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Antibacterial activity of Azadirachta indica, Moringa oleifera and Punica granatum extracts against Methicillin-Resistant Staphylococcus aureus, "the super bug"

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ABSTRACT

Prevention and treatment of infections have become a major challenge for healthcare. Many regulatory organisations are continuously monitoring the evolution of bacteria on a day-to-day basis. The pathogens are gaining more armour every day and becoming resistant to multiple drugs. Methicillin-resistant *Staphylococcus aureus* is creating major challenges in the field of healthcare. Methicillin-resistant Staphylococcus aureus (MRSA) poses a significant challenge to global healthcare systems. Its ability to resist multiple β-lactam antibiotics limits treatment options and contributes to prolonged hospital stays, increased morbidity and mortality, and higher healthcare costs. MRSA infections can affect skin, soft tissues, lungs, bloodstream, and surgical sites, and they spread rapidly in both healthcare settings and the community, making early detection, stringent infection-control measures, and the development of novel antimicrobials essential. To challenge Antimicrobial Resistance (AMR), the screening of plants that are rich sources of bioactive compounds is necessary. Along with the advent of new antimicrobial agents, maintenance of hygienic practices is also necessary.

Keywords: *MRSA, Antimicrobial agents, Plant extract, Azadirachta indica, Moringa oleifera, Punica granatum*

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1. INTRODUCTION

Plants are rich sources of bioactive molecules, which are mainly used to produce medicines for various diseases. The majority of the drugs available today are obtained from natural sources or are semi-synthetic derivatives of natural products used in the traditional systems of medicine [1]. Hence, the screening of traditional natural products is significant. A large number of antibiotics available in the market are obtained from natural or semi-synthetic resources obtained from screening of plants [2]. In the current scenario, medicinal plants are widely used in pharmaceuticals, cosmetics, and nutraceuticals. Medicinal plants are generally used in the pharmaceutical field for a wide range of substances that can be used for the treatment of several diseases [3]. The extensive and inappropriate use of antibiotics has led to the development of antibiotic resistance in microorganisms, which challenges the world in many fields now [4-6]. Therefore, there is a significant importance of plants in the development of new antimicrobials and in coping with resistance [7-9].

The World Health Organisation (WHO) also has stated that over 80% of the developing world still profits from oriental medicines obtained from medicinal plants [10-12]. Out of the total estimated number of plants, which amounts to 374,000, 28,187 plants are used by humans [7,8]. WHO has over 20,000 species of medicinal plants recorded and has identified the medicinal plants as one of the potential sources of new drugs [13,14].

Over 1340 plant species have been identified for antimicrobial activity and more than 30,000 antimicrobial candidates have been isolated from them [15]. Additionally, an estimated 14-28% of higher plant species are considered medicinal, with 74% of bioactive compounds from plants being discovered through the knowledge of traditional medicine [16].

Plants are constantly exposed to changing and potentially harmful environmental conditions. Since they cannot move, they have developed complex defence mechanisms, primarily through producing a wide range of chemical compounds to cope with stress [17,18].

Their ability to perform combinatorial chemistry by modifying and evolving genes involved in secondary metabolite production. Results in a vast array of chemical substances. Humans have harnessed this chemical diversity for both traditional and modern medicine, utilizing the very strategies plants evolved for their own survival [19].

The therapeutic potential of plants stems from specific chemical constituents known as phytochemicals, which exert distinct physiological effects on the human body [20]. They are non-nutritional and possess protective and disease-preventive properties. Thanks to the advancements in phytochemical analysis, numerous active ingredients from medicinal plants have been identified and incorporated into modern medicine [21].

Among the most significant bioactive compounds are alkaloids, flavonoids, tannins, and phenolic compounds, which serve as an essential raw material in drug development. Additionally, many plants naturally produce antimicrobial agents that help defend against harmful microorganisms [22,23].

In this study, we have used Neem (*Azadirachta indica*), Moringa leaves (*Moringa oleifera*) and Pomegranate Peel (*Punica granatum*) to study their antimicrobial activity against Methicillin-Resistant Staphylococcus aureus (MRSA) superbug. Neem is revered as ‘nature's pharmacy’ due to its wide-ranging medicinal benefits, like antibacterial and antifungal properties. Moringa leaves are often referred to as the miracle tree due to their high levels of vitamins and minerals, and they are often known for their anti-inflammatory and antioxidant property. Pomegranate Peel is a medicinal gold mine. It is known for its high antioxidant content and its benefits in skin health and cardiac health, and metabolism.

2. METHODS

Bacterial strains

Staphylococcus aureus (ATCC 700699) and Methicillin-resistant *Staphylococcus aureus* (MRSA) from SDM Medical College and hospitals, Sattur, Dharwad, Karnataka [29] are the bacterial strains used in this study. These strains were grown routinely overnight in Brain Heart Infusion Broth at 37°C. Bacterial isolates were preserved in Glycerol stocks and stored at -80 °C for future use.

Sample collection and preparation

A total of 14 samples were collected from the local markets of Sidhartha Nagar, Mysore, from January 2020 to March 2020. The food samples considered for the study are Milk Peda, Mysore Pak, Black Forest pastry, Milk, Egg, Chicken, Fish, Mutton, and Vegetables like Cabbage, Radish, Spinach, Capsicum, Potato, and Coriander. The samples were carried to the laboratory in sterile conditions and preserved at the required temperature. All the samples were analysed within one to four hours of collection. Samples were aseptically chopped and smashed into smaller pieces using a sterile stainless-steel knife before weighing.

Table 1: List of food samples collected for the study

SAMPLE NO:	NAME OF SAMPLE	PLACE OF COLLECTION
1	Milk Peda	Siddhartha Nagar, Mysore
2	Mysore Pak	Siddhartha Nagar, Mysore
3	Black Forest Pastry	Siddhartha Nagar, Mysore
4	Chicken	Siddhartha Nagar, Mysore
5	Mutton	Siddhartha Nagar, Mysore
6	Fish	Siddhartha Nagar, Mysore
7	Milk	Siddhartha Nagar, Mysore
8	Egg	Siddhartha Nagar, Mysore
9	Cabbage	Siddhartha Nagar, Mysore
10	Radish	Siddhartha Nagar, Mysore

11	Spinach	Siddhartha Nagar, Mysore
12	Capsicum	Siddhartha Nagar, Mysore
13	Potato	Siddhartha Nagar, Mysore
14	Coriander	Siddhartha Nagar, Mysore

Isolation of MRSA from food samples

10 g of solid samples and 10 ml of liquid samples were inoculated into 90 ml Brain Heart Infusion broth (BHI) and incubated at 37 ºC in a shaking incubator for bacterial enrichment. The overnight cultures were serially diluted, and 100 µL of the sample was inoculated onto Baird Parker Agar (BPA) supplemented with egg yolk emulsion, incubated for 24 - 48 hours at 37 ºC. Jet black colonies with opaque zones on BPA were selected and streaked on Mannitol Salt Agar (MSA) and were incubated at 37 ºC for a period of 24 hours. Positive colonies on MSA were further characterized by Gram Staining. The positive colonies were grown in MeReSa and CCLM media formulated and reported earlier, for confirmation. A standard culture of MRSA and *S. aureus* was also compared with the isolates.

Bacterial identification using BD PHOENIX M50

The positive cultures were then identified and screened for antibiotic resistance with the BD PHOENIX M50 system. Identification is done by screening of the samples using various conventional, chromogenic, and fluorogenic biochemicals embedded in the ID panel. Antibiotic sensitivity is determined by using the minimum inhibitory concentration (MIC) of different antimicrobial agents embedded in the AST panel to classify them as Susceptible, Intermediate, or Resistant (SIR)

Molecular identification by triplex PCR

The genomic DNA of isolates was isolated with a genomic DNA isolation kit. PCR was done for molecular-level confirmation. A triplex PCR with *nuc*, *mec*, and *fem* primers was optimised. The reactions were optimized with 1X PCR buffer, 2.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTP mixture, and 1.2 units of *Taq* polymerase. The primer concentrations were optimized to 0.5 µ mol l^{-1} of *nuc* F and *nuc* R and 0.6 µ mol $l^{-1}$of *mec* A F and *mec* A R and 0.6 µ mol $l^{-1}$of *Fem* F and R.

PCR cycle was optimized for an Initial denaturation at 94 ºC for 4 minutes, then thirty cycles of denaturation at 94 ºC for 1 minute, annealing for 1 minute at 56 ºC, followed by extension at 72ºC for 1 minute and a final extension at 72 ºC for 8 minutes.

Table 2: List of Primers used in the study

Genes Targeted	Primer Sequence 5'-3'	Amplicon size (bp)	Amplicon size (bp)
mec A	F-GGATTGCTTCACTGTTTTG R-GAGTAGCACTCGAATTAGGC	800 bps	JF778650.1
Fem	F-GCTTGCTTACTTACTGCTGTAC R-TGACGTATCTTCCATAAATGAT	474 bps	DQ352467.1
Nuc	F-GCTGGCATATGTATGGCAATT R-GCTTCAGGACCATATTTCTCTAC	385 bps	DQ507382.1

Antimicrobial activity testing

Aqueous and Ethanolic extract of *Azadirachta indica*, *Moringa oleifera* leaves, and *Punica granatum* peels are used in this study for testing their antimicrobial activity against MRSA.

Aqueous and ethanolic leaf extraction

Collected leaves were cleaned and allowed to dry completely. They were then ground into fine powder. The pomegranate peels were dried in a hot air oven for 48 hours. The dried peels were then ground to a fine powder. 10 g of each powder was dissolved in separate tubes with 40 mL of distilled water and 70% ethanol to prepare the aqueous and ethanolic extract,

respectively. The tubes were continuously agitated at room temperature for about seven days in the case of moringa leaves, two days for neem leaves, and 30 minutes in the dark for pomegranate peel.

The mixture is filtered with Whatman filter paper and concentrated in a rotary vacuum evaporator. The crude leaf extract is then stored at 4 °C.

Screening for antimicrobial activity by agar well diffusion method.

BHI tubes were inoculated with MRSA and incubated overnight at 37 ºC. 100µL from this culture is spread onto BHI plates. Wells were made with a cork borer, and 100 µL of each crude extract was added to the well. This was done with all three extracts. The plates are incubated for 24 hours at 37 ºC. The zone of inhibition can be observed if the respective crude extracts have antimicrobial activity against MRSA.

3. RESULTS

Isolation of MRSA from various food samples

Of the 14 samples studied, 12 were found to harbour *S. aureus,* of which four were MRSA. All the samples had luxuriant growth of *S. aureus* in BPA plates. MeReSa and CCLM media confirmed the four MRSA isolates.

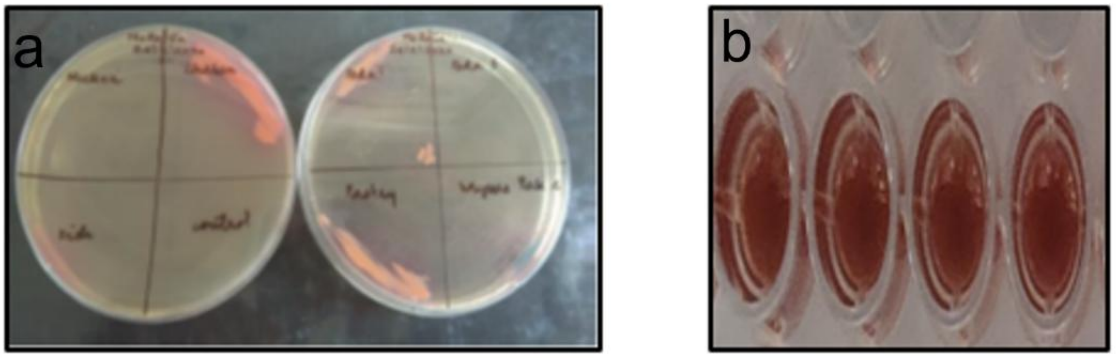

Figure 1: a) The MRSA colonies in MeReSa agar plates, b) MRSA isolates in CCLM broth after overnight incubation.

Table 3: Cultural characteristics of the isolates on BPA and MSA media

Sample No	Sample Name	Growth on BPA media	Growth on MSA media
1	Milk Peda	Jet black colonies with opaque zone	Yellow colonies
2	Mysore Pak	Jet black colonies with opaque zone	Yellow colonies
3	Black Forest Pastry	Jet black colonies with opaque zone	Yellow colonies
4	Milk	No Jet-black colonies	No yellow colonies
5	Egg	No Jet-black colonies	No yellow colonies
6	Chicken	Jet black colonies with opaque zone	Yellow colonies
7	Fish	Jet black colonies with opaque zone	Yellow colonies
8	Mutton	Jet black colonies with opaque zone	Yellow colonies
9	Cabbage	Jet black colonies with opaque zone	Yellow colonies
10	Radish	Jet black colonies with opaque zone	Yellow colonies
11	Spinach	Jet black colonies with opaque zone	Yellow colonies

12	Capsicum	Jet black colonies with opaque zone	Yellow colonies
13	Potato	Jet black colonies with opaque zone	Yellow colonies
14	Coriander	Jet black colonies with opaque zone	Yellow colonies

Molecular Identification of MRSA

Four isolates were confirmed to be MRSA by the presence of *nuc*, *Mec* A and *fem* gene.

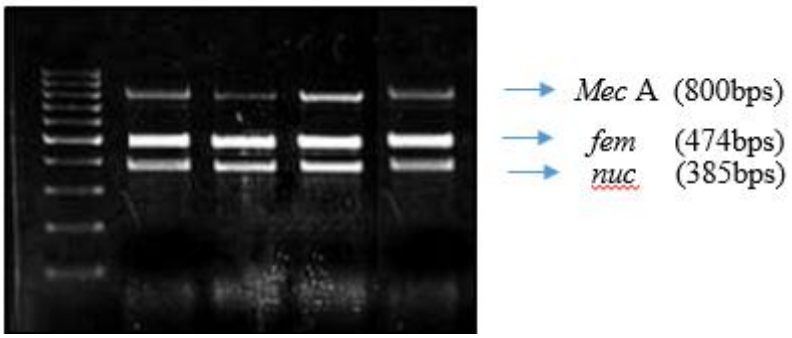

Figure 2: Agarose gel electrophoresis of PCR amplified products.

Automated identification and antibiotic susceptibility study

All the isolates were identified as *S. aureus*, and four isolates were found to be MRSA based on the AST studies done with BD PHONIEX M50.

Table 4: Results of Identification of the isolates obtained from the BD Phoenix M50 Sample

No.	Sample Name	Name of the Organism	Confidence Value
1	Milk Peda	Methicillin-Resistant *S. aureus* (MRSA Isolate 1)	91%
2	Mysore Pak	Methicillin-Resistant *S. aureus* (MRSA Isolate 2)	99%
3	Black Forest Pastry	Methicillin-Resistant *S. aureus* (MRSA Isolate 3)	99%
4	Chicken	Methicillin-Resistant *S. aureus* (MRSA Isolate 4)	99%

The four MRSA isolates were found to be resistant to Penicillin G, Oxacillin, Chloramphenicol, Ciprofloxacin, Trimethoprim, Amoxicillin Clavulanate, Ampicillin, and Cefazolin.

Antimicrobial Screening of Plant Extracts

Crude aqueous and ethanolic extracts of *Azadirachta indica*, *Moringa oleifera* leaves, and *Punica granatum* were tested against MRSA strains isolated from Milk Peda. Preliminary screening revealed that all three plant extracts exhibited antibacterial effects against MRSA, though the intensity varied. The ethanolic extracts demonstrated significantly stronger antibacterial activity with *Moringa oleifera* and *Punica granatum.* Both of them showed a zone of inhibition of 20mm, while the aqueous and ethanolic extract of *Azadirachta indica* developed only a 16mm zone of inhibition. The aqueous

extract of *Moringa oleifera* and *Punica granatum* developed zones of inhibition of 18 and 16mm, respectively.

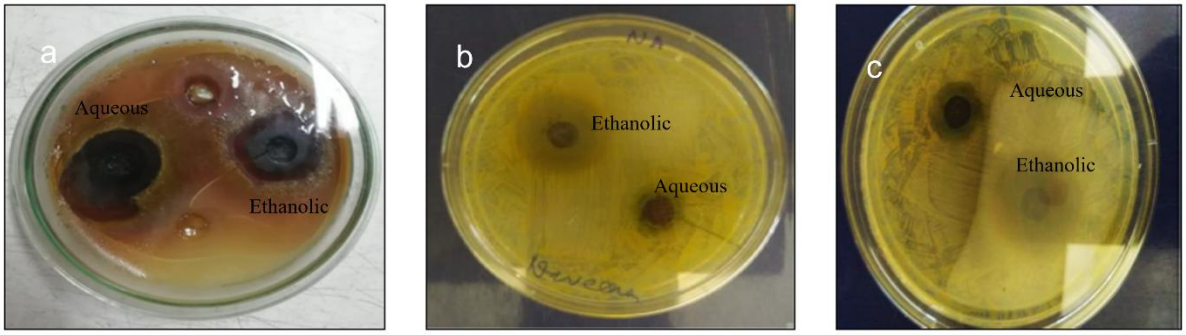

Figure 3: a) Antibacterial activity of *Punica granatum* against MRSA isolated from Milk Peda, b) Antibacterial activity of *Azadirachta indica* against MRSA isolated from Milk Peda, c) Antibacterial activity of *Moringa oleifera* against MRSA isolated from Milk Peda.

4. DISCUSSION

The current study focused on studying the protective efficacy of candidate plant extracts in the inhibition of MRSA. MRSA, due to its antibiotic resistance, has emerged as a superbug. The non-judicious use of antibiotics is the major reason for the antibiotic resistance gain by bacteria. Hence, there is a need to explore new compounds that can inhibit these bacteria. In the current scenario, they are transmitted through many modes. The most dangerous among the modes is the food [25]. The study made an effort to identify the common sources of dispersion of MRSA. Of 14 food samples, 12 harboured *S. aureus,* which is a food-borne pathogen that can cause severe infections once ingested. Out of 12 *S. aureus,* 4 were identified to be MRSA. The majority of the samples positive for MRSA were ready-to-eat food items.

Three plants were selected for screening their antimicrobial capacity, which is known for their medicinal properties. Many Researchers have reported the use of these plants in antimicrobial studies. This study is a humble effort to compare the three plant extracts against MRSA. The study also compared the efficacy of the solvent system used for the extraction of the crude bioactive compounds. The ethanolic extracts of *Moringa oleifera* and *Punica granatum* have shown the highest inhibition zones.

Numerous researchers have explored the potential of plant extracts and their active compounds as antimicrobial agents to inhibit the growth of foodborne pathogens and spoilage bacteria. Some studies propose that bioactive components such as terpenoids, alkaloids, and phenolic compounds disrupt microbial cell membranes by interacting with their enzymes and proteins. This disruption leads to a proton imbalance across the membrane, ultimately causing cell death or interfering with essential enzyme functions involved in amino acid synthesis (26, 27).

Other researchers emphasize the role of the extracts' hydrophobic nature, which allows them to bind with membrane and mitochondrial proteins, altering their structure and permeability (28). Pomegranate peel and Moringa oleifera (drumstick) extracts show notable antibacterial activity against Methicillin-resistant Staphylococcus aureus (MRSA). Rich in polyphenols, flavonoids, tannins, and other bioactive compounds, they damage bacterial cell walls, disrupt biofilm formation, and inhibit growth. As natural products, they help limit resistance development and can be combined with conventional antibiotics for enhanced efficacy. These findings suggest their potential as safe, affordable adjuncts or leads for new anti-MRSA therapies. Based on the current findings, the study suggests that these effective plant extracts could serve as natural preservatives, helping to prevent food-borne illnesses, and extend shelf life without the health risks associated with synthetic chemical preservatives [30].

5. CONCLUSION

Food spoilage and food-borne illness are a great threat to the public. The presence of antibiotic-resistant bacteria in food is a major issue that cannot be overlooked. In this situation, the evolution of bacteria can be dealt with only by the introduction of new antimicrobials. Since plants are continuously subjected to harsh environments and new challenges, they are equipped with several combat molecules. This can be identified by screening the plants, which are nature's treasure chest. The plant candidates suggested by the study are promising and can be further studied to identify the fractions responsible for the inhibition of growth.

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Conflict Of Interest

The authors declare that there is no conflict of interest.

Authors' Contribution

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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None.

Data Availability

All datasets generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

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