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PHYTOCHEMICALS AND ANTIMICROBIAL STUDIES ON STEM EXTRACTS OF *TINOSPORA CORDIFOLIA* (WILLD.)

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ABSTRACT

Medicinal plants represent one of the most important fields of traditional medicine all over the world. In light of the recent emergence of pathogenic bacterial and fungal strains which are resistant to antibiotics posing a challenge for the treatment of infections, the need to discover new antimicrobial substances for use in combating such pathogens become pertinent. Therefore, the aim of the present study is to analyze the composition of phytochemicals and their antimicrobial activity from stem of *T. cordifolia* by using agar well diffusion method against seven bacterial and three fungal strains. Methanol and aqueous extracts exhibited the high antimicrobial activity while chloroform exhibited moderate activity and hexane showed low antimicrobial activity. Methanol and aqueous extracts showed antimicrobial activity is higher than standard antibiotics against *Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae* and *Proteus vulgaris*. Methanol extract was found to be strong MIC against *Enterococcus faecalis, Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Proteus vulgaris* whereas aqueous extract against *Staphylococcus aureus* and *Sterptococcus pneumoniae*, at concentration of 31.2 µg/ml. When the concentration of the extracts was increased the zone of inhibition also increased.

KEYWORDS: *Tinospora cordifolia*, medicinal plant, phytochemicals, antimicrobial activity.

INTRODUCTION

The development of drug resistant in human pathogenic microorganisms against commonly used antibiotics has necessitated a search for new antimicrobial substances from medicinal

plants. They have curative properties due to the presence of various complex chemical substances of different composition which are found as secondary metabolites in one or more parts of these plants.[1] In India, from ancient times, different parts of medicinal plants have been used to cure skin diseases mainly associated with pathogenic bacteria and fungi. The use of plant compounds for medicinal purposes has gradually increased in India. About 80% of individuals from developed countries use traditional medicine, which involves compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. Hence, studies involving the use of medicinal plants as therapeutic agents should be emphasized, especially those related to the antibiotic resistant microorganisms.

Tinospora cordifolia (Willd.) is known by the common names Heart-leaved Moonseed, Guduchi and Giloy, is an herbaceous vine of the family Menispermaceae, indigenous to the tropical areas of India, Myanmar and Sri Lanka. It is a large, deciduous, climbing shrub ascending to an altitude of 300 m. It being a rasayana drug from Ayurbeda is widely used in the Ayurvedic system of medicine for its general tonic, anti-inflammatory, antiarthritic, antiallergic, anti-malarial, anti-diabetic and aphrodisiac properties.[2] The whole plant is harvested due to its medicinal value. However, the stem is the official medicine as listed by the Ayurvedic pharmacopoeia of India. It is one of the constituents of several Ayurvedic preparations used in skin diseases, jaundice, anemia, diabetes, general debility, dyspepsia, fever and urinary diseases.[3]

It is a general practice in herbal practice in tribal areas to resort to only herbal medicinal formulations for most clinical disorders and thus, traditionally, the crude extracts of *T. cordifolia* is extensively used as topical application for infectious wounds and other bacterial and fungal infections. It would be essential to evaluate the phytochemical composition of this plant because of its widespread utilization as a folk medicine, to ensure that the phytoconstituents are novel and may offer a possible role to play as effective antimicrobial agent. Therefore, the aim of the present study is to analyse the composition of phytochemicals from stem of *T. cordifolia*, to asses the potential use of these extracts for their antimicrobial activity against human pathogenic bacterial and fungal strains.

MATERIAL AND METHODS

Collection, identification and extraction

Stem was collected from Kambalakonda forest area, Visakhapatnam, Andhra Pradesh, India. The collected plant material was identified by Prof. M. Venkaiah, Department of Botany, Andhra University, Visakhapatnam, India. The collected sample of stem was kept in the shadow until it gets dried completely. Then it was powdered in the mixture grinder and packed in Soxhlet apparatus. Sequential extraction of it was done by using hexane, chloroform, followed by methanol.[4] The filtrates were concentrated by removing the solvents under reduced pressure at 40^0C using a rotary evaporator. The concentrated crude extracts were labeled and stored at 4^0C.

Simultaneously, the aqueous extract of the stem was prepared by adding boiled water to the powder in a beaker on water bath, with occasional stirring for 4 hrs. The aqueous extract was then filtered and reduced under pressure.

Bacterial and fungal strains

The following microbial strains were collected from microbial type culture and collection (MTCC), Chandigarh, India. Seven bacterial strains including both Gram-positive and Gram-negative, namely *Bacillus subtilis* MTCC B2274, *Enterococcus faecalis* MTCC B3159, *Escherichia coli* MTCC B1560, *Klebsiella pneumoniae* MTCC B4030, *Micrococcus luteus* MTCC B1538, *Pseudomonas aeruginosa* MTCC B2297, *Proteus vulgaris* MTCC B7299, *Staphylococcus aureus* MTCC B3160, *Streptococcus pneumoniae* MTCC B2672, and three fungal strains such as *Aspergillus niger* MTCC F4325, *Candida albicans* MTCC F7315 and *Saccharomyces cerevisiae* MTCC F2567.

Antimicrobial screening

The lyophilized culture was sub cultured and concentration of working stock culture was assessed as 10^{-6} CFU/ml. Specified quantity of nutrient agar was prepared and plated in aseptic condition. The agar well diffusion method[5] was followed for antimicrobial susceptibility test for crude extracts and DMSO (negative control) whereas agar disc diffusion method was followed for standard antibiotic disc. The extracts were dissolved in DMSO to get the known concentrations of 10 mg/ml, 25 mg/ml and 50 mg/ml. The activity was compared with tetracycline disc (10 mcg/disc). After 24 h of incubation at 37^0C the zone of inhibition was measured by using an antibiotic zone reader scale (HiAntibiotic ZoneScale-c) and tabulated. For the antifungal activity, the same method as for bacteria was adopted of

nutrient agar, saboraud dextrose agar was used. The inoculated medium was incubated at 25^0C for two days for the *C. albicans*, *S. cerevisiae* and three days for *A. niger*. About 500 µg of nystatin was dissolved in 1 ml of sterile de ionized water. About 10 µl of 0.5 mg/ml nystatin (equivalent to 5 µg dose).

The extracts that exhibited inhibition zones were subjected to minimum inhibition concentration (MIC) assay by using serial two-fold dilution.[6] A quantity of 0.6 g of each extract was dissolved in 300 ml sterile nutrient broth which yields initial concentration of 2000 µg/ml. Subsequently, two-fold serial dilution was made from the stock to obtain 1000, 500, 250, 125, 62.5, 31.2 µg/ml concentrations. One ml of standardized inoculums of each test organism was introduced into each extract nutrient broth mixture and then incubated at 37^0C. The lowest concentration inhibiting growth was regarded as the MIC of the extracts. For the fungi, the inoculated medium was incubated at 25^0C for two (*C. albicans*, *S. cerevisiae*) to three (*A. niger*) days.

Statistical analysis

Each experimental data from triplicates was subjected to one way ANOVA using Minitab version 15. A significant level of $p < 0.01$ was used for all statistical analyses.

RESULTS AND DISCUSSION

The antimicrobial activity of the stem extracts were assayed by agar well diffusion method against seven bacterial and three fungal strains. Table 1 shows the antimicrobial activity in the form of growth inhibition zones of hexane, chloroform, methanol and aqueous extracts of screened *T. cordifolia*. Polar extract methanol and aqueous extracts exhibited the high antimicrobial activity while semi polar solvent chloroform exhibited moderate activity and non polar solvent hexane showed low antimicrobial activity. When the concentration of the extract was increased the zone of inhibition also increased.

Aqueous extract showed the maximum antimicrobial activity against *S. pneumoniae* followed by *S. aureus*, *K. pneumoniae* and *P. vulgaris* whereas methanol extract against *S. aureus* followed by *E. faecalis*, *S. pneumoniae* and *P. vulgaris*. The chloroform extract exhibited the maximum antimicrobial activity against *E. faecalis*, *C. albicans* whereas hexane extract against *S. aureus* followed by *E. faecalis*. Methanol and aqueous extracts showed inhibition zones higher than standard antibiotics against *B. subtilis*, *E. faecalis*, *S. aureus*, *S. pneumoniae*, *K. pneumoniae* and *P. vulgaris*. Methanol extract also showed the high value

against *A. niger* whereas chloroform extract shown similar value against *E. faecalis*. This broad spectrum antimicrobial activity displayed by some of phytochemicals could be attributed to the presence of pronounced antimicrobial compounds in the extracts. The negative control, DMSO had no effect on microbial growth of bacterial and fungal strains. MIC values of all the extracts tested against bacterial and fungal strains were summarized in Table 2. Methanol extract was found to be the strong MIC against *E. faecalis, S. aureus, S. pneumoniae, K. pneumoniae, P. vulgaris* whereas aqueous extract against *S. aureus* and *S.*

Table 1 Inhibition zones of stem extracts of *T. cordifolia.*

Organisms	Zone of inhibition (mm)[a]												S	D
	Hexane extract			Chloroform extract			Methanol extract			Aqueous extract				
	10	25	50	10	25	50	10	25	50	10	25	50		
B. subtilis	12±0.1	14±0.1	16±0.1	12±0.1	14±0.9	16±0.1	19±0.1	21±0.9	24±0.9	15±0.1	18±0.1	20±0.1	18^{T}	–
E. faecalis	11±0.2	15±0.4	18±0.2	17±0.7	20±0.5	21±0.4	19±0.5	24±0.9	26±0.5	19±0.1	20±0.1	23±0.1	21^{T}	–
M. luteus	-	-	10±0.1	13±0.9	14±0.1	14±0.1	14±0.4	17±0.1	18±0.4	11±0.1	14±0.5	16±0.1	24^{T}	–
S. aureus	18±0.9	20±0.3	21±0.9	14±0.4	17±0.1	20±0.3	23±0.4	25±0.7	28±0.2	21±0.9	26±0.9	28±0.9	24^{T}	–
S. pneumoniae	12±0.1	14±0.4	16±0.1	12±0.9	14±0.7	19±0.4	19±0.2	23±0.4	26±0.5	27±0.9	28±0.4	30±0.9	22^{T}	–
E. coli	-	-	10±0.1	10±0.6	12±0.4	14±0.1	16±0.1	18±0.5	19±0.2	13±0.4	16±0.1	20±0.2	22^{T}	–
K.pneumoniae	-	-	-	-	10±0.1	12±0.1	21±0.2	23±0.1	24±0.1	21±0.1	23±0.1	26±0.1	24^{T}	–
P. aeruginosa	-	-	-	-	-	-	-	-	12±0.2	-	-	-	25^{T}	–
P. vulgaris	13±0.1	15±0.1	16±0.1	16±0.2	18±0.2	19±0.1	21±0.1	24±0.5	26±0.1	21±0.4	23±0.2	24±0.4	22^{T}	–
A. niger	-	10±0.1	12±0.1	14±0.4	16±0.2	18±0.1	14±0.5	18±0.2	20±0.5	14±0.2	15±0.5	18±0.9	18^{N}	–
C. albicans	12±0.1	15±0.1	16±0.1	16±0.1	17±0.4	21±0.1	16±0.2	18±0.5	20±0.2	13±0.9	14±0.4	16±0.5	23^{N}	–
S. cerevisiae	-	-	10±0.7	-	11±0.2	13±0.1	13±0.1	15±0.9	16±0.2	11±0.5	13±0.2	15±0.9	20^{N}	–

a: Each value is the mean of three replicates with standard deviation; *P* value is <0.001 extremely significant when compared with standard, S: Standard antibiotics.

pneumoniae, at concentration of 31.2 µg/ml. Crude extracts are generally a mixture of active and non active compounds of and MIC of less than 100 µg/ml demonstrating good antimicrobial activity.[7]

Table 2. MIC values of *T. cordifolia* against various pathogens.

Organisms	Hexane extract	$ChCl_3$ extract	Methanol extract	Aqueous extract
B. subtilis	500	500	62.5	125
E. faecalis	500	125	31.2	62.5
M. luteus	>1000	500	250	250
S. aureus	125	250	31.2	31.2
S. pneumoniae	500	500	31.2	31.2
E. coli	1000	1000	250	125
K. pneumoniae	-	1000	31.2	62.5
P. aeruginosa	-	-	1000	-
P. vulgaris	500	500	31.2	62.5
A. niger	1000	250	125	250
C. albicans	500	250	125	500
S. cerevisiae	>1000	500	500	500

The phytochemical studies revealed the presence of alkaloids, phenols, flavonoids, saponins, tannins, steroids, terpenoids etc. in the all extracts (Table 3). Methanol and aqueous are found to be effective against all tested microorganisms. antimicrobial compounds such as flavonoids and terpenoids are polar constituents and they can't be extracted using non-polar solvent system. Hexane and chloroform extracts however displayed mild activity against both bacteria and fungi, with few number of phytochemicals are present in the extracts. The broad spectrum antimicrobial activity displayed by these extracts could be attributed to the presence of various complex chemical substances of different composition which are found as secondary metabolites in one or more parts of these medicinal plants.[1]

Table 3. Phytochemical constituents of stem of *T. cordifolia*.

Phytochemicals	hexane extract	$ChCl_3$ extract	Methanol extract	Aqueous extract
Alkaloids	-	+	+	-
Aminoacids	+	-	-	+
Anthraquinone	+	-	-	-
Carbohydrates	+	+	+	+
Cardiac glycosides	+	+	+	+
Flavonoids	-	-	+	+
Glycosides	-	+	+	+
Phenols	+	+	+	+
Saponins	+	+	+	+
Steroids	-	-	+	+
Tanins	+	+	+	+
Terpenoids	-	-	+	+
Volatile compounds	-	-	-	-

As phytochemicals often play an important role in plant defense against prey, microorganisms, stress as well as interspecies protections, medicinal plant components have been used as antimicrobial agents for millennia. Hence, phytochemicals screening serve as the initial step in predicting the types of potential active antimicrobial compounds from stem from *T. cordifolia*. Therefore, medicinal plants are a promising source of antimicrobial drugs. For this reason, WHO has encouraged studies that evaluate traditional medicinal practices to treat infectious diseases caused by bacterial and fungal strains. The present study showed that hexane, chloroform, methanol and aqueous extracts of stem of *T. cordifolia* exhibited a significant antimicrobial activity at dose dependent manner.

CONCLUSION

In can be concluded, that our results offer a scientific basis for the traditional use of *T. codifolia*. However, *in vivo* studies on this plant is warranting determining toxicity of active

constituents, their side effects and pharmacokinetic properties. It also be assumed that this plant could be a potential source for a novel 'lead' discovery for antimicrobial drug development.

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