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Modulation of steroid hormone synthesis by methanolic extracts of *Boerhavia diffusa*

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Abstract
Steroid hormones such as oestrogen and progesterone play multidimensional role in regulation of reproduction, metabolic and neuroendocrine functions in humans and animals. Phytoestrogens bind to oestrogen receptors and mimic the role of oestrogen, thus play a role in the management of fertility as well as therapy of hormone dependent cancers. The whole plant of *Boerrhavia diffusa* was collected locally from the campus of College of Veterinary and Animal sciences, Mannuthy, Thrissur. The qualitative phytochemical analysis was performed on the methanolic extracts of *Boerrhavia diffusa* for the presence of steroids, alkaloids, tannins, phenolic compounds, flavonoids, diterpenes, triterpenes and saponins [1]. In the present study, methanolic extract of whole plant of *B. diffusa* was analysed for its modulatory activity on steroidogenesis. The cytotoxicity of methanolic extract of *B. diffusa* was explored using MTT assay in MCF-7 cells and IC_{50} was calculated. The cells were also cultured in six well plates and exposed to (IC_{50}, 2 times IC_{50} and half dose of IC_{50}) for 96 hours. The culture media were collected every 48 and 96 hours, was stored at -80 °C and used for the estimation of progesterone and oestrogen by ELISA. The phytochemical analysis revealed the presence of alkaloids, glycosides, tannins and flavonoids. Through the MTT assay the IC_{50} of methanolic extract of *B. diffusa* was found to be 170 µg/mL. The methanolic extract of *B. diffusa* showed significant increase in the progesterone concentration with maximum increase at 340 and 170 µg/mL. There was a significant decrease in oestrogen concentration when exposed to extracts at 96 hours post treatment with maximum decrease at 340 µg/mL. Hence it could be concluded that the methanolic extract of *B. diffusa* down regulated oestrogen synthesis independent of the interconversion of cholesterol to progesterone. Hence the effect may be in the pathway of conversion of progesterone to oestrogen.

Keywords: *B. diffusa*, oestrogen, progesterone, phytoestrogens, steroidogenesis

1. Introduction
An estimate of World Health Organization (2003) states around 80 per cent of the world's population of developing countries are unable to afford pharmaceutical drugs and primarily depend on traditional medicine to cater their primary health care needs and management. India being one of the medico-culturally diverse countries where the use of medicinal plants, is an integral part of traditional medicine systems of medicine like Ayurveda, Unani and Siddha. The earliest known mention of the utility of medicinal plants is in Rigveda and dates back to period between 4500 and 1600 BC [2]. There has been an increase in the acceptance of alternative and complementary medicine globally leading to the sharp upsurge in demand and supply of medicinal plants from countries biologically diverse countries like India. In a country like India, which comprises of people belonging to various ethnic groups from different regions, herbal medicine is practiced to cure a variety of diseases. Medicinal plants are much safer and proved elixir in the treatment of various ailments. The isolation of chemical components from plants and their phytochemical and pharmacological screening would pave way for developing new lead molecules in natural product drug discovery.
Oestrogen is a major steroid hormone synthesized from the granulosa cells under the influence of follicle stimulating hormone (FSH). Progesterone is the precursor of all steroid hormones and is formed from cholesterol via the action of cytochrome P450scc, which is the rate-limiting step in steroid hormone formation [3]. Oestrogen and Progesterone stimulate proliferation of cells and induce receptor protein and DNA synthesis both in stromal and glandular organs, stimulating the development and growth of tumours, there by acting on the target cells by binding the steroid-receptor complex to DNA, altering the transcription of genes.

With the advent of new drug molecules for hormone replacement therapy, it is evident that they are neither safe nor as effective as presumed, that paved way for the increase in usage of plant derived oestrogens. Phytoestrogens bind with classical oestrogen receptors and bring about actions similar to that of endogenous oestrogens. The MCF-7 cell line was oestrogen receptor (ER) +ve, progesterone receptor (PR) +ve and human epidermal growth factor receptor (HER) -ve. MCF-7 cells are widely used in studies of tumor biology and to elucidate hormonal mechanism of action of various chemicals. MCF-7 cells are human oestrogen dependent breast carcinoma cell line. H295 R, MCF-7 cells are widely used as screening systems to study the effects of hormones, endocrine disruptors and its role in steroidogenesis. Ovarian hormones progesterone and oestrogen are known to play a crucial role in mammary tumorigenesis. Spontaneous mammary tumors account for the most widespread type of neoplasms in women as well as in female dogs [4].

B. diffusa belonging to family *Nyctaginaceae* is used traditionally for its hepatoprotective, immunomodulatory, anti-inflammatory, laxative, diuretic activities [5].

2. Materials and Methods

2.1. Plant extraction

The whole plant of *B. diffusa* were collected locally, from Mannuthy and was dried in shade until they were dry. The plant was coarsely powdered using an electric pulveriser and the powder obtained was extracted using a Soxhlet extraction apparatus with methanol. The methanol extract was then concentrated using a rotary vacuum evaporator under reduced pressure and temperature (40 °C). The yield of the extract was calculated and kept under refrigeration in an airtight container after complete evaporation of the solvent until further use.

2.2. Phytochemical analysis

The qualitative phytochemical analysis was performed according to [1].

2.3. Assessment of effect of extracts on viability of MCF-7 cells and calculation of IC_{50}

The MTT assay was done using methanolic extracts of *B. diffusa* in MCF-7 cells as per [6]. The T25 flask with MCF-7 cells on attaining 70-80 per cent confluency was trypsinized and seeded in a 96 well plate and exposed to 1280, 640, 320, 160, 80, 40, 20 and 10 µg/mL concentrations of the extract. After 24 hours of incubation with the extract, the media were carefully pipetted out and ten microliters of MTT (5 mg/mL prepared in DPBS) was added to all wells including blanks and covered with aluminium foil and incubated at 37 °C for 4 hours, in CO_2 incubator. After incubation, the media containing MTT was removed. Added 200 µL of DMSO to all the wells to dissolve to formazan crystals formed. The plates were gently agitated on orbital shaker for 10 minutes. The absorbance was measured using microplate reader (Varioskan Flash, Thermofischer Scientific, Finland) at a wavelength of 570 nm. Per cent cell viability was found out using the formulae and IC_{50} was calculated using online software My curvefit.com.

2.4 Culture of cells for steroid analysis

Adherent human breast adenocarcinoma cell line, MCF-7 received as a gift from Amala Cancer Research Centre, Thrissur was used for *in vitro* hormone assays. Cells were adapted to grow in Rosewells Park Memorial Institute (RPMI-1640) media supplemented with 10 per cent 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). Charcoal stripped FBS was used for studies involving modulation of oestrogenic activity. Cell lines were sub cultured by enzymatic digestion with one per cent trypsin/1mM EDTA solution when they reached approximately 70 to 80 percent confluency and these trypsinized cells were used for the studies. 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. The collected media was stored at -80 °C and used for the estimation of Progesterone and Oestrogen.

2.5. Assay for hormones

The MCF-7 cells were exposed to extracts of *B. diffusa* in the concentrations 340, 170 and 85 µg/mL (IC_{50}, 2 times 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 containing the extract. The assay was done in triplicates.

The total progesterone level in the cell culture media was estimated using Progesterone ELISA kit provided by Abnova Coorpartion, USA as per manufactures protocol. The absorbance was measured using microplate reader (Varioskan Flash, Thermofischer Scientific, Finland) at a wavelength of 450 nm. The mean absorbance values were calculated.

The total oestrogen level in the cell culture media was estimated using Enzyme-Immunoassay kit provided by Omega diagnostics as per manufactures protocol. 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 for both hormones using the mean absorbance of each standard on Y-axis and the concentration on X-axis. Online curve fitting software [7] AAT Bioquest was used for plotting the 4 Parameter logistic Curve [8] for ELISA and the regression equation was derived.

2.6 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.

3. Results and Discussion

3.1. Phytochemical analysis

The qualitative phytochemical analysis revealed the presence of alkaloids, glycosides, tannins, flavonoids, saponins and phenols.

3.2. Assessment of effect of extracts 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 1280 µg/mL (table 1). The graph showing the analysis of IC_{50} is depicted in Figure 2. The IC_{50} of methanolic extract of *B. diffusa* was 170 µg/mL as obtained from MTT assay. The cytotoxicity studies on the ethanolic root extract of *B. diffusa* in HeLa cell line and the IC_{50} was 300 µg/mL causing 30 per cent cell death. The lower IC_{50} in the present study may be due to the type of extract and cell line used [9].

Table 1: Percent viability of cells exposed to methanolic extract of *B. diffusa*

Concentrations (µg/mL)	Percent viability (Mean+SE)
10	97.3873±1.083718
20	97.64344±1.71885
40	94.67213±3.510461
80	85.91189±6.406507
160	83.35041±2.423038
320	76.07582±2.2
640	53.68852±2.426474
1280	53.48361±2.3
IC_{50} (µg/mL)	170

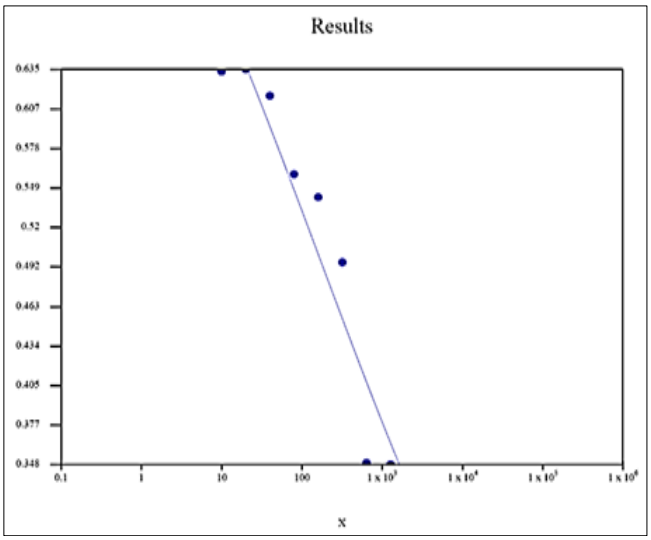

Fig 1: IC_{50} of *B. diffusa*

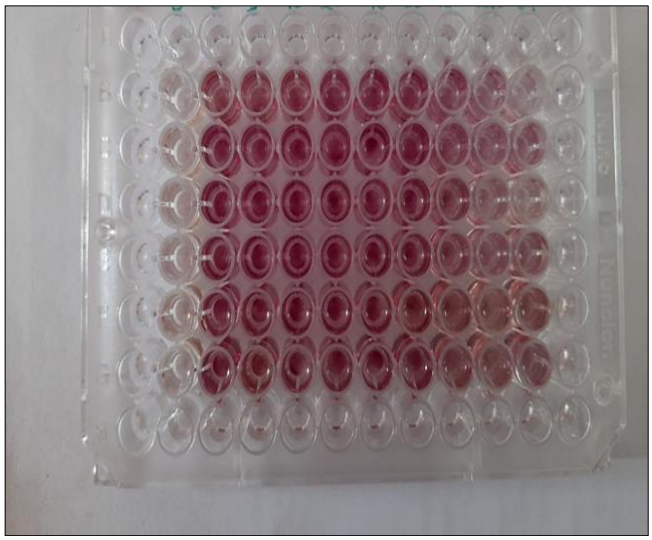

Fig 2: Depicts the picture of MCF-7 cells assayed for MTT in 96 well plates

3.3. Effect on methanolic extract of *B. diffusa* on oestrogen concentration

The effect of methanolic extract of *B. diffusa* on the oestrogen secretion by MCF-7 cells is depicted in Figure 3. There was significant decrease in the concentration of oestrogen when cells were treated with methanolic extract of *B. diffusa* at dose of 340, 170 and 85 µg/mL at 48 and 96 hours compared to control. It was also evident that the decrease was more pronounced compared to control as well as 48 hour concentration after 96 hours of treatment. Thus it was found that the methanolic extracts of *B. diffusa* down regulated the synthesis of oestrogen. The methanolic extracts of *B. diffusa* inhibited proliferation in MCF-7 cells signifying its probable antiestrogenic role.

The methanolic extracts of *B. diffusa* inhibited oestrogen induced proliferation in MCF-7 cells suggesting its possible antiestrogenic role. The competitive radioactive binding studies were carried out to study the interaction of methanolic extracts of *B. diffusa* with Oestrogen receptor (ER). At the dose range of (20–320 µg/mL) the binding of [3H]-E2 to cytosolic ER was inhibited in a dose dependent manner and the IC_{50} was found to be is 320±25 µg/mL [10].

The aqueous leaf extract of *B. diffusa* induced an irregular pattern in the oestrous cycle which may be accountable to the high amount of phytoestrogens like saponins. Alkaloids and other phytochemicals reduced the plasma concentrations of follicle stimulating hormone and leuteinizing hormone. The reduction in serum oestrogen concentration may be attributed to the decrease in the aromatase activity or by replacement of the substrate during the oestrogen biosynthesis [11].

Oestrogens are known to be the stimulants of proliferation of breast cancer whereas antioestrogens arrest the proliferation of cells. As oestrogens are associated with the aetiology of hormone dependent breast cancer and inhibition of oestrogen dependent cell growth by plant extracts like *B. diffusa* as shown by MTT assay, may aid in becoming a good therapeutic strategy/lead molecule in the treatment of hormone dependent breast cancer.

The phytoconstituents in the plant extract may compete indirectly with the oestrogen receptors resulting in a functionally inactive complex and may act directly by endogenously decreasing the concentration of oestrogen by causing a restrain in the biosynthesis of oestrogen either by downregulating the aromatase enzyme or by rapid degradation/metabolism of the secreted oestrogen. The antiestrogenic activity exhibited by the extract may be accountable to the presence of the diverse phytochemicals. However, further studies are to be undertaken to ascertain the antioestrogenic activity of methanolic extracts of *B. diffusa.*

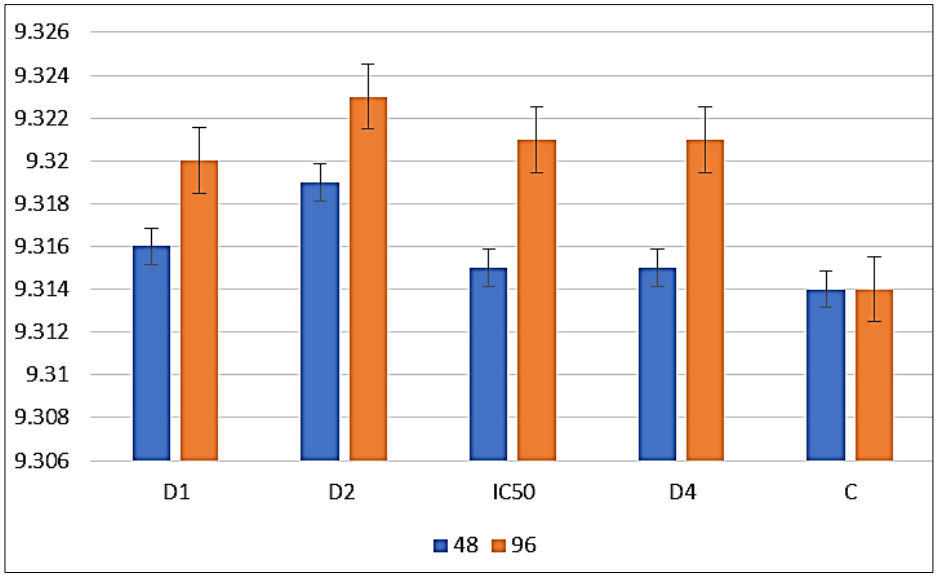

Fig 3: The effect of methanolic extract of *B. diffusa* on oestrogen secretion by MCF-7 cells

3.4. Effect on methanolic extract of *B. diffusa* on progesterone concentration

The effect of methanolic extract of *B. diffusa* on the progesterone secretion by MCF-7 cells is depicted in Figure 4. The methanolic extract of *B. diffusa* showed significant increase in the progesterone levels when treated with 340 and 85µg/mL compared to control at all-time intervals, whereas there was no significant difference between the concentration of progesterone in cells treated with of 170µg/mL.

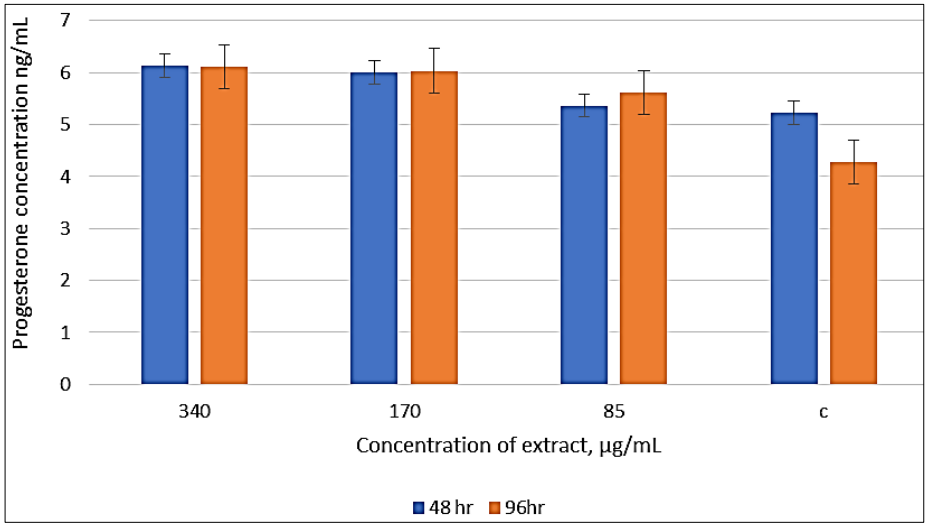

Fig 4: The effect of methanolic extract of *B. diffusa* on progesterone secretion by MCF-7 cells

From the study, it could be seen that, even though the progesterone levels in the extract treated cells were increased, the oestrogen levels decreased over the period of 96 hours, indicating the modulatory effect of *B. diffusa* on steroidogenesis. Since there was an increase in the progesterone levels and a decrease in the oestrogen levels, it could be inferred that the effect of *B. diffusa* may be somewhere in the interconversion steps of progesterone to oestrogen, most probably at aromatase which is the key rate limiting step.

Progesterone is utilized in the biosynthesis of oestrogen wherein our study the methanolic extracts of *B. diffusa* drastically reduced the concentrations of oestrogen inferring progesterone may be subutilized in the biosynthesis of oestrogen conferring to higher concentrations of progesterone or the significant increase in the progesterone level indicates that progesterone was not used for steroidogenesis, hence accumulated in the cells. The further identification of the active components of the extract and validation of antiestrogenic and health promoting effects *in vivo* is required to demonstrate its suitability of the plant in hormone replacement therapy and treatment of hormone dependent breast cancer.

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