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Antimicrobial activity of fixed oils found in Brazil nuts and sunflower seeds against microorganisms isolated from bovine subclinical mastitis

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Concern with traces of antibiotics found in milk and resistant bacteria has led to the search for alternatives, mainly natural products, to treat mastitis. This study aimed at evaluating the antimicrobial activity of fixed oils found in Brazil nuts and sunflower seeds against microorganisms isolated from mastitic milk and at analyzing their fatty acids. The following microorganisms were isolated from milk produced by cows with subclinical mastitis and selected for the antimicrobial test: *Staphylococcus aureus*, *Enterococcus* spp., *Candida* spp. and *Cryptococcus laurentii.* The oil components and the antimicrobial activity were determined by gas chromatography and by the broth microdilution technique, respectively. The following fatty acids were identified: myristic, palmitic, palmitoleic, margaric, stearic, oleic, linoleic, linolenic, arachidic, gadoleic, behenic and lignoceric. Averages of minimum inhibitory concentrations (MIC) for *S. aureus*, *Enterococcus* spp., *Candida* spp. and *C. laurenti* were 36.30; 21.35; 15.62 and 10.41 µg.mL^{-1} (Brazil nut oil) and 23.5; 19.21; 7.81 and 1.2 µg.mL^{-1} (sunflower seed oil), respectively. These values show that these oils, in low concentration, have antimicrobial activity against the microorganisms under study. Therefore, this study shows that these fixed oils may be used as therapeutic resources for the control of mastitis.

Key words: Mastitis, antimicrobial activity, fixed oils.

INTRODUCTION

Mastitis, an inflammatory process in the mammary glands, causes major economic losses to dairy farms due to the decrease in milk production and quality, to the increase in medication use and to the risk of animal death. The etiology of this disease has shown that it may have toxic, traumatic, allergic, metabolic and mainly infectious origins (Melchior et al., 2006).

Even though several other microorganisms may affect the intramammary region and cause an infection, *Staphylococcus aureus* is the main etiologic agent of chronic mastitis in dairy cows (Roberson et al., 1994). Despite the fact that bacteria are the agents which are more likely to be isolated, there are some cases of environmental microorganisms in the literature, mainly yeast from the genera *Candida* spp. and *Cryptococcus* spp. (Spanamberg et al., 2009).

Antibiotic therapies have been the most common procedures in the treatment of mastitis in dairy cows. However, concern with traces of antibiotics in milk and with resistant bacterial strains has led to the search for alternatives that may mitigate or eliminate such problems (Freitas et al., 2005). Researchers have become increasingly interested in natural products as sources of new drugs, such as medicinal plants (Pinto et al., 2006).

Therefore, this study aimed at evaluating the antimicrobial activity of fixed oils found in Brazil nuts and sunflower seeds against microorganisms isolated from milk from mammary quarts with subclinical mastitis.

MATERIALS AND METHODS

Chromatographic

Chromatographic analyses of Brazil nut and sunflower seeds oils were carried out by a GC-2010 chromatographer (Shimadzu, Japan) with an Elite-WAX capillary column (0.25 µm x 30 m x 0.25 mm). The injection of 1 µL of the sample was carried out in split mode (1:25). The carrier gas was H_2 (1.2 mL.min^{-1}). The initial temperature of the column was 140°C for 5 min; then, it was increased to 4°C per minute, up to 230°C for 10 min.Quantification was performed by the area normalization method proposed by GCSolution software whereas the identification of fatty acids was carried out by comparison with FAME MIX 37 standards (Sigma-Aldrich).

Antimicrobial tests

In this study, microorganisms that had been previously identified by the Vitek 2 system were selected: *S. aureus* (n=10), *Enterococcus* spp. (n=4), *Candida* spp. (n=4) and *Cryptococcus laurentii* (n=3) from bovine subclinical mastitis. Before the realization of the tests, it was recommended that bacteria which had resistance *in vitro* to at least five antibiotics be used in the routine of veterinary medicine.

The determination of the minimum inhibitory concentrations (MIC) of the oils was carried out by the broth microdilution technique, in agreement with CLSI 2008 (protocols M7-A6 and M27-A3, for bacteria and yeasts, respectively), adapted to a phytopharmacological agent.

The oils were prepared with dimethylsulphoxide (DMSO) as a dispersing solvent. In order to carry out the bacterial test, sterile microdilution plates that were previously filled with 100 µL Müeller-Hinton broth; ten successive oil dilutions, whose concentration varied from 170.8 to 0.33 µg.mL^{-1}, were made. Afterwards, plates were inoculated with 50 µL of the bacterium to be tested. It was been prepared to comply with 0.5 turbidity in the McFarland scale and adjusted in a cultivation media. After incubation at 36°C for 18 h, the plates had their MIC determined. In order to determine their minimum bactericidal concentrations (MBC), an aliquot of 5 µL from the first well of the microplate on which there was growth and from two preceding ones – were planted on a plate with Brain Heart Infusion Agar and incubated for 24 h so that bacterial growth could be observed.

Regarding yeasts, ten successive dilutions of oils were made on the microdilution plates; concentrations varied from 83.3 to 0.16 µg.mL^{-1} in RPMI media. The microplates were inoculated with 100.0 µL of the microorganism to be tested and incubated at 36°C for 48h. Then, the MIC was determined. In order to determine their minimum fungicidal concentrations (MFC), an aliquot of 5 µL - from the first well of the microplate on which there was growth and from two preceding ones - was planted on a plate with Sabouraud dextrose agar and incubated for 24 h so that yeast growth could be observed.

RESULTS

Results of the chromatographic analysis of Brazil nut and sunflower seed oils are shown in Table 1. The following fatty acids were found in the oils under study: myristic acid (C 14:0), palmitic acid (C 16:0), palmitoleic acid (C 16:1), margaric acid (C 17:0), stearic acid (C 18:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c), linolenic acid (C18:3n3), arachidic acid (C20:0), gadoleic acid (C20:1n9), behenic acid (C22:0) and lignoceric acid (C24:0). Results showed that oleic acid (C18:1n9c) and linoleic acid (C18:2n6c) are the fatty acids with the highest concentrations in Brazil nut oil. The former is composed of 30.61%, whereas the latter is composed of 47.26% of Brazil nut oil. Myristic acid (C 14:0) is the one with the lowest concentration (0.044%) in the composition of the fatty acids found in the sample.

The MIC of the oils, in relation to the microorganisms under study are shown in Table 2. In the case of Brazil nut oil, the averages of these concentrations were 36.30; 21.35; 15.62 and 10.41 µg.mL^{-1} for *S. aureus*, *Enterococcus* spp., *Candida* spp. and *C. laurentii*, respectively. In the case of the 19 isolated microorganisms, MIC was similar to MBC/MFC. Regarding the sunflower seed oil, MIC's were 23.5; 19.21; 7.81 and 1.2 µg.mL^{-1} for *S. aureus*, *Enterococcus* spp., *Candida* spp. and *C. laurentii*, respectively. MBC/MFC of this oil was equal to MIC's of 18 microorganisms.

DISCUSSION

Venkatachalam and Sathe (2006) determined the fatty acids found in Brazil nut oil in 2006: their values were similar to the ones found in this study, that is, 45.43% of

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Table 1. Identification and concentration of fatty acids found in Brazil nut and sunflower seed oils.

Compound	Brazil nut oil (%)	Sunflower seed oil (%)
Myristic acid (C 14:0)	0.044	0.071
Palmitic acid (C 16:0)	13.467	5.791
Palmitoleic acid (C 16:1)	0.232	0.073
Margaric acid (C 17:0)	0.129	0.039
Stearic acid (C 18:0)	7.741	4.106
Oleic acid (C 18:1n9c)	30.606	35.805
Linoleic acid (C 18:2n6c)	47.256	53.638
Linolenic acid (C 18:3n3)	0.095	0.043
Arachidic acid (C 20:0)	0.194	0.244
Gadoleic acid (C 20:1n9)	0.070	0.096
Behenic acid (C 22:0)	0.099	0.0866
Lignoceric acid (C 24:0)	0.065	0.228
Total	100.00	100.00

Table 2. Minimum inhibitory concentrations and minimum bactericidal/fungicidal concentrations of fixed oils found in Brazil nuts and sunflower seeds.

Microorganism	Brazil nut oil		Sunflower seed oil	
	MIC (µg.mL^{-1})	MBC/MFC (µg/mL) (µg/mL)	MIC (µg.mL^{-1})	MBC/MFC (µg/mL)
S. aureus	42.71	42.71	21.35	21.35
	42.71	42.71	21.35	21.35
	21.35	42.71	21.35	21.35
	42.71	42.71	21.35	21.35
	85.42	85.42	42.71	42.71
	21.35	42.71	21.35	42.71
	21.35	21.35	10.68	21.35
	42.71	42.71	21.35	21.35
	21.35	21.35	42.71	42.71
	21.35	21.35	10.68	21.35
Enterococcus spp.	21.35	21.35	21.35	21.35
	21.35	21.35	21.35	21.35
	21.35	21.35	21.35	21.35
	21.35	21.35	21.35	21.35
Candida spp.	10.41	10.41	5.21	5.21
	20.83	20.83	5.21	5.21
	20.83	20.83	10.41	10.41
	10.41	10.41	10.41	10.41
Cryptococcus laurentii	10.41	10.41	0.65	0.65
	10.41	10.41	2.60	2.60
	10.41	10.41	0.33	0.33

linoleic acid and 28.75% of oleic acid. Other authors, who also studied the content of fatty acids in this oil, found similar results (Santos and Marin, 2005; Spanamberg et al., 2009). Gas chromatography was employed to quantify the content of fatty acids in both previously mentioned studies.

The sunflower seed oil has the same main compounds of the Brazil nut one, that is, oleic acid represents 35.80% and linoleic acid is 52.64% of the total amount of fatty acids. Margaric acid (C 17:0) comprises only 0.039% of this oil. It is worth mentioning that linoleic acid is the most abundant unsaturated fatty acid in both samples under study.

Studies reported in literature have shown that linoleic acid is able to inhibit the growth of *S. aureus*, since it affects the protein synthesis on cell walls and on the nucleic acids (Mandelbaum et al., 2003). MIC values were higher against *S. aureus* (36.6 and 23.5μg.mL^{-1} for Brazil nut and sunflower seed oils, respectively) for both fixed oils under study. Increase in the prevalence of multi-resistant *S. aureus* which causes bovine mastitis is a serious problem worldwide since antimicrobial agents have less effective and morbidity rates and costs in fighting the disease have increased. There is considerable genetic heterogeneity in natural populations of *S. aureus*. Besides, it is an important pathogen which causes diseases that result from food ingestion, that is, the intake of toxins; thus, it may lead to a public health problem (Zafalon et al., 2008). These facts explain the stimulus for the search for alternative means to reduce or eliminate such problems.

Several studies demonstrate the resistance profile of agents causing bovine mastitis against antimicrobial agents available in the market for the treatment of this disease, for example, in a study conducted by Laport et al. (2012), 49 *Staphylcoccus* spp. strains were isolated from bovine mastitis cases from 21 different dairy herds kept at farms in Southeast Brazil. Strains were analyzed for antimicrobial susceptibility. Fifty-nine percent of the bacteria strains were resistant to at least one of the drugs tested and 12.2% were classified as multiresistant.

MBC was equal to MIC in 80% of the isolated microorganisms in the Brazil nut oil and in 70% of the sunflower seed oil ones. It shows that the oils exerted bactericidal/fungicidal and bacteriostatic/fungistatic activities against these strains. In a study carried out by Aquino et al. (2010), the fixed oil of *Ocimum basilicum* had bactericidal and bacteriostatic activity against *S. aureus* strains. Five out of eight strains under study were inhibited and killed when MIC's and MBC's were 3.12 and 6.25 μg.mL^{-1}, respectively. All isolates selected for this work, in a previous study, presented *in vitro* resistance to at least five antibiotics, which does not mean the MICs can be compared between them, since each active ingredient has specific concentrations to act.

Table 2 shows that the fixed oils under study exerted activity against yeasts in low concentrations; they varied from 10.41 to 20.83 μg.mL^{-1} in the case of Brazil nut oil and from 0.33 to 10.41 μg.mL^{-1} in the case of sunflower seed oil. The activity fixed oils exert against these microorganisms has been emphasized due to the scarcity of antimycotic pharmacological agents available in an adequate way to treat mycotic mastitis, mainly when antimicrobial medicines often used to fight the bacterial disease are compared.

High sensitivity was observed for *Cryptococcus laurentii* when sunflower seed oil was used; average MIC and MFC were 1.2 μg.mL^{-1}.

Regarding isolated microorganisms of *Candida* spp., the oils under analysis also had inhibitory activity in low concentrations; this fact is relevant since *Candida* represents the most commonly isolated pathogen from infections found in mammary glands of dairy cows, among all yeasts (Santos and Marin, 2005; Wunder, 2007). Besides, these results are also important due to the development of resistance against azolic agents which may cure candidiasis (caused by this yeast) in animals (Cleff et al., 2012).

Given these results, it was evidenced that the oils studied showed antimicrobial activity against microorganisms isolated from mastitis milk in low concentrations. Considering the current problem of difficulty in control and treatment of bovine mastitis due to resistance of these pathogens to antimicrobials routinely used in the treatment of mastitis and the concern of the presence of residues of these drugs in milk, the data demonstrated the potential use of these oils as a therapeutic resource in the control of this disease.

Conflict of Interests

The authors have not declared any conflict of interests.

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