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Antimicrobial activity of *Moringa oleifera* and its synergism with *Cleome viscose*

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ABSTRACT

The phytochemical study, antioxidant activity and *in vitro* antibacterial activity of acetone, methanol and chloroform extracts of *Moringa oleifera* roots were investigated. Phytochemical analysis revealed the presence of tannins, saponins, flavonoids and alkaloids in roots of *Moringa oleifera*. The extracts were screened for *in vitro* antibacterial activity against selected otitis media pathogens *S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *S. pneumoniae* by disc diffusion method. Most prominent activity was shown by acetone extract against *P. aeruginosa* and *S. pneumoniae* followed by *E. coli*, *S. aureus, and K. pneumoniae*. Effective zone range was 19-35 mm and MIC range was 4-16 mg/ml. Antioxidant activity of *Moringa oleifera* in acetone extract was 84.23 %, 60.00 % and 43.30 % at 10µg/ml, 50µg/ml and 100µg/ml respectively. *Moringa oleifera* and *Cleome viscosa* combination demonstrated strong synergistic effect towards *K. pneumoniae, E. coli, S. pneumoniae* and *S. aureus*. The antibacterial effects of *Moringa oleifera* alone as well as in combination with *Cleome viscosa* against all tested pathogens suggest their potential use as alternative tools for controlling otitis media.

KEYWORDS

Otitis media, Antimicrobial Activity, *Moringa oleifera, Cleome viscosa,* Synergistic activity.

INTRODUCTION

The diversity of plants growing with their known ethno- pharmacological uses is wealth of India. According to World Health Organization, medicinal plants are the best source to obtain a variety of newer herbal drugs. About 80 % of individuals from developed and developing countries use traditional medicine, which has compounds derived from medicinal plants. The extracts of many plant species have become popular in recent years and attempts to characterize their bioactive principles have gained momentum for varied pharmaceutical applications (Lis-Balchin and Deans, 1997). There is an increasing prevalence of antibiotic resistant pathogens in hospitals and home. Deliberate research is in progress for alternative treatment to combat further spread for antibiotic resistant pathogens (Olukoya *et al.,* 2003). In recent time, the search of potent antimicrobial agents has been shifted to plants. The antimicrobial compounds from plants may inhibit bacteria by different mechanism than the presently used antibiotics and may have clinical value in treatment of resistant microbial strain (Eloff, 1998). Formulating new synergistic combinations using different commer- cially available antibiotics or to combine an active phytochemical having antimicrobial properties is one approach (Braga, 2005).

Otitis media is inflammation of the middle ear drum and the inner ear including a duct known as the Eustachian tube. It is known to be the most common childhood infections which lead annually to death of over 50,000 children under 5 years (Rovers *et al*., 2006). The effect of early otitis media causes hearing loss on auditory skills, language development, social development and other aspects of children's behavior is extensive (Bowd, 2005). The hearing loss is a significant sequel of chronic suppurative otitis media among the school children and it had adverse effect on their academic performance (Olatoke *et al*., 2008).

The "Moringa" tree is considered one of the world's most useful trees, as almost every part of the Moringa tree can be used for food or has some other beneficial properties (Devendra *et al*., 2011). *Moringa oleifera* also known as Drumstick in India belongs to family Moringaceae is a well-documented world renowned plant herb for its extraordinary nutritional and medicinal properties. It is a natural antihelmintic, antibiotic, detoxifier, outstanding immune builder and is used in many countries to treat malnutrition and malaria. The plant was shown to possess antimicrobial activity against wide array of pathogens (Rahman *et al*., 2009).

Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to human against infections and degenerative diseases. (Rahmat *et al.,* 2004). Phytochemical is a natural bioactive compound found in plants, such as vegetables, fruits, medicinal plants, flowers, leaves and roots that work with nutrients and fibers to act as an defense system against disease or more accurately, to protect against disease. (Sudhanshu *et al.,* 2012) Herbal medicines are often prepared from a combination of different plant species. The effects of such mixtures could be due to synergistic action of various phytochemicals (Williamson, 2001) The use of synergistic combinations in antimicrobial chemotherapy is often used commercially for the treatment of various infections.

MATERIALS AND METHODS

1) Ethno botanical survey

Plants were selected for this study based on their medicinal use. Roots of *Moringa oleifera* were collected from the waste fields and road sides in Amravati. Material was washed thoroughly, shade dried and then powdered with the help of blender. The powdered material was kept in airtight bottles until further use. The ethno botanical data gathered at medicinal and aromatic plants unit, Dr. P.D.K.V. Akola.

2) Preparation of plant extracts

Dry powdered plant material was extracted with solvents petroleum ether, chloroform, methanol and acetone with Soxhlet's extractor for 6 hrs or till the plant material gets colourless. The solvent was removed using a rotary vacuum evaporator to give a concentrated extract, which was then frozen and freeze-dried until use.

3) Otitis media pathogens

i) Specimen collection : Clinical specimens of 100 patients suffering from otitis media infection from Shri Daryao Clinic, Amravati were collected by swabbing the affected area of ear using sterile cotton swab and immediately taken to the laboratory for bacteriological investigation.

ii) Isolation of Pathogens: Samples were inoculated on blood agar and incubated aerobically at 37°C for 24 hours. Isolates obtained were maintained on nutrient agar slants at 4°C until required.

iii) Identification and biochemical characterization of bacterial isolates: Cultures from nutrient agar slants were streaked on different selective media such as EMB agar, Baird Parker Agar, *Klebsiella* selective agar, cetrimide agar and *Streptococcus* selective agar. Identification of the bacterial cultures was done using staining motility, biochemical tests such as indole test, methyl red test, VP test, citrate utilization test, oxidase test, urease test, coagulase test, catalase test and bile solubility test (Cheesbrough, 1984).

4) Preparation of inoculum

To prepare bacterial inoculum, pure culture of test organism was inoculated into 5 ml of sterile nutrient broth and incubated at 37° C for 2 to 8 hrs till moderate turbidity developed. The inoculum was standardized by matching with 0.5 McFarland turbidity standard, which corresponds to cell density approximately 10^8 CFU/ ml.

5) Antibacterial sensitivity testing

Antibacterial susceptibility testing of antibiotics was performed by disc diffusion method (Bauer *et al*., 1966). Antibiotic discs included gentamycin (10 µg), amoxicillin (10 µg) and ciprofloxacin (5 µg). For susceptibility testing, a sterile cotton swab was dipped into the standardized inoculum and rotated firmly against the upper inside wall of the test tube to remove excess inoculum from swab. Entire sterile and dried Mueller Hinton agar surface of the plate was streaked with the cotton swab. For antibacterial susceptibility testing of plant extracts the sterile disc of 6 mm diameter (SD067, Hi-Media, Mumbai)

was impregnated with 20µl of plant extract (200 mg/ ml). The discs were then placed on the seeded agar. The standard discs of gentamycin, amoxicillin and ciprofloxacin were used as a reference control. The plates were incubated at 37° C for 24 hrs. The assessment of antibacterial activity was done by measuring the diameter of the growth inhibition zone formed around disc. Test was done in duplicate.

6) Determination of MIC

Minimum inhibitory concentration of acetone extract of *Moringa oleifera* was determined by NCCLS method (NCCLS, 2003). In brief stock solution of plant extract (1024 mg/ml) was prepared in respective solvent and vigorously shaken for about 1 min. The stock solution was then stored in refrigerator until use. Seventeen well characterized clinical isolates of otitis media pathogens were selected for MIC determination by broth macrodilution method. The clinical isolates included *Klebsiella pneumoniae* (03 isolates), *Staphylococcus aureus* (04 isolates), *Pseudomonas aeruginosa* (03 isolates), *Streptococcus pneumoniae* (04 isolates) and *Escherichia coli* (03 isolates). Each isolate was originated from a different patient with clinical manifestations and was maintained on nutrient agar.

7) Phytochemical Analysis

The freshly prepared extracts were subjected to standard preliminary phytochemical analysis for the presence of alkaloids, flavonoids, tannin and saponins as described elsewhere (Jane and Patil, 2012).

8) Antioxidant activity

Antioxidant activity of *Moringa oleifera* was measured by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity. The extracts were mixed with methanol to get various concentrations as 400, 200, 100, 50, and 10µg/ml. From each concentration, 2 ml of extract was mixed with 1 ml of methanolic solution containing DPPH radicals, with final concentration of 0.2 mM DPPH. The contents were shaken vigorously and kept in dark for 30 min. Absorbance was measured at 517 nm. Absorbance of control was determined by replacing the sample with methanol. The scavenging activity was calculated using formula,

$$\% \text{ Abtioxidant Activity} = \frac{1 - \text{Absorbance of Sample(extract)}}{\text{Absorbance of DPPH}} \times 100$$

9) Synergistic activity of *Moringa oleifera* with *Cleome viscosa*

To assess synergistic effect of the *Moringa oleifera* and *Cleome viscosa* extracts,10µl of each plant extract of combination to be tested was impregnated on separate sterile disc (6 mm diameter). A third sterile disc was impregnated with 1:1 mixture (10µl one extract +10µl another extract of combination) of both extracts. The discs were then placed on the surface of seeded agar, aseptically and plates were incubated at 37° C for 24 hrs and zone of inhibition surrounding the paper discs was measured.

RESULTS AND DISCUSSION

Phytochemical analysis of *Moringa oleifera* revealed the presence of tannins, alkaloids, saponins and flavonoids in acetone and methanol extract but absent in chloroform extract (Table 1). Antioxidant activity of *Moringa oleifera* in terms of radical scavenging activity in acetone extract was found to be 84.23 %, 60.00 %, 43.30 % at 10µg/ml, 50µg/ml and 100µg/ml concentration respectively (Table 2).

Antimicrobial activity of standard reference antibiotics is summarized in Table 3.The results indicate that otitis media pathogens were inhibited by gentamicin with inhibition zone 10-20mm, amoxicillin with 8-15 mm and ciprofloxacin with 20-35mm. The root extracts of *Moringa oleifera* showed varying antimicrobial activity against tested clinical isolates with inhibition zone range of 19-35mm. Acetone extract yielded very good antibacterial activity by inhibiting 66 % isolates of *K. pneumoniae,* 80% isolates of *S. aureus*, 100% isolates of *P. aeruginosa*, *S. pneumoniae* and 86% isolates of *E. coli.* Methanol extract on the other hand inhibited 89% and 47% isolates of *S. pneumonia* and *P. aeruginosa* respectively. However chloroform extracts exhibited poor antibacterial activity and inhibited only *E. coli* (23% isolates). Petroleum ether extract did not show any activity (Table 4, Fig 1). *S. pneumoniae* and *E. coli* isolates each required minimum inhibitory concentration of 16 mg/ml of *Moringa oleifera* acetone extract. *P. aeruginosa* isolates were inhibited at MIC of 8 mg/ml. *K. pneumoniae* required MIC range of 8-16 mg/ml for inhibition. Among 03 isolates, 02 isolates of *K. pneumoniae* inhibited at lower concentration of 8 mg/ml while

01 isolate required higher concentration of 8 mg/ml of *Moringa oleifera* extract. Isolates of *S. aureus* were inhibited at MIC range of 4-8 mg/ml. Among 04 isolates, 03 isolates of required higher concentration of 8 mg/ml as compared to 01 isolate that required lower concentration of 4 mg/ml of *Moringa oleifera* extract for inhibition (Table 5).

Synergistic interactions of *Moringa oleifera* and *Cleome viscosa* are presented in Table 6. Tremendous increase in bioactivity was recorded against *K. pneumoniae* isolates as average inhibition zone value was increased from 20mm theoretical value to 35mm. However, the weak potency recorded when the two plant extracts were tested independently. Moderate synergistic action was observed against *S. aureus* and *E. coli* isolates while weak synergism was observed in *S. pneumoniae* isolates where the combination inhibition zone of 24 mm was increased to a level of 24.8 mm. Among *P. aeruginosa* isolates the effect has been shown to possess antagonistic action. Synergy was seen in 100% isolates of K. *pneumoniae* and *E. coli* followed by 89% isolates of *S. pneumoniae* and 64% isolates of *S. aureus*.

Table 1: Phytochemical screening of *Moringa oleifera*

Sr. no.	Constituent	Name of test/ Reagent	Acetone	Methanol	Chloroform
1.	Tannins	$FeCl_3$	+	+	-
2.	Alkaloids	Mayer's reagent	+	+	-
3.	Saponins	Frothing test	+	+	-
4.	Flavonoids	Shinoda's test	+	+	-

Table 2: Antioxidant activity of acetone extract of *Moringa oleifera*

Concentration	Radical scavenging activity
10 µg/ml	84.23 %
50 µg/ml	60.00 %
100 µg/ml	43.30 %

Table 3: Sensitivity of otitis media pathogens against antibiotics

Organism (No. of isolates)	Zone of inhibition range (mm)		
	Gentamicin	Amoxycillin	Ciprofloxacin
K.pneumoniae (09)	17-18	12-14	28-30
S. aureus (25)	10-18	12-15	20-26
P. aeruginosa (19)	18-20	12-15	30-35
S.pneumoniae (28)	18-20	08-10	26-30
E. coli (22)	15-18	10-12	25-27

Table 4: Sensitivity of otitis media pathogens to *Moringa oleifera* extracts

Organism (No. of isolates)	Methanol		Petroleum ether		Acetone		Chloroform	
	S	R	S	R	R	S	R	S
K.pneumoniae (09)	00	09	00	09	06	03	00	09
S. aureus (25)	00	25	00	25	20	05	00	25
P. aeruginosa (19)	09	10	00	19	19	00	00	19
S.pneumoniae (28)	25	03	00	28	28	00	00	28

S- Sensitive; R- Resistant

Fig 1: Sensitivity of bacterial isolates to *Moringa oleifera* extracts

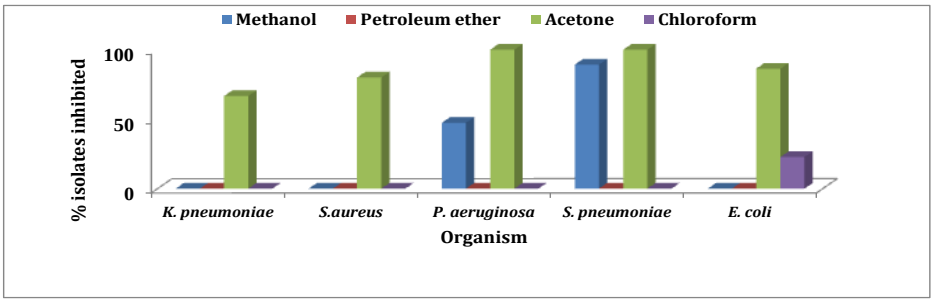

Table 5: MIC of acetone extract of *Moringa oleifera* against Otitis Media pathogenes

Organism	Isolate No.	MIC (mg/ml)
K.pneumoniae	14	08
	66	08
	84	16
S. aureus	09	08
	10	08
	62	04
	85	08
P. aeruginosa	12	08
	13	08
	19	08
S.pneumoniae	14	16
	65	16
	88	16
E. coli	10	16
	64	16
	93	16

Table 6: Average values of inhibition zone(mm) of acetone extract of *Moringa oleifera* alone and in combination with *Cleome viscosa*

Organism (No. of isolates)	*Moringa oleifera* (10µl/disc)	*Cleome viscosa* 10µl/ disc)	Combination (20µl/ disc)	Synergistic action on percent isolates
K. pneumoniae (09)	10.00	10.00	35.00	100
S. aureus (25)	07.20	10.08	21.44	64.00
P. aeruginosa (19)	16.01	11.30	22.10	26.00
S.pneumoniae (28)	09.00	15.00	24.80	89.00
E. coli (22)	10.00	15.00	39.00	100

DISCUSSION

Phytochemicals are present in virtually all plant tissues of *Moringa oleifera* e.g. leaves, roots, stem and fruits (Siddiqui *et al.*, 2009). The antimicrobial activities of phytochemical compounds may involve multiple modes of actions for e.g. oil degrades the cell wall, interact with the composition and disrupt cytoplasmic membrane (Khanahmadi *et al.,* 2010) and damage membrane protein, interfere with membrane integrated enzymes, cause leakage of cellular components, coagulate cytoplasm, deplete the proton motive force, change fatty acid and phospholipid constituents, alter nutrient uptake and electron transport, impair enzymatic mechanism for energy etc (Baldemir *et al.,* 2006). Sharma *et al.,* (2011) revealed the prominent presence of alkaloids, phenolics, flavonoids and tannins in hydro-ethanolic extract of *Moringa oleifera* pods. In present study alkaloids, tannins, saponins and flavonoids are detected in *Moringa oleifera*. This is

in analogy with other workers (Doughari *et al.,* 2007; Krishnaiah *et al*., 2009) who also reported presence of alkaloids, tannins, saponins and flavonoids in *Moringa oleifera.* The antimicrobial activity of *M. oleifera* seed is due to the presence of an array of phytochemiacals, but most importantly due to the activity of a short polypeptide named 4 (ά– L – rhamnosyloxy) benzyl-isothiocyanate (Eilert *et al*., 1981; Guevara *et al*., 1999). The peptide may act directly on microorganisms and result in growth inhibition by disrupting cell membrane synthesis or synthesis of essential enzymes (Silvestro *et al*., 2000; Suarez *et al*., 2003).

The free-radical scavengers (antioxidants) have potential to prevent, delay or ameliorate many of human chronic and ageing diseases such as cancer, diabetes, heart disease, stroke, malaria, rheumatoid arthritis. Free radical scavenger is an important mechanism for the inhibitory activity towards lipid peroxidation and can be a good marker for antioxidant activity (Mariutti *et al*., 2008). In the present study highest antioxidant activity of *Moringa oleifera* found to be 84.23%. This result is in agreement with other workers Reddy *et al*., (2005), Lako *et al*., (2007) who reported high antioxidant activity *Moringa oleifera. Moringa oleifera* leaves are potential source of natural antioxidants due to their marked antioxidant activity. Methanol and ethanol extracts of *Moringa oleifera* possess the highest antioxidant activities of 65.1% and 66.8%, respectively (Siddhuraju and Becker, 2003).

Acetone extract of *Moringa oleifera* showed prominent activity against all tested organisms causing otitis media. However, methanol extract showed antibacterial activity only against *S. pneumoniae* and *P. aeruginosa*. Various researchers reported antimicrobial activity of *Moringa oleifera* against variety of pathogens including *S. aureus*, *S. albus, S. pyogenes P. aeruginosa, Salmonella gallinarum, B.subtilis* and *E. coli* (Thilza *et al*., 2010, Dewagan *et al*., 2010, Kumar *et al*., 2012). Crushed seed extract of *Moringa oleifera* have been reported to possess bactericidal activity against *S. pyogenes* and P. *aeruginosa* (Suarez *et al*., 2005). Doughari *et al*., (2007) reported acetone extract of *Moringa oleifera* against *Salmonella typhi* with inhibition zone of 16 mm. In the present investigation chloroform extract inhibited only *E. coli.* Such activity was also reported by Devendra *et al* (2011). Further our results do not match with the reports of Devendra *et al* (2011) for the pathogens *P. aeruginosa* and *S. aureus* as they reported the inhibitory activity of chloroform extract against these organisms. Such variation may be attributed to climatic conditions, verity of plant, geographic distribution and source of pathogen used. In present investigation petroleum ether extract was totally inactive in suppressing the bacterial growth. The inactivity of petroleum ether extract may be due to the fact that the active compound which posses the antimicrobial properties are polar in nature and not possibly extracted by petroleum ether (Saadabi and Zaid, 2011). Minimum inhibitory concentration was lowest in case of *S. aureus* (4 mg/ml). The highest sensitivity of *S. aureus* may be due to its cell wall structure and outer membrane (Zaika, 1988).

Ample evidence of individual plant extracts as antimicrobial agent are available, but research on combined effect of two or more plants is very limited (Prakash *et al*., 2006). However, to our knowledge there is no data available on combination effects of *Moringa oleifera* and *Cleome viscosa*. The aspect of synergistic mechanisms becomes the apparent strategy employed by the plants. It is well known that most of the independent plant - derived extracts possess weak potency against pathogenic bacteria compared to antibiotics. Hence the combination of plant extracts that demonstrate improved efficacy for killing the drug resistant microbes may form a strong basis for control of such pathogens. Combination of *Moringa oleifera* and *Cleome viscosa* showed synergistic effect against all tested pathogens except *P. aeruginosa.*

CONCLUSION

The antimicrobial activity of *Moringa oleifera* was found to be consistent with the folk uses of this plant by local people. Synergistic action with *Cleome viscosa* may also provide valuable information on remedy for otitis media.

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