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IN-VITRO ANTIMICROBIAL ACTIVITY AND *IN-VIVO* TOXICITY OF *MORINGA OLEIFERA* AND *ALLAMANDA CATHARTICA* AGAINST MULTIPLE DRUG RESISTANT CLINICAL PATHOGENS

KARTHIC RAJAMANICKAM AND SINGARAM SIVASUBRAMANIAM SUDHA*

Dept of Microbiology, Dr.N.G.P. Arts and Science College, Kalapatti, Coimbatore, Tamilnadu, India.

ABSTRACT

Based on the traditional usage, *Moringa oleifera* and *Allamanda cathartica* plants were selected. The present study is to find out its antimicrobial potential against clinical isolates of KMCHospital. The resistant patterns of the isolated pathogens were studied against commercial standard antibiotics. By using the different polar solvents (Methanol, Ethanol, Chloroform, Water, Petroleum ether and Ethyl acetate), the plants material were extracted and studied their phytochemical constituents. Antimicrobial activity of plants extract were studied by well diffusion assay in muller hinton agar and minimum inhibitory concentration of the significant extracts were studied by tube dilution assay. The results of *A. cathartica* antimicrobial property revealed that petroleum ether extract showed good inhibition (*Staphylococcus aureus* (20mm), *Escherichia coli* (13mm), *Pseudomonas aeruginosa* (19mm), *Acinetobacter sp* (20mm), *Proteus sp* (18mm)) than that of remaining extracts. *M. oleifera* ethanol extract showed significant inhibition (*S.aureus* (20mm), *E.coli* (20mm), *Bacillus sp* (18mm), *P. aeruginosa* (14mm), *Cornebacterium sp* (12mm), *Klebsiella pneumonia* (12mm), *Acinetobacter sp* (14mm)). Acute toxicity was performed in albino mice and up to 1000mg/kg, no toxic symptoms were observed. These results suggested that both the plants showed relatively broad spectrum of antimicrobial activity and are non toxic.

Key words: **Moringa oleifera, Allamanda cathartica, pathogens, toxicity, antimicrobial**

SINGARAM SIVASUBRAMANIAM SUDHA
Dept of Microbiology, Dr.N.G.P. Arts and Science College, Kalapatti, Coimbatore, Tamilnadu, India.

Corresponding author

INTRODUCTION

Pathogenic bacteria resistant towards antibiotics play a major concern for the treatment of human and animal diseases. Mishandling of antimicrobial agents leads to easiest adaptation of bacteria and, as a result, resistant pattern of pathogens gets increased[1]. Thus the scientists are much interested in developing safe and effective drugs from natural source with least toxicity to the host. In addition, the formation of oxidant free radicals leads to damage of essential biomolecules like proteins, lipids, DNAs and RNAs[2]. Medicinal plants have many significant bioactive metabolites to cure various highly pathogenic infection of mankind. Numerous approaches are available to know the active principle of medicinal plants[3]. In most of developing countries, billions of people are using medicinal plants for various remedies. This is because of high cost of modern medicine, economically cheaper, locally available, without side effects as well as traditional beliefs and preferences[4,5]. The human interactions with plants have intensively increased and led to habitat loss, degradation and fragmentation with subsequent loss of plant species in the natural environment. As a result of urbanization, the demand rate for medicinal plants gets increased[6].

The "Moringa" tree is found to have many beneficial properties. It is a medium sized tree, about 10m high, found in all over India[7]. Leaves contain flavonoid pigments such as kaempferol, kaempferitrin, isoquercitrin and rhamnatin. It is also rich in alkaloids, vitamins and has α-linolenic acid[8]. The leaves have high antioxidant property and whole parts of plant can be used as food or beneficial to some other purpose. In tropical region it is used as livestock food and various ailments are prepared with the micronutrients of *Moringa* leafs. In Asia and African countries the fruit of the tree is quite popular as vegetable in their normal diet. The leaves have more nutrient than other dietary foods like more Vitamin than carrot, more calcium than milk, more iron than spinach, more Vitamin C than oranges, more potassium than bananas, and the protein quality of Moringa leaves more than that of milk and eggs[9]. Since it is rich in nutritive value, the leaves were recommended as food supplement among malnutrition children's and nursing mothers[10]. Its leaves can be widely used as many pharmaceutical applications few are emmenagogue, diuretic, ophthalmic, expectorant, stimulant, scury, wounds, tumors, inflammation and helminthasis[11].

South and Central America is the native of *Allamanda cathartica* Linn. It is widely growing perennial shrub, the leaves are smooth and thick. Traditionally it is used for treating various disease includes jaundice, complications with malaria and enlargement of spleen. Yellow allamanda has produced antibacterial activity against the pathogen *Staphylococcus*[12]. The bioactive metabolites of plumericin and isoplumericin showed cytotoxic activity against Madison lung carcinoma (M109)[13]. It was reported that *Allamanda* of various extracts showed good antimicrobial activity against various bacterial and dermatophytic infections[13, 14]. The main aim of the study was to collect *Moringa oleifera* and *Allamanda cathartica* leafs and extracted the secondary metabolites by using various solvents. The extracts were studied for phytonutrient study and antimicrobial activity with clinical pathogens.

MATERIALS AND METHODS

2.1. Materials

Double distilled water from Millipore unit was used for the synthesis and measurements. All the chemicals were purchased from HIMEDIA Chemicals Private Limited, Mumbai, India. Methanol (CH_3OH), Chloroform, Ethyl Acetate, were obtained from SIGMA chemicals; Ethanol (CH_3CH_2OH -99.7% purity) was obtained from Changshu Yangyuan chemicals, China; Petroleum ether (C6H14) from Rankem chemicals and Chitosan (95% deacetylated)

from India Seafood's, Kerala. The commercially available standard antibiotics were purchased from Poorani Hospital suppliers.

2.2. Isolation and Identification of clinical pathogens

2.2.1. Collection of samples

The clinical samples were collected in aseptic container for laboratory analysis from the Department of Laboratory Medicine-Microbiology Laboratory, Kovai Medical Centre and Hospital (KMCH), Coimbatore.

Isolation and Characterization of Pathogens

The samples were observed under microscopic view by performing gram's staining and motility staining. Then the samples were cultured in medium includes nutrient agar, macconkey agar, manitol salt agar and blood agar. The isolated colonies were subject to all basic biochemical tests and to evaluate the results based on standard protocols.

2.3. Collection and extraction of M.oleifera and A.cathartica extracts

The leaves of *M.oleifera* and *A.cathartica* were collected from in and around Coimbatore[15]. Collected leaves were allowed to shade dry in room temperature to reduce the moisture content. Then the collected dried leaves were ground into fine powdered by Mechanical shearing, and sieving. 20g of each leaves powder was weighed and mixed with 100ml of respective solvents (Ethanol, Methanol, Petroleum ether, Chloroform, Ethyl acetate and water) and kept for overnight. The filtrate was obtained by filtering these contents twice using No 1 Whatman filter paper. The clear filtrate was condensed using rotary vacuum evaporator (SUPER FIT make) at 50°C for about 15 minutes.

2.4. Preliminary Phytochemical Screening

Above mentioned all the extracts were then treated with various reagents which revealed the presence of various phyto constituents[16].

2.5. Antibacterial activity of crude extracts

Sterile Muller Hinton agar plates were prepared. The isolated clinical pathogens were heavily inoculated with the young bacterial culture (16-20hrs) by means of sterile cotton swab to ensure efficient growth of the organisms equal to that of 0.5McFarland standard. Four wells were made in each plate using cork borer. 50µl of above prepared extracts were added to the each of the wells, which is pre swabbed with the culture to observe the antagonistic effect of various microorganisms. The plates were incubated for 24 to 48 hours. Similar procedure was carried out for the rest of the isolates. The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (mm).

2.6. Acute toxicity studies

Albino mice weighing 22-25 g selected by random sampling technique were used in the study. Acute oral toxicity was performed as per OECD- 423 guidelines (acute class method) [17] at KMCH college of Pharmacy, Coimbatore. The animals were fasted overnight, provided only water after which extracts were administered to the groups orally at the dose level of 5 mg/kg body weight by gastric intubation and the groups were observed for 14 days. If mortality was observed in 2 or 3 animals among 6 animals then the dose administered was assigned as a toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher doses such as 50, 300 and 1000 mg/ kg body weight. The animals were observed for toxic symptoms such as behavioral changes, locomotion, convulsions and mortality for 72 hours.

3. RESULT AND DISCUSSION

3.1. Characterization of isolated clinical pathogens

From the collected clinical samples, pathogens were isolated; they were further identified and

characterized by using microscopic observation, biochemical characterization and culture characteristics and finally confirmed as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter sp.*, *Corynebacterium sp., Salmonella sp.*, *Bacillus sp.* and *Proteus sp.* (Table.1). The isolated strains were sub cultured periodically, and maintained in the nutrient broth for the further analysis.

Table 1
List of identified pathogens

S. No	Isolate	Organism
1	Isolate 1	*Escherichia coli*
2	Isolate 2	*Klebsiella pneumoniae*
3	Isolate 3	*Pseudomonas aeruginosa*
4	Isolate 4	*Staphylococcus aureus*
5	Isolate 5	*Acinetobacter sp.*
6	Isolate 6	*Salmonella sp.*
7	Isolate 7	*Bacillus sp.*
8	Isolate 8	*Proteus sp.*
9	Isolate 9	*C. albicans*
10	Isolate 10	*Corynebacterium sp*

3.2. Antibiotic sensitivity testing by Kirby-Bauer's disc diffusion method

All the isolated pathogens were subjected to antibiotic sensitivity test. The resistant pattern of Gentamycin, Colistin, Cftazidime, Vancomycin, Ciprofloxacin, Ampicillin, Cefpodoxime were studied**.** Because of continuous evolution of antibiotic resistance, regular monitoring of this phenomenon appears to be necessary to improve guidelines for empirical antibiotic therapy, which must consider the most probable microorganism, their susceptibilities according to the characteristics of the population concerned, without forgetting side effects, and ecological and economic consequences. From the characteristics of the population, risk factors of infections caused by resistant organisms can be determined (Table.2).

Table 2
Antimicrobial pattern among the clinical isolates.

S. No	Standard Antibiotics	Isolated Pathogens	Zone of Inhibiton in(mm)
1	Ciprofloxacin	*Escherichia coli*	12
2	Ampicillin	*Klebsiella pneumoniae*	16
3	Gentamycin	*Pseudomonas aeruginosa*	16
4	Vancomycin	*Staphylococcus aureus*	8
5	Colistin	*Acinetobacter sp.*	10
6	Vancomycin	*Salmonella sp.*	8
7	Cefpodoxime	*Bacillus sp.*	12
8	Gentamycin	*Proteus sp.*	7
9	Amphotericin B	*C. albicans*	13
10	Tetracycline	*Corynebacterium sp*	11

3.3. Phytochemical Analysis of plant extracts

The phyochemical analysis of ethanolic extract of *M.oleifera* and *A.cathartica* were studied using standard protocols. The major phytochemicals present in these plants were Tannins, Flavanoids, Carbohydrates, Proteins, Phenols, Steroids and Glycosides. The Alkaloids is absent in both the case of plant extracts. All these phytochemical constituents help the plants to exhibit their various types of activities like antimicrobial, anti-inflammatory and many other activities (Table.3).

Table 3
Qualitative analysis of phytochemical constituents of Moringa oleifera and Allamanda cathartica extracts

Phytochemical constituents	Ethanolic Extracts of *Moringa oleifera*	Petroleum Ether Extracts of *Allamanda cathartica*
Alkaloids	-	-
Flavonoides	+	+
Saponins	+	+
Carbohydrates	+	+
Proteins	+	+
Phenols	+	+
Steroids	+	+
Glycosides	+	+
Tannins	+	+

3.4. Screening of medicinal plants for antibacterial activity

3.4.1. Preliminary Screening of medicinal extracts for antibacterial activity

Both the plant extracts were prepared from ethanol, methanol, ethyl acetate, chloroform; petroleum ether and water assessed for their antibacterial activity using agar well diffusion method. The extent of zone of inhibition represent the antibacterial activity of each solvent extracts. Among all the extracts only ethanol extract of *M.oleifera* showed good antimicrobial activity (*S.aureus*(20mm), *E.coli*(15mm), *Bacillus sp*(15mm), *P. aeruginosa*(14mm), *Corynebacterium sp*(12mm), *Acinetobacter sp*(14mm) and *K. pneumoniae*(12mm)) (Table. 4) other solvent extracts showed not much significant inhibitions. In case of *A.cathartica* petroleum ether extract showed good antimicrobial activity than that of other extracts (*S.aureus*(20mm), *E.coli*(13mm), *P. aeruginosa*(19mm), *Acinetobacter sp*(20mm) and *Proteus sp*(18mm)) (Table. 4).

Table 4
Screening of Moringa oleifera (M.o) and Allamanda cathartica (A.c) for antimicrobial activity (Inhibitions in (mm))

Isolated Pathogens in (mm)	Methanol		Ethanol		Ethyl Acetate		Chloroform		Water		Petroleum Ether	
	M.o	A.c	M.o	A.c	M.o	A.c	M.o	A.c	M.o	A.c	M.o	A.c
S.aureus	-	6	20	8		13	7	7	15	-	-	20
E.coli	-	-	15	-	-	-	-	-	-	6	-	13
Bacillus sp	10	-	15	-	12	-	7	-	-	-	13	-
P. aeruginosa	10	12	14	-	12	10	-	8	13	-	10	19
Corynebacterium sp	-	-	12	-	-	-	-	-	-	-	9	-
Acinetobacter sp	-	18	14	20	11	19	-	-	10	10	-	20
K. pneumoniae	-	-	12	-	-	-	-	-	-	-	-	-
Proteus sp	-	9	-	-	-	-	-	-	-	-	-	18
Salmonella sp	-	-	-	-	-	-	-	-	-	-	-	-
C. albicans	-	-	-	6	-	-	-	-	-	-	-	-

3.5. Antimicrobial susceptibility testing- Minimum inhibitory concentration

Petroleum ether extracts of *A.cathartica* was tested for its minimum inhibitory concentration for all the pathogens. The minimum inhibitory concentrations of plant extract against various pathogens were shown in table.5. The MIC range was observed for the extracts from 25mg/ml to 50mg/ml for *S.aureus*, *Acinetobacter sp*, *P.aeruginosa*. The ethanolic extract of *M.oleifera* inhibits the *Staphylococcus aureus* MRSA with a MIC of 50mg/ml, *E.coli* with a MIC of 50mg/ml (Table.6). Mashiar Rahman reported that ethanol extract of *M.oleifera* showed more susceptible to *S. shinga, P. aeruginosa, S. sonnei, Pseudomonas* spp., *B. cereus, B. subtilis, S. lutea*, and *B. megaterium* and their respective MIC values were ranged from 458-916 µg/ ml[18]. B.N.Devendra et al., reported that chloroform extract of *M.oleifera* chloroform extracts showed significant inhibition on *E.coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pyogenes*[19].

Table 5
MIC value of the crude petroleum ether extract of Allamanda cathartica

TEST ORGANISM	Antimicrobial susceptibility testing (Minimum Inhibitory concentration mg/ml)				
	Allamanda cathartica extracts				
	100	50	25	12.5	6.25
Staphylococcus aureus	-	-	+	+	+
Acinetobacter sp	-	-	+	+	+
Pseudomonas aeruginosa	-	-	-	+	+

3.6. Toxicity studies

The Petroleum ether extracts of *A.cathartica* and ethanolic extract of *M.oleifera* were administered at a dose of 100mg/kg orally to albino mice. From the time of administration, the mice were under observation for the next 72 hours. No toxic symptoms such as behavioral changes, locomotion, convulsions and mortality were observed. For further confirmation, increasing concentrations of both plant extracts were administered up to a dose of 1000mg. The mice were under observation for the next two weeks. Upon completion, however the mice showed no toxic symptoms. Thus the *in-vivo* studies suggested that *A.cathartica* and *M.oleifera* are non toxic up to the dose of1000mg/kg.

Table 6
MIC value of the crude ethanolic extract of Moringa oleifera

TEST ORGANISM	Antimicrobial susceptibility testing (Minimum Inhibitory concentration mg/ml)				
	Moringa oleifera extracts				
	100	50	25	12.5	6.25
E.coli	-	-	+	+	+
Staphylococcus aureus MRSA	-	-	+	+	+
Staphylococcus aureus	-	-	-	+	+

CONCLUSION

The present study was conducted to obtain the preliminary information on the phytonutrients, toxicity, and antimicrobial property of *A.cathartica* and *M.oleifera* against clinical pathogens. The results of antibiotic sensitivity test showed that petroleum ether extract of *A.cathartica* and ethanolic extract of *M.oleifera* showed good antimicrobial property against most of pathogens than that of other extracts. In general, natural drugs are much less side effects when compare to that of synthetic and other chemical drugs. This experiment might be helpful to develop the new natural medicine to combat the emergence of resistant developing pathogens.

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