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EVALUATION OF ANTITUMORAL ACTIVITY OF WHOLE PLANT EXTRACT OF *PHYLLANTHUS ACIDUS* IN A549 CELL LINES

***[1]S. Sri Vikram, [2]S. Parthiban, [3]S. Mohan**

*[1]Department of Pharmacology, Karpagam College of Pharmacy, Coimbatore – 32, India.

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***Corresponding Author**

S. Sri Vikram

Department of Pharmacology, Karpagam College of Pharmacy, Coimbatore – 32, India.

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ABSTRACT

Cancer is a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body. The pathogenesis of lung adenocarcinoma is complex, involving multiple mutations of proto-oncogenes and the activation of various intracellular molecular signaling pathways. The aim of the present study is to evaluation of antitumoral activity of whole plant extract of *Phyllanthus acidus* in A549 cell line. The study employs an integrative approach encompassing whole plant of extraction material with ethanol and Preliminary Phytochemical analysis with Quantification of total Phenol and Flavonoid content and futher Cell line studies were evaluated such as Cell viability assay, Scratch healing assay, Apoptosis assay. Results include that Total phenolic and Total flavonoid content in EPA was found to be 71.37mg/g and be 40.64 mg/g of extract calculated as Gallic acid and Quercetin equivalent respectively. In Cell viability assay, the percentage of cell viability is decreased at 44% at 300 µM concentration. Mostly the reduction happens at identified IC50 value246.5 µM respectively. A549 cells reductions in the viability of cells were identified with high concentration of the extract. In Apoptosis assay, also seen in other concentration also, but high apoptotic index is not noticed in any concentration. A549 cells treated with EPA (25-200 µM) of *phyllanthus acidus* extract. In Scratch healing assay particularly noticeable in the (50-200 µM) concentration range compared to the control. The anticancer activity of the *phyllanthus acidus* extract also inhibits migration of A549 cells and it doesn't promote invasion. Isolation and characterization of the chemical constituents present in the *phyllanthus acidus* extract and exploration of molecular targets in cancer cells will be the

efficient process to eradicate cancer in future.

KEYWORDS: Cancer, Phyllanthus acidus, Antitumoral, IC50, Cell viability, Scratch healing, Apoptosis.

INTRODUCTION

Cancer is a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body. Cancer is a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumours and neoplasms.[1]

GLOBOCAN 2020 estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer. Worldwide, an estimated 19.3 million new cancer cases (18.1 million excluding non melanoma skin cancer) and almost 10.0 million cancer deaths (9.9 million excluding non melanoma skin cancer) occurred in 2020. The disease is an important cause of morbidity worldwide, and region,and irrespective of the level of human development.[2]

The gender gap for overall cancer mortality worldwide is twice that for incidence, with death rates 43% higher in men than in women (120.8 and 84.2 per 100,000, respectively) partly because of differences in the distribution of the cancer types. Death rates per 100,000 persons varied from 165.6 per 100,000 in Eastern Europe to 70.2 per 100,000 in Central America among men and from 118.3 per 100,000 in Melanesia to 63.1 per 100,000 in Central America and South Central Asia among women. Notably, the cumulative risk of dying from cancer among women in 2020 was higher in Eastern Africa (11.0%) than in Northern America (8.2%), Western Europe (8.8%), and Australia/New Zealand (7.4%).[3]

The number of newly diagnosed cancer cases and deaths, the incidence and mortality ASR, and the cumulative risk of developing and dying from cancer overall and for the 36 cancer types separately in men and women. One in 5 men or women develop the disease, and 1 in 8 men and 1 in 11 women die from it. Below, we describe and discuss the variations in sex-specific incidence and mortality rates by world region for 16 of these cancer types.[4]

Lung cancer is the most frequently occurring cancer and the leading cause of cancer death in men, followed by prostate and colorectal cancer for incidence and liver and colorectal cancer for mortality.[5]

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020.

The most common in 2020 (in terms of new cases of cancer) were: Breast (2.26 million cases); Lung (2.21 million cases); Colon and rectum (1.93 million cases); Prostate (1.41 million cases); Skin (Non – melanoma) (1.20 million cases); and Stomach (1.09 million cases).[6]

The most common causes of cancer death in 2020 were: Lung (1.08 million deaths); Colon and rectum (935 000 deaths); Breast (685 000 deaths) Stomach (769 000 deaths); and Liver (769 000 deaths).[7]

Lung cancer is occurs when abnormal cells develop in one or both lungs and then grow so quickly that the body's immune system cannot keep up. The abnormal cells can form tumors that keep your lungs from working. Left untreated, the cancer can spread to nearby lymph nodes and other parts of your body.[8]

An depth study of the pathogenesis of lung adenocarcinoma and the search for new therapeutic targets for lung adenocarcinoma to improve the attendant chemotherapy sensitivity will greatly improve the prognosis of patients with the disease and reduce the economic burden on both society and the patients.[9]

TYPES OF LUNG CANCER[10]

A Lung cancer is divided into 2 main types

Such as

I) SMALL CELL LUNG CANCER (SCLC) AND

II) NON-SMALL CELL LUNG CANCER (NSCLC)

And, therefore the category of the cancer determines the treatment options.

Small cell lung cancer

Small cell lung cancer (SCLC) accounts for about 15% of all lung cancers. Also known as oat cell carcinoma or small cell undifferentiated carcinoma, SCLC tends to be aggressive. The cancer often grows rapidly and spreads to other parts of the body including lymph nodes, bone, brain, adrenal glands, and the liver. Risk of developing SCLC is highly associated with tobacco smoking. Less than 5% of patients diagnosed with the disease have never smoked.

Non-small cell lung cancer

Non-small cell lung cancer (NSCLC) is divided into three categories, based on appearance and other characteristics of the cancerous cells.

Squamous cell carcinoma (Scc)

SCC accounts for approximately 25-30% of all lung cancer cases. SCC is highly associated with tobacco smoking and usually develops in the central region of the lungs.

Adenocarcinoma

Adenocarcinomas account for approximately 40% of all lung cancer cases. This cancer type usually develops in the outer region of the lungs.

Large cell carcinoma (Lcc)

LCC accounts for approximately 10-15% of all lung cancer cases. LCC is associated with rapid tumor growth and poor prognosis.

Other, less common types of lung cancers include carcinoid tumors, adenoid cystic carcinomas, hamartomas, lymphomas, and sarcomas.

LUNG CANCER EPIDEMIOLOGY

Lung cancer is the leading cause of cancer morbidity and mortality in men, whereas, in women, it ranks third for incidence, after breast and colorectal cancer, and second for mortality, after breast cancer. Incidence and mortality rates are roughly 2 times higher in men than in women, although the male to female ratio varies widely across regions, ranging from 1.2 in Northern America to 5.6 in Northern Africa.[11]

LUNG CANCER TRENDS IN THE UNITED STATES

In the United States, lung cancer remains the sec- ond most common cancer and the leading cause of cancer death. According to the Surveillance, Epidemiology, and End Results pro- gram, lung cancer currently accounts for approximately 12.9% of all new cancer cases in the United States and 538,243 people in the United States were estimated to be living with lung cancer in 2016. Data from 2016 revealed deaths from lung cancer in men and women were 80,775 and 68,095, respectively, which exceeded the combined number of deaths for breast cancer, prostate cancer, colon cancer, and leukemia.[12]

Although lung cancer incidence and mortality rates are higher in males, they continue to

decrease more rapidly in men compared with women, possibly attributed to earlier decreases in smoking prevalence among men and women's increased uptake of smoking around World War.[13]

HISTORY OF LUNG CANCER

Having a personal or family history of lung cancer raises your risk of developing the disease. Talk with your doctor if lung cancer runs in your family, especially if you experience other risk factors.

Cancer research is constantly evolving, and we may learn of additional causes in the future. The following substances may raise your risk for lung cancer, but there is not yet enough evidence to be sure

- Smoking marijuana
- E-cigarettes
- Talc and talcum powder.[14]

PATHOPHYSIOLOGY OF LUNG CANCER

Pathophysiology is the study of the functional changes that cause, result from, or are otherwise associated with the disease. In the case of lung cancer, the changes occur in the lungs. As the disease progresses, other areas of the body may be involved as well. Gene mutations, either inherited or acquired, may raise your risk of developing lung cancer.[15]

TREATMENT OF LUNG CANCER

Treatment for lung cancer usually begins with surgery to remove the cancer. If the cancer is very large or has spread to other parts of the body, surgery may not be possible. Treatment might start with medicine and radiation instead. Your healthcare team considers many factors when creating a treatment plan. These factors may include your overall health, the type and stage of your cancer, and your preferences.[16]

PATHWAYS INVOLVED IN LUNG CANCER LUNG CANCER SIGNALING PATHWAY MAIN SIGNALING PATHWAYS INVOLVE IN LUNG CANCER

RTK signaling cascade

Whereas receptor tyrosine kinases （RTKs） activity in normal resting cells is tightly regulated, mutations or deregulated expression might cause them to function as potent

oncogenes. Ligands, such as EGF, VEGF, HGF or others, bind to the homo- and heterodimer kinase domain, resulting in activation and receptor trans phosphorylation. This creates docking sites for the adaptor proteins, Grb2 and Sos, which recruit Ras and phosphatidylinositol 3-kinase (PI3K), leading to the formation of two major signalling pathway branches, Ras/MAPK and PI3K/Akt and PLC-PKC pathways.

The PI3K-AKT signaling phosphorylates and inhibits several apoptosis-inducing genes. NF-kB is convergently activated by several distinct stimuli including growth factors. So the activation of the RTK pathway can thus have noteworthy downstream effects on angiogenesis, evasion of apoptosis, cell growth.[17]

Growth inhibitory signaling cascade

p53 is a sensor of cell stress, such as DNA damage and oncogenic stimuli, and functions as a transcription factor. Its target genes play roles in the control of the G1 arrest pathway, cyclin-dependent kinases (CDKs) are key regulatory enzymes, each consisting of a catalytic CDK subunit and an activating cyclin subunit. CDKs regulate the cell's progression through the phases of the cell cycle by modulating the activity of key substrates. Downstream targets of CDKs include transcription factor E2F and its regulator Rb.[18]

LUNG CANCER DIAGNOSIS

Molecular markers for lung cancer

Molecular diagnostics-guided targeted therapies have become a standard treatment for patients with lung cancer. Here, we grouped molecular biomarkers into three categories-mutations, gene rearrangements, and amplifications.[19]

Targeted therapy for lung cancer

The numerous molecular mechanisms implicated in the pathogenesis of lung cancer present exciting avenues for target-specific approaches to therapy. Major components of cell signaling pathways, such as the receptor tyrosine kinases (RTKs), protein kinase C (PKC), and the ras/mitogen-activated protein kinase (MAPK) systems, are altered in lung cancer cells by oncogenes through overexpression or mutation, leading to dysregulated cell signaling and cell proliferation. Here, we summarize potentially viable targets and new agents that have been developed and employed in recent, ongoing and future clinical trials to attempt to improve clinical outcomes in this disease.[20]

SCREENING METHODS FOR LUNG CANCER

Key points

- Tests are used to screen for different types of cancer when a person does not have symptoms.
- Three screening tests have been studied to see if they decrease the risk of dying from lung cancer.[21]
- Screening with LDCT scans has been shown to decrease the risk of dying from lung cancer in heavy smokers.
- Screening with chest x-rays and/or sputum cytology does not decrease the risk of dying from lung cancer.
- Screening tests for lung cancer are being studied in clinical trials.[22]

Tests are used to screen for different types of cancer when a person does not have symptoms

Scientists study screening tests to find those with the fewest harms and most benefits. Cancer screening trials also are meant to show whether early detection (finding cancer before it causes symptoms) helps a person live longer or decreases a person's chance of dying from the disease. For some types of cancer, the chance of recovery is better if the disease is found and treated at an early stage.

Three screening tests have been studied to see if they decrease the risk of dying from lung cancer

The following screening tests have been studied to see if they decrease the risk of dying from lung cancer:

Low-dose computed tomography (LDCT): A procedure that uses low-dose radiation to make a series of very detailed pictures of areas inside the body using an x-ray machine that scans the body in a spiral path. This procedure is also called spiral scan or helical scan.

Chest x-ray: An x-ray of the organs and bones inside the chest. An x-ray is a type of energy beam that can go through the body and onto film, making a picture of areas inside the body.

Sputum cytology: Sputum cytology is a procedure in which a sample of sputum (mucus that is coughed up from the lungs) is viewed under a microscope to check for cancer cells.

Screening with ldct scans has been shown to decrease the risk of dying from lung cancer in heavy smokers

The National Lung Screening Trial studied people aged 55 years to 74 years who had smoked at least 1 pack of cigarettes per day for 30 years or more. Participants were either current smokers or former smokers who had quit within the last 15 years. The trial used chest x-rays or LDCT scans to check for signs of lung cancer.[23]

RISKS OF LUNG CANCER SCREENING

Key points

The risks of lung cancer screening tests include the following:

- Finding lung cancer may not improve health or help you live longer.
- False-negative test results can occur.
- False-positive test results can occur.
- Chest x-rays and CT scans expose the chest to radiation.
- Talk to your doctor about your risk for lung cancer and your need for screening tests.[24]

HALLMARKS OF LUNG CANCER

- Sustaining proliferative signaling
- Evading growth suppressors
- Enabling replicative immortality
- Activating invasion and metastasis
- Inducing angiogenesis
- Resisting cell death.[25]

SYMPTOMS OF LUNG CANCER

Lung cancer typically doesn't cause symptoms in early stages, and some symptoms can be caused by other conditions. Lung cancer is sometimes discovered after a chest X-ray for another condition. Talk with your doctor if you're concerned about:

- Chest discomfort or pain
- Bone pain
- A cough that doesn't go away or worsens
- Shortness of breath
- Wheezing
- Blood coughed up from the lungs
- Hoarseness

- Loss of appetite
- Loss of appetite
- Weight loss for no known reason
- Severe fatigue
- Headaches
- Trouble swallowing
- Swelling in the face and/or neck veins.[26]

CELL LINES OF A549 CELLS IN LUNG CANCER

A549 cells are adenocarcinomic human alveolar basal epithelial cells, and constitute a cell line that was first developed in 1972 by D. J. Giard, et al. through the removal and culturing of cancerous lung tissue in the explanted tumor of a 58-year-old caucasian male.

The cells are used as models for the study of lung cancer and the development of drug therapies against it.[27]

HERBAL MEDICINES

Herbal medicines which formed the basis of health care throughout the world since the earliest days of mankind are still widely used, and have considerable importance in international trade. Recognition of their clinical, pharmaceutical and economic value is still growing, although this varies broadly between countries.[28]

P. acidus is an ornamental shrub or tree that can grow up to 10 m tall, with phyllanthoid dense branching. The bark is rough, grey, and with prominent lenticels. The leaf is in pinnate shape with 20–40 cm long. Its leaflets are alternate, ovate or ovate lanceolate and short petiole up to 7.5 cm. It is green and smooth on the upper surface, bluegreen with a bloom on the underside; and the pinnate leaf is with numerous leaflets with two tiny pointed stipulate at the base of each leaf. The flower is small, reddish-pink cushion-shaped cymules at the nodes of leafless branches on older wood.[29]

Database search method

The present review of P. acidus on its traditional uses, phytochemistry and pharmacological activity is based on several popular databases such as ACS, PubMed, Scopus, Web of Science, SciFinder, Science Direct, Google Scholar, Springer, Wiley, Taylor, Mendeley, and other published materials such as books and dissertations. The literature was searched and

accessed using the keywords 'Phyllanthus acidus', 'Phyllanthus distichus', 'Averrhoa acida' and 'Cicca acida' that related to the present review.[30]

MATERIALS AND METHODS

Extraction of the plant materials

- The coarsely powdered Plant (200gm) were taken in a round bottom flask and with ethanol for 24 hours at room temperature.
- After extraction the extracts were evaporated or concentrated by using rotary evaporator and dried at room temperature.
- The obtained crude extracts were weighed and stored at 40^{o}C for the further analysis**.**

QUALITATIVE PHYTOCHEMICAL ANALYSIS OF EPA

Preparation of test sample

A small quantity of the extract was dissolved in 5ml of distilled water and filtered. The filtrate was tested to detect the presence of various phytochemical constituents in the sample.

TEST FOR CARBOHYDRATES

Molisch's test

Few drops of Molisch's reagent was added to 2-3ml of filtrate, followed by addition of concentrated sulphuric acid along the sides of the test tube. Formation of violet colour ring at the junction of two liquids indicates the presence of carbohydrates.[53]

Fehling's test

1ml Fehling's-A (copper sulphate in distilled water) was added to 1ml of Fehling's-B (potassium tartarate and sodium hydroxide in distilled water) solution, boiled for one minute. To this added 1ml of filtrate and heated gently. Formation of brick red precipitate indicates the presence of reducing sugars.

Benedict's test

Few ml of filtrate was mixed with equal volume of Benedict's reagent (alkaline solution containing cupric citrate complex) and heated in boiling water bath for 5min. Formation of reddish brown precipitate infers the presence of reducing sugars.

TEST FOR ALKALOIDS

Small amount of extract mixed with few ml of dilute hydrochloric acid. Shaken well and filtered. Following tests were performed with the obtained filtrate.

Dragendorff's test

A few drops of Dragendorff's reagent (potassium bismuth iodide solution) was added to 2-3ml of filtrate. Orange red precipitate indicates the presence of alkaloids.

Mayer's test

A few drops of Mayer's reagent (potassium mercuric iodide solution) was added to 2-3ml of filtrate. Cream (dull white) precipitate was formed.

Wagner's test

A few drops of Wagner's reagent (solution of iodine in potassium iodide) was added to 2-3ml of filtrate. Reddish brown precipitate was obtained.

Hager's test

A few drops of Hager's reagent (Picric acid) was added to 2-3ml of filtrate. Yellow precipitate was obtained.

TEST FOR TRITERPENOID

Libermann-burchard test

A small quantity of extract was treated with few drops of acetic anhydride, followed by a few drops of concentrated sulphuric acid. A brown ring was formed at the junction.[53]

Salkowski test

A small quantity of the extract was treated with chloroform and few drops of concentrated sulphuric acid and allowed to stand for few minutes. Yellow colour at the lower layer indicates the presence of triterpenoids.

TEST FOR GLYCOSIDES

Legal's test

1ml of pyridine and 1ml of sodium nitroprusside was added to 1ml of extract. Pink to red colour indicates the presence of glycosides.

Keller-killiani test

Glacial acetic acid was added to 2ml extract, followed by the addition of trace quantity of ferric chloride and 2 to 3drops of concentrated sulphuric acid. Reddish brown colour appears at the junction of two liquid indicates the presence of cardiac glycosides.

Baljet test

2ml of extract was added to sodium picrate solution. Yellow to orange colour formation indicates the presence of glycosides.

TEST FOR STEROIDS AND STEROLS

Liebermann- burchard reaction

2ml of extract was mixed with chloroform. To that mixture added 1-2ml of acetic anhydride and 2drops of concentrated sulphuric acid along the sides of the test tube. The solution becomes red, then blue and finally bluish green colour.

Salkowski reaction

2ml of extract was mixed with 2ml chloroform and 2ml concentrated sulphuric acid. Shaken well. Chloroform layer appears red and acid layer shows greenish yellow fluorescence.

TEST FOR PHENOLS

Ferric chloride test

1ml of the alcoholic solution of the extract was added to 2ml of distilled water followed by few drops of 10% ferric chloride. Formation of blue or green colour indicates the presence of phenols

Lead acetate test

Diluted 1ml of alcoholic solution of extract with 5ml distilled water and to this added few drops of 1% aqueous solution of lead acetate. Formation of yellow colour precipitate indicates the presence of phenols**.**

TEST FOR TANNINS

Lead acetate test

A few drop of lead acetate was added to 5ml of aqueous extract. Formation of yellow or red colour precipitate indicates the presence of tannins.

TEST FOR SAPONINS

Foam test

1ml of test sample was diluted with 20ml of distilled water and shaken it in a graduated cylinder for 3minutes. Foam of 1cm after 10min indicates the presence of saponins.

Froth test

5ml of test sample was added to sodium bicarbonate solution. After vigorous shaking the mixture, kept it for 3minutes. A honey comb like froth formation indicates the presence of saponins.

TEST FOR FLAVONOIDS

Alkaline reagent test

A few drop of sodium hydroxide solution was added to the extract. Formation of an intense yellow colour, which turns to colourless on addition of few drops of dilute hydrochloric acid, indicates the presence of flavonoids.

Shinodas test [Magnesium Hydrochloride Reduction Test]

Alcoholic solution of extract was treated with a small piece of magnesium ribbon and a few drops of concentrated HCl was added and heated. Appearance of crimson red or occasionally green to blue colour infers the presence of flavonoid.

TEST FOR PROTEINS AND AMINO ACIDS

Biuret test

3ml of test solution was added to 4% sodium hydroxide and few drops of 1% copper sulphate solution. Formation of violet colour indicates the presence of proteins.

Ninhydrin test

A mixture of 3ml test solution and 3drops of 5% Ninhydrin solution was heated in a boiling water bath for 10min. Formation of purple or bluish colour indicates the presence of free amino acids.

QUANTIFICATION OF TOTAL PHENOLICS AND FLAVONOIDS ESTIMATION OF TOTAL PHENOLICS

Reagents

- Folin-Ciocalteu's reagent
- Gallic acid (1mg/ml)
- 20% sodium carbonate

Preparation of standard

Standard solution of was prepared by adding 10mg of accurately weighed Gallic acid in 10ml of distilled water.

Preparation of sample

10mg of the accurately weighed EPA extract were separately dissolved in 10ml ethanol and used for the estimation.

Procedure

The total phenolic content of the EPA were determined by Folin-Ciocalteu assay method. To an aliquot 100µl of EPA (1mg/ml) or standard solution of Gallic acid (10, 20, 40, 60, 80, 100µg/ml) added 50µl of Folin-ciocalteu reagent followed by 860µl of distilled water and the mixture is incubated for 5min at room temperature. 100µl of 20% sodium carbonate and 890µl of distilled water were added to make the final solution to 2ml. It was incubated for 30min in dark to complete the reaction. After that absorbance of the mixture was measured at 725nm against blank. Distilled water was used as reagent blank. The tests were performed in triplicate to get mean the values. The total phenolic content was found out from the calibration curve of Gallic acid. And it was expressed as milligrams of Gallic acid equivalents (GAE) per gram of extract.

ESTIMATION OF TOTAL FLAVONOIDS

Reagents

- Ethanol
- 10% Aluminium chloride
- 1M Potassium acetate

Preparation of standard

Standard solution of was prepared by adding 10mg of accurately weighed Quercetin in 10ml of ethanol.

Preparation of sample

10mg of the accurately weighed EPA extracts were separately dissolved in 10ml ethanol and used for the estimation.

Procedure

The total flavonoid content of the EPA was determined by using Aluminium chloride colorimetric method. To an aliquot of 100µl of extract (1mg/ml) or standard solutions of Quercetin (10, 20, 40, 60, 80, 100µg/ml) ethanol was added separately to make up the solution upto 2ml. The resulting mixture was treated with 0.1ml of 10% aluminium chloride,

0.1ml of 1M potassium acetate and 2.8ml of distilled water. Shaken well and incubated at room temperature for 30minutes. The absorbance was measured at 415nm against blank, where a solution of 2ml ethanol, 0.1ml potassium acetate, 2.8ml distilled water and 0.1ml of aluminium chloride serve as blank solution. The total flavonoid content was determined from the standard Quercetin calibration curve. And it was expressed as milligrams of Quercetin equivalents (QE) per gram of extract**.**

CELL LINE STUDIES CELL VIABILITY ASSAY

1X104 A549 cells were seeded in 96 well plates and kept at 37oC in a 5% CO2 humidified incubator for cells to be attached. After confluency is attained, the existing serum media were removed. The treatment grouping and plan is provided as follows: Plant extract was treated with 20 µM-200 µM concentration at 24 hours to check the efficacy. At the end of treatment hours, the morphological changes were observed in "Zoe fluorescent microscope Biorad" at bright field. 100µl of MTT solution were added after the condition media were removed and the plates were incubated at 37oC for 3hours in dark. Purple colour formazan crystals were formed based on the mitochondrial enzyme activity. After 3 hours of incubation, the existing MTT reagent was aspirated and dimethyl sulfoxide (DMSO) was utilized to dissolve the formazan crystals developed. The plates were read at 590 nm in ELISA reader (BioTek, Synergy H1). The readings obtained were plotted in a graph and represented as percentage viability.

SCRATCH HEALING ASSAY

5x105 A549 cells/well were plated in 12 well plates and were allowed to attain confluency. A gentle scratch was created after removing the media. PBS was used to remove the detached cells and the cells were treated with plant extract. The pattern of migration was recorded time dependently (0,24, 48 hours) by photographing the wounded area (Muthusami *et al.*, 2014).

APOPTOSIS ASSAY

A549 cells were seeded 1x104 cells/well in 96 well plates and treated with Different concentration of plant extracts dependently. The existing media was aspirated after 24 hours of treatment and washed with PBS. AO/EtBr working solution was added in each well. After staining, the cells were immediately visualized and the images were captured using a fluorescent microscope. AO stain was captured using a green channel and the EtBr stain was captured using a red channel. Finally, both the photomicrographs were merged to analyze the results obtained.

RESULTS AND DISCUSSION

PRELIMINARY PHYTOCHEMICAL ANALYSIS

Table: Phytochemical analysis of *Phyllanthus acidus*.

S. No.	Phytochemical constituents	Name of the test	Result
1	Alkaloids	Mayer's & Wagner's test	Positive
2	Flavonoids	Alkaline & Shinodas test	Positive
3	Triterpenoids	Salkowski test	Positive
4	Tannins	Lead acetate test	Positive
5	Glycosides	Legal's & Keller-killiani test	Positive
6	Phenols	Lead acetate test	Positive
7	Saponins	Foam test	Positive

QUANTIFICATION OF TOTAL PHENOL AND FLAVONOIDS ESTIMATION OF TOTAL PHENOL CONTENT OF EPA

Table: Estimation of total phenolic content of EPA.

Sample	Concentration (µg/ml)	Absorbance
Standard (Gallic acid) 1mg/ml	10	0.087
	20	0.118
	40	0.205
	60	0.336
	80	0.401
	100	0.495
EPA	100	0.3185

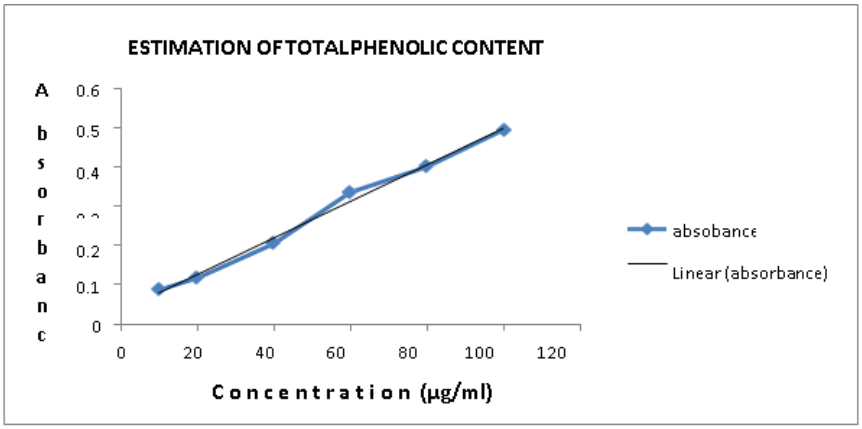

Figure: Estimation of total phenolic content of EPA

The total phenolic content in EPA was found to be 71.37mg/g of extract calculated as Gallic acid equivalent**.**

ESTIMATION OF TOTAL FLAVONOID CONTENT OF EPA

Table: Estimation of total flavonoid content of EPA.

Sample	Concentration (µg/ml)	Absorbance
Standard (Quercetin) 1mg/ml	10	0.031
	20	0.085
	40	0.26
	60	0.5026
	80	0.776
	100	1.053
EPA	100	0.7046

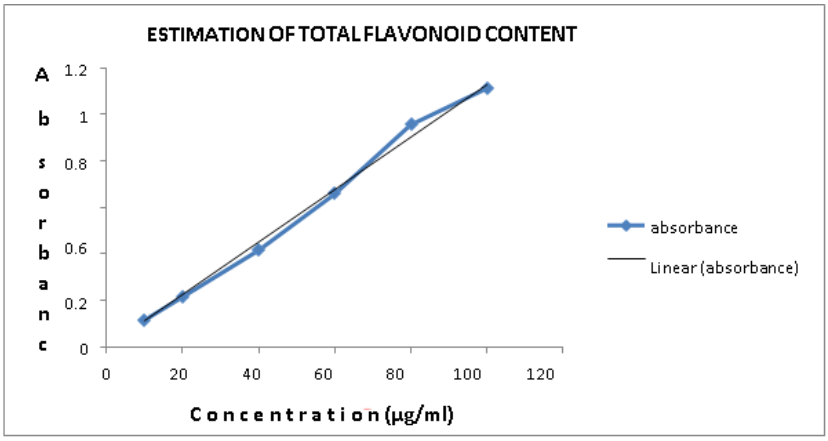

Figure: Estimation of total flavonoid content of EPA.

The total flavonoid content in EPA was found to be **40.64mg/g** of extract calculated as Quercetin equivalent.

CELL LINE STUDIES CELL VIABILITY ASSAY

***Phyllanthus acidus* extract reduces A549 cell viability and controls proliferation of non small cell lung cancer**

- A preliminary investigation done on a wide range of concentration (25–300 µM) of *phyllanthus acidus* extract on A549 cells. The percentage of cell viability is decreased at 44% at 300 µM concentration.
- The identified IC50 value is 246.5 µM respectively Reduction in the viability of A549 cells were identified with high concentration of the extract.

Table: Estimation of total extract values.

Si no	Concentration	% of inhibition
1	25	77.60336
2	50	70.65603
3	100	63.80064

4	150	59.74673
5	200	55.47543
6	250	54.05815
7	300	44.58225

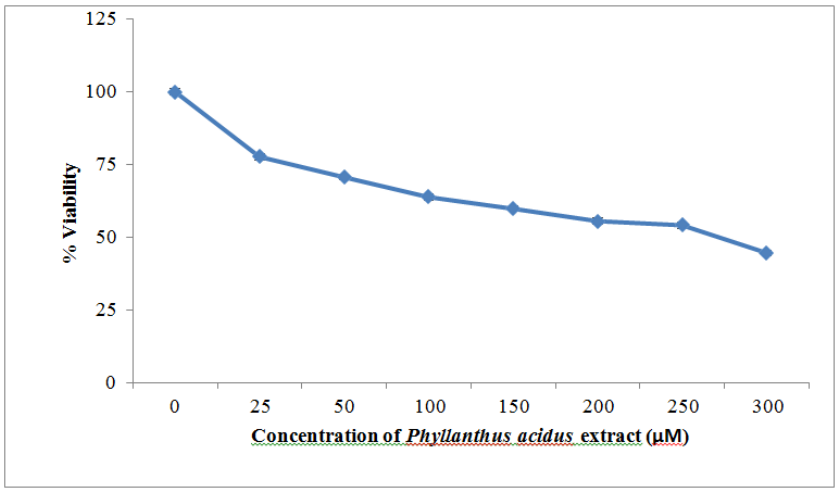

Figure: Cell viability of A549 cells treated with different concentration (µM) of *Phyllanthus acidus.*

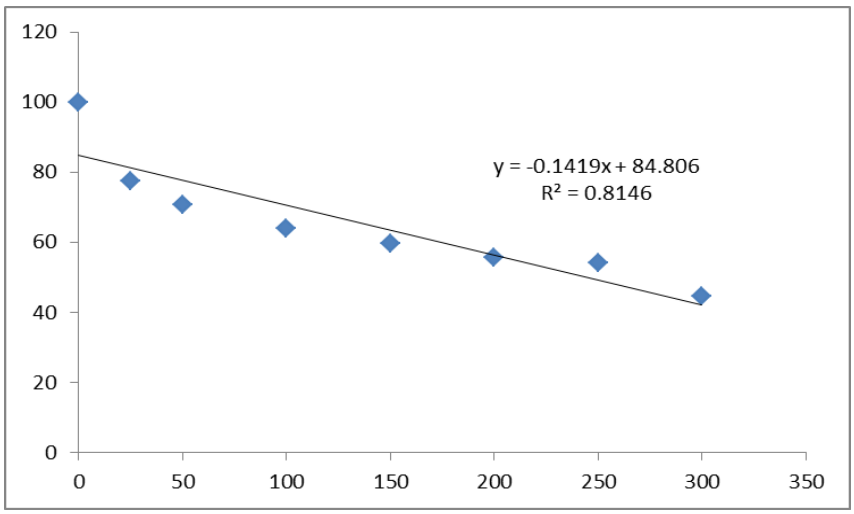

Figure: IC 50 values of % cell viability.

IC50=246.5 µM

APOPTOSIS ASSAY

***Phyllanthus acidus* extract enhances A549 cell apoptosis and controls proliferation of non small cell lung cancer**

- The reduction in the cell viability is the index of early/late apoptosis. Thus, apoptotic index was identified upon the staining of A549 cells with AO/EtBr, when treated with (25–200 µM) of *phyllanthus acidus* extract.

- The live cells tend to emit green fluorescence, the early apoptotic cells emit yellowish orange nucleus, and the late apoptotic cells shows orange/red nucleus.
- The increased apoptosis was noted in the 200µM concentration treated A549 cells.
- The apoptosis index also seen in other concentration also, but high apoptotic index is noticed in 200 µM concentration sequentially.

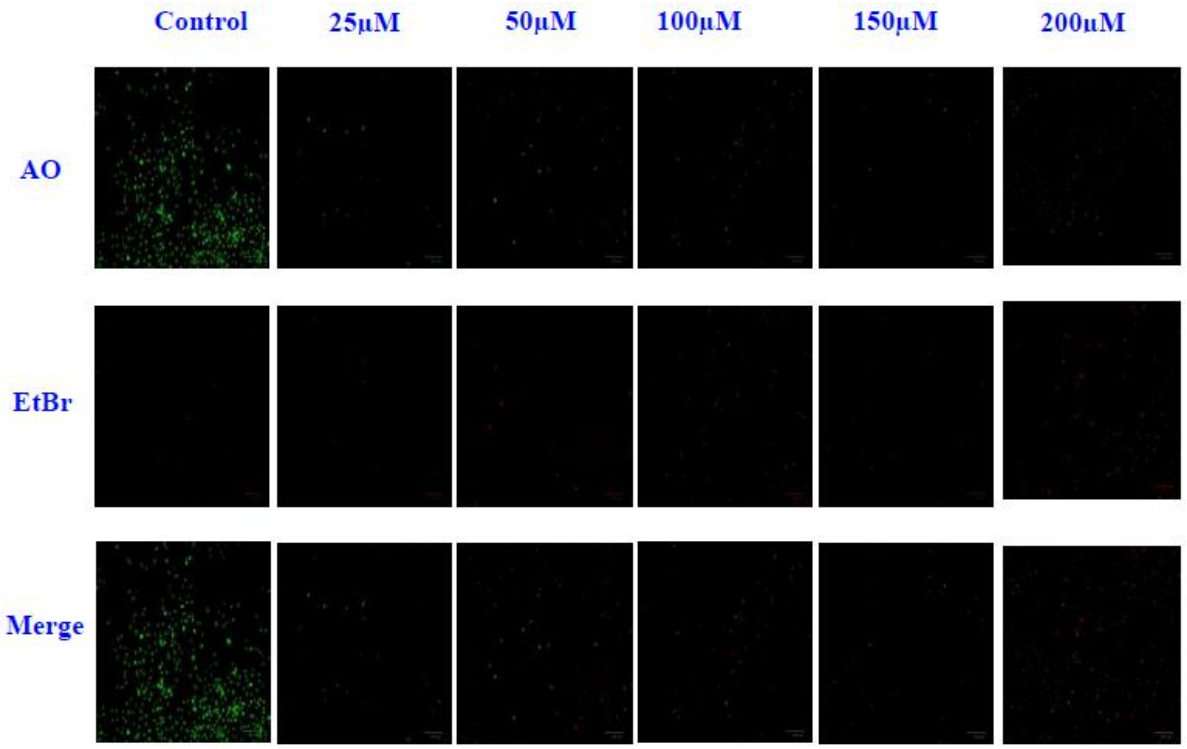

Figure: Apoptotic index of A549 cells treated with *Phyllanthus acidus* in different concentration (µM).

SCRATCH HEALING ASSAY

- *Phyllanthus acidus* extract Inhibited A549 cells migration and controls invasion of non small cell lung cancer.
- The ability of *phyllanthus acidus* extract in attenuating the migration of A549 cells were then evaluated using wound healing assay. The A549 cells treated with different concentration of extract like at low 50µM, medium 100µM and high 200µM.
- The migration of A549 cells were inhibited by low, medium, and high concentration of *phyllanthus acidus* extract. The extract doesn't allow the cancer cells to migrate towards the matrix. The migration is inhibited by the doses 50, 100, 200µM concentration of *phyllanthus acidus* extract.

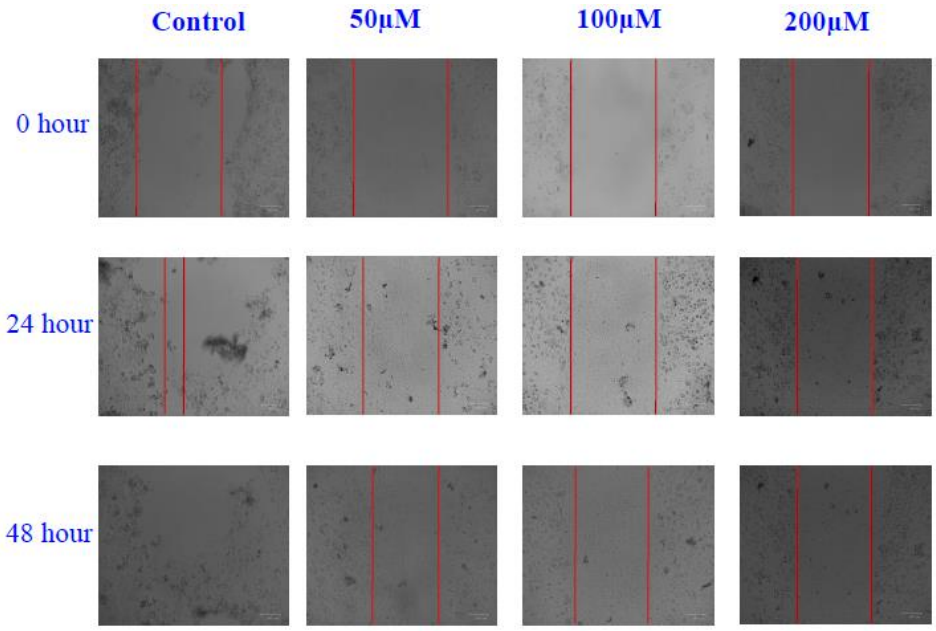

Figure: Migration of A549 cells analysed with the treatment of *Phyllanthus acidus* in different concentration (µM).

CONCLUSION

Natural plant extracts are more effective for treatment of various cancers. In this *phyllanthus acidus* plant has anti-oxidant activity and their role is well defined in various conditions. The Cell viability assay performed using *phyllanthus acidus* plant extract in different concentrations (25-200µM) in A549 cells showed significant reduction in cell proliferation at 200 µM. This analysis is supported by an apoptosis assay using AO/EtBr staining, which is also inferred that *phyllanthus acidus* in 200 µM concentration induced apoptosis. The anti-cancer activity of the *phyllanthus acidus* extract also inhibits migration of A549 cells and it doesn't promote invasion. Isolation and characterization of the chemical constituents present in the *phyllanthus acidus* extract and exploration of molecular targets in cancer cells will be the efficient process to eradicate cancer in future.

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