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Research Article

Modulation of Steroid Hormone Synthesis by Methanolic Extract of *Mallotus Phillippensis*

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ABSTRACT

The presence of oestrogen and progesterone receptors in breast cancer cell lines can be exploited to find the modulatory potential of phytochemicals on steroid hormone synthesis in MCF -7 cell lines. The current study was conducted to examine the modulatory potential methanolic extract of *Malllotus philippensis* leaves on steroid hormone synthesis in MCF-7 breast cancer cell line. The locally collected leaves of *M. phillippensis* were shade dried, pulverized and extracted using methanol in a Soxhlet apparatus, which was concentrated and stored. The phytochemicals were identified qualitatively. MTT assay was carried out to assess the *in vitro* cytotoxic effects of methanolic extract of *M. philippensis* in MCF-7 cells at dosage rates of 320, 160, 80, 40, 20, 10 µg/mL. From the results of MTT assay, per cent cell viability, inhibition and IC_{50} were determined. The cells at concentration $1x10^5$ cells/mL were seeded in 6 well plate and incubated with the extract at doses of IC_{50}, half and twice IC_{50} for 96 hrs, media was collected after 48 and 96hrs and used to quantify the concentration of oestrogen and progesterone using ELISA. Alkaloids, tannins, flavonoids, steroids, diterpenes and phenols were identified by qualitative phytochemical analysis of extract of *M. philippenisis.* The MTT assay revealed a dose dependent cytotoxicity of the extract in MCF-7 cell lines with an IC_{50} of 190µg/mL. Methanolic extract of *M. phillippensis* showed a dose and time dependent increase in oestrogen concentration as well as a dose dependent decrease in progesterone concentrations in the culture media. Based on the findings of the present study, *M. phillippensis* induced a positive modulation of oestrogen production which might be the reason for low progesterone levels.

Keywords: *M. phillippensis,* MCF-7 cells, Progesterone, Oestrogen.

INTRODUCTION

Oestrogen and progesterone, the sex steroids in females have a major role in metabolic, neurological, reproductive and endocrine functions in humans and animals. The ovary is the primary site of oestradiol generation, which occurs in granulosa cells via the action of follicular stimulating hormone (FSH)[1]. Oestrogen deficiency causes infertility, atrophy, poor healing and postmenopausal symptoms. Hot flushes, osteoporosis, cardiovascular disease and mood swings are all linked to low oestrogen levels during the menopausal phase. Excess of progesterone induces ovarian cyst or ovarian cancer.[2] Oestrogen and progesterone bind to their respective receptors, then operate on transposable elements in genes to change gene expression.

Phytoestrogens are secondary metabolites found in plants that have a similar structure to oestrogen. As a result, they can bind to oestrogen receptors and act as an alternative to oestrogen.[3] The plant derived oestrogens include flavonoids, isoflavonoids, lignans, coumestans, stilbenes and certain mycotoxins like zearalenol also are having biological actions in humans and animals. The oestrogen-responsive human mammary adenocarcinoma MCF-7 cell line is frequently utilised for studies on the estrogenic potential of phytoestrogens. *Mallotus philippensis* is a huge woody multipurpose medicinal tree native to India that belongs to the *Euphorbiaceae* family. Various components of the plant are used to cure skin problems, bronchitis, antifungal, tape worm, eye-disease, cancer, diabetes, diarrhoea, jaundice, malaria, urinogenital infection, and other conditions[4]. Previous studied reported flavonoids, triterpenes, benzopyrans, and flavonolignans in *M. phillippensis* were cytotoxic to human cancer cell lines.[5]

Since phytoestrogens play a role in the management of postmenopausal syndromes as well as hormone replacement therapy[6], the current study investigates the potential use of *Mallotus philippensis* as a medication or as an adjuvant to such therapy

Materials and Method

Plant Extraction

The leaves of *M. phillippensis* was obtained from the campus of College of Veterinary and Animal Sciences, Pookode, Wayanad, authenticated at MSSRF and dried under finely and was extracted with methanol using a Soxhlet extraction apparatus. The methanol extract, obtained after exhaustive extraction, was filtered and concentrated in rotary vacuum evaporator (Rotavapor,

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Buchi, Switzerland)) at 40°C and 100 mbar pressure. The residue obtained was first kept open at room temperature for complete evaporation of solvent and then stored in the sealed airtight container in refrigerator for further use.

Phytochemical analysis

The qualitative phytochemical analysis was performed according to Harborne (1998).[7]

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 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 *M. Philippensis* was assessed in MCF-7, using MTT reduction assay as per Riss *et al.,* (2004).[8] 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 wells 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 steriodgenic activity. The cells at a concentration of $1x10^5$ 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 *M. phillippensis* 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.* (1988)[9]. 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

The qualitative phytochemical analysis revealed the presence of alkaloids, diterpenes, tannins, flavonoids, steroids and phenols.

Table 1: Depicted phytochemicals identified in *M. phillippensis*

Phytochemicals	Test	Inferences
Steroids	Salkowski's test	+
Alkaloids	Dragendroff's test	+
Glycosides	Sodium hydroxide test	-
Tannins	Ferric chloride test, Gelatin test	+
Flavonoids	Ferric chloride test, Lead acetate test	+
Phenolic compounds	Ferric chloride test	+
Saponins	Foam test	-
Diterpenes	Diterpenes test	+

Assessment of effect of extract on viability of MCF-7 cells and calculation of IC_{50}

There was a dose dependent decrease in the viability of cells exposed to different concentrations of extract with the viability being least at 320 µg/mL with value 25.58 ±0.056 per cent. Highest viability seen at dose of 10µg/ml, which was found to be 66.57 ±0.042 percent (table 2, figure.1.).

Table 2: Percent viability of cells exposed to methanolic extract of *M. phillipensis*

Concentrations(µg/ml)	Percent cell viability (Mean±SE)
320	25.58 ±0.056
160	60.92 ±0.248
80	64.31 ±0.193
40	64.72 ±0.181
20	62.67 ±0.105
10	66.57 ±0.042
IC_{50} µg/ml	190

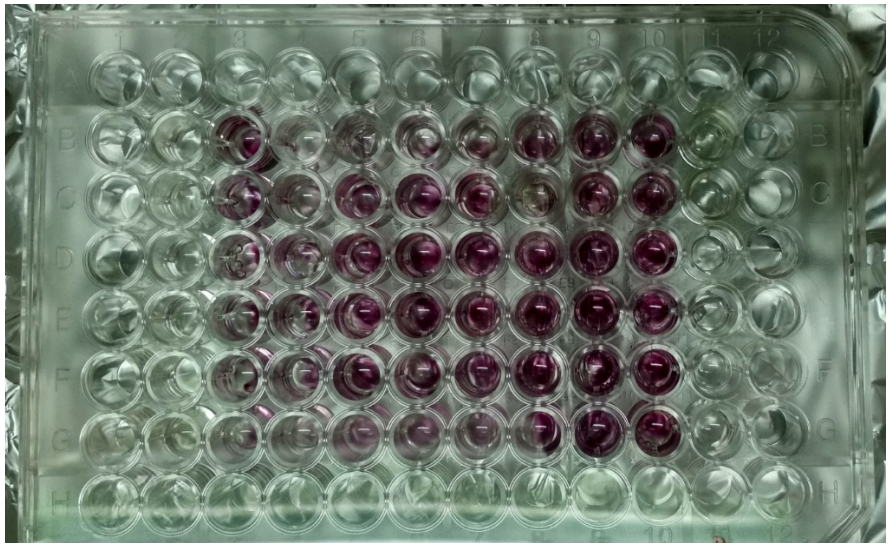

Figure 1: MTT assay of MCF-7 cells treated with *M. phillippensis*

The graph showing the analysis of IC_{50} is depicted in Figure 2. The IC_{50} of methanolic extract of *M. phillippensis* was 190 µg/mL as obtained from MTT assay.

Effect on methanolic extract of *M. phillippensis* on Progesterone concentration

The effect of methanolic extract of *M. phillippensis* on the progesterone secretion by MCF-7 cells is depicted in Figure 2.

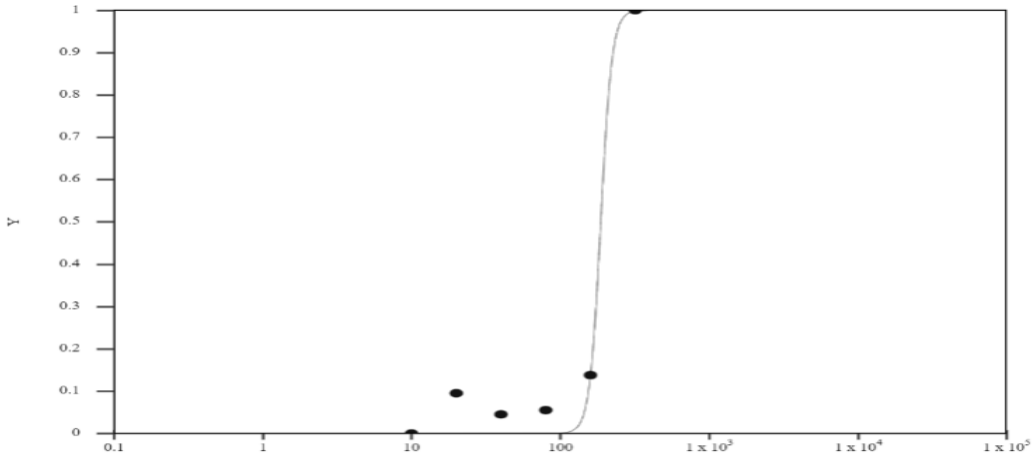

Figure 1: IC_{50} of *M. phillippensis*

There was a substantial drop in progesterone levels after 96 hours of treatment with a methanolic extract of *M. phillippensis*. It produced 5.022±0.072, 2.466±0.0325 and 4.97±0.1655 ng/mL after 48 hours of extract treatment at dosages of 380, 190, and 95 µg/mL, respectively. At the end of 96 hours of incubation with *M.phillippensis*, progesterone concentration decreased at dosages of 380 and 95µg/mL, whereas it increased somewhat at doses of 190 µg/mL. However, as compared to the control, there was a significant drop in progesterone concentration.

Effect on methanolic extract of *M.phillippensis* on Oestrogen concentration

The effect of methanolic extract of *M. philippensis* on the oestrogen secretion by MCF-7 cells is depicted in Figure 3.

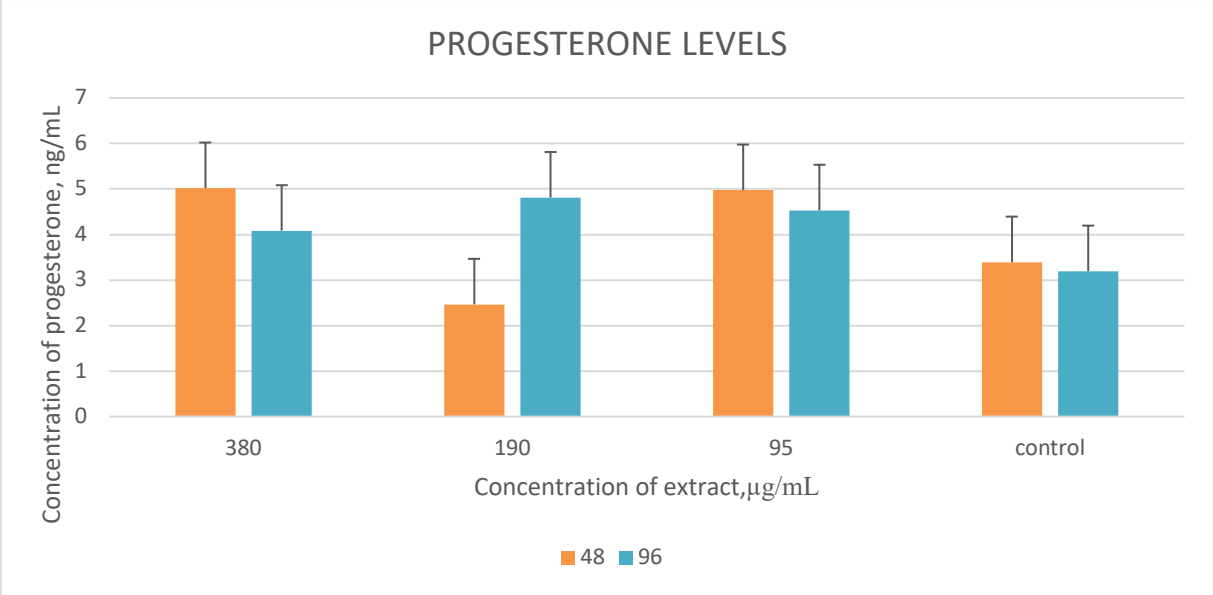

Figure 2: The effect of methanolic extract of *M. phillippensis* on progesterone secretion by MCF-7 cells

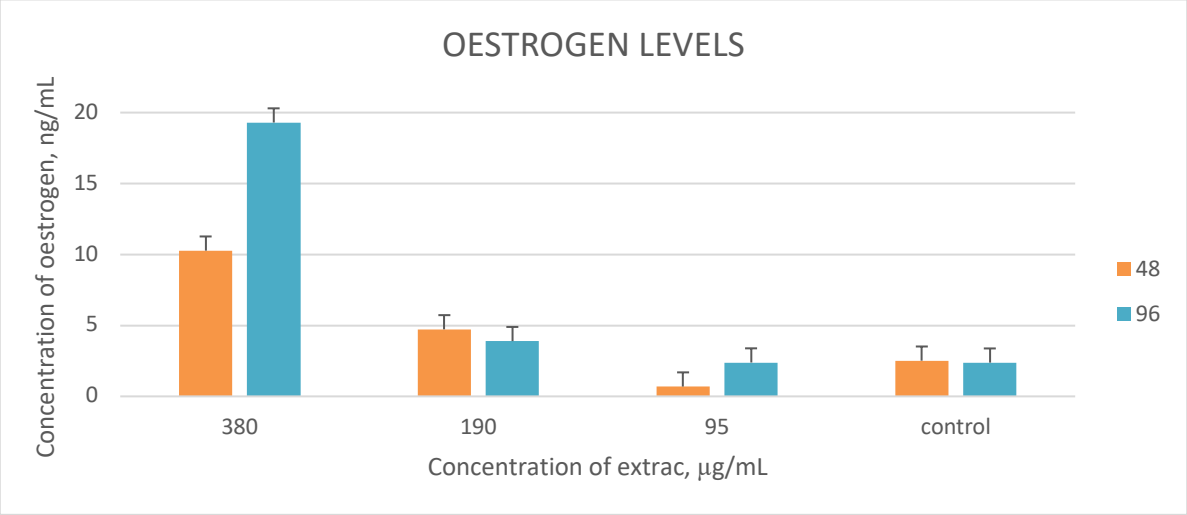

Figure 3: The effect of methanolic extract of *M. phiilippensis* on oestrogen secretion by MCF-7 cells

There was a significant increase in the secretion of oestrogen after 48 hours of treatment with *M. phillippensis* at a dose of 380 µg/mL, which was found to be 10.27±0.67 ng/mL in MCF-7 cells. At doses of 190 and 95 µg/mL, the oestrogen concentrations were 4.72±1.49 and 0.69+0.0 ng/mL, respectively. When cells were exposed to *M. phillippensis*, oestrogen levels gradually increased. After 96 hours, the oestrogen concentrations were 19.30±1.51and 2.38±0.10 ng/mL for 380 and 95µg/mL doses, respectively. After 96 hours of treatment at a dosage of 190µg/mL, the oestrogen levels were reduced to 3.89±0.41 ng/mL. However, as compared to the control and the 48-hour concentration after 96 hours of treatment, the rise was more substantial.

DISCUSSION

Oestrogen and progesterone are major sex steroids generated in ovary, both are generated from same precursor cholesterol by the action of p 450 enzymes. The cholesterol was subsequently transformed into progesterone, which was then converted into androgen. Finally, androgen is converted into oestrogen by enzyme aromatase.[10] In addition to its reproductive function, oestrogen has a significant role in neuroprotection, bone metabolism, and cardiovascular disease. A lack of oestrogen resulted in infertility, menopause syndromes while an excess resulted in hormone-dependent cancers[11]. Phytoestrogens are non-steroid compounds obtained from plants that mimic the activities of oestrogens. Phytoestrogens have the ability to both stimulate and

disrupt reproductive processes. Genistein, an isoflavone, has the ability to promote animal ovarian progesterone, oestradiol, and cAMP production, as well as oocyte maturation and preimplantation zygote development[12]. Pre-and postmenopausal exposure to phytoestrogens (isoflavones, lignans, and coumestans from various botanical sources) may prevent menopausal symptoms caused by decreased endogenous oestrogen production[13]. Oestrogen exerts its influence via the cell membrane receptors ER alpha ER beta and nuclear receptor GPER[14]. Progesterone is primarily involved in maintenance of pregnancy[15].

Alkaloids, flavonoids, and phenolic compounds are the most significant bioactive substances found in medicinal plants[16]. The presence of flavonoids, alkaloids, phenolic compounds, tannins, terpenes, and steroids were detected in the current study. Flavonoids and phenolic compounds are phytoestrogens with anti-proliferative and antioxidant properties.[17] Using the MTT assay, the antiproliferative potential of *M.phillippensis* was determined. The presence of phytosterols in *M.phillippensis* could be linked to the modulation of steroid production in MCF-7 cells. The study revealed that progesterone levels in the extract-treated cells were reduced at half and twice IC_{50} concentrations whereas oestrogen levels increased at same doses over a 96-hour period, demonstrating that *M. phillippensis* has a modulatory impact on steroidogenesis. Because there was a reduction in progesterone levels and an increase in oestrogen levels, it is possible that *M. phillippensis* had an influence somewhere in the interconversion stages of progesterone to oestrogen, most possibly at aromatase, which is the critical rate limiting step. The reduction in progesterone production at higher doses over time may be attributed to increased hormone metabolism or increased oestrogen synthesis. Previous study reported administration of flavanone 8-prenyl naringenin 6 at a concentration of 100mg/mL produced estrogenic effects in ovariectomized rats.[18]

Oestrogenic effects of *M. phillippensis* is seen also in the present study. At IC_{50} concentrations *M.phillippensis* showed decreased oestrogen level and increased progesterone level, which indicated the biphasic response of *M.phillippensis* on steroidogenesis. Several studies have been published on the biphasic effect of phytochemicals on oestrogen secretion. Genistein has been demonstrated to exhibit oestrogenic effects at concentrations less than 1µM, although antagonist action is shown at doses more than 10 µM, which was found in the present study also[19]. Certain phytoestrogens, such as biochanin A and genistein, were found to considerably boost progesterone production at lower concentrations while inhibiting it at greater concentrations. This can be due to the fact that at lower concentrations the phytochemicals are proliferative and higher concentrations, metastasis inducing whereas at intermediate doses like IC_{50}, they are more of cytostatic nature[20].

CONCLUSION

According to the findings of this study, methanolic extract of *M. phillippensis* modulated both oestrogen and progesterone production. Further identification of the active components of the extract, as well as validation of estrogenic and health-promoting effects in vivo, are required to demonstrate the plant's suitability in hormone replacement therapy and the treatment of infertility, as well as acting as an oestrogen alternative.

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