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Original Article

Anti-biofilm activity against *Staphylococcus aureus* MRSA and MSSA of neolignans and extract of *Piper regnellii*

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ABSTRACT

Many infections worldwide are associated with bacterial biofilms. The effects of isolated neolignans (conocarpan and eupomathenoid-5) and the dichloromethane extract of *Piper regnellii* (Miq.) C. DC., Piperaceae, were tested against isolates of methicillin-resistant *Staphylococcus aureus* and methicillin-sensitive *S. aureus* biofilms and *S. aureus* planktonic cells. The dichloromethane extract presented better results than isolated neolignans against all of the biofilms tested, with a minimum inhibitory concentration <400 μg/ml for preformed biofilms and minimal biofilm inhibitory concentration of 15.6 μg/ml for biofilm formation. The minimum inhibitory concentration to planktonic cells was <12.5 μg/ml. These results indicate a good effect of the dichloromethane extract against methicillin-resistant *S. aureus* and methicillin-sensitive *S. aureus* biofilms and efficient prophylaxis.

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Introduction

Infections that are associated with antimicrobial resistance are a primary challenge in public health, resulting in high rates of morbidity and mortality, increased length of hospitalization, and higher healthcare costs (Neidell et al., 2012). According to the National Institutes of Health (2002), approximately 80% of all infections worldwide are associated with biofilms, especially those that involve biomaterials.

Biofilms are communities of microorganisms that are embedded in an extracellular matrix that is composed of proteins, lipids, polysaccharides, and nucleic acids. The members of a biofilm are protected from environmental factors (*e.g.*, ultraviolet light and dehydration) and host immune cells (*e.g.*, neutrophils and other phagocytes) (Hall-Stoodley et al., 2004). Biofilm-associated bacteria are also much more resistant to antimicrobial agents (Stewart and Costerton, 2001).

Studies that investigate biofilm physiology are important. Searching for new strategies to control this complex mode of bacterial life is extremely challenging (Trentin et al., 2013). Plants with antibacterial activity, such as the genus *Piper*, have shown promise.

Piper regnellii (Miq.) C. DC., popularly known in Brazil as "pariparoba," is an herbaceous plant found to exist in tropical and subtropical regions of the world (Cronquist, 1981). Leaves and roots are used as crude extracts, infusions or platers to treat wounds, reduction of swellings and skin irritations (Corrêa, 1984). Extracts of the leaves of *P. regnellii* have shown good antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* (Pessini et al., 2003) and antifungal activity against the yeasts *Candida albicans*, *Candida krusei*, and *Candida parapsilosis* (Pessini et al., 2005). The extract of the leaves and neolignan eupomatenoid-5 have also shown activity against methicillin-resistant *S. aureus* (Marçal et al., 2010). Neolignans that are isolated from the leaves of *P. regnellii* also have activity against the parasites *Trypanosoma cruzi* (Luize et al., 2006) and *Leishmania amazonensis* (Vendrametto et al., 2010).

Because of the reported effect of *P. regnellii* against *S. aureus*, the aim of the present study was to test a dichloromethane extract of *P. regnellii* and isolated neolignans (conocarpan and eupomathenoid-5) against methicillin-resistant *S. aureus* biofilms and methicillin-sensitive *S. aureus* biofilms that were obtained from clinical isolates.

Materials and methods

Plant material

Leaves from *Piper regnellii* (Miq.) C. DC., Piperaceae, were collected in June 2012 in the Prof[a]. Irenice Silva Medicinal Plant

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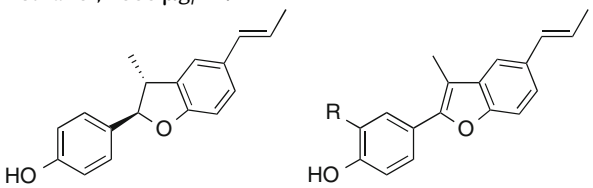
Garden on the campus of the Universidade Estadual de Maringá and identified by Marilia Borgo of the Botany Department of the Universidade Federal do Paraná. A voucher specimen (HUM 8392) was deposited in the Herbarium of the Universidade Estadual de Maringá, Paraná, Brazil.

HPLC analysis

The analyses were carried out using a Waters Binary HPLC Pump 1525, equipped with UV-VIS detector 2489, an autosampler 2707 with a 20 μl loop, and controlled by Breeze 2 Software. Chromatographic separations were carried out in a Phenomenex ODS (C18) Luna column, 5 μm, 250 × 4.6 mm, maintained at room temperature, in an isocratic system, using acetonitrile–water acidified with 2% acetic acid (70:30, v/v) with a flow rate of 1 ml/min. The detection was carried out at 280 nm and the run time was 20 min. Conocarpan (**1**), eupomathenoid-6 (**2**) and eupomathenoid-5 (**3**) were quantified by external standardization, in method previously validated being linear, precise and accurate. Samples were diluted in methanol, 1000 μg/ml.



Isolation of the constituents

Dried and powdered leaves of *P. regnellii* (300 g) were extracted by maceration with ethanol:water (9:1) at room temperature in a leaf:solvent ratio of 1:10 (w/v). The solvent was then removed under vacuum at 40 °C to give an aqueous extract and dark green residue that was washed with dichloromethane, yielding 24 g of the dichloromethane extract (DE), and dried at room temperature, with yield of 8.2%. The DE (12 g) was subjected to vacuum column chromatography with silica gel (230–400 mesh) and eluted with hexane (1000 ml), dichloromethane (1400 ml), ethyl acetate (1000 ml), acetone (700 ml), and methanol (1000 ml). The hexane fraction resulted in the isolation of eupomathenoid-5 (**3**, 1.05 g). The dichloromethane fraction (6 g) was chromatographed by column chromatography on silica gel 60 (230–400 mesh) with hexane:dichloromethane:ethyl acetate (12:7:1, v/v/v) to yield conocarpan (**1**, 0.53 g) and eupomathenoid-6 (**2**), but in very small quantity, 0.002 g. The column chromatography procedure was monitored by thin layer chromatography (TLC) and used as the mobile phase, hexane:dichloromethane:ethyl acetate (12:7:1) and vanillin sulfuric 2%. Structures were identified by spectroscopy (UV, ^{1}H NMR, ^{13}C NMR, H–H COSY, gNOE, HETCOR, HMBC) and comparisons with the literature (Achenbach et al., 1987; Chauret et al., 1996; Snider et al., 1997).

Bacterial strains

The organisms used in this study were obtained of laboratory collection and were originated and identified in University Hospital of Maringá. Ten clinical isolates of methicillin-resistant *S. aureus* (MRSA), strains 72, 73, 74, 76, 77, 78, 79, 81, 83 and 90 (with strains 72 and 73 obtained from blood and strains 74, 76, 77, 78, 79, 81, 83 and 90 obtained from secretion), and three clinical isolates of and methicillin-sensitive *S. aureus* (MSSA), strains 97, 170, and 212, obtained from urine (Marçal et al., 2010). It was also used *S. aureus* ATCC 25923. Test strains were preserved in glycerol 10% at −80 °C and were cultured on nutrient agar and incubated for 24 h at 37 °C prior to determination of the minimum inhibitory concentration (MIC).

Antibacterial susceptibility testing

The MIC of conocarpan, eupomathenoid-5, and the DE were determined by a microdilution method in sterile flat-bottom microplates according to CLSI (2012) using Mueller-Hinton broth (Merck S.A., São Paulo, Brazil). Inoculates were prepared in the same medium at a density that was adjusted to a 0.5 McFarland turbidity standard (10^{8} colony-forming units [CFU]/ml) and diluted 1:10 for the broth microdilution procedure. Conocarpan, eupomathenoid-5 and DE were diluted and transferred into the first well, and serial dilutions 1:2 were performed so that concentrations in the range of 100–1.56 μg/ml were obtained. Vancomycin was used as the reference antibacterial control in the range of 50–0.8 μg/ml. Positive control of strains (without presence of drugs) and negative control (with medium solely) was performed. Microtiter trays were incubated at 37 °C, and the MICs were recorded after 24 h of incubation. Three susceptibility endpoints were recorded for each isolate. The MIC was defined as the lowest concentration of the compounds at which the microorganism did not exhibit visible growth. The minimum bactericidal concentration (MBC) was performed in Mueller-Hinton agar, incubated at 37 °C during 24 h. MBC was defined as the lowest concentration that yielded negative subcultures or only one colony. The *in vitro* results for the drugs were classified as the following. MIC <100 μg/ml (good antibacterial activity), MIC = 100–500 μg/ml (moderate antibacterial activity), MIC = 500–1000 μg/ml (weak antibacterial activity), and MIC >1000 μg/ml (no activity) (Pessini et al., 2003).

In vitro *preformed biofilm assay*

Biofilms of *S. aureus* ATCC 25923, 10 clinical isolates of MRSA, and three clinical isolates of MSSA were formed on polystyrene 96-well microtiter plates. A 100 μl suspension that contained 10^{8} cells/ml in TSB medium with 1% glucose was seeded in wells and incubated at 37 °C for 24 h. The well content was discharged, and the wells were washed with phosphate-buffered saline (PBS). Several dilutions of the DE, conocarpan, eupomathenoid-5, and vancomycin (standard reference drug) (15.6–1000 μg/ml) were then added to each well. After incubation at 37 °C for 24 h, the wells were rinsed with PBS. The MTT reduction assay was performed to evaluate the viability of the biofilms. Three independent assays were performed. The MTT reduction assay was a slight modification of the method reported by Schillaci et al. (2008). The MTT solution (50 μl; 2 mg/ml in PBS) was added to each well, and the plates were incubated at 37 °C for 2 h. After staining, the MTT solution was removed from each well, and 100 μl of dimethylsulfoxide (DMSO) was added to dissolve the MTT formazan product. The DMSO (100 μl) was transferred to a new plate, and the optical density was measured at 570 nm using a microplate reader. Positive control of biofilm formation (without treatment) and negative control (with medium solely) was performed. The results are expressed as the MIC_{50}, at which 50% of the sessile *S. aureus* cells were inhibited compared with the control (without treatment) for the MTT assays.

Inhibiting biofilm formation in vitro

This assay investigated the drugs ability to prevent biofilm formation. Briefly, 100 μl of drug (DE, conocarpan and eupomathenoid-5) at different concentrations (15.6–1000 μl/ml) and standard drug, vancomycin in concentration 7.8–500 μl/ml in sterile TSB medium with 1% glucose was added to selected wells of

a 96-well microtiter plate, seeded with 100 μl of a suspension that contained 10^8 cells/ml, and incubated for 24 h at 37 °C to allow biofilm formation. The contents of the wells were aspirated and washed three times in sterile PBS. The extent of biofilm formation was assessed by the MTT reduction assay as described above. The results are expressed as the minimal biofilm inhibitory concentration (MBIC) compared with the control (without treatment) for the MTT assays.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on glass coverslips by dispensing 400 μl of the standardized cell suspensions that contained 1.0×10^8 cells/ml of TSB supplemented with 1% glucose into the wells of 24-well flat-bottomed polystyrene plates. To view preformed biofilm, the plates were incubated at 37 °C for 24 h. After washing with PBS, conocarpan (110 μg/ml) and the DE (370 μg/ml) were added to preformed biofilms, and the plates were incubated for 24 h at 37 °C. To view biofilm formation, standardized cell suspensions and drug (15.6 μg/ml conocarpan, 31.5 μg/ml eupomathenoid-5, 15.6 μg/ml DE, and 7.8 μg/ml vancomycin) were added and incubated at 37 °C for 24 h. The coverslips were then washed twice with PBS and fixed with 2.5% glutaraldehyde overnight at 4 °C. The coverslips were then washed twice with 0.1 M cacodylate buffer for 15 min and dehydrated by replacing the buffer with increasing concentrations of ethanol (30, 50, 70, 80, 90, 95, and 100%) for 10 min each. After critical-point-drying and coating with gold sputter, the samples were examined with a scanning electron microscope, in Shimadzu SS-550 (Probe 4.0 and AccV 15.0 V).

Results and discussion

Isolation of the constituents

The extraction of *P. regnellii* (Miq.) C. DC. resulted in an 8.2% yield of dichloromethane extract. From this extract, the use of silica gel chromatographic columns and a gradient system with increasing degrees of polarity allowed the isolation of two major compounds that were present in the extract: conocarpan (**1**) and eupomathenoid-5 (**3**), and eupomathenoid-6 (**2**) in minor amount, not allowing use in biological assays. Conocarpan, with stereochemistry (+)-conocarpan is the most abundant in nature, produced naturally in plants of several families, and these molecules exhibit a trans-dihydrobenzofuran heterocycle as a key structural element (Chen and Weisel, 2013). These substances were isolated and identified by comparing spectroscopic data of ^{1}H and ^{13}C NMR spectra with those previously published (Achenbach et al., 1987; Chauret et al., 1996; Snider et al., 1997).

HPLC analysis

Fig. 1 shows dichloromethane extract chromatogram. HPLC analysis allowed the neolignans quantification. Dichloromethane extract DE was standardized in 135.32 μg/ml of conocarpan (**1**), 91.90 μg/ml of eupomathenoid-6 (**2**) and 106.98 μg/ml of eupomthenoid-5 (**3**).

Antibacterial effect in planktonic cells

The MIC results obtained in the study are shown in Table 1. The dichloromethane extract had good activity against MRSA and MSSA, with MIC <15 μg/ml and MBC <30 μg/ml. These values were very similar to those reported by Marçal et al. (2010). Conocarpan also had good activity against MRSA and MSSA (Table 1), with a maximum MIC = 50 μg/ml and maximum MBC = 100 μg/ml. Eupomathenoid-5 had a MIC ≤6.25 μg/ml and MBC <25 μg/ml.

The DE and isolated neolignans were tested against the standard strain of *S. aureus*, with a MIC <15 μg/ml and MBC <30 μg/ml (Table 1). These values are similar to those reported in the literature (Pessini et al., 2003; Felipe et al., 2008).

Antibacterial effect in preformed biofilm

Resistance mechanisms of biofilms are multifactorial and depend on each organism. Although not fully understood, these mechanisms can be attributed to such factors as a reduction of the penetration of antibiotics through the biofilm matrix, the presence of slow-growing or non-growing cells in the biofilm, a heterogeneous bacterial population with the presence of phenotypic subpopulations with different levels of resistance, and the persistent presence of cells (Mah and O'Toole, 2001; Harrison et al., 2005).

Table 1 shows good activity of the isolate neolignans against planktonic cells, but the activity against the biofilm was not the same (Table 2). Euphomatenoid-5 showed no activity against *S. aureus* ATCC 25923 biofilm. However, conocarpan and the DE had a satisfactory MIC_{50} against the biofilm, 110 and 370 μg/ml, respectively (Table 2). These MTT assay results demonstrate the metabolic activity of the cells in the biofilm, which was confirmed by SEM (Fig. 2).

The photomicrographs in Fig. 2A show the control biofilm with a large amount of adhered cells and an extracellular matrix. The treated biofilms (Fig. 2B and C) presented cellular lysis regions and biofilm detachment, suggesting the good action of conocarpan and the DE against preformed biofilms.

Although the concentration of conocarpan that was required to combat the biofilm was less than the concentration of the DE, the isolated substance showed no activity against clinical isolates of MRSA and MSSA biofilms (Table 2). The dichloromethane extract was effective against all of the tested clinical isolates of MRSA and MSSA biofilms (MIC_{50} < 400 μg/ml). In many parts of the world, MRSA is responsible for a high rate of community- and hospital-acquired *S. aureus* infections. The most important classes of antibiotics that are used to prevent and treat *S. aureus* infection are ineffective (Köck et al., 2010). In the present study, dichloromethane extract of *P. regnellii* had better activity against *S. aureus* biofilms.

Antibacterial effect in inhibition biofilm formation

An alternative to the control of biofilm formation is to prevent colonization. Thus, we evaluated the ability of different concentrations of the DE, conocarpan, and eupomathenoid-5 to inhibit the formation of biofilms when added to the medium at the same time as the cells. The results were compared with vancomycin. If an agent is added at the beginning of the experiment, then the agent might act before the biofilm is formed and inhibit its development. This could be of interest for combating recalcitrant infections (Khan and Ahmad, 2012). Concentrations as low as 15.6 μg/ml resulted in a significant reduction of the metabolic activity of adherent cells, with >95% inhibition of MRSA and MSSA biofilm formation compared with untreated control cells (Table 3). The DE had results that were very close to vancomycin for the inhibition of *S. aureus* biofilm formation.

These results were confirmed by SEM. Fig. 3 shows the positive control with a large number of cells that adhered to each other and the presence an extracellular matrix (Fig. 3A). Photomicrographs of cells that were treated with conocarpan (15.6 μg/ml; Fig. 3B), eupomathenoid-5 (31.2 μg/ml; Fig. 3C), and the DE (15.6 μg/ml;

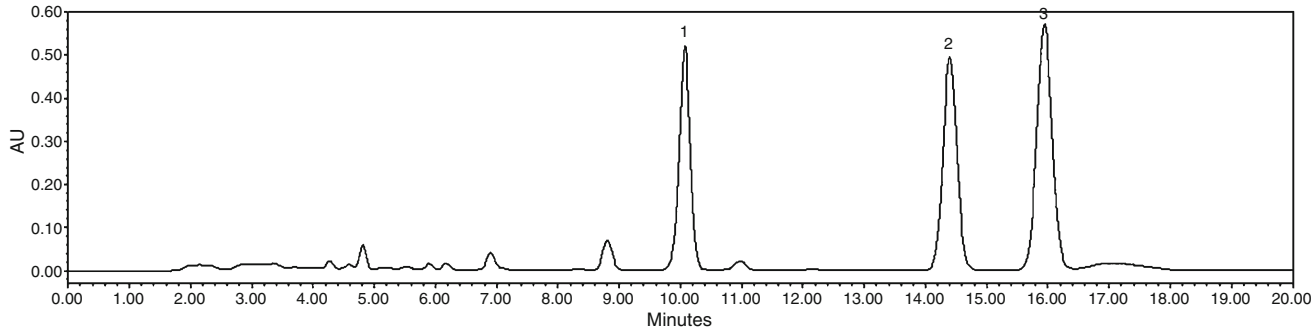


Fig. 1. Chromatographic profile of dichloromethane extract of *Piper regenelli* at 1000 μg/ml. (1) Conocarpan; (2) eupomatenoid-6; (3) eupomatenoid-5. Conditions: mobile phase – acetonitrile:water with 2% acetic acid (70:30); flow: 1 ml/min, detection at 280 nm.

Table 1
MIC and MBC of *Piper regnellii* dichloromethane extract, eupomathenoid-5, conocarpan and Vancomycin in *S. aureus* planktonic cells.

MIC (MBC) μg/ml				
Strains	Dichloromethane extract	Eupomathenoid-5	Conocarpan	Vancomycin
72	12.5 (12.5)	6.25 (25)	50 (100)	1.56 (1.56)
73	12.5 (12.5)	6.25 (25)	50 (100)	1.56 (1.56)
74	6.25 (12.5)	6.25 (6.25)	25 (25)	1.56 (1.56)
76	6.25 (25)	6.25 (12.5)	25 (25)	1.56 (1.56)
77	12.5 (25)	6.25 (12.5)	25 (25)	1.56 (1.56)
78	6.25 (12.5)	3.13 (6.25)	12.5 (100)	1.56 (1.56)
79	12.5 (25)	3.13 (6.25)	25 (25)	1.56 (1.56)
81	12.5 (25)	3.13 (6.25)	25 (50)	1.56 (1.56)
83	6.25 (12.5)	3.13 (6.25)	25 (100)	1.56 (1.56)
90	6.25 (25)	3.13 (6.25)	25 (100)	1.56 (1.56)
97	12.5 (25)	6.25 (6.25)	12.5 (12.5)	1.56 (1.56)
170	12.5 (25)	6.25 (12.5)	12.5 (12.5)	1.56 (1.56)
212	12.5 (25)	6.25 (12.5)	12.5 (25)	1.56 (1.56)
ATCC 25923	6.25 (12.5)	6.25 (12.5)	12.5 (25)	1.56 (1.56)

The MIC results were considered good antibacterial activity (MIC < 100 μg/ml).

Table 2
MIC_{50} of *Piper regnellii* dichloromethane extract, eupomathenoid-5, conocarpan and vancomycin to *S. aureus* preformed biofilms.

MIC_{50} (μg/ml)				
Strains	Dichloromethane extract	Eupomathenoid-5	Conocarpan	Vancomycin
72	300	–	>1000	–
73	170	–	–	–
74	50	–	–	–
76	360	–	–	–
77	390	–	>1000	–
78	180	–	>1000	–
79	190	–	>1000	–
81	210	–	>1000	–
83	230	–	>1000	–
90	130	–	>1000	–
97	260	–	>1000	–
170	260	–	>1000	–
212	370	–	>1000	–
ATCC 25923	370	–	110	–

MIC_{50}, minimal inhibitory concentration at which 50% of the sessile *S. aureus* cells were inhibited.

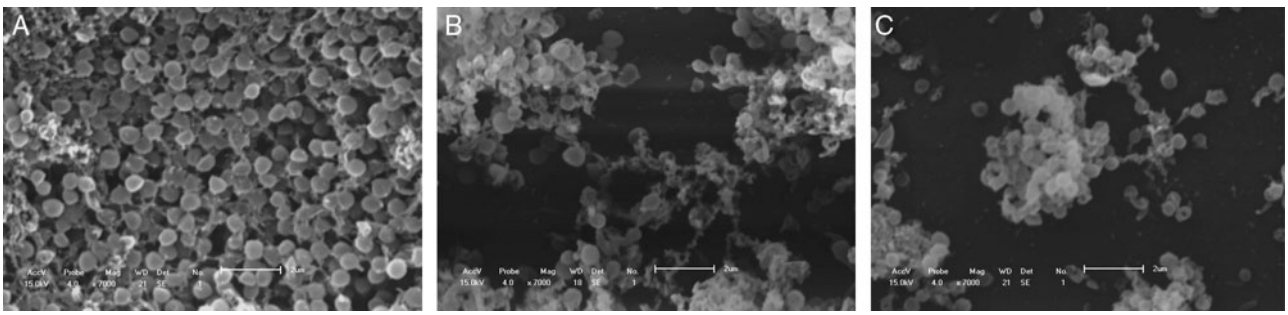


Fig. 2. Scanning electron micrographs of randomly chosen areas of *S. aureus* ATCC 25923 biofilm at 24 h. (A) Positive control. (B) Biofilm treated with 370 μg/ml of dichloromethane extract. (C) Biofilm treated with 110 μg/ml of conocarpan. 7000 magnification.

Table 3
Minimal biofilm inhibitory concentration (MBIC) values for the *Piper regnellii* dichloromethane extract, eupomathenoid-5, conocarpan and vancomycin to *S. aureus* biofilms formation.

MBIC (μg/ml)				
Strains	Dichloromethane extract	Eupomathenoid-5	Conocarpan	Vancomycin
72	15.6	15.6	15.6	7.8
73	15.6	15.6	15.6	7.8
74	15.6	31.2	15.6	7.8
76	15.6	31.2	15.6	7.8
77	15.6	250	125	7.8
78	15.6	15.6	15.6	7.8
79	15.6	15.6	15.6	15.6
81	15.6	15.6	15.6	7.8
83	31.2	31.2	31.2	62.5
90	15.6	15.6	15.6	7.8
97	15.6	15.6	15.6	7.8
170	15.6	15.6	15.6	7.8
212	15.6	15.6	15.6	7.8
ATCC 25923	15.6	31.2	15.6	7.8

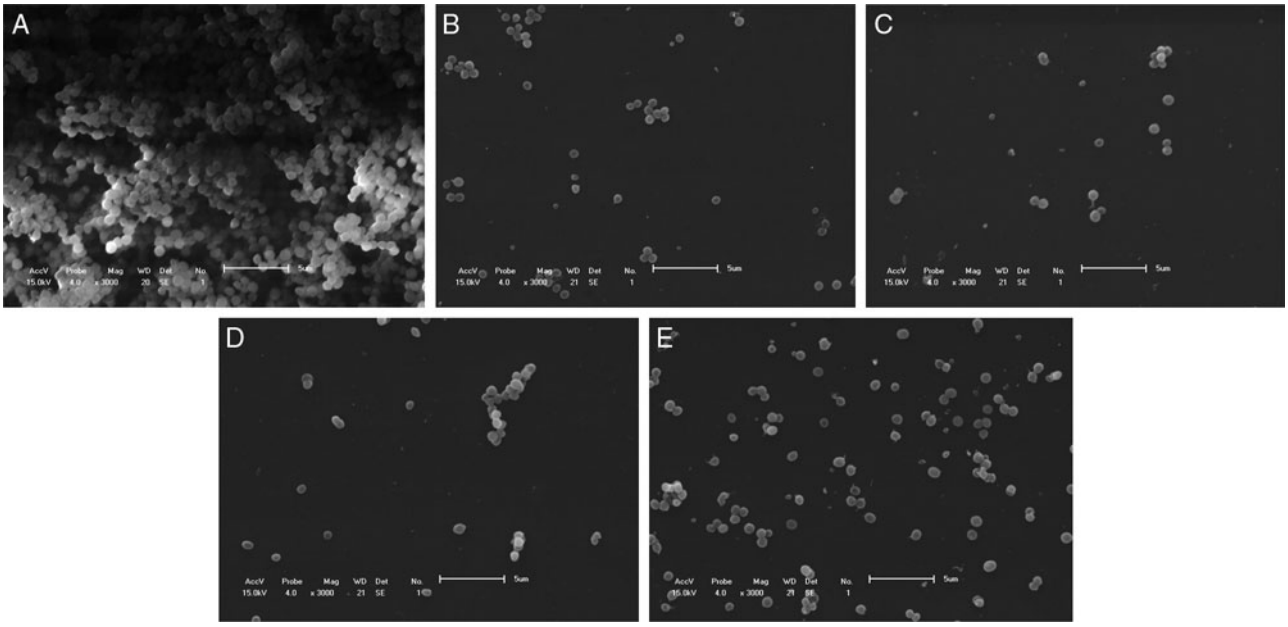


Fig. 3. Scanning electron micrographs of randomly chosen areas of *S. aureus* ATCC 25923 biofilm formation. (A) Positive control. (B) Biofilm treated with conocarpan (15.6 μg/ml). (C) Biofilm treated with eupomathenoid-5 (31.25 μg/ml). (D) Biofilm treated with dichloromethane extract (15.6 μg/ml), € Biofilm treated with vancomycin (7.8 μg/ml). 3000 magnification. This study was performed according to the international, national and institutional rules considering clinical studies and biodiversity rights.

Fig. 3D) showed isolated and fewer cells compared with vancomycin (Fig. 3E).

Biofilm-related infections are an important cause of healthcare-associated infections (Darouiche, 2004). Biofilm-embedded bacteria are challenging to treat because they display tolerance to antibiotics and the host's immune system (John et al., 2011; Leite et al., 2011). Traditional antibiotics that were developed to kill planktonic bacteria often have limited effects on sessile bacteria that are encased within a biofilm. Additionally, the development of antimicrobial resistance is common in sessile bacteria. Thus, there is an urgent need to develop non-antimicrobial treatment strategies to prevent or treat biofilm-associated infections (Kuehn, 2011).

Conclusion

Good results were achieved with the dichloromethane extract of *P. regnellii* (Miq.) C. DC. and isolated neolignans conocarpan and eupomathenoid-5 against planktonic cells of *S. aureus* MRSA and MSSA, similar to the standard drug vancomycin. Similar good effects were found against biofilm formation. The dichloromethane extract and isolated neolignans also showed activity that was close to vancomycin and appeared to be effective as a prophylactic alternative. The DE was also effective against *S. aureus* MRSA and MSSA preformed biofilms. These results support the application of this extract of *P. regnellii* as an alternative agent against biofilm-associated bacterial infections.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Author's contribution

LZSB responsible for isolation of substances and performed inhibitions assays, biofilm formation and preformed biofilm. EHE performed inhibitions assays, biofilm formation and preformed biofilm. DAGC supervised the laboratory work and contributed to the isolation and identification of substances. BPDF supervised the laboratory work and contributed to the biological studies. All the authors have read the final manuscript and approved its submission.

Conflicts of interest

The authors declare no conflicts of interest.

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