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Investigation on antimicrobial activity of root extracts of *Thespesia populnea* Linn

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Abstract. Many medicinal plants have been used for centuries in daily life to treat microbial diseases all over the world. In this study, the *in vitro* antibacterial activity of aqueous and ethanol root extracts of *Thespesia populnea* Linn were investigated. Antimicrobial properties of *T. populnea* Linn was evaluated against five pathogenic bacteria and two fungi. Disc diffusion method and minimum inhibitory concentration (MIC) were determined by broth serial dilution method. The ciprofloxacin (5 µg/ml) and flucanozole (100 units/disc) were used as positive controls for bacteria and fungi respectively. Different concentrations (50, 100, 150 µg/ml) of ethanolic and aqueous root extracts of *T. populnea* were checked for the dose dependent antibacterial activity. *Thespesia populnea* showed broad spectrum antimicrobial activity against gram positive and gram negative bacteria and maximum inhibition by ethanolic extract was observed at higher dose (250 µg/ml) as 27±0.2mm. The MIC of the ethanol extract was 10 µg/ml for *Staphylococcus aureus* and 750 µg/ml for *Candida albicans*. The antifungal activity offered against *S. aureus* by the ethanolic extract is more than the aqueous extract. The results concluded that the anti-microbial activity of *T. populnea* was dose dependent. As the concentration increased the inhibition zone also increased. Flavonoids and tannins present in the extracts may be responsible for the antimicrobial activity.

INTRODUCTION

Antimicrobial resistance is one of the most significant future threats to public health due to the exploitation of synthetic antibiotic drugs and is threatening to undo decades of advances in our ability to treat disease through the natural products. Because of the resistance and side effects that pathogenic micro-organisms build against the antibiotics, it causes major therapy failure. A feasible way to combat the problem of microbial resistance is the development of new natural antibacterial agents for replacement with ineffective ones. The *in vitro* and *in vivo* screening of extracts or isolated compounds from different natural sources is a common way to discover biological active metabolites. According to WHO (2000), the primary health care for 80% of the world's inhabitants depend on traditional medicines. Pharmaceutical industries and clinicians are interested to pay attention on plant extracts and biologically active compounds isolated from plant species used in herbal medicine.

Thespesia populnea Linn (Malvaceae family) is a small tree and found in coastal regions of India, tropical Asia, Africa and West Indies. It probably originated from the Asiatic tropics and now occurs throughout the tropics. *Thespesia populnea* is a 20 m tall medium-sized evergreen tree with long petiolate leaves. The major phytoconstituents

reported in this plant include anthraquinones glycosides, cardiac glycosides, flavonoids, alkaloids, tannins, mansonone-D, mansonone-H, gossylpol and thespone (Akhila & Rani,1993; Johnson *et al.*, 1999). Various parts of *T. populnea* has been scientifically reported to possess hepatoprotective activity (Shirwaikar & Sreenivasan, 1996), antiviral (Vohora & Mishra, 1998), wound healing activity (Nagappa & Cheriyan, 2001) Alzheimer's disease (Vasudevan & Parle, 2006) and anti-steroidogenic acivity (Kavimani *et al.*, 1999). However, there is no scientific report justifying the traditional use of T. populnea root as antimicrobial activity. The objective of the present study was to perform phytochemical analysis, antibacterial and antifungal properties of *T. populnea* root extracts.

MATERIALS AND METHODS

Collection of Plant Material

The roots of *T. populnea* were collected from the botanical garden at the Campus of Padmavathi College of Pharmacy, Dharmapuri. The plant was identified and authenticated by Dr. P. Jayaraman, Botanist, Plant Anatomy Research Centre (PARC), Chennai, India. The roots were collected and dried in the shade. The dried roots were subjected to pulverization to get coarse powder. The coarsely powdered roots of *T. populnea* were used for extraction.

Preparation of aqueous extract

Hundred grams (100 g) of the powdered roots were mixed with 500 mL of distilled water in a 1 L flask and boiled for 1.5 hours. The homogenate was allowed to cool for about 6 hr before it was rapidly filtered through a piece of clean white cloth The filtrate was concentrated in a rotary evaporator under vacuum (40°C) and the concentrated extract was stored at 4°C until use. The percentage yield of extract was found to be 7.36%. From this, a fresh stock was reconstituted in distilled water at a concentration of 100 mg/mL, whenever needed.

Preparation of ethanolic extract

Crude ethanolic extract of powered roots of *T. populnea* was prepared according to the standard method (Gilani *et al.*, 2004). Briefly, 200gm of ground root material was soaked in sufficient quantity of 90% ethanol by cold maceration at room temperature for 72 h after which the filtrate was collected through a piece of muslin cloth and then the filter paper and the plant material was resoaked twice. The filtrate was concentrated in a rotary evaporator at 40°C under reduced pressure to yield crude extract and was stored at 4°C until use. The percentage yield of extract was found to be 5.64%.

Test microorganisms

The test microorganisms used in this investigation (bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Klebsiella aerogenes*, fungi: *Candida albicans*, *Aspergillus niger*) were obtained from the P.G. Department of Biotechnology, North Orissa University, Baripada, Orissa. All the strains were confirmed by cultural and biochemical characteristics and maintained in slants for further use.

Phytochemical analysis of the plant extract

Phytochemical studies of aqueous and ethanol root extracts of *T. populnea* were performed using standard procedures (Harborne, 1998).

Antimicrobial Activity

The antimicrobial activities of the ethanol extracts of plants were analysed by the disc diffusion method (Collins *et al.*, 1995; Martinez Vazquez *et al.*, 1999). The crude ethanol extract was dissolved in dimethyl sulfoxide (DMSO) and anti-microbial effect was measured in difference concentration.

The microorganism viz., *S. aureus*, *E. coli*, *K. aerogenes*, *C. albicans* and *A. niger* were activated by inoculating a loop full of the strain in the Nutrient broth medium (25 ml) and incubated at 37°C on a rotary shaker overnight. Next day, the old inoculated culture was mixed with Muller-Hinton agar medium

and when the temperature reached to 45ºC was poured into the sterilized plates. All plates were placed at room temperature under laminar flow to solidify.

Approximately, 100 µl of the test bacteria/ fungi were grown in 10 ml of fresh media until they reached a count of approximately 10^8 cells/ml for bacteria, 100 µl of microbial suspension was spread onto the nutrient agar plates.

The extracts were tested using 5 mm sterilized filter paper discs. Discs were impregnated with 25 µl (10mg/mL concentration) of the test extract samples, allowed to dry and placed onto inoculated plates for 30 min incubation. The plates were allowed to stand at 4ºC for 2 hours before incubation with the test microbial agents.

Plates inoculated with *S. aureus*, *E. coli*, *K. aerogenes*, *C. albicans* and *A. niger* were incubated at 37ºC for 24 hours, than the diameters of the inhibition zones were measured in millimetres.

Standard antibiotics, ciprofloxacin (Ranbaxy Pharmaceuticals India, 5 µg/ disc) and fluconazole (Greenlife; pharmaceuticals India, 100 units/disc) served as positive controls for antimicrobial activity. Filter discs impregnated with 10 µl of distilled water were used as a negative control. Solvent control disc (DMSO) was also placed with the test, positive control.

Dose dependent antibacterial activity

Ethanolic and aqueous root extracts of *T. populnea* was investigated for its dose dependent antibacterial activity. Different concentration of extract (50, 100, 150 µg/ml) was impregnated on to the disc and the same procedure was followed as mentioned before. Each assay was performed in triplicate and mean of all the three experiments were taken.

Minimum inhibitory concentration (MIC)

Both serial dilution method was used to determine MIC for each of the *T. populnea* extracts that were tested in Muller non broth for bacteria and Sabouraud's dextrose broth for fungus (Baron & Finegold, 1990). *Thespesia populnea* extracts were dissolved in 5% DMSO to obtain 128 mg/ml stock solutions. *Thespesia populnea* extracts was prepared at different concentrations (0-250 µg/ml and 0-2000 µg/ml for bacterial and fungal strains respectively). Fifty µl of standardized suspension of the test organism was transferred on to test tube. The control tube contained only organism and devoid of the extracts. By using antibiotics ciprofloxacin and fluconazole were used for bacteria and fungi respectively were repeated on the test organisms. The culture tubes incubated at 37ºC for 24 hours. Turbidity was taken as an indication of growth, and the lowest concentrations which did not show any growth of tested organism after careful observation of turbidity was determined as minimum inhibitory concentration.

Statistical Analysis

All antimicrobial assays were performed in triplicates and the mean values of the diameter of clear zones with ± standard deviation were estimated.

RESULTS

The preliminary phytochemical study of *T. populnea* showed the presence of carbohydrates, glycosides, steroids, tannins, polyphenols in aqueous extract and alkaloids, carbohydrates, glycosides, saponins, steroids, triterpenoids, tannins, polyphenols, flavonoids, anthocyanins and proteins in the ethanol extract of *T. populnea* root.

The antimicrobial activity of aqueous and ethanolic root extracts of *T. populnea* against human pathogenic bacteria, *S. aureus*, *E. coli* and *K. aerogenes*, *C. albicans*, *A. niger* were measured. Antibacterial activity at different doses was done by disc diffusion method. Concentration was in the range of 50 to 250 µg/disc for antibacterial activity and 50 to 2000 µg/disc for antifungal activity. The pattern of inhibition varied with the type of solvent used for extraction and the microorganism tested for susceptibility assay. Antimicrobial activity was dependent on the dose of the test material. As the concentration increased the inhibition zone also increased.

Test sample per disc was about 25 µl (10mg/mL concentration). The organisms used and zone of inhibition to the corresponding extracts are shown in Table 1. The zones inhibition ranged from 7±0.4 to 22±0.1 mm and 11±0.3 to 27±0.2 mm for aqueous and ethanolic root extracts respectively.

Against ethanol root extract of *T. populnea*, *S. aureus* and *E. coli* showed a highest inhibition zone of 27±0.2 and 24±0.5 mm respectively whereas *P. aeruginosa* showed a lesser inhibition zone of about 22±0.2 mm (Table 1, Figure 1).

The ethanolic extract of *T. populnea* produced a maximum zone of inhibition 22±0.4 mm radius at 250 µg/ml against *C. albicans* followed by aqueous extract of 17±0.1 mm radius at 250 µg/ml. Whereas the antifungal activity against *A. niger* at the concentration of 250 µg/ml is almost equal for the both extracts (15 mm radius). However the antifungal activity of aqueous extract at the concentration of 50 µgm/ml and 150 µgm/ml is lesser when compared to effect exhibited by ethanolic extract of *T. populnea* in both the fungal organisms (Table 2, Figure 2). The capability of the extracts to inhibit the growth of both bacterial and fungal organisms is an indication of the broad spectrum anti-microbial potential of *T. populnea* extract, which makes the plant a potential candidate to replace synthetic drugs for controlling microbial infections.

Staphylococci are among the most commonly encountered pathogens in clinical practice (Rubin, 1999). *Staphylococci aureus* is a major cause of *nosocomial* infections, food poisoning, osteomyelitis, pyoarthritis, endocarditis, toxic shock syndrome, and a broad spectrum of other disorders (Todd, 1998; Hajjeh *et al*., 1999).

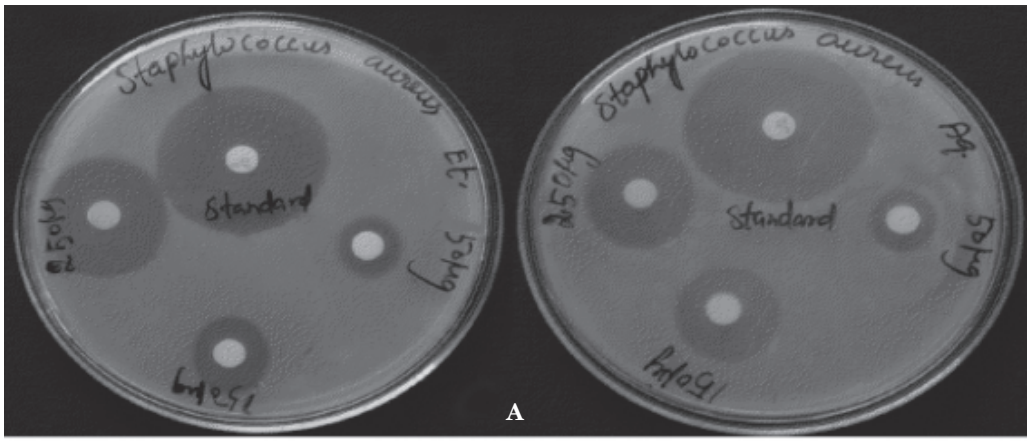
The MICs of the extracts observed against the sensitive strains ranged from 10 to 250 mg/mL (for bacterial strains) and 750 to 2000 mg/mL (for fungal strains). In case of bacterial strains, ethanol extract showed potent activity against the *S. aureus* and *E. coli* having MICs 10 and 25 mg/mL, respectively (Table 3).

Similarly the ethanol extracts of *T. populnea* showed the lowest MICs against *C. albicans* (750 mg/mL) compared to aqueous (1250 mg/mL). As for the ethanolic extracts, MIC values was 1750 mg/mL against A. *niger* compared to aqueous extract of 2000 mg/mL.

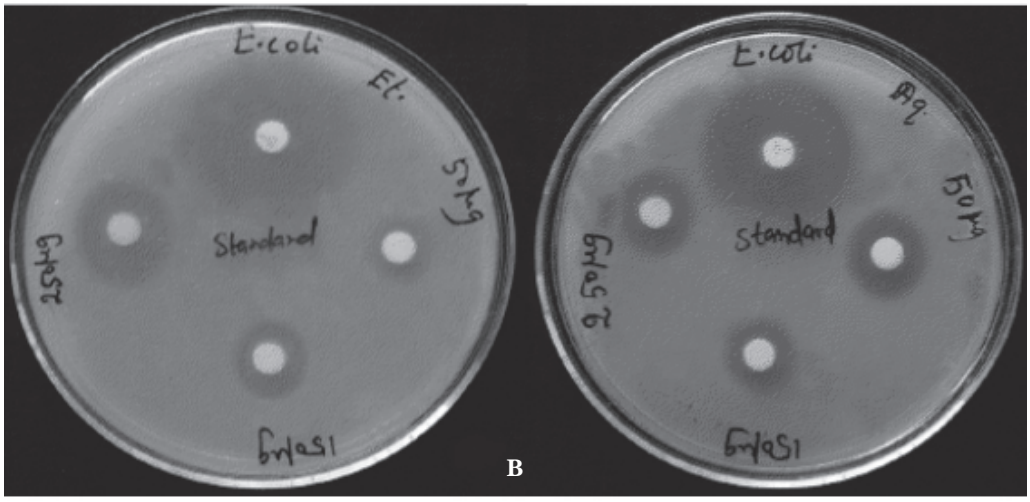
Table 1. Antibacterial activities of aqueous and ethanol extracts of *Thespesia populnea*

Name of the Micro organisms	Diameter of zone of inhibition in mm at different concentration levels						Standard Ciprofloxacin 5 µg /disc
	Aqueous extract			Ethanol extract			
	50 µg/ml	150 µg/ml	250 µg/ml	50 µg/ml	150 µg/ml	250 µg/ml	
Staphylococcus aureus (NCIM 2079)	11±0.4	14±0.2	19±0.3	14±0.1	18±0.2	27±0.2	35±0.1
Escherichia coli (NCIM 2065)	12±0.1	15±0.1	22±0.1	16±0.2	19±0.3	24±0.5	32±0.2
Pseudomonas aeruginosa (NCIM 2036)	10±0.3	13±0.4	17±0.5	14±0.2	18±0.1	22±0.2	35±0.4
Bacillus subtilis (NCIM 2063)	9±0.1	15±0.3	18±0.1	13±0.4	20±0.2	26±0.6	28±0.1
Klebsiella aerogenes (NCIM 2098)	7±0.4	12±0.5	15±0.2	11±0.3	16±0.4	21±0.5	32±0.3

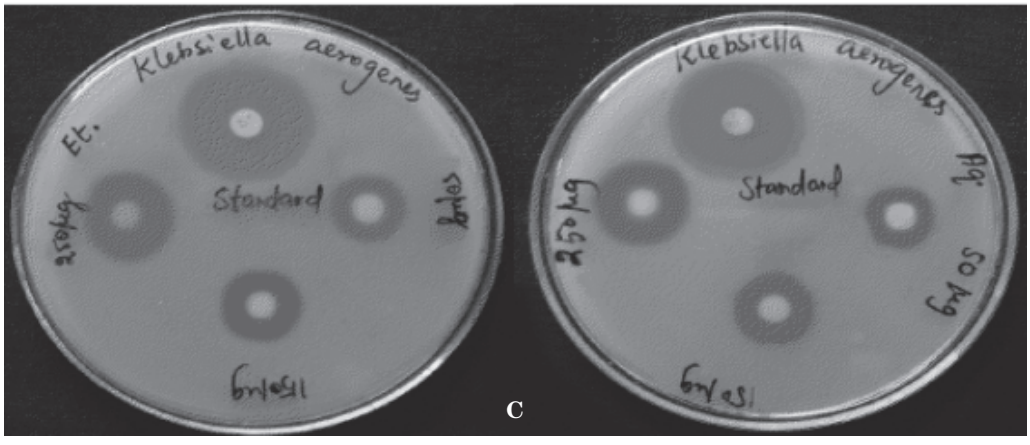
Data are means (n=3) ± standard deviation of three replicates



Staphylococcus aureus



Escherichia coli



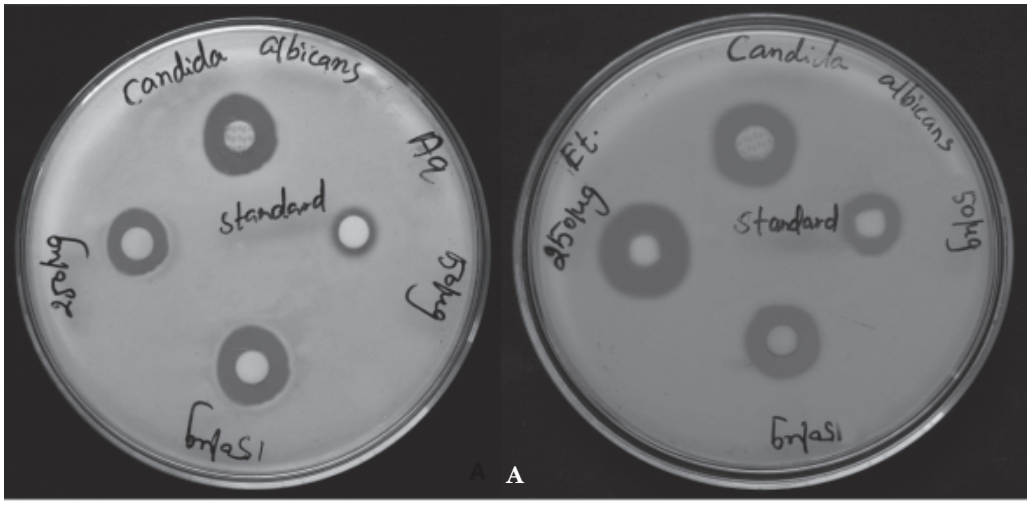
Klebsiella aerogenes

Figure 1. Zone of inhibition of bacterial isolates (A) *Staphylococcus aureus*; (B) *Escherichia coli*; (C) *Klebsiella aerogenes*; (D) *Bacillus subtilis*; (E) *Pseudomonas aeruginosa*

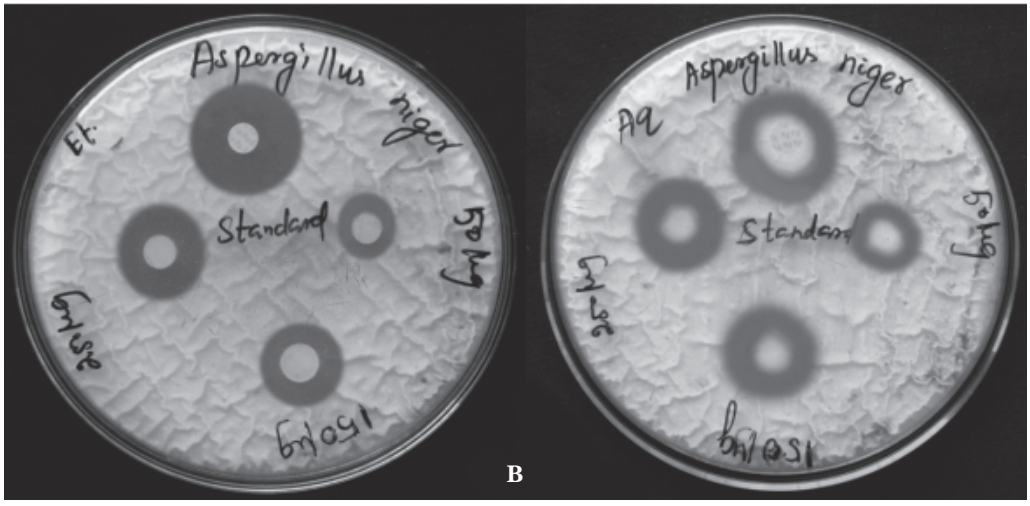
Table 2. Antifungal activities of aqueous and ethanol extracts of *Thespesia populnea*

Name of the micro organisms	Diameter of zone of inhibition in mm at different concentration levels						Standard Fluconazole 100 units/disc
	Aqueous extract			Ethanolic extract			
	50 µg/ml	150 µg/ml	250 µg/ml	50 µg/ml	150 µg/ml	250 µg/ml	
Candida albicans (NCIM 3102)	8±0.3	12±0.2	17±0.1	16±0.2	21±0.3	22±0.4	30±0.1
Aspergillus niger (NCIM 105)	13±0.2	14±0.1	15±0.3	11±0.3	12±0.2	15±0.1	32±0.2

Data are means (n=3) ± standard deviation of three replicates



Candida albicans



Aspergillus niger

Figure 2. Zone of inhibition of fungal isolates (A) *Candida albicans*; (B) *Aspergillus niger*

Table 3. Minimum inhibition concentration of aqueous and ethanol extracts of *Thespesia populnea*

Micro organisms	MIC (µg/ml)		
	Aqueous extract	Ethanol extract	Standard drug
Staphylococcus aureus	200	10	0.035
Escherichia coli	75	25	0.058
Pseudomonas aeruginosa	250	150	0.078
Bacillus subtilis	100	75	0.043
Klebsiella aerogenes	500	250	0.124
Candida albicans	1250	750	0.028
Aspergillus niger	2000	1750	0.032

Standard drug used: Ciprofloxacin for gram positive and negative bacteria and Fluconazole for fungi.
MIC: Minimum inhibition concentration

DISCUSSION

This strong antibacterial effect could be due to flavonoids, which have been shown to be active against *S. aureus* (Xu & Lee, 2001; Sato *et al.*, 2004). On the other hand, the extract had lesser activity against *E. coli*, Gram-negative bacteria. This could be related to the outer membrane acting as a permeability barrier in these bacteria (Denyer & Russell, 2004).

During the past decade, *S. aureus* has developed resistance to many commonly used antibiotics (Alanis, 2005). In this study, both extracts of *T. populnea* showed activity against *S. aureus* and can be used as raw materials for phytotherapy.

According to the literature there is no scientific data available on the microbial activity on the root extract of *T. populnea*. The findings of this research work have shown clearly that the ethanolic extract of *T. populnea* has potential anti-microbial activity compared to the aqueous extract.

Root extract can be classified as antimicrobial agents based on MIC values. Extract with MIC values less than 100mg/ml are classed as strong inhibitors, at 100-500 mg/ml as moderate inhibitor, at 500-1000 mg/ml as weak inhibitor and at more than 1000 100mg/ml as inactive inhibitor (Zubia *et al.*, 2008). According to this literature, ethanolic extract of *T. populnea* has proven to be strong inhibitor with low MIC values and higher values of zone of inhibition in antibacterial activity. On the other hand, aqueous extract has shown to be strong inhibitor with low MIC values and higher values of zone of inhibition against antifungal activity.

Aqueous and ethanol root extracts of *T. populnea* contains various compounds including terpenoids, tannins and polyphenolic compounds, as well as flavonoids. The latter being more likely to possess antimicrobial activity.

Flavonoids' activity is probably due to their ability to form complex with extracellular and soluble proteins, as well as bacterial cell wall. Lipophilic flavonoids may also disrupt bacterial membranes (Tsuchiya *et al.*, 1996).

The present investigation data support the traditional medicinal use of this plant and its future aspects in developing novel antimicrobials. Furthermore, active plant extracts can be subjected to various chemical evaluations by several methods such as GC-MS, nuclear magnetic resonance, mass spectrometry, etc. for the isolation of the therapeutic antimicrobials.

Further phytochemical evaluations are essential to determine the purified bioactive constituents responsible for the antibacterial activities of *T. populnea*, which could serve as useful sources for establishment of new antimicrobial agents.

According to this result it may suggest that the ethanolic extracts of these plants possess compounds with antimicrobial properties which can be used as new drugs for therapy of infectious diseases in human.

Thespesia populnea can potentially be used in the treatment of various infectious diseases caused by microorganisms that are showing resistance to currently available antibiotics.

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