



# ***In vitro* Antibacterial Activity of n-Hexane Fraction of Methanolic Extract of *Alstonia scholaris* L. R.Br. Stem Bark against Some Multidrug Resistant Human Pathogenic Bacteria**

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## **ABSTRACT**

Plants are a source of wide range of bioactive molecules. *Alstonia scholaris*, a tree species of Apocynaceae family is being reported as a well known herbal remedy of various diseases. In the present investigation, n-hexane fraction of crude methanolic extract of *Alstonia scholaris* Linn. R.Br. stem bark was evaluated for antibacterial activity using four human pathogenic multi-drug resistance bacterial strains *Enterobacteriaceae bacterium IK1\_01*, *Shigella dysentery*, *Enterobacter cloacae* and *Serratia marcescens*. Antibiotic susceptibility tests were also performed to evaluate the multi-drug resistance patterns of these strains. All the strains showed multi-drug resistance against several commercially available antibiotics. The n-hexane fraction showed significant inhibitory activities against all the strains by agar-diffusion assay. The n-hexane fraction of crude methanolic extract of stem of *A. scholaris* showed MICs of 5.5 mg/ml, 5 mg/ml, <5.5 mg/ml and 8 mg/ml, and induced a maximum of 85.7%, 95.6%, 89.3% and 94.4% growth inhibition against *E. bacterium IK1\_01*, *S. dysentery*, *E. cloacae* and *S. marcescens*, respectively.

**Keywords:** *Alstonia scholaris*; n-hexane; methanolic extract; antibacterial; agar-diffusion assay; multi-drug resistance.

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## 1. INTRODUCTION

Development of multi drug resistance to the antibiotics is a major cause of concern. Emergence of newer disease causing pathogens and evolution of existing microorganisms are responsible for human morbidity and mortality. Plants being a rich source of novel biologically active molecules may serve as a natural remedy against these pathogens. Also the high cost of conventional antimicrobial treatments particularly in Asian and African countries necessitates the use of plant based natural medicine for therapeutic purpose.

*Alstonia scholaris* Linn. R.Br. (Apocynaceae) is an evergreen tree of the Indian subcontinent and South East Asian countries. Different parts of the plant have long been used as a potent phytomedicine in traditional as well as Ayurvedic, Unani, Homoeopathy and Sidhha/Tamil types of alternative medicinal systems. Traditionally the plant is being used against a number of human ailments such as asthma, epilepsy, fever, malaria, diarrhea, dysentery, skin diseases, wound, earache, leprosy, leucorrhea, hepatitis, snakebite etc. Pharmacological efficacy of the plant species has been investigated for anticancerous, anti-inflammatory, analgesic, antipsychotic, wound healing, anti asthmatic, expectorant, antioxidant, free radical scavenging, immunostimulating, hepatoprotective, broncho-vasodilatory, antiparasitic, antidiarrhoeal, larvicidal, molluscicidal, piscicidal, antiplasmodial, antomalarial, antileishmanial, schizonticidal, antiparasitic and phytotoxic properties (Dey, 2011). Several phyto-constituents have been isolated from the bark of the plant (Gupta et al., 2002; Feng et al., 2009). Pharmacological efficacy of the bark of the plant species is being reported by many authors (Gupta et al., 2002; Jahan et al., 2009; Jahan and Goyal, 2010). Crude extract of the plant has also shown biological activity (Kulkarni and Juvekar, 2009; Shah et al., 2010).

Antibacterial (Khan et al., 2003; Khyade and Vaikos, 2009; Dutta et al., 2010) antimycobacterial (Macabeo et al., 2008), antidiarrhoeal (Shah et al., 2010) and antifungal (Riaz et al., 2010) activities of the plant have been reported. The present paper demonstrates *in vitro* bioactivity of n-hexane fraction of methanolic extract of stem bark of *A. scholaris* against four human pathogenic bacterial strains such as *Enterobacteriaceae* bacterium *IK1\_01*, *Shigella dysentery*, *Enterobacter cloacae* and *Serratia marcescens* which had shown multi-drug resistance against several commercially available antibiotics. The results support ethnomedicinal use of the plant species against several microbial ailments and also open up a possible remedy against multi drug resistance bacteria.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

The stem bark of 3.5 years old *A. scholaris* plant was collected during the month of January, 2011 from Sonarpur-Subhashgram, South 24 Parganas, West Bengal, India. The plant was of 8 ft in height and has shown seasonal flowering last year (September). The plant samples were identified by a Taxonomist and a voucher specimen was preserved in Molecular Biology Laboratory, Department of Zoology and Molecular Biology & Genetics, Presidency University.

## 2.2 Bacterial Strains

Four bacterial strains were used in this study. All of these strains were environmental isolates and common human pathogens. SN3b was *E. bacterium IK1\_01*, SN8a was *E. cloacae*, SN4a and SN8b were *S. dysentery* and *S. marcescens* respectively. A battery of biochemical tests like MR (Methyl Red Test), VP (Voges-Proskauer Test), TSI (Triple Sugar Iron Agar Test), tests for gelatinase, degradation of starch, indole production, citrate, oxidase and motility tests were performed and the bacterial strains were identified using Bergey's Manual of Determinative Bacteriology. Sequencing of 16srDNA (Busse et al., 1992), followed by phylogenetic analysis was done as confirmatory tests.

*E. bacterium IK1\_01* and *E. cloacae* are members of the Enterobacteriaceae, which are Gram negative, facultative-anaerobic, rod shaped bacteria. Enterobacteriaceae, particularly *E. cloacae* is important nosocomial pathogen responsible for various infections, including bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, and ophthalmic infections. This can also cause various community-acquired infections, including UTIs, skin and soft-tissue infections, and wound infections, among others.

*S. dysentery* is a Gram-negative, non-spore forming, facultative-anaerobic, non-motile bacteria, spread by contaminated water and food, causes the most severe dysentery because of its potent and deadly Shiga toxin.

*S. marcescens* is a species of Gram-negative, rod-shaped bacterium in the family Enterobacteriaceae. A human pathogen, *S. marcescens* is involved in nosocomial infections, particularly catheter-associated bacteremia, urinary tract infections and wound infections. It is commonly found in the respiratory and urinary tracts of hospitalized adults and in the gastrointestinal system of children.

## 2.3 Extraction

The chopped stem bark segments were air dried at ambient temperature (27-29°C) for seven days and powdered using a mixer grinder. The fine powder (to 40 mesh size) (1kg) was soaked thrice in methanol at room temperature (26°C) for 7 days. The material was filtered each time using Whatman No. 1 filter paper and the filtrate was concentrated and evaporated to dryness in vacuum at 40°C. Thus a crude methanolic extract of *A. scholaris* stem bark was prepared. The crude extract (125 g) was suspended in distilled water (100ml). Partitioning was done in n-hexane (3x100ml) and the yield was 100g. The concentrations of n-hexane fraction were prepared as 0.05, 0.1, 0.25 and 0.5 mg/ml by dissolving the appropriate amount of residue in the solvent. The dry extracts were refrigerated at -4°C for future use.

## 2.4 Antibiotic Susceptibility Test

Antimicrobial susceptibility testing was performed by the disc diffusion method (Thakurta et al., 2007) with commercially available disks (HiMedia, Mumbai, India) of ampicillin (10 µg); chloramphenicol (30µg); co-trimoxazole (25µg); erythromycin (15µg); gentamicin (10µg); kanamycin (30µg); ofloxacin (5µg); streptomycin (10µg); tetracycline (30µg); vancomycin (30µg). Culture suspensions were obtained after incubation at 37 °C in 5 ml Mueller–Hinton broth (MHB) (HiMedia) for 4–5 h and spread on Mueller–Hinton agar (MHA) (HiMedia). The

plates were incubated at 37 °C for 24 h. Isolates were considered susceptible, reduced susceptible, or resistant to a particular antimicrobial agent on the basis of the diameters of the inhibitory zones that matched the criteria of the manufacturer's interpretive table, which followed the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 2002). The American Type Culture Collection (ATCC) strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used for quality control.

## 2.5 Screening for Antimicrobial Activity

Antibacterial activity of the extract was determined by agar-diffusion assay (Reeves, 1989). Bacterial strains were first grown in MHB under shaking condition for 4 h at 37 °C and after the incubation period, 1ml of culture were spread on MHA plate. In the inoculated MHA plate, wells were made by using sterile 6mm cork borer. The wells were filled with 200µl of the plants extracts (re-suspended in n-hexane) and blanks (n-hexane). The concentrations of extract employed were 10, 25, 50, and 100 mg/ml. Tetracycline (150 µg/ml, 200µl) was used as antibacterial positive control and the ATCC strain *E. coli* ATCC 25922 was included for quality assurance. Zone diameter was measured after 24 h incubation at 37 °C.

## 2.6 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC of the extract were assessed using the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997, 1999). An inoculum of the microorganism was prepared from 24 h MHB cultures and suspensions were adjusted with turbidity equivalent to that of a 0.5 McFarland standard. Suspensions were further diluted 1:10 in sterile MHB to obtain a final inoculum of  $5 \times 10^5$  CFU/ml. The 96-well round bottom sterile plates were prepared by dispensing 180 µl of the inoculated broth into each well. A 20 µl aliquot of the plant extract was added. The concentrations of plant extract tested were 0.05, 0.1, 0.25 and 0.5 mg/ml. Dilutions of tetracycline served as positive control, while broth with 20 µl of n-hexane was used as negative control. The ATCC strain *E. coli* ATCC 25922 was included for quality assurance purposes. Plates were covered and incubated for 24 h at 37 °C. After incubation, minimum inhibitory concentrations (MIC) were read visually; all wells were plated to nutrient agar (Hi-Media) and incubated. The minimal bactericidal concentration (MBC) was defined as a 99.9% reduction in CFU.

## 2.7 Statistical Analysis

Values are expressed as mean  $\pm$  S.D. Statistical significance was determined using Student's *t*-test. Values with  $p < 0.05$  were considered significance.

# 3. RESULTS

## 3.1 *In-Vitro* Antibacterial Activity of *A. scholaris* Stem Bark Extract

Strains of four bacterial cells, *E. bacterium* IK1\_01, *S. dysentery*, *E. cloacae* and *S. marcescens* were tested to evaluate the antibacterial activity of the n-hexane fraction of crude methanolic extract of *A. scholaris* stem bark. Antibiotic susceptibility tests were performed with these strains to evaluate the multi-drug resistance patterns of these strains.

All the strains showed multi-drug resistance against several commercially available antibiotics (Table 1).

**Table 1: Antibiotic Susceptibility Test results of the strains used in this study**

Strains	Bacterial Strain	Sensitive	Resistant
SN3b	<i>Enterobacteriaceae</i> bacterium IK1_01	A, C, G, OF, S, T,	E, CO, K, VA,
SN4a	<i>Shigella dysentery</i>	A, C, G, K, OF, T	E, CO, S, VA,
SN8a	<i>Enterobacter cloacae</i>	C, G, K, OF, S, T,	A, CO, E, VA,
SN8b	<i>Serratia marcescens</i>	A, C, G, K, OF, T	E, CO, S, VA,

[A, ampicillin (10 µg); C, chloramphenicol (30µg); CO, co-trimoxazole (25µg); E, erythromycin (15µg); G, gentamicin (10 µg); K, kanamycin (30µg); OF, ofloxacin (5µg); S, streptomycin (10µg); T, tetracycline (30µg); VA, vancomycin (30µg)]

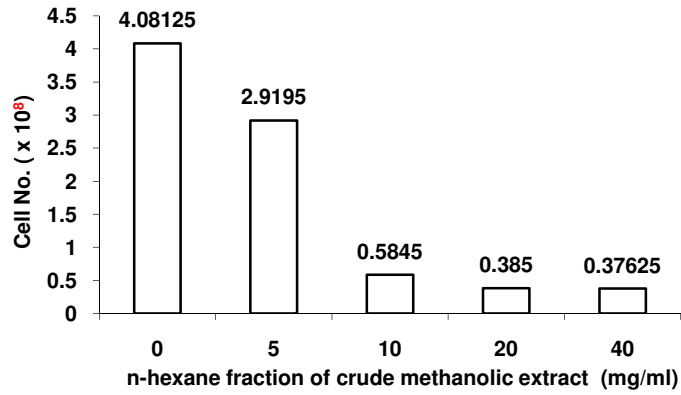
However, the n-hexane fraction of crude methanolic extract of stem of *A. scholaris* showed inhibitory activities against all the strains by agar-diffusion assay with significance ( $p < 0.05$ ) (Table 2). Thus the active plant extract in this study showed antibacterial activities against all the multi-drug resistant strains, all of which can cause serious clinical problems.

**Table 2: Antibacterial activity of *A. scholaris* stem bark extract on four bacterial strains**

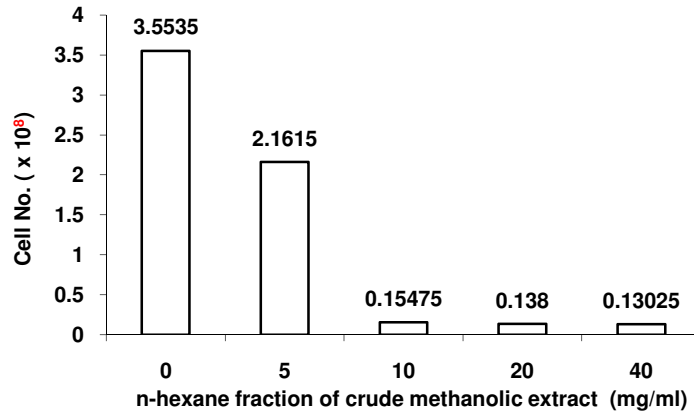
Bacterial strain	Zone of inhibition diameter (mm)				
	n-hexane fraction of methanolic extract (200 µl/ well)				Control (n-hexane)
	200mg/ml	100mg/ml	50mg/ml	25 mg/ml	
SN3b	15.7 ± 0.5	12.8 ± 0.6	10.9 ± 0.8	8.8 ± 0.7	0.00
SN4A	16.3 ± 0.9	13.3 ± 0.8	11.5 ± 1.2	8.5 ± 0.9	0.00
SN8A	17.5 ± 0.8	14.5 ± 1.1	12.1 ± 0.8	9.4 ± 0.9	0.00
SN8B	17.5 ± 0.5	14.8 ± 0.7	12.7 ± 0.9	9.8 ± 1.3	0.00

Antibacterial activity was expressed in terms of diameter of zone of inhibition (mean ±S.D., n = 3).  $p < 0.05$  compared to control (n-hexane) is considered significant.

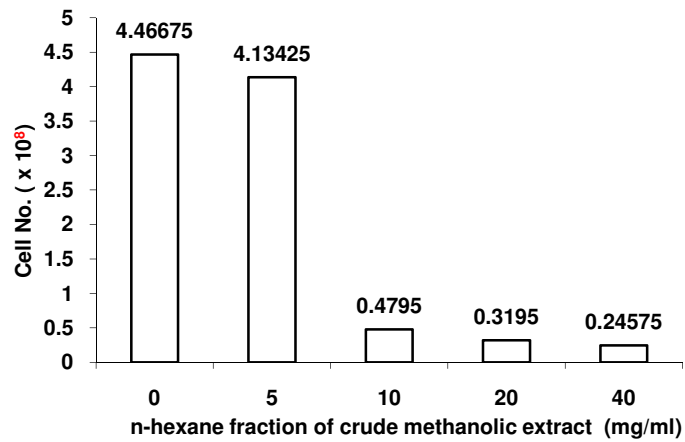
For determination of MIC and MBC for the n-hexane fraction of crude methanolic extract against the bacterial strains, the concentration ranges tested were from 0.05 to 40 mg/ml. The n-hexane fraction of crude methanolic extract of stem of *A. scholaris* showed MICs of 5.5 mg/ml, 5 mg/ml, <5.5 mg/ml and 8 mg/ml, and induced a maximum of 85.7%, 95.6%, 89.3% and 94.4% growth inhibition against *E. bacterium IK1\_01*, *S. dysentery*, *E. cloacae* and *S. marcescens* respectively (Fig. 1 and 2), The minimum bactericidal concentration for the extract was 12 mg/ml, 10 mg/ml, 11 mg/ml and 15 mg/ml respectively.



a)



b)



c)

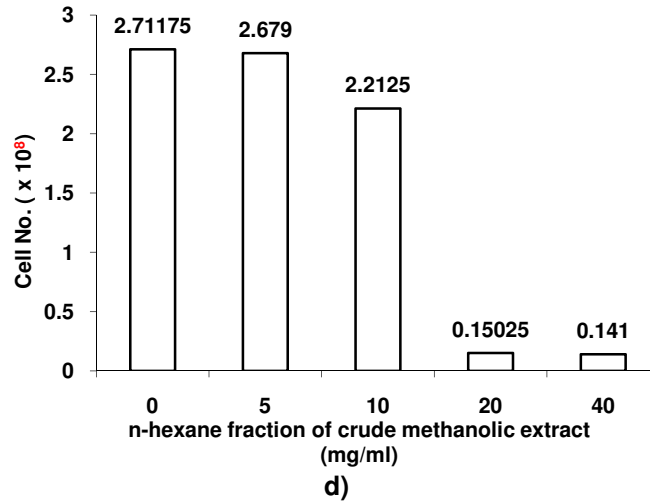


Figure 1: Bar Diagram showing decrease of a) *E. bacterium* IK1\_01, b) *S. dysentery*, c) *E. cloacae* and d) *S. marcescens* cell number in liquid Mueller- Hinton Broth with increased concentration of n-hexane fraction of crude methanolic extract of *A. scholaris* stem bark.

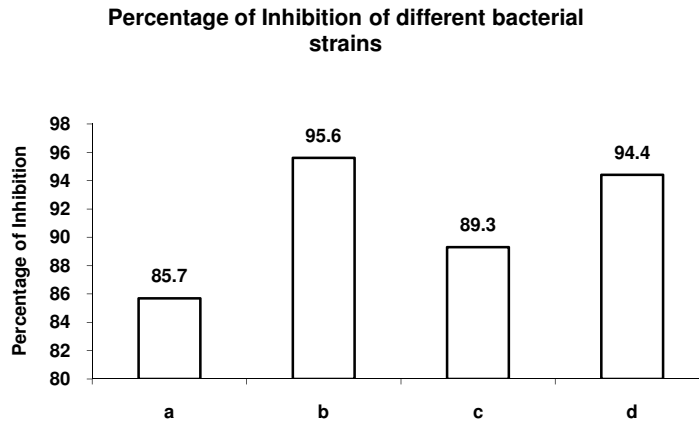


Figure 2: Bar Diagram showing percentage of inhibition of different bacterial strains by n-hexane fraction of the crude methanolic extract of *A. scholaris* stem bark. a) *E. bacterium* IK1\_01, b) *S. dysentery*, c) *E. cloacae* and d) *S. marcescens*

#### 4. DISCUSSION

Butanol fraction of methanolic extract of *A. scholaris* had shown broad spectrum antimicrobial activity (Khan et al., 2003). Methanolic leaf extract was found to inhibit *Bacillus subtilis* the most followed by *Escherichia coli* and *Staphylococcus aureus* (Khyade and Vaikos, 2009). Mature seed oil extracted in hexane had shown to contain saturated and unsaturated fatty acids. Antibacterial property of the oil was also demonstrated (Dutta et al., 2010). Crude extract of the plant was found to protect castor oil-induced diarrhea in mice. The antidiarrheal efficacy was noted to be mediated by calcium channel blockade (Shah et

al., 2010). Antibacterial activity of n-hexane fraction of different plant extracts has been evaluated by many authors (Akhter et al., 2008; Walter et al., 2011; Bulbul et al., 2011). Chemical characterization of n-hexane fraction (Green et al., 2011) has been reported to contain non polar compounds (Ahmad et al., 2011). Different types of terpenoids, fatty acids and sterols have been reported from the hexane extracts of botanicals (Seca and Silva, 2008). Antimicrobial activity of terpenoids (Ulubelen et al., 2003), fatty acids (Skalicka-Woźniak et al., 2010), volatile oils (Dorman and Deans, 2000) and sterols (Zhao et al., 2005) has been investigated. Terpenoids, fatty acid and sterols have also been reported in *A. scholaris* (Wang et al., 2009; Khyade and Vaikos, 2009; Dutta et al., 2010). Antibacterial efficacy of the hexane extract could have been contributed by the non-polar compounds.

Development of drug resistance in bacteria is a common clinical problem. Use of plant derived bioactive molecules have been reported in many cases (Ahmad and Beg, 2001) such as methicillin resistant *Staphylococcus aureus* (MRSA) (Linuma et al., 1996; Aqil et al., 2005), multi-drug resistant tuberculosis (MDR-TB) (Jimenez-Arellanes et al., 2003; Camacho-Corona Mdel et al., 2008), multidrug-resistant enteric bacteria (Ahmad and Aqil, 2007) etc.

## 5. CONCLUSION

The four bacterial strains used in this experiment are responsible for an array of human diseases such as infections of skin, wound, gastro intestine, urinary and respiratory tract. However, these multi drug resistance human pathogenic strains were significantly inhibited by the n-hexane fraction of the methanolic stem extract of *A. scholaris*. Therefore, the study provides support to the plants' traditional and alternative use against skin diseases, wound, asthma, diarrhea, dysentery, other gastrointestinal diseases and urinary tract infections. Further, the active biomolecules present in the extract has to be characterized against these multi drug-resistant bacteria. Use of natural products has been encouraged due to less or no side effects, cost effectiveness and development of resistance to conventional synthetic antibiotics. *A. scholaris* has been investigated both *in vitro* and *in vivo*. Crude extract of the plant and isolated compounds have shown pharmacological activity. The efficacy of crude extract should be compared with that of isolated compounds in order to reveal the possible role of synergistic interaction of the phyto-constituents against different ailments. Positive results from the experiments might incorporate the herbal medicine to future drug discovery programs through the rigors of clinical trials.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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