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ORIGINAL ARTICLE

Phytochemical Evaluation and Cytotoxic Potential of Chloroform Soluble Fraction of Methanol Extract of *Thespesia populnea* in Human Breast Cancer Cell Lines

Anu Gopalakrishnan, Bibu John Kariyil, Reni John, P. T. A. Usha

Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala, India

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ABSTRACT

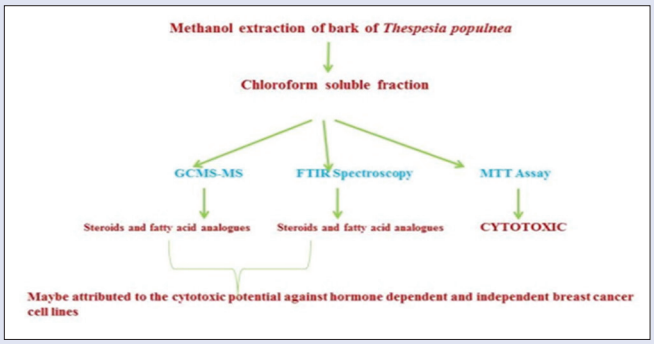
Background: *Thespesia populnea* (Poovarasu), belonging to family Malvaceae, is a tropical evergreen tree found abundantly in the coastal regions of India. It is traditionally known for its hepatoprotective, antitumor, antioxidant, and wound healing activities. **Objective:** The present study was undertaken to find out different phytochemical principles of chloroform-soluble fraction of *T. populnea* (CSFTP) using gas chromatography mass spectrometry-mass spectrometry (GCMS-MS) and Fourier-transform infrared (FTIR) spectroscopy and to analyze *in vitro* cytotoxic potential of the fraction in MDA-MB-231 and MCF-7 human breast carcinoma cell lines. **Materials and Methods:** Shade-dried bark of *T. populnea* was extracted using methanol and fractionated to obtain CSF. The fraction was subjected to GCMS, FTIR, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. **Results:** GCMS-MS and FTIR analysis showed the presence of various steroids and fatty acid analogs. Cell growth inhibition was noted for CSFTP in MDA-MB-231 and MCF-7 cell lines. **Conclusion:** CSFTP revealed the presence of various active principles which could be attributed for its cytotoxic potential.

Key words: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, chloroform-soluble fraction, fourier-transform infrared, gas chromatography mass spectrometry-mass spectrometry, phytochemical principles, *Thespesia populnea* methanol extract

SUMMARY

- Phytochemical analysis of chloroform-soluble fraction (CSF) of *Thespesia populnea* revealed the presence of various steroids and fatty acid derivatives on gas chromatography mass spectrometry-mass spectrometry and Fourier-transform infrared spectroscopy
- The CSF showed cytotoxic potential with an IC_{50} of 23.97 ± 2.66 and 20.62 ± 3.47 μg/mL in MDA-MB-231 and MCF-7 breast cancer cells, respectively, hormone-independent and hormone-dependent breast cancer cell lines.

Further studies and isolation of these phytochemicals could lead to the development of specific compounds for the treatment of hormone-independent and hormone-dependent breast cancers.



Abbreviations used: CSFTP: Chloroform-soluble fraction of methanol extract of bark of *Thespesia populnea*; GCMS-MS: Gas chromatography mass spectrometry-mass spectrometry; FTIR: Fourier-transform infrared; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Correspondence:

Dr. Bibu John Kariyil,
Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur - 680 651, Kerala, India.
E-mail: bibujohn@kvasu.ac.in
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INTRODUCTION

Breast cancer is one of the most dreaded diseases of the 20th century and spreading further with continuance and increasing incidence in the 21st century. It is considered as an adversary of modernization and advanced pattern of sociocultural life dominated by Western style along with hereditary changes. Among the various breast cancers, hormone-independent breast cancers are aggressive in nature as they lack receptors for the drugs to act. A greater emphasis has been given toward the researches on natural products on all types of cancer treatment. Several studies have been conducted on herbs under a multitude of ethnobotanical grounds.

With the advancement of molecular analysis and gene sequencing methods, the diversity of human breast tumors has been explored deeply. Accurate grouping of breast cancers into clinically relevant subtypes is of particular importance for its successful therapeutic management.[1] Hence, breast cancers have been classified into four major intrinsic subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2-enriched) and basal-like (or triple negative), based on the expression of estrogen, progesterone, and human epidermal growth factor receptors. Cancer cell lines have been widely developed as an essential experimental tool in cancer research. *In vitro* culture of breast cancer cell lines were suggested as the most popular

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model for preclinical evaluation studies.[2] MDA-MB-231 breast carcinoma cell line was named after its isolation from M. D. Anderson Cancer Center in 1973. They are spindle-shaped cells with an *in vitro* invasive behavior. It is tumorigenic, has the ability to transform and form colonies. They can also form mammary fat pad tumors in nude mice. They are usually referred as triple-negative cell lines due to lack of estrogen, progesterone, and HER2/Neu receptors. MCF-7 breast cancer cell lines were evolved after isolating from pleural effusion of a metastatic breast cancer patient in Detroit and developed at Michigan Cancer Foundation, Detroit.[3] They are non-invasive, luminal-type ductal breast carcinoma cells presenting estrogen and progesterone receptors and lack HER2/Neu receptors.

Thespesia populnea, belonging to the family Malvaceae, is a medium-sized evergreen tree, used traditionally for ailments of skin and liver diseases, dysentery, tumors, hemorrhoids, and wound healing.[4] It has been scientifically proved for its astringent, antibacterial, antioxidant, hepatoprotective, anti-inflammatory, and antinociceptive activities.[5] However, the scientific validation for its cytotoxic potential has not been carried out in hormone-independent breast cancers. Hence, the present study is aimed at the evaluation of various phytoconstituents and the cytotoxic potential of chloroform-soluble fraction (CSF) of methanol extract of *T. populnea* stem bark (CSFTP) in hormone-independent and hormone-dependent types of human breast cancer cell lines, MDA-MB-231 and MCF-7.

MATERIALS AND METHODS

Collection of plant material and authentication

Bark of *T. populnea* was collected during August 2014–September 2014 from Thiruvananthapuram district of Kerala. The plant material was taxonomically identified and authenticated by The Head, Raw Materials, Herbarium and Museum Division, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (India). Voucher specimen (NISCAIR/RHMD/2014/2794/173-2) of the plant has been deposited at the herbarium of NISCAIR.

Methanol extraction

Fresh bark of *T. populnea* was chopped into small pieces, shade–dried, and powdered coarsely in a temperature-controlled plant sample pulverizer. Powdered bark (1 kg) was extracted with methanol in Soxhlet extractor at room temperature. After exhaustive extraction, the methanol extract was filtered and concentrated in rotary vacuum evaporator (Evator, Equitron EV11.ABI.029) at 50°C, 30 mmHg pressure. A reddish-brown-colored residue obtained was kept open at room temperature for complete evaporation of solvent. This crude methanol extract of *T. populnea* bark was stored in sealed airtight container for further use.

Preparation of chloroform soluble fraction

Methanol extract of *T. populnea* was fractionated using chloroform and water in a separating funnel, to obtain CSF, aqueous fraction, and insoluble residual fraction of the extract. Solvents were removed from various fractions using rotary vacuum evaporator and then dried completely by keeping at room temperature. Crude extracts and fractions were stored in sealed containers for further experiments.

Gas chromatography mass spectrometry-mass spectrometry analysis

The active phytochemical principles of CSFTP was analyzed using gas chromatography mass spectrometry-mass spectrometry (GCMS-MS) system of the Central Instruments Laboratory of College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala. GCMS-MS analysis was carried out on TSQ 8000 GCMS-MS. The compounds were separated on TSQ-2MS capillary column (30 m × 0.25 mm; i.d., 0.25 µm film). The sample dissolved in methanol, filtered in 0.22-micron syringe filter, was used for analysis. The column oven temperature was programmed from an initial temperature of 40°C (1 min) to 100°C (1 min), then to 150°C (1 min) and finally to 250°C (1 min) each at 10°C min^{-1} with a final time of 10 min. The oven run time was 25 min with prep-run time out at 10 min. The injection temperature and ion source temperature were 290 and 230°C, respectively. Helium was used as the carrier gas at flow rate of 1 ml/min. The ionizing energy was 70 eV. All the data were obtained by collecting the full-scan mass spectra within the scan range 40–350 amu. Compounds were identified using the National Institute of Standards and Technology MS Search 2.0 library.[6]

Fourier-transform infrared spectroscopy

Fourier-transform infrared (FTIR) spectra of the fraction were obtained by conventional potassium bromide (KBr)/pellet disc method using Perkin–Elmer FTIR spectrometer with some modifications.[7] Two mg of extract and 298 mg of KBr was taken in a mortar and mixed. The mixture was placed into an evacuable die on hydraulic laboratory press and compressed under eight ton pressure to form a transparent pellet. The KBr pellet was placed in the pellet holder and spectrum was taken from 4000 cm^{-1} to 400 cm^{-1} wave number range using IR spectrophotometer against the blank. Three hundred mg KBr pellet was used as blank. The spectrum obtained was compared with FLUKA library provided by Perkin–Elmer.

Culturing of cell lines

MDA-MB-231 and MCF-7 cell lines were procured from the National Centre for Cell Science, Pune, India. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 4% antibiotic-antimycotic solution containing penicillin-streptomycin and amphotericin B. The cells were maintained in a humidified incubator at 37°C with 5% CO_2. After attaining 70% confluency, the cells were subcultured by enzymatic digestion with 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid solution, and these trypsinized cells were used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

MTT assay was done to assess the cytotoxicity.[8] Trypsinized breast cancer cells were seeded at a density of 5×10^3 cells per well in 200 µL medium and were allowed to attach for overnight in a CO_2 incubator. Cells were treated with CSFTP at concentrations of 2.5, 5, 10, 20, 40, 80, 160, and 320 µg/mL for a period of 24 h. After the treatment, 20 µL of MTT (5 mg/mL) in 150 µL medium was added and incubated at 37°C for 4 h after removing the medium with CSFTP. Then, the media with MTT was removed and 200 µL of DMSO was added and read at 570 nm in an ELISA plate reader (Varioskan flash, ThermoFischer Scientific, Finland).

The percent inhibition was calculated using the formula below:

Percent inhibition = (Average absorbance of control cells − average absorbance of treated cells/average absorbance of untreated cells) × 100

The net absorbance from the control wells was taken as 100% viable. The half maximal inhibitory concentration (IC_{50}) values of fractions were calculated by plotting the concentration against percentage cell viability using the online software "very simple IC_{50} tool kit."

RESULTS

Yield of chloroform-soluble fraction of methanol extract of bark of *Thespesia populnea*

The percent yield of methanol extract of *T. populnea* was found to be 18.6% and that of CSF was found to be 39.5%.

Gas chromatography mass spectrometry-mass spectrometry and Fourier-transform infrared analysis of chloroform-soluble fraction of methanol extract of bark of *Thespesia populnea*

The chromatogram obtained on phytochemical analysis using GCMS-MS is given in Figure 1. Phytoconstituents of significance obtained on GCMS-MS analysis of CSFTP are listed in Table 1. GCMS-MS analysis showed the presence of dichloroacetic acid tetradecylester, cis-13-octadecenoic acid, phthalic acid, decyl isobutyl ester, 17-octadecynoic acid, 9, 12, 15-octadecatrienoic acid, 2[(trimethyl silyl) oxy]-1-[[(trimethyl silyl) oxy] methyl] ethyl ester, (Z, Z, Z)-, cyclopropane butanoic acid, 2[[2-[[2-(2-pentyl cyclopropyl) methyl] cyclopropyl methyl]-, methyl ester, benzene propanoic acid, 3,5-bis (1,1-dimethyl ethyl)-4-hydroxy-, methyl ester, estra-1,3,5 (10)-trien17à-ol, phthalic acid, butyl oct-3-yl ester, 1-monolinoleoyl glycerol trimethylsilyl ester, cis-vaccenic acid, pregan-20-one, 2-hydroxy,-5,6-epoxy-15-methyl-and butanoic acid, and 1a,2,5,5a,6,9,10,10a-octahydro-5a-hydroxy-4-(hydroxymethyl)-1,1,7,9-tetramethyl-6,11-dioxo-1H. The most probable compounds obtained by comparing the spectra of the CSFTP using FTIR spectroscopy with FLUKA library are enlisted in Table 2 and the obtained spectrum is given in Figure 2. FTIR spectroscopy showed the similarity to structures of cis-androsterone, digitoxigenin, methyl linoleate, butyl stearate, methyl elaidate,5-amino tetralin, citronellal, ethyl palmitate, diethylenetriamine pentaacetic acid, and 2-(methyl thio) ethylamine.

Cytotoxic potential of chloroform-soluble fraction of methanol extract of bark of *Thespesia populnea*

The data of MTT reduction assay of CSFTP in MDA-MB-231 and MCF-7 cells are presented in Table 3. The percent cell growth inhibition of CSF showed a drastic increase from 20 µg/mL and was in a constant range for further concentrations in a similar pattern in MDA-MB-231 cells. In MCF-7 cells, a gradual increase in percent cell growth inhibition with a 100% inhibition at 160 and 320 µg/mL, respectively. The IC_{50} values for CSF were obtained to be 23.97 ± 2.66 and 20.62 ± 3.47 µg/mL for MDA-MB-231 cells and MCF-7 cells, respectively.

DISCUSSION

Phytochemical screening of CSFTP using qualitative chemical tests revealed the presence of steroids, alkaloids, flavonoids, triterpenes, and tannins.[9] The compounds obtained by GCMS-MS analysis were steroids and fatty acid derivatives. FTIR spectroscopy also showed the similarity to structures of steroids and fatty acid derivatives. Thus, these phytoconstituents could be attributed for the cytotoxic potential of CSFTP. Phytosterols, isoflavones, saponins,[10] many unsaturated, and a few saturated branched-chain fatty acids have been reported to exhibit anticancer activity.[11] In the present study, the cytotoxic potential of CSFTP was revealed by reducing yellow MTT to insoluble

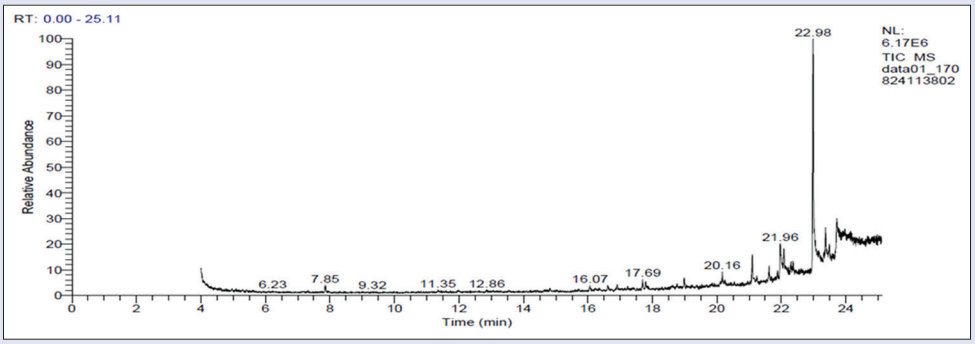

Figure 1: Gas chromatography mass spectrometry-mass spectrometric chromatogram of chloroform-soluble fraction of methanol extract of the bark of *Thespesia populnea*

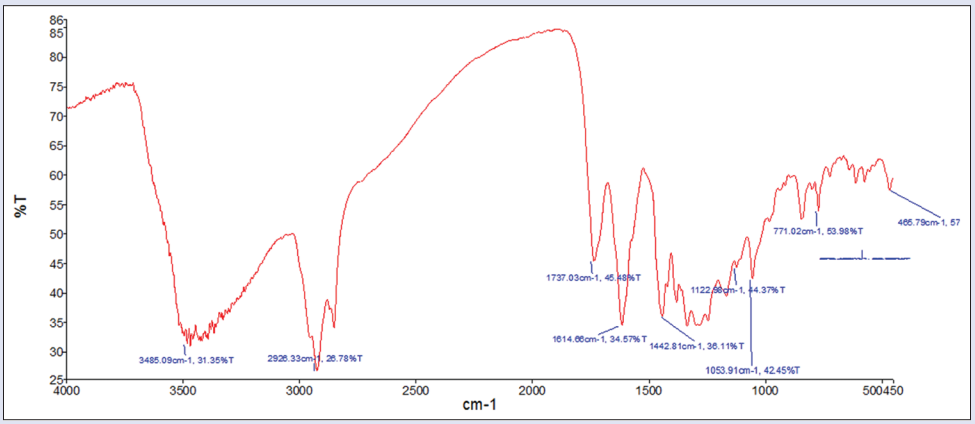

Figure 2: Fourier-transform infrared spectrum of chloroform-soluble fraction of methanol extract of the bark of *Thespesia populnea*

Table 1: Gas chromatography mass spectrometry-mass spectrometric analysis of chloroform-soluble fraction of *Thespesia populnea* revealing different phytoconstituents

RT (min)	Name of compound	MW (g/mole)	Peak area (%)	Class	Probability %
17.69	Dichloroacetic acid tetradecylester	142	0.58	Halogenated organic acid	2.77
20.16	Cis-13-octadecenoic acid	282	1.07	Fatty acid derivative	5.75
21.09	Phthalic acid decyl isobutyl ester	362	3.91	Aromatic dicarboxylic acid	4.7
28.33	17 Octadecynoic acid	280	910,486.45	Fatty acid analogue	8.48
21.23	9, 12, 15-octadecatrienoic acid, 2[(trimethyl silyl) oxy]-1-[[(trimethyl silyl) oxy] methyl] ethyl ester,(Z, Z, Z)-	496	0.42	Fatty acid derivative	31.65
21.62	Cyclopropane butanoic acid, 2[[2-[[2-(2-pentyl cyclopropyl) methyl] cyclopropyl methyl]-, methylester	374	1.98	Fatty acid ester	9.51
21.88	Benzene propanoic acid, 3,5- bis (1,1-dimethyl ethyl)- 4- hydroxy-, methyl ester	292	0.76	Fatty acid ester	18.91
21.96	Estra-1,3,5 (10)-trien17à-ol	256	9.75	Steroid	18.91
22.08	Phthalic acid, butyl oct-3-yl ester	334	4.67	Aromatic dicarboxylic acid	15.17
22.23	1-Monolinoleoyl glycerol trimethylsilyl ester	498	0.89	Fatty acid ester	33.8
23.72	cis-Vaccenic acid	282	10.10	Fatty acid	10.34
23.85	Pregan-20-one, 2-hydroxy,-5,6-epoxy-15-methyl-	346	0.11	Steroid	6.67
24.32	Butanoic acid, 1a, 2,5,5a, 6,9,10,10a-octahydro-5a-hydroxy-4-(hydroxymethyl)-1,1,7,9-tetramethyl-6,11-dioxo-1H	416	0.17	Fatty acid analogue	9.11

MW: Molecular weight; RT: Retention time

Table 2: Structurally similar compounds in chloroform-soluble fraction of *Thespesia populnea* revealed using Fourier-transform infrared spectroscopy

Plant extract	Structurally similar compounds	Class
CSF of methanol extract of stem bark of *Thespesia populnea*	Cis-androsterone	Steroids
	Digitoxigenin	Cardiac glycoside
	Methyl linoleate	Fatty acid
	Butyl stearate	Fatty acid
	Methyl elaidate	Fatty acid derivative
	5-amino tetralin	Amines
	Citronellal	Monoterpenoid
	Ethyl palmitate	Fatty acid derivative
	Diethylenetriamine pentaacetic acid	Amino polycarboxylic acid
	2-(methyl thio) ethylamine	Amine

Table 3: The percent inhibition of MDA-MB-231 and MCF-7 cells after 48 h treatment with chloroform soluble fraction of *Thespesia populnea* determined by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay

Concentration (µg/mL)	Percent inhibition of MDA-MB-231 cells	Percent cell inhibition of MCF-7 cells
2.5	18.48±1.59	2.73±2.01
5	24.10±1.13	7.87±1.54
10	35.29±0.81	15.4±6.28
20	46.09±4.04	46.15±4.69
40	102.7±1.28	64.39±2.87
80	106.22±1.24	98.98±0.39
160	109.06±1.08	100
320	100.45±0.63	100
IC_{50} (µg/mL)	23.97±2.66	20.62±3.47

Values expressed as mean±SEM (n=3). IC_{50}: Half maximal inhibitory concentration; SEM: Standard error of mean

purple formazan with an IC_{50} of 23.97 ± 2.66 and 20.62 ± 3.47 µg/mL for MDA-MB-231 and MCF-7, respectively. The MDA-MB-231 cell lines are absent for estrogen and progesterone receptors, whereas MCF-7 cell lines present receptors for estrogen and progesterone and both lacks HER-2/Neu receptors. Hence, studies on these cell lines will provide a comprehensive idea on the effect of CSFTP on both hormone-dependent and hormone-independent breast cancer cells. As per the National Cancer Institute guidelines, the IC_{50} limit for selecting the plant extracts for anticancer studies is <30 µg/mL after 72 h of exposure.[12,13] Oestra-1,3,5 (10) trien-17á-ol, having structural similarity to estradiol was obtained on GCMS-MS analysis. On FTIR spectroscopy, the fraction was showing a major structural resemblance to cis-androsterone, which is a precursor in estrogen synthesis. Thus, it could be seen that phytoestrogens were commonly obtained in both GCMS-MS and FTIR analysis. Phytoestrogens can exert cytotoxicity by means of other pathways such as MMP, Akt, NF-κB, and MAPK regardless of estrogen receptor expression which has to be explored for the present fraction.[14] Thus, from the present study, it could be concluded that CSFTP could be further subjected for the development of potent anticancer compounds in hormonal dependent and independent breast cancers.

CONCLUSION

GCMS-MS analysis and FTIR spectroscopy of CSFTP revealed the presence of various steroids and fatty acid derivatives which could be ascribed for the cytotoxic potential possessed by the plant fraction in MDA-MB-231 cells. It could be concluded that CSFTP is a promising fraction for the elucidation of potent anticancer compounds, especially for hormonal-dependent and hormonal-independent breast cancers. Further studies and isolation of these phytochemicals could lead to the development of specific compounds in the process of anticancer drug discovery.

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Conflicts of interest

There are no conflicts of interest.

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