Evaluation of in vitro antibacterial effect of essential oil and some herbal plant extract used against mastitis pathogens

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Abstract
Background: Mastitis in dairy cattle is a highly prevalent infectious disease, caused by various pathogens, mainly Staphylococcus aureus and Escherichia Coli, considerable economic loss worldwide.

Objectives: The aim of this study was to evaluate the in vitro activity of Herbal plants used against S. aureus and E. coli bacteria which are the causative agents of mastitis.

Methods: Therefore, in this study we investigate the antimicrobial effect of plant to evaluate the in vitro antibacterial activity of squaw mint (Mentha pulegium L., Lamiaceae family), catnip (Nepeta cataria L., Lamiaceae), lemon balm (Melissa officinalis L., Lamiaceae), for mastitis treatment. Solutions prepared in fixed oils, against S. aureus and E. coli bacteria which are the main agents of mastitis. Isolation and antibiotic susceptibility analyses of milk samples taken from 100 subclinical mastitis dairy cows were performed. The antibacterial properties of the solutions were analysed by a disk diffusion method.

Results: In the bacterial isolation, S. aureus was determined 97.7% and E. coli 53.5% positive of cows with mastitis. Antibacterial susceptibility test of the Lemon balm extract and essential oil showed maximum zone of inhibition against S. aureus 30 µl (23 mm), followed by 20 µl (19 mm), E. coli (19 mm) and 10 µl (5–7 mm), of the same extract against the Gram-positive bacteria. The ethanol extracts show the similar activity against the Gram-negative bacteria at 30, 20, and 10 µl (18–20 mm). Followed by S. aureus, when the zone areas for the susceptible solutions (Lemon balm, and essential oil) and the control group were compared, determined that there was little difference between for S. aureus and E. coli.

Conclusions: This study hence indicated that in vitro cultured plantlets of lemon balm and peppermint oil can be used as the alternative method for production of mastitis and cheap source its precursor with antimicrobial activities.

KEYWORDS
essential oil, herbal plants, in vitro, subclinical mastitis

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Mastitis is a complex disease caused by various bacterial pathogens, mainly *Staphylococcus aureus*, and *Escherichia coli*. It is also reported to be the most common reproductive disease in dairy cattle and a heavy burden disease in dairy farms worldwide (Alonso et al., 2020; Harjanti & Sambodho, 2020). It is believed that the bacterial infection in mastitis cases is related to the disruption of alveolar cell-integrity, sloughing of cells, induced apoptosis and increase of poorly differentiated cells. Since the ability of ruminant mammary glands to produce milk is determined by the number and activity levels of milk-secreting cells, the amount of milk produced and the protein, lactose, and fat concentrations in milk can be affected by the level of inflammation in the mammary gland (Harjanti & Sambodho, 2020).

Drug residues in milk cause allergic reactions for the consumer, interference in the intestinal flora, and resistant bacterial populations, and accordingly, they can destroy the effect of antibiotic treatment (Amber et al., 2018). The World Health Organization (WHO) stated that *E. coli* and *S. aureus* are the priority pathogens for overcoming antimicrobial resistance and for the research and development of new antibiotics (Arbab et al., 2021a; Arbab et al., 2022; Klaas & Zadoks, 2018; Tepeli, 2020). The main treatment of mastitis is commonly administered by intramammary infusion of an ointment or intramuscular or intravenous injection of antibiotics, such as beta-lactams (Tepeli, 2020). However, the treatment is anticipated to become problematic in the near future owing to the rapid increase in antibiotic-resistant pathogens (Milik, 2014; Oliver & Murinda, 2012).

There are an increasing number of published studies in the field of antimicrobial therapy using natural products (Boldbaatar et al., 2014; Rios & Recio, 2005), including studies on the antimicrobial effect of plant products on pathogens isolated from BM (Dorman & Deans, 2000; Gopinath et al., 2011). However, the majority of these studies are focused on plants which have a natural distribution, specific to certain geographical areas (Taemchuay et al., 2009). Despite the encouraging results of these studies, more studies including indigenous or acclimatised plants are required to cover distinct geographical areas in order to have a great availability and a low manufacturing cost for these products.

Essential oils and plant extracts are rich with a wide variety of metabolite compounds (Taemchuay et al., 2009). Peppermint oil is recommended for mastitis therapy has been proven to be effective against a wide variety of microorganisms (Grzesiak et al., 2018), but there are only a few studies available that describe the antimicrobial effects of essential oils (Vlase et al., 2014). Onion bulbs contain a good number of phytochemical properties, most of which are hydrocarbons and their derivatives. Several studies have proved that the plant extracts and essential oils have antimicrobial effects (Zajmi et al., 2015). However, a large number of plant species have not been studied for their potential medicinal value (Duda et al., 2015).

Previous studies have evaluated the antimicrobial effect of several medicinal plants on different collection strains of pathogens (Duda et al., 2015). The aim of this study was to evaluate the in vitro activity of solutions of essential oils, squaw mint (*Mentha pulegium* L., Lamiaceae family), catnip (*Nepeta cataria* L., Lamiaceae) and lemon balm (*Melissa officinalis* L., Lamiaceae) against *S. aureus* and *E. coli* bacteria which are the causative agents of mastitis.

## 2 | MATERIAL AND METHODS

### 2.1 | Animal’s clinical data

The lactating breed udder secretions were the test subjects in this study and clinical data were recorded from Cattle with Clinical mastitis (CM). In the study, 100 cows evaluated as CMT +/- 1, ++: 2, +++: 3 and without clinical endometritis, laminitis were accepted as the experimental group. All animal experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People’s Republic of China, and all efforts were made to minimise suffering. This work was supported by grants from the National Natural Science Foundation of China (No: 31872520).

### 2.2 | Collection of plants

Medicinal aromatic plants used in the study are the extract of squaw mint (*Mentha pulegium* L., Lamiaceae family), catnip (*Nepeta cataria* L., Lamiaceae), lemon balm (*Melissa officinalis* L., Lamiaceae), and the plants were washed with water and kept in an oven at 40°C for 3 days their weight stabilised and turned into powder and macerated was first filtered through doubled layered muslin cloth and then centrifuged at 4000 × g for 30 min. The supernatant fluid was filtered through Wattman No. 1 filter paper and heat sterilised. The extract was preserved aseptically in a brown bottle at 5°C until used.

### 2.3 | Extraction of plant and preparation of the solution

#### 2.3.1 | Extraction of essential oil

Herb parts of squaw mint (*Mentha pulegium* L., Lamiaceae family), catnip (*Nepeta cataria* L., Lamiaceae), lemon balm (*Melissa officinalis* L., Lamiaceae), the region with the highest essential oil, were used, and then powdered. Ten grams of this powder was soaked in 100 ml of solvents namely ethanol, and essential oil for 24 h. The contents were then filtered through Whatman filter paper no. 1 and the filtrate was evaporated to dryness. This dried extract was further powdered and then dissolved in distilled water to make the working solution having 10 mg/ml concentration. Solvent controls (Ethanol) were prepared in the similar manner.

### 2.4 | Bacterial isolation from milk sample

Bacterial strain isolation from milk samples was carried out following aseptic procedures as described by National Mastitis Council.
A loopful of milk sample was streaked on blood agar (Oxoid) supplemented with 5% sheep red blood cells and then subcultured on selective media, Mannitol Salt Agar, Salmonella Shigella Agar, Edwards medium and MacConkey Agar. All plates were then incubated aerobically at 37°C for 24 h. The plates were examined for colony morphology, pigmentation, and haemolytic characteristics at 24–48 h. Catalase test was applied for distinguishing between staphylococci and other Gram-positive cocci, mannitol fermentation test, coagulase test (either positive or negative). Furthermore, Gram-negative bacterial isolation was carried out according to the standard microbiological procedures described by Clinical Laboratory Standards Institute (Humphries et al., 2021).

The isolates were confirmed by biochemical tests and sub-cultured on differential and selective medium. The biochemical tests were oxidase activity, acid production (lactose sucrose and glucose fermentation), indole production, Voges–Proskauer and hydrogen sulphide production.

### 2.5 Determination of antibiotic resistance profile

Antibiogram potential of the isolates were determined according to the modified Kirby-Bauer disc diffusion method using Muller-Hilton agar following Clinical Laboratory Standards Institute guidelines (CLSI Performance Standards for Antimicrobial Susceptibility Testing) (Zhang et al., 2018).

### 2.6 Determination of antimicrobial activities of plant extracts and essential oil

#### 2.6.1 Disc diffusion method

This method was done according guidelines mentioned by guidelines mentioned by CLSI (Arbab et al., 2021b; Humphries et al., 2021). Bacterial isolates were diluted at a concentration to McFarland 0.5. The suspension was poured on plate with Mueller–Hilton agar after 1 h. Fifty discs of 6 mm Whatman filter paper were obtained by punching and placing in bottle and sterilising in hot air oven at 170°C for 30 min. The filter paper with 6 mm diameter discs was impregnated with 20 µl of each essential oil was diluted in 1 ml of distilled water (v/v) and compared with reference antibiotics and solvent or double-distilled water as negative control aseptically placed on Mueller–Hilton agar plates. The plates were incubulated at 37°C for 24 h (Perez, 1990). The number of visible growth in minimum inhibitory concentration disc diffusion assay was subculture using a 10 µl inoculating loop onto a 5% sheep BAP and incubated at 37°C for 24 h were found (Table 1).

#### 2.6.2 Statistical analysis

The table’s representation was performed using the program (Microsoft Office Excel, 2007). Descriptive statistics of the data obtained from the study are given with mean, standard deviation, frequency and percentage analysis.

### 3 RESULTS

#### 3.1 Bacteria isolation data

The overall percentage of positive bacteria isolates from dairy mastitis cattle. A total of 100 cattle were examined and 73 were recorded positive for different organisms. The percentage prevalence of organisms is presented in Table 2. Out of 100 samples, S. aureus was isolated and identified positive sample in cattle 43 (97.7%) and E. coli 30 (53.5), respectively. All organisms were identified on their morphological, cultural characteristics and staining reactions. Organisms were further confirmed by their biochemical reactions.

#### 3.2 Bacterial zone diameters

While some of the materials were found to have different levels of antibacterial activity on the tested microorganisms, some were found to be ineffective (Table 3 and Figure 1).

#### 3.3 Sensitivity of plant extract

Table 3 shows the summarised results of the antibacterial susceptibility test of the ethanol extracts of squaw mint (Mentha pulegium L., Lamiaceae family), lemon balm (Melissa officinalis L., Lamiaceae), catnip (Nepeta cataria L., Lamiaceae) and essential oil, respectively, against the

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**TABLE 1** Zone diameters used in the evaluation of inhibition activity (Åobankara et al., 2004)

| Zone diameter | Inhibition activity |
|---------------|---------------------|
| Not seen      | (–)                 |
| <10           | (+)                 |
| 10–14         | (++)                |
| 15–19         | (+++)               |
| 20            | (+++++)             |

(−): resistant; (+): less resistant; (++): medium sensitive, (+++): sensitive; (++++) very sensitive.

**TABLE 2** Clinical mastitis microorganism determination results

| Microorganisms isolated in cattle | Number percentage (%) |
|----------------------------------|------------------------|
| Staphylococcus aureus Negative (–) | 1                      | 2.2                    |
| Positive (+)                     | 43                     | 97.7                   |
| Escherichia coli Negative (–)    | 26                     | 86.6                   |
| Positive (+)                     | 30                     | 53.5                   |
**TABLE 3** Antibacterial activities of plant extract on test microorganisms

| Plant extract | Zone of inhibition (in mm) different concentration | Staphylococcus aureus | Escherichia coli |
|---------------|---------------------------------------------------|-----------------------|------------------|
|               | 30 µl Act | 20 µl Act | 10 µl Act | 30 µl Act | 20 µl Act | 10 µl Act |
| Squaw mint    | 19 (++++) | 12 (++)  | 5 (–)    | 18 (++++) | (–)  (++++) | 3 (–)   |
| Lemon balm    | 23 (+++++)| 19 (++)  | 7 (–)    | 19 (+++++)| 10 (++)  | (–) (–) |
| Cantnip       | 8 (+)     | 5 (–)    | 2 (–)    | (–) (–)  | (–) (–) |
| Essential oil | 21 (+++++)| 15 (++++) | 3 (–)    | 20 (+++++)| 14 (++++) | 3 (–)   |
| Amoxicillin/ clavulanic acid | 15 (+++) | | |
| Cefoperazone  | 19 (++++) | | | |

(-): resistant; (+): less resistant; (++): medium sensitive, (+++): sensitive; (++++): very sensitive.

**FIGURE 1** Bacterial zone of inhibition diameters (a) shows 30 µl ethanol extract of lemon balm and (b) shows essential oil solution against *S. aureus* and *E. coli*

Bovine mastitis is a serious disease causing considerable economic loss worldwide (Halasa et al., 2007). Based on previous studies and the recommendations of the National Mastitis Council, DCT is considered to

| S. aureus | Group | Number (%) | Control Number (%) |
|-----------|-------|------------|--------------------|
| Resistance | 5 | 45.5 | 0 | 0 |
| Sensitive | 3 | 27.2 | 1 | 50 |
| Medium sensitive | 1 | 9.0 | 0 | 0 |
| Very sensitive | 2 | 18.1 | 1 | 50 |

| E. coli | Group | Number (%) | Control Number (%) |
|---------|-------|------------|--------------------|
| Resistance | 10 | 62.5 | 1 | 33 |
| Sensitive | 3 | 18.7 | 0 | 0 |
| Medium sensitive | 1 | 6.2 | 2 | 66 |
| Very sensitive | 2 | 12.5 | 0 | 0 |

**3.4 | Sensitivity of solutions**

Among the solutions, five solutions were found resistant for *S. aureus*, while six solutions were found to be sensitive (moderate and very sensitive). For *E. coli*, 10 solutions were found to be resistant, while six solutions were found to be sensitive (moderate and very sensitive) as shown in the Table 4.

When the zone diameters were compared between the two antibiotics groups and solution used as the control group in the study, it was determined that was little statically significant difference as showed in the Table 5 and Figure 2.
be one of the most effective methods for preventing mastitis during the dry period (Schukken et al., 1993). Given the various arguments that discourage widespread antibiotic use, development of alternative strategies using compounds not subject to limitations associated with antibiotics is needed. Herein, historically, plant extracts, or their derivate, have been used as a safe, effective and natural remedy for ailments and diseases in traditional medicine; we present data indicating the efficacy of several plant-derived compounds for killing mastitis pathogens in vitro.

The present study increases the number of data regarding the antimicrobial activity of plants pathogens isolated from BM. The obtained results have shown an inhibition of bacterial growth for all tested plants, with better results for squaw mint (Mentha pulegium L., Lamiaceae family), lemon balm (Melissa officinalis L., Lamiaceae), catnip (Nepeta cataria L., Lamiaceae) and essential oil. These results could be explained by the active compounds determine in these plants. The results of the evaluation of antibacterial activity of both plants showed that only one of each plant extracts (ethanol extract of squawmint, lemon balm and catnip) has a significant activity against the strain of Gram-positive bacteria S. aureus. This could suggest that the opportunistic bacterial pathogens that affect animals infected by mastitis disease in our study area could be due to Gram positive bacteria and Gram-negative bacteria. Results in this work are similar to the ones found by Aliyu et al. (2008), Arbab et al. (2020) and Arbab et al. (2021b).

S. aureus, which is known to be highly resistant to antibiotic treatment in mastitis, lives in the host’s cells and becomes chronic by forming micro abscesses or granulomas in the mammary gland tissues (Azadi et al., 2011). In the bacterial isolation results, S. aureus bacteria were found to be 97.7% and E. coli 53.5% positive. It was determined that the effects of these two bacteria in the formation of mastitis were intense in the cows studied. Especially, in vitro solution trials were conducted against these two bacteria. These findings of these studies were agreement to the previous study by Arbab et al. (2021c). The values found were similar to other research results (Arbab et al., 2021d).

Although all extracts showed antimicrobial activity against nearly all of the microorganisms tested, lemon balm and essential oil were found to exhibit broad-spectrum activity against selected bacterial pathogens isolated from clinical mastitis in dairy cows. The study found that extract of lemon balm and essential oil showed maximum inhibition against S. aureus (21–23 mm) and E. coli (19–20 mm). E. coli, which is already known to be multiresistant to drugs, was susceptible only to the extract from lemon balm essential oil.

The essential oils contain many compounds that act synergistically and induce strong anti-algal effects. The active substances, including various polyphenols, are capable of dissolving the algal cell wall and penetrating into the cell, where they affect the cell metabolism (Bouari et al., 2011). Another researcher study conducted to check the antibacterial activity of ethanol extract of medicinal plant exhibited maximum inhibition against S. aureus (Arbab et al., 2020; Jothi et al., 2014). The present study also supported by Paiano et al. (2020).

Researchers have found that peppermint oil is recommended for mastitis therapy (Grzesiak et al., 2018). It was determined that cinnamon, clove, oregano and thyme essential oils showed inhibition zone diameters of 36 mm, E. coli 20 mm, especially for S. aureus causing endometritis (Paiano et al., 2020). Peppermint oil showed positive activity maximum 30 µl 21 mm inhibition zone against S. aureus and 30 µl 20 mm for E. coli, respectively. There was no indication of inhibition against E. coli. In the study conducted by the researchers, the antioxidant and antimicrobial activities of the phenolic components of the phenolic extracts of olive oil varieties obtained from 11 Algerian varieties against various bacteria were investigated.

Peppermint oil is used as a natural medicine for multiple therapeutic purposes in animals. It has been observed that peppermint essential oil inhibits the growth of Gram-negative microorganisms, especially

![Comparison of antibacterial Activities of solutions and control group against S. aureus and E. coli](image)

**TABLE 5** Zone diameters of the solution and control groups that were found susceptible to S. aureus and E. coli

| Variable   | Solution Mean ± SD | Control Mean ± SD |
|------------|--------------------|-------------------|
| S. aureus  | 11.58 ± 6.79       | 16.5 ± 14.5       |
| E. coli    | 12.42 ± 8.64       | 19.5 ± 17.5       |
Gram-positive bacteria. It has been reported that the biological activity of the oil is due to its phenolic content (Azadi et al., 2011).

When the zone diameters were compared between the two antibiotic groups and solutions used as the control group in the study, it was determined that there was little statistically significant difference. It was determined that ±6.79 to 14.5 SD of the solutions and control were susceptible to S. aureus and ±8.64 to 17.5 SD were susceptible to E. coli. These findings determined that there was a little difference between S. aureus and E. coli. Various workers throughout the world did high-rate antibacterial activity against S. aureus (zone of inhibition 8.0 mm), but less active against E. coli (Arbab et al., 2022; Zhang et al., 2018). The results of this study show that the two antibiotics, peppermint oil and lemon balm, have an antibacterial effect against S. aureus ‘high priority’, which is the causative agent of subclinical mastitis and for the production of new herbal mastitis drugs alternative to antibiotics in the intramammary application. It shows that it will form the basis of further research in veterinary medicine in vitro as well as in vivo. Current research will also provide scientific validation of traditional knowledge and increase local farmers’ confidence in ethno veterinary practice.

5 | CONCLUSION

In this study, considering the significant antibacterial effects of plant extracts, the separation of the ethanol extracts of squaw mint (Mentha pulegium L., Lamiaceae family), lemon balm (Melissa officinalis L., Lamiaceae), catnip (Nepeta cataria L., Lamiaceae) and essential oil, in comparison with various antibiotic discs, it can be concluded that the peppermint oil and lemon balm of this plant have an antimicrobial activity better than some antibiotics and can be used combined with other preservatives as a natural herbal drug in the treatment of mastitis. Therefore, it is suggested that further research is needed to evaluate the effects of the essential oil and herbal plant extract as a natural source on animal models for controlling diseases and also in the food industry as antimicrobial inhibitors and flavours.

AUTHOR CONTRIBUTIONS

Safia Arbab: Conceptualisation, formal analysis, manuscript writing. Hanif Ullah: Writing – review and editing. Jiyu Zhang: Funding acquisition, investigation, project administration, supervision. Iqra Bano, Ka Li, Weiwei Wang, Inam Ul Hassan and Abdul Qadeer: Review and editing.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ETHICAL STATEMENT

All animal experiment was conducted according to the guidelines for animal use in toxicology Lab. All experiments were approved by the Animal Administration and Ethics Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences. The certificate number was SCXX (Gan) 2019–001.

DATA AVAILABILITY STATEMENT

All the relevant data are available within the manuscript.

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PEER REVIEW

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