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Plant phenolic extracts as an effective strategy to control *Staphylococcus aureus*, the dairy industry pathogen

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ABSTRACT

Staphylococcus aureus is one of the most common contagious mastitis pathogens. Bovine mastitis is considered an important reservoir for dairy industry contamination, and therefore to ensure *S. aureus* control has gained a pivotal importance. Natural matrices present multiple biological effects, being its antimicrobial potential increasingly reported. Thus, the present study aims to assess the antibacterial activity of several methanol:water extracts, obtained from plants, against *Staphylococcus aureus*. Moreover, the most effective extract was characterized in terms of phenolic compounds, by using high performance liquid chromatography coupled to diode array and mass spectrometer detectors. Among the tested extracts, *Eucalyptus globulus* was the most effective against all tested *S. aureus* strains, followed by *Juglans regia* and *Foeniculum vulgare*. Inhibition halos of the plant extracts varied between 8.0–16.0 mm, excepting for *F. vulgare* in which two evident halos were observed: one with growth inhibition (5.0–7.0 mm) and a second one with visible cell density reduction (13.0–14.0 mm). Susceptibility assays evidenced that *E. globulus* extract exerted the highest antibacterial activity (MICs = 0.195–0.39 mg/mL), being effective against all the tested strains. Among the phenolic compounds identified in this extract, gallotannins, ellagic acid glycoside, and quercetin derivatives, were the most abundant; and therefore, may exert a positive and contributive effect to the observed antibacterial effect. Overall, the use of plant extracts to control bovine mastitis caused by *S. aureus* is a promising solution that could contribute to the reduction of the occurrence of dairy food industry contaminations, providing considerable benefits to agro-industries on the formulation of high-quality and safety dairy products.

1. Introduction

Bovine mastitis is the most expensive disease for the worldwide dairy industries. The management of this pathology is mainly based on the extensive use of antibiotics/disinfectants (Pieterse and Todorov, 2010), which has triggered the development of complicated scenarios of antimicrobial resistance (Motlagh et al., 2013). Beyond the poor efficacy of the antibiotic treatment, bovine mastitis has become increasingly difficult both to control and mainly to eradicate in many herds (Carter and Kerr, 2003; Sutra and Poutrel, 1994).

The increasing rates of antibiotic resistance hamper an urgent and effective bovine mastitis management, at the same time that motivate the search for effective antimicrobials (Rossi et al., 2011). Among the etiological agents for this complicated infection, *Staphylococcus aureus* is considered the most prevalent; moreover, and due to its zoonotic potential, a pivotal attention has driven an increasing solicitude by dairy industries (Kummel et al., 2016). Recently, Kummel et al. (2016) showed that *S. aureus* from bovine mastitis can enter in the dairy chain production via contaminated milk, which is in accordance with the previous study carried out by Sabour et al. (2004), who described the presence of antibiotic-resistant *Staphylococcus* species in milk processing lines, associated with chronic mastitis. Based on these findings, it becomes of the utmost importance to discover more effective, safer and selective control strategies, not only to reduce the number of microorganisms present in milking installations, but also to reduce the likelihood of bovine mastitis and milk contamination occurrence.

Natural matrices have been increasingly reported as effective alternatives to the current antimicrobial agents. In fact, the use of botanical preparation dates back from the beginning of human civilization, being effectively used in a wide variety of health conditions (Saranraj and Sivasakthi, 2014). As a rich source of bioactive molecules, among them phenolic compounds, natural matrices are commonly defined as

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"prototypes of new antimicrobial agents" (Meléndez and Capriles, 2006). Several studies have supported the effective use of botanical preparations against bovine mastitis pathogens, being aqueous and alcoholic extracts the most commonly used (Diaz et al., 2010; Doss et al., 2012; Mubarack et al., 2011; Rossi et al., 2011). However, to the authors' best knowledge no previous studies have assessed the antibacterial activity of methanol: water plant extracts against *S. aureus* isolated from bovine mastitis. In this sense, the present work aims to evaluate the antibacterial activity of fourteen methanol: water extracts against different *S. aureus* strains isolated from bovine mastitis, towards providing new insights for an effective control of dairy industry contaminations; as also to perform the characterization in terms of phenolic compounds of the most effective plant extract.

2. Materials and methods

2.1. Plant samples

A total of fourteen plant species were used: four of them were wild samples, harvested in Trás-os-Montes − Bragança, North-Eastern Portugal, namely petals of *Rosa canina* L. (rose hips/dog rose), leaves of *Juglans regia* L. (walnut), flower buds and fully opened flowers of *Rubus ulmifolius* Schott (elm-leaved blackberry), and leaves and roots of *Fragaria vesca* L. (wild strawberry). The other ones were commercial samples, namely fruits of *Pimpinella anisum* L. (anise) and *Coriandrum sativum* L. (coriander); leaves of *Melissa officinalis* L. (lemonbalm), *Eucalyptus globulus* Labill. (blue gum) and *Tabebuia impetiginosa* (Mart. ex DC) Standley (pau d'arco); aerial parts of *Foeniculum vulgare* Miller (fennel), *Matricaria recutita* L. (chamomile) and *Echinacea purpurea* (L.) Moench (purple coneflower), and lastly flowering parts of *Pterospartum tridentatum* (L.) Willk (carqueja). Plant scientific nomenclature is according The Plant List (2013), version 1.1 (2013).

2.2. Standards and reagents

Acetonitrile (99.9%, HPLC grade) and methanol (99%, PA) were from Fisher Scientific (Lisbon, Portugal). Phenolic standards were from Extrasynthèse (Genay, France). Formic acid was from Sigma-Aldrich (St. Louis, MO, USA). Tryptic Soy Broth (TSB) and Agar were purchased from Liofilchem (Roseto degli Abruzzi, Italy) and Merck (Darmstadt, Germany), respectively. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Carrollton, USA).

2.3. Preparation of the extracts

Methanol: water extracts were obtained by extracting each plant sample (1 g) with 30 mL of methanol: water (80:20, v/v) mixture at 25 °C and 150 rpm for 1 h, and filtering through Whatman No. 4 paper. Final residue was then extracted with an additional 30 mL portion of the methanol: water mixture. Each one of the combined extracts was evaporated at 35 °C under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). The lyophilized methanol: water extracts were re-dissolved in water to obtain stock solutions at 50 mg/mL, from which several dilutions were prepared.

2.4. Evaluation of the antibacterial activity

2.4.1. Disc diffusion assay

Seven *S. aureus* strains were used in this study (Table 1), one from the American Type Culture Collection (ATCC 25923), and six clinical isolates from cows with mastitis (North region of Portugal). The clinical isolates were provided by Segalab (Laboratório de Sanidade Animal e Segurança Alimentar, S. A.).

All strains were inoculated into 15 mL of TSB from Tryptic Soy Agar (TSA) plates not older than 2 days, and grown for 24 h at 37 °C in an orbital shaker at 120 rpm. Cells were harvested by centrifugation (for 5 min at 9500*g* at 4 °C), resuspended in TSB, adjusted to an optical density (640 nm) equivalent to 1×10^6 cells/mL and, then used in the subsequent assays. An aliquot of each strain (300 µL) was spread in TSA plates. An aliquot (25 µL) of each plant extract, with a known concentration (50 mg/mL), was placed on a sterile blank disc. Sterile water was used as negative control. Then, plates were incubated at 37 °C, during 24–48 h, and the determination of inhibitory effects was performed measuring the corresponding zones of inhibition (inhibition halo diameter).

2.4.2. Determination of minimal inhibitory concentrations (MICs)

Minimal inhibitory concentrations (MICs) were determined by microbroth dilution technique, to the plant extracts in which most pronounced effects were observed, considering the results obtained in the disc diffusion assay. MIC values were determined to the selected plant extracts by serial two-fold dilutions method, at concentrations ranging from 0.049 mg/mL to 6.25 mg/mL, adjusting final cellular concentration to 5×10^5 cells/mL. The 96-wells plates (Orange Scientific, Braine-l'Alleud, Belgium) were incubated at 37 °C for 24–48 h. Sample and bacteria-free controls were also included. After visualization of the resultant plate, MIC values corresponded to the concentration used in which no visible growth was observed, or a bacteriostatic effect was observed by comparison with positive controls (cells grown without extracts). Then, the number of viable cells was assessed by determination of the number of colony forming units (CFUs), after 24 h of incubation at 37 °C. The number of colonies formed was counted and the results presented as the total of CFUs (Log CFUs). Experiments were carried out in triplicate, and repeated in three independent occasions.

2.5. Phenolic compounds analysis

The most effective extract was characterized regarding its phenolic composition. Therefore, it was re-dissolved at a concentration of 5 mg/mL with 80% methanol, filtered through a 0.22-µm disposable LC filter disc before the chromatographic analysis. The phenolic profile was determined by HPLC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), following a procedure previously described by Bessada et al. (2016). Detection was achieved with DAD (280, 330 and 370 nm as preferred wavelengths) and in a mass spectrometer (MS). The MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. The identification of the phenolic compounds was performed using standard compounds, when available, by comparing their retention times, UV–vis and mass spectra; as also, comparing the obtained information with available data reported in literature giving a tentative identification. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard, such as for compounds. The results were expressed as mg per g of extract.

2.6. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and means were compared using Tukey's honestly significant difference (HSD) multiple comparisons test. All statistical tests were performed using Prism software package (GraphPad Software version 6.0 for Macintosh). Results were considered statistically significant when $P < 0.05$.