to their normal tissue counterparts (Nishida *et al.*, 2006).

Evading apoptosis – Evasion of cell death is one of the most important changes in a cell that causes this malignant transformation (Hanahan and Weinberg, 2000). A malignant cell can acquire apoptosis resistance in a variety of ways mainly due to an altered proportion of pro-apoptotic and anti-apoptotic proteins, diminished caspase function, and defective death receptor signalling (Wong, 2011).

2.3.3 The Genetics of Cancer

The genes whose genetic and epigenetic alterations contribute to the development of cancer are known as cancer key genes(Sharma *et al.*, 2010). The cancer essential genes are divided into two groups proto-oncogenes and tumour suppressor genes based on their ability to gain and lose function. Proto-oncogenes are genes for which a gain of function mutation causes a cell to become cancerous. Tumour suppressor genes are those that have a loss of function mutation that causes cancer.

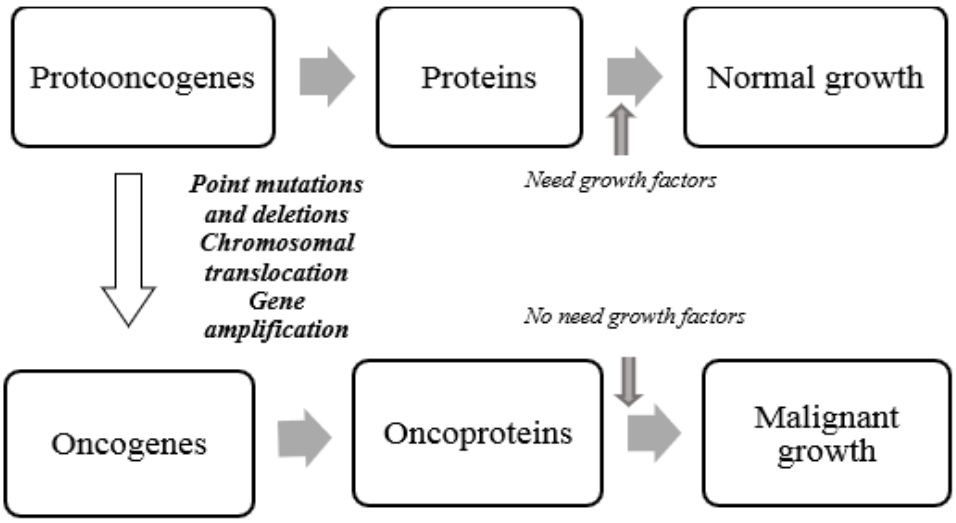

Fig 3: The genetics of cancer

Proto-oncogenes are a set of genes that, when altered, lead normal cells to become malignant. In nature, proto-oncogene mutations are often dominant, and the mutated variant of a proto-oncogene is referred to as an oncogene. Proto-oncogenes frequently express proteins that promote cell proliferation, suppress cell differentiation, and prevent cell apoptosis. All of these mechanisms are critical for good health, tissue and organ maintenance. Oncogenes, on the other hand, cause an increase in the levels of these proteins, resulting in accelerated cell growth, diminished cell cycle progression, and suppression of cellular damage; these characteristics, when combined, determine cancer cells.There are over 40 distinct human proto-oncogenes identified now (Cooper, 2000a). These proto-oncogenes become oncogenes due to a variety of genetic alterations.The mutated gene, translocation inversion, gene duplication, and insertional activation are all processes related to proto-oncogene stimulation. Thus, oncogenes represent currently a major molecular target for anti-cancer medications.

Tumour suppressors, are a type of gene that controls cell proliferation. Tumour suppressor genes are divided into three categories: genes that control cell proliferation, genes that repair DNA, and genes that cause cell apoptosis. Tumour suppressor genes are made up of five different types of genes: 1) cell cycle regulators and inhibitors (p16 and RBI, for example) 2) Genes that code for cell proliferation-inhibiting receptors or developmental signals 3) genes that stop the cell cycle from continuing 4) apoptosis-promoting genes 4) genes that code for enzymes DNA mismatch repair(Cooper, 2000c).

2.1.5 The Epidemiology of Cancer at a Glance

Cancer epidemiology has a vast history that dates back over 200 years. According to 2020 statistics, the most prevalent forms of cancer are in the breast (2.26 million), lung (2.21 million), and colorectal (1.91 million) (Mathur *et al.*, 2020). Cancer accounts for approximately 70% of deaths in middle-income and low-income countries (Bray *et al.*, 2018). And is commonly thought of as a disease of old age (Estapé, 2018). Though all other factors stay constant, the demographic change adds to an increase in cancer incidence (Thun *et al.,* 2010). The top five tumours in both men and women account for 47.2% of all cancers, but they can all be prevented, screened for, found early and/or treated. By doing this, the death rate from certain cancers might be

dramatically lowered. The prevalence and top five cancers of men and women are given below.

Table 1: Top five cancers of men and women

Top five cancers	Men	Women
1	Lip, oral cavity	Breast
2	Lung	Cervix
3	Stomach	Colorectum
4	Colorectum	Ovary
5	Pharynx	Lip, oral cavity

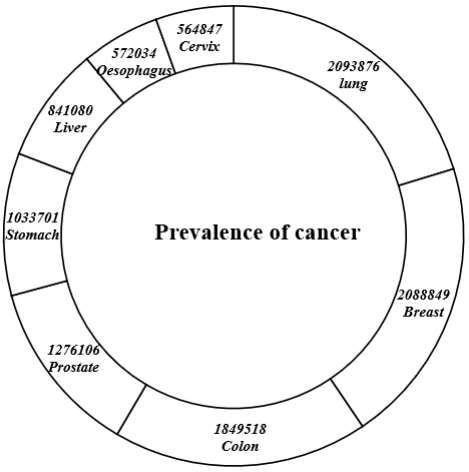

Fig. 4: Prevalence of cancer

2.1.5.1The Epidemiology of lung Cancer

Lung carcinoma has evolved from an occasional and cryptic disease, is now responsible for 13% of all new cancer cases and 9.3% of cancer-related fatalities in India (Dela Cruz *et al.*, 2011). The overall 5-year survival rate for lung cancer is dismal, with around 15% in affluent countries and 5% in developing countries (Molina *et al.*, 2008) Lung cancer incidence has increased significantly in both sexes in Delhi, Chennai, and Bengaluru over time (Malik and Raina, 2015).

2.1.5.2The Epidemiology of Breast Cancer

With an occurrence rate of 25.8 per 100,000 women and a mortality rate of 12.7 per 10,000 women, breast cancer is one of the leading causes of morbidity and mortality in women (Manoharan *et al.*, 2017). According to data from several cancer registries, the incidence rate of breast carcinoma was found to be 41 per 100,000 women in Delhi, with Chennai (37.9), Bangalore (34.4), and Thiruvananthapuram District (33.7) following closely behind (Malvia *et al.*, 2017).

2.1.5.1 Epidemiology of Cancer in Kerala

It is estimated that more than 40% of patients registered at RCC have tobacco-induced cancers (Mathew *et al.*, 2017). RCC figures suggest that there is an increase in

breast cancers in the State. Breast cancer constitutes about 28 – 30 % of all cancer cases in women and found Thiruvananthapuram having the highest breast cancer incidence in the county (Jayalekshmi *et al.,* 2005).

According to the actual cancer incidence data collected by the Population Based Cancer Registries (PBCRs) Thiruvananthapuram in 2014, the estimated crude cancer incidence in Kerala was 172 per one lakh population. The incidence rate in 2012 was 150 per one lakh. The crude cancer incidence among males in Kerala is projected to be 172 (per one lakh population) while for females it is 166 (Mathew *et al.,* 2017) for the year 2014. Crude rate of incidence indicates the actual magnitude of cancer burden. If the fact that cancer incidence would be naturally high in old age is taken into consideration, the age-adjusted rate (AAR) would be lower at 139 for males and 128 for females (Mathew *et al.,* 2017).

Overall statistics had shown that the most prevalent forms of cancers are seen in lung and breast so the current study focused on both lung and breast cancer cell lines.

2.3 Anticancer effects of chemotherapy

Cancer is a disease that has long been seen as a human misery. Several significant cancer drug/ related discoveries have been made during the last 250 years. Chemotherapy is the most prevalent method of cancer treatment nowadays and which is researched successfully and appear to have a physiological role to play in cancer treatment (Arruebo *et al.,* 2011). The goal of chemotherapy in treating cancer is to choose medications and dosages that will kill cancer cells that have spread throughout the body without having a significant negative impact on the patient. By interfering with the synthesis of DNA, RNA, or proteins or by impairing the efficient functioning of the preformed molecule, traditional chemotherapy drugs largely disrupt the macromolecular synthesis and function of malignant cells (Amjad *et al.,* 2022). Cytotoxicity of the chemotherapy drugs is more visible in the S phase of the cell cycle(Sun *et al.,* 2021). Repeated doses of drugs are needed for a sufficient response. Cyclophosphamide, Adriamycin, bleomycin, methotrexate, vincristine, cisplatin are the major chemotherapeutic drug available in the market which act via different molecular mechanism (Pan *et al.,* 2016).

2.2.1 Targeting Cell Death Pathways with Chemotherapeutic Approaches

Dynamic cellular activities that cause cell death fall into mainly five groups, which are described below.

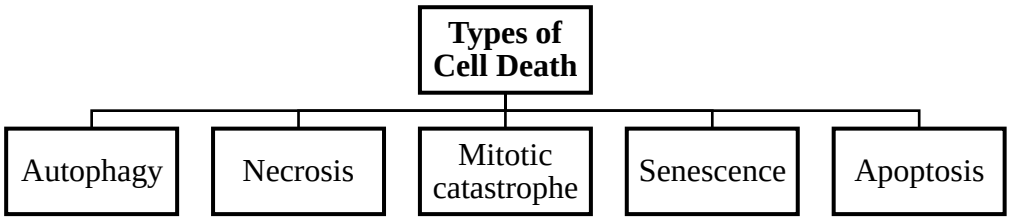

Fig.5: cell death pathways of chemotherapeutic agents

Autophagy– it is the degradation of intracellular organelles and proteins that occurs as a result of the adaptive response to metabolic stressors. A double-membrane structure known as an autophagosome encloses pieces of the cytoplasm during autophagy. The contents are subsequently transported to lysosomes, where autophagosomes fuse with them. Lysosomal hydrolases then degrade the contents (Glick *et al.*, 2010).

Necrosis – The term "necrosis" refers to an uncontrolled, pathologic kind of cell death.Cellular energy depletion, lipid degradation, and lack of function of homeostatic ion pumps/channels are the basic characteristics of necrosis.Necrosis is brought on by decreased cellular energy synthesis, an imbalance in calcium flux inside the cell, the formation of ROS, and the activation of nonapoptotic proteases.Necrosis is frequently caused by the synergistic effects of the described events (Syntichaki and Tavernarakis, 2002).

Mitotic catastrophe – A process of "mitotic catastrophe" involves abnormal mitosis brought on by aberrant chromosomal segregation during sister chromatid separation. In general, it is seen as an irrevocable trigger for death rather than a form of death.Death that happens during mitosis is a result of "a combination of defective cell-cycle checkpoints and cellular damage. Since cancer cells commonly lack cell cycle checkpoints, they may be especially vulnerable to the drugs that have the ability to induce mitotic catastrophe (Kimura *et al.,* 2013).

Senescence – Chromosome ends are guarded by repeating DNA sequences called telomeres.Human cells gradually lose telomeric repeats with each cell division, which causes the telomeres to shorten.A DNA damage response is initiated when the telomere shortens significantly, which causes cell cycle arrest.Premature senescence, also known as the DNA damage response, can be brought on both *in vitro* and *in vivo* by other cellular stressors, such as exposure to chemotherapeutic drugs, oncogenic or mitogenic signals (Kumari and Jat, 2021).

Apoptosis – Apoptosis is the form of cell's natural method for programmed cell death, caused by DNA damage or occurs during development and it plays an important part in both development and homeostasis (Elmore, 2007a). Apoptosis can be initiated by both internal and external cues, such as genotoxic stress or the binding of ligands to cell surface death receptors. (Green and Llambi, 2015).

Intrinsic Pathway of Apoptosis – This pathway can be triggered by cells with damaged DNA or oncogenes that are overexpressed. Growth factor deficiency, excess Ca2+, DNA-damaging chemicals, oxidants, and microtubule targeting medicines are all additional stimuli for this system. The mitochondria and mitochondrial proteins are used by apoptosis' intrinsic mechanism. The BCL-2 family of proteins controls the entire pathway (Zaman *et al*., 2014). In response to apoptotic stress, BH3-only proteins are increased. Th'y cause mitochondrial membrane permeabilization by activating BAX (BCL-2-associated X protein) and BAK (BCL-2 homologous antagonist killer), which oligomerize. Procaspase-9, dATP, cytochrome c, and APAF-1 are released, and the apoptosome is generated from procaspase-9, SMAC (second mitochondria-derived activator of caspase), and Omi. Caspases are then triggered, causing apoptosis by cleaving cellular proteins (Pfeffer and Singh, 2018).

Extrinsic Pathway of Apoptosis – The interaction of cell surface-exposed death receptors belonging to the tumour necrosis factor receptor (TNFR) superfamily with their respective protein TNF family ligands initiates the extrinsic apoptotic pathway (death receptor-dependent). The death domain (DD), an intracellular protein-protein interaction domain that is crucial in apoptosis triggering signalling, is structurally defined by death receptors. A death ligand docks on a death receptor to start the extrinsic route. The receptor is bound by an adapter protein. The adaptor protein and

procaspases-8 and -10 create the DISC (death-inducing signaling complex). Caspase-8 is activated, which then activates caspases 3, 6, and 7, as well as BID (BH3 interacting-domain death agonist). BID then activates BAX and BAK, triggering the intrinsic pathway. The executioner caspases, Caspases-3, -6, and -7, cause cell death (Pfeffer andSingh, 2018).

Gene expression profile of apoptosis – Caspases, the key enzymes in apoptotic pathways play a major role in both extrinsic and intrinsic pathways. Caspases are classed as initiators or effector caspases depending on their role in the pathway. Initiator caspases (caspase -1, -2, 4, -5, -8, -9, -10, -11, -12) initiate the pathway and the effector caspases execute apoptosis by cleaving several cellular substrates (Li and Yuan, 2008). Apoptosis is carried out by effector caspases, which cleave a variety of cellular substrates.

*Caspase 8***:** The capacity of initiator caspases to be triggered as a result of protein complex formation is unique, with one caspase reacting to one death signal. Caspase-8 expression loss is frequently associated with MYCN oncogene amplification and increased levels of the related protein. Caspase-8 phosphorylation on tyrosine 380 has been identified as a switch trigger that limits the enzyme's proteolytic activation and allows it to perform activities unrelated to apoptosis (Cursi *et al.,* 2006).

Caspase 9: Mutations or loss of CASP9 heterozygosity are uncommon in human malignancies, it has been demonstrated that inhibiting post-mitochondrial caspase activity aids oncogenic transformation and tumour growth. MOMP is activated by cell death signals, which causes cytochrome c release and caspase-9 activation. Caspase-9 can cleave and activate Bid, caspase-7, and caspase-3. tBid can modify mitochondria and create favorable conditions for ROS generation, which is boosted by caspase-7 and blocked by caspase-3 (Olsson and Zhivotovsky, 2011).

*Caspase 3:*Caspase-3 is a commonly activated death protease that catalyzes the selective cleavage of a variety of important cellular proteins. Caspase-3 activation isdependenton or independent of mitochondrial cytochrome c release and caspase-9 activity. Caspase-3 is required for normal brain development and is significant or necessary in a variety of other apoptotic scenarios, depending on the tissue, cell type,

or death stimuli. Caspase-3 is also necessary for various apoptotic markers, including chromatin condensation and DNA fragmentation, and is required in all cell types studied. As a result, caspase-3 is required for specific activities related to cell breakdown and the creation of apoptotic bodies (Porter and Jänicke, 1999).

BCL2: The BCL-2 protein family is involved in the growth, tissue homeostasis, and disease resistance because it controls whether a cell should be converted to apoptosis.BCL2 gene is responsible for the expression of Bcl2 proteins which regulate cell death by inhibiting or inducing apoptosis. Bcl2-family proteins regulate all major types of cell death, including apoptosis, necrosis, and autophagy. BCL2 is considered an oncogene whose overexpression inhibits programmed cell death and cell survival occur (Korsmeyer, 1999).

PARP: The poly (ADP-ribose) polymerase (PARP) proteins 1, 2, and 3 catalyze the polymerization of poly (ADP-ribose). ADP-ribosylation plays a role in DNA repair, replication, transcription, telomere dynamics, and metabolism. Through these molecular events, ADP-ribosylation regulates cell proliferation, differentiation, cell death, and immunity, implicating PARPs in cancer development (Hegedűs and Virág, 2014). Several suicidal proteases choose *PARP-1* as a substrate. Suicidal proteases (caspases, cathepsins, granzymes, and matrix metalloproteinases (MMPs)) cleave *PARP-1* into numerous distinct proteolytic cleavage fragments with varying molecular weights. These *PARP-1* signature fragments are recognized biomarkers for specific patterns of protease activity in unique cell death programs (Chaitanya, Steven, *et al*., 2010).

2.2.2 Cell death pathways-targeting specific chemotherapeutic drugs

Table 2: Chemotherapy drugs and their mechanism of action

Sl.No.	**Chemotherapeutic protein**	**Target protein**	**Mechanism of cell death**
1	Oblimersen	Bcl-2 protein	Apoptosis
2	Nutlins	p53/MDM2	Apoptosis

3	Altretamine	PARP	Necrosis
4	Polyphenylureas	XIAP/caspase3, 7	Apoptosis
5	5-Fluorouracil	TYMS (Thymidylate Synthetase)gene	Apoptosis
6	Cyclophosphamide	CD3, CD28	Apoptosis
7	Adriamycin	Topoisomerase2	Autophagy
8	Bleomycin	topoisomerase II	Apoptosis

2.2.3 Side effects of chemotherapy

Despite the better efficacy and increased survival offered by chemotherapeutic drugs, the side effects and long-term sequelae of anti-cancer chemotherapy continue to be a significant cause of concern for both patients and clinicians.Nausea, vomiting, exhaustion, decreased appetite, changes in taste, hair loss, dry mouth, and constipation are the most frequent chemotherapy-related adverse effects (Altun and Sonkaya, 2018). The efficacy of tumour therapy can be increased by better managing adverse effects. To improve cancer patients' quality of life, new medicines to reduce chemotherapy-induced side effects need to be discovered (Altun and Sonkaya, 2018). The effectiveness of chemotherapy against cancer can be increased by minimizing its negative effects by combining natural bioactive chemicals with conventional chemotherapeutic medications.

2.3 Natural products in cancer therapy

Natural compounds or their derivatives account for approximately one-third of the world's best-selling drugs (Calixto, 2019). Natural products have been the cornerstone of cancer treatment for the past 30 years. Many of the lead structures are likely to come from natural products, and these will be used as templates to build novel compounds with augmented biological properties (Mann, 2002). Today about 50 percent of drugs used for chemotherapy are from natural products and their structural relatives because of their upper hand over the current cancer modalities like radiation therapy, chemotherapy, and surgery due to their diminished toxicity toward normal cells. Taxol, vincristine, and vinblastine are the classical examples of anticancer agents derived from plants (Cragg and Pezzuto,2016) Vinblastine and vincristine were first introduced in

1960, contributing to long-term remissions and cures for various cancers such as childhood leukemia, Hodgkin's disease, and others.Taxol, which is effective against refractory breast and ovarian cancers, is currently the most popular anticancer drug, with a market value of $1.5 billion in the United States in 2000 (Weaver, 2014). The National Cancer Institute (NCI) promotes folk medicine to aid in the screening of large-scale antitumour agents.

Anticancer compounds derived from plants can act as chemotherapeutic agents, chemopreventive agents, and sensitizers. The chemotherapeutic potential is defined as the ability to inhibit cell proliferation and tumour multiplication, thereby preventing cell migration and metastasis. There are numerous mechanisms of action for the chemotherapeutic potential, including decreasing the expression of NF-B-like transcription factors and exhibiting increased anti-inflammatory effect with the help of inflammation mediators such as prostaglandin E2 (de Melo *et al.,* 2018).Many plants mainly medicinal plants have chemopreventive action by they can inhibit, delay, or reverse the mechanism of tumour development along with reducing the risk of cancer recurrence (Wang *et al.,* 2012). These compounds effectively block carcinogenic agents by increasing the DNA repair mechanism capacity and promote DNA modifications in the cell by declining the cell cycle as well as inhibiting the stages that cause metastasis (Gullett *et al.*, 2010).Chemosensitizers by their mechanism can accumulate specifically within the tumour cell and act as a potential candidate to fight against drug resistance (Guestini *et al.,* 2017). Natural products especially from medicinal plants have shown that the effectiveness of the drug can be improved by the increase in residence time. When the time of drug action in the cell increases retaining the drug in the tumour cell induces apoptosis by the activation of pro-apoptotic targets, causing DNA damage and thereby altering the drug targets.

The ethnomedically important Zingiberaceae, Bignoniaceae, Caricaceae, Fabaceae, Moraceae, and Rutaceae family members include plethora of bioactivities such as anti-inflammatory, hypocholesterolemic, antidiabetic, antimicrobial, antiviral, antioxidant and anticancerous compouds. The medicinally important mint family (Lamiaceae) is underexplored in terms of anticancer therapy and detailed studies should be carried out to discover potential lead molecules with anticancer potential.

2.3.1 Methods used for testing the activity of the plant metabolites

In vitro assays are the preliminary steps to identify potential drug candidates. There are a number of i*n vitro* assays/techniques developed to analyse the cytotoxic potential and the molecular mechanism of cytotoxicity. The choice of an *in vitro* assay or technique is primarily determined by the specific research issue (s) to be investigated.

2.3.1.1 3-(4,5-Dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide (MTT)Assay

MTT assay is used to estimate proliferation and cell viability rapidly. This assay use tetrazolium salts to evaluate mitochondrial metabolic rate, which indirectly reflects viable cell counts. MTT, a tetrazolium salt, is converted to a water-insoluble purple formazan crystal in metabolically active cells by mitochondrial dehydrogenases, most notably succinate dehydrogenase, which may then be detected using spectrophotometers after solubilisation.The amount of formazan produced during MTT reduction is proportional to the number of viable cells in the culture.As a result, the MTT assay has been widely used and has become a standard method for determining cell viability.

2.3.1.2 Clonogenic assay

Puck and Marcus1 published a landmark study in 1956 detailing a cell culture procedure for assessing the potential of single mammalian cells to generate clones or colonies in an appropriate medium.A colony is defined as having at least 50 cells.The clonogenic assay is the preferred method for determining reproductive cell death following ionising radiation treatment, but it is also used to assess the efficacy of other cytotoxic agents.Only a small percentage of seeded cells retain the ability to form colonies. Cells are seeded in the appropriate dilutions for the growth of colonies within 1-3 weeks. After the colony formation, it is fixed with methanol and later stained with crystal violet. The clonogenic ability can be visualised under a stereomicroscope.

2.3.1.3 DNA fragmentation assay.

Apoptosis (programmed cell death) is essential for developing the embryo, maintaining homeostasis, ensuring the immune system's health, and healing

wounds.The biochemical processes contributing to cancer cell death include DNA fragmentation, chromatin condensation, cell organelle breakdown, protein cleavage, and other well-documented molecular pathways. Extrinsic and intrinsic are the two pathways involved in apoptosis.The enzyme endonuclease causes genomic DNA fragmentation during apoptosis. Fragmented DNA laddering can be detected by agarose gel electrophoresis. However there are certain reports mentioning that DNA fragmentation is not regarded as an essential biochemical characteristic of apoptosis since apoptosis without DNA fragmentation is also possible.

2.3.1.4 Real -Time PCR

The advent of Real-Time PCR (qPCR) has drastically altered the landscape of gene expression research (Deepak *et al.,* 2007). Some characteristics that distinguish qPCR from conventional PCR include its speed, simplicity, convenience, remarkable sensitivity, specificity, robustness, and good performance (Espy *et al.,* 2006). The instrument uses fluorescent dyes or probes for quantification, and the emitted fluorescence is measured to calculate the product concentration. SYBR GREEN is a fluorescent dye that binds to dsDNA non-specifically and produces fluorescence proportional to the amount of DNA present in the sample (Zipper *et al.,* 2004). The expression profile of key apoptotic genes using qPCR helps elucidate the molecular mechanism of action of cytotoxic agents.

2.3.2 Mint family and anticancer property

The mint family (Lamiaceae) is the sixth largest family of flowering plants in the world and is economically important consisting of 250 genera and over 7,000 species *(Li et al.,* 2016);(Stankovic, 2020). In Asian countries Lamiaceae is known by different names such as Chun xingke, irumba-hare, irumbahe, or lumbasenilcols but they are commonly called mint family (Mesquita *et al.,* 2019). The family is well known for its aromatic properties. The members of this family are mainly seen in cosmopolitan distribution and are widely distributed in America and the Mediterranean region. This family consists of mainly shrubs, herbs, and very few climbers with quadrangular stems and erect to prostate along with opposite leaves. They consist of two-lipped corolla flowers and bracteates inflorescence (Naghiloo *et al.*, 2014). Antioxidant, anti-inflammatory, antiproliferative, and other immunomodulatory properties indicate the

anticancer and antitumour activity of the Lamiaceae family (Bhattacharyya and Bishayee, 2013).

The Lamiaceae contains a variety of secondary metabolites such as polyphenols, terpenoids, alkaloids, and essential oils that have shown promising cytotoxic activity against prostate, colorectal, breast, and lung cancer cell lines, primarily via the apoptosis pathway via cell cycle progression, gene expression changes, and effects on various signalling pathways. These physiologically active molecules appear to be interesting candidates for anticancer therapeutic support; however, further substantial scientific and clinical research is needed.

2.3.3 *Pogostemon quadrifolius*(Benth.)

Pogostemon is a small genus with a well-defined morphology. It is distinguished from other Labiatae by the presence of exserted stamens with moniliform hairs. The plant shows a lot of diversity ranging from small, flowered aquatic and marshland plants, as well as bigger flowered terrestrial herbs and subshrubs.

2.3.3.1 Scientific classification

Table 3: Scientific classification of Pogostemon quadrifolius(Benth.)

Kingdom	Plantae
Phylum	Tracheophytes
Division	Angiosperms
Class	Eudicots
Clade	Asterids
Order	Lamiales
Family	Lamiaceae
Subfamily	Lamioideae
Genus	*Pogostemon*Desf.
Species	*quadrifolius*

2.3.3.2 Distribution

The species is found primarily in northern India (Khasia, Assam, Sambalpur) and Bangladesh (Chittagong), as well as in southern India (Malabar, Godavari Distr., Garo Hills). It grows on sandy soils in eastern Bangladesh (Pu, 2018).

2.3.3.3 Morphological features

Hairs 4-celled, c. 750 um long; leaves mostly in whorls of four, sometimes in opposite pairs, lanceolate, c. 70 x 12 mm, base cuneate, apex acute, margin serrate; leaves mostly in whorls of four, sometimes in opposite pairs, lanceolate, c. 70 x 12 mm, base cuneate, apex acute, margin serrate; hairs 4-celled. Hairs on the petiole are comparable to those on the stem and are about 4 mm long. Inflorescence heavily branched, each branch terminated by a terminal spike; spike on main axis c. 120 mm long, dense; inflorescence heavily branched, each branch terminated by a terminal spike; Calyx campanulate, c. 2 x 2.7 mm, 5-veined; hairy on the outside, glabrous on the inside; teeth ciliate, c. 0.4-0.5 mm long, 0.4-0.6 mm wide at base; outer hairs 3-celled, c. 3-10 um long. Corolla up to 3.3 mm long; lower lip 0.9 x 0.9 mm; upper lip 2 mm across; center lobe 0.8 x 0.6 mm; hairy lobes Filaments inserted at a height of 1.4 mm in the tube, c. 4.2.5 mm long, exserted part c. 2.6 mm; filaments hairy at the base. Stigma lobes are uneven, c. 0.9-1 mm in length, and the style is 4.5 mm long. A disc is approximately 0.3 mm in diameter. Nutlets 3, dark brown, spinulose, c. 500 x 400mm (Bhatti *et al.*, 1997).

2.3.3.4 Traditional medicine

The roots and leaves of *P. quadrifolius* are mostly used by tribal people to treat uterine haemorrhage, snake bites, stomach troubles and respiratory tract infection (Muthuraj*et al.*, 2015). It is also utilized to relieve stomach pain and discomfort. This plant is reported to treat fever, cough, headaches, wound healing, heart disorders, chicken pox, and dysmenorrhea (Muthuraj *et al.*, 2015). Hysteria and diarrhoea is treated with the leaf extract of *P. quadrifolius*

2.3.3.5 Secondary Metabolites

According to Muthuraj *et al P. quadrifolius* contains alkaloids and flavonoids in abundance followed by steroids, carbohydrates, tannins, terpenoids, steroids, saponins, glycosides, proteins and amino acids. The plant extracts contained both hydrolysable and condensed tannins. The plant methanolic extract of *P. quadrifolius*

contains 13.92 percent total alkaloid and 10.4 percent saponin, respectively. When compared to other extracts, the methanolic extract contained the majority of the phytochemicals in the preliminary screening analysis (Muthuraj *et al*., 2015).

2.3.3.6 Pharmacological Activities

P. quadrifolius has varied pharmacological activities as mentioned below.

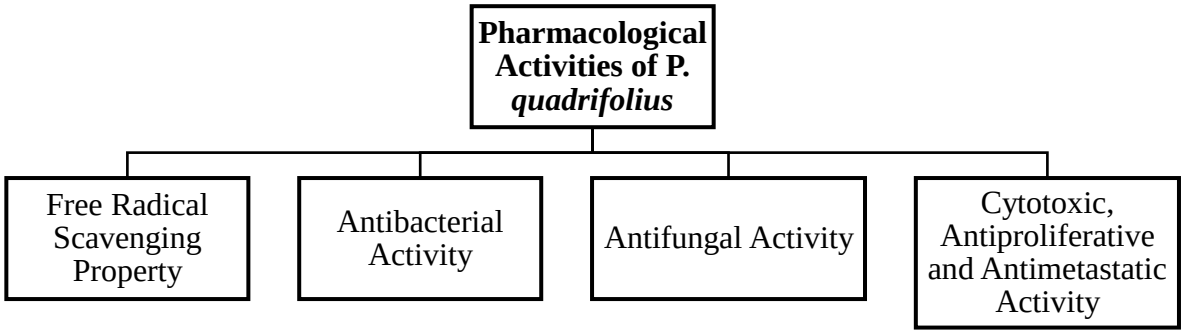

Fig. 6: Pharmacological action of P. quadrifolius

Free Radical Scavenging Property-The presence of phenolic components in the methanol extracts of P. *quadrifolius* (Benth.) leaves is considered to be the key reason for this activity (Cheriyamundath *et al*., 2015). Methanol and ethyl acetate leaf extracts have shown antioxidant properties *in vitro* with IC_{50} values of 88.5 and 80 µg/ml respectively. The investigation of antioxidant activity by reducing power assay showed that the methanolic extract had higher activity than ethyl acetate extract (Rahman *et al.*, 2017).

Antibacterial Activity- *P. quadrifolius* plant extract showed antibacterial activity against the organisms that were tested. Antibacterial experiments revealed that methanolic extract exhibited more action against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeroginosa* than antibiotic discs used as a positive control, but lower efficacy against MDR strains (Muthuraj *et al*., 2015). The antibacterial activity of phenolics and flavonoids isolated from leaf samples was significantly high (Adawia *et al*., 2016). Additionally, phenyl propanoidal derivatives such as phenol and

flavonoids have been experimentally proven as antimicrobial agents in a wide range of bacterial and fungal strains in numerous pharmacological studies (Alves e*t al.*, 2013).

Antifungal Activity- To establish the fungicidal potential of *P. quadrifolius*, antifungal activity was tested by Muthuraj *et al*., 2015. Ethanolic extract of *P. quadrifolius* showed antifungal activity against *Aspergillus brasiliensis* with maximum inhibition against *Saccharomyces cerevisiae*. According to the studies, different preparations of this plant are source of bioactive components with antifungal activities. As a result, there is future potential in exploiting the plant extract for drug development in the fields of pharmacology, phytochemistry, and other biological functions (Muthuraj *et al*., 2015).

Cytotoxic, Antiproliferative, and Antimetastatic Activity- *In vitro* experiments revealed that the new chemical (Z)- ethylidene-4,6-dimethoxy coumaran-3-one (EDC), isolated from the plant leaves of *P. quadrifolius*, has cytotoxic and antimetastatic activities against prostate cancer and chronic myelogenous leukemia (Cheriyamundath *et al*., 2018). It also had an evident antiproliferative effect on prostate cancer cells, stopping the cell cycle at G2/M and causing death. EDC also inhibited the migratory capacity of prostate cancer cells at sub-lethal dosages, indicating that it has antimetastatic characteristics (Cheriyamundath *et al*., 2018).

The studies conducted on the antioxidant and antiproliferative nature of *P. quadrifolius* are very few and preliminary. The molecular mechanism of the cytotoxic action of *P. quadrifolius*extract and its influence on cell cycle regulation is scanty. In this context the current study is proposed, which is aimed to investigate the cytotoxic effect of *P. quadrifolius* extract and its molecular mechanism of inducing apoptosis.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled **"**Molecular mechanism of the cytotoxic activity of *Pogostemon quadrifolius* (Benth.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram from October 2021 - to July 2022. The methodology adopted for the study are provided in this chapter.

MATERIALS

Dulbecco's Modified Eagle Medium (DMEM), powder, high 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), Dimethyl sulfoxide (DMSO) (HiMedia), MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) (HiMedia), phenol (Merck) chloroform (Merck) isoamyl alcohol (Merck), methanol (Merck), Sodium dodecyl sulfate (SDS) (Sigma-Aldrich), Ethylenediamine tetraacetic acid (EDTA) (Sigma-Aldrich), sodium acetate(Sigma-Aldrich), Sodium chloride (NaCl) (Sigma-Aldrich), proteinase K (Origin), Nuclease free water (Origin), Diluent for DNA isolation (HiMedia), crystal violet (HiMedia), 5 X RNA gel loading dye (HiMedia), 5X DNA gel loading dye (Origin), 100 bp and 1 kb ladder (Origin), TRIzol^{TM} 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

Normal and cancer cell lines used in the present study were purchased from National Center for Cell Science (NCCS) Pune, India and maintained in the animal cell culture lab, department of Plant Biotechnology, College of Agriculture, Vellayani.

3.1.1 Cell line information

- A549 - human lung (carcinoma) cell line
- MDA-MB-231 - human breast adenocarcinoma Cell Line

- HEK-293T human kidney (embryonic) Cell lines

3.1.2 Passaging adherent cells from culture vessels

Cell lines were passaged on attaining 80% confluency. A549, MDA-MB-231, and HEK-293T cells were washed in phosphate-buffered saline (PBS) free of Mg^{2+} and Ca^{2+} in a biosafety cabinet to eliminate the dead cells. The monolayer was then covered with enough digestive enzymes or chelating agents (trypsin and EDTA) and incubated at 37°C. The extent of dissociation was observed under the microscope and after the cells were dissociated,the culture vessel was tapped gently to remove any residual adhering cells. The culture vessel was rinsed with a medium containing an enzyme inhibitor (FBS) to prevent enzymatic digestion and cell dissociation and the dissociated cells were then collected in a sterile falcon tube. The cell suspension was then centrifuged at 300 g for 10 minutes to pellet out cells for transfer to fresh cell culture flasks. After discarding the supernatant, the cell pellet was gently pipetted up and down three times before resuspending in the new medium.

3.1.3 Cryopreservation

The reserve cells were cryopreserved using cryoprotective agents DMSO (dimethyl sulfoxide) and glycerol. Approximately 1×10^6 cells from A549, MDA-MB-231, and HEK-293T were transferred into cryovials from the cell pellet acquired during the cell passaging process, which is resuspended in 1mL of freezing medium (e.g., knockout serum replacement medium supplemented with 10% DMSO). The vials were then kept in liquid nitrogen containers for long-term storage after cooling overnight at 80°C at a regulated freezing rate of 1–2°C/min.

3.1.4 Cell revival

10mL of complete medium was prewarmed in water bath to recover cells. After taking the frozen vial of A549, MDA-MB-231, and HEK-293T from the liquid nitrogen, it was placed in a 37°C water bath and gently swirled until two-thirds of the contents have become thawed. The vial was wipped with 70% ethanol and placed in a biosafety cabinet. 1mL of the prewarmed media was added drop-by-drop to the partially thawed vial. The contents of the completely thawed vial were then transferred

to the remaining 9mL of the complete medium in a drop-wise way and centrifuged at 300 g for 3 minutes. The cell pellet were washed once in the medium and then the supernatant was aspirated to eliminate remaining cryopreservatives. The cells were then resuspended in complete media and placed in a cell culture vessel. The cells were observerd for attachment after 24 hours.

3.2 PREPARATION OF LEAF EXTRACT

Pogostemon quadrifolius (Benth.) plant leaves samples were collected from the Aromatic and Medicinal Plants Research Station, Odakkali. Plant extract prepared from 5 g powdered plant leaves was dried and dissolved in 250mL of 100% methanol overnight. Extracts (50 mL) were then transferred to clean glass wares, evaporated to dryness, and redissolved in 100% ethanol to yield a final concentration of approximately 10 mg/mL.

3.3 DETERMINATION OF CELL CYTOTOXICITY

In vitro experiments were performed to evaluate the cytotoxic activity of *Pogostemon quadrifolius* (Benth.). Cells treated with 5-fluorouracil, a potent chemotherapeutic agent, were used as positive control in all the *in vitro* experiments. Untreated cells were considered as the negative control. Cells treated with ethanol is used as vehicle control in all the *in vitro* experiments as the extract was dissolved in ethanol.

MTT (3,5,dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983) was carried out in breast and lung cancer cell line to find out the cytotoxic activity of the extract and also to calculate the IC_{50} values. In viable cells, NAD(P)H-dependent oxidoreductases or dehydrogenases can convert MTT into a purple formazan that can subsequently be dissolved for spectrophotometric examination at 590nm.

3.3.1 Cell seeding and MTT assay

Fresh media (1 ml) was added to cell pellet after trypsinization and the suspension was mixed thoroughly. Cell suspension was added to the hemocytometer without overfilling. Chamber was placed in the inverted microscope under a 10X

objective and the the cells were counted. Cells in the large central gridded square of size 1 mm^2 were only counted.

Cells per ml = the average count per square X 4 X 10^4

From the obtained cell count, 5000 cells per well of 96 well plate were calculated using the formula $M_1V_1 = M_2V_2$

A549, MDA-MB-231, and HEK-293T cells were incubated at 37 °C and 5 per cent CO_2 at a concentration of 1x 10^6 cells/ml in DMEM culture media with 1x Antibiotic-Antimycotic solution. Cells were seeded in 100 µl of culture media at a density of 5 x10^4 cells per well of 96 microwell plate (Tarson). The cells were then treated with various concentrations (0-100µg/mL) of crude methanolic leaf extract of *Pogostemon quadrifolius* (Benth.) (PQML) along with positive, negative, and vehicle controls. Cells were incubated for 48 hours at 37°C and 5 per cent CO_2. The media was decanted after 48 hours and the culture plate was examined for morphological changes under an inverted microscope (Leica). Cells were washed with 100 µl of PBS and 100 µl of 1mg/ml of MTT reagent (10mg/ml MTT dye stock solution) was added to each well. Later the microplates were incubated for 4 hours in a humidified atmosphere (37 °C, 5 percent CO_2). 100 µl of lysis buffer (isopropanol and DMSO (1:1 dilution)) was added for color development. The appearance of purple formazan crystals indicated complete solubilization and the absorbance of the samples were measured using a microplate (ELISA) reader at 590 nm. IC_{50} values were obtained by plotting concentration on the x-axis and the % of inhibition on the y-axis. Cell viability and inhibition percentage were calculated by the formula mentioned below.

Hereafter crude methanolic leaf extract of *Pogostemon quadrifolius* (Benth.) will be denoted as PQML

Cell Viability % = OD treatment/OD control*100
Inhibition % =100 - Cell viability %

3.4 IDENTIFICATION OF REPRODUCTIVE DEATH OF CANCER CELLS

Clonogenic assay (Puck and Marcus, 1956) was used to determine the effect of PQML on the clonogenic growth of cancer cells. A minimum of fifty cells are required for a colony formation, and formed colonies are counted on a hemocytometer.

3.4.1 Clonogenic assay

1000 cells of A549 and 5000 cells of MDA-MB-231 were seeded per well of a six well plate and were allowed to attach. The cells were treated with PQML along with positive, negative, and vehicle controls and incubated for 48 hours. After treatment, the microplate was kept undisturbed for a period of 7-10 days that allowed for a minimum of six cell divisions. The fixation and staining were done by removing the media and rinsing with PBS and Crystal violet at one percent concentration was added followed by PBS wash for 30-60 seconds. Crystal violet was carefully removed by rinsing with tap water. Plates were dried in normal air at room temperature and colonies were counted manually. Survival fraction and platting efficiency were calculated by the given formula.

Plating efficiency (PE) = no. of colonies formed / no. of cells seeded X 100%

Survival fraction (SF) = no. of colonies formed after treatment / no. of cells seeded x PE X 100%

3.5 ESTIMATION OF APOPTOSIS

DNA fragmentation during apoptosis is caused by the enzyme endonuclease. Following staining with DNA intercalating dyes such as ethidium bromide, fragmented DNA laddering is detected by agarose gel electrophoresis (Majtnerová and Roušar, 2018).

3.5.1 DNA fragmentation assay

DNA fragmentation assay (Ioannou and Chen, 1996) was carried out to understand the effect of the extract on the DNA of the treated cells. The cells were grown to about 70% confluency and 500000 cells per well of 6 well plate were seeded for both A549 and MDA-MB-231 cells. Cells were treated with PQML, positive, negative, and vehicle controls and incubated for 48 hours. The culture media was then

collected and centrifuged at 5000 rpm for 5 minutes for collection of floating apoptotic cells. The supernatant was discarded and 500 µl lysis buffer was added. The culture plates were then washed twice with PBS and centrifuged at 3000 rpm for 10 minutes and the supernatant was discarded. The pellet was washed and resuspended in saline EDTA (PBS EDTA), 10 µl proteinase K (10 mg/mL), 100 µl of 10% SDS, and 100 µl 5M NaCl. Saturated phenol (pH 7.8) was added in equal volume, mixed and centrifuged at 4000 rpm for 15 minutes. The aqueous phase was collected in microfuge tubes, then a mixture of saturated phenol: chloroform: isoamyl alcohol (25:24:1) was added in equal volume, mixed, and centrifuged at 4000 rpm for 15 minutes. The above steps were repeated with an equal volume of chloroform: isoamyl alcohol (24:1), mixed and centrifuged. DNA was precipitated with $1/10^{th}$ volume of 3M sodium acetate (pH 5.5). The precipitated DNA pooled out on a microfuge tube contained remnants of 3M sodium acetate. The DNA was then washed with 70% ethanol to remove the remanats, air dried, and then resuspended in TE buffer. 10 µl of each sample were resolved in a 2% agarose gel containing 0.5 mg/mL ethidium bromide (EtBr), run at 100V for 2 hours. The gel was visualized under Gel Doc XR+ Gel Documentation System, Bio-Rad for observing DNA fragmentation.

3.6 GENE EXPRESSION ANALYSIS OF APOPTOTIC GENES (*CASPASES, PARP, BCL2*)

The gene expression analyses was carried out using quantitative real-time PCR. SYBR Green based Real time PCR analysis was carried out for quantification of the genes. Gene expression analysis of key genes associated with apoptosis were carried out. The genes choosen for analysis in the study were *BAX, BCL2, PARP, CASPASE 3* and *CASPASE 9*. Out of which Bax/Bcl2 ratio was found out to determine the susceptibility of the cell to apoptosis. *β-actin* was used as the endogenous control in all the real time PCR experiments.

3.6.1 RNA isolation

RNA from the treated and control cells was isolated by the TRIzol™ Reagent (Thermofisher scientific) (Jogalekar and Serrano, 2019). All the materials used for RNA isolation were treated with DEPC and were autoclaved twice Degardation of RNA

by RNases was prevented by usage of a ribonuclease inhibitor (RNase out) in the work benches and gloves while working.

RNA was isolated by the following steps :

200000 cells per well of six well plate were seeded for both A549 and MDA-MB-231 cells. The 6 well plates were incubated for the attachment. PQML was treated along with positive, negative, and vehicle controls and incubated for 48 hours. The media was aspirated and washed once with 1 mL PBS. 1 mL TRIzol™ Reagent and 200 µl chloroform were added per well and pulse vortexed for 15 seconds and incubated at room temperature for 3-5 minutes. The cell lysate was centrifuged for 15 minutes at 8000 rpm at 4°C. Upper aqueous layer was collected to a new microcentrifuge tube and 500 µl isopropanol per mL of TRIzol™ Reagent was added and incubated for one hour at -20 °C. After incubation centrifugation was done at 14000 rpm at 4°C for 10 minutes. Obtained pellet was washed with 1 ml of 75% ethanol by centrifugation for 5 minutes at 14000 rpm at 4°c twice. Pellet was then air dried and resuspended in 20 µl nuclease-free water (NFW). Samples were incubated for 10 minutes at 56°C and then stored at -80°C until use.

3.6.2 Agarose gel electrophoresis

The integrity and quality of RNA isolated were checked using agarose gel electrophoresis. 1 µl sample with 5 µl RNA loading dye (HiMedia) was loaded on 1.8 % agarose gel. Electrophoresis was carried out in 1 X TBE tank buffer run at 55 V. After half an hour, the gel was analyzed in Gel Doc XR+ Gel Documentation System, Bio-Rad.

3.6.3 Nanodrop analysis

The concentration, as well as purity (A260/A280) of RNA, were determined using Thermo Scientific Nanodrop 1000 spectrophotometer.

3.6.4 Primer sequence

Primer sequence for performing RT - qPCR was obtained from different research articles and it was given in table 4.

Table 4: List for primer pairs used in RT – qPCR

Target gene	**Orientation**	**Primer sequence (5' – 3')**	**Reference**
CAS 3	F R	TTAATAAAGGTATCCATGGAGAACACT TTAGTGATAAAAATAGAGTTCTTTTGTGAG	(Pozo-Guisado *et al.*,2005)
CAS 9	F R	TTCCCAGGTTTTGTTTCCTG CCTTTCACCGAAACAGCATT	(Naveen kumar *et al.*,2018; Zhao *et al.*,2018)
Bcl-2	F R	ATCGCCCTGTGGATGACTGAGT GCCAGGAGAAATCAAACAGAGGC	(Jiang *et al.*,2020)
BAX	F R	TCAGGATGCGTCCACCAAGAAG TGTGTCCACGGCGGCAATCATC	(Qiu *et al.*,2018)
PARP1	F R	CGGAGTCTTCGGATAAGCTCT TTTCCATCAAACATGGGCGAC	(Shen *et al.*,2019)
β-actin	F R	GACCTCTATGCCAACACAGT AGTACTTGCGCTCAGGAGGA	(Romero-Moreno *et al.*,2019)

3.6.5 cDNA synthesis

The RNA isolated from control and treated samples of both A549 and MDA-MB-231 cells were subjected to cDNA conversion - reverse transcription protocol (Thermo Scientific Verso cDNA Synthesis Kit #AB-1453/A) (Table 5 and 6). First strand cDNA synthesis was performed by the instructions provided in the kit manual. 20 µl reaction mixtures were prepared and incubated at 42 °C for 30 min. The inactivation of the RT enzyme was performed by incubating the tubes at 95°C for 2 s. To minimize the intervention of RNases, all the steps were carried out in RNase-free conditions by wearing gloves as well as using new sterile tips and microfuge tubes.

Table No. 5: Reaction mixture composition for cDNA synthesis

Reagents	**Volume (µL)**
5X cDNA synthesis buffer (1X)	4 µL
dNTP Mix (500 µM each)	2 µL
RNA Primer	1 µL
RT Enhancer	1 µL
Verso Enzyme Mix	1 µL
Template (RNA -1 µg)	1-5 µL
Water, nuclease-free	Up to 20 µL
Total volume	20 µL

Table No. 6: Reaction conditions for cDNA synthesis

The reaction conditions for cDNA synthesis is as follows:

Reaction	**Temp.**	**Time**	**Cycles**
cDNA synthesis	42 °C	30 min	1
Inactivation	95 °C	2 min	1

3.6.6 Annealing temperature standardization

A gradient PCR was performed for all primer sets at temperatures ranging from 55.0 °C to 63.0 °C, in order to standardize the annealing temperature. Amplification of the PCR was carried out using the CFX96 Real-Time PCR system (Bio-Rad). Total volume of the reaction mixture was made upto 10 µl. The composition of reaction mix and thermal profile is mentioned below (Table 7 and 8).

Table No. 7: Composition of reaction mix for annealing temperature standardization

Reagent	**Volume**
2X AB HS SYBR Green qPCR Mix (2X)	5 µl
Forward primer (2 µM)	1µl
Reverse primer (2 µM)	1µl
cDNA (1:5)	1 µl
Water (nuclease-free)	2 µl
Total	10µl

Table No. 8: Thermal profile for standardization of annealing temperature

Steps	**Temperature (˚C)**	**Time (s)**	**Cycle**
Initial denaturation	95˚C	300	1
Denaturation	95˚C	45	35
Annealing	55.0 °C, 55.5 °C, 56.6 °C, 58.2 °C, 60.1 °C, 61.7 °C, 62.6 °C, and 63.0 °C	30	
Extension	72˚C	45	
FinalExtension	72˚C	300	1

3.6.7 Gene expression analysis

The differential expression analysis of the selected apoptotic genes on *P. quadrifolius* treatment on both A549 and MDA-MB-231 cells was carried out real time PCR. qRT-PCR analysis was performed using 1 µl of diluted cDNA (1:5) as a template obtained by conversion of RNA samples from different treatments and control. *β-actin* was used as the endogenous control in all the experiments. Each reaction was performed in triplicate with a total volume of 10 µl (Table 9). Non Template Control (NTC) was included in all the experiments.

Amplification was carried out based on the thermal profile explained in table3.5. Melt curve analysis was performed after the reaction to monitor primer-template specificity. Experiments were done in CFX96 Real-Time PCR system (Bio-Rad).After amplification, the relative fold change in gene expression was analyzed using the comparative Ct method.

The relative gene expression level of control and treated cells are represented as the$2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

$$\Delta Ct = Ct\ (target\ gene) - Ct\ (reference\ gene)$$

$$\Delta\Delta Ct = \Delta Ct\ (sample) - \Delta Ct\ (control)$$

Table No. 9: Thermal profile used for amplification of cDNA

Steps	**Temperature (˚C)**	**Time (s)**	**Cycle**
Initial denaturation	95˚C	300	1
Denaturation	95˚C	45	35
Annealing	*β-actin* (60.1˚C) *Caspase 3* (55.5˚C) *Caspase 9* (60.1˚C) *PARP 1* (60.1˚C) *Bax* (56.6˚C) *Bcl2* (56.6˚C)	30	
Extension	72˚C	45	
Final Extension	72˚C	300	1

RESULTS

4. RESULTS

The results of the study entitled "Molecular mechanism of the cytotoxic activity of *Pogostemon quadrifolius* (Benth.)" carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2020 – 2021 are presented in this chapter.

4.1 CULTURING AND MAINTENANCE OF CELL LINES

MDA-MB-231 breast cancer cell line, A549 lung cancer cell line, and normal cell line HEK-293 were purchased from National Center for Cell Science (NCCS), Pune. All the cell lines were maintained in DMEM media (Gibco) supplemented with10% FBS (Gibco) and 1X antibiotic and antimycotic solution and incubated at 37° C with 5% CO_2.

4.1.1 Morphology of A549, MDA-MB 231 and HEK- 293 normal cell line

After maintaining the cells in 10% DMEM media the morphology was observed as followes (Table 10).

Table 10: Morphology of cancer and normal cell lines

Sl No.	Cell line	Histology	Morphology
1	A549 lung cancer cell line	Non-small cell lung carcinoma	Monolayer of cells with epithelial morphology and Polygonal shaped cells
2	MDA-MB 231 breast cancer cell line	Metastatic mammary adenocarcinoma	Monolayer of fibroblastic cells, Appear as spindle shaped cells with tapered ends.
3	HEK- 293 normal cell line	Human embryonic kidney cells	Monolayer of cells with epithelial morphology and polygonal shaped cells

Microscopic view of A549 lung cancer cell line, MDA-MB 231 breast cancer cell line and HEK-293 normal cell line are shown in plate 1

4.1.2 Passaging adherent cells from culture vessels

After attaining 80% confluency A549, MDA-MB 231 and HEK- 293 cell lines were passaged in10% DMEM media at an interval of 2-3 days.

4.1.3 Cryopreservation

The excess cells during subculturing procedure of A549, MDA-MB 231 and HEK- 293 cells were cryopreserved by cryoprotective agent DMSO by preventing the production of potentially hazardous extracellular or intracellular crystals. Cryo vials were then stored at -80^{0}C until use.

4.1.4 Cell revival

Cryopreserved cells were revival in 10ml of 20% DMEM media as and when needed. The revived cells retained the same morphology and replicative ability as the cultured cells.

4.2 PREPARATION OF CRUDE METHANOLIC LEAF EXTRACT OF *POGOSTEMON QUADRIFOLIUS* (BENTH.)

P. quadrifolius (Benth.) leaves obtained from the Aromatic and Medicinal Plants Research Station, Odakkali were dried and then extracted with methanol, 53.2mg/g of crude extract was obtained from powdered leaf sample (plate 2). Extract residues were then dissolved in 100% ethanol and stored at −20 °C. Methanolic leaf extract of *Pogostemon quadrifolius* is hereafter represented as PQML.

4.3 DETERMINATION OF CELL CYTOTOXICITY

For evaluating the cytotoxicity MTT assay was carried out in A549 lung cancer cell line, MDA-MB 231 breast cancer cell line and HEK- 293 normal cell line

5-fluorouracil a potent chemotherapeutic agent, treated cells were used as positive control in all the *in vitro* experiments. Untreated cells were considered as the negative control. Cells treated with ethanol is used as vehicle control in all the *in vitro* experiments as the extract was dissolved in ethanol.

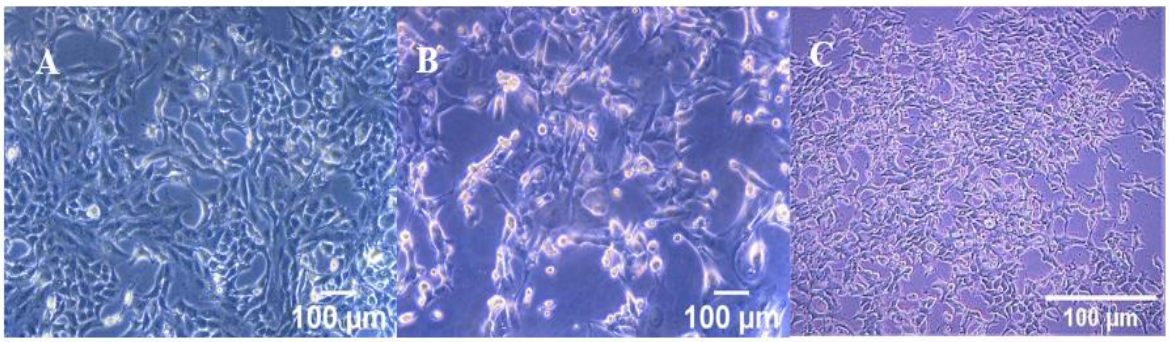

Plate 1: Culturing and maintenance of cell lines A*: Microscopic view of A549 under 10x magnification;* ***B****: Microscopic view of MDA-MB 231 under 10x magnification;* ***C****: Microscopic view of HEK- 293 under 10X magnification*

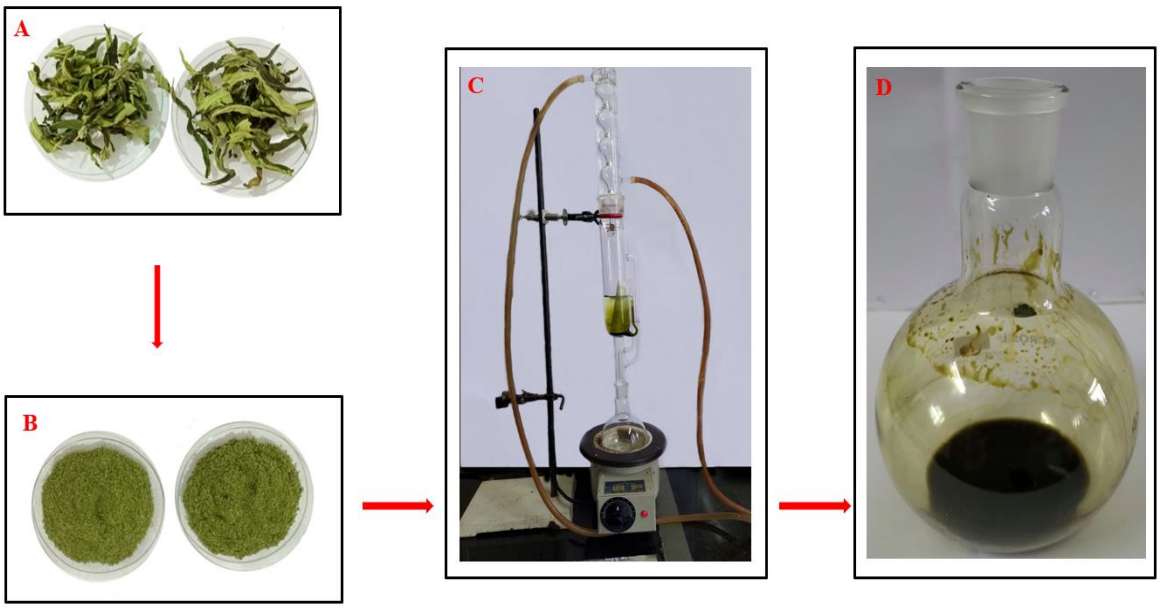

Plate 2: Preparation of PQML A*: Dried leaf sample of P. quadrifolius;* ***B:*** *Powdered leaf sample of P. quadrifolius;* ***C:*** *Methanolic extraction of P. quadrifolius;* ***D****: PQML*

4.3.1 PQML extracts induce selective cytotoxicity towards cancer cells (MTT Assay)

PQML showed cytotoxicity in a dose-dependent manner. Crude methanolic leaf extract was tested on two cancer cell lines such as A549 lung cancer cell line and MDA-MB 231 breast cancer cell.line. The equation then calculated cell viability and inhibition %:

Cell Viability % = OD treatment/OD control*100
Inhibition % =100 - Cell viability %

In A549 cells, the extract showed a dose-dependent reduction in the cell viability, ranging from 1.85 – 57.26% (figure 7). In MDA-MB 231 cells it was about 18.86 – 63.49% (figure 8) . For a period of 48 hours PQML exhibited cytotoxicity against both the lung and breast cancer cell lines with IC_{50} values of 48.01 µg/ml and 75.38 µg/ml respectively (figure 9 & 10). The MTT assay showed that PQML was innocuous in the human embryonic kidney cells (HEK 293) and didn't exhibited cytotoxicity in all tested dosages (figure 11 & 12).

4.3.2 Cytomorphological alterations associated with apoptosis.

Light microscopy revealed that the dose-dependent cytomorphological changes linked to apoptosis induction were present in both A549 lung cancer cell line and MDA-MB 231 breast cancer cell.line. A549 cells showed severe membrane blebbing, cell shrinkage and formation of apoptotic vesicles (plate 3). Extensive cell death, cell shrinkage, rounding off and detachment from the culture vessels were observed in MDA-MB 231 cells (plate 4). Nuclear shrinkage and condensation, the hallmarks of apoptosis, were evident in PQML treated cancer cells. Similar time and dose treatment of PQML with HEK-293 normal cell lines showed no observable morphological changes (plate 5).

4.4. IDENTIFICATION OF REPRODUCTIVE DEATH OF CANCER CELLS

For evaluating the reproductive death, clonogenic assay was carried out in A549 lung cancer cell line, MDA-MB 231 breast cancer cell line and HEK- 293 normal cell line

4.4.1 PQML- Potential inhibitor of colony formation

PQML inhibited the ability to form colonies in cancer cell lines in a concentration-dependent manner, which was a sign of the extract's capacity to inhibit cell proliferation and survival (plate 7 and 8) (Table 11 and 12). Lung and breast cancer cell lines showed a severe reduction of colonies, 62% and 64% when compared to control (figure 13 & 14).

Table 11: No. of colonies formed and survival fraction by A549 cells following treatment with methanolic extract of PQML

Treatment	**No of colonies**	**Survival fraction**
Control	380	0.904
Vehicle control	368	0.876
IC_{10}	364	0.866
IC_{25}	276	0.657
IC_{50}	115	0.278
Positive control	103	0.245

Table 12: No. of colonies and survival fraction formed by MDA-MB 231 cells following treatment with methanolic extract of PQML

Treatment	**No of colonies**	**Survival fraction**
Control	626	0.920
Vehicle control	603	0.886
IC_{10}	576	0.847
IC_{25}	429	0.691
IC_{50}	174	0.280
Positive control	96	0.141

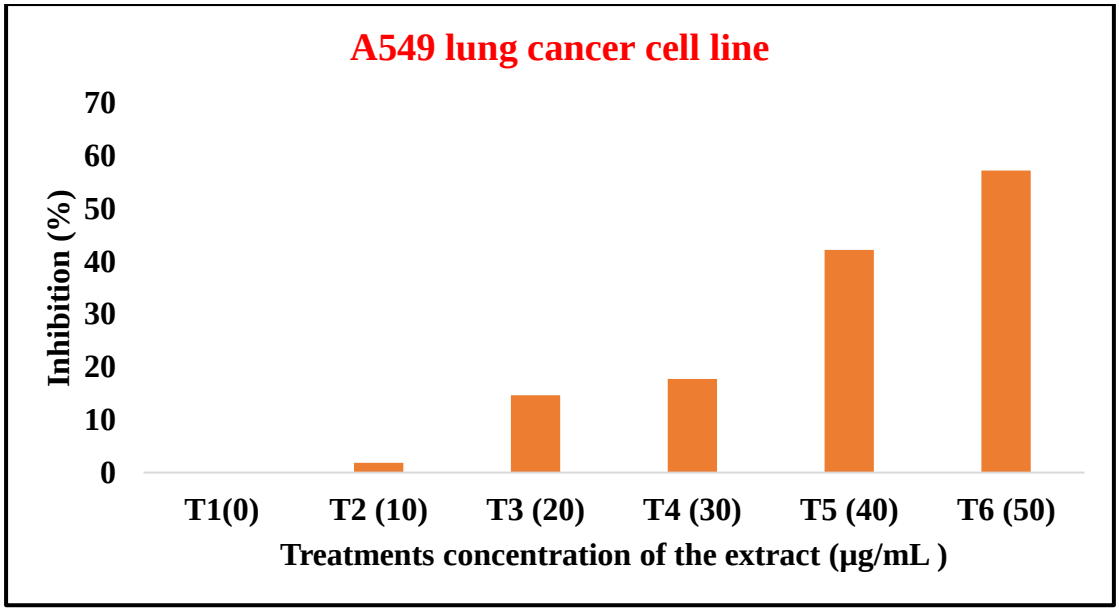

Figure 7: Number of non-viable cells of A549 lung cancer cell line treated with PQML

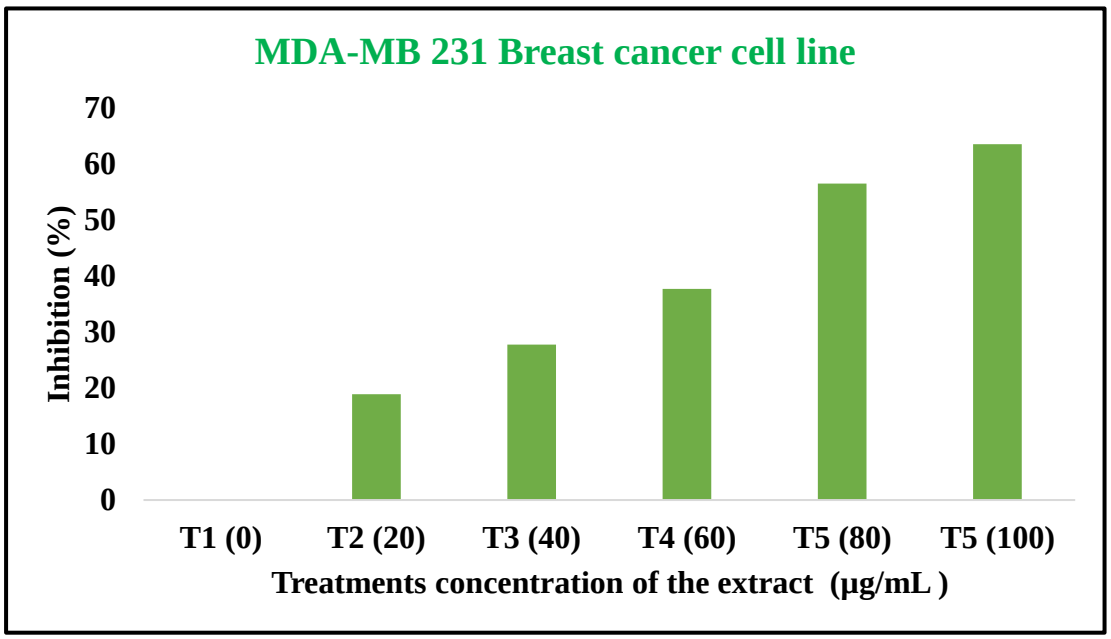

Figure 8: Number of non-viable cells of MDA-MB 231 breast cancer cell line treated with PQML

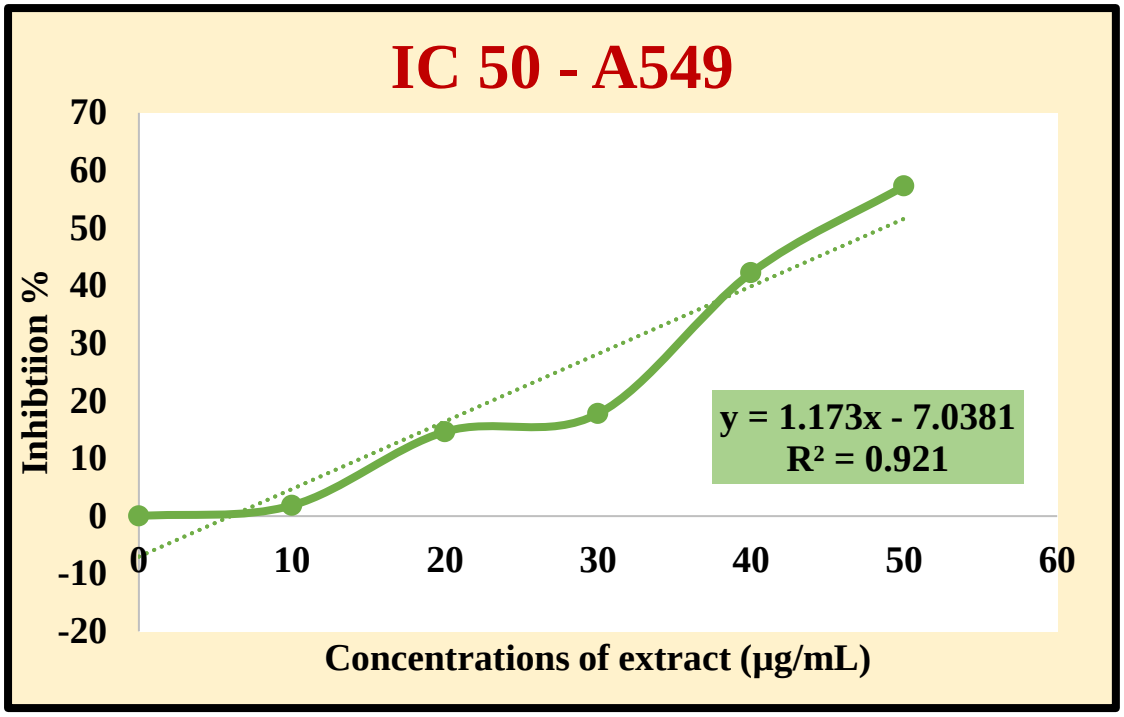

Figure 9: Percentage of inhibition of A549 lung cancer cell line treated with PQML

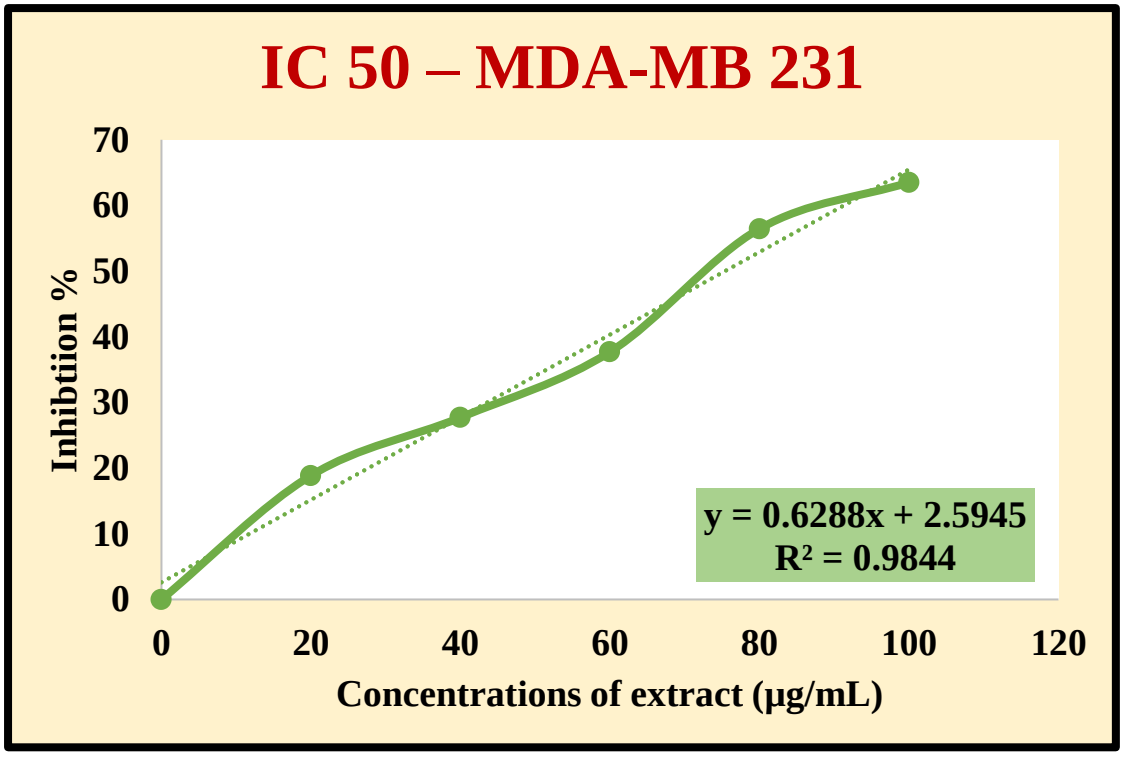

Figure 10: Percentage of inhibition of MDA-MB 231 breast cancer cell line treated with PQML

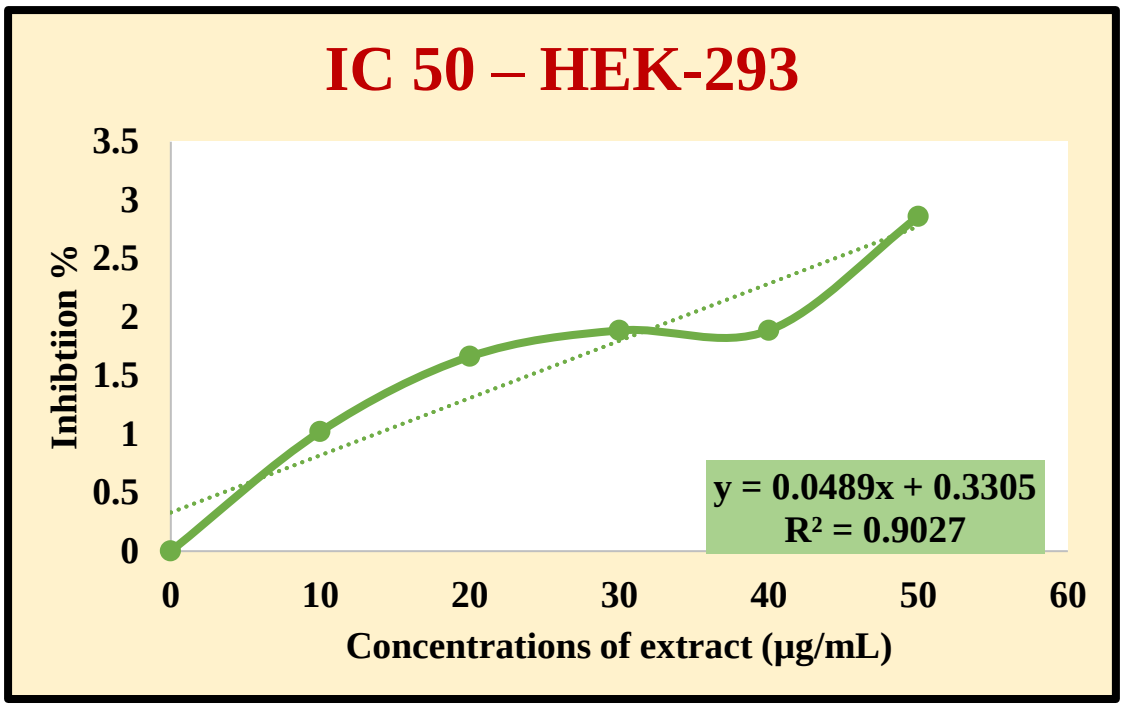

Figure 11: Percentage of inhibition of HEK-293 normal cell line treated PQML

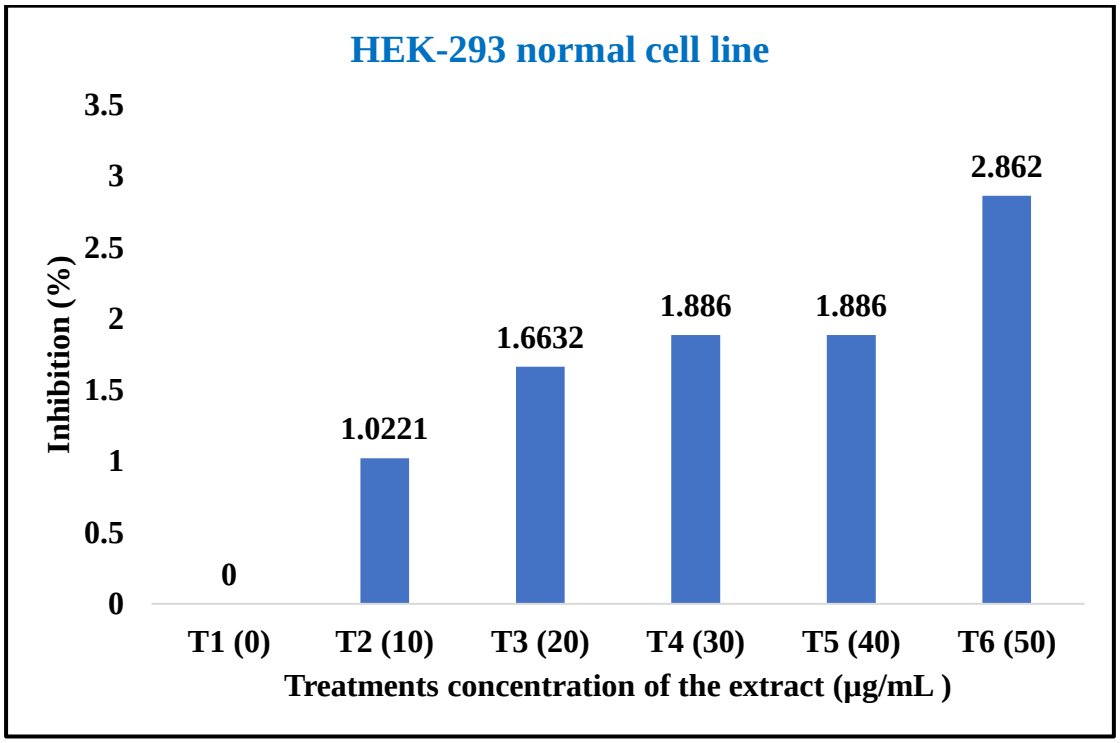

Figure 12: Number of non-viable cells of HEK-293 normal cell line treated PQML

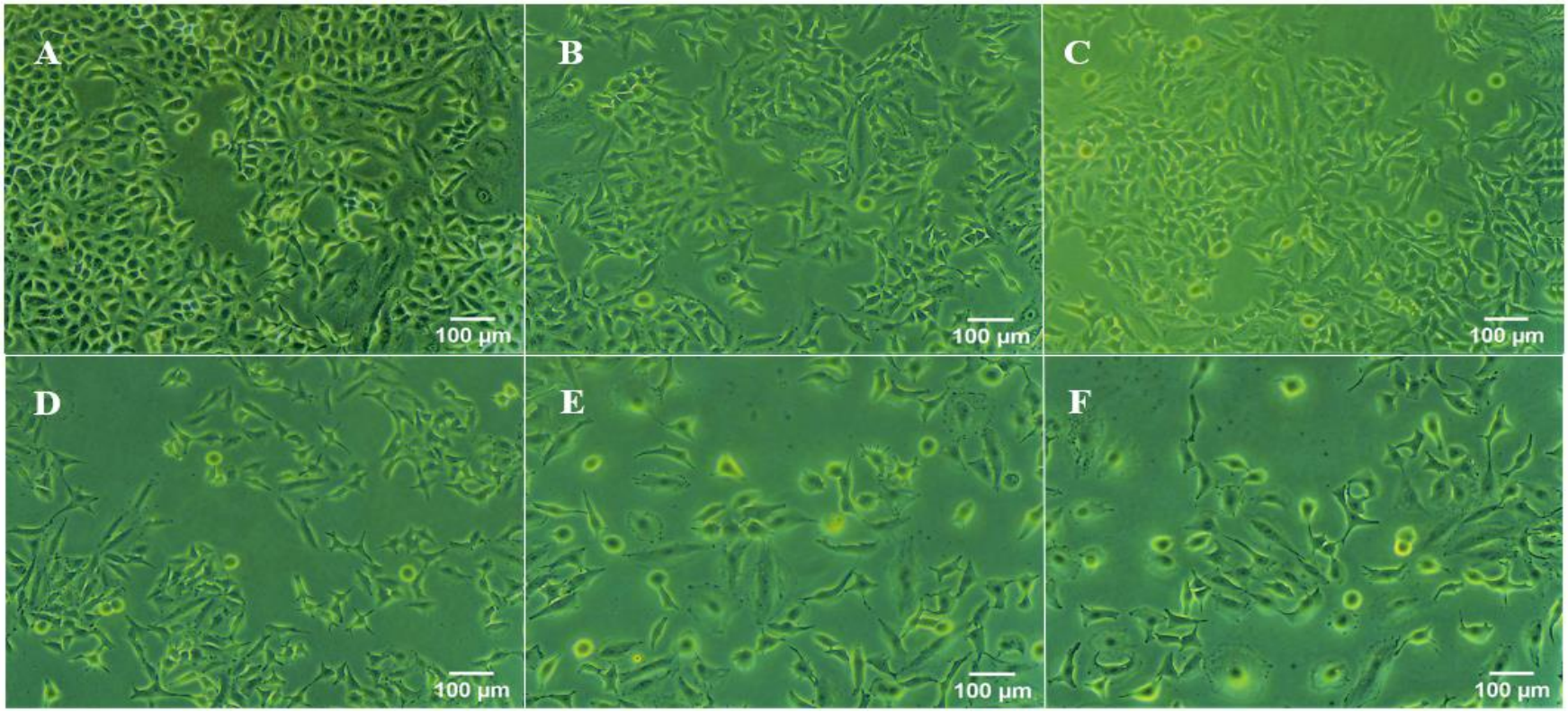

Plate 3: Morphological changes of A549 lung cancer cells treated with PQML. ***A****: Control A549 cells;* ***B****: A549 cells treated with vehicle control;* ***C****: A549 cells treated with* IC_{10} *of PQML;* ***D****: A549 cells treated with* IC_{25} *of PQML;* ***E****: A549 cells treated with* IC_{50} *of PQML;* ***F****: A549 cells treated with positive control*

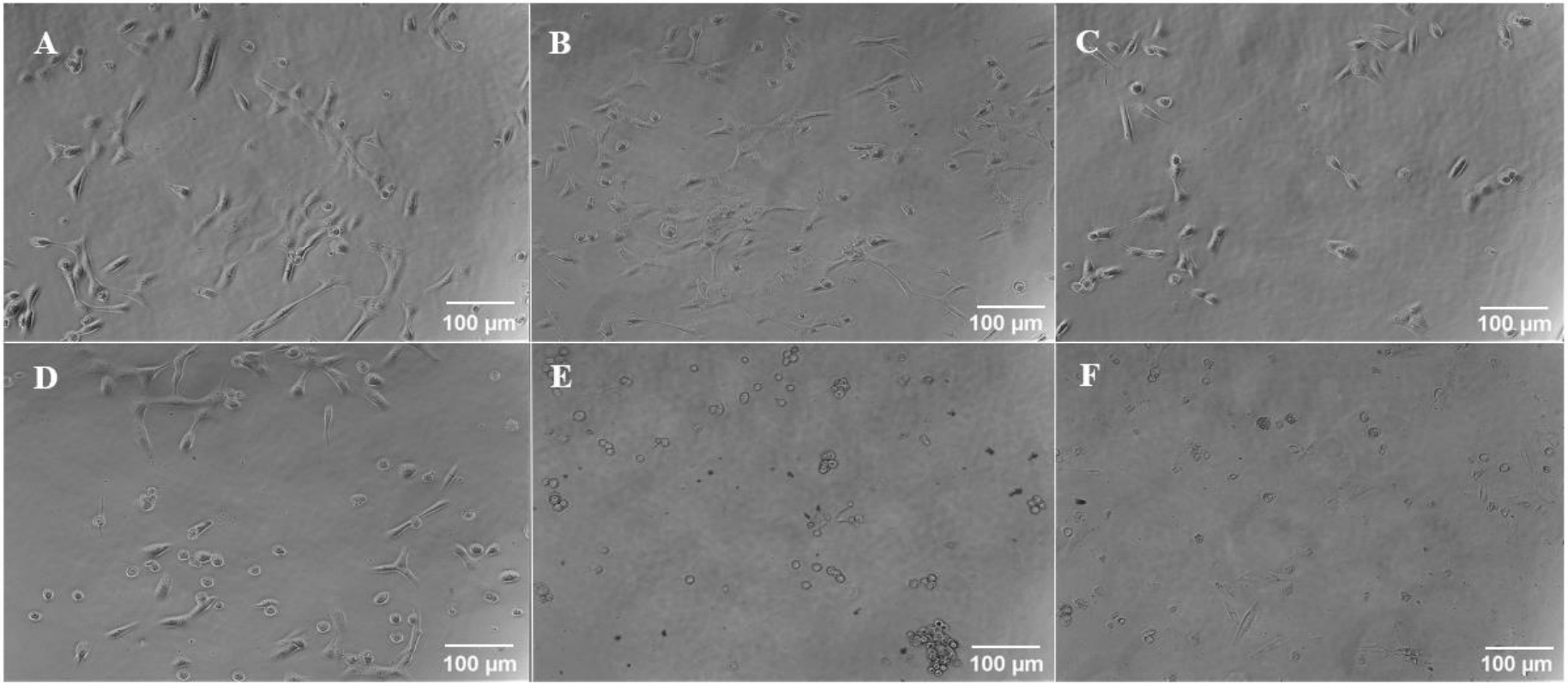

Plate 4: Morphological changes of MDA-MB 231 breast cancer cells treated with PQML*. **A**: Control MDA-MB 231 cells; **B**: MDA-MB 231 treated with vehicle control; **C**: MDA-MB 231 cells treated with IC_{10} of PQML;**D**: MDA-MB 231 cells treated with IC_{25} of PQML; **E**: MDA-MB 231 cells treated with IC_{50} of PQML ;**F**: MDA-MB 231 cells treated with positive control.*

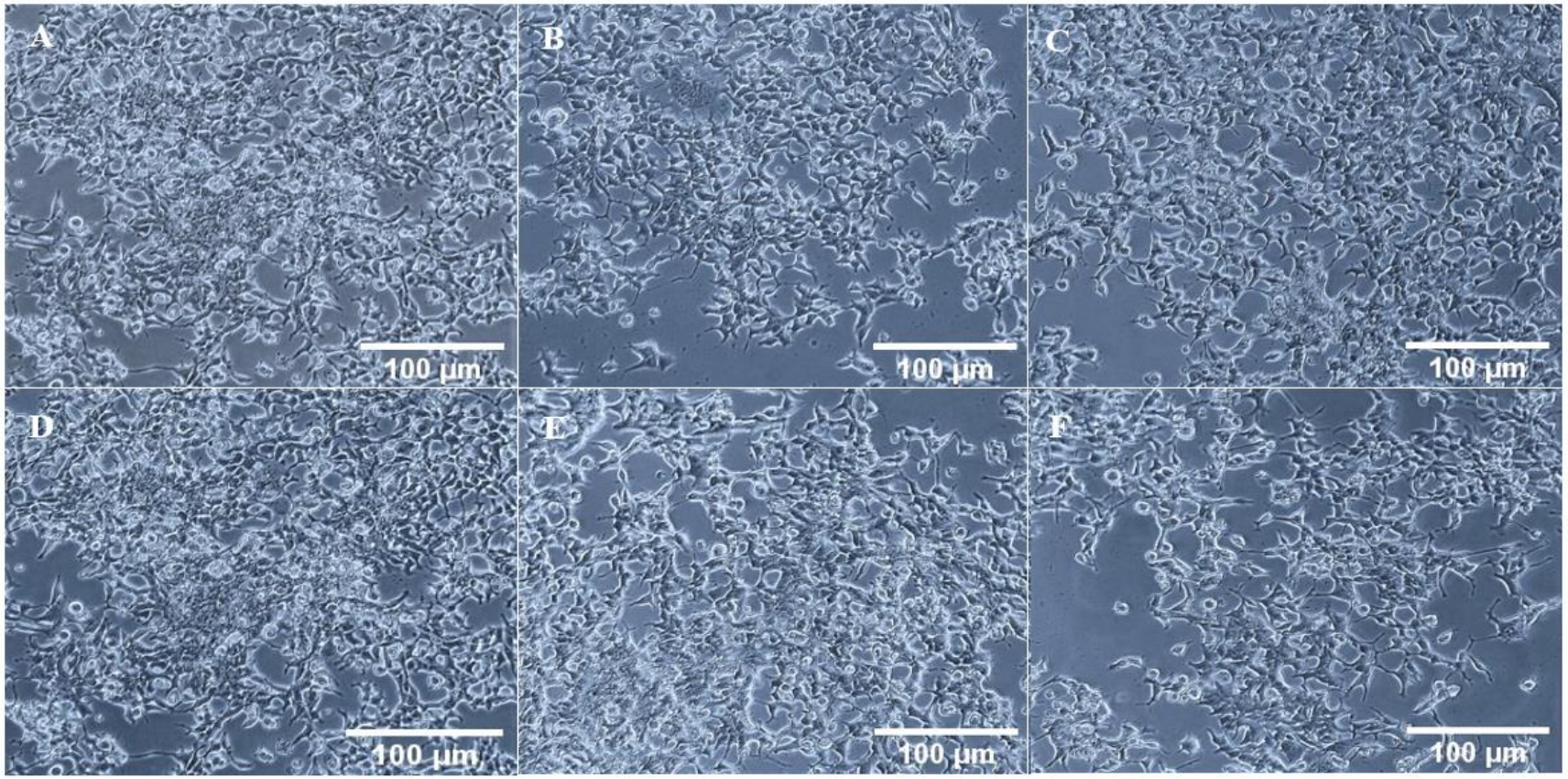

Plate 5: Morphological changes of HEK-293 normal cells treated with PQML A*: Control HEK-293 cells;* ***B****: HEK-293 treated with 10 µg/ml extract;* ***C****: HEK-293 cells treated with 20 µg/ml extract;* ***C****: HEK-293 cells treated with 30 µg/ml extract;* ***D****: HEK-293 cells treated with 40 µg/ml extract;* ***E****: HEK-293 cells treated with 50 µg/ml extract.*

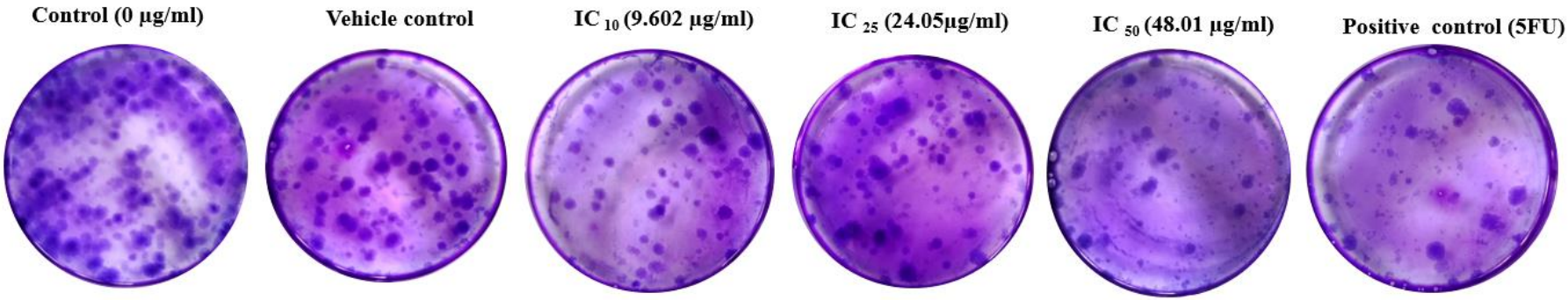

Plate 6: Clonogenic capacity of A549 cells following treatment with PQML

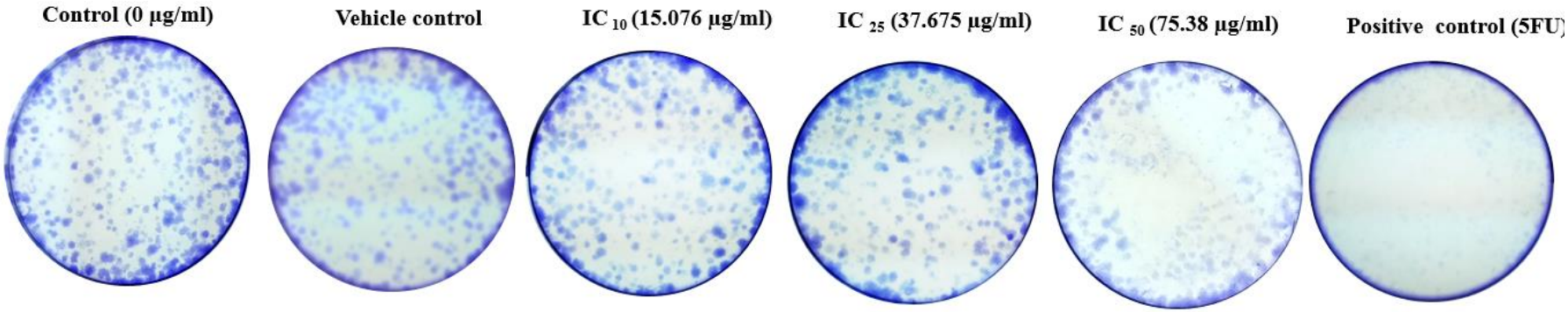

Plate 7: Clonogenic capacity of MDA-MB 231 cells following treatment with PQML

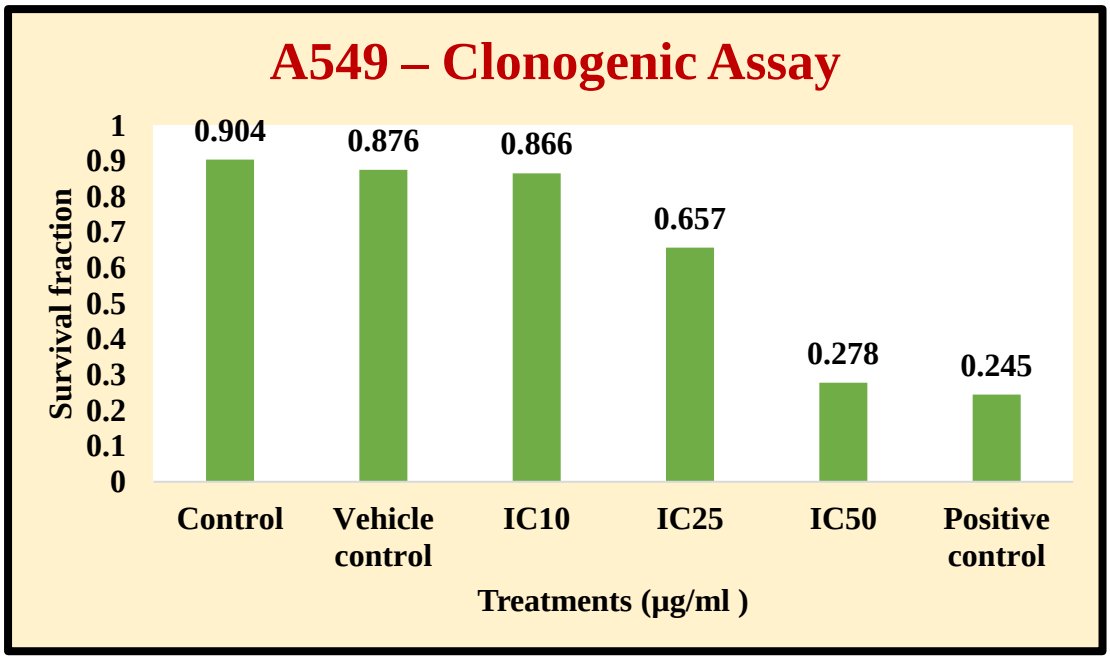

Figure 13: Graph showing significant percentage reduction in number of colonies in A549 compared to untreated and vehicle controls

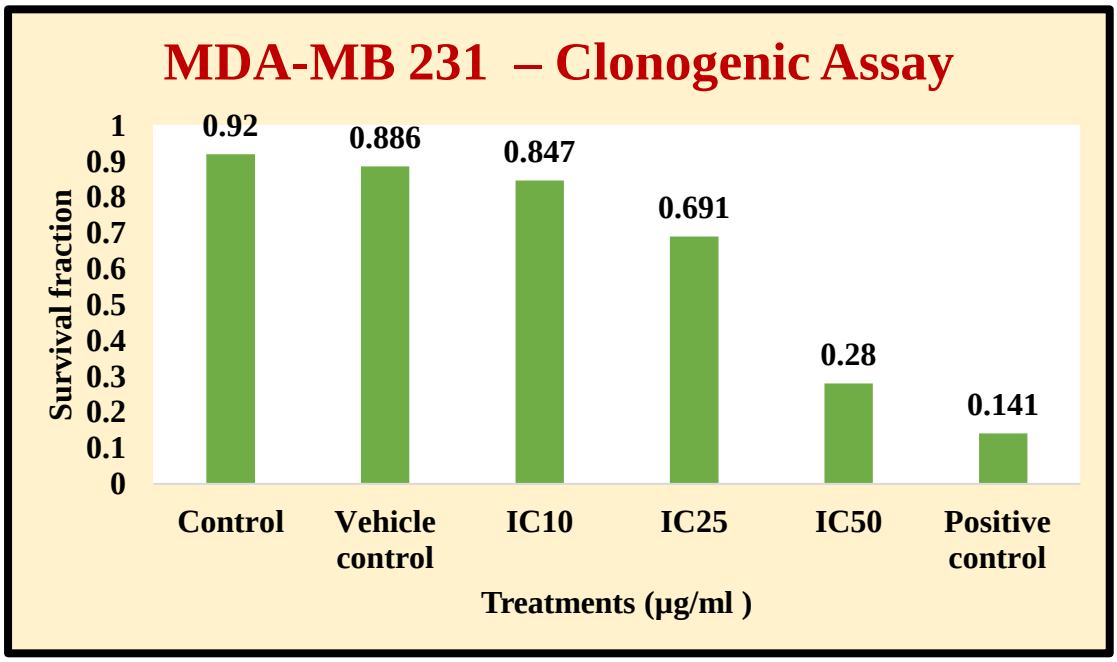

Figure 14: Graph showing significant percentage reduction in number of colonies in MDA-MB 231 compared to untreated and vehicle controls.

4.5 ESTIMATION OF APOPTOSIS

For evaluating the apoptotic death DNA fragmentation assay was carried out in A549 lung cancer cell line, MDA-MB 231 breast cancer cell line .

4.5.1. PQML induced apoptosis by DNA damage (DNA fragmentation assay)

DNA fragmentation/laddering, a biochemical sign of apoptosis, was analyzed on 1.8 percent agarose gels. after isolating DNA from the PQML-treated cells. The gel profile showed that the DNA from treated cells (both A549 and MDA-MB-231) showed shearing, which is an indicator of DNA damage; whereas untreated cells showed sharp bands. (Plate 8a and 8b).

4.5 DIFFERENTIAL EXPRESSION ANALYSIS OF APOPTOTIC GENES ON TREATMENT WITH PQML USING REAL-TIME PCR

4.5.1 RNA isolation

Good quality RNA was isolated from both control and treated cells of A549 and MDA-MB 231 cancer cells using trizol method. Samples were eluted out to a final volume of 20 µl and stored at -80 °C.

4.6.2 Agarose gel electrophoresis

The isolated RNA was seen as two intact bands (28 S and 18 S) without degradation in 1.8 % AGE (Plate 9a and 9b). No apparent genomic DNA contamination was noted.

4.6.3 Nanodrop analysis

Concentration as well as purity values of the isolated RNA was found out using Thermo Scientific Nanodrop 1000 spectrophotometer and which is represented in Table 13 and 14

Table No.13: Quality and quantity of the isolated RNA from A549 cell line

Sl. no.	**Sample**	**A_{260}/A_{280} value**	**Concentration (ng/µl)**
1	Control	2.08	93.8
2	Vehicle control	1.90	72.3
3	IC_{50}	1.88	83.4
4	Positive control	1.86	75.2

Table No. 14: Quality and quantity of the isolated RNA from MDA-MB 231 cell line

Sl. No.	**Sample**	**A_{260}/A_{280} value**	**Concentration (ng/µl)**
1	Control	1.97	313.4
2	Vehicle control	1.83	312.1
3	IC_{50}	1.87	177.1
4	Positive control	1.89	209.6

4.6.4 Primer sequence

The length, GC content, melting temperature, and self-complementarity of the primers obtained from different research articles were verified in OligoCalc: Oligonucleotide Properties Calculator software *(http://biotools.nubic.northwestern.edu/).*

A total of six sets of primers were used for studying differential expression of apoptotic genes. β-actin was used as the endogenous control.

4.6.4.1 Primer reconstitution and dilution

All the primer sets (six for qPCR studies) were reconstituted according to manufacturer's instructions and was stored at -20 °C. Working solutions of 10pM concentration were prepared from these stocks, for gene expression studies.

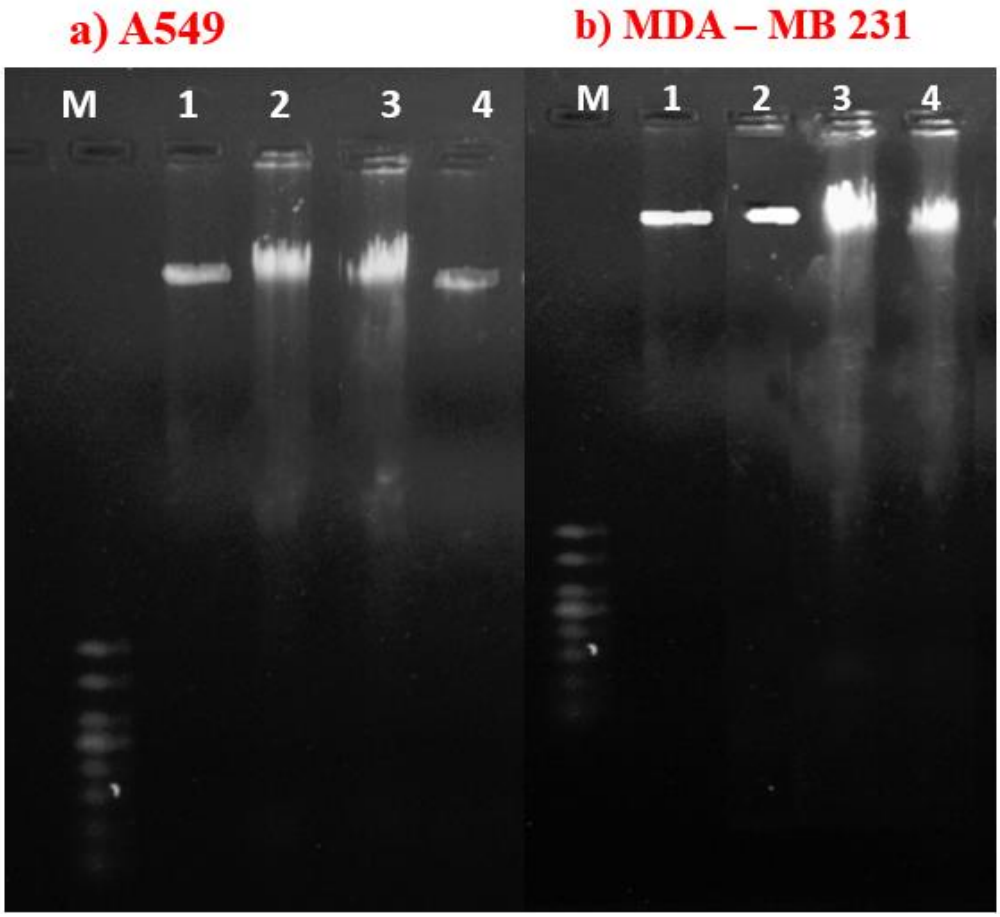

Plate 8a: Assessment of PQML induced DNA damage in A549 cancer cells; *Lane M: 100 bp DNA ladder; Lane 1: DNA from control cells (negative control); lane 2: DNA from positive control; lane 3: DNA from PQML treated cells (48.01(*IC_{50}*) µg/ml for A549); lane 4: DNA from vehicle control*

Plate 8b: Assessment of PQML induced DNA damage in MDA-MB 231 cancer cells*; Lane M: 100 bp DNA ladder; Lane 1: DNA from control cells (negative control); lane 2: DNA from vehicle control; lane 3: DNA from PQML treated cells (75.38 µg/ml (*IC_{50}*) for A549); lane 4: DNA from positive control*

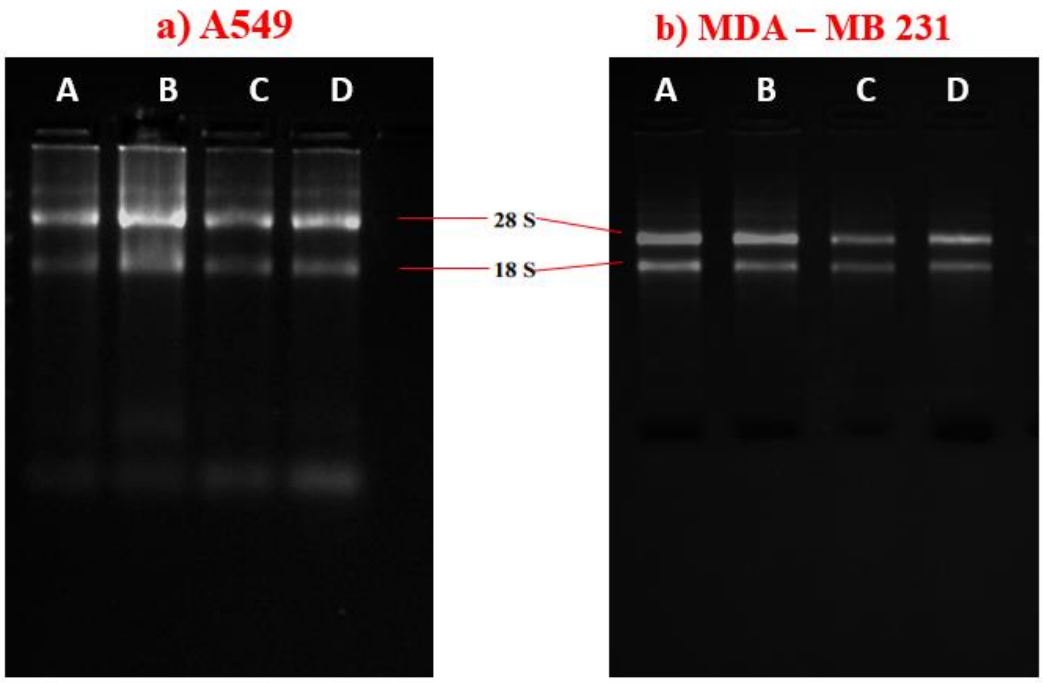

Plate 9a : Gel profile of RNA isolated from A549 lung cancer cell line *;Lane A: RNA from control cells; lane B: RNA from IC* $_{50}$ *treated cells; lane C: RNA from vehicle control treated cells; lane D: RNA from positive control treated cells.*

Plate 9b: Gel profile of RNA isolated from MDA-MB 231 breast cell line*; Lane A: RNA from control cells; lane B: RNA from IC* $_{50}$ *treated cells; lane C: RNA from vehicle control treated cells; lane D: RNA from positive control treated cells*

4.6.5 cDNA synthesis

The isolated RNA samples were subjected to first strand cDNA synthesis using oligo dT primers and random hexamers in the presence of Verso Enzyme Mix (Thermo Scientific). For each 20 µl cDNA reaction performed, 1 µg of RNA was used. The cDNA synthesized were diluted to a final volume of 50 µl in 1:5 ratio using nuclease free water.

4.6.6 Annealing temperature standardization

Standardization of annealing temperature was performed in CFX96 Real-Time PCR system (Bio-Rad) by setting a gradient at temperatures ranging from 55.0 °C to 63.0 °C. The best annealing temperature was then confirmed by melt curve analysis (plate 10) (Table 15).

Table No.15:List for primer pairs and their annealing temperature.used in RT – qPCR

Target gene	**Orientation**	**Primer sequence (5' – 3')**	**Ta**
Caspase 3	F R	TTAATAAAGGTATCCATGGAGAACACT TTAGTGATAAAAATAGAGTTCTTTTGTGAG	55.5˚C
Caspase 9	F R	TTCCCAGGTTTTGTTTCCTG CCTTTCACCGAAACAGCATT	60.1˚C
Bcl-2	F R	ATCGCCCTGTGGATGACTGAGT GCCAGGAGAAATCAAACAGAGGC	56.6˚C
BAX	F R	TCAGGATGCGTCCACCAAGAAG TGTGTCCACGGCGGCAATCATC	56.6˚C
PARP1	F R	CGGAGTCTTCGGATAAGCTCT TTTCCATCAAACATGGGCGAC	60.1˚C
β-actin	F R	GACCTCTATGCCAACACAGT AGTACTTGCGCTCAGGAGGA	60.1˚C

4.6.7 PQML induces change in apoptosis-related gene expression.

Expression analysis of *CASPASES, PARP, BCL2* and *BAX* were performed using *β-actin* as endogenous control. By using the resultant Ct values (Table 16) (figure 15 and 16), relative mRNA expression at different treatments were calculated using Comparative Ct method. In both A549 and MDA-MB cell lines, all the apoptotic genes studied, *BAX, CASPASE 9* and *CASPASE 3* were upregulated in PQML (IC_{50}) treated cells and positive control and downregulated in vehicle control with respect to the negative control. Where as anti-apoptotic genes *PARP* and *BCL2* were downregulated in PQML(IC_{50}) treated cells, positive control and negative control (Table 17). The *BAX/BCL2* ratio was found to be greater than 1 (Table 18) in both cell lines which confirms that active apoptosis is happening in cancer cells upon treatment with PQML.

In A549 cells, exposed to PQML (IC_{50}) showed a 1.665, 1.326 and 1.479 folds increase in C*ASPASE 9, CASPASE 3* and *BAX* expression relative to controls. Notably, PQML treatment was found to cause 0.049 and 0.0052 reduction in *PARP* and *BCL2* expression (figure 17).

Expression profiling data from MDA-MB cells treated with PQML, normalized to the endogenous control showed that *CASPASE 9, CASPASE 3* and *BAX* were upregulated by 1.316, 9.651, and 3.512 folds respectively. On the other hand, *PARP* and *BCL2* were downregulated by 0.574 and 0.398 folds (figure 18).

The ratio between major markers of apoptosis *BAX* and *BCL2* in A549 and MDA-MB cells after treated with PQML were 284.547 and 54.678 respectively. Since the BAX/BCL2 ratio is significantly greater than 1 in both cell lines (figure 19), it indicates that active apoptosis is occurring in PQML treated cells.

Table 16: Mean Ct values of apoptotic genes in A549 lung cancer cell line and MDA-MB 231 cell lines at different treatments

Cell lines	**Treatments**	**Ct average**				
		Apoptotic genes			Anti-apoptotic genes	
		CAS 9	**CAS 3**	**BAX**	**BCL2**	**PARP**
A549	Control	31.1	25.32	25.52	25.04	28.34
	Vehicle control	27.8	24.42	23.96	19.4	22.98
	Positive control	30.33	24.89	22.86	30.81	30.03
	IC_{50}	26.86	21.42	21.46	29.13	29.19
MDA-MB 231	Control	28.13	28.06	18.24	21.66	25.34
	Vehicle control	27.17	30.22	16.73	22.05	24.69
	Positive control	27.72	25.55	16.04	23.35	25.56
	IC_{50}	28.07	20.57	16.77	21.11	26.14

Table 17: Relative fold change in gene expression

Cell lines	**Treatments**	**Relative fold change**				
		Apoptotic genes			Anti-apoptotic genes	
		CAS 9	***CAS 3***	***BAX***	***BCL2***	***PARP***
A549	Control	1	1	1	1	1
	Vehicle control	0.033	0.134	0.0177	0.01	0.95
	Positive control	1.783	1.42	6.635	0.0192	0.324
	IC_{50}	1.665	1.326	1.479	0.0052	0.049
MDA-MB 231	Control	1	1	1	1	1
	Vehicle control	0.529	0.224	0.774	0.776	1.561
	Positive control	1.326	5.689	3.508	0.236	0.653
	IC_{50}	1.316	9.651	3.512	0.398	0.574

Table 18: BAX: BCL2 ratio A549 lung cancer line and MDA-MB 231 breast cancer cell lines

Cell lines	**Treatments**	**BAX/BCL2 Ratio**		
		BAX	***BCL2***	***BAX/BCL2***
A549	Control	1	1	1
	Ethanol control	0.0177	0.01	0.05914
	Positive control	6.635	0.0192	344.3128
	IC_{50}	1.479	0.0052	284.547
MDA-MB 231	Control	1	1	1
	Ethanol control	0.774	0.776	0.27
	Positive control	3.508	0.236	74.66
	IC_{50}	3.512	0.398	54.678

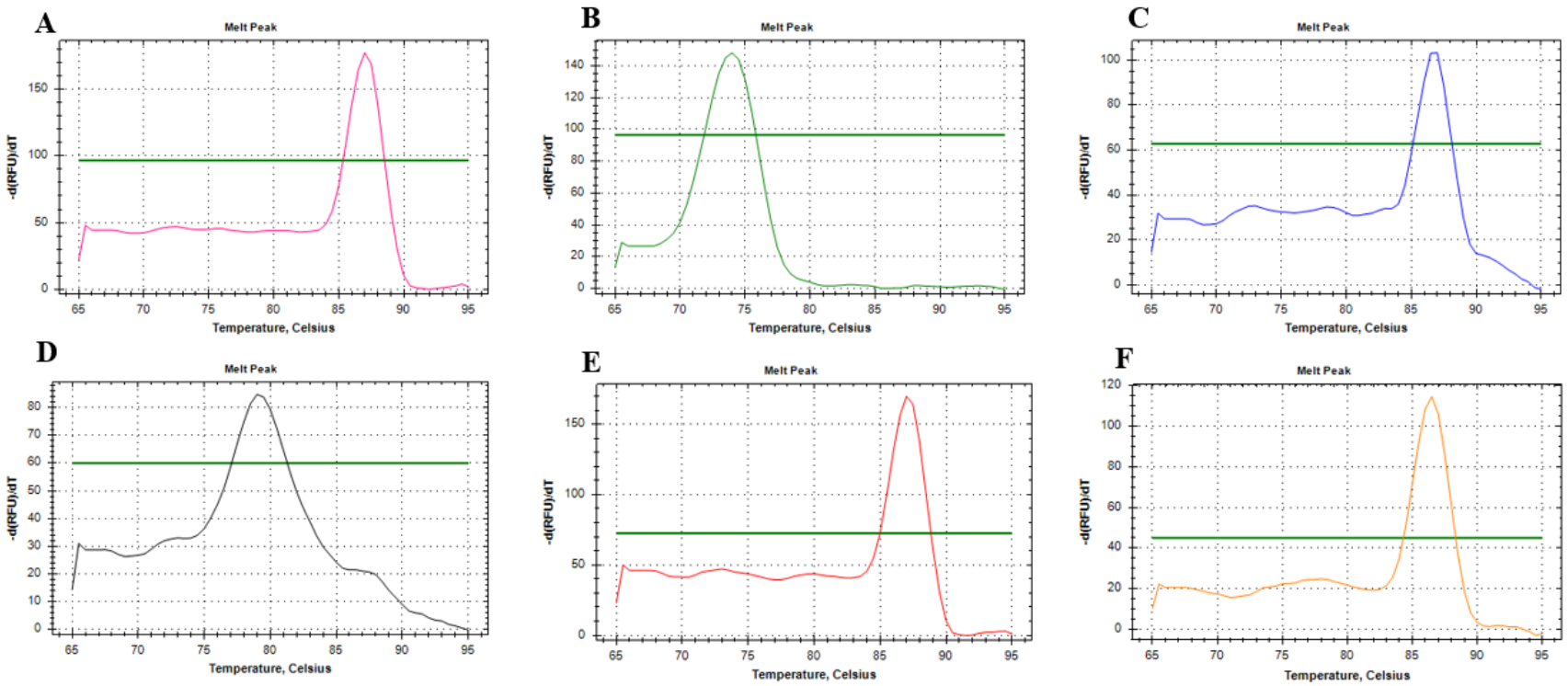

Plate 10: Melt curve analysis : *(A) β-actin at 60.1˚C; (B) Caspase 3 at 55.5˚C; (C) Caspase 9 at 60.1˚C; (D) PARP at 60.1˚C;(E) Bcl2 at 56.6˚C; (F) BAX at 56.6˚C*

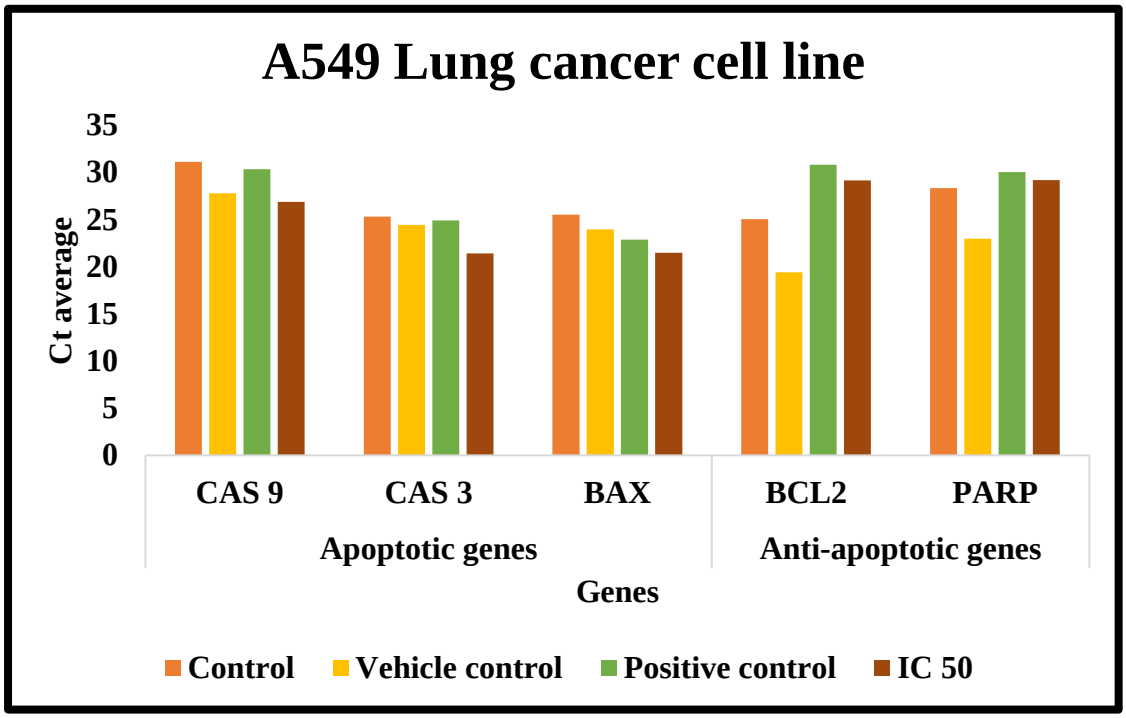

Fig.15: Mean of Ct values of apoptotic genes in A549 lung cancer cell line at different treatments

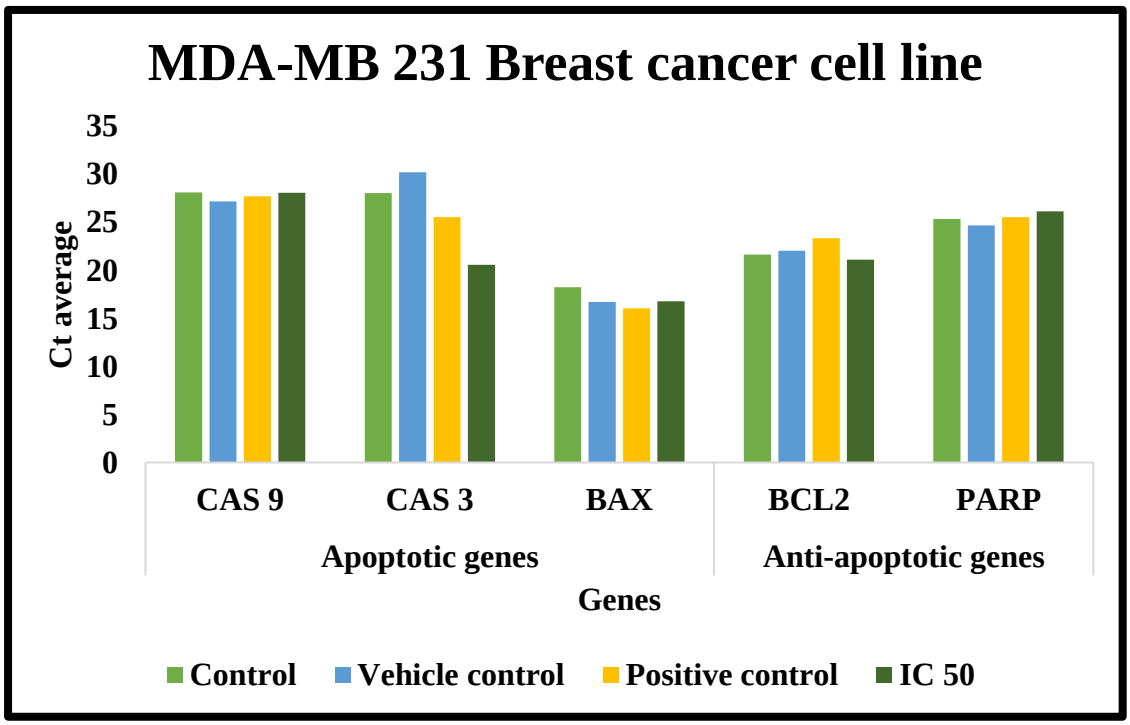

Fig.16: Mean of Ct values of apoptotic genes in MDA-MB 231 breast cancer cell line at different treatments

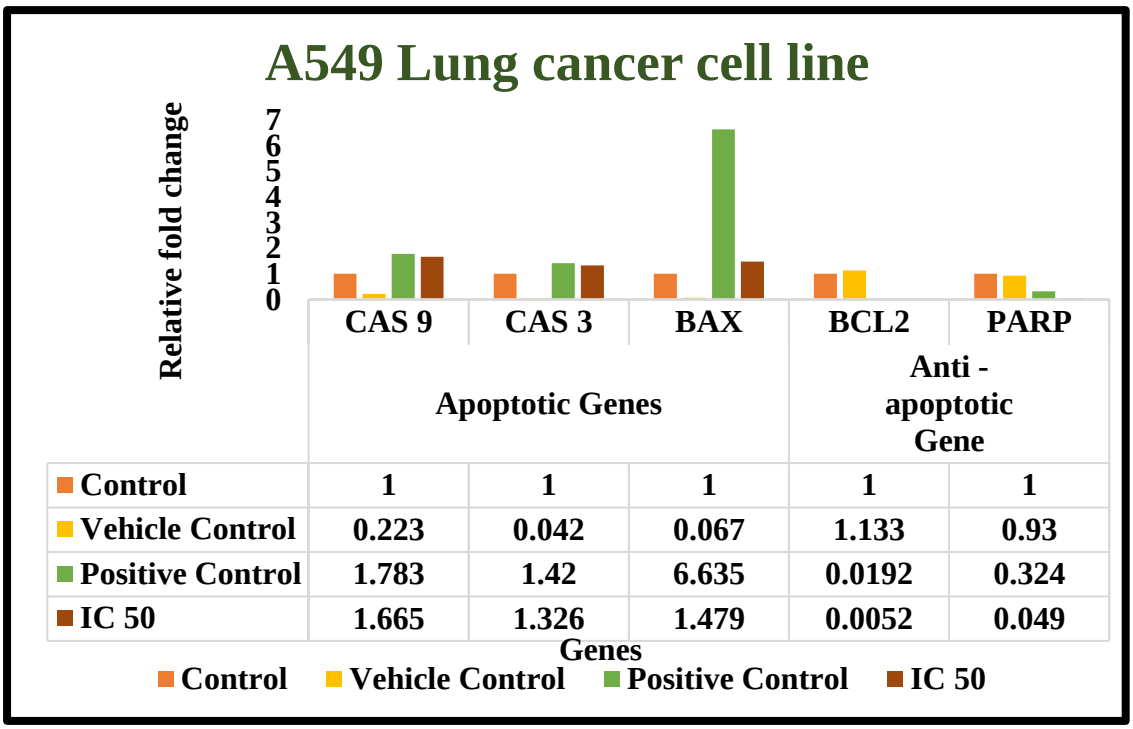

Fig.17: Differential expression of apoptotic genes at different treatments of A549 lung cancer cell line.

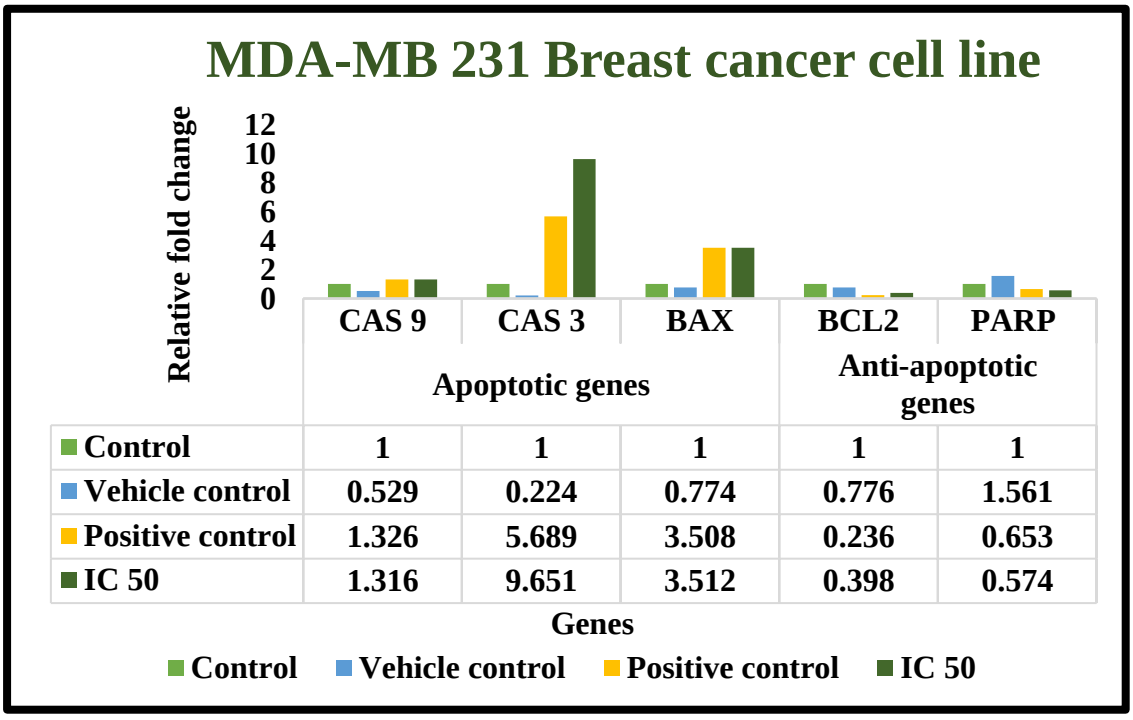

Fig.18: Differential expression of apoptotic genes at different treatments of MDA- MB 231 breast cancer cell line

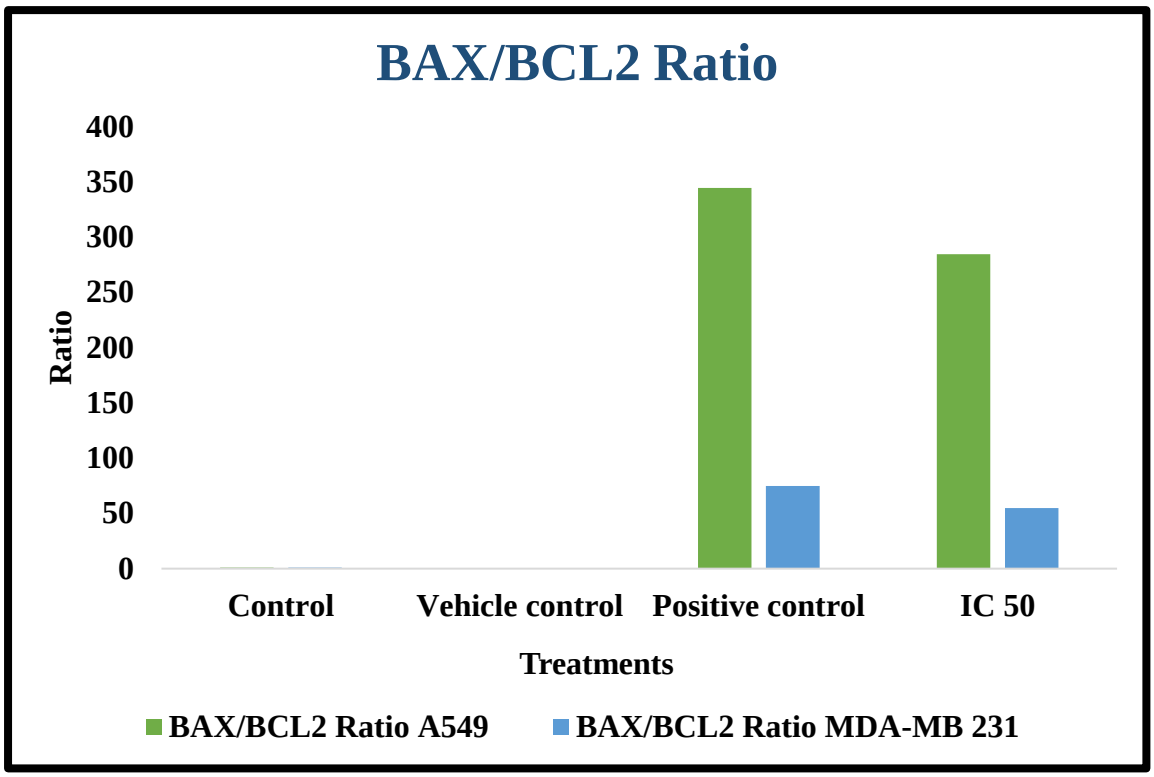

Fig.19: BAX /BCL2 ratio of both A549 lung cancer cell line and MDA MB 231 breast cancer cell line

DISCUSSION

5. DISCUSSION

The quest for novel anticancer drugs without adverse side effects has been there since time immemorial. Most of the drugs used currently for cancer treatment have been derived from plants (Newman and Cragg, 2016). The plant-derived anticancer agents have the upper hand over the current cancer treatment modalities like radiation therapy, chemotherapy and surgery due to their diminished toxicity toward normal cells.

Pogostemon quadrifolius (Benth.) is a shrub belonging to the family *Lamiaceae* and has various medicinal properties. The ethnomedically important mint family (*Lamiaceae*) is underexplored in terms of anticancer therapy. Therefore, the current objective of the study was to evaluate the cytotoxic effect of *P. quadrifolius* (Benth.) leaf extract on cancer cells and to elucidate its action on apoptotic pathway genes. There are preliminary reports on the antiproliferative effect of *P. quadrifolius* (Benth.) leaf extract in chronic myelogenous leukemia and prostate cancer cells *(Cheriyamundath et al*., 2018). However, the molecular mechanism of its cytotoxicity is not yet understood. This is the first study to demonstrate preliminary information on the molecular mechanism of the cytotoxic action of *P. quadrifolius* (Benth.).

The leaf samples collected from the Aromatic and Medicinal Research Station, Odakalli were dried, powdered and ground for extraction. Specific extraction methods and extraction solvent needs to be employed to recover the anticancer compounds from plants with variable chemical properties and polarity. Active compounds are commonly retrieved from plant matrices using polar solvents (Altemimi *et al*., 2017). Aqueous solutions comprising ethanol, methanol, chloroform, acetone, and ethyl acetate are the best solvents for extraction. In the present study methanol was used as the solvent. Primarily it is used to extract various polar compounds, but a specific group of non-polar compounds are relatively soluble in methanol if not readily soluble. Also, methanol can often extract lower molecular weight molecules more effectively. 53.2mg/g of crude extract was obtained from *P. quadrifolius* (Benth.) by methanolic extraction, and the final 10mg/ml stock solution was prepared in ethanol for *in vitro* studies.

The most popular approach for high throughput screening of a compound's antiproliferative effect on cultured cells is a metabolic viability-based test utilizing tetrazolium salts like MTT (3-(4,5-Dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide) (van Tonder *et al.,* 2015). While studying the cytotoxic effect of PQML on lung and breast cancer cell lines, the MTT assay showed the growth inhibition potential of the extract. Treated cells demonstrated a reduction in the amount of formazan generated per cell which indicates that PQML reduces the metabolic viability of cells. The extract showed a dose-dependent reduction in cell survival percentage, and IC_{50} values were found to be 48.01 µg/mL and 75.38 µg/mL for lung and breast cancer, respectively. The IC_{50} values indicates the effectiveness of the drug (Aykul and Martinez-Hackert, 2016). A lower IC_{50} score implies the drug is safe and potent at low concentration and will cause minimal cytotoxic effects when administered to the patient (Berrouet *et al*., 2020). At the same time, it was non-toxic to HEK-293 normal human embryonic kidney cells. This result is in accordance with the findings of Cheriyamundath *et al.* (2015), who reported that crude leaf extracts of *P. quadrifolius* were shown to elicit antiproliferative effects on chronic myelogenous leukemia cancer cells *in vitro* with an IC_{50} of 77.7±0.21 µg mL^{-1}. Results of the current study suggest that PQML is more effective in lung cancer cell lines. 5 Fluro uracil (5FU), a potent chemotherapy drug was used as negative control in MTT assay. The IC_{50} value of 5FU is about 8.625 µM (3.45 µg/mL) in lung cancer cell lines (Țigu *et al.*, 2020). Compared to the 5FU the cytotoxic effect of PQML on lung cancer cell line is low. A crude extract with an IC_{50} value less than 50 µg/mL is comparable with current ethnopharmacological studies (Minh *et al*., 2019;Nordin *et al.*, 2018), which indicate that PQML is a potential source of bioactive compounds.

The effect of PQML on reproductive death of A549 and MDA MB was identified through clonogenic assay. The colony forming ability of the PQML treated cells was identified by calculating both plating efficiency (PE) and survival fraction (SF). Variable plating efficiencies exist between cell lines. PE measures the ratio of colonies developed to the cells seeded (Rafehi *et al*., 2011). The SF is the number of colonies that form after cell treatment, represented in terms of PE (Franken *et al.*, 2006). PQML hindered the ability of cancer cell lines to form colonies in a concentration-

dependent manner, which was proof of the extract's potential to reduce cell survival and proliferation. Compared to the control, lung and breast cancer cell lines showed a significant drop in the number of colonies of approximately 62 and 64 per cent, respectively. 5FU , a potent chemotherapy drug for breast cancer (Cameron *et al.*, 1994) showed a 77.9% clonogenic capacity in breast cancer cell line which was comparatively higher than PQML. The SF and the inhibition of colony formation was comparable for both the 5-FU and PQML in lung cancer cell line. However this drug is not used for treatment of lung cancer in clinical practice.

Apoptosis is one among the major pathways that induces cell death by means of cancer therapeutics. A549 lung cancer cells appeared as a monolayer of cells with epithelial morphology and polygonal shaped cells. After treating with PQML(IC50) severe membrane blebbing, cell shrinkage and formation of apoptotic vesicles were visible in A549 cell lines under phase contrast microscope. During apoptosis the plasma membrane will first blebs (form circular bulges), and a transient stage arises that will quickly progress to bleb separation and the formation of apoptotic bodies (Zhang *et al.*, 2018). The morphological changes observed in A549 cell in this study indicated induction of apoptosis after PQML treatment. MDA-MB 231 cells appeared as spindle shaped cells with tapered ends. On treatment with PQML, these cells showed nuclear shrinkage and condensation which are the hallmarks of apoptosis (He *et al.*, 2009).

DNA damage, a biochemical marker of apoptosis was analysed through DNA fragmentation assay. The biological sign of apoptosis is the cleavage of chromosomal DNA into the fragment of oligonucleosomal size (Elmore, 2007). Cancer cells treated with PQML at doses of 48.01 and 75.38 g/ml showed DNA shear and degradation, but there was no laddering of DNA into fragments. The earlier findings by (Meggyeshazi *et al.*, 2014)indicates that apoptosis without DNA fragmentation is also possible and hence this assay cannot be always considered as an indicator of apoptosis.

Results of *in vitro* assays indicate that PQML extract can cause cytotoxicity and apoptosis. To have a better understanding of the effect of PQML on apoptosis molecular analysis was carried out to study the expression of some of the key genes in apoptotic pathway. namely *CASPASE 3, CASPASE 9, PARP, BCL2*.The expression

pattern of selected apoptotic genes were investigated using β-actin as endogenous control and the expression of apoptotic genes in treated samples with respect to control, vehicle control after 48 hour time period were analyzed using Comparative ΔΔCq method (Livak and Schmittgen, 2001)

Transcription profiling by RT-qPCR provided additional molecular evidence confirming apoptosis induction by upregulation of *CASPASES 3, 9*, pro-apoptotic *BAX* and downregulation of anti-apoptotic *BCL-2* and *PARP*. *BAX/BCL2* ratio is a key indicator of apoptosis because *BAX* has a direct role in the induction of apoptosis and the loss of function of *BAX* leads to *in vitro* tumour development (Pawlowski and Kraft, 2000). However, *BCL2* is considered to be one of the significant apoptosis suppressor genes and its overexpression will inhibit apoptosis (Czabotar *et al*., 2014). The *BAX/BCL2* ratio should be greater than 1 to induce apoptosis (Azimian *et al*., 2018; Khodapasand *et al.*, 2015). In accordance with the findings in the current study the *BAX/BCL2* ratio was greater than one in both breast and lung cancer cell lines, confirming that active apoptosis occurs in cancer cells after PQML treatment.

In the intrinsic pathway of apoptosis, cellular stress results in programmed cell death by the downregulation of *BCL2* and further activation of *BAX, CASPASE 9* and *CASPASE 3* (Fulda and Debatin, 2006). *PARP* has an indirect role in the apoptosis pathway. DNA breaks formed during the cellular stress lead to the activation of *PARP*, which may attempt cell repair by adding poly (ADP ribose) (Chaitanya *et al.*, 2010). During programmed cell death, the PARP cleaves and gets inactivated; therefore, no DNA repair occurs (Herceg and Wang, 1999). PQML treated A549 cells showed upregulation of *CASPASE 9, CASPASE 3,* and *BAX* expression by 1.665, 1.326, and 1.479 fold compared to untreated cells. PQML treatment also reduced *PARP* and *BCL2* expression by 0.049 and 0.0052 fold in A549 cells. Expression profiling data from MDA-MB cells treated with PQML revealed that *CASPASE 9, CASPASE 3,* and *BAX* were upregulated by 1.316, 9.651, and 3.512 fold respectively. At the same time, *PARP* and *BCL2* were downregulated by 0.574 and 0.398 folds. Our results suggests that PQML treated MD-MB-231 and A549 cells undergo apoptosis and there is upregaulation of genes involved in intrinsic pathway of apoptosis.

Preliminary gene expression analysis of PQML in A549 and MDA-MB cancer cells is given in figure 20.

On the basis of our results, it can be speculated PQML has potent cytotoxic effect against lung and breast cancer and can be used in the development of cancer chemotherapeutics. PQML may contain a plethora of bioactive compounds and it can potentially target multiple cellular pathways. In the present study, only a limited number of gene expression patterns were conducted to understand the molecular mechanism. Preliminary results suggest a possibility towards intrinsic pathway of apoptosis. However further research is essential to understand the molecular mechanism of cytotoxic action of *P. quadrifolius.*

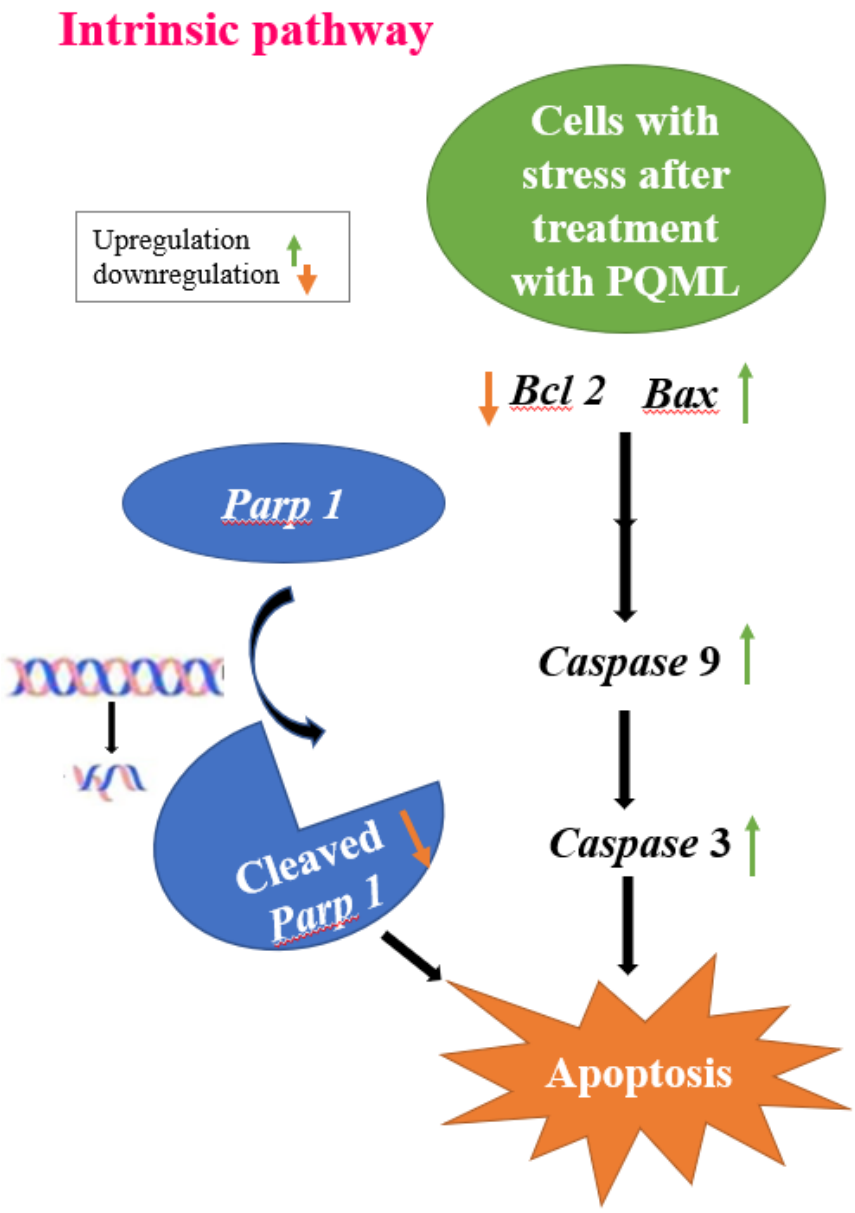

Fig. 20: Preliminary gene expression analysis of PQML in A549 and MDA-MB cancer cells

SUMMARY

6. SUMMARY

The study entitled "Molecular mechanism of the cytotoxic activity of *Pogostemon quadrifolius* (Benth.)" was undertaken in the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2021 - 2022.

The use of traditional or ethnomedicinal plants continue to contribute towards cancer cure due to their diminished toxicity toward normal cells. Lamiaceae plants are well known for their use in ethnomedicine, but it is underexplored in terms of anticancer therapy. *P. quadrifolius* (Benth.) is a medicinal plant belonging to the family of Lamiaceae. There are only very limited studies conducted on the apoptotic and antiproliferative nature of *P. quadrifolius*. The current study is the first report on the cytotoxic effect of *P. quadrifolius* extract on lung and breast cancer cell lines and its molecular mechanism of cytotoxicity.

In the present study preliminary in vitro assays were carried out followed by gene expression studies to understand the cytotoxic potential of *Pogostemon quadrifolius* (Benth.) leaf extract. The leaf samples were collected from AMPRS, Odakkali dried, powdered and extracted in methanol. A549 lung cancer cell line and MD-MB-231 breast cancer cell lines were used in the current study as they are the most prevalent forms of cancer in men and women respectively. MTT assay was carried out to determine the cytotoxic action of PQML on the cancer cell lines and it was found that the extract can render cytotoxicity to both lung and breast cancer cell line. IC_{50} values were found to be 48.01 µg/mL and 75.38 µg/mL for lung and breast cancer, respectively. A lower IC_{50} value in lung cancer cell line indicates that the extract shows higher cytotoxicity in lung cancer cell line when compared to breast cancer cell line. Interestingly, it did not show any signs of cytotoxicity in HEK293 cell line, specifying its safety profile to be used as a cancer therapeutic.

The colony-forming ability of A549 and MDA-MB cells after treatment with PQML was determined by clonogenic assay. After calculating plating efficiency and survival fraction, the reduction of colonies in A549 and MDA-MB cells after treatment with PQML was calculated by comparing it with the control which implicated that

PQML inhibited 62% and 64% colony-forming ability of lung and breast cancer cells. Hence it is evident from the results of MTT and clonogenic assay that PQML is a potent cytotoxic agent.

Based on the results of *in vitro* assays and gene expression analysis, we can conclude that PQML is a potent cytotoxic agent against lung and breast cancer cell line and the possible mechanism of its action is by apoptosis. An upregulation of apoptotic genes (*CASPASE 9, CASPASE 3* and *BAX*) and downregulation of anti-apoptotic (*PARP* and *BCL2)* genes clearly indicates the apoptotic action is modulated by differential expression of genes. However the results are not sufficient to elucidate the exact pathway of apoptosis, advanced research is needed in this area for better understanding of the cytotoxic potential of *P. quadrifolius*.

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7. REFERENCES

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APPENDICES

8. APPENDICES

APPENDIX I

Reagents required for maintenance of normal and cancer 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
   - Na_2HPO_4 1.44g/L
   - KH_2PO_4 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 *P. quadrifolius*, 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 required for DNA fragmentation assay

1. ***DNA extraction buffer***

- PBS EDTA
- proteinase K 10 mg /ml
- SDS 10%
- NaCl 5M

2. ***Phenol: chloroform: isoamyl alcohol*** – 25:24:1
3. ***70% ethanol***

- 100% ethanol 70 mL
- Distilled water 30 mL

APPENDIX V

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

ABSTRACT

9. ABSTRACT

The study entitled "Molecular mechanism of the cytotoxic activity of *Pogostemon quadrifolius* (Benth.)" was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2021 - 2022. The objective of this study was to evaluate the cytotoxic effect of *P. quadrifolius* (Benth.) leaf extract on cancer cells and to elucidate its action on genes of apoptotic pathway. *P. quadrifolius* (Benth.) is a shrub belonging to the family *Lamiaceae* and there are preliminary reports on antiproliferative effect of *P. quadrifolius* (Benth.) leaf extract in chronic myelogenous leukemia cancer cells and prostate cancer cells. However, the molecular mechanism of its cytotoxicity is not yet understood.

P. quadrifolius (Benth.) leaves were dried and then extracted with methanol, 53.2 mg/g of crude extract was obtained from powdered leaf sample. Cytotoxic effect of the leaf extract was evaluated by MTT assay in human lung (A549), breast cancer cell line (MDA-MB -231) and normal cell line HEK 293. The extract showed dose dependent reduction in cell survival percentage and IC_{50} values were found to be 48.01 µg/ml and 75.38 µg/ml for lung and breast cancer respectively, while it was non-toxic towards normal cells in the above dosage. The leaf extract also inhibited the colony-forming ability of cancer cells in the clonogenic assay which was indicative of the extract's ability to affect cell survival and proliferation. DNA isolated from the methanolic leaf extract treated cells when subjected to DNA fragmentation assay was seen sheared which is an indication of apoptosis in those cells. Expression profiling of the key apoptotic genes *BAX, CASPASE 3, CASPASE 9, PARP and BCL2* showed upregulation of *BAX, CASPASE 9* and *CASPASE 3* in the treated cells. The anti-apoptotic genes *BCL2* and *PARP1* were downregulated and also the *BAX/ BCL2* ratio was found to be greater than 1 which confirms that active apoptosis is happening in cancer cells upon treatment with leaf extract.

Cytotoxic effect of methanolic leaf extract of *P. quadrifolius* (Benth.) on lung and breast cancer cells were elucidated and the gene expression analysis revealed that the mechanism of cytotoxicity is via apoptosis. The findings suggest that the leaf extract of *P. quadrifolius* (Benth.) can be a potent candidate for development of cancer chemotherapeutics after further advanced research.

സംഗ്രഹം

"*പോഗോസ്റ്റിമോൻ കാഡ്രിഫോളിയസ്* (ബെന്ത്.) ന്റെ സൈറ്റോടോക്സിക് പ്രവർത്തനത്തിന്റെ മോളിക്യുലർ മെക്കാനിസം" എന്ന തലക്കെട്ടിലുള്ള പഠനം 2021 - 2022 കാലയളവിൽ തിരുവനന്തപുരം വെള്ളായണി കാർഷിക കോളേജിലെ സസ്യ ജൈവസാങ്കേതികവിദ്യാവിഭാഗത്തിൽ നടത്തപ്പെട്ടു. *പി. കാഡ്രിഫോളിയസ്* (ബെന്ത്.) ഇല സത്ത് കാൻസർ കോശങ്ങളിൽ പ്രകടമാകുന്ന സൈറ്റോടോക്സിസിറ്റി വിലയിരുത്തുകയും അതിനോടൊപ്പം അപ്പോപ്ടോട്ടിക് പാതയിലെ ജീനുകളുടെ പ്രവർത്തനം വ്യക്തമാക്കുക എന്നതായിരുന്നു പഠനത്തിന്റെ ലക്ഷ്യം. *പി. കാഡ്രിഫോളിയസ്* (ബെന്ത്.) ലാമിയാസിയ യിൽപെട്ട ഒരു കുറ്റിച്ചെടിയാണ്, കൂടാതെ *പി. കാഡ്രിഫോളിയസ്* (ബെന്ത്.) ഇല സത്തിൽ ക്രോണിക് മൈലോജെനസ് ലുക്കീമിയ കാൻസർ കോശങ്ങളിലും പ്രോസ്റ്റേറ്റ് കാൻസർ കോശങ്ങളിലും ആന്റിപ്രൊലിഫെറേറ്റീവ് ഫലത്തെക്കുറിച്ച് പ്രാഥമിക റിപ്പോർട്ടുകൾ ഉണ്ട്. എന്നിരുന്നാലും, അതിന്റെ സൈറ്റോടോക്സിസിറ്റിയുടെ മോളിക്യൂലർ മെക്കാനിസം ഇതുവരെ മനസിലാക്കിയിട്ടില്ല.

പി. കാഡ്രിഫോളിയസ് (ബെന്ത്.) ന്റെ ഇലകൾ കഴുകി, ഉണക്കി, പൊടിച്ച്, സോക്സ്ലെറ്റ് എക്സ്ട്രാക്ഷൻ വഴി ഇലയുടെ മെത്തനോളിക് സത്ത് തയ്യാറാക്കപ്പെട്ടു. 1g പൊടിച്ച ഇലയുടെ സാമ്പിളിൽ നിന്ന് 53.2 mg/g ക്രൂഡ് എക്സ്ട്രാക്റ്റ് ലഭിച്ചു. മനുഷ്യ ശ്വാസകോശത്തിലെ (A549), സ്തനാർബുദ സെൽ ലൈൻ (MDA-MB -231), സാധാരണ സെൽ ലൈൻ HEK 293

എന്നിവയിലെ MTT പരിശോധനയിലൂടെ ഇല സത്തിൽ സൈറ്റോടോക്സിക് പ്രഭാവം വിലയിരുത്തി. കോശങ്ങളുടെ അതിജീവന ശതമാനത്തിലും IC_{50} മൂല്യങ്ങളിലും ഡോസ് ആശ്രിത കുറവ് കാണപ്പെട്ടു. ശ്വാസകോശത്തിനും സ്തനാർബുദത്തിനും യഥാക്രമം 48.01 µg/ml ഉം 75.38 µg/ml ഉം ആണെന്ന് കണ്ടെത്തി, അതേസമയം മുകളിൽ പറഞ്ഞ അളവിൽ സാധാരണ കോശങ്ങൾക്ക് സൈറ്റോടോക്സിസിറ്റി കാണപ്പെട്ടില്ല. കോശങ്ങളുടെ നിലനിൽപ്പിനെയും വ്യാപനത്തെയും ബാധിക്കാനുള്ള സത്തിന്റെ കഴിവിനെ സൂചിപ്പിക്കുന്ന ക്ലോനോജെനിക് അസ്സെയിൽ കാൻസർ കോശങ്ങളുടെ കോളനി രൂപീകരണ കഴിവിനെ ഇല സത്ത് തടയുന്നതായി കാണപ്പെട്ടു. ഡിഎൻഎ ഫ്രാഗ്മെന്റേഷൻ പരിശോധനയ്ക്ക് വിധേയമായപ്പോൾ മെത്തനോളിക് ഇല സത്ത് നൽകിയ കോശങ്ങളിൽ നിന്ന് വേർതിരിച്ചെടുത്ത ഡിഎൻഎ യിൽ വിഘടനം നിരീക്ഷിച്ചു. ഡിഎൻഎ വിഘടനം അപ്പോപ്ടോസിസിന്റെ സൂചകമായാണ് കണക്കാക്കുന്നത്. *BAX, CASPASE 3, CASPASE 9, PARP, BCL2* എന്നീ കീ അപ്പോപ്റ്റോട്ടിക് ജീനുകളുടെ എക്സ്പ്രഷൻ പ്രൊഫൈലിംഗ് നടത്തിയപ്പോൾ, മെത്തനോളിക് ഇല സത്ത് നൽകിയ സെല്ലുകളിൽ *BAX, CASPASE 9, CASPASE 3* എന്നിവയുടെ വർധനവ് കാണിക്കുന്നു. ആന്റി- അപ്പോപ്റ്റോട്ടിക് ജീനുകളായ *BCL2, PARP1* എന്നിവ നിയന്ത്രിക്കപ്പെട്ടു, കൂടാതെ *BAX/BCL2* അനുപാതം 1-ൽ കൂടുതലാണെന്ന് കണ്ടെത്തി, ഇത് ഇല സത്ത് നൽകിയ കാൻസർ കോശങ്ങളിൽ സജീവമായ അപ്പോപ്റ്റോസിസ് സംഭവിക്കുന്നുവെന്ന് സ്ഥിരീകരിക്കുന്നു.

ശ്വാസകോശത്തിലും സ്തനാർബുദ കോശങ്ങളിലും *പി. കാഡ്രിഫോളിയസ്* (ബെന്ത്.) ന്റെ മെത്തനോളിക് ഇല സത്തിൽ നിന്നുള്ള സൈറ്റോടോക്സിക് പ്രഭാവം വ്യക്തമാക്കുകയും ജീൻ എക്സ്പ്രഷൻ വിശകലനം ചെയ്തപ്പോൾ സൈറ്റോടോക്സിസിറ്റിയുടെ സംവിധാനം അപ്പോപ്റ്റോസിസ് വഴിയാണെന്ന് വെളിപ്പെടുത്തുകയും ചെയ്തു. കൂടുതൽ വിപുലമായ ഗവേഷണത്തിന് ശേഷം കാൻസർ കീമോതെറാപ്യൂട്ടിക്സ് വികസിപ്പിക്കുന്നതിനുള്ള ശക്തമായ സ്ഥാനാർത്ഥിയാകാൻ *പി. കാഡ്രിഫോളിയസ്* (ബെന്ത്.) ന്റെ ഇല സത്തിൽ കഴിയുമെന്ന് കണ്ടെത്തലുകൾ സൂചിപ്പിക്കുന്നു.