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Anti Ovarian and Cervical Cancer Potential of *Tamarindus Indica* Leaf Ethanol Crude Extract and Its Bioactive Fraction, Apigenin

Shirisha Rao and Varalakshmi Kilingar Nadumane*

Department of Biotechnology, Center for Research in Pure and Applied Sciences, School of Sciences, Jain (Deemed to be University), Bangalore, 560027.

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Abstract

Cervical and ovarian cancers account for the majority of deaths among women. Due to the failure of conventional therapies to completely cure these diseases, research has shifted focus to traditional and plant-based medicines. This study aimed to assess the potential anticancer activity of tamarind leaf extract on ovarian cancer PA-1 and cervical cancer HeLa cell lines. The extract was prepared using Soxhlet equipment with ethanol as the solvent and fractionated using Thin Layer Chromatography (TLC) under bioassay guidance. Anticancer activity was determined through *in vitro* tests, including MTT assay, fluorescence microscopy, trypan blue assay, LDH activity assay, caspase-9 activity assay, analysis of DNA fragmentation, Annexin V/PI staining and flow cytometry. Bioactive compound characterization was performed using gas chromatography-mass spectrometry (GC-MS) followed by NIST library search, to understand the underlying principles behind the anticancer attributes of tamarind leaf. The results revealed that treatment with tamarind leaf ethanol extract drastically reduced HeLa and PA-1 cell viabilities to 26.8% and 11.9%, respectively, after 72 hours. The yellow fraction (TLYF) of the extract was identified as the bioactive component through bioassay-guided fractionation. Trypan blue assay results showed cell concentrations reduced to 1.3×10^5 and 1.2×10^5 cells/mL compared to about 3.6×10^5 cells/mL in control HeLa and PA-1 cells. Cell cycle analysis demonstrated that TLYF reduced overall cell count, with a significant decrease in S and G2/M phases, and increased percentage of cells (50.9 and 18.8%) in early and late apoptotic stages as per annexin V/PI staining. Caspase assay confirmed apoptosis initiation through caspase-9 activation in cancer cells induced by TLYF. According to GC-MS data, apigenin, an anticancer compound reported in several fruits, was found in tamarind leaf. It can be assumed that apigenin in tamarind leaf might be the reason for its profound antiproliferative effects against cancer cells.

Keywords: Anticancer, apoptosis, caspase 9 activation, cell cycle, tamarind leaf, yellow fraction.

1. Introduction

Despite the availability of contemporary therapeutic options, cancer is one of the most feared diseases due to the high mortality rate linked with it (Jeong et al., 2009; Garg et al., 2015). Cervical cancer is the foremost reason for deaths due to cancer in several countries including India, sub-Saharan Africa, and Latin America (Sung et al., 2021; Arbyn et al., 2018). Inflammation is a key factor in the development of cancer, and the presence of an inflammatory response is a prominent characteristic of cancer (Hanahan and Weinberg, 2011). Research indicates that persistent inflammation can heighten the likelihood of cancer. Additionally, inflammation is significantly involved in the process leading to cervical cancer, besides the infection with human papilloma virus (HPV) (Wei et al., 2022). Chemotherapy plays a crucial role in the conventional treatment of cervical cancer and is usually given as an adjuvant therapy after surgery or in combination with radiotherapy. Cisplatin, a platinum-based drug, has been the most effective single-agent treatment for cervical cancer over the past thirty years (Tewari and Monk, 2005). Despite its initial effectiveness, patients often develop resistance to cisplatin during treatment. Therefore, it is vital to explore and create new and better therapies to address the issue of multidrug resistance in cancer cells, as this resistance significantly affects the success of chemotherapy. Recently, Complementary and Alternative Medicine (CAM) as therapeutic options are adopted more often to treat cancer, besides modern allopathic drugs and biological therapies in order to reduce the side effects of chemotherapy, which is known as integrative oncology. By all means, the less invasive therapeutic options with the use of natural compounds wherever possible are better to manage cancer ideally (Chizenga and Abrahamse, 2021). Therefore, during the past few decades, research on cutting-edge, better, and tumor-specific treatments has accelerated. Several nutritional and medicinal plants have so far contributed numerous anticancer compounds to this cause (Patel et al., 2010; Dev et al., 1999; Faridi et al., 2023).

The tamarind, or *Tamarindus indica* L., is an important dietary plant, with most of its parts, like the bark, leaves, seed pulp and seeds, having one or the other nutritional or medicinal property (Kumar and Bhattacharya, 2008). Its origins can be traced to the old tribal people who made decoctions of the components and utilised them as

* Corresponding author. e-mail: kn.varalakshmi@jainuniversity.ac.in; namavarapusiri@yahoo.com.

medicines. To treat wounds, the leaves and bark of tamarind tree was used in West and East Africa, as per a report (Havinga et al., 2010). Fruit and leaves possess laxative properties; dried seed paste was used to repair broken bones, and the bark, and leaf decoction was used to treat jaundice (El-Siddig et al., 2006; Vaidyanathan et al., 2013; Caluwe et al., 2010). According to certain reports, the tamarind tree's bark has analgesic, anti-inflammatory, and wound-healing qualities (Irvine et al., 1961; Morton, 1987).

Macerated fresh bark of tender twig was used in West Africa as a purgative and a remedy for stomach pain (Bhat et al., 1990). In a previous study, the anticancer potential of tamarind bark dichloromethane extract was reported on cervical and ovarian cancer cell lines, where cantharidin was identified as the bioactive component (Rao and Varalakshmi, 2016). The anti-inflammatory properties of the seeds, bark, leaves, and other parts of *T. indica* have been reported in traditional medicine and Ayurvedic medicine, and by some researchers (Havinga et al., 2010; Kuru, 2014). The anticancer activity of tamarind bark and seeds was also reported. However, the anticancer efficacy of the leaves of the tamarind plant has not been scientifically investigated, despite its anti-inflammatory property and the established causal relationship between inflammation and cancer. In this present study, due to the medicinal properties of tamarind leaves (along with their traditional use for treating inflammation, pain, and wounds), we focused on scientifically investigating and validating the anticancer efficacy of tamarind leaf ethanol extract, characterizing the bioactive principle, and evaluating its anticancer mechanism on in vitro ovarian and cervical cancer cell lines.

2. Materials and Methods

2.1. Cell Lines

The National Centre for Cell Sciences (NCCS) in Pune, India, provided the ovarian cancer (PA-1), and cervical cancer (HeLa) cell lines. The media used to culture HeLa cells was Dulbecco’s Modified Essential Medium (DMEM from HIMEDIA, India), while Minimum Essential Medium with 10% foetal bovine serum was used to culture PA-1 cell line. Cell lines were used in the investigations of the study during the linear phase of growth, and were kept for up to passage 20. The anticancer experiments were conducted using both these cancer cell lines.

2.2. Lymphocyte isolation

Lymphocytes were extracted from the blood of five healthy individuals, both male and female, aged approximately 20 years. These individuals were visibly unaffected by pathogenic infections and had not undergone any medical treatments in the preceding six months. Blood collection process adhered to the ethical guidelines established by the Indian Council of Medical Research for research purposes (ICMR, 2006). The isolation process employed HiSep medium (HIMEDIA, India). The cells were then suspended in complete RPMI 1640 medium, which was enriched with 10% Fetal Bovine Serum (HIMEDIA, India) and 5 g ml−1 phytohemagglutinin (PHA). Subsequently, the lymphocytes were cultured at 37°C in a 5% CO2 humidified incubator. These lymphocytes served as normal control cells for evaluating the cytotoxicity of the plant extracts.

2.3. Crude Extract Preparation and Cytotoxicity Screening

Tamarind leaves were harvested in Bengaluru, inside the campus of Indian Institute of Science, and the plant sample was identified by Prof. Shivakumar Swamy, a Botanist and Adjunct Professor at Jain University, Bangalore. For future reference, the specimen samples of the tamarind plant were stored in the Jain University herbarium (voucher no. JUH-34). The leaves were dried under shade conditions. In order to isolate the metabolites from the leaf samples (50 gm), ethanol solvent was used and extracted in a Soxhlet apparatus (Hayashi et al., 1998). The extracted material was concentrated with the help of a rotary evaporator and was kept at 4ºC until further usage. Dimethyl sulphoxide (DMSO) was used to make the stock solution (at 1 mg/mL concentration), which was further diluted to the required quantities using phosphate buffered saline (PBS).

2.4. Assay of cytotoxicity (MTT)

The cytotoxic impact of tamarind leaf extract on cancer cell lines was evaluated using the MTT [3- (4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] test (Mosmann, 1983). In 96-well microplates, 2×10^4 cells per well were seeded and incubated for 24 hours at 37°C. From the stock solution (1000 µg/ml) of tamarind leaf ethanol extract serial dilutions were made, and the sample was added in various concentrations (1, 10, 50 and 100 µg/ml). Simultaneously, as a positive control the cancer drug, camptothecin was used at 5 and 10 µg/ml concentrations. The microplates were then incubated for an additional 24, 48, and 72 hours. 20 µl of MTT was added after the predetermined amount of incubation time and incubated for 3 hours in the dark. After three hours, 200 µl of DMSO was added to each well, and an ELISA plate reader (LISA Plus, Aspen Diagnostics, India) was used to measure the absorbance at 540 nm. Utilising the following formula, the % viability was calculated:

Viability (%) = (ODs/ OD) × 100

With, OD being Optical Density of the control, and ODs is the Optical Density of the sample.

2.5. Calculation of IC_{50} value

The IC_{50} values of the samples (tamarind leaf ethanol extract and its bioactive fraction) were determined from the dose response curves obtained after MTT results, through the application of the four parameter logistics (4PL) equation, a statistical model frequently employed for fitting sigmoidal curves (AAT Bioquest, Inc., 2023).

2.6. Thin Layer Chromatographic (TLC) separation of crude extract

Identification of the bioactive compound from the crude extract of tamarind leaf was done through bioassay guided fractionation using TLC sheets. Merck Specialties Private Limited in Mumbai provided the TLC plate (TLC Silica Gel 60 F 254). The optimal solvent combination was utilised to separate the fractions from tamarind leaf extract after trying various solvent combinations with various concentrations (Ciesla and Hajnos, 2010). By using preparative TLC, the fractions were collected, then dried

after being dissolved in methanol. The dried fractions underwent another MTT assay test for cytotoxicity, and the fraction showing maximum cytotoxicity was chosen for further studies.

2.7. Assessment of fragmentation of DNA

In 25cm^2 roux culture flasks, PA-1 and HeLa cells (2×10^4 cells/ml) were cultivated for 24 hours. The bioactive fraction from tamarind leaf (TLYF) was added at 10 µg/ml concentration, and afterwards the period of incubation was for 48 hours. Untreated cells were used as the negative controls. After 48 hours of treatment, cells were harvested by trypsinizing, followed by centrifuging, and the cells thus precipitated were lysed using a solution having 10mM Tris HCl, 10mM EDTA, and 0.5% Triton X-100. Afterwards, addition of 200 µg/ml of proteinase K and 200 µg/ml of RNAse was followed, to prevent contamination with RNA and proteins (DNA isolation kit, Bangalore Genei). The DNA was then precipitated by adding ice-cold ethanol and suspended in a Tris-EDTA solution. The DNA samples stained with ethidium bromide were loaded into the wells of 0.8% agarose gel, and electrophoresed at 50v, till the samples reached almost 3/4th of the gel. The samples were visualised using a UV Trans illuminator and were photographed (Lee and Shacter, 1999).

2.8. Cell count by trypan blue staining method

Using phosphate buffer (PBS), trypan blue stain was prepared (at 0.4% concentration). About 500 µl of this stain and 500 µl of PA-1 and HeLa cells were mixed thoroughly to avoid clump formation and allowed to stand for about 5 minutes. This sample (20µl) was loaded on the sample induction point on both sides of the chamber of a haemocytometer, and cells were counted with the help of a binocular microscope (Labomed, India).

2.9. Fluorescence Microscopy

TLYF (10µg/ml) was treated to the cancer cells, PA-1 and HeLa, for 24 hours along with the simultaneous maintenance of untreated controls. The control and treatment cells were collected separately and were stained using a 1:1 mixture of acridine orange (100µg/ml) and ethidium bromide (100µg/ml). The stained cells, after mounting on glass slides, were examined using a fluorescence microscope (Thermoscientific) (Roy et al., 2008).

2.10. Assay of Caspase 9 enzyme

Caspase 9 apoptosis assay kit (G-Biosciences, Missouri, USA) was used in accordance with the manufacturer's instructions to carry out the experiment. Along with untreated controls, cervical and ovarian cancer cells were given a 24 hour treatment with TLYF at a concentration of 5 µg/mL. Cells were harvested by trypsinization after 24 hours and then lysed with 50 µl of lysis buffer. To ensure that the cells were evenly suspended in PBS solution, the lysed cells were thawed and frozen thrice. Later, this solution was centrifuged, and the supernatant was collected. The lysate was mixed with 50 µl of 1M DTT (dithiothreitol)-containing caspase assay buffer. AFC conjugated substrate (5 µl) was added, and the mixture was incubated at 37 °C for 2 hours. Using an ELISA plate reader, absorbance was measured once in every 15 minutes, at 405 nm. We calculated the percentage caspase activity as per the formula given below:

OD_{405} control/sample – OD_{405} blank / OD_{405} blank X 100

2.11. Propidium iodide (PI) and Annexin V staining based analysis through Flow cytometry

The most popular method for detecting apoptosis using a flow cytometer is through annexin V combined with propidium iodide (PI) staining (Dicker et al., 2005). This assay can be used to determine the cell population going through apoptosis, necrosis, as well as the population of viable cells. HeLa cells were cultured in a roux flask (1×10^6 cells/mL), and 24 hours later were again incubated for 24 hours post treatment with the TLYF. Later, the trypsinized cells were collected, labelled with PI and Annexin V staining, and examined with the help of a flow cytometer.

2.12. Characterization through Gas chromatography and Mass Spectrometry

Tamarind leaf's bioactive fraction (TLYF) was analysed using GC-MS. Thermo GC - Trace Ultra ver. 5.0 and a Thermo MS DSQ II mass spectrometer were used for the GC-MS analysis. A non-polar DB 5 - MS Capillary Standard column with dimensions of 30 mm x 0.25 mm and a film of 0.25 µm thickness, was employed. The carrier gas, helium gas with 1 mL/min of flow rate was employed. The 70°C initial temperature of the oven was raised slowly to 260°C following a steady pace with 6°C increase per minute. After injecting 1 µl of the material onto the column, mass spectra with a mass range of 50-650amu were produced. The resulting mass spectra were analysed with the mass spectra obtained through the NIST collection, and the compounds' structure and molecular weight were established (Belayachi et al., 2014).

2.13. Statistical Analysis

Each experiment was performed three times. Standard error (SE) values were determined for the outcomes. The one-way analysis of variance (ANOVA), and Dunnett's multiple comparison tests were used to determine the statistical significance. The results deemed significant were with p values <0.05.

3. Results

3.1. Cytotoxicity of tamarind leaf ethanol extract

When we treated HeLa cells with the ethanol extract of tamarind leaf, a significant reduction in cancer cell viability was observed, following a time- and dosage-dependent pattern. At concentrations of 1, 10, 50, and 100 µg/ml, the cell viabilities were 71.4%, 61.9%, 48.8%, and 35.9% respectively after 24 hours. When the exposure duration was extended to 72 hours, the viabilities further decreased to 53.6%, 41.4%, 31.7%, and 26.8% respectively (Figure 1a). Similar trends were observed in PA-1 cells. At 1.0 µg/ml concentration of tamarind leaf ethanol extract, the viability was 65.5%, at 5 µg/ml it was 58.6%, at 10 µg/ml it was 53.4%, and at 15 µg/ml it was 50% after 24 hours. After 72 hours, viability decreased as the treatment concentration increased (Figure 1c). All these results were statistically significant according to ANOVA (p values < 0.05 and < 0.001).

3.2. Fractionation by TLC and bioassay

Due to the potent cytotoxicity of tamarind leaf extract against cancer cells, the crude ethanol extract was fractionated using thin-layer chromatography with different solvents. Using a solvent mixture of toluene:ethyl acetate: formic acid in the ratio 2.5:1:1 (v/v), seven distinct fractions were separated from the tamarind leaf ethanol extract when observed under UV light (Figure 1e). The yellow fraction (TLYF) exhibited the highest cytotoxicity against cervical cancer HeLa and ovarian cancer PA-1 cells. This TLYF fraction reduced HeLa cell viability to 84.0%, 69.0%, 51.1%, and 42.8% at concentrations of 1, 10, 50, and 100 µg/ml respectively after 24 hours of treatment (Figure 1a, 1c). A similar trend was observed in PA-1 cell line. At concentrations of 1.0, 2.5, 5.0, and 7.5 µg/ml, cell viabilities were 67.2%, 53.4%, 50%, and 41.3% respectively after 24 hours of incubation. The lowest viability (11.9%) was observed 72 hours after treatment with a 5 µg/ml concentration of TLYF. TLYF exhibited a superior antiproliferative effect on both HeLa and PA-1 cells, comparable to that of Camptothecin, the positive control (Figure 1b). Neither the crude extract nor the TLYF showed any cytotoxic effects on normal lymphocytes (Figure 1d). The IC_{50} value of TLYF was determined as 10.45 µg/mL for HeLa and 4.3 µg/mL for PA-1 cell lines after 72 hours, very much nearing to the IC_{50} value of camptothecin, which is 3.7 µg/mL for both the cell lines (Table 1).

Table 1. IC_{50} Value of Tamarind leaf yellow fraction on HeLa and PA-1 cells

Bioactive fraction	IC_{50} Values (µg/ml)					
	HeLa			PA-1		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
TLYF	40.46	93.85	10.45	4.33	4.31	4.3
Camptothecin	3.92	3.23	3.67	3.9	3.19	3.7

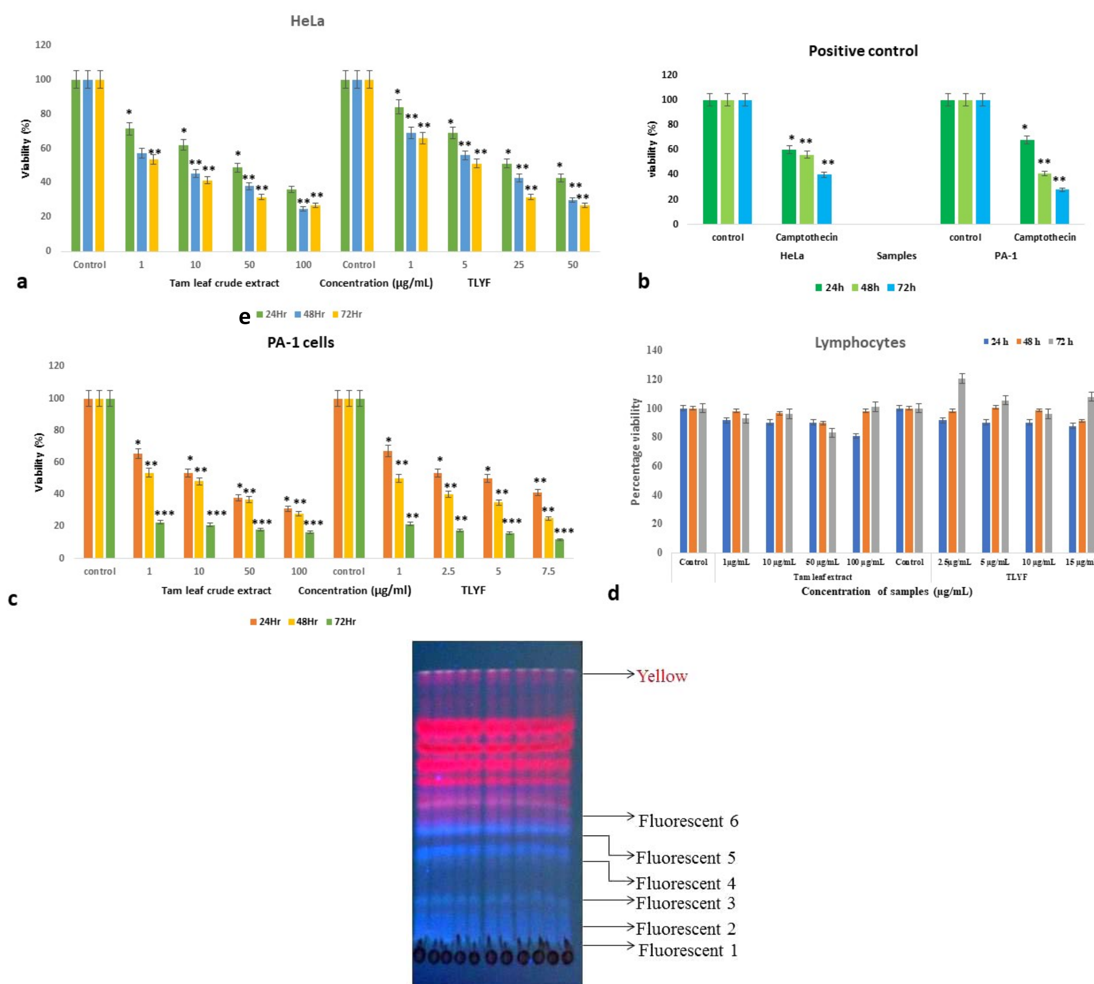

Figure 1: a) Effect of Tamarind leaf ethanol extract and TLYF on HeLa cells **b)** Effect of Camptothecin (10 µg/ml) on HeLa and PA-1 cells. **c)** Effect of Tamarind leaf ethanol extract and TLYF on PA-1 cell line. **d)** Effect of TL(et) and TLYF on lymphocytes. **e)** TLC chromatogram of tamarind leaf ethanol extract as observed under the UV light. Data represented as mean ± SE. Data were analyzed by one way ANOVA. * denotes significance at $p<0.05$, **denotes significance at $p<0.01$, *** significance at $p<0.001$

3.3. Cell Count Using Trypan Blue Dye Exclusion

Assessing total cell concentration through trypan blue staining is a traditional method wherein nonviable cells appear distinctly blue under the microscope. TLYF demonstrated effectiveness in reducing the viable cell count and viability of PA-1 and HeLa cancer cell lines. At

5 µg/ml of TLYF, the total cell count was 1.5×10^5 cells/mL for PA-1 and 1.6×10^5 cells/mL for HeLa, compared to the control counts of 3.6×10^5 cells/mL and 3.8×10^5 cells/mL respectively (Table 2). Similarly, at 10 µg/ml of TLYF, the total cell count was 1.2×10^5 cells/mL for PA-1 and 1.3×10^5 cells/mL for HeLa. These results were found to be statistically significant with $p<0.05$. The viable cell count decreased with increasing treatment concentrations.

Table 2. Total cell count of HeLa and PA-1 cell lines by trypan blue staining

Plant Extract	Concentration of the extract ((µg/ml)		Total Cell Count (cells/ml)		Percentage Viability of the cells (%)	
	HeLa	PA-1	HeLa	PA-1	HeLa	PA-1
Control	0.4% DMSO	0.4% DMSO	$3.8 \pm 0.5 \times10^5$	$3.6 \pm 0.45 \times10^5$	92.3	96.2
	5	5	$1.6 \pm 0.29 \times10^5$	$1.5 \pm 0.3 \times10^5$	43.5	49.6
TLYF	10	7.5	$1.3 \pm 0.27 \times10^5$	$1.2 \pm 0.28 \times10^5$	31.6	28.6

3.4. Fluorescence Microscopical observation

Because this particular fraction exhibited anti-proliferative effects in both cancer cell types, we aimed to investigate how it works by examining the appearance of cells under a fluorescence microscope. Our observations revealed that the treated cells underwent nuclear breakdown, unlike the control cells. The control cells emitted a vibrant green fluorescence, indicating that they were alive and had intact membranes, whereas the treated cells emitted a vivid orange fluorescence (Figure 3). These results have obviously proven that TLYF induced apoptotic changes in the cells.

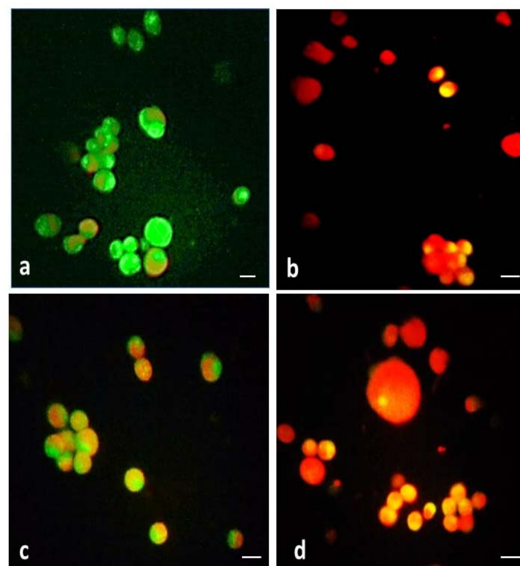

Figure 3: Fluorescence microscopic photographs of HeLa and PA-1 cells. **a**: untreated HeLa cells, **b**: TLYF treated HeLa cells, **c:** Control PA-1 cells, **b**: TLYF treated PA-1 cells. Scale bar: 20 µm. Magnification-100X.

3.5. Phytochemical screening

To identify the chemical groups in tamarind leaf crude extract and its active fraction TLYF, phytochemical screening was performed and the results are given in the Table 3. As we can see from the table, the yellow fraction from tamarind leaf indicated the presence of tannins and flavonoids.

Table 3. Phytochemical screening of the selected extracts and their bioactive fractions

TEST	TLE	TLYF
Alkaloids	+	-
Tannins	+	+
Flavonoids	+	+
Steroids	+	-
Terpenoid	+	-
Phenols	+	-
Glycosides	+	
Saponins	+	-

3.6. Fragmentation of DNA Visualization by Agarose Gel

DNA fragmentation is a characteristic feature of cells undergoing apoptosis. The apoptotic potential of TLYF was evident from the DNA banding pattern observed during gel electrophoresis. Treated cells exhibited a smear of fragmented DNA when viewed under a UV transilluminator, whereas control cells showed intact DNA bands (Figure 4).

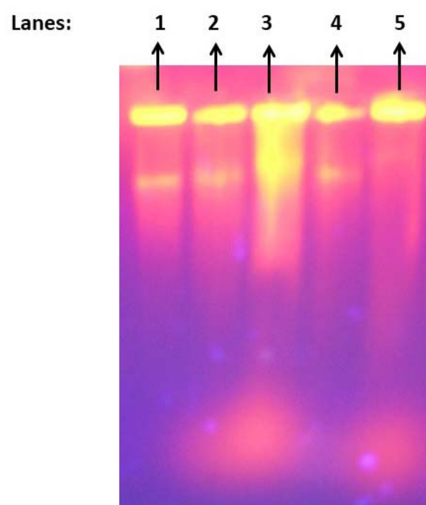

Figure 4: Analysis of DNA by gel electrophoresis in cancer cells treated with 5µg/ml of TLYF. **Lanes: 1 & 2**. DNA of untreated control HeLa cells, **3**. DNA of HeLa cells treated, **4**. DNA of untreated PA-1 cells. **5**. DNA of TLYF treated PA-1 cells.

3.7. LDH Release Assay

The release of LDH into the cytoplasm occurs as a consequence of damage due to apoptotic cell death or the cytotoxicity of the test compound. In cancer drug development and research, a test compound's activity can be determined by the LDH release assay in treated cancer cells, as it is a simple, reliable, and quick technique for estimating cell death. When HeLa cells were treated with TLYF at concentrations of 5 and 10 µg/mL, the percentage cytotoxicity was 39.5% and 53.7% respectively. Similarly, on PA-1 cells, the cytotoxicity percentages were 51.9% and 68.2% respectively (Figure 5). Thus, the bioactive fraction was responsible for membrane damage due to apoptosis or cytotoxicity in HeLa and PA-1 cancer cell lines.

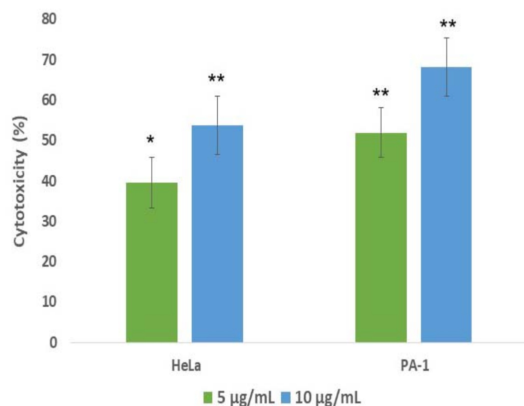

Figure 5: Percentage cytotoxicity as a measure of LDH release in PA-1 and HeLa cells treated with TLYF. Data was analyzed using one way ANOVA and *denotes significance at $p<0.05$, ** $p<0.01$.

3.8. Caspase 9 activity

The initiator caspase, caspase 9, can activate procaspases and induce apoptosis in living cells. Increased caspase 9 activity in any cell indicates that the cell is undergoing apoptosis. The initial caspase 9 activity in HeLa cells treated with TLYF was 89.9%, and in PA-1 cells it was 104.2% (Figure 6). After a 90-minute incubation period, caspase 9 activity increased to 146.3% in HeLa cells and 158.0% in PA-1 cells. Thus, there was a 56.4% increase in caspase 9 activity in HeLa cells and a 53.8% increase in PA-1 cells when comparing initial and final activity. These results were found to be statistically significant ($p<0.05$, $p<0.01$ and $p<0.001$ as per one-way ANOVA). Overall, a significant increase in caspase 9 activity was observed after TLYF treatment in cancer cells.

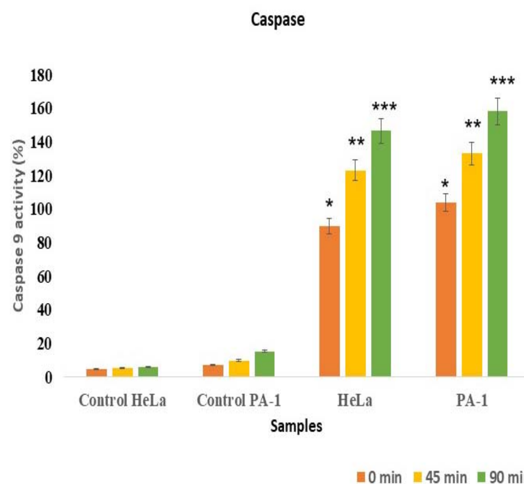

Figure 6: Activity of caspase- 9 (%) in the PA-1 and HeLa cells treated with TLYF. Data was analyzed by one way ANOVA. *Denotes significance with $p<0.05$, ** significance with $p<0.01$, *** significance with $p<0.001$.

3.9. Analysis of Cell cycle through Annexin V/ PI labelling and flow cytometry

The cancer cells become insensitive to normal cell signals, and hence avoid apoptosis and thereby proliferate faster. Therefore, quantitative measurement of the cell cycle has emerged as a key factor in identifying cell death. After Annexin V/PI labelling, we analysed the cell cycle stages of the TLYF treated PA-1 and HeLa cancer cells by flow cytometry, to ascertain the impact of the treatment on the cancer cells. Tamarind leaf yellow fraction was found to induce apoptosis in the cancer cells as shown by the cell populations in different quadrants. There were 18.84% of late apoptotic cells, 50.92% of early apoptotic cells and live cells were found to be 26.94%, while in untreated HeLa cell line, the percentage of viable cells were 95.97%, 2.53% early apoptotic cells, and a mere 0.61% cells were in late apoptotic phase (Figure 7). Thus, there was a clear indication of cells undergoing apoptosis by tamarind leaf fraction treatment.

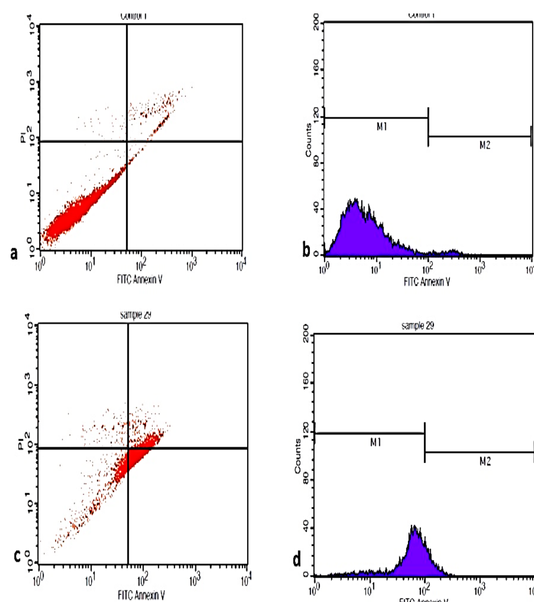

Figure 7: Annexin V/PI analysis of HeLa cells treated TLYF and control HeLa cells. **a) &b)** untreated control HeLa cells distributed in different phases (quadrants) of cell cycle, **c) & d)** treated HeLa cells distributed in different phases (quadrants) of cell cycle.

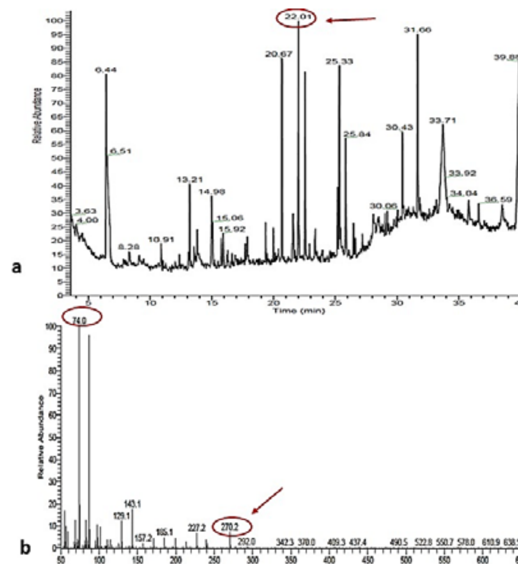

Figure 8: GC-MS depiction of TLYF **a**) The GC chromatogram **b)** Mass spectrum of the highest peak at 22.01 min. Arrow indicating a fragment ion having mol. wt. of 270.2 corresponding to apigenin.

3.10. GC-MS analysis of TLYF

The potent anticancer activity of the bioactive fraction from tamarind leaf prompted us to further characterize the components underlying this activity through GC-MS analysis.

The GC-MS chromatogram of the tamarind leaf yellow fraction exhibited a major peak with 100% abundance at retention time (RT) 22.01 minutes (Figure 8a), indicating the presence of a compound with a mass-to-charge ratio (m/z value) of 74 (Figure 8b). The corresponding compound with a molecular weight of 270.2 was identified as Apigenin from the database (http://bioinf-data.charite.de/cancerresource), where it is listed as an anticancer compound. Apigenin, a flavonoid derived from edible plants, has been linked to various experimental and biological investigations suggesting its anticancer properties (Imran et al., 2020). We propose that apigenin could be the reason for the profound antitumor property of tamarind leaf ethanol extract, as it is documented in the literature for its anticancer activity (Luo et al., 2008). Notably, this is the first report of the presence of apigenin in tamarind leaf and its anticancer activity.

4. Discussion

Cancer continues to dominate the diseases that cause significant morbidity and mortality, despite recent advancements in technologies for its detection and treatment. Among all the different types of cancers, ovarian and cervical cancers are the two most common cancers among women. Though surgery and other treatments are available, ovarian cancer has the highest mortality rate and the worst prognosis (Momenimovahed et al., 2019; Bray et al., 2018). Cervical cancer, the fourth most frequent female cancer globally, results in more than 300,000 deaths worldwide. Therapeutic approaches include surgery, radiotherapy, and/or chemotherapy either alone or in combination, all of which result in severe toxicity to the cells (Burmeister et al., 2022; Koppikar et al., 2010). Despite advancements in cancer diagnosis and treatment options, mortality rates due to cancer have not significantly declined. Synthetically prepared chemotherapeutic agents have a series of undesirable side effects on patients (Parveen and Nadumane, 2022). Recently, people are turning towards Complementary and Alternative Medicine more frequently for cancer treatment, alongside advanced allopathic drugs and other biological medicines, a practice known as integrative oncology. Less invasive therapeutic options using natural compounds wherever possible are ideal for managing cancer, with complementary and alternative medicines becoming the most sought-after treatment for ovarian and cervical cancers currently (Chizenga and Abrahamse, 2021; Adams and Jewell, 2007).

Different parts of the tamarind tree (*T. indica*) have traditionally been used for their high therapeutic properties, along with their nutritional components, attracting the attention of modern researchers (Kumar and Bhattacharya, 2008). Tamarind seeds are useful in treating bladder stones, boils, dysentery, ulcers, eye conditions, jaundice, chronic diarrhea, acne, and diabetes. Tamarind leaves can treat conjunctivitis, constipation, hemorrhoids, diarrhea, jaundice, boils, and liver disorders. When applied to boils, tamarind leaves reduce swelling and inflammation. Tamarind flowers are utilized as a pesticide and also used to cure eye conditions, jaundice, and bleeding piles. The bark is employed to treat ocular inflammations and digestive system conditions and has demonstrated anticancer potentials (Caluwe et al., 2010; Shirisha and Varalakshmi, 2016).

We hypothesized that tamarind leaves might possess anticancer potentials based on its reported therapeutic properties, anti-inflammatory action, and the known link between inflammation and cancer. Chronic inflammatory conditions are typically associated with a higher risk of developing cancer, as reported in several epidemiological studies. Cancer progression is often linked to inflammation, with both intrinsic and extrinsic types of inflammation leading to immunosuppression and creating a conducive environment for tumor development. Cancer-related inflammation cells are genetically stable, making them less prone to rapid drug resistance. Therefore, specifically targeting inflammation can be a viable strategy for cancer therapy or prevention (Singh et al., 2019; Coussens and Werb, 2002). We chose to investigate the leaves of the tamarind plant based on this idea, as numerous epidemiological findings point to the therapeutic benefits of dietary sources in cancer treatment, and tamarind leaves have been traditionally used in treating inflammations.

The potential anticancer efficacy of tamarind leaf was scientifically investigated in the current study. The ethanol extract of tamarind leaf was examined on the cervical cancer (HeLa) and ovarian cancer (PA-1) cell lines through the MTT assay. The results demonstrated a dose- and time-dependent decrease in cell density. Confirmation of this activity was obtained from trypan blue test results. Additionally, TLC was employed to guide the selection of the active component based on bioactivity. This method allowed the identification of the yellow fraction from tamarind leaf (TLYF) as having significant inhibitory effects on PA-1 and HeLa cancer cell lines. Viabilities decreased from 43% at 24 h to 27% at 72 h (in HeLa) and from 42% at 24 h to 11.9% (in PA-1) after 72 h of treatment. These effects were inversely proportional to treatment concentrations and duration of exposure, pointing to the antiproliferative property of the TLYF fraction. The IC_{50} value of TLYF was very low (0.1 μg/mL), within permissible limits for natural anticancer compounds suggested by the FDA. The TLYF fraction indicated the presence of both tannins and flavonoids through phytochemical screening.

Despite the enormous number of anticancer drugs developed and currently in use in clinical settings, they are not frequently chosen due to their impact on both tumor and healthy cells. Therefore, it is crucial to evaluate the safety of potential medicines before clinical trials in the development of anticancer drugs (Srivastava et al., 2005). In the current study, the safety evaluation of the tamarind leaf extract and the bioactive fraction TLYF was conducted on human peripheral lymphocytes. This choice is significant, as many anticancer drugs affect the survival of blood cells, leading to patients developing neutropenia and anemia after chemotherapy. Observations revealed that at all tested concentrations for 24, 48, and 72 hours, the percentage viability of lymphocytes remained above 90% and sometimes approached 100%, suggesting that this natural compound does not adversely affect blood cells. Consequently, we proceeded to analyze the mechanism of TLYF's anticancer activity on cervical and ovarian cancer cells using various *in vitro* assays.

Apoptotic cell death maintains tissue homeostasis and controls cell growth. Any deviation in the cell's ability to undergo apoptosis results in the formation of tumors. Hence, researchers in the field of anticancer drug development have been concentrating on identifying apoptotic inducers. In the current research, we aimed to investigate the apoptosis-inducing properties of TLYF on cancer cells. Apoptotic cells exhibit morphological and physiological changes such as cell shrinkage, the formation of apoptotic bodies, and chromatin condensation, observable directly under an inverted microscope or through staining for visualization under a fluorescence microscope (Loannou and Cheng, 1996). In this regard, when we treated the cancer cells with TLYF, we observed that the treated cells fluoresced bright orange, indicating apoptosis, while the untreated control cells fluoresced bright green, indicating viability, under fluorescence microscopy. Furthermore, we observed a significant increase of over 50% in caspase activity in PA-1 and HeLa cells treated with TLYF, clearly suggesting the apoptosis-inducing ability of this fraction. Caspases, members of the cysteine protease family, are well-known for their crucial role in apoptosis. Caspase 9, one of the most crucial apoptosis executors among various caspases, initiates and activates other apoptotic proteins. An increasing level of Caspase 9 indicates cell death.

Apoptosis in TLYF-treated cancer cells was also supported by the visualization of the DNA fragmentation pattern in agarose gel electrophoresis of DNA from treated cells. The DNA of TLYF-treated cells appeared as a smear upon gel electrophoresis, signifying a higher degree of apoptosis in these cancer cells (Mathi et al., 2014). Thus, there was strong evidence for the induction of apoptosis in cancer cells by TLYF. Moving forward, we estimated cell membrane damage through the LDH release assay. This assay, believed to be indicative of cytoplasmic release resulting from treatment with any cytotoxic compound, revealed that TLYF treatment caused the highest release of LDH compared to untreated control cells of both HeLa and PA-1.

Apart from inducing apoptosis, cell cycle progression is one of the effective mechanisms in cell proliferation. Blocking the cell cycle at various stages and inhibiting DNA replication were considered as some of the effects exerted by efficient or most successful anticancer drugs (Loniakan et al., 2023). In view of this we studied cell cycle phases of the cervical cancer cells by flow cytometry method following Annexin V and PI staining. The results have shown that TLYF treatment resulted in a significant decrease in S-phase cells along with a decline in the total cell population compared to the controls, indicating that it

had arrested the cell cycle at the G_0/G_1 phase. Thus, it can be interpreted that TLYF treatment resulted in direct cytotoxicity in the cells, as evidenced by the increased percentage of LDH release in the cells. Taken together, it appears that TLYF exerts its anticancer effects through inhibiting cell proliferation, initiating apoptosis, causing cell cycle arrest, and inducing direct cytotoxicity. This demonstrates its significant anticancer potential.

The characterization of TLYF was carried out by phytochemical analysis followed by LC-MS and GC-MS methods. The presence of flavonoids and tannins in the bioactive fraction of tamarind leaf was confirmed by phytochemical analysis of TLYF. In GC-MS results, it was observed that the mass-to-charge (m/z) ratio of the highest peak at the retention time (RT) 22.01 minutes of the LC-MS chromatogram indicated a compound with 100% relative abundance, having an m/z value of 74. The corresponding compound with a molecular weight of 270.2 was found to be apigenin during the library search. Apigenin, the flavonoid, was isolated from different sources, such as fruits and vegetables, and was reported to have anticancer activities (Luo et al., 2008). The identification of apigenin in tamarind leaf, as reported in this study, is the first of its kind according to our literature survey. Therefore, we assert that this compound is responsible for the appreciable anticancer activity observed *in vitro*.

5. Conclusion

In conclusion, the results of this study highlight the potential of dietary plants with medicinal significance, such as the tamarind leaf, to play a crucial role in anticancer drug discovery. Future research efforts should concentrate on the comprehensive chemical and structural characterization of the identified bioactive fraction, paving the way for subsequent clinical and drug development studies.

Declarations

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Conflicts of interest/Competing interests

The authors disclose that they do not have any conflicts of interest.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All the data generated or analysed during this study are included in this published article or available from the corresponding author upon reasonable request.

Code availability

Not applicable

Authors' contributions

SR and VKN contributed equally to the study conception and design. Material preparation, data collection, analysis was performed by SR. Supervision was by VKN. The initial write-up of the draft manuscript was by SR. The review and corrections for the previous version of the manuscript were carried out by VKN. All authors read and approved the final manuscript.

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