Small Ruminant Research 130 (2015) 229–235

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Treatment of goat mastitis experimentally induced by *Staphylococcus aureus* using a formulation containing *Hymenaea martiana* extract

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ARTICLE INFO

Article history:
Received 25 December 2014
Received in revised form 4 July 2015
Accepted 10 July 2015
Available online 20 July 2015

Keywords:
Antibacterial activity
Dairy goat
Mastitis
Natural extract
Therapy

ABSTRACT

The aim of the present study was to assess the *in vivo* potential of therapy based on *Hymenaea martiana* Hayne extract in mastitis cases among goats that were experimentally induced with strains of *Staphylococcus aureus*. The phytochemical characterization of the plant extract was performed using HPLC–DAD. Ten female goats (20 mammary glands) were experimentally infected with a clinical strain of *S. aureus*. The experimental infection was performed in four groups, each consisting of five mammary glands. One group was treated with a commercial antimicrobial and another with ointment from natural plant extract. The other two groups were controls. The diagnosis of mastitis was performed using a bacterial culture, SCC, CFU/mL and CMT. The animals were monitored at nine experimental periods. Three milk samples were subjected to chromatography to survey the constituents present in the ointment prepared with the plant extract. Phytochemical screening of crude ethanol extract confirmed the presence of phenolic compounds, flavonoids, steroids and terpenoids. The results of the HPLC confirmed the presence of phenolic compounds in the chloroform and ethyl acetate. Concerning SCC and CFU/mL, the analysis of variance performed between the experimental periods confirmed that there was a higher score at the time of the first collection after infection (P1) in all of the experimental groups. After treatment with ointment containing the extract of *H. martiana* and commercial antimicrobials, these variables returned to values indicative of an absence of intra-mammary infection, based on the international standards for raw goat's milk. In the present study, milk collected on the 32nd day post treatment with ointment containing the extract of *H. martiana* exhibited a significant reduction in CFU/mL, when compared with the control group. Antibacterial activity may be related to the classes of secondary metabolites found.

1. Introduction

For a long time, antibiotic therapy has provided a manner of controlling numerous bacterial diseases (Kümmerer, 2004). However, bacteria are known for their ability to adapt and they have developed a set of mechanisms that enable them to become resistant to antibiotics. The spread of resistance to antibiotics among bacteria has become inevitable. It is currently considered a serious problem for public health services (Levy, 2002).

A number of studies have focused on alternative therapies in different areas of the health sector. In the area of veterinary medicine, several studies have demonstrated the antibacterial activity of natural plant extracts and some of their compounds (Poppenga, 2002; Viegi et al., 2003; Cos et al., 2006; Schuch et al., 2008; Baskaran et al., 2009; Mubarack et al., 2011) in relation to isolates obtained from mastitis cases. A number of breeders and veterinarians have

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http://dx.doi.org/10.1016/j.smallrumres.2015.07.009
0921-4488/

used phytotherapy to prevent and treat mastitis. There is a predominance of practices focused on treatment, with herbal solutions or medicinal ointments commonly employed for local use, as well as the oral administration of green or dry plants (Schuch et al., 2008).

Silva et al. (2004) noted that *Staphylococcus aureus* was involved in 37% of subclinical cases of mastitis in dairy goats. The same authors reported the occurrence of multi-resistance in microorganisms isolated from these small ruminants. Concerning the treatment of mastitis, very few drugs are specifically licensed for use in small ruminants, especially goats. The use of antibiotics or other drugs for cattle in small ruminants is very risky. The safety and efficiency involved in using these products with goats is unknown (Mavrogianni et al., 2011).

In vitro studies seeking to determine the anti-microbial potential of Brazilian flora have provided satisfactory results (Fernandes et al., 2005; Gonçalves et al., 2005; Oliveira et al., 2007; Ushimaru et al., 2007), although there is a discontinuity in these studies and fragmentation of the results, thereby hindering advances in the area.

Hymenaea is a genus that is distributed throughout tropical America, with one species also found in coastal East Africa (Rates, 2001). Plants of the genus *Hymenaea* are commonly used in traditional medicine to treat several bacterial infections and inflammatory processes (Agra et al., 2007). Phytochemical screening of the crude ethanolic extract (CEE) confirmed the presence of phenolic substances, flavonoids, steroids and terpenoids. Flavonoids are secondary metabolites and can be found in moderate concentrations in the extract (Silva et al., 2012).

The use of phytotherapeutic medication has provided satisfactory results in the therapy of mastitis cases, although the studies involved mainly used cattle. Therefore, the aim of the present study was to assess the *in vivo* potential of *Hymenaea martiana* Hayne extract in the therapy of mastitis among goats that were experimentally infected with *S. aureus*.

2. Materials and methods

The present study received approval from the Ethics Committee for Human and Animal Studies of the *Universidade Federal do Vale do São Francisco* under protocol number 0005/131211.

2.1. Obtaining the ethanol extract of Hymenaea martiana Hayne

The plant material was collected in the district of Petrolina-PE and identified in the Reference Center for the Recovery of Degraded Areas (CRAD) of the *Universidade Federal do Vale do São Francisco*. One specimen of the plant (21868) was deposited in the Herbarium of the *Vale do São Francisco* (HVASF).

After drying and pulverization, the bark of the plant was submitted to exhaustive maceration with ethanol 95% in a stainless steel container. Several extractions were performed (interval of 72 h between each extraction), until the plant material was completely depleted. The solvent of the solution that was extracted was distilled in a rotary evaporator with the pressure reduced and a mean temperature of 50 °C. After the solvent had been evaporated, the crude ethanolic extract (CEE) was obtained. A small portion of this CEE was partitioned with solvents using an increasing gradient of polarity (hexane, chloroform and ethyl acetate) (Silva et al., 2012). The respective phases were used to investigate the main chemical compounds involved.

2.2. Phytochemical screening

For the phytochemical screening of the CEE, chromatography analysis was conducted using thin layer chromatography on plates of silica gel 60, with aluminum support and fluorescence F254 in different solvent systems. This was performed following the methodology described by Wagner and Bladt (1996) to investigate alkaloids, anthracene derivatives, coumarins, flavonoids, tanins, lignans, monoterpenes, diterpenes, naphtoquinones, triterpenes and steroids.

2.3. Analysis by high-performance liquid chromatography with diode array detector (HPLC–DAD)

HPLC–DAD analysis was performed to identify the profile of the phenolic compounds of the fractions obtained in the partition. Analysis of the phenolic compounds was conducted using a Shimadzu Prominence HPLC LC2OAT chromatograph, equipped with an SPDM2O diode array detector (DD) and a reverse phase column (Luna – Phenomenex (250 mm × 4.6 mm, 5 μm). In the analysis, the mobile phase involved water: formic acid (99:1, solvent A) and methanol (solvent B). The chromatographic condition was as follows: 0–15 min 20% B; 15–20 min 30% B; 20–30 min 40% B; 30–40 min 40% B. The flow was 1.0 mL/min and the temperature was 35 °C. The injection volume was 10 μL and a wavelength of 290 nm was used for the monitoring.

2.4. Animals and experimental infection with S. aureus

Ten female Saanen goats were used. They were all aged between 1.5 and 4 years and between the first and second lactation period. In total, 20 mammary glands were experimentally infected. Prior to the inoculation, the bacteriological and cellular characteristics of the milk were established. The animals were negative in three consecutive lacto cultures, with intervals of seven days between collections (pre-infection period).

The goats were milked manually, following the regular sanitary control standards. After obtaining the indices of the variables of the initial period, the two halves of each female mammary gland were infected, using the intra-mammary route and a number 4 urethral probe, coupled to a sterilized plastic syringe. Prior to inoculation, complete milking and antisepsis were carried out on both teats using 70% alcohol. After the inoculation (1 mL), a massage involving upward movements was conducted so that the inoculum would be distributed throughout the mammary gland. A strain of *S. aureus* was used at an infectious dose of 1.2×10^8 CFU/mL. This field strain was taken from a case of subclinical goat mastitis, identified by its biochemical characteristics. The DNA of the sample used in the infection of animals was extracted and submitted to DNA sequencing using the ABI PRISM® Big Dye terminator v. 3.1 cycle sequencing kit (Applied Biosystems) and processed in an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems). The analysis of the alignment of the sequencing of the strain used to infect animals confirmed the species as *S. aureus* subsp. *aureus*.

The following experimental periods were established: P0 – prior to infection; P1 – 24 h post-infection; P2 – 48 h post-infection; P3 – 72 h post-infection/start of treatment; P4 – 6 days post-infection/03 days after starting treatment; P5 – 09 days post-infection/end of treatment; P6 – 12 days post-infection/03 days after end of treatment; P7 – 25 days post-infection/16 days after end of treatment; P8 – 32 days post-infection/23 days after end of treatment; P9 – 41 days post-infection/32 days after end of treatment.

The animals were monitored daily through clinical assessments (rectal temperature, heart rate, respiratory rate and rumen dynamics).

2.5. Preparation of the intra-mammary ointment

The compatibility of the extract was assessed with cream and ointment, with better results obtained for the ointment. This base was confirmed as a vehicle of a number of commercial formulations of intra-mammary drugs.

Technical pharmaceutical strategies were used in an attempt to incorporate the extract in the base ointment of vaseline/lanolin. One of these strategies involved the use of propylene as a cosolvent for later incorporation in the ointment base (vehicle). Subsequently, the gradual incorporation of the EEC of *H. martiana* was conducted.

Wet sieving was carried out to ensure uniformity in the formulation and enable the withdrawal of residues. The pH of the formulation was between 5.0 and 6.0.

Formula:

Components	Quantity (g)
Crude ethanol extract of *H. martiana* (*jatobá*)	2.5
Lanolin	15.0
Solid Vaseline q.s.p.	50.0

2.6. Diagnosis of mastitis

Bacterial cultures, the somatic cell count (SCC) and the California Mastitis Test (CMT) were used to diagnose subclinical mastitis. The following values were considered as indicators of the illness: SCC: $>1 \times 10^6$ and CMT: ≥1+ (Souza et al., 2012). The bacterial culture was used as a confirmatory test and was carried out according to the criteria established by the National Mastitis Council (1999). Plates that exhibited one or more colonies with the characteristics of the previously identified strain were considered positive.

The California Mastitis Test (CMT) was used to detect subclinical mastitis, as described by Schalm and Noorlander (1957). The results of the CMT provided a score (negative, trace, 1+ or 2+). In order to determine the SCC, milk samples were collected in vials containing Bronopol. Flow cytometry was used in this process to measure fluorescence (SomaScope MKII, Delta Instruments).

The total bacterial count was conducted using automated equipment that uses flow cytometry to count bacteria in raw milk (Bactocount IBC, Bentley Instruments®). The result was expressed in CFU/mL. This analysis was performed in the official laboratory of milk quality in the Department of Animal Science at the *Universidade Federal Rural de Pernambuco* (UFRPE).