ANTIBACTERIAL PROPERTIES OF *LAWSONIA INERMIS* AGAINST BIOFILM PRODUCING METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* IN AHMADU BELLO UNIVERSITY TEACHING HOSPITAL ZARIA, NIGERIA

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ABSTRACT

Background: The treatment options for MRSA are getting narrower by the day, which is associated with MRSA turning multi-drug resistance organisms, causing increased mortality around the globe. Biofilm formation by MRSA worsens the situation by rendering it impenetrable, making the treatments more complex.

Aim: To evaluate the antibacterial properties of *Lawsonia inermis* against Biofilm producing MRSA among burns/wound patients and health-care workers at Ahmadu Bello University Teaching Hospital Zaria.

Materials and Methods: The study was conducted on 300 participant, 94 Health Care Workers and 206 Burn Wound Patient. *S. aureus* was cultured on Mannitol salt agar while MRSA was detected using Cefoxitin disc. The plant was extracted using Soxhlet technique and antibacterial activity of the plant was tested using Kirby-Bauer technique by testing 3 different concentrations of the extract (2400, 2800, and 3200) µg. the biofilm formation studies was done using TCP technique.

Results: From the 94-samples collected from HCW 32 and 17 *S. aureus* and MRSA was detected respectively while from the 206 samples collected from BWP 36 and 26 *S. aureus* and MRSA was detected respectively, among the MRSA isolated all were Biofilm producers. Increase in zone of inhibition was observed when the isolates were tested against increasing concentration of *L. inermis* (2400µg 7±4mm, 2800µg 10±3mm, 3200µg 12±4mm).

Conclusion: The extracts of *L. inermis* have shown antibacterial activity against Biofilm producing MRSA.

Keywords: Health Care Workers. Burn. Wound Patient. *Lawsonia inermis*. Biofilm

INTRODUCTION

The use of herbal medicine is a practice that provides the basis for modern-day treatment Nigussie *et al.,* (2021). To date, scientists continue to exploit its significance in providing efficient and affordable medicinal products to treat several infectious disease Dewan *et al.* (2018). *Lawsonia inermis* Linn (henna) is a *Lythraccae* family genus *Lawsonia* Nigussie *et al.* (2021). Henna was reported to show activity against several infectious agents such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus epidermidis* among others Kouadri (2018).

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a bacterium of interest after first been reported in 1961 by a United Kingdom-based scientist Emaneini *et al*., (2018). In the recent past, different researchers have documented the prevalence of MRSA in different samples ranging from 6.8% to 78% (Onanuga *et al*., 2005 Akerele *et al.*, 2015; Baba *et al.*, 2015; Yusuf and Airauh, 2015; Aminu *et al.,*2017; Olowo *et al.,*2017). The mechanism of the bacterial resistance was reported to be through the Production of penicillin-binding protein 2a (PB2a) encoded in the bacterial DNA and excessive production of β-lactamases Shahi *et al.* (2018); Osiyemi *et al.* (2018)). The organism's ability to spread rapidly in the hospital ward through contact poses a significant risk to managing burn and wound patients during hospital admission Emaneini *et al*., (2018). Methicillin-resistant *Staphylococcus aureus* has been the Centre of concern due to its persistence and ubiquitous potential in healthcare delivery (Eftekhar *et al*., 2017). It was isolated from hospital curtains, surfaces, and equipment (Shek *et al*., 2018). The treatment options for MRSA are getting narrower by the day, which is associated with MRSA turning multi-drug resistance organisms, causing increased mortality around the globe (Fasihi*et al.,* 2017). Biofilm formation by MRSA worsens the situation by rendering it impenetrable, making the treatments more complex (Ansari *et al*., 2015). *L. inermis* was 75% more active than standard *Gentamicin* (Kulkarni *et al*., 2018). Therefore, this study aimed at evaluating the antibacterial properties of *Lawsonia inermis* against Biofilm producing MRSA among burns/wound patients and health-care workers at Ahmadu Bello University Teaching Hospital Zaria.

MATERIALS AND METHODS

Study Area

The study was conducted at Ahmadu Bello University Teaching Hospital Shika Zaria Nigeria.

Study population

This was a cross sectional studies conducted on 300 participants comprising of 206 burn/wound patient and 94 healthcare workers at

Ethical Consideration

Permission for the research was obtained from the health research ethics committee (HREC) of the Ahmadu Bello University Teaching Hospital, Zaria, before the commencement of the study. HREC number ABUTHZ/HREC/W38/2020. Informed consent/assent was sought and obtained from each participant before enrollment into the study.

Preparation of *Lawsonia inermis leaves* Extract

Lawsonia inermis leaves were collected in a farmland at Bindawa Local Government of Katsina State. Nigeria then was authenticated at the herbarium unit of the Plant Biology Department, Bayero University Kano, crushed to a powdered form using pestle and mortar as described by (Usman and Rabiu, 2018). While the extract preparation was performed as previously described, (Kouadri 2018) Soxhelation 500 ml ethanol were placed in a one neck flask roundbed flat of 1000 ml. fifty grams of *L. inermis* leaves powder was prepared and put in the filter paper which was placed in the soxhlet accordingly. It was heated at 80 ^{0}C. The extraction occurred until the solvent in the soxhlet become clear or colorless. The soxhletation extraction was counted to be one cycle when the solvent filled in soxhlet and then turned back to the one neck flask roundbed. Percentage yield (PY) of the extract was calculated and a stock solution was prepared to obtain a 300mg/ml final concentration and stored at 4^{o}C until use.

PY= (weight of the dissolved extract/weight of the powder dissolved) *100

Swab Sample collection

The sample was collected aseptically. The swab was taken from burn and wound patients at sites with the highest deep tissue exposure.

The area was cleaned with sterile saline, after which the wound was swabbed; also, a nasal swab was collected aseptically from healthcare workers. The samples were transported to Medical Microbiology Laboratory, ABUTH Zaria for microbial culture and identification (Goudarzi *et al*., 2017).

Phenotypic identification of *Staphylococcus aureus*

The sample collected was cultured on Mannitol Salt agar (MSA) then incubated for 24hrs at 37^{o}C. The isolates were identified using the following conventional biochemical tests: gram staining, growth patterns on MSA (yellow colonies), hemolysis on Blood agar, catalase test, rabbit plasma coagulase test (slide test) and DNAse test (Goudarzi *et al*., 2017).

Detection of MRSA

Phenotypic screening of MRSA isolates was done using a cefoxitin disc (30 µg) on Mueller Hinton agar plates supplemented with 4% NaCl resistance against cefoxitin (30µg) was considered as positive test for MRSA by subjecting each organism to a sensitivity test using Kirby-Bauer method. CLSI guideline was used for the determination of resistance (Goudarzi *et al*., 2017).

Determination of Phenotypic Biofilm formation by MRSA

Tissue culture plate (TCP) technique was carried out using the method described by (Ansari *et al*. 2015). Ten millilitres of tryptic soy broth (TSB) with 1% glucose were inoculated with a loop-full of test organism from overnight culture on nutrient agar in a test tube. The test tube was incubated at 37°C for 24 hrs, and then a dilution of 1:100 with the fresh medium was made. After gentle mixing, the 96 wells flat bottom TCPs were filled with 200µl of diluted cultures each. The sterile broth was used to serve as blank. Similarly control organisms were also diluted and incubated. The culture plates were incubated at 37°C for 24 hrs. After incubation, gentle tapping of the micro titer plates was done. The wells were washed with 200µl of phosphate buffer saline at pH 7.2 four times to remove free-floating bacteria. While, the Biofilm, which remained adherent to the wells' walls and bottoms, was fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with de-ionized water, and plates were appropriately dried. An optical density (OD) of stained adherent biofilm was obtained with a microtiter plate reader at wavelength 570 nm. The experiment was performed in triplicate and repeated thrice. The average OD values of the sterile medium were calculated and subtracted from all test values.

Antibacterial effect of *Lawsonia inermis* against biofilm-producing MRSA

Minimum Inhibitory Concentration (MIC) Determination

The experiment was carried out as follows; The MIC of the *L. inermis* extract against MRSA was determined by dilution method in Tryptic soy broth. A concentration of 3000µg/ml to 2050µg/ml was prepared from the stock solution of the extract. The tubes were inoculated with 10µl of microorganism cultures—un-inoculated tubes containing growth medium and extract ware used as negative controls. The test tubes were then incubated overnight at 37^{0}C. The MIC was defined as the lowest concentration that showed no turbidity (Kouadri, 2018).

Antimicrobial Activity Assay (Disc Diffusion Method

This was performed as per the method described by (Kouadri, 2018) the antibacterial activities of three different concentrations (2400, 2800 and 3200) µg of the *L. inermis* extract was evaluated by disc diffusion method (Kirby and Bauer, 1966) the microorganisms' suspensions (prepared in peptone water) with turbidity equivalent to that of 0.5 McFarland standard, was seeded uniformly with sterile swabs onto Muller Hinton Agar (MHA).

The filter paper discs (6mm in diameter) was impregnated with 20 µl of the extracts (3) 2400, 2800 and 3200) µg, dried and carefully laid on the surface of the agar plates inoculated with test microorganisms, the inoculated plates were incubated overnight at 37°C inhibition zone of test microorganisms around the paper. Antibacterial disc with Gentamicin (10 µg/disc) was used as a positive control. All assays were carried out in triplicate.

RESULTS

Demographic Characteristics of the Study Participants

The study was conducted on 300 subjects comprising 68.7% (206) BWP and 31.3% (94) HW; among the BWP, 51.5% (106) are male, and 48.5% (100) are females. also, according to age categorization, the age group of 3-12 years had 22 (10.7%), 13-22 years had 50 (24.3%), 23-32 years had 51 (24.8%), 33-42 years had 45 (21.8) and 43 years above had 38 (18.4 %) of the study participant, on the other hand, HW comprised Doctors with 12.8 % (12) participants, Nurses having 55.3% (52) participants and Health Assistant having 31.9% (30) participants all these characteristics are listed in Table 1.

Table 1: Demographic Characteristics of the Participants Recruited.

Variable	Frequency of sample collected (%)	95% Confidence interval (Lower – Upper) Limit
Study Groups		
BWP	206 (68.7)	63.1-73.9
HCW	94 (31.3)	26.1-36.9
Total	300	
HCW; Cadre		
Doctors	12 (12.8)	6.80-21.2
Nurses	52 (55.3)	44.7-65.6
Health Assistant	30 (31.9)	22.7-42.3
Total	94	
BWP; Sex		
Male	106 (51.5)	44.4-58.5
Female	100 (48.5)	41.5-55.6
Total	206	
BWP; Age Groups (Years)		
3-12	22 (10.7)	6.8-15.7
13-22	50 (24.3)	18.6-30.7
23-32	51 (24.8)	19.0-31.2
33-42	45 (21.8)	16.4-28.1
43 above	38 (18.4)	13.4-24.4
Total	206	

BWP: Burn and wound patient; HCW: Healthcare workers.

Distribution of Study Participants

A total of 206 samples were collected from BWP and 94 HCW. Of the 94 samples collected from HCW, 12 (13%), 52 (55%) and 30 (32%) samples were collected from doctors, nurses and hospital attendants, respectively (Figures 1 and 2).

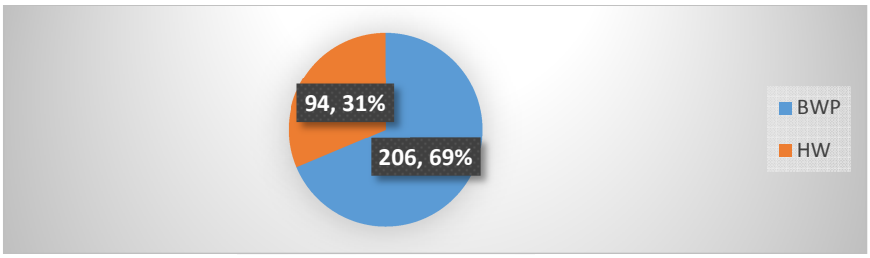

Figure 1: Samples collected from BWP and HCW (n=300).

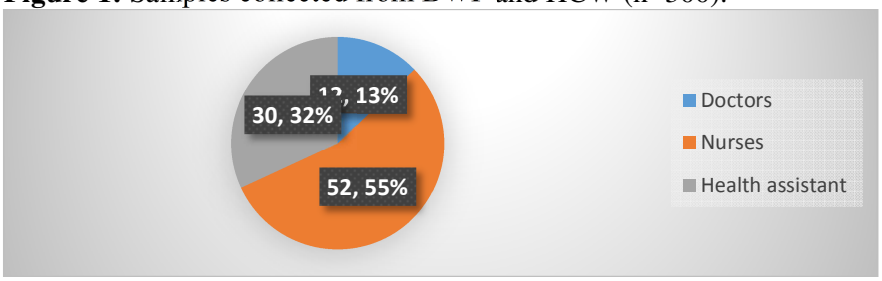

Figure 2: Distribution of health care workers recruited in the study (n=94).

Characterization of *S. aureus* isolated from BWP and HCW

A total of 68 *Staphylococcus aureus* isolates were phenotypically characterized from the 300 samples collected from both BWP 12% (36) and HCW 11% (32). The distribution of *S. aureus* is shown in Figure 3.

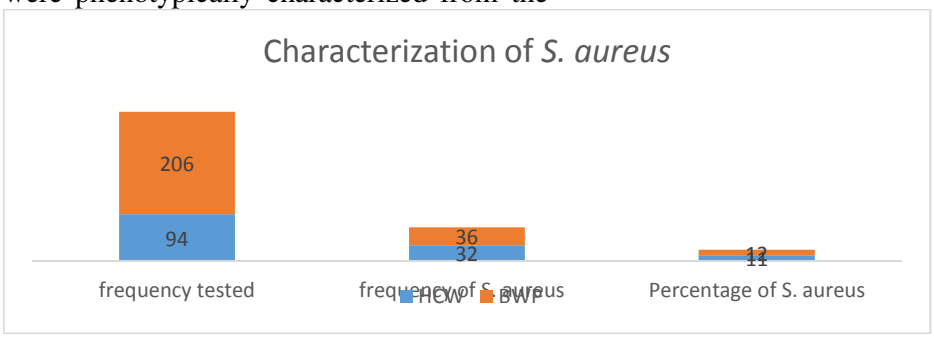

Figure 3: Frequencies of *S. aureus* identified by phenotypic characterization (n=68).

Characterization of *S. aureus* isolated from HCW

From the 32 *S. aureus* isolated from HCW, doctors have a prevalence of 41% followed by nurses with 34% then health assistants with 30% (Figure 4.4).

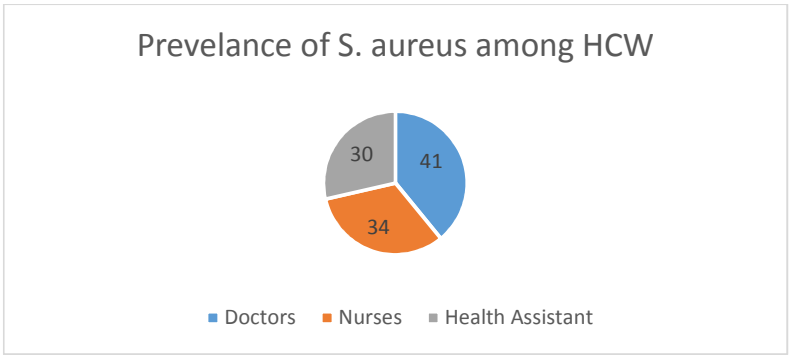

Figure 4: Percentage *S. aureus* isolated from HCW according to cadre. (n=32)

Characterization *S. aureus* isolated from BWP and HCW into MRSA

Phenotypic analysis of the isolated *S. aureus* revealed a total of 43 (62.3%) MRSA among which 26 (38%) and 17 (25%) are from BWP and HCW respectively.

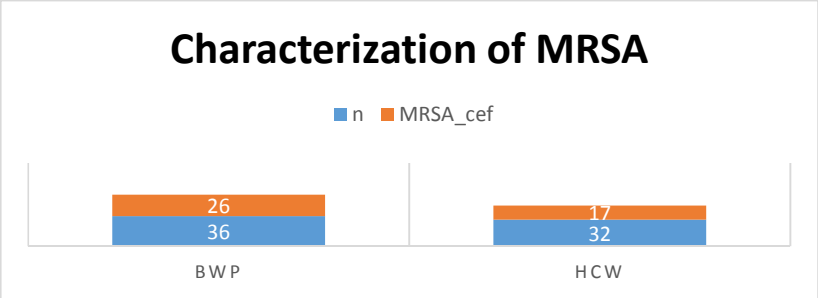

Figure 5: Phenotypic characterization of *S. aureus* into MRSA (n=43). Cefoxitin resistant *S. aureus*.

Determination of phenotypic Biofilm formation of MRSA

Among the MRSA tested all were found to be moderate biofilm producers when tested using Tissue Culture Plate Technique. As shown in Table 2.

Table 2: Distribution of biofilm production across the study population using TCP technique

Study population	Frequency of MRSA Tested	Frequency of Biofilm producing MRSA (%)
HCW	17	17 (100)
BWP	26	26(100)
Total	43	43 (100)

Key: MRSA; Methicillin Resistant *Staphylococcus aureus*, TCP; Tissue culture plate

Minimum inhibitory concentration of *Lawsonia inermis* against the Biofilm producing MRSA isolated from BWP and HCW

The *L. inermis* leaves were extracted successfully and a percentage yield of 16% was obtained by dividing the weight of the dissolved extract (8) with the weight of the dissolved powder (50) multiplied by 100, the extract was then stored at 4^0C for further studies. Minimum Inhibitory concentration of *L. inermis* against the Biofilm producing MRSA isolated from HW and BWP were found to be 2350 µg/ml in all isolates.

Antibacterial activity of *Lawsonia inermis* against the Biofilm producing MRSA isolated from BWP and HCW

When the three different concentrations of *L. inermis* leaves extract was tested against the Biofilm producing MRSA there was a statistical significance with respect to increase in the zone of inhibition as the concentration of the extract increased (p=0.0044) compared with Gentamycin. As shown in Table 3.

Table 3: Antibacterial Sensitivity result of *L. inermis* against biofilm producing MRSA isolated from BWP and HCW.

Different Conc. of *L. inermis*(µg)	Z.I (±SD) mm
2400	7 (4)
2800	10 (3)
3200	12 (4)
Gentamycin 30	17 (5)

Key; Z.I- Zone of Inhibition.

DISCUSSION

Methicillin-resistant *Staphylococcus aureus* has been the center of concern due to its persistence and ubiquitous potential in healthcare system (Eftekhar *et al*., 2017). Biofilm formation by MRSA worsens the situation by rendering it impenetrable, making the treatments more complex (Ansari *et al*., 2015). *L. inermis* was 75% more active than standard *Gentamicin* (Kulkarni *et al*., 2018).

From the result obtained 68 were phenotypically characterized as *S. aureus* of which 32 are from the 94 samples collected from HCW which is in agreement to the findings of (Giri *et al*., 2021) in india and Egypt respectively Allam *et al.,* 2021, while from the 206 samples collected from BWP 36 *S. aureus* were isolated which is low compared to the isolation rate among HCW.

Phenotypically 18% of the *S. aureus* isolated from HCW were characterized as MRSA which is relatively similar to the findings of (Wu *et al.,* 2019) were he isolated 22% and 7.8% from 204 HCW but is higher compared to the findings from Ethiopia were the *S. aureus* and MRSA were reported to be 12% and 5.8% respectively (Legese *et al.,* 2018).

On the other hand, the finding of this research agrees with the result obtained by (Khan *et al.,* 2018) as regards to the *S. aureus* and MRSA isolated from BWP

The findings of Gatta *et al*. (2021) Agrees with our findings were the detected 89.28% of the MRSA isolated to be biofilm producers but disagrees with the findings of (Senobar *et al.,* 2021) were they found that 39% of the MRSA tested were biofilm producers.

It has been demonstrated as the concentration of *L. inermis* crude extract increases the diameter for the zone of inhibition increases with the highest Zone of Inhibition at 3200µg while yet Gentamycin 30µg exhibit the highest antibacterial activity this may be due to the fact that crude extract of *L. inermis* was used not the active compound found in the plant (Kumar *et al.,* 2016).

CONCLUSION

From the findings of our study it can be concluded that indeed there is antibacterial agent contain in the biological active component of *L. inermis*

RECOMMENDATION

Future studies should focus on isolation of the active compound responsible for the antibacterial properties of *L. inermis* leaves extract.

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