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Research Article

Antibacterial Activity of Plant Extracts of *Alstonia scholaris*

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ABSTRACT
The present study aims to investigate the antibacterial activity of leaves of *Alstonia scholaris* against bacterial pathogens by disc diffusion method, well method and incorporating the extract in the media before solidifying. The result of disc diffusion technique showed that fractions of leaf extract had pronounced antibacterial activity against Methicillin Resistant *Staphylococcus aureus* (MRSA) and the clinical strain *Providence stuartii*. Antibacterial activity was also tested against a large group of Gram positive and Gram negative bacteria and it was found to reside maximum in the butanol and ethyl acetate fractions of methanol extract of leaf and bark.

Keywords: *Alstonia scholaris*, Antibacterial Activity, bark, clinical strain, leaf.

INTRODUCTION

Approximately 20% of plant products found in the world have been subjected to pharmacological or biological tests[1]. The systematic screening of antimicrobial plant extracts represents a continuous effort to find new compounds with the potential to act against multi-resistant pathogenic bacteria and fungi. A special feature of higher Angiosperm plants is their capacity to produce a large number of organic chemicals of high structural diversity. The accumulation of phytochemicals in the plant cell cultures has been studied for more than thirty years and the generated knowledge has helped in realization of using cell cultures for production of desired phytochemicals[2]. The antimicrobial activity of Solananceae and Apocynaceae members has been well documented in the literature[3]. These include *Cestrum diurnum*[4], *Capsicum annum*[5], *Withania* spp[6], *Picralima nitida*[7-8], *Alstonia macrophylla*, *Alstonia scholaris*, *Voacango foetida*, *Wrightia* spp[9] and *Rauvolfia serptentina*[10]. In ethno pharmacology research the antimicrobial susceptibility test (AST) is used to determine the efficacy of potential antimicrobials from biological extracts against a number of different microbial species. AST standard tests can be conveniently divided into diffusion and dilution methods. Agar diffusion techniques have been widely used to assay plant extracts for antimicrobial activity[11].

MATERIALS AND METHODS

Materials tested: Extracts (sequential extraction) of *Alstonia scholaris* (Leaf and Stem bark) - Hexane, Chloroform, Butanol, Ethyl acetate and water were tested for Anti bacterial activity.
Alstonia scholaris –leaf and stem bark were exclusively subjected to Methanol extraction followed by fractionation with Hexane, Chloroform, Ethyl acetate, Butanol and water, and were tested for anti bacterial activity.

Media used for Anti Bacterial studies
Muller Hinton Agar -(MHA)

Beef infusion	300 ml
Casein hydrolysate	17.5 g
Starch	1.5 g
Agar	10 g

Starch was emulsified in a small amount of cold water, then beef infusion, casein hydrolysate and the agar was added. Volume was made up to 1000 ml with distilled

Figure 1: Influence of concentration on inhibition resulting in increase in zone size
Ethyl acetate extract of Alstonia scholaris – bark tested against ATCC E coli strain 1µl of Ethyl acetate extract = 1.26µg, 10µl = 12.6µg, 20µl = 25.2µg, 30µl = 37.8µg. Solvent Control = 20µl Ethy acetate ; DMSO control = 20µl

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Table 1: Anti bacterial Testing by Disc Diffusion Method

Sl No	Test material & concentration	Organisms & zones of inhibition (mm)				
	Extracts incorporated on 6mm sterile discs	ATCC *E coli*	ATCC *S.aureus*	MRSA	*Providence stuartii*	ATCC *Ps aeruginosa*
1.	DMSO control	-	-	-	-	-
2.	Hexane –ASL(1 mg)	-	-	-	7 mm	-
	Hexane –ASB(0.5 mg)	-	-	-	-	-
	Hexane solvent control	-	-	-	-	-
3.	Chloroform –ASL(0.5 mg)	-	-	-	-	-
	Chloroform –ASB(0.5mg)	-	-	-	-	-
	Chloroform solvent control	-	-	-	-	-
4.	Butanol –ASL(0.25 mg)	-	-	8 mm	7 mm	-
	Butanol –ASB (0.25 mg)	-	-	-	-	-
	Butanol solvent control	-	-	-	-	-
5.	Ethy Acetate –ASL(0.25 mg)	10 mm	-	7 mm	7 mm	7mm
	Ethyl Acetate -ASB(0.25 mg)	10 mm	-	-	-	7mm
	Ethyl Acetate control	-	-	-	-	-
6.	Water -ASL (1 mg)	-	-	7 mm	7 mm	-
	Water -ASB (0.5 mg)	-	-	-	-	-
7.	Antibiotic control –Gentamycin	15 mm	15 mm	-	15 mm	16mm

Table 2: Anti bacterial activity against gram negative bacteria by well method

Test Organism		Solvent	20 µl/well - Solvent extracts & conc: (mg) & zone of inhibition in mm				
Sl no :	Bacteria	DMSO 20 µl/well	Hexane L 5 mg B 2 mg	Chloroform L 2mg B 2mg	Butanol L 1mg B 2mg	EA L 1mg B 1mg	Water L 4mg B 2mg
1.	ATCC *Esch. coli*						
	Leaf	-	-	-	5	5	-
	Bark		-	-	7	5	-
2.	*A.calcoaceticus*						
	B 309/11-Leaf	-		-	7	8	9
	Bark		7	-	10	7	7
3.	*Achromobacter*						
	C 70/11- Leaf	-	-	-	7	6	7
	Bark		-	-	8	6	7
4.	*Burkholderia*						
	B 196/11- Leaf	-	-	-	6	6	5
	Bark		-	-	6	6	5
5.	*Morganella morganii*						
	P 294/11-Leaf	-	-	-	5	6	-
	Bark		-	-	6	6	-
6.	*Proteus vulgaris*						
	Leaf	-	-	-	6	6	-
	Bark		-	-	5	5	-
7.	*Burkholderia maltophila*						
	P 198/10-Leaf						
	Bark	-	-	-	7	6	5
			-	-	7	6	-
8.	*Pantoea sp.*						
	S 73/11-Leaf	-	-	-	-	-	-
	Bark		-	-	7	7	-
9.	*Salmonella typhimurium*						
	Leaf						
	Bark	-	-	-	-	-	-
			-	-	-	-	-
10.	*Chryso indologenes*						
	E 579/11- Leaf	-	-	-	-	-	-
	Bark		-	-	9	7	-
11.	*Serratia marscesens*						
	B 159/11- Leaf	-	-	-	+/-	+/-	-
	Bark	-	-	-	6	6	-

Table 3: Anti bacterial activity against Gram positive bacteria by well method

Test Organism		Solvent	20 µl/well - Solvent extracts & conc: (mg) & zone of inhibition in mm				
Sl no:	Bacteria	DMSO 20 µl/well	Hexane L 5 mg B 2 mg	Chloroform L 2mg B 2mg	Butanol L 1mg B 2mg	EA L 1mg B 1mg	Water L 4mg B 2mg
1.	ATCC						
	S.aureus-Leaf	-	-	-	7	7	7
	A.aureus Bark	-	5	-	7	7	7
2.	*S.citreus*						
	Leaf	-	10	10	13	11	25
	Bark	-	8	7	30	10	14
3.	*Coryn pseudo diptheriticum*						
	B 398/11-Leaf	-	-	-	-	-	14
	Bark	-	-	-	8	-	-
4.	Diptheroids			-	-	-	-
	Leaf	-	15	12	12	12	15
	Bark		20	15	30	14	-
5.	*Enterococcus faecium*						
	Leaf	-	-	-	-	-	-
	Bark	-	8	9	15	10	10
	Streptococcus pyogenes						
6.	Leaf	-	-	-	-	-	-
	Bark	-	-	-	9	8	-

Table 4: *Alstonia scholaris* Bark-Ethyl acetate extract showing gradations of inhibition with increasing concentrations

Organism	Solvent (Ethyl acetate) control	Solvent (DMSO) control	Ethyl acetate extract of AS bark 10µl 12.6µgms	20µl 25.2µgms	30µl 37.8µgms
E. coli ATCC	-	-	8mm	12mm	14mm
S.aureus ATCC	-	-	8mm	8mm	9mm
Ps.aeruginosa ATCC	-	-	9mm	9mm	10mm
Serratia marscescens	-	-	7mm	8mm	9mm
Aeromonas SCTIMST O-756/03	-	-	8.5mm	10mm	11mm

water. All the constituents were dissolved by heating gently at 100ºC with agitation.

It was filtered and pH adjusted to 7.4. The medium was distributed into stock bottles and autoclaved at 121lbs for 20 minutes. Autoclaved medium was poured into sterile flat bottomed Petri plates in a laminar flow hood, allowed to solidify and stored in a cold room (4^0C) for later use.

<u>Nutrient Broth</u>

Beef extract	3 g
Peptone	5 g
Sodium chloride	5 g
Distilled water	1000 ml
pH	6.8

Broth was distributed in tubes and sterilized in the autoclave for 15 minutes at 15 pounds pressure (121^0C).

Nutrient broth was used in the tube dilution method for testing the sensitivity of microorganisms to antibiotics.

0.85% Normal Saline (Sodium chloride 8.5 g and Distilled water 1000ml) was distributed into tubes and autoclaved at 15 pounds pressure (121^0C) for 15 minutes.

Techniques Used: Antibacterial studies were done by 3 methods -

1. Disc diffusion[12].
2. Well method - cutting wells of 3 mm diameter and adding the various extracts[13].
3. Incorporating the various extracts into the medium before solidifying[14].

Preparation of sterile discs: Whatman filter paper No: 1 was cut into discs of 6mm diameter, dispersed in Petri plates, and sterilized by dry heat in an hot air oven for 160^0C for 60 minutes.

Table 5: Anti bacterial studies by disc diffusion using ethyl acetate extracts of leaf and bark of *Alstonia scholaris*

No:	Organism	Test sample, conc.:, Zone of inhibition (mm) ASL-Ethyl acetate (extract dried and dissolved in DMSO) 40 µg/disc	ASB-Ethyl acetate (extract dried and dissolved in DMSO) 25.2 µg/disc
1.	ATCC *Ps aeruginosa*	10	8
2.	ATCC S aureus	7	7
3.	ATCC Kl pneumoniae	13	9
4.	ATCC S aureus P-R strain	8	7
5.	ATCC E coli	12	8
6.	*Salmonella group B*	8	7
7.	*Aeromonas*	14	10
8.	*Pantoea*	9	10
9.	*Brevun dimunita*	14	9
10.	*Moraxella lacunata*	12	10
11.	*Ps putida*	14	8
12.	*Kl pneumoniae*	13	9
13.	*Citrobacter koseri*	-	9
14.	*E coli*	9	8
15.	*Serratia marscens*	12	10
16.	*Enterobacter cloacae*	11	8
17.	*S typhi*	12	9
18.	*Proteus mirabilis*	14	9
19.	*Aeromonas salmonicida*	13	13
20.	*Atypical E coli*	15	8
21.	*Stenotrophomonas maltophila*	8	9
22.	*Staph aureus (MRSA)*	8	7

ASL = *Alstonia scholaris* leaf, ASB = *Alstonia scholaris* bark

Table 6: Different clinical strains inhibited on the Ethyl acetate extract plate ASL.

No	Organism tested	ASL EA fraction1 ml (315mg)in 10 ml Nutrient agar	Control with 1 ml Ethyl acetate in Nutrient agar
1.	*ATCC 27853 Ps aeruginosa*	NG	4+
2.	*ATCC 25923 S aureus*	1+ growth	4 + growth
3.	*ATCC 700603 Kl pneumoniae*	No growth	4+ growth
4.	*ATCC 25022 E coli*	No growth	4+ growth
5.	*P573 CNStaph SCTIMST isolate*	1+ growth	4+ growth
6.	*P571 Klebsiella SCTIMST isolate*	1+ growth	4+ growth
7.	*E 1568 Ps aeruginosa SCTIMST isolate*	No growth	4 + growth
8.	*E 1570 Ps aeruginosa SCTIMST isolate*	No growth	4+ growth
9.	*E 1571 Kl pneumoniae SCTIMST isolate*	No growth	4+ growth
10	*E 1572 Klebsiella SCTIMST isolate*	No growth	4+ growth
11	*U1103 E coli SCTIMST isolate*	No growth	4+ growth
12	*U 1077 Acinetobacter SCTIMST isolate*	No growth	4+ growth
13	*E 1552 S aureus SCTIMST isolate*	1+ growth	4+ growth
14	*Qc 10 Salmonella typhi*	No growth	4+ growth

NG- No growth; 1+ to 4+ relative density of growth

Disc diffusion method: Agar diffusion methods employing dried filter paper discs impregnated with specific concentrations of antimicrobial agents/extracts were developed in the 1940's. In order to eliminate or minimize variability in the testing, Bauer et al in 1966 developed a standardized procedure in which Mueller Hinton Agar (MHA) was selected as the test medium[15]. Necessary MHA plates were dried for about 30 minutes in a 37^0C incubator just before use. Bacterial strains to be tested were freshly sub cultured, tested for purity and suspensions in one ml nutrient broth were made, incubated at 37^0C for 2 hours to obtain cultures in a log phase, opacity was checked with 0.5 McFarland turbidity standard (approximately 1 to 2 x 10^8 colony forming units per ml) and plated on the entire surface of the MHA plates using sterile swabs. The entire agar surface was

Table 7: Growth density in 5% extracts in the medium

No	Organism (Test strain)	10 ml MHA in the control plate	10 ml MHA+.5 ml DMSO	10 ml MHA+.5 ml (150mg) ASL extract - Butanol fraction	10 ml MHA+.5 ml (175mg) ASL extract-EA fraction	10 ml MHA+.5 ml (0mg) ASL extract-water fraction	10 ml MHA+.5 ml (150mg) ASB extract Butanol fraction
1	*ATCC - S. aureus*	4+	4+	0	0	3+	0
2	*ATCC - E coli*	4+	4+	0	0	2+	0
3	*ATCC –Ps aeruginosa*	4+	4+	0	0	3+	0
4	*ATCC – Kl pneumoniae*	4+	4+	0	0	4+	0
5	*E-579/11 Chrys indologenes*	4+	2+	0	0	1+	0
6	*Corynebacterium*	1+	+/+	0	0	0	0
7	*U-497/11 Acinetobacter baumannii*	4+	4+	0	0	4+	0
8	*B1238/10 Kodomea*	3+	0	0	+/-	2+	0
9	*B594/10 Candida*	3+	0	0	1+	4+	0
10	*U 611/10 Candida albicans*	4+	1+	0	2+	2+	1+
11	*P 70/10 Nocardia*	4+	3+	0	0	0	0
12	*Staph citreus*	4+	4+	0	0	3+	0

0 (no Growth); 1-4 + gradation of relative density of growth

streaked, turning the plate 60^0C between streaking to obtain an even inoculation. Sterile discs incorporated with 5 µl of the various *Alstonia scholaris* leaf and bark extracts along with the respective solvents were placed on the plated MHA plates in an equidistant manner and incubated at 37^0C incubator for 18 hours. Zone of inhibition was measured.

Well method: MHA plates dried in the 37^0C incubator were used. Six 3 mm (dia) wells equidistant were aseptically punched on the agar surface using a sterile cork borer, pure cultures of test strains in the log phase and turbidity adjusted were swabbed on the surface of MHA plates under aseptic conditions. Twenty µl of each extract and 20µl of respective solvents was filled in one of the wells to rule out nonspecific inhibition using sterile micro tips and the plates were incubated at 37^0C for 18 hours. The zones of inhibition were noted in mm.

Extracts incorporated in the media**:** Antibacterial studies were done by incorporating the various extracts in autoclaved MHA cooled to 50^0C in a water bath and plating the various strains of bacteria. Control plates with the respective solvents incorporated in a similar manner were prepared and the same test strains of bacteria were streaked on the plate to rule out non specific inhibition by the solvents.

RESULTS AND DISCUSSION

In our study (Table 1) fractions of *Alstonia scholaris* leaf extracts seemed to show antibacterial activity, especially against Methicillin Resistant *Staphylococcus aureus* (MRSA) and the clinical strain – *Providence stuartii*. Hussain et al in 2010 reported antibacterial activity mainly from Methanolic extracts of bark of *Alstonia scholaris* by disc diffusion[16].

Larger quantities of the extract could be introduced into the wells and most of the anti bacterial activity was spread out in the butanol and ethyl acetate fractions of the Methanol extract of *Alstonia scholaris* (Table 2 and Table 3) and our results are comparable to that done by Khan et al in 2003 who has also reported broad spectrum antimicrobial activity from the butanol fraction[14]. In our study the commensals like *Staphylococci citreus* and Diptheroids were most susceptible to all the fractions of methanol extracts. It was an interesting finding to note that aerial and environmental bacteria are highly susceptible to *Alstonia scholaris* fractions because it was some of these environmental bacteria that aid spoilage of stored foods and grains.Toxic and harmful insecticides and chemicals now sprayed to check spoilage could in future be replaced by non toxic but bactericidal plant extracts.

Figure 1 and Table 4 show results of direct sequential extraction with ethyl acetate of *Alstonia scholaris* bark where detectable antimicrobial activity was noted in very small quantities and clear gradations could be noted with increasing quantities. Ethyl acetate extracts were dried and dissolved in DMSO and hence Ethyl acetate and DMSO were included as controls to rule out any nonspecific activity.

The ethyl acetate extract showed significant anti bacterial activity when compared to the control plate by complete inhibition or only 1+ growth. When extracts are incorporated in the medium inhibition of growth due to antibacterial moieties are more marked and evident than disc diffusion or well method.

Experiment on extracts incorporated in the Muller Hinton agar was repeated with another batch of *Alstonia scholaris* leaf extract to see if reproducibility in inhibition was obtained. Growth was graded from 0 to 4+ (complete inhibition to good growth). Bacterial cultures in the log phase were used for testing and turbidity was adjusted to

0.5 on the Density meter. Almost complete inhibition was seen in the Butanol fraction of both leaf and bark.

Medically important bacteria isolated from various sites such as urinary tract, respiratory tract and pus aspirates (Table 7) were tested against different extracts of *Alstonia scholaris* leaf and bark using solvents from Butanol to water incorporated in the medium and Ethyl acetate and Butanol extracts generally inhibited bacterial growth when compared to controls.

In all our studies on anti bacterial activity of *Alstonia scholaris* extracts maximum activity was obtained in the Butanol and Ethyl acetate fractions of either leaf or stem bark. The antibacterial effects of extracts when incorporated into the medium and different strains of test organisms were plated with corresponding controls; the inhibitory effect had been very marked. Khan et al in 2003 has reported similar findings that Butanol fraction exhibited antimicrobial activity compared to other solvent systems[14]. Goyal and Varshney in 1995 have stated that it is the chemical constituents such as alkanes, sterols and alkanols of *Alstonia scholaris* which were responsible for the antimicrobial property[17]. Thankamani et al and Misra et al in 2011 have found potent antimicrobial activity from flower and root extracts of *Alstonia scholaris*[18-19]. Khyade and Vaikos in 2009 have demonstrated antibacterial properties in the methanol extracts of *Alstonia scholaris* leaves as compared to chloroform and acetone while no inhibitory effect by petroleum ether extract[20]. The same group in 2010 has compared the phytochemical and antibacterial activities of bark extracts of *Alstonia scholaris* and *Alstonia macrophylla* and has demonstrated that chloroform extracts of *A. macrophylla* possessed broader spectrum of antibacterial activity when compared to *A.scholaris*[21]. However *Alstonia scholaris* is the widely used medicinal plant[21]. There are reports of *Alstonia boonei* to have antimicrobial properties in the ethanol and aqueous extracts of stem bark[22]. In our study we noted that commensal bacteria found in the natural environment such as Diptheroids and *Staph. citreus* to be highly inhibited by *Alstonia scholaris*, showing wide zones of inhibition. The phytochemical screening of the plant extracts showed not only the presence of bioactive components like alkaloids, flavonoids and steroids but also contained macro elements like calcium, magnesium, sodium, potassium and copper to varying degrees[22]. The medicinal property of *Alstonia scholaris* is by virtue of the presence of several phytochemical constituents which exhibit antimicrobial activity against several pathogenic micro-organisms[19]. The anti diarrhoea effects of the aqueous and the alcoholic bark extracts of *Alstonia scholaris* in mice have been reported by Patil et al in 1999[23].

CONCLUSION

The present study describes the antibacterial activity against various Gram positive and Gram negative bacteria which are important pathogenic strains opening the new field of sourcing out suitable antibiotics from plant extract of *Alstonia scholaris*. However to reach the firm conclusion about their potency of anti-bacterial activity, more detailed bioassays needs to be performed.

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