

IN VITRO ANTIBACTERIAL PROFILE OF ALSTONIA VENENATA R. BR- A COMPARATIVE STUDY**SHIRLY K. THOMAS, ROSHIN ELIZABETH GEORGE, MANESH KUNJUMON, V. THANKAMANI***

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ABSTRACT**Objective:** To investigate the antibacterial efficacy of leaves, stem-bark, root- bark, flowers and fruits of *Alstonia venenata* R. Br.**Methods:** The antimicrobial efficacy of butanol and methanol solvent extracts was evaluated by agar well diffusion against selected pathogenic bacterial strains. Gram negative strains like *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enteric typhimurium*, *Salmonella typhi*, *Salmonella paratyphi A*, *Shigella* spp. were tested. Gram positive strains tested were *Micrococcus luteus* and *Staphylococcus aureus*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using macro broth dilution method against clinical isolate of *Staphylococcus aureus*. Streptomycin 0.125mg/ml was used as positive control and Dimethyl sulfoxide (DMSO) as solvent control.**Results:** Butanol and methanol extracts of all the plant parts are highly effective against Gram positive strains and show moderate inhibition against Gram negative strains. Stem-bark and root-bark butanol were the most effective fractions followed by fruit, flower and leaf extracts. The inhibition zones of *Micrococcus luteus* and *Staphylococcus aureus* were 24-26 mm and 18-20 mm respectively. The observed zone size was equal to or greater than the positive control used. The MIC value for stem-bark butanol and root-bark butanol were 0.98 mg/ml and the MBC values were 7.8 mg/ml and 3.9 mg/ml respectively. For fruit, flower and leaf butanol the MIC values were 15.6, 31.25 and 125mg.**Conclusion:** The extracts were highly active against Gram positive strains than Gram negative strains. The butanol extracts were the most active fraction followed by the methanol extracts. Highest activity was observed for root-bark and stem-bark followed by fruit, flower and leaf extracts.**Keywords:** *Alstonia venenata*, Anti bacterial, Leaf, Stem- bark, Root-bark, Fruit, Flower.**INTRODUCTION**

Plants are recognized for their ability to produce a wealth of secondary metabolites and are considered as novel templates of drug discovery for innumerable human ailments. Today a substantial number of drugs are developed from plants that reveal new arena in medical research and which are in high demand. Acceptance of traditional medicine as an alternative form of health care has lead researchers to investigate the bioactivity of plant extracts. Around 70,000 plants species from lichens to towering trees have been used for medicinal purposes[1]. Indiscriminate use of commercially available antimicrobial drugs has lead to the development of multiple drug resistance strains which has led researchers to search for clinically useful antimicrobial compounds in the last century [2,3].

Alstonia venenata R. Br, a medium-sized evergreen shrub is a member of the family Apocynaceae. It is known for its medicinal value. In India it is seen on Ganja hills, Northern circars & Godavari up to 2000 feet, in Western Ghats, Nilgiri hills, Anamalai, Coimbatore, Pulneys and Tirunalvelly hills up to 4000 feet [4]. The plant was commonly known as Analivegam (Malayalam) or Vishagni (Sanskrit) since the ancient texts attribute anti-venom activities to the plant [5]. It has been used in the treatment of fever, epilepsy, malaria, fatigue, otalgia and a variety of skin diseases [5]. The plant is an active ingredient of Tri Health Ayurvedic formulations like 'Mahathiktakam Kwath' and 'Gritham'. Govindachari reported the isolation of twenty new alkaloids belongs to the class of Yohimbine, Aspirdofractinine and Vincadiformine [6]. Venenatine, Isovenenatine, Alstovenine are some of the reported alkaloids[7]. Psychopharmacological investigations of the 4-Methoxyindole alkaloids have been reported by Battacharia S. K, 1975 [8]. Singh *et al* also reported the antifungal activity of root bark alkaloids Venenatine in 2000 [9]. The antibacterial and anti-fungal effect of the crude leaf and stem bark extracts were also investigated in a few studies [10].

In the present study butanol and methanol extracts from the root-bark, stem-bark, leaf, flower and fruit of *Alstonia venenata* were tested and compared for its antibacterial activity.

MATERIALS AND METHODS**Plant material collection and authentication**

Plant was collected from interior parts of Ponmudi hills, Kerala, India. The plant was taxonomically identified and authenticated at the Herbarium, Department of Botany, University of Kerala, Thiruvananthapuram (Voucher specimen accession no. KUBH 5847).

Preparation of plant extracts

Different plant parts viz. the leaves, stem- bark, root-bark, flowers, fruits were separately washed with distilled water several times, air dried in shade, powdered and stored in dry polythene bags. The powdered materials were extracted successively with hexane, butanol, methanol and water for 12 hours using a Soxhlet apparatus. The excess solvent in the extracts were removed by distillation and concentrated extracts were then weighed kept in screw capped bottles and stored at room temperature.

Screening for Antibacterial activity**Tested Microorganisms and preparation of test cultures**

Bacterial stock cultures of *Pseudomonas aeruginosa* (MTCC 424), *Proteus vulgaris* (MTCC 426), *Escherichia coli* (MTCC 40), *Klebsiella pneumoniae* (MTCC 3384), *Salmonella enteric typhimurium* (MTCC 98), *Micrococcus luteus* (MTCC106) were obtained from the Microbial Type Culture Collection (MTCC), The Institute of Microbial Technology, Chandigarh, India. The stock cultures of *Staphylococcus aureus*, *Salmonella typhi*, *Shigella* spp., *Salmonella paratyphi A* were clinical isolates maintained at the Department of Biotechnology, University of Kerala.

Stock cultures were maintained at 4°C on nutrient agar by regular subculture. Overnight grown cultures were adjusted to 0.5 McFarland turbidity standards and were used for testing antibacterial activity [11].

Preparation of culture media

The Nutrient agar (NA) was used for the regular maintenance of test culture, Peptone water for inoculum preparation, Mueller Hinton

agar (MHA) used for antimicrobial testing and Nutrient broth (NB) for testing MIC were obtained from Hi Media Laboratories Pvt. Ltd., Mumbai. The culture plates were prepared by pouring 20 ml of MHA and were kept at 37°C for 24 hours for sterility checking.

Preparation of plant extracts as test samples

A single concentration of 500 mg of different test extracts dissolved in 1 ml DMSO (40mg/80 µl in each well) were used for the entire test.

Agar well diffusion

Agar-well diffusion method as described in European pharmacopeia with slight modification was used for antimicrobial testing [12]. Wells were cut using sterile well bore of 6 mm diameter and 100 µl of each of the prepared culture of test bacteria was loaded on the agar plate. The plates were swabbed uniformly using a sterile swab and allowed to dry for 5 minutes [12]. 80 µl of various test extracts dissolved in DMSO were transferred to each wells. Streptomycin (0.125mg/ml) was taken as positive control and DMSO as solvent control [12]. The antibacterial activities were observed after incubating the plates for 24 hours at 37°C as evidenced by the zone of inhibition surrounding the well.

Minimum inhibitory concentration [MIC] for bacteria

For determining the MIC broth dilution method was used. MIC values are determined as the lowest concentration of antimicrobial agent that which inhibit the growth of the organism at the end of the incubation period. For the present study method as described by CLSI 2006[13] with slight modification was used. The plant extracts were diluted in the range of 0.48-125mg/ml in Nutrient broth, positive control Streptomycin was also prepared simultaneously. After overnight incubation at 37°C the lowest concentration of the extract that produces no visible turbidity (growth) was recorded as MIC values.

Minimum bactericidal concentration [MBC] for bacteria

A modified method as described by British Society for Antimicrobial Chemotherapy Guide to Sensitivity Testing was followed. Subcultures were done from all the tubes showing no visible growth.

An aliquot of 0.1 ml was spread on MH agar plates uniformly by using sterile cotton swab and incubated overnight at 37°C. MBC values were noted as the lowest concentration that kills >99.9% of the initial bacterial population where no visible growth was observed on the MH plate.

RESULTS

Antibacterial assay

The antimicrobial activity of the methanol and butanol extracts from different parts of *Alstonia venenata* against ten different bacterial strains by agar well diffusion are shown in Table 1. Almost all extracts from different plant parts showed varying degrees of antibacterial potential. The extracts exhibited highest inhibition against Gram positive and moderate inhibition against Gram negative bacteria. The butanol extracts from different plant parts were more active when compared to the corresponding methanol extracts.

Butanol extracts showed an average zone of inhibition 19 mm with a range of 12 to 26 mm. Highest activity was observed for root-bark, stem-bark, fruit extract followed by flower and leaf. Maximum activity was observed for stem-bark and root-bark butanol extract with a zone inhibition diameter of 24-26 mm and 18-20 mm against *Micrococcus luteus* and *Staphylococcus aureus* respectively. The fruit and flower butanol extracts showed 23 mm and 26 mm zone of inhibition against *Micrococcus luteus*. The test extracts showed greater or equivalent zone size when compared with that of the control. Zone size of the control was 26 mm and 17 mm respectively for *Micrococcus luteus* and *Staphylococcus aureus*.

MIC and MBC

The MIC value of butanol extracts of stem and root-bark were 0.98 mg/ml and MBC values were 7.8mg/ml and 3.9mg/ml respectively. MIC values of fruit, flower and leaf butanol extracts were 15.6, 31.25, 125 and MBC values were 125 mg/ml for fruit and flower and greater than 125mg/ml for leaf. MIC and MBC value for streptomycin was 0.125mg/ml for the tested strains of *Staphylococcus aureus*.

Table 1: Antibacterial activity of butanol and methanol extracts from different plant part against ten different bacterial strains

| Plant parts | Extracts | con: 500 mg/ml Zone of inhibition (mm) | | | | | | | | | |
|--------------|----------|--|----|----|----|----|----|----|----|----|----|
| | | a | b | c | d | e | f | g | h | I | j |
| Leaf | Butanol | 12 | - | - | - | - | 17 | 12 | - | - | - |
| | Methanol | - | - | - | - | - | 17 | 12 | - | - | - |
| Fruit | Butanol | 15 | 11 | 11 | 10 | 12 | 26 | 10 | 13 | 12 | 11 |
| | Methanol | 11 | 10 | 11 | - | 12 | 24 | 12 | 10 | 11 | 11 |
| Flower | Butanol | 11 | 9 | 11 | - | 11 | 23 | 10 | 10 | 15 | 13 |
| | Methanol | 11 | - | - | - | - | 19 | - | - | 12 | - |
| Stem-bark | Butanol | 10 | - | 12 | - | 10 | 26 | 18 | 9 | 10 | 10 |
| | Methanol | 8 | - | 10 | - | 9 | 23 | 16 | 9 | 10 | 10 |
| Root-bark | Butanol | - | - | 13 | - | 11 | 24 | 20 | 11 | 10 | 11 |
| | Methanol | - | - | 11 | - | - | 24 | 16 | - | - | 10 |
| Streptomycin | | | | | | | | | | | |
| 0.125mg/ml | | 17 | 26 | 25 | 13 | 24 | 26 | 17 | 18 | 17 | - |
| DMSO | | - | - | - | - | - | - | - | - | - | - |

Values are inhibition zones in mm and average of triplicate.

Pseudomonas aeruginosa (MTCC 424)(a), *Proteus vulgaris* (MTCC 426)(b), *E. coli* (MTCC 40)(c), *Klebsiella pneumonia*(MTCC 3384)(d), *Slamonella enteric typhimurium* (MTTC 98) (e), *Micrococcus luteus* (MTTC106 (f), *Staphylococcus aureus* (g), *S. typhi*(h), *S. paratyphi A* (i) *Shigella* sps. (j).

Table 2: Mic of the root-bark, stem-bark, leaf, flower, fruit butanol extracts against staphylococcus aureus strain

| Butanol extracts | Concentration (mg/ml) | | | | | | | | | |
|------------------|-----------------------|------|-------|------|-----|-----|------|------|------|---|
| | 125 | 62.5 | 31.25 | 15.6 | 7.8 | 3.9 | 1.95 | 0.98 | 0.48 | |
| Root-bark | - | - | - | - | - | - | - | - | - | + |
| Stem-bark | - | - | - | - | - | - | - | - | - | + |
| Fruit | - | - | - | - | + | + | + | + | + | + |
| Flower | - | - | - | + | + | + | + | + | + | + |
| Leaf | - | + | + | + | + | + | + | + | + | + |

(-) Inhibition of organism, (+) Growth of organism

Table 3: Mbc of the root-bark, stem-bark, leaf, flower, fruit butanol extracts against *staphylococcus aureus* strain.

| Butanol extracts | Concentration (mg/ml) | | | | | | | | |
|------------------|-----------------------|------|-------|------|-----|-----|------|------|------|
| | 125 | 62.5 | 31.25 | 15.6 | 7.8 | 3.9 | 1.95 | 0.98 | 0.48 |
| Root-bark | - | - | - | - | - | - | + | + | + |
| Stem-bark | - | - | - | - | - | + | + | + | + |
| Fruit | - | + | + | + | + | + | + | + | + |
| Flower | - | + | + | + | + | + | + | + | + |
| Leaf | + | + | + | + | + | + | + | + | + |

(-) Inhibition of organism, (+) Growth of organism

DISCUSSION

Root and stem- bark butanol extracts showed similar MIC value which suggests the presences of similar phytoconstituents in the extracts. Isopropanol and methanol leaf extracts and benzene stem bark extract of *A. venenata* reported in another work also showed activity against pathogens and *Bacillus* spp. [10]. The activity of the leaf extract was comparatively less in our study and this may be due to the difference in the extraction procedure. Antibacterial activity of crude stem-bark extract and some purified compounds which were more effective against Gram positive bacteria was also reported by Chinmayee M D and Deepthi S R [14].

Antibacterial activity of *Alstonia scholaris* reported by Chandra Shekhar Misra also point out that methanol extract of the root was found to exhibit maximum antimicrobial activity against tested strains followed by stem bark and least by leaf extract. Here also Gram positive are more sensitive than Gram negative strains [15]. When compared to other solvent system like petroleum, dichloromethane, and ethyl acetate the butanol fractions of leaves, stem bark and root bark of *Alstonia scholaris* exhibited broad spectrum of antimicrobial activity in a similar work [19]. The flowers of *Alstonia scholaris* were also reported as potent antimicrobial agent by Thankamani *et al* where significant activity was observed against gram positive (20-25 mm) than against Gram negative (14-18 mm) organisms [12]. The benzene and the butanol extract from the fruit of *Alstonia scholaris* exhibited highest activity with a zone of 25 mm against *Lactobacillus lactis* and *Staphylococcus aureus*. The butanol extract also showed significant activity against *Salmonella typhi* with a zone size of 23 mm [16]. It was reported by A Hussain and co-workers that Gram-positive bacteria are more susceptible to *Alstonia scholaris* aqueous extract followed by methanolic extract but in our work, aqueous extracts were least active [17]. Antibacterial activity of the fruit and flower of *Alstonia venenata* are reported here for the first time. Since both the plants belong to same genera it showed similarity in antibacterial properties. The above results supports the traditional use of the dried stem bark powder for curing scabies and boils as well as the ripe fruit for the treatment of Syphilis among the tribal groups [18,19].

All the plant parts are rich in alkaloids and presence of alkaloids may be one of the main reasons for antibacterial activity. However, it is difficult to attribute the activity to a single or particular constituent as major or trace compounds might also contribute to the antimicrobial activity. Possible synergistic and antagonistic effect of phytochemicals in the crude should also be taken into consideration. Developing new antimicrobial against *Staphylococcus* is extremely important as Methicillin Resistant *Staphylococcus aureus* (MRSA) and Vancomycin Resistant *Staphylococcus aureus* (VISA) have emerged and scientists are in search of newer alternatives against these extremely drug resistant strains. In this scenario result of this investigation is highly significant to explore the potential of the bioactive compound present in the extract.

CONCLUSION

The current investigation proved the antibacterial efficacy of the plant against different bacterial strains and thus validating the traditional use of the plant against different diseases. Activity of the extracts against *Staphylococcus aureus*, the causative of skin and soft tissue infections, strongly supported its traditional use against skin diseases.

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