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Research Article

In-Vitro Antineoplastic Potential of *Aristolochia indica* and *Psidium guajava* Leaves in MCF-7 Cell Line

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Abstract

Cancer stands as the foremost contributor to mortality globally and poses a substantial impediment to increasing life expectancy across all regions. Despite being the focus of multimodal treatments, cancer remains the primary cause of death worldwide. The undesirable repercussions linked with traditional therapies, along with the emergence of resistance to them, necessitate exploration into alternative therapeutic options. Hence, this current investigation was initiated to assess the *in-vitro* antineoplastic potential of *Aristolochia indica* and *Psidium guajava* leaves in the MCF-7 cell line and to compare the effects with those of doxorubicin.

The leaves of *A. indica* and *P. guajava* were collected, cleaned, shade dried, extracted using methanol with Soxhlet apparatus and concentrated using a rotary vacuum evaporator. The concentrated extract was then fractionated based on the ascending polarity of solvents such as hexane (HF), dichloromethane (CF), n-butanol (BF), and water (WF), before being preserved for future use. The phytochemical constituents of methanolic extract of the plants were analyzed qualitatively.

The cytotoxicity potential of methanolic extract and other fractions were assessed using MTT assay at the doses of 10, 20, 40, 80 and 160 µg/mL from which per cent cell viability, per cent inhibition and half-maximal inhibitory concentration (IC_{50}) were calculated and the fraction showing lowest IC_{50} from both plants were selected for further studies. Doxorubicin was used as a positive control. Following the determination of the IC_{50} concentration for the most potent fraction, apoptotic changes were examined using dual acridine orange (AO)-ethidium bromide (EB) staining. The effect of the most potent fraction on the expression of *Bcl-2* and *GPER* genes were studied using real time-quantitative polymerase chain reaction (RT-qPCR) with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as house-keeping gene. The phytochemical constituents of the most potent fraction were analyzed qualitatively and with Gas Chromatography-tandem Mass Spectrometry (GC-MS/MS) and Fourier Transform Infrared (FTIR) spectroscopy.

Keywords: Antineoplastic; *Aristolochia Indica*; *Psidium Guajava*; MTT Assay; GC-MS; FTIR

Abbreviations

A. Indica: Aristolochia Indica; P. Guajava: Psidium Guajava; MCF-7: Michigan Cancer Foundation-7; HF: Hexane Fraction; CF: Dichloromethane Fraction; BF: Butanol Fraction; WF: Water Fraction; IC_{50}: Half Maximal Inhibitory Concentration; AO: Acridine Orange; EB: Ethidium Bromide; Bcl-2: B- Cell Lymphoma 2; GPER: G Protein Coupled Estrogen Receptor; GAPDH: Glyceraldehyd-3-Phosphate Dehydrogenase; RT-qPCR: Real Time-Quantitative Polymerase Chain Reaction; GC-MS/MS: Gas Chromatography-Tandem Mass Spectrometry; FTIR: Fourier Transform Infrared Spectroscopy

Introduction

Cancer remains a pervasive threat to human and animal health, exerting a significant toll worldwide despite advances in early detection. It is a leading cause of mortality, striking over one-third of the world's population. It is the cause of more than 20 per cent of all deaths. Mammary tumour is the primary cause of cancer death in women globally. In dogs, it occurs twice as frequently as in humans, have histopathological features and biological behaviour similar to human tumours, and progress more rapidly than in humans.

Chemotherapy is resorted to the maximum among the different modalities of cancer therapy. These chemicals are capable of destroying cancer cells, keeping them from growing and spreading, shrinking the size of the tumour, or relieving the cancer symptoms. However, chemotherapy can destroy or slow down the growth of normal cells, including those of the hair, mouth, and digestive system, as well as those of the blood. Therefore, the search for novel anticancer agents with enhanced potency and reduced toxicity remains a priority in oncological research.

Cell lines serve as invaluable tools, offering reproducibility and scalability in laboratory investigations, particularly, as *in vitro* models in cancer research. Michigan Cancer Foundation-7 (MCF-7) cell line, derived from human breast adenocarcinoma, is a widely utilized model in cancer research, particularly in assessing the efficacy of potential anticancer agents.

Plants have emerged as promising candidates for various ailments, including cancer due to their diverse chemical composition, potential pharmacological activities, high safety margins and lower costs. *Aristolochia indica* (*A. indica*) and *Psidium guajava* (*P. guajava*), commonly known as Indian birthwort and guava, respectively, have been traditionally used in various medicinal systems for their purported therapeutic properties. Among their diverse bioactivities, their potential anticancer effects have gained considerable attention in recent years. The phytoconstituents present in *A. indica* and *P. guajava* likely contributes to their anticancer properties by targeting various hallmarks of cancer, including proliferation, apoptosis evasion, angiogenesis and metastasis.

In recent years, advancements in molecular biology have shed light on the intricate mechanisms underlying cancer development and progression. Among these, the *B-cell lymphoma 2* (*Bcl-2*) and the *G protein-coupled oestrogen receptor* (*GPER*) have emerged as pivotal players in regulating cellular processes associated with cancer, including apoptosis and signalling pathways involved in cell proliferation. Understanding the interplay between the natural compounds and key cancer-associated genes such as *Bcl-2* and *GPER* holds promise for uncovering novel therapeutic strategies and enhancing comprehension of the molecular mechanisms underlying their anticancer effects.

Hence the present study was designed to explore the anticancer potential of *A. indica* and *P. guajava* extracts with the following objectives

- Identification of potent fractions from methanolic leaf extracts of *Aristolochia indica* (Eswaramooli) and *Psidium guajava* (Guava) with anticancer properties in MCF-7 breast cancer cell line.
- Assessing the effect of potent fractions on apoptosis and relative expression of *Bcl-2* and *GPER* genes.
- Phytochemical analysis of the potent fractions by Gas Chromatography-Mass Spectroscopy (GC-MS) and Fourier Transform Infrared Spectroscopy (FTIR).

Materials and Methods

Plant materials

Leaves of *Aristolochia indica* (Eswaramooli) and *Psidium guajava* (Guava) were locally collected from Kannur and Wayanad, Kerala. They were identified and authenticated by a botanist at Kannur University, Mananthavady Campus, Wayanad, Kerala.

Preparation of methanolic extract of *A. indica* (MAI) and *P. guajava* (MPG) leaves

Leaves of *A. indica* and *P. guajava* were collected, cleaned, and shade-dried on paper. The dried leaves were coarsely pulverized with an electric pulverizer, weighed, and packed in a thimble for Soxhlet extraction with methanol (99%, v/v) at 67°C. The methanolic extracts were concentrated using a rotary vacuum evaporator at 40°C, dried, and scraped off. The dried extracts were stored in a wide-necked container at -20°C until further use.

Preparation of different fractions of methanolic extract of *A. indica* and *P. guajava* leaves

The methanolic extracts of both plants were mixed with hexane (150 mL) in a separating funnel, and the soluble fractions were collected. The insoluble fractions were then mixed with dichloromethane (150 mL), and the soluble fractions were collected. The remaining insoluble portions were mixed with n-butanol (75 mL) and distilled water (75 mL), separated, and the fractions were extracted in ascending polarity. After fractionation, the solvents were removed using a rotary vacuum evaporator at 172 mbar and 40°C. Hexane fraction (HF), dichloromethane fraction (CF), butanol fraction (BF), and water-soluble fraction (WF) were obtained.

Screening of phytochemicals

The methanolic extracts from the leaves of *A. indica* and *P. guajava* were analyzed for phytochemical components, including steroids, phenolic compounds, alkaloids, flavonoids, diterpenes, triterpenes, and saponins.

In vitro antineoplastic activity of methanolic leaf extracts and fractions of *A. indica* and *P. guajava*

The human breast cancer cell line MCF-7 was procured from the National Centre for Cell Science (NCCS), Pune. Cells were incubated in a CO_2 incubator at 37°C with 95% air and 5% CO_2. When 70-90% confluent, cells were passaged. Methanolic extracts and fractions of *A. indica* and *P. guajava* were prepared in 1% DMSO at 5 mg/mL, then diluted to working solutions of 160, 80, 40, 20, and 10 µg/mL in the growth medium. Doxorubicin served as the standard control.

3-(4,5- dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

Cytotoxicity was assessed using the MTT assay. Cells were seeded and observed under an inverted microscope for even attachment. The medium was removed, and cells were treated with the extracts, fractions, and Doxorubicin at various concentrations for 24 hours. After treatment, the medium was removed, and 10 µL of MTT (5 mg/mL) in 100 µL RPMI-1640 without FBS was added. Cells were incubated at 37°C with 5% CO_2 for four hours in the dark. The medium with MTT was then removed, and the purple formazan crystals were dissolved in 200 µL of DMSO. Absorbance was read at 570 nm using an ELISA plate reader. Cell viability and inhibition were calculated using the following formulas

$$\text{Per cent cell inhibition} = \frac{\text{Mean OD Control x Mean OD Treatment}}{\text{Mean OD Control}} \times 100$$

Per cent cell viability = 100 - per cent cell inhibition

The half-maximal inhibitory concentration (IC_{50}) for the treatments was calculated using GraphPad Prism version 5.00. Fractions showing lower IC_{50} values were selected for further studies.

Dual Acridine orange/ethidium bromide (AO/EB) staining

Cells from a sub-confluent flask were trypsinized and seeded into six-well plates at a concentration of 1 x 10^7 cells/well, then allowed to grow for 24 hours. The cells were treated with the IC_{50} concentration of the hexane fraction (HF) of *A. indica* and *P. guajava*, as well as doxorubicin, for 24 hours. One well was left as a negative control. After 24 hours, the spent medium was removed, and the cells were trypsinized (250-500 µL of 0.25% Trypsin-1 X EDTA/10 cm^2 surface area), centrifuged, and the pellet resuspended in the medium. Twenty-five microliters of the cell suspension were mixed with AO/EB stain solution (10 µg/mL each of AO and EB) and examined under a trinocular research fluorescence microscope (DM 2000 LED, Leica) with blue excitation (488 nm) and emission (550 nm) filters at 20X magnification.

Relative gene expression study

The technique of real-time - quantitative polymerase chain reaction (RT- qPCR) was employed to study the relative expression of *B-cell lymphoma-2* (*Bcl- 2*) and *G protein-coupled oestrogen receptor 1* (*GPER*) in cell culture samples, for which *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as the housekeeping gene.

The MCF-7 cells were seeded onto a six-well plate and treated with HF of *A. indica* and *P. guajava* at their IC_{50}, half IC_{50}, and double

the IC_{50} concentrationsagainst the control cells. Doxorubicin at its IC_{50} was used as the positive control. After 24h of treatment, the cells were trypsinized (250-500 µL of 0.25% Trypsin- 1X EDTA/10 cm^2 of surface area) and the cell suspension was centrifuged at 3000rpm for three to five minutes, washed thrice with PBS (pH 7.4) and then used for ribonucleic acid (RNA) isolation.

The total RNA was isolated from prepared cell culture samples using TRIzolor TRI Reagent. One millilitre of ice-cold TRI reagent® was added to the cell pellet, mixed thoroughly by pipetting, and incubated for five minutes at room temperature, for complete dissociation of nucleo-protein complexes. After incubation 0.2 mL of chloroform (per millilitre of TRI reagent® used) was added to each sample and thetubes were shaken vigorously for 15 sec and allowed to stand at room temperaturefor 10 min. The resultant mixture was centrifuged at 12,000 x g for 15 min at 4°C. The upper clear aqueous phase rich in RNA was transferred to a fresh microcentrifuge tube and 0.5 mL of ice-cold isopropanol (per millilitre of TRI reagent® used) was added for precipitation of RNA. The tubes were incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was carefully decanted and the RNA pellet was washed withone millilitre of 75 per cent ethanol (per millilitre of TRI reagent® used). The samples were then centrifuged at 12,000 x g for five minutes at 4°C. The supernatant was discarded and the pellet was air-dried by inverting the tube on fresh paper towels for five minutes to remove traces of ethanol. The RNA pellet was dissolved in nuclease-free water (40µL).

After isolation, the RNA was quantified by spectrophotometric analysis with a Nanodrop spectrophotometer, using the principle that one absorbance unit at260 nm wavelength equals 40 µg RNA per mL. The purity of total RNA was asseszed by using the ratio of absorbance of the samples taken at 260 nm, 280 nm, and 230 nm in a spectrophotometer. The samplesshowing A_{260}/A_{280} and A_{260}/A_{230} ratios between 1.8 - 2.0 or above were consideredof ideal purity and taken for further study.

The cDNA synthesis was carried out from total RNA using a verso cDNA synthesis kit as per the manufacturer's protocol. After thawing, mixed and briefly centrifuged all components of the kit andkept in ice. Ten microlitres of nuclease-free water were added to a 0.2 mL PCR tube. The template RNA (volume to get 500 ng) and one microlitre random hexamerto each tube, were added and mixed gently and the tubes were snap chilled on ice. To this, four microlitres of 5X cDNA synthesis buffer, two microlitres of dNTP mix, and one microlitre each of RT enhancer and enzyme mix for each tube were added. The total reaction volume was made to 20 µL using sterile nuclease-free water, mixed gently, and spun at 1000 rpm for about two minutes. Then the tubes were incubated at 42 °C for 60 minutes. The reaction was terminated by heating the reaction mixture at 95°C for five minutes. The product was stored at -80 °C until use.

The optimum annealing temperature for the primers to amplify the Bcl-2, GPER, and GAPDH genes was determined by gradient PCR (Biorad, M/s Thermal cycler, USA). The conditions were optimized by setting different temperatures for the annealing process. The temperature that gave the best result for amplification was taken for further studies. The details of gradient PCR conditions for amplification are given in table 1.

Sl. No.	Steps	Temperature		Time
1	Initial denaturation	95°C		5 min
2	Denaturation	95°C		45 sec
3	Annealing	*Bcl-2*	58.6°C	30 sec
		GPER	63.3°C	
		GAPDH	54.65°C to 64.65°C	
4	Extension	72°C		1 min
5	Step 2 to 4 repetition → 34 cycles			
6	Final extension	72°C		10 min

Table 1: Gradient PCR conditions for amplification.

The expression of *Bcl-2, GPER,* and *GAPDH* was studied using SYBR green chemistry (Maxima SYBR green qPCR master mix) (M/s Thermo Scientific,USA). The reaction was carried out in triplicates (Table 2). The expression of target gene *Bcl-2* and *GPER* was compared with a reference gene *GAPDH* to calculate ΔCq and the expression of the same gene in treatment samples versus control samples to calculate ΔΔCq. The method used is referred to as the comparative Cq method.

Components	Volume (µL)
Template (cDNA)	1.0
Maxima SYBR Green qPCR Master Mix (2X)	6.25
Forward Primer (10 *pM*/µl)	0.5
Reverse primer (10 *pM*/µl)	0.5
Nuclease free water	4.25

Table 2: Optimized concentrations of RT-qPCR Master Mix (12.5 µL) for *Bcl-2,GPER* and *GAPDH* genes.

Separate PCR reactions were set up for genes. Each sample was amplified in triplicate (technical replicates). In addition, one non-template control (NTC) for each gene, Reverse Transcription minus (RT minus) control for each sample, and negative control (with only nuclease-free water) were also included in the reaction.A suitable plate setting was done before the start of the experiment. Master Mix with template DNA was loaded into the designated well of PCR strips (8 tubes/strip). The strips were centrifuged at 250 g for 30 seconds and were placed ina StepOne Plus® Realtime Thermal cycler. Fluorescence signals were measured ineach cycle. For each sample, the curve was generated after the completion of amplification and was analyzed in positive and negative controls to detect the specificity of the PCR reaction. The thermal cycler was pre-programmed fortemperature and cycling conditions specified in table 3.

Steps		Temperature		Time
Initial denaturation		95˚C		3 min
34 cycles of	Denaturation	94˚C		30 sec
	Annealing	*Bcl-2*	58.6˚C	30 sec
		GPER	63.3˚C	
		GAPDH	62.9˚C	
Extension		72˚C		60 sec

Table 3: qRT-PCR conditions for *Bcl-2, GPER,* and *GAPDH* gene.

A melt curve analysis was performed after the reaction to check the specificity of amplification. The programme for melt curve analysis consisted of denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec followed by 95°C for15 sec. Data acquisition was performed during the final denaturation step.

Relative quantification describes the change in expression of the target samplerelative to some reference group such as an untreated control. *Bcl-2 and GPER* quantification was done by Ct (Cycle Threshold) comparative method and was expressed as 'n' fold up-regulation of the transcribed gene in relation to the calibrator. For relative quantification by comparative method, the values were expressed relative to the control sample called the calibrator. *GAPDH* was used as the internal control. The Cq of the target gene and Cq of the control gene were determined for each sample and calibrated (ΔΔCq method).

$$\Delta Cq = Cq \text{ (target gene)} - Cq \text{ (reference gene)}$$

$$\Delta\Delta Cq = \Delta Cq \text{ (test sample)} - \Delta Cq \text{ (control sample)}$$

$$\text{Relative quantification, } RQ = 2^{-\Delta\Delta Cq}$$

Analysis of potent fraction of leaves of *A. indica* and *P. guajava*

The phytochemical constituents and structurally similar molecular compounds in the most potent extract from leaves of *A. indica* and *P. guajava* were analyzed using GC-MS/MS and FTIR respectively.

Results and Discussion

The methanolic extracts of *A. indica* (MAI) and *P. guajava* (MPG) were prepared using a hot continuous extraction method employing a Soxhlet extraction apparatus. The extraction yield, as outlined in table 4, was calculated relative to the initial dry mass of the plant material. In the current investigation, the methanolic extract of *A. indica* leaves (MAI) yielded 20.09%, higher than the 16% [10], likely due to seasonal and geographical variations. The methanolic extract of *P. guajava* leaves (MPG) yielded 33.48%, consistent with the 35.44% [7] using a 70% methanol solution.

SL No.	Sample	Sample weight (g)	Extract weight (g)	Extraction yield (%)
1	MAI	69.98	14.06	20.09
2	MPG	72.45	24.26	33.48

Table 4: Extraction yield of MAI and MPG using the hot continuous extraction method using Soxhlet apparatus.

The methanolic extracts obtained from *A. indica* and *P. guajava* leaves were fractionated based on increasing polarity of solvents using hexane, dichloromethane, n-butanol and aqueous fractions. The per cent yield obtained fromMAI and MPG are outlined in Table

6. In the current study, yields of hexane, dichloromethane, n-butanol, and aqueous fractions from *A. indica* were 16.8%, 14.2%, 13.6%, and 17.2%, respectively. Bhatnagar and Maharana [10] reported 4% for the hexane fraction and 2% for the chloroform fraction. The higher hexane yield in this study may be due to collection time and geographical variations. For *P. guajava*, yields were 19.0%, 15.0%, 11.6%, and 18.3%, respectively. Metwally., *et al*. [24] reported yields of 6% for petroleum ether, 2.7% for chloroform, 9.7% for ethyl acetate, and 28.5% for n-butanol. The lower n-butanol yield in this study might be due to differences in collection time and location.

FRACTIONS	PER CENT YIELD (%)	
	Aristolochia indica	***Psidium guajava***
Hexane	16.8	19.0
Dichloromethane	14.2	15.0
n-Butanol	13.6	11.6
Aqueous	17.2	18.3

Table 5: Extraction yield of fractions from MAI and MPG.

The outcome of the phytochemical analysis of MAI and MPG is depicted in Table 6. In the present study, qualitative analysis of the methanolic extract of *A. indica* leaves (MAI) demonstrated the presence of alkaloids, phenolic compounds, tannins, flavonoids, saponins, and triterpenes. Murugan and Mohan [27] recorded similar compounds along with coumarin, sugars, terpenoids, and xanthoprotein. Janani and Prasanna [19] also identified additional compounds such as quinines, cardiac glycosides, steroids, and phytosteroids. These findings align with the current study. For *P. guajava* leaves (MPG), the qualitative screening revealed steroids, alkaloids, glycosides, phenolic compounds, tannins, flavonoids, and saponins. Growther and Sukirtha [16] reported tannins, alkaloids, flavonoids, and saponins. These results are consistent with the current study. The anticancer activity of phytochemicals such as steroids, alkaloids, tannins, flavonoids, and terpenes has been established [13]. Therefore, the presence of these compounds in MAI and MPG could account for the extracts' cytotoxic effects, inducing apoptosis in cancerous cells.

The MTT assay was used to evaluate the *in vitro* cytotoxicity of methanolic extract and fractions of *A. indica* and *P. guajava* in MCF-7 cells. The study showed that all extracts and fractions exhibited concentration-dependent cytotoxic effects. The IC_{50} values for *A. indica*'s methanolic extract, hexane, dichloromethane, n-butanol, and aqueous fractions were 39.68 ± 1.43, 78.9 ± 1.80, 87.67 ± 1.48, 86.07 ± 1.03, and 118 ± 1.10 µg/mL, respectively. For *P. guajava*, the values were 64 ± 1.61, 80 ± 1.30, 104.4 ± 1.09, 105.6 ± 1.20, and 154 ± 1.03 µg/mL, respectively.

Sl. No.	Phytochemical screened	Screening test	MAI	MPG
1	Steroids	Salkowski's test	+	+
2	Alkaloids	Dragendorff's test	+	-
		Mayer's test	+	+
		Wagner's test	+	+
		Hager's test	-	-
3	Glycosides	Sodium hydroxide test	-	+
4	Phenolic compounds	Ferric chloride test	+	+
5	Tannins	Ferric chloride test	+	+
		Gelatin test	-	+
6	Flavonoids	Ferric chloride test	+	+
		Lead acetate test	+	-
7	Diterpenes	Copper acetate test	-	-
8	Triterpenes	Salkowski's test	+	-
9	Saponins	Foam test	+	+
+ indicates presence; - indicates absence				

Table 6: Qualitative phytochemical screening of MAI and MPG.

The *in-vitro* cytotoxicity of *A. indica* leaves against MCF-7 cells were studied using the MTT assay and found that the methanolic extract had an IC_{50} of 32.0 µg/mL [19]. The cytotoxicity of the chloroform extract of *A. indica* leaves in MCF-7 cells was assessed, with an IC_{50} of 347 µg/mL, using Taxol as a standard [41]. The antitumor potential of *A. indica* leaves on HT29 colon cancer cells was examined and reported an IC_{50} of 28.56 µg/mL [20]. These results indicate that the methanolic leaf extract of *A. indica* has significant cytotoxic activity. The anticancer potential of Indonesian guava leaf fractions on MCF-7 cells was investigated, reporting IC_{50} values of 22.61 µg/mL for ethanol, 52.88 µg/mL for ethyl acetate, and 4.28 µg/mL for n-Hexane [35]. The variance in the IC_{50} value

for hexane might be due to geographical differences in plant material. Braga., *et al*. [11] found an IC_{50} of 15.6 ± 0.8 µg/mL for the ethanolic extract of *P. guajava* leaves in HeLa cells. Sul'ain., *et al*. [42] reported IC_{50} values of 18.60 µg/mL and 55.69 µg/mL for the methanol and aqueous extracts of *P. guajava* leaves in MDA-MB-231 cells, respectively. Variations in IC_{50} values could be associated with differences in plant material, extraction techniques, extract purity, concentration, cell line variability, assay conditions, concentration fluctuations and the quality of reference compounds. Findings from this investigation indicated that the hexane fractions from both plants exhibited the lowest IC_{50} values, thus demonstrating their potent efficacy and warranting further examination in subsequent studies. Table 7 represents the IC_{50} values of various concentrations of *A. indica, P. guajava* and doxorubicin.

Plant	Extracts/fractions	IC_{50} (µg/mL)
Aristolochia indica	Methanolic extract	39.68 ±1.43
	Hexane fraction	78.9 ± 1.80
	Dichloromethane fraction	87.67 ± 1.48
	n- Butanol fraction	86.07 ± 1.03
	Aqueous fraction	118 ± 1.10
Psidium guajava	Methanolic extract	64 ± 1.61
	Hexane fraction	80 ± 1.30
	Dichloromethane fraction	104.4 ± 1.09
	n- Butanol fraction	105.6 ± 1.20
	Aqueous fraction	154 ± 1.03
Positive control	Doxorubicin	15.27 ±1.13

Table 7: IC_{50} values of various concentrations of *A. indica, P. guajava* and doxorubicin.

The antiproliferative potential of hexane fraction from *A. indica* and *P. guajava* on MCF-7 cell lines was assessed by examining morphological alterations in the cytoplasm and nucleus. The staining method employed was acridine orange/ethidium bromide. Doses were chosen as half IC_{50}, IC_{50} and double IC_{50} of most potent fraction, as presented in table 8.

Extract/Fraction	Dose (µg/mL)		
	Half IC_{50}	IC_{50}	Double IC_{50}
HF *A. indica*	39.45	78.9	157.8
HF *P. guajava*	40.0	80.0	160.0
Doxorubicin	7.63	15.27	30.54

Table 8: Different doses selected for microscopic studies.

In this study, control cells exhibited green fluorescence with centrally located nuclei. Doxorubicin-treated cells showed orange nuclei, indicating late apoptosis, with some necrosis. Cells treated with half IC_{50} of HF of *A. indica* showed early apoptosis with green fluorescence. At IC_{50}, cells appeared green with peripheral nuclei, nuclear fragmentation, and chromatin condensation. Double the IC_{50} concentration led to late apoptosis with yellow-orange fluorescence and some necrosis. Similarly, cells treated with HF of *P. guajava* at half IC_{50} and IC_{50} concentrations showed early apoptosis with green fluorescence, while double IC_{50} treatment resulted in both early and late apoptotic changes. The results of this study align with Benny., *et al*. [9], who used AO/EB staining to demonstrate different apoptotic stages in MCF-7 cells after exposure to methanolic extract of *Mallotus philippensis* leaves. Plant steroids exhibit antineoplastic effects by promoting apoptosis and targeting hormone-signaling pathways that regulate cellular growth [23]. Tannins possess potent anticancer properties by inducing apoptosis, halting the cell cycle, and inhibiting invasion and metastasis [12]. Alkaloids trigger autophagy, necroptosis, and apoptosis by modulating the expression of proteins involved in cell death mechanisms, demonstrating their anticancer efficacy [25]. The inclusion of steroids, alkaloids, flavonoids, and tannins in the plant extracts likely accounts for the apoptosis induction observed. Therefore, the current study suggests that the hexane fractions from *A. indica* and *P. guajava* induce apoptosis, leading to cytotoxic effects, possibly due to these compounds.

Real-time quantitative PCR was conducted using the standardized annealing temperature for the genes *Bcl-2, GPER* and *GAPDH*. With *GAPDH* serving as the control gene, the relative expression of *Bcl-2* and *GPER* in MCF-7 cells is presented in Table 10 and Table 11 respectively. In the current study, treatment of MCF-7 cells with IC_{50} concentrations of hexane fractions from *A. indica*

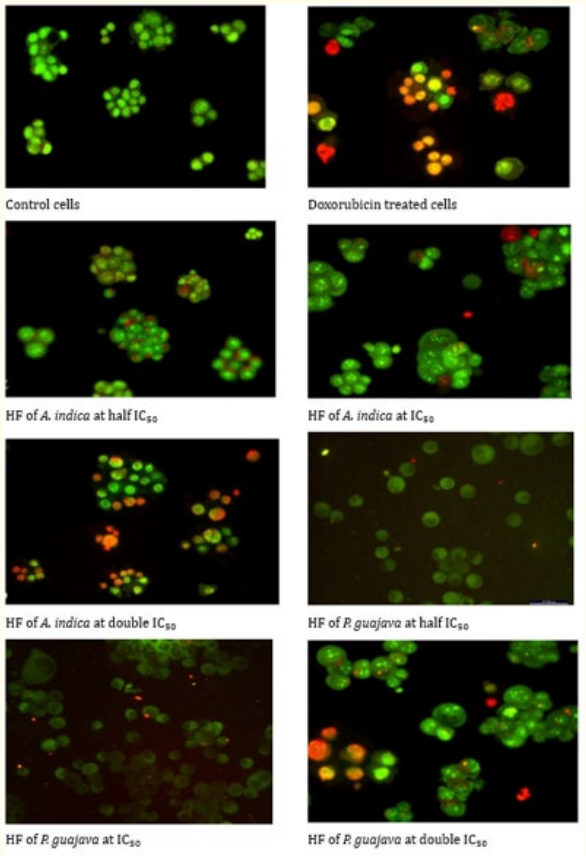

Figure a

and *P. guajava* resulted in downregulation of the antiapoptotic gene *Bcl-2*, with reductions of 0.57-fold and 0.72-fold, respectively, compared to 0.85-fold with doxorubicin. This aligns with Pilco-Ferreto and Calaf [32], who reported significant downregulation of *Bcl-2* in MCF-7 cells exposed to doxorubicin, supporting the findings of this study. In HepG2 cells, guava leaf extract was found to enhance the expression of apoptotic pathway proteins, including cleaved caspase-3, -8, and -9, and facilitated the translocation of Bax and Cytochrome c (cyt-c) from the mitochondria to the cytosol, while downregulating *Bcl-2* expression [28], consistent with the current study. Similarly, the guava seed fraction (GSF-3) significantly decreased Bcl-2 mRNA expression and increased the Bax/Bcl-2 mRNA ratio in PC-3 human prostate cancer cells [22], which aligns with the findings of the present study.

Treatment	Fold change in expression
Control	1
Doxorubicin	0.85
HF *A. indica* double IC_{50}	1.25
HF *A. indica* IC_{50}	0.57
HF *A. indica* half IC_{50}	0.12
HF *P. guajava* double IC_{50}	0.29
HF *P. guajava* IC_{50}	0.72
HF *P. guajava* half IC_{50}	1.85

Table 9: Relative gene expression of *Bcl-2* in response to treatment.

In the current study, MCF-7 cells treated with IC_{50} concentrations of hexane fractions from *A. indica* and *P. guajava* showed upregulation of *GPER* by 1.75-fold and 1.49-fold, respectively, compared to 0.62-fold with doxorubicin. This finding contrasts with Ariazi., *et al*. [4], who reported that *GPER* depletion reduced growth in ER-negative SKBr3 breast cancer cells but potentiated estrogen-stimulated growth in ER-positive MCF-7 cells, suggesting a dual role of GPER in different breast cancer contexts. In a study investigating the *GPER*-specific agonist G-1 in MCF-7 cells found that G-1 inhibited cell proliferation in a concentration-dependent manner by inducing cell cycle arrest and promoting apoptosis, which supports the potential antiproliferative role of *GPER* activation [46]. The guajadial from guava leaves exhibited antiproliferative and antiestrogenic effects against MCF-7 breast cancer cell lines via *GPER*, similar to tamoxifen [8]. However, these conclusions do not align with the current study's findings regarding the upregulation of GPER by hexane fractions from *A. indica* and *P. guajava*. These studies collectively indicate complex roles of GPER in breast cancer, suggesting context-dependent effects that may vary based on cell type, treatment, and specific agonists or antagonists involved.

The outcome of the phytochemical analysis of hexane fraction of *A. indica* and *P. guajava* is depicted in Table 11. The qualitative analysis of phytochemical screening of hexane fraction of *A. indica* and *P.guajava* showedthat both plants contain steroids, phenols, diterpenes and triterpenes. Flavonoids were found exclusively in hexane fractions of *P. guajava*. while saponins were detected only

Treatment	Fold change in expression
Control	1
Doxorubicin	0.62
HF *A. indica* double IC_{50}	0.83
HF *A. indica* IC_{50}	1.75
HF *A. indica* half IC_{50}	1.46
HF *P. guajava* double IC_{50}	0.28
HF *P. guajava* IC_{50}	1.49
HF *P. guajava* half IC_{50}	0.73

Table 10: Relative gene expression of *GPER* in response to treatment.

in *A. indica.* A study on the antibacterial properties of *A. indica* on analysis of the phytochemical composition of leaf extracts revealed the existence of glycosides, terpenoids, and steroids in the hexane fraction [36]. The assessment of phytochemical, cytotoxic, and antioxidant activities of leaf extracts from *Aristolochia indica* reported the presence of flavonoids in the hexane fraction of the leaves [10].

S.no	Phytochemical	Screening test	*A.indica*	*P. guajava*
1	Steroids	Salkowski's test	+	+
2	Alkaloids	Dragendorff's test	-	-
		Mayer's test	-	+
		Wagner's test	+	+
		Hager's test	-	-
3	Glycosides	Sodium hydroxide test	-	+
4	Phenolic compounds	Ferric chloride test	+	+
5	Tannins	Ferric chloride test	-	+
		Gelatin test	-	-
6	Flavonoids	Ferric chloride test	-	+
		Lead acetate test	-	+
7	Diterpenes	Copper acetate test	+	+
8	Triterpenes	Salkowski's test	+	+
9	Saponins	Foam test	+	-
+ indicates presence; - indicates absence				

Table 11: Qualitative phytochemical screening of HF of *A. indica* and *P. guajava.*

In the present study, the GC-MS/MS analysis of hexane fraction revealed the presence of 38 compounds. 9,12,15-Octadecatrienoic acid, Z, Z)-, n-Hexadecanoic acid, Caryophyllene, 9,12,15-Octadecatrienoic acid, ethyl ester, (Z, Z, Z)-(8.70, alfa.-Copaene, Hexadecanoicacid, ethylester, Caryophyllene oxide, 9,12,15-Octadecatrienoic acid, methyl ester,(Z, Z, Z)-, Pentadecanoic acid, 14-methyl-thyl ester, Linoleic acid ethyl ester, Phytol, 1Pyrrolidinebutanoicacid,2-[(1,1 dimethylethoxy) carbonyl]-. alpha.-nitro-, 2,6-bis (1,1dimethylethyl)-4 methoxyphenylester,[S-(R*,R*)]-,1,4,7,-Cycloundecatriene,1,5,9,9-tetramethyl-,Z,Z,Z-,Aromandendrene,1-Methoxy-3-(2 hydroxyethyl)nonane,Naphthalene,1,2,3,5,6,8a- hexahydro-4,7-dimethyl-1-(1-methyl ethyl)-,(1S-cis)- were found to be the major phytocompounds present in the hexane fraction of *A. indica*.

In the present study, the GC-MS/MS analysis of hexane fraction revealed the presence of 36 compounds. Caryophyllene, dl-.alpha.-Tocopherol, alfa.-Copaene, 1,6,10-Dodecatrien-3-ol,3,7,11-trimethyl-, Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-(1S-cis), Naphthalene, decahydro-

4a-methyl-1-methylene-7-(1-methylethenyl)-,[4aR (4a.alpha.,7. alpha.,8a.beta.)], cis-.alpha.- Bisabolene, Alloaromadendrene, Eicosane, Squalene, 1,4,7,-Cycloundecatriene,1,5,9,9- tetramethyl-, Z,Z,Z-,.beta.-Bisabolene, Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methyl ethyl), n-Hexadecanoic acid, beta.-Panasinsene (1.63 per cent), Bicyclo [3.1.1] heptane, 2,6,6-trime thyl-, (1.alpha.,2.beta.,5.alpha.)(1.56 per cent), Phytol, acetate (1.44 per cent), Naphthalene,1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-(1.37 per cent), Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl) -, [1R-(1.alpha.,3a. beta.,4.alpha,7.beta.)],gamma.-Muurolene, Alloaromadendrene, Hexadecanoic acid, methyl ester, Caryophyllene oxide, Naphthalene, 1,2,4a,5,6,8a- hexahydro-4,7-dimethyl-1-(1-methylethyl)-, Cholesteryl benzoate, alfa.-Copaene were found to be the major phytocompounds present in the hexane fraction of *Psidium guajava*. The chromatograms obtained from phytochemical analysis of HF of *A. indica* and *P. guajava* through GC-MS/MS are presented in Fig 1 and 2, respectively.

A study was conducted to assess the chemical composition, antioxidant potential, and enzyme inhibitory effects of the n-hexane extract obtained from *Psidium guajava*. The results of GC-MS/MS analysis of the n- hexane extract revealed the presence of 40 different compounds. The extract was notably abundant in hydrocarbons, with aromatic compounds comprising 34.01 per cent and aliphatic compounds 10.93 per cent. Additionally, oxygenated sesquiterpenes accounted for 13.65 per cent of the extract, while sesquiterpene hydrocarbons constituted 8.75 per cent. Among the identified compounds in the n-hexane extract, squalene and α-tocopherol were predominant, making up 9.76 per cent and 8.53 per cent of the extract, respectively. Other notable compounds included D-limonene (4.83 per cent), 1-epicubenol (4.51 per cent), n-dodecane (4.15 per cent), γ-sitosterol (3.90 per cent), and β-caryophyllene (3.80 per cent) [3]. Therefore, these findings suggest that comparable results were achieved in the present investigation.

Among the diverse compounds identified, alloaromadendrene[43], caryophyllene [21], copaene [45], 9,12,15-Octadecatrienoic acid, Z, Z and 9,12,15-Octadecatrienoic acid, ethyl ester, (Z, Z, Z[33], caryophyllene and caryophyllene oxide [14], 1,4,7,-Cycloundecatriene,1,5,9,9- tetramethyl-, Z,Z,Z-[30], 9,12,15-Octadecatrienoic acid, methyl ester,(Z, Z, Z)- [6], n- Hexadecanoic acid [37], phytol [2], Squalene [15], phytol [18], aromandendrene [31], Linoleic acid ethyl ester [26], Eicosane [40], Pentadecanoic acid [44], dl-.alpha.-Tocopherol [5], bisabolene [39], 1,6,10- Dodecatrien-3-ol,3,7,11-trimethyl[1], Hexadecanoic acid, ethyl ester [29] were reported to have anticancer activity. The presence of these compounds in the hexane fraction of *A. indica* and *P. guajava* could be responsible for the antiproliferative action of the plants.

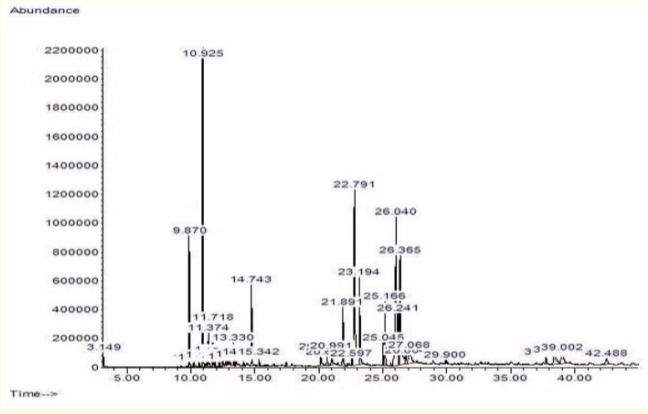

Figure 1: GC-MS chromatogram of HF of A. indica with time along (X) axis and relative abundance along (Y) axis.

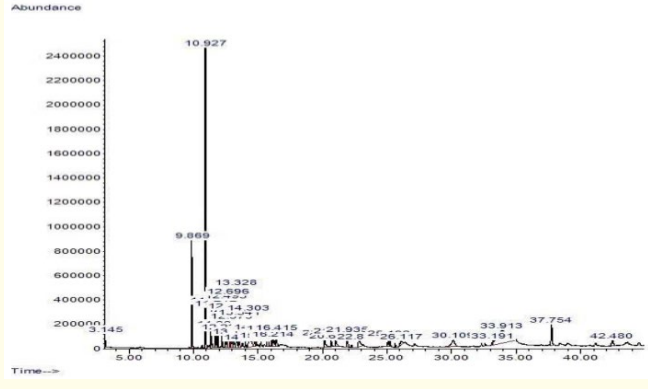

Figure 2: GC-MS chromatogram of HF of P. guajava with time along (X) axis and relative abundance along (Y) axis.

The structurally similar compounds identified in HF of *A. indica* and *P. guajava* based on spectra analysis using ATR-FTIR with the FLUKA library are depicted in Table 12 and 13, respectively. The absorbance spectra of HF *of A. indica* and *P. guajava* with ATR-FTIR spectroscopy are displayed in Fig 3 and 4 respectively.

The FTIR spectroscopic examination of the hexane fraction derived *from A. indica* leaves in the current investigation unveiled the presence of diverse functional groups. These encompassed carboxylic acids (O-H stretching), methylamino compounds (C-H

stretching), alkenyls (C=C stretching), alkyl/alkenes (C-H bending), phenol/tertiary alcohols (O-H bending), aliphatic fluoro compounds (C-F stretching) and aliphatic iodo compounds (C-I stretching). These results further validated the presence of flavonoids (O-H stretching), alkaloids (N-H group), saponins (C-H group), sterols (C=C and aromatic group) and phenol (O-H stretching) in the sample previously evaluated through qualitative screening.

The methanolic extract obtained from *A. indica* leaves were analysed using FTIR spectroscopy, confirming the presence of hydroxyl, carbonyl and nitro groups [34]. In a separate study, *A. indica* leaves extracted with de-ionized water, identifying hydroxyl (OH) and NH stretching, as well as aliphatic and aromatic C-H stretching, carboxylic and C-N stretching and C-O groups of aliphatic amines [38]. The present study yielded similar bonding patterns as those previously reported.

Absorption (cm^{-1})	Group	Compound class
3369.27	O-H Stretching	Carboxylic acids
2916.69	C-H Stretching	Alkanes
2849.15	C-H Stretching	Alkyl
1645.34	C=C Stretching	Conjugated alkene
1447.65	C-H Bending	Alkane
1375.97	O-H Bending	Phenol
1020.78	C-O Stretching	Primary alcohol
565.63	C-S Stretching	Disulphides

Table 12: FTIR spectroscopy analysis of HF of *A. indica* leaves.

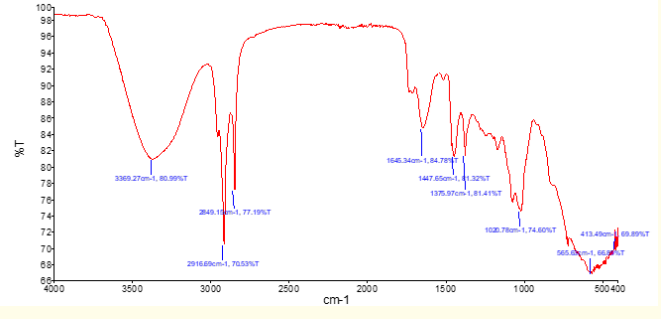

Figure 3: FTIR spectra of HF of *A. indica* depicting the absorption peaks corresponding to chemical groups present, with wave number along X axis and absorbance along Y axis

The FTIR spectroscopic analysis of the hexane fraction obtained from *P. guajava* leaves in the present investigation revealed the presence of various functional groups. These included alcohols (O-H stretching), carboxylic acids (O-H stretching), phenols (O-H stretching), alkanes (C-H stretching), alkyls (C-H stretching), aromatic compounds (C-C stretching), ethers (C-O stretching), aliphatic phosphates (P-O-C stretching) and aryl disulfides (S-S stretching). These findings served to corroborate the existence of flavonoids (O-H stretching), alkaloids (N-H group), saponins (C-H group), sterols (C=C and aromatic groups) and phenol (O-H stretching) in the sample previously subjected to qualitative screening.

The methanolic extract from guava leaves using FTIR spectroscopy observed the presence of hydroxyls (O-H stretching), aliphatic functional groups (C-H stretching), aromatic compounds (C-C stretching), esters, carboxyls, ethers and hydroxyls (C-O stretching), [17] which aligns with findings in the current study.

Absorption (cm^{-1})	Group	Compound class
3354.75	O-H Stretching	Carboxylic acids
2924.27	C-H Stretching	Alkanes
2852.350	C-H Stretching	Alkene
1613.69	C-C Stretching	Aromatic compounds
1448.88	C-H Bending	Alkyl
1375.61	C-H Bending	Alkene
1193.42	C-O Stretching	Alkyl substituted ether
1018.78	P-O-C Stretching	Aliphatic phosphates
578.12	C-S stretching	Disulphides
405.58	S-S stretching	Aryl disulphides

Table 13: FTIR spectroscopy analysis of HF of *A. indica* leaves.

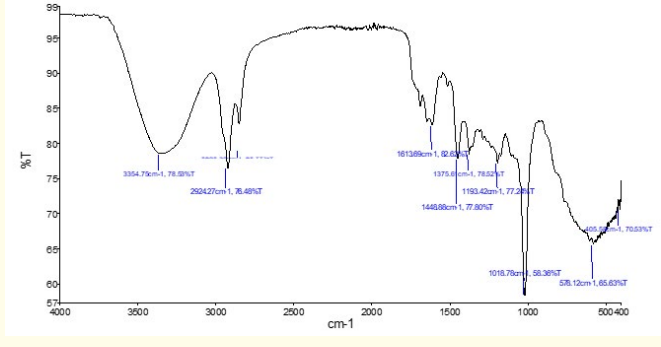

Figure 4: FTIR spectra of HF of *P. guajava* depicting the absorption peaks corresponding to chemical groups present, with wave number along X axis and absorbance along Y axis.

Conclusion

In conclusion, findings from the current study demonstrated that the hexane fractions of *A. indica* and *P. guajava* exhibited dose-dependent cytotoxic effects on the MCf-7 breast cancer cell line. Dual AO/EB staining revealed early apoptotic changes induced by both plant fractions. Additionally, relative gene expression analysis indicated the downregulation of the *Bcl-2* gene and the upregulation of the *GPER* gene. The observed cytotoxicity of these plant fractions may be attributed to the presence of phytochemicals within them. Nonetheless, further research is imperative to develop novel therapeutic candidates against cancer utilising *A. indica* and *P. guajava.*

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