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Research Paper

Evaluation of Antimicrobial and Anti-biofilm activities of *Anacardium occidentale* stem bark extract

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ABSTRACT

The antimicrobial and anti-biofilm activities of the cashew tree stem bark hydroethanolic extract (SBHE) were evaluated against clinical isolates of *Staphylococcus* species. We determined the minimal bactericidal concentration (MBC) of the SBHE against planktonic cells and biofilms through macrodilution and microdilution assays, and phytochemical analyses were conducted. The MBC for planktonic cells was seen at 15.2mg/ml, and inhibition of biofilm formation and biofilm eradication were seen at 30.5 and 61.0mg/ml, respectively. Flavonoids, saponins and tannins were detected on the SBHE. Despite the high concentrations, our results are consistent with previous researches and suggest that the SBHE exhibited potential antimicrobial activity, what may be useful for anti-staphylococcal chemotherapy.

Keywords: *Staphylococcus;* Biofilms; *Anacardium occidentale.*

INTRODUCTION

Staphylococcus aureus and *Staphylococcus epidermidis* have been frequently reported in hospital and community-acquired infections (Howden, et al., 2011). Although they are part of the comensal microflora found in skin and mucous membranes, they can be opportunistic pathogens, regarding mostly premature newborns and immunosupressed patients. If inoculation happens, pathogenic staphylococci may adhere to host tissues or implanted foreign bodies like catheters, evade the immune response and multiply, causing severe chronic infections because of virulence factors expression and biofilm formation (Davis, et al., 2005). Biofilms are naturally more resistant to drugs and the immune response, and can attach to biological or abiotic surfaces. Genes like *sar* A

play a crucial role in *S. aureus* biofilm formation, whereas *S. epidermidis* tend to be more dependent on operon *ica* ADBC transcription, which encodes the enzyme N-acetylglucosaminyltransferase, involved in the matrix biosynthesis (Arciola, et al., 2001).

Staphylococci currently represent serious therapeutic challenges for drug therapy-since the use of transient or permanent medical implanted devices and procedures like surgery and dialysis are rising, so are infections (Howden, et al., 2011). Evidences of multidrug resistance among staphylococci suggest that this is because of the incorrect use of antimicrobials (Davis, et al., 2005), and rises a demand of new bioactive compounds for drug therapy. Natural products represent an important source of innovative therapeutic agents for infectious diseases.

There is a growing interest of the scientific community in using natural products (NP) for antimicrobial purposes: when compared to synthetic compounds, NP can have privileged structures that have evolved to bind unspecifically to biological targets and elicit pharmacological effects which hardly induce resistance, considering that classical resistance pathways like efflux pumps or enzymatic systems are avoided (Davis, et al., 2005).

The cashew tree (*Anacardium occidentale*, Linn) is an angiosperm plant found in Brazil (mainly the northeast area), African countries and India, and has been investigated for is varied medicinal value (Kubo, et al., 2011; Abreu, et al., 2013; Olajide, et al., 2013). Many parts are popularly used, for instance, for diabetes, asthma, inflammatory diseases and wound healing treatments, and the cashew apple juice is largely consumed by the population (Konan and Bacchi, 2007; Abreu, et al., 2013). The chemical constituents of the cashew tree stem bark hydroethanolic extract (SBHE) have been described, and pharmacological and toxicological studies have provided evidence of its safety and antimicrobial activity (Akinpelu, 2001; Ayepola and Ishola, 2009; Kubo, et al., 2011). However, there are few reports in the literature of the SBHE effects on *Staphylococcus* species and on biofilm inhibition, although it is commonly prescribed by physicians for clinical treatments, particularly in Brazil (Silva, et al., 2007).

Therefore, the present research was undertaken to evaluate the antimicrobial and anti-biofilm activities of the SBHE on clinical isolates of *S. aureus* and *S. epidermidis*. Besides the effects on planktonic cells, we demonstrate the inhibition of staphylococcal biofilm formation and its eradication by this extract by the first time. Despite the high concentrations, which have also been reported by others, our findings suggest that the SBHE may contribute to anti-staphylococcal chemotherapy, and open doors for its exploration in clinical studies.

MATERIALS AND METHODS

***Plant Material*:** The SBHE was purchased from All Chemistry (Brazil). Aliquots of 100ml were filtered and sterilized in a 220nm vacuum bomb (Milipore Stericup & Steritop, Milipore, U.S.A.) and the filtrate was concentrated in a vacuum process at 45°C to yield a dark brown paste, which weighed an average of 3g and was stored in a refrigerator (4°C) until used.

Phytochemical Analysis: The SBHE was qualitatively analyzed through classical methods for flavonoids, saponins and tannins detection. For flavonoids, Shinoda test

was performed as follows: the SBHE was mixed with few fragments of magnesium ribbon and concentrated HCl was added drop wise. The pink color indicated the presence of flavonoids (Harborne, 1973; Trease and Evans, 1989). For phenols, the SBHE was mixed with 2ml of a 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols. For saponins detection, the SBHE was mixed with 5ml of distilled hot water in a test tube and shaken vigorously. The formation of stable foam indicated the presence of saponins. For terpenoids, the SBHE was mixed in chloroform, and concentrated H_2SO_4 was carefully added to form a layer. A red brown color indicated the presence of terpenoids.

***Bacterial Strains*:** The clinical isolates were kindly provided by The Samaritan Hospital Clinical Laboratory (Governador Valadares, Minas Gerais, Brazil). They were isolated from indwelling catheters (*S. aureus*) and nasal cavities (*S. epidermidis*) of haemodyalisis patients. Reference strains of the organisms were obtained from the American Type Culture Collection (*S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228). All strains were stored in glycerol phosphate buffer and sub cultured in Brain Heart Infusion (BHI) broth (Difco) before testing. Isolates were identified as described (Kloss, 1990). All procedures were performed in triplicate.

Biofilm Formation Screening: Adherence of the clinical isolates and ATCC strains was determined in triplicate for each strain by optical density analysis (OD) as described (Li, et al., 2003), with some modifications. Aliquots (300µl) of overnight cultures of all strains in pure BHI broth and BHI broth containing 1% glucose were dispensed in eight wells of untreated sterile 96-well polystyrene micro titer plates, using pure BHI broth as negative control. Untreated polystyrene plates were chosen because they are uncharged and have hydrophobic surface, favoring the biofilm formation. The concentration of the suspensions was standardized by adjusting the optical density to 0.5 at 600nm wavelength. The plates were then incubated in humidified conditions at 35±2°C overnight. Following incubation, the wells were gently aspirated, washed three times with 100µl of PBS buffer (pH 7.4) for removal of planktonic cells and left to air dry at room temperature. The biofilms were then fixed with 300µl of 99% methanol for 15min, air dried and then stained with 200µl of 1% crystal violet (CV) for 20min in room temperature, rinsed under running tap water, and dried. The fixed dye was extracted with 98% ethanol and dispensed in new sterile 96-wells plates (Kartell, Italy). The OD was measured by reading the absorbance the wells at 620nm in a micro titer plate reader (Biorad). The strains were defined as non-adherent, weakly adherent, moderately adherent or strongly adherent according to the reference values (Table 1).

Determination of Minimum Bactericidal Concentration (MBC) for Planktonic cells: The antimicrobial tests were performed as suggested by the CLSI standards (CLSI, 2010), the formed paste was dissolved in sufficient Dimethyl sulfoxide (DMSO) to a final concentration of 122mg/ml and serial dilutions were performed, using DMSO as a diluent. The SBHE was tested at seven concentrations that ranged from 122 to 1.9mg/ml. These concentrations correspond to the serial dilutions, performed from 1:2 v/v to 1:64 v/v. Therefore, the concentrations were not directly chosen, but they were a consequence of the quantity of DMSO needed for dissolving the paste, reflected at the serial dilutions. The MBC of the SBHE was determined in triplicate by broth macrodilution for planktonic cells by the procedures recommended

by the Clinical and Laboratory Standards Institute (CLSI, 2010). Overnight cultures of all strains were used for preparing bacterial suspensions in Mueller-Hinton broth and the turbidity was spectrophotometrically standardized at 0.5 McFarland Scale (600nm). An aliquot of 1ml was added to serial dilutions of the SBHE in Mueller-Hinton broth, such that the extract concentration ranged from 122 to 1.9mg/ml. All dilutions were incubated overnight at 35±2°C. Following, 300µl of each dilution were dispensed in Mueller-Hinton agar (Difco) plates and inoculated through spread plate technique, and the extract was inoculated as a negative control. All plates were incubated overnight at 35±2°C and bacterial growth was observed. After incubation, the number of CFUs for each dilution was counted. Plates with a number of colonies ranging from 1 to 200 were used for counting. MBC was established as the lowest concentration that yielded no bacterial growth in all strains.

Determination of Minimal Biofilm Inhibitory Concentration (MBIC): The micro titer plate essay for biofilm growth inhibition and bactericidal effects on formed biofilms was carried out in untreated 96-wells plates by the procedures recommended by the CLSI (2010), with some modifications. For biofilm growth inhibition, aliquots of 150µl of each 0.5 McFarland bacterial suspension were added to 150µl of each serial dilution of the SBHE ranging from 122 to 1.9mg/ml, and incubated overnight at 35±2°C as described in previous sections. Following, 10µl of each well were dispensed in Mueller-Hinton agar plates and the SBHE was inoculated as a control. All plates were incubated overnight at 35±2°C and bacterial growth was observed. The lowest concentration that yielded no bacterial growth in all strains was taken as the MBIC.

Determination of Minimal Biofilm Eradication Concentration (MBEC): For assessing the bactericidal effects of the SBHE on formed biofilms, dry biofilms were prepared as described in *Biofilm Screening* section. Aliquots of 300µl of each SBHE dilution were then dispensed in each well, and the plates were again incubated in humidified conditions at 35±2°C overnight. Conventional viability staining tests could not be performed because of the brown color of the extract, which caused false positive results. To overcome this technical problem, after incubation, 10µl of each well were dispensed in Mueller-Hinton agar plates and the extract was inoculated as a control. All plates were incubated overnight at 35±2°C and bacterial growth was observed. The lowest concentration that yielded no bacterial growth in all strains was taken as the MBEC. All procedures were performed in triplicate.

RESULTS

Biofilm Formation: CV staining analysis showed that the 13 isolates formed biofilm following overnight culture. The quantity of biofilm varied from isolate to isolate. Absorbance (parameters at table 1) at OD_{620} among the isolates ranged from a minimum of 0.7039 to a maximum of 1.069 (normal media) and a minimum of 1.030 to a maximum of 1.145 (glucose supplemented media). Our observations indicated that all strains were moderately adherent to the polystyrene plates (table 2).

Qualitative Phytochemistry Analysis and Antimicrobial Activity of the SBHE on Planktonic Cells: The qualitative analysis of the SBHE extract revealed the presence of tannins, flavonoids and saponins. The effect of planktonic cells exposure to the SBHE was assessed through the macrodilution test. MBC assays were performed in

triplicate, and the results (table 3) indicated that the SBHE varied for each strain; however, full inhibition of planktonic cells was seen at 15.2mg/ml.

SBHE activity against Biofilms: The effect of the SBHE against *S. aureus* and *S. epidermidis* biofilm formation and formed biofilms was evaluated in triplicate. The concentrations of the SBHE ranged like in macrodilution assay (table 4). The growth of 4 strains was seen at 15.2mg/ml however, complete growth absence was observed at the concentration of 30.5mg/ml, considered the MBIC. Full eradication of biofilms was seen at 61mg/ml (data not shown), and this was considered the MBEC value.

DISCUSSION

The employment of phytocompounds as alternatives for antimicrobial drug therapy remains an active area of research. *A. occidentale* is widely distributed in most tropical and subtropical countries, and have long been used in folk medicine to treat several diseases. The SBHE is popularly used in Brazil, African countries and India for varied purposes such as infectious diseases, asthma and wound healing (Abreu, et al., 2013). In Brazil, the SBHE is commonly prescribed by physicians in drops for dilution in water prior to administration, given that low concentrations of the SBHE are considered safer for consumption (Silva, et al., 2007). However, the efficacy of this plant extract is poorly explored, and data related to its anti-biofilm activity are scarce, and although there are reports of the SBHE antimicrobial activity on some bacterial species, few reports are related to staphylococci. Hence, in order to investigate the bactericidal properties of the *A. occidentale* SBHE in staphylococci, this study used different concentrations of the extract in macrodilution and micro titer plate techniques.

Among the concentrations used in the experiments, 122, 61, 30.5 and 15.2mg/ml showed biological activity on all planktonic cells, while lower concentrations affected few strains (table 3). For biofilms, 122, 61 and 30.5mg/ml were effective against all strains, but this was not noticed at lower concentrations (table 4). Full eradication of strains was also seen, and to our knowledge, this potential of *A. occidentale* SBHE is described by the first time.

Our results are consistent with the observation of others that the SBHE has antimicrobial activity on *S. aureus* species, and have some overlapping features with others in relation to planktonic cells. SBHE concentrations of 20mg/ml (Silva, et al., 2007) and 32mg/ml (Ayepola and Ishola, 2009), have been described as effective for *S. aureus*. However, no data has been provided for *S. epidermidis*, and anti-biofilm activity was not directly addressed by these studies. Most of the antimicrobial research has been carried out against planktonic bacteria, although the majority of the bacteria in natural habitats live as biofilms, which should be the target included already in primary discovery for antimicrobial drugs. Here we have demonstrated biofilm inhibition and biofilm eradication by this extract for both species by the first time and our data suggests that the SBHE may be useful in the clinical management of staphylococcal infections.

Despite considered high for natural products, our SBHE sample was effective at the concentration of 15.2mg/ml. Possible explanations for such high values described in our work and in literature may be related to seasonal influences on the plant, extraction and/or concentration methods (what may interfere on the levels of

active phytomolecules during experimental assays) and to the presence of multiple phytomolecules instead of a single active molecule.

Phytochemical analysis conducted on the SBHE indicated the presence of compounds such as, tannins, saponins, flavonoids and phenols, which have been described as the main active molecules of the SHBE (Masuoka and Kubo, 2004; Konan and Bacchi, 2007, Kubo, et al., 2011). The compounds identified at our SBHE samples have varied biological activities, but their antimicrobial mechanism of action remains unclear (Kubo, et al., 2011). Nevertheless, complexation with extracellular and soluble proteins and to bacterial cell wall (Masuoka and Kubo, 2004), and membrane injury and inhibition of varied enzymes (Green, et al., 2008) have been suggested as possible pathways.

A synergic mechanism of action of the detected molecules for the whole antimicrobial potential of the SBHE is suggested, given that all concentrations lower than the MBC were active for some strains. However, as previously mentioned, this may be also one of the reasons for such high values of effective antimicrobial concentrations. More studies of phytochemical characterization of the SBHE are being conducted by our group to gain insights into possible different phytomolecules and their potential antimicrobial and anti-biofilm activities.

CONCLUSIONS

Thus SBHE showed antimicrobial activity on staphylococcal planktonic cells, and biofilm eradication and inhibition of biofilm production by this extract were also demonstrated. Further, some studies described that the SBHE bioactive tannins and phenolic compounds also show anti-inflammatory and antioxidant activities, which suggests that more protocols could be established for its clinical usage.

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Table-1: Reference values for Optical Density (OD).

OD Parameter	OD Value
Control Value	0,88
Non-adherent (–)	OD < 0,880
Weakly adherent (+)	0,880 < OD ≤ 1,760
Moderately adherent (++)	1,760 < OD ≤ 3,520
Strongly adherent (+++)	OD ≥ 3,520

Table- 2: Strains OD after 24 h incubation (medium of three independent experiments).

Strain	OD (24h) NM	PAR	OD (24h) SM	PAR
S. epidermidis 1	1.035±0.064	+	1.0146±0.088	+
S. epidermidis 2	1.052±0.032	+	1.0408±0.048	+
S. aureus 4	1.030±0.033	+	1.009±0.083	+
S. aureus 5	1.044±0.028	+	1.0695±0.019	+
S. aureus 6	1.054±0.028	+	1.054±0.027	+
S. aureus 7	1.056±0.017	-	0.8721±0.081	-
S. aureus 8	1.145±0.042	+	1.0444±0.019	+
S. aureus 9	1.136±0.094	+	1.0021±0.095	+
S. aureus 10	1.053±0.023	+	1.0029±0.081	+
S. aureus 11	1.071±0.048	+	1.0082±0.060	+
S. aureus 14	1.045±0.029	+	0.8915±0.065	+
S. epidermidis ATCC 12228	1.054±0.023	+	0.9806±0.063	+
S. aureus ATCC 29213	1.049±0.018	-	0.7039±0.068	-

- GSM-glucose supplemented media. NM-normal media. OD-Optical density. PAR - Resulting parameter.

Table -3: SBHE MBC for Planktonic cells.

Stains	SBHE concentration (mg/ml)						
	122	61	30.5	15.2	7.6	3.8	1.9
S. epidermidis 1	–	–	–	–	+	+	+
S. epidermidis 2	–	–	–	–	–	+	+
S. aureus 4	–	–	–	–	–	–	+
S. aureus 5	–	–	–	–	–	–	+
S. aureus 6	–	–	–	–	–	–	–
S. aureus 7	–	–	–	–	+	+	+
S. aureus 8	–	–	–	–	–	–	–
S. aureus 9	–	–	–	–	–	+	+
S. aureus 10	–	–	–	–	–	+	+
S. aureus 11	–	–	–	–	–	+	–
S. aureus 14	–	–	–	–	–	–	–
S. epidermidis ATCC 12228	–	–	–	–	+	+	+
S. aureus ATCC 29213	–	–	–	–	–	–	+

- Positive and negative signs indicate presence or absence of bacterial growth, respectively.

Table - 4: SBHE MBIC.

Stains	SBHE concentration (mg/ml)						
	122	61	30.5	15.2	7.6	3.8	1.9
S. epidermidis 1	–	–	–	+	+	+	+
S. epidermidis 2	–	–	–	–	–	+	+
S. aureus 4	–	–	–	–	–	–	+
S. aureus 5	–	–	–	–	–	–	+
S. aureus 6	–	–	–	–	–	–	–
S. aureus 7	–	–	–	–	+	+	+
S. aureus 8	–	–	–	–	–	–	–
S. aureus 9	–	–	–	–	–	+	+
S. aureus 10	–	–	–	–	–	+	+
S. aureus 11	–	–	–	–	–	+	–
S. aureus 14	–	–	–	–	+	+	+
S. epidermidis ATCC 12228	–	–	–	–	–	–	+
S. aureus ATCC 29213	–	–	–	–	–	–	+

- Positive and negative signs indicate biofilm growth or inhibition, respectively.

