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Azadirachta indica as an Alternative Treatment Source for Methicillin-Resistant *Staphylococcus Aureus* Infections

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ABSTRACT

The emergence and spread of antibacterial strains of pathogenic bacteria pose a significant global health threat. This study assessed the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) and its susceptibility to *Azadirachta indica* (neem) leaf extract. A total of 300 *S. aureus* isolated from wounds and burns were collected from two hospitals (Yusuf Dan Tsoho general hospital and Barau Dikko teaching hospital) within Kaduna metropolis and were screened for methicillin resistance. Occurrence rates of MRSA were found to be 28% and 18 % for wound and burn isolates, respectively. Evaluation of antibacterial activity of ethanolic and aqueous extracts of *Azadirachta indica* leaf against MRSA isolates using the agar well diffusion method shows positive antibacterial activity, with mean zones of inhibition ranging from 9.97 ± 1.54 mm to 21.94 ± 2.63 mm for wound isolates and 8.53 ± 3.34 mm to 19.31 ± 3.85 mm for burn isolates with The MIC and MBC of the ethanolic extract ranging from 250 mg/ml to 500 mg/ml, indicating bactericidal activity. However, no significant antibacterial activity was recorded against the aqueous extract. The results obtained from this study suggest leaf extracts of *Azadirachta indica* could serve as a potential source of antibacterial agents for treating methicillin-resistant Staphylococcus aureus (MRSA) infections**.**

Key words: Antibacterial activity; methicillin-resistant *Staphylococcus aureus* (MRSA); *Azadirachta indica; In vitro.*

INTRODUCTION

The constant emergence and spread of antimicrobial-resistant strains of pathogenic microorganisms creates a continuing global health concern. World Health Organisation (WHO) Global Antimicrobial Surveillance System (GLASS) report revealed [alarming resistance rates among common bacterial pathogens. The resistance rates of great global concern were 42% for third-generation cephalosporin-resistant *E. coli* and 35% for methicillin-resistant *S. aureus* as median reported rates in 76 countries (Report, 2022). The rapid emergence and global spread of antibiotic resistance threaten our capacity to treat common infectious diseases, leading to severe and prolonged morbidity and increased mortality. This is frightening because effective antimicrobials are not only important for treatment and prevention of infectious diseases, but are particularly essential in reducing the risks of many medical procedures such as organ transplants, cancer therapy, and major surgery like caesarean section; through infection control (Factsheet, 2018). Generally, antimicrobial resistance occurs naturally through mutations. However, this process is enhanced by the abuse and misuse of antimicrobials in humans and animals for growth promotion and disease protection (Levy and Marshall, 2005).

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β -lactamase enzymes produced by Gram-negative and Gram-positive bacteria, which hydrolyse β -lactam antibiotics contribute substantially to the antimicrobial resistance threat (Fisher and Knowles, 1978). Specifically, the production of such enzymes by *Staphylococcus aureus* confers multidrug resistance and thus the organism is generally termed methicillin-resistant *S. aureus* (MRSA). This strain majorly constitutes *S. aureus*-associated public health problem as it is responsible for significant morbidity and mortality (Zetola, Francis, Nuermberger, and Bishai, 2005).

Fortunately, as is evident in the rarity of infection in medicinal plants, it is believed that such plants possess antimicrobial properties. Consistently, advances in biotechnology have led to the identification of many compounds from different plants with therapeutic potentials against various infectious agents (Kolli, Laouer, Kolli, Akkal, and Sahli, 2016; Onivogui, Letsididi, Diaby, Wang, and Song, 2016; Sagbo, Afolayan, and Bradley, 2017; Teanpaisan, Kawsud, and Pahumunto, 2017). Considering the rapidity with which antibiotic resistance evolves in pathogenic bacteria and the persistence of its determining factors, it is expected that the bacteria will in future develop resistance against the active antibiotics used for the treatment of their infections. It is therefore against this background that this research was designed to determine the antibacterial activity of *Azadirachta indica* against MRSA. It is expected that any plant extract that shows antibacterial activity; by either inhibiting the growth or killing the target bacterial strains, can serve as a potential candidate for the alternative treatment of MRSA infections.

MATERIALS AND METHODS

Test Organisms Collection and Re-Identification

Using aseptic techniques, a total of 300 *S. aureus* isolates of wound and burn origins were collected from Barau Dikko Teaching Hospital Kaduna and Yusuf Dantsoho Memorial Hospital Kaduna on mannitol salt agar. In addition to the ability of the isolates to grow on Mannitol salt agar, the bacterial identity was further reconfirmed by Gram's staining, coagulase, and catalase tests.

Screening for Methicillin Resistance

The *S. aureus* isolates were screened for methicillin resistance using Kirby-Bauer's' disc diffusion method based on Clinical and Laboratory Standards Institute (CLSI) antimicrobial susceptibility testing standards (CLSI, 2020). The concentration of the bacterial suspension was calibrated to 0.5 McFarland units and inoculated on Mueller-Hinton agar by spread plate method. The Cefoxitin disk (30 µg) (Oxoid, Basingstoke, UK) was appropriately placed at the center of the plate and then incubated at 33-35 ^{o}C. Zones of inhibition around the cefoxitin disc were observed for 16-18 hours. A zone of inhibition around the cefoxitin disc ≤ 21 mm in diameter was recorded as positive for MRSA.

Collection and Identification of Plant Materials

The *Azadirachta indica* was identified within Kaduna metropolis and their leaves were plucked accordingly. The leaf's identity was further authenticated at the herbarium of the Biological Science Department, Kaduna State University, Kaduna, Nigeria and assigned voucher number 2241. The leaves were then dried and ground into fine powdery form.

Phytochemical Extraction

The plant leaves phytochemicals were extracted in ethanol and aqueous solvents, using the maceration method as described in (Padhi and Panda, 2015). 100 g of the powdered leaves was dissolved in 400 ml of solvent and left at 28 ^{O}C for 2 days after which it was filtered through Whiteman No 2. Filter paper. The extraction was repeated three times and the solvent was evaporated

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and removed under a vacuum using rotary evaporator at about 45 °C to generate dry crude extract.

Phytochemical Screening

Phytochemical components of the leaf extracts such as saponins, tannins, alkaloids, phlorotannins, and glycosides were determined according to the methods described by (Egbe, Garba, Adamu, and Aliyu, 2022; Hassan, Musa, Adamu, and Gabi, 2022).

Antibacterial Activity Assay of the Extracts Against Methicillin-Resistant *S. aureus* Isolates

The antibacterial activity of the plant extracts was carried out using the agar well diffusion (agar cup) method as described by (Padhi and Panda, 2015). Overnight Mueller Hinton broth culture of the isolates were standardised with 0.5 % McFarland standard solution and then evenly seeded on Mueller Hinton agar. Using sterile borer wells of about 6 mm diameter and 2.5 mm depth were dug on the agar plates. Each well was filled with 40 µl of varying concentrations, 500 mg/ml, 250 mg/ml, 125 mg/ml, and 62.5 mg/ml of extract dissolved in DMSO in separate wells. Respective solvents without extract were used as standard control and a standard antibiotic, 30 µg/ml linezolid as a reference control. The plates were incubated for 24 hours, after which the zones of inhibition were measured.

Determination of Minimum Inhibitory Concentration (MIC) and Bactericidal Concentration (MBC)

The MIC was determined using broth microdilution technique in 96 well microtiter plate and 2,3,5-triphenyl tetrazolium chloride (TTC) as an indicator. crude extracts was added to the second column (A2-H2) wells of the plate and serially diluted across the rows with 100 µl of the 0.5 McFarland adjusted activated culture in MH broth as described in (Panda, 2014). The plates were sealed with foil paper and incubated for 24 hours at 37 °C in an incubator shaker (130 rpm) and observed for growth of the bacteria. A visible colour change to pink indicates growth of bacteria. The MIC value of the extract were taken as the lowest concentration that showed no growth for individual test bacteria.

MBC was determined as described in (Padhi and Panda, 2015). Samples of 10 mL of the broth from wells of the 96-well microtiter plate exhibiting MIC and from control, wells were taken aseptically and inoculated on MH agar plates as a spot inoculum under the laminar flow hood. The plates were then sealed and incubated at 37 °C for 24 hours and observed for growth. Absence of bacterial growth indicated the MBC for the respective bacteria. However, if growth is observed at MIC, a higher concertation is examined in similar manner until a concertation reaches a value that records no growth.

RESULTS AND DISCUSSION

Susceptibility of *S. aureus* Isolates to Methicillin

The identity of all the 300 wound and burn isolates of *S. aureus* collected were confirmed based on their ability to grow in the presence of salt, in Mannitol salt agar and their appearance in the same medium. Biochemically, *S. aureus* is distinguished from other Gram-positive cocci by the production of two enzymes, coagulase, and catalase, and thus is identified by same. Table 1 summarises the cultural, morphological, and biochemical characteristics of *S. aureus* used in this study.

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Table1: Cultural, morphological, and biochemical characteristics of *Staphylococcus aureus* isolates

Source	Cultural Appearance	Morphology		Biochemical Characteristics			Organism	Frequency
		Cell shape	Arrangement	Gram's reaction	Catalase	Coagulase		
Wound	Golden yellow and round	Cocci	Grape-like in clusters	+	+	+	*S. aureus*	150
Burn	Golden yellow and round	Cocci	Grape-like in clusters	+	+	+	*S. aureus*	150

The results of susceptibility testing of the *S. aureus* isolate to methicillin (Table 2) indicate that a significant proportion of the isolates were resistant to methicillin (MRSA). Specifically, 28% of the isolates from wounds and 18% of the isolates from burns were MRSA. The proportion is alarming considering the healthcare implications of this resistant organism, which include necessitation to use stronger and controlled drugs, increased treatment costs, prolonged morbidity, and increased mortality. However, the prevalence determined by this study is not extreme, as compared to those reported by various studies for different regions of the world. A study conducted among 26,310 *S. aureus* isolated from 15 tertiary health care in India within two years period between January 2008 and December 2009, to determine the susceptibility pattern of the isolates reported a prevalence of 41 % for MRSA among *S. aureus* (Joshi et al., 2013). In 2010, the national prevalence rate of MRSA in the USA was reported to be 66.4 per 1,000 (Jarvis, Jarvis, and Chinn, 2012). This value is equivalent to 6.64 %, which is significantly lower than the values reported in this study.

An epidemiological review study of the occurrence of MRSA reported nasal colonization ranges from 2%-16% in the Gulf Cooperation Council, 1-9% in the Levant, and 0.2%-9% in North African Arab states (Tabaja, Hindy, and Kanj, 2021). Another study that determined the antibiotic susceptibility pattern of *S. aureus* in eight large hospitals in Africa found that the prevalent rate of MRSA was relatively high in Nigeria, Kenya, and Cameroon (21-30%), and below 10% in Tunisia, Malta, and Algeria (Kesah et al., 2003). Comparing this to the findings of the current study indicates no significant increase/decrease in the MRSA prevalence in Nigeria.

Table 2: Susceptibility *S. aureus* to Methicillin and Prevalence of MRSA in Wound and Burn

Clinical isolates	Number of isolates	Sensitive (MSSA)	Resistant (MRSA)	Occurrence of MRSA
Wound	150	108	42	28 %
Burn	150	123	27	18 %
Total	300	231	69	46 %

Key: MSSA= Methicillin sensitive *Staphylococcus aureus*, MRSA= methicillin resistant *Staphylococcus aureus,* Resistant ≤ 21mm, above 21mm is Sensitive (CLSI, 2020).

Antibacterial Activity of *Azadiracta indica* Leaf Extract

The phytochemicals that tested positive in the ethanolic extract of *Azadirachta indica* were alkaloids, saponins, terpenoids, flavonoids, and tannins. In aqueous extract, the same phytochemicals were detected except flavonoids. Table 3 demonstrates the antibacterial activities of ethanolic and

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aqueous leaf extracts of *Azadirachta indica* against MRSA isolates from wound and burn cases. The ethanolic extract exhibited promising antibacterial activity, with mean zones of inhibition ranging from 21.94 ± 2.63 mm to 9.97 ± 1.54 mm for wound isolates and 19.31 ± 3.85 mm to 8.53 ± 3.34 mm for burn isolates, depending on the extract concentration. The aqueous extract showed weaker antibacterial activity compared to the ethanolic extract, probably due to variations in the phytochemical concentrations and the flavonoid that is absent in the aqueous extract.

Table 3: Antibacterial Activities Profile of *Azadirachta indica* Leaf Extract against MRSA

Source	Solvent	Mean Zone of Inhibition ± SD (mm)			
		500mg/ml	**250mg/ml**	**125mg/m**	**62.5mg/m**
Wound	Ethanol	21.94 ± 2.63	18.78 ± 2.57	14.32 ± 2.65	9.97 ± 1.54
Burn		19.31 ± 3.85	14.60 ± 3.59	13.09 ± 4.44	8.53 ± 3.34
Wound	Aqueous	11.69 ±2.37	8.49 ±2.50	6.00 ± 00	6.00 ± 00
Burn		8.38 ± 4.06	9.81 ± 3.60	6.00 ± 00	6.00 ± 00
Wound	Linezolid 30 µg/ml	24			
Burn	Linezolid 30 µg/ml	21			

Table 4 presents the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanolic leaf extracts of *Azadirachta indica* against MRSA isolates. The results indicate that the ethanolic extract exhibited bactericidal activity against MRSA isolates, with MBC values ranging from 250 mg/ml to 500 mg/ml. This activity is expected particularly because many studies demonstrated *Azadirachta indica* to have antimicrobial activities against many microorganisms including malaria parasites, and fungi (Mistry, Sanghvi, Parmar, and Shah, 2014)(Verma et al., 2009) as well as anti-cancer properties (Al Saiqali, Tangutur, Banoth, and Bhukya, 2018).

Table 4: Bactericidal Activity of Ethanolic Leaf Extracts *Azadirachta indica*. *n=10*

Source	MIC				MBC			
	500 mg/ml	**250 mg/ml**	**125 mg/m**	**62.5 mg/m**	**500 mg/ml**	**250 mg/ml**	**125 mg/m**	**62.5 mg/m**
Wound	0	7	3	0	6	4	0	0
Burn	0	9	1	0	8	2	0	0

CONCLUSION

In general, the results indicate significant prevalence of MRSA in wound (28 %) and burn (18 %) cases. This stresses the need for effective antimicrobial strategies to reduce the rates. The ethanolic leaf extract of *Azadirachta indica* demonstrated promising antibacterial and bactericidal activities against MRSA isolates with MIC of 250 – 500 mm/mg, suggesting its potential as a natural source for the development of alternative antimicrobial agents against drug-resistant *S. aureus* strains.

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