The Journal of Phytopharmacology 2022; 11(6):407-413
Online at: www.phytopharmajournal.com

Research Article

ISSN 2320-480X
JPHYTO 2022; 11(6): 407-413
November- December
Received: 05-10-2022
Accepted: 28-11-2022
Published: 20-12-2022

doi: 10.31254/phyto.2022.11606

A Sandeshkrishna
MSc Scholar, Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India, Pin code: 680651

Bimitha Benny
MSc Scholar, Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India, Pin code: 680651

Sujith Samraj
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India, Pin code: 680651

Preethy John
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode, Kerala India, Pin code: 673576

Uma Radhakrishnan
Associate professor, Department of Veterinary Biochemistry, College of Veterinary and animal Sciences, Mannuthy, Kerala, India, Pin code: 680651

Correspondence:
Dr. Sujith Samraj
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India, Pin code: 680651
Email: sujith@kvasu.ac.in

Modulatory potential of *Tamarindus indica* on steroidogenesis by targeting *in vitro* expression of *CYP 19* and *StAR* genes in MCF-7 cell lines

A Sandeshkrishna, Bimitha Benny, Sujith Samraj, Preethy John, Uma Radhakrishnan

ABSTRACT

The use of medicinal plants for treating different ailments dates back to thousands of years through Ayurveda. They increased the efficacy of current chemotherapy while lowering the risk and side effects in cancer patients. The current study was carried out to identify the effect of methanolic extract of *Tamarindus indica* seed coat on the expression of *CYP 19* and *StAR* gene in MCF -7 cell lines. The phytochemical analysis of *T. indica* seed coat extract was done through Gas chromatography- mass spectrometry and Fourier transform infrared spectroscopy. The half maximal inhibitory concentration (IC_{50}) of the extract was discovered in a previous study using MTT assay and was found to be 32 µg/mL. The cells after treatment with extract at concentrations of 8, 16 and 32µg/mL produced a differential expression of *CYP 19*. There was a fold increase at IC_{50} concentration of the extract mean while the gene expression was decreased at higher and lower doses of IC_{50}. On the other hand, a dose dependent fold increase was observed in the expression of *StAR* gene after treatment with the extract. The presence of Phyto steroids, fatty acid analogues and terpenoids were identified by gas chromatography mass spectrometry analysis. Fourier transform infrared spectroscopy analysis results supports the finding from GC-MS analysis. Even though there was an increase in the expression of *StAR* gene, there was a biphasic response with *CYP19*, which indicated a downregulation in the synthesis of oestrogen.

Keywords: CYP 19, StAR, MCF-7, *Tamarindus indica*, Phytoestrogens.

INTRODUCTION

Oestrogen is the major steroid hormone produced by the theca cells of ovary, the biosynthesis of which is stimulated by the action of follicular stimulating hormone (FSH) [1]. Oestrogen is having an important role in the various physiological processes including energy regulation, stress responses and sexual differentiation [2]. The steroidogenic acute regulator (*StAR*) facilitates the transfer of cholesterol to the inner mitochondrial membrane which is the precursor of all steroid hormones. *CYP11A* catalyzes the synthesis of pregnenolone from cholesterol followed by the conversion pregnenolone to androgen by *CYP17*. Finally, aromatization of androgen by *CYP19*, which belongs to the cytochrome 450 enzyme family expressed in gonads, brain, adipose tissue, placenta, blood vessels, skin, bone and breast cancer tissue, leads to the formation of estrogen [3]. The optimum level of oestrogen is important for the regulation various physiological processes. Prolonged exposure to oestrogen due to late menopause, hormone therapy, oral contraceptive and exogenous oestrogen can increase the risk and aetiology of breast cancer development [4, 5]. When compared to normal cells the oestradiol levels of breast cancer cells are relatively higher [6]. The mechanisms that contribute to carcinogenicity due to oestrogen receptor-mediated hormonal action and metabolic activation mediated by cytochrome P450 (CYP), both elicit direct genotoxic effects by increasing mutation rates [7]. The over expression of hormones involved in oestrogen biosynthesis has been proven to have an important role in pathogenesis and development of hormone dependent breast cancer [8]. Thus, understanding a mechanism that regulate aromatase activity have key role in preventing hormone dependent breast cancer development.

Phytoestrogen are a class of plant derived phenolic compounds which are structurally similar to 17 β oestradiol and are mainly classified as flavones, isoflavones, lignans and coumestans [9]. Isoflavones and lignans undergoes complex enzymatic conversions after consumption that results in the formation of phenols structurally similar to oestradiol. Due to their structural similarity to oestradiol, they are capable of binding to the alpha and beta oestrogen receptors, there by inducing oestrogenic or antioestrogenic activity [10]. In addition, phytoestrogens are capable of suppressing aromatase mRNA expression, the general mechanisms by which phytoestrogen inhibit aromatase include reducing promoter utilisation, inhibiting aromatase itself and by reducing cell proliferation [11].

Tamarindus indica, a tropical tree belonging to the family Fabaceae, is primarily used for its fruit that is consumed all around the world. It is also used in traditional medicine; the fruit pulp is used for treating

diseases like malaria and rheumatism. Studies suggest that phytochemicals present in *T. indica* seed coat possess anti-bacterial, antioxidant and hepatoprotective activities [12-14]. It is also found that polysaccharides isolated from *T. indica* seed coat possess antiproliferative activity against mammalian cancer cell lines [15], [16]. Previous studies from our laboratory confirmed that the methanolic extract of seed coat of *T. indica* produced a reduction on the secretion of oestrogen from MCF-7 cells [17]. In order to ascertain the effect of the extract on the key regulators of oestrogen secretion, current study was undertaken to evaluate expression of *StAR* and *CYP 19* genes in MCF-7 breast cancer cell lines.

MATERIALS AND METHODS

Chemicals and reagents

Agarose-low melting, 0.25 per cent Trypsin-EDTA, bovine serum albumin, gentamicin sulphate, ceftriaxone sodium, Rosewells Park Memorial Institute (RPMI), antibiotic-antimycotic solution (100x), Foetal bovine serum (FBS), were purchased from Invitrogen Life Technologies USA. Tris buffer, dimethyl sulphoxide (DMSO), methanol, Dulbecco's phosphate buffered saline (DPBS) were purchased from Sisco Research Laboratories (SRL) Maharashtra. Polymerase Chain reaction (PCR) kit was purchased from M/s Promega. Realtime quantitative polymerase chain reaction (qRT-PCR) kit was procured from M/s Thermofischer.

Collection of *T. indica* seed coats and authentication

The seed coat of *T. indica* was obtained from the campus of College of Veterinary and Animal Sciences, Mannuthy, Thrissur, authenticated at St Thomas College Thrissur

Cell lines culture

For *in vitro* studies, adherent human breast cancer cell lines, MCF7, were obtained from the National Centre for Cell Science (NCCS) in Pune, Maharashtra, India. MCF-7 was oestrogen receptor (ER) positive, progesterone receptor (PR) positive and human epidermal growth factor receptor (HER) negative. These adherent cells were cultured in RPMI1640 supplemented with 10% charcoal stripped FBS and 1% Gentamicin and kept in a humidified incubator at 37°C with 5% CO2. Trypsinization was performed on the cells using a 0.25 percent trypsin/1 mM EDTA solution. Cell suspensions with a viability of 95% (as assessed by trypan blue vital stain in an automated cell counter (CountessTM, Invitrogen, Van Allen Way, Carlsbad, California) were seeded in culture plates for different *in vitro* research.

Methanol extraction

The seeds of *T. indica* were shaded dried, seed coat were removed and coarsely ground in a temperature-controlled plant sample pulveriser. At 60 ºC, the powdered plant material (500 g) was extracted with methanol in a Soxhlet extractor. After thorough extraction, the methanol extract was evaporated and extract was concentrated in a rotary vacuum evaporator (Rotavapor, Buchi, Switzerland) at 40°C and 10000 pascal pressure. The residue was first kept open at room temperature to allow the solvent to completely evaporate before being stored in an airtight container in the refrigerator for later use.

Sample preparation

To get the appropriate concentrations of the extract, a stock solution of the methanol extract was prepared in RPMI-1640 medium and then diluted with the prepared culture media.

The effect of methanolic extract of *T. indica* seed coat on *CYP 19* and *StAR* genes expression

The cells were cultured for 96 hours with IC_{50} (16), double (32) and half dosages of IC_{50} (8) extract and appropriate medium, with media replacement every 24 hours. The cells were collected by trypsinisation after 96 hours and utilised to isolate total RNA for expression experiments. Total RNA was extracted separately using the 'TRIzol' reagent (Thermo Scientific) according to the manufacturer's instructions [19].

The quality and quantity of total RNA were determined spectrophotometrically using the Nano drop (Thermo Scientific NanoDropTM1000 Spectrophotometer) technique. Using a Nano Drop spectrophotometer, the purity of total RNA was evaluated using the A260/A280 and A260/A230 ratios.

cDNA was synthesized by reverse transcription of 500 ng of RNA, the reaction was set up according to the maxima first strand cDNA synthesis kit's standard procedure (Table 1)

The product of first strand cDNA synthesis was stored at -20^{0}C until use. Then 1 µL of cDNA was used for further qPCR experiments.

Exon spanning primers (Table 2) were developed using NCBI primer design software (Primer3, http://bioinfo.ut.ee/primer3/), and the primer's specificity was confirmed using BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Gradient PCR was used to amplify the *CYP19*, *StAR*, and *GAPDH* genes and optimize the PCR condition. Table 3 shows the PCR reaction mix and cycle conditions used.

SYBR green chemistry (Maxima SYBR green qPCR master mix (Thermo scientific, USA)) was used to investigate the expression of *CYP19*, *StAR* and *GAPDH*. The reaction was performed in triplicate (Table 4). The expression of target gene *CYP19*, *StAR* was compared with a reference gene *GAPDH* to calculate ΔCq and the expression of same gene in treatment sample versus control samples to calculate ΔΔCq. The method used is known as comparative Δ Cq method [20].

Following the reaction, a melt curve analysis was done to ensure that the amplification was specific. The melt curve analysis method included denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and denaturation at 95°C for 15 seconds. During the final denaturation stage, data was collected.

Gas chromatography – Mass spectrometry (GC-MS) analysis of *T. indica*

The active phytochemicals present in *T.indica* seed coat was analysed using Gas chromatography Mass Spectrometer (Shimadzu GC-MS, Japan, QP2010S) with a mass range of 1.5-1000 m/z was used. Helium at flow rate of one mL/ min was the carrier gas with oven temperature of 80ºC for four min and then increased to 280ºC in six min. The injector temperature was 260ºC and total analysis time was 50 min. Aliquots of extract (0.4 µL) were injected into the chromatographic column after a clear baseline was obtained. Major compounds were detected using mass spectrum library (NIST 11 and WILEY 8).

Fourier transform infrared spectroscopy (FTIR) analysis

ATR-FTIR (Attenuated Total Reflectance- Fourier transform infrared) analysis was performed, using a Perkin- Elmer spectrum two FTIR spectrometer with attenuated total reflectance. The sampling station was equipped with an overhead ATR accessory. A small quantity of the sample was carefully placed on the diamond crystal surface to cover the ATR diamond window in order to focus the laser beam. Each spectrum was recorded as absorbance under 60 N.

Statistical Analysis

All results were presented as Mean ± SE with 'n' equal to the number of replicates. SPSS software version 24 is used for the intergroup comparison was analysed using one-way analysis of variance (ANOVA) accompanied by Duncan Multiple Range Test (DMRT) for paired analysis.

RESULTS

Total RNA concentration and purity determination

For gene expression studies, the quality and concentration of RNA samples are chosen as shown in Table 5. The A260/280 and A260/230 ratios are in the 1.9 to 2.0 range, suggesting that the RNA samples are pure and of high quality.

Analyses of relative gene expression

The parameters required for the amplification of human *CYP 19* and *StAR* genes were standardised using a gradient PCR reaction. For real-time qPCR, the primer concentrations and annealing temperature that produced the best results were used. The gradient PCR products were run on 1.5 per cent gel. Figure 1 depicts the amplification plot and melt curve of *CYP 19* and *StAR* genes in RT-qPCR.

The effect of methanolic extract of *T. indica* seed coat on *CYP 19* gene expression

Methanolic extract of *Tamarindus indica* seed coat exerted differential expression on *CYP 19* gene. Both twice and lower IC_{50} doses of extract decreased *CYP 19* gene expression 0.014 and 0.211 folds respectively. But IC_{50} dose increased *CYP 19* gene expression by 3.43 folds (Table 6).

The effect of methanolic extract of *T. indica* seed coat on *StAR* gene expression

Significant fold increase of 460 was seen in gene expression after treatment with the extract at concentration of half IC_{50}. At IC_{50} and twice IC_{50} doses of the extract the fold increase was found to be 88.03 and 29.24 respectively (Table 7).

Gas chromatography -Mass spectroscopy (GC-MS) analysis of *T. indica*

Chromatograms obtained on phytochemical analysis of *T. indica* using GC-MS are given in figure 2. Phytoconstituents obtained on GC-MS analysis of *T. indica* is listed in Table 8.

Fourier transform infrared spectroscopy (FTIR) analysis

Table 9 lists functional groups present in the *T. indica* by comparing the FTIR spectra of the extract with the FLUKA library. Figure 3 depicts the spectrum of *T. indica* methanolic

DISCUSSION

Most of the current researchers are focussing on different genes involved in oestrogen biosynthesis as a way to regulate breast cancer proliferation, as 80 percent of breast cancers are positive for ER receptors and they mainly depend on the steroid hormone for growth and differentiation [19]. Aromatase and *StAR* have vital role in oestrogen biosynthesis as *StAR* facilitates the movement of cholesterol into the mitochondrial membrane and aromatase convert androgen finally to oestrogen [20]. In this present study, methanolic extract of seed coat of *Tamarindus indica* at higher and lower doses down regulated the expression of *CYP19* gene while at IC_{50} doses stimulated *CYP 19* gene expression in MCF-7 cell lines. On the other hand, methanolic extract of seed coat of *Tamarindus indica* upregulated the expression of *StAR* gene in a dose dependent manner, which is the key regulator of steroidogenesis.

Isoflavones such as genistein, flavones, and quercetin promoted aromatase activity in H295R cells in a dose-dependent manner, which reduced as cytotoxicity increased [21]. Rotenone inhibited *CYP19*, aromatase inhibition was caused by the oxo-group on the 4th position of the carbon ring on flavonoids interacting with the haem group of *CYP19* [21].

Apigenin, quercetin, biochanin, genistein and daidzein reduced the expression of *CYP* 19 [22]. Soy isoflavones are capable of decreasing aromatase enzyme activity there by inhibiting oestrogen biosynthesis and exerting cancer preventive effect [23]. Daidzein and genistein were also found to be decreasing aromatase activity in MCF-7 cell lines there by decreasing the oestrogen levels [24].

StAR promoter activity was increased in MA-10 cells after treatment with 10µM of quercetin whereas higher concentrations of diverse phytochemicals like naringenin (NAR), 8-prenylnaringenin (8-PN), genistein (GEN), coumestrol (COU), quercetin (QUE) and resveratrol (RSV) upregulated the *StAR* mRNA levels in KGN human granulosa-derived tumor cells [25, 26].

All the findings of the current study on gene expression are in accordance with previous studies with different phytochemicals such as steroids, glycosides, phenolic compounds, tannins, flavonoids, alkaloids and saponins. which are particularly found in *T. indica* seed coat.

The GC-MS analysis of the methanolic extract of seed coat of *T. indica* indicated the presence of terpenes, phytosterols and fatty acid analogues. Among the identified compounds, 3,7,11,15-Tetramethyl-2- hexadecen-1-ol possess anticancer, antimicrobial, anti-inflammatory and antioxidant potential [27]. Gamma sitosterol claims to have anticancer activities in MCF-7 cells and a potent inhibitor of tumour as confirmed by *in vivo* studies: furthermore, consumption gamma sitosterol through diet also lowers cholesterol levels in humans [28, 29]. The anticarcinogenic and other therapeutic effects of Stigmast-4-en-3-one is demonstrated by [30]. The most known phytoestrogen compounds such as naringenin, apigenin, epicatechin, catechin are identified by previous studies conducted in *Tamrindus indica*, these phenolic compounds accounts for the phytoestrogenic potential of *T. indica* in the current study by modulating *CYP 19* and *StAR* genes expression. [31] Methanolic extract of *Tamrindus indica* comprise of numerous bioactive compounds, which are known for their anticancerous activities.

Table 1: Master Mix for cDNA synthesis

Components	Volume (µL)
5X Reaction buffer	4
dnTP mix	2
Oligo dT primer	1
Enzyme Mix	1
Ribolock	1
Total RNA	To get 500 ng
Nuclease free water	Added to make 20 µL of total mix

Table 2: Description of primers used

Gene Name	Primer sequence (5”- 3”)	Primer Length (bp)	Product Size (bp)
CYP19	TGCTTCCTCACTGGCCTTTTT CCATGATGGACCAAAATCCCAA	20 22	121
StAR	GCTGCTAGCGACATTCAAGC AGCCGAGAACCGAGTAGAGA	20 20	186
GAPDH	TCGGAGTCAACGGATTTGGT TTCCCGTTCTCAGCCTTGAC	20 20	181

Table 3: PCR conditions for amplification

Steps		Temperature	Time
Initial denaturation		95°C	5 min
30 cycles of	Denaturation	95°C	45 sec
	Annealing *CYP19* *StAR* *GAPDH*	62.1° C 58.6° C 62.9° C	30 sec
	Extension	72°C	1 min

Table 4: qRT-PCR conditions for *CYP19 and StAR* genes

Steps		Temperature	Time
Initial denaturation		95°C	3min
30 cycles of	Denaturation	94°C	30sec
	Annealing *CYP19* *StAR* *GAPDH*	62.1° C 58.6° C 62.9° C	30 sec
	Extension	72°C	30 sec

Table 5: Showed the concentration and purity of RNA samples is chosen for gene expression studies

RNA samples	Concentration(µg/µL)	$A_{260\backslash280}$	$A_{260/230}$
Untreated	2002	2.01	2.1
Cells treated with half IC_{50}	1750	1.90	2.02
Cells treated with IC_{50}	1200	1.90	2.02
Cells treated with double IC_{50}	756	2.0	2.03

Table 6: Relative *CYP19* expression in MCF-7 cells in response to treatment with different doses of *T. indica* Values expressed as Mean± Standard error. (n=3)

Concentration	$^{\delta}$ CT	$^{\delta\delta}$ CT	Fold changes
Control	-5.67	0^{d}	1±0.73
8	-3.42	2.24^{a}	0.211±2.36
16	7.45	-1.78^{b}	3.43±1.14
32	0.42	6.09^{c}	0.014±4.46

Table 7: Relative *StAR* expression in MCF-7 cells in response to treatment with different doses of *T. indica*. Values expressed as Mean± Standard error. (n=3)

Concentration	$^{\delta}$ CT	$^{\delta\delta}$ CT	Fold changes
Control	8.36	0^{d}	1±.02
8	-0.48	-8.84^{a}	460±2.94
16	1.90	-6.46^{b}	88.03±1.11
32	3.49	-4.87^{c}	29.24±3.94

Table 8: GC-MS analysis of phytochemicals in *T. indica*

Retention Time (Min)	Name of the Compound	Molecular formula	MW(g/mole)	Peak area	Class	Probability (%)
26.570	Neophytadiene	$C_{20}H_{38}$	278.5	1654214	Diterpene	95
27.466	3,7,11,15-Tetramethyl 2- hexadecen-1-ol	$C_{20}H_{40}O$	296.5	356168	Acyclic diterpene alcohol	91
29.249	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.3	1033816	Phthalate ester	96
42.133	Gamma- sitosterol	$C_{29}H_{50}O$	414.7	2485013	Phytosterol	80
42.339	Cycloartenyl acetate	$C_{32}H_{52}O_2$	468.8	10872416	Triterpenes	79
44.002	Methyl commate D	$C_{31}H_{50}O_4$	486.7	10803897	Methyl esters	82
45.782	Triacontyl acetate	$C_{32}H_{64}O_2$	480.8	2419875	Ester	95
47.025	Stigmast 4-en-3-one	$C_{29}H48O$	414.7	12289017	Phytosterol	89

Table 9: Functional groups obtained from FTIR absorption spectra of seed coat of *Tamarindus indica*

Wave number (cm^{-1})	Bond	Functional group
3250	O-H	Alcohol
1606	C=C	α, β unsaturated ketone
1442	C-H	Alkane
1283	C-O	Aromatic ester
1105	C-O	Secondary alcohol
764	C-H	1,2,3 trisubstituted

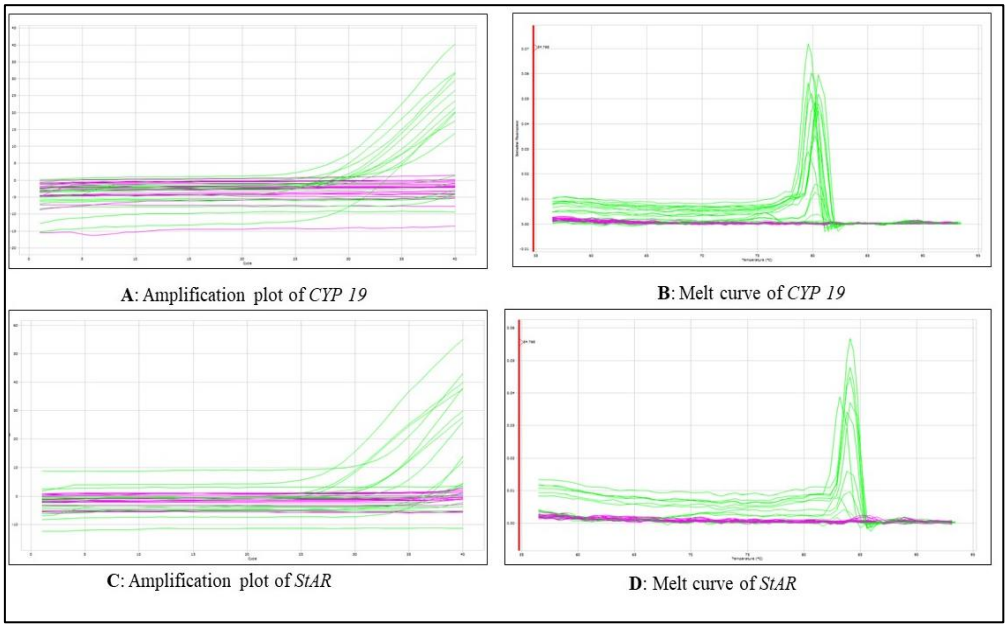

Figure 1: Amplification plot and melt curve of the *CYP 19* and *StAR* genes, respectively. A: *CYP19* gene amplification plot. B: *CYP 19* gene melt curve. C: *StAR* gene amplification plot. D: *StAR* gene melt curve

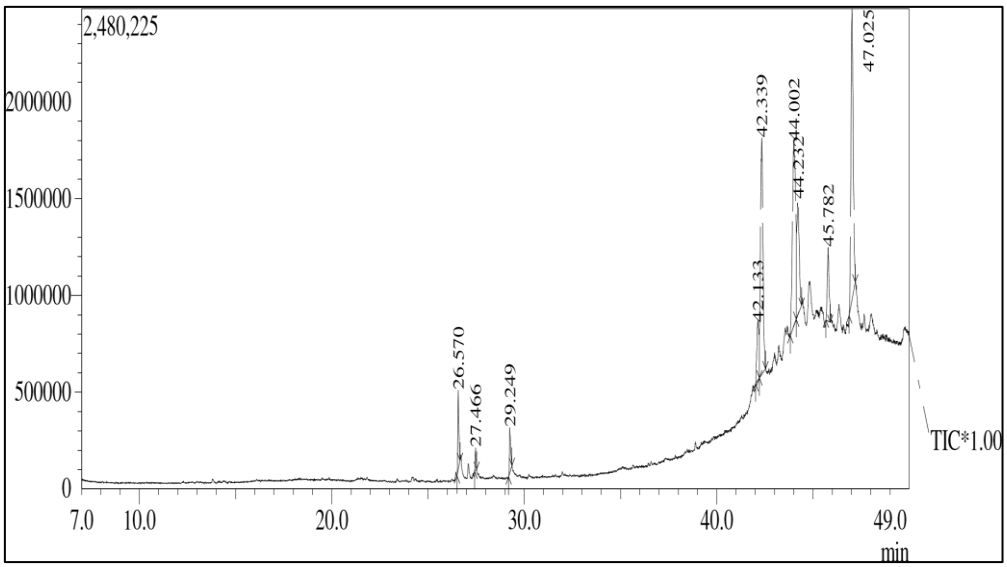

Figure 2: Gas chromatography mass spectroscopy chromatogram of methanolic extract of *T. indica* seed coat

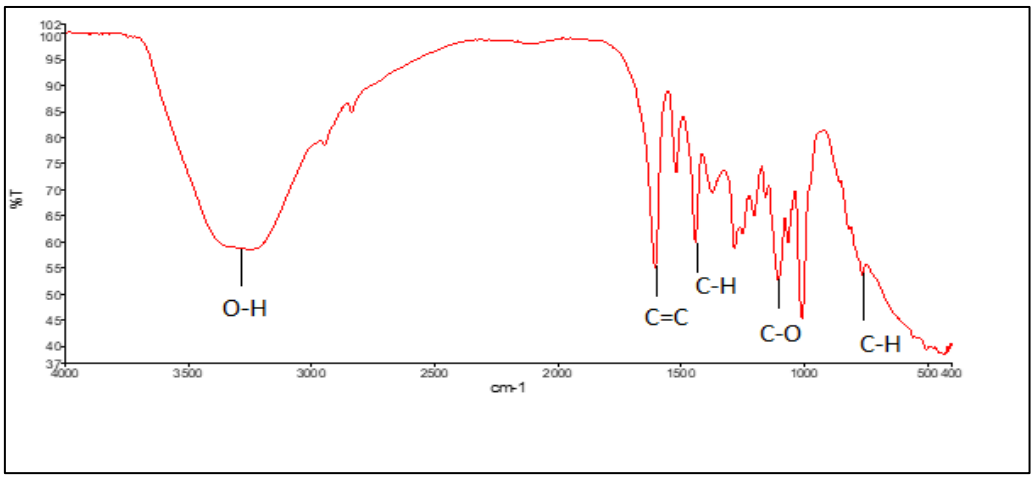

Figure 3: FTIR spectrum of methanolic extract of *T. indica*

CONCLUSION

From this study it can be concluded that the star gene expression was found to be increased at all doses on the other hand the *CYP 19* expression was found to be inhibited at twice and half IC_{50} concentrations. Furthermore, at IC_{50} doses the extract significantly stimulated *CYP19* expression. Since there was an upregulation of *StAR*, the production of pregnenolone or testosterone might have been enhanced which can be beneficial in treatment of hormone dependent malignancies. The changes in the oestrogen concentrations were induced by the differential expression of these genes and may in part or fully responsible for the antiproliferative effect in MCF-7 cells as they are hormone dependent. Hence, the inhibitory effect on *CYP 19* can be utilized for the treatment of hormone dependent breast cancer.

Acknowledgements

The authors are thankful to the College of Veterinary and Animal Sciences, Mannuthy, under Kerala Veterinary and Animal Sciences University for providing the facilities financial assistance provided.

Conflict of Interest

None declared.

ORCID ID

Bimitha Benny: NA

A Sandeshkrishna: NA

Sujith Samraj: NA

Preethy John: NA

Uma Radhakrishnan: NA

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HOW TO CITE THIS ARTICLE
Sandeshkrishna A, Benny B, Samraj S, John P, Radhakrishnan U. Modulatory potential of *Tamarindus indica* on steroidogenesis by targeting *in-vitro* expression of *CYP 19* and *StAR* genes in MCF-7 cell lines. J Phytopharmacol 2022; 11(6):407-413. doi: 10.31254/phyto.2022.11606