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Research Article

Cytotoxicity of Methanolic Extract of Seed Coat of *Tamarindus indica*

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ABSTRACT

Medicinal herbs provided the way for the generation of large number of pharmaceuticals used in cancer therapy. They are expected to improve the therapeutic efficacy of chemotherapy, potentially reducing adverse effects in cancer patients. Tamarind is used as traditional medicine and the seed coat possess antioxidant, antibacterial properties. The current study was performed to assess cytotoxicity of methanolic extract of seed coat of T. indica in MCF-7 human breast cancer cell line. The seeds of T. indica were obtained locally, shade dried, coat removed pulverised and extracted with methanol and concentrated using rotary vacuum evaporator and used for research. The qualitative phytochemical analysis of the methanolic extract of T. indica was performed. Cytotoxicity of T. indica was evaluated by 3-(4,5-dimethyl thazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay in MCF-7, at dose rates of 320, 160, 80, 40, 20, 10 µg/mL and the half maximal inhibitory concentration (IC50) was calculated using online software AAT Bioquest. The cells cultures in 6 well plates at 1x105 cells/mL and were treated for 24 hours with extracts of T.indica at concentrations of half, IC50 and double IC50 concentrations. The cells were trypsinised and subjected to Acridine orange - Ethidium bromide staining (AOEB) and JC-1 staining. The results were analysed statistically. Qualitative phytochemical tests revealed the presence of alkaloids, flavonoids, steroids, phenolic compounds, diterpenes, saponins, glycosides and tannins. T.indica reduced cell viability in dose dependent manner and IC50 value is found to be 16µg/mL. AO/EB staining detected apoptotic cells with orange- red fluorescence in the extract treated cells which were increasing in a dose dependent manner while normal cells shown green fluorescence. Control cells stained with JC-1 dye showed red-orange fluorescence whereas there was a dose dependent shift from red -green fluorescence in cells treated with T.indica. Methanolic extract of T.indica showed cytotoxicity in vitro against MCF-7 cell line and induced apoptosis in dose dependent manner, so it can be used as novel compound against breast cancer.

Keywords: T.indica, MCF-7 cells, MTT, apoptosis, Breast cancer

INTRODUCTION

Breast cancer is an invasive disease which mainly affects women. It is one of the most common types of cancer and ranked second among cancer-related causes of mortality in women. Age, gender, inheritance, mutations, menopause, obesity, food, smoking, and radiation exposure have all been identified as key risk factors for breast cancer. Breast cancers are often treated with radiation, chemotherapy and surgery One of the major problems encountered with anticancer therapy is the evolution of resistance to commonly used drugs along with their high risk of organ toxicities. Plants have been widely studied for their antitumor potential and many have shown to possess potent anticarcinogenic agents. Medicinal herbs are viable options for cancer treatment due to their low cost and lack of side effects.

Polyphenols are a large and diverse family of natural substances that are utilised in the prevention and treatment of cancer. They also act as adjuvants in therapy of cancer. MCF-7 was estrogen receptor (ER) positive, progesterone receptor (PR) positive, and human epidermal growth factor receptor (HER) negative cell line commonly utilized in hormone dependent tumor biology research. Tamarind is a leguminous tree, which belongs to family Fabaceae and the fruit pulp is widely used in cuisine around the world and also as traditional medicine.[2]Recent studies suggest that polyphenols present in tamarind seed coat possess anti-inflammatory hepatoprotective, anthelmintic, antimicrobial and antioxidant properties.[3] Hence, a study was designed to evaluate the anticancer activity of methanolic extract of seed coat of *T indica* in MCF-7 cell lines.

MATERIALS AND METHODS

Plant material and extract preparation

The seeds of *Tamarindus indica* were collected locally, from Mannuthy, authenticated by Botanist at St. Thomas College, Thrissur, separated seed coat was used for the study. The seed coat was coarsely powdered using an electric pulveriser and the powder obtained was extracted using a Soxhlet extraction apparatus with methanol, which is followed by the concentration of the extract in a rotary vacuum evaporator under reduced pressure and temperature (40˚C).

Cell lines

MCF-7, an adherent human breast adenocarcinoma cell line obtained from the National Centre for Cell Sciences in Pune, was used for *in vitro* investigations. Cells were adapted to grow in Rosewell Park Memorial Institute (RPMI) -1640 media supplemented with 10 per cent foetal

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bovine serum and 1 per cent gentamicin (50 mg/mL). The cells were maintained in a humidified incubator at 37° C with five per cent carbon dioxide (CO_2).

Phytochemical analysis

The qualitative phytochemical analysis was performed according to Harborne (1998). [4]

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

In-vitro cytotoxic potential of the extract of seed coat of *T. indica* was assessed in MCF-7, using MTT reduction assay as per Riss *et al.*, (2004).[5] The extract was diluted to 320,160,80,40,20 and 10µg/mL and used for the study. 96 well plates were seeded with $1x10^5$ cells/mL and was allowed to proliferate for 24 hours. Then the extract was added to the cells in the above-mentioned concentrations and incubated for 24 hrs. Then MTT at a concentration 5mg/mL was added to each well at 10µL, incubated for 4 hours with serum free media. The reaction was stopped by adding 100 µL of DMSO and the absorbance was read at 570 nm in a Varioscan ELISA Plate reader.

The per cent cell viability and per cent cell inhibition were calculated using the following formulae:

Per cent cell viability = (Average absorbance of treated cells /Average absorbance of untreated cells) × 100

Per cent cell inhibition = 100 - per cent cell viability

The net absorbance from the control wells was taken as 100 per cent viable. The IC_{50} values of extracts were calculated by plotting the concentration against per cent cell inhibition using AAT Bioquest

Acridine orange / Ethidium bromide (AO/EB) staining

A concentration of 1×10^5 cells were seeded into a six well cell culture plate and treated with of extract for 24 h. The acridine orange / ethidium bromide (AO/EB) staining procedure was followed to differentiate the live, apoptotic and necrotic cells. Twenty-five microlitres of the treated or untreated cells were stained with five microlitres of acridine orange (10 µg/mL) and ethidium bromide (10 µg/mL) and analysed under Trinocular Research fluorescence microscope, DM 2000 LED, Leica with blue excitation (488 nm) and emission (550 nm) filters at 20X magnification (Ribble *et al.*, 2005).[6]

Analysis of mitochondrial transmembrane potential (MMP)

MCF-7 cells were plated at a seeding density of 1×10^5 cells per well in six well plates. After 24 h of treatment with extracts at concentrations 320, 160, 80, 40, 20, 10µl/ml and the cells were incubated with five µM fluoroprobe, 5. 5', 6, 6'- tetrachloro- 1, 1', 3,3'-tetra ethyl 2enzimidazole-carbocyanine iodide (JC-1) for 30 min at room temperature in the dark. The cells were analysed using fluorescent microscope with filters having blue excitation/ emission of 540/ 570 nm and red excitation/ emission of 590/ 610 nm filters (DM 2000 LED, Leica) (Ovadje *et al*., 2011)[7].

Table 1. Per cent viability of cells exposed to methanolic extract of T.indica, presented as Mean±SE, with n=3 replicates

Concentration(µg/mL)	Percent viability (Mean±SE)
320	19.15±0.033
160	21.91±0.075
80	20.60±0.00
40	24.93±0.00
20	38.23±0.06
10	56.79±0.03

Table 2. Percent inhibition of cells exposed to methanolic extract of *T.indica*, presented as Mean±SE, with n=3 replicates

Concentration(µg/mL)	Percent inhibition (Mean±SE)
320	80.85±0.03
160	78.08±0.07
80	79.40±0.00
40	75.06±0.00
20	61.76±0.06
10	43.20±0.03
IC_{50}(µg/mL)	16

RESULTS

Phytochemical Analysis of Methanolic Extract Of Seed COAT OF *Tamarindus indica*

The phytochemical analysis of *T.indica* revealed the presence of steroids, glycosides, phenolic compounds, tannins, flavonoids, alkaloids and saponins.

Cytotoxic evaluation of methanol extract of seed coat of *Tamarindus indica* in MCF-7 cancer cell lines.

The per cent viability is tabulated in table 1. Figure 1depicted MTT assay of MCF-7 cells after treatment with *T. indica*. There was a dose dependent inhibition of MCF-7 cells with an IC_{50} of 16µg/mL. From per cent viability values, per cent inhibition is calculated showed in the table 2. The graph showing the analysis of IC_{50} is depicted in Figure 2.

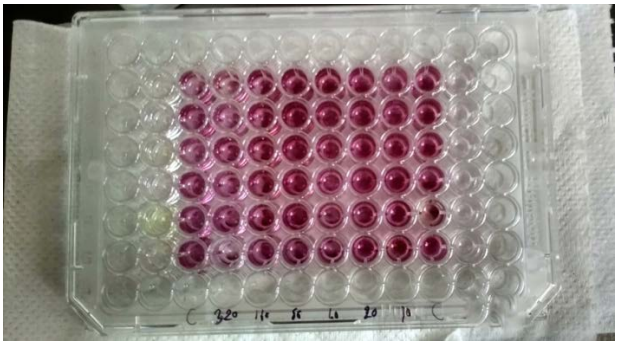

Figure 1: shows MTT assay of MCF-7 cells after treatment with T.indica extract

Acridine Orange/ Ethidium Bromide staining

After treatment with extract, live, necrotic, early, and late apoptotic cells were found. Figure 3 show representative pictures of cells from different treatments after AO/EB staining. Cells in the control population were alive and fluoresced greenish with a circular pattern and the nucleus was evenly dispersed across the centre. In the early apoptotic cells, the nuclei were crescent shaped or granular, which where stained yellow or green and were detected in the cell lines 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. Cells that had been treated also revealed morphological changes like nuclei fragmentation, membrane blebs and apoptotic bodies.

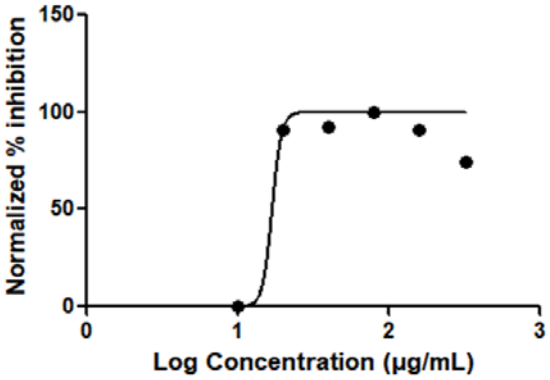

Figure 2: IC_{50} of T. indica

JC-1 staining

Figure 4 show representative pictures of cells from different treatments after JC-1 staining. JC1 aggregates with reddish/orange fluorescence were found in control cells, indicating a greater mitochondrial membrane potential. After 24 hours, both cell lines showed a dose dependent shift from red to green fluorescence for *T.indica*, indicating a concentration dependent reduction of mitochondrial membrane potential.

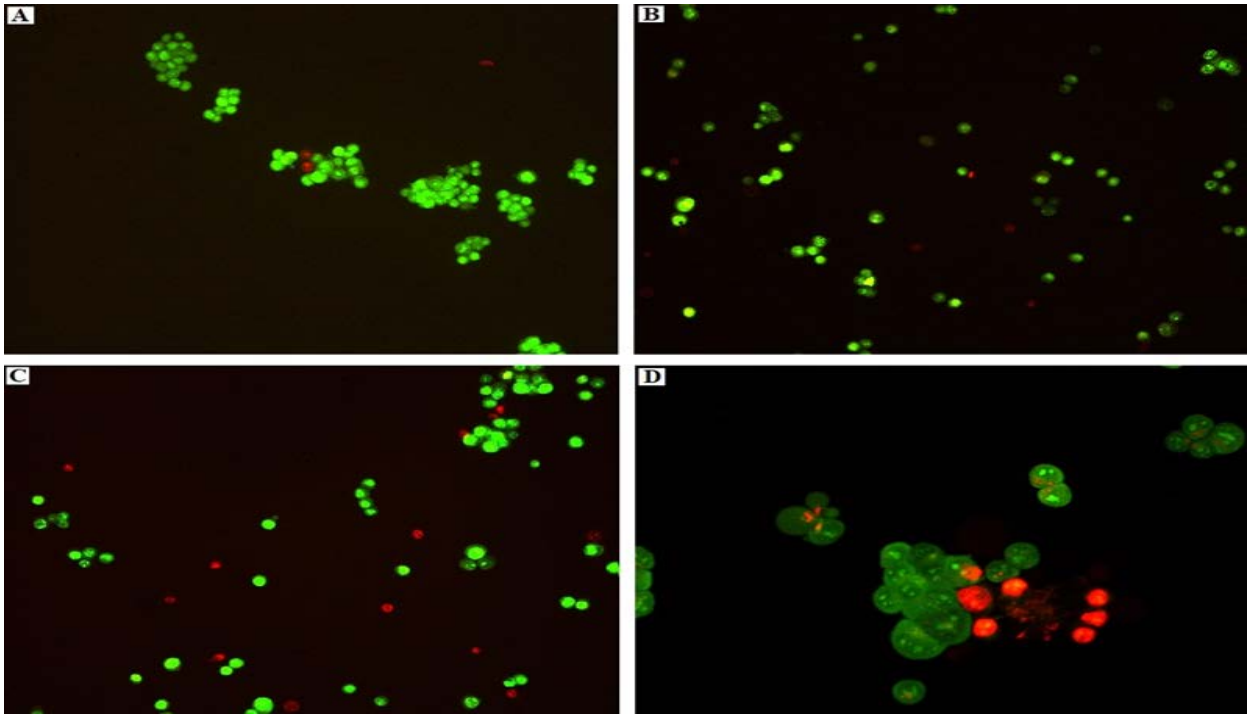

Figure 1: AO/EB staining of MCF-7 cells after treatment with various concentrations of *T. indica* 20X A-control cells, B-cells treated with half IC_{50}, C-cells treated with IC_{50} and D-cells treated with twice IC_{50} (highest dose)

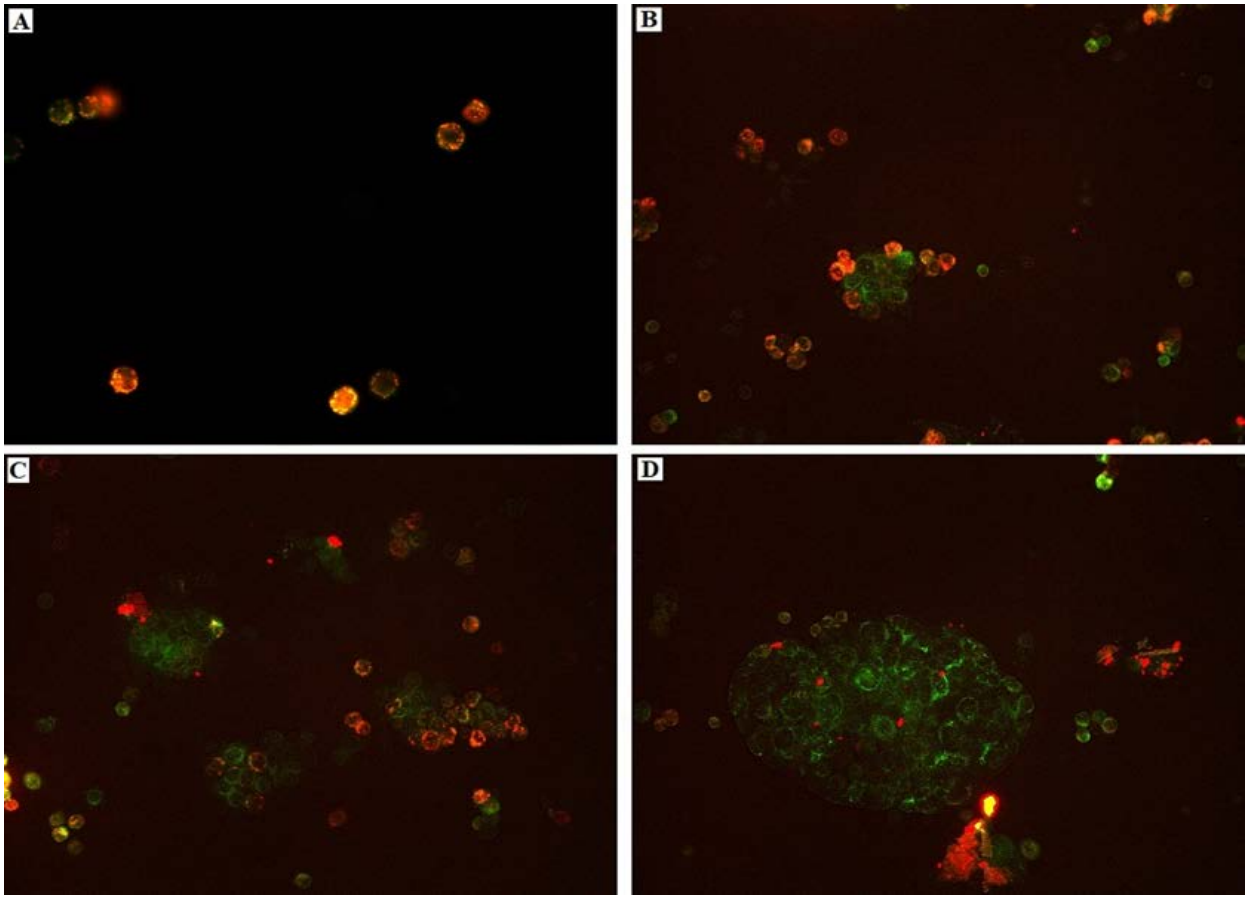

Figure 4: JC-1 staining of MCF-7 cells after treatment with various concentrations of T. indica 20X A-control cells, B-cells treated with half IC_{50}, C-cells treated with IC_{50} and D-cells treated with twice IC_{50} (highest dose).

DISCUSSION

Breast cancer is the most common type of cancer found in women after skin cancer, and is very invasive. Phytochemicals present in plants are known for their anti-inflammatory, antioxidant and antiproliferative activities. [8] The phytochemical analysis revealed the presence of phenolic compounds which is known for its cytotoxicity against cancer cells. The presence of phenolic compounds in *T.indica* extract might have caused the antiproliferative and apoptotic effect in MCF-7 cell lines.[9] Preliminary in vitro anticancer activity of *T.indica* was assessed in MCF-7 cell line using the MTT assay. MTT is reduced to a purple formazan product by cells that are in active metabolism that have a maximum absorption at 570 nm. There was a dose dependent decrease in the viability of cells exposed to different concentrations of the extract and from the study, the IC_{50} was found to be 16µg/mL. The polysaccharide (PST001) from seed coat of *T. indica* had anticancer effect on murine cancer cell lines DLA and EAC which inhibited the cell proliferation significantly with an IC_{50} value of 91.14 µg/mL in DLA cells. [10] Different cells behave in different fashion against noxious stimuli and some cells tolerate them more which can be the reason for the low IC_{50} in this experiment. The MTT assay cannot distinguish between apoptosis and necrosis as the cause of cell growth inhibition. [11] Apoptosis is one of the major mechanisms through which cytotoxic drugs produce their effect in cancerous cells. The AO/EB staining was done to assess morphological and apoptotic mechanism of cell, which provided a clear contrast between live, early, and late apoptotic cells. [12] The nuclei of normal cells stained by AO penetration which green via attaching to DNA, EB, on the other hand, dyes the nuclei of late apoptotic and necrotic cells red and the result shows that *T.indica* is induced apoptosis in a dose dependent manner. The intrinsic pathway of apoptosis is initiated when there is an increase in the mitochondrial membrane permeability, which subsequently release the apoptotic factors initiating the apoptotic cascade. JC-1 accumulates in the membrane at high transmembrane potential producing red fluorescence and changes from red to green at lower trans membrane potential. [13] In current study *T.indica* decreased the transmembrane potential in a dose dependent manner, showing possibility for activation of intrinsic apoptotic pathway.

CONCLUSION

According to the findings of the study, the methanolic extract of *T. indica* induces apoptosis in cancer cells in a dose dependent manner and hence can be a lead molecule for development of an anticancer drug.

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