

**Understanding microbial pathogens:  
current knowledge and educational  
ideas on antimicrobial research**

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## Antimicrobial action of propolis extracts against staphylococci

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One Health is a worldwide strategy of healthcare for humans, animals, and the environment. Antimicrobial resistance is a serious global problem, recently recognised by the World Health Organization. Prudent, responsible use of antimicrobials should be a concern for both human and veterinary doctors.

Concerning animal health and its repercussion in human health, antimicrobials and disinfectants have been widely used for the control of mastitis. This practice induces a selective pressure for resistant bacterial strains, which is deleterious for public health associated with milk consumption. Antimicrobial resistance genes have been detected in pathogens associated to small ruminants' mastitis. These genes may be transferred to the indigenous microbiota of humans. The presence of disinfectant resistance genes has also been reported in staphylococci from both ovine and caprine milk.

Propolis is a resinous substance produced by honeybees using different types of plants. It is used to seals holes and cracks in the beehive, contributes to an aseptic internal environment, maintains the hive's internal temperature, and prevents predators from entering the beehive. Propolis has been used as a natural medicine for its antiseptic, antimicrobial, antioxidant, anti-inflammatory, and other immunomodulatory properties.

The aim of the present study was to investigate the *in vitro* activity of propolis ethanol extracts (PEE) against staphylococci isolated from mastitic milk of sheep and goats.

Antimicrobial susceptibility was assessed for 16 antimicrobials (ampicillin, gentamicin, lincomycin, trimethoprim/sulfamethoxazole, penicillin, streptomycin, tetracycline, cloxacillin, neomycin, cefazolin, cefoperazone, cephalexin, amoxicillin+clavulanic acid, oxacillin, ceftriaxone, and ciprofloxacin) by the disk diffusion method.

Ten PEE from propolis samples collected both in Portugal (three brown), and Brazil (one green, two red, and seven brown) were evaluated for their antimicrobial action against 146 staphylococci: 35 *S. aureus*, and 104 coagulase-negative staphylococci isolates, together with seven reference strains. Antimicrobial activity of PEE was assessed on polystyrene flat-bottomed microtiter plates in triplicate by the microdilutions methodology for concentrations between 0.05 and 214 mg/mL.

All staphylococci isolates revealed susceptibility to all but one of the studied PEE. Minimal bactericidal concentration for most isolates was 3.34 mg/mL.

According to our results, propolis may be an important alternative to the use of antimicrobials, with remarkable advantages for public health.

**Keywords:** *Staphylococcus*; antimicrobial resistance; One Health

### 1. Introduction

One Health is a worldwide strategy of healthcare for humans, animals, and the environment, shared by the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the World Organisation for Animal Health (OIE). Addressing the rising threat of antimicrobial resistance (AMR) requires a holistic and multisectoral approach, because antimicrobials used to treat various infectious diseases in animals may be the same or similar to those used for humans. Resistant bacteria arising in humans, animals or the environment may spread from one to the other, and from one country to another.

According to the WHO, antimicrobial resistance is the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals, and antimalarials) from working against it. As a result, standard treatments become ineffective, infections persist, and may spread to others.

Antimicrobials and disinfectants have been widely used for the control of mastitis in small ruminants, a practice with severe consequences for human health. The massive use of antimicrobials induces a selective pressure for resistant bacterial strains, which is deleterious for public health associated with milk consumption.

Mastitis, the inflammation of the mammary gland, which is mostly caused by bacteria, is highly prevalent in dairy herds [1]. *Staphylococcus* spp. are the main aetiological agents of mastitis in small ruminants [2, 3].

The prophylaxis and treatment of mastitis is currently mostly dependent on the use of antimicrobials, which exert pressure selection over resistant and multi-resistant strains [4-6]. A recent study showed a noticeable increase in antimicrobial resistance, over a period of ten years, in *Staphylococcus aureus* isolates from goats and sheep milk [7].

These resistant bacteria may worsen the problem of mastitis, but in addition, they may be threatening for human health. Their access to the consumer through milk and dairy products may be responsible for the transfer of resistance genes to the microorganisms of the indigenous microbiota in the gut of humans [8].

The search for control measures and antimicrobial alternatives to decrease intramammary infections are needed to reduce losses in the dairy sector; to increase the motivation and profitability of farmers and, last, but not least, to protect public health.

One possible alternative is propolis, a natural antimicrobial. Propolis is a resinous mass produced by *Apis mellifera* bees by manipulating resins harvested in several plants with their salivary gland secretions, which they use to close the hive to difficult the access of invaders. Its biological properties are related to the chemical composition, which differs in its structure and concentration depending on the region of production, availability of sources for resin harvesting, genetic variability of the queen bee and technique used for production [9].

The biological activity of propolis has been used in traditional medicine since ancient times. Different solvents have already been tested for extracting propolis components and to produce extracts [10, 11]. Propolis has been shown to possess several properties, such as: antioxidant action, anti-inflammatory [12], antiulcerogenic [13], antitumoral [14], antidiabetogenic [15], antiatherogenic and anti-angiogenic [16], immunomodulatory [17], antifungal [18, 19], antiviral [20], namely for human immunodeficiency virus (anti-HIV) [21], anti-bacterial [22, 23] and acting versus some virulence factors, such as antibiofilm [24, 25] and antimotility [26, 27].

The aim of the present study was to evaluate the *in vitro* activity of propolis ethanol extracts (PEE) against staphylococci isolated from mastitic milk of sheep and goats.

## 2. Materials and methods

### 2.1 Staphylococci isolates

Thirty-five *Staphylococcus aureus* and 104 coagulase-negative staphylococci (CNS) were isolated from small ruminant milk samples. Seven reference strains were also used: five *S. aureus* (ATCC 25923, ATCC 29213, COL, FRI 472, and FRI 913) and two *S. epidermidis* (ATCC 12228, and ATCC 35984).

### 2.2 Antimicrobial Sensitivity Test (AST) - disk diffusion method

The AST followed the performance standard M02-A11 CLSI [28] using the Kirby Bauer diffusion method. Bacterial cultures were suspended in sterile saline solution whose turbidity was adjusted at 0.5 of the MacFarland scale ( $1 \times 10^8$  cfu/mL) and confirmed in turbidimeter (DensiChek, bioMérieux). The suspension was inoculated onto the surface of Muller-Hinton agar (MHA) plates (Oxoid, CM0337) and after 5 minutes at room temperature, antimicrobial disks were applied with the aid of a disk dispenser (Oxoid, ST 6090). The plates were incubated at 37 °C for approximately, 24 hours and the inhibition zone diameter was measured. The list of 16 antimicrobials studied, used in intramammary antibiotics preparations, are grouped according to their chemical structure and their mode of action in **Table 1**.

**Table 1** Antibacterial agents, their classes, and their corresponding mode of action.

Antimicrobials	Class	Mode of action
Penicillin	$\beta$ -LACTAMS	Cell wall inhibitors
Ampicillin	Semisynthetic penicillins	
Cloxacillin		
Amoxicillin + Clavulanic Acid		
Oxacillin		
Cephalexin	Cephalosporins	
Cefazolin	Generation II	
Ceftriaxone	Generation III	
Cefoperazone		
Streptomycin		
Gentamycin	Aminoglycosides	
Neomycin		
Lincomycin	Lincosamides	
Tetracycline	Tetracyclines	
Ciprofloxacin	Fluoroquinolones	DNA replication inhibitor
Cotrimoxazole (Sulfamides + Trimethoprim)	Chemotherapeutic agents	Competitive inhibitor

### 2.3 Propolis Ethanol Extracts (PEE)

Ten propolis samples, seven from Brazil (Green, Red1, Red2, Brown1, Brown2, Brown3, and Brown4), and three from Portugal (Brown5, Brown6, and Brown7) were collected from apiaries located in different regions.

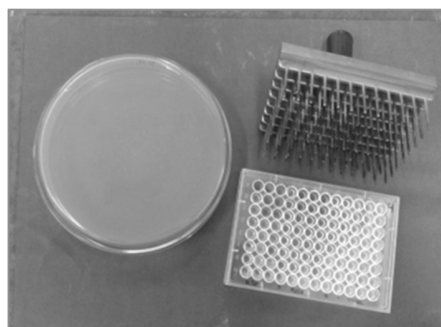
Propolis ethanol extracts were prepared according to the official standards for extracts production in Normative Instruction N° 3, 19/01/2001, published by the Department of Inspection of Animal Products of the Ministry of Agriculture, Livestock and Food Supply, Brazil [29]. Cold maceration of 300 g of raw propolis in 700 mL of 70% ethanol was performed, resulting in a 30% PEE. The preparations were kept at room temperature, protected from light, for 45 days. After this period, extracts were filtered with a sterile funnel and filter paper. Extracts were kept under refrigeration at 4 °C, in amber bottles, until use.

#### 2.4 *In vitro* antimicrobial activity of propolis extracts

*In vitro* antimicrobial activity of propolis ethanol extracts was performed according to the CLSI protocol M07-A9 [30].

A total of 150 µL of 30% PEE were added to 150 µL of Mueller-Hinton Broth (MHB) (Oxoid, CM0405), in polystyrene flat-bottomed 96-well microtiter plates, in triplicate, by the microdilutions methodology for concentrations between 0.05 and 214 mg/mL. Ethanol (70%) was used as a control, to ensure that the resulting antimicrobial action was not due to the ethanol used for extract production.

A suspension with a turbidity equivalent to a 0.5 McFarland standard ( $1 \times 10^8$  cfu/mL) was made for each bacterial culture using a turbidimeter (DensiChek, bioMérieux), and was further diluted to reach a concentration of  $5 \times 10^5$  cfu/mL. Microplates were incubated at 37°C for approximately 24 hours. In order to determine the minimum bactericidal concentration (MBC), a 96-pin microplate replicator (Boekel Scientific) was used to inoculate approximately 10 µl of each dilution onto a 150 mm diameter Petri dish with Mueller-Hinton Agar (MHA) (Fig. 1). Analyses were performed in triplicate, and Petri dishes incubated for 24 hours at 37 °C. The MBC is defined as the lowest dilution that inactivated the inoculum.



**Fig. 1** Minimum Bactericidal Concentration (MBC) methodology: polystyrene flat-bottomed 96-well microtiter plates, 96-pin microplate replicator; and 150 mm diameter Petri dish.

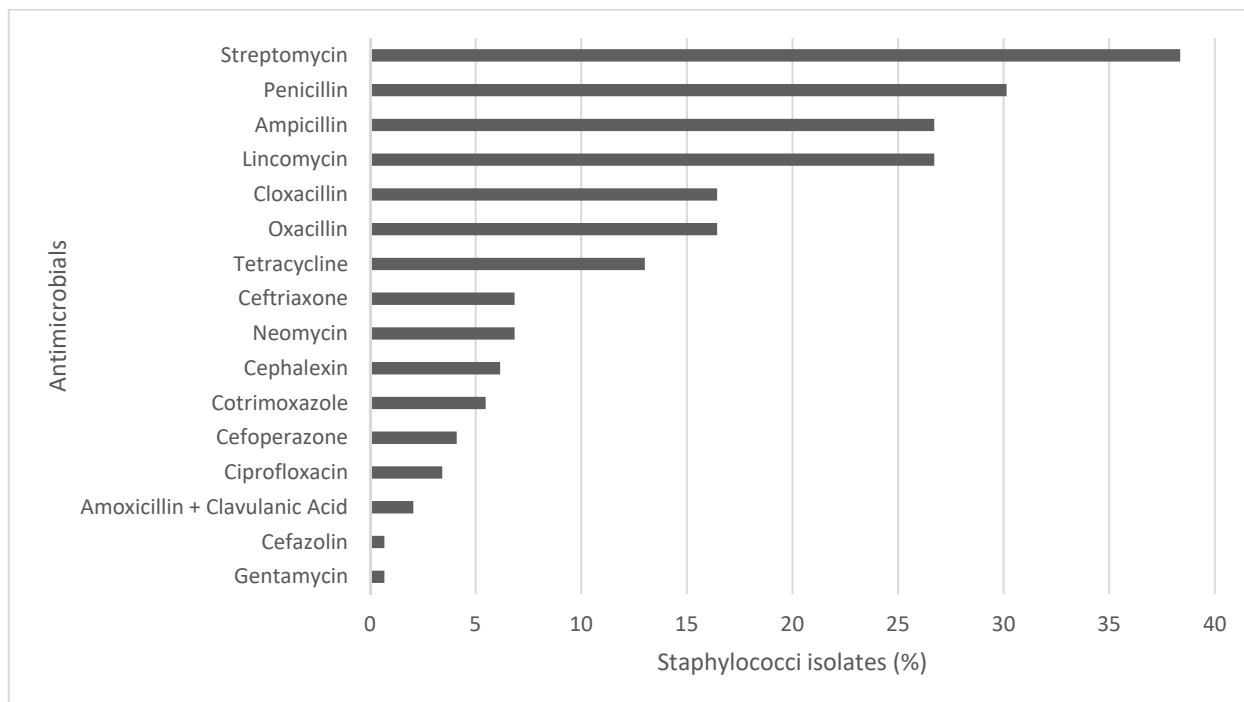
### 3. Results

#### 3.1 Antimicrobial susceptibility test of staphylococci

Staphylococci isolates and reference strains have shown distinct resistance rates to the tested antimicrobials. Thirty eight percent of all 146 staphylococci were resistant to streptomycin, 30% to penicillin, 27% to ampicillin and lincomycin, 16% to cloxacillin and oxacillin and 13% to tetracycline. Less than 10% of all staphylococci were found to be resistant to all other antimicrobials (Fig. 2). Nevertheless, at least one resistant staphylococcus to each antimicrobial was found.

Substituir por:  
non-susceptible

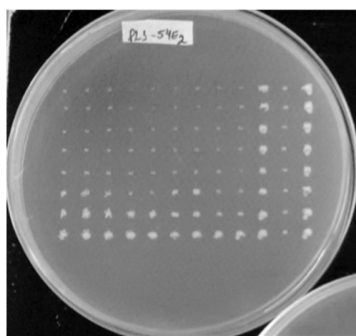




**Fig. 2** Antimicrobial resistance of 146 staphylococci isolates and reference strains.

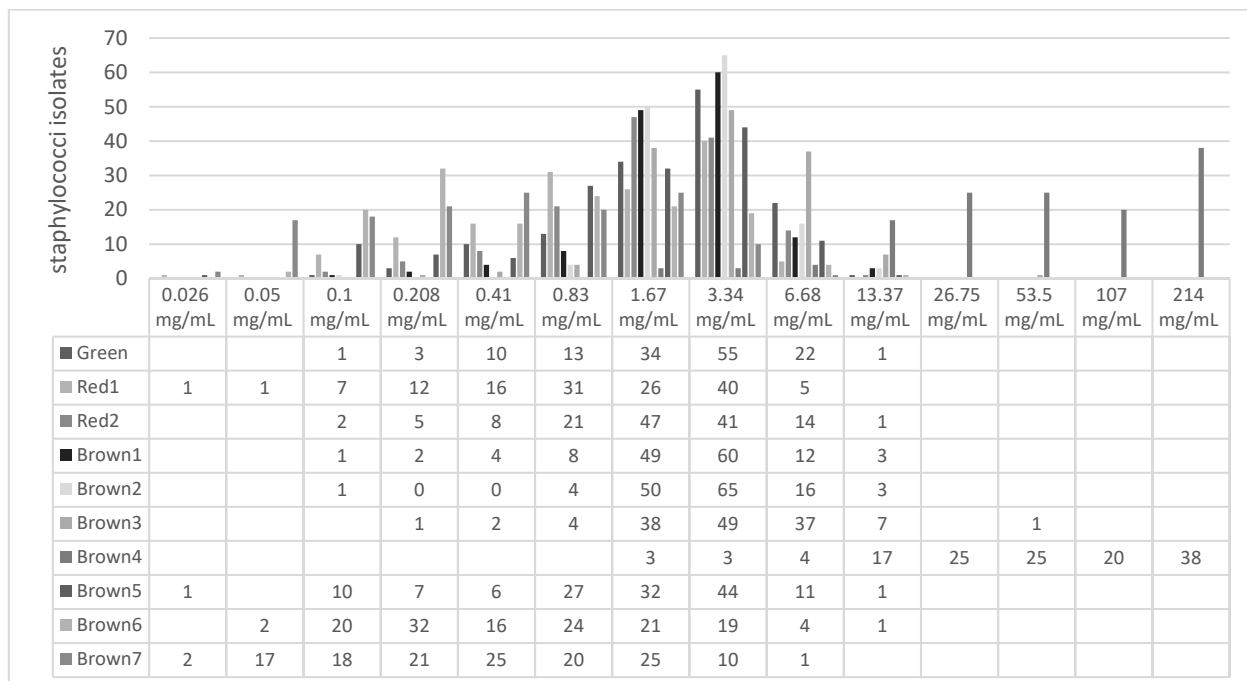
### 3.2 Antimicrobial activity of PEE against staphylococci

Bacterial activity is shown in **Fig. 3**. Identifications per column are shown from left to right: 1st, 2nd and 3rd are triplicates of dilutions of Brown1 extract; 4th, 5th and 6th are triplicates of dilutions of the Red2 extract; 7th, 8th and 9th are triplicates of Brown3 dilutions; 10th is ethanol control (70% ethanol dilutions with inoculum); 11th negative control (MHB culture medium only) and 12th positive control (culture medium with inoculum). Different dilutions of each PEE are positioned in different rows.



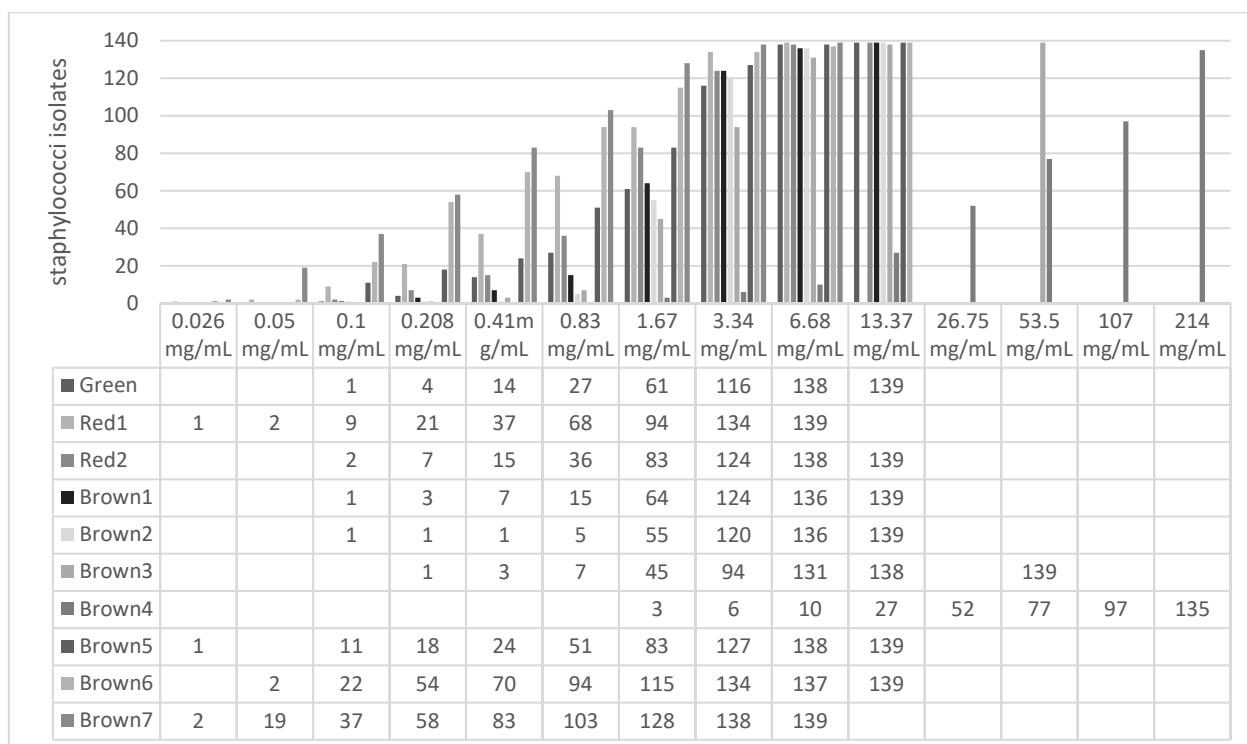
**Fig. 3** Minimum Bactericidal Concentration (MBC): Petri dish showing the growth of a bacterial isolate in the presence of Brown1, Red2 and Brown3 PEE.

All 139 staphylococci isolates revealed susceptibility to all but one of the studied PEE, in concentrations ranging between 0.026 and 13.37 mg/mL. Furthermore, the inhibitory activity was bactericidal. Most PEE minimal bactericidal concentration for most isolates was 3.34 mg/mL. Brown4 was the only PEE that showed less antimicrobial activity against the studied isolates (**Fig. 4**).



**Fig. 4** Minimum inhibitory concentration of 10 PEE against 139 staphylococci isolates.

All but one isolate were inhibited with concentrations between 0.208 and 13.37 mg/mL of the nine PEE. The concentration of 13.37 mg/mL, of these nine PEE, is adequate to inhibit *Staphylococcus* isolates. Red1 and Brown7 PEE were bactericidal to all isolates at the concentration of 6.68 mg/mL. Red1, Brown5, 6 and 7 extracts showed bactericidal activity with the lowest concentrations. This last one, Brown7, collected in PT, showed to inhibit more isolates at all concentrations (**Fig. 5**), showing the best performance. Following best performance was for Brown6, then Red1 followed by Brown5. Brown1 and Brown2 PEE showed quite similar antimicrobial activity. Green PEE showed an inhibitory activity not much different from Brown1, Brown2 and Brown3 PEE.



**Fig. 5** Antimicrobial activity of 10 PEE against 139 staphylococci isolates.

Regarding the antimicrobial activity of the PEE against the studied reference strains (Fig. 6), all strains showed sensitivity to the same nine PEE, in concentrations between 0.1 and 13.37 mg/mL, and 5 *S. aureus* (71.4%) revealed sensitivity to Brown4 extract, between 107 and 214 mg/mL concentrations. Two reference strains (*S. epidermidis* ATCC 12228 and 35984) showed Brown4 resistance, which was the one that showed less antimicrobial activity against all the strains tested. PEE Brown6 and 7 showed antimicrobial activity at lower concentrations.

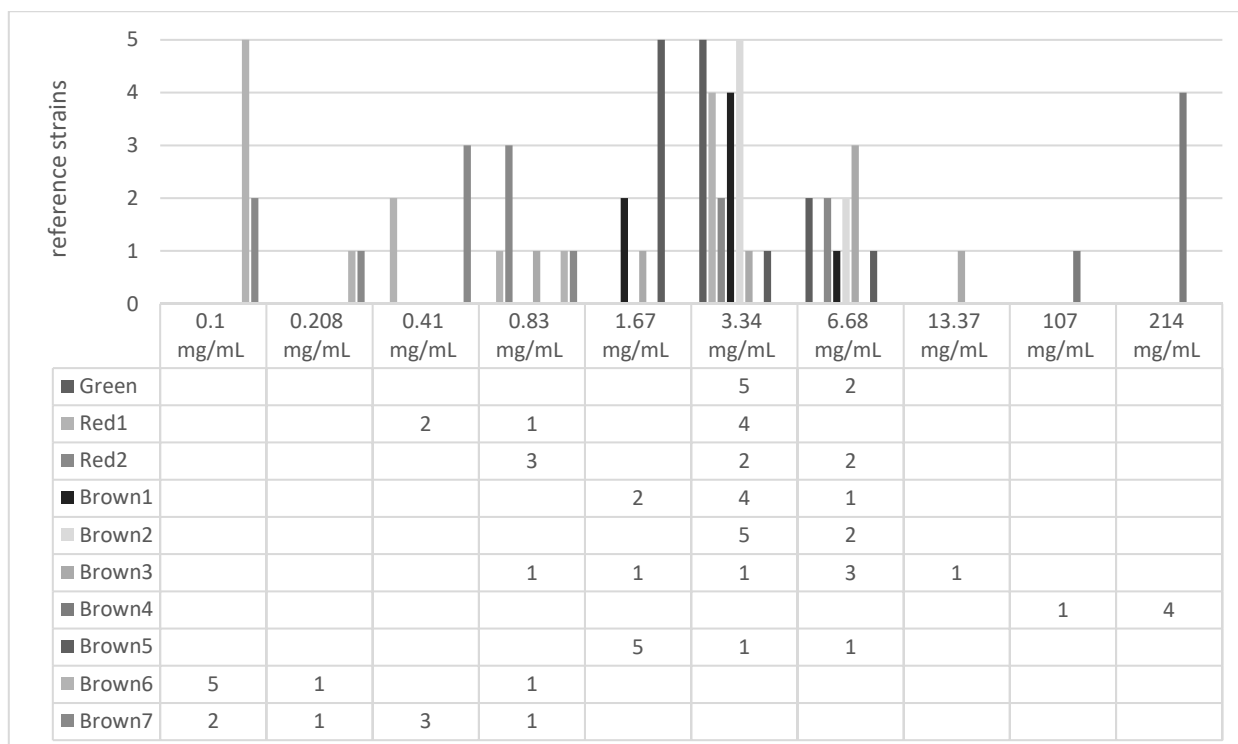


Fig. 6 Minimum inhibitory concentration of 10 PEE against reference strains.

#### 4. Discussion

In this study, resistance rate was higher to streptomycin (38%), followed by penicillin (30%), ampicillin and lincomycin (27%). Although antimicrobial resistance is not particularly high for all tested antimicrobials, it is noteworthy that resistant isolates were found to all antimicrobials and that 16% isolates showed resistance to cloxacillin and oxacillin, which is mostly relevant, as methicillin resistant staphylococci are a real scourge for public health. Staphylococci isolated from the milk of small ruminant with mastitis with low resistance patterns have been mentioned by other researchers [4, 31]. Nevertheless, some reports indicate *S. aureus* from ewes' subclinical mastitis with a high rate of resistance to ampicillin, cefalotin, cephalixin, gentamicin, streptomycin, erythromycin, oxytetracycline and sulphonamide with a percentage from 50.0 to 100.0 [32]. Additionally, Onni *et al.* [33] reported 38% *S. epidermidis* associated with ovine mastitis (n = 50) resistant to penicillin.

Except for Brown4 PEE, all the other PEE showed inhibitory activity against all staphylococci isolates. Furthermore, all ten PEE are bactericidal, which is a very important feature of propolis. For example, essential oils (EO) of Aromatic and Medicinal Plants (AMP) have been tested for their antimicrobial effect against staphylococci, and they were found to be only bacteriostatic [34].

Due to bacterial resistance to antimicrobials, researchers are exploring the natural components of propolis for their antimicrobial properties [35]. Plant compounds have been shown not to be prone to develop acquired bacterial resistance even after prolonged exposure [36].

The antimicrobial activity of propolis has been addressed before by other authors [22-24, 37-39]. The antibacterial activity of PEE over the bacterial cell wall was confirmed through atomic force microscopy (AFM) images. Furthermore, it is possible to differentiate the degenerative ability of propolis against Gram-negative and Gram-positive bacteria. In fact, PEE were found to be more effective against the Gram-positive *S. aureus* [40].

Propolis extracts can be used alone or in combination with antimicrobials [35]. This mixture represents a synergy that potentiates the antimicrobial power of antibiotics against various microorganisms. Minimal inhibitory concentrations (MIC) of penicillin G, doxycycline, streptomycin, cloxacillin, chloramphenicol, cephradine, ampicillin, and polymyxin B were established, in the absence of PEE, against *S. aureus*. When PEE was added at concentrations of up to 600 µg/ml

to antimicrobial solutions, a high synergistic effect was observed in the anti-bacterial activity of streptomycin and cloxacillin, and a moderate synergistic effect with other antibiotics, but no action was noticed when at the junction with ampicillin [41]. On the contrary, the good performance of a mixture of propolis with ampicillin, was observed by Ismael et al. [42], *in vivo*, when treating sheep and goats affected with *Listeria monocytogenes*. The animals treated with this mixture showed the best result, compared to the mixture of cefotaxime antibiotics, cefotaxime alternative with gentamicin and trimethoprim-sulfadimethoxine combination. The author also noted that treatments with combinations of propolis and antibiotics were more effective than those treatments with only antibiotics. Better synergistic results between PEE and antimicrobials were observed with chloramphenicol, gentamicin, netilmicin, tetracycline and clindamycin, antibiotics (causers interference in protein synthesis of bacteria), than those antibiotics that show other forms of action in the inactivation of the bacterium [43].

Resistant and multi-resistant microorganisms to antibiotics were submitted to analysis with sub-inhibitory concentrations of PEE together with antimicrobials. The results of the synergistic action demonstrated the potential of propolis to improve the action of certain antimicrobials, which had been previously undetected [44]. Scazzocchio *et al.* [45] also evaluated sub-inhibitory concentrations of PEE added to different antimicrobials. These showed divergent activities against *S. aureus*. When sub-inhibitory concentrations were added to ampicillin, gentamicin and streptomycin showed high antimicrobial potency against this strain, when added to chloramphenicol, ceftriaxone and vancomycin showed moderate activity and when added to erythromycin did not show antimicrobial action.

Our results have clearly demonstrated that propolis may be highly efficient as antimicrobial for *Staphylococci* spp. Its use alone or in combination with antimicrobials may be an important alternative for the control of small ruminant mastitis, with remarkable advantages for public health.

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