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ORIGINAL RESEARCH

Evaluation of the antibacterial activity of *Moringa oleifera* leaf extract against Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Objectives: Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most significant hospital-acquired pathogens, responsible for numerous life-threatening infections globally. The increasing resistance of this bacterium to commonly used antibiotics poses a major challenge in managing infections effectively. . This study aimed to evaluate the antibacterial properties of hydro alcoholic *Moringa oleifera* extract against clinical MRSA isolates. The primary goals were to determine the minimum inhibitory concentration (MIC) of the extract, assess its bactericidal activity, and investigate the correlation between antibiotic resistance genes and MIC values.

Materials and Methods: Clinical MRSA isolates were collected from hospitalized patients *Moringa oleifera* extract was prepared using maceration in a hydro alcoholic solvent (70% ethanol). The antibacterial activity of the extract was assessed via the agar diffusion method, using disc placement on Mueller-Hinton agar. Each test was repeated three times, and inhibition zone diameters were measured and recorded to calculate the antibacterial activity. The MIC was determined using the broth microdilution method. Additionally, the presence of antibiotic resistance genes, including mecA, was evaluated in relation to MIC values. Statistical analyses were conducted using specialized software to ensure accuracy and reliability.

Results: Out of 100 samples studied, 23 were confirmed as MRSA. Statistical comparisons revealed significant differences between the inhibition zones produced by *Moringa oleifera* leaf extract and the standard antimicrobial agent (Linezolid) against MRSA isolates (p-value < 0.001). Concentrations below 8 µg/mL significantly reduced the killing time and exhibited superior efficacy in controlling MRSA infections. Furthermore, all MRSA isolates were found to carry the mecA gene, which encodes methicillin resistance.

Conclusion: This study demonstrated the strong antibacterial effects of hydro alcoholic *Moringa oleifera* extract against clinical and standard MRSA isolates. The findings highlight the potential of *Moringa oleifera* as a source for developing new and effective antibiotics to treat MRSA-associated infect *Moringa oleifera* ions. This research underscores the importance of exploring plant-based therapeutic options to address the growing challenge of antibiotic resistance.

Keywords: Methicillin-resistant *Staphylococcus aureus* (MRSA), *Moringa oleifera,* minimum inhibitory concentration (MIC).

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) ranks among the most significant and problematic hospital-acquired pathogens, causing various infectious diseases such as skin and soft tissue infections, endocarditis, osteomyelitis, bacteremia, pneumonia, and central nervous system infections [1]. MRSA infections are typically associated with adverse clinical outcomes, including prolonged hospital stays, increased mortality rates, and higher treatment costs [2]. Antibiotics are valuable tools for treating many human diseases, yet excessive use leads to microbial resistance. As a result, researchers prioritize investigating medicinal plants to discover new plant-derived drugs [3]. MRSA is one of the most prevalent bacteria resistant to multiple antibiotics, capable of colonizing and surviving in hospital environments. *Staphylococcus aureus* achieves this through acquiring resistance genes from various organisms in its surroundings [4]. Shortly after methicillin was introduced in 1959 to combat penicillinase-producing Staphylococcus strains, the first methicillin-resistant isolate was reported in Europe. A decade later, MRSA became widespread in a Boston hospital in the United States [5]. To date, various reports highlight different prevalence rates of MRSA in multiple regions. Studies indicate MRSA accounts for over 25% of bacteremia cases across European countries [6].

MRSA infections are among the most common hospital-acquired infections in Iran, contributing to various opportunistic infections resistant to existing antibiotics [7]. Despite strategies for antibiotic use, bacterial infections remain a major challenge in healthcare-associated infections. Excessive antibiotic use in hospitals can increase bacterial disease severity and lead to more complex clinical issues [8]. Additionally, widespread antimicrobial use reduces bacterial sensitivity to these compounds, potentially supporting the survival of resistant strains and facilitating the transfer of resistance genes [9]. A systematic review and meta-analysis of Iranian children estimated MRSA prevalence to be approximately 22% [10]. A global study has issued urgent warnings regarding the rising prevalence of MRSA-related infections [11]. Given the continued spread of MRSA, introducing novel treatment options is essential as infections often lead to severe clinical complications, including prolonged hospitalization, increased disease severity, side effects, higher treatment costs, and increased mortality rates [11]. Medicinal plants have traditionally been used for microbial infections, and recent studies demonstrate their antimicrobial efficacy against drug-resistant pathogens [12-15]. These plants offer potential solutions for developing new treatments [16, 17]. Medicinal plants, with diverse bioactive compounds, effectively combat drug-resistant infections, acting as alternatives or complements to chemical treatments [18]. Active compounds such as phenols, flavonoids, alkaloids, and terpenes contribute to plants' antimicrobial properties [19]. Research indicates that plants like turmeric (curcumin), garlic (allicin), and thyme (thymol and carvacrol) inhibit resistant bacteria such as *Staphylococcus aureus* [20-22]. Furthermore, medicinal plants can reduce side effects of chemical drugs and lower the risk of drug resistance [23].

Materials and Methods

Study sample

Study Design and Sample Collection

This experimental laboratory-applied study was conducted from June to July 2024, targeting 100 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. The isolates were obtained from various clinical specimens, including blood, skin, urine, and sputum, collected from selected teaching hospitals in Tehran. Biochemical tests such as mannitol sugar fermentation on Mannitol Salt Agar, DNase, and phenotypic tests (e.g., Gram staining, catalase test) were performed to confirm MRSA isolates. Screening with 30 µg cefoxitin discs using the disk diffusion method identified 23 isolates as MRSA. Plant Collection and Extract Preparation Flowering branches of *Moringa oleifera* were collected from Sistan and Baluchistan and identified in the Herbarium of the Faculty of Pharmacy, Mashhad University of Medical Sciences (Code: 13573). Leaves were separated, and their hydro alcoholic extract was prepared using the maceration method. Agar Screen Method for Antibiotic Susceptibility The sensitivity of MRSA isolates to oxacillin was assessed using the agar screen method. Mueller-Hinton Agar, supplemented with 4% NaCl and 6 µg/ml

oxacillin, served as the medium. ATCC25923 and ATCC33591 strains were used as negative and positive controls, respectively.

Minimum Inhibitory Concentration (MIC) Determination MIC of the *Moringa oleifera* leaf extract was determined using the broth microdilution method. Serial dilutions (256 to 0.12 µg/ml) of the extract were added to 96-well microplates. Microbial suspensions standardized to 0.5 McFarland and diluted in Mueller-Hinton broth (1:100; 1×10^6 CFU/ml) were introduced. Plates were incubated at 35°C for 20-24 hours. Turbidity indicated bacterial growth, while clarity indicated inhibition. The lowest concentration with no bacterial growth was recorded as MIC. DNA Extraction and PCR Amplification Bacterial DNA was extracted using a commercial kit (GTP, Tehran, Iran) according to the manufacturer's guidelines. PCR was performed to assess DNA concentration, followed by agarose gel electrophoresis (1.5%) in TAE buffer for DNA analysis. The 16S rRNA gene was amplified via PCR using universal primers for the molecular identification of isolates (Table 1).Phylogenetic Analysis Amplified 16S rRNA gene sequences were aligned with related sequences from *Staphylococcus aureus* retrieved from GenBank using NCBI BLAST. A phylogenetic tree was constructed using the Neighbor-Joining method and the Kimura 2-parameter model with 1000 bootstrap replications using MEGA 4.1 software. Statistical Analysis Data were analyzed using SPSS version 22. Chi-square tests were applied to determine relationships between groups.

Results

Antibiotic Susceptibility Patterns:

Antibiotic resistance and sensitivity patterns were assessed for penicillin, gentamicin, erythromycin, doxycycline, ciprofloxacin, clindamycin, trimethoprim-sulfamethoxazole, and linezolid using the disk diffusion method (Figure 1). Among 23 MRSA isolates, the highest resistance was observed for penicillin (98.4%), followed by erythromycin (55.6%), doxycycline (50.8%), ciprofloxacin (38.1%), trimethoprim-sulfamethoxazole (25.4%), and gentamicin (12.7%). The highest sensitivity was recorded for linezolid, with 100% susceptibility. Statistical analysis revealed significant differences between resistance to penicillin and cefotaxime compared to other antibiotics (P value = 0.015), and between tetracycline resistance and other antibiotics (P value = 0.026). No statistically significant differences were observed between gender, presence of *Staphylococcus aureus*, or type of infection (P value < 0.05).

Minimum Inhibitory Concentration (MIC):

The MIC of *Moringa oleifera* hydro alcoholic extract against MRSA isolates ranged from 0.5 to 4 µg/ml. Statistical analysis showed no significant association between sample type, age, gender, and MIC values of the *Moringa oleifera* leaf extract in MRSA isolates (P value > 0.05). However, the mean inhibition zone diameter of *Moringa oleifera* leaf extract was significantly greater compared to the standard antimicrobial agent, linezolid (P value < 0.001).

Genetic Analysis of Antibiotic Resistance Genes: Based on PCR analysis of mecA, VanS, and VanR genes, mecA was the most prevalent gene in MRSA isolates, present in 100% of samples. VanS and VanR genes were detected in 30.4% and 26% of isolates, respectively (Table 1). No significant association was observed between the presence of mecA, VanS, or VanR genes and antibiotic resistance (P value > 0.05).

Molecular Confirmation and Phylogenetic Analysis: For enhanced sensitivity in MRSA identification, the 16S rRNA gene was utilized. All 23 confirmed isolates had this gene (Figure 2). Trimmed sequences were evaluated for similarity using the Nucleotide BLAST database. Comparative analysis of the 16S rRNA gene sequences of MRSA isolates revealed 99.8-100% similarity with *Staphylococcus aureus* ATCC 25293 (Figure 3). Phylogenetic proximity with ATCC 25293 was confirmed through bootstrap analysis and phylogenetic tree construction using the Neighbor-Joining method (Figure 4).

Time Kill Assay Results

The Time Kill assay performed on two MRSA isolates with high MIC values demonstrated that *Moringa oleifera* leaf extract exhibited bactericidal effects at both MIC and 2×MIC concentrations. These effects were observed within 120 minutes, leading to a reduction in CFU counts. At sub-MIC concentrations, the

bactericidal effect required more time; although a reduction in CFU was initially observed, no significant decrease was achieved, and CFU counts eventually increased (Figure 5).
Additionally, the results indicated that while both extract-treated and control samples showed bacterial counts exceeding 2 Log CFU/ml, the bacterial counts in the extract-treated samples were significantly lower than those of the controls (p-value > 0.05).

Discussion

The spread of antibiotic-resistant bacteria in healthcare settings has become a major challenge, causing hospital-acquired infections that lead to prolonged treatments, extended hospital stays, and increased healthcare costs. Among the most significant pathogens in Iran are Methicillin-resistant *Staphylococcus aureus* (MRSA) strains, which are responsible for various infections. Evidence indicates that the indiscriminate use of antibiotics has led to the emergence and spread of multidrug-resistant strains. Therefore, developing alternative therapeutic options to treat infections caused by drug-resistant hospital pathogens is crucial. This study aimed to evaluate the antimicrobial activity of *Moringa oleifera* leaf extract against MRSA isolates and investigate antibiotic resistance patterns and kill-time assay results.
The highest antibiotic resistance rates were observed for penicillin (98.4%), followed by erythromycin (55.6%) and doxycycline (50.8%). All isolates were 100% sensitive to linezolid. Similar findings were reported in studies conducted in Marand, Sari, and Tehran, where erythromycin resistance rates ranged between 83.6% and 85.1%, slightly higher than the resistance observed in this study [24, 25]. A study in Egypt by Youssef et al. (2021) showed that linezolid had the highest sensitivity (98.3%), with high resistance to gentamicin (56.4%) and ciprofloxacin (53.8%) [26]. Another Egyptian study revealed higher erythromycin resistance (93.5%) compared to our findings, and only 76% of isolates were sensitive to linezolid [27]. Overall, antibiotic resistance levels in Egyptian studies were notably higher than those in this study [26, 27]. Mohammadi et al. examined 150 MRSA isolates from community and hospital settings, reporting high resistance to tetracycline and co-trimoxazole, while no resistance was observed to dalbavancin or teicoplanin, emphasizing the need for new antibiotics [28]. Similarly, a US study analyzing 300 MRSA isolates identified vancomycin as the most effective option [29]. In Europe, resistance to fifth-generation cephalosporins in *Staphylococcus aureus* isolates is reportedly increasing [30]. Sadari et al. reported a penicillin resistance rate of 90.8% in 87 isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from the nose in 2002-2003 [31]. T. Wadros in 1984 estimated the penicillin resistance rate in 109 strains of *Staphylococcus aureus* isolated from nasal secretions in hospital patients to be 86.2% [32].
In 2001, Tavakoli et al. reported that all strains of *Staphylococcus aureus* isolated in their study were resistant to penicillin [33]. The susceptibility of *Staphylococcus aureus* isolates to cephalexin in the present study was determined to be 96.67%. This is while in the study by Safdari et al. in 2012 in Ghaem Hospital, Mashhad, this rate was reported to be 57% [34], in the study by Tavakoli et al. it was 81.6%, and in the study by Ghasemian et al. in 2004 it was 69.4% [33, 35]. The isolates of *Staphylococcus aureus* in the present study showed 95% susceptibility to erythromycin and 99.5% susceptibility to vancomycin. Ghasemian et al. estimated the susceptibility of *Staphylococcus aureus* isolates to vancomycin at 44.4% in 2004 [35].
In the case of other antibiotics studied, similar or different results were found with other researchers from Iran, and these differences could be due to the local characteristics of each region, the method used to determine the level of sensitivity, and factors involved in the development of drug resistance, including transferable plasmids, etc. In general, all *Staphylococcus aureus* isolates studied in the present study have multiple resistance to different antibiotics, which in itself doubles the risk of excessive use of antibiotics at the community level.
In another part of this research, genes encoding antibiotic resistance in *Staphylococcus aureus* were investigated and the PCR method was used to detect the studied genes. The results showed that the mecA gene (encoding methicillin resistance) was present in all isolates of *Staphylococcus aureus* isolated from clinical samples.
Stromenger et al. in 2003 in Germany used multiplex PCR to detect antibiotic resistance

genes in *Staphylococcus aureus* and showed that tetM, tetK, aacA-D and mecA genes were present in 26.66, 16.66, 66.33 and 93.33% of the isolates, respectively, but none of them carried the vatA and vatB genes [36]. Heidari et al. in 2001 reported the frequency of mecA, msrA, msrB, aacA-D, tetK and tetM genes in *Staphylococcus aureus* strains isolated from human clinical infections and bovine mastitis as 18.85, 29.46, 07.49, 33.33, respectively. They determined 50/80 and 66/66 percent [37].

The antimicrobial activity of *Moringa oleifera* leaf extract demonstrated promising inhibitory effects on MRSA growth, with significant inhibition observed at a concentration of 25 mg/ml. While no similar studies have measured the MIC of *Moringa oleifera* against MRSA isolates, research has shown its strong antibacterial properties against other pathogens [38–39]. For instance, Abalaka et al. reported MIC values ranging between 10–20 µg/ml for *Moringa oleifera* leaf extract against bacterial pathogens [38]. Differences between studies may result from variations in extraction methods or experimental protocols.

In conclusion, the hydro alcoholic of *Moringa oleifera* leaf extract exhibited notable antimicrobial activity against MRSA isolates, highlighting its potential as a candidate for developing next-generation antimicrobial agents. Future studies should focus on broader sample populations, test *Moringa oleifera* against other hospital pathogens, and evaluate its effects in animal models.

Table 1: The Primers used for the PCR:

Primer	Primer Sequence (5'-3')	Product Size	Reference
mecA F	AGAAGATGGTATGTGGAAGTTAG	583 bp	40
mecA R	ATGTATGTGCGATTGTATTGC		
vanR F	AGCGATAAAATACTTATTGTGGA	425 bp	41
vanR R	CGGATTATCAATGGTGTCGTT		
vanS F	TTGGTTATAAAATTGAAAAATAA	1155 bp	41
vanS R	TTAGGACCTCCTTTTATC		

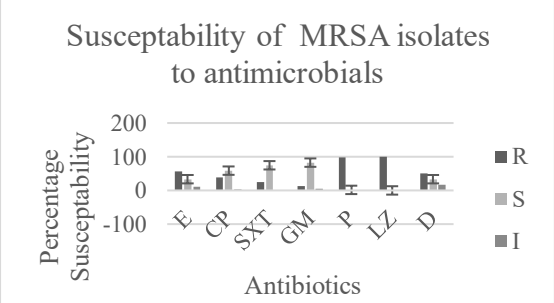

Figure 1: Antibiotic resistance rates among MRSA isolates : E: Erythromycin, CP: Ciprofloxacin, SXT: Sulfamethoxazole-Trimethoprim, GM: Gentamicin, P: Penicillin, , LZ: Linezolid, D: Doxycycline.

Conflict of Interest

Author declares no conflict of interest.

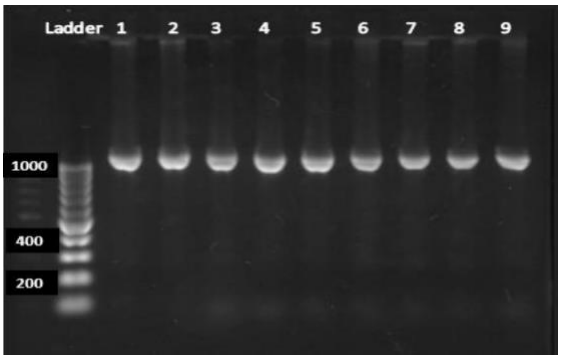

Figure 2: Amplification of the 16S rRNA gene (1028 nucleotides) from MRSA isolates. Column 1: 100 bp marker, Columns 2 onward: clinical MRSA isolates.

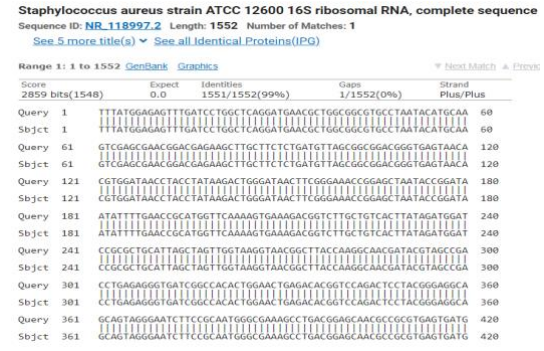

Figure 3: BLAST results for the **16S rRNA gene** of methicillin-resistant *Staphylococcus aureus* isolates in the NCBI database.

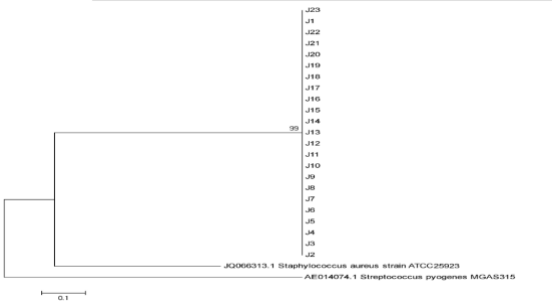

Figure 4: Phylogenetic tree based on mecA gene sequences for methicillin-resistant *Staphylococcus aureus* isolates and evolutionarily close species, constructed using the NJ method with the K2P model.

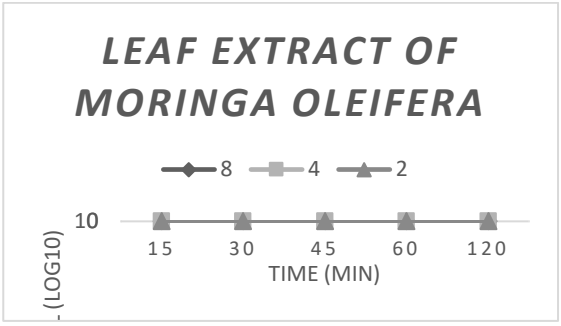

Figure 5: Killing time of *Moringa oleifera* leaf extract on methicillin-resistant *Staphylococcus aureus* (MRSA). The extract demonstrated bactericidal effects within 120 minutes at MIC and 2×MIC concentrations, significantly reducing CFU. At sub-MIC concentration, a longer duration was required for bacterial reduction, but no significant killing effect was observed, and CFU eventually increased.

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