

ANTIPROLIFERATIVE EFFECT OF ETHANOL EXTRACT OF *SOLANUM TRILOBATUM* ON HUMAN OVARIAN CANCER AND COLON CANCER CELL LINES

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ABSTRACT

Ovarian and Colon cancer accounts for inflated tumour-related mortality. As most of the patients are diagnosed with ovarian and colon cancer at an advanced stage this provokes an emergency to discover a new therapeutic strategy. In recent decades, the use of plant life as a natural remedy has been extensively carried out in the Ayurvedic treatment of various diseases, including cancer. The aim of this study was to evaluate the antiproliferative activity of an ethanolic extract obtained from *Solanum trilobatum* on ovarian and colon cancer cells. The antiproliferative activity of the extract was assessed using MTT assays on ovarian cancer cell lines (PA1) and colon cancer cell lines (HCT116). Apoptotic induction was measured using the AO/EB dual staining method. A reverse transcriptase-polymerase chain reaction was done to determine the expressions of BAX, BCL2, Casp9 and mTOR genes to establish apoptosis. The extract significantly inhibited cell growth in a dose-dependent manner in the cell lines tested. The IC₅₀ values for PA1 and HCT116 cells were 361 ±24,160 µg/ml and 247.857 ± 10.545 µg/ml, respectively. Treatment with the extracts changed the morphology of the cells, as cells diminished and distorted in shape. Gene expression was analysed in PA1 cell lines on ACT, BAX genes and in HCT116 cell line on Casp9 and mTOR genes. The expression in treated cells and the untreated control indicated the activity of the extract against ovarian cancer and colon cancer. Further investigations are needed to identify the active compounds responsible for the observed effects and to elucidate the underlying mechanisms of action. Nevertheless, the findings suggest that the ethanolic extract from this plant has the potential as a natural antiproliferative agent for the treatment of ovarian and colon cancers.

Introduction

Cancer is a complex and heterogeneous disease characterized by uncontrolled cell growth and proliferation, which can form malignant tumours (Ferlay et.al., 2020). Ovarian and colon cancers are among the most prevalent types of cancer and are associated with high morbidity and mortality rates (de Martel et.al., 2018). Despite the advances in cancer treatment, the development of effective and safe therapies for these cancers remains a major challenge (Carlotta Pucci et.al., 2019). In recent years, there has been increasing interest in using natural products, including plant extracts, as potential sources of new anticancer agents (Greenwell et.al., 2015).

Plant extracts have been widely studied for their potential as anticancer agents due to their bioactive compounds that can modulate various cellular pathways involved in cancer development and progression (Shrihastini et.al., 2021). *Solanum trilobatum* is a shrub commonly found in Southern India that has been traditionally used for treating respiratory diseases, asthma, chronic infections, tuberculosis, and liver and heart ailments (Ramachandran (Venugopal et.al., 2007). In Siddha medicine, it is regarded as a specific remedy and is used to prepare ghee for treating tuberculosis and as food for lung diseases (Nadkarni, 1979). Studies have shown that the extract of *Solanum trilobatum* has free radical scavenging activity and can inhibit skin carcinogenicity by reducing papilloma formation in response to exposure to DMBA (Mohanani et al., 1997). Additionally, the extract has been found to potentiate the protective effect of cyclophosphamide against toxicity. Furthermore, studies have shown that *Solanum trilobatum* extract does not produce any cytogenetic effects in mice bone marrow cells (Mohanani et al., 1996). However, the effect of leaf extracts on ovarian carcinoma cells PA1 cells and colon cancer cells HT116 is still not known. Therefore, in this, we investigated the anticancer activity of leaf extracts study, in PA1 and HCT116 cell lines in order to evaluate the potential of extract for the treatment of Ovarian and Colon cancer.

Materials and Methods

Sample collection

Solanum trilobatum was amassed from Tenkasi (Tamil Nadu, India). The plant has been identified utilizing plant taxonomy methodology. The leaves of the *Solanum trilobatum* were taken and cleaned by utilizing water to abstract the soil residues, patted dry and stored for further extraction analysis.

Plant extraction

The leaves of *Solanum trilobatum* were cleansed with tap water first, then with distilled water, and last they were carefully chopped into little bits. The leaves were then pulverised after being shade-dried. The ethanol extract was made by blending 500 g of fresh leaves with 1000 mL of analytical-grade ethanol for 15 minutes at room temperature. The mixture was then filtered using analytical filter paper. The alcohol extract was condensed to a semisolid paste using a rotavapor after being centrifuged at 30,000 g for 15 min. After being lyophilized, this paste produced 5.6 g of a light-green powder that was kept at -20°C.

Cell culture maintenance

Ovarian Cancer (PA1) and Colon cancer (HCT116) cell lines were procured from the cell repository of the National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco's Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 U/ml), and streptomycin (100 µg/ml) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO₂ at 37°C.

Subculturing

Subculturing involves transferring a small number of cells into a new vessel. A confluent plate was taken for subculturing. For subculturing, firstly, remove media from the flask. The flask was then washed twice with PBS and then 200 µl of trypsin (0.25% in 0.5 Mm EDTA) was added to the flask. Incubation was done for 2 min in a CO₂ incubator. Observe through a microscope for analysing whether the cells have detached or not. 7ml of sample culture medium with 10% serum to the trypsinized cells and resuspend cells up and down. Transfer the cell suspension to a 50µl conical tube. Place the tube in the centrifuge at 2000 rpm. The supernatant was removed and add 5 ml of fresh prewarmed culture medium to the tube. Add to the new culture media according to the cell count.

Cell viability assay

The cytotoxicity of ethanolic extract of *Solanum trilobatum* on cells was performed by MTT (3-(4, 5-dimethylthiazol 2-yl)-2, 5-diphenyl tetrazolium bromide) (Mosmann T, 1983). Cells PA1 and HCT116 were seeded at a density of 1x10⁴ cells per well in 96-well plates each in complete media. Then, cells were divided into two groups; the control group and the treatment group. For the treatment group, cells were incubated with complete media containing different concentrations of plant extract ranging from 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml and 600 µg/ml. The control group was left as such without adding the plant extract. After 24 h of incubation, after incubation, the drug-containing cells

were washed with fresh culture medium and the MTT (5 mg/ml in PBS) dye was added to each well, and the plate was incubated for 4 hours at 37 °C until the purple colour of formazan crystal is visible. The suspension was aspirated, and 100 µl of DMSO was added to stop the reaction and dissolved the formazan crystal. Finally, the intensity of the MTT reaction was measured by Microplate Reader at 570 nm of absorbance. The results were expressed at the percentage of stable cells with respect to the control. The half-maximal inhibitory concentration (IC₅₀) values were calculated. The IC₅₀ values were determined from the sample dose-responsive curve where inhibition of 50% cytotoxicity compared to vehicle control cells. All experiments were performed in triplicate.

Morphological analysis

Morphological analysis was done to determine the morphological changes, like cell shrinkage, membrane blebbing etc, as per (Srivastava *et al.*, 2011). PA1 and HCT 116 cells were seeded and the next day the cells were treated with ethanol extract for 24 h. The ethanol effect on the PA1 and HCT116 cells on their morphology was assessed under the phase-contrast microscope inverted after 24 h (at 10 X magnification). The cells which were not treated with plant extract served as control.

Measurement of apoptotic induction

The fluorescence microscopic analysis of apoptotic cell death was carried out according to Baskic *et al.* (2006). PA1 and HCT116 cells were seeded at 5 x 10⁴ cells/well in a 6-well plate each and incubated for 24 hours. After treatment with ethanolic extract 300 and 400 µg/ml for 24 h, the cells were detached, washed with cold PBS and then stained with a mixture of AO (100µg ml⁻¹)/ EB (100µg ml⁻¹) ratio (1:1) at room temperature for 5 min. The stained cells were observed by a fluorescence microscope at 20x magnifications. At the end of treatment, the cells were collected and washed three times with PBS. The plates were stained with acridine orange/ethidium bromide (AO/EB 1:1 ratio; 100 µg/ml) for 5 minutes and examined immediately under a fluorescent microscope with 20x magnification. The number of cells showing the feature of apoptosis was counted as a function of the total number of cells present in the field.

Gene expression study

Total RNA was isolated using a total RNA isolation kit according to the manufacturer's instructions (RNeasy Mini Kit). cDNA synthesis was performed using the Thermo scientific Verso cDNA synthesis kit and amplification was performed using the QuantiNova SYBR Green PCR kit according to the manufacturer's protocol.

Results

Effect on cell viability

The *Solanum trilobatum* leaf extract showed the most potent cytotoxic effects against the HCT116 cancer cell line, with an IC₅₀ value of 247.857 ± 10.545 $\mu\text{g/ml}$ (Fig.1). In contrast, the extract showed a noticeable cytotoxic effect against PA1 cells, with IC₅₀ values of $361 \pm 24,160$ $\mu\text{g/ml}$.

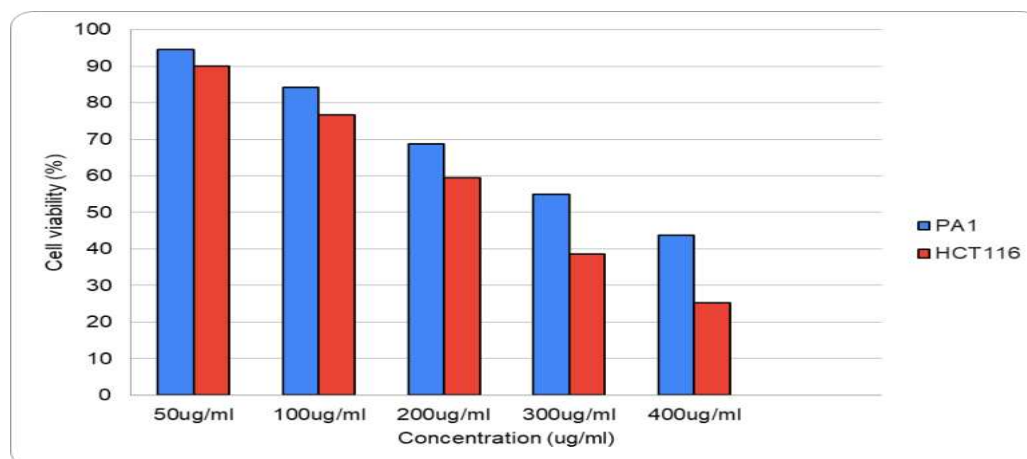


Figure 1. Effect of different concentrations of *Solanum trilobatum* root extracts on cell viability using MTT assay in PA1 and HCT116 cell line. The blue bar represents PA1 cell line and red bar cell viability.

Morphological changes in control and sample-treated

The photomicrograph, captured by a fluorescent microscope at a magnification of 20X, shows that PA1(Fig.2.) and HCT116 (Fig.3.) cells treated with ethanolic extracts at concentrations of 300 $\mu\text{g/ml}$ and 400 $\mu\text{g/ml}$ for 24 hours exhibited morphological changes such as shrinkage, detachment, membrane blebbing, and distorted shape. In comparison, the control group showed normal and intact cell morphology. This indicates that the ethanolic extract treatment induced significant changes in the structure of cell lines.

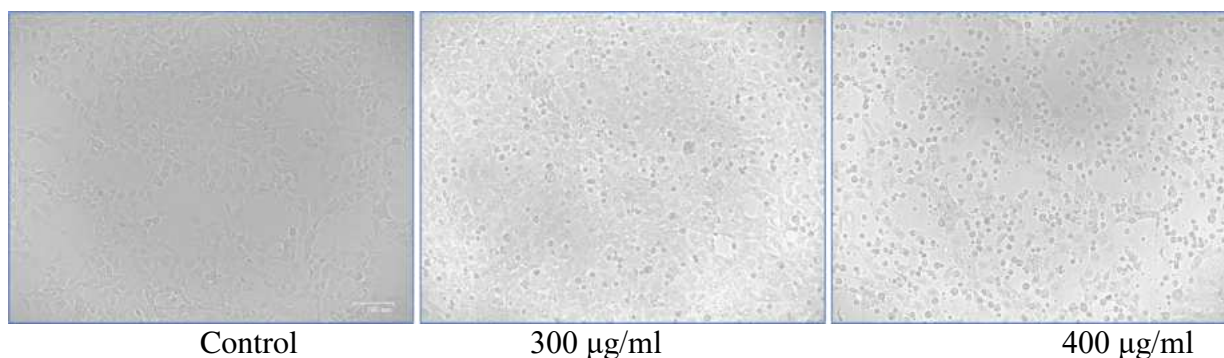


Figure 2. Effect of different concentrations of *Solanum trilobatum* leaf extract on the morphology of PA1 cell line.

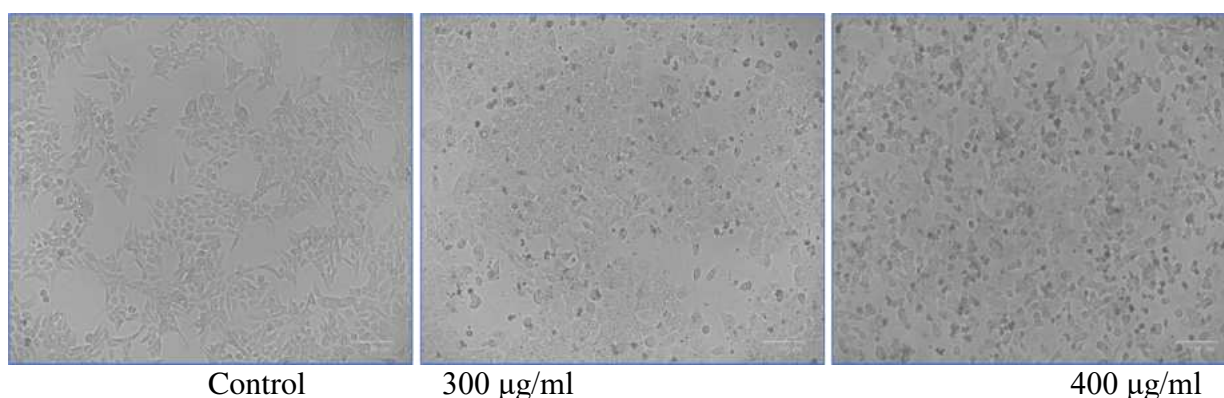
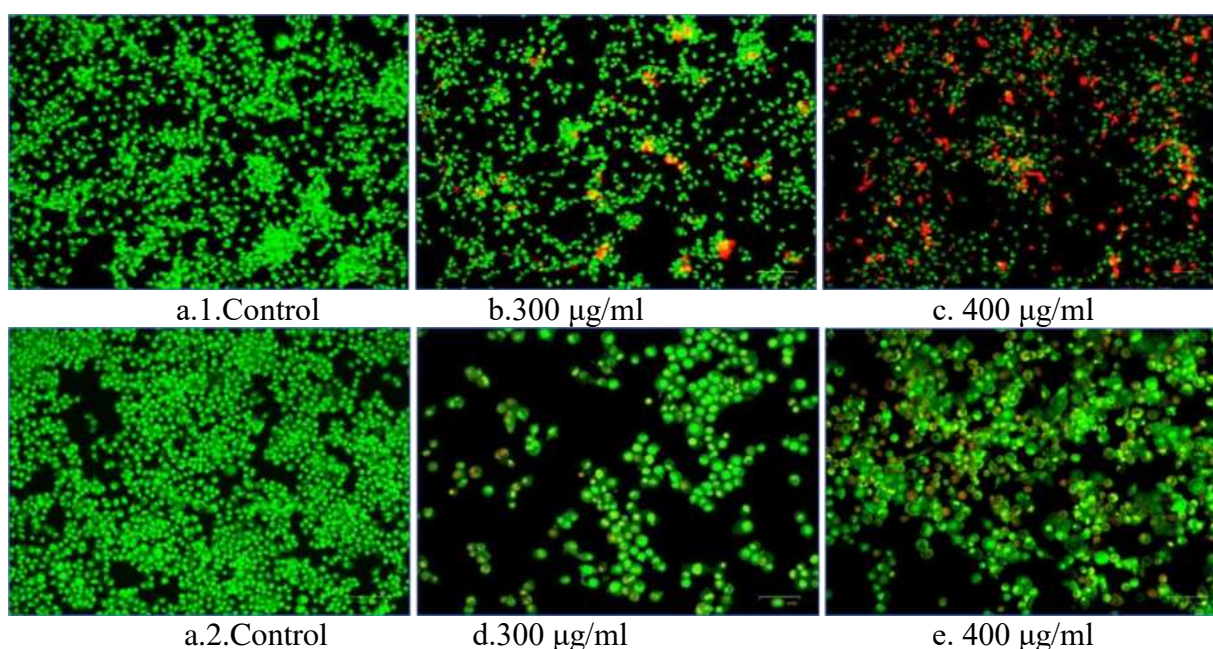


Figure 3. Effect of different concentrations of *Solanum trilobatum* leaf extract on the morphology of HCT116 cell line.

Measurement of apoptotic induction

The aquas extract Sample was utilized to treat PA1 and HCT116 cells for a duration of 24 hours at concentrations of 300µg/ml and 400 µg/ml. The cells were subsequently examined via fluorescence microscopy utilizing a dual dye AO/EB, utilizing the Zoe Fluorescent Cell Imager by Biorad. Living cells displayed green fluorescence with a typical nuclear appearance. Apoptotic cells showed yellow fluorescence with fragmented nuclear structures, whereas late apoptotic cells displayed orange fluorescence with chromatin condensation or fragmentation producing uniformly red/orange-stained cell nuclei. It is observed that the concentration of 400µg/ml induces more cell death than the concentration of 300µg/ml in both cell lines.



In Fig.3 of this experiment, the control of PA1(a.1) and HCT116 (a.2) contains live cells which are all in green. However, (b), (c) depicts the PA1 and (d) and (e) depict the HCT116 cell line treated with different concentrations of plant extract respectively.

Gene expression studies

Gene expression studies were conducted in mRNA isolated from the cells treated with the *Solanum trilobatum* extract. Significant targets including BAX & BCL2 in the PA1 cell line and in HCT116 targets including Casp9 and mTOR were analysed for mRNA expression level using RT PCR. BAX was upregulated and BCL2 was downregulated in the PA1 cell line and in the HCT116 cell line Casp9 was upregulated and mTOR was downregulated upon treatment with plant extract when compared with the untreated control samples. The complete data for all the targets were depicted in Figures 4 and 5 and their respective ct values were represented in Tables 1 and 2.

Discussion

Ovarian and colon cancer are two of the most common cancers affecting women (Momenimovahed *et al.*, 2017) and men (Maria Abancens *et al.*, 2020) worldwide, respectively. The search for effective treatments for these cancers is ongoing, with traditional plant-based medicine proving to be a valuable source of potential therapeutics (Atanasov *et al.*, 2021). *Solanum tribolactum*, a plant commonly found in Southern India, due to the availability of phytochemicals that may be used to treat numerous ailments, represents a potential source of valuable adulticidal medicine. *Solanum tribolactum* is a member of the Solanaceae family and is commonly used in traditional medicine to treat a variety of ailments. The plant has been shown to possess a range of biological activities, including antiproliferative, antioxidant, and anti-inflammatory properties. These properties are attributed to the presence of various phytochemicals, including alkaloids, flavonoids, and terpenoids (Ranjith *et al.*, 2010; Balakrishnan *et al.*, 2015).

The ovarian teratocarcinoma cell line PA-1 demonstrated a single chromosomal aberration and is reported to be a useful model cell line used to demonstrate the anticancer efficacy of the investigated agent in vitro studies (Ranjith *et al.*, 2010; Balakrishnan *et al.*, 2015; Sahu *et al.*, 2013; Balakrishnan *et al.*, 2015).

HCT116 is a commonly used cell line for colorectal cancer studies and the target gene responsible for the function of miR-224 has been identified to be elevated in colorectal cancer (Gordon *et al.*, 2018). Our studies showed a decrease in cell viability as per the method of cell viability assay which is a common method to find cell viability (Moongkarndi *et al.*, 2004), IC₅₀ values for PA1 and HCT116 cells were 361 ± 24,160 µg/ml and 247.857 ± 10.545 µg/ml, respectively. The values denoted that the plant extract has an antiproliferative effect against ovarian as well as colon cancer cell lines, and their effect more is found in colon cancer cells.

House keeping Gene - ACT					Gene of Interest - BAX							
Sample	ct1	ct2	ct3	Avg ct	Sample	ct1	ct2	ct3	Avg ct	Δct	$\Delta\Delta ct$	$2^{-\Delta\Delta ct}$
Control	8.45	8.72	8.54	8.57	Control	22.07	22.17	23.01	22.42	13.85	0.00	1.00
T1	8.72	8.62	8.61	8.65	T1	19.09	19.05	20.01	19.38	10.73	-3.11	8.65
										Avg Δct	13.85	
House keeping Gene - ACT					Gene of Interest - BCL2							
Sample	ct1	ct2	ct3	Avg ct	Sample	ct1	ct2	ct3	Avg ct	Δct	$\Delta\Delta ct$	$2^{-\Delta\Delta ct}$
Control	8.45	8.72	8.54	8.57	Control	15.09	14.98	15.05	15.04	6.47	0.00	1.00
T1	8.72	8.62	8.61	8.65	T1	18.07	18.14	18.13	18.11	9.46	2.99	0.13
										Avg Δct	6.47	

Table1. Cycle threshold value of PA1 cell line using RTPCR

House keeping Gene - ACTS					Gene of Interest - Casp 9							
Sample	ct1	ct2	ct3	Avg ct	Sample	ct1	ct2	ct3	Avg ct	Δct	$\Delta\Delta ct$	$2^{-\Delta\Delta ct}$
Control	18.56	18.57	18.56	18.56	Control	17.09	17.21	17.2	17.17	-1.40	0.00	1.00
T1	18.62	18.61	18.65	18.63	T1	16.19	16.21	16.08	16.16	-2.47	-1.07	2.10
										Avg Δct	-1.40	
House keeping Gene - ACTS					Gene of Interest - mTOR							
Sample	ct1	ct2	ct3	Avg ct	Sample	ct1	ct2	ct3	Avg ct	Δct	$\Delta\Delta ct$	$2^{-\Delta\Delta ct}$
Control	18.56	18.57	18.56	18.56	Control	11.02	11.05	11.02	11.03	-7.53	0.00	1.00
T1	18.62	18.61	18.65	18.63	T1	14.21	14.2	14.3	14.24	-4.39	3.14	0.11
										Avg Δct	-7.53	

Table1. Cycle threshold value of HCT116 cell line using RTPCR

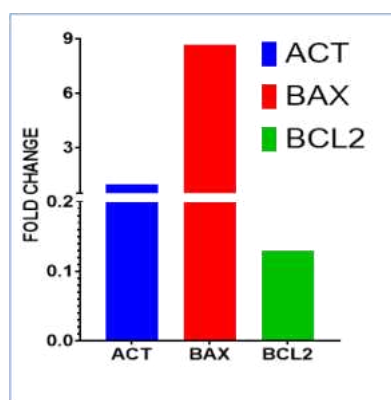


Figure 4

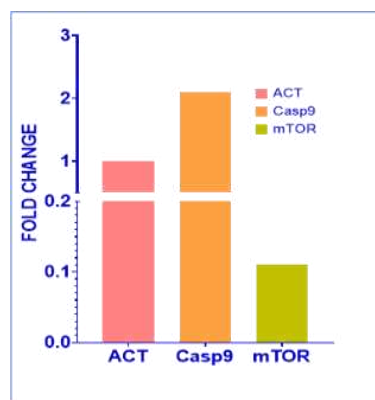


Figure 5

Figures 4 and 5: Gene expression analysis graph of PA1 and HCT116 cell lines respectively

Morphological changes in the cell are an indicator of apoptosis. In PA1 and HCT116 cells, morphological alterations including shrinkage, detachment, membrane blebbing, and deformed shape were seen. This is because the ethanolic extract caused considerable alterations in the cell lines' structural membrane. The control group, in contrast, had healthy and undamaged cell morphology. This shows that the treatment with ethanolic extract resulted in apoptotic induction of cell lines.

Reverse transcriptase PCR was done to check the effect of plant extract on the expression of BAX and BCL2 in PA1 cell lines and in HCT116 the expression was analysed on Casp9 and mTOR genes. Bax is found in homodimers, and an apoptotic signal results in

cell death, suggesting a crucial role of the Bax/Bcl-2 balance in the regulation of proliferation or cell suicide (Marx *et al.*, 2001).

Conclusion

The results indicate that the ethanolic extract obtained from the plant has potent antiproliferative activity against ovarian and colon cancer cells and that this activity may be mediated by the induction of apoptosis. These findings support the potential use of the extract as a natural anticancer agent for the treatment of ovarian and colon cancers. Further studies are needed to identify the active components responsible for the observed effects and to evaluate the extract's safety and efficacy in preclinical and clinical studies.

Abbreviations

FBS: Fetal bovine serum; DMEM: Dulbecco's Modified Eagle Media; IC50: Inhibitory concentrations 50%; PA1: Ovarian cancer cell line; HCT116: Colon cancer cell line.

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