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Modulatory potential of *Tamarindus indica* seed coat on oestrogen and progestogen secretion in MCF-7 cell lines

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ABSTRACT

According to epidemiological research, the consumption of phytoestrogen rich foods has been shown to reduce the development of hormone dependent breast cancer. Phytoestrogens improved the efficacy of ongoing chemotherapy. Recent research indicates that polyphenols found in the tamarind seed coat have anti-inflammatory, hepatoprotective, antibacterial and antioxidant activities. The current study was undertaken to evaluate the modulatory potential of methanolic extract of seed coat of *T. indica* on oestrogen and progesterone production in MCF-7 cancer cell line. *T. indica* seeds were procured locally, shade dried and the seed coat was removed and extracted with methanol, followed by concentration of the extract using a rotary vacuum evaporator. The qualitative phytochemical analysis of seed coat extract was performed. The cytotoxicity of *T. indica* seed coat was assessed in MCF-7 cells using MTT assay and the IC_{50} was determined. The presence of alkaloids, flavonoids, steroids, phenolic compounds, diterpenes, saponins, glycosides and tannins were discovered by qualitative phytochemical analysis. T. indica seed coat decreased cell viability in a dose dependent manner, with an IC_{50} value of 16 µg/mL. There was dose dependent decrease in oestrogen concentration, whereas the progesterone concentration was found to be increased after 96hrs of treatment with the extract. From the study it could be concluded that methanolic extract of *T. indica* showed cytotoxicity in vitro against MCF-7 cell lines and it positively modulated progesterone secretion and negatively modulated oestrogen concentration in a time dependent fashion in MCF-7 cell lines. Present *in vitro* study shows that methanolic extract seed coat of *T. indica* may have promising role in breast cancer prevention, hence it can be used to develop novel compounds against hormone dependent breast cancer.

Keywords: *Tamarindus indica*, Phytoestrogens, Breast Cancer, Oestrogen, Progesterone.

INTRODUCTION

The steroid hormones oestrogen and progesterone have important role in proliferation, aetiology and treatment of breast cancer. In the early stages of breast cancer treatment oophorectomy was used as an effective treatment, as three quarter of all breast cancers are dependent on steroid hormone for their differentiation and development. Extended exposure to steroid hormones due to late menopause, usage of oral contraceptives and obesity has transiently increased the risk of causing breast cancer. Thus, researchers are targeting the oestrogen (ER) and progesterone (PR) receptors and their selective modulation as an effective method for breast cancer treatment [1].

Phytochemicals have been known for their role in preventing cancer even back in ancient times through Ayurveda. The modern cancer treatment is very expensive and have many side effects. Phytoestrogens are plant derived compounds which possessed a phenolic ring and capable of binding to oestrogen receptor, they usually generate weak oestrogenic or antioestrogenic activity in mammals, by affecting the expression of oestrogen genes as they are structurally similar to mammalian 17 β estradiol. The most well-known phytoestrogens are isoflavones and flavones and are capable of binding to both ERα and ERβ receptors whose subsequent activation or repression of their transcriptional pathways [2]. The phytochemicals present in plants are also capable of inducing antiprogestin effects. Epidemiological studies imply that consuming a phytoestrogen-rich diet, as found in traditional Asian countries is connected with a decreased risk of breast and prostate cancer, as well as cardiovascular disease.

Tamarindus indica belongs to the family Fabaceae, the fruit pulp of which are consumed widely and used in traditional medicines as laxatives, treatment of wounds, diarrhea, fever and malaria [3]. However limited studies have been conducted to find the modulatory potential of seed coat of *T.indica* on steroid hormone synthesis, the current study was conducted for the assessment of modulatory effect of *T.indica* seed coat extract on oestrogen and progesterone secretion in MCF-7 cells.

MATERIALS AND METHODS

Plant Extraction- Methanolic extract of seed coat of *Tamarindus indica* (MTI)

Tamarindus indica seeds were obtained locally from Mannuthy validated by a botanist at St Thomas College in Thrissur the seed coat was removed and used for the study The seed coat was finely crushed using an electric pulveriser and extracted with methanol using a Soxhlet extraction apparatus The methanol extract was then dried using a rotating vacuum evaporator at 40℃ and stored under refrigerator till use.

Phytochemical analysis

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

Cell lines

The 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 charcoal stripped foetal 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).

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

In-vitro cytotoxic potential of the extract 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 10 µg/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 at the desired concentrations was added to the cells, again incubated for 24 hrs. Then MTT 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.

Culture of cells for steroid analysis

MCF-7 cells were cultured as described in RPMI-1640 media supplemented with Charcoal stripped FBS for studies involving modulation of steroidogenic activity. The cells at a concentration of $3x10^5$ cells/mL of media was plated into six well plates and incubated at 37°C for 24 hours. Once the cells reached confluency, they were treated with the extracts of the plant.

Assay for hormones

The MCF-7 cells were exposed to extracts of *T.indica* in the concentrations 380, 190 and 95µg (twice IC_{50}, IC_{50} and half dose of IC_{50}) for 96 hours. The culture media were collected every 48 and 96 hours and replaced with fresh media. The assay was done in duplicates. The collected media was stored at -80°C and used for the estimation of Progesterone and oestrogen

The total progesterone level in the cell culture media was estimated using Progesterone ELISA kit provided by Abnova Coorpartion, USA. The absorbance was measured using microplate reader (Varioskan Flash, Thermofischer Scientific, Finland) at a wavelength of 450 nm. The mean absorbance values were calculated. Standard curve was obtained by plotting mean absorbance of each standard on Y- axis and the concentration on X-axis. Online curve fitting software AAT Bioquest was used for plotting the 4 Parameter logistic Curve for ELISA and the regression equation was derived. The mean absorbance values of each media were used to determine the corresponding concentration of progesterone from the standard curve.

The total oestrogen level in the cell culture media was estimated using Enzyme-Immunoassay kit provided by Omega diagnostics as per Ratcliffe *et al.* [6]. The absorbance was measured using microplate reader (Varioskan Flash, Thermofischer Scientific, Finland) at a wavelength of 450 nm. The mean absorbance values of standard and samples were calculated. The standard curve was plotted using the mean absorbance of each standard on Y-axis and the concentration on X-axis. Online curve fitting software AAT Bioquest was used for plotting the 4 Parameter logistic Curve for ELISA and the regression equation was derived.

Statistical Analysis

The results were analysed using repeated measures ANOVA using SPSS V 24 and post hoc analysis was done by Latin Square Design. Data on cell viability was analysed using student ‘t’ test.

RESULTS

Phytochemical analysis of methanolic extract of seed coat of *Tamarindus indica* (MTI)

The phytochemical analysis of MTI revealed the presence of steroids glycosides phenolic compounds tannins flavonoids alkaloids and saponins (Table 1).

Table 1: Phytochemical present in MTI

Phytochemicals	Type of test	Inference
Alkaloids	Dandruff’s Test	+
Steroids	Salkowski’s Test	+
Phenolic compounds	Ferric chloride test	+
Flavonoids	Ferric chloride test	+
Glycosides	Sodium hydroxide test	+
Tannins	Ferric chloride test	+
Saponins	Foam test	+
Diterpenes	Ferric chloride test	+

Cytotoxicity studies of methanolic extracts of seed coat of *T. indica*

When the cells were treated with the extract for 24 hours, there was a dose dependent inhibition of cell proliferation with maximum inhibition when cells were exposed to 320µg/mL with values of 80.85±0.03 per cent. Table 2 depicted per cent inhibition of Mcf-7 cells treated with MTI and Fig 1 represented per cent cell viability of cells after treatment with MTI. The percent cell inhibition obtained from MTT assay were used to find the IC_{50} of MTI. A curve was plotted using the values in ATT Bioquest.com and the graph obtained is represented in the fig 2. The IC_{50} value was identified to be 16µg/mL.

Table 2: Per cent inhibition of cells exposed to MTI, presented as Mean±SEM, with n=3 replicates

Concentration (µg/mL)	Percent inhibition (Mean±SEM)
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

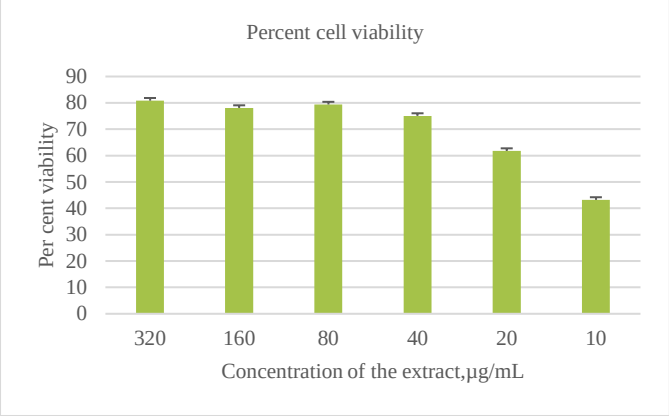

Figure 1: Percent cell viability of MCF-7 cells after treatment with MTI, Values expressed as Mean ±Standard error of mean (n=3)

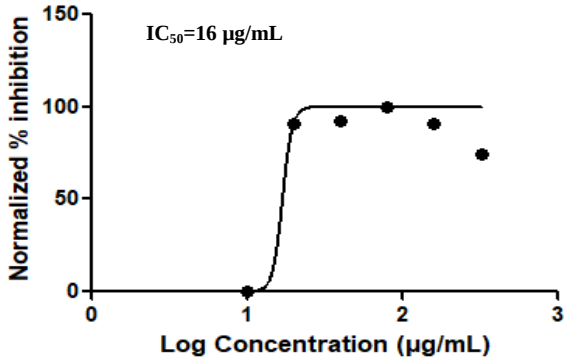

Figure 2: IC_{50} of *T. indica*

Effect of methanolic extract of seed coat *T. indica* on Oestrogen concentration of MCF-7

When the cells were treated with MTI at concentration 32µg/mL, the oestrogen concentration after 48 hours was found to be 1.32± 0.26 ng/mL. At the doses of 16 and 8µg/mL the oestrogen concentrations were found to be 1.14± 0.01 and 1.04± 0.01 ng/mL respectively. After 96 hours, the oestrogen concentration was observed to be 0.41± 0.18, 1.55± 0.00, 1.35± 0.00 ng/mL at the doses of 32, 16 and 8µg/mL respectively and the concentration of control cells at 48 and 96 h were 1.56±2.99 and 1.76±0.00 ng/mL respectively. There was a dose dependent decline in the concentration of oestrogen after treatment with MTI compared to the control cells. Figure 3 showed the graph of oestrogen levels.

Effect of methanolic extract of seed coat *T. indica* on progesterone concentration of MCF-7

After 48hrs of treatment with MTI at concentrations IC_{50}, half and twice IC_{50}, the concentration of progesterone was found to be 2.79±0.72, 3.63±0.23 and 3.17±0.01 ng/mL respectively. At 96hrs the concentrations were found to be 4.08±0.28, 4.81±0.08 and 4.53±0.48 ng/mL respectively. When compared to the control cells there was a significant increase in progesterone concentration in half IC_{50} concentration ($P \leq 0.05$) after 48hrs treatment and the increase was more profound after 96hrs of treatment with the extract at all the doses. Figure 4 showed the graph of progesterone levels.

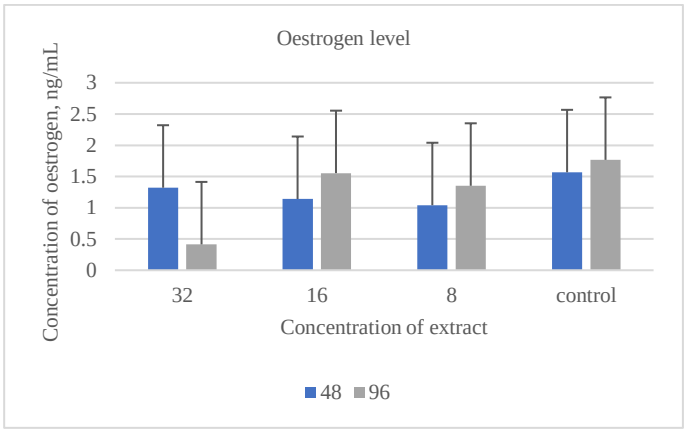

Figure 3: Effect of oestrogen concentration after treatment with MTI, values expressed as Mean± standard error of mean (n=2)

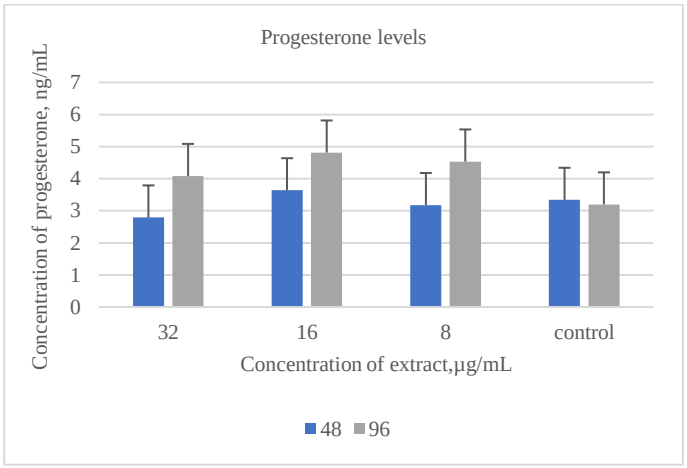

Figure 4: Effect of progesterone concentration after treatment with MTI, values expressed as Mean± standard error of mean (n=2)

DISCUSSION

Oestrogen and progesterone are steroid hormones having major roles in the regulation of differentiation, growth and maintenance of female reproductive tissues. Cholesterol is the precursor of all steroid hormones, which is first converted to progesterone, then testosterone and finally oestrogen [7]. Oestrogen deficiency causes infertility, atrophy, poor healing and postmenopausal symptoms. Two third of the breast cancer are positive for oestrogen and progesterone receptors (ER and PR) and these can be treated through hormone therapy by decreasing the steroid hormone concentration or by blocking their receptors. Phytoestrogens are plant derived compounds which are structurally similar to oestrogen. Phytoestrogens are capable of binding to oestrogen receptors (ERs) and show affinity towards ERβ which can inhibit the transcription and growth-promoting activity of ERα. Bilal *et al.* [8].

The qualitative phytochemical analysis of seed coat of *T.indica* shown the presence of glycoside, diterpenes, phenolic, steroids, tannins, flavonoids and saponins. Which can be the reason for the steroidogenic modulatory potential of MTI. The IC_{50} limit for selecting plant extracts for anticancer investigations, according to NCI recommendations, is fewer than 30 µg/mL [9]. In the current study, the IC_{50} of methanolic extract of the seed coat of *T. indica* was found to be 16 µg/mL; MTI ca be used as potent anticancer compound. The

high cytotoxicity and antiproliferative property of the extract can be due to the presence of terpenes [10].

The cells after treatment with *T.indica* at concentration 32, 16 and 8µg/mL for 48hrs indicated a decrease in the oestrogen concentration when compared to the control wells furthermore *T. indica* also prevented the proliferation of MCF-7 cells confirming antioestrogenic role of *T. indica* in oestrogen production. The decreased concentration of oestrogen might be caused by the action of phytoestrogens present in the extract. Previous studies reported that phytoestrogens (lignans, isoflavones, stilbenes) inhibited ERα and ERβ expression in breast cancer cell line and also found inhibitory effect on oestrogen metabolizing enzymes and a concomitant decrease in oestrogen concentration which is in line with the current study [11].

An increase in progesterone concentration was seen at all doses of *T. indica* after 96hrs of treatment. The increase in progesterone concentration can be an indication that progesterone was not utilized for the synthesis of oestrogen. Methanolic extract of *Boerhavia diffusa* in human breast cancer cell line showed an increase in progesterone concentration after 48hrs of treatment with the extract [12].

CONCLUSION

From the results of the present study, it could be inferred that the methanolic extract of *T. indica* seed coat had cytotoxic potential and the extract also possess antiestrogenic activity with a positive modulation progesterone. Since most of the oestrogen dependent tumours can be treated with progesterone this extract is having a double advantage that it decreased oestrogen synthesis and upregulated progesterone secretion. Hence further studies may be undertaken to isolate and characterise the active compound in the extract.

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Conflict of Interest

None declared.

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