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Original Research

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

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Abstract: *Alstonia venenata* is a plant commonly found in South India and used in traditional medicine. The aim of this study was to characterize the phytochemicals present in *A. venenata* leaf 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 content and analyzed by thin layer chromatography. The antibacterial, antifungal and antiviral activities of crude extracts were measured by *in vitro* methods. Alkaloids, carbohydrates, tannins, phenolic compounds, terpenoids, cardiac glycosides and amino acids were found in the different crude extracts analyzed. Isopropanol extracts showed antifungal activity and it was more pronounced in the bark extract than the leaf extract. Moreover, the isopropanol extract exhibited antibacterial and antiviral activity. In conclusion, the leaves and bark of *A. venenata* have antimicrobial components which are more present in the isopropanol fraction.

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

Introduction

Phytochemicals are considered as novel templates of drug discovery for human ailments (1-3). Taking advantage of the knowledge of traditional medicine is a strategy used by some researchers to find new therapeutical components in the different extracts of the different part of the medicinal plants. Currently, drug resistance is a worldwide problem that need to be solved with the development of new drugs.

Alstonia venenata R.Br. (Apocynaceae) is a large shrub or a small tree that grows wildly in the hilly regions of South India (4, 5). In the Glossary of Indian Medicinal Plants (6) this plant is reported to be used in the treatment of epilepsy and insanity. Commonly, the plant was known as Analivegam (Malayalam) or Vishagni (Sanskrit) and the ancient texts attributed anti-venom activities to the plant (7). Several compounds have been identified in the plant, e.g., Sutha et al. (8) identified 23 compounds in ethanol extract by gas chromatography-mass spectrometry (GC-MS) analysis. Moreover, several alkaloids have been isolated from this plant such as venenatine, isovenenatine and alstovenine (9, 10). Venenatine is a 4-methoxyindole alkaloid and a major constituent of *A. venenata* bark (11) and the antifungal activity of this compound was reported by Singh et al. (12). Also, the antibacterial and antifungal activities of the leaf and bark extracts were also investigated (13, 14).

The aim of this study was characterizing the chemicals present in *A. venenata* leaf and bark extracts and study their antimicrobial activities.

Materials and Methods

Collection of samples

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

Solvent extraction

The dry powdered material was subjected to successive organic solvent extraction by refluxing in the

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

Soxhlet apparatus each for 12 h. The solvents used were nonpolar to polar (hexane, benzene, isopropanol, methanol, and water) and the collected extracts were subjected to vacuum drying and stored in sterile containers in the refrigerator.

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 (15).

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.

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 $FeCl_3$ 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

- **Ferric chloride test:** to the mixture of 200 µL of the extract and 2 mL of distilled water, was added a few drops of 5% $FeCl_3$ 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 isopropanol extract of *A. venenata* was subjected to silica gel thin layer chromatographic (TLC) separation using ready-made TLC plates (silica gel G 60 F^{254}, Merck). 1 mg of dried active extract was dissolved in isopropanol and 10 µL of the extract was spotted on the ready-made Silica gel G TLC plate using capillary tubes. For spotting single extract of *A. venenata,* TLC plates with a dimension of 3.0 × 10 cm were used. The spotted TLC plate was resolved using *n*-hexane: chloroform: methanol (5:4:1, v/v) solvent system in a chromatographic chamber. The resolved plate was examined under UV light at 356 nm and photographed without derivatization. Since all bands were not visible, TLC plate was developed by spraying with anisaldehyde-sulfuric acid reagent and heated at 60ºC.

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 the 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* sp, Shigella sp, *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella* sp, *Salmonella typhi*, *Salmonella paratyphi A,* MDR strain of *Klebsiella* sp, and MDR strain of *Esherichia coli.* In addition, the antibacterial activity of the different extracts against *Staphylococcus haemolyticus* (C 330/12), *S. aureus* (ATCC 25923) and *E. coli* (ATCC 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

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 1 mL 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 checkup. Sterile plates only were selected for bacterial cultures (16).

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 checkup. Only sterile plates were selected for bacterial cultures.

5% sheep blood agar

Sheep blood agar plates were prepared by dissolving trypticase soy agar base (Hi-media) and autoclaved. After cooling to 45°C - 50°C aseptically were added 50 mL of sterile defibrinated sheep blood, mixing thoroughly to avoid accumulation of air bubbles. The plates were 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 sp, Candida sp, Penicillium sp, Epidermophyton sp, Microsporum sp, Fusarium sp, Aspergillus flavus, Aspergillus niger, *Rhizopus* sp and *Aspergillus fumigatus.*

Preparation of the media for fungal culture

SDA slants were prepared by dissolving SDA (Himedia- 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 was 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. venenata* were collected and weighed 360 g, and on drying it, approximately 120 g powder was obtained. Similarly, 380 g of stem bark of the *A. venenata* on drying gave around 127 g. Yields of different extracts are shown in Table 1. The yield in *A. venenata* leaf was maximum in water extract (9.5 g) followed by methanol (5.6 g), isopropanol (4.9 g), hexane (4.3 g) and least in benzene (2.9 g). In *A. venenata* stem bark the yield was maximum in water and least in benzene extraction. The nature of the crude extracts is shown in Table 2.

Phytochemical screening

The results of qualitative phytochemical screening of leaf and stem bark of *A. venenata* revealed the presence of alkaloids, carbohydrates, tannins, terpenoids, cardiac glycosides, amino acids, saponins, flavonoids, steroids and fixed oils and fats as mentioned in Table 3.

Thin layer chromatography

Figure 2 showed the chromatogram of isopropanol

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

Sample	Leaf	Bark
Hexane	3.5%	2.6%
Benzene	2.4%	2.0%
Isopropanol	4.0%	4.0%
Methanol	4.6%	4.1%
Water	7.9%	6.8%

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

	Odor		Color		Consistency	
Sample	**Leaf**	**Bark**	**Leaf**	**Bark**	**Leaf**	**Bark**
Hexane	Pungent	Pungent	Dark Brown	Dark Brown	Sticky	Sticky
Benzene	Chocolate	Chocolate	Dark Brown	Dark Brown	Sticky	Sticky
Isopropanol	Pungent	Pungent	Dark Brown	Dark Brown	Sticky	Sticky
Methanol	Chocolate	Chocolate	Dark Brown	Dark Brown	Sticky	Sticky
Water	Light Pungent	Light Pungent	Brown	Brown	Powder	Powder

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

	Leaf					Bark				
Name of the test	**Hex**	**Ben**	**Iso**	**Met**	**Wat**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**
Alkaloid Wagner's test	++	-	-	++	++	++	-	-	++	++
Tannin 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).

extract of *A. venenata* leaf under UV (356 nm) (Fig 2A) and white light (after developing) (Fig 2B), respectively. The isopropanol extract showed 15 fluorescent compounds while after derivatization with anisaldehyde-sulfuric acid reagent the isopropanol extract showed 13 individual components under white light.

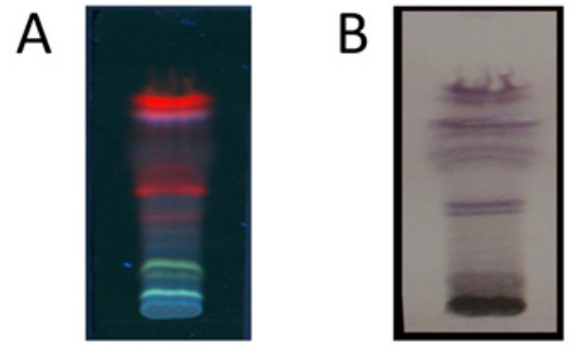

Figure 2. TLC profile of isopropanol extract of *Alstonia venenata* leaf under UV (A, 356 nm) and white light (B).

Antibacterial activity of crude extracts

Results of antibacterial activity of *A.* venenata leaf and bark is shown in Table 4 (nutrient agar) and Table 5 (Muller Hinton agar). The results of different solvent extracts of leaves show that isopropanol extract has very weak antibacterial activity against the selected strains (*Proteus* sp, *Shigella* sp*, P. aeruginosa, E. coli, Klebsiella* sp*, S. aureus* and MDR strains of *E. coli* and *Klebsiella* sp). On the other hand, our findings in bark extracts showed that isopropanol extract was the only active against the selected strains tested (*Shigella* sp, *P. aeruginosa, E. coli, S. aureus* and MDR strains of *E. coli* and *Klebsiella* sp).

Hexane, benzene, isopropanol and methanol fractions of *A. venenata* leaf shows activity against *Enterobacter cloacae* (Table 6). Hexane fractions of *A. venenata* bark shows activity whereas benzene, isopropanol, and methanol fraction show partial inhibition. Water extract is not active in any of the above tested plant part.

Antifungal activity of crude extracts

The antifungal potential of different extracts of leaf and bark of *A. venenata* are presented in Table 7. Isopropanol and benzene extracts of leaf and bark showed antifungal activity against selected human pathogenic fungi. The isopropanol leaf extracts had activity against *Cryptococcus* sp, *Candida* sp, *Fusarium* sp*, A. flavus* and *A. fumigatus.* The benzene leaf extract demonstrated the inhibition of growth against human pathogenic fungus includes *Cryptococcus* sp, *Candida* sp, *Fusarium* sp*, A. flavus* and *A. niger.* The leaf water extract showed inhibition against *Penicillium* sp, *Epidermophyton* sp*,*

Table 4. Inhibition zone of antibacterial activity of *Alstonia venenata* 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 sp	13	R	R	R	9	R	R	R	R	R	R	R
MDR of *Escherichia coli*	10	R	R	R	12	R	R	R	R	7	R	R
Shigella sp	R	R	R	R	5	R	R	R	R	11	R	R
Salmonella paratyphi A	R	R	R	R	R	R	R	R	R	R	R	R
Pseudomonas aeruginosa	6	R	R	R	10	R	R	R	R	5	R	R
Klebsiella sp	11	R	R	R	7	R	R	R	R	R	R	R
Escherichia coli	11	R	R	R	12	R	R	R	R	10	R	R
Salmonella typhi	7	R	R	R	R	R	R	R	R	R	R	R
MDR of *Klebsiella* sp	R	R	R	R	14.5	R	R	R	R	14.5	R	R
Staphylococcus aureus	18	R	R	R	12	R	R	R	R	7	R	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 5. Inhibition zone of antibacterial activity of *Alstonia venenata* 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	9	8	R/+	R	R	R	R	R
ATCC *S. aureus* strain no: 25923	R	R	R	7	9	R	R	R	R	R	R
ATCC *E. coli* strain no: 25922	R	R	R	R	R	10	R	R	R	R	R

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

Table 6. Neutralizing activity of various solvent extracts of *Alstonia venenata* against Gram negative bacilli – *Enterobacter cloacae.*

Leaf					Bark				
Hex	**Ben**	**Iso**	**Met**	**Wat**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**
S	S	S	S	4+	S	3 CG	1 CG	3 CG	4+

Hexane (Hex), benzene (Ben), isopropanol (Iso), methanol (Met), water (Wat), sensitive (S), growth (+), colonies of growth (CG), confluent growth (4+).

50 µL of solvent extracts incubated over night with 50µL of 10^5/mL of the test organism.

Table 7. Antifungal activity *Alstonia venenata* leaf and bark (concentration 80 mg/mL).

	Leaf					Bark				
Name of the fungus	**Hex**	**Ben**	**Iso**	**Met**	**Wat**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**
Penicillium marneffei	-	-	-	-	-	-	-	+	+	-
Cryptococcus sp	-	+	+	-	-	-	-	+	+	-
Candida sp	-	+	+	-	-	+	-	+	-	+
Penicillium sp	-	-	-	-	+	+	-	+	+	+
Epidermophyton sp	-	-	-	-	+	-	-	-	-	-
Microsporum sp	-	-	-	-	+	-	-	-	-	-
Fusarium sp	-	+	+	-	+	-	-	-	-	-
Aspergillus flavus	-	+	+	-	-	-	-	-	-	-
Aspergillus niger	-	+	-	-	-	+	-	-	-	+
Rhizopus sp	-	-	-	-	+	+	-	-	+	+
Aspergillus fumigatus	-	-	+	-	-	-	-	-	+	-

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

Table 8. Neutralizing activity of various solvent extracts of *Alstonia venenata* leaf and bark against an isolate of atypical *Mycobacterium.*

Control			**Leaf**				**Bark**			
DMSO	**Hex**	**Ben**	**Iso**	**Met**	**Wat**	**Hex**	**Ben**	**Iso**	**Met**	**Wat**
4+	S	3+	1+	+/-	4+	1+	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 9. Neutralizing activities of various fractions of solvent extracts of the plant extracts of *Alstonia venenata* 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	0.01	0.03	0.39	3.27	12.67	0.11	0.07	0.01	0.02	11.43
Interpretation	P	P	N	N	P	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.

Microsporum sp*, Fusarium* sp*,* and *Rhizopus* sp.

The bark isopropanol extract shows activity against *P. marneffei, Cryptococcus* sp*, Candida* sp*,* and *Penicillium* sp. The bark methanol extract demonstrate inhibition against human pathogenic fungus including*, P. marneffei, Cryptococcus* sp*, Penicillium* sp*, Rhizopus* sp*,* and *A. fumigatus.* The bark hexane and water extracts showed inhibition against *Candida* sp, *Penicillium* sp*, A. niger,* and *Rhizopus* sp.

Anti-mycobacterial activity of the plant extracts of *A. venenata* is represented in Table 8. Benzene, isopropanol and methanol fraction of the *A. venenata* bark showed activity. Hexane fraction of *A. venenata leaf* is active whereas benzene, isopropanol and methanol fractions are weakly active. Water extract is not at all active for any of the above tested plant parts.

Anti-Hepatitis B virus of crude extracts

Table 9 shows results against Hepatitis B virus. Hexane, benzene, isopropanol, and methanol fractions of *A. venenata* bark shows activity against Hepatitis B virus. In the case of A. venenata leaf, hexane and benzene fractions were active.

Discussion

A. venenata is an ethnobotanical plant scarcely studied and the majority of reports are about alkaloid presence in the plant (9, 17-20) and antifungal properties of these (12, 21). In a previous report, our group analyzed antioxidant and *in vitro* cytotoxic activities of *A. venenata* leaf and bark extracts (22). Moreover, Bhattacharya and Ray and Dutta (4) reported psychopharmacological activity of alstovenine and venenatine, an epimeric pair of 4-methoxyindole alkaloids isolated from the *A. venenata* bark.

Sutha et al. (23) reported that the ethanol extracts of the *A. veneneta* leaf shows the presence of alkaloids, terpenoids, coumarin, tannin, saponin, flavonoids, phenols, anthraquinones, quinones, carbohydrate, glycosides and starch. According with our results obtained, we also detected alkaloids, carbohydrates, tannins, phenolic compounds, terpenoids, cardiac glycosides and amino acids in the different crude extracts analyzed of *A. venenata* leaf and bark.

Several plant extracts presented antibacterial potential against human pathogenic strains (24-26). In a previous study, Shirly and Roshin and Manesh and Thankamani (5) tested antibacterial efficacy of butanol and methanol extracts of leaves, stem-bark, root-bark, flowers and fruits of *A. venenata* plant. The results of

this study showed that the extracts were highly active against Gram positive strains (*Micrococcus luteus* and *S. aureus*) than Gram negative strains (*Pseudomonas aerugenosa*, *Proteus vulgaris*, *E. coli*, *Klebsiella pneumoniae*, *Salmonella enteric typhimurium*, *S. typhi*, *S. paratyphi A*, and *Shigella* sp), butanol extracts were most active fractions than methanol extracts, and the bark parts presented higher activity than leaf extracts. In our study, only the isopropanol fraction showed antibacterial activity against the selected strains tested, and as in the study performed by Shirly and Roshin and Manesh and Thankamani (5) we also observed a highest activity from the bark extract than the leaf extract. The activity of the water extract against microbes investigated in this study is not in agreement with previous works which showed that aqueous extracts of plants generally exhibited little antimicrobial activities (27, 28).

A number of chemical compounds isolated from plant extract sources are antifungal (25) and alkaloids are a kind of these compounds (29). 3-Dehydroalstovenine is a 4-methoxyindole alkaloid and its psychopharmacological activity was studied along with its naturally occurring dihydro derivatives, alstovenine and venenatine (12). Singh and Sarma and Mishra and Ray (12) isolated these 4-methoxyindole alkaloids from A. venenata and reported their antifungal activity of against plant pathogenic fungi. Moreover, alstovenine, showed antifungal activity against pigmented and non-pigmented spores of several fungi (21, 30). In our study, isopropanol and benzene extracts showed antifungal activity but the Wagner's test not indicated the presence of alkaloid in these extracts. So, another kind of compounds such as tannins or phenols may be responsible of its antimicrobial activity (31). According to our knowledge we are the first to report the *in vitro* antiviral activity, anti-Hepatitis B virus, of *A. venenata*.

In conclusion, isopropanol fraction of *A. venenata* leaf and bark extracts presented antifungal, antibacterial and antiviral activities. More studies are needed to characterize which specific compounds perform this antimicrobial function and if there is synergy between these.

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Conflicts of Interest
The authors declare no conflict of interest.

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