International Pharmacy Acta. 2023;6(1):e3
Original Article
OPEN ACCESS
ISSN: 2645-3266

Antibacterial Effect of Aqueous and Ethanol Extracts of *Moringa oleifera* Leaves on Methicillin Resistant *Staphylococcus aureus* (MRSA) Isolates obtained from a University Community

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Article Info:
Received: May 2022
Accepted: November 2022
Published online: January 2023

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Abstract:

Methicillin Resistant *Staphylococcus aureus* (MRSA) are strains of *Staphylococcus aureus* (*S. aureus*) that are not susceptible to the methicillin and related beta-lactam antibiotics. The dominance of MRSA has been linked to infectious diseases that has increased morbidities and mortality in patients. Failed attempts in the use of most antibiotics have initiated the search of suitable drugs that are effective, low cost, and with lesser side effects. The study focus on the Effect of Aqueous and Ethanol Extracts of *Moringa oleifera* Leaves on MRSA Isolates. The results reveal that *S. aureus* prevalence was 143 (48.3%), while MRSA was 105(35.5%). The sample types are; Armpit, Ear, High(upper?) Vaginal, Mouth, Nose swabs, Urine and Semen. Statistically at P>0.05 there was association between *S. aureus* and MRSA. Thirty five (35) selected isolates of MRSA where analyzed for their antibiotic susceptibility and effect of Aqueous and Ethanol Extracts of *Moringa oleifera* Leaves. They were highly susceptible to Ciprofloxacin 26(74.3%), Gentamicin 31(88.6%), Streptomycin 32(91.4%) and Levofloxacin 32(91.4%). However, low levels of MRSA susceptibility for Vancomycin 21(60%) and Ampiclox 31(88.6%) were noted. Multidrug resistant (isolates resistant to drug agents ≥ 4) samples recorded was 9(25.7%) of the 35 isolates studied. Comparison of the zones of inhibitions between aqueous plant extract and 70% ethanol extracts on the 35 selected MRSA isolates using Mann Whitney test reveal that it was Statistically significant with P= 0.047. Meaning aqueous extract mean zone of inhibition was higher than that of 70% ethanol extracts. Therefore, aqueous extraction of *M. oleifera* leaves should be studied intensively as a probable source for drug development for the treatment of MRSA infections.

Keywords: *Moringa oleifera*; Multidrug; Resistant; Susceptibility; Prevalence; Antibacterial.

Please Cite this article as: Iwetan Benedict B., Okwu M. U., Enwa F. O. Antibacterial Effect of Aqueous and Ethanol Extracts of *Moringa oleifera* Leaves on Methicillin Resistant *Staphylococcus aureus* (MRSA) Isolates obtained from a University Community. Int. Pharm. Acta. 2023;6(1): e3
DOI: https://doi.org/10.22037/ipa.v6i1.38373

1. Introduction

Staphylococcus aureus (*S. aureus*) is among the most prevalent easy spreading bacteria among human with a very high disease-causing ability. It is morphologically identified as Gram's positive cocci clusters and biochemical considered as catalase, coagulase, deoxyribonuclease and mannitol fermentation positive [1]. The trending concern is the possible development of multi-resistant bacteria to conventional antibiotics by this organism. This makes treatment of *S. aureus* caused diseases such as septicemia, pneumonia, wound sepsis, septic arthritis and post-surgical toxic shock syndrome

challenging, culminating into morbidity and sometimes death [2]. *S. aureus* strains resistant to the Beta-lactam antibiotics such as methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin are referred to Methicillin resistant *Staphylococcus aureus* (MRSA) [3]. The hospital, Community and the Environment can be potential reservoir for MRSA. Their prevalence is multifaceted in nature ranging from patient noncompliance to prescribed regimen, mode of transmission, drug adulteration, and lack of the bacterial sensitivity test before treatment with the appropriate antibiotics as well as biofilm formation of some strains of MRSA among others [4]. In spite of the well-established conventional antibiotic actions, some basic challenges of their usage are side effects, cost, availability and counterfeit drugs overflowing the drug-counters in the market space. On this light there is extraordinary demand for newer agents devoid of these shortcomings of conventional drugs [5].

Medicinal Plants have historical and outstanding beneficial roles in tackling infectious diseases with lesser or no side effects. The plant *Moringa oleifera*, Lam (*M. oleifera*), is also refers to as "miracle tree" or "drumstick", "natural gift" or "horseradish" or "mother's best friend".

As a plant, the roots, leaves, green pods, flowers, and seeds of *M. oleifera have been useful as additives to food, purification of water, bioenergy generation, nutraceuticals and as medicine* [6,7,8].

The phytochemicals linked to *M. oleifera* that tend to have some kinds of bioactivities are glucosinolates, flavonoids, phenolic acids, carotenoids, tocopherols, polyunsaturated fatty acids (PUFAs), minerals and folate with high bioavailability [9].

The multiple biological activities namely, antiproliferation effect, liver protective potential, anti-inflammatory, antinociceptive, amelioration of cardiovascular system related diseases, protection against oxidative stress DNA damage, and antiperoxidative, are the folk medicinal uses of *M. oleifera* which may be ascribed to the availability of bioactive compounds, such as vitamins, phenolic acids, flavonoids, alkaloids, minerals, phytosterols, natural sugars, and organic acids. The low molecular weight of *M. oleifera* cationic proteins (MOCP) are extracted from the seeds [10,11].

M. oleifera *plant extraction with* solvents like hexane, petroleum either, butanol, chloroform, acetone, ethyl acetate, methanol, and water extract of pod husks have been effective against microorganisms such as *S. aureus*, S. epidermidis, S. typhimurium, E. coli, *and* K. *pneumoniae* [12].

These bacteria that are pathogenic to human can cause diseases such as dysentery, diarrhea resulting from oxidative diseases, Cancer, organ transplant infections in immunocompromised individuals and complications in Acquired Immune Deficiency Syndrome [13, 14]. The fact remains that antibiotic activities of the plant may largely been attributed to its active biomolecules. However it's proposed antibacterial potentials on MRSA which are sparely studied may serve as alternative for antibiotic resistant bacteria that are deleterious to human and other animals, hence the relevance of the study.

2. Materials & Methods

2.1. Samples Collection

This study was done over a period of Six month between January 2015 to June 2015. Clinical samples were collected from patients attending the Health Centre of the Delta State University Abraka and the participants' consent were sought. Analysis was done in Pharmaceutical microbiology laboratory. Two hundred ninety-six (296) samples were collectedas urine, semen/urethral specimens, and armpit, ear, mouth, and high vaginal swabs. The samples were labelled and conveyed to the laboratory for analysis [15].

2.2. Bacterial Isolates

Sample swabs were inoculated in nutrient broth and were incubated overnight 37ºC. The growth media were then plated on mannitol salt agar (MSA). Bacterial isolates obtained subjected to microbiological analysis such as; Gram staining, Coagulase and Catalase test [16, 17, 18, 19, 20].

2.3. Oxacillin Susceptibility Test

With a sterile swab the broth culture of *S. aureus* cell suspension of the respective isolates was made whose turbidity was balanced with 0.5% McFarland's standard and were inoculated using 4% sodium chloride impregnated into the Mueller Hinton Agar plate. Antibiotic discs (single oxacillin disc) are placed after 5 minutes, to allow the agar surface to dry. The inoculated plates were incubated at 35°C for 24 hours in an inverted position and the zone of inhibition was recorded in millimeters (mm) [21, 22].

2.4. Antibiotic Susceptibility Test

The *S. aureus* isolates whose turbidity were balanced with 0.5% McFarland's standard and were inoculated the Mueller Hinton Agar plate (without 4% sodium chloride). Antibiotic discs were placed after 5 minutes, to allow the agar surface to dry. The inoculated plates were incubated at 37°C for 24 hours in an inverted position and the zone of inhibition was recorded [21] [22]. The antibiotics used are Vancomycin (1µg),

Ciprofloxacin (10µg), Norfloxacin (10µg), Gentamicin (10µg), Amoxacillin (20µg), Streptomycin (30µg), Rifampicin (20µg), Erythromycin (30 µg), Chloramphenicol (30 µg), Ampiclox (20 µg), and Levofloxacin (20 µg).

2.5. Plant Collection and Preparation

A sample of *M. oleifera* leaves was collected at Akoku village close to Obiaruku in Ukwani Local Government Area of Delta State, Nigeria. It was identified by Dr. Akinnibosun Henry Adewale of the Department of Plant Biology and Biotechnology, Herbarium Unit, with Specimen Voucher Number: UBH-M340. The freshly collected plant leave were rinsed and air dried under the shade. The dried leaves were pulverized and kept in dry air tight container.

2.6. Plant extraction

Crude extraction was done by maceration as described by the United State Pharmacopeia (USP). Fifty grams (50 g) of the dried powdered leaves of *M. oleifera* was submerged with distilled water and left for 24 hours for proper percolation. It was filtered with whatman No.1 filter paper, the filtrate was evaporated to dryness with heating mantle at regulated temperature of 40^0C. The extract obtained was weighed and percentage yield was calculated. The extract was kept in an air tight container and preserved in the refrigerator for further analysis.
In the ethanol extraction the above procedure for the aqueous extraction was adopted except for the extracting solvent which was 70% ethanol.

2.7. Antibacterial effect of *M. oleifera* leaves extract on MRSA isolates

Sterile cork-borer of 6mm was used to bore well on the 25 milliliters Mueller Hinton agar plates after inoculation with 0.5% McFarland standard inoculum. The prepared concentrations of both aqueous and ethanolic extracts (200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, and 12.5 mg/ml) were introduced into the wells. A well containing 2.5% Dimethyl Sulfoxide (DMSO) which was the dissolving solvent also served as a negative control. The plates were allowed to dry for 30 minutes before they were incubated at 37˚C for 24 hours. Clear zones around the well (zones of inhibition) were measured and the diameter of the well (6mm) was subtracted from the zones of inhibition. The mean zones of inhibition were calculated and recorded [23].

2.8. Statistical Analysis

Frequencies were obtained and percentages were calculated for study variables. Fisher's Exact test was used to calculate significance ($P < 0.05$) by Instat Graphpad Demo – [DATASET 1.ISD] and Prism 7 version software.

3. Results and Discussion

Table 1 shows the colonial morphology and biochemical reactions of the bacterial isolates. Analysis showed that the isolates were *S. aureus.*

Table 1. Colonial Morphology and Biochemical Reactions of *Staphylococcus aureus*

Characteristics	*Staphylococcus aureus*
Cultural	
Shape	Circular
Color	Golden – yellow
Consistency	Sticky
Elevation	Slightly raised
Morphology	
Gram reaction	
Gram type	Gram positive cocci
Arrangement	Clusters
Biochemical test	
Catalase	Positive
Coagulase	Positive
Mannitol Fermentation	Positive (Golden – yellow)

Table 2 depicts the prevalence of *S. aureus* and MRSA isolates with respect to age distribution. The prevalence of S. aureus and MRSA in this study were 48.3% and 35.5% respectively. The statistical analysis showed that there was no significant difference ($p > 0.05$) in prevalence with respect to age distribution [26].

Table 2. Prevalence of *S. aureus* and MRSA isolates with respect to age distribution

Age	0-24	25-50	51-76	Total
Sample Number	176(59.46%)	109(36.82%)	11(3.71%)	296(100%)
S. aureus	83(28.04%)	54(18.24%)	6(2.02%)	143(48.31%)
MRSA	61(20.61%)	39(13.17%)	5(1.69%)	105(35.47%)

Chi-square test; P value = 0.9906
% = Percentage.
MRSA = Methicillin resistance *Staphylococcus aureus*.

As designated in Table 3, 176 subjects (59.5%) were female while 120 (40.5%) were male. The prevalence of S. aureus and MRSA isolates among the female sampled were 26.0% and 17.6% respectively while the male sampled were 22.3% and 17.9%. Statistically, there was no significant difference in prevalence regarding sex. Meaning sex is not a determinant in distribution of *S. aureus* and MRSA which is also in accordance with other work [24]. The clinical samples worked with which had the higher number of isolations of *S. aureus* and MRSA were pus, wound swabs and high vaginal swabs. The collections of samples were non-invasively done. Though the sample types are not the same in both studies, it points out that *S. aureus* and MRSA can be isolated from the different sample types. This concept suggests the possibility of easy spreading of the organism and its nosocomial infection ability.

Table 3. Prevalence of *S. aureus* and MRSA isolates with respect to sex

Sex	Male	Female	Total
Sample Number	120(40.54%)	176(59.46%)	296(100%)
S. aureus	66(22.29%)	77(26.01%)	143(48.31%)
MRSA	53(17.91%)	52(17.56%)	105(35.47%)

Chi-square test, P = 0.1743
% = Percentage.
MRSA = Methicillin resistance *Staphylococcus aureus*.

The **Table 4**, shows the various sample types from which *S. aureus* and MRSA were isolated. The highest sample was urine, 77(24.3%) followed by nose swab, 60 (20.3%) then ear swab, 49 (16.6%). S. aureus and MRSA isolates obtained from these samples are: urine, 10.5% and 7.4%; nose swabs, 11.1% and 9.1%; ear swabs, 7.4% and 5.4%. The prevalence statistically indicates no association (no significant difference), meaning sample types are not determinant in the distribution of *S. aureus* and MRSA.

Table 5 depicts the antibiotic susceptibility profiles of 35 selected MRSA isolates. High susceptibility values were obtained from ciprofloxacin (74.3%), chloramphenicol (77.1%), gentamicin (88.6%), streptomycin (91.4%), rifampicin (91.4%) and levofloxacin (91.4%). The least value was recorded for ampiclox (11.4%). MRSA simply means not susceptibility to oxacillin. The highly susceptible drugs; Rifampicin, Streptomycin and Gentamicin are likely the drug of choice to be considered when MRSA is implicated in an infection. A study pointed out that the initial low-dose of gentamicin as part of the therapy for *S. aureus* bacteremia was probable cause of valve infective endocarditis, even nephrotoxicity might occur as a result of routine used that should be cautioned, given the minimal data supporting its benefit [25]. However, in the choice of drug may also be considered other rationales, for instance drug interactions, availability, side effects and a lot more. MRSA Isolates that considered to be multi-drug resistant are those that are resistant to 4 and above antibiotic agents which amount to 9(25.71%) as presented in Table 6. However, definition of some text considered more than one drug resistant by the organism. This work has similar multi-drug resistant > 60% as reported [26].

Table 4. Prevalence of *S. aureus* and MRSA Isolates with respect to Sample Types.

Sample Type	Armpit	Ear	HVS	Mouth	Nose	Urine	Semen	Total
Sample Number	46(15.54%)	49(16.55%)	25(8.45%)	31(10.47%)	60(20.27%)	72(24.32%)	13(4.39%)	296(100%)
S. aureus	22(7.43%)	22(7.43%)	13(4.39%)	12(4.05%)	33(11.14%)	31(10.47%)	10(3.38%)	143(48.31%)
MRSA	17(5.74%)	16(5.41%)	8(2.7%)	8(2.7%)	27(9.12%)	22(7.43%)	7(2.37%)	105(35.47%)

Chi-square test, P value = 0.9765
% = Percentage. HVS = High Virginal Swab.
MRSA = Methicillin resistance *Staphylococcus aureus*.

Table 5. Antibiotics Susceptibility Profile of 35 MRSA Isolates.

Antibiotics	% Resistance (R)	% Intermediate (I)	% Susceptible (S)
Oxacillin (1µg)	35 (100%)	0(0%)	0(0%)
Vancomycin (1µg)	21 (60%)	0(0%)	14 (40%)
Ciprofloxacin (10µg)	2 (5.7%)	7 (20%)	26 (74.3%)
Norfloxacin (10µg)	10 (28.6%)	6 (17.1%)	19 (54.3%)
Gentamicin (10µg)	2 (5.7%)	2 (5.7%)	31 (88.6%)
Amoxacillin (20µg)	3 (8.6%)	10 (28.6%)	22 (62.8%)
Streptomycin (30µg)	2 (5.7%)	1 (2.9%)	32 (91.4%)
Rifampicin (20µg)	1 (2.9%)	2 (5.7%)	32 (91.4%)
Erythromycin (30µg)	3 (8.6%)	14 (40%)	18 (51.4%)
Chloramphenicol (30 µg)	4 (11.4%)	4 (11.4%)	27 (77.1%)
Ampiclox (20 µg)	31(88.6%)	0(0%)	4(11.4%)
Levofloxacin(20µg)	3 (8.6%)	0(0%)	32 (91.4%)

Key; µg = microgram, % = Percentage

Table 6. Prevalence of Multiple Drug Resistance among 35 Isolates of MRSA.

Parameter	Frequency of multi-drug resistant MRSA	
	Number of Isolates	Percentage (%)
Fully Sensitive	0	0
Resistant to 1 agent	2	5.71
Resistant to 2 agents	10	28.57
Resistant to 3 agents	14	40
Resistant to 4 agents	2	5.71
Resistant to 5 agents	2	5.71
Resistant to 6 agents	1	2.86
Resistant to 7 agents	3	8.57
Resistant to 8 agents	1	2.86
Resistant to 9 agents	0	0

Key: The agents are; Oxacillin, Vancomycin, Ciprofloxacin, Norfloxacin, Gentamicin, Amoxacillin, Streptomycin, Rifampicin, Erythromycin, Chloramphenicol, Ampiclox and Levofloxacin. **Note:** Isolates may be resistant to a specific number of antibiotics, but different antibiotic types.

Fig. 1, Key; A; Aqueous extract of *M. oleifera*, E; Ethanol extract of *M. oleifera*. Results shows more antibacterial effect with the aqueous extract of *M. oleifera* leaves when compared with the ethanol extract of same concentration. 200 mg/ml aqueous extract compared to all concentrations of both aqueous and ethanol extract, there is significant difference at $P < 0.001$ except for 100 mg/ml aqueous extract in which there is no significant difference since $P > 0.05$ ($P= 0.6023$). The phytochemicals associated with the plant *M. oleifera* as well as other natural products are known for their antibacterial actions as reported by Yenda and colleagues [20].

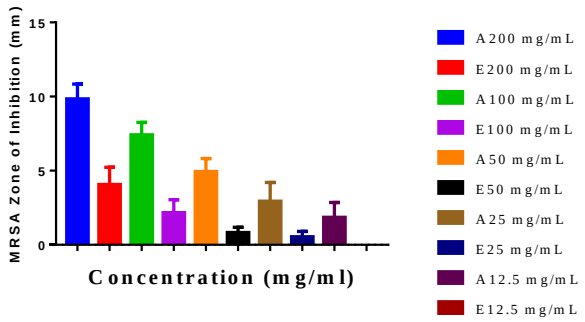

Figure 1. Zones of inhibition of various concentrations of Aqueous and Ethanol Extracts of *M. oleifera* on 35 isolates of MRSA.

4. Conclusion

The study of *S. aureus* becomes pertinent, sequel to the emergence of MRSA that can be associated with hospitalized patients as well as asymptomatic individuals who are carriers and are on the rise in recently. The consequential rise in the failure of most antibacterial agents are generating serious health challenges that might lead to morbidity and mortality. Amidst some of the adverse effects of these antibacterial agents, necessitate the search for newer and safer agents, which most often natural products such as plant are indicated.

The findings from this experimental research on the plant *M. oleifera* lives no doubt that potential antibacterial agents can be soughed. However, the need to evaluate the molecular activities of both the isolates of MRSA and the plant extract should be on focus.

Acknowledgements

The data of this research were extracted from PgD dissertation of Department of Biological Sciences (Microbiology Option) College of Natural and Applied Sciences, Igbinedion University Okada, Edo State, Nigeria. I appreciate the lecturers and also the laboratory technologist Mr. Mick Oghenejebo of the Department of Pharmaceutical Microbiology, Faculty of Pharmacy Delta State University, Abraka, Delta State Nigeria for their support.

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