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Original Research

Phytochemical screening of *Alstonia scholaris* leaf and bark extracts and their antimicrobial activities

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Abstract: *Alstonia sholaris* is an evergreen tree commonly found in South East Asia. In traditional medicine pharmacological activities are attributed to the leaves and bark of this plant. The aim of this study is characterizing the chemicals present in *A. sholaris* leaves and bark extracts and study their antimicrobial activities. Solvent extractions with Soxhlet apparatus of leaves and bark were obtained using hexane, benzene, isopropanol, methanol, and water. The crude extracts were concentrated and screened for qualitative phytochemical analysis and thin layer chromatography, and the antibacterial, antifungal an antiviral activity of crude extracts were measured by *in vitro* methods. Isopropanol and methanol extracts showed significant antibacterial activity and it was more pronounced against Gram positive than against Gram negative bacteria. Hexane, benzene, isopropanol and methanol fractions of *A. scholaris* bark and leaf showed activity against *Enterobacter cloacae*. Isopropanol extract showed maximum activity against selected human pathogenic fungus. In conclusion, the leaves and bark of *A. scholaris* are rich in phytochemicals with antimicrobial activities against human pathogens, being the isopropanol fraction the one with the highest antibacterial, antifungal, antiviral and anti-mycobacterial activities.

Key words: *Alstonia scholaris*; Phytochemical; Antimicrobial; Antibacterial; Antifungus.

Introduction

Microbes are very important microorganisms of human pathogens, which cause diseases and death. One of the most important therapeutic discoveries of the 20th century was the antimicrobial agents. However, with the "antibiotic era" barely five decades old, mankind is now faced with the global problem of emerging resistance in virtually all pathogens (1). Resistance has been developed in antivirals, antimycotics and almost all groups of antibiotics (2). This is indeed quite alarming when considering that in 1990, out of the 39.5 million of death in the developing world, 9.2 million were estimated to have been caused by infectious and parasitic diseases and that 98% of death in children in developing countries resulted mostly from infectious diseases (3). Antibiotics are very much prevalent now a day due to indiscriminate use and abuse of antibiotics (1). In human medicine alone, the US Centre for Disease Control and Prevention estimates that approximately one-third of the 150 million prescriptions for antibiotics written each year were not needed. It is of utmost importance to find appropriate solutions associated with drug resistance (4).

New antimicrobial agents are needed to treat diseases in humans caused by drug resistant microorganisms. In addition, there is a continuing consumer demand for cosmetic products as well as natural, preservative-free, microbiologically safe foods now days (5-9).

The antimicrobial properties of extracts from natural sources have been studied for nearly 60 years. During the past 20-25 years, interest in their antimicrobial nature has expanded due to increased resistance of pathogenic microbes to currently employed antimicrobial drugs, and toxicity or adverse host reactions of other anti-infectives (10). The antimicrobial extracts exhibit their activity either by lysis or disruption of the outer membrane of microbes. Others interact with specific internal targets or cause pore formation and leakage (11).

Plants are complex chemical storehouses of undiscovered biodynamic compounds with unrealized potential for use in modern medicine (12-16). It has long been established that naturally occurring substances in plants have anti-bacterial, anti-viral and anti-fungal activities(17). In India, secondary metabolites or medicinal plants, for centuries, have been used for the treatment of

a wide range of ailments, many of which are still in use today and hold favored positions among local traditional practitioners (18).

Alstonia scholaris R.Br. (Apocynaceae) is a tropical evergreen native to South and Southeast Asia. Different plant parts have been used for a wide spectrum of ailments in traditional medicinal systems such as China, India, Thailand, Malaysia, Philippines, Africa and Australia (19, 20); however, *A. scholaris* is traditionally used to treat infectious diseases. Several researchers have evaluated the antimicrobial potential of different parts of *A. scholaris* such as leaves, stembark, root, and flower to evaluate its antimicrobial potential (21, 22) and cytotoxic and antioxidant activities (23). The aim of the present study is characterizing the chemicals present in *A. sholaris* leaves and bark extracts and investigate their antibacterial, antiviral and antifungal activities.

Materials and Methods

Collection of samples

Samples of fresh leaves and bark of *A. scholaris* (Fig. 1) were collected from the university campus in Kariavattom, Trivandrum (India). The samples were processed by shade drying for 4 days. The sample was finely powdered in a blender, weighed and stored in dry polythene bags. Weight of the powder was taken.

Solvent extraction

The dry powdered material was subjected to successive organic solvent extraction by refluxing in the Soxhlet apparatus each for 12 h. The solvents used were nonpolar to polar consisting of hexane, benzene, isopropanol, methanol, and water. Each fraction was collected when no further elution of compounds was observed. The collected extracts were subjected to vacuum drying and stored in sterile containers in the refrigerator till further analysis.

Phytochemical analysis of plant extracts

Prior to starting of the experiment the phytochemical extracts were dissolved in dimethyl sulfoxide (DMSO) except water extract, which was dissolved in distilled water (24).

Chemical test for carbohydrate

Fehling solution test: 200 µL of the extract was boiled over water bath at 60ºC. 200 µL of Fehling A and 200 µL of Fehling B solutions were added to the test tube. A red precipitate indicates the presence of carbohydrate.

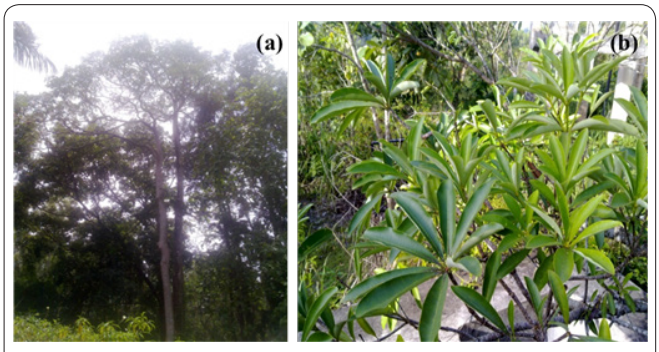

Figure 1. *Alstonia scholaris*: (a) plant and (b) leaves.

Chemical test for proteins and amino acids

Ninhydrin test: the test is used to detect the presence of alpha-amino acids and proteins containing free amino groups. To 200 µL of the extract few drops of ninhydrin reagent was added and boiled over water bath, formation of purple color indicates a positive test.

Chemical test for alkaloids

Wagner's test: to 200 µL of the extract add few drops of Wagner's reagent (dilute iodine solution) to the sides of the tube. Formation of reddish-brown precipitate indicates a positive result.

Chemical tests for steroid and triterpenoid glycosides

Salkovaski test: alcoholic extract of drug was evaporated to dryness and extracted with $CHCl_3$, add conc. H_2SO_4 from sidewall of test tube to the $CHCl_3$ extract. Formation of yellow colored ring at the junction of two liquids, which turns red after 2 min indicates positive test.

Chemical tests for cardiac glycosides

Keller Killiani test: to 200 µL of the drug add 100 µL of glacial acetic acid containing 1 drop of ferric chloride solution followed by 100 µL of con. H_2SO_4. A brown ring at the interface indicates a deoxysugar characteristic of cardienolides. A violet ring may appear below the brown ring, while in acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Test for oils and fats

Spot test: a small quantity of the extract was pressed between two filter papers. Oil stain on the paper indicated the presence of fixed oil and fats.

Chemical tests for phenolic compounds

chloride test: to the mixture of 200 µL of the extract and 2 mL of distilled water, was added a few drops of 5% ferric chloride along the sides of the test tube. A dark green color showed the presences of phenolic compounds.

Phytochemical screening by thin layer chromatography (TLC)

The n-hexane and isopropanol extracts of *A. scholaris* were subjected to silica gel layer chromatographic (TLC) separation using ready-made TLC plates (silica gel G 60 F^{254}, Merck). 1 mg of dried active extracts (n-hexane and isopropanol extracts of *A. scholaris*) was dissolved in isopropanol or n-hexane separately and 10 µL of the respective extract was spotted on TLC plate using capillary tubes. For spotting two extracts (n-hexane and isopropanol) of *A. scholaris,* TLC plate with a dimension of 4.5 x 10 cm was used. The spotted TLC plates were resolved using n-hexane: chloroform: methanol (5:4:1, v/v) solvent system in a chromatographic chamber. The resolved plates were examined under UV light at 356 nm and photographed without derivatization. Since all bands were not visible, TLC plates were developed by spraying with anisaldehyde-sulphuric acid reagent and heated at 60ºC.

Phytochemical screening by *GC-MS analysis*

Homogenized A. scholaris bark isopropanol frac-

tion was subjected to GC-MS analysis. GC-MS analysis was performed using Agilent GC 7890A & MSD 5975C system equipped with an Elite-I, fused silica capillary column; J&W DB-5 (30 m x 0.25 mm x 0.25 µm). For GC-MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1.2 mL/min. Oven temp. 60°C – hold (5 min), 5°C raise to 250°C – hold (10 min), in let temp. 250°C, auxiliary temp. 275°C. MSD source temp. 230°C, MSD quad temp. 150°C. The elutes were automatically passed into a mass spectrometer with a dictator voltage set at 1.8 kV and sampling rate of 0.1 s. The spectrum of the unknown component was compared with the spectrum of the known component stored in the NIST (National Institute Standard and Technology) library.

Bioactivity assays

The crude extract of each plant part was subjected to *in vitro* methods like antibacterial, antifungal and antiviral activities.

Antibacterial activity of crude extracts by well diffusion method

Crude extracts were tested to detect their antibacterial property against a group of human pathogens by well diffusion method. The bacterial cultures used were obtained from the Collections of Standard Microorganisms maintained at Department of Biotechnology, University of Kerala, Trivandrum. They consisted of *Proteus spp*, Shigella *spp*, *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella* spp, *Salmonella typhi*, *Salmonella paratyphi A,* MDR strain of *Klebsiella* spp, and MDR strain of *Escherichia coli.* In addition, the antibacterial activity of the different extracts against *Staphylococcus haemolyticus* (C 330/12), *S. aureus* (ATCC no: 25923) and *E. coli* (ATCC no: 25922), was compared with standard antibiotic streptomycin.

Stock cultures were maintained at 4°C on slopes of nutrient agar. A pure single colony grown on an agar plate was transferred to 5 mL of peptone water and incubated for 2 h at 37°C.

Antibacterial activity against Enterobacter cloacae dissolvens

For neutralizing activity testing for various solvent extracts, 50 mg each of the various dried plant extracts was dissolved in 1 mL of DMSO. The test organism used was a 4 h young culture containing 10^5/mL colonies. After overnight incubation of the extract and test organism, a loop full of it was plated on MacConkey agar to check for growth.

Anti-Mycobacterial activity against atypical Mycobacterium

Neutralizing activity of various solvent extracts was tested using 50 mg of the various dried plant extracts, which was dissolved in 1000 µL of DMSO. The test organism used was a 5 days old culture containing 10^5/mL colonies of *Mycobacterium*. After overnight incubation of the extract and test organism a loop full of it was plated on 5% sheep blood agar to check for growth.

Media for bacterial culture

Nutrient agar media

Nutrient agar plate (Hi-media) was prepared by dissolving nutrient agar (37 g/L) in distilled water. The media were sterilized in an autoclave at 121°C for 15 min and poured in sterile Petri dishes. The Petri dish was dried, kept for 24 h for sterility check up. Sterile plates only were selected for bacterial cultures (25).

Muller Hinton agar (MHA)

Starch was emulsified in a small amount of cold water and then beef infusion, casein hydrolysate and the agar were added. Volume was made up to 1 L with distilled water. All the constituents were dissolved by heating gently at 100°C with agitation. It was filtered and pH adjusted to 7.4. The media was then distributed into stock bottles and autoclaved at 121ºC for 20 min. Autoclaved medium was then poured into sterile flat bottomed petri plates in a laminar flow hood and allowed to solidify and stored in a cold room (4ºC) for later use.

Plates were prepared and wells of 3 mm, 6 mm and 8 mm diameter were cut using a sterile borer. 100 µL of each of the 2 h culture of test bacteria was placed on the nutrient agar. The inoculum was swab bed uniformly over the entire agar surface and allowed to dry for 5 min. 80 µL of various extracts dissolved in DMSO was loaded into the wells. Plates were incubated at 37°C for 24 h. DMSO was used as negative control and streptomycin (10 µg/80 µL) as positive control. At the end of the incubation period, inhibition zones were measured.

MacConkey agar

MacConkey agar plates (Hi-media) were prepared by dissolving MacConkey agar (55.07 g/L) in distilled water. The medium was heated to boiling to dissolve the medium completely sterilized by autoclaving at 121°C for 15 min and poured in to sterile Petri dishes. The Petri dishes were dried, kept for 24 h for sterility check up. Only sterile plates were selected for bacterial cultures.

5% sheep blood agar

Dissolved trypticase soy agar base (Hi-media) and autoclaved. Cool the steriled blood agar base to 45°C to 50°C. Aseptically added 50 ml of sterile defibrinated blood. Mixed thoroughly, to avoid accumulation of air bubbles. Dispensed in to sterile tubes or plates while in liquid.

Antifungal activity of crude extracts

Crude extracts of plants were subjected to fungal studies to detect their fungicidal properties against human pathogens, plant pathogens and industrially important strains of fungi by incorporating crude extracts in the Sabouraud dextrose agar (SDA) media used for fungal culture. The following standard strains of fungi were used for the study: *Penicillium* marneffei, Cryptococcus spp, Candida spp, Penicillium spp, Epidermophyton spp, Microsporum spp, Fusarium spp, Aspergillus flavus, Aspergillus niger, *Rhizopus and Aspergillus fumigatus.*

Preparation of the media for fungal culture

SDA slants were prepared by dissolving SDA (Hi-

media, 67 g/L) in distilled water The media were sterilized in an autoclave at 121°C and 1.05 kg/cm^2 and poured in to sterile culture tubes (25 mL capacity), 5 mL in each tube. To each tube 0.5 mL of particulate crude extract was added. Contents were mixed well by shaking the tubes and allowed to set to form slants. The slants were kept for sterility check before use. Negative control tubes were treated with solvents only. Fungal culture were inoculated on SDA slopes and incubated at room temperature at 30-32°C for 5 to 7 days. The results were compared with standard fungicide (imidazole). Fungal cultures were inoculated to SDA crude extract slants and kept at room temperature for 5 to 7 days.

Antiviral activity of plant extracts

In vitro antiviral activity against Hepatitis B virus by neutralization test

HepG2.2.15 cells were cultured in MEM (Hi-media) containing 10% fetal calf serum (FBS) and gentamycin 20 µg/100 mL medium at 37°C in a humidified incubator gassed with 5% CO_2. 50 mg of the extract was dissolved in 1 mL of DMSO and in the Hepatitis neutralization test 500 µL of the extract containing 25 mg was used in the test.

500 µL of various fractions of plant extracts were added to 500 µL of the MEM medium in which HepG2.2.15 cell line established growth was taken in various tubes and incubated overnight for neutralization to occur. Each of the tubes was tested for quantitating the Hepatitis B surface antigen after 24 h of incubation at room temperature using ELFA test.

Principle of ELFA test (enzyme-linked fluorescent immunoassay)

The solid phase receptacle (SPR) serves as the solid phase as well as the pipetting device for the assay. At each stage of the reaction, it aspirates the reagents in and out, thus preventing any inter-reagent or inter-sample contamination. The reagents for the assay are ready to use and pre-dispensed in the sealed reagent strips. The strip consists of 10 wells covered with a labeled foil seal. The label comprises a bar code, which mainly indicates the assay code, kit lot number, etc. The foil of the first well is perforated to facilitate the introduction of the sample. The last well of each strip is a cuvette in which the fluorometric reading is performed. The wells in the center section of the strip contain various reagents required for the assay. The interior of the SPR is coated during production with monoclonal anti-HBsAg antibody (mouse). Each SPR is identified by the HBS code. All the steps of the assay were performed automatically by the instrument, VIDAS®–Auto immuno analyser (Bio Merieux). The reaction medium is cycled in and out of the SPR several times. After a preliminary washing step, the antigen present in the sample will bind simultaneously to the monoclonal antibody coating the interior of the SPR and to the antibody conjugated with biotin. Unbound sample components are washed away. The antigen bound to the solid phase and to the biotynilated antibody is in contact with streptavidine conjugated with alkaline phosphatase, which will bind with biotin. Another wash step follows and removes unbound components. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolyses of the substrate into a fluorescent product (4-methyl-umbelliferone). The fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of antigen present in the sample. At the end of the assay, results are expressed as an index calculated using a standard. The sensitivity of the assay is with 0.12 ng/mL.

Once the assay is completed, the computer analyzes the results automatically. Fluorescence is measured twice in the Reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The relative fluorescence value (RFV) is calculated by subtracting the background reading from the final result. RFV < 0.13 is taken as negative and RFV > 0.13 is considered positive.

Results

Yield from extracts

Fresh leaves of A. scholaris were collected and weighted 350 g and on drying it, approximately 125 g powder was obtained similarly the 460 g of stem bark of the A. scholaris on drying give around 185 g. Yield of different extract is shown in Table 1. The yield in A. sholaris leaf was maximum in water extract (10.3 g) followed by methanol (6.5 g), isopropanol (5. 5 g), hexane (4.8 g) and least in benzene (3.2 g). In A. scholaris stem bark the yield was maximum in water and least in benzene extraction. The nature of the crude extracts is shown in Table 2.

Table 1. Percentage of yield of *Alstonia scholaris* leaf and bark different extracts.

Sample	Leaf	Bark
Hexane	3.8%	3.1%
Benzene	2.5%	1.7%
Isopropanol	4.4%	3.7%
Methanol	5.2%	4.2%
Water	8.2%	5.9%

Table 2. Nature of the crude extract of *Alstonia scholaris* leaf and bark.

	Odour		Colour		Consistency	
Sample	**Leaf**	**Bark**	**Leaf**	**Bark**	**Leaf**	**Bark**
Hexane	Pungent	Pungent	Dark Brown	Dark Brown	Sticky	Sticky
Benzene	Pungent	Pungent	Light green	Brownish	Powder	Powder
Isopropanol	Sharp tingling	Pungent	Dark Brown	Dark Brown	Sticky	Sticky
Methanol	Pungent	Chocolate	Dark Brown	Dark Brown	Sticky	Sticky
Water	Pungent	Chocolate	Dark Brown	Dark Brown	Sticky	Sticky

Table 3. Phytochemical analysis of *Alstonia scholaris* leaf and bark.

	Leaf					Bark				
Name of the test	**Hex**	**Ben**	**Iso**	**Met**	**Wat**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**
Alkaloid Wagner's test	++	++	++	++	++	++	++	++	++	++
Tanin and Phenolic Compounds $FeCl_3$ Test	++	-	++	++	++	-	-	-	++	-
Cardiac Glycosides Keller Killiani	-	-	-	-	-	-	++	-	-	-
Carbohydrate Fehling's Test	-	-	+	+	+	-	-	+	+	+
Amino acids Ninhydrin	-	-	-	-	-	-	-	-	-	-
Oil and fat Spot test	-	-	-	-	-	-	-	-	-	-
Terpenoids Salkowski test	-	-	++	-	+	++	-	-	++	++

Hexane (Hex), benzene (Ben), isopropanol (Iso), methanol (Met), water (Wat).

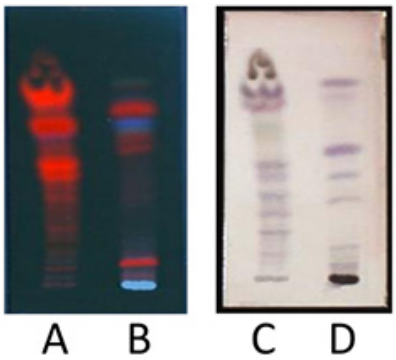

Figure 2. TLC profile of isopropanol (A, C) and hexane (B, D) extracts of *Alstonia scholaris* bark under UV (A, B, 356 nm) and white light (C, D).

Phytochemical screening

The results of qualitative phytochemical screening of hexane, benzene, isopropanol, methanol and water extracts of *A. scholaris* leaf and bark revealed the presence of alkaloids, carbohydrates, tannins, terpenoids, saponins, flavonoids, steroids and fixed oils and fats as mentioned in Table 3.

Thin layer chromatography

Figure 2 shows the class of compounds present in hexane and isopropanol extracts of *A. scholaris* bark. UV fluorescence (at 356 nm) of components present in the hexane and isopropanol extracts of *A. scholaris* was given in the fig B, A. The hexane extract showed 13 number florescent components while isopropanol extracts contains 18 fluorescent compounds. The fig D and C showed various bands (components) present in the hexane and isopropanol extracts of *A. scholaris* bark after developed with anisaldehyde-sulphuric acid reagent. A total of 11 components present in hexane extract while-15 number of compound were present in isopropanol extract of *A. scholaris* bark.

GC-MS analysis

Table 4 represents the active principles with their molecular formula, molecular weight (MW), retention time, probability percentage, total percentage content and fragment peak of compounds identified in A. scholaris. The prevailing compounds were hexadecanoic acid methyl ester, 1,4-benzenedicarboxylic acid dimethyl ester, and dodecanoic acid methyl ester.

Antibacterial activity of crude extracts

Results of antibacterial activity with different solvent extracts of *A. scholaris* leaf and bark are presented in Table 5 (nutrient agar) and Table 6 (Muller Hinton agar).

Results showed that except isopropanol and methanol extract none of the other extracts showed no activity against the selected strains. On nutrient agar, bark extracted with isopropanol, methanol, exhibited comparatively strong activity against most of the gram positive and MDR stains. The isopropanol extracts had activity against *Proteus* spp, *Shigella* spp, *Salmonella typhi, Pseudomonas aeruginosa, E.coli, Klebsiella* spp *Salmonella paratyphi A, Staphylococcus aureus* and MDR strains of *E.coli* and *Klebsiella* spp. The methanol extract of bark showed antibacterial activity against all of selected organism except *Shigella* spp.

Hexane, benzene, isopropanol and methanol fractions of A. scholaris bark and leaf showed activity against *Enterobacter cloacae* (Table 7). Water extract was not active.

Antifungal activity of crude extracts

The antifungal potential of different extracts of leaf of *A. scholaris* are presented in Table 8. The hexane, benzene, isopropanol, methanol and water extracts of leaf strongly inhibited the growth of fungus include human pathogenic strains. It was observed that the isopropanol, methanol and water extract of leaf inhibited the growth of *P. marneffei*, *Cryptococcus* and *Candida*. Hexane, benzene and water extract inhibited *Aspergillus niger*. The water and hexane extract showed inhibition against the growth of *Rhizopus* spp.

Hexane, benzene, isopropanol and methanol fractions of A. scholaris bark shows activity against atypical *Mycobacterium* (Table 9). Isopropanol and methanol fractions of A. scholaris leaf as well as benzene, isopropanol shows activity. Hexane and benzene fraction of A. scholaris leaf hexane fraction was not active.

Table 4. GC-MS Analysis and Mass Spectral Data of *Alstonia scholaris* bark isopropanol fraction

Peak	Compound	Molecular formula	Molecular weight (g/mol)	Retention time (min)	Probability (%)	Content (%)	Fragment peaks (m/z)							
							1	%	2	%	3	%	4	%
1	Nonadecan-1-ol trimethylsilyl ether	$C_{22}H_{48}OSi$	356	11.116	20.6	7.11	73	100	341	76.98	74	53.35	342	26.83
2	1-Pentene, 4,4-dimethyl-1,3-diphenyl-1-(trimethylsilyloxy)-	$C_{22}H_{30}OSi$	338	12.920	19.3	1.86	73	100	281	64.86	147	54.15	327	24.22
3	1,4-Benzenedicarboxylic acid, dimethyl ester	$C_{10}H_{10}O_4$	194	13.099	72.7	11.76	163	100	194	23.32	135	21.32	103	12.81
4	Dodecanoic acid, methyl ester	$C_{13}H_{26}O_2$	214	13.256	72.9	9.97	74	100	87	61.26	55	18.41	143	12.31
5	Diethyl phthalate	$C_{12}H_{14}O_4$	222	14.27	57.5	2.10	149	100	177	24.22	150	12.51	176	10.21
6	1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trimethylsiloxy) tetrasiloxane	$C_{14}H_{42}O_5Si_6$	458	15.291	27.6	1.01	73	100	355	72.47	147	42.24	221	37.93
7	Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$	228	16.354	32.8	5.68	74	100	87	64.9	55	19.2	143	17.3
8	4-Hydroxyphenylpyruvic acid	$C_9H_8O_4$	180	19.331	56.9	1.64	163	100	181	69.9				
9	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	22.417	71.4	15.93	74	100	87	72.6	55	23.6	75	20.2
10	9-Dodecenoic acid, methyl ester, (E)-	$C_{13}H_{24}O_2$	212	29.257	59.4	4.90	55	100	69	73.7	83	59.7	57	59.3
11	Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$	228	30.056	29.7	2.39	74	100	87	76.9	55	26.9	75	22.1

Table 5. Inhibition zone of antibacterial activity of *Alstonia scholaris* leaf and bark (concentration 50 mg/mL) on nutrient agar.

			Leaf					Bark				
Name of the bacteria	**Con**	**DMSO**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**
Proteus spp.	13	R	R	R	9	12	R	R	R	10	9	R
MDR of *Escherichia coli*	10	R	R	R	R	9	R	R	R	12	6	R
Shigella spp	R	R	R	R	R	9	R	R	R	5	R	R
Salmonella paratyphi A	R	R	R	R	7	15	R	R	R	9	12	R
Pseudomonas aeruginosa	6	R	R	R	10	10.5	R	R	R	12	10	R
Klebsiella spp.	11	R	R	R	14	8	R	R	R	15	7	R
Escherichia coli	11	R	R	R	5	8	R	R	R	10	9	R
Salmonella typhi	7	R	R	R	12	13	R	R	R	13	12	R
MDR of *Klebsiella*	R	R	R	R	12	10	R	R	R	14	7	R
Staphylococcus aureus	18	R	R	R	R	9	R	R	R	13	9	R

Control (Con, NO 12 Streptomycin), DMSO (dimethyl sulfoxide), hexane (Hex), benzene (Ben), isopropanol (Iso), methanol (Met), water (Wat), multidrug-resistant (MDR), resistance (R).

Table 6. Inhibition zone of antibacterial activity of *Alstonia scholaris* leaf and bark (concentration 50 mg/mL) on Muller Hinton agar.

		Leaf					Bark				
Name of the bacteria	**DMSO**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**
C 330/12 *S. haemolyticus*	R	R	R	R	R	R/+	R	+/R	+/R	+/R	R
ATCC *S. aureus* strain no: 25923	R	R	R	R	R	6	R	R	R	R	R
ATCC *E. coli* strain no: 25922	R	R	R	7	5	7	R	R	R	4	R

Table 7. Neutralizing activity of various solvent extracts of *Alstonia scholaris* against Gram negative bacilli – *Enterobacter cloacae* dissolvens.

Leaf					Bark				
Hex	**Ben**	**Iso**	**Met**	**Wat**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**
S	S	S	S	4+	S	S	S	S	4+

Hexane (Hex), benzene (Ben), isopropanol (Iso), methanol (Met), water (Wat), sensitive (S), growth (+), confluent growth (4+). 50 µL of solvent extracts incubated over night with 50 µL of 10^5/mL of the test organism.

Table 8. Antifungal activity *Alstonia scholaris* leaf (concentration 80 mg/mL).

Name of the fungus	**Hex**	**Ben**	**Iso**	**Met**	**Wat**
Penicillium marneffei	-	-	+	+	+
Cryptococus	-	-	+	+	+
Candida	-/+	-	+	+	+
Penicillium spp	-	-	-	+	-
Epidermophyton	-	-	-	-	-
Microsporum	-	-	-	-	-
Fusarium	-	-	-	-	-
Aspergillus flavus	-	-	-	-	-
Aspergillus niger	+	+	-	-	+
Rhizopus	+	-	-	-	+
Aspergillus fumigatus	-	-	-	-	-

Hexane (Hex), benzene (Ben), isopropanol (Iso), methanol (Met), water (Wat). No growth (-), growth (+).

Table 9. Neutralizing activity of various solvent extracts of *Alstonia scholaris* leaf and bark against an isolate of atypical *Mycobacterium.*

Control	Leaf					Bark				
DMSO	**Hex**	**Ben**	**Iso**	**Met**	**Wat**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**
4+	4+	4+	S	S	4+	S	S	S	S	4+

Hexane (Hex), benzene (Ben), isopropanol (Iso), methanol (Met), water (Wat), sensitive (S), growth (+), confluent growth (4+). 50 µL of solvent extracts incubated over night with 50 µL of 10^5/mL of the test organism. Reading on 5th day.

Table 10. Neutralizing activities of various fractions of solvent extracts of the plant extracts of *Alstonia scholaris* leaf and bark against Hepatitis B virus produced in HepG2.2.15 cell line.

	Hep G 2.2.15		Leaf					Bark				
	Con	DMSO	Hex	Ben	Iso	Met	Wat	Hex	Ben	Iso	Met	Wat
ELFA Reading (RFV)	14.91	7.56	0.04	0.08	0.03	0.38	5.50	0.22	0.01	0.03	0.03	12.98
Interpretation	P	P	N	N	N	P	P	N	N	N	N	P

Control (Con), hexane (Hex), benzene (Ben), isopropanol (Iso), methanol (Met), water (Wat), relative fluorescence value (RFV), positive (P), negative (N). 500 µL of medium from bottles in which Hep G2.2.15 cell line was growing and 500 µL of extracts of plants incubated at room temperature.

Anti-Hepatitis B virus of crude extracts

Hexane, benzene and isopropanol fractions of A. scholaris leaf and bark showed activity against Hepatitis B virus (Table 10). Methanol fractions of A. scholaris bark also is active whereas methanol and water of leaf A. scholaris does not inhibit the growth of the Hepatitis B virus.

Discussion

A. scholaris is one of the highly-investigated plants and nearly 400 compounds have been isolated and characterized (26-31). The *Alstonia* species are rich in alkaloids, steroids, flavanoids and triterpenoids (32-34). The results reported by Misra, Pratyush (21) supported the results showed in present study, reporting the presence of alkaloids, carbohydrates, terpenoids, saponins, cardiac glycosides, flavonoids, steroids in various steam bark extract of *A. scholaris.*

Previous studies on *A. scholaris* have revealed the antibacterial and antioxidant properties (21, 22, 33, 35-39). Khyade and Vaikos (33) and Misra, Pratyush (21) supported the same activity of the methanol extract of *A. scholaris* leaf against the human pathogens. As the present study, several other studies have also reported similar antibacterial potential of the leaf methanolic extract (21, 33). Molly, Shekhar (22) tested hexane, chloroform, butanol, ethyl acetate and water fractions of methanol extracts of *A. scholaris* leaf and bark for antibacterial activity observing that fractions of leaf extract had pronounced antibacterial activity against methicillin resistant *S. aureus* and *Providence stuartii.* Moreover, in this study antibacterial activity of these extracts was tested against several Gram positive and Gram negative bacterial strains and was found to reside maximum in the butanol and ethyl acetate fractions of methanol extract of leaf and bark. Cowan (40) reported that the differences in the observed activities of the various extracts may be due to varying degree of solubility of the active constituents in the four solvents used. It has been documented that different solvents have diverse solubility capacities for different phytochemical constituents.

Thankamani (26) has reported the maximum inhibition, and it was activity against all the bacteria tested. Bioactive compounds in *A. scholaris* include alkanes, alkanols and sterols that possess antibacterial activity against certain Gram-positive and Gram-negative bacteria, further suggesting the broad-spectrum effect (41, 42). Various extract of *A. scholaris* possess antibacterial activity and contained tannins, flavonoids and alkaloid (43, 44) which are known to have antimicrobial activity (45, 46).

Khan, Omoloso (38) have reported that the extract of the leaf of A. scholaris fractions were ineffective against the moulds Aspergillus niger, A. rubrum, A. versicolor, A. vitis, Candida albican, C. tropicalis, Cladosporium cladosporioides, Penicillium notatum, Trichophyton mentagrophytes and T. tronsurum. A. scholaris showed fungicidal activity against pathogenic fungi. This may be due to phytoconstituents like flavanoids (47), phenolics and polyphenols (48), tannins (49), terpenoids (50), sesquiterpenes (51) etc., that are effective antimicrobial substances against a wide range of microorganisms. In another report A. scholaris showed fungicidal activity against pathogenic fungi (38).

The leaves and bark from A. scholaris are rich in phytochemicals with antimicrobial activities against human pathogens. On the basis of the results obtained in the present study it can be concluded that the isopropanol fraction of *A. scholaris* have antibacterial, antifungal, antiviral, and anti-mycobacterial activities. The present study may serve to be the basis of new studies to found new antimicrobials agents to be used in phytotheraphy or development of new drugs.

Conflicts of Interest

The authors declare no conflict of interest.

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