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PHYTOCHEMICAL AND ANTIBACTERIAL OF *MORINGA OLEIFERA* LEAVES

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ABSTRACT

Background and Objective: The aim of this study was the determine *in vitro* antibacterial activity of different extracts of *Moringa oleifera* leaves against clinical isolates and standard bacteria, determine the Minimum Inhibitory Concentrations (MICs) and identify the major chemical components of the plants extracts. **Material and Method:** The cup agar plate diffusion method was used to screen the antibacterial activity of plant extracts and antibiotic. The Minimum Inhibitory Concentrations (MICs) of the most active extracts were determined using the agar plate dilution method. All data were expressed as mean ± standard Deviation mean. Analysis of variance was performed by ANOVA using the SPSS software. Significant differences between means were determined by Least Significant Difference (L.S.D) and student t-test. A significant difference was considered at the level of $P < 0.05$. **Results:** The results indicated that most extracts exhibited inhibitory effect against stranded bacteria and isolates. The minimum inhibitory concentrations (MICs) ranged between 25 mg/ml and 50mg/ml for all the organisms. The methanolic extract of *Moringa oleifera* leaves is active against the tested gram positive and gram negative microorganism. The phytochemical screening reveal the presence of flavonoids, sterols, coumarins tannins, alkaloids, saponins, triterpenes and anthraquinones.

KEYWORDS: Antibacterial, Phytochemical, *Moringa oleifera*, Extract, leaves.

INTRODUCTION

Antimicrobial agents include naturally occurring antibiotics, semi-synthetic derivatives of naturally occurring antibiotics and chemical antimicrobial compounds.[1] An ideal antimicrobial agent exhibits selective toxicity. This term implies that a drug is harmful to parasite without being harmful to the host.[2] Infectious diseases caused by bacteria, fungi, viruses and other pathogenic parasites are still a major threat to public health, despite the tremendous progress in human medicine. Antimicrobial activity of natural extracts and pure compounds can be detected by observing the growth response of various microorganisms to samples that are placed in contact with them. Several methods for detecting antimicrobial activity are available, but since they are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method.[3] Medicinal and aromatic plants and their derivatives represent an integral part of life in Sudan (Khalid et al., 2012).[4] In Sudan, people have been tapping their herbal remedies for medication for time immemorial. For this purpose they use a vast variety of plants ranging from the rain forest vegetation in the south, to the desert vegetation of the north, and from the semi-Mediterranean climatic zone of the red sea, to the rich savanna of the west.[5] Sudan folklore-medicine represents a unique blend of indigenous cultures with Egyptian, Indian. Arabian, East and West African cultures. This in view of a number of factors, such as draught, desertification, expansion of agricultural schemes and the introduction of health services to primitive areas, which initiated astonishingly rapid changes, leading to the least use of native medicines, which would eventually disappear.[6] *Moringa olifera* belonging to the family Moringaceae. The leaves of *Moringa oleifera* Lam. are eaten in African countries, such as Ghana, Ethiopia, Nigeria, East Africa and Malawi. Moringa tree is cultivated for foods and medicinal purposes.[7] The leaves and pods are helpful in increasing breast milk in nursing mothers during breastfeeding and leaf decoction has been found useful in the treatment of asthma, back pain and rheumatism. *M. oleifera* tree has in recent times been advocated as an outstanding indigenous source of highly digestible protein, carotenoids and vitamin C suitable for utilization in many of the so called developing regions of the world where undernourishment is a major concern.[8]

MATERIALS AND METHODS

Plant material: *Moringa Oleifera* was obtained from the market, Sudan at 2013 and authenticated by Mr.Yahia Suliman at herbarium of Medicinal and Aromatic Plants and

Traditional Medicine Research Institute (MAPTMRI), National Center for Research, Khartoum, Sudan.

Preparation of the extracts: Extraction was carried out according to method descried by Sukhdev *et al.*[9]

The *Moringa Oleifera* leaves sample was grounded using mortar and pestle 500g of *Moringa Oleifera* leaves was successively extracted with petroleum ether and methanol using soxhelt extractor apparatus. Extraction carried out for about eight hours for each solvent till the colour of solvents as the last siphoning time returned colourless. Solvents were evaporated under reduced pressurs using rotary evaporator apparatus. Finally extracts allowed to air in petri dishes till complete dryness.

Organisms: Clinical isolates (*Bacillus subtillus ,Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* were collected randomly from lab of Khartoum Hospital and lab of Soba hospital Khartoum, Sudan. The clinical isolates were subjected to identification procedures which were based on the cultural characteristic, the microscopical examination and the biochemical characteristics**.** The standard bacterial strain used *Bacillus subtilis*(NCTC 7596), *Staphylococcus aureus*(ATCC 25923), *Escherichia coli*, (ATCC 25922), *Pseudomonas aeruginosa*(ATCC 27853) and *Salmonella typhi*(NCTC 0650) were obtained from the Department of Microbiology and Parasitology, Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), National Centre for Research, Sudan.

Determination of minimum inhibitory concentration (MIC): The agar plate dilution method was used to determine the least concentration of plant extract that completely inhibits the growth of microorganism[7]at concentrations (50, 25, 12.5,6.25 and 3.125mg/ml).[10]

Antimicrobial screening: The cup-plate agar diffusion method[11] was adopted, to assess the antibacterial activity of the prepared extracts[12] In accordance with this method one ml of the isolated standardized and bacterial stock suspension (10^8-10^9 C.F.U per ml) were thoroughly mixed with 100 ml of sterile molten Mueller- Hinton agar which was maintained at 45°C. Twenty ml aliquots of the inoculated Mueller-Hinton agar were distributed onto sterile Petri-dishes. The agar was left to set, and in each of these plates, four cups (10 mm in diameter) were cut using a sterile cork borer (NO.4) and the agar discs were removed. Alternate cups

were filled with 100µL of samples of each of the extract, using standard fine adjustable automatic pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position, at 37°C for 18 hours. Two replicates were carried out for each extract against each of the tested organisms. Upon the completion of incubation the diameter of the resultant inhibition zones were measured, averaged and then the mean values were tabulated.

Antibacterial activity of reference drug: In the present work, ciprofloxacin was used as reference drugs anti Antibacterial drug was tested at different concentrations obtained by taking 0.1 g of each powdered drug and dissolved in 100 ml sterile distilled water to give a concentration of 1000 µg/ml followed by serial dilutions to give concentrations of 100, 50, 25 and 12.5 µg/ml. These drug tested against reference bacteria i.e *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtillus* and *Salmonella typhi.*

Phytochemical screening: Phytochemical screening for the active constituents was carried out on methanolic extracts using the methods.[13]

Statistical analysis

All data were expressed as mean ± standard Error mean. Analysis of variance was performed by ANOVA using the SPSS software (version 16.0 for windows, 2007). Significant differences between means were determined by Least Significant Difference (L.S.D) and student t-test. A significant difference was considered at the level of $P < 0.05$.

RESULTS

The inhibition zones obtained in the preliminary screening of the antibacterial activity of *Moringa oliefera* leaves of the petroleum ether extracts is presented in Table (1), the petroleum ether showed no inhibition in all of bacteria used in the study (*Escherichia coli., Pseudomonas aeruginosa., Salmonella typhi, Bacillus subtilis* and *Staphylococcus aureus).* Table (2) showed that the antimicrobial activity of *Moringa oliefera* leaves methanolic extract against standard bacteria. The lower significance ($p<0.05$) inhibition zone value had been marked against *Bacillus subtilis* on concentration 100mg/ml. On the concentration of 200mg/ml the higher significance ($p<0.05$) inhibition zone values had been observed against *Pseudomonas aeruginosa* and *Escherichia coli* were (22.5 ± 0.29mm and 21.5 ± 0.29mm) respectively, which were higher significant ($p<0.05$) than *Salmonella typhi, Bacillus subtilis and Staphylococcus aureus* were (18.5 ± 0.29mm).

Table (1): Diameters (in mm) of inhibition zones obtained in the preliminary screening of the antibacterial activity of *Moringa oliefera* leaves of the petroleum ether extract.

Petroleum ether extracts	Concentration µg/ml	Standard Bacteria use				
		Mean Diameter of growth Inhibition Zone in mm (MDIZ*)				
		E.c	P.s	Sal.	B.s	S.a
Moringa oliefera leaves	100mg/ml	-	-	-	-	-

Data in table were given as mean ± standard Error mean (Std error) of five species of pathogenic bacteria (triple replications for each). Statistical comparison between groups applied using Post hoc (LSD) test in rows and t-test in columns.

[a,b,c,d] Values with different superscripts in the same row and column are significantly different at ($P<0.05$).

***MDIZ**= Mean diameter of growth inhibition zone in mm.

Where:- **E.c:** *Escherichia coli*, **P.s:** *Pseudomonas aeruginosa*, **Sal.:** *Salmonella typhi*, **B.s:** *Bacilus subtilis* and **S.a:** *Staphylococcus aureus*.

Table (2): Diameters (in mm) of inhibition zones obtained in the preliminary screening of the antibacterial activity of *Moringa oliefera* leaves of the methanolic extract.

Moringa oliefera	Concentration mg/ml	Standard Bacteria use				
		Mean Diameter of growth Inhibition Zone in mm (MDIZ*)				
		E.c	P.s	Sal.	B.s	S.a
leaves Methanol extract	100mg/ml	15.5 ± 0.29[a]	15.0 ± 0.0 [a]	15.5 ± 0.29 [a]	14.0 ± 0.0 [b]	15.5 ± 0.29 [a]
	200mg/ml	21.5 ± 0.29[c]	22.5 ± 0.29 [d]	18.5 ± 0.29 [b]	18.5 ± 0.29 [b]	18.5 ± 0.29 [b]

***MDIZ**= Mean diameter of growth inhibition zone in mm.

- = No inhibition.

Interpretation of results: **MDIZ** (<18 mm: Sensitive, 14-18 mm, Intermediate: >14 mm: Resistant.

Where:- **E.c:** *Escherichia coli*, **P.s:** *Pseudomonas aeruginosa*, **Sal.:** *Salmonella typhi*, **B.s:** *Bacilus subtilis* and **S.a:** *Staphylococcus aureus*.

The minimum inhibitory concentrations of methanolic extract was determined against five standard microorganisms(*B.subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi)* by agar dilution method at concentrations (50, 25, 12.5, 6.25 and 3.125mg/ml). Table(3). The lowest minimum inhibitory concentration (MIC) values

was obtained was 25 mg/ml for the extract methanolic extract against *Staphylococcus aureus* and *Salmonella typhi*. The range of MICs values was obtained was 50 mg/ml for the methanolic extract against *Escherichia coli* and *Pseudomonas aeruginosa*.

Table (3): Minimum Inhibition Concentration (MIC) obtained in the Preliminary Screening of the Antibacterial activity of *Moringa oleifera* leaves of the different Concentrations of methanolic extract.

Concentration	Standard Bacteria use				
	Mean Diameter of growth Inhibition Zone in mm (MDIZ*)				
	E.c	P.s	Sal.	B.s	S.a
50 mg/ml	14 ± 0.12^{a}	14 ± 0.12^{a}	15 ± 0.17^{b}	13 ± 0.17^{c}	15 ± 0.17^{b}
25 mg/ml	13 ± 0.17^{b}	13 ± 0.17^{b}	14 ± 0.12^{c}	12 ± 0.12^{d}	14 ± 0.12^{c}
12.5 mg/ml	12 ± 0.12^{c}	12 ± 0.12^{c}	13 ± 0.17^{a}	0.0^{e}	13 ± 0.17^{a}
6.5 mg/ml	11 ± 0.12^{f}	0.0^{e}	12 ± 0.12^{d}	0.0^{e}	12 ± 0.12^{d}

Data in table were given as mean ± standard Error mean (Std error) of five species of pathogenic bacteria (triple replications for each). Statistical comparison between groups applied using Post hoc (LSD) test in rows and t-test in columns.

a,b,c,d,e,f Values with different superscripts in the same row and column are significantly different at ($P<0.05$).

***MDIZ**= Mean diameter of growth inhibition zone in mm.

0.0 = No inhibition.

Interpretation of results: **MDIZ** (<18 mm: Sensitive, 14-18 mm, Intermediate: >14 mm: Resistant.

Where:- **E.c:** *Escherichia coli*, **P.s:** *Pseudomonas aeruginosa*, **Sal.:** *Salmonella typhi*, **B.s:** *Bacilus subtilis* and **S.a:** *Staphylococcus aureus*.

Showed the comparable of MIC of the *Moringa oliefera* leaves of methanolic extracts antibiotic ciprofloxacin in different concentrations; in (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonellatyphi*, *Bacillus subtilis* and *Staphylococcus aeruginosa)*, taking account significantly ($P<0.05$) different.

Table (4): Comparable of Minimum Inhibition Concentrations (MICs) of the of *Moringa oliefera* leaves of Methanolic extractsand antibiotic Ciprofloxacin in different concentrations.

Standard Bacteria use		Mean Diameter of growth Inhibition Zone in mm (MDIZ*)		
		50mg/ml	25 mg/ml	12.5 mg/ml
E.c	Methanol extract	14 ± 0.12^{a}	13 ± 0.17^{a}	12 ± 0.12^{a}
	Ciprpofloxcin	0.0^{b}	0.0^{b}	0.0^{b}
P.s	Methanol extract	14 ± 0.12^{a}	13 ± 0.17^{a}	12 ± 0.12^{a}
	Ciprpofloxcin	40 ± 0.17^{b}	39 ± 0.12^{b}	37 ± 0.12^{b}
Sal.	Methanol extract	15 ± 0.17^{a}	14 ± 0.12^{a}	13 ± 0.17^{a}
	Ciprpofloxcin	35 ± 0.17^{b}	33 ± 0.12^{b}	29 ± 0.12^{b}
B.s	Methanol extract	13 ± 0.17^{a}	12 ± 0.12^{a}	0.0^{a}
	Ciprpofloxcin	31 ± 0.12^{b}	29 ± 0.12^{b}	27 ± 0.12^{b}
S.a	Methanol extract	15 ± 0.17^{a}	14 ± 0.12^{a}	13 ± 0.17^{a}
	Ciprpofloxcin	40 ± 0.17^{b}	35 ± 0.17^{b}	31 ± 0.12^{b}

Data in table were given as mean ± standard Error mean (Std error) of five species of pathogenic bacteria (triple replications for each). Statistical comparison between groups applied using t-test incolumns.

[a,b] Values with different superscripts in the same column and column are significantly different at ($P<0.01$).

***MDIZ**= Mean diameter of growth inhibition zone in mm.

Where:- **E.c:** Escherichia coli**, P.s:** Pseudomonas aeruginosa, **Sal.:** Salmonella typhi, **B.s:** Bacilus subtilis and **S.a:** Staphylococcus aureus.

4.4: Phytochemical screening

Table (5) and pictures (1) showed the results of the Phytochemical screening of the crude extract of *Moringa oliefera* leaves. The results reveal the presence of flavonoids, sterols, coumarins and tannins with high concentrations, while alkaloids and saponins with moderate concentration and triterpenes and anthraquinones with low concentration in the methanol extract of *Moringa oliefera* leaves.

Table (5): Phytochemical screening of methanolic extract of *Moringa oliefera* leaves.

Test	Observation	Result
Alkaloids	Turbidity	++
Sterols	Green colour	+++
Triterpenes	Pink colour	+
Flavonoids	Yellow colour	+++
Saponins	Foam	++
Coumarins	UV absorption	+++
Tannins	Blue colour	+++
Anthraquinones	Pinkcolour	+

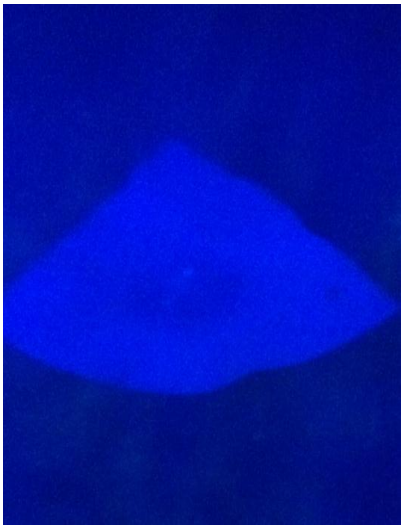
Where:-

+ : Low concentration

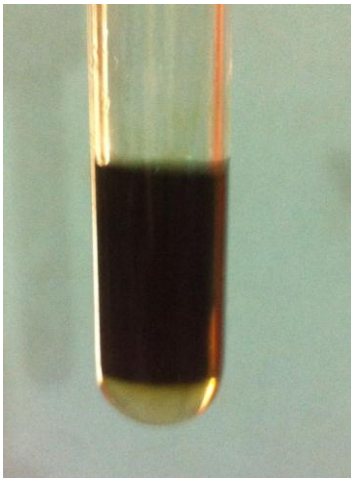
++ : Moderate concentration

+++ : High concentration

- : Negative



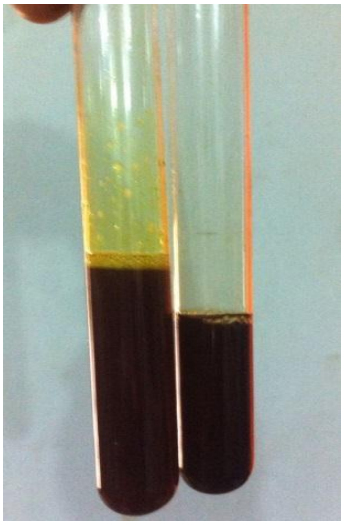
(1)



(2)

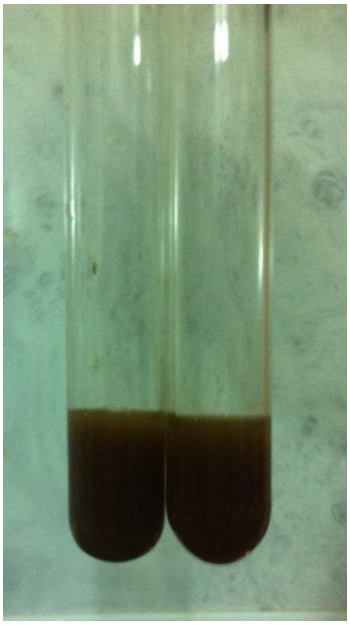
(3)

(4)



(5)

(6)



(7)

Pictures (1): Showed the Phytochemical screening of secondary metabolites present in the methanolic extract of *Moringa oliefera* leaves. (1) Coumarins (2) Sterols and Triterpenes (3) Tannins (4) Anthraquinones (5) Flavonoids (6) Saponins (7) Alkaloids.

DISCUSSION

All extracts showed effect on types of standard bacteria at concentration 100mg/ ml, there was significant difference ($P<0.05$) between the zones of inhibition of different extracts. The methanol extract of leaves showed effect on all types of standard bacteria at concentration 100mg/ ml. highest values recorded *in Escherichia coli, Salmonella typhi* and *Staphylococcus aureus*, which were (15.5 ± 0.29mm), while the value of *Pseudomonas aeruginosa* (15.0 ±

0.0 mm) and *Bacillus subtilis*(14.0±0.0 mm) as the lower value, so the negative bacteria affected more than positive bacteria. Similarly, Bukar *et al*.[14]; the results showed that it had activity against four bacterial isolates *S. aureus* (08mm), *P.aeruginosa* (07mm) and *E. coli* (07mm) were sensitive at concentration of 200mg /ml.

The zones of inhibition of the Antibacterial activity of *Moringa oleifera* leaves of the Methanolic extracts at concentration 200ml; highest value recorded in *Pseudomonas aeruginosa* (22.5±0.29mm) and *Escherichia coli* (21.5±0.29mm), followed by *Salmonella typhi, Bacillus subtilis* and *Staphylococcus aureus* (18.5 ± 0.29mm); accordingly the gram negative bacteria affected more than positive bacteria. Similarly, Jackson *et al*.[15] found that the ethanolic extracts produced halos measuring 9–23mm. Disks with 400 mL extract displayed the largest halos: 22.3 mm (S.aureus), 17.0 mm (E. faecalis), 21.2 mm (A. caviae) and 17.8 mm (V. parahaemolyticus).

Table (3). The minimum inhibitory concentrations (MICs) ranged between 25 mg/ml and 50mg/ml for all the organisms except *Bacilus subtilis*. Similarly Napolean *et al*.[16] research that reported resistant of *P. aeruginosa* to all concentrations of methanol used apart from the highest concentration of 200mg/ml. *P. aeruginosa* is well known as a hardy and difficult organism that constitutes problems to researchers Brooks *et al*.[17] The leaves extract demonstrated none antibacterial activity of the petroleum ether extracts, as the growth inhibition zones were 0.0 mm. This indicates that M. oleifera leaf has no effect on these organisms (*Staphylococcusaureus*, Bacillus subtilis, *Escherichia coli, Pseudomonas aeruginos* and *Salmonella* typhi) at the concentration used (100 mg/ml).

The extracts have varying degree of antibacterial activities against the test organisms. Ciprofloxacin (the control) had the highest zones of inhibition on the all test organisms. However, there was significant difference ($P<0.05$) between the zones of inhibition of extracts and the control. The strong activities of the leaf suggest that the plant they may be used for treatment of infections caused by these organism except *Escherichia coli* and *Bacillus subtilis* (12.5mg/ml) which were found to be resistant to the activity of the methanol leaf extract.

The results obtained from the phytochemical screening of the methanolic extract indicated the presence of tannins, flavonoids, alkaloid, sterol, saponins, anthraquinones and triterpens.

Similarly Singh *et al*.[18] found that presence of gallic acid, chlorogenic acid, ellagic acid, ferulic acid, kaempferol, quercetin and vanillin from the aqueous extracts of leaves.

CONCLUSION

Findings of the present study suggested that methanolic extracts of *Moringa oleifera* leaves have potential as antibacterial compounds against pathogens and their ability to either block or inhibit resistance mechanisms of bacteria could improve treatment and eradication of bacterial strains. Thus this plant extracts could be used in the treatment of infectious diseases caused by resistant bacteria. The high activity of the methanolic extract of *moringa oleifera* leaves could be due to their active constituents which were detected by Phytochemical screening.

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