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Antibacterial and Anti-Biofilm Activities of *Neocarya Macrophylla* Against Clinical Bacterial Isolates

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: The increasing threat of bacteria resistant to current antibiotics underscores the need for an alternative source of antimicrobial agents. This study was designed to investigate the antibacterial and antibiofilm activity of *Neocarya macrophylla* against two important human pathogens commonly associated with biofilm-related infections.

Methods: The methanolic extract of *N. macrophylla* leaves and its n-butanol and ethylacetate fractions were screened *in-vitro* for their antimicrobial activity using agar well diffusion technique while the antibacterial and antibiofilm activity of the extract and its fractions were investigated against clinical isolates of *S. aureus* and *P. aeruginosa* using microbroth dilution technique and microtiter plate method respectively.

Results: The methanolic leaf extract and its fractions exhibited substantial antimicrobial activity. The n-butanol and ethylacetate fractions showed highest activity against *P. aeruginosa* and *S. aureus* respectively. The MIC and MBC of the extract and its fractions against both *S. aureus* and *P. aeruginosa* ranged from 3.125-37.5 mg/ml and 6.25-75 mg/ml respectively. In addition, a concentration-dependent antibiofilm activity against the test organisms was also observed with the ethylacetate fraction exhibiting the highest antibiofilm activity.

Conclusion: The extracts and fractions of *N. macrophylla* exhibited remarkable antibacterial and antibiofilm activities against *S. aureus* and *P. aeruginosa*. The plant thus can be considered as a potential source of bioactive principles in the continuous fight against bacterial virulence and resistance.

Keywords: *Neocarya macrophylla*, Antimicrobial activity, Antibiofilm activity, Antimicrobial resistance.

INTRODUCTION

The emergence of antibiotic resistant strains of bacteria dampened the initial optimism that followed the discovery of antibiotics (Lowy, 2003; O'Neill, 2014). Antibiotic resistance was reported immediately after successful use of antibiotics for treatment of infections (Davies and Davies, 2010). In some cases, the resistance development predated the introduction of antibiotics in medicine (Bradford, 2001; Dzidic *et al.,* 2008; Cundliffe *et al.,* 2010; Davies *et al.,* 2010). While antibiotics resistance can occur naturally even with the most appropriate use of the antibiotics, several anthropogenic activities such as the use of antibiotics as feed and growth promoter in agriculture and aquaculture, indiscriminate discharge of industrial wastes containing heavy metals into the environment, disposal of millions of tons of manufactured antibiotics and their metabolites from human excreta into the environment, facilitate its emergence and spread (Davies *et al.,* 2010; O'Neill, 2014; WHO, 2014).

Staphylococcus aureus is one of the most common causes of healthcare and community-associated infections (Taddesse *et al.,* 2014). It is inherently susceptible to several antibiotics except those with purely Gram-negative spectrum (Lindberg *et al.,* 2004). However, acquisition of resistant genes has led to the emergence of multidrug resistant *S. aureus* including methicillin resistant *S. aureus* (Stefani *et al.,* 2010; Frieri *et al.,* 2017).

Methicillin Resistant *Staphylococcus aureus* (MRSA) is a clinically important pathogen posing a serious public health threat in both community and hospital settings (Lowy, 2003; WHO, 2014; Mottola *et al.,* 2016). Methicillin Resistant *Staphylococcus aureus* (MRSA) has been implicated as a causative agent in many skin and wound infections, pneumonia, blood stream infections (BSI), meningitis and toxic shock syndrome (TSS) (WHO, 2014; Jo *et al.,* 2016; Mottola *et al.,* 2016).

Among the Gram-negative bacteria, *Pseudomonas aeruginosa* is remarkable with intrinsic resistance to several classes of antimicrobial agents (Strateva *et al.,* 2009). It is also equipped with a number of mechanisms for horizontal acquisition of antibiotic resistant genes (Strateva *et al.,* 2009). Extensively and Pan-drug resistant strains of *P. aeruginosa* have been reported (Livermore, 2002; CDC, 2013; Mutalib *et al.,* 2015)..

Furthermore, the resistance of some strains of bacteria to antibiotics and persistence of bacterial infections is enhanced by their ability to form biofilms (Araujo *et al.,* 2015). Biofilm is a surface-associated behaviour of bacteria in which bacteria grow and attach to each other and to the substratum and are being protected by the Extra-cellular Polymeric Substance (EPS) (Kouidhi *et al.,* 2015; Brambilla *et al.,* 2017; Das *et al.,* 2017). This enables single cell organisms to assume a multicellular lifestyle thereby facilitating survival of the organism in an adverse environment (Kostakioti *et al.,* 2013; Oliver *et al.,* 2015). Biofilms serve as a physico-chemical barrier, protecting the embedded cells from external stress and elements of the hosts immune system (Maggs *et al.,* 2015; Brambilla *et al.,* 2017). As against its planktonic counterpart, bacteria exhibiting biofilm mode of growth show remarkable recalcitrance towards antibiotics (Kouidhi *et al.,* 2015). The resistance of biofilm-forming bacteria can be as much as 100-1000 times higher than that of their planktonic counterparts (Maggs *et al.,* 2015). This phenomenon, named "recalcitrance of biofilm bacteria toward antibiotics", is a complex process of serious clinical concern as the infections become difficult to manage often leading to chronic infections.

In the last decades, amidst increasing threat of resistance of bacteria to many of the existing antibiotics and the difficulty in eradicating biofilm, interest in the discovery and development of novel antimicrobials saw a huge decline (Kanaan *et al.,* 2017). However, with the problem of antimicrobial resistance being recognized as a global health emergency, there is now a renewed interest in antimicrobial drug discovery with natural products offering hope for novel lead compounds (Toner *et al.,* 2015; Piper *et al.,* 2016).

Since prehistoric time, the medicinal properties of plants have been exploited to treat and prevent multitude of health problems in different population throughout the world (Raskin *et al.,* 2002; Halberstein, 2005). Notwithstanding the concern about toxicity, lack of standardization in preparation and dosing, contamination and potential drug or food interactions, the use of plants for medicinal purposes is still prevalent particularly in developing countries where about 80% or more of the populace lack access to conventional pharmacotherapy (Halberstein, 2005; Gbonjubola *et al.,* 2014). Nigeria's diverse flora offers a wide spectrum of medicinal plants (Mann *et al.,* 2009). Several of which have been demonstrated to possess varying degree of antimicrobial activity (Garba *et al.,* 2012; Opara *et al.,* 2015; Nduche *et al.,* 2016).

Neocarya macrophylla is a shrub or tree, commonly called gingerbread plum or neou oil tree belonging to the family Chrysobalanaceae (Sirajo, 2015; Yusuf, *et al.,* 2015). It is well distributed in many coastal Savanna region of Africa, including Nigeria (Yusuf *et al.,* 2015). In most parts of Northern Nigeria, the plant has been used for many years in the treatment

of snakebites, skin infections, cancer, asthma, diarrhea, dysentery, tooth decay, pain and inflammation (Yusuf *et al.*, 2015). In 2005, Audu *et al.* reported the susceptibility of *S. aureus* and other important human pathogens to the hexane, ethyl-acetate and methanolic extracts of the plant (Audu *et al.*, 2005). This finding was recently corroborated by other researchers (Yusuf *et al.*, 2015; Isaka *et al.*, 2017). However, despite that biofilm formation is the predominant mode of bacterial growth in nature and its clinical significance in the prognosis of many infections, there is dearth of information on the inhibitory activities of this important medicinal plant against biofilm forming-bacterial isolates. Thus, the present study was designed to investigate the antibacterial and antibiofilm activities of fractions and methanolic extracts of *Neocarya macrophylla* against *S. aureus* and *P. aeruginosa.*

MATERIALS AND METHODS

Collection and Identification of Plant material

The leaves of *Neocarya macrophylla* were collected in October, 2015 at Jega Local Government Area of Kebbi State. They were authenticated at the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University, Zaria, by comparing with herbarium reference voucher specimen (No. 3197). The leaves were shade dried, pulverized to powder, labelled and stored at room temperature for use.

Preparation of Plant material

The powdered leaves (1961 g) was extracted with 70 % methanol using maceration method for 6 days. The extract was evaporated *in-vacuo* using rotary evaporator to yield a green residue (288. 69 g) subsequently referred to as the crude methanol leaf extract (MEL).

Some part of MEL (200 g) was suspended in distilled water, filtered and partitioned successively with n-hexane (1 L), chloroform (1 L), ethylacetate (2.5 L) and n-butanol (2.5 L) to obtain hexane (HFL), chloroform (CFL), ethylacetate (EFL), n-butanol (BFL) and the residual aqueous (AFL) fractions, respectively. MEL, EFL and BFL were used for the study.

Bacteria strains

The bacterial isolates used in this study were obtained from the Department of Medical Microbiology, Usmanu Danfodiyo University Teaching Hospital, Sokoto. The bacterial isolates were *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The isolates were identified using conventional biochemical tests and were maintained on nutrient agar slant in a refrigerator at 4°C.

Antimicrobial Screening

Antimicrobial screening was carried out using the agar diffusion method as previously described (Mutalib *et al.*, 2015). Briefly, the suspension of the test organism in sterile saline [0.85% NaCl (w/v)] photometrically adjusted to an optical density of 0.1 at 620 nm (equivalent to 0.5 McFarland standard turbidity) was swabbed evenly over an entire surface of Mueller Hinton agar plate. Then, 0.1 ml of the extract and its fractions were added into wells bored with a standard sterile 6 mm cork borer while ofloxacin (10µg) disc that was placed at the centre of the plate served as control. The plates were incubated aerobically at 37ºC for 24 hours. After an overnight incubation, the plates were observed. The zones were measured with a transparent ruler and recorded. The experiments were conducted in triplicate.

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

Minimum Inhibitory Concentration (MIC) was determined by the microbroth dilution method using serially diluted plant extracts according to the CLSI guideline (CLSI, 2015). The extract and fractions of *N. macrophylla* was diluted in 5% DMSO and a 100µl aliquot added into wells of microtiter plate containing 100µl of Mueller Hinton broth. This was serially diluted to give final concentrations ranging from 150 mg/ml to 1.17 mg/ml. Then, 100µl of a 5×10^5 CFU/ml suspension of the two test organisms was afterwards added into each well of microtiter plate. The plates were covered and incubated aerobically at 37ºC for 18-24 hours. The negative control consisting the broth and bacterial cell suspension without the extract and the blank control containing only the medium were maintained for each test. After overnight incubation at 37°C, absorbance was measured at 600 nm using a microtiter plate reader (Optic Ivymen System, Model 2100C, Bioteck SL, Madrid, Spain). The MIC end-point was defined as the lowest concentration of the test agent that produced at least 90% reduction of absorbance in comparison with the negative control as previously described (Teanpaisan *et al.*, 2014). All experiments were performed in triplicate and the average values were reported as MIC.

The minimal bactericidal concentration (MBC) was defined as the lowest concentration of the test agent that did not allow visible growth when 10 µl culture

from the wells with no visible growth was sub-cultured into a freshly prepared Mueller Hinton agar and grown 24-48 h at 37 °C.

Evaluation of Antibiofilm Activity

The inhibitory effect of the extract and its fractions on the biofilm biomass of the test organisms was investigated by the microtiter plate method as previously described (Araujo *et al.,* 2015). Two-fold serial dilutions of the plant extracts were made and added into sterile 96 wells microtiter plates containing 50 µl of sterile trypticase soy broth supplemented with 0.5% glucose already inoculated with 50 µl of an overnight culture of the test organisms standardized to an optical density of 0.1 at 620 nm (equivalent to 0.5 McFarland standard turbidity). Positive control (bacterial suspension in broth) and negative control (extract in broth) were maintained for each concentration. Following an incubation at 37°C for 24 hours, the content of each well was gently removed by tapping the plates. The wells were washed with 200 µl of sterile distilled water to remove free floating planktonic bacteria. Biofilms formed by adherent cells in plate was stained with 0.1% crystal violet and incubated at the room temperature for 30 minutes. Excess stain was rinsed off by thorough washing with distilled water and plates were then fixed with 200 µl of ethanol 70% and glacial acetic acid. Optical densities (OD620 nm) of stained adherent bacteria was then measured using an ELISA microplate reader and the results expressed as percentage inhibition of biofilm formation by comparing the OD of the adhered cells with the optical density of the negative control.

Statistical analysis

Statistical analysis of the results obtained was done with the GraphPad Prism® 7.0 software (GraphPad Sofware Inc.). All data were expressed as means ± standard errors of triplicate measurements.

RESULTS

The screening of the extract and fractions of *N. macrophylla* against two important human pathogens showed that the extracts and its fractions exhibited significant antimicrobial activity (Table 1). Among the three fractions tested, n-butanol (18.25±1.1) exhibited the highest activity against *P. aeruginosa* while the least activity was observed in the ethyl-acetate fraction (16.00±0.70). *Staphylococcus aureus* however was most susceptible to inhibitory effect of ethyl acetate fraction (21.00±1.40) followed by n-butanol fraction while the least activity was observed in methanol extract. The activity of the extracts and its fractions were comparatively lower than what was observed for ofloxacin (positive control).

The MIC and MBC of the extracts and its fractions against both *S. aureus* and *P. aeruginosa* ranged from 3.125-37.5 mg/ml and 6.25-75 mg/ml respectively. As shown in Table 2, *P. aeruginosa* was the most susceptible.

The extracts and its fractions exhibited remarkable antibiofilm activity against the test organisms (Figures 1, 2 and 3). Similar to the antibacterial activity of the extracts and its fractions, the anti-biofilm activity was also concentration dependent. The ethyl-acetate and n-butanol fractions exhibited the highest biofilm inhibition activities against *S. aureus* (92%) and *P. aeruginosa* (80.33%) respectively at 150 mg/ml.

Table 1: Antibacterial activity of the extracts and fractions of *N. macrophylla*

		Zone of inhibition (mm)			
Bacteria strains	Fractions	200 mg/ml	100 mg/ml	50 mg/ml	Ofloxacin 10µg
P. aeruginosa	MEL	17.50±0.70	16.00±0.70	15.00±0.70	20.25±0.35
	NBF	18.25±1.1	16.75±1.10	15.50±0.70	22.00±1.40
	EAF	16.00±0.70	14.50±0.70	12.50±0.70	20.50±0.70
S. aureus	MLE	14.00±0	13.00±0.14	12.50±0.70	29.00±1.40
	NBF	16.50±0.70	14.00±0.00	12.00±0.00	20.00±0.00
	EAF	21.00±1.40	20.00±0.00	17.00±1.40	26.50±0.70

Key: MLE, Methanol Leaf Extract; NBF, n-butanol fraction; EAF, Ethyl acetate fraction

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts and fractions of *N. macrophylla*

Test Organisms	Fractions	MIC (mg/ml)	MBC (mg/ml)
P. aeruginosa	MLE	3.13	6.25
	NBF	4.69	18.75
	EAF	4.69	18.75
S. aureus	MLE	25.00	50.00
	NBF	37.50	75.00
	EAF	18.75	18.75

Key: MLE, Methanol Leaf Extract; NBF, n-butanol fraction; EAF, Ethyl acetate fraction

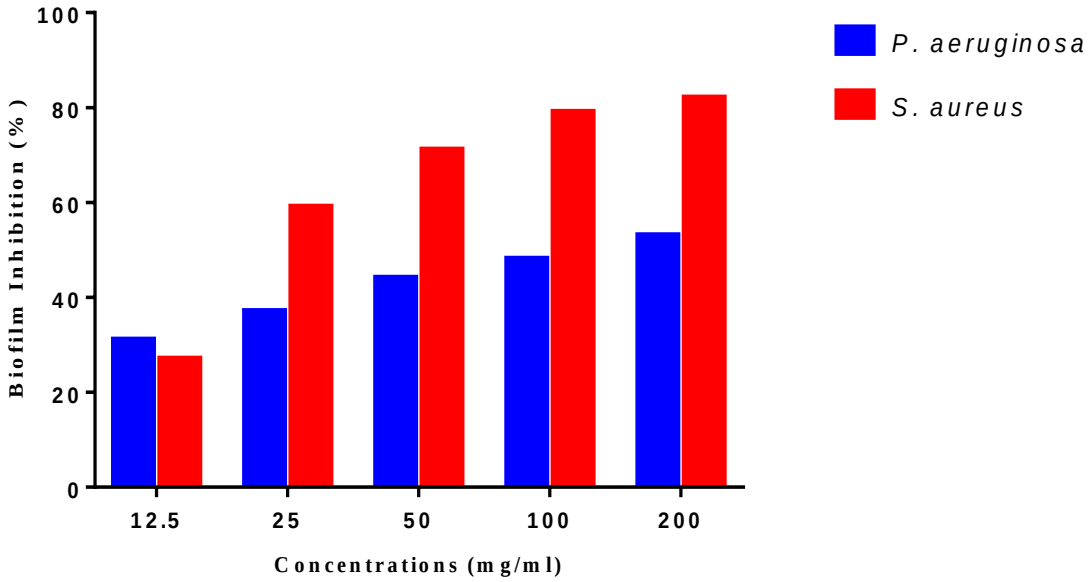

Figure 1: The anti-biofilm activity of methanol fraction of *N. macrophylla* leaves against *P. aeruginosa* and *S. aureus*

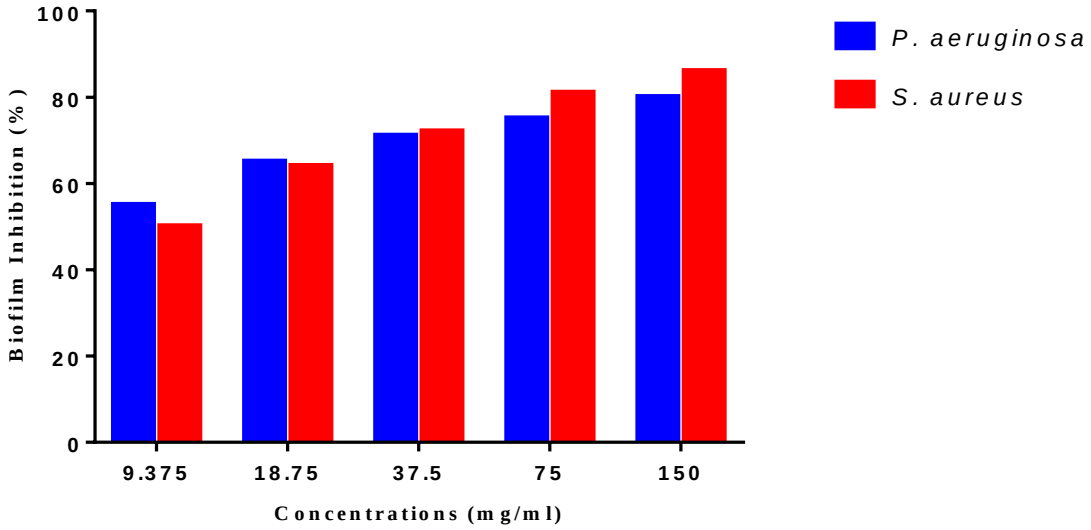

Figure 2: The anti-biofilm activity of N-butanol fraction of *N. macrophylla* leaves against *P. aeruginosa* and *S. aureus*

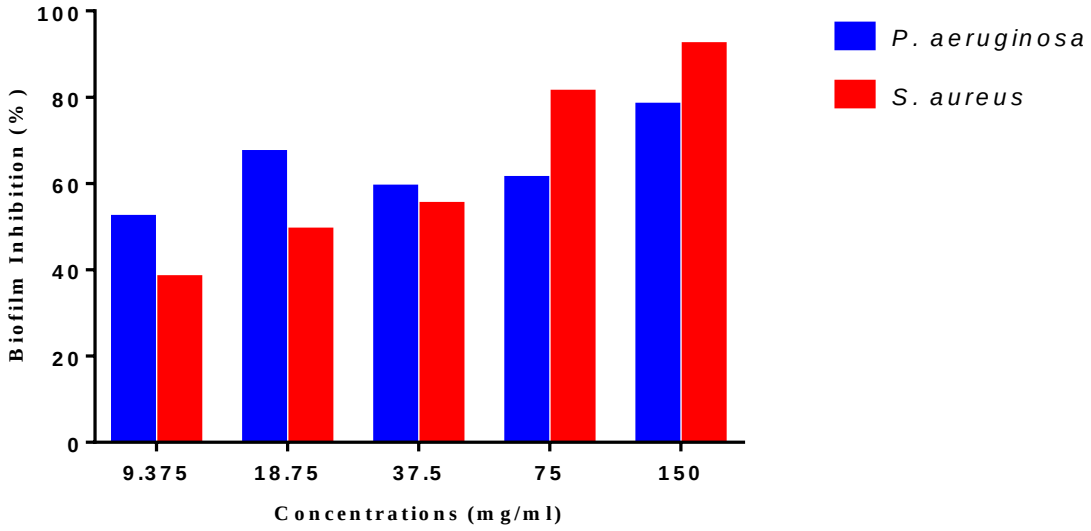

Figure 3: The anti-biofilm activity of ethylacetate fraction of *N. macrophylla* leaves against *P. aeruginosa* and *S. aureus*

DISCUSSION

The marked antibacterial activity displayed by the extracts and fractions of *N. macrophylla* leaves against *S. aureus* and *P. aeruginosa* corroborated the results of the previous studies on the extracts and fractions from stem-bark and fruits of the plant. It thus validates the efficacy of the plant as an ethnomedicinal remedy in combating a number of infections in Northern Nigeria (Yusuf *et al.*, 2015; Isaka *et al.*, 2017).

Similar to the findings of this study, Peni *et al.* (2010) has reported higher susceptibility of *S. aureus* to the stem-bark extract of *Parinari curatellifolia* (a member of Chrysobalanaceae family). The higher susceptibility of *S. aureus* to the inhibitory effect of

N. macrophylla observed in this study may be due to the differences in the organisation of cell envelope of Gram-positive and Gram-negative bacteria (Selim *et al.*, 2014). The higher resistance of Gram-negative bacteria has been ascribed to the low permeability of phospholipidic Gram-negative cell envelope to lipophilic compounds (Selim *et al.*, 2014).

Previous phytochemical studies on this plant have revealed the presence of saponins, tannins, flavonoids, alkaloids, glycosides, steroids and triterpenes (Sirajo, 2015; Yusuf *et al.*, 2015; Isaka *et al.*, 2017). These phytochemicals have been demonstrated in several literatures to exert antimicrobial activities via a number of diverse mechanisms like intercalation of DNA, destruction of cell membrane, inactivation of microbial adhesions and enzymes (Selim *et al.*, 2014; Dar *et al.*, 2016). The antibacterial properties of this plant may therefore be attributed to the presence of these secondary metabolites.

To the best of our knowledge, this is the first report on the antibiofilm activity of *N. macrophylla.* The methanolic extract and its fractions exhibited antibiofilm activity at all the tested concentrations. The concentration-dependent antibiofilm activity reported in this study is consistent with the report of Teanpaisan *et al*., (2014). However, it is in contrast to the finding of Damiano and colleagues where sub-MIC concentrations of secondary metabolites from *Ziziphus jujuba* leaves was shown to exhibit excellent antibiofilm activities (Damiano *et al.*, 2017).

Since control of biofilms through eradication of established biofilm is difficult, prevention of its formation through the interference with the various stages of its formation is a good alternative strategy (Brambilla *et al.*, 2017). The significant inhibitory effect of the extract and fractions of *N. macrophylla* on the biofilm formation by the two test organisms may be due to interference with the first stage of biofilm formation, the cell adhesion stage (Romero *et al.*, 2016). From the finding of this work, the extract from the *N. macrophylla* and its fractions have shown promise for use in the management of biofilm related infections as a significant proportion of existing antibiotics are ineffective against bacteria embedded in biofilms.

This result provides a baseline data on the antibiofilm activity of the plant and it may serve as a basis for further studies on the acute toxicity, compound characterization and investigation of the antimicrobial and antibiofilm of secondary metabolites from other parts of the plant. Also, formulation of the most active fractions into various dosage forms for application in medical practice should be considered in future studies.

CONCLUSION

This study revealed remarkable antibacterial and antibiofilm activity of *N. macrophaylla* against *S. aureus* and *P. aeruginosa.* The plant thus can be considered as a potential source of bioactive principles in the continuous fight against bacterial virulence and resistance.

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