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Plant Extracts Display Synergism with Different Classes of Antibiotics

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Abstract: One manner in which plant-derived compounds exert their antibiotic potential is the synergism, a positive interaction between two compounds. Studies indicate that the use of plant extracts combined with antimicrobials may promote a significant reduction of the minimum inhibitory concentrations of antibiotics for bacterial strains. This study aimed to evaluate the activity of plant extracts and antibiotics as well as their combination on *Staphylococcus aureus*. The activity of 15 plant extracts was evaluated using diffusion assay. The minimum inhibitory concentrations (MICs) and the interactions between the extracts and antibiotics as well as compound emodin were evaluated with the checkerboard method. The active extracts were a hexane extract of the leaves of *Baccharis dracunculifolia* and the ethanol extracts of the leaves of *Plectranthus ornatus*, *Inga edulis, Salvia officinalis* and *Senna macranthera*. The Plectranthus ornatus extract displayed synergism with ampicillin (a β-lactam), kanamycin and gentamicin (aminoglycosides), with 8-fold reductions in the MIC. The same reduction was observed for the extracts of *Salvia officinalis* and *Senna macranthera*, which displayed the lowest MIC. Using these combinations resulted in a reduction in the minimum dose required for effective antimicrobial effects, which is interesting because it may decrease both the risk of side effects and the costs of treatment.

Key words: antibiotics, bovine mastitis, plant extract, *Staphylococcus aureus*.

INTRODUCTION

The increase in bacterial resistance to currently known drugs is an issue of global concern and reinforces the need for new classes of antibacterial substances. Compounds of natural origin are potential sources of new chemical scaffolds for antibiotic development (Ortholand and Ganesan 2004) but unlike synthetic drugs, they are not associated with side effects, are cheaper, come from renewable sources and have greater acceptance due to a long history of use (Chanda and Rakholiya 2011). One manner in which plant-derived compounds exert their potential as antibiotics is synergism, a positive interaction created when two agents are combined (González-Lamothe et al. 2011) that results in an inhibitory effect greater than the sum of their individual effects (Chanda and

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Rakholiya 2011). Many currently used drugs are based on synergistic interactions between different antibiotics with different targets (Sibanda and Okoh 2007). Studies indicate that the use of plant extracts combined with antimicrobials promotes a significant reduction of the minimum inhibitory concentrations of antibiotics for bacterial strains (Darwish et al. 2002, Betoni et al. 2006, Souza et al. 2011). Darwish et al. (2002) observed that the efficacy of the antibiotics gentamicin and chloramphenicol was increased against isolates of *Staphylococcus aureus* when the drugs were combined with extracts produced from Jordanian plants. Betoni et al. (2006) also observed synergistic interactions between eight Brazilian herbal extracts with antibiotics against *S. aureus* isolated from human infections. Sousa et al. (2011) reported the antibacterial activity and the positive interference of extracts from the leaves and roots of *Lantana camara* and *Lantana lantana* on the activity of aminoglycosides against Gram-positive and Gram-negative strains.

Previous studies conducted by our group demonstrated that various extracts of plants were effective in inhibiting the growth of bovine-origin *S. aureus* (Diaz et al. 2010, Rossi et al. 2011). This study aimed to evaluate the activity of plant extracts and antibiotics as well as their combination on *S. aureus* isolates from animals affected with bovine mastitis.

MATERIALS AND METHODS

PLANT MATERIAL

Plectranthus ornatus (voucher 39644), *Solanum cernuum* (voucher 011654), *Calendula officinalis* (voucher 15593), *Cymbopogon citrates* (voucher 30283), *Symphytum officinale* (voucher 24060), *Inga edulis* (voucher 2173), *Jacaranda cuspidifolia* (voucher 21419), *Azadirachta indica, Salvia officinalis* (voucher 1240), *Bixa Orellana* (voucher 31977), *Baccharis dracunculifolia* (voucher 31322), *Ocimum basilicum, Senna macranthera* (voucher 1237), *Helianthus annuus* (voucher 7650) and *Acrocomia aculeata* specimens were collected in Viçosa, Minas Gerais, Brazil. A voucher of each specimen used was deposited at the herbarium of the Department of Botany of the Universidade Federal de Viçosa. The specimens (*Azadirachta indica*, *Ocimum basilicum* and *Acrocomia aculeate*) that do not have voucher are still up identification. The plant parts were dried at 40 °C for 24 h in an air circulation oven before the extracts were prepared.

EXTRACT PREPARATION

The leaves (500 g) of *P. ornatus, Solanum cernuum, C. officinalis, C. citrates, S. officinale, I. edulis, J. cuspidifolia, A. indica, S. officinalis, B. orellana* and *O. basilicum* (500 g) were extracted with ethanol (2 L) and the roots of *S. macranthera* (500 g) with dichloromethane for 120 h at room temperature. The solvent was removed under vacuum at 40 °C to obtain an ethanolic extract of each plant. The leaves of *B. dracunculifolia, A. aculeate* and seeds of *H. anus* were extracted with hexane by the same procedure. All extracts were stored at 4 °C.

BACTERIAL STRAINS

S. aureus 3993 and 4125 strains, (identified by the Embrapa Dairy Cattle from the Milk Microbiology Laboratory) as an infectious reference microorganism which were isolated from animals with mastitis infections, were kindly provided by Embrapa/CNPGL, Juiz de Fora, MG, Brazil. The bacterial cultures were streaked on plates containing brain heart infusion agar (BHA; Himedia ®) and incubated for 16 h at 37 °C. Müeller Hinton broth (Himedia ®) was used for testing antibacterial activities. The cell concentration was adjusted to 10^6 CFU mL^{-1} with an optical density set at 600 nm. Stock cultures were maintained in BHI agar containing 25% glycerol at -80 °C.

ANTIMICROBIAL ACTIVITY

Antimicrobial activity was assessed using the agar diffusion assay. For this purpose, 100 µL of a suspension containing 10^6 CFU.mL^{-1} was spread in Petri dishes containing Müeller Hinton agar (Himedia®). Holes of approximately 5 mm in diameter and 3 mm in height were created in the agar and were filled with 30 µL of the extracts at a concentration of 50 mg.mL^{-1}.The controls were prepared with 30 µL of DMSO and 5 mg.mL^{-1}of ampicillin (Sigma®, A9518). The plates were incubated for 24 h at 37 ºC, and the subsequent inhibition zones were measured in mm. Inhibition zones greater than 7 mm were considered positive (Nascimento et al. 2000). Student's t-test ($p<0.05$) was performed to compare the results of the inhibition zones obtained from the extracts with the positive control. Tests were performed twice in triplicate.

MINIMAL INHIBITORY CONCENTRATION (MIC) ASSAY

The activity of the extracts on bacterial growth was determined using the microdilution method described in CLSI (2003). The microorganisms were initially grown on BHI agar plates (Himedia®) and pre-incubated for 24 h at 37 ºC. Isolated colonies were then inoculated into Müeller-Hinton broth (Himedia®) and incubated at 37 ºC with shaking at 180 rpm until the exponential phase was reached. The culture was subsequently diluted to an optical density corresponding to the standard 0.5 on the McFarland scale (OD_{620}=0.10). Microplate wells were filled with 100 µL of Müeller-Hinton broth that had extracts concentrations ranging from 0.1mg.mL^{-1} to 10 mg.mL^{-1} and 10^6 CFU.mL^{-1}of bacterial suspensions. Whereas the DMSO could be bactericidal a control of microbial growth in this solvent was done with 100 µL of bacterial suspension and 100 µL of Müeller-Hinton broth with DMSO at the highest concentration used in the preparation of the extract. After 24 h at 37 ºC, 4µL of *p*-iodonitrotetrazolium (INT, I8377, Sigma®) was added to each well, and the plate was incubated for an additional 2 h at 37 ºC. A change in the color of the medium from yellow to pink-violet was used as an indication of bacterial growth. The minimal inhibitory concentration of the antibiotics was determined by the same procedure, with concentrations ranging from 0.1 mg.mL^{-1} a 500 mg.mL^{-1}.

EVALUATION OF THE INTERACTIONS BETWEEN PLANT EXTRACTS AND ANTIBIOTICS

The checkerboard method, which is commonly used for measurement of interactive inhibition, was used to determine the interactions between the antibiotics and the natural antimicrobials (Palaniappan and Holey 2010). Synergistic interactions involving the plant extracts (drug A) plus the antibiotics (drug B) were tested. The concentrations of the agents used started from twice their MIC value and were serially diluted in five-fold steps. The effects of combinations were evaluated by calculating the FIC (Fractional Inhibitory Concentration) index for each combination using the formulas displayed below:

FIC of drug A (FIC_A) = MIC of compound A in combination/MIC of compound A alone

FIC of drug B (FIC_B) = MIC of compound Bin combination/MIC of compound B alone

$$\text{FIC index} = FIC_A + FIC_B$$

Synergy was defined as an FIC index ≤0.5. An FIC ≥4.0 indicated antagonism between the two agents, between 4.0 and 1.0 indicated indifference, and between 1.0 and 0.5 was evaluated as an additive interaction.

RESULTS

This study evaluated the antimicrobial potential of plant extracts from Viçosa, Minas Gerais,

Brazil. The activity of these extracts was tested on strains of *S. aureus* isolated from cows affected with mastitis. Five of the 15 plant extracts tested displayed antimicrobial activity, as indicated by inhibition zones greater than 7 mm (Table I). The active extracts were the hexane extract of the leaves of *B. dracunculifolia* and the ethanol extracts of the leaves of *P. ornatus*, *I. edulis*, *S. officinalis* and *S. macranthera*.

The MICs for the active extracts were determined (Table II). Among the plant extracts tested, *S. macranthera* displayed the lowest MIC (0.2 $mg.mL^{-1}$), followed by *S. officinalis* (0.3 $mg.mL^{-1}$). The highest MICs were observed for the extracts of *P. ornatus* (2.0 $mg.mL^{-1}$) *B. dracunculifolia* (5.0 $mg.mL^{-1}$) and *I. edulis* (7.0 $mg.mL^{-1}$).

In addition to the antimicrobial potential of plant extracts, in this study, we investigated the combined effect of the active extracts with antibiotics traditionally used to treat bovine mastitis. First, the MICs of five antibiotics, namely ampicillin, kanamycin, chloramphenicol, gentamicin, and tetracycline were determined (Table III). The checkerboard method was used to determine the interactions between the antibiotics and the active extracts. The concentrations of the extracts and antibiotics varied between 1/8 MIC to 2MIC when combined. Based on the lower combined concentrations where bacterial growth inhibition was observed, the fractional inhibitory concentration index (FIC) was calculated and the type of interaction was determined (Table IV).

In almost all combinations of extracts and antibiotics tested, additive or synergistic interactions were observed. Only the interaction between *S. officinalis* and chloramphenicol was indifferent. No antagonistic interactions were detected.

The *P. ornatus* extract displayed synergism with ampicillin (β-lactam), kanamycin and gentamicin (aminoglycosides), with 8-fold reductions in the MIC. The same reduction was observed for *S. officinalis* extract, which demonstrated synergism with ampicillin, kanamycin, gentamicin and tetracycline but displayed an indifferent interaction with chloramphenicol. The same results observed for the *S. officinalis* extract were observed for the *S. macranthera* extract, except for the interaction between chloramphenicol and *S. macranthera* extract, which was additive.

DISCUSSION

S. aureus is an important human pathogen and also a relevant pathogen that causes diseases in animals.

TABLE I
***Staphylococcus aureus* inhibition zones of the active extracts (mm).**

Extracts	*S. aureus* 4125		*S. aureus* 3993	
	Inhibition zones ± SD	*p value* t-test (0.05)	Inhibition zones ± SD	*p value* t-test (0.05)
Baccharis dracunculifolia	10.16 ± 0.71	0.001	9.83 ± 0.24	0.01
Inga edulis	14.00 ± 0.94	0.001	16.66 ± 1.41	0.001
Plectranthus ornatus	12.49 ± 1.65	0.005	9.16 ± 0.71	0.003
Salvia officinalis	21.83 ± 2.59	0.001	20.33 ± 0.47	0.012
Senna macranthera	15.49 ± 0.23	0.005	12.66 ± 0.47	0.027
DMSO	0.00 ± nd	nd	0.00 ± nd	nd
Ampicillin	24.25 ± 0.35	0.005	24.10 ± 0.28	0.003

Nd: Not determined.

TABLE II
Values of minimum inhibitory concentrations (mg.mL^{-1}) obtained for the active plant extracts.

Extracts	Minimum inhibitory concentration (MIC) (mg.mL^{-1})	
	S. aureus 3993	*S. aureus* 4125
Baccharis dracunculifolia	5	5
Plectranthus ornatus	1.2	1.2
Salvia officinalis	0.3	0.3
Inga edulis	7	7
Senna macranthera	0.2	0.2

TABLE III
Values of minimum inhibitory concentrations (mg.mL^{-1}) obtained for the tested antibiotics.

Antibiotic	Minimum inhibitory concentration (MIC) (mg.mL^{-1})	
	S. aureus 3993	*S. aureus* 4125
Ampicillin	0.3	7
Kanamycin	2	2
Chloramphenicol	8	8
Gentamicin	0.8	0.6
Tetracycline	0.3	0.25

In dairy cattle this bacteria causes mastitis, an inflammation of the udder that causes significant economic losses worldwide. Antibiotic therapy is commonly used by veterinarians to treat infection increasing the chance of antimicrobial resistance over the years (Oliver and Murinda 2012). Saini et al. (2012) showed that intramammary administration of a penicillin-novobicin combination used in the treatment of mastitis was associated with increased antimicrobial resistance, in particular to ampicillin. Other studies conducted in different countries showed that resistance of bovine *S. aureus* strains can range between 5.2% and 77.3 % for ampicillin (Oliveira et al. 2012, Li et al. 2009) between 0% and 44.2% for gentamicin (Persson et al. 2011, Jumarnović et al. 2011) and between 3% and 60% for tetracycline (Li et al. 2009, Jumarnović et al. 2011). In this sense, new methods to reduce the development of resistance to antibiotics are urgently needed.

A large variety of molecules with potential antimicrobial activity are produced from plant secondary metabolism. It should be highlighted that most of these molecules have a weak antibiotic activity when compared to antibiotics produced by bacteria or fungi (Hemaiswarya et al. 2008). However, these compounds can act in synergism with antimicrobials to potentiate their effect and help the host to overcome the infection.

This study evaluated the antimicrobial potential of fifteen plant species on bovine-origin *S. aureus*. Although the antimicrobial activity of most of the species tested here has been already described for other bacterial species (Parente et al. 2009, Sharma et al. 2009, Vargas et al. 2010) this is the first time their activity was assayed against *S. aureus*.

Extracts of *B. dracunculifolia, P. ornatus, I. edulis, S. officinalis,* and *S. macranthera* displayed biological activity. *S. officinalis* extract displayed the largest inhibition zones (Figure 1). The antimicrobial potential of the plant extracts of *S. officinalis* on Gram-positive bacteria other than *S. aureus* and Gram-negative bacteria has been previously reported (Bara and Vanetti 1998, Haida et al. 2007).

There is no consensus on the acceptable level of inhibition when comparing natural products with antibiotic standards. Some authors consider natural products only effective when they have levels of inhibition similar to antibiotics. Others, however, accept compounds as efficacious with levels of inhibition lower than the normal levels observed with commercial antimicrobials (Duarte 2006). A plant material classification based on MIC results was proposed by Aligiannis et al. (2001) where extracts with strong inhibition have an MIC of 0.5 mg.mL^{-1}; moderate inhibition, between 0.6 to 1.5 mg.mL^{-1}; and weak inhibition, above 1.6 mg.mL^{-1}. Using these criteria, the extracts of *S. officinalis* (MIC of 0.3 mg.mL^{-1}) and *S. macranthera* (MIC of

TABLE IV
Interaction between plant extracts and antibiotics on *Staphylococcus aureus* strains.

Extract	Antibiotic	*S. aureus* 3993				*S. aureus* 4125			
		FIC ant	FIC ext	∑FIC	Interaction	FIC ant	FIC ext	∑FIC	Interaction
1	Ampicillin	1/8 MIC	1/8 MIC	0.25	Synergism	1/8 MIC	1/4 MIC	0.375	Synergism
	Kanamycin	1/8 MIC	1/8 MIC	0.25	Synergism	1/8 MIC	1/8 MIC	0.25	Synergism
	Chlo	1 MIC	1/8 MIC	0.75	Additive	1 MIC	1/8 MIC	0.75	Additive
	Gentamicin	1/8 MIC	1/4 MIC	0.375	Synergism	1/8 MIC	1/8 MIC	0.25	Synergism
	Tetracycline	1/2 MIC	1/4 MIC	0.75	Additive	1 MIC	1/8 MIC	0.875	Additive
2	Ampicillin	1/8 MIC	1/8 MIC	0.25	Synergism	1/2 MIC	1/8 MIC	0.625	Additive
	Kanamycin	1/8 MIC	1/4 MIC	0.375	Synergism	1/8 MIC	1/4 MIC	0.375	Synergism
	Chlo	1/2 MIC	1 MIC	1.5	Indifferent	1/2 MIC	1 MIC	1.5	Indifferent
	Gentamicin	1/8 MIC	1/8 MIC	0.25	Synergism	1/4 MIC	1/8 MIC	0.375	Synergism
	Tetracycline	1/4 MIC	1/8 MIC	0.375	Synergism	1/4 MIC	1/8 MIC	0.375	Synergism
3	Ampicillin	1/2 MIC	1/8 MIC	0.5	Synergism	1/4 MIC	1/8 MIC	0.375	Synergism
	Kanamycin	1/8 MIC	1/4 MIC	0.375	Synergism	1/8 MIC	1/4 MIC	0.375	Synergism
	Chlo	1/2 MIC	1/8 MIC	0.625	Additive	1/2 MIC	1/4 MIC	0.75	Additive
	Gentamicin	1/4 MIC	1/8 MIC	0.325	Synergism	1/4 MIC	1/8 MIC	0.325	Synergism
	Tetracycline	1/4 MIC	1/8 MIC	0.325	Synergism	1/4 MIC	1/8 MIC	0.325	Synergism

[1]*Plectranthus ornatus;* [2]S*alvia officinalis;*[3]*Senna macranthera;* [Chlo.]Chloramphenicol.

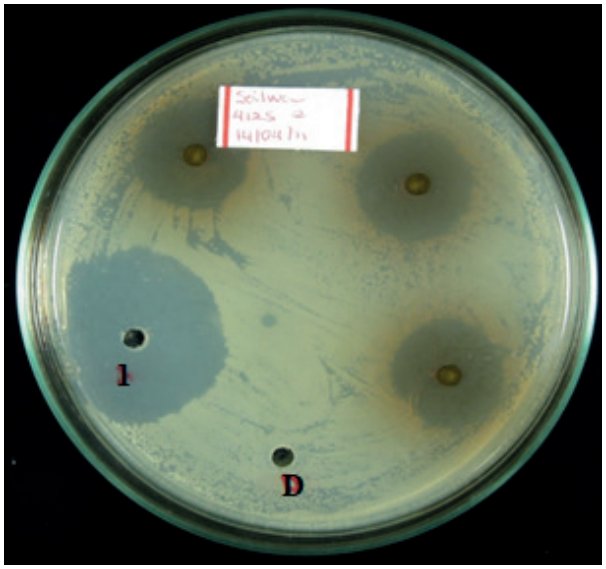

Figure 1 - Inhibition zones of sage (*S. officinalis*) extract on S. aureus 4125. The positive control of growth inhibition antibiotic ampicillin was identified in the figure by number 1. The negative control of inhibition was done with DMSO (D).

0.2 mg.mL^{-1}) can be considered to have displayed strong inhibition. In contrast, extracts of *P. ornatus* (MIC of 1.2 mg.mL^{-1}) can be considered to have displayed moderate inhibition*, B. dracunculifolia* and *I. edulis,* with an MIC of 5.0 mg.mL^{-1} and 7.0 mg.mL^{-1}, respectively, are classified as displaying weak inhibition.

Based on the checkerboard assay, synergism was observed between the *P. ornatus* extract and ampicillin, kanamycin or gentamycin, with an 8-foldreductionin the MIC. The extract of *S. officinalis* also displayed synergism with ampicillin, kanamycin, gentamicin, and tetracycline, though the extract displayed an indifferent interaction with chloramphenicol. An 8-fold decrease in the MIC value was also detected. The same reduction was observed for the extract of *S. macranthera*; however, its interaction with chloramphenicol was considered additive.

The main components of *S. officinalis* and *P. ornatus* extracts are the essential oils caryophyllene, eugenol, and thymol, although lower concentrations of rosmarinic acid, caffeic acid, carnosol, and flavonoids are also found in the former (Velickovic

et al. 2003, De Albuquerque et al. 2007). *S. macranthera* is rich in phenolic compounds as emodin, physcione and chrysophanol and have been used by some farmers to treat bovine mastitis, a chronic infection that produces an inflammatory response in cow's udders and that is caused by *S. aureus* (Andrade 2015), suggesting that compounds present in this extract could be responsible for the decrease observed in MIC combined with β lactams antibiotic.

No specificity to a group of antibiotics was observed which suggests that the crude extract of these plants might contain a mixture of compounds that potentiate the activities of different antibiotics. These compounds facilitate a reduction in the minimum dose required for effective antimicrobial activity. This is interesting because smaller doses can reduce the chance of side effects as well as reduce the costs of treatment. However, for the purposes of therapeutic use, the mode of action of plant extracts should be explored to gain a comprehensive view of the molecular mechanisms involved in their interactions.

CONCLUSIONS

Synergism against the two strains of *S. aureus* was observed between the extracts of *P. ornatus*, *S. officinalis* and *S. macranthera* and different antibiotics used in the treatment of bovine mastitis. The interactions displayed between the extracts of *S. officinalis* and *S. macranthera* and chloramphenicol were additive and between the extract of *P. ornatus* and chloramphenicol was indifferent. The present results are promising and may enhance the use of natural products instead of antibiotics or these can be used in association with active extracts to reduce the use of existing antibacterial drugs. Using these combinations can result in a reduction in the minimum dose required for effective antimicrobial effects, which is interesting because it may decrease both the risk of side effects and the costs of treatment of infectious diseases.

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AUTHOR CONTRIBUTIONS

Danielle Mendes Silva, Priscilla Almeida da Costa and Gislaine Aparecida Purgato: wrote the article; Andréa de Oliveira Barros Ribon and Gaspar Diaz-Muñoz: statistical analyzes; Marisa Alves Nogueira Diaz: statistical analysis and final supervision of the text.

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