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Vol. 11(39), pp. 1484-1494, 21 October, 2017
DOI: 10.5897/AJMR2016.7986
Article Number: E77E78266484
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Comparative evaluation of the antimicrobial profile of *Moringa* leaf and seed oil extracts against resistant strains of wound pathogens in orthopedic hospitals

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Received 27 February, 2016; Accepted 9 May, 2016

The antimicrobial profile of oil extracts from *Moringa oleifera* leaves and seeds on orthopaedic wound pathogens was tested and compared with the antimicrobial activity of some antibiotics. The pathogens were characterized using biochemical and morphological tests. Antimicrobial susceptibility test was done on the pathogens using paper discs diffusion method. Plasmid curing was done on the isolates that showed resistance to antibiotics and the *Moringa* extracts. Time of kill assay was done with modified plating technique. *Staphylococcus aureus* accounted for 43% of the pathogens followed by *Proteus* spp. (16%), *Klebsiella* spp. (15%), *Citrobacter* spp. (11%), *Escherichia coli* (8%) and *Pseudomonas aeruginosa* (6%). Over 80% of the organisms were resistant to the tested antibiotics and their resistance were of plasmid origin. The methanolic leaf oil extract showed highest antimicrobial activity. The activity of the aqueous seed oil extract was significantly higher than the methanolic and ethanolic seed oil extracts ($P > 0.05$). Viable cell counts of *S. aureus* and *Klebsiella* spp. were reduced by the methanolic leaf and aqueous seed oil extracts. The antimicrobial activity of the methanolic leaf and aqueous seed oil extracts compared favourably with the reference antibiotics and can be used as alternatives for treatment of orthopaedic wound infections.

Key words: Orthopeadic, antibiotics, plant extracts, plasmid, wound, infection, antimicrobial, *Moringa oleifera.*

INTRODUCTION

Antibiotic resistance, especially among wound containing bacteria is an important issue of discussion in the

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treatment of chronic wound infections. Most wounds have been reported to be contaminated by pathogens and body commensals ranging from bacteria, fungi to other parasites (Sani et al., 2012). The common gram positive organisms found in most wounds include β- haemolytic *Streptococcus- Streptococcus pyogenes* and *Staphylococcus aureus*. Others such as the gram negative aerobic rods, *Pseudomonas aeruginosa* have also been implicated in wound infections (Enweani, 1991). The facultative anaerobes like the Enterobacteriaceae species, such as *Escherichia coli*, *Klebsiella* and *Proteus* spp. have also been reported to constitute a serious problem in several wound infections (Mulugeta and Bayeh, 2011). The widespread use of antibiotics, together with the length of time over which they have been available, have led to major problems of resistant organisms, contributing to high levels of morbidity and mortality in patients (Akinjogula et al., 2010). The control of wound infections is becoming more challenging due to widespread bacterial resistance to antibiotics and to a greater incidence of infections caused by methicillin resistant *S. aureus* (MRSA) and polymicrobic flora (Cheesbrough, 2006). Increased antimicrobial resistance among pathogens of wound infections can result to complications and increase in the cost associated with procedures and treatments. Due to this rising resistance among wound pathogens, there is a need to search for alternative sources of treatment of wound infections in natural compounds from medicinal plants such as *Moringa oleifera*.

M. oleifera is an ancient tree that is historically known to possess numerous medicinal qualities (Posmontier, 2011). Previous studies have reported the antimicrobial properties of the various parts of *Moringa* roots, flowers, barks and stem including the seeds (Lockett et al., 2000; Walter et al., 2011). The seed kernel of *M. oleifera* has been employed in the treatment of bronchial asthma and found to show an appreciable decrease in severity of symptoms of asthma and also simultaneous improvement in respiratory functions (Fahey et al., 2001).The family Moringaceae has been found to be rich in compounds containing rhamnose, and it is rich in a fairly unique group of compounds called glucosinolates and isothiocyanates (Bennett et al., 2003). *Moringa* is also rich in a number of vitamins and minerals as well as other more commonly recognized phytochemicals such as carotenoids (Siddhuraju and Becker, 2003). *M. oleifera* has been reported to have natural antioxidant properties and thus enhance the shelf-life of fat-containing foods (Gilani et al., 1994). The roots of *Moringa* plants have been reported to possess antispasmodic activity through calcium channel blockade which forms basis for its traditional use in diarrhoea (Ghebremichael et al., 2005). The flowers show effective hepatoprotective effect due to the presence of quercetin (Costa-Lotufo et al., 2005). The seed of *Moringa* is also effective against skin papillomas in mice and the seed ointment had similar effect to neomycin against *S. aureus* pyodermia in mice (Adegoke et al., 2010). Finding an alternative source of treatment for wound infections using *M. oleifera* plant extracts could provide the needed replacement for conventional antibiotics since most wound pathogens have appeared to have developed resistance to most of the available antibiotics. The aim of the present study was to investigate the antimicrobial activity of *M. oleifera* seed and leaf oil extracts against broad spectrum antibiotic resistant microorganisms isolated from wound infections. The study also investigate if the resistance to the conventional antibiotics by some wound pathogens is plasmid associated.

MATERIALS AND METHODS

Sample collection *(M. oleifera),* identification and preparation of the plant extracts

The fresh leaves and seeds of *M. oleifera* plant (Supplementary Figure 1a and b) were collected from Dekina Local Government Area, Kogi State Nigeria. The plant samples were identified at the Department of Botany, University of Nigeria, Nsukka, Nigeria. For the methanol and ethanol extractions, 50 g each of *M. oleifera* leaves and seeds were crushed and fed to a lab-scale Soxhlet extractor fitted with a 1 L round-bottom flask and a condenser. About 250 mL of both methanol and ethanol were added to the set up and the extraction was done for 6 h. The solvents were then evaporated under reduced pressure using a rotary evaporator at 45°C.

Aqueous extracts of the fresh leaves and seeds for antimicrobial examination

One hundred grams (100 g) of fresh leaves and seeds of *M. oleifera* were weighed and crushed directly using electric blender and missed in 400 mL distilled water into a conical flask stoppered with rubber corks and shook for 30 min, after which they were left to stand for 72 h and were shaken intermittently. The mixture was filtered off using sterile filter paper into a clean conical flask. The solvents were allowed to evaporate leaving the concentrated filtrates, which were then stored at 4°C for antimicrobial activity test.

Isolation, characterization and identification of orthopaedic wound samples

Wound samples were collected from one hundred (100) patients at National Orthopaedic Hospital Enugu, using a sterile swab stick and sterile distilled water. Out of the 100 samples, 45 were from post-operative wounds while 55 were from other wound sources. The samples were transferred to the Microbiology Laboratory of the University of Nigeria within 24 h for further analysis. The collected samples were streaked on freshly prepared nutrient agar and MacConkey agar and incubated aerobically at 37°C for 24 h. Discrete colonies differing in size, shape and colour were selected from the different plates and were sub cultured into fresh nutrient agar plates. The colonies were characterized using conventional biochemical tests (indole, catalase, urease, coagulase, sulphide indole motility, sugar fermentation and oxidase tests) following established protocols (Doughari et al., 2007). Further characterization was done using colonial and cell morphology.

Antibiotic susceptibility test

Antimicrobial susceptibility of the bacterial isolates was determined using the discs diffusion method, as described by Doughari et al. (2007). Briefly, the isolates were picked using sterile inoculating loop and inoculated into a sterile test tube with 5 mL of phosphate-buffered saline. The optical density of the organisms at 600 nm wasadjusted to 0.5 McFarland (0.08 to 0.1). Sterile cotton swabs were used to spread the growth evenly on the surface of Mueller-Hilton (MH) agar. The antibiotic discs which contained the following antibiotics, streptomycin, erythromycin, cloxacillin, chloramphenicol, amoxycillin, nitrofurantoin, nalidixic acid, ofloxacid, tetracycline, augmentin, gentamycin and cotrimoxazole were placed on the surface of MH agar plates. The MH agar plates were incubated for 18 to 24 h at 37°C. All the antibiotics were tested on both the gram positive and gram negative organisms. The antibiogram was read and recorded as the diameter of zone of the inhibition (ZI). The resistant isolates were separated from the susceptible ones and further analysis was carried out on them.

Antibacterial activity of the leaf and seed oil extracts

Antimicrobial activity of the aqueous, ethanolic and methanolic oil extracts of the leaves and seeds were assayed using the paper discs diffusion method (Oluma et al., 2000). The concentrated leaf extracts were dissolved in 5% dimethyl sulfoxide (DMSO) and sterile discs, 6 mm (Hi-media, India) in size were each impregnated with 30 µL of 50 µg/mL of each extract. The discs were carefully and firmly placed on the Muller Hinton Agar (MHA) plates earlier seeded with standardized bacterial suspensions (approximately 1.5×10^6 CFU/mL). Filter paper discs dipped into sterile distilled water and allowed to dry were used as control. The plates were then incubated at 37°C for 24 h. Antibacterial activity was determined by measurement of ZI around each paper disc.

Determination of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Equal volumes of each oil extract (with different concentrations) and nutrient broths were mixed in 8 test tubes and 0.1 mL of the standardized inoculum was added to each of the tubes. The tubes were incubated aerobically at 37°C for 18 to 24 h. Two control tubes were maintained for each test batch. The MIC was taken as the lowest concentration that prevented bacterial growth. The MBC was determined by sampling all the macroscopically clear tubes and the first turbid tube in the series. Before being sampled, the tubes were gently mixed by flushing them with a sterile pipette, and a 100 mL aliquot was removed from each test tube. Each aliquot was placed on a single antibiotic-free nutrient agar plate in a single streak down the centre of the plate in accordance with the method established by Irkin and Korukluoglu (2007). The samples were allowed to stand for 30 min so that they can be absorbed into the agar until the plate surface appeared dry. The aliquots were then spread over the plate by lawn technique. The growth and sterility controls were sampled in the same manner. The MBC lawn plates were incubated for 24 h at 35°C. After the incubation periods, the lowest concentrations of the extract that did not produce any bacterial growth on the solid medium were regarded as MBC values for the extract (Cruishank et al., 1975).

Time of kill assay

An assay on the rate of killing of *S. aureus* and *Klebsiella* spp. by the methanol and aqueous seed oil extracts was carried out using a modified plating technique (Zhou et al., 1990). Briefly, the extracts were incorporated into 10 mL Mueller Hinton broth in McCartney bottles at MIC. Two controls, one Mueller Hinton broth without extract inoculated with test organisms and Mueller Hinton broth incorporated with the extract at the test concentrations without the test organisms, were included. Inoculum density, approximately 10^5 CFU/mL further verified by total viable count was used to inoculate 10 mL volumes of both test and control bottles. The bottles were incubated at 37°C on an orbital shaker at 120 rpm. A 100 µL aliquot was removed from the culture medium at 0, 1, 2, 3 and 4 h for the determination of CFU/mL by the plate count technique (Zhou et al., 1990). About 25 µL of each of the dilutions was plated out and incubated at 37°C for 24 h for emergence of bacterial colonies. The cell counts from the experimental cultures were compared with the controls.

Plasmid isolation and profiling

The bacterial isolates that were resistant to the antibiotic susceptibility test were selected for plasmid isolation and curing. Preparations of plasmid DNA of the resistant isolates was done by alkaline lysis method as described by Taylor and Brose (1988). A single bacterial colony was picked from the clones and grown in nutrient broth at 37°C for 24 h. The cell suspension was transferred to Eppendorf tube using a micropipette and centrifuged at 6000 rpm for 5 min at 4°C. Pelleted cells were re-suspended in 300 µL of the lysis solution (25 mM Tris, 10 mM EDTA, 0.1 N NaOH and 0.5% SDS) and incubated at room temperature for 5 min. Sodium acetate solution was added to the mixture and the solution was mixed properly by whirling before incubation at room temperature for 5 to 10 min. Cold absolute ethanol was used to precipitate the $_P$DNA and the mixture was spun for 10 min at 12000 rpm. The pelleted DNA was washed with 1 mL 70% cold ethanol, air dried for at least 10 min and re-suspended in 20 to 40 µL of distilled water for further analysis. Electrophoresis was done with 2 µL of the extracted plasmid in 0.8% agarose gel for 1 h at 90 V. The gel was stained with ethidium bromide (0.5 µg/mL) and visualized with UV light (302 nm UV transilluminator).

Plasmid DNA curing

Plasmid curing was done with acridine orange (AO) and sodium dodecyl sulphate (SDS) as described in previous studies (Bhalakia, 2005; Isibor et al., 2008). Nutrient broth was prepared and supplemented with 1g of SDS in one batch of 99 ml and 10 g of SDS in a second batch of 90 mL to achieve final concentrations of 1 and 10% (w/v) SDS, respectively. Each of the solutions was adjusted to pH 7.6 with 1 M NAOH and autoclaved at 121°C for 15 min. Selected overnight (O/N) cultures of the isolates were standardized to 0.5 McFarland turbidity standard using sterile saline. In each 5 mL SDS supplemented nutrient broth, 0.1 mL of each O/N culture was inoculated and incubated at 37°C for 24 h. Cultures were later subjected to the same antibiotics to which they were resistant to previously. The curing process was repeated with acridine orange following the standard protocols.

RESULTS

Characterization and antibiotic susceptibility test of the bacterial isolates

A total of 100 wound samples were collected from the National Orthopaedic Hospital Enugu and were identified. From the biochemical test, 43% of the bacterial isolates were *S. aureus,* 16% were *Proteus* spp., 15% were

Table 1. Biochemical characterization and the identification of bacterial pathogens from orthopaedic wound infections.

Organism	Gram reaction	Oxidase	Catalase	Urease	H_2S	Motility	Coagulase	Indole	Citrate
S. aureus	+	-	+	+	-	NT	+	-	+
Citrobacter spp.	-	NT†	+	NT	NT	+	NT	NT	+
Proteus spp.	-	NT	NT	-	+	+	NT	-	-
Klebsiella spp.	-	NT	+	+	-	-	NT	NT	+
E. coli	-	NT	+	-	-	+	NT	+	-
P. aeruginosa	-	+	NT	+	-	+	NT	+	+

Different biochemical tests were carried out to characterize and identify each orthopaedic wound samples. The biochemical analysis were able to group the othorpedic wound samples into gram positive (*S. aureus*) and gram negative organisms (*Citrobacter spp*, *Proteus spp, Klebsiella spp, E. coli* and *P. aeruginosa*). NT = Not tested; - = Negative; + = Positive.

Table 2. Descriptive statistics of the antibiotic resistance profile of the different bacterial groups identified in this study.

Antibiotic†	Concentration (µg/mL) ‡	No of resistant isolates††	Gram (-)	Gram (+)	Percentage
Cotrimoxazole	25	31	ND	31	75.6
Gentamicin	10	69	35	35	70.4
Augmentin	30	94	56	38	95.9
Tetracycline	25	91	53	38	92.8
Ofloxacin	5	46	46	ND	80.7
Nalidixic acid	30	47	47	ND	82.4
Nitrofurantoin	200	44	44	ND	77.1
Amoxycilin	25	56	56	ND	98.2
Chloramphenicol	10	32	ND	32	78.0
Cloxacillin	5	41	ND	41	100
Erythromycin	5	41	ND	41	100
Streptomycin	10	38	ND	38	92.6

† = Antibiotics used for the susceptibility study; ‡ = Concentrations of the antibiotics; †† = Number of isolates that were resistant to the antibiotics. ND = Not determined.

Klebsiella spp., 11% were *Citrobacter* spp. while *Escherichia coli* and *P. aeruginosa* were 8 and 6% respectively (Table 1). The result from the biochemical test was further confirmed with the analysis of the colonial and cell morphology of the organisms. Out of the 100 wound isolates used in this study, 98 of them were used for antibiotic susceptibility test, 88.7% of them were resistant to at least 4 or more antibiotics (Table 2). All the organisms were resistant to amoxycillin and cloxacillin.

Bacterial resistance and plasmid association

All the gram negative bacterial wound pathogens (*Proteus* spp., *P. aeruginosa*, *Klebsiella* spp. and *E. coli*) had 3 plasmid bands each. The first set of bands lie in the region of 23 kb while the two smaller sized plasmids lie within the regions of 2 kb and 500 bp (Figure 1a). For the gram positive wound pathogens which were mainly *S. aureus*, no bands were observed in two strains (lanes 13 and 72), while the three remaining strains (lanes 16, lane 22 and 45) had 3 plasmid bands each. The first set of bands was 23 kb in size while the other bands lie between the regions of 500 bp and 2 kb (Figure 1b). To analyse if the resistance observed in some multidrug resistant isolates was plasmid associated, a subset of the isolates that showed resistance to the tested antibiotics were subjected to plasmid curing. All the gram negative organisms became susceptible to most of the antibiotics they were previously resistant to after the curing procedure. However, *S. aureus* remained resistant to all the antibiotics even after plasmid curing.

Antimicrobial activity of the *M. oleifera* leaves and seed oil extracts

All the leaf oil extracts showed inhibitory effects at the tested except on *Proteus* spp. whose growth was not

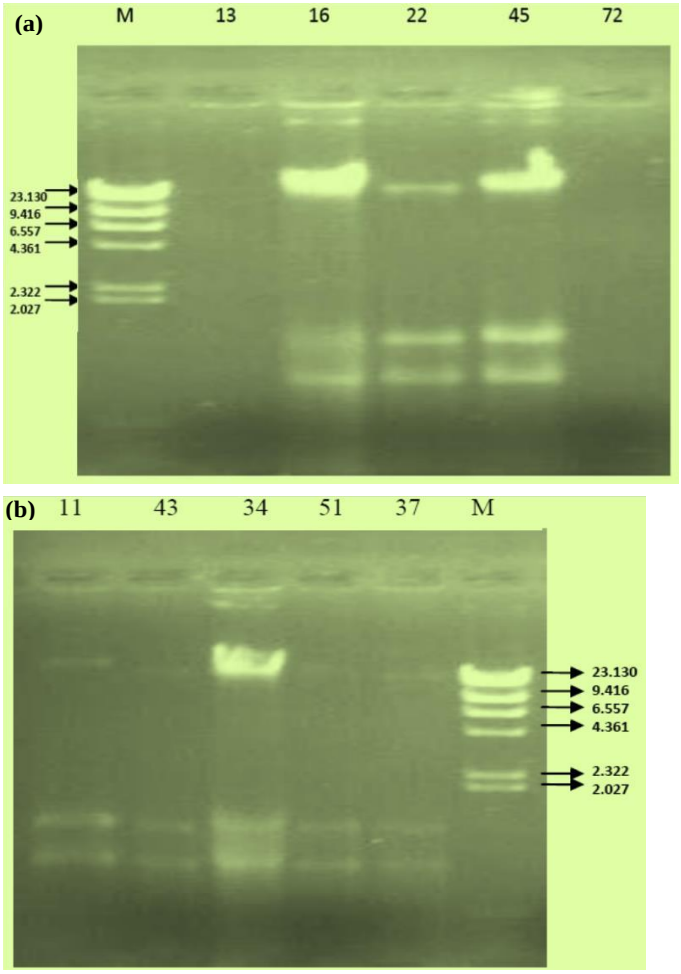


Figure 1. Plasmid profile of the bacterial species isolated from orthopedic wound infection. (a) pDNA from gram negative organisms, lanes (11 = *Proteus spp,* 43 = *Klebsiella spp,* 34 = *Pseudomonas* spp*.,* 51= *E. Coli,* 37= *Klebsiella spp*), (**b**) pDNA from gram positive bacteria, lanes (13, 16, 22, 45 and 72 represent different strains of *S. aureus*). Electrophoresis was done with 2 µl of the plasmid from each sample in 0.8% agarose gel for 1 h at 90 V. The gel was stained with ethidium bromide (0.5 µg/mL) and visualized with UV light (302 nm UV transilluminator), 23 kb marker was used to determine the size of the isolated pDNA.

concentration of 50 µg/mL on all the microorganisms inhibited by the aqueous and ethanolic oil extracts at the tested concentration. The aqueous leaf oil extract had ZI ranging from 3.0 ± 0.0 to 20.3 ± 1.5 mm in *Proteus* spp. and *E. coli* respectively (Table 3). The methanolic leaf oil extract had the highest ZI, 22.7 ± 2.1 mm in *P. aeruginosa.* However, it showed a reduced ZI of 11.0 ± 2.0 mm in *Klebsiella* spp. The ethanolic leaf oil extracts showed the lowest ZI ranging from 3.0 ± 0.0-12.3 ± 1.5 mm in *Proteus spp* and *E. coli* respectively. In all the extracts, all the isolates tested were more susceptible to the methanolic leaf oil extract (Figure 2) while the ethanolic leaf oil extract had the least antimicrobial activity against the tested organisms (Table 3). The antimicrobial activity of the aqueous oil extract, methanolic and ethanolic oil extracts of *M. oleifera* seeds respectively is presented in Table 4. All the seed oil extracts showed inhibitory effects at the concentration of 50 µg/mL. The aqueous oil extract of the seeds had more inhibitory activity with ZI ranging from 15.3 ± 2.2 to 22.0 ± 1.3 mm in *S. aureus* and *E. coli* respectively. This was followed by methanolic seed oil extract with the ZI ranging from 11.0 ± 3.0-18.7 ± 1.5 mm in *S. aureus* and *E. coli* respectively. However, the sensitivity of the isolates to ethanolic seed oil extract was relatively low in most of the isolates tested (Table 4). Three isolates, *Citrobacter* spp., *Proteus* spp and *E. coli* were not sensitive to the ethanolic seed oil extract (Table 4).

Minimal inhibitory concentration of *M. oleifera* leaves and seeds oil extracts

The MIC for the methanolic leaf oil extract was 20 µg/mL, at this concentration, the growth of all the microorganisms were inhibited. The aqueous leaf oil extract had inhibitory effect on *Citrobacter* spp and *E. coli* at a concentration of 20 µg/mL. At a concentration of 30 µg/mL the aqueous leaf oil extract also had inhibitory effect on *S. aureus and Klebsiella* spp*,* but did not have any inhibitory effect on *P. aeruginosa.* A slightly different trend was observed for the seed oil extracts. At the concentration of 20 µg/mL, the aqueous seed oil extract inhibited the growth of all the microorganisms tested except *P. aeruginosa* that was inhibited at 30 µg/mL. At the concentration of 30 µg/mL, the methanolic seed oil extract had inhibitory effect on *Proteus* spp, *Klebsiella* spp and *E. coli* but it showed inhibition on the growth of *S. aureus* and *P. aeruginosa* at concentration of 40 µg/mL. At a higher concentration of 50 µg/mL it inhibited the growth of *Citrobacter* spp. However, for the ethanolic seed oil extract, the MIC was quite high in most of the bacterial species, 60 µg/mL, 40 µg/mL and 50 µg/mL for *S. aureus, Klebsiella* spp*.* and *P. aeruginosa* respectively (Figure 3).

Minimal bactericidal concentration of *M. oleifera* leaves and seeds oil extracts

The aqueous leaf oil extract had the highest bactericidal effect, with concentration as low as 20 µg/mL, all the organisms except *P. aeruginosa* were killed (Figure 4). The methanolic leaf oil extract also had effect on all the tested organisms although at variable concentrations. However *M. oleifera* ethanolic leaf oil extract had no bactericidal activity on half of the tested bacterial species although at a concentration of 40 µg/mL, *Klebsiella* spp. was observed to be killed by the extract. A similar trend was observed for the seed oil extracts (data not shown). The aqueous seed oil extract showed the highest bactericidal effect at concentration of 20 µg/mL. At this concentration, *Citrobacter* species, *Proteus* spp. and *E. coli* were killed while *S. aureus*, *Klebsiella* spp. and *P.*

Table 3. Zones of inhibition of *M. oleifera* leaf oil extracts on different bacterial species.

Organism	Aqueous (Mean ± SD)	Methanolic (Mean ± SD)	Ethanolic (Mean ± SD)
S. aureus	13.2± 2.3	18.0 ± 2.6	9.0 ± 2.4
Citrobacter spp.	16.0 ± 2.0	17.7 ± 4.0	7.7 ± 1.8
Proteus spp.	3.0 ± 0.0	15.0 ± 1.0	3.0 ± 0.0
Klebsiella spp.	13.7 ± 3.5	11.0 ± 2.0	8.3 ± 2.1
E. coli	20.3 ± 1.5	16.3± 2.3	12.3 ± 1.5
P. aeruginosa	16.3 ± 2.5	22.7 ± 2.1	11.0 ± 2.0

Data are presented as mean ±SD as measurement of inhibition zone (mm); Means and standard deviations determined from 3 biological replications; [a]

Table 4. Zones of inhibition of *M. oliefera* seed oil extracts on different bacterial species.

Organism	Aqueous (Mean ± SD)	Methanolic (Mean ± SD)	Ethanolic (Mean ± SD)
S. aureus	15.3± 2.2	11.0± 3.0	8.0± 2.8
Citrobacter spp.	16.0± 2.0	13.7± 1.1	0.0± 0.0
Proteus spp.	17.3± 1.5	13.0± 1.0	0.0± 0.0
Klebsiella spp.	16.7± 2.1	12.0± 1.7	7.0± 1.4
E. coli	22.0± 1.3	18.7± 1.5	0.0± 0.0
P. aeruginosa	19.0± 1.0	13.7± 2.5	9.0± 2.3

Data are presented as mean ±SD as measurement of inhibition zone (mm); Means and standard deviations determined from 3 biological replications.

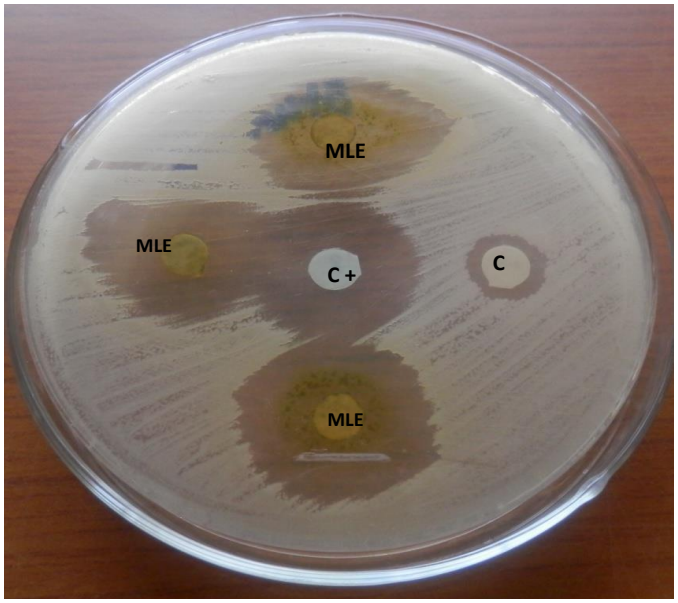


Figure 2. Susceptibility test of the methanolic leaf oil extract on *S. aureus*. The susceptibility tests were done using paper discs diffusion methods. Discs were placed on Muller Hinton Agar (MHA) plates earlier seeded with standardized bacterial suspensions (approximately 1.5×10^6 CFU/mL). C = Paper discs impregnated with 30 µL of a solution of 50 µg/mL of ciprofloxacin and gentamycin were used as control for comparison. MLE= Methanolic leaf extract, C+ = cotrimoxazole, C= gentamycin.

aeruginosa were killed at a concentration of 30 µg/mL. The methanol oil extract had bactericidal effect at 30 µg/mL for *E. coli* and 50 µg/mL for *S. aureus*, *Proteus* species and *P. aeruginosa.* While ethanol seed oil extract had the lowest bactericidal activity with concentration ranging from 50 µg/mL to 70 µg/mL.

Comparison of the MIC and the MBC from different extraction sources

A comparison of the MIC between leaf and seed oil extracts from the three extraction methods showed that the leaf oil extracts from ethanolic, aqueous and methanolic extractions had more inhibitory activity when compared with the seed extracts from the three sources. The MIC of the leaf oil extracts for the three extraction sources were the same as the control. However a different trend was observed for the MBC of the leaf and seed oil extracts from the three extraction methods. The seed and leaf oil extracts from the three sources showed variable MBC at relatively high concentrations. For the ethanol seed oil extract, there was no MBC for *Citrobacter* spp., *Proteus* and *E. coli* even at concentrations as high as 70 µg/mL. No MBC was recorded for *Proteus* spp. for the leaf oil extract as well. A similar trend was observed for the methanolic seed oil extracts. In addition, the methanolic leaf oil extract could not show any observable MBC on *Proteus* spp. For the aqueous seed oil extract, the leaf oil extract had no MBC on *Proteus* and *Pseudomonas* spp.

Reduction of viable bacterial counts by the extracts

The time of kill estimates the time it took the extracts to kill the bacterial pathogens, hence showing a progressive reduction in the number of colonies that survive over a given time frame. The time of kill assayed at a maximum time interval of 0 to 5 h showed a reduction of the *Klebsiella* spp. from 8.2 to 2.3 CFU/mL by the methanolic leaf oil extract and 9.0 to 4.8 CFU/mL by the aqueous leaf oil extract (Figure 5). For *S. aureus,* the colonies reduced from 9.4 to 3.2 CFU/mL with the methanolic seed oil extract and 9.6 to 6.4 CFU/mL with the aqueous seed oil extracts at 0 to 5 h (Figure 5).

DISCUSSION

Microorganisms such as bacteria, fungi and other parasites are common pathogens associated with wound infections (Sani et al., 2012). However, the resistance of these microbial agents to conventional antibiotics has

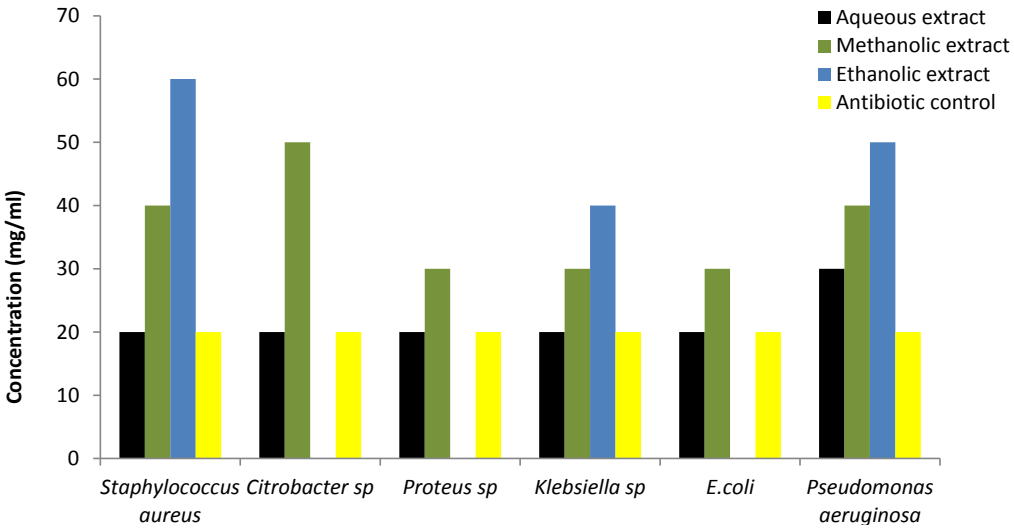


Figure 3. The minimal inhibitory concentration of *M. oleifera* seed oil extracts against orthopedic wound infection pathogens. Black bars indicate the MIC of *M. oleifera* aqueous seed oil extracts, green bars indicate the MICs of the methanolic seed oil extract, blue bars show ethanolic seed oil extracts and the yellow bars are the antibiotic control.

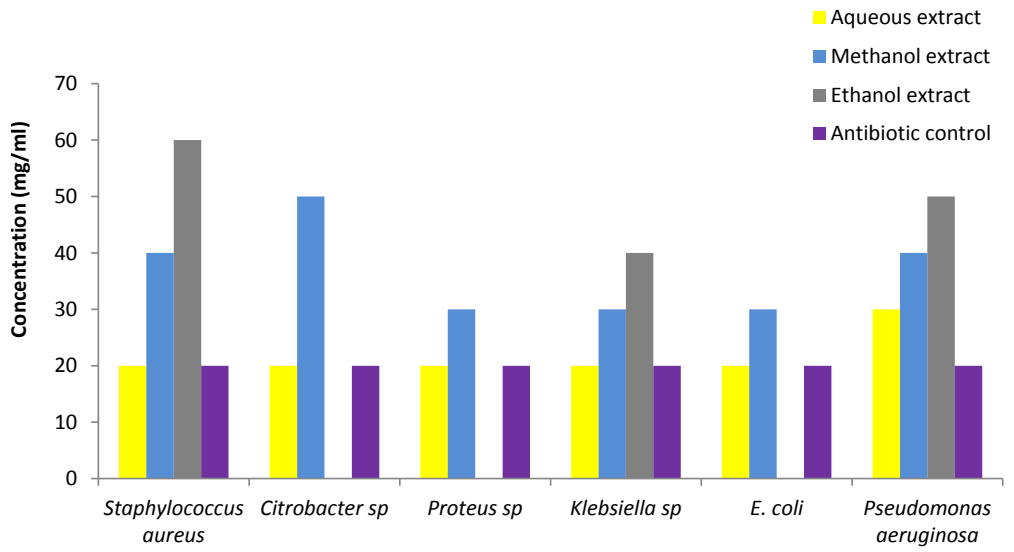


Figure 4. The MBC of *M. oleifera* leaf oil extract: yellow bars represent the MBC of the aqueous leaf oil extract, blue bars indicate the methanol leaf oil extract, ash bars represent the ethanolic leaf oil extract while the purple bars are the antibiotic controls.

become very alarming. In the present study, an evaluation of the antibiotic susceptibility pattern of microorganisms isolated from orthopaedic wounds and also the antimicrobial potential of *M. oleifera* leaf and seed oil extracts against orthopaedic wound pathogens were tested. The results from our biochemical analysis showed that gram negative organisms such as *Citrobacter* spp., *Proteus* spp., *Klebsiella* spp., *E. coli* and *P. aeruginosa* were the predominant organisms present in the infected wounds. However, *S. aureus* was the only gram positive organism identified in the samples. This observation corroborates with the findings from other work where *S. aureus* was reported as the predominant gram positive bacterial isolate in wound infections (Samuel et al., 2010; Magiorakos et al., 2012). The antibiogram of the isolates screened in this study showed that over 90% of the bacterial species showed high level of resistance to most of the tested antibiotics (cloxacillin, erythromycin, amoxycilin, augmentin and tetracycline). However, gentamicin and cotrimoxazole having resistance levels of 70.4 and 75.6% respectively were the antibiotics with the least resistance profiles. The high

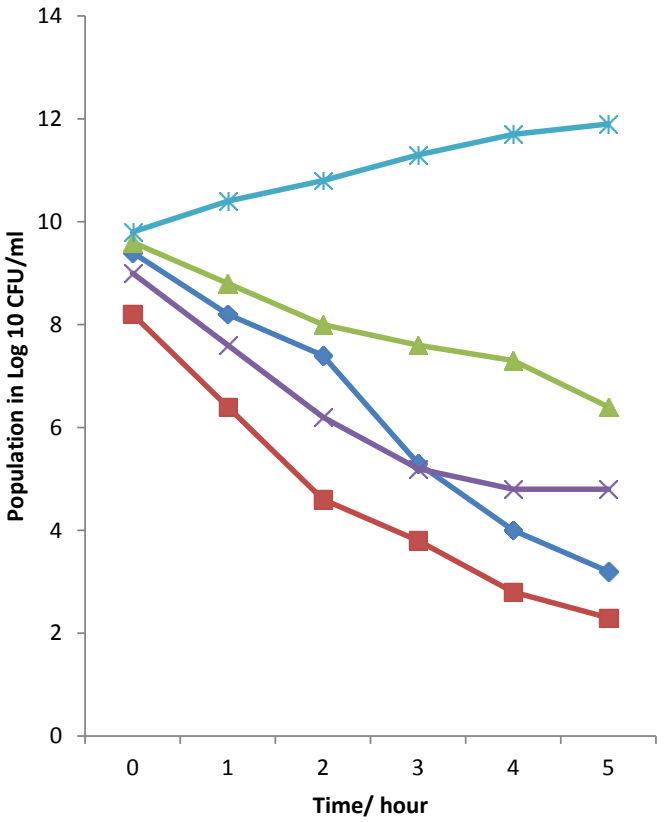


Figure 5. Time of kill of the aqueous and methanolic oil extracts on *S. aureus* and *Klebsiella* spp. The blue line represents the time of kill of the methanolic leaf oil extracts on *S. aureus*, the red line represents the time of kill for the *Klebsiella* spp., the green line represents the time of kill of the methanolic seed oil extract on *S. aureus,* the purple line represents the time of kill of the aqueous seed oil extracts on *Klebsiella* spp., while the light blue one represents the antibiotic control (cotrimazole). The time of kill was measured as the reduction in population of viable cells per hour.

level of resistance observed among the tested bacterial isolates in this study could be as a result of indiscriminate use of the tested antibiotics. It could also be due to the transfer of resistant genes among organisms as has been reported in microbial communities like biofilms (Andhoga et al., 2002). Although the resistance observed in gentamicin and cotrimoxazole are relatively high, the two antibiotics proved to be the candidates of choice for treatment of orthopaedic wound infections in this study. Other studies have also reported that gentamicin was the most effective antibiotic against most isolates of gram negative organisms isolated from wound infection (Rathi et al., 2006). The antimicrobial activity of the aqueous, methanolic and ethanolic leave oil extracts of *M. oleifera* against individual isolates of the bacteria revealed that the zones of inhibition (ZI) of the extracts varied from one bacterial species to the other. Methanolic leaf oil extract showed appreciable antibacterial activity on the orthopedic wound organisms, an indication of its high anti-bacterial potential and effectiveness in the treatment of wound infections. Evaluation of the wound healing property of aqueous oil extracts of leaves of *M. oleifera* on male Swiss albino mice showed a significant increase in wound closure rate, skin breaking strength, granuloma breaking strength, granuloma dry weight and decrease in scar area (Srinivasan et al., 2001). However, in our study, the aqueous and ethanolic leaf oil extracts showed no activity against *Proteus* spp., with both extracts showing no ZI against the organism. The variations in the activity of the different leaf oil extracts against individual organisms might be as a result of the differences in the concentration and the amount of phytochemical constituents of each extract. These differences in the phytochemical constituents might have been caused by the difference in polarity of the solvents used in the extraction process (Faizi et al., 1995). Several studies have characterised the chemical compounds present in *M. oleifera* plant leaves (Fuglie, 1999). Two nitrile glycosides from the ethanolic extracts of *M. oleifera* leaf and three mustard oil glycosides have been identified (Fuglie, 1999). Although the ethanolic and aqueous leaf oil extracts showed a considerable reduction in the growth of the tested organisms, the methanolic leaf oil extract showed a broader ZI on all the organisms, which could suggest that the method of extraction has a huge effect on the efficacy of the extracts. Additionally, it is also possible that due to the different methods of extraction employed in this work, each extract could possibly have a different biochemical pathway during metabolism in the organisms. For the seed oil extracts, the activity of the individual extracts (ethanolic, methanolic and aqueous) varied amongst the tested organisms. A comparison of the leaf and seed oil extracts showed that the methanolic and ethanolic leaf oil extracts showed relatively higher activity than their corresponding counterparts from the seed oil extracts. A possible explanation for this could be due to the presence of more bioactive phytochemicals in the methanolic and ethanolic leaf oil extracts when compared to the seed extracts. It could also be that the phytochemicals in the two extracts were more soluble due to the polarity of the solvents. Furthermore, the methanol and ethanol have probably eluted more bioactive components from the leaf than in the seed. In contrast to this result, a higher activity of ethanolic leaf oil extract of *M. oleifera* and a relatively lower activity of the aqueous oil extract was observed at a concentration of 100 µg/mL (Shanholtzer et al., 1992). The reason for this discrepancy in results could not be explained. However, the MIC of the extracts showed that ethanolic leaf oil extract had the lowest activity on the organisms but showed the highest MBC values. MBC values have been shown to be consistently higher than MIC values probably because higher concentrations of antibiotics may be required to kill bacterial organisms than that required to inhibit their growth. The MIC values for the seed oil extracts followed a similar trend with the leaf extracts.

Plasmid profiling of the isolates showed the presence of plasmids in all the gram negative organisms tested. These plasmids may be responsible for the observed resistance in the tested isolates as a reasonable number of the gram negative organisms lost their resistance after curing. Most bacterial plasmids harbour antibiotic resistant genes some of which are acquired through horizontal gene transfer. Interestingly, 60% of the gram positive organisms tested in this study had plasmids and they were resistant to the tested antibiotics. However, 40% of the gram positive organisms (*S. aureus*) had no plasmids but they still showed resistance to most of the tested antibiotics. The presence of resistance in the *Staphylococcus* strains that had no plasmids could suggest that antibiotic resistance in *Staphylococcus* spp. and perhaps other bacteria could be of both chromosomal and plasmid origin. From this study, it has been demonstrated that the application of extracts from natural plants such as *M. oleifera* for the treatment of wound infections can be a promising alternative to conventional antibiotics. The methanolic leaf and aqueous seed oil extracts showed the highest antimicrobial activity when compared to the other extracts. The potential of using these extracts for the treatment of wound infection should be fully harnessed.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are grateful to the technical staff of the Department of Pharmacology, Faculty of Pharmaceutical Sciences and the Department of Microbiology, Faculty of Biological Sciences, University of Nigeria Nsukka.

REFERENCES

Adegoke AA, Iberi PA, Akinpelu DA, Aiyegoro OA, Mboto CI (2010). Studies on phytochemical screening and antimicrobial potentials of *Phyllanthus amarus* against multiple antibiotic resistant bacteria. Int. J. Appl. Res. Nat. Prod. 3:6-12.

Andhoga J, Macharia AG, Maikuma IR, Wanyonyi ZS, Ayumba BR, Kakai R (2002). Aerobic pathogenic bacteria in post- operative wounds at Moi teaching and referral hospital. East Afr. Med. J. 79:640-644.

Akinjogula OJ, Eghatona NO, Enabulele IO, Mboto CI, Ogbemudia FO (2010). Antibacterial activity of ethanolic extracts of *Phyllanthus amarus* against extended spectrum β-lactamase producing *Escharichia coli* isolated from stool samples of HIV sero-positive patients with or without diarrhea. Afr. J. Pharmacol. 4 (6):402-407.

Bhalakia N (2005). Isolation and plasmid analysis of vancomycin – resistant *Staphylococcus aureus*. J. Young Investig. 13(4):1-6.

Bennett RN, Mellon FA, Foid N (2003). Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *Moringa oleifera* L. (Horseradish tree) and *Moringa stenopetala* L. J. Agric. Food Chem. 51:3546-3553.

Costa-Lotufo LV, Khan MT, Ather A, Wilke DV, Jimenez PC, Pessoa C (2005). Studies of the anticancer potential of plants used in Bangladeshi folk medicine. J. Ethnopharmacol. 99:21-30.

Cheesbrough M (2006). Medical Laboratory Manual for Tropical Countries Vol. II. Elsevier Health Sciences. pp. 255- 275.

Cruishank R, Duguid JP, Marmion BP, Swain RHA (1975). Medical Microbiology. The Practice of Medical Microbiology, 12th ed.; Churchill Livingstone: London, UK. 587p.

Doughari JH, Pukuma MS, De N (2007). Antibacterial effects of *Balanites aegyptiaca* L. Drel. and *Moringa oleifera* Lam. on *Salmonella typhi*. Afr. J. Biotechnol. 6(19):2212-2215.

Enweani UN (1991). Surgical wound sepsis in clean orthopaedic procedure: Bacteriology and sensitivity pattern in a regional specialist center. Orient. J. Med. 3:1-6.

Fahey JW, Zalcmann AT, Talalay P (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 6:5-51.

Faizi S, Siddiqui BS, Saleem R, Siddiqui S, Aftab K, Gilani AH (1995). Fully acetylated carbamate and hypotensive thiocarbamate glycosides from *Moringa oleifera*. Phytochemistry 38(4): 957-963.

Fuglie LJ (1999). The Miracle Tree: *Moringa oleifera*: Natural Nutrition for the Tropics. Church World Service; Dakar, Senegal. No. 633.85/F958

Gilani AH, Aftab K, Suria A (1994). Pharmacological studies on hypotensive and spasmodic activities of pure compounds from *Moringa oleifera*. Phytother. Res. 8:87-91.

Ghebremichael KA, Gunaratna KR, Henriksson H, Brumer H, Dalhammar G (2005). A simple purification and activity assay of the coagulant protein from *Moringa oleifera* seed. Water Res. 39:2338-2344

Irkin R, Korukluoglu M (2007). Control of *Aspergillus niger* with garlic, onion and leek extracts. Afr. J. Biotechnol. 6:384-387.

Isibor JO, Oseni A, Eyaufe A, Ahmadu T (2008). Incidence of aerobic bacteria and *Candida albicans* in post-operative wound infections. Afr. J. Microbial. Res. 2:288-291.

Lockett CT, Calvert CC, Grivetti LE (2000). Energy and micronutrient composition of dietary and medicinal wild plants consumed during drought. Study of rural Fulani, Northeastern Nigeria. Int. J. Food Sci. Nutr. 51:195-208.

Mulugeta K, Bayeh A (2011). Bacteriology and antibiogram of pathogens from wound infections at Dessie Laboratory, North East Ethiopia. Tanzan J. Health Res. 13:1-10.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG (2012). "Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance." Clin. Microbiol Infect. Clin. Microbiol. Infect. 18(3):268-281.

Oluma HO, Umeh EU, Onekutu A Okolo J (2000). Antibacterial potentials of eight medicinal plants from the lower Benue valley of Nigeria. Niger J. Bot. 17:1-11.

Posmontier B (2011). The medicinal qualities of *Moringa oleifera*. Holistic Nurs. Pract. 25:80-87.

Rathi BS, Bodhankar SL, Baheti AM (2006). Evaluation of aqueous extract of *Moringa oleifera* for wound healing in albino rats. Indian J. Exp. Biol. 44:898-901.

Samuel SO, Kayode OO, Musa OI, Nwigwe GC, Aboderin AO, Salami TAT (2010). Nosocomial infections and the challenges of control in developing countries. Afr. J. Clin Exp. Microbiol. 11:102-110

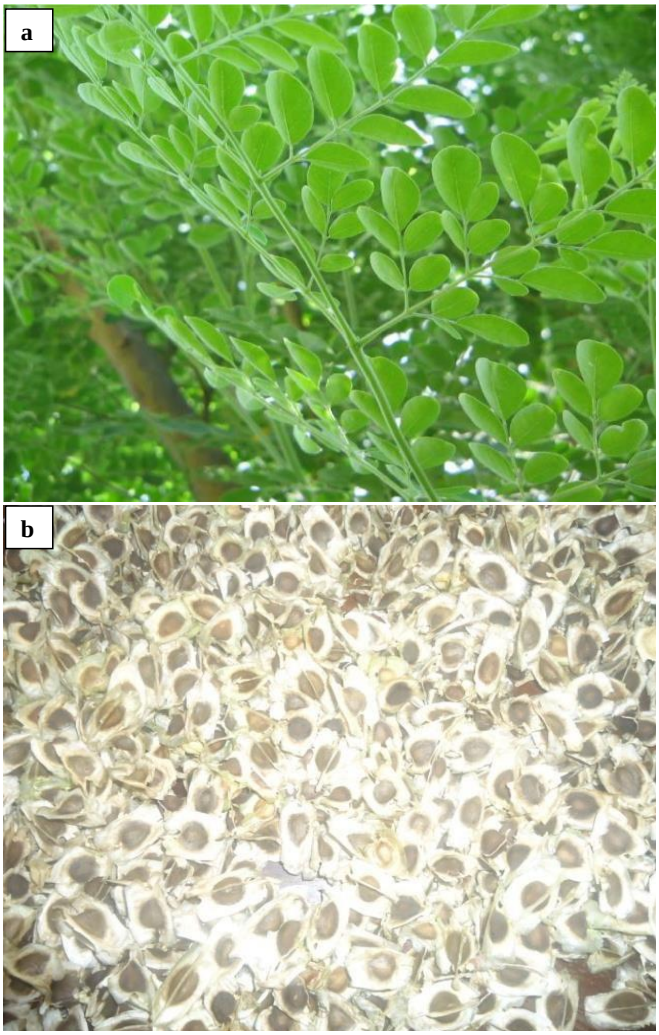
Sani RA, Garba SA, Oyewole OA (2012). Antibiotic resistance profile of gram negative bacteria isolated from surgical wounds in Minna, Bida, Kontagora and Suleja Areas of Niger State. Am. J. Med. Med. Sci. 2(1):20-24.

Shanholtzer CJ, Willard KE, Holter JJ, Olson MM, Gerding DN, Peterson LR (1992). Comparison of the VIDAS *Clostridium difficile* toxin A immunoassay with *Clostridium difficile* culture and cytotoxin and latex tests. J. Clin. Microbiol. 30(7):1837-1840.

Siddhuraju P, Becker K (2003). Antioxidant properties of various solvent extracts of total phenolic constituents from three different agro-climatic origins of drumstick tree (*Moringa oleifera* Lam.). J. Agric. Food Chem. 15:2144-2155

Srinivasan DS, Nathan T, Suresh O Perumalsamy P (2001). Antimicrobial activity of certain Indian medicinal plants used in Claudia Folkloric medicine. J. Ethnopharmacol. 74:217-220.

Taylor DE, Brose EC (1988). Modified Birnboim-Doly method for rapid detection of plasmid copy number. Nucleic Acids Res. 16(18):9056.
Walter A, Samuel W, Peter A, Joseph O (2011). Antibacterial activity of *Moringa oleifera* and *Moringa stenopetala* methanol and n-hexane seed extracts on bacteria implicated in water borne diseases. Afr. J. Microbiol. Res. 51:153-157.
Zhou QY, Grandy DK, Thambi L, Kushner JA, Van Tol HH, Cone R, Pribnow D, Salon J, Bunzow JR, Civelli O (1990). Cloning and expression of human and rat D1 dopamine receptors. Nature 347:76-80.



Supplementary Figure 1. Pictures of *M. Oleifera* plants. The plant samples obtained from Dekina Local Government Area of Kogi State Nigeria and identified at the Department of Botany, University of Nigeria, Nsukka (a) *M. oleifera* leaves, (b) *M. oleifera* seeds.