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RAUVOLFIA GRANDIFLORA (APOCYNACEAE) EXTRACT INTERFERES WITH STAPHYLOCOCCAL DENSITY, ENTEROTOXIN PRODUCTION AND ANTIMICROBIAL ACTIVITY

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ABSTRACT

Staphylococci bacteria are involved in many human and animal infections and development of alternative antimicrobial drugs against pathogenic bacteria is of great interest to the pharmaceutical industry. This study investigated the *in vitro* effect of *Rauvolfia grandiflora* methanol extract (root bark fraction) (RGE) on the density of ATCC strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*, and a clinical enterotoxin-producer, *S. aureus* bovine strain. The alkaloid, isoreserpiline, obtained from dichloromethane extract of *R. grandiflora* was ineffective against the strains tested. After incubation of staphylococci strains in the presence of 1.2 $\mu g.mL^{-1}$ RGE, a significant inhibition of cell growth was observed using both spectrophotometry and ELISA assays. Twelve drugs were evaluated for their antimicrobial effects on culture RGE-treated cells using the disk diffusion method. Penicillin resistant strains became sensitive to the drug after RGE treatment. Furthermore, enterotoxin production by RGE-treated *S. aureus* was evaluated using a standardized ELISA method. Although staphylococcal LSA 88 bovine strain cells remained viable after exposure to the extract, enterotoxin production was precluded in 20% after RGE treatment. Significant interference in staphylococci cell density, drug sensitivity and enterotoxin secretion was observed after treatment. The study highlights the necessity to find new methods of disease prevention and new antibiotic therapies against staphylococcal infections.

Key words: *Rauvolfia grandiflora*, *Staphylococcus aureus*, enterotoxin, antimicrobial activity.

INTRODUCTION

Staphylococcus aureus is a human pathogen encapsulated bacterium with anti-phagocytic activity (26,15,30) which can invade and survive within a wide variety of mammalian cells (2,14) and causes intramammary infections in animals (27,34). The detection of a major *S. aureus* clone A (63%), infecting dairy herds, has demonstrated its geographical spread among farms in Rio de Janeiro State, Brazil, affecting five different animal species and it has been suggested that all but one of the clones was animal specific (1,34). *S. aureus* causes food poisoning epidemics in humans (5,24) by secreting

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staphylococcal enterotoxins (SEs). However, their role in intoxications has yet to be clarified, and descriptions of the bacterial pathogenicity continues to grow as more proteins with the same or similar properties are isolated or characterized (20,16,18,25,31). SEs are known to function as potent super-antigens that stimulate non-specific clonal T-cell proliferation, followed by a state of hyporesponsiveness to subsequent antigen stimulation and, thus, hinder the development of protective immunity, while promoting the persistence of bacteria in the host (11). SEs may also aggravate bovine intramammary infections throughout local cytokine release (40). SEs are produced by *S. aureus* after *agr* activation quorum-sensing system (QS), which consists of a sophisticated system to ensure that some bacteria functions only take place when a specific population density has been reached (3). This *agr* commanded system, however, is negatively influenced by the octapeptide RNAIII Inhibiting Peptide (RIP) *in vivo* by interfering in staphylococcal infections (4) and *in vitro* by polyclonal affinity purified antibodies anti-recTRAP, resulting in staphylococcal enterotoxins inhibition in liquid culture (35). Several plant extracts containing phenolics and alkaloid compounds may interfere with bacterial (QS) and biofilm formation (13,29).

As a group, the coagulase-negative staphylococci are among the most frequently-isolated bacteria in clinical microbiology and are becoming increasingly important, especially as causes of hospital-acquired infections, including *S. epidermidis* (37), considered a normal inhabitant of human skin and mucous membranes, but they also infect animals (32,38).

Several plant-derived compounds possess medicinal properties with activity against important medical microorganisms (17,29). A new indole alkaloid isolated from root bark was described in *Rauvolfia grandiflora,* however, its biological activity has not as yet been tested (7,8). Previous studies have shown that plant extracts can inhibit enterotoxin production by *Staphylococcus aureus* strains (22,6). A natural product found in the bark of witch hazel, hamamelitannin, had no effect on staphylococcal growth *in vitro*, but inhibited the (QS) regulator RNAIII and, in a rat graft model, prevented device-associated infections *in vivo*, including infections caused by MRSA and MRSE strains (19). The present study describes the biological activity of methanol extract from *Rauvolfia grandiflora* against staphylococci strains, and discusses the interference of enterotoxin production by *S. aureus*, cell growth inhibition and antibiotic interactions with the bacteria.

MATERIAL AND METHODS

Test organisms and plant extract

Root bark of *R. grandiflora* was collected in São Francisco do Itabapoana City, Rio de Janeiro State, Brazil. A voucher specimen was deposited in the herbarium of the Agricultural University of wageningem, Netherlands. Root bark samples were dried, powdered, and extracted using methanol and after solvent evaporation, all samples were dissolved in DMSO (Sigma, USA) at a concentration of 100000 $\mu g.mL^{-1}$. The assays were performed using the extracts at a final concentration of 1.2 $\mu g.mL^{-1}$ at RT.

Reference bacterial strains: *S. aureus* Wood 46 (ATCC10832) a capsule-negative strain, *S. aureus* Smith Diffuse (SD) (ATCC 13709) a capsule-producer strain, *S. aureus* ATCC 25923 strain, and *S. epidermidis* ATCC 12228 a non-biofilm-forming strain. Bovine *S. aureus* LSA88 (34) is a SEC/SED producing strain previously studied (35,36) and used in the development of the recombinant *sec* gene (33). All strains were re-activated in Brain Heart Infusion broth.

Fractionation of RGE

The methodology used to obtain RGE followed a previously alkaloid extraction from *R. grandiflora* with minor modifications (8). Dried and powdered root bark (1.67 kg) from *R. grandiflora* was extracted with methanol at room temperature, resulting in 36.0g of residue, after solvent evaporation. About 95% of this residue was chromatographed on a silica gel column eluted with a methanol gradient in dichloromethane, and 10 fractions collected. Nine of these

samples were re-fractionated but the concentrations were insufficient to carry out the tests. The isolated alkaloid compound isoreserpiline, obtained from dichloromethane fraction, was tested against all strains.

Experimental procedures

Growth Inhibition: An aliquot of 0.1 mL from *Rauvolfia grandiflora* root bark methanol extraction (RGE) was added to 1.8 mL of BHI and 0.1 mL of bacteria inoculum (OD_{550nm} 0.5 McFarland), determined using a photometer (Densimat, bioMérieux, France). The density readings of treatments and controls were recorded every sixty minutes. Controls employed medium and inoculums with DMSO added as a dispersing solvent. After 12 h incubation period at 37°C, 0.1 mL aliquots of cultures were streaked on BHA agar (Acumedia, USA) and incubated on 37ºC.

Antibiotic activities after extract treatments

Antibiograms were performed following exposure of strains to RGE. Pure colonies of each strain were sub-cultured on blood agar plates for antimicrobial standard disk diffusion tests, performed in agar Muller Hinton, according to the guidelines of the National Committee for Clinical Laboratory Standards (21), and the halo formed around each disk was recorded. The disks contained the following drugs: penicillin G (PEN, 10U), oxacillin (OXA, 1 μg), amoxicillin (AMO, 10 μg), ampicilin (AMP, 10 μg), cephalothin (CFL, 30 μg), cefoxitin (CFO, 30 μg), trimethoprim-sulfamethoxazole (SUT, 25 μg), clindamycin (CLI, 2 μg), erythromycin (ERI, 15 μg), gentamicin (GEN, 10 μg), tetracycline (TET 30 μg), and vancomicin (VAN, 30 μg) (Laborclin, PR, Brazil).

Growth and Enterotoxin inhibition assays

In order to evaluate the inhibition of SEC and SED secretion by LSA 88 bovine strain after treatment with RGE, ELISA assays were performed as previously described (35). After ON reactivation at 37ºC, cells were harvested by centrifugation at 12 000 x *g* and washed twice in PBS. Cells were then diluted in fresh TECRA® Staphylococcal growth medium (Bioenterprises Pty. Ltd., Australia) to a density of 10^8 cells mL^{-1} at OD_{550nm} 0.5. Tubes with cells plus DMSO were used as negative controls. Kit-positive and -negative controls were used. A proportional cell growth assay was conducted over a 2h duration, and growth of cells was interrupted by adding a stop solution. A second experiment was conducted, where *S. aureus* cell growth was monitored using a controlled incubation at 37ºC for 3h for controls and 4h for treatments in order to standardize the values for cell growth and subsequent comparison of enterotoxin production. After centrifugation, supernatants were harvested and tested for SEs using the TECRA® Visual Immunoassay VIA™ kit (Bioenterprises Pty. Ltd., Australia), according to the manufacturer's instructions. This assay allows for the specific and sensitive detection of enterotoxins A to E (lower limit 1.0 $ng.mL^{-1}$). Color development readings (OD_{405nm}) (Biorad, USA) were realized during two intervals at the end of the assay. All the experiments described were performed in triplicate.

RESULTS

Following purification of RGE, the only alkaloid compound obtained, isoreserpiline, when tested alone against the bacterial strains selected here, showed no antimicrobial activity. The structure of isoreserpiline is shown in Figure 1. The other 9 fractions resulted in very low concentrations of the alkaloids darcyribeirine and β-yohimbine obtained from methanol extract and were not tested. ELISA results, as expected, showed that RGE-treated *S. aureus* LSA88 (bovine strain) cells were inhibited and directly accompanied by a proportional decreased enterotoxin production (Figure 2), *i.e.* the lower the cell density, the lower the amount of enterotoxin detected. RGE also caused growth inhibition of all other strains when cultured on BHA agar (Tukey, $p<0.05\%$). Controls exhibited CFU counts above 300 colonies/mL, while RGE-treated cells presented CFU counts that were less than 300 and above 30 CFU.

ELISA experiments to determine enterotoxin production after RGE treatment revealed 20% inhibition in SE production, compared to controls, even with similar cell populations (Figure 3).

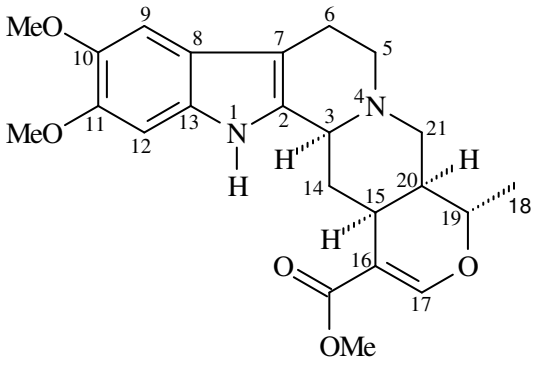

Figure 1. Structure of isoreserpiline obtained from dichloromethane fraction.

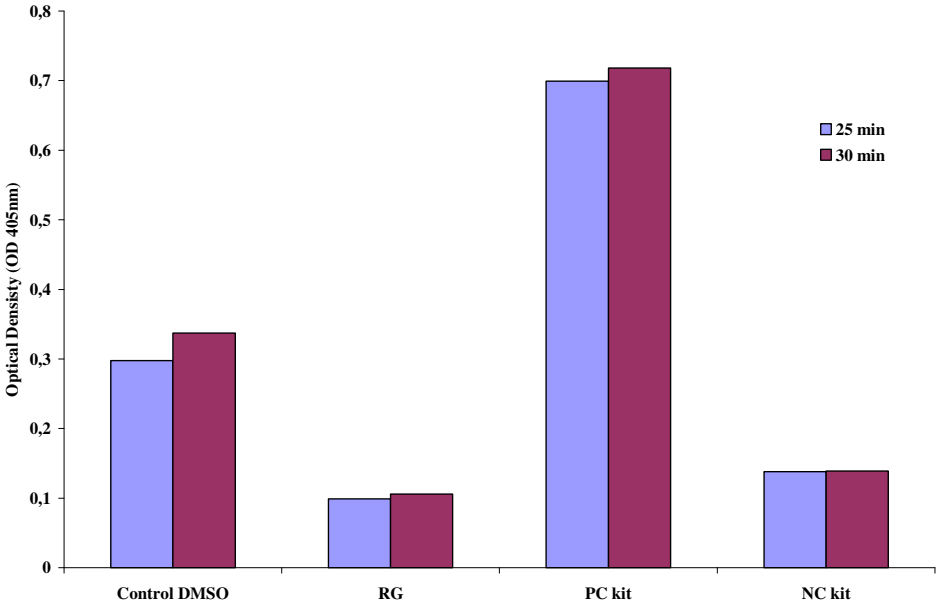

Figure 2. Density of *Staphylococcus aureus* (bovine strain) cells. Color development intervals (25 and 30 minutes readings). Production of enterotoxins by LSA 88 *Staphylococcus aureus* (bovine strain) was directly dependent/proportional on cell population. RG (treatment with *Rauvolfia grandiflora* extract); PC = positive control; NC= negative control.

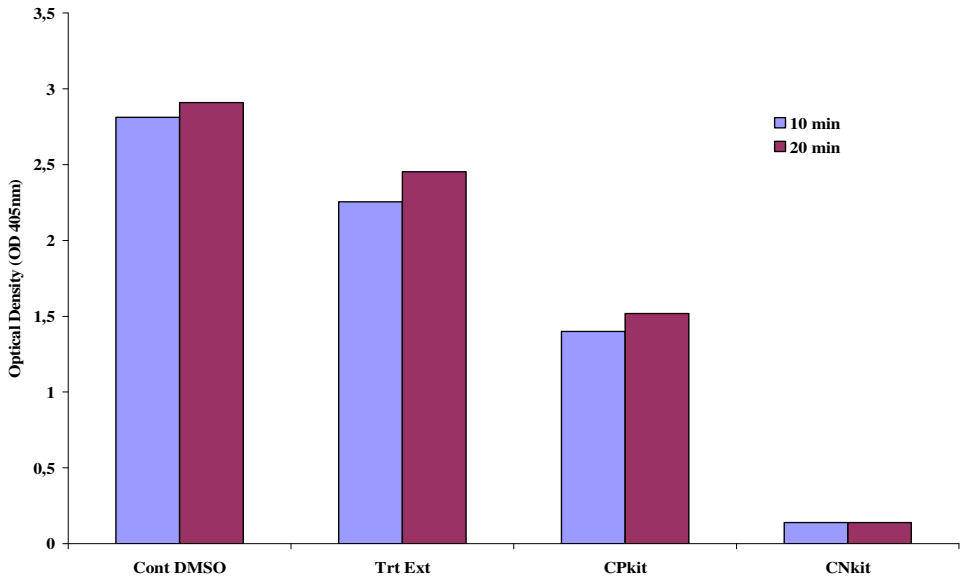

Figure 3. Color development intervals (10 and 20 minutes readings). Inhibition of enterotoxins produced by LSA 88 *Staphylococcus aureus* (bovine strain) cultured in liquid medium in the presence of *R. grandiflora* methanol extract, in proportional cell density (OD_{550nm}) of both, treatment (Trt Ext incubated for 4h) and control (Cont DMSO incubated for 3h). PC and NC indicate positive and negative controls, respectively.

The results of antibiograms of RGE treated cells are shown in Tables 1A-2B.

Individually, after antibiotic exposure of bacteria, comparing RGE treated cells and controls, the results show larger inhibition zones than those of the respective controls for most strains ($p<0.05$). In contrast, *S. aureus* 25923 and Wood 46 strains, did not present larger inhibition zones after exposure to oxacillin (Table 1A) and ampicillin (Table 1B), respectively, suggesting no effect of the treatment. For SD strain, treatment did not affect the activity of drugs, except for amoxicillin, oxacillin, and trimethoprim-sulfamethoxazole, where RGE augmented the inhibition zones. Comparing results between drugs, cephalosporin (CFL and CFO) demonstrated greater activity than the other drugs against most of the strains tested. Tables 2A and 2B show that the SD strain remained less sensitive to drugs, except for OXA and SUT. All other strains were responsive to RGE treatment and exhibited higher sensitivity towards the drugs tested, as confirmed by the augmentation of the inhibition zone ($p<0.05$).

Table 1A. Antibiogram (measured in mm) of staphylococcal strains, treated with *Rauvolfia grandiflora* extract, and submitted to six drugs. Evaluated by agar diffusion method using Gram-positive A kit.

	Strains									
	ATCC12228		**LSA88**		**ATCC25923**		**Wood46**		**Smith Diffuse**	
GrA	**Treat**	**Cont**	**Treat**	**Cont**	**Treat**	**Cont**	**Treat**	**Cont**	**Treat**	**Cont**
PEN	24.0Ad	15.0Bc	23.7Ac	15.7Bd	36.3Aa	21.3Bb	16.7Ad	14.7Bd	24.3Ae	23.3Ad
CFL	40.0Aa	32.3Ba	32.3Aa	32.0Aa	33.Ab	26.3Ba	34.0Aa	29.7Ba	38.0Aa	37.0Aa
CLI	40.0Aa	33.0Ba	27.0Ab	20.7Bb	28.3Ad	19.7Bc	31.7Ab	27.3Bb	25.3Ade	25.3Ac
AMO	28.3Ac	15.0Bc	24.3Ac	14.7Bd	31.3Abc	22.3Bb	17.0Ad	14.7Bd	36.7Ab	34.3Bb
TET	19.3Ae	11.3Bd	18.0Ad	12.3Be	29.7Acd	25.7Ba	16.3Ad	13.7Bd	26.3Ad	25.7Ac
OXA	36.3Ab	26.3Bb	23.7Ac	18.3Bc	25.0Ae	21.7Bb	21.0Bc	25.3Ac	28.0Ac	26.0Bc

GrA- Kit used for antibiogram to analyze six anti-Gram-positive A drugs; Means followed by the same small letter in the column (drug) and capital letter in the line (extract treatment), do not differ among themselves. Tukey test, 5% probability.

Table 1B. Antibiogram (measured in mm) of staphylococcal strains, treated by *Rauvolfia grandiflora* extract, and submitted to six drugs. Evaluated by agar diffusion method using Gram-positive B kit.

	Strains									
	ATCC12228		**LSA88**		**ATCC25923**		**Wood46**		**Smith Diffuse**	
GrB	**Treat**	**Cont**	**Treat**	**Cont**	**Treat**	**Cont**	**Treat**	**Cont**	**Treat**	**Cont**
VAN	27.7Ad	21.3Bb	20.0Ad	14.7Bd	20.7Ad	14.7Bc	18.7Ad	14.7Bd	16.7Ae	16.0Ae
AMP	23.7Ae	13.7Bc	31.7Aa	23.7Bb	37.0Ba	40.0Aa	15.7Ae	14.3Ad	39.3Aa	35.7Aa
ERI	31.3Ac	23.7Bb	21.7Acd	13.3Bd	25.7Ac	20.3Bb	22.0Ac	17.7Bc	17.7Ae	16.7Ae
SUT	34.3Ab	22.7Bb	28.3Ab	17.7Bc	32.0Ab	20.7Bb	28.3Ab	23.0Bb	25.7Ac	24.0Bc
GEN	33.0Abc	24.0Bb	23.3Ac	17.0Bc	25.0Ac	18.7Bb	21.3Ac	15.7Bcd	22.0Ad	21.0Ad
CFO	45.3Aa	41.0Ba	29.7Aab	26.7Ba	34.0Aab	37.7Ba	37.3Aa	35.3Ba	32.7Ab	31.7Ab

GrB- Kit used for antibiogram to analyze six anti-Gram-positive B drugs; Means followed by the same small letter in the column (drug) and capital letter in the line (extract treatment), do not differ among themselves. Tukey test, 5% probability.

Table 2A. Comparison of inhibition (measured in mm) of staphylococcal strains, treated by *Rauvolfia grandiflora* extract, and submitted to antibiogram towards six drugs by agar diffusion method using Gram-positive A kit.

Drugs / Strains	PEN		CFL		CLI		AMO		TET		OXA	
	Treat	Cont	Treat	Cont	Treat	Cont	Treat	Cont	Treat	Cont	Treat	Cont
ATCC12228	24.0Ab	15.0Bc	40.0Aa	32.3Bb	40.0Aa	33.0Ba	28.3Ac	15.0Bc	19.3Ac	11.3Bc	36.3Aa	26.3Ba
LSA88	23.7Ab	15.7Bc	32.3Ac	32.0Ab	27.0Ac	20.7Bd	24.3Ad	14.7Bc	18.0Acd	12.3Bbc	23.7Ac	18.3Bc
ATCC25923	36.3Aa	21.3Bb	33.0Ac	26.3Bd	28.3Ac	19.7Bd	31.3Ab	22.3Bb	29.7Aa	25.7Ba	25.0Ac	21.7Bb
Wood46	16.7Ac	14.7Bc	34.0Ac	29.7Bc	31.7Ab	27.3Bb	17.0Ae	14.7Bc	16.3Ad	13.7Bb	21.0Bd	25.3Aa
Smith Diffuse	24.3Ab	23.3Aa	38.0Ab	37.0Aa	25.3Acd	25.3Ac	36.7Aa	34.3Ba	26.3Ab	25.7Aa	28.0Ab	26.0Ba

GrA- Kit used for antibiogram to analyze six anti-Gram-positive A drugs; Means followed by the same small letter in the column (strains) and capital letter in the line (extract treatment), do not differ among themselves. Tukey test, 5% probability.

Table 2B. Comparison of inhibition (measured in mm), among staphylococcal strains, treated by *Rauvolfia grandiflora* extract, and submitted to antibiogram towards six drugs by agar diffusion method using kit Gram-positive B.

Drugs / Strains	VAN		AMP		ERI		SUT		GEN		CFO	
	Treat	Cont	Treat	Cont	Treat	Cont	Treat	Cont	Treat	Cont	Treat	Cont
ATCC12228	27.7Aa	21.3Ba	23.7Ac	13.7Bd	31.3Aa	23.7Ba	34.3Aa	22.7Bab	33.0Aa	24.0Ba	45.3Aa	41.0Ba
LSA88	20.0Abc	14.7Bb	31.7Ab	23.7Bc	21.7Ac	13.3Bd	28.3Ab	17.7Bc	23.3Abc	17.0Bcd	29.7Ad	26.7Bd
ATCC25923	20.7Ab	14.7Bb	37.0Ba	40.0Aa	25.7Ab	20.3Bb	32.0Aa	20.7Bbc	25.0Ab	18.7Bc	34.0Bc	37.7Ab
Wood46	18.7Ac	14.7Bb	15.7Ad	14.3Ad	22.0Ac	17.7Bc	28.3Ab	23.0Bab	21.3Ac	15.7Bd	37.3Ab	35.3Bb
Smith Diffuse	16.7Ad	16.0Ab	39.3Aa	35.7Ab	17.7Ad	16.7Ac	25.7Ab	24.0Ba	22.0Ac	21.0Ab	32.7Acd	31.7Ac

GrB- Kit used for antibiogram to analyze six drugs anti-Gram-positive B; Media followed by the same small letter in the column (strains) and capital letter in the line (extract treatment), do not differ among themselves. Tukey test, 5% probability.

DISCUSSION

Staphylococcus aureus and *S. epidermidis* may represent a threat to human health and animals by surpassing host immune response with an arsenal of virulence factors, most of them based on genetic regulation, i.e. quorum sensing (QS) (3,38). Many other pathogenic bacteria use the same regulation system to secrete their virulence factors, however, polyphenols such as flavonoids from plants, can affect biofilm formation by interfering with QS in *B. cepacia* (13). Recently, epigallocatechin gallate (EGCG) from green tea, was tested for its ability to inhibit QS in *Chromobacterium violaceum*, and even following an increase in bacteria growth due to the addition of EGCG, the production of QS-dependent violacein by the organism was lower (28). Other workers have observed that hamamelitannin, a natural product found in the bark of *Hamamelis virginiana*, had no effect on drug resistance and growth *in vitro* but inhibited the quorum-sensing regulator, RNAIII, of staphylococcal strains by interfering in the competition with RAP (19). Different products, including RNAIII inhibiting peptide (RIP) and anti-TRAP IgY polyclonal antibodies were able to inhibit the growth of *S. aureus* strains (35) as observed in the present work. Inhibition of enterotoxin production by *S. aureus,* at the QS level, as suggested herein, may be due to interference with surface receptors for RAP activation and with subsequent transcription of virulence genes, including SE genes as proposed previously (4). Here, enterotoxin production was precluded in 20% by treatment of LSA 88 (bovine strain) with RGE. The method used in the present study may represent real inhibitory activity

on staphylococcal enterotoxin secretion, if one compares this to SE inhibition using an agglutination test (22), which may cause misinterpretation of the results. Also, enterotoxin A inhibition was assessed using a membrane over agar method and measured by immunodifusion assay (6), which is less sensitive than ELISA SEs screening used in the present work. Alkaloid-containing extracts from different plants also show antimicrobial activity, including activity against *S. aureus* (29,9). Although we have not totally identified the constituents of RGE, alkaloids are known to be produced by this plant (7,8). Apocynaceae are known to be alkaloid-producing plants, but the alkaloids vary in their chemical structures (7,8,29). The lack of activity of the purified compound, isoreserpiline, on the staphylococci tested may be explained by the interference of others compounds present in RGE (10) or did not present activity alone. The results of the present study, using low concentrations of RGE, showed significant growth inhibition on *S. aureus* strains. In order to investigate any variation among staphylococci from different origins, the antibiogram analysis showed that most of the RGE-treated strains were affected by exposure to drugs. However, in the case of Wood46 and ATCC 25923, where inhibition zones of (OXA) and (AMP), both beta-lactamic drugs, presented inverted results for treatments compared with all other strains. Although nearly all *S. aureus* strains are resistant to PEN (12), in the present study some resistant strains after RGE treatment became sensitive to the drug, demonstrating inhibition zones between 28 and 29 mm, for resistant and sensitive β-lactamase positive *Staphylococcus*, respectively (Adapted from CLSI, M100-S17, Jan 2007). The ATCC 12228 *S. epidermidis* strain, a MSSE (Methicillin Susceptible *Staphylococcus epidermidis*), remained susceptible to all drugs but with a larger halo after RGE treatment. Results suggest that differences in antibiotic zone inhibition of treated staphylococci growth could result from the increased sensitivity of *S. aureus*. This may occur in a QS-dependent manner by interfering in *sae*, a locus related to resistance and susceptibility towards drugs in the bacteria (23,39). Taken together the results reported in the present investigation may contribute to the understanding of plant extract activities in the strains tested, although further studies are needed.

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