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MODULATION OF THE EXPRESSION OF *CYP11A1, StAR* AND *CYP19* GENES BY NARINGENIN AND THE EXTRACTS OF *Boerhaevia diffusa* AND *Asparagus racemosus*

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ABSTRACT

Initial trials on MCF-7 breast carcinoma cells with the crude methanolic extracts of *Asparagus racemosus* tubers (MAR) and *Boerhaevia diffusa* leaves (MBD) revealed pro-oestrogenic and anti-oestrogenic activities, respectively. To evaluate their effects on steroidogenic pathway, the present study assessed the modulation of expression of steroidogenic acute regulatory protein (*StAR*), cytochrome P45011A1 (*CYP11A1*), and aromatase (*CYP19*) genes in extract-treated MCF-7 cells. Naringenin, a known CYP450 inhibitor, served as the positive control. Shade-dried plant materials were extracted in methanol, and cytotoxicity was evaluated by MTT assay to determine IC_{50} values. The IC_{50} of MAR, MBD and naringenin were 267, 170, and 395 µg mL^{-1}, respectively. Cells were treated with IC_{50}, half, and double IC_{50} doses of the test materials for 96 h, followed by RNA extraction and qRT-PCR analysis using GAPDH as a reference gene. MBD and naringenin significantly downregulated *StAR* and *CYP19*, indicating antioestrogenic effects, whereas MAR upregulated *CYP11A1* expression, suggesting enhanced steroidogenesis. The findings indicate that *A. racemosus* promotes oestrogen biosynthesis and may be beneficial in hormone deficiency, while *B. diffusa* exhibits antioestrogenic and potential anticancer activity in hormone-responsive cancers.

Keywords: *Asparagus racemosus, Boerhaevia diffusa, CYP11A1, CYP19,* MCF-7, qRT-PCR, *StAR*

INTRODUCTION

Cholesterol, synthesized from acetyl-coenzyme A or derived from plasma, serves as the principal substrate for steroid hormone biosynthesis. Its translocation to the inner mitochondrial membrane is facilitated by the steroidogenic acute regulatory protein (StAR) and the translocator protein complex at the outer mitochondrial membrane (Stocco, 2001a; Gazouli *et al*., 2002). Within mitochondria, cholesterol is converted to pregnenolone by the side-chain cleavage enzyme encoded by *CYP11A1*. Aromatase, a cytochrome P450 enzyme located in the endoplasmic reticulum, catalyzes the conversion of C19 androgens such as androstenedione and testosterone to oestrogens (Ghosh *et al*., 2009). Its activity is predominant in gonadal tissue and also occurs in the adrenals, bone, brain, and liver (Kamat *et al*., 2002).

Phytoestrogens are plant-derived polyhydric compounds structurally similar to 17β-oestradiol that can bind to oestrogen receptors and modulate oestrogen dependent gene expression (Sirotkin and Harrath, 2014). They act as natural agonists or antagonists of endogenous hormones and thus influence reproductive, metabolic, and proliferative processes. Given the health risks associated with synthetic oestrogens and endocrine disruptors, phytoestrogen-rich medicinal plants have attracted significant attention for their potential role as natural modulators of steroidogenic gene expression and their applications in cancer prevention and hormone therapy (Dhanusha *et al.,* 2021a; Ghosh *et al.,* 2025).

Boerrhavia diffusa is known for its hepatoprotective, immunomodulatory, anticancer, hormone modulation, and antiproliferative activities (Muntgantiwar *et al.*, 1997; Sreeja and Sreeja, 2009; Dhanusha *et al.*, 2021b). *Asparagus racemosus* Willd. is known for antioxidant, hepatoprotective, antiulcer, galactagogue and oestrogen modulatory activities (Bhatnagar and Sisodia, 2006; Visavadiya *et al.*, 2009; Dhanusha *et al.*, 2021c; Ghosh *et al.,* 2025). Naringenin is a flavone, commonly found in citrus fruits (Duda-madej *et al.,* 2022), and possesses antioxidant, anti-inflammatory, and anticancer activities (Faramarzi *et al.,* 2022). Naringenin and its enantiomers were found to be potent inhibitors of CYP 450 group of enzymes (Kaci *et al.,* 2023). This study was aimed to evaluate the expression of *StAR, CYP11A1,* and *CYP19)* genes in MCF-7 cells treated with the methanolic extract of above plants.

MATERIALS AND METHODS

Drugs/chemicals

Agarose-low melting, 0.25% trypsin-EDTA, bovine serum albumin (BSA), thiazolyl blue tetrazolium, ethylene diaminetetraacetic acid (EDTA), gentamicin sulphate, ceftriaxone sodium (Rosewells Park Memorial Institute, RPMI), antibiotic-antimycotic solution (100x) and foetal bovine serum (FBS) were purchased from Invitrogen Life Technologies USA. Tris buffer, naringenin, dimethyl sulphoxide (DMSO), methanol, Dulbecco's phosphate buffered saline (DPBS) and Naringenin were purchased from Sisco Research Laboratories (SRL) Maharashtra. Polymerase chain reaction (PCR) kit was purchased from M/s Promega. Real time quantitative polymerase chain reaction (qRT-PCR) kit was procured from M/s ThermoFischer.

Cell lines

Adherent human breast adenocarcinoma cell line, MCF-7, purchased from National Centre for Cell Sciences Pune (India), was used for *in vitro* anticancer studies. The MCF-7 cell line was oestrogen receptor (ER) +ve, progesterone receptor (PR) +ve and human epidermal growth factor receptor (HER) -ve. Cells were adapted to grow in Rosewell Park Memorial Institute (RPMI) media supplemented with 10% foetal bovine serum and 1% gentamicin (50 mg mL^{-1}). The cells were maintained in a humidified incubator at 37ºC with 5% CO_2. Cell lines were subcultured by enzymatic digestion with 1% trypsin/1mM EDTA solution when they reached 70-80% confluence and these trypsinized cells were used for further studies.

Preparation of extracts

The tubers of *A. racemosus* and leaves of *B. diffusa* were completely dried under shade and coarsely powdered using an electric pulveriser. The powder obtained was extracted with methanol by using a Soxhlet extraction apparatus. The methanol extract was then concentrated using a rotary vacuum evaporator under reduced pressure and temperature (40°C). The yield of extract was noted and extract kept under refrigeration in an airtight container after complete evaporation of the solvent until further use (Dhanusha *et al.*, 2021c).

Determination of IC_{50} concentration of the extracts in MCF-7 cells

The cells were assayed for inhibition using MTT assay (Dhanusha *et al.*, 2021a-c) and IC_{50} values of extracts and naringenin were calculated by plotting the concentration against per cent cell inhibition using Graphpad prism version 8. Briefly MCF-7 cells from an 80% confluent flask was collected by

trypsinisation and the concentration was adjusted to 10^5 cells mL^{-1}. Added 200 µL of cell suspension into all wells of a 96 well sterile tissue culture plate except the first column of wells which were kept as blank. The cell plates were incubated for 24 hrs at 37°C in CO_2 incubator. Stock solution of the methanolic extract of *A. racemosus* (MAR), *B. diffusa* (MBD) and naringenin was prepared in plain RPMI-1640 medium and vortexed for 5 min to completely dissolve the chemical. The stock was diluted to 640, 320, 160, 80, 40, 20, 10 and 5 µg mL^{-1} using complete RPMI-1640 medium. After 24 h incubation, the plates were taken out of the incubator and the media containing the extract were carefully pipetted out. Then 200 µL each of fresh medium was added to all the wells including blanks. Ten microliters of MTT (5 mg mL^{-1} prepared in DPBS) was added to all wells including blanks. The plates were covered with aluminium foil and incubated at 37°C for 4 h in CO_2 incubator. After incubation, the media containing MTT was removed and 200 µL DMSO added to all the wells to dissolve to formazan crystals formed. The plates were gently agitated on orbital shaker for 10 min. The absorbance was measured using microplate reader (Varioskan Flash, Thermofischer Scientific, Finland) at a wavelength of 570 nm. The per cent cell viability and per cent cell inhibition were calculated using the following formulae:

$$\text{Cell viability (\%)} = \frac{\text{Average absorbance of treated cells}}{\text{Average absorbance of untreated cells}} \times 100$$

$$\text{Cell inhibition (\%)} = 100 - \text{Cell viability}$$

The net absorbance from the control wells was taken as 100% viable. The IC_{50} values of extracts were calculated by plotting the concentration against %inhibition using Graphpad prism version 8 (Nair and Sujith, 2023). All the experiments were done in triplicates.

Gene expression studies

Isolation of total RNA from cells and cDNA synthesis: The IC_{50} values of MAR, MBD and naringenin were 267, 170 and 385 µg mL^{-1}, respectively. The half IC_{50} and double IC_{50} of MAR, MBD and naringenin were calculated. The cells were cultured in 6 well plates and exposed to half, IC_{50} and double IC_{50} concentrations of MAR, MBD and naringenin for 96 h. The spent media was removed every day and fresh media containing the extract was added till 96 h of study. The cells after the exposure were harvested by trypsinisation using 0.25% w/v trypsin EDTA and collected by centrifugation for 5 min under 3000 rpm in a 15 mL sterile centrifuge tube (Tarson) RNA was isolated using TRIzol method as per the manufacturers protocol and the extracted RNA checked for quality and quantity by nanodrop method (Benny *et al.*, 2022a). Those showing a purity beyond 1.8 for both 260/280 and 260/230 ratios were used for cDNA synthesis using maxima first strand cDNA synthesis kit as per the manufacturer‘s protocols (Benny *et al.*, 2022a).

Quantitative real time PCR (qRT-PCR): Designing and selection of primers and standardisation of PCR conditions. Exon spanning primers were designed using online NCBI primer design software (Primer3, *http://bioinfo.ut.ee/primer3/*) and specificity of primer was checked by BLAST (*http://www.ncbi.nlm.nih.gov/tools/primer-blast/*). Gradient PCR was carried out to amplify *CYP11A1, CYP19, StAR* and *GAPDH* gene. The conditions were optimized by setting different temperatures for annealing processes. The PCR amplified products were checked in 1.5% agarose gel (1X TBE) electrophoresis and visualized under UV transilluminator and documented in a gel documentation system (BioRad, Gel Doc 2000TM) [Benny *et al.*, 2022a].

Quantitative real time PCR: The expression of *CYP11A1, CYP19, StAR* and *GAPDH* was studied by using SYBR green chemistry (Maxima SYBR green qPCR master mix, (Thermo scientific, USA). The reaction was carried out in triplicates. The expression of target gene *CYP11A1, CYP19,* and *StAR* was compared with reference gene *GAPDH* to calculate ΔCq and the expression of same gene in treatment sample versus control samples to calculate ΔΔCq.

Melt curve analysis: A melt curve analysis was performed after the reaction for checking specificity of amplification. The programme for melt curve analysis consisted of denaturation at 95°C for 15 sec,

annealing at 55°C for 15 sec, followed by 95°C for 15 sec. Data acquisition was performed during the final denaturation step (Benny *et al.*, 2022b).

Relative quantification: Relative quantification describes the change in expression of target sample relative to some reference group such as an untreated control and is depicted below (Livak and Schmittgen, 2001):

$$\Delta Cq = Cq \text{ (target gene)} - Cq \text{ (reference gene)}$$
$$\Delta\Delta Cq = \Delta Cq \text{ (test sample)} - \Delta Cq \text{ (reference sample)}$$
$$RQ = 2^{-\Delta\Delta Cq}$$

Gas chromatography and mass spectrometry analysis

The active phytochemical principles of MBD analysed using gas chromatography mass spectrometry (Shimadzu GC-MS, Japan, QP2010S) with a mass range of 1.5-1000 m/z. Helium was used as the carrier gas at flow rate of 1 mL min^{-1}. The oven temperature was maintained at 80ºC for 4 min and then increased to 280ºC in 6 min. The injector temperature was 260ºC and total analysis time was 50 min. Aliquots of extracts (0.4 µL) were injected into the chromatographic column after a clear baseline was obtained. Major constituents were identified using mass spectrum library (NIST 11 and WILEY 8). The active principles of MAR have already been published elsewhere ((Dhanusha *et al.*, 2021c).

Statistical analysis

The results of the gene expression studies were analysed individually by student t test and group wise comparison was conducted using one-way analysis of variance.

RESULTS AND DISCUSSION

Gene expression studies

The MCF-7 cells grown in six well plates were continuously exposed to different concentrations of MAR, MBD and naringenin. The cells were trypsinized after 96 h exposure. The RNA was isolated from these cells, cDNA synthesized and used for expression of *CYP11A1*, *StAR* and *CYP19* genes keeping *GAPDH* as house-keeping gene.

Modulation of steroid hormone synthesis is mainly through the regulation of key enzymes involved, which depends on the transcription and translation of specific genes. *CYP11A1* controls a rate limiting step in the synthesis of steroids, i.e., the side chain cleavage of cholesterol to pregnenolone. The *StAR* gene product is the protein that regulates the transfer of cholesterol to inner membrane of mitochondria whereas aromatase is the enzyme that converts testosterone to estrogen. The changes in expression of various genes of steroid hormone synthesis like *CYP19, CYP11A1* and *StAR* genes affect the synthesis and release of reproductive hormones. Modulation of oestrogen synthesis and gene expression by various xenobiotics including environmental pollutants are known; and phytochemicals may have their own ameliorative role, reducing the ill effects of such changes.

Table 1: Relative expression of various steroidogenic genes in *MCF-7* cells after exposure MAR, MBD and naringenin

Extract/ Treatment	MAR			MBD			Naringenin		
	StAR	*CYP11A1*	*CYP19*	*StAR*	*CYP11A1*	*CYP19*	*StAR*	*CYP11A1*	*CYP19*
Control	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}
Half IC_{50}	0.92 ± 0.06^{a}	2.14 ± 0.14^{b}	1.49 ± 0.09^{b}	0.26 ± 0.03^{b}	3.83 ± 0.31^{b}	0.43 ± 0.04^{b}	0.31 ± 0.03^{b}	0.32 ± 0.03^{b}	0.37 ± 0.03^{c}
IC_{50}	1.18 ± 0.09^{a}	2.03 ± 0.16^{b}	1.13 ± 0.08^{a}	0.22 ± 0.02^{b}	4.04 ± 0.28^{b}	0.65 ± 0.05^{c}	0.27 ± 0.02^{b}	0.67 ± 0.02^{c}	$0.37+ 0.03^{c}$
Double IC_{50}	1.02 ± 0.08^{a}	2.00 ± 0.16^{b}	1.35 ± 0.12^{b}	0.27 ± 0.02^{b}	4.19 ± 0.30^{b}	0.55 ± 0.02^{c}	0.18 ± 0.06^{b}	0.45 ± 0.06^{c}	$0.22 0.02^{c}$

Means having the same superscripts in the same column do not differ significantly at $P < 0.05$.

Relative expression of steroidogenic genes

The effect of various treatments on expression of *CYP19*, *StAR* and *CYP11A1* genes are presented in Table 1. It was observed that both MBD and naringenin down regulated the expression of *CYP19* and *StAR*; whereas the treatment of MAR at double and half IC_{50} caused an upregulation of *CYP19* with no effect on *StAR*. It can also be noted that both MBD and MAR upregulated *CYP11A1* while naringenin downregulated the expression.

Cholesterol synthesized from acetyl-coenzyme A or derived from plasma, forms the primary substrate for steroid hormone synthesis The translocation of cholesterol to the inner mitochondrial membrane occur by the activation of a complex, including the steroidogenic acute regulatory protein (StAR) and the translocator protein (TSPO), at the outer mitochondrial membrane (Stocco, 2001b, Gazouli *et al.*, 2002). Inside mitochondria it is converted to pregnenolone by side chain cleavage enzyme encoded by *CYP11A1.* Aromatase enzyme converts c-19 sex steroids androstenedione and testosterone having unsaturated A rings in their structure to oestrogen and occurs at the endoplasmic reticulum (Ghosh *et al.*, 2009). Its enzyme activity is predominantly seen in the gonadal tissue along with adrenals, bone, brain, liver, etc. (Kamat *et al.*, 2002).

Isoflavones and flavonoids modulate aromatase expression and activity in various models. Sanderson *et al*. (2004) demonstrated that genistein, flavones, and quercetin induce aromatase activity in a dose-dependent manner in H295R cells, and simultaneously propose that inhibition of CYP19 by compounds such as rotenone may involve interaction of oxo group at the 4th carbon position of flavonoid ring with haem moiety of CYP19. Similarly, 7-hydroxyflavone (4 µM), chrysin (7 µM), apigenin (20 µM), and naringenin (85 µM) inhibited *CYP19* expression in H295R cells. Apigenin, quercetin, biochanin A, genistein, and daidzein also reduce *CYP19* expression at higher concentrations (Rice *et al*., 2006). Moreover, biochanin A suppressed *CYP11A1* transcription, leading to decreased sex steroid synthesis and endocrine disruption in H295R cells (Ohno *et al*., 2002).

Several mycotoxins have also been reported to modulate the expression of steroidogenic genes. Deoxynivalenol, a *Fusarium* derived mycotoxin inhibited action of *CYP19, StAR* and *CYP11A1* at 1000 ng mL^{-1} (Ranzenigo, 2008). Lephart *et al.* (2015) reviewed that lignans, flavones, flavonoids and isoflavonoids were weak inhibitors of *CYP19* whereas stilbenes were strong inhibitors of *CYP19*. Chrysin, a naturally occurring flavonoid in honey, passion flowers, proplois, etc. inhibited aromatase and the proliferation of MCF-7 breast cancer cells *in vitro* (Balam *et al.*, 2020). Resveratrol and genestein downregulated *StAR* gene expression in MA-10 cells while quercetin upregulated it (Cormier *et al.,* 2017). The ethanolic extract of *Zingiber officinale* restored the expression of *StAR* and *CYP11A1* in testis of rats treated with toxic doses of arsenic (Seif *et al.,* 2021) suggesting a possible protective role in steroidogenic regulation.

Gas chromatography-mass spectroscopy (GC-MS) analysis

Chromatograms obtained on phytochemical analysis of MBD using GC-MS is given in Fig. 1. Phytoconstituents

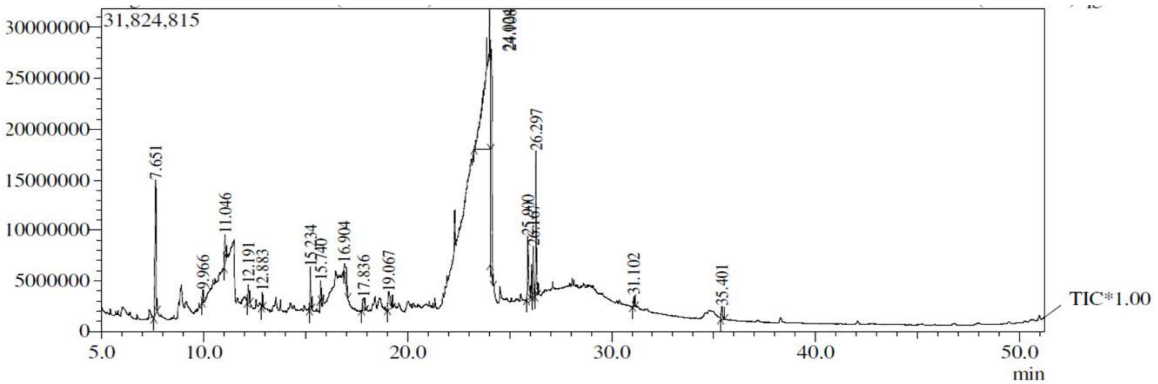

Fig. 1: GC-MS chromatogram of the phytochemicals of MBD

Table 2: GC-MS analysis of phytochemicals in MBD

Selected compounds	Retention time (min)	Similarity (%)
2-Cyclopenten-1-one, 2-hydroxy-	7.651	7.72
(D)-pantolactone	9.966	0.48
2-methoxyphenol	11.046	1.72
1,3-Diisopropoxy-1,3-dimethyl-1,3-disilacyclobutane	12.191	1.22
1,3-Propanediol, 2-methyl-2-(1-methyl propyl)-, dicarbamate	12.883	0.52
2-methoxy-4-vinylphenol	15.234	1.65
2-Acetamido-2-deoxy-d-mannolactone	15.740	0.68
Butane-1,2,3,4-tetraol	16.904	2.06
Methyl 4-O-methyl-d-arabinopyranoside	17.836	1.47
D-Allose	19.067	2.23
Hexadecanoic acid, methyl ester	24.024	44.19
Mome inositol	24.108	21.97
14-beta-h-pregna	25.900	4.23
Methyl linolenate	26.167	2.47
Phytol	26.297	5.73
2-Monopalmin	31.102	0.65
Tetracosanoic acid, methyl ester	35.401	1.02

obtained on GC-MS analysis are listed in Tables 2. The phyto-chemicals reported from MAR included 5-hydroxy-methyl-furfural, 1,2,3-propanetriol, guanosine, hexadecanoic acid, methyl ester, linoleic acid, methyl ester, 9-octadecenoic acid (Z)-, methyl ester, β-sitosterol, γ-sitosterol and stigmasterol (Dhanusha *et al.,* 2021c). Among these γ-sitosterol, stigmasta-5,22-dien-3-ol are potent phytoestrogens which may be the major cause of upregulation of various genes by the extract (Grattan, 2013; Bakrim *et al*., 2022). Siloxanes and linoleic acid methyl ester can also influence the biosynthesis of steroids, especially oestrogen. Phytol and myo-insoitol were found to be regulating the oestrogen biosynthesis and the presence of these can be the cause of anti-oestrogenic effect of MBR (Guo *et al*.,2014; Merveil *et al*., 2021). Many compounds listed in Table 2 also have potent anti-inflammatory and antioxidant properties.

From the study it may be concluded that MBD and naringenin downregulate the steroid hormone synthesis at the levels of cholesterol transport and conversion of testosterone to oestrogen, indicating a very strong anti-estrogenic effect. On the other hand, MAR upregulated *CYP11A1* and *CYP19* without significantly affecting the activity of *StAR*. The study concludes that the extracts were able to modulate the expression of key steroid-genic genes that are valuable in modulating the fertility and managing the hormone- dependent cancers.

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