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ANTIMICROBIAL ACTIVITY OF DIFFERENT EXTRACTS OF *AZIMA TETRACANTHA* ROOT

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ABSTRACT

Hexane, chloroform, ethyl acetate and methanol extracts of *Azima tetracantha* were screened for their antimicrobial activity against human pathogenic bacterial and fungal strains. Antimicrobial activity was carried out by disc diffusion method, determination of minimum inhibitory concentrations (MIC), minimum bacterial concentrations (MBC) and minimum fungicidal concentrations (MFC) against three strains of Gram positive bacteria, four strains of Gram negative bacteria and three species of fungi. The antimicrobial activity of various extracts of *A. tetracantha* showed varied levels of antimicrobial activity against the studied bacterial and fungal pathogens. The mean zone of inhibition produced by all the tested extracts ranged from 6.5±0.20 to 19.1±0.15 mm. The MIC, MBC and MFC values were between 15.6 and 1000 µg/ml. The methanol extract of *A. tetracantha* showed good antimicrobial activity with the highest mean zone of inhibition (19.1+0.15 mm), lowest MIC (15.6 µg/ml), MBC (31.2 µg/ml) MFC (125 µg/ml) and values followed by other extracts.

KEY WORDS: **Antibacterial activity; Antifungal activity; *Azima tetracantha*; Successive extracts**

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INTRODUCTION

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress 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]. Among the infectious diseases, diseases caused by fungal infections account for a larger proportion of health problems in human, particularly among women and children[2]. Skin diseases are a significant high frequency problem of certain developing countries. In low and middle income countries, skin diseases are dominated by bacterial and fungal infections. Microbial infections are the cause of a large burden of diseases and bacteria are listed in the first position among the common microorganisms responsible for opportunistic diseases[3]. Besides the usage of crude plant extracts by human beings for their antimicrobial activity, plants can also produce antimicrobial compounds to protect themselves from biotic attacks that could be essential for microbial infection. Therefore, it is worthwhile to study plants and plant products for activity against microorganisms[4]. The substances that can inhibit the growth of microorganisms or kill them and have little or no toxicity to human cells are considered candidates for developing new antimicrobial drugs. Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties[5]. In recent years antimicrobial properties of medicinal plants have been increasingly reported worldwide[6]. *Azima tetracantha* Lam. locally known as “Mulsangu”, is a rambling spinous shrub flowering throughout the year found in peninsular India, West Bengal, Orissa, African countries and extends through Arabia to tropical Asia. The juices of the leaves were used to relieve the cough phthisis and asthma. In western India, juices of the leaves were applied as eardrops against earache and crushed leaves were placed on painful teeth. In India and Sri Lanka the root, root bark and leaves were administered with food as a remedy for rheumatism[7]. This plant is considered as a powerful diuretic and is also used to treat rheumatism, dropsy, dyspepsia, chronic diarrhoea and as a stimulant tonic for woman after confinement[8]. It is also used as food for various herbal medicines in Africa, India and Madagascar[9]. Locally, the traditional healers from Tirunelveli district of Tamilnadu are using root bark (Paste with butter milk) of this plant as a potent remedy for jaundice. This plant has been reported to possess different biological activities like anti-inflammatory, wound-healing, diuretic and analgesic activities[10]. In the present investigation, various solvent extracts viz. hexane, chloroform, ethyl acetate and methanol of root of *Azima tetracantha* were studied for its antimicrobial activity against human pathogenic bacteria viz. *Staphylococcus aureus, Bacillus cereus, B. subtilis, Escherichia coli, Klebsiella pneumonia, Pesudomonas aeruginosa* and *Salmonella typhi* and fungal strains viz. *Aspergillus niger, A. flavus* and *A. fumigatus.*

MATERIALS AND METHODS

(i) Collection of plant material

The root of *Azima tetracantha* was collected from Athamangalam (Lat, 10.46 ºN; Long, 79.49 ºE), Nagapattinam District, Tamil

Nadu, India during months of August to September 2012. Herbarium was deposited in Department of Botany, Annamalai University (AUBOT 262). The root was washed with tap water, then surface sterilized with 10 % sodium hypo-chloride solution to prevent contamination of any microbes. The samples were rinsed with distilled water and allowed to shade dried under room temperature followed by oven drying at 50 ºC and then ground into powder using electric blender.

(ii) Preparation of extraction

One hundred grams of powdered materials of root samples were extracted in a soxhlet apparatus for 8 hours with different solvents system like hexane, chloroform, ethyl acetate and methanol[11]. The extracts were filtered, pooled and the solvents were evaporated with the help of rotary evaporator (Heidolph, Germany) under reduced pressure at 40 ºC and the crude extracts were kept at 4 ºC in refrigerator for further analysis.

(iii) Antimicrobial assay

a) Microorganisms

Antibacterial activity was tested against three strains of Gram positive bacteria viz. *Staphylococcus aureus, Bacillus cereus, B. subtilis,* four strains of Gram negative bacteria viz. *Escherichia coli, Klebsiella pneumonia, Pesudomonas aeruginosa* and *Salmonella typhi* and three fungal species viz. *Aspergillus niger, A. flavus* and *A. fumigates.* The stock cultures were maintained on nutrient agar medium (for bacteria) and Sabouraud dextrose agar medium (for fungi) at 4 ºC.

b) Disc diffusion assay

Antimicrobial susceptibility test of the crude extracts were tested against the above mentioned Gram positive, Gram negative bacteria and fungi by disc diffusion method[12]. Petri plates were prepared with 20 ml of sterile Muller Hinton Agar (Himedia, Mumbai) for bacteria and 20 ml of Sabouraud Dextrose Agar (SDA) for fungi. The twenty four hours prepared test inoculums were swabbed on the top of the solidified media and allowed to dry for 10 minutes. Previously prepared extracts were impregnated with discs at concentrations of 1000, 500, 250 µg/ml and were placed aseptically on plates with were placed on with appropriate controls. The loaded discs were placed on the surface of the medium and left for 30 minutes at room temperature. Negative control was prepared using 10 % DMSO. For bacteria, Ciprofloxacin (5µg / disc) and for fungi, Ketaconozole (10µg/disc) were used as positive controls. Finally, the inoculated plates were incubated at 37 °C for 24 h (for bacteria) and 35 °C for 48 h (for *Aspergillus*). The inhibition zones were observed including the diameter of the disc (6mm).

c) Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations of the crude extracts were tested in Muller Hinton Broth for bacteria and Sabouraud Dextrose Broth for mycelia fungi to get the concentrations of 1000 – 15.6 µg/ml by the broth macro dilution method[13]. The culture tubes were incubated at 37 °C for 24 h (for bacteria) and 35 °C for 48 h (for mycelia fungi).

d) Minimum Bacterial Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

The MBC and MFC of the crude extracts were determined[14] by plating 100 µl samples from each MIC assay with growth inhibition into freshly prepared Muller Hinton Agar (for bacteria) and

Sabouraud Dextrose Agar (for mycelia fungi). The plates were incubated at 37 °C for 24 h (for bacteria) and 35 °C for 48 h (for mycelia fungi).

e) Statistical Analysis

All the data of microbial activities were examined as mean ± SD. One-way analysis of variance (ANOVA) was carried out to determine the significant difference ($P < 0.005$) between the means. The analyses were carried out using SPSS package software, 11.5 (SPSS Inc., Chicago, IL).

RESULTS

In the present investigation, different solvent extracts of *Azima tetracantha* showed varied levels of antimicrobial activity (Tables 1 and 2) against the studied bacterial and fungal pathogens. The mean zone of inhibition produced by all the extracts ranged from 6.5±0.20 to 19.1±0.15 mm. The MIC, MBC and MFC values were between 15.6, and 1000 µg/ml. The methanol extract of *Azima tetracantha* showed good antibacterial activity with the highest mean zone of inhibition (19.1±0.15 mm) against *Staphylococcus aureus*, lowest MIC (15.6 µg/ml) and MBC (31.2 µg/ml) against *Staphylococcus aureus, Bacillus cereus* and *Bacillus subtilis* and methanol extract of *A. tetracantha* showed antifungal activity with the highest mean zone of inhibition ranged from (10.1±0.15 mm) against *A.fumigatus*, lowest MIC (62.5 µg/ml) and MFC (125 µg/ml) against *A.fumigatus* and *A. flavus.* The ethyl acetate extract of *A. tetracantha* showed good antibacterial activity with the highest mean zone of inhibition (13.1±0.11 mm) against *Staphylococcus aureus*, lowest MIC (31.2 µg/ml) and MBC (62.5 µg/ml) against *Staphylococcus aureus, Bacillus cereus* and *Bacillus subtilis* and ethyl acetate extract of *A. tetracantha* showed antifungal activity with the highest mean zone of inhibition ranged from (9.6±0.76 mm) against *A.fumigatus*, lowest MIC (125 µg/ml), and MFC (250 µg/ml) against *A.fumigatus* and *A. flavus.* The chloroform of *A. tetracantha* showed good antibacterial activity with the highest mean zone of inhibition (13.0±0.0 mm) against *Staphylococcus aureus*, lowest MIC (62.5 µg/ml) and MBC (125 µg/ml) against *Staphylococcus aureus* and *Bacillus cereus* and chloroform extract of *A. tetracantha* showed antifungal activity with the highest mean zone of inhibition ranged from (9.1±0.28 mm) against *A.fumigatus*, lowest MIC (250 µg/ml) and MFC (500 µg/ml) against *A. niger*, *A.fumigatus* and *A. flavus.* The hexane extract of *A. tetracantha* showed good antibacterial activity with the highest mean zone of inhibition (11.3±0.28 mm) against *Staphylococcus aureus*, lowest MIC (125 µg/ml) and MBC (250 µg/ml) against *Staphylococcus aureus* and *Bacillus cereus* and hexane extract of *A. tetracantha* showed antifungal activity with the highest mean zone of inhibition ranged from (9.0±0.50 mm), lowest MIC (500 µg/ml) and MFC (1000µg/ml) against *A. niger*, *A.fumigatus* and *A. flavus.*

Table 1
Antimicrobial activity of different solvent root extracts of Azima tetracantha (250, 500, 1000 µg / disc)

S. No	Microorganism	Mean Zone of inhibition[a] (mm)[b]											
		Hexane			Chloroform			Ethyl acetate			Methanol		
		250	500	1000	250	500	1000	250	500	1000	250	500	1000
1	*Staphylococcus aureus*	7.6±0.28	9.0±0.11	11.3±0.28	8.6±0.28	9.1±0.28	13.0±0.0	11.3±0.28	12.5±0.28	13.1±0.11	12.1±0.10	15.3±0.15	19.1±0.15
2	*Bacillus cereus.*	8.0±0.05	8.6±0.11	9.5±0.20	8.1±0.10	9.0±0.10	11.3±0.28	8.1±0.11	10.3±0.15	12.0±0.50	8.1±0.10	10.3±0.15	14.6±0.76
3	*Bacillus subtillis*	7.2±0.17	7.3±0.28	8.2±0.11	7.6±0.28	9.0±0.0	11.3±0.11	8.0±0.11	9.0±0.11	11.4±0.20	8.3±0.15	10.0±0.11	12.5±0.10
4	*Escherichia coli*	NA	NA	NA	7.2±0.20	8.1±0.10	9.2±0.20	7.2±0.10	8.1±0.15	9.5±0.20	8.1±0.15	9.3±0.15	10.6±0.28
5	*Klebsiella pneumonia*	7.2±0.20	7.6±0.28	8.0±0.11	9.5±0.20	10.2±0.25	10.7±0.10	9.6±0.10	10.3±0.17	12.3±0.28	9.7±0.25	10.6±0.20	12.3±0.28
6	*Pseudomonasae ruginosa*	7.2±0.10	7.6±0.28	8.2±0.25	8.3±0.15	9.5±0.20	11.1±0.10	9.6±0.15	10.2±0.20	12.5±0.0	10.5±0.20	10.8±0.15	13.1±0.28
7	*Salmonella typhi*	7.0±0.10	8.1±0.10	9.1±0.10	8.0±0.11	9.5±0.15	10.1±0.1	9.7±0.10	10.3±0.28	12.6±0. 1	12.1±0.36	14.7±0.20	16.1±0.15
8	*Aspergillus niger*	6.5±0.20	7.1±0.28	7.2±0.05	7.0±0.0	7.5±0.0	8.0±0.0	7.2±0.25	7.6±0.28	8.1±0.28	8.1±0.15	8.4±0.11	8.5±0.20
9	*Aspergillus fumigatus*	7.2±0.05	8.2±0.05	9.0±0.50	7.6±0.28	8.6±0.15	9.1±0.28	8.9±0.11	9.6±0.15	10.1±0.15	9.3±0.10	10.0±0.0	12.3±0.10
10	*Aspergillus flavus*	6.6±0.28	7.0±0.11	7.1±0.11	7.1±0.10	8.3±0.28	8.6±0.10	8.0±0.11	8.4±0.10	9.3±0.15	8.0±0.20	8.5±0.25	9.3±0.15

a – diameter of Zone of inhibition (mm) including disc diameter of 6 mm, b – mean of three assays; ± standard deviation; Ciprofloxacin (5µg/disc) – antibacterial drug between 22.6±0.20 and 29.5±0.50 mm; Ketoconazole (10µg/disc) – antifungal drug between 11.1±0.70 and 13.0±0.11 mm.

Table 2
MIC, MBC and MFC values of different solvent root extracts of Azima tetracantha

S. No	Microorganisms	Minimum Inhibitory Concentrations (µg/ml)				Minimum Bacterial Concentrations (µg/ml)			
		Hexane	Chloroform	Ethyl acetate	Methanol	Hexane	Chloroform	Ethyl acetate	Methanol
1	*Staphylococcus aureus*	125	62.5	31.2	15.6	250	125	62.5	31.2
2	*Bacillus cereus.*	125	62.5	31.2	15.6	250	125	62.5	31.2
3	*Bacillus subtilis*	250	125	31.2	15.6	500	250	62.5	31.2
4	*Escherichia coli*	NA	250	125	62.5	NA	500	250	125
5	*Klebsiellapneumonia*	250	125	62.5	31.2	500	250	125	62.5
6	*Pseudomonasaeruginosa*	250	125	125	62.5	500	250	250	125
7	*Salmonella typhi*	500	250	125	62.5	1000	500	250	125
8	*Aspergillus niger*	Minimum Inhibitory Concentrations (µg/ml)				Minimum Fungicidal Concentrations (µg/ml)			
		500	250	125	62.5	1000	500	250	125
9	*Aspergillus fumigatus*	500	250	125	62.5	1000	500	250	125
10	*Aspergillus flavus*	500	250	250	125	1000	500	500	250

DISCUSSION

The hexane, chloroform, ethyl acetate and methanol root extracts of *A. tetracantha* showed a broad spectrum of antimicrobial activity against all the microorganisms tested. In the present study, Gram positive bacteria were more susceptible than Gram negative

and fungal pathogens. The differences in the antimicrobial activity of crude extracts may be due to the amount of antimicrobial agent present in the extracts[15]. However, six selected plants belonging to Apocynaceae family were collected and their extract was tested for their antimicrobial activity against gram positive bacteria were found to be more susceptible than the gram negative bacteria. While the gram positive bacteria *Bacillus subtillis* (MIC 250 µg/ml) and *Staphylococcus aureus* (MIC 500 µg/ml) was the most resistant and was inactive against the gram negative bacteria *Escherichia coli* and *Pseudomonas aureuginosa* [16].The evaluation of antimicrobial potential by disc diffusion method indicates that all the bacterial tested organisms showed growth inhibition towards the plant extract, with differing sensitivity. Among the bacterial pathogens, *S. aureus* is more sensitive when compared to other bacteria. Gram-positive bacteria were exhibited more sensitive to plant extracts when compared to Gram-negative bacteria[17]. The methanol extract of pomegranate peels were more active than over water extract against *E.coli, S. aureus* and *B. subtilis*[18]. The disc diffusion bioassay showed that the methanol leaf and stem extracts have the higher activity against all gram positive bacteria than the gram negative bacteria and antifungal activity of different concentration viz., 100 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml to assess the antifungal activity against various fungal strains. Among all the fungal isolates tested interestingly all the isolates exhibited maximum percentage growth inhibition at 1000 µg/ml concentration[19]. The methanol extract showed higher antifungal activity than that of aqueous extract. This may be due to the solvent to extract the different constituents having antifungal activity. The crude aqueous seed extract of *Syzygium jambolanum* showed antifungal activity against *A. flavus, A. fumigates* and *A. niger*[20]. The methanol extracts of leaf and root exhibited high antibacterial activities against the gram-positive bacteria. Similar results were observed in previous studies. Methanolic and ethanolic extracts of *Punica granatum* were effective against *Bacillus cereus, Escherichia* coli and *Staphylococcus aureus* [21, 22]. In addition, these results confirmed the evidence in previous studies reported that the methanol is a better solvent for more consistent extraction of antimicrobial substances from medicinal plant compared to other solvents, such as water, ethanol and hexane[23, 24, 25,26].

CONCLUSION

It is concluded that methanol root extract of *Azima tetracantha* had a potential antimicrobial activity against all the microorganisms tested. Based on this study, isolation and identification of antimicrobial compound from methanol extract of *A. tetracantha* will fetch a new natural antimicrobial agent.

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