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Antiproliferative effect of methanolic extract of *Tamarindus indica* in C127I cell line

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ABSTRACT

Investigation of natural bioactive compounds that possess antioxidant and anticancer properties have drawn interest in scientific and industrial perspectives. The current study was conducted to assess the antiproliferative action of methanolic extract of seed coat of *Tamarindus indica* in C127I mouse mammary tumour cell line. 3-(4,5-dimethyl thazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay is performed to assess the cytotoxicity of methanolic extracts of *T. indica* in the C127I cell line at concentrations of 320, 160, 80, 40, 20, and 10 µg/mL. The half maximal inhibitory concentration (IC_{50}) was then calculated using Graph Pad Prism 5.0. Doxorubicin was used as positive control. After determining IC_{50}, the cells were treated with extract of *T. indica* at half, IC_{50} and double IC_{50} concentrations after being seeded at a concentration of $1x10^5$ cells/mL in 6 well plates. After 24 hours the cells were trypsinised and stained with acridine orange-ethidium bromide (AOEB) to assess the morphology of apoptosis. When the cells were exposed to various doses of the extract, a dose-dependent decrease in cell viability was observed, and the IC_{50} value of *T. indica* was estimated to be 13 µg/ml. In the control cells, AO/EB labelling revealed proliferating cells with green fluorescence, but the *T. indica* extract-treated cells displayed dose dependent shift from orange to red fluorescence. In conclusion, methanolic extract of *Tamarindus indica* was found to possess antiproliferative effect in C127I cell line comparable to doxorubicin.

Keywords: *T. indica*, C127I, Antiproliferative effect, Mammary tumour cell line.

INTRODUCTION

Breast cancer is reported with highest incidence in women and animals and is responsible for cancer mortality in females throughout the world [1]. Even though chemotherapy being one of the main therapeutic techniques for cancer, these conventional chemotherapeutic agents are ineffective in achieving complete tumour remission and inhibition of metastasis, as they are often prone to resistance and hazardous to normal tissues. For the past few years plant-based compounds have been the fundamental component of cancer treatment and are expected to be the lead structures that can be employed as templates to create new compounds with improved biological features [2]. *Tamarindus indica (T. indica),* a member of the Leguminosae family and the most prevalent and significant large evergreen tree and is widely found in arid regions throughout Central and South Indian states as well as other South East Asian nations. Tamarind is grown primarily for its soft, flavourful pulp, which is chiefly used in food and confectionery and is one of the traditional herbal remedies[3]. The plant seeds are frequently discarded as waste because of their brittleness and blandness, despite the fact that they are nutrient-rich. Water, acetone, ethanol and methanol extracts of seeds of *T. indica* were found to have potent anti-diabetic, antihyperlipidemic and antioxidant potential. Tamarind seeds are also reported to contain polymeric tannins, fatty acids, flavonoids, saponins, alkaloids, glycosides and phenolic antioxidants which have been shown to reduce lipid peroxidation *in vitro* [4]. With this background the present study was designed to explore the antiproliferative potential of *T. indica* seed coat extract in C127I cell lines.

MATERIAL AND METHODS

Plant: The *Tamarindus indica* obtained locally from Mannuthy, authenticated at St. Thomas College Thrissur.

Preparation of extract: The seeds of *Tamarindus indica* were isolated, gently roasted and seed coat was separated and coarsely ground to powder employing an electric pulverizer, and methanol-based extraction was performed using soxhlet extraction equipment to extract the powder and further concentrated in rotary vacuum evaporator at temperature 40°C under reduced pressure.

Cell lines: Mouse mammary tumor cell line C127I, used in this study, procured from the National Centre for Cell Sciences in Pune. During the conduct of study, the obtained cells were cultured in 25 cm^2 tissue culture flaks with the addition of Rosewell Park Memorial Institute (RPMI) -1640 medium containing 10% foetal bovine serum and penicillin-streptomycin (100 U/mL penicillin and 100 g/mL streptomycin) kept in humid incubator with 5% carbon dioxide (CO_2) at 37° C.

Estimation of Phytoconstituents in *T. indica*:

The qualitative phytoconstituent analysis was performed to test the presence of the bioactive ingredients in methanolic extract of seed coat of *T. indica* [5].

Cytotoxicity (MTT) assay

The cytotoxic ability of the methanolic extract of seed coat *T. indica* was evaluated in C127I, using (MTT) 3-(4,5- dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide reduction assay[6]. $5x10^4$ cells/mL were seeded into 96-well microtiter plates, which were then left to attach for 24 hours. The cells were further treated with extract at doses of 320, 160, 80, 40, 20 and 10µg/mL for 24 hrs. Then, each well received 10 µL of MTT at a concentration of 5 mg/mL, which was incubated for 4 hours with supplementation of serum-free media. By adding 100µL of DMSO to each well to dissolve the formed formazan crystals, the reaction was stopped. At a wavelength of 570 nm, the absorbance was determined using a microplate reader (Varioskan Flash, Thermofischer Scientific, Finland). Cells with active metabolism reduce MTT to a purple formazan product that show absorbance at 570 nm. The IC_{50} values of extract was calculated by Graph Pad Prism 5.0.

Estimation of phytocomponents by gas chromatography-mass spectrometry (GC-MS):

The GCMS machine at the Kerala Forest Research Institute (KFRI), Peechi, Kerala, was used to examine the active phytochemical components of methanolic extract of the seed coat of *T. indica*. A Shimadzu GC-MS (Japan, QP2010S) gas chromatography mass spectrometer with a mass range of 1.5-1000 m/z was employed. Helium as the carrier gas, was employed at a flow rate of 1mL/min. The oven's temperature was held at 80^0C for 4 minutes and then raised to 280^0 C in 6 minutes. 50 minutes were allotted for the analysis, and the injector's temperature was 260^0 C. After a distinct baseline was obtained, extract aliquots were injected onto the chromatographic column. Principal components were determined using mass spectrum library (NIST 11 and WILEY 8).

Estimation of phytocompounds by FTIR analysis:

ATR-FTIR (Attenuated Total Reflectance- Fourier transform infrared) investigation was carried out using Fourier transform infrared spectroscopy (FTIR). The FTIR spectrometer was used to identify molecular groupings. For FTIR analysis, a Perkin-Elmer spectrum two FTIR spectrometer with attenuated total reflectance was used. An overhead ATR attachment was installed at the sample station. Between measurements, the ATR diamond crystal was meticulously cleaned with pure isopropanol. The seed coat extract was analysed using FTIR. In order to focus the laser beam, a little amount of the sample was carefully applied to the surface of the diamond crystal to cover the ATR diamond window. Each spectrum's absorbance under 60 N value was recorded.

AO/EB staining: After determining IC_{50}, the cells were treated with extract of *T. indica* at half, IC50 and double IC_{50} concentrations after being seeded at a concentration of $1x10^5$ cells/mL in 6 well plates. After 24 hours the cells were trypsinised and stained with acridine orange-ethidium bromide (AOEB) to assess the morphology of apoptosis to distinguish between living, apoptotic, and necrotic cells. Acridine orange (10 µg/mL) and ethidium bromide (10 µg/mL) were used to stain 25 microliters of the treated or untreated cells and examined under a Trinocular Research fluorescence microscope, DM 2000 LED, Leica, with blue excitation (488 nm) and emission (550 nm) filters at 10X magnification [7].

RESULTS

Phytochemical analysis of *T. indica:*

Alkaloids, steroids, glycosides, flavonoids, tannins, phenolic compounds and saponins were revealed in the phytochemical analysis of *T. indica* and the results were tabulated in the Table 1.

Table 1: Phytoconstituents present in methanolic extract of seed coat of *T. indica:*

PHYTO-CONSTITUENTS	Methanolic extract of seed coat of *T. indica*
STEROIDS (Salkowski's test)	+
ALKALOIDS (Dragendorff's test)	+
GLYCOSIDES (Sodium hydroxide test)	+
TANINS (Ferric chloride test)	+
FLAVONOIDS (lead acetate test)	+
PHENOLIC COMPOUNDS (Ferric chloride test)	+
SAPONINS (Foam test)	+
DITERPENS (Ferric chloride test)	+

GC-MS analysis:

Chromatogram produced following a phytochemical study of methanolic extract of seed coat of *T. indica* using GC-MS is given in Figure 1.

Table 2 lists the phytoconstituents identified by GC-MS analysis.

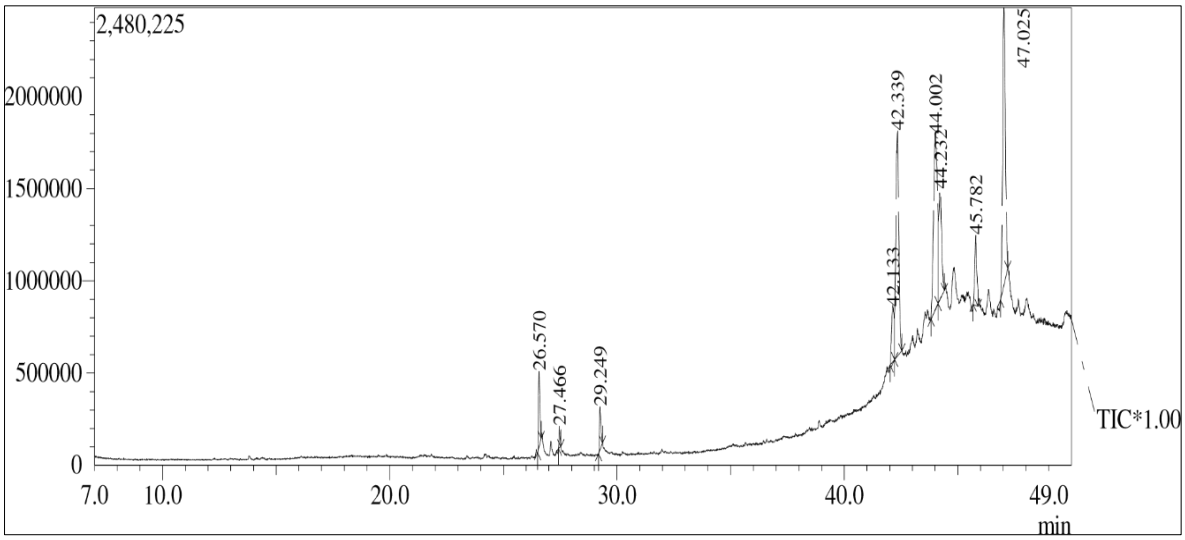

Figure 1: GC-MS chromatogram of methanolic extract of seed coat of *T. indica*

Table 2: *T. indica* seed coat methanolic extract phytochemical GC-MS analysis

Peak#	R. Time	Area	Name	Probability (%)
1	26.570	1654214	Neophytadiene	95
2	27.466	356168	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	91
	29.249	1033816	Dibutyl phthalate	96
4	42.133	2485013	Gamma. -Sitosterol	80
5	42.339	10872416	Cycloartenyl acetate	79
6	44.002	10803897	Methyl commate D	82
7	44.232	5315622	2,3-Dehydro-4-oxo-. beta. -ionol	71
8	45.782	2419875	Triacontyl acetate	95
9	47.025	12289017	Stigmast-4-en-3-one	89

The chemical Compounds identified by comparing the extract's FTIR and FLUKA library spectra are reported in table 3 along with the spectrum of the methanolic extract of *T. indica* seed coat in figure 2.

Table 3: List of structurally similar compounds of *T. indica* seed coat methanolic extract

Wave number (cm^{-1})	Bond	Functional group
3250	O-H	Alcohol
1606	C=C	α, β unsaturated ketone
1442	C-H	Alkane
1283	C-O	Aromatic ester
1105	C-O	Secondary alcohol
764	C-H	1,2,3 trisubstituted

FTIR analysis:

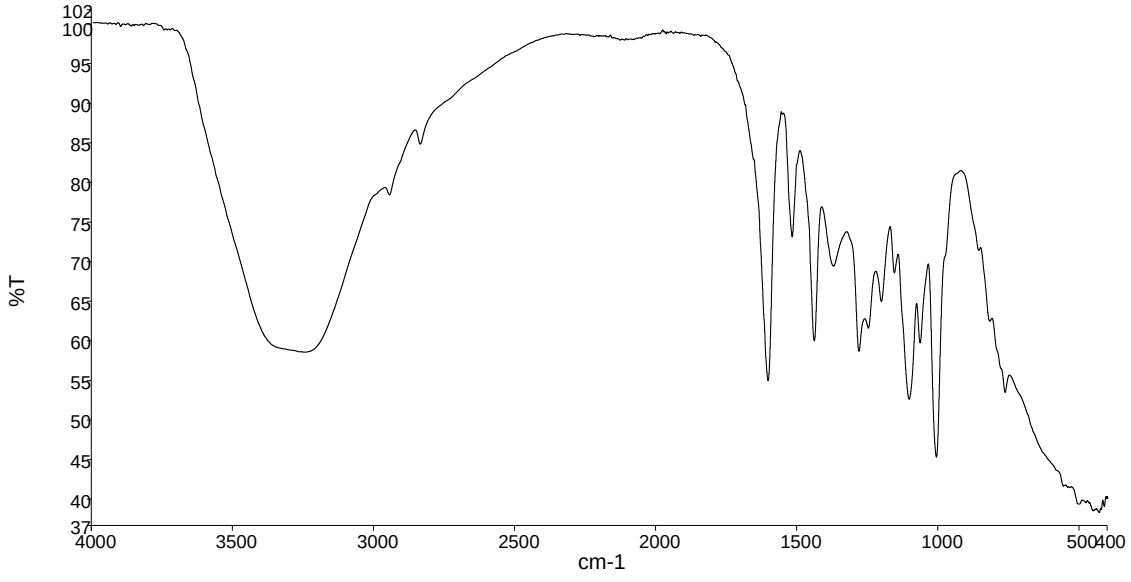

Figure 2: FTIR spectrum of methanolic extract of seed coat of *T. indica*

Cytotoxic testing in *Tamarindus indica* in C127I cell line:

Tamarindus indica methanolic extract of seed coat was tested for cytotoxicity using the C127I cell line. When the cells were exposed to various extract concentrations, a dose-dependent decrease in cell viability was observed. The IC_{50} value of *T. indica* was determined to be 13 µg/mL using Graph Pad Prism 5.0. Table 3 lists the values of the concentration of inhibition of cells exposed to various concentrations of methanolic extract of *T. indica*.

AO/EB staining:

After treatment of cells with various doses of the extract living, apoptotic, and necrotic cells were distinguished using the acridine orange ethidium bromide (AO/EB) staining. Figure 3 shows images of cells that had undergone various treatments. Fluoresced greenish cells with a circular pattern and an evenly spaced nucleus were visible in control cells. Early apoptotic cells showed granular or crescent-shaped nuclei that were stained yellow or green following treatment with doses below the IC_{50} of the extract the late apoptotic stage of orange-to-red luminous cells was studied in IC_{50} and above IC_{50} concentrations. Treatment-exposed cells also displayed morphological alterations such as fragmented nuclei, membrane blebs, and apoptotic bodies.

Table 3: Mean±SE, with n=3 replicates, depicting the inhibition of cells subjected to various doses of methanolic extract of *T. indica*

Concentration of MTI (µg/mL)	Cell Inhibition
320	81. 4796±1.059544
160	77.399±0.173156
80	73.6398±0.165287
40	72.973±0.297297
20	73.3022±0.133542
10	0.520413±0.156693
IC50(µg/mL)	13

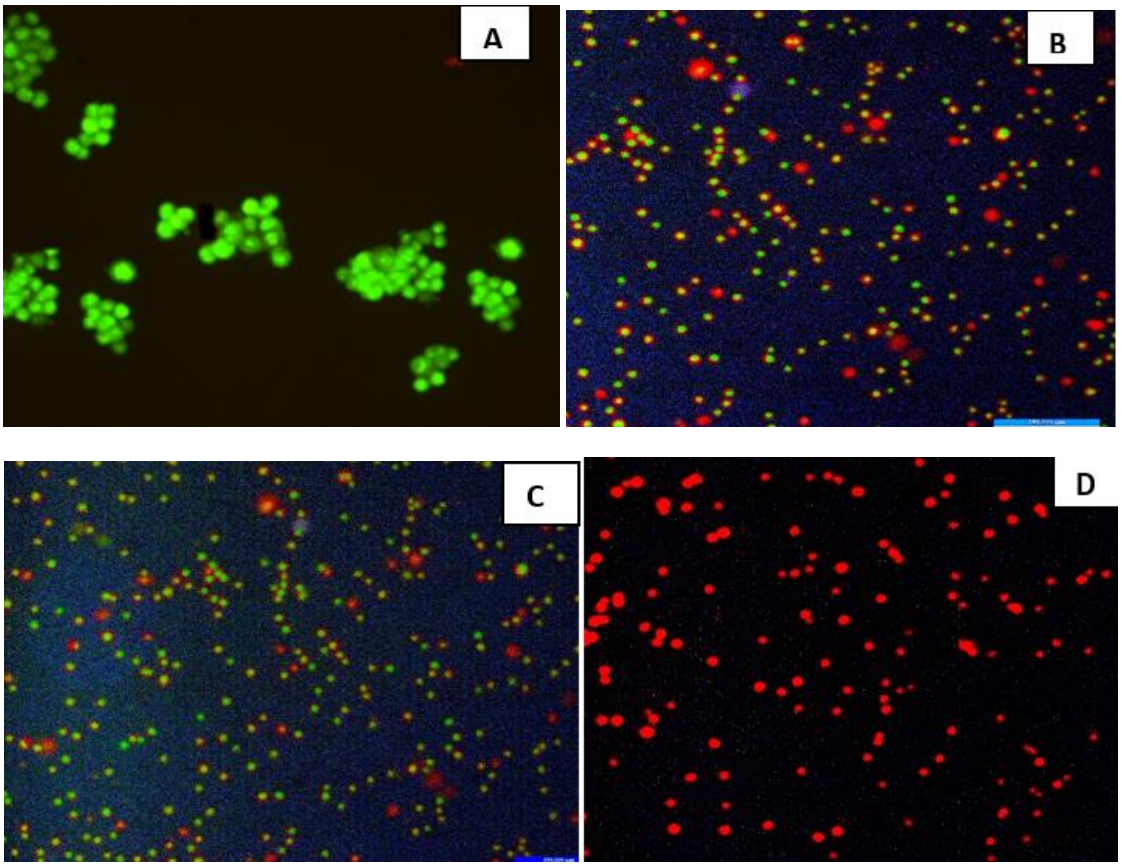

Figure 3: AO/EB staining of C127I cells after treatment with various concentrations of *T. indica* 10X A-control cells, B- cells treated with half IC_{50}, C-cells treated with IC_{50} and D-cells treated with twice IC_{50} (highest dose)

DISCUSSION

Breast cancer is the most prevalent malignancy in women worldwide and the primary reason for cancer deaths. Investigation of natural bioactive compounds that possess antioxidant and anticancer properties have gained attention in scientific and industrial approaches. Extracts from the whole seed, seed kernel, and seed coat of *T. indica* are known for their antioxidant, cytotoxic, and immuno-protective effects [4]. Various phytoconstituents, including alkaloids, tannins, steroids, phenolic compounds, saponins, glycosides and flavonoids were found in current study of methanolic extract of *T. indica* after phytochemical investigation. Phytocompound analysis of *T.indica* seed coat-hydromethanolic and aqueous extract were found to possess polyphenols, saponins, alkaloids, flavonoid and tannins. Flavanoids and phenolic compounds act as reducing agents and singlet oxygen scavengers[8]. Tannins have to reported to show anticancer properties [9]. Thus, potentially useful bioactive components for the treatment and prevention of cancer can be found in *Tamarindus indica*. In our present study GCMS of methanolic extract of seed coat *T. indica* revealed the presence of Neophytadiene,

3,7,11,15-Tetramethyl-2-hexadecen-1-ol, Dibutyl phthalate, Gamma. -Sitosterol, Cycloartenyl acetate, Methyl commate D, 2,3-Dehydro-4-oxo-beta-ionol, Triacontyl acetate and Stigmast-4-en-3-one. FTIR analysis confirmed the presence of Secondary alcohol, aromatic ester, α, β unsaturated ketone, alcohols and alkanes. Alcohols, esters/carboxylic acid, alkyne, alkene, and amines were reported in the FTIR spectrum analysis of hydroethanol extract of *T. indica* [10]. In our present study the *in-vitro* cytotoxic assay of the methanolic extract of seed coat *T. indica* was evaluated in C127I cell line using MTT reduction assay. Dose dependent decrease in cell viability was seen when the cells were treated with various concentrations of the extract and calculated IC_{50} value of *T.indica* was 13 µg/mL. The cytotoxicity of *Tamarindus indica* seed methanolic extract was studied on Rhabdomyosarcoma cancer (RD) and Human Lymphoma cell line (SR) cancer cell lines and it was reported that the extract has potent anticancer properties because of the presence of caffeic acid and other polyphenols that can boost the antioxidant activities of treated cancer cells, assisting in defense against oxidative damage and also having an impact on gene expression, cell viability, cell cycle, migration and other processes[11].Polysaccharide (PST001) from seed kernel of *T. indica* showed antitumor activity on murine cancer cell lines, Daltons Lymphoma Ascites and Ehrlich Ascites Carcinoma and could inhibit the cell proliferation significantly [12]. Dual staining with ethidium bromide and acridine orange is a technique for identifying apoptotic cells that are alive, early in the process, and late in the process. *T. indica* induces apoptosis in a dose-dependent way. The nuclei of control cells stained by AO penetration which emit green fluorescence via binding to DNA, EB stains the nuclei of late apoptotic and necrotic emitting red fluorescence. As a result, each detected phytochemical in the methanolic extract of the *T. indica* seed coat has a unique bioactivity and has therapeutic potential, making it possible to use it in therapeutic formulations in a secure and economical manner.

CONCLUSION

In conclusion, the study revealed that the methanolic extract of *T. indica* exerts apoptosis in cancer cells in a dose dependent way suggesting that it might be developed as a lead drug for the treatment of cancer after conducting clinical trials.

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Conflict of Interest

None declared.

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