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Research Article

In vitro antibacterial evaluation of *Moringa oleifera* against wound infecting bacteria: Phytochemical based approach

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Abstract
Background: The rising trend of antibiotic resistance has made it essential to explore alternative antibacterial agents. Medicinal plants have been found to be a valuable source of natural antimicrobials, and this study sought to screen the antibacterial properties of methanolic extracts from leaves of medicinal plant *Moringa oleifera* against pathogenic bacteria *Staphylococcus aureus* (a Gram-positive bacterium) and *Escherichia coli* (a Gram-negative bacterium).
Methods: Clinical wound samples containing Gram-positive and Gram-negative bacteria were obtained, sub-cultured and identified using Gram staining and standard biochemical tests. Methanolic extracts of *M. oleifera* leaves were prepared and screened for phytochemicals using standard qualitative assays. Antibacterial activity was evaluated by agar well diffusion and broth microdilution methods to determine zones of inhibition and minimum inhibitory concentrations. Data was analyzed using GraphPad Prism software.
Results: Phytochemical analysis of *M. oleifera* methanolic leaf extract confirmed the presence of flavonoids, steroids and saponins, while quinones were absent. Bacterial isolates were identified as *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) through morphological and biochemical tests. The extract exhibited concentration-dependent antibacterial activity, with negligible inhibition at lower concentrations and significant zones of inhibition at 1200 µL against both organisms. The minimum inhibitory concentration was determined to be as low as 300 µL, indicating effective antibacterial potential of the extract.
Conclusion: This study proves the antibacterial properties of *M. oleifera* and indicates that the plant can serve as sources of natural antibacterial for treating bacterial infections.
Key Words: *Moringa oleifera*, Medicinal plant, Antibacterial activity, Phytochemicals

INTRODUCTION

Gram-positive and gram-negative bacteria are major source of bacterial infections worldwide and mainly found in wounds because it is a very favorable medium for bacterial colonization [1]. Infections with wounds are superficial or deep depending on involvement of tissues and the severity is graded accordingly. Acute wounds are associated with an intense immune response, while chronic wounds are difficult to heal, often related to biofilms [2]. Burns compromise skin integrity, therefore increasing the risk of infections [3].

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Burn wounds are initially sterile but rapidly become populated by bacteria, with severe infections resulting in dead tissue excision and delayed wound closure. Surgical wounds can cause infections, which increase the cost of healthcare. The symptoms of wound infections include redness, swelling, pain, pus, fever, and systemic complications such as sepsis if left untreated [4]. Common pathogens for wound infection include *Staphylococcus aureus* (*MRSA*), *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Escherichia coli* [5]. Treatment involves antimicrobial dressings and antibiotics targeting bacterial mechanisms, such as β-lactams (cell wall inhibitors), aminoglycosides to inhibit protein synthesis and quinolones for DNA disruptors [6]. Antibiotic choice is important in wound infections and

thus healing. Antibiotic resistance, the ability of bacteria to develop mechanisms that help them evade the effects of antimicrobial drugs reduce efficacy. Overuse and misuse of antibiotics, such as using antibacterial drugs to treat viral infections, have accelerated resistance in bacteria [7]. With increased resistance, the treatments pose significant public health concern. Bacteria employ various mechanisms to overcome antibiotics including decreased drug permeability, expel antibiotics using efflux pumps, enzymatically alter or degrade antibiotics and change drug targets [8]. Gram-negative bacteria limit the entry of drugs by modifying porins in their outer membrane, which decreases permeability. Efflux pumps actively pump out antibiotics before they become effective. Multi-drug efflux pumps can pump out more than one antibiotic. All these mechanisms help bacteria survive and continue spreading despite antimicrobial treatment [9].

New antibiotics produced are often the modifications of existing drugs, hence vulnerable to resistance via mutations in the bacterium. As a result, enzyme-mediated resistance remains a significant health crisis particularly for critical pathogens [10]. Infections, therefore, are becoming more challenging to treat because of increased resistance and as a matter of fact, infections that were previously treatable are now considered untreatable. Under these conditions, medicinal plants can serve as possible antibiotic agents in an antibiotic-resistant world [11]. According to World Health Organization, 70% of the world's population now uses traditional medicine. Most modern drugs also owe their origin often to plant-based compounds. Medicinal plants have both antibacterial and anti-inflammatory effects [12]. They are promising substitutes in the quest against bacterial infections. Among the several medicinal plants, *Moringa oleifera* is an option with antimicrobial potential. *M. oleifera* exhibits notable antibiotic properties due to rich content of bioactive compounds including flavonoids, tannins and alkaloids [13]. Extracts from its seeds, leaves and roots have shown broad-spectrum activity against the Gram-positive and Gram-negative bacteria. Compounds in *M. oleifera* act by disrupting bacterial cell membranes, inhibit enzyme activity and interfere with microbial replication. The plant has also demonstrated anti-inflammatory and antioxidant properties that may support body's response to infections [14]. Current study aimed at extraction of crude extracts from leaves of *M. oleifera* and examined for presence of various compounds and subsequent antibacterial activity.

MATERIALS AND METHODS

Sample Collection and Bacterial Isolation

This experimental study was conducted at Riphah International University, Islamabad. The work was carried out in the Hematology and Biochemistry laboratories. Clinical bacterial samples were obtained from Alkhidmat Raazi Diagnostic Lab, Rawalpindi. Only wound-infected samples with Gram-positive and Gram-negative bacterial isolates were included. Isolates were sub-cultured and identified using Gram staining and standard biochemical tests such as oxidase, catalase, and coagulase assays.

Preparation of Plant Extracts and Biochemical Tests

Fresh leaves of *M. oleifera* were collected, washed and air-dried at room temperature. Dried leaves (~20g) were macerated in 100 mL methanol and incubated at 40°C on a magnetic stirrer for 24 hours. Extracts were filtered and stored at 4°C for further use. Qualitative phytochemical tests were performed to detect the presence of bioactive compounds using standard protocols. For flavonoids (Alkaline Reagent Test), a few drops of sodium hydroxide (NaOH) were added to the plant extract. Formation of a yellow coloration that becomes colorless upon acidification indicated the presence of flavonoids. For steroids (Salkowski Test), 1 mL of the extract was mixed with 10 mL chloroform, followed by the addition of 1 mL concentrated sulfuric acid. A red upper layer and a yellow sulfuric acid layer with green fluorescence indicated the presence of steroids. For quinones, few drops of sodium hydroxide were added to the extract. The appearance of any color indicated the presence of quinones. For saponins (Froth Test), the extract was shaken vigorously with water. Persistent formation of foam indicated the presence of saponins.

Antibacterial Assay

The antibacterial activity of plant extracts was evaluated using the agar well diffusion method. Bacterial suspensions were spread on nutrient agar plates and wells were loaded with plant extracts at amounts of 300, 600, 900, and 1200 µL. Plates were incubated at 37°C for 24 hours and zones of inhibition were measured in millimeters. Minimum Inhibitory Concentration (MIC) values were determined using the broth microdilution method. Plant extracts were serially diluted in nutrient broth and inoculated with bacterial strains. After incubation at 37°C for 24 hours, the lowest concentration showing no visible growth was recorded as the MIC. Zone of inhibition measurements were expressed as mean ± standard deviation. Data was analyzed using GraphPad Prism software.

RESULTS

Plant Collection and Methanolic Extract Preparation

M. oleifera collected from different places and their methanolic extracts were prepared for the purpose of analyzing their phytochemical constituents and determining their antibacterial activity on bacterial strains used for conducting this research. The leaves of *M. oleifera* were vibrant green in color with the extract showing a deep dark green color as shown in Figure 1.

Detection of Phytoconstituents

Phytochemical screening of *M. oleifera* revealed the presence of several bioactive compounds. The extract tested positive for flavonoids, steroids, and saponins, as evidenced by the respective colorimetric reactions. However, the test for quinones was negative. Positive results were indicated by specific color changes as per standard protocols. Negative results were indicated by the absence of such changes. Figure 2 presents the results of qualitative phytochemical tests performed on an extract of *M. oleifera*.

Culturing of Bacterial Strains

The bacterial strains collected were labelled as wound samples with sample number 139, 155 and 189. Among them, sample 139 was of *S. aureus* and sample 155 and 189 was *E. coli* as these samples were already identified when received. Sample 139 was sub-cultured to preserve the colonies of *S. aureus* on nutrient agar giving golden-yellow colonies. Sample 155 and 189 were sub-cultured on nutrient agar giving grayish-white colonies of *E. coli* as shown in Figure 3.

Figure 1: Leaves of *Moringa oleifera* with their methanolic extracts.

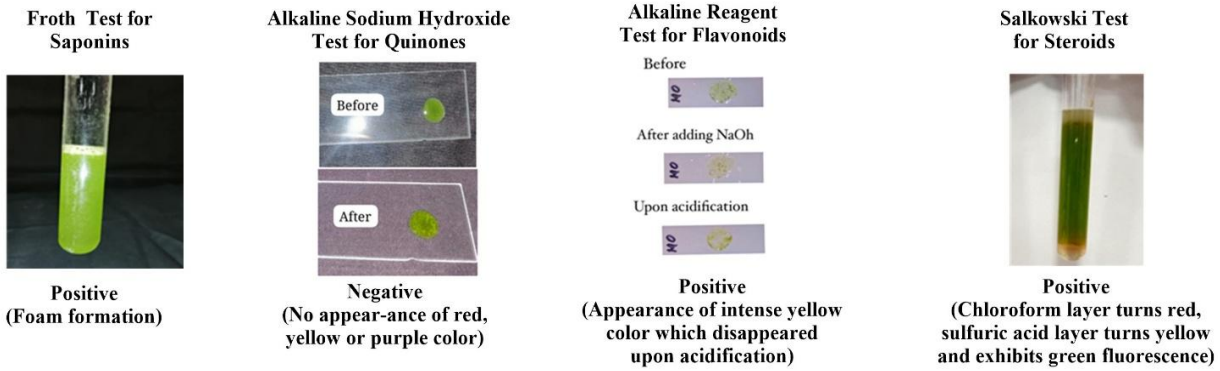

Figure 2: Phytochemical test results of *Moringa oleifera.*

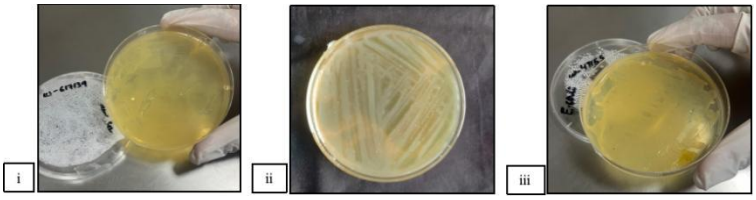

Figure 3: Isolation of bacteria from wound infected samples and sub-culturing on nutrient agar media i- Sample139 (*S. aureus*) ii- Sample 189 (*E. coli*) iii- Sample 155 (*E. coli*)

Identification of Bacterial Strains

The bacterial cultures received were sub-cultured on nutrient agar media. To confirm the observations of bacterial cultures already identified at Alkhidmat Raazi Diagnostics lab, we performed Gram staining in our laboratory. Staining was done on all samples, and the staining results were given. Sample 139 demonstrated the morphology of Gram-positive bacteria as the colonies were seen in spherical, or cocci in shape while sample 155 and 189 were found to have the characteristics of Gram-negative bacteria having rods. As an additional step to culture identification, an oxidase test, a biochemical test, was undertaken together with Gram staining. The results indicated that for oxidase activity, sample 139 was negative and likewise sample 155 and 189 were also negative. The catalase test was also performed as a part of our biochemical tests. This allowed us to get a more refined insight into the identification. Results indicated that in all the three samples, catalase activity was present. Lastly, coagulase test was performed. Results indicated that sample 139 was positive for Coagulase whereas sample 155 and 189 were negative for coagulase. Overall results of staining and biochemical tests are shown in Figure 4.

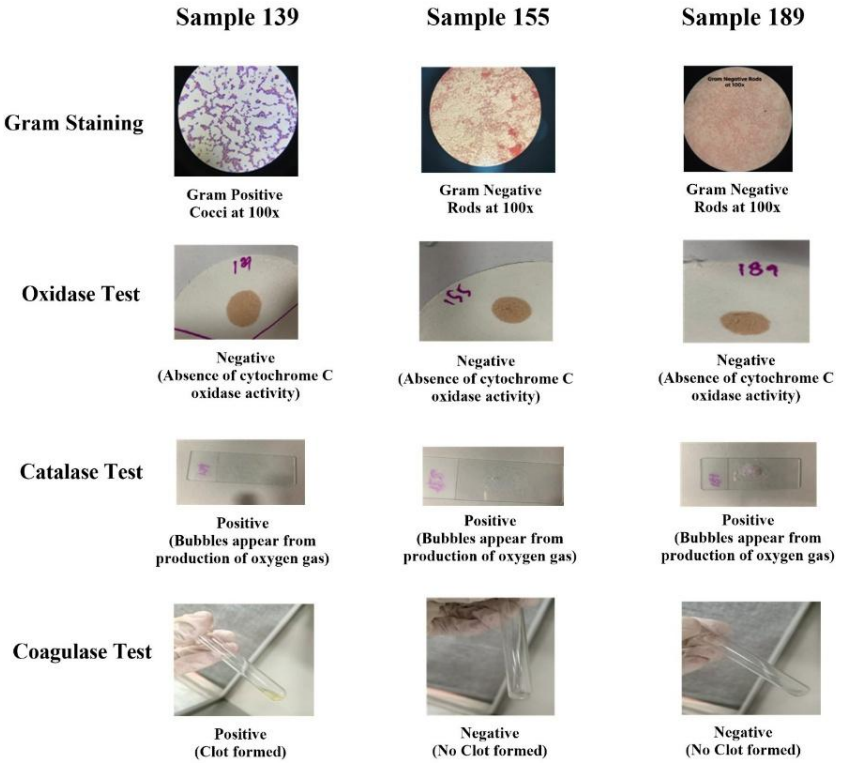

Figure 1: Gram staining and biochemical test results of Sample 139 (*S. aureus*), 155 (*E.coli*) and 189 (*E. coli*).

Antibacterial Activity of *Moringa oleifera*

The antibacterial activity of methanolic extract of *M. oleifera* against *S. aureus* and *E. coli* was tested by using agar well diffusion method. The concentrations of the plant extracts tested were 600, 900 and 1200 µL (~20g leaves in 100 mL) and the zones of inhibition produced were measured in millimeters (mm). Preliminary screening was conducted using 200 µL concentrations of plant extract in which no clear zone of inhibition was seen (results not shown). Subsequent experiments were conducted using higher concentrations of the extract in which little zone of inhibition was observed, prompting further investigation. At concentration of 1200 µL, significant zone of inhibition was seen Figure 5.

For sample 139 (*S. aureus*), the methanolic extracts of *M. oleifera* demonstrated varying degrees of antibacterial activity depending on the concentration applied. At 600 µL, it was a very small zone of inhibition. While at 900µL concentration, larger clear zone of inhibition was observed. At 1200µL concentration, *M. oleifera* extract showed clear zone of inhibition measuring (29x26mm), while control showed no zone of inhibition. Similar kinds of results were observed against the samples 155 and 189. More specifically, there were almost no zones of inhibition for 200 µL of the extracts, while for higher volumes, considerable zones of inhibition were noticed. For the highest concentration (1200 µL), a considerable zone of inhibition was observed in sample 155 (~35x32mm) and 189 (~30x32mm).

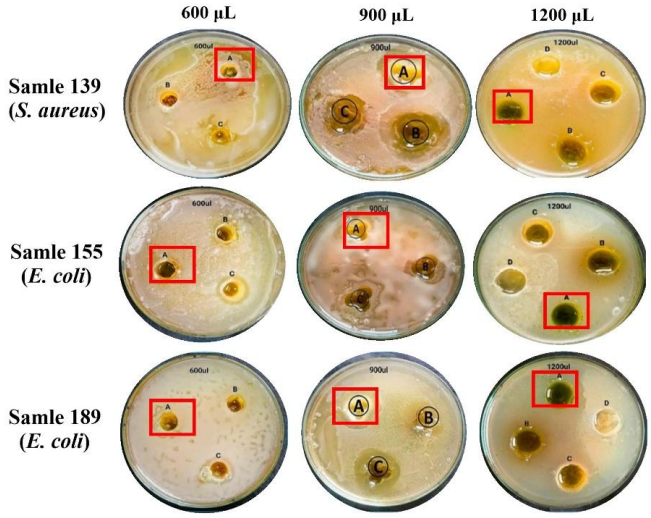

Figure 5: Antibacterial activity of medicinal plants against sample 139 (*S. aureus*), 155 (*E. coli*) and 189 (*E. coli*) after applying three different concentrations of *M. oleifera* methanolic extracts. *M. oleifera* extract showed clear zone of inhibition highlighted in red boxed in comparison to control where no zone of inhibition was shown.

Minimum Inhibitory Concentration

A microdilution assay was performed to determine the MIC of the plant extracts. Initially, agar plate method was used to evaluate the antibacterial activity of the plant extracts against three different bacterial isolates. Later, we used the broth dilution method to further investigate the antibacterial activity, although focused on a limited scale as on only two species of bacteria *S. aureus* (sample 139) and *E. coli* (sample 189) were considered. Four different concentrations (300, 600, 900, and 1200 µL) were tested for the plant. Positive and negative controls were included in experiment. The MIC values for the plant extract were found to be 300 µL, as shown in Figure 6, indicating effective inhibition of bacterial growth at this concentration.

Graphical representation was employed by GraphPad prism to illustrate antimicrobial effectiveness of the plant extracts against three bacterial species. The x-axis lists the concentration of the plant extract used, while y-axis demonstrated the zone of inhibition measured in millimeters (mm). Evaluation of the antimicrobial properties of extract against the three bacterial species (*S. aureus* sample 139, *E. coli* sample 155 and *E. coli* sample 189) and the resulting zone of inhibition values are displayed on the graph. This visual format allows for an easy comparison of the antimicrobial activity of the plant extract against the bacterial species, helping to pinpoint the most effective extract concentration and its associated zone of inhibition value (Figure 7).

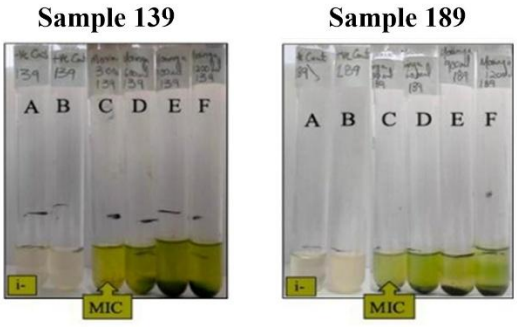

Figure 6: Analysis of MIC of *Moringa oleifera* against sample 139 (*S. aureus*) and 189 (*E. coli*) of wound infection: A: Negative control, B: Positive control, C: 300 µL concentration, D: 600 µL concentration, E: 900 µL concentration, F: 1200 µL concentration.

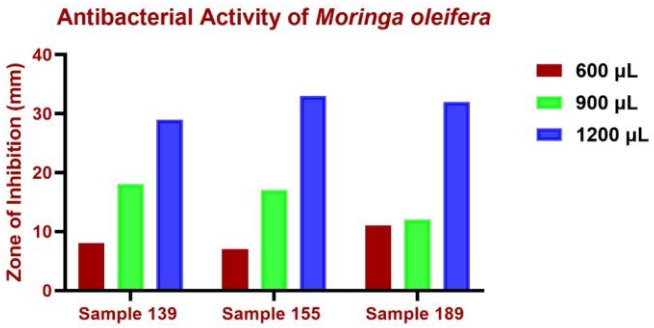

Figure 7: Graphical representation of antibacterial activity of *Moringa oleifera* on sample 139 (*S. aureus*), 155 (*E. coli*) and 189 (*E. coli*). Three different concentrations (600, 900 and 1200µL) were applied and subsequent zone of inhibitions were monitored.

DISCUSSION

The increasing problem of antibiotic resistance has led to the need to reconsider natural products as potential sources of antimicrobial agents. In this study, we focused on the antibacterial activity of *M. oleifera* on two bacterial isolates; Gram-positive *S. aureus* and Gram-negative *E. coli*, which are largely associated with wound infection. The results demonstrated the presence of bioactive compounds with antibacterial properties in the methanolic extracts of this plant, which were tested against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative). *M. oleifera* plant is another name for the drumstick tree or horseradish tree. It is a rapidly growing, drought-resistant tree characterized by feathery compound leaves which are hydrophobic in nature. The leaves alternate on the branches, formed by many small oval leaflets. They are very green in color with the appearance of being delicate and feathery [15].

Methanol extracts of many plants have shown improved antibacterial activity compared to the extracts prepared using other solvents like water or ethanol [16]. This is due to the better solubility of diverse phytochemical constituents in methanol. In previous studies, methanol extracts report greater zones of inhibition compared with a broad spectrum of bacterial pathogens, showing how effective it is for application purposes in antibacterial purposes [17]. Furthermore, in terms of phytochemical analysis, our results are in parallel with the previous findings as studied in the literature review, indicating presence of phytochemicals like saponins, quinones, flavonoids and steroids [18]. The phytochemical analysis in our research study revealed the presence of flavonoids, steroids and saponins, while quinones were absent in *M. oleifera*. Flavonoids, known for their antimicrobial activity, are believed to disrupt bacterial membranes and inhibit nucleic acid synthesis, as corroborated by a study conducted by Cushnie and Lamb (2005), which identified flavonoids as potent antibacterial agents [19]. The presence of saponins, which can increase cell membrane permeability and induce bacterial lysis, further supports the antibacterial activity observed in this study. These findings are consistent with research by Zaynab et al. (2021), who reported that saponins in plant extracts exhibit strong activity against Gram-positive bacteria [20].

The results of the agar well diffusion assay highlighted the dose-dependent antibacterial activity of the plant extracts. *M. oleifera* demonstrated significant antibacterial activity. The methanolic extract of *M. oleifera* produced inhibition zones of 29×26mm for *S. aureus* and 35×32mm for *E. coli* at 1200 µL concentration. These findings are consistent with research by Shamim et al. (2019), who highlighted the antibacterial potential of *M. oleifera* extracts due to the presence of isothiocyanates and polyphenols [21].

The broth dilution method confirmed the MIC of the extract at 300 µL, indicating that relatively low concentrations of the extracts are sufficient to inhibit bacterial growth. This finding is particularly noteworthy, as it highlights the potential of the plant extracts as cost-effective and sustainable alternatives to synthetic antibiotics. Similar MIC values for plant extracts have been reported in studies by AlSheikh et al. (2020), emphasizing potency of natural products in bacterial inhibition [22].

The differential response of Gram-positive and Gram-negative bacteria to the plant extracts is noteworthy. *S. aureus*, a Gram-positive bacterium, was generally more susceptible to the extracts than *E. coli*, a Gram-negative bacterium. This observation is consistent with the findings of Breihyeh et al. (2020), who reported that the outer membrane of Gram-negative bacteria acts as a barrier to many antibacterial agents, thereby reducing their susceptibility [23].

The lack of activity at lower concentrations (200 µL) in the agar well diffusion assay raises important considerations regarding the optimization of extract dosage for therapeutic applications. The dose-dependent nature of antibacterial activity observed in this study aligns with findings of Hari et al. (2024), who reported similar trends in the antibacterial activity of plant-derived compounds [24].

Comparing the efficacy of the plant with conventional antibiotics, the zones of inhibition produced by the plant extracts at higher concentrations are comparable to those reported for standard antibiotics like amoxicillin and ciprofloxacin. For instance, research by Zafar et al. (2022) showed that *M. oleifera* extracts could produce inhibition zones of similar magnitude to those of ciprofloxacin against *E. coli* [25]. This comparative efficacy highlights potential of the plant as adjuncts or alternatives to conventional antibiotics, particularly in the context of rising antibiotic resistance.

The phytochemical composition of the plant studied also provides insights into the potential mechanisms of action. The presence of steroids in all the plants suggests an additional pathway for antibacterial

activity, as steroids are known to interact with bacterial membranes and disrupt their integrity [26]. The absence of quinones in *M. oleifera*, however, raises questions about specific compounds responsible for the antibacterial activity, warranting further investigation.

The implications of these findings are particularly significant in the context of wound infections, which are often polymicrobial and involve both Gram-positive and Gram-negative bacteria. The broad-spectrum activity of the plant extracts studied suggests potential utility in managing wound infections, especially in resource-limited settings where access to conventional antibiotics may be restricted. Our study provides strong evidence for the antibacterial potential of *M. oleifera*. The plant could serve as valuable sources of natural antimicrobial agents, particularly in context of increasing antibiotic resistance. Further research is necessary to elucidate the precise mechanisms of action of the plant and to develop effective formulations for therapeutic applications. This study has several limitations, making areas for improvement in future research. A major limitation of this research is that it was conducted only on two strains of bacteria. Hence the scope and generalization can be improved by testing several other types of bacteria. However, by harnessing the power of nature, we may be able to develop novel and sustainable solutions to combat bacterial infections.

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Competing Interests: Authors declare no competing interests.
Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.