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ANTIMICROBIAL EFFICACY OF AQUEOUS MORINGA OLEIFERA LEAF EXTRACT AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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Abstract

The primary objective was to investigate the potential antibacterial efficacy of Moringa oleifera against Methicillin-resistant Staphylococcus aureus (MRSA). A well-known medicinal plant valued for its nutritional benefits. Given the escalating challenge of antibacterial resistance, there is a pressing need to explore alternative treatments, thus providing impetus for this investigation. The antibacterial effects of aqueous Moringa oleifera leaf extract at concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 mg/ml were tested against MRSA using the standardized broth microdilution technique and a confirmatory modified disc diffusion method. Strains of MRSA were isolated from blood, wound and pus swabs, sputum, urine, cerebro-spinal fluid and synovial fluid. The broth microdilution method revealed mean inhibitions of 34.3% (1.5) at 25 mg/ml and 10.7% (0.3). The disc diffusion method indicated inhibitory zones of 14 mm, 13 mm, 12 mm, 10 mm, and 9 mm at concentrations of 200 mg/ml, 100 mg/ml, 50 mg/ml, and 25 mg/ml, respectively. The inhibition zones demonstrate a proportional increase with the rising concentration of the extract, confirming a direct correlation. No effective inhibition or zone formation was observed at 6.25 mg/ml. The minimum inhibitory concentration determined by both techniques concurred at 12.5 mg/ml.

Keywords: *Broth micro dilution, Crude extraction, Kirby-Bauer disc diffusion, Maceration, Moringa oleifera*

INTRODUCTION

Moringa oleifera, a member of the Moringaceae family, is a botanical species renowned for its multifaceted medicinal properties. Native to India, Africa, Southeast Asia, Arabia, and South America, *Moringa oleifera* is a versatile plant with anti-inflammatory, anticancer, antidiabetic, and antibacterial agents (1). The "Miracle Tree" surpasses common foods in nutritional value. Its leaves contain more calcium, iron, vitamin C, potassium, and vitamin A than carrots, oranges, and milk (2-3). It is also rich in antioxidants that fight free radicals and prevents diabetes, heart disease, and Alzheimer's. The plant's antioxidant and cellular health properties make it as a super food (4).

Using natural resources is important, and leaves are abundant in biologically active substances. One of these resources is the leaf extract from the *Moringa oleifera* plant, which has shown antibacterial effectiveness (5). Aqueous *Moringa oleifera* extract has a considerable effect on MRSA strains by disrupting cell membranes, interfering with cellular processes, and limiting biofilm development, indicating potential for eradicating resistant MRSA infections (6). Methicillin-resistant *Staphylococcus aureus* (MRSA), also known as multidrug-resistant *S. aureus*, is a bacterial species exhibiting pronounced antibiotic resistance. It harbors the mecA gene, which encodes PBP 2a and confers high methicillin resistance levels. This pathogen forms golden-yellow colonies on blood agar and exhibits catalase and coagulase positivity with a gram-positive cell wall morphology (7).

MRSA is an opportunistic pathogen linked to both hospital-acquired (HA-MRSA) and community-acquired (CA-MRSA) infections (8). MRSA causes various illnesses, including skin abscesses, upper respiratory tract infections, and postoperative wound infections, which can lead to severe and life-threatening conditions and pose significant challenges to modern medicine (9). Rising concerns about *Staphylococcus aureus*'s potential for multidrug resistance underscore the imperative for innovative therapeutic approaches.

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This study aimed to investigate the antimicrobial efficacy of aqueous *Moringa* leaf extract against pathogenic *S. aureus* and determine its minimum inhibition concentration (MIC) using the broth microdilution technique according to Clinical and Laboratory Standards Institute guidelines (10).

METHODOLOGY

STUDY DESIGN AND SETTING

This Quasi-experimental study was conducted in the Microbiology department of Shifa International Hospital, Islamabad, Pakistan over a six-month period, from August 2023 to February 2024.

INCLUSION CRITERIA

1. Diagnosed MRSA patient's sample.
2. Patients of IPD and OPD.

EXCLUSION CRITERIA

1. Immunocompromised or individuals going through organ transplantation.
2. Patients receiving antibiotic therapy.

PLANT COLLECTION

Moringa oleifera leaves were sourced from Pir Mehr Ali Shah Arid Agriculture University, Rawalpind and Soil and Water Testing Laboratory, Rawalpindi. Following collection, processing of leaves involved thorough rinsing with distilled water to remove the dirt and debris and shade-drying for 14 days to remove impurities. Heat and sunlight containing ultra-violet rays can deteriorate the phytochemical compounds present in the leaves (11). Prior to extraction, leaves were pulverized using an ordinary coffee bean grinder. Leaf powder was stored in various sterile containers at 4℃ for further use.

EXTRACT PREPARATION

Crude extraction was done using the Maceration technique according to the States of Pharmacopeia (12) and 40 g of leaf powder was macerated with one liter of distilled water. The solvent was autoclaved to eliminate impurities. The solute was immersed for 24 hours before being filtered with sterile Whatman no. one (pore size 11 µm). A rotatory evaporator (13) at 50°C was utilized to concentrate the extract to 100% purity. To constitute 400 mg/ml concentration, four grams of pure extract was dissolved in ten milliliters of distilled water. The extract was then transferred to sterilized containers for storage at 4°C.

SAMPLE SIZE, COLLECTION AND PROCESSING

Patient samples were obtained from Shifa International Hospital, Islamabad, following informed consent. Based on sample size calculations using OpenEpi, 384 bacterial isolates were collected for six months and cultured on blood agar at 37.5°C. The sample set comprised various clinical specimens, including pus, nasal swabs, wound swabs, blood, urine, and other bodily fluids. MRSA identification was confirmed through biochemical tests, including DNase production, mannitol salt agar sensitivity, gram staining, catalase activity, and coagulase production.

ANTIBIOTIC ACTIVITY AGAINST MRSA

The cell suspension of the grown isolates was standardized against the 0.5% McFarland turbidity standard (1.5×10^8 CFU/ml). Each isolate was inoculated onto Mueller Hinton Agar. Cefoxitin (30 µg) and vancomycin (30 µg) discs were placed on the agar surfaces. The plates were incubated at 37.5°C for 24 hours in an inverted position. Resistivity to cefoxitin and sensitivity to vancomycin confirmed the presence of MRSA (14).

ANALYZING THE ANTIBACTERIAL EFFECTS OF MORINGA ON MRSA

Following inoculation, four concentration sections were labeled on the agar plates: 400 mg/ml, 200 mg/ml, 100 mg/ml, and 50 mg/ml. Dried antibiotic discs of varying concentrations were positioned within

their respective sections. The plates were incubated at 37°C for 24 hours in an inverted orientation. The inhibition zones were measured using a calibrated vernier caliper.

DETERMINATION OF THE MIC

To determine the MIC, Ten MRSA isolates were analyzed using two techniques including, Broth microdilution technique and Kirby Bauer disc diffusion technique.

BROTH MICRODILUTION TECHNIQUE

The MIC of *Moringa* aqueous extract was determined according to Clinical and Laboratory Standards Institute (15). The McFarland solution was prepared by diluting the bacterial suspension to a concentration of 1.5×10^8 CFU/ml in normal saline. Then, 100 µl of the bacterial inoculum and 100 µl of Mueller Hinton broth were dispensed into 71 out of 96 sterilized wells on a microtiter plate. One well was designated as the growth control containing MRSA and Mueller Hinton broth, and another well served as the sterility control, containing only Mueller Hinton broth. To the first wells, 100 µl of the extract was added at a concentration of 400 mg/ml. Serial dilution was performed by transferring 100 µl from each well to the subsequent well, resulting in a series of dilutions: 400 mg/ml, 200 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, and 6.25 mg/ml. The prepared microtiter plate was then incubated at 37°C for 24 hours. Following incubation, the optical density of the samples was analyzed at 620 nm using an ELISA microplate reader after 24 hours to assess the bacterial growth and inhibition properties of the *Moringa* extract at different concentrations. The percentage reduction in bacterial growth (GR) was estimated using the growth control treatment as the reference:

$$GR\% = O.D(C) - \frac{O.D(T)}{O.D\ (C)} x\ 100$$

Where, O.D = optical density calculated by ELISA plate reader, C = cell concentration of control, and T = cell concentration after extract treatment.

KIRBY BAUER DISC DIFFUSION TECHNIQUE

Ten MRSA isolates were inoculated onto Mueller Hinton Agar plates using the lawn culture technique, standardized against a 0.5% McFarland turbidity standard equivalent to 1.5×10^8 CFU/ml. seven concentration sections were labeled: Control (C), 400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, and 12.5 mg/ml. Two control discs were placed separately: Cefoxitin (30 µg) and vancomycin (30 µg).

STATISTICAL ANALYSIS

Data analysis was performed using SPSS version 23, with a 95% confidence interval (CI = 0.95) and a significance threshold of $p < 0.05$. Categorical variables were expressed as percentages, with results obtained through the chi-square test. Continuous variables, such as age and zone of inhibition measurements, were reported as mean (standard deviation). An independent t-test was applied to evaluate age-related differences.

RESULTS

This study examined 384 participants, revealing a predominance of 208 (54%) males compared to 176 (45%) women. The mean age was 44.4 (11.2) for men and 43.9 (10.6) for women. The most prevalent infection in this study was cellulitis with 47 (12.2%) cases. Among the men, diabetic foot ulcer was predominant with 14 (82.4%) cases, but among women, endo-prosthetic hip wound infection was more common with 19 (68%) cases. The least frequent infections in patients included infective endocarditis 12 (3.5%) and septic arthritis 16 (4.2%), as shown in Table I.

Table I. Association of MRSA with demographics and infection types based on gender

Parameters	Male	Female	Total	P-value
Age, mean (SD)	44.38 (11.23)	43.94 (10.63)	44.18 (10.95)	0.694
Gender (%)	208 (54%)	176 (45%)	384 (100%)	0.102
Cystoscopy-induced cystitis	34 (75.6)	11 (24.4)	45 (11.7)	0.011
Infective endocarditis	8 (66.7)	4 (33.3)	12 (3.5)	

Hematogenous osteomyelitis	10 (62.5)	6 (37.5)	16 (4.2)
Endoprosthetic knee wound infection	7 (35)	13 (65)	20 (5.4)
Endoprosthetic hip wound infection	9 (32)	19 (68)	28 (7.3)
Postoperative meningitis	9 (39)	14 (61)	23 (5.2)
Lung abscess	21 (46.7)	24 (53.3)	45 (11.7)
Lower UTIs	21 (56.8)	16 (43.2)	37 (9.6)
Cellulitis	22 (46.8)	25 (53.2)	47 (12.2)
Diabetic foot ulcers	14 (82.4)	3 (17.6)	17 (4.4)
Septic arthritis	9 (56.3)	7 (43.7)	16 (4.2)
Impetigo	12 (54.5)	10 (45.5)	22 (5.7)
Postoperative pelvic abscess	11 (55)	9 (45)	20 (5.5)
Sepsis	9 (69.2)	4 (30.8)	13 (3.4)
Hospital-acquired pneumonia	12 (52.2)	11 (47.8)	23 (6.0)

*SD=Standard Deviation

The predominant source of specimens for MRSA is pus swabs with 151 (39.9%) samples, showing a slightly higher prevalence in 80 (53%) men than in 71 (47%) women. The second most frequent source is urine with 82 (21.4%) samples, exhibiting a higher prevalence in men (67%) than in women (33%). Synovial fluid with 16 (4.2%) samples was the least common source, as shown in Table II.

Table II. Association of MRSA prevalence with sample types

Sources of Specimen (%)	Male	Female	Total	P-value
Blood	27 (65.9)	14 (34.1)	41 (100)	
Pus swab	80 (53)	71 (47)	151 (100)	
Sputum	12 (52.2)	11 (47.8)	23 (100)	0.005
Wound	16 (33.3)	32 (66.7)	48 (100)	
Urine	55 (67)	27 (33)	82 (100)	
Synovial fluid	9 (56.2)	7 (43.8)	16 (100)	
CSF	9 (39.1)	14 (60.9)	23 (100)	

*CSF=Cerebrospinal Fluid

The Fig. 1 illustrates a pie chart about *Moringa* leaf extract concentration against the zone of inhibition demonstrates a direct relationship between concentration and sensitivity. As the *Moringa oleifera* leaf concentration declines, sensitivity decreases and resistivity increases. There is a gradual decrease as 400 mg/ml showed 15 mm, 200 mg/ml showed 13 mm, 100 mg/ml showed 11 mm, and 50 mg/ml showed 10 mm of inhibition.

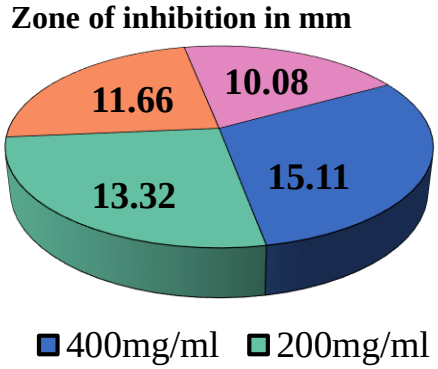

Fig. 1. Inhibition zones of MRSA demonstrated by different concentrations of *Moringa oleifera* aqueous extract

The Fig. 2 depicts the various concentrations of extract and their inhibitory zones that is 14 mm, 13 mm, 12 mm, 10 mm, and 9 mm, indicating that high concentrations are effective in inhibiting MRSA growth. Sensitive zones are formed at 400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, and 25 mg/ml.

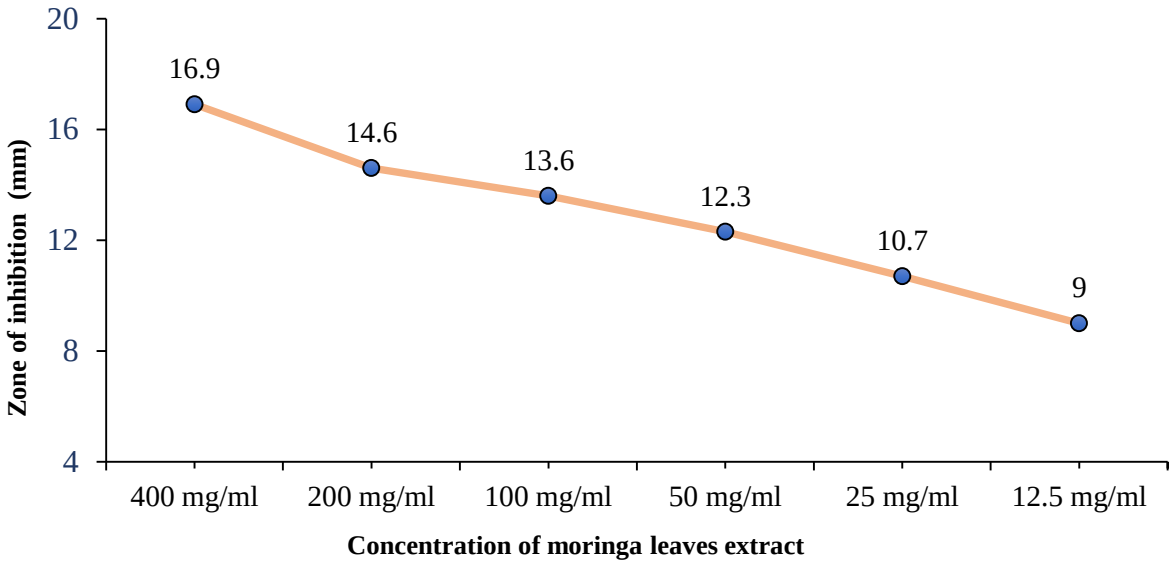

Fig. 2. Minimum inhibitory concentration of aqueous extract of *Moringa oleifera* on MRSA isolates**.**

The MIC was calculated using the broth microdilution method and the disc diffusion technique. The mean inhibition in percentage shows a correlation between the two methods. At 400 mg/ml, 83.9 (1.3) growth reduction and 16.9 (0.2) diameter in mm of zone of inhibition were observed. At 200 mg/ml, 71.5% (0.9) and 14.6 (0.4) were recorded. At 100 mg/ml, 60.6 (1.1) and 13.6 (0.3) were recorded. At 6.25 mg/ml, 4.6 (0.3) and no zone formation were observed, as shown in Table III.

Table III. Growth inhibition by aqueous *Moringa* extracts on MRSA isolates using broth microdilution and disc diffusion method

Concentrations of Moringa leaf extract	Mean inhibition in % by broth micro dilution method	Mean diameter of halo in mm by disc diffusion method
400 mg/ml	83.9 (1.3)	16.9 (0.2)
200 mg/ml	71.5 (0.9)	14.6 (0.4)
100 mg/ml	60.6 (1.1)	13.6 (0.3)
50 mg/ml	48.5 (1.9)	12.3 (0.4)
25 mg/ml	34.3 (1.5)	10.7 (0.3)
12.5 mg/ml	22.2 (1.1)	9.00 (0.2)
6.25 mg/ml	4.6 (0.3)	No zone information

*mm=Millimeter

DISCUSSION

MRSA is a prevalent infection causing skin and subcutaneous infections, contaminating post-surgical wounds, and leading to nosocomial infections and infectious endocarditis. Cellulitis, a severe bacterial skin infection affecting the lower legs and feet, accounts for 12.2% of cases, with serious complications such as abscesses or sepsis. There was a significant association between infection in male and female patients $P = 0.011$ ($P < 0.05$). Rodriguez et al. (16) found that cellulitis affected 43 (81.1%) patients with lower limb lymphedema and 10 (19.9%) of those with upper limb lymphedema. MRSA-related infections include post-cystoscopy cystitis (11.7%), lung abscess (11.7%), and cystoscopy-induced cystitis, causing bladder inflammation after a cystoscopy procedure. Gajdács et al. (17) suggested that *S. aureus* and CoNS were once considered causes of UTIs similar to bacteremia or low sterility of cystoscopy equipment. Diabetic foot ulcers, a prevalent infection among 14 (82.4%) men, are caused by neuropathy and impaired circulation and are linked to MRSA infections due to delayed healing. Stanaway et al. (18) isolated 42% *S. aureus* and 19% MRSA from diabetic foot ulcers. Meo et al. (19) suggested that the higher prevalence of diabetes in men may be due to their higher susceptibility to risk factors. Women are frequently infected with endoprosthetic hip wound infections (68%) despite strict sterilization. Hospital-acquired MRSA is a significant source of nosocomial infections due to

higher arthritis incidence. A study by Akhter et al. (20) found that osteoarthritis was more prevalent in women, with a female-to-male ratio of 4:1. MRSA detection is primarily done using pus swabs (39.3%) and urine samples (21.3%), with the majority of samples collected from diabetic foot ulcers and lung abscesses in men (53%) and women (47%). The study found that male urine samples (67%) were more prevalent than female samples (33%). Synovial fluid is rarely used for detecting MRSA-induced septic arthritis due to invasive procedures, with a low prevalence (21%). The *Moringa* extract exhibits inhibition zones that increase with concentration, with the largest zone measuring 16 mm at 400 mg/ml, and concentrations of 200, 100, and 50 mg/ml show diminishing zones. The experiment determined the minimum inhibitory concentration of MRSA isolates using the broth microdilution method and the Kirby-Bauer disc diffusion method. At 400 mg/ml, 83.9% of MRSA isolates were inhibited. Turbidity increased significantly at 6.25 mg/ml, but growth decreased by only 4.6%. The Kirby-Bauer disc diffusion method showed that as *Moringa oleifera* extract concentration decreased, so did its sensitivity. The sensitive zone is at concentrations of 400 mg/ml, 200 mg/ml, 100 mg/ml, and 50 mg/ml, and the intermediate zone is 12.5 mg/ml, where MRSA becomes resistant. No zone of inhibition forms at 6.25 mg/ml, making 12.5 mg/mL the MIC.

CONCLUSION

The study findings have demonstrated that aqueous extracts of *Moringa oleifera* showed significant antibacterial activity against MRSA in various infections, suggesting potential use in infection therapy. *M. oleifera* leaves contain bio-components with antibacterial potentials similar to those of antibiotics against gram-positive *Staphylococcus aureus*. Its efficacy against MRSA may indicate a broad range of bioactive chemicals in the leaf. *M. oleifera* may be a viable natural antibacterial agent with prospective uses in the pharmaceutical industry. These results are encouraging, but further research is essential to optimize extraction methods, determine effective dosages, explore mechanisms of action, and evaluate its safety and efficacy in vivo. This study underscores the importance of exploring plant-based antimicrobials as a sustainable solution to the growing issue of antibiotic resistance.

Limitations:

Aqueous extracts of *Moringa oleifera* may not fully represent the plant's bioactive compounds. Alternative solvents such as ethanol or methanol could extract additional antibacterial components, highlighting the importance of varied extraction methods. Bioactivity varies based on factors such as plant age, geographic origin, and extraction protocols, underscoring the complexity of studying bioactive compounds. Although the study demonstrates MRSA inhibition, the precise mechanisms remain unclear. Further research is needed to elucidate the molecular basis of this effect. Findings remain preliminary without animal trial data, limiting the translational potential to clinical efficacy.

Authors' contribution:

RI Conceived & designed the study; AR and TT Performed the experiments; ZB and AS Analyzed the data & contributed to the interpretation of results; KN Assisted in literature review & manuscript formatting. All authors read and approved the final manuscript.

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