Original Article

Anticancer effect of *Phyllanthus reticulatus* methanolic leaf extract on HCT-116 colon cancer cell line using GC-MS and FTIR analysis

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ABSTRACT

Phyllanthus reticulatus is a plant species belonging to the family Euphorbiaceae. The plant is native to tropical areas of India, Bangladesh, China, and the Malay Islands. The plant is known for its medicinal properties and has been traditionally used as a diuretic, cooling medicine, and remedy for spongy and bleeding gums. Literature revealed the plant's hepatoprotective, hypolipidemic, antinociceptive, analgesic, anti-inflammatory, antimalarial, cytotoxic, and antimicrobial, anticancer properties. A plant species with significant medicinal potential and its leaves have been studied for their potential therapeutic applications in managing diabetes and diarrhea. This study highlights the chemical profile of *P. reticulatus* methanolic leaf extract by phytochemical and GC-MS analysis, revealing nine major peaks with active chemical components. FTIR analysis showed the presence of six biologically active functional groups. The methanolic leaf extract showed the existence of 93.44 mg/g, a significant amount of phenolic content, and 55.35 mg/g of flavonoid content. The elemental concentration of plant leaf revealed the presence of 12 elements. The DPPH and PM assay showed the antioxidant properties of the leaf extract, as evidenced by its anticancer property of the leaf extract with 28.56 % cell growth inhibition on the HCT-116 colon cancer cell line at 24 h in a dose-dependent manner. This is the first report on *Phyllanthus reticulatus*, which reports the spectroscopic and anticancer properties of the leaf extract on colon cancer. The study opens avenues for further investigation into the bioactive constituents of *Phyllanthus reticulatus* leaves and their mechanisms of action, paving the way for future studies and potential drug development.

Keywords: *Phyllanthus reticulatus* Methanolic leaf extract (PRM), GC-MS, FTIR, antioxidant, HCT-116 colon cancer.

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1. Introduction

Medicinal plants occupy a significant place throughout the world. There are multipotential plants like *Phyllanthus reticulatus*, which can cure many ailments. The tribal community, Malasar of Nilgiri Biosphere, is an efficient tribal community that uses this plant as a remedy to cure diseases. Malasars are a highly respectable tribe in this locality, owing to their traditional knowledge about the medicinal values of specific medicinal plants grown in their ecosystem. The medicinal properties of this plant may be due to the presence of many phytochemicals in it. Traditional medicine is an essential resource of beneficial compounds for developing phytotherapeutic agents (1). The consumption of plants as remedies for countless diseases is stated in Ayurvedic books like Charaka Samhita. Subsequently, medicinal plants were utilized for centuries to treat innumerable ailments. *Phyllanthus reticulatus* plant is a five-meter-tall, deciduous shrub or small tree with a distinct smell. *P. reticulatus* has narrow leaves with reddish-brown net-like veining that is more noticeable from above. *P. reticulatus* produces black and purple-black berries, fruits known for inflammation. Phyllanthus reticulatus bark is used as an astringent and diuretic, and leaves are used for antidiarrheal purposes. Plant roots are used for treating asthma (2). The plant is known for its medicinal properties. It has traditionally been used as a diuretic and cooling medicine, including hepatoprotective, hypolipidemic, analgesic, anti-inflammatory, and rheumatic properties. Traditional medicine is also utilized to treat gastrointestinal disorders due to its presumed positive effects on the digestive system. The identification of its active constituents allows for the potential development of new pharmaceuticals, such as germanic acid derivatives, which have been shown to have antinociceptive and anti-hyperglycemic effects, and its leaves have been studied for their potential therapeutic applications in managing diabetes and diarrhea that may be more effective and have fewer side effects than existing treatments. Colon cancer is one of the fourth primary cancer because of its high incidence and fatality rates in affluent nations. Colon cancer has up to 97% cure rate when detected at an early stage (3). When considering other malignancies, colon cancer has a more extended latency period, which offers the potential for early detection and a better prognosis (4). The plant has been found to contain various bioactive compounds with significant medicinal potential. To explore this plant more, its phytochemical analysis using methanolic leaf extract was carried out to identify its bioactive components through GC-MS analysis and FTIR spectroscopic study to determine its active functional groups. The results demonstrate its antioxidant and anticancer properties on the HCT-116 colon cancer cell line in a dose-dependent manner. This is the first report on *Phyllanthus reticulatus* to reveal the antioxidant activity of the methanolic leaf extract due to the presence of active biological constituents such as phenols and flavonoids. Phytol, the principal constituent of GC-MS analysis of *P. reticulatus* leaf extract, is known for its anticancer activity. Further, isolation and purification of the active chemical components are required to explore the medicinal value of this plant.

2. Materials and Methods

2.1 Materials

Benedict's reagent, Molisch's reagent, Fehling's A and B reagents, Molybdate reagent, Millions reagent, Mayer's and Wagner's reagents, Folin-ciocalteau's reagent, 1 % aqueous HCl, Ferric Chloride, Ammonia solution, Potassium hydroxide, Sodium phosphate, Conc. HCl, Lead acetate, Iodine solution, conc. H2SO4, Glacial acetic acid, 0.2 % Ninhydrin solution, Fecl3 solution, Gallic acid, Quercetin, Sodium hydroxide, Chloroform, Acetic acid, Sodium carbonate, Aluminum chloride, Sodium acetate.DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), Methanol, Ascorbic acid, Phosphomolybdenum reagent, DMSO (Dimethyl sulfoxide), Ascorbic acid, 3(4, 5-Dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide, Foetal Bovine Serum (FBS), Dulbecco's Phosphate Buffered Saline (DPBS), Trypsin, EDTA (Ethylenediaminetetraacetic acid), MTT, DMSO (Dimethyl sulfoxide), Dulbecco's Modified Eagle's Medium(DMEM). All the chemicals used were of analytical grade. Cell culture: The HCT-116 human colon carcinoma cells were grown in Dulbecco's Modified Eagle Medium (DMEM), which was enhanced with 10% FBS, 100 µg/ml streptomycin, and 100 units/ml penicillin (full media). The cells were incubated with 95% humidity and 5% CO2 at 37°C in the incubator.

2.1.1. Plant source material

The leaves of *Phyllanthus reticulatus* were collected from Karnatak University, Dharwad, Karnataka, India.

2.2. Methods

2.2.1. Preparation of extracts

Drying the plant leaves involved separating them and placing them in the shade to dry. Afterward, they were ground into a coarse powder using a blender and dried for 24 hours at 35° C in hot air. 10 grams of each material were then individually extracted with 100 milliliters of methanol using a Soxhlet extractor at 55-60° C for 8 hours. The resultant extracts were employed in the experiment after being dehydrated in a rotary evaporator at 40° C with reduced pressure to extract excess solvent.

2.3. Elemental Analysis

Elements like Nitrogen, Phosphorus, Potassium, Sulphur, Calcium, Magnesium, Sodium, Zinc, Iron, Manganese, and Copper are estimated by different methods for different elements using the standard protocols (5, 6). Boron is estimated by a spectrophotometric method using the standard protocol (7).

2.4. Quantification of total phenolics

Though with some modifications, the Folin-Ciocalteau method was employed to evaluate the total phenol content by the protocol described by Singleton et al. in 1999 (8). 1.5 ml of 7 % Na2CO3 and 0.5 ml of 50% FCR were incubated with gallic acid solutions at concentrations of 25, 50, 75, and 100 µg/ml for 30 minutes at room temperature.

At 760 nm, the absorbance was calculated about the blank. Similarly, the plant extracts were appropriately diluted and processed using the same method as for gallic acid. The total phenols present in the plant extract was calculated by extending the standard calibration curve created with gallic acid to the absorption of unidentified samples.

2.5. Quantification of total flavonoid

The extraction of total flavonoids in the sample was quantified according to Phuyal et al. in 2020 (9) with some alterations. To create the standard curve, 1 ml of quercetin at varying concentrations (20 to 200 µg/ml) was mixed with 1 ml of 95 % methanol, followed by the addition of 0.1 ml of 10 % Aluminum chloride, 0.1 ml of 1.0 M Sodium acetate, and 2.8 ml of distilled water. After 30 minutes of room temperature incubation, the absorbance was measured at 415 nm. The blank was distilled water. To determine the concentration of total flavonoids in the extract, the obtained absorbance was interpolated from the standard curve by replacing quercetin with the appropriately diluted extract samples and following the same procedure.

2.6. Gas chromatography and mass spectrometry (GC-MS) analysis

Using gas chromatography and mass spectrometry (Model: QP2010S; Shimadzu Corporation, Japan), an advanced analytical instrument equipped with a Rxi-5Sil MS capillary column (dimensions: 30 m×0.25 mm ID, film thickness: 0.25 µm), the chemical compounds present in the methanolic extracts of the samples were separated. Helium (99.9995%) was the carrier gas, flowing at 1 ml/min constant. After being kept at 60 °C for five minutes, the temperature in the column oven was raised to 260 °C at a rate of 5 °C per minute. One µl diluted samples were injected with a 4-minute solvent delay in the split injection method. Thirty minutes was the whole run time. Two temperatures were set: 200°C for the ion source and 280°C for the interface line. The separated components were identified by comparing the retention periods of natural compounds with mass spectra from NIST 11 & WILEY 8 mass spectral libraries.

2.6. FTIR analysis of PRM

The methanolic leaf powder Soxhlet extraction was used for the Fourier transform infrared (FT-IR) spectrum of Phyllanthus reticulatus was obtained using a spectrometer (Nicolet IN – 10) in the 400–4,000 cm-1 range.

Antioxidant Tests

2.7. DPPH free radical-scavenging activity

Using the DPPH radical as a reagent, the radical scavenging activities of several *Phyllanthus reticulatus* leaf extracts were ascertained using the techniques described by Rice-Evans in 1997 (10). 100 µl of a sample solution in ethanol and 100 µl of a DPPH radical solution in ethanol (60 µM) were combined. After 30 minutes of dark incubation at room temperature, the absorbance was measured at 517 nm using a UV-visible spectrophotometer (Eppendorf Bio Spectrometer Basic Model #6135). The following formula was used to determine the DPPH scavenging activity: Inhibition percentage = Ac- At/Ac x 100

Where At is the test sample's absorbance, and Ac is the control reaction's absorbance (100 µl of ethanol with 100 µl of the DPPH solution). For every sample that was used, the IC50 value was determined. The reaction mixture's lower absorbance suggested higher levels of free radical activity.

2.8. Phospho-molybdenum (PM) assay

The total antioxidant activity was calculated for the PM experiment by applying the Prieto et al. standard approach in 1999 (11). The leaf extracts of *Phyllanthus reticulatus* were placed in test tubes with 3 ml of distilled water, 1 ml of molybdate reagent solution, and a concentration range of 100 µl to 500 µl. For ninety minutes, these tubes were incubated at 95 °C. After incubation, these tubes were allowed to reach room temperature, and the reaction mixture absorbance was measured at 695 nm.

2.9. MTT ASSAY

A 96-well plate was seeded with 10×104 cells/ml of subconfluent HCT-116 cells in full DMEM, and the cells were cultured for 48 hours. Following a PBS wash, cells were incubated in an incomplete DMEM medium for 24 hours at varying doses of PRM (62.5 to 1000µg/ml). 50 µl of MTT (5 mg/ml) in DMEM was added to each well, and the mixture was incubated for two hours at 37 °C. After that, 100 µl of DMSO was used to dissolve the formed formazan crystals. A repetition of this procedure is followed at each time point. At 570 nm and 630 nm wavelengths, the absorbance was measured using a microplate reader (Tecan i-200 Pro). The background and blank were subtracted to determine the percentage growth inhibition. It was compared the PRM-treated cells' % cell vitality with the untreated as control. Statistical analysis: Every experiment was carried out three times in duplicate with three distinct lots. Microsoft Office Excel 2007 was used to calculate the mean and percentage standard deviations.

3. Results

3.1 Phytochemical analysis

Phyllanthus reticulatus methanolic leaf extract contains secondary metabolites such as flavonoids, phenols, alkaloids, steroids, tannins, and other biologically active compounds as summarized in Table 1.

3.2. Elemental analysis

The human body requires a wide variety of minerals and trace elements, which are abundant in the leaves of *Phyllanthus reticulatus.* The leaves of *Phyllanthus reticulatus* contain a variety of minerals. The amount of nitrogen (N), phosphorous (P), potassium (K), sulfur (S), sodium (Na), boron (B), copper (Cu) magnesium (Mg), zinc (Zn), iron (Fe), calcium (Ca), and manganese (Mn) were identified and summarized in Table 2.

3.3. Determination of total phenolics and flavonoid contents

The entire phenolic content of the methanolic leaf extract was found to be an average value of 93.44 mg/g of

phenolics in higher proportion followed by 55.06 mg/g of flavonoids in the leaves of methanolic leaf extract of *Phyllanthus reticulatus* was calculated.

3.4. Gas chromatography and mass spectrometry (GC-MS) analysis

To get insight into the chemical composition of *Phyllanthus reticulatus* leaves, GC-MS analysis was carried out. PRM chromatogram showed the presence of nine primary spectral peaks as shown in Figure 1, each of which had active biological activity as represented in Table 3.

3.4 FTIR Analysis

The PRM used in FTIR spectroscopy analysis investigated six significant peaks, each corresponding to a separate functional group. The peaks in the ethanol extract are located within the spectral range of 3306 to 610 cm -1 as shown in Figure 2. The methanol extract of the leaf showed the presence of total of six active functional groups. These functional groups include alkanes, aromatic, carboxylic acids, esters, ethers, aliphatic amines, and alkyl halogenated compounds shown in Table 4.

Table 1. Phytochemical Analysis of *Phyllantus reticulatus*

Constituents	Test	Present/Absent
Proteins	Millon's	+
	Ninhydrin	+
	Fehling's	+
Carbohydrates	Benedict's	+
	Molisch's	+
	Iodine	-
Phenols	Phenols	+
Tannins	Tannins	+
Flavonoids	Shinoda	+
	Alkaline reagent	+
Saponins	Foam	+
Glycosides	Liebermann's	+
	Salkowskis	+
	Keller-Kilani	+
Steroids	Liebermann-Burchard	+
Terpenoids	Terpenoids	-
Alkaloids	Mayer's and Wagner's	+

Table 2. Elemental concentrations of *Phyllantus reticulatus* leaves

Sl.no	Sample	PRL
1	N	1.475 %
2	P	0.210 %
3	K	1.69 %
4	S	0.193 %
5	Ca	1.7 %
6	Mg	0.66 %
7	Na	0.078 %
8	Zn	480.5 PPM
9	Fe	1132 PPM
10	Mn	84 PPM
11	Cu	13.9 PPM
12	B	26.9 PPM

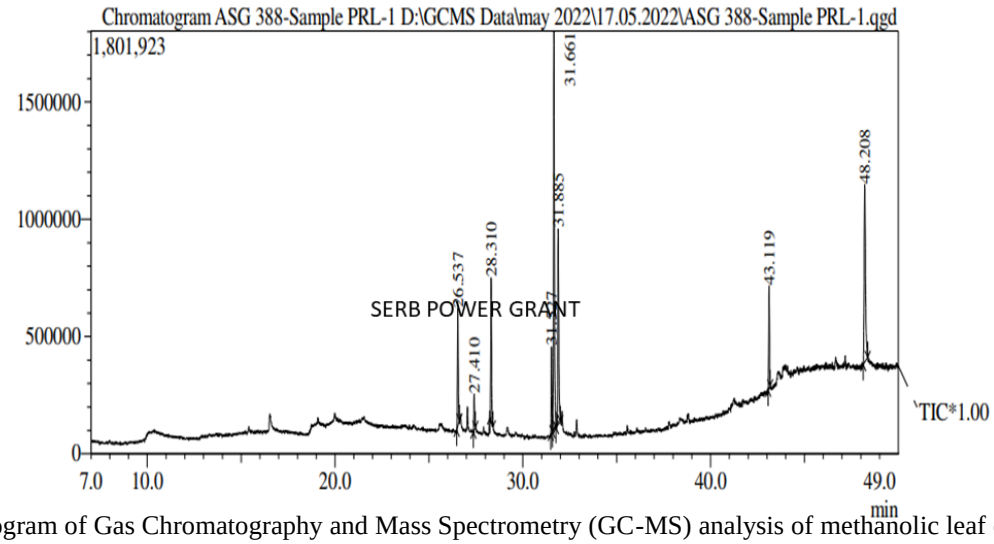

Figure 1. Chromatogram of Gas Chromatography and Mass Spectrometry (GC-MS) analysis of methanolic leaf extract of *Phyllanthus reticulatus.*

Table 3. GC-MS analysis of methanolic leaf extract of *Phyllanthus reticulatus*

Sl. No.	Chemical compounds	Retention Time	Area%	Biological activity
1	Neophytadiene	26.537	8.84	Anxiolytic-like activity (10)
2	3,7,11,15-Tetramethyl-2-Hexadecen-1-ol	27.410	2.43	Possess antimicrobial, anti-inflammatory activity (11).
3	Methyl Palmitate	28.310	9.46	anti-inflammatory (12)
4	Methyl Octadeca-9,12-Dienoate	31.527	5.07	antimicrobial and antioxidant activities (13)
5	Alpha-LinolenicAcid Methyl Ester	31.661	29.87	Used to cure heart diseases (14).
6	Phytol	31.885	16.55	for the treatment of rheumatoid arthritis, antiflammatory, antimicrobial, anticancer, diuretic, (15,16)
7	Squalene	43.119	6.80	Antibacterial, antitumor and anticancer preventive agent (15).
8	Vitamin-E	48.208	20.98	Antibacterial, antioxidant, immunostimulant, anticancer (17).

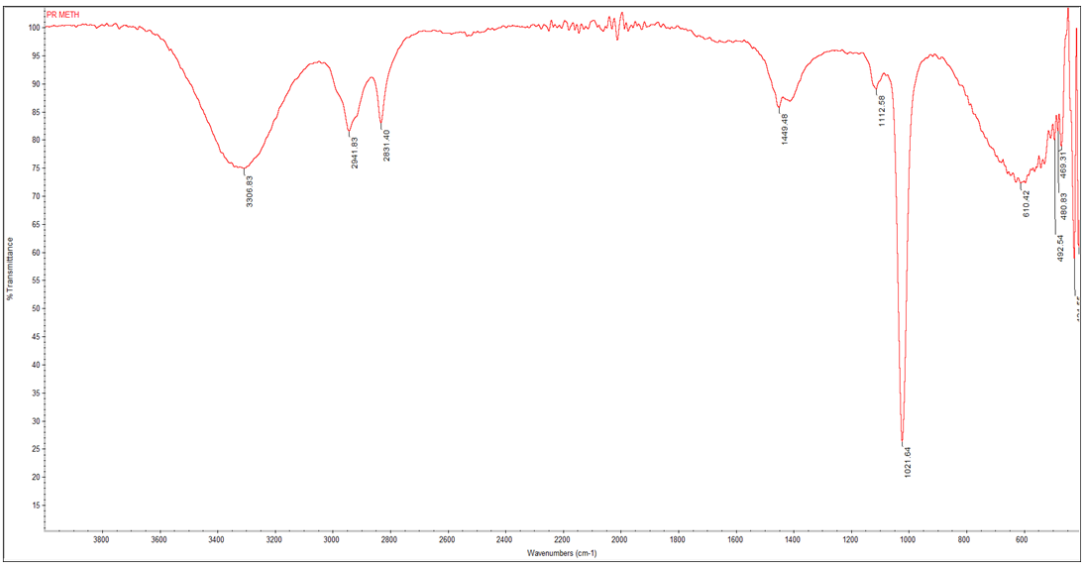

Figure 2. FTIR Spectrum of the *Phyllanthus reticulatus* methanolic leaf extract.

Table 4. FTIR analysis of methanolic leaf extract of *Phyllanthus reticulatus*

Sl. No.	Wave Number (cm-1)	Bond/Mode of Vibration	Functional Group
1	3306	–C–H stretch	Alkanes
2	2941	-C–H stretch	Alkanes
3	1449	-C–H bend or scissoring	Aromatics
4	1112.58	C-O-C	Saccharides
5	1021.64	-C-N stretching	Carboxylic acids, Esters, Ethers, Aliphatic amines
6	610.42	-C-Br stretch	Alkyl halides

In-vitro antioxidant activities

3.5. DPPH radicals scavenging activity

Because of their lone pair of electrons, DPPH free radicals had a strong purple color. The antioxidants in the samples scavenged the lone pair of electrons, which caused the DPPH-containing solution to become less pigmented. As a result, the methanolic extracts from the samples' antioxidant potential is closely related to the decrease in purple hue. The µg/ml value indicated the concentration-dependent scavenging ability against DPPH radicals. Methanolic leaf extract's DPPH radical scavenging capacity was demonstrated by its IC_{50} value, which was 50. 75 µg/ml as shown in the Figure 3A. This value was less than that of ascorbic acid.

3.6. Antioxidant activity by PM Assay

In this case, we have observed the antioxidant activity of PRM was moderate as compared to ascorbic acid as shown in Figure 3B. The moderate increase in activity was observed as the concentration of PRM increases. This confirms the antioxidant activity of PRM increases with increase in the concentration of the extract similar to the DPPH assay.

3.7. Effect of PRM on HCL-116 colon cancer cell line by MTT assay

PRM-induced cell growth inhibitory effects on HCL-116 colon cancer cells in a dose-dependent manner. PRM (1000 µg/ml) inhibits the growth of HCL-116 cells by 28.56% at 24 h as shown in Figure 4A. The PRM is effective at higher concentration, as the concentration decreases, percentage cell growth inhibition decreases as shown in the Figures. As it's visible in the image, at a higher concentration of PRM, cells look like stressed as visible in Figure 4B, which may be due to the release of reactive oxygen species compared to a lower concentration of PRM.

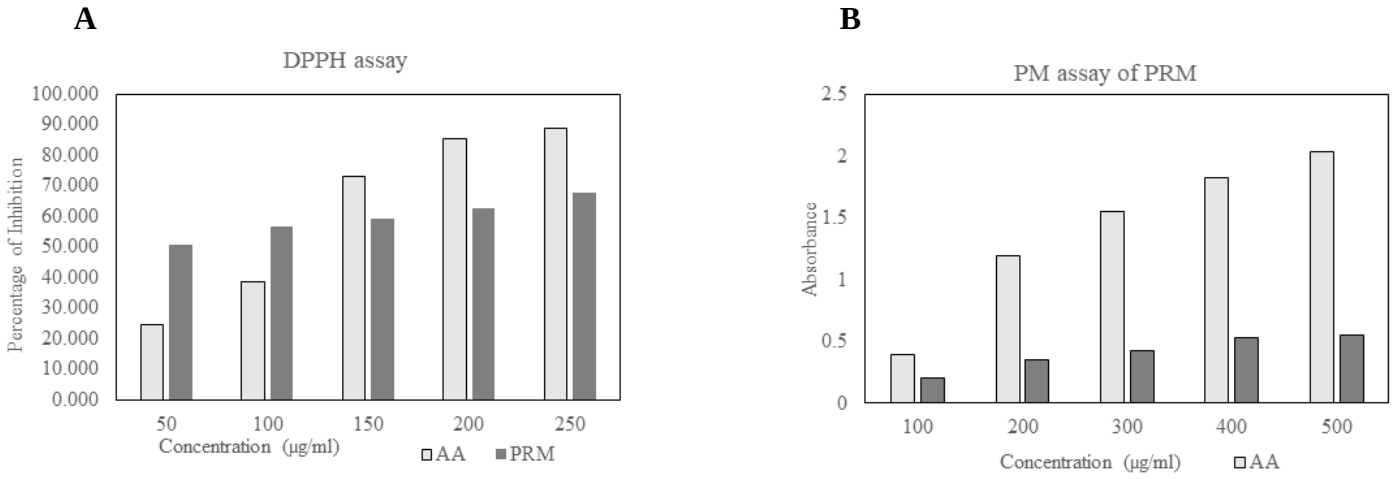

Figure 3. (A). DPPH radical scavenging activity of PRM. **(B).** Phospho-molybdenum (PM) activity of PRM

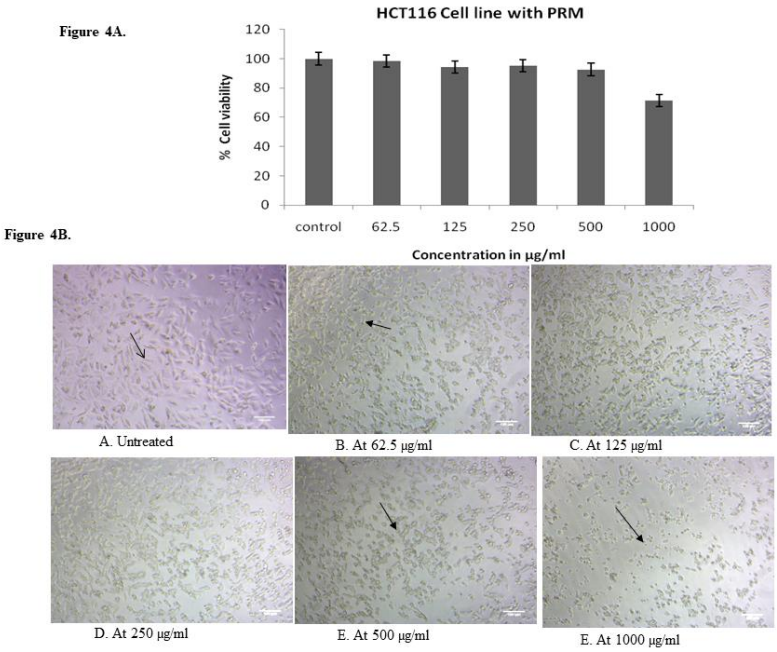

Figure 4. PRM shows colon cancer cell growth inhibition by MTT assay:

A. Bar graph shows the % of cell growth inhibition at different concentration of PRM at 24 h.

B. HCT-116 colon cancer cells under stress at various concentrations of PRM at 24 h. Arrows mark pointing shows the stressed and normal cells at different concentrations.

4. Discussion

Medicinal plants offer a new perspective on the search for novel pharmaceuticals. Traditional and alternative medicines have recently gained momentum as novel, scientifically convenient selections (20). Natural phytochemical elements from medicinal herbs have significantly controlled several human medical ailments, such as cancer (21, 22). The literature review revealed the potential medicinal properties of *Phyllanthus reticulatus* leaves, particularly in managing diabetes and diarrhea. The leaf extract was given orally at doses of 150 mg/kg and 300 mg/kg, according to the in-vivo investigations, and it showed a substantial antidiabetic effect in comparison to the control (23). *P. reticulatus* demonstrated protective effects against gastric ulcers through anti-inflammatory and immunomodulatory activities, as evidenced by improvements in histopathological parameters and reduction in TNF-α and IL-8 levels (24). *Phyllanthus reticulatus* methanolic leaf extract showed over 90% inhibitory effect on HIV-1 ribonuclease H (RNase H) at a concentration of 50 mg/mL (25). The methanolic extract of leaves of *Phyllanthus reticulatus* has demonstrated anti-inflammatory, analgesic, and CNS depressant properties (26). The study conducted in the foothills of Velliangiri Hills, part of the Western Ghats in Tamil Nadu, provides valuable insights into using *Phyllanthus reticulatus* for oral health in the specific region. *Phyllanthus reticulatus* as a natural remedy for oral health issues. It also emphasizes the importance of traditional practices, such as chew sticks and dried leaf powders, in maintaining oral hygiene (27). Ethanolic extract of *Phyllanthus reticulatus* leaves showed good antidermatophytic activity on *Malassezia pachydermatis* and *Trichophyton rubrum* at 62.5 and 250µg/mL respectively (28). The iron nanoparticles synthesized from leaf extract of *phyllanthus reticulatus* showed potent antibacterial and antifungal activity including biofertilizer for the growth of green gram seeds (29). Ethyl acetate extract of *Phyllanthus reticulatus* reveled antiproliferative effect on human liver tumor cell line BEL-7404 and Hep-G2 cancer cell line at 2.48 and 6.34 mg/mL concentration by inducing apoptosis in HepG2 cancer cell line through PI3K/Akt pathway (30). The scientific understanding of the pharmacological activities of *Phyllanthus reticulatus* leaves, as a traditional medicinal plant, and their potential health benefits made this study successful in identifying biologically active chemical components of the plants such as phenols, flavonoids, saponins, tannins, steroids, alkaloids were identified. As our results support the antioxidant and anticancer property of the leaf extract is due to the presence of active phytochemicals like phenols and flavonoids including GC-MS and FTIR analysis identified bio-constituents. The GC-MS analysis chromatogram exhibited the existence of 9 major compounds with active biological functions such as enzyme inhibitory activity with anti-inflammatory, antimicrobial, anti-angiogenic antihelmintic, antimutagenic, antiprotozoal, antioxidant, anticancer, diuretic, immunostimulant. Also, some of the identified constituents are used for the treatment of rheumatoid arthritis, heart diseases, and other chronic inflammatory diseases, including inhibition of platelet aggregation as summarized in results. This will be evidenced by FTIR spectroscopic

analysis for identifying 7 major peaks with active functional groups. The chromatogram of FTIR analysis showed the presence of 7 major peaks, each consistent for the presence of specific functional groups. These groups are known to be involved in antiinflammation, antioxidant, anticancer, and other properties, as verified from our results except antiinflammation (31). The cell viability assay by MTT using methanolic leaf extract showed 28.51 % cell growth inhibition of HCT-116 colon cancer cell line at 24 h in a dose-dependent manner, exhibiting the anticancer effect of *Phyllanthus reticulatus*. The PRM is effective only at higher concentrations, less effective at lower concentrations is the limitation of PRM for its anticancer activity. The study's findings suggest that the methanolic extract of *Phyllanthus reticulatus* leaves has potential therapeutic applications as antioxidant and anticancer properties. The extract's observed pharmacological effects may be attributed to its bioactive constituents, which certify further investigation into developing novel natural medicinal plant drugs. The future experiments are identifying and purifying a particular active novel molecule to understand its chemical principle by spectroscopic studies and discovering the mechanism involved in its active principles. Further this purified biologically active novel molecules studied for its effect in animal models by in vivo experiments to explore in cancer biology studies.

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Authors' Contribution
Study concept and design: K.Y.H, S.S, U.J, A.A.P, A.B, B.N.A, V.G, S.M, S.S.H, S.C.T
Acquisition of data: K.Y.H, S.S, U.J, A.A.P, A.B, B.N.A, V.G, S.M, S.S.H, S.C.T
Analysis and interpretation of data: K.Y.H, S.S and U.J
Drafting of the manuscript: K.Y.H, U.J
Critical revision of the manuscript: K.Y.H, U.J

Ethics
We have not used any animals in this study to take ethical approval and we have used standardised protocols and procedures in this study by citing the referred articles.

Conflict of Interest
The authors declare that they have no conflict of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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