Antibacterial Activity of Black Soap (Sabulun Salo) against Methicillin Resistant *Staphylococcus aureus* (MRSA) and Coagulase Negative Staphylococi (CoNS) Isolated from Skin and Soft Tissue Infections

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Abstract: Methicillin resistant *Staphylococcus aureus* (MRSA) and Coagulase negative Staphylococci (CoNS) especially *Staphylococcus epidermidis* cause skin and soft tissue infections that are often challenging to treat. This study determines the antibacterial activity of locally made black soap on MRSA and CoNS isolated from skin and soft tissue infections. Two hundred (200) swab samples were collected from patients with skin/wound infections. *Staphylococcus aureus* and CoNS were isolated and identified using standard microbiological tests. MRSA was detected phenotypically using 30µg Cefoxitin discs. Also *MecA* and *blaZ* genes were detected from some of the samples using Polymarase Chain Reaction (PCR). Susceptibility of the isolated organisms to locally made black soap was determined using agar well diffusion method. The results revealed that 16% of the samples were identified as MSSA, 6% were MRSA and 10% were *S. epidermidis*. The highest prevalence of *S. epidermidis* of 24.3% and 17.6% was recorded from surgical and diabetic wounds. The result revealed that patients aged 50-59 and 20-29 years had the highest prevalence of MRSA with 50% each (P=0.134). Whereas the highest prevalence of *S. epidermidis* of 60% was recorded among patients aged 50-59 years. There was a slightly higher prevalence of MRSA in female patients (28.6%) compared to male patients (27.2%) (P=0.100) and a higher prevalence of *S. epidermidis* among males (37.8%) than among females (22.2%). The study revealed that black soap had antibacterial activity both on MRSA and *S. epidermidis* at all concentrations tested, with higher activity of 18mm and 17.55mm zone of inhibitions against MRSA and *S. epidermidis* at 50% concentration respectively. The study recommends further studies on the safety of using Black soap against wound infections.
Key words: Antibacterial activity, Black soap, MRSA, *Staphylococcus epidermidis*

INTRODUCTION

Staphylococcus aureus is the most common bacterial pathogen responsible for acute as well as many chronic skin infections in humans (Taylor and Unakal, 2021). The emergence of antibiotic resistance among this group of bacteria is an issue of global concern, among them; Methicillin resistant *Staphylococcus aureus* (MRSA) shows multiple drug resistance to a number of antimicrobials including commonly prescribed β-lactam antibiotics (Penicillins and cephalosporins) (Felson, 2021; Simou *et al*., 2006). *Staphylococcus aureus* skin infections are classified as primary or secondary. Primary infections are those occurring on apparently normal skin, and mainly comprise impetigo, ecthyma, folliculitis, furuncles, *Sycosis barbae*, cellulitis, abscesses, paronychia and whitlows. Secondary infections are those arising in damaged skin (traumatized skin, or a pre-existing skin disease) (Del-Giudice *et al*., 2006; Simou *et al*., 2005).

Coagulase-negative Staphylococci (CoNS) are mainly important components of the normal skin and mucosal flora and *Staphylococcus epidermidis* has been identified as the most clinically significant CoNS that have emerged as an important pathogen (Vandecasteele *et al*., 2003; Kolawole *et al*., 1997). The CoNS develop from commensal to pathogenic lifestyle only when external barriers, such as skin, are damaged due to wounds or implantation of foreign bodies (e.g. indwelling medical devices) (Longauerova, 2006). They also exhibit certain degree of antibiotic resistance (Bresco *et al*. 2017; Augustinsson *et al*., 2004).

The ability of MRSA to evade the actions of not only the penicillins but also other classes of antibiotics such as Vancomycin is of global health concern (Osiyemi, *et al.*, 2018). Similarly up a greater percentage of *Staphylococcus epidermidis* strains are resistant to methicillin, aminoglycosides, macrolides, ciprofloxacin, clindamycin and levofloxacin (Elaidi *et al*., 2019).

Additionally, the CoNS have long been considered contaminants rather than true clinical pathogens, as such there are few systematic studies describing their epidemiology in human infections. Thus, identifying local remedies could contribute in no small measure towards addressing the increasing antibiotic resistance among this group of pathogenic Bacteria.

Black soap, widely used by different tribes in Nigeria such as Hausa (*sabulun salo*), Yoruba (*ose dudu*) and Nupe (*eco zhiko*), has been used for centuries in many African homes especially in Nigeria and Ghana (Getradeghana, 2000). The soap is made from a mixture of vegetable oils (palm karnel oil and shea butter) that made the soap to have antimicrobial properties recognized in traditional African households (Getradeghana, 2000). The major fatty acids in palm karnel oil are; lauric acid (C12, 48 %), myristic acid (C14, 16%) and Oleic acid (C18, 15%) (Dayrit, 2014). Certain fatty acids (medium chain saturates) have adverse effects on various microorganisms (Kabara, 1978), monolaurin is known to be able to inhibit various pathogenic bacteria in the form of vegetative cells and bacterial spores; (Schlievert *et al.*, 2013). The attributes of the soap includes gentleness on skin, rich lather, protection against skin disorders (including rashes, eczema and scabies) protection of even skin toning and smoothness of the skin (Getradeghana, 2000).

This study evaluates the antibacterial activity of indigenous black soap against MRSA and CoNS.

MATERIAL AND METHODS

The research was conducted at Aminu Kano Teaching Hospital (AKTH), Kano and the study population comprised of all patients with skin and soft tissue infections that reported to Accident and emergency unit (A and E), surgical specialty ward, Female and Male Surgical wards as well as paediatric wards of hospitals. Ethical clearance for the study was obtained from the Research Ethics Committee of AKTH (AKTH/MAC/SUB/12A/P-3/VI/32-62).

Demographic data and additional information from the consented patients werecollected using a questionnaire in accordance with the declaration of Helsinki.

The sample size was calculated based on the 80% prevalence of MRSA as reported by Awoke *et al.* (2019) and according to the formula for sample size determination;

$$\boldsymbol{n = \frac{z^2 pq}{d^2}}$$

Where;

n= number of samples

Z= standard normal deviation at 90%, confidence limit = 1.645 from z table

P= Prevalence from most recent past study (P=80% i.e; 0.80, Awoke, *et al;* 2019)

Q= 1-p

d= allowable error of 5%=0.05

Therefore, $n = \frac{(1.645)^2 \times 0.80\ (1-0.80)}{(0.05)^2} = 173.18$

The sample size was rounded up to 200.

The BBL culture Swab were used to collect aseptically 200 swab samples from the consented patients with skin infections, labeled appropriately and transported to the laboratory within one hour of collection in cold biosafety boxes and processed for isolation and identification of MRSA and CoNS.

Isolation of Staphylococci was done according to the methods of Nwankwo *et al.* (2010). The Swab samples were aseptically inoculated on Mannitol Salt Agar (MSA) and incubated aerobically at 37°C for 24 hours. Colonies that developed were identified using morphological, Gram staining reaction and biochemical tests according to the methods of Nwankwo *et al.* (2010); Cheesbrough (2000). The identified colonies were subcultured on MSA plates and incubated at 37°C for 24 hours and further identified as mannitol fermenters and non-mannitol fermenters. Samples identified as Coagulase Negative Staphylococci were subjected to Novobiocin sensitivity testing according to the methods of Naqvi *et al.* (2007).

The standardized inoculum (0.5 McFarland standards) was used to inoculate the surface of Mueller Hinton agar plates and Novobiocin discs were placed on the center of each plate and incubated at 37°C for 24hrs. Zones equal to or wider than 20mm were recorded as resistant, while zones smaller than this were recorded as sensitive to Novobiocin (Saber *et al*., 2020; Naqvi *et al*., 2007). CoNS that appeared as Novobiocin sensitive are identified as *S. epidermidis*.

To identify MRSA, samples previously identified as *S. aureus* were tested for resistance to cefoxitin. This was done using standardized (0.5 McFarland standards) bacterial suspension to inoculate the surface of Mueller Hinton agar plates (90mm in diameter) using sterile cotton swab. Cefoxitin discs were carefully placed on the center of the plates and then incubated at 37°C for 24hrs after which zones of inhibition around the disc were measured. Zones equal to or wider than 22mm were recorded as sensitive, while zones smaller than this were recorded as resistant to Cefoxitin Naqvi *et al*. (2007).

Further identification of MRSA was done using Polymerase Chain Reaction by the detection of virulence *mecA* and *blaZ* genes which are associated with resistance. Genomic DNA isolation was carried out using solution-based DNA extraction methods that employ organic solvents (phenol and chloroform). The procedure was carried out according to the manufacturer instructions (Biorad USA).

The amplification using Polymerase Chain Reaction (PCR) was carried out in a total volume of 20ml and the primer sequences and predicted sizes used in the PCR were shown in Table 1. Briefly, 2.0 µl of genomic DNA sample (obtained from DNA extraction) was added to 18 µl of PCR mixture (1µl of primers and 17µl of distilled water) for *blaZ*. Another 2.0 of genomic DNA sample was added to 18µl of PCR mixture (1µl of primers and 17µl of distilled water) for *mecA*. The amplification process was started with an initial pre-denaturation step (94^0C for 5 minutes) for both *blaZ* and *mecA*. Each cycle consisted of three steps (denaturation, annealing and extension) and each PCR reaction consisted of 35 cycles of amplification.

After amplification of the genes, 8µl of the PCR products and 8µl of molecular weight marker were loaded directly onto a 1.5% agarose gel in 1 x Tris-acetic acid -EDTA buffer (TAE) containing 10 µl Green nucleic acid stain. The DNA bands are of the molecular weight 400 and 336 base pairs in comparism with molecular weight marker. DNA amplicons were visualized using a gel imaging system. Positive control used was *S. aureus* (Staph756F). Also, negative control was used by adding DNA of fungi (Wichelhaus *et al.,* 2001).

Table 1: The primer sequences and predicted sizes used in the multiplex PCR

Gene	Oligonucleotide sequence(5'-3)	Expected amplicon size (bp)
MecA	5'-GTTGTAGTTGTCGGGTTTGG-3 5'CTTCCACATACCATCTTCTTTAAC'3	336
BlaZ	5'CAAAGATGATATAGTTGCTTATTC'3 5'TGCTTGACCACTTTTATCAGC'3	400

Adapted from Ayepola (2012) and Deneeling *et al*. (1998)

CoNS were further identified using API kit (API Staph- bioMérieux Inc) based on the guidelines provided by the manufacturers. Briefly, colonies of the CoNS were subcultured onto blood agar and incubated for 24 hours at 37°C after which a homogeneous bacterial suspension with turbidity equal to 0.5 McFarland was prepared. API STAPH consists of a strip containing dehydrated test substrates in individual microtubes. The tests were reconstituted by adding to each tube an aliquot of API STAPH Medium that has been inoculated with the standardized inoculum.

The strip was then incubated for 18-24 hours at 35-37°C after which the results were read and interpreted with reference to the information contained in the package insert.
The identification was facilitated by the use of the API STAPH Analytical Profile Index and the identification software.
Staphylococcus aureus control Organism (ATCC 25923) was collected from the Department of Microbiology, Aminu Kano Teaching Hospital and reconfirmed according to the methods of Cheesbrough (2000).
The susceptibilities of the test organisms to African Black soap was determined using agar-well diffusion method (Aliyu *et al.*, 2009; Ndukwe *et al*., 2005). Different concentrations (50%, 25%, 12.5%, and 6.25% w/v) of the black soap were prepared using sterile distilled water according Aliyu *et al*. (2009) as follows:

$$w/v = \frac{Mass\ of\ solute\ (g)}{Volume\ of\ solution\ (ml)} \times 100.$$

For 50% w/v, $\frac{w}{v} = \frac{5g}{10ml} \times 100 = 50\%$

For 25% w/v, $\frac{w}{v} = \frac{2.5g}{10ml} \times 100 = 25\%$

For 12.5% w/v, $\frac{w}{v} = \frac{1.25g}{10ml} \times 100 = 12.5\%$

For 6.25% w/v, $\frac{w}{v} = \frac{0.625g}{10ml} \times 100 = 6.25\%$

Four (4) wells were made on each plate using sterilised cork borer (8mm) and 0.3 ml of each concentration prepared above was transferred in to four of the five wells (appropriately labelled) and distilled water into the fifth well as the negative control. An antibiotic disc containing Vancomycin (30µg) was carefully placed on the centre of the plates, this served as positive control. The plates were incubated using standardized inoculum at 37^0C for 24 hours after which the plates were observed for zones of inhibition around the wells which were measured and recorded.
The minimum inhibitory concentration and minimum bactericidal concentration were determined according to the Clinical and Laboratory Standards Institute (2009). Two (2) mls of sterile Mueller Hinton broth was transferred into a set of 4 tubes and 2ml of each concentration (50%, 25%, 12.5% and 6.25% w/v) of the black soap was added to obtain final concentrations of 25%, 12.5% and 6.25%, 3.125% w/v respectively. Standardized inocula of the wound isolates were inoculated into the labelled tubes containing various concentration of the black soap except the control; the tubes were incubated at 37^0C for 18 hours, the lowest concentration that prevents visible growth of the isolates was taken as the MIC. Tubes from MIC test that showed no visible growth were subcultured into freshly prepared Mueller Hinton agar plates and incubated at 37^0C for 24 hrs, the lowest concentration at which the isolates do not recover and grow was taken as the MBC.

RESULTS

Table 1 revealed that out of the 200 samples studied, 32 (16%) were identified as MSSA, 12 (6%) were MRSA, 20 (10%) were *S. epidermidis* and 136 (68%) had no growth.
Samples from furuncles and those obtained from wounds sustained as a result of Road traffic accidents had the highest prevalences of MRSA of 11.8% and 10% respectively (Table 2). The highest prevalence of *S. epidermidis* of 24.3% and 17.6% was recorded from surgical wounds and diabetic wounds respectively (Table 2). Table 2 also revealed that 80% and 100% of samples from impetigo and abrasions had no growth.
Table 3 revealed that the highest prevalence of *S. aureus* of 83.3 % and 78.8% was obtained from those aged 20-29 and 10-19 years and the lowest was from those aged 50-59 years. The highest prevalence of *S. epidermidis* of 40% and 60% was obtained from those aged 0-9 and 50-59 years, while the lowest prevalence of *S. epidermidis* of 16.7% was recorded in those aged 20-29 years (Table 3). The statistical analysis indicated that there was no significant difference in the prevalence of both infections among the studied subjects with respect to their age (P=0.672). The study further revealed that a higher prevalence of *S. aureus* and *S. epidermidis* among males (37.8%) than in Females (22.2%) (P=0.275) (Table 3).

Table 3 also revealed that subjects in the age groups of 20-29 years had the highest prevalence of MRSA of 41.7%, while those in age groups ≥60 years had lower prevalence of MRSA of 16.7% (P=0.134).

Table 4 revealed that Black soap had antibacterial activity both on MRSA and *S. epidermidis* at all concentrations tested except at the concentration of 6.25%. The highest activity was exhibited at higher concentrations of 50% and 25% of the soap. At 50%, the soap produced zones of inhibition of 18mm and 17.55mm against MRSA and *S. epidermidis* respectively, while at 25% it produced zones of inhibition of 12.55mm and 12.75mm against MRSA and *S. epidermidis* respectively (Table 4).

Similarly, compared with the standard drug used (Vancomycin), the soap produced higher activity of 18mm and 17.55mm zones of inhibition against MRSA and *S. epidermidis* respectively at 50% concentration, whereas Vancomycin produced zones of inhibition of 17.50mm and 15.75mm against MRSA and *S. epidermidis* respectively (Table 4).

Also, the black soap had appreciable activity on the isolated organisms at all concentrations compared with the standard strain (ATCC25923) used, for instance, at 50% concentration of the black saop, it produced an inhibition zone of 18mm and 17.55mm against MRSA and *S. epidermidis* respectively compared with the 19.00mm zone of inhibition it produced against the standard strain (ATCC25923) at the same concentration (Table 4).

Table 5 revealed that the Minimum Inhibitory Concentration (MIC) of the black soap against MRSA and *S. epidermidis* was 12.5%, whereas the Minimum Bactericidal Concentration (MBC) of the black soap against both MRSA and *S. epidermidis* was 25%.

The result of PCR products shows that, out of the 9 MRSA isolates 4 (44.4%) amplified at 336bp indicating the presence of *mecA* gene (Plates 1) while 5 (55.6%) amplified at 400bp (Plates 2) indicating the presence of *blaZ* gene

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Table 2: Distribution of MRSA, MSSA and *S. epidermidis* among the different sources of samples

Sources	Sample Examined No (%)	MSSA No (%)	MRSA No (%)	*S. epidermidis* No (%)	No growth No (%)
Abrasions	5 (2.5)	1 (20)	0	0	4 (80)
Boils	26 (13)	8 (30.8)	2 (7.7)	0	16 (61.5)
Burns	39 (19.5)	4 (10.3)	1 (2.6)	2 (5.1)	32 (82)
Diabetic Wds	17 (8.5)	1 (5.9)	0	3 (17.6)	13 (76.5)
Furuncles	17 (8.5)	6 (35.3)	2 (11.8)	2 (11.8)	7 (41.2)
Impetigo	1 (0.5)	0	0	0	1 (100)
Orthopedic Wds	18 (9.0)	4 (22.2)	0	1 (5.6)	13 (72.2)
RTA Wounds	40 (20)	3 (7.5)	4 (10)	3 (7.5)	30 (75)
Surgical Wds	37 (18.5)	5 (13.5)	3 (8.1)	9 (24.3)	20 (54)
Total	**200 (100)**	**32 (16%)**	**12 (6%)**	**20 (10%)**	**136 (68%)**

Key; MRSA- Methicillin Resistant *Staphylococcus aureus* MSSA- Methicillin Sensitive *S. aureus.* Wds-wounds, RTA - Road Traffic Accident.

Table 3: Distribution of *S. aureus* and *S. epidermidis* according to the age and sex of studied Patients with skin/wound infections

Black Soap Concentration (%)	Susceptibility status of the isolates (Zones of inhibition-mm)		
	MRSA	***S. epidermidis***	**ATCC25923**
50	18.00	17.55	19.00
25	12.55	12.75	17.00
12.5	11.43	10.50	12.00
6.25	8.00	8.00	8.00
Vancomycin (30 µg)	17.50	15.75	20.00

Table 4: Antibacterial activity of Back Soap on MRSA and *Staphylococcus epidermidis*

Demographic Parameters	No. Examined	No. Positive (%)	Isolated Staphylococci				
			S. aureus No (%)	***S. epidermidis*** No (%)		**MRSA** No (%)	
Age (Years)					**P-value**		**P-value**
0 – 9	20	10 (50)	6 (60.0)	4 (40.0)	0.672	2 (20)	0.134
10 – 19	33	9 (27.3)	7 (77.8)	2 (22.2)		0 (0)	
20 – 29	45	12 (26.7)	10 (83.3)	2 (16.7)		5 (41.7)	
30 – 39	34	12 (35.3)	8 (66.7)	4 (33.3)		3 (25)	
40 – 49	34	10 (29.4)	7 (70.0)	3 (30.0)		0 (0)	
50 – 59	23	5 (21.7)	2 (40.0)	3 (60.0)		1 (20)	
≥60	11	6 (54.5)	4 (66.7)	2 (33.3)		1 (16.7)	
Total	**200**	**64 (32)**	**44 (22)**	**20 (10)**		**12 (6.0)**	
Gender							
Male	113	37 (32.7)	23 (62.2)	14(37.8)	0.275		
Female	87	27 (31)	21 (77.8)	6 (22.2)			
Total	**200**	**64 (32)**	**44 (68.8)**	**20 (31.3)**			

Note: Well diameter=8mm

Table 5: MBC and MIC of locally made black soap against MRSA and *S. epidermidis*

Isolates	MIC	MBC
MRSA	12.5%	25%
S. epidermidis	12.5%	25%

Key: MIC = Minimum Inhibitory Concentrations, MBC = Minimum Bactericidal Concentration,

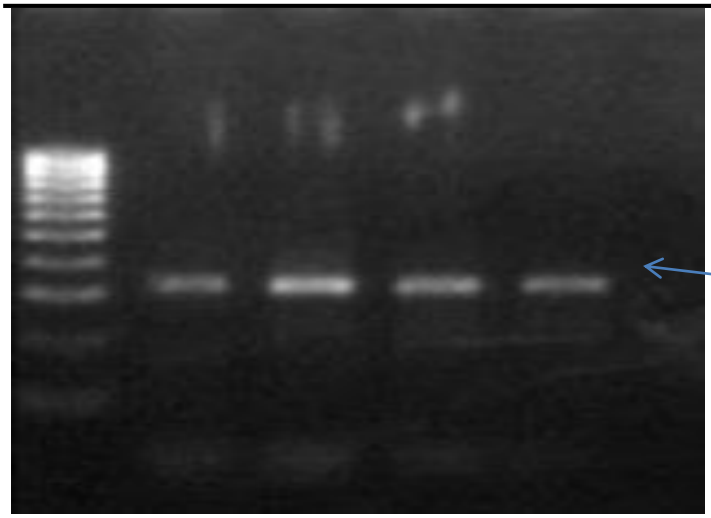

Plate 1: Detection of *MecA* gene from MRSA
Key: L- Ladder, Lanes 1-4 *mecA* gene from MRSA isolates, Lane 5- Negative control

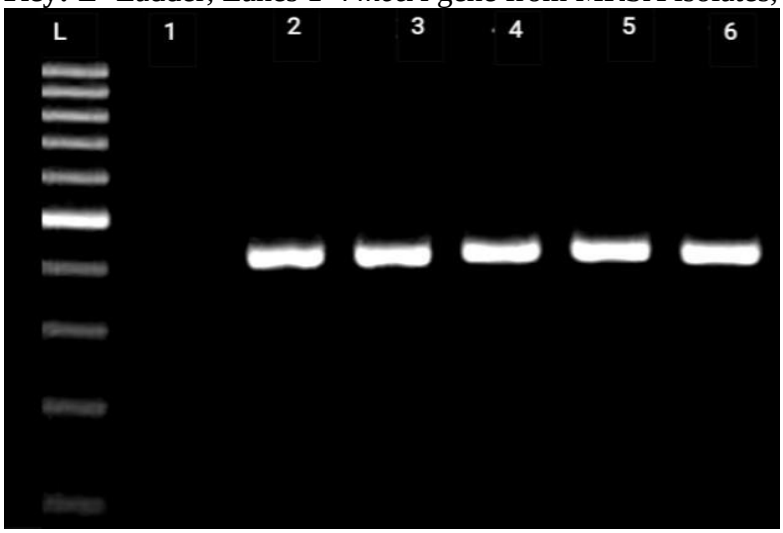

Plate 2: Detection of *blaZ* gene from MRSA
Key: L- Ladder, Lane 1- Negative control, Lanes 2-6 *blaZ* gene from MRSA isolates

DISCUSSION

The findings of this study indicated there is decrease in the prevalence of MRSA (6.0%) in the study area compared to 10.7% prevalence reported by Nwankwo and Nasir (2011) in the same study area and 10% in Maiduguri (Adelowo *et al*., 2014). Other studies reported higher MRSA prevalence of 37.5% at University of Calabar Teaching Hospital (Azeez *et al.*, 2008); 34.7% in Ilorin (Taiwo *et al*., 2004). This decline in the prevalence of MRSA skin infections in the study area could be due to the improvement in hygiene recorded across healthcare facilities which was triggered by the COVID-19 pandemic.

The Study findings revealed that *S. epidermidis* was the only CoNS isolated in the study area and its prevalence was found to be 30.1%. This observation supports the assertion by Becker *et al.* (2014) that *S. epidermidis* is the most frequently recovered member of the CoNS group. *Stahylococcus epidermidis* was also the most common CoNS isolated from skin infections in Hospitals in Northern Tehran (Al-tayyar *et al.*, 2015). Narayani *et al.* (1999) earlier reported a prevalence of 49.1% of *S. epidermidis* from skin of health personnel and surgical patients in India. Sheik *et al.* (2011) also recorded a 66.7% prevalence of CoNS from skin infections in Iran, most of which are strains of *S. epidermidis*.

The findings of this study imply that *S. epidermidis* could be the most important CoNS especially in the study area and hence possess a health threat especially to patients with skin infections.

The findings of this study showed that locally made Black soap (*Sabulun salo*) had antibacterial activity against MRSA and *S. epidermidis*, producing zones of inhibition of 18.0mm and 17.55mm comparable to those by the standard drug used (Vancomycin), which also is the drug of choice for the treatment of MRSA infections. Ugbogu (2006) earlier expounded that peptidoglycan is found to be distorted by long chain fatty acids that are present in palm kernel oil (which is an active ingredient of Black soap), as such the antibacterial activity of the soap against MRSA and *S. epidermidis* could be attributable to the palm kernel oil present in the soap.

The study further demonstrated that even the standard bacterial strain (ATCC25923) used in the study was found to be susceptible at similar concentrations of the Black soap just like the isolated MRSA and *S. epidermidis* and this implies that the black soap could be regarded as a potential antibacterial agent in the continued search for therapeutic agents against skin infections.

CONCLUSION

The study identified the presence of CoNS in the study area, among which only *S. epidermidis* was detected with a prevalence of 10%. The Prevalence rate of MRSA was 6% and was found to be lower than the findings of previous studies conducted in the same study area. Black soap was found to have antibacterial activity against MRSA and *S. epidermidis* producing wider zones of inhibition even greater than the standard drug used (Vancomycin). The study identifies black soap as a potential therapeutic agent against skin infections caused by MRSA and *S. epidermidis.* The study recommends further studies on the safety of using Black soap against wound infections and the possibility of enhancing the antibacterial activity of local Black soap, so that its antibacterial potentials can be fully harnessed.

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