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Antimicrobial, Anticancer and Cytotoxic Activities of Acetone Extract Fraction from Stem Bark of *Thespesia populnea* (Linn.).

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ABSTRACT

The fraction from the acetone extract of stem bark of *Thespesia populnea* (Linn.) was investigated to explore the antimicrobial, anticancer and cytotoxic properties. The screening of antimicrobial activity of the fraction was carried out by cup plate method against four standard bacterial [*Staphylococcus aureus* (MTCC 96), *Micrococcus luteus* (MTCC 1541), *Klebsiella pneumonia* (MTCC 109) and *Escherichia coli* (MTCC 118)] and three fungal strains [*Candida albicans* (MTCC 183), *C. parapsilosis* (MTCC 2509) and *C. tropicalis* (MTCC 184)]. The anticancerous activity against HEp2 cell line and cytotoxicity assay against HEL cell line were carried out by standard protocols. The results indicated that the fraction possess comparatively higher antibacterial activity than antifungal activity. While the fraction inhibited all the other three bacteria at the concentration of $\geq$500 µg/ml, it was ineffective against *K. pneumonia*. A moderate antifungal activity against all the Candidal species at the concentration of $\geq$750 µg/ml was recorded. The fraction exhibited satisfactory anticancerous activity against HEp2 cell line with the concentration of 5 mg/ml (14.6% of cell viability). The cytotoxic activity of the fraction on normal cell line by MTT assay indicated that a concentration of 5 mg/ml was required to cause 50% (IC_{50}) of inhibition of HEL cell line. Thus, the findings of the present study vividly present the potential of acetone extract fraction from stem bark of *T. populnea*, to be a promising antimicrobial and anticancerous with low cytotoxicity on normal cells.

Keywords: *Thespesia populnea*, Antimicrobial activity, Anticancerous activity, Cytotoxicity

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INTRODUCTION

India has a rich flora that is widely distributed throughout the country. Herbal medicines have been the basis of treatment and cure for various diseases and physiological conditions in traditional methods practiced such as Ayurveda, Unani and Siddha. Several plant species are used by many ethnic groups for the treatment of various ailments [1,2,3]. The past three decades have seen a dramatic increase in microbial resistance to antimicrobial agents that lead to repeated use of antibiotics and insufficient control of the disease. New prototype antimicrobial agents are needed to address this situation. Recently considerable attention has been paid to utilize eco-friendly plant based products for prevention and cure of different human diseases since they are safe and effective [4,5].

Thespesia populnea is a ever green tree belonging to the family Malvaceae and it is commonly known as Indian tulip tree. All parts of the tree are used in Indigenous system of medicine. The leaves, flower, bark and fruits are useful in cutaneous infection such as eczema, scabies and psoriasis. The fruit is used to control diabetes in the Ayurveda system of medicine. The fruits, bark and root were used for the treatment of cholera, dysentery and hemorrhoids [6]. The oil obtained from leaves and bark used for the treatment of fracture wounds and as an anti-inflammatory poultice applied to ulcers and boils [7]. Gossypol the major compound from T. populnea found to be anti-fertility effect in rats[8]. It has been reported to possess a number of medicinal properties such as hepatoprotective, antinociceptive and antidiarrheal. The phytochemical study of bark shows the presence of tannin, acacetin, quercetin and gossypol. Dichloromethane extract of bark of *T.* populnea produced antitumor, antioxidant and cytotoxic activity [9].

Therefore the present study has been carried out to explore the antimicrobial and cytotoxic potential of the stem bark of *T. populnea* so as to render a basis for the discovery of a promising drug of the future.

MATERIALS AND METHODS

Collection and processing of plant part

The stem bark of *T. populnea* (Linn.) was collected from Mysore and was authenticated by Dr.M.S. Sudarshana, Department of studies in Botany, University of Mysore, Mysore (Specimen Number BOT-0004)

Extraction and isolation of the fraction

The collected stem bark of *T. populnea* was shade dried, powdered and sieved in mesh 40. The powdered material was extracted with 5 L of 70% acetone at 60^{o} C for 2 hrs. Extraction was repeated twice with 5 L of acetone. The acetone extracts were combined and evaporated under reduced pressure.

The concentrated acetone extract was portioned between n-hexane and methanol. The methanol part was evaporated under reduced pressure to obtain a semi solid extract. This extract was poured into cold diethyl ether to precipitate the crude mixture. This was repeated several times and the precipitate was collected by filtration. The filtered crude mixture (400g) was subjected to column chromatography (Silica gel, 60-120 mesh) and eluted with n-hexane, n-hexane : ethyl acetate mixture, ethyl acetate : methanol mixture, methanol.

All the eluted fractions were monitored by thin layer chromatography using pre coated aluminium plates. Three fractions were obtained. The major fraction (350 g) was taken for further study and other two fractions were not considered as they were negligible amount. The major fraction was subjected for repeated HPLC on a reverse phase C-18 semi preparative column using Acetonitrile:water (7:3) as mobile phase with flow rate of 10 ml/min. The eluted peak of fraction was collected and concentrated to dry mass. The isolated compound was recrystallized to get pure compound (1.5 g). The working concentrations of fraction were prepared in 1% DMSO (Sisco Research Laboratories, India).

Test organisms and inoculums preparation

In order to study the antimicrobial activity of fraction from the acetone extract of stem bark of *T. populnea*, standard strains were obtained from Microbial Type Culture Collection Center from Institute of

Microbial Technology, Chandigarh, India. For demonstrating antibacterial activity four bacterial strains namely *Staphylococcus aureus* (MTCC 96), *Micrococcus luteus* (MTCC 1541), *Klebsiella pneumonia* (MTCC 109) and *Escherichia coli* (MTCC 118) were used. Parallel study on antifungal activity included the standard stains of Candida such as *Candida albicans* (MTCC 183), *C. parapsilosis* (MTCC 2509) and *C. tropicalis* (MTCC 184).

The stock cultures of standard bacterial and fungal strains were maintained at 4^{o}C on nutrient agar and sabourauds dextrose agar (HiMedia, India) respectively. A working culture of test organism was prepared by inoculating a loop full of the clinical isolate into a 3 ml sterile nutrient broth (bacteria) and sabourauds dextrose broth (fungi) tube and incubated at 37^{o}C for 24 h. The turbidity was matched with 0.5 Mc Facland's Nephelometer Standard [10].

Antimicrobial susceptibility test

For the purpose of demonstrating antimicrobial activity of fraction from the acetone extract of stem bark of *T. populnea*, the Cup plate technique [11]. Briefly, suspension (0.1 ml) of the test organisms (bacteria and fungi) from the 18 h cultures was thoroughly mixed with 20 ml of sterile Mueller Hinton agar (HiMedia, India) maintained at 45-50 ^{o}C. The seeded M.H. agar was poured in pre-sterilized petriplates and set aside. After solidification, the seed agar was punched with a flamed (sterile) cork borer in order to obtain 5 wells. Care was taken to make each well to possess a size of 10 mm dia and to maintain a well-well distance of 35 mm.

For the screening study, the central well loaded with 100 µl acetone served as control. The surrounding wells were loaded with the 100 µl each of varying concentrations (250, 500, 750 and 1000 µg/ml) of fraction from the acetone extract of stem bark of *T. populnea*. The petriplates were delicately handled and kept in refrigerator for 30 min and then at room temperature for 30 min which facilitated diffusion of the fraction. The petriplates were then incubated at 37^{o}C for 24 h [12]. The zones of growth inhibition were measured in mm with HiAntibiotic zone scale (PW 096), HiMedia, India.

Anticancer and Cytoxicity tests

The anticancer activity of fraction from the acetone extract of stem bark of *T. populnea* was tested against human cancerous cell line by a modified method [13]. The HEp2 cell line was obtained from National Centre for Cell Sciences (NCCS), Pune, India. The cells were treated with tripsin (Sisco Research Laboratories, India) and maintained in Minimal Essential Media (HiMedia, India) supplemented with 10% phosphate-buffered saline (PBS) (Cistron Laboratories), sodium bicarbonate (3.7 g/L), glucose (4.5 g/L), penicillin (100 U/ml) and streptomycin (100 µg/ml) (HiMedia, India) in a humidified atmosphere of 50 µg/ml CO_2 at 37^{o}C.

Different aliquots of cell line were seeded with 100 µl of varying concentrations (0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10 mg/ml of 1% DMSO) of fraction. The control cell line was seed with 1% DMSO. The cell cultures were incubated for 24 – 48 h and observed under inverted tissue culture microscope (Olymbus) for observing cytopathic effect.

The *in vitro* cytoxicity of the fraction from the acetone extract of stem bark of *T. populnea* on Human embryonic lung (HEL) cell line was determined by MTT assay [9]. Cells (1 x 10^{5}/well) were plated in 1 ml of medium / well in 24 well plates (Costar Corning, Rochester, NY). After 48 h of incubation the confluent cells growth was obtained. Then, the cells were incubated in the presence of various concentrations of the samples prepared in 0.1% DMSO for 48h at 37°C. After the incubation a part of the sample was removed and washed with PBS (pH 7.4). Then the cells were flooded with 200µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) prepared in PBS was added. After 4 h of incubation, 0.04M HCl/ isopropanol was added to the set up. The absorbance of cell suspension at 570 nm was measured with a UV-Spectrophotometer maintain a well without cells as blank. Viable cells were determined by the absorbance at 570 nm. The concentration required for a 50% inhibition of cell viability (IC_{50}) was determined graphically. The effect of the samples on the proliferation of HEpG2 cells was expressed as the % cell viability, using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100%.

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Cytoxicity was expressed as minimal cytotoxic concentration required to cause microscopically detectable alteration of normal cell morphology of the confluent cell cultures that were exposed to the fraction.

RESULTS

Antimicrobial activity

The results of antibacterial activity of fraction from the acetone extract of stem bark of *T. populnea* indicated that the fraction could inhibit the growth of three of the four bacterial strains tested (Table 1). Although the fraction at the concentration of 250 µg/ml did not inhibit the growth of these bacteria, a minimum concentration of 500 µg/ml was inhibitory to *S. aureus, M. luteus* and *E. coli.* Comparatively better antibacterial activity of the fraction was observed against *S. aureus* (12 mm) and *M. luteus* (10 mm) than *E. coli* (9 mm). However, the fraction was observed to be ineffective against *K. pneumoniae*.

Antifungal activity

The screening of fraction from the acetone extract of stem bark of *T. populnea* for antifungal activity revealed its inhibitory activity against all the three species of Candida namely *Candida albicans, C. parapsilosis* and *C. tropicalis* (Table 2). Compared to the antibacterial activity, the fraction showed only a moderate inhibitory activity against fungi as it could prevent the growth at the concentration of ≥750 µg/ml.

Anticancer and Cytotoxic activity

The results of anticancer activity of fraction from the acetone extract of stem bark of *T. populnea* are as given in Table 3. Although maximum anticancer activity (cell growth inhibition) of the fraction was obtained with 5 mg/ml (14.6% of cell viability), the fraction could continue to exhibit its activity even at the lowest concentration of 0.156 mg/ml (77% of cell viability). The neat undiluted solution of the test fraction exhibited higher anticancer activity, which made only 6.25% of the cells to survive. However, complete anticancer activity was not obtained.

The MTT assay to determine the cytotoxicity indicated that a concentration of 5 mg/ml was required to cause 50% (IC_{50}) of inhibition of HEL cell line. While the minimum concentration of fraction required to cause cytotoxicity was 1 mg/ml, proportionate cytotoxic effects were obtained with increasing concentrations of the test extract (Fig. 1)

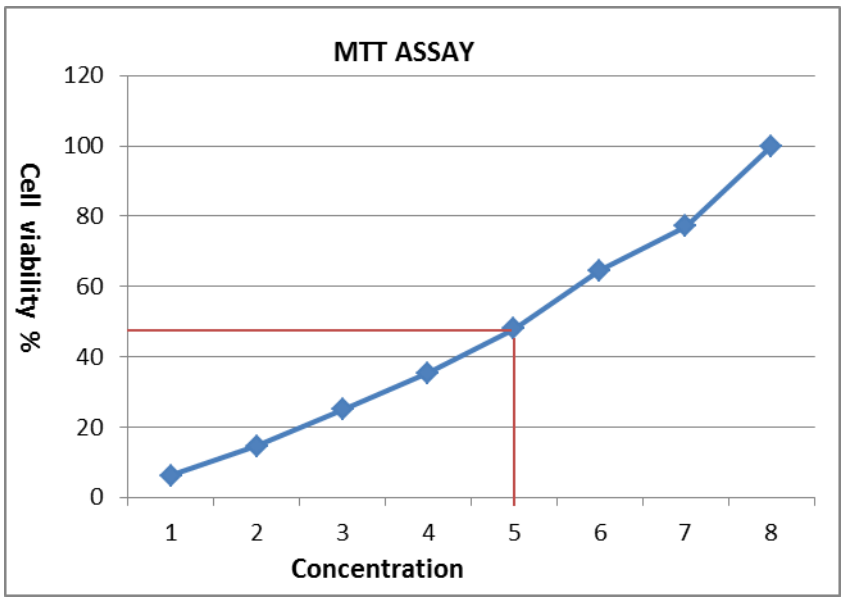

Fig. 1. Cytotoxic Effect of fraction from the acetone extract of stem bark of *Thespesia populnea* on HEp2- Cell line

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Table 1: Antibacterial activity of fraction from the acetone extract of stem bark of *Thespesia populnea*

Organism	Concentration of Fraction (µg/ml) and size of Zone of Growth inhibition (mm)			
	250	500	750	1000
S. aureus MTCC 96	-	12	12	14
M. luteus 1541	-	10	13	14
K. pneumoniae MTCC 109	-	-	-	-
E. coli MTCC 118	-	9	13	13

Table 2: Antifungal activity of fraction from the acetone extract of stem bark of *Thespesia populnea*

Organism	Concentration of fraction (µg/ml) and size of zone of growth inhibition (mm)			
	250	500	750	1000
C. albicans MTCC 183	-	-	12	14
C. parapsilosis MTCC 2509	-	-	13	15
C. tropicalis MTCC 184	-	-	12	12

Table 3: Anti Cancer Effect of fraction from the acetone extract of stem bark of *Thespesia populnea* on HEp2- Cell line

S.No	Concentration (mg/ml)	Dilution	Absorbance (OD)	Cell viability (%)
1	10	Neat	0.03	6.25
2	5	1:1	0.07	14.58
3	2.5	1:2	0.12	25.0
4	1.25	1:4	0.17	35.41
5	0.625	1:8	0.23	47.91
6	0.312	1:16	0.31	64.58
7	0.156	1:32	0.37	77.08
8	Control	-	0.48	100

DISCUSSION

Emerging antibiotic resistant infections are one of the most serious problems faced by the medicinal professionals. The misuse and overuse of antimicrobial agents causes spread and selection of resistant strains during and after treatment. Control of improper distribution and prescription of antibiotics is required foremost in developing countries where other health issues overshadow the threat of antibiotic resistance. Comprehensive surveillance of resistance is also lacking in a global perspective and the overall picture is incomplete even though new reports are being published. The occurrence of ESBL producting and muti-drug resistant organisms among bacteria had been reported [14]. In order to circumvent this alarming situation, there is a necessity to find an alternative drug, preferable from a natural source. The herbal medicines have been a part of traditional medicines followed in India with higher efficiency and least or no side effects. In recent years there is a growing interest in discovering drugs of herbal origin with higher potential for treating infectious diseases.

The objective of antimicrobial activity was to analyze the antimicrobial, anticancerous and cytotoxic properties of the plant *T. populnea* and to suggest this medicinal plant as a potential source for promising future drug.

The test organisms used in this study are implicateed with various forms of human infections. From a clinical point of view, *Escherichia coli* is the most important member of the Escherichia genus of enterobacteriaceae capable of causing septicemias and can infect the gall bladder, meninges, surgical wounds, skin lesions and the lungs especially in debilitate and immunodeficient patients[15]. Infection caused by *Staphylococcuis aureus* is a serious public health problem in developing countries as it is involved in causing pyogenic and respiratory tract infections. In recent years the emergence of resistant varieties of these bacteria, MRSA and VRSA, has become a matter of global concern [16]. The bacteria *Micrococcus luteus*,

although not a common pathogen, its ability of causing opportunisitic infections in immunocompromised individuals has raised greater attention in the field of control of infectious diseases. The yeast Candida has occupied as a significant member among the list of fungal pathogens that cause serious and life threatening infections of humans and animals.

As the methonolic extract of *T. populnea* had been proved to be more potent [14,17] than the other solvent counterparts, the same has been employed in this study. The antibacterial activity of the fraction tested in the present study has showed a significant inhibitory activity on gram positive bacteria such as *S. aureus* and *M.luteus* than the gram positive bacteria. There was only 50% of activity against gram negative bacteria tested. While there was a moderate antibacterial activity against *E. coli*, the fraction was observed to be inefficacious against *K. pneumoniae*. However, the overall antibacterial activity of the fraction requires a minimum concentration of $\geq$500 µg/ml. It had been demonstrated the antibacterial activity of methanolic leaf extract of *T. populnea* (L.) against *S. aureus*, *E. coli* and *K. pneumonia* at a concentration of 500 µg/ml[17]. The lacking of antibacterial activity of fraction from the acetone extract of stem bark of *T. populnea* employed in the present study could be due to the absence of important antibacterial phytoconstituent. It had been reported substantial antibacterial activity of ethanolic and aqueous extracts of stem bark of *T. populnea* (Linn.) against *S. aureus*, *Streptococcus pyogenes*, *E. coli* and *Pseudomonas aeruginosa*[18]. These findings suggest the need for comprehensive analysis of antibacterial activity of stem bark using different solvent extracts.

The inhibition of growth of Candida species tested in this study indicates the antifungal activity of fraction from the acetone extract of stem bark of *T. populnea* against all the strains tested in the study. A concentration of $\geq$750 µg/ml of the fraction has been proved to be effective in controlling the candidal growth.It had been reported significant antifungal activity of methanolic extract of leaf of *T. populnea* against *Aspergillus niger* and lacking of activity against *C.albicans* and *Saccharaomyces cereviceae*[19]. This infers that the antifungal activity of the fraction of this plant is better exhibited for filamentous fungi than yeasts. The reports on the antifungal activity of *T. populnea* are scanty. However the demonstration of anticandidal activity of fraction from the acetone extract of stem bark of *T. populnea* could be considered as new information to the literature.

The demonstration of activity against both Gram negative and Gram positive bacteria and against Candida is an indication that the fraction from the acetone extract of stem bark of *T. populnea* can be a source of bioactive substances which could be attributed to its broad spectrum of activity.

The anticancerous activity of fraction from the acetone extract of stem bark of *T. populnea* against HEp2 demonstrated in the present study could be considered as a first of its kind attempt. The anticancerous potential of the fraction to the extent of 94% (viability of 6.25%) clearly advocates its potential of being a promising drug (Table 3). The cytotoxicity activity of the fraction against the normal human cell line, HEL, demonstrated in the present study reveals its low cytotoxic efficiency. The IC_{50} obtained at a concentration of 5 mg/ml clearly indicates that only at higher concentrations of fraction from the acetone extract of stem bark of *T. populnea* would be toxic to normal cells.

CONCLUSIONS

Thus the findings of the present study vividly present the potential of fraction from the acetone extract of stem bark of *T. populnea* to be a promising antimicrobial and anticancerous with low cytotoxicity on normal cells. Further investigations are required to explore the action of individual phytoconstituents of this plant so as to obtain a comprehensive report in this line of study.

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