Full Length Research Paper

Aquatic plants as potential sources of antimicrobial compounds active against bovine mastitis pathogens

Ciro César Rossi, Ananda Pereira Aguilar, Marisa Alves Nogueira Diaz and Andréa de Oliveira Barros Ribon*

Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, Minas Gerais, Brazil.

Resistance of pathogens to common veterinary antibiotics hampers mastitis treatment and motivates the discovery of new antimicrobials. In this study, extracts from two aquatic plants, *Salvinia auriculata* and *Hydrocleys nymphoides,* were assayed against bovine mastitis pathogens. Selected parts of plants were extracted with different solvents. The extracts showed activity only against the Gram-positive strains tested and the largest inhibition zones were seen for hexane extracts. The minimum inhibitory concentration values ranged from 0.2 to 1.0 mg/ml. Growth of *Streptococcus agalactiae* in the presence of different extracts with concentrations below MIC reduced the number of CFU/ml by more than 90%. Sub-MIC concentrations of the hexane extracts prepared from roots of *S. auriculata* inhibited approximately 50% of biofilm formation. Greater reduction was achieved for ethanol extract prepared from leaves of *H. nymphoides.* We concluded that these aquatic plants are potential sources for the investigation of new antimicrobial compounds.

Key words: *Staphylococcus aureus*, *Streptococcus agalactiae*, aquatic plants, *Salvinia auriculata*, *Hydrocleys nymphoides,* antimicrobial activity.

INTRODUCTION

Bovine mastitis, an inflammatory response in cow's udder, is the main infecto-contagious disease affecting dairy cattle and is considered a limiting factor in many dairy properties (LeBlanc et al., 2006). The huge economic losses associated with mastitis worldwide motivate researches focused on several aspects of the disease and since the middle sixties a successful 5-point Mastitis Control Plan has been adopted in different countries aiming to reduce the incidence of mastitis (Hillerton and Berry, 2005). The disease is often classified as clinical, when it is easily detected by abnormal milk or detectable changes in the udder, or subclinical, which shows out as a low grade infection that affects 10 to 15% cows. In this case, the animal becomes a potential reservoir and infections are spread among cows during the milking process.

Bacteria are among the main etiologic causative agents of mastitis, and *Staphylococcus aureus* and *Streptococcus agalactiae* are two of the most common species that have infected udder as main reservoir (Watts, 1988). Infections caused by *S. aureus* frequently turn into chronic and cows with such infections have to be culled. *S. agalactiae* also causes chronic and subclinical mastitis but opposed to *S. aureus* is susceptible to treatment with many antibiotics (Keefe, 1997).

Biofilms are structured community of bacterial cells associated with a surface, enclosed in an extracellular polysaccharide matrix (Costerton et al., 1999). By 1990, it was assumed that biofilms could be related with chronic

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*Corresponding author. E-mail: abribon@ufv.br. Tel: +55 31 38992837. Fax: +55 31 38992373.

Abbreviations: HRSA, Extract prepared in hexane from roots of *Salvinia auriculata;* **DRSA,** extract prepared in dichloromethane from roots of *S. auriculata;* **ARSA,** extract prepared in ethyl acetate from roots of *S. auriculata;* **ERSA,** extract prepared in ethanol from roots of *S. auriculata;* **HLSA,** extract prepared in hexane from leaves of *S. auriculata;* **DLSA,** extract prepared in dichloromethane from leaves of *S. auriculata;* **ALSA,** extract prepared in ethyl acetate from leaves of *S. auriculata;* **ELSA,** extract prepared in ethanol from leaves of *S. auriculata;* **HLHN,** extract prepared in hexane from leaves of *Hydrocleys nymphoides;* **DLHN,** extract prepared in dichloromethane from leaves of *H. nymphoides;* **ALHN,** extract prepared in ethyl acetate from leaves of *H. nymphoides;* **ELHN,** extract prepared in ethanol from leaves of *H. nymphoides.*

infections and in recent years several studies show that biofilm formation is a key factor in the establishment and persistence of infections caused by *S. aureus* and *S. agalactiae* in animals (Karaolis et al., 2005; Melchior et al., 2006; Ghiorghi et al., 2009). Due to its aggregate form, biofilms are not susceptible to phagocytosis by macrophages and sessile bacteria are 10 to 10,000 fold less susceptible to antimicrobials when compared to planktonic cells (Monzon et al., 2002; Davies, 2003; Hall-Stoodley et al., 2004). Six veterinary pathogenic bacteria, including *S. aureus* and *S. agalactiae*, that were resistant to twelve antibiotics when allowed to form biofilms showed increased susceptibility when grown as plank-tonic cells (Olson et al., 2002). Despite these findings, antibiotics are still used in the treatment of bovine mastitis but the prognosis of a complete cure is usually poor (Sandholm et al., 1990). Another difficulty in mastitis treatment is the increasing resistance of pathogens to most common antibiotics what limits treatment options (Werckenthin et al., 2001; Acar and Moulin, 2006).

In the last few decades, the number of researches on plants antimicrobial properties has increased as they are considered sources of pharmacologically active compounds that can become prototypes of new antibiotics (Meléndez et al., 2006). Plants that live in nutrient-rich environment, as well as plants living in an environment with a very high bacterial cell density will become overwhelmed by microbial biofilms if they lack any means of biofilm control (Hu et al., 2006; Vattem et al., 2007). For this reason, aquatic plants have attracted the interest of researchers and have shown themselves as promising sources of antimicrobial agents (Özbay and Alim 2009). *Hydrocleys nymphoides* (Limnocharitaceae), popularly known as water poppy and *Salvinia auriculata* (Salviniaceae), also known as eared water moss, are aquatic plants native from South America, commonly found in sweet watered lakes (Kissmann, 1997; Lorenzi, 2000). Studies have shown the potential of *S. auriculata* in the remediation of polluted water contaminated with heavy metals (Peixoto et al., 2005; Soares et al., 2008). However, studies have not yet investigated their potential as antimicrobial compounds producers. This study aimed to evaluate for the first time the antimicrobial potential of extracts obtained from these plants over Gram-positive and Gram-negative bovine mastitis causative bacteria.

MATERIALS AND METHODS

Plant material

S. auriculata and *H. nymphoides* were collected in a pond located in Recanto das Cigarras, Universidade Federal de Viçosa, Minas Gerais State, Brazil, from April to June, 2009. The material was exhaustively washed in water and roots and leaves of *S. auriculata* were separated. Only leaves of *H. nymphoides* were studied due to small length of the roots. The plant parts were dried at 40°C for 24 h in an air circulation oven and 100 g of each part were extracted using 1.0 L of solvent for 2 days and repeated at least five times. The solvents used were hexane, followed by dichloromethane, ethyl acetate, and ethanol (Vetec®). The extract was concentrated with a rotary evaporator, redissolved in dimethyl sulphoxide (DMSO) to final stock concentration of 50 mg ml^{-1} and stored at 4°C for further experiments.

Bacterial strains and culture media

The bacterial strains used in this study were kindly provided by Embrapa/CNPGL, Juiz de Fora, Minas Gerais, Brazil, and were isolated from animals with mastitis manifestation by standard procedures. Twenty one (21) *S. aureus* strains (3828, 4119, 3008, 3007, 3022, 4163, 4784, 3019, 4766, 4313, 3703, 3820, 4347, 4082, 4098, 3992, 4118, 4072, 3993, 2221, and 4052), five *Escherichia coli* strains (23, 24, 25, 26, and 27) and three *S. agalatictae* strains (3866, 3867, and 3868), were used to determine antimicrobial activity of the extracts. Bacteria were routinely cultured on brain heart infusion (BHI) at 37°C for 16 h. Prior to experiments, cell concentration was adjusted to 10^6 CFU ml^{-1} by optical density at 600 nm. Stock cultures were maintained in BHI containing 25% glycerol at -80°C.

Antibacterial screening assay

Hole-plate diffusion assay was initially performed to test for antibacterial activity of the twelve extracts produced. For such purpose, bacteria were cultivated overnight and suspension containing 10^6 CFU ml^{-1} was spread in plates containing Müeller-Hinton agar (Himedia®). Holes of approximately 5 x 3 mm were made in the agar and filled with 30 µl of the stock solutions (50 mg ml^{-1}) of the extracts. After incubation at 37°C for 24 h, inhibition zones were measured in millimeters and compared to the controls. The minimum inhibitory concentration (MIC) of the effective extracts was determined by a broth microdilution method ranging the concentrations from 5 to 3 mg ml^{-1}, followed by incubation at 37°C for 24 h and observation of media turbidity (Caetano et al., 2002). The antibiotic ciclopyrox olamine (Uci-Farma®) was used as the positive control due to its antibacterial properties. Dimethyl-suphoxide (DMSO) was used as negative control. Tests were performed twice in triplicate.

Cell viability assay

Cell viability was observed 6 h after inoculation of 1.0 ml bacteria culture containing 10^8 CFU ml^{-1} in tubes with 9 ml of phosphate buffer 5 mM, pH 6.5, containing concentrations of the active extracts equivalent to 2X MIC, 1.5X MIC, MIC, 1/2 MIC, and 1/4 MIC. The tubes were incubated in water bath at 37°C. Aliquots were taken from the tubes and serially diluted (ten-fold dilutions) in microplates containing BHI that were incubated at 37°C for 24 h, following visual inspection of turbidity of the medium. This method allowed the estimation of the order of magnitude of the cells that remained viable after incubation with plant extract. DMSO was used in the positive control, in the extract's corresponding concentrations. Tests were realized twice in triplicate.

Effect of plant extracts on cell adherence

The effect of sub-inhibitory concentrations of active extracts on established biofilms was evaluated according to Nostro et al. (2007) with few modifications. Bacterial suspensions were inoculated into microplate containing 180 µl of BHI to final concentration of 10^6 CFU ml^{-1} and incubated at 37°C for 24 h. The supernatant was withdrawn, wells were washed three times with saline 0.85% and filled again with BHI containing different concentrations of the active

Table 1. Inhibition zones (mm) ± SD of the active extracts on *Staphylococcus aureus* and *Streptococcus agalactiae* cultures.

Bacteria		Extract (Inhibition zones (mm) ± SD)							
		HRSA	HLHN	DLSA	DLHN	ALSA	ALHN	ELHN	Control[a]
S. agalactiae	3866	10.67±1.33	-	11.33±0.33	12.00±1.15	10.33±0.88	10.67±0.33	9.67±0.67	14.00±0.58
	3867	9.33±0.67	-	9.67±0.88	11.67±0.88	9.00	10.00±0.58	9.33±0.33	15.33±0.88
	3868	10.33±0.33	-	9.67±0.33	12.00±0.58	10.67±0.33	10.00±1.15	9.00±0.58	14.67±0.88
S. aureus	4163	20.00±0.58	15.33±0.33	-	-	-	-	-	21.00±0.58
	3019	15.67±0.33	8.33±0.67	-	-	-	-	-	21.67±0.88
	4347	23.00±0.58	12.00±0.58	-	-	-	-	-	21.00±0.58

[a] Ciclopyrox olamine; - no inhibition zone.

extracts (MIC, 1/2 MIC,1/4 MIC, 1/8 MIC, and 1/16 MIC), followed by incubation at 37°C for 24 h. The biofilm inhibitory concentration (BIC) was defined as the concentration at which no visible microbial growth was observed. The assay was realized twice in triplicate.

Effect of plant extracts on biofilm formation

Bacterial suspensions were inoculated on microplate containing 180 µl of BHI with different concentrations of the active extracts (MIC, 1/2 MIC, 1/4 MIC, 1/8 MIC, and 1/16 MIC) to final concentration of 10^6 CFU ml^{-1} and incubated at 37°C for 24 h. The supernatant was withdrawn and wells were washed three times with saline 0.85%. The remaining bacterial mass was dried at 37°C for 15 min and stained with 200 µl of crystal violet 0.1% for 30 min. Wells were rewashed and dried as described previously, followed by addition of 300 µl of ethanol and measurement of absorbance at 630 nm. The test was realized twice in triplicate.

RESULTS

In this study, the antimicrobial activity of extracts obtained from roots of *S. auriculata* prepared in hexane (HRSA), dichloromethane (DRSA), ethyl acetate (ARSA) and ethanol (ERSA); leaves of *S. auriculata* prepared in hexane (HLSA), dichloromethane (DLSA), ethyl acetate (ALSA) and ethanol (ELSA); and leaves of *H. nymphoides* prepared in hexane (HLHN), dichloromethane (DLHN), ethyl acetate (ALHN), and ethanol (ELHN) were evaluated. Among all extracts tested, HRSA was the only one to show activity against all the Gram-positive strains (Table 1), while no activity was detected for other extracts obtained from *Salvinia* roots, that is, DRSA, ARSA, and ERSA. Among the four extracts prepared from leaves of *S. auriculata,* DLSA and ALSA inhibited *S. agalactiae* but no *S. aureus* growth. HLSA and ELSA displayed no inhibitory activity. HLHN, prepared from leaves of *H. nymphoides*, showed biological activity against *S. aureus* only (Table 1). However, three extracts (DLHN, ALHN, and ELHN) with different polarities displayed activity against *S. agalactiae* strains. Since the results obtained for all *S. aureus* strains were essentially the same, Table 1 shows the data obtained for the strains 4347 and 3019, which displayed the highest and lowest inhibition zones from the HRSA extract, respectively, and 4163 and 3019, which displayed the highest and lowest inhibition zones from the HLHN extract.

No inhibitory halo was observed for *E. coli* irrespective of the extract assayed. Minimum inhibitory concentration of each active extract was determined and there was no difference in MIC values between strains of the same species (Table 2). HRSA was more effective in inhibiting the growth of *S. aureus* of bovine origin when compared to HLHN. Among the biological active extracts tested against *S. agalactiae*, DLSA showed the lowest MIC value (0.3 mg ml^{-1}). ALSA was not further evaluated due to high MIC value detected (> 3.0 mg ml^{-1}). Since the same extract had equal effect on the 21 strains of *S. aureus* and three strains of *S. agalactiae* one strain of each species were chosen for further assays. To investigate the effect of the extracts listed on Table 2 on cell viability, *S. aureus* 3828 and *S. agalactiae* 3866 were incubated for 6 h in different extract concentrations. In all cases, concentrations below MIC reduced the number of CFU ml^{-1} by more than 90% (more than one log cycle) (Figure 1). Concentrations equal to 2X MIC of the extracts HRSA and HLHN decreased in 100% the viability of *S. aureus* 3828 cells (Figure 1B), the same was observed for extract HRSA against *S. agalactiae* 3866 (Figure 1A). The effect of the same extracts tested in Figure 1 on cell viability was estimated in intervals equal to 0, 6, 12, and 24 h, with extract concentrations ranging from MIC to 1/4 MIC.

Table 2. Minimum inhibitory concentration (MIC) and biofilm inhibitory concentration (BIC) values obtained from the active extracts on *Staphylococcus aureus* and *Streptococcus agalactiae*.

Organism	Extract	MIC (mg ml^{-1})	BIC (mg ml^{-1})
S. aureus	HRSA	0.3	0.075
	HLHN	1.0	0.5
	Positive control[a]	0.05	0.025
S. agalactiae	HRSA	0.5	0.25
	DLHN	0.5	0.5
	DLSA	0.3	0.3
	ALHN	1.0	0.5
	ALSA	>3.0	-
	ELHN	0.6	0.3
	Positive control[a]	0.05	0.025

[a]Ciclopyrox olamine.

Again, a logarithmic reduction value greater than one was observed after 6 h. As expected, in the period of 24 h, only concentrations equal to MIC were sufficient to inhibit the growth of 100% of the cells.

The active extracts were tested in subinhibitory concentrations (1/2 MIC, 1/4 MIC, 1/8 MIC, and 1/16 MIC) to evaluate BIC on pre-formed biofilms (Table 2). HRSA presented the lowest BIC values for either *S. aureus* or *S. agalactiae*, equivalent to, respectively 1/4 and 1/2 of the MIC values obtained. The HLHN extract was the best that inhibited the growth of adherent cells of *S. aureus.* Figure 2 shows the quantitative analysis of the effect of *S. auriculata* extracts on biofilm formation. Concentrations correspondent to 1/4 MIC and 1/8 MIC of HRSA were enough to inhibit approximately 50% of biofilm formation by *S. aureus* 3828 and *S. agalactiae*, respectively (Figure 2A). The same was observed for 1/8 MIC of the DLSA extracts on *S. agalatiae* cultures (Figures 2B). Figure 3 shows the effect of different concentrations of extracts obtained from leaves of *H. nymphoides* on the tested strains. The best result was obtained with the extract produced from *H. nymphoides* by maceration in ethanol, since a concentration equivalent to 1/8 MIC inhibited more than 50% of biofilm formation by *S. agalactiae* 3866 (Figure 3D). Only 1/2 MIC of the other extracts obtained from *H. nymphoides* leaves, HLHN, DLHN and ALHN, inhibited biofilm formation by more than 50% *S. aureus* 3828 and *S. agalactiae* 3866 cultures (Figures 3A to C).

DISCUSSION

The great concern with the resistance of pathogens to antimicrobials used in veterinary practices stimulates research on new compounds that have antimicrobial activity. It is believed that there are many producers of natural compounds unexplored in aquatic environment that could be potential sources to the reduction or control of bacterial diseases (Özbay and Alim, 2009). Recently, the antibacterial activity of some water plants has been described to motivate researches with other species (Morales et al., 2006; Al-Bayati and Al-Mola, 2008; Fareed et al., 2008). One advantage in studying aquatic plants is their high growth rate, which facilitates the large-scale production of extracts for the purpose of purification of active compounds (Martins et al., 2004; Wolff et al., 2008). *S. auriculata* and *H. nymphoides* are water plants distributed in several regions of Brazil. *S. auriculata* is an aquatic bioindicator and has a great capacity to absorb metals, a property that is explored in bioremediation processes (Oliveira et al., 2001). As far as we know, there are no reports focused on their antimicrobial properties neither in their chemistry.

Our results showed the antimicrobial potential of specific parts of both plants against Gram-positive strains belonging to two species, *S. aureus* and *S. agalactiae*. Under the conditions tested, the extracts had no effect on *E. coli*, a Gram-negative bacteria. It is well known that Gram-positives are susceptible to antibiotics, like vancomycin, that do not work or work poorly against Gram-negatives, a difference related to the ability of antibiotics to be blocked by the pore size of porin proteins in the outer membrane of the later (Koebnik et al., 2000). In this study, the hexane extract from roots of *S. auriculata* showed relatively low inhibitory concentration values against Gram-positive bacteria, what indicates its potential as an antimicrobial compound producer. The roots are in close contact with water and probably more susceptible to invasion by microorganisms in the case of microbial water contamination. Extracts prepared with several solvents and, therefore, with different polarities of *H. nymphoides* were active against *S. aureus* and *S. agalactiae*, suggesting the existence of more than one substance with different chemical characteristics with antimicrobial activity. Also, the presence of the same
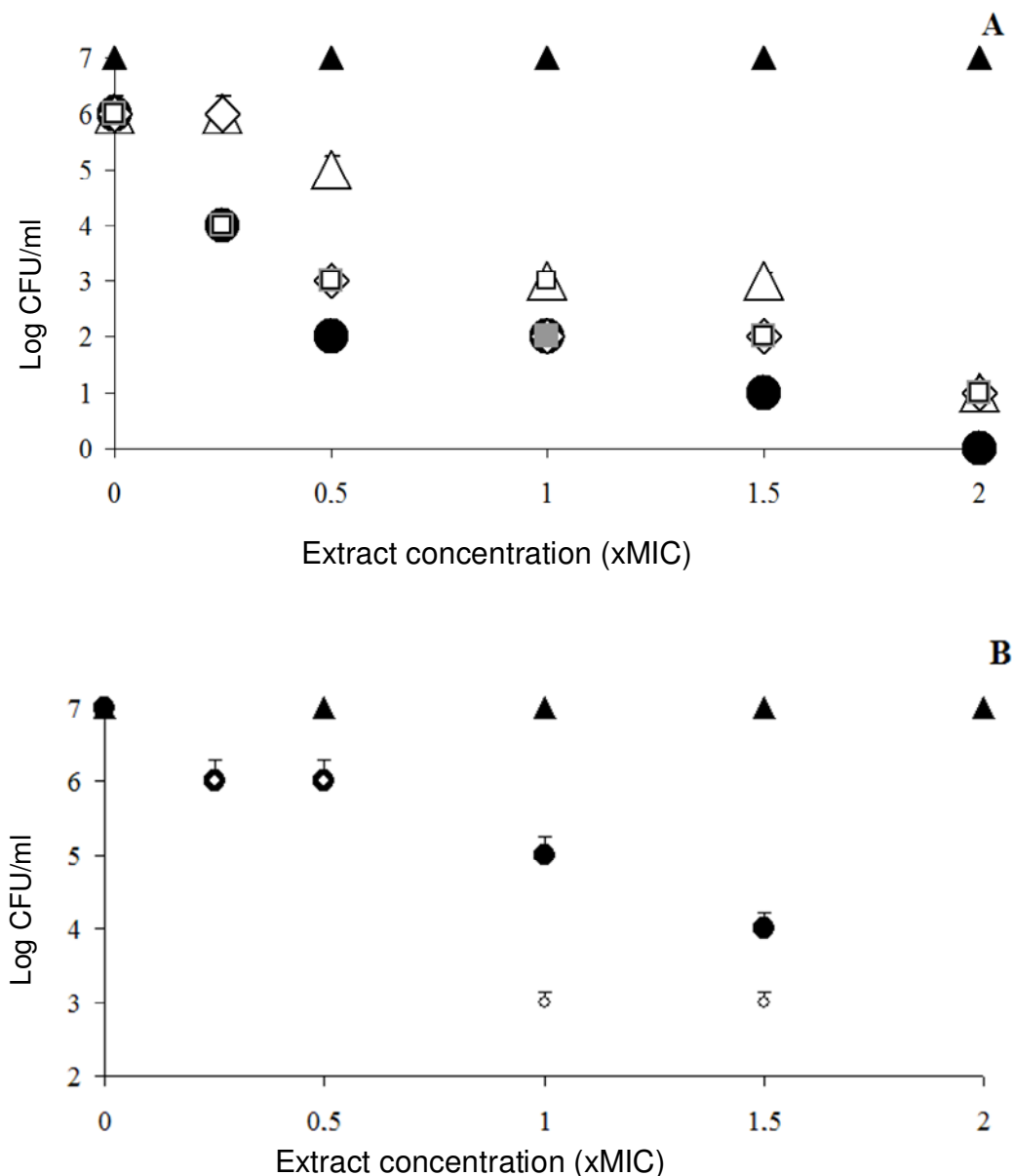
Figure 1. Cell viability of bacterial strains incubated for 6 h in the presence of extract concentrations equivalent to 1/2 MIC, 1.0X MIC, 1.5X MIC, and 2X MIC. (A) Viability of *Streptococcus agalactiae* 3866 was determined in presence of the extracts HRSA (●), HLSA (■), DLHN (□), ALHN (◊), and ELHN (Δ), (B) viability of *Staphylococcus aureus* 3828 was determined in presence of the extracts HLHN (○), and HRSA (●). The filled triangle represents the negative control (DMSO in the highest concentration among the other tubes, present in the plant extracts).

active molecule in different solvents cannot be excluded.

The MIC values obtained are lower than some values previously found for plant extracts with antimicrobial activity (Duarte et al., 2004; Virtuoso et al., 2005). Based on MIC values, extracts can have strong (0.05 to 0.5 mg ml^{-1}), moderate (0.6 to 1.5 mg ml^{-1}) or weak activity (> 1.5 mg ml^{-1}) (Aligiannis et al., 2001). Using the afore-mentioned criteria, the extracts evaluated in this study can be considered moderate and strong inhibitors except

for the ethyl acetate extract prepared from leaves of *S. auriculata* that showed weak activity. However, the minimum concentrations for bacterial inhibition are still very high compared to those for commercial antibiotics. If we consider that plant extracts contain few substances responsible for bactericidal activity among a mixture of compounds, the results are still very promising and reinforce the need for identification of the substance with antibiotic property. Also, the extracts can be used for the
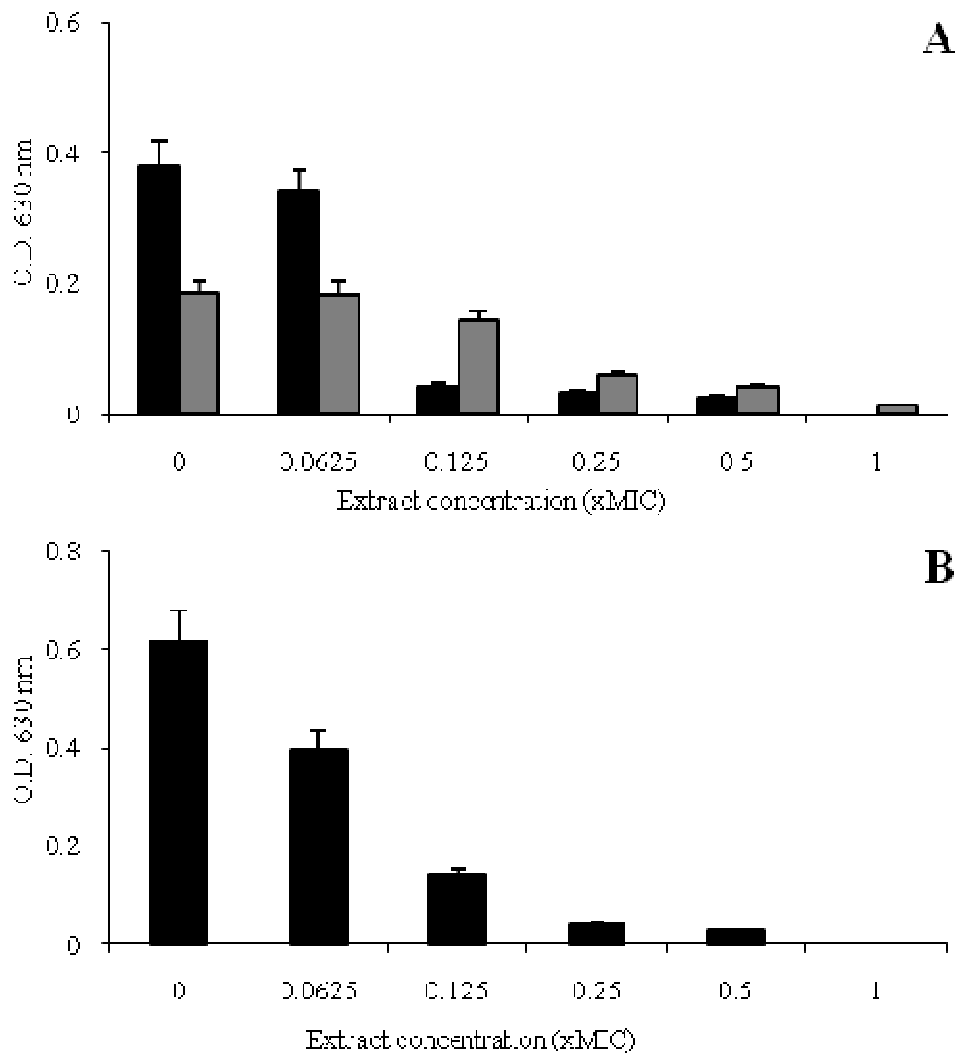
Figure 2. Effect of different concentrations of extracts obtained from *Salvinia auriculata* on bacterial film formation. *Streptococcus agalactiae* 3866 (black bars) and *Staphylococcus aureus* 3828 (gray bars). Extracts: (A) HRSA and (B) DLSA.

production of therapeutic soaps to clean animal´s teats before milking, improving udder health and milk quality as long as no general cell toxicity is detected.

The cell viability assays showed that subinhibitory concentrations affected the bacterial cell in some way since, in all cases, concentrations equal to 1/2 MIC reduced the viability of at least 90% of cells. Again this is another strong motivation to promote research on extract fractionation followed by purification and identification of the active component.

The pharmaceutical industry has major interest in the identification of novel antimicrobials that target microbial biofilms, since their role in pathogenesis was recognized. Adhesion and formation of biofilms were two factors affected by the extracts of *S. auriculata* and *H. nymphoides*. The values determined for the BIC were similar to the ones found for antibiotic substances

reported elsewhere (Castillo et al., 2006; Nostro et al., 2007). The best results on biofilm inhibition were found for ethanol extracts from *H. nymphoides* and hexane extracts from roots of *S. auriculata*. In resume, the results shown here prove the efficiency of secondary metabolites produced by the aquatic plants *S. auriculata* and *H. nymphoides* in inhibiting the growth of bacteria of bovine origin. Further studies for the isolation of active substances for control of pathogens and the study of their mode of action on the microbial cell are underway.
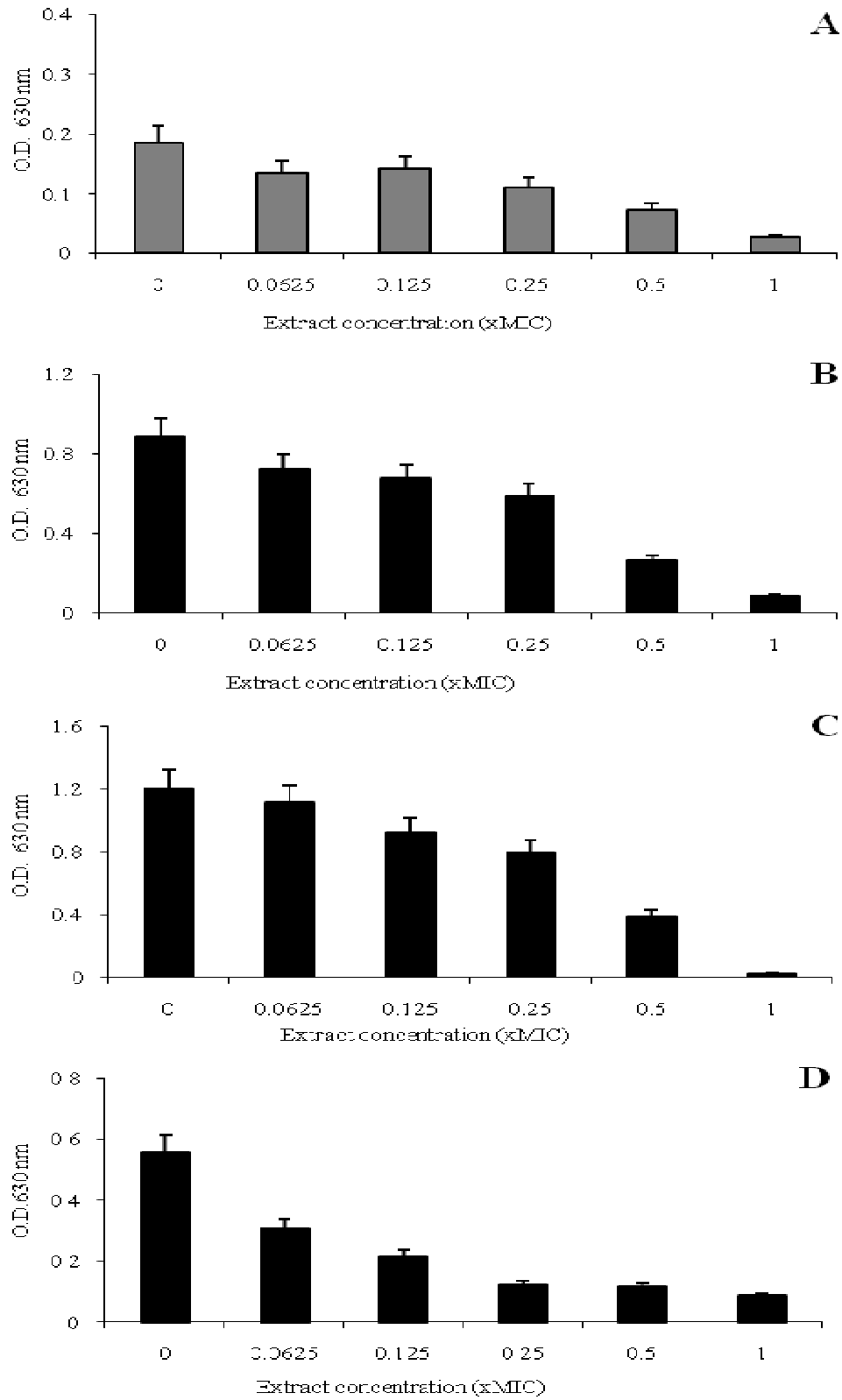
ACKNOWLEDGMENT

Figure 3. Effect of different concentrations of extracts obtained from leaves of *Hydrocleys nymphoides*. Extracts were prepared on hexane (HLHN; A), ethyl acetate (ALHN; B), dichloromethane (DLHN; C), and ethanol (ELHN; D) and the effect on bacterial film formation by *Streptococcus agalactiae* 3866 (black bars) and *Staphylococcus aureus* 3828 (gray bars) were assayed.

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