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Antibacterial Activity of Sage Essential Oil against Common Mastitis Causing Bacterial Isolates from Bovine Clinical Mastitis

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Abstract: This study was conducted to evaluate the *in vitro* antibacterial activity of Sage (*Salvia officinalis*) essential oil (EO) against common mastitis pathogens isolated from clinical bovine mastitis. A total of 130 quarter milk samples collected from cases of clinical mastitis were used in the study. Bacterial isolation and identification was performed according to standard laboratory methods. Agar well-diffusion method was used to test the antibacterial activity of Sage EO at concentration of 60µl against all isolated strains of bacteria. Amoxicillin at concentration of 25µg was used as a positive control. A total of 119 bacterial strains were recovered from bovine clinical mastitis cases. The most prevalent bacterial groups were coagulase-negative *Staphylococci* (CNS) (35.3%) followed by coliforms (33.6%), *Staphylococcus aureus* (19.3%) and environmental *streptococci* (11.7%). Sage EO showed similar activity against *Staphylococcus epidermidis, Proteus vulgaris* and *Corynebacterium* with inhibition zone diameter of 12mm, followed by *Staphylococcus chromogenes* (11 mm). The lowest inhibition zones of Sage EO were reported for *E. coli* (7 mm), *Pseudomonas* and *Staphylococcus hyicus* (8 mm each) and *Acinetobacter*, *Streptococcus uberis* and *Staphylococcus aureus* (9 mm each). Results of this study indicated that Sage EO has promising antibacterial activities against some udder pathogens in dairy cows. Further *in vivo* studies are therefore warranted to investigate its efficacy in treating clinical mastitis.

Key words: Antibacterial Sensitivity · Alternative Antibacterial Therapy · Udder Pathogens · Udder Inflammation

INTRODUCTION

Mastitis is considered as one of the most economically important diseases affecting dairy cattle worldwide [1, 2]. Mastitis leads to significant changes in the physical and chemical composition of milk and thus its wholesomeness. It also greatly impacts cow general wellbeing and health. In the USA, the prevalence of clinical mastitis in dairy herds has been estimated to be around 15% [3] while in Jordan, estimates suggested 13 to 16% [4, 5]. *Staphylococcus aureus* is the most frequent udder pathogen isolated from dairy cattle with clinical and subclinical mastitis [4, 6].

Uncontrolled use of antibacterial agents for the treatment of mastitis can lead to significant human health hazard by consuming raw milk containing antibiotic residues [7, 8]. The overzealous use of antibacterial agents for the treatment of mastitis in dairy cattle has also led to the appearance of resistant bacteria against commonly available antibiotics [9]. This has created a serious challenge in management, prevention and treatment of mastitis [10].

Several natural alternative therapies have been evaluated for treatment of mastitis [11-13]. Essential oils (EO) are the main components of many aromatic plants. They are classified as “Generally regarded as safe” natural

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products. Sage (*Salvia Officinalis*) is a common herb that can grow wildly or in cultivation. It has been long known for its broad medicinal effects including anti-inflammatory, antiseptic and antibacterial medicinal effects [14-16]. The active component of Sage EO is 1, 8-cineole which was particularly found effective against *Escherichia coli* and *Staphylococcus aureus* [17, 18]. This study was conducted to evaluate the *in vitro* antibacterial activity of Sage (*Salvia officinalis*) EO against common mastitis pathogens isolated from clinical bovine mastitis in Jordan.

MATERIAL AND METHODS

Ethical Approval: All procedures performed in this study were approved by the Jordan University of Science and Technology Animal Care and Use Committee. Signed consents were obtained from animal owners before milk sample collection.

Milk Samples: A total of 130 quarter milk samples were collected from 94 cows affected with clinical mastitis that were presented to the Veterinary Health Canter of the Faculty of Veterinary Medicine at Jordan University of Science and Technology. Cows were subjected to a comprehensive examination including udder palpation and gross milk examination before milk sample collection. Milk samples were only collected from cows with systemic clinical signs (Depression, fever, abnormal respiration and heart rates), abnormal milk physical appearance (Bloody, watery and clotted) and abnormal udder appearance (Swelling, hot and painful).

Milk samples were collected aseptically in sterile test tubes and were submitted to the diagnostic laboratory for bacterial culture and sensitivity testing immediately after collection [19].

Bacterial Culture and Identification: Bacterial culture from milk samples was performed according to previously published methods [20]. Briefly, a sterile “hockey stick” was used to spread 1 ml of milk on the surface of culture plates containing blood agar and MacConkey agar. Plates were then incubated aerobically at 37°C for 48 hours. Bacterial identification was performed initially according to their Gram-stain reaction, haemolytic features and colony morphology. Biochemical tests were used to identify isolated strains of bacteria using commercially available identification kits Microbact™ Gram-negative system (Thermo Scientific, UK), Microbact™ Staph 12S system (Thermo Scientific, UK) and HiStrep™ Identification Kit (HiMedia Laboratories, India).

Essential Oil Extraction: Sage aerial parts including both flowers and leaves were collected from Irbid in the Northern part of Jordan during September, October and November of 2016. The collected plant material was dried in the shade away from direct sunlight. The EO was extracted by hydro-distillation process using Cleveger's apparatus (Standard Scientific Glass Industries, India) as described previously [21]. Briefly, about 220 g of dried aerial parts were mixed with 1.5 litre of distilled water and heated up to 100 °C for 3 h. The EO was then collected and dried using sodium sulphate (Sigma-Aldrich, Inc. Germany) according to the European Pharmacopeia [22]. The EO was stored in an amber glass vials at -20 °C until used.

Antibacterial Sensitivity Test: The agar well-diffusion method on Mueller-Hinton agar was used to evaluate the antibacterial activity of Sage EO [23]. Initially, inoculates of each isolated micro-microorganism were prepared by growing on nutrient agar slant cultures for 24 h and suspending colonies in a sterile solution of Mueller-Hinton broth (Sigma-Aldrich, Inc. Germany) [24]. Suspensions were adjusted to the turbidity of 0.5 McFarland standards (approx. 10^8CFU/ml) [25]. At first, 100µl of each bacterial suspension was spread onto Mueller-Hinton agar plate. After 15 min, 60 µl of Sage EO (1ml sage EO dissolved into 0.2ml of Dimethylsulphoxide (DMSO; Sigma-Aldrich, Inc. Germany) was poured in wells (6mm diameter) made in the centre of each agar plate. The addition of DMSO, an aprotic organic solvent, had the purpose of facilitating the solubilisation of EO in the culture media [24]. Different DMSO concentrations were tested to avoid any antibacterial effect that could be attributed to DMSO. The final concentration of DMSO was never exceeded 2%. Amoxicillin (25 µg/disc, BioRad, USA) was used as a positive control. DMSO was used as a negative control. All tests were performed in triplicates. After the plates were incubated at 37 °C for 48 h, the inhibition zones were measured in millimetres.

RESULTS

Sage EO Extraction: Extraction of the Sage EO produced 1.5–2.2% (w/w) depending on the dry weight of the plant.

Bacterial Isolates: Of all the 130 milk samples, 119 bacterial isolates were recovered (Table 1). The most prevalent bacterial groups were coagulase-negative *Staphylococci* (CNS) (35.3%) followed by coliforms (33.6%), *Staphylococcus aureus* (19.3%) and environmental streptococci (11.7%).

Table 1: Classification of bacterial isolates from milk obtained from cases of bovine clinical mastitis.

Bacterial groups	N	Frequency (%)
Coliforms	40	33.6
Environmental streptococci	14	11.7
Staphylococcus aureus	23	19.3
Coagulase-negative staphylococci	42	35.3

Table 2: Frequency (%) and types of bacterial strains isolated from milk obtained from cases of bovine clinical mastitis.

Bacterial strains	Number	Frequency (%)
Escherichia coli	34	28.5
Pseudomonas spp.	1	0.84
Proteus vulgaris	2	1.68
Acinetobacter spp.	6	5
Streptococcus uberis	2	1.68
Corynebacterium spp.	6	5
Staphylococcus aureus	23	19.3
Staphylococcus hyicus	2	1.68
Staphylococcus chromogenes	41	34.4
Staphylococcus epidermidis	2	1.68

Table 3: Zones of inhibition (mm) of Sage EO oil, amoxicillin and DMSO against different bacterial isolates from milk obtained from cases of bovine clinical mastitis.

	Treatment		
Bacterial isolates	Sage EO (60 µl)	Amoxicillin (25 µg/disc)	DMSO (2%)
Escherichia coli	7	30	6
Pseudomonas spp.	8	0	0
Proteus vulgaris	12	7	0
Acinetobacter spp.	9	18	0
Streptococcus uberis	9	34	14
Corynebacterium spp.	12	45	6
Staphylococcus aureus	9	20	0
Staphylococcus hyicus	8	30	0
Staphylococcus chromogenes	11	21	6
Staphylococcus epidermidis	12	17	6

Table 2 shows the most prevalent bacterial strains isolated from cases of clinical mastitis in cows. The most common bacterial species isolated were *Staphylococcus chromogenes* (34.4%), *Escherichia coli (E. coli*) (28.5%) and *Staphylococcus aureus* (19.3%).

Antibacterial Sensitivity Patterns of Sage EO: The inhibition zones (mm) for Sage EO, amoxicillin and DMSO against different strains of isolated bacteria from clinical cases of bovine mastitis are shown in Table 3. The largest inhibition zones of Sage EO (12 mm) were reported against *Staphylococcus epidermidis, Proteus vulgaris* and *Corynebacterium*, followed by *Staphylococcus chromogenes* (11 mm). The lowest inhibition zones of Sage EO were reported for *E. coli* (7 mm), *Pseudomonas* & *Staphylococcus hyicus* (8 mm each) and *Acinetobacter*, *Streptococcus uberis*, & *Staphylococcus aureus* (9 mm each).

DISCUSSION

Aromatic and medicinal plants have been long used as natural alternative therapies for the treatment of many different diseases [21]. In recent years, many anecdotal reports were published regarding the effectiveness of using different aromatic plants for the treatment of common diseases, including mastitis [25]. Nevertheless, scientific investigations to evaluate the antimicrobial activity of essential oils contained in these plants against common bacteria isolated from clinical mastitis cases are limited. Therefore, this study was conducted to evaluate the antibacterial sensitivity of common bacterial isolates from clinical mastitis cases to Sage EO.

Sage plant has been used commonly in Jordan as a traditional medicine for its antimicrobial, antiseptic, anti-scabies, antisyphilitic, antipyretic and anti-inflammatory properties [21]. The chemical composition of Sage from different parts of Jordan has been determined previously [21]. Twenty five different chemicals have been identified in Sage accounting for almost 99% of its EO composition. The major compounds were 1,8-cineole (39.5–50.3%) and camphor (8.8–25.0%) while thujones were present in a very low concentrations (0.9–9.9%) [21].

In Jordan, *Staphylococcus aureus* is the most prevalent pathogen isolated from clinical bovine mastitis cases, followed by *Corynebacterium bovis* and *E. coli* [26]. In this study, *Staphylococcus chromogenes* was the most prevalent bacteria isolated from bovine clinical mastitis, followed by *E. coli* and *Staphylococcus aureus*. Coagulase-negative Staphylococci are now predominant over *Staphylococcus aureus* in most countries; therefore it could be described as emerging mastitis pathogens [27]. The high prevalence of *Staphylococcus chromogenes* may be due to its ability to cause considerable damage to udder tissues [27]. Moreover, *Staphylococcus chromogenes* can cause persistent infections, which results in increased milk somatic cell count (SCC) [27].

The emergence of resistant bacterial strains is most problematic in Jordan dairy industry [6]. Therefore, research examining alternative or unconventional therapies is most warranted. Several recent studies have revealed a promising antimicrobial activity of Sage against many bacterial isolates including *Escherichia coli,*

Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis and others [15, 16]. In this study, Sage EO showed moderate antibacterial activity especially against environmental bacteria. These findings are in congruent with those reported by Gulluce *et al*. [15]. The antibacterial effects of Sage EO were reported previously against 23 bacterial isolates [15]. Moreover, Rami and Li [16] proved a bacteriostatic effect of Sage EO again*st Escherichia coli, Salmonella typhi and Pseudomonas aeruginosa.* Pafica *et al*. [28] estimated effective "*In vitro*" antibacterial activity of Sage EO oil at 12mm inhibition zone against *Staphylococcus aureus*. Similar results were reported in this study against *Staphylococcus epidermidis, Proteus vulgaris, Corynebacterium spp.* and *Staphylococcus chromogenes*.

CONCLUSIONS

In this study, it could be concluded that the antibacterial activity of Sage EO is highest against environmental Streptococci, followed by CNS and least active against coliforms and *Staphylococcus aureus*. Further *in vivo* studies are therefore warranted to investigate its efficacy in treating clinical mastitis cases. In the future, an easy to apply gel or suspension formulation for intramammary use could be developed for use in the field.

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