Antimicrobial Activity of Propolis on the Bacterial Causes of Mastitis

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Abstract: The purpose of the current study was to isolate and identify mastitis causing bacteria from milk samples of mastitic cows and to evaluate the antimicrobial activity of propolis in relation to antibiogram profile. A total of 100 quarter milk samples were collected from 25 dairy cows in two farms. California Mastitis Test (CMT), Somatic Cell Counts (SCC) and microbiological examination of milk were used to identify and confirm mastitis cases. Major bacterial isolates were Gram positive as *Staphylococcus aureus* (33.1 %), coagulase-negative staphylococci (CNS) (10.8%), *Streptococcus agalactiae* (2.3%), *Streptococcus dysgalactia* (3.8%), *Corynebacterium bovis* (0.8%) and *Enterococcus faecalis* (5.4%), and Gram-negative rods as *Escherichia coli* (35.4%), *Pseudomonas aeruginosa* (3.1%), *Klebsiella* spp. (1.5%), *Pasteurella* spp. (2.3%) and *Proteus* spp. (1.5%). The antibiogram profile of different bacterial isolates indicated that norfloxacin, doxycycline, enrofloxacin and gentamicin proved to be the most effective antimicrobial agents against mastitis causing bacteria in this study. Propolis exhibited potent antimicrobial activity against ≈41% of the pathogens isolated from mastitis. The most susceptible strains to propolis were Gram-positive bacteria except *C. bovis* and *E. faecalis* while the least susceptible strains were Gram-negative bacteria including *Pasteurella* spp. and some isolates of *E. coli*.

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1. Introduction

Bovine mastitis cause great economic losses in dairy herds [1] due to its damaging effect on the production and processing of milk [2]. Mastitis is characterized by physical, chemical and bacteriological changes in the milk and pathological changes in the glandular tissue of the udder [3,4].

Staphylococcus aureus, an important pathogen for both humans and animals [5], has been one of the etiological agents of bovine mastitis in dairy farms beside other pathogens. The most infectious of the staphylococcal pathogens, is often referred to as contagious mastitis [6].

Propolis is a natural resinous mixture produced by honeybee (*Apis mellifera*) from substances collected from parts of plants, buds and exudates [7-9]. Bees gather propolis from different plants, in the temperate climate zone mainly from poplar [10]. Ancient Egyptians knew propolis very well for its anti-putrefactive properties and used bee glue to embalm their cadavers [11,12].

Propolis contains a variety of chemical compounds, such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids, and amino acids [9]. Current biological applications of propolis include antiviral [13, 14], antibacterial [15], antifungal [16], immunostimulatory [17, 18], anti-cancer [11], anti-oxidant [19] and anti-inflammatory [20]. The objective of this investigation was to diagnose mastitis cases by using CMT and SCC and then evaluate the antimicrobial activity of propolis on bacteria that cause mastitis as well as their antibiogram profile using the commonly used antibiotics.

2. Material and Methods

Samples

A total of 100 quarter milk samples were collected from 25 dairy cows in two farms. The cows were either showing clinical signs of mastitis or having low milk production. Milk samples were collected according to the National Mastitis Council [21]. After a quarter had been cleaned up by removing any possible dirt and washed with tap water, the teat end was dried and swabbed with cotton soaked in 70% ethyl alcohol. Quarter milk samples collected were subjected to CMT following the methods described by Dhakal [22]. Somatic cell counts were done according to Atasever [23]. Milk samples were simultaneously cultured, and the bacteria were isolated and identified by the method described by Guha *et al.* [24] and tested for their antimicrobial susceptibility. If SCC of a cow or of a quarter exceeded 300,000 cell/mL, the cow was defined as mastitic [25,26].

Propolis

Propolis material was collected from apiary farm near El-Mansoura City, Dakahlia Province, Egypt. The resinous materials were kept in dark bag in the refrigerator till being extracted with ethyl alcohol.

Extraction and sample preparation

One gram of propolis sample was cut into small pieces and extracted at room temperature with 50 mL of 70% ethanol according to Hegazi *et al.* [8]. Ethanol was evaporated under vacuum at 50 °C until dryness. The percentage of extracted matter was 0.8 g/dry weight.

Microbiological procedures

The samples collected from individual quarters of the udder according to the National Mastitis Council [21] were cultured on Nutrient agar, Blood agar, MacConkey agar plates and Columbia agar plates containing 5% of defibrinated ovine blood, incubated aerobically for 24-48h at 37 °C, supporting the growth of various types of bacteria for their study and isolation [27,28]. Bacterial species were identified according to Bergey's manual [29]. The pure cultures of bacterial isolates were obtained by sub culturing on differential and selective media. The bacterial isolates further subjected to biochemical tests for confirmation including, sugar fermentation, indole, methyl red, Voges-Proskauer, hydrogen sulphide, citrate and catalase tests. The isolates were tested *in vitro* for their antimicrobial susceptibility by agar disk diffusion method in accordance with the standard in National Mastitis Council guidelines [21]. Sensitivity test was done for all strains using the following antimicrobial agents: norfloxacin (10 µg), doxycycline (30 µg), enrofloxacin (5 µg), gentamicin (10µg), tetracycline (30 µg), sulfamethoxazole/trimethoprim (25µg), colistin (10µg), streptomycin (10µg), ampicillin (10µg), and penicillin (10 units) by the agar disc diffusion technique on Mueller-Hinton agar (Bio-Rad) and Oxoid test disc [30]. In another experiment, sensitivity test was done for all strains using propolis (25 µg) as antibacterial agent.

Statistical analysis:

The results obtained in the present study were represented as means ± standard error, and were analyzed using analysis of variance (ANOVA). The significance of difference between means at $P<0.05$ was calculated using the Duncan Multiple Range Test [31].

3. Results

Mastitis cases were identified by the CMT, SCC and microbiological examination of milk. The SCC count was of 10 power 3 (10^3). The SCC in normal cows was 328.67 ± 64.20 viz affected cows 934.01 ± 157.17. Out of 100 milk samples, 130 isolates were obtained from clinical and sub-clinical cases of mastitis (Table-1). The majority of the isolates were Gram positive bacteria including *Staphylococcus aureus* (33.1%), coagulase-negative staphylococci (CNS) (10.8%), *Streptococcus agalactiae* (2.3%), *Streptococcus dysgalactia* (3.8%), *Corynebacterium bovis* (0.8%) and *Enterococcus faecalis* (5.4%), and Gram negative rods including *Escherichia coli* (35.4%), *Pseudomonas aeruginosa* (3.1%), *Klebsiella* spp. (1.5%), *Pasteurella* spp. (2.3%) and *Proteus* spp. (1.5%). The antibiogram profiles of different bacterial isolates were listed in Table-2. Because all the used antibiotics have broad spectrum activity, the number of sensitive isolates were reported based on the highest susceptibility (the biggest zone of inhibition recorded). The results indicated that norfloxacin, doxycycline, enrofloxacin, and gentamicin proved to be the most effective antimicrobial agents against mastitis causing bacteria in this study. Where propolis exhibited potent antimicrobial activity against ≈41% of pathogens isolated from mastitis cases (Table-2). The most susceptible strains to propolis were Gram positive bacteria including *S. aureus*, coagulase-negative staphylococci, *S. agalactiae*, *S. dysgalactia*, while *Corynebacterium bovis* and *E. faecalis* were resistant to propolis. Meanwhile, Gram negative bacteria showed resistance against propolis including some isolates of *E. coli* and *Pasteurella* spp.

Table 1. Frequency of different bacterial species isolated from mastitis milk samples

Bacterial Species	No of isolates	% Isolation
Staphylococcus aureus	43	33.1
coagulase-negative staphylococci	14	10.8
Streptococcus agalactiae	3	2.3
Streptococcus dysgalactia	5	3.8
Corynebacterium bovis	1	0.8
Enterococcus faecalis	7	5.4
Escherichia coli	46	35.4
Pseudomonas aeruginosa	4	3.1
Klebsiella spp.	2	1.5
Pasteurella spp.	3	2.3
Proteus spp.	2	1.5
Total	130	100

Table 2. *In vitro* antimicrobial sensitivity of different antibiotics and propolis against bacterial isolates recovered from mastitic quarters' milk.

	n	Nor	Dox	Enr	Gen	Tet	Sul	Col	Str	Amp	Pen	Pro
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
S. aureus	43	11(25.6)	15(34.9)	8(18.6)	6(13.9)	6(13.9)	2(4.6)	1(2.3)	1(2.3)	0(0)	1(2.3)	19(44.2)
CNS	14	8(57.1)	7(50.0)	2(14.3)	2(14.3)	1(7.1)	1(7.1)	0(0)	0(0)	0(0)	0(0)	11(78.6)
S. agalactiae	3	1(33.3)	1(33.3)	1(33.3)	1(33.3)	0(0)	0(0)	1(33.3)	0(0)	0(0)	0(0)	2(66.7)
S. dysgalactia	5	2(40.0)	1(20.0)	1(20.0)	1(20.0)	0(20.0)	1(20.0)	0(0)	0(0)	0(0)	0(0)	4(80.0)
C. bovis	1	0(0)	0(0)	1(100.0)	0(0)	1(100.0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. faecalis	7	2(28.6)	4(57.1)	3(42.8)	0(0)	1(14.3)	1(14.3)	1(14.3)	0(0)	0(0)	0(0)	0(0)
E. coli	46	8(17.4)	1(2.2)	1(2.2)	2(4.3)	0(0)	3(6.5)	2(4.3)	1(2.2)	1(2.2)	0(0)	9(19.6)
P. aeruginosa	4	1(25.0)	1(25.0)	1(25.0)	2(50.0)	0(0)	0(0)	2(50.0)	0(0)	0(0)	0(0)	3(75.0)
Klebsiella spp.	2	1(50.0)	0(0)	1(50.0)	0(0)	1(50.0)	0(0)	1(50.0)	0(0)	0(0)	0(0)	2(100.0)
Pasteurella spp.	3	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Proteus spp.	2	1(50.0)	0(0)	1(50.0)	1(50.0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(50.0)

Nor = Norfloxacin (10µg) Dox = Doxycycline (30µg) Enr = Enrofloxacin (5µg) Gen = Gentamicin (10µg) Tet = Tetracycline (30µg) Sul = Sulfamethoxazole (25µg) Col = Colistin (10µg) Str = Streptomycin (10µg) Amp = Ampicillin (10 µg) Pen = Penicillin (10 units) Pro = Propolis (25 µg)

4. Discussion

Mastitis cases were identified by the CMT and SCC to confirm clinical and detect subclinical cases. The use of CMT in conjunction with SCC was proved to be efficient as a diagnostic tool for the detection of subclinical mastitis [25]. The direct microscopic method is inexpensive and most commonly used in India [32]. The results revealed that cows suffering from clinical and sub-clinical cases of mastitis showed an increase in SCC. Normally, in milk from a healthy mammary gland, the SCC is lower than 1×10^5 cells/mL, while bacterial infection can cause an increase in SCC to above 1×10^6 cells/mL [33]. The results in this experiment were in agreement with the results obtained by various workers as illustrated by Gonzalo *et al.* [34].

The contagious pathogens, *S. aureus* and *S. agalactiae* generally cause the greatest SCC increase. While the infection by environmental pathogens, *S. dysgalactiae*, *Streptococcus uberis*, *C. bovis* and coagulase negative *Staphylococcus* usually causes considerably less SCC elevation. Rise in the leukocyte number in milk and in the mammary gland, as a response to the assaulting pathogens or to their metabolites leads to an increase in SCC [35]. It is also evident that SCC is significantly higher (p<0.01) in subclinical mastitis caused by different bacteria. The National Mastitis Council defines subclinical mastitis in cows as a quarter with SCC of 200×10^3 cells /mL of milk or more, with a normal quarter having counts around 100×10^3 cells/mL of milk [22]. In the present study, the cut-off value of SCC for the detection of subclinical mastitis in milk was 215×10^3 cells/mL or Log10 5.34 cells/mL of milk which is marginally higher than the observation of Dhakal [22]. Though SCC is the only test to have likelihood ratio > 10, yet, SCC alone is diagnostically insufficient sometimes due to latent and nonspecific infections [26].

A total of 130 bacterial isolates belonging to 11 various genera of bacteria were isolated from 100 milk samples collected from 25 dairy cows. The results indicated that *E. coli* was the most frequently isolated bacterium (35.4 %) followed by *S. aureus* (33.1 %). Meanwhile *S. aureus* and coagulase-negative staphylococci combined together (43.9%) were the most significant isolates. These results were in agreement with those previously observed by Haghkhah *et al.* [36]. They isolated eight various genera of bacteria from subclinical mastitis and *S. aureus* was the most significant bacterium (27.9% of all isolated bacteria). Furthermore, it was found that Staphylococcus spp. (47.37%) and Streptococcus spp. (33.68%) were the most prevalent etiological agents isolated from subclinical mastitis in cattle [25,26]. In a separate study, to determine the prevalence of mastitis in dairy heifers, the most common isolates were *S. aureus*, *Staphylococcus hyicus*, and *Staphylococcus chromogenes* [37].

Taking in consideration that the majority of authors have noted the increase in the resistance of pathogens isolated from mastitis to antibiotics [4,37-44], the aim of the current study was to evaluate the antibacterial activity of propolis, as a natural alternative to some of the commonly used antibiotics, against bacteria isolated from clinical and subclinical cases of mastitis. Propolis exhibited potent antibacterial activity against ≈41% of the isolated bacteria with more efficiency than some of the tested antibiotics. Such activity was documented earlier against various microbial strains [11,12,15]. This antimicrobial activity is due to the unique chemical composition of propolis [15, 19]. Although, the chemical composition of propolis extracted with different solvents is different, Ivancajic *et al.* [45]

found that propolis extracted by five different solvents exhibited a significant antibacterial activity against different bacterial strains including exotic pathogenic bacteria such as *S. aureus* and *Bacillus cereus*. Although propolis was more effective against Gram-positive bacteria than Gram-negative bacteria, some isolates of both groups showed resistance against propolis including *C. bovis* and *E. faecalis* (Gram-positive) and *E. coli* and *Pasteurella* spp. (Gram-negative). These findings were in agreement with those found in earlier studies [15,46]. Although, this study confirmed the efficacy of propolis as antibacterial agent against bacterial strains isolated from mastitis, further investigation is needed to standardize its application as a single antibacterial agent or in combination with other antibacterial agents.

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