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DETERMINATION OF THE ANTIBACTERIAL EFFECTS OF ETHANOLIC EXTRACT OF *MORINGA OLEIFERA* LEAVES ON BACTERIAL ISOLATES FROM WOUND INFECTIONS

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ABSTRACT
Moringa oleifera is a medicinal substance that is used in the treatment of many infectious and non infectious ailments. In this study, the antibacterial activities of the crude ethanolic extracts of *Moringa oleifera* leaves on four bacterial wound isolates were determined, namely; *S. aereus, P. aeruginosa, E. coli* and *P. mirabilis.* The activity patterns of the crude extract was determined using Kirby-Bauer disc diffusion and Punched Holes techniques. Similarly, minimum inhibitory concentrations (MICs) of the extract were determined using Macrobroth dilution techniques as well as minimum bactericidal concentrations (MBCs). Phytochemical screening of the crude extract of *Moringa oleifera* was also carried out. Highly significant activity was observed with the crude extract of *M. oleifera* on *P. mirabilis* (at 125mg/ml, 250mg/ml and 500mg/l, $p = 0.037$), followed by its effect on *S. aureus* (125mg/ml, 250mg/ml and 500mg/ml, $p = 0.046$). Lower activity was observed with the plant on *P. aeruginosa* and *E. coli.* The overall results of the present study support that, the crude extracts of *M. oleifera* leaves could be considered as a potential source of novel antibacterial agents which may be employed to forestall the present antibiotic resistance menace.

KEYWORDS: Moringa, Bacteria, Wound, Ethanol, Infection.

INTRODUCTION
A wound is an interruption or break in the continuity of the external surface of the body or of an internal organ, caused by surgical or other forms of injury or trauma. Small numbers of bacteria usually gain access even to clean surgical wounds; larger number of bacteria invariably contaminate open wound incurred by accident.[1] Wound infections have however become a leading cause of frequent hospital visits and the use of antimicrobial agents is crucial in their management.[2] Regrettably, the conventional antimicrobial therapy has been seen posing problem in that the most incriminating bacteria are largely resistant to the readily available antibiotics.[3]

Many of these natural preparations have been described as natural God-given foods for the good health of the body.[3] As such, *Moringa oleifera* have been identified among other natural substances, to have antimicrobial effects on some bacterial isolates from wound infections.[3,4] The increasing prevalence of chronic wounds together with the emergence of antibiotic resistant bacteria warrants further investigation to improve wounds management practices and prevent complicated wound infection.[5]

On the other hand, *Moringa oleifera* is the widely cultivated species in the family Moringaceae.[6] It has been reported that *Moringa oleifera* is considered to have its origin in Agra and Oudh in the northwest region of India, south of the Himalayan Mountains.[7] In addition to its compelling water purifying powers and high nutritional value, *Moringa oleifera* is very important for its medicinal values. Various parts of this plant such as leaves, roots, seeds, bark, fruit, flowers and immature pods, act as cardiac and circulatory stimulant, antibacterial and antifungal activities among others and are being used for the treatment of different ailments.[8]

The medicinal value of *Moringa oleifera* cannot be over emphasized. Odebiyi and Sofowora[9] stressed that nearly every part of this plant, has been used for various

ailments in the indigenous medicine. The Indian ancient tradition of ayurveda says that Moringa leaves prevent 300 diseases.[10] Various parts of Moringa plants possess anti-inflammatory, anti-asthmatic and analgesic properties.[11] Moringa has a high nutritional value and contains carbohydrate, fat, and protein. The leaves are rich in vitamin A, vitamin B, vitamin C and minerals.[12]

Several research works had however been carried out on the antibacterial effect of *Moringa oleifera* plant on bacterial isolates. Eze *et al.*[8] in Abia, Nigeria worked on this and concluded that *Moringa oleifera* contained phytochemicals which confer antibacterial activity. On the other hand, Lar *et al.*[13] carried out research on the antibacterial activity of *Moringa oleifera* seed extracts on some selected gram negative bacterial isolates. They found out that ethanolic extract of the plant, at certain concentrations showed significant clear zones of inhibition against some selected bacteria namely: *Escherichia coli, Shigella flexneri* and *Salmonella typhi.* Dike-Ndudim *et al.*[4] used extracts of fresh and dried *Moringa oleifera* leaves and found that both aqueous and ethanolic extracts of *Moringa oleifera* exhibited antibacterial effect against all the test organisms. Their test organisms were *Escherichia coli, Streptococcus pyogenes, Salmonella typhi and Pseudomonas aeruginosa.*

MATERIALS AND METHODS

Study Design

It is a cross sectional observational study.

Study Area

Usmanu Danfodiyo University Teaching Hospital, (UDUTH), Sokoto is a Federal Government owned Hospital located within the Sokoto metropolis. Sokoto metropolis lies between latitude $13^0 3^1$ 49^0N, longitude $5^0 14^1$ 89^0E and at an altitude of 272m above sea level. UDUTH is located in Wammako local government area of Sokoto state. Sokoto metropolis was estimated to have a population of 427,760 people. The major occupations of the inhabitants are; trading, commerce and farming with reasonable proportion of the population working in both public and private sectors.[15]

Sample Size Determination

The sample size was determined according to Cochran[14] using the formula;

$$n = \frac{Z^2 pq}{d^2}$$

Where n =Sample Size, z =Standard normal deviates, p = prevalence factor,
q = complimentary proportion of P (1-p), d =Tolerable margin of error or confidence interval.
Therefore, taken a confidence level of 95% Z =1.96
p =12% =0.12 **(**Abdurrahman *et al*., 2012**).**
q = (1-0.5), d = (5%) =0.05,
n =?
Thus,

$$n = \frac{(1.96)^2 \times 0.12 \times (1-0.5)}{(0.05)^2}$$

$$= \frac{3.8416 \times 0.12 \times 0.5}{0.0025}$$

$$= \frac{0.230496}{0.0025}$$

= 92 + 9.2 (10% attrition rate)
= 101

Source of Test Organisms

A total of 101 bacterial isolates from wound infections were collected from the Medical Microbiology Laboratory, Usmanu Danfodiyo University Teaching Hospital, UDUTH, Sokoto. They comprise of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Proteus mirabilis.*

Plant Collection and Identification

The *Moringa oleifera* leaf was purchased from Yabo local government, Sokoto state. The leaf was identified and authenticated in the herbarium of Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto by head of department, Pharmacognosy, Dr. Halilu E. Mshelia. The voucher specimen with specimen number; PCG/UDUS/Mori/0001 was preserved at the herbarium.

Preparation of Plant Sample for Extraction

Fresh leaf of *Moringa oleifera* plant was extracted by maceration process. About 200g of the fresh leaves were grinded using a clean sterile mortar and pestle. The leaves were then soaked in 1L of 98% ethanol[16] for 24 hours at room temperature and in an air tight plastic container. The leaves were then filtered with Whatman's No.1 filter paper. The ethanol extract was then concentrated at 40^0C under reduced pressure using rotary evaporator (R100); and when it completely dried, the phytochemical analysis was carried out and the final extract was weighed.

Bacterial Isolation

The organisms of interest were *Staphylococci aureus, Pseudomonas aeruginosa, Proteus mirabilis and Escherichia coli*. Bacteria biochemical tests were performed to confirm the identity of all the isolates. The isolates were each sub cultured on nutrient agar, incubated at 37^oc for 24 hours. This was done to produce discrete colonies of the isolates.

Preparation of extract Concentrations

The resultant extract concentrations 500mg/ml, 250mg/ml, 125mg/ml and 63mg/ml were prepared.

Preparation of Inoculum

Direct colony suspension method was the technique employed in the preparation of the inoculums in this study as recommended by CLSI.[17] After overnight subculture, discrete colonies of the isolates were picked with a sterile inoculating loop and suspended in 5mL of sterile normal saline to make a suspension. The turbidity

of the inoculum suspension was adjusted to that of 0.5 McFarland standard (10^5 CFU/ml) against a card with a white background and contrasting black lines under an illuminated surface.

Inoculation of Tests Plate

Mueller Hinton agar plates were prepared aseptically, allowed to set and dry. The carefully adjusted inoculum suspension was allowed to stand for 15 minutes and a sterile cotton swab dipped into the adjusted suspension, rotated several times and press firmly on the inside wall of the tube above the fluid to remove the excess fluid from the swab.[17] Thereafter, the swab was streaked over the entire sterile surface of the dried Mueller Hinton agar plate. This procedure was repeated twice by rotating the plate at approximately 60^o each time to ensure an even distribution of the inoculums.[17]

Determination of Antibacterial Activity of Ethanolic Extracts of *Moringa oleifera* Leaves

Agar Diffusion Test (Punched Hole Method)

This was done with the aid of the sterile standard cork borer. Five wells of 6mm in diameter were punched at different sites on the plates. The bottoms of the wells were sealed with a drop of the sterile Mueller Hinton agar to prevent diffusion of the honey under the agar. The first well was filled with 63 **mg/ml**, second well with 125 **mg/ml**; third well with 250 **mg/ml** and the fourth well with 500 **mg/ml** (well 1 to 4). A prepared ciprofloxacin disc (5µg/disc) was used as positive control at the centre of the agar.

The plates were allowed on the bench for 40 minutes, for pre-diffusion and then incubated at 37^oC overnight. The resulting zones of inhibition were measured in millimeters. The diameters of the zones of inhibition of the bacterial isolates in question were taken at a particular concentration of the test honey.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration gives the lowest (highest dilution) of the *Moringa oleifera* leaves extracts that can inhibit the growth of the test bacteria. This was determined by using the broth tube dilution method as described by Ceyhan and Ugar.[18]

Freshly prepared nutrient broth was used in sterile tubes. 1ml of nutrient broth was put into test tubes number two (2) to test tube number twelve (12). 1ml of the extract concentration was added to tubes 1 and 2. The extract in tube 2 was therefore diluted 1:2. It was properly mixed then 1ml was transferred to tube 3 giving 1:4 dilution. This was continued until the 11^{th} tube from which 1ml was discarded. The tube 12 which contained only nutrient broth, served as control. 1ml of the standard inoculum of each of the organism was then added to all tubes. The entire procedure was repeated for all the test organisms that might be susceptible to extract. The tubes were thoroughly mixed and incubated at 37^oC for 24hrs. Thereafter, they were visually observed for turbidity after incubation by comparing with control tube.

Determination of the Minimum Bactericidal Concentrations (MBCs) of the Crude Extract of *Moringa oleifera*

The MBC was determined by sub-culturing 0.01ml (10µL) of the highest concentrations of the dilutions which showed visible growth and all the tubes showing no visible sign of growth in the MIC tube dilution test onto a solid media.[19]

Ethical Clearance

Prior to the commencement of clinical isolates collection, ethical approval was obtained from the ethical committee under the Chairman Medical Advisory Committee (CMAC), UDUTH. The approval of the hospital management was sought to allow the use of some of the bacterial clinical isolates from wound swabs of patients having wound/burns isolated in medical microbiology laboratory unit of the hospital.

RESULTS

The amount of residues obtained after the extraction of *M. Oleifera* leaves was 15.2g (7.6%) of the original 200g of the leaves. The yield obtained from the ethanolic extraction was 7.6%. One hundred and one (101) bacterial wound isolates were collected and identified using the standard microbiological methods,[17,19,20] out of which 33(32.7%) were *Staphylococcus aureus*, 29(28.7%) *Pseudomonas aeruginosa*, 21(20.8%) *Escherichia coli* and 18(17.8%) *Proteus mirabilis* (Table 1).

A significant difference ($p < 0.05$) was observed between the mean inhibitory zone diameters of the ethanol extract of *M. oleifera* when compared with the standard antibiotic (Table 2). The highly significant difference was observed on *Staphylococcus aureus* [20.3±0.49, 15.6±0.36, and 10.7±0.34 at 500mg/ml, 250mg/ml and 125mg/ml respectively, in contrast to ciprofloxacin (34.7±0.47) p=0.046] and the lowest was observed on *Proteus mirabilis* [12.5±0.20, 11.1±0.26, and 8.0±0.18 at 500mg/ml, 250mg/ml and 125mg/ml respectively, in contrast to Ciprofloxacin (38.3±0.24) p =0.037].

No significant difference ($p > 0.05$) was observed between the inhibitory zone diameters of the other two isolates; *Pseudomonas aeruginosa* [18.3±0.44 and 9.9±0.29 at 500mg/ml and 250mg/ml respectively, in contrast to Ciprofloxacin (23.2±0.34) p=0.68] and *Escherichia coli* [19.7±0.58 and 11.1±0.37 at 500mg/ml and 250mg/ml respectively, in contrast to Ciprofloxacin (26.4±0.39) p =0.49].

Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations of Crude Ethanol Extract of *M. oleifera* on Bacterial Isolates

The results of the minimum inhibitory concentration (MIC) of the ethanolic extracts of *M. oleifera* leaves

showed that *S. aureus* and *P. aeruginosa* were susceptible or sensitive at a concentration of 250mg/ml which is the lowest concentration of the extract that inhibited bacterial growth resulting in visually clear tubes after 24hrs incubation. *E. coli* was susceptible at a concentration of 63mg/ml while *P. mirabilis* was resistant at all concentrations (Table 3).

The minimum bactericidal concentration (MBC) of the ethanolic extract on *S. aureus, P. aeruginosa* and *E. coli* was 500mg/ml. *P. mirabilis* was resistant at all concentrations (Table 3).

Results Obtained from the Preliminary Phytochemical Screening of the Crude Extract of *M. oleifera*

The results of the preliminary phytochemical screening of the extracts are shown in Table 4. The ethanol extract of *M. oleifera* leaves revealed the presence of carbohydrates, phenols, flavonoids, tannins, alkaloids, steroids, anthraquinones, cardiac glycosides, saponins.

Table 1: The Identified Bacterial Isolates and their Source.

Bacterial	No. Isolated	Source	Percentage(%)
Staphylococcus aureus	33	Wound swab/pus/aspirate	32.7
Pseudomonas aeruginosa	29	,, ,,	28.7
Escherichia coli	21	,, ,,	20.8
Proteus mirabilis	18	,, ,,	17.8
Total	**101**		**100**

Table 2: Comparison of the Inhibitory Zone Diameters of the Crude Ethanol Extracts of *M. oleifera* with Standard Antibiotic against the Clinical Bacterial Isolates.

	Zones of inhibition (mm)							
	Ethanol extracts (mg/ml)				Neg. Control		Std drug (µg/disc)	
Isolates	500	250	125	63	DW	Cipro (5)	F	P
S. aureus	20.3±0.9	15.6±0.6	10.7±0.4	6.0±0.00	6.0±0.00	34.7±0.47	2.4	0.046
P.aeruginosa	18.3±0.44	9.9±0.29	6.0±0.00	6.0±0.00	6.±0.00	23.1±0.34	0.7	0.68
E. coli	19.7±0.58	11.1±0.37	6.0±0.00	6.0±0.00	6.0±0.00	26.4±0.39	1.1	0.49
P. mirabilis	12.1±0.20	11.1±0.26	8.0±0.18	6.0±0.00	6.0±0.00	38.3±0.24	3.7	0.037

Data are presented as mean ±SEM by using ANOVA. Values greater than 6±SEM indicate some activity. ***Key:*** *Std drug = Standard antibiotics, Neg. = Negative, Cipro. = Ciprofloxacin, S.= Staphylococcus, P.= Pseudomonas E. = Escherichia, P.= proteus DW = Distilled water.*

Table 3: The MICs and MBCs of the Crude Ethanol Extract of M. oleifera against the Bacterial Isolates.

Isolate	MIC *M. oleifera* (mg/ml)	MBC *M. oleifera* (mg/ml)
S. aureus	250	500
P. aeruginosa	250	500
E. coli	63	500
P. mirabilis	--	_

Key *- = No concentration could affect the MBC*

Table 4: Preliminary Phytochemical Screening Results of Ethanol Extracts of *M. oleifera*.

Constituents	Type of Test	EE of *M. oleifera*
Carbohydrates	Mollisch's test	+
	Fehling's test	+
Phenols	Ferric Chloride test	+
Flavonoids	NaOH test	+
	Schinoda's test	+
Tannins	Lead acetate test	+
	Ferric Chloride test	+
Alkaloids	Meyer's test	+
	Wagner's test	+
Steroids	Salkowki's test	+
	Liebermann-Buchard's test	+
Anthraquinones	Borntrager's test	-
Cardiac glycosides	Kella-Killiani's test	+
Saponins	Froathing test	+

Table 5: Comparison between the Efficacies of *M. oleifera* and Standard Antibiotic.

Isolates	ZIM(mm)	ZIA(mm)	P-value
S. aureus	20.3±0.49	34.7±0.47	0.046
P. aeruginosa	18.3±0.44	23.1±0.34	0.68
E. coli	19.7±0.58	26.4±0.39	0.49
P. mirabilis	12.1±0.20	38.3±0.24	0.037

Keys: *ZIM = zone of inhibition at the highest concentration of M. oleifera,*
ZIA = zone of inhibition of the antibiotic

DISCUSSION

In this study, the antibacterial activities of the crude extracts of *M. oleifera* leaves were tested against four wound associated bacteria viz; *S. aureus, P. aeruginosa, E. coli* and *P. mirabilis.* The antibacterial activity of the extracts was recorded when the inhibition zone was greater than 6mm.

This study revealed a significant antibacterial activity of the crude ethanol extract of *M. oleifera* to all the tested bacteria at varying concentrations. A highly significant difference was observed between the inhibitory zone diameters of the crude ethanol extracts of *M. oleifera* when compared with the standard antibiotic on *S. aureus* (p = 0.046) and the lowest was observed on *P. aeruginosa* (p = 0.68). The susceptibility of some bacteria strains to the extract *M. oleifera* may be a pointer to its potential as a drug that can be used against these susceptible bacterial strains.[21] It was noted that the ethanol extract of *M. oleifera* leaves exhibited antimicrobial effect against both Gram-positive and Gram-negative bacteria (broad spectrum activity) and this corroborates with the findings of Moyo *et al.*[21] Noteworthy, is the ability of the *M. oleifera* ethanol extract to inhibit the growth of *E. coli* at 63mg/ml which is the lowest MIC value in comparison to the other bacterial strains. This suggests that *E. coli* was very sensitive to the *M. oleifera* ethanol extract and could be used as an antibiotic against diseases that are caused by *E. coli* although Ciprofloxacin antibiotic was more effective.

In this experiment, it was found that ethanol is a good solvent capable of extracting constituents like tannin and phenol which are reported to possess antibacterial activity.[22] The phytochemical results of the ethanolic extracts of *M. oleifera* leaves, in this study, corroborate the reports by Bukar *et al.*[23] This work also showed that the ethanolic extracts of fresh *M. oleifera* leaves extract have inhibitory effect against *S. aureus, E. coli* and *P. aeruginosa* which was supported by the study done by Dike-Ndudim *et al.*[4] and Nepolean *et al.*[24]

CONCLUSION

The results of this research have demonstrated that *M. oleifera* leaf extracts have potential antibacterial effects on wound bacterial pathogens. Significant antibacterial activity was observed on the bacterial isolates with the crude ethanol extracts despite their antibiotic resistance antecedents. Inhibition of both Gram-positive and Gram-negative organisms by this plant extract depicts that it can serve as a source of broad spectrum antibiotic; this justified the traditional use of this plant for therapeutic purposes.

RECOMMENDATIONS

1. Due to its broader antibacterial activity coupled with less or non toxic effects, *M. oleifera* plant materials may become an alternative source of antibacterial agents that would complement the effort of the existing antibiotics or provide a novel or lead compound that may be employed to forestall antibiotic resistance menace pending further work on drug development processes.
2. Higher concentrations of *M. oleifera* above 500mg/ml would be more efficacious on bacterial pathogens and this is recommended for further clinical trials.

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