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Original Research

Phytochemical screening of *Moringa oleifera* leaf extracts and their antimicrobial activities

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Abstract: *Moringa oleifera* is a tree native to tropical and subtropical regions of South India and used in traditional medicine. The aim of this study was characterize the phytochemicals present in *M. oleifera* leaf extracts and study their antimicrobial activities. Solvent extractions with Soxhlet apparatus of leaves were obtained using hexane, benzene, isopropanol, methanol, and water. The crude extracts were concentrated and screened for qualitative phytochemical analysis, and the antibacterial, antifungal and antiviral activities of crude extracts were measured by *in vitro* methods. Alkaloids, carbohydrates, tannins, phenolic compounds, terpenoids, cardiac glycosides, amino acids, oils and fats were found in the different crude extracts analyzed. Water and methanol extracts showed antibacterial activity against all selected bacteria, hexane and benzene extracts showed antifungal activity against all fungi tested, and hexane, benzene and isopropanol extracts showed activity against Hepatitis B virus. In conclusion, the leaves of *M. oleifera* have antimicrobial phytochemicals.

Key words: *Moringa oleifera*; Phytochemical; Antimicrobial; Antibacterial; Antifungus.

Introduction

Medicinal plant therapy has been shown to be beneficial for treatment of various human and animal diseases (1-3). *Moringa oleifera* Lam, also known as drumstick tree, possesses a wide range of medicinal and therapeutic properties (4, 5). This perennial tree is indigenous to foothills of Himalayas, is small, grows quickly, and its leaves are rich in amino acids, vitamins, minerals, and antioxidants (6-8). This plant has been consumed by humans in diverse culinary ways (9). The leaves could be eaten fresh, cooked, or stored as a dried power (10). The edible *M. oleifera* leaves contain essential provitamins including ascorbic acid, carotenoids (11) and tocopherols (12), and due to its nutritional value the powdered leaves are consumed by pregnant women and lactating mothers in under developed countries suffering from malnutrition (13). Moreover, for centuries this medicinal plant has been used to treat skin infections, anaemia, asthma, bronchitis, diabetes, epilepsy, diarrheas, and many other illness (4, 14). A chemical composition study of *M. oleifera* leaf performed by gas chromatography-mass spectrometry revealed a total of 35 compounds, the most important were n-hexadecanoic acid, tetradecanoic acid, cis-vaccenic acid, octadecanoic acid, palmitoyl chloride, β-l-rhamnofuranoside, 5-O-acetyl-thio-octyl, γ-sitosterol, and pregna-7-diene-3-ol-20-one (15).

Antimicrobial agents present in plants are a promising target for new drug discovery and new human therapies (16-20). Ethanolic (21-25) and chloroform (26, 27) extract of *M. oleifera* leaf showed antibacterial and antifungal activities. Moreover, methanolic extract of leaves demonstrated antimicrobial activity against urinary tract pathogens, such as *Escherichia coli*, *Klebsiella pneumoniae, Staphylococcus aureus*, and *Staphylococcus saprophyticus* (28-30). The aim of this study was characterize qualitatively the chemicals present in *M. oleifera* leaf extracts and study their antimicrobial activities.

Materials and Methods

Plant material

Fresh leaves of *M. oleifera* (Fig. 1) were collected from the university campus in Kariavattom, Trivan-

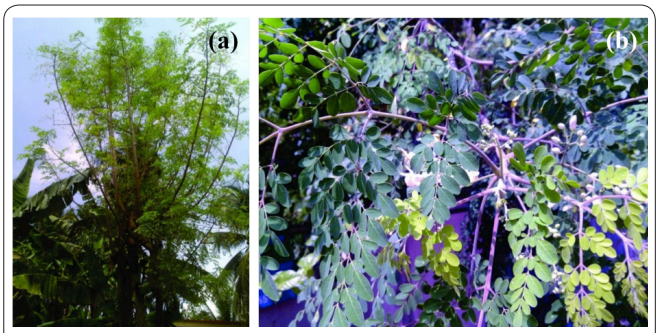

Figure 1. *Moringa oleifera*: (a) plant and (b) leaves.

drum (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 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 (31).

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 glycoside

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.

Bioactivity assays

The crude extract of *M. oleifera* leaf was subjected to *in vitro* methods like antibacterial and antifungal activities.

Anti-bacterial 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., *S. aureus, Pseudomonas aeruginosa, E. coli, Klebsiella* spp., *Salmonella typhi*, *Salmonella paratyphi A,* multidrug-resistant (MDR) strain of *Klebsiella* spp., and MDR strain of *E. 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 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 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 (32).

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 dis-

tilled 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

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 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., *Aspergillu 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 cultures were inoculated on sabouraud dextrose agar (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 enzyme-linked fluorescent immunoassay (ELFA) test.

Results

Yield from extracts

Fresh leaves of *M. oleifera* were collected and weighted 390 g and on drying it, approximately 115 g powder was obtained. Yield of different extract is shown in Table 1, the yield was maximum in water extract (8.7 g) followed by methanol (5.1 g), isopropanol (4.4 g), hexane (3.9 g) and least in benzene (2.2 g). The nature of the crude extracts is shown in Table 2.

Phytochemical screening

The results of qualitative phytochemical screening of hexane, benzene, isopropanol, methanol, and water extract of *M. oleifera* leaf revealed the presence of alkaloids, tannins, phenolic compounds, terpenoids, carbohydrates, amino acids, oil and fats fixed oils (Table 3).

Table 1. Yield of *Moringa oleifera* leaf different extracts.

Sample	Percentage
Hexane	3.4%
Benzene	1.9%
Isopropanol	3.8%
Methanol	4.4%
Water	7.5%

Table 2. Nature of the crude extract of *Moringa oleifera* leaf.

Sample	Odour	Colour	Consistency
Hexane	Sharp tingling	Dark Brown	Sticky powder
Benzene	Sharp tingling	Black	Sticky
Isopropanol	Pungent	Dark Brown	Sticky
Methanol	Alcoholic	Dark Brown	Sticky
Water	Pungent	Brown	Powder

Table 3. Phytochemical analysis of *Moringa oleifera* leaf.

Name of the test	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).

Table 5. Inhibition zone of antibacterial activity of *Moringa oleifera* leaf (concentration 50 mg/ml) on nutrient agar.

Name of the bacteria	Con	DMSO	Hex	Ben	Iso	Met	Wat
Proteus spp.	13	R	R	R	8	8	9
MDR of *Escherichia coli*	10	R	R	R	R	8	12
Shigella spp.	R	R	R	R	R	8	12
Salmonella paratyphi A	R	R	R	R	12	8	13
Pseudomonas aeruginosa	6	R	R	R	R	9	8
Klebsiella spp.	11	R	R	R	R	7	6
Escherichia coli	11	R	R	R	10	6	9
Salmonella typhi	7	R	R	R	12	5.5	9
MDR of *Klebsiella*	R	R	R	6	13	14.5	12
Staphylococcus aureus	18	R	R	R	12	8.5	9

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 *Moringa oleifera* leaf (concentration 50 mg/mL) on Muller Hinton agar.

Name of the bacteria	DMSO	Hex	Ben	Iso	Met	Wat
C 330/12 *S. haemolyticus*	R	R/+	R/+	R/+	8	R
ATCC *S. aureus* strain no: 25923	R	R	R	7	9	R
ATCC *E. coli* strain no: 25922	R	R	R/+	6	R	R

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

Alkaloids were present in all fractions.

Antibacterial activity of crude extracts

Results of antibacterial activity with different solvent extracts of *M. oleifera* leaf are presented in Table 5 (nutrient agar). The water extract demonstrated reasonable activity more than benzene, isopropanol, and methanol against human pathogenic bacteria including Gram negative and positive strains, and MDR strains. The isopropanol extract of leaf *M. oleifera* showed inhibitory activity against all of selected bacteria except *Klebsiella* spp., *P. aeruginosa, Shigella* spp. and MDR strains of *E. coli*. It was interesting to note that crude water extracts and methanol extract showed inhibitory activity all selected bacteria.

Antimicrobial activities of hexane, benzene, isopropanol, methanol, and water extracts of leaf of M. oleifera against different human pathogens grown up in Muller Hinton agar are given in Table 6. Results showed that isopropanol and methanol extracts were active against *S. aureus,* and also the isopropanol extract was active against *E. coli* and methanol was active against *S. haemolyticus*.

Hexane, isopropanol and methanol fractions of *M.*

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

Hex	Ben	Iso	Met	Wat
S	2 CG	S	S	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 105/mL of the test organism.

Table 8. Antifungal activity *Moringa oleifera* 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 (+).

oleifera leaf showed activity against *E. cloacae* (Table 7). Benzene and water extracts are not active.

Antifungal activity of crude extracts

Antifungal activity obtained with different solvent extract of leaf of *M. oleifera* is presented in Table 8. Among the different extract of *M. oleifera*, hexane and benzene extracts of leaf inhibited the growth of all tested fungal strains. Water extract inhibited the growth of all fungi tested except *Microsporum* and *A. flavus*. Isopropanol extract only inhibited the growth of *P. marneffei Candida*, *Penicillium* spp. and *A. fumigatus* and methanol extract only inhibited *Candida*, *Epidermophyton* and *A. fumigatus* growth.

Anti-mycobacterial activity of the plant extracts of *Alstonia scholaris*

Hexane, benzene, isopropanol and methanol fractions of *M. oleifera* leaf shows activity against *Atypical Mycobacterium* (Table 9).

Anti-Hepatitis B virus of crude extracts

Hexane, benzene and isopropanol fractions of M. oleifeira showed activity against Hepatitis B virus (Table 10).

Table 9. Neutralizing activity of various solvent extracts of *Moringa oleifera* leaf against an isolate of *Atypical Mycobacterium.*

DMSO Control	Hex	Ben	Iso	Met	Wat
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 50µL of 105/mL of the test organism. Reading on 5th day.

Discussion

M. oleifera leaves are a good source of nutrition and several studies exhibited their anti-tumor, antiinflammatory, anti-ulcer, anti-atherosclerotic and anti-convulsant activities (33-35). These biological activities are related with the phytochemical composition of *M. oleifera* leaves. Oluduro, Idowu (36) reported the presence of alkaloids, tannins, saponins, flavonoids and phenols in leaf extract of *M. oleifera*. Kasolo, Bimenya (37) reported that ethanol and water extracts of *M. oleifera* leaves contained catechol tannins, gallic tannins, steroids and triterpenoids, flavonoids, saponins, anthraquinones, alkaloids and reducing sugars. Shahriar, Hossain (38) investigated the phytochemical constitution of five leaf extracts of *M. oleifera* (n-hexane, chloroform, petroleum ether, methanol, ethanol and water) and their results support the results obtained in the present study.

Several phytochemical compounds such as flavonoids, tannins, alkaloids and steroids were found to have antimicrobial activity (23, 39, 40). Peixoto, Silva (41) reported that in their study, the aqueous and ethanolic *M. oleifera* leaf extracts indicated promising potential as a treatment for certain bacterial infections. In this study, the antibacterial activity of the *M. oleifera* extract was observed to be greater against gram-positive species (*S. aureus* and *Enterococcus faecalis*) than against gram-negative species (*E. coli*, *Salmonella*, *P. aeruginosa*, *Vibrio parahaemolyticus* and *Aeromonas caviae*) which was also indicated in several other studies (42-44). In our study, water and methanol extracts showed antibacterial activity against all selected bacteria, and isopropanol extract showed inhibitory activity against all of selected bacteria except four strains. 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 or no antimicrobial activities (45, 46). Fadeyi, Raheem (47) demonstrated the antibacterial activities of ethanolic extract of *M. oleifera* leaf against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *Acinetobacter bauamanii* (ATCC 19606). Our results showed that isopropanol and methanol were active against the same *S. aureus* strain and also the isopropanol extract was active against the same *E. coli* strain, but we not observed antibacterial activity of methanol extract against this *E. coli* strain.

Also, several authors were reported the antifungal activity of various extract of *M. oleifera* against human

Table 10. 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				
	Con	DMSO	Hex	Ben	Iso	Met	Wat
ELFA Reading (RFV)	14.91	7.56	0.09	0.04	0.02	0.78	8.63
Interpretation	P	P	N	N	N	P	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

pathogenic fungus (23, 24, 48). In our study, the hexane and benzene extracts showed the highest antifungal potential inhibiting the growth of all fungi tested, and water extract inhibited the growth of all fungi tested except *Microsporum* and *A. flavus*. A number of phytochemicals isolated from plant extract sources are antifungal (49, 50) and alkaloids are a kind of these compounds (51). In all the extracts obtained in our study, alkaloids were detected. Moreover, in water extract we detected another kinds of compounds which could have antimicrobial activity, such as tannins (52) and terpenoids (53). Moreover, hexane, benzene and isopropanol fractions of M. oleifeira showed activity against Hepatitis B virus. *In vitro* studies also reported the protective effects of *M. oleifera* against Hepatitis B (54, 55).

In conclusion, phytochemical compounds present in *M. oleifera* presented antifungal, antibacterial and antiviral activities. More studies are needed to characterize which specific compounds perform this antimicrobial function and if exist a synergy between these.

Conflicts of Interest

The authors declare no conflict of interest.

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