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ORIGINAL ARTICLE

In Vitro Assessment of the Antibacterial Potential and Minimum Inhibitory Concentration of *Moringa oleifera* Extract Against Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT

Objective: The plant *Moringa oleifera* serves as a popular medicinal herb which people use for its health advantages. The rising problem of antibacterial resistance requires us to find new treatment options. The main objective of the study was to examine how *Moringa oleifera* performed as an antibacterial agent against Methicillin-resistant *Staphylococcus aureus (*MRSA*)* bacteria.

Methodology: An in vitro experimental laboratory study was conducted in the Microbiology department of Shifa International Hospital, Islamabad, Pakistan, over a six-month period, from August 2023 to February 2024. The study evaluated the antibacterial activity of *Moringa oleifera* leaf extract, which was tested at various concentrations between 6.25 mg/ml and 400 mg/ml against MRSA through standardized broth microdilution method and through confirmed results of modified disc diffusion testing. Scientists obtained MRSA strains from blood, wound, pus, sputum, urine, cerebro-spinal and synovial fluids.

Results: The broth microdilution method detected 34.3% (1.5) inhibition at 25 mg/ml and 10.7% (0.3) inhibition at another concentration. 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.

Conclusion: The experimental results show that increasing extract concentrations lead to larger inhibition zones, which establish a direct relationship between extract concentration and inhibition zone measurement. The results showed that there was no effective inhibition when testing at 6.25 mg/ml. The minimum inhibitory concentration determined by both techniques concurred at 12.5 mg/ml.

Keywords: Broth microdilution, Crude extraction, Kirby-Bauer disc diffusion, Maceration, *Moringa oleifera.*

Authors' Contribution:
[1,2]Conception; Literature research; manuscript design and drafting; [3,4]Critical analysis and manuscript review; [5,6]Data analysis; Manuscript Editing.

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Introduction

The Moringaceae family includes *Moringa oleifera* which serves as a medicinal plant with multiple therapeutic uses. *Moringa oleifera* originates from India Africa Southeast Asia Arabia and South America as a plant that possesses anti-inflammatory and anticancer and antidiabetic and antibacterial properties.[1] The "Miracle Tree" provides better nutritional value than ordinary food items. Its leaves contain more calcium, iron, vitamin C, potassium, and vitamin A than carrots, oranges, and milk.[2, 3] The plant contains antioxidants which protect against free radicals while it prevents diabetes and heart disease and Alzheimer's disease. The plant's ability

to protect cells and its antioxidant capacity establish it as a superfood.[4]

Natural resources should be utilized because leaves serve as a resource which contains numerous active biological compounds. The leaf extract from *Moringa oleifera* functions as a resource which demonstrates antibacterial properties.[5]

The aqueous *Moringa oleifera* extract shows strong effects against Methicillin-resistant *Staphylococcus aureus* (MRSA) strains because it disrupts their cell membranes and blocks their cellular functions and prevents biofilm formation. The extract shows potential as a treatment to eliminate MRSA infections that are resistant to standard treatments.[6]

MRSA exists as multidrug-resistant S. aureus, which represents a bacterial strain that demonstrates exceptional resistance against multiple antibiotics. The bacterium contains the mecA gene, which produces PBP 2a that enables the organism to achieve elevated levels of methicillin resistance. The pathogen produces golden-yellow colonies when grown on blood agar and shows both catalase and coagulase activity while its cells display a gram-positive cell wall structure.[7]

MRSA, therefore, is a bacteria which is harmful in itself and can harm those who have weak resistance, turning into a real risk to patients.[8] MRSA causes different illnesses which include skin abscesses and upper respiratory tract infections and postoperative wound infections that can result in severe and life-threatening conditions while creating major difficulties for contemporary medical practice.[9]

Several studies from Pakistan have investigated the antibacterial potential of *Moringa oleifera* against different pathogenic organisms. The study conducted in Karachi tested *Moringa oleifera* leaf extracts for their antibacterial properties against clinically important bacterial species through in vitro testing.[10] The study conducted in Multan demonstrated that *Moringa oleifera* extracts showed strong antibacterial properties and non-biofilm formation capacity against human pathogens, which proved its potential as a substitute for standard antibiotics.[11] The research conducted in Khyber Pakhtunkhwa examined the phytochemical makeup and antibacterial effects of different *Moringa oleifera* plant parts, which proved the plant's ability to kill a wide range of bacteria.[12]

The increasing number of multidrug-resistant *Staphylococcus aureus* infections especially *MRSA* strains has generated an immediate requirement for new effective antimicrobial treatments. Plant-derived substances show antibacterial activity yet there exists insufficient standardized research data that assesses *Moringa oleifera's* effectiveness against MRSA strains which have been clinically discovered. The research has not yet investigated its minimum inhibitory concentration (MIC) testing through conventional laboratory methods.[13]

The present study evaluated the antibacterial properties of *Moringa oleifera* leaf extract against MRSA strains which were obtained from clinical samples and determined its minimum inhibitory concentration (MIC) through standardized testing methods that followed Clinical and Laboratory Standards Institute (CLSI) guidelines. The research adds to existing scientific knowledge by delivering region-specific clinical proof which includes minimum inhibitory concentration (MIC) measurements that increase its value for scientific research on antimicrobial treatments.

Methodology

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

We conducted their study using non-probability consecutive sampling method. The study included all MRSA isolates which doctors confirmed as clinical cases and which the Microbiology Department of Shifa International Hospital received during the

research period until the we collected their desired sample size.

Plant Collection: The Arid Agriculture University and Water Testing Institute in Rawalpindi provided *Moringa oleifera* leaves for our research. The leaves underwent complete washing followed by 14 days of shade drying which maintained their phytochemical content because both high temperatures and ultraviolet rays could damage their bioactive substances. The dried leaves were ground into a fine powder which we kept in sterile containers at 4°C until we needed them for further testing.[14]

Preparation of Aqueous Extract: Crude aqueous extraction was performed using the maceration technique according to the United States Pharmacopeia method.[15] We used one liter of sterile distilled water to extract active compounds from 40 grams of powdered leaves through a 24-hour maceration process. The mixture was filtered using sterile Whatman No. 1 filter paper (pore size 11 µm), and the filtrate was concentrated using a rotary evaporator at 50°C. [16] We prepared a stock solution at 400 mg/ml and used sterile distilled water to create serial dilutions that produced concentrations of 200 100 50 25 12.5 and 6.25 mg/ml.

Sample Size, Collection and Processing: Clinical specimens were collected from patients at Shifa International Hospital, Islamabad, after obtaining informed consent. The sample size was calculated using OpenEpi [17] version 3.01, assuming a population size of 1,000,000, a 95% confidence level, and an expected frequency of 50%, resulting in a required sample size of 384 bacterial isolates. These isolates were collected over a six-month study period. Clinical specimens included pus, nasal swabs, wound swabs, blood, urine, and other body fluids. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were identified using standard microbiological and biochemical methods, including Gram staining, catalase and coagulase tests, DNase production, and growth on mannitol salt agar.

Antibiotic Activity against MRSA: The cell suspension of the grown isolates was standardized against the 0.5% McFarland turbidity standard (1.5 × 108 CFU/ml). We inoculated each isolate onto Mueller Hinton Agar. We applied cefoxitin (30 µg) and vancomycin (30 µg) discs onto the agar surfaces. We incubated the plates at 37.5°C for 24 hours while they remained in an inverted position. The presence of MRSA was confirmed through the testing which showed resistance to cefoxitin and sensitivity to vancomycin.[18]

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. Antibiotic discs, in various dilutions, were put in their respective compartments. 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: The zone of inhibition refers to the clear area surrounding the impregnated disc on Mueller–Hinton agar where bacterial growth is completely suppressed, and its diameter (measured in millimeters) is used as an indicator of the antibacterial activity of the tested extract.

The minimum inhibitory concentration testing for MRSA isolates followed CLSI guidelines [19] through the application of the broth microdilution testing method. The extract was diluted through serial two-fold dilutions which ranged from 400 mg/ml to 6.25 mg/ml in Mueller–Hinton broth within 96-well microtiter plates. The experiment included both growth control and sterility control wells. The plates spent 24 hours at 37°C before we used an ELISA microplate reader to measure optical density at 620 nm for bacterial growth assessment.

The analysis used SPSS version 23 for data analysis which established a confidence level of 95% (CI = 0.95) and set the significance level at $p < 0.05$. The study presented categorical variables through percentage values which the we obtained from chi-square test results. The study reported continuous variables through age and zone of inhibition measurements as mean values together with their

standard deviation. We used an independent t-test to assess age-based differences between participants.

Ethical approval was obtained from the Institutional Review Board and Ethics Committee, Shifa International Hospital (IRB#0285-23, dated 28 August 2024). All participants gave written informed consent.

Results

The study involved 384 participants who were divided into 208 male participants and 176 female participants. Table I demonstrates that cellulitis was the most frequently observed infection, accounting for 47 (12.2%) cases. The gender distribution revealed that males experienced diabetic foot ulcer more frequently than females who had endoprosthetic hip wound infection. The least frequently reported infections were infective endocarditis (12, 3.5%) and septic arthritis (16, 4.2%). The study found a statistically significant difference between males and females regarding their distribution of infection types. The statistically significant p-value (0.011) indicates that the pattern of MRSA-associated infections differs significantly between males and females. The results indicate that different clinical conditions and behavioral factors together with healthcare procedures lead to different infection susceptibility patterns between genders 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. 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 as shown in figure 01.

Table I: Association of MRSA with infection types

Type of Infection	Male, n (%)	Female, n (%)	Total, n (%)	P - value
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)	

Table II: Association of MRSA prevalence with clinical sample types

Sources of Specimen (%)	Male	Female	Total	P-value
Blood	27 (65.9)	14 (34.1)	41 (100)	0.005
Pus swab	80 (53)	71 (47)	151 (100)	
Sputum	12 (52.2)	11 (47.8)	23 (100)	
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)	

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

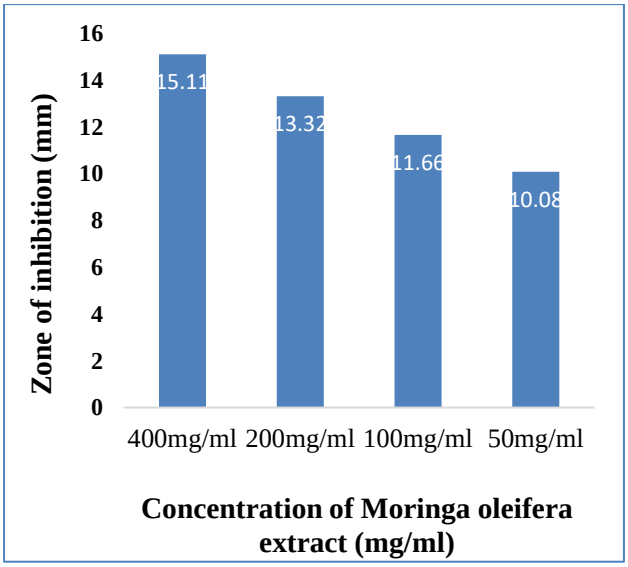

Figure I: Mean zone of inhibition (mm) of MRSA produced by different concentrations of aqueous *Moringa oleifera* leaf extract.

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 ($P < 0.05$). Rodriguez et al [20] 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 [21] 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. Qi et al [22] isolated 27.7% *S. aureus* and 33.7% MRSA from diabetic foot ulcers. Meo et al [23] 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 Segal et al [24] found that Osteoarthritis occurs more commonly in women, predominating excessively in 60 percent of cases, and showing a marked prevalence with age, especially at 40 years of age and above. 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 because its invasive procedures are needed and its detection rate is low at 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. The 400 mg/ml concentration of the substance led to 83.9% of MRSA isolates being 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. The MIC of 12.5 mg/mL is established because no zone of inhibition forms at 6.25 mg/ml.

Study Strengths: The research investigates the increasing problem of antimicrobial resistance through testing the antibacterial effects of *Moringa oleifera* against clinical MRSA strains. The study results become more applicable to wider populations because the research team used extensive clinical sample collection to confirm their findings. The research results gain better reliability and validity through the implementation of standardized CLSI-guided methods which include both broth microdilution and Kirby-Bauer disc diffusion techniques. The extract's antibacterial activity demonstrates evidence through dose-dependent inhibitory effects which also provide essential data about Pakistan's specific geographic region.

Limitations of the Study: The study evaluated only aqueous extracts of *Moringa oleifera*, which may not fully represent all bioactive compounds present in the plant; however, this approach aligns with traditional and safer extraction practices. The research findings exist as an initial stage because additional studies need to take place in both in vivo and animal testing environments to verify their medical use. The study did not investigate specific molecular mechanisms that produce antibacterial effects, which creates a need for more research in this particular area.

Conclusion

The study shows that aqueous *Moringa oleifera* leaf extract displays antibacterial properties which increase with higher concentrations against clinically isolated MRSA strains tested in laboratory conditions. The standardized broth microdilution and Kirby-Bauer disc diffusion methods produced matching results which determined a minimum inhibitory concentration of 12.5 mg/ml thus proving the test results to be accurate. The research demonstrates that M. oleifera contains bioactive compounds which show potential to fight MRSA pathogens making it a possible natural substitute or supplementary treatment for standard antibiotic medications. The research requires further investigation to determine the phytochemical profile and biological effects and safety profile and actual effectiveness of the treatment in clinical use.

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