



ANTIBACTERIAL & ANTIOXIDANT ACTIVITY OF DIFFERENT EXTRACT OF *MORINGA OLEIFERA* LEAVES – AN *IN-VITRO* STUDY

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ABSTRACT

Moringa oleifera is a common plant and known for various medicinal properties. The research work was conducted to investigate the *in vitro* antimicrobial and antioxidant activity of aqueous, ethanolic and methanolic extract of leaf *Moringa oleifera*. *In-vitro* antimicrobial activity was performed by disc diffusion method in MH agar medium and MIC test. The extract showed significant effect on the tested organisms. The methanolic extract showed maximum zone of inhibition against *S.aureus* (23.6±1.15), whereas aqueous extract showed lowest against *B.subtilis* (12.0±1.73). Methanolic extract showed maximum relative percentage inhibition against *S.aureus* (90.34 %) and aqueous extract showed lowest relative percentage inhibition against *B.subtilis* (27.86 %). Minimum Inhibitory Concentration (MIC) was measured by tube dilution method. Extract showed 2.4 mg/ml for *B.subtilis*, *E.coli* and 7.4 mg/ml MIC values for *S.aureus*. The antioxidant activities of different concentrations of methanol extracts of the leaves were determined by the three assay techniques i.e. DPPH radical scavenging assay, Nitric oxide scavenging assay, and Hydrogen peroxide scavenging assay method. Methanol extract of leaves has shown effective antioxidant activity in all assay techniques. The results obtained in the present study indicate that the leaves of *Moringa oleifera* are a potential source of natural antibacterial and antioxidants.

Keywords: *Moringa oleifera*, Antibacterial activity, Antioxidant activity, Disc diffusion method.

INTRODUCTION

In current scenario of medical and pharmaceutical advancement, microbes involve in the change of their metabolism and genetic structure to acquire resistant against the drugs used in the treatment of common infectious disease^{1, 2}. These drug resistant candidates are more pathogenic with high mortality rate and become a great challenge in the pharmaceutical and healthcare industry. To overcome microbial drug resistant, scientists are looking forward for the development of alternative and novel drugs. Natural sources such as plants, algae and animals provide an array of natural medicinal compounds for the treatment of various infectious diseases. Plants are exploited as medicinal source since ancient age. The traditional and folk medicinal system uses the plant products for the treatment of various infectious diseases. In recent times, plants are being extensively explored for harboring medicinal properties. Studies by various researchers have proved that plants are one of the major sources for drug discovery and development³⁻⁵. Plants are reported to have antimicrobial, anticancer, anti-inflammatory, antidiabetic, hemolytic, antioxidant, larvicidal properties etc. In the continuation of this strategy of new drug discovery we have studied the leaf parts of the plant *Moringa oleifera* for their antibacterial and antioxidant activities. *Moringa oleifera* Lam. is the most widely cultivated species of a monogeneric family, the Moringaceae that is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan. It is a perennial softwood tree with timber of low quality, but which for centuries has been advocated for traditional medicinal and industrial uses. All parts of the Moringa

tree are edible and have long been consumed by humans. *Moringa oleifera* is called 'miracle vegetable' because it is not just a food, but also a medicine and it may therefore be a functional food⁶. Phytochemicals such as vanillin, omega fatty acids, carotenoids, ascorbates, tocopherols, beta-sitosterol, kaempferol and quercetin have been reported from the flowers, roots, fruits and seeds⁷. The leaves are highly nutritious, being a significant source of beta-carotene, vitamin C, protein, iron and potassium⁸. Leaves of this plant have been studied extensively for various biological activities including hypolipidemic, antiatherosclerotic, immune-boosting agents, and tumor-suppressive effects⁹⁻¹¹. According to Fuglie¹² the many uses for Moringa include: alley cropping (biomass production), animal forage (leaves and treated seed-cake), biogas (from leaves), domestic cleaning agent (crushed leaves), blue dye (wood), fencing (living trees), fertilizer (seed-cake), foliar nutrient (juice expressed from the leaves), green manure (from leaves), gum (from tree trunks), honey- and sugar cane juice-clarifier (powdered seeds), honey (flower nectar), medicine (all plant parts), ornamental plantings, biopesticide (soil incorporation of leaves to prevent seedling damping off), pulp (wood), rope (bark), tannin for tanning hides (bark and gum), water purification (powdered seeds). In particular, this plant family is rich in compounds containing the simple sugar, rhamnose, and it is rich in a fairly unique group of compounds called glucosinolates and isothiocyanates.^{13, 14} For example, specific components of Moringa preparations that have been reported to have hypotensive, anticancer, and antibacterial activity include 4-(4'-O-acetyl- α -L-rhamnopyranosyloxy) benzyl isothiocy-



anate, 4-(α -L-rhamnopyranosyloxy) benzyl isothiocyanate, niazimicin, pterygospermin, benzyl isothiocyanate and 4-(α -L-rhamnopyranosyloxy) benzyl glucosinolate¹³. The antioxidant activity and the total phenolic contents of *Moringa oleifera* leaves in two stages of maturity responsible for preventing the deleterious effects of oxidative stress were disclosed in a recent study¹⁵. Due to the wide uses of *Moringa oleifera*, the present study was focused to evaluate their antibacterial and antioxidant activities.

MATERIALS AND METHODS

Plant Material

The leaves of *Moringa oleifera* was collected from Jalandhar in the month of July 2010.

Plant Material Extraction

The fresh leaves were collected, surface sterilized, sun dried for seven days and ground. The ground leaf (20g) were extracted in a round bottom flask in 500ml solvent (water, ethanol and methanol) at room temperature and loaded on an orbit shaker at a speed of 120 rpm for three days. After three days the extract was filtered through filter paper and dried by using rotary evaporator. Then the crude extract was dissolved in solvent and stored in a refrigerator at 4°C for antibacterial activity and antioxidant activity test.

Antibacterial Screening

The antibacterial assay was performed by disc diffusion technique and minimum inhibitory concentration.

Disc diffusion analysis

Antibacterial activity of the four different samples; 1: Ethanol extract, 2: Methanol extract, 3: Water extract, 4: Mixed extract were individually tested against three bacteria. In vitro antibacterial test was carried out by disc diffusion method using 25 μ l of standardized suspension of tested bacteria (10^8 cfu ml⁻¹) spread on MH plates. The discs (6 mm in diameter) were impregnated with 10 μ l of leaf extract, followed by air-drying and were placed on seeded agar plates then petridishes left in a refrigerator at 4°C for 24 hrs in order to diffuse the material from the discs to the surrounds media in the Petri dishes. The Petri dishes were then incubated at 37°C for overnight to allow the bacterial growth. Antimicrobial activity was evaluated by measuring the zones of inhibition against the tested bacteria. Each assay was carried out in triplicate¹⁶.

Positive and negative control

Amoxycillin and ciprofloxacin standard disc was used as positive control for *B.subtilis*, *E.coli* and *S.aureus*. Sterilized distilled water, ethanol and methanol was used as negative control.

Determination of relative percentage inhibition

The relative percentage inhibition of the test extract with respect to positive control was calculated by using the following formula^{17, 18}.

$$\text{Relative percentage inhibition of the test extract} = 100 \times \frac{(x-y)}{(z-y)}$$

Where,

x: total area of inhibition of the test extract

y: total area of inhibition of the solvent

z: total area of inhibition of the standard(ciprofloxacin) drug

The total area of the inhibition was calculated by using area = πr^2 ; where, r = radius of zone of inhibition.

Statistical analysis

The value of antimicrobial activity of leaves extracts of *M.oleifera* were expressed as mean \pm standard deviation (n= 3) for each sample.

Determination of minimum inhibitory concentration (MIC)

MIC of methanol leaf extract was determined using Serial tube dilution technique. In this technique the tubes of broth medium, containing graded doses of compounds are inoculated with three the test organisms. After suitable incubation, growth will occur in those tubes where the concentration of compound is below the inhibitory level and the culture will become turbid (cloudy). Therefore, growth will not occur above the inhibitory level and the tube will remain clear.¹⁹

Preparation of the sample solution

200 mg/ml of methanol extract was taken in vials.

Preparation of inoculums

The test bacteria grown at 37 degree Celsius in nutrient agar medium was diluted in sterile nutrient broth medium and used only if suspension contain about 10⁷ cells /ml. This suspension was used as the inoculums.

Procedure:

- Twelve test tubes were taken, nine of which were marked 1, 2, 3, 4, 5, 6, 7, 8, 9, and the rest three were assigned as T_M(medium), T_{MC}(medium + compound) and T_{MI}(medium + inoculums)
- 2 ml of nutrient broth medium was poured to each of the 12 test tubes.
- Then test tubes were cotton plugged and sterilized in an autoclave for 15 lbs /sq. inch pressure.
- After autoclave, 1 ml of the sample solution was added to the 1st test tube and mixed well and then 1ml of this content was transferred to the 2nd test tube.



- The content of the second test tube was mixed well and again 1 ml of this mixture was transferred to the 3rd test tube. This process of serial dilution was continued up to the 9th test tube. So concentration of extract is obtained as 66.6, 22.2, 7.4, 2.4, 0.82, 0.27, 0.09, 0.03 and 0.01 mg/ml.
- 10 µl of properly diluted inoculums was added to each of 9 test tubes and mixed well.
- To the control test tube T_{MC} , 1 ml of the sample was added, mixed well and 1 ml of this mixed content was discarded to check the clarity of the medium in presence of diluted solution of the compound.
- 10µl of the inoculum was added to the control test tube T_{MI} , observed the growth of the organism in the medium used.
- The control test tube T_M , containing medium only was used to confirm the sterility of the medium.
- All the test tubes were incubated at 37°C for 18 hours.

Antioxidant Activity

DPPH free radical scavenging assay

The free radical scavenging activity of the fractions was measured in vitro by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. 0.3 mM DPPH was prepared in 100% ethanol and 1 ml of this solution was added to 1 ml plant extract of different concentration (20 - 100 µg/ml) and 1ml of methanol. Control reaction was carried out without plant extract. The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm by spectrophotometer. The percentage scavenging inhibition was determined and compared with Gallic acid, which was used as the standard²⁰.

% DPPH radical-scavenging = [(Absorbance of Control - Absorbance of test Sample) / (Absorbance of Control)] x 100

Then % inhibitions were plotted against respective concentrations used.

Hydrogen peroxide scavenging assay

Hydrogen peroxide solution (2 mM) was prepared with standard phosphate buffer (pH 7.4). Different concentration of the methanol extracts (20 - 100 µg/ml) added to 1.0 ml of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage inhibition of different concentrations of the extracts was determined and compared with the standard, ascorbic acid²¹.

Nitric oxide free radical scavenging (NO) assay

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured

using the Griess reaction reagent. 3.0 ml of 10 mM sodium nitroprusside in phosphate buffer was added to 2.0 ml of extract and reference compound in different concentrations (20 - 100 µg/ml). The resulting solutions were then incubated at 25°C for 60 min. A similar procedure was repeated with methanol as blank, which serves as control. To 5.0 ml of the incubated sample, 5.0 ml of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_3) was added and absorbance of the chromophore formed was measured at 540 nm. Percent inhibition of the nitrite oxide generated was measured by comparing the absorbance values of control and test preparations. Curcumin used as a positive control²².

RESULTS AND DISCUSSION

The Results of Antibacterial Screening

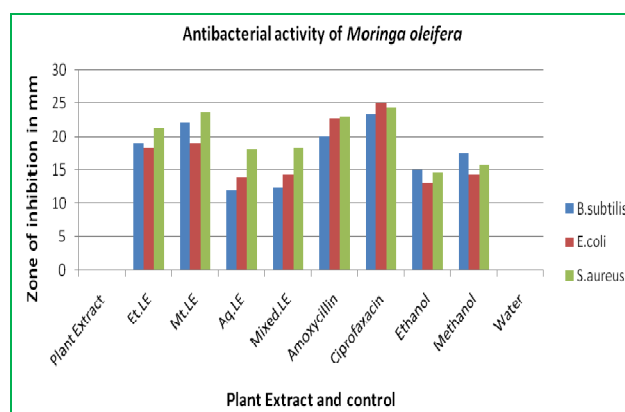
Moringa oleifera are being probed as an alternate source to get therapeutic compounds based on their medicinal properties. All extract of leaves exhibited the antibacterial activity against three isolates of bacteria (Tables 1 and Figure 1) and the results were expressed as mean ± standard deviation (n=3). Methanolic Extract showed maximum antibacterial activity against *S.aureus* (23.6±1.15) and aqueous extract showed lowest activity (12.0±1.73) against *B.subtilis*.

Table 1: Antimicrobial activity of *Moringa oleifera*

Plant Extracts	Zone of inhibition in mm		
	<i>B.subtilis</i>	<i>E.coli</i>	<i>S.aureus</i>
Et.LE	19.0±1.58	18.3±1	21.3±0.36
Mt.LE	22.0±.75	19.0±1.46	23.6±1.15
Aq.LE	12.0±1.73	14.0±.64	18.0±.89
Mixed.LE	12.3±1.15	14.3±1.2	18.3±.25
Amoxycillin	20.0±1.52	22.6±1.73	23.0±2.08
Ciprofloxacin	23.3±0.57	25.0±1.15	24.3±1.73
Ethanol	15.0±1.15	13.0±1.52	14.6±0.57
Methanol	17.5±0.36	14.3±1.73	15.6±1.15
Water	0	0	0

Et: ethanolic extract, Mt: methanolic extract, AE: aqueous extract LE: leaf extract; Values are expressed as mean ± standard deviation of the three replicates; Zone of inhibition not include the diameter of the disc.

Figure 1: Antimicrobial activity of *Moringa oleifera*

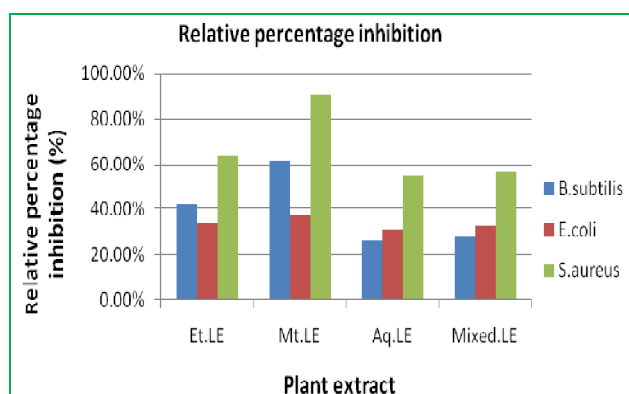


The results of antimicrobial activity of crude extract were compared with the positive control (Ciprofloxacin) for evaluating their relative percentage inhibition (Table 2 and Figure 2), while the Methanolic extract exhibits maximum relative percentage inhibition against *S.aureus* (90.34 %). Results of MIC are reported in Table 3. The crude extract showed 2.4 and 7.4 mg/ml MIC values *B.subtilis*, *E.coli* and *S.aureus* respectively.

Table 2: Relative percentage inhibition of *Moringa oleifera* compare to standard antibiotics

Test organisms	Relative percentage inhibition (%)			
	Et.LE	Mt.LE	Aq.LE	Mixed.LE
<i>B.subtilis</i>	42.21%	61.34%	26.52%	27.86%
<i>E.coli</i>	33.99%	37.21%	31.36%	32.71%
<i>S.aureus</i>	63.74%	90.34%	54.87%	56.71%

Figure 2: Relative percentage inhibition of *Moringa oleifera*



The Results of Antioxidant Activity

In antioxidant investigation, the crude methanolic extract of the plant and the standard showed the antioxidant activity.

DPPH radical scavenging method

The antioxidants react with the stable free radical DPPH (deep violet colour) and convert it to 1,1-diphenyl-2-picryl hydrazine with decoloration. The scavenging effects of

extract increased with their concentrations to similar extents. The percentage inhibitions of concentration 20, 40, 60, 80 and 100 µg/ml are 53.65, 64.13, 74.17, 85.02 and 95.52 % respectively (Table 4). The standard gallic acid presented a scavenging effect of 93.69% at the concentration of 100 µg/ml. So increased scavenging of DPPH radicals was dose dependent manner due to the scavenging ability of the *Moringa oleifera* methanolic extract and comparable to gallic acid.

Hydrogen Peroxide scavenging method

Extracts of leaves of *Moringa oleifera* scavenged hydrogen peroxide in a concentration dependent manner. The percentage inhibitions of concentration 20, 40, 60, 80 and 100 µg/ml are 15.08, 47.16, 50.55, 58.12 and 64.49 % respectively (Table 5). The standard ascorbic acid presented a scavenging effect of 65.92% at the concentration of 100 µg/ml. So increased antioxidant activity was dose dependent manner due to the scavenging ability of the *Moringa oleifera* methanolic extract and comparable to ascorbic acid.

Nitric oxide free radical scavenging activity

Nitric oxide or reactive nitrogen species formed during its reaction with oxygen or with superoxide such as NO₂, N₂O₄, N₃O₄, nitrate and nitrite are very reactive. The scavenging effects of extract increased with their concentrations to similar extents. The percentage inhibitions of concentration 20, 40, 60, 80 and 100 µg/ml are about 4.34, 18.30, 49.88, 57.66, and 71.85 % respectively (Table 6). The standard curcumin presented a scavenging effect of 84.21% at the concentration of 100 µg/ml. So increased scavenging of Nitric oxide radicals was dose dependent manner due to the scavenging ability of the *Moringa oleifera* methanolic extract and comparable to curcumin.

Increased scavenging of DPPH, Hydrogen peroxide and Nitric oxide free radicals in dose dependent manner due to the scavenging ability of the *Moringa oleifera* methanolic extract and comparable to gallic acid, ascorbic acid and curcumin.

Table 3: MIC values of methanol and aqueous extracts of *Moringa oleifera* on test organisms

Marked No. of the test tubes	Nutrient broth medium added (ml)	Diluted solution of extract (mg/ml)	Inoculums added (µl)	Bacterial growth observed against		
				<i>B.subtilis</i>	<i>E.coli</i>	<i>S.aureus</i>
1	2	66.6	10	-	-	-
2	2	22.2	10	-	-	-
3	2	7.4	10	-	-	-
4	2	2.4	10	-	-	+
5	2	0.82	10	+	+	+
6	2	0.27	10	+	+	+
7	2	0.09	10	+	+	+
8	2	0.03	10	+	+	+
9	2	0.01	10	+	+	+
TMC	2	66.6	10	-	-	-
TMI	2	0	10	+	+	+
TM	2	0	10	-	-	-

'+' Indicates 'growth'

'-' Indicates 'no growth'



Table 4: Results of DPPH radical scavenging assay

Extract concentration ($\mu\text{g/ml}$)	% inhibition	Gallic acid concentration ($\mu\text{g/ml}$)	% inhibition
20	53.65	20	56.44
40	64.13	40	62.40
60	74.17	60	75.29
80	85.02	80	87.11
100	92.52	100	93.69

Table 5: Results of Hydrogen peroxide scavenging assay

Plant extract concentration ($\mu\text{g/ml}$)	% inhibition	Ascorbic acid concentration ($\mu\text{g/ml}$)	% inhibition
20	15.08	20	50.72
40	47.16	40	57.18
60	50.55	60	61.80
80	58.12	80	65.92
100	64.49	100	67.98

Table 6: Results of Nitric oxide free radical scavenging (NO) assay

Extract concentration ($\mu\text{g/ml}$)	% Inhibition	Curcumin concentration ($\mu\text{g/ml}$)	% Inhibition
20	4.34	20	19.45
40	18.30	40	33.86
60	49.88	60	57.89
80	57.66	80	80.77
100	71.85	100	84.21

CONCLUSION

Due to increasing prevalence of antibiotic-resistant pathogens in hospitals and homes, research is in progress for alternative treatments to combat further spread of antibiotic resistant pathogens. Plants have been screened for their potential antimicrobial properties but studies on the antimicrobial properties and activities of lower plants are very scanty. All the extracts showed varying degrees of antimicrobial activity on the microorganism tested. The chance to find antimicrobial activity was more apparent in methanol and ethanol than water extracts of the same plant. This plant can be source of new antibiotic compounds. Further work is needed to isolate the secondary metabolites from the extracts studied in order to test specific antimicrobial activity. This *in vitro* study demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. The millenarian use of these plants in folk medicine suggests that they represent an economic and safe alternative to treat infectious diseases.

Apart of antimicrobial activity leaf extract has got profound antioxidant activity. So the plants may be considered as good sources of natural antioxidants for medicinal uses such as against aging and other diseases related to radical.

It may be concluded from this study that the extract of *Moringa oleifera* is active against the tested microorganisms and also have antioxidant effects. In addition, the results confirm the use of the plant in traditional medicine. Now our study will be directed to explore the lead compound responsible for aforementioned activity from this plant.

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