Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
20(5): 724-728, Out./Nov. 2010
Received 13 Aug 2009; Accepted 21 Nov 2009, Available online 27 Aug 2010.

Article

Screening of medicinal plants for antibacterial activities on *Staphylococcus aureus* strains isolated from bovine mastitis

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RESUMO: "Screening de plantas medicinais com atividade antimicrobiana contra cepas de *Staphylococcus aureus* isoladas de mastite bovina". *Staphylococcus aureus* é o principal agente causador de mastite bovina. A atividade de diversos extratos de dez plantas medicinais tradicionalmente usadas no Brasil como anti-sépticas foi investigada contra quinze cepas de *Staphylococcus aureus* isoladas de animais com manifestação de mastite pelo método de difusão em ágar e ensaio de microdiluição. A interferência dos extratos na célula bacteriana em forma de colônias aderidas também foi avaliada. Os valores de MIC variaram de 0.5 mg/mL a 1.0 mg/mL e a concentração inibitória de biofilme (BIC) variou de 0.25 mg/mL a 0.8 mg/mL. Os resultados revelaram o potencial dos extratos de *Senna macranthera, Artemisia absinthium, Cymbopogon nardus* e *Baccharis dracunculifolia* como agentes antibacterianos contra cepas de S. aureus isolados de mastite bovina e suportam o possível uso destas plantas no manejo clínico da doença.

Unitermos: *Staphylococcus aureus,* mastite bovina, plantas medicinais, medicina tradicional, inibição de biofilme.

ABSTRACT: *Staphylococcus aureus* is the main causative agent of bovine mastitis. The activity of several extracts from ten medicinal plants traditionally used in Brazil as antiseptic was investigated against fifteen strains of *Staphylococcus aureus* isolated from animals with mastitis manifestation by the disc diffusion method and broth microdilution assay. The interference of the extracts on cell in the form of adherent colonies was also evaluated. MIC values ranged from 0.5 mg/mL to 1.0 mg/mL and biofilm inhibitory concentration (BIC) were between 0.25 mg/mL and 0.8 mg/mL. Results revealed the potential of extracts of *Senna macranthera, Artemisia absinthium, Cymbopogon nardus* and *Baccharis dracunculifolia* as antibacterial agents against S. aureus strains isolated from bovine mastitis and support the possible use of these phytotherapic agents in the clinical management of the disease.

Keywords: *Staphylococcus aureus,* bovine mastitis, medicinal plants, folk medicine, biofilm inhibition.

INTRODUCTION

Medicinal plants have been used for ages in developing countries as alternative treatment to health problems. Brazil has a diverse flora and a rich tradition in the use of medicinal plants for antimicrobial applications (Sartoratto et al., 2004). Many plant extracts have been shown to exert biological activity *in vitro* and *in vivo*, justifying research on traditional medicine focused on the characterization of antimicrobial activity of these plants (Gullece et al., 2006). The importance of using medicinal plants can be attributed to affordability as well as the trust in herbal medicine as an outcome from the witnessed positive results when applying herbs. Many medicinal plants are also used to treat cows, sheep, poultry, horses and pigs (Marinho et al., 2007). Viçosa is a small town located in the state of Minas Gerais, Southeastern Brazil, where dairy activities play an important role in the livelihood of the community from rural area. In this area, mastitis is also the most prevalent dairy health problem. *Staphylococcus aureus* is the main causatitive agent of bovine mastitis and it's commonly associated to chronic infections (Hebert et al., 2000). *S. aureus* presents high resistance to adverse environmental conditions, which makes it a very important

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ISSN 0102-695X
DOI: 10.1590/S0102-695X2010005000013

pathogen. Many chronic infections are associated with bacterial growth in the form of adherent colonies involved by an exopolysaccharide matrix, constituting a biofilm (Cucarella et al., 2004). Due to its aggregated form, some biofilms are not susceptible to phagocytosis and become resistant to some antibiotics (Monzon et al., 2002). It is a key role in the establishment of persistent infections caused by *Staphylococcus aureus* and it is a limiting factor in the treatment of bovine mastitis. Conventional therapeutics is usually based in antibiotics that are not always effective as bacteria become resistant. Besides, its residues can affect human health and interfere in fermentation processes in the milk industry. This motivated the search for new antimicrobial agents and the study of natural plant products as substitutes for chemical antimicrobial agents (Pandian et al., 2006). Medicinal plants were reported by local farmers to be used for increased production of milk and for treatment of bovine mastitis. This work is a step towards exploring the potential use of these medicinal plants indicated. Likewise, other plants traditionally used for treatment of tropical diseases and antimicrobial application for infectious diseases were also screened against strains of *Staphylococcus aureus* isolated from bovine mastitis to evaluated their use in the clinical management of the disease as phytotherapic agent.

MATERIALS AND METHODS

Aerial parts of the medicinal plants *Artemisia absinthium* L. (voucher 15614); *Cymbopogon nardus* L. Rendle (voucher 30283); *Symphytum officinale* L. (voucher 24060); *Baccharis dracunculifolia* DC. (voucher 31322); *Solanum asperolanatum* Ruiz & Pav (voucher 11609); *Salvia officinalis* L. (voucher 1240); *Bauhinia forficata* Link (voucher 15128); *Calendula officinalis* L (voucher 15593) and *Chenopodium ambrosioides* L. (voucher 11762) were collected in Viçosa-MG, Brazil, identified and deposited at the herbarium of the Department of Botany of the Federal University of Viçosa. *Senna macranthera* (Collad.) Irwin et Barn. flowers (voucher 1237) were colleted in Cascavel-PR, Brazil, identified and deposited at the herbarium of the Department of Botany of at State University of West of Paraná. The plants were dried under shade in open air to reduce deterioration of the plant drug material.

The air-dried plant material was weighed and 250 g of each plant sample was extracted exhaustively and consecutively by using *n*-hexane, dichloromethane and ethanol, and 250 g of the seven plants indicated by the farmers were also extracted, additionally, using ethanol/water 80:20 as suggested by them. The extracts were dried under reduced pressure at about 40 ºC. Stock solutions of the extracts were prepared in dimethylsuphoxide (DMSO).

The extracts produced were tested against fifteen *Staphylococcus aureus* strains isolated from animals with occurrence of mastitis cases reported in southeastern Brazil. The isolates were previously grown in 3 mL of brain heart infusion broth (BHI) for 16 h at 37 ºC for the biological tests realization.

In order to make the screening of antibacterial activity of the extracts, suspension of the bacteria containing 10^6 UFC/mL was spread on Müeller Hinton agar and dried at room temperature. Filters paper disks impregnated with each plant extract stock sollution were then placed on the plates, followed by 24 h of incubation at 37 ºC. After incubation, the inhibition zones were measured in millimeters. Clear inhibition zones around the disks with 7 mm or higher of diameter were considered as positive susceptibility (Leite et al., 2000). Ciclopirox olamine (10 mg/mL) was used as positive control and DMSO as negative control. The experiments were repeated twice in triplicate.

The broth microdilution method was used for determination of the minimum inhibitory concentration (MIC) of those extracts which showed activity. The 96 well plates were prepared by dispensing an aliquot of 180 µL of Müeller Hinton broth followed by 20 µL inoculum containing 10^6 UFC/mL. The stock extracts solutions were dispensed in corresponding positions to concentrations from 5 µg/mL to 3.0 mg/mL. The microplates were incubated at 37 ºC for 24 h. The MIC was defined as the lowest concentration of the extract at which the microorganism didn't show visible growth. Ciclopirox olamine (10 mg/mL) was used as positive control and DMSO as negative control. Experiments were conducted in triplicate.

The effect of subinhibitory concentrations of the active extracts on established biolfilms was evaluated according to Johnson et al. (2002) and Nostro et al. (2007) with some modifications. The isolates were grown in BHI in a polystyrene flatbottomed microtitre plate in order to form biofilm after 24 h of incubation at 37 ºC. The supernatant cells were removed with a micropipette and the wells were washed with saline solution 0,85% for three times and filled with 200 mL twofold dilutions of the extracts, ranging from the MIC to a 16-fold dilution of the MIC. Ciclopirox olamine (10 mg/mL) was used as positive control. The plates were incubated for 24 h at 37 ºC. The biofilm inhibitory concentration (BIC) was determined as the lowest concentration at which no visible growth was observed in the supernatant fluid.

Statistical analysis was performed according to Student's t-test ($p<0.05$) in order to compare the results of the inhibition zones obtained from the oils to the ones obtained from the positive control.

RESULTS AND DISCUSSION

The antimicrobial activity of ten medicinal plants was evaluated in this work (Table 1). Seven of them have been used by local farmers in mastitis treatment while the others where chosen due to previous reports.

Table 1. Plants used as antimicrobial in folk veterinary medicine.

Plants	Popular names	Indication
Baccharis dracunculifolia DC	Alecrim-do-campo	Farmers; Alencar et al., 2005; Filho et al., 2008.
Chenopodium ambrosioides L.	Erva-de-santa-maria	Farmers, Albuquerque et al. 2007.
Cymbopogon nardus L. Rendle.	Citronela	Farmers; Koba et al., 2003; Nogueira et al., 2007; Simic et al., 2008.
Symphytum officinale L.	Confrei	Farmers; Tarle et al., 1982; Fenner et al., 2006; Pinto et al., 2006.
Artemisia absinthium L.	Losna	Farmers; Juteau et al., 2003; Michelin et al., 2005; Albuquerque et al., 2007.
Solanum asperolanatum Ruiz & Pav	Jurubeba	Bento et al., 2004; Albuquerque et al., 2007.
Salvia officinalis L.	Salvia	Farmers; Albuquerque et al., 2007; Alvarenga et al., 2007.
Bauhinia forficata Link	Pata-de-vaca	Silva & Cechimel filho 2002; Albuquerque et al. 2007.
Calendula officinalis L.	Calendula	Farmers; Viegi et al., 2003; Lans et al., 2007; Rozwalka et al., 2008.
Dendranthema grandiflorum L.	Crisantemo	Rahman et al., 2007.
Senna macranthera (Collad.) Irwin et Barn.	Pau-fava	Data not published

Among the 37 plant extracts produced, only four inhibited the growth of all *S. aureus* strains evaluated. The results showed that *C. nardus* ethanol/water 80% extract, *S. macranthera* ethanol extract, *A. absinthium* dichloromethane extract and *B. dracunculifolia* ethanol/water 80% extract could inhibit the growth of *S. aureus* strains isolated from bovine mastitis. Minimum inhibitory concentrations (MIC) of the active extracts are shown in Table 2.

Table 2. Minimum inhibitory concentrations of extracts determined by the broth microdilution method.

Plant extracts (mg/mL)	MIC (mg/mL)
S. macranthera (ethanol extract)	1.0
A. absinthium (dichloromethane extract)	1.0
C. nardus (ethanol/water 80% extract)	0.5
B. dracunculifolia (ethanol extract)	0.8
Positive control*	0.05

*ciclopirox olamine

C. nardus showed the strongest antistaphylococcal activity with MIC values of 0,5 mg/mL, followed by *B. dracunculifolia,* MIC of 0,8 mg/mL, and *S. macranthera* and *A. absinthium*, both with MIC equal to 1,0 mg/mL. According to literature results, it is considered a strong activity when MIC values are between 0.05-0.50 mg/mL, moderate activity for MIC values between 0.6-1.50 mg/mL and weak activity above 1.50 mg/mL (Aligiannis et. al, 2001). Therefore, *C. nardus* showed strong activity, while *B. dracunculifolia, S. macranthera* and *A. absinthium* displayed moderate activity. When compared to the positive control, MIC values obtained are still low. However, the extracts produced contain not only one but a mixture of compounds among a single one or a few responsible for the biological activity, what makes necessary the identification of the most relevant substances in later studies. The values of biofilm inhibitory concentrations (BIC), determined over pre-formed biofilms, are presented in Table 3.

Table 3. Biofilm inhibitory concentrations of the active extracts.

Plant extracts	BIC (mg/mL)
S. macranthera (ethanol extract)	0.25
A. absinthium (dichloromethane extract)	0.25
C. nardus (ethanol/water 80% extract)	0.25
B. dracunculifolia (ethanol extract)	0.8
Positive control*	0.025

*ciclopirox olamine.

The active extracts were tested in concentrations correspondent to MIC, ½ MIC, ¼ MIC, ⅛ MIC and $^{1/16}$ MIC. The values of BIC obtained were between 0.25 and 0.8 mg/mL. *S. macranthera, A. absinthium* and *C. nardus* showed BIC values equal to 0.25 mg/mL, lower than the BIC obtained to *B. dracunculifolia*, 0.8 mg/mL. None of the BIC values found were lower than ¼ MIC of the respective extract. Therefore, *C. nardus* was the extract that displayed the biggest inhibition zones and the lowest MIC and BIC values.

CONCLUSIONS

Many of the plants used by farmers in southeastern Brazil have popular indication of antibacterial activity. Our results, although, revealed only the potential of the extracts obtained from *Senna macranthera, Artemisia absinthium, Cymbopogon nardus* and *Baccharis dracunculifolia* against *S. aureus* of bovine origin. Among the seven plants indicated by the farmers for the treatment of mastitis (Table 1), *B. dracunculifolia* and *C. nardus* were the only ones that validated the popular use of medicinal plants in the management of the disease. Our results validate the use in folk Brazilian culture of some plants that can be easily found in our flora and some worldwide. The results support the possible use of these in the clinical management of this disease as phytotherapic agent.

ACKNOWLEDGEMENT

The authors are thankful to Embrapa Gado de Leite, which provided the bacterial strains used in this work.

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