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Original Article

A COMPARATIVE STUDY ON THE IN-VITRO ANTIMICROBIAL ACTIVITY OF THE ROOTS OF FOUR THOTTEA SPECIES

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ABSTRACT

Objective: The main objective of the present study was to investigate the antimicrobial activity of the methanol extract of the roots of four *Thottea* species.

Methods: The root extracts of four *Thottea* species were subjected to antimicrobial assay by Minimum Inhibitory Concentration (MIC) and Agar Disc diffusion Assay against various medically important pathogens.

Results: It is evident from the study that. Significant antibacterial activity was recorded by *Thottea sivarajanii* and highest activity was recorded against *Pseudomonas aeruginosa* and *Staphylococcus epidermis* (64 µg/ml). Out of the four extracts tested for antifungal activity, *Thottea barberi* and *Thottea ponmudiana* recorded significant antifungal activity and the highest activity was recorded by *T. barberi* against *Trichophyton rubrum* (16 µg/ml).

Conclusion: Results offer a scientific basis for the traditional use of *Thottea* species in the treatment of microbial infections, showing that the plant extract has an enormous potential as a prospective alternative drug against microbial pathogens. The present study lays the basis for future studies, to validate the possible use of *Thottea* species as a candidate in the treatment of microbial infections.

Keywords: Antimicrobial activity, *Thottea* species, MIC, Methanol extract.

INTRODUCTION

Infectious diseases are still a major threat to public health, despite the tremendous progress made in human medicine. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread drug resistance [1]. Contrary to synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to treat many infectious diseases [2]. Plants and plant products have been used extensively to treat various ailments. Numerous studies have been carried out to extract various natural products for screening antimicrobial activity [3]. It has been estimated that between 60-90% of the population in developing countries use traditional and herbal medicines exclusively and consider them to be a normal part of primary healthcare [4]. The Indian subcontinent is unique in its richness of plant wealth. Over 15000 species of higher plants occur in India, of which 9000 are economically useful. The therapeutic potentials of a large number of plants are yet to be explored [5]. Plant-based antimicrobials and anti bacterials represent a vast untapped source for medicines and hence have enormous therapeutic potential. Therefore, interest in higher plant extracts exhibiting antimicrobial activity has increased in recent years [6-8].

The genus *Thottea* belongs to the family Aristolochiaceae. This small genus of shrubby understory species is occurring in tropical forests of India and South East Asia [9]. The genus consists of about 35 species distributed in India, Sri Lanka, Bangladesh, China, Myanmar, Thailand and Malaysia [10-11]. Twelve species are reported from India [12] of which eight are distributed in the southern Western Ghats [13-14]. Various therapeutic properties were attributed to some species of *Thottea*. *T. siliquosa* is credited with alexiteric properties [15]. The roots of *T. duchartrei* are crushed and applied externally for abscess, inflammation and poisonous bites by the Kani and Malapandaram tribes of Kerala. The Malappandaram tribe used the root of *T. duchartrei* against malaria [16]. The root powder of *T. duchartrei* is given in hot water as an antidote to poison and externally applied on swellings [17]. Tribal communities in Pathanamthitta district, Kerala are using the fresh roots of *T. dinhoui* for the treatment of dysentery [18]. The pounded leaves of *T. dependens* are used for skin complaints in peninsular Malaysia. The decoction of the roots and rhizomes is used to treat cough, bronchitis and asthma. Rhizomes of *T. parviflora* and the crushed stem and leaves of *T. tomentosa* are also used to cure cough. The crushed leaf of *T. tomentosa* is used in peninsular Malaysia as antivenom against snake bite. In Java, *T. tomentosa* is used as an emmenagogue and for treating boils. The stem and root are diuretic and the juice is given for cough [19]. Rhizome of *T. grandiflora* is an excellent tonic. The decoction of the rhizome of *T. rhizantha* is used to treat gonorrhoea by natives of Sarawak. The decoction of *T. parviflora* and *T. tomentosa* are used as a diuretic and also for the treatment of prostatitis by the people of Thailand. *T. grandiflora* and *T. corymbosa* are also diuretic [20].

The species selected for the present study are *Thottea barberi* (Gamble) Ding Hou., *T. ponmudiana* Sivar., *T. siliquosa* (Lam.) Ding Hou. and *T. sivarajanii* Santhosh, Shanavas and Binu. Its roots are pungently aromatic. Among the candidate species, *T. siliquosa* is used in Ayurvedic System of Medicine and also by various tribal communities of Kerala, India for treating a number of ailments, while the rest of the species are under exploited. Paste prepared from the root of *T. siliquosa* is applied externally for headache and also against spider, scorpion and snake poison. Decoction of root is administered internally for chest pain and cough [21].

The Paniyar community is using the fruits of *T. siliquosa* to cure stomach ache [22]. The Malappandaram tribe is using the root juice and paste of *T. siliquosa* as an anti inflamatory drug, against fever, intestinal colic, diarrhoea and dysentery [23]. They also used the root juice mixed with coconut water for diarrhea [24]. The Mannan tribes as well as Uralis of Idukki district are using the root of *T. siliquosa* to reduce pain during pregnancy by applying a paste over the abdomen [25]. In Ayurveda the roots of ***T. siliquosa*** are used against cholera, diarrhoea and dysentery. Ointment made using the plant with oil is said to be beneficial for carbuncles, chronic ulcers, psora or invertebrate ulcers [26]. Roots are powdered and given

with warm water as an antidote to venomous bites. Leaves crushed and boiled with coconut oil is applied to cure itches [27]. *T. siliquosa* is one of the main components in a medicinal preparation developed for inducing apoptosis [28].

However, these species were so far not subjected to a comprehensive antimicrobial screening. The present study was aimed to investigate the comparative antimicrobial activity of methanol extracts of the roots of four *Thottea* species.

MATERIALS AND METHODS

Plant Material

The roots of the four *Thottea* species *viz. Thottea barberi, T. ponmudiana*, *T. siliquosa*, and *T. sivarajanii* were collected from its natural habitats from Kerala, India *viz.* Chemunji (Thiruvananthapuram), Ponmudi (Thiruvananthapuram), Palode (Thiruvananthapuram) and Thariode (Wayanad). The taxonomic identity of all the species was certified by the concerned taxonomist and also confirmed by matching with the authentic live and herbarium specimens at Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Thiruvananthapuram, Kerala, India.

Voucher Specimen

Anilkumar 40639, Chemunji, Thiruvananthapuram (TBGT); Anilkumar 40623, Ponmudi, Thiruvananthapuram (TBGT); Anilkumar 40606, Palode, Thiruvananthapuram (TBGT); Anilkumar 40628, Thariode, Wayanad, (TBGT).

Preparation of the extracts

The methanolic extracts of the dried root of the four *Thottea* species were filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extracts were stored at -20°C for further studies. The extracts were dissolved in 5% dimethyl sulphoxide (DMSO) for the experiments.

Antimicrobial activity

Microorganisms and media

The following bacteria were used as test organisms in this study: Gram positive bacteria: *Bacillus subtilis* MTCC 2756, *Staphylococcus aureus* MTCC 902, *S. epidermidis* MTCC 435 and *S. simulans* MTCC 3610; Gram negative bacteria: *Escherichia coli* MTCC 2622, *Klebsiella pneumoniae* MTCC 109, *Proteus mirabilis* MTCC 425, *Vibrio cholerae* MTCC 3905, *Pseudomonas aeruginosa* MTCC 2642 and *Salmonella typhi* MTCC 3216. Bacterial cultures were maintained on Müller Hinton agar substrates (Hi-media, Mumbai, India). Fungal pathogens used in the present study were *Aspergillus flavus* MTCC 183, *Candida tropicalis* MTCC 184, *Candida albicans* MTCC 277, *Trichophyton rubrum* MTCC 296 and *Fusarium oxysporum* MTCC 284. All cultures were stored at 4°C and subcultured every 15 days.

The sensitivity of microorganisms to methanol extracts of the investigated species was tested by determining the Minimal Inhibitory Concentration (MIC). Bacterial inoculi were obtained from bacterial cultures incubated for 24 hours at 37°C on Mueller-Hinton agar substrate and brought up by dilution according to the 0.5 McFarland standards to approximately 10^8 CFU/ml and then further diluted to approximately 10^6 CFU/ml according to the procedure recommended by NCCLS.

Minimal Inhibitory Concentration (MIC)

The Minimal Inhibitory Concentration (MIC) was determined by the broth microdilution method using 96-well micro-titer plates [29]. A series of dilutions with concentrations ranging from 1 to 2000 µg/ml for extracts was used in the experiment against every microorganism tested. The starting solutions of extracts were obtained by measuring of a certain quantity of extract and dissolving it in DMSO. Two-fold dilutions of extracts were prepared in Mueller-Hinton broth for bacterial cultures and SD broth for fungal cultures. The MIC was determined with resazurin. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The boundary dilution without any changing color of resazurin was defined as the MIC for the tested microorganism at the given concentration. As a positive control of growth inhibition, ciprofloxacin was used. A DMSO solution was used as a negative control for the influence of the solvents. All experiments were performed in triplicate.

Antimicrobial assay by agar diffusion

Agar disc diffusion technique was used to determine the antibacterial activity of oils using Mueller Hinton agar medium [30]. The test cultures maintained in nutrient agar slant at 4°C were sub-cultured in nutrient broth to obtain the working cultures approximately containing 1×10^6CFU/ml. The methanol extract (50 µg/ml) were incorporated in a 6 mm sterile disc. Mueller Hinton agar plates were swabbed with each bacterial strain and the test discs were placed. Ciprofloxacin discs (5 µg/disc) were used as positive control. Plates were incubated overnight at 37°C. Clear, distinct zone of inhibition was visualized surrounding the discs. The determinations were done in duplicates. After 24 hours of incubation, the plates were examined if there was any inhibition zone. The diameters of the inhibition zones produced by each of the concentrations of the solutions were measured in millimeters and interpreted using the CLSI zone diameter interpretative standards.

Antifungal activity

MIC of the extract was determined using potato dextrose agar media against the standard fungicide bavistin by the poisoned food technique [31] against *Aspergillus flavus*, *Fusarium oxysporum*, and *Trichophyton rubrum*. A stock solution of 2000 µg/ml of the test extracts was prepared, which was further diluted with methanol to give the required concentrations 1000 to 1 µg/ml. One tube was used as solvent control. For *Candida albicans* and *C. tropicalis*, the broth dilution method was adopted using potato dextrose broth against the standard fungicide fluconazole. All experiments were done in triplicate for each treatment against each fungus.

Agar disc diffusion method

In vitro antifungal activity of the extracts was measured using agar disc diffusion assay against the test bacteria and fungi. The sterile discs were impregnated with MIC concentration of test compounds. The fluconazole (10 µg/disc) was used as positive reference standards. The antimicrobial activity was evaluated by measuring the zone of growth inhibition surrounding the discs. All the assays were carried out in triplicate.

Statistical analysis

Statistical analyses were performed with the EXCEL and SPSS software packages. To determine the statistical significance of antioxidant activity, student's t-test was used. All values were expressed as mean ± SD of three parallel measurements.

RESULTS AND DISCUSSION

The antimicrobial activities of the extracts of four *Thottea* species are shown in Table 1-4. Significant antibacterial activity was recorded by *T. sivarajanii* (Table 1 and 2) and highest activity was recorded against *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (64 µg/ml). *T. ponmudiana* recorded poor antibacterial activity (Table 1).

Antifungal activity of the extracts of the four species is shown in Table 3 and 4. Out of the four extracts tested, *T. barberi* and *T. ponmudiana* recorded significant antifungal activity and the highest activity was recorded by *T. barberi* against *Trichophyton rubrum* (16 µg/ml).

Emergence of multiple drug resistance in human pathogenic organisms has given momentum to search new antimicrobial substances from alternative sources. There have been several mechanisms proposed for the antibacterial activity of potent drugs including plant extracts [32]. In many cases phytochemicals can be more effective than chemically synthesized pure compounds because they are a complex mixture of compounds. Their complexity enables them to interact with multiple molecular targets and thus it

becomes more difficult for targeting microorganisms to develop resistance because of multiple response sites [33].

Some of the test extracts (*T. sivarajanii*, *T. barberi* and *T. siliquosa*) in the current work exhibited considerable antibacterial activity. Some of the test bacteria (*S. epidermis*, *P. mirabilis* and *V. cholerae*) also exhibited resistance to a few extracts (*T. barberi*, *T. ponmudiana*, and *T. siliquosa*) at test concentration. Gram-negative bacteria are frequently reported to have developed multidrug resistance to many of the currently available antibiotics [34]. Therefore, it is not surprising to learn that Gram-negative bacteria are the least responding bacterial strains to some of the tested extracts.

Table 1: MIC values of the methanol extract of *Thottea* species against bacteria

Test bacteria	MIC (µg/ml)				
	T. barb	*T. ponm*	*T. sili*	*T. siva*	ciprofloxacin
B. subtilis	125	-	250	-	2
S. aureus	1000	-	125	-	2
E. coli	500	-	500	125	2
P. aeruginosa	250	-	125	64	4
S. epidermidis	-	-	-	64	4
P. mirabilis	-	-	-	125	8
V. cholerae	-	-	-	250	4
K. pneumonia	500	2000	250	250	4
S. simulans	125	2000	-	125	8
S. typhi	-	1000	-	1000	1

Values represent mean of three replications. -no MIC up to 2000 µg/ml

Table 2: Disc diffusion values of the methanol extract *Thottea* species against bacteria

Test bacteria	Diameter of zone of inhibition (mm)				
	T. barb	*T. ponm*	*T. sili*	*T. siva*	ciprofloxacin
B. subtilis	15.00	-	11.52	-	30.00
S. aureus	9.12	-	12.72	-	31.00
E. coli	11.00	-	9.00	14.00	32.12
P. aeruginosa	9.72	-	11.15	17.00	29.72
S. epidermis	-	-	-	19.12	28.51
P. mirabilis	-	-	-	13.72	28.12
V. cholerae	-	-	-	12.52	24.72
K. pneumonia	8.00	7.52	12.77	11.12	27.51
S. simulans	13.12	8.00	-	12.72	29.58
S. typhi	-	10.77	-	12.1	33.72

Values represent mean of three replications. –no zone of inhibition.

Table 3: MIC values of the methanol extract of *Thottea* species against fungi

Test fungi	MIC (µg/ml)				
	T. barb	*T. ponm*	*T. sili*	*T. siva*	fluconazole
A. flavus	-	-	-	-	1
C. tropicalis	32	32	64	125	0.5
C. albicans	64	64	250	250	1
T. rubrum	16	32	16	34	1
F. oxysporum	-	-	-	-	8

Values represent mean of three replications. -no MIC up to 2000 µg/ml.

Table 4: Disc diffusion values of the methanol extract *Thottea* species against fungi

Test fungi	Diameter of zone of inhibition (mm)				
	T. barb	*T. ponm*	*T. sili*	*T. siva*	fluconazole
A. flavus	-	-	-	-	23.72
C. tropicalis	13.12	16.00	15.00	14.12	25.00
C. albicans	16.72	18.72	14.00	10.00	20.00
T. rubrum	21.00	17.52	22.52	18.52	25.12
F. oxysporum	-	-	-	-	22.52

Values represent mean of three replications. –no zone of inhibition

CONCLUSION

The demonstration of antibacterial activity against gram-positive bacteria is an indication that the selected *Thottea* species are potential sources for the production of drugs with a broad spectrum of activity.

The results of the study also supports the traditional application of the plant and suggest that plant extracts possess compounds with antibacterial properties which can be used as antibacterial agents in novel drugs for the treatment of several ailments and infections.

Further pharmacological evaluations, toxicological studies and possible isolation of the therapeutic antibacterial compounds from these species are the future challenges.

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