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Antibacterial Effects of Ethanol Extract of Aloe vera and Ceftiofur Against *Streptococcus dysgalactiae* and *Streptococcus uberis* Isolated from Mastitic Milk of Buffalo

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Abstract: *Aloe vera is often referred to as a "miracle plant" due to its extensive range of antibacterial, antifungal, antiviral, and antiparasitic properties. This study investigated the antibacterial activity of pure Aloe vera and its ethanol extract and compared it with Ceftiofur. A total of 50 clinically positive mastitic milk samples from buffaloes were collected from dairy farms. These samples underwent microbial culture analysis. Following the isolation and*

identification of organisms, the minimum inhibitory concentration (MIC) was determined using 96-well plates. Concentrations of pure Aloe vera, its ethanol extract, and Ceftiofur were used: 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 µg/µl, and 0.009 µg/µl to evaluate their MICs against Streptococcus dysgalactiae and Streptococcus uberis. Results indicated that 27 (54%) samples were positive for Streptococcus dysgalactiae, while 5 (10%) were positive for Streptococcus uberis. The mean susceptibility values were 3.75 µg/µl for pure Aloe vera, 1.87 µg/µl for the ethanol extract, and 0.19 µg/µl for Ceftiofur against Streptococcus dysgalactiae. For Streptococcus uberis, MICs were 7.5 µg/µl for pure Aloe vera, 3.75 µg/µl for ethanol extract, and 0.4 µg/µl for Ceftiofur. All treatments displayed susceptibility to these organisms, suggesting Aloe vera as a potential alternative to combat antibiotic resistance.

Keywords: *Aloe vera; Antibacterial effect; Ceftiofur; Ethanol Extract; Streptococcus dysgalactiae*

Introduction

Mastitis is defined as inflammation of the udder, characterized by chemical and physical alterations in the milk, the presence of bacteria, and pathological changes in the mammary gland tissue (Reshi et al., 2015). Key indicators of mastitis include milk discoloration, clot formation, and elevated leukocyte counts. This condition is categorized into clinical and subclinical forms (Hussain et al., 2012). Both forms contribute to economic losses, including reduced milk yield, increased veterinary expenses, culling of affected animals, and heightened labor costs (Hossain et al., 2017). The complex etiology of mastitis involves various pathogens, primarily bacteria, but also includes fungal and algal organisms. Notable bacterial pathogens linked to mastitis include *Streptococcus dysgalactiae*, *Staphylococcus aureus*, *E. coli*, *Klebsiella pneumoniae*, *Streptococcus agalactiae*, *Corynebacterium pyogenes*, *Streptococcus parauberis*, and *Streptococcus uberis* (Dharajiya et al., 2012). While antibiotic therapy can mitigate economic losses associated with mastitis, extended use may lead to multidrug resistance among pathogens, raising health concerns for humans (Nazir et al., 2021). Furthermore, the high cost of synthetic medications and their ineffectiveness against certain bacterial diseases have sparked interest in alternative treatments, particularly those derived from plant sources, which are often more accessible and affordable (Muhammad et al., 2024).

Plant-derived active ingredients have demonstrated efficacy in treating a broad range of diseases affecting both humans and livestock. Among these, Aloe vera has garnered attention for its therapeutic properties (Radha & Laxmipriyia, 2015).

Aloe vera, belonging to the Liliaceae family, is one of the oldest medicinal plants, native to North Africa, with over 400 species identified worldwide. Characterized by its thick, fleshy leaves that store water in gel form, Aloe vera possesses numerous therapeutic properties, including antimicrobial, anti-inflammatory, antifungal, and antioxidant activities (Shrestha et al., 2015). The latex derived from Aloe vera is rich in anthraquinones and phenolic compounds, contributing to its laxative and antibacterial effects. Additionally, the plant's leaves contain a variety of beneficial compounds, including amino acids, vitamins, minerals, and enzymes (Abro et al., 2024). Research has shown that Aloe vera exhibits antibacterial activity against a range of bacteria, including *E. coli*, *Proteus vulgaris*, *Enterococcus bovis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae* (Keerio et al., 2024). Crude Aloe vera extracts have also demonstrated antimicrobial activity against both fungal and bacterial pathogens, including *Aspergillus niger*, *Candida albicans*, and *Staphylococcus aureus* (Begum et al., 2016). Furthermore, studies indicate that both ethanol and aqueous extracts of Aloe vera possess antimicrobial effects, with the ethanol extract exhibiting lower minimum inhibitory concentrations compared to other extracts (Karpagam & Devaraj, 2011). The ethanol extract has shown greater activity against clinical isolates of common mastitis pathogens, such as *Staphylococcus aureus* and *E. coli* (Soomro et al., 2022).

Ceftiofur, a broad-spectrum semi-synthetic antibiotic, is particularly favored for its safety, cost-effectiveness, and broad spectrum of antimicrobial activity (Lin et al., 2021). As a beta-lactam antibiotic, ceftiofur inhibits bacterial cell wall synthesis, specifically targeting peptidoglycan (Hussan et al., 2016). Studies have shown that ceftiofur can effectively treat clinical mastitis caused by pathogens such as *E. coli*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and various *Staphylococci* when administered intramammary (Ubaidullah et al., 2021).

While studies have examined the antibacterial properties of Aloe vera, there is limited research specifically on its effectiveness against mastitis in buffaloes. Considering the

economic impact of mastitis in buffalo populations, this study seeks to assess the antibacterial effects of pure Aloe vera and its ethanol extract in comparison to Ceftiofur against gram-positive bacteria linked to mastitis in buffaloes. Therefore, this research is designed to evaluate the antibacterial activities of pure Aloe vera, its ethanol extract, and Ceftiofur against mastitic organisms isolated from the milk of affected buffalo. Additionally, it aims to compare the efficacy of these treatments in addressing mastitic pathogens and to identify an alternative therapy for drug-resistant infections.

Materials and methods

1. Sampling, isolation, and identification of bacteria

A total of 50 mastitic milk samples from buffaloes were collected from dairy farms in the Tandojam area and transported to the postgraduate research laboratory of the Department of Veterinary Pharmacology, Sindh Agriculture University, Tandojam. The milk samples were initially cultured on nutrient agar for primary isolation. Subsequent purification of the isolates involved subculturing onto both nutrient agar and blood agar, which were then incubated at 37°C for 24 hours. Further purification continued with additional subculturing onto blood agar medium. Following this, pure bacterial colonies were aseptically transferred to nutrient agar slants, which were incubated for 24 hours at 37°C and subsequently stored in a refrigerator at 4°C for future use.

For the identification of *Streptococcus dysgalactiae*, samples were subcultured onto blood agar. The petri dishes were examined for colonies exhibiting a greenish hue, and further confirmation was obtained through Gram staining and biochemical testing of the isolates. Similarly, for the detection of *Streptococcus, translucent*, circular colonies with smooth edges and entire margins, surrounded by a zone of β-hemolysis, were noted, with final confirmation also achieved through Gram staining and biochemical reactions of the isolates (Arain et al., 2024).

2. Gram staining procedure

Smears were prepared from isolated colonies of pure culture and subjected to Gram staining, with the organisms classified as either Gram-negative or Gram-positive based on their staining characteristics.

3. Biochemical tests

Biochemical tests, including Catalase, Oxidase, and Coagulase, further validated the identification of bacterial organisms.

4. Extraction of gel from Aloe vera leaves

Aloe vera leaves were sourced from a local nursery in Tandojam. The leaves were disinfected with 70% alcohol, then cut open with a sterile knife to extract the gel. The gel was then blended to achieve a uniform consistency, filtered through muslin cloth, and sterilized in an autoclave at 121°C and 15 lbs of pressure for 15 minutes. The sterilized filtrate was prepared as a stock solution at 100% concentration, from which the following dilutions were made: 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019, and 0.009 µl using a micropipette.

5. Ethanol extraction of Aloe vera gel

To prepare the ethanol extract, the Aloe vera gel was air-dried in an oven at 100°C for 48 hours. The dried gel was then ground into a powder using a mortar and pestle. 10 g of Aloe vera powder was immersed in 100 ml of ethanol for 24 hours. After soaking, the mixture was filtered through Whatman No. 1 filter paper, and the filtrate was evaporated to dryness. The resulting dried extract was ground further into a powder, dissolved in 10 ml of distilled water, and then sterilized. The final solution was stored at 4°C as a stock solution.

6. Preparation for antibiotic solution

A Ceftiofur stock solution was prepared by dissolving 15 mg of Ceftiofur in 15 ml of distilled water. The solution was thoroughly mixed and then autoclaved at 121°C for 15 minutes at 15 lbs of pressure. Following autoclaving, the solution was stored at -4°C under refrigeration until further analysis. The following dilutions from the stock solution were prepared using a micropipette: 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019, and 0.009 µl (Ubaidullah et al., 2021).

7. Antibacterial susceptibility test

Aloe vera, its ethanol extract, and Ceftiofur were used to evaluate antibiotic susceptibility**.** The antibiotic susceptibility test was performed. 1:1000 dilutions were prepared for MIC. For this, 6 µl of bacterial culture in Tryptic Soy Broth (TSB) was added to 6 ml of Muller-Hinton (MH) medium. 96-well plates were used to determine the minimum inhibitory concentration of Aloe vera, its ethanol extract, and Ceftiofur against *Streptococcus uberis* and *Streptococcus dysagalactiae*. 100 µl of the concentration was added to all wells, then 90 µl of culture was added to the 1st and 2nd wells, and 10 µl of Aloe vera was added to the 2nd well. Then, 100 µl

of 2% Aloe vera was taken from the 2nd well and added to the 3rd well of the microtiter plate. For Ceftiofur, a concentration of 15.69mg/15ml (stock solution) was used against the isolates. Afterward, the same MIC procedure was followed for the ethanol extract and Ceftiofur. The MIC plates were incubated at 37^0C °C overnight. After that, the breakpoints were recorded as turbidity in cultured wells to assess the minimum inhibitory activity of Aloe vera, its ethanol extract, and Ceftiofur.

8. Statistical Analysis

The collected data, presented in percentages, were systematically tabulated and analyzed using a one-way ANOVA statistical package to assess the differences among group means and determine whether these differences were statistically significant. The analysis aimed to evaluate the effect of the independent variable on the dependent variable, with a significance level set at $\alpha = 0.05$.

Results

The current study aimed to compare the antibacterial effects of pure Aloe vera, its ethanol extract, and Ceftiofur against *Streptococcus dysgalactiae* and *Streptococcus uberis* isolated from mastitic milk of buffaloes.

1. Prevalence of isolated pathogens

A total of 50 mastitic milk samples from buffaloes were collected and examined. Out of these samples, 27 (54%) were positive for *Streptococcus dysgalactiae*, 5 (10%) were positive for *Streptococcus uberis*, and 18 (36%) showed mixed colonies (Table 1). The mastitic pathogens were identified based on their morphological, cultural, and staining characteristics, and were further confirmed through biochemical tests.

Table 1: Prevalence percentages of organisms isolated from mastitic buffalo milk

Bacterial organisms	Total no of samples	No. of positive samples	Percentage (%)
Streptococcus dysgalactiae		27	54%
Streptococcus uberis	50	05	10%
Mixed Colonies		18	36%

2. Susceptibility of *Streptococcus dysgalactiae*

The susceptibility of *Streptococcus dysgalactiae* was assessed using various concentrations of pure Aloe vera, its ethanol extract, and Ceftiofur: 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019, and 0.009 µg/µl. The mean susceptibility values for *Streptococcus dysgalactiae* were 3.75 µg/µl for pure Aloe vera, 1.87 µg/µl for the ethanol extract, and 0.19 µg/µl for Ceftiofur (Figure 1). The highest and lowest concentrations at which growth inhibition of *Streptococcus dysgalactiae* was observed were 5, 2.5, 0.31 µg/µl for pure Aloe vera, 2.5, 1.25, 0.15 µg/µl for its ethanol extract, and Ceftiofur, respectively (Figure 1). The data revealed significant differences ($P<0.05$) among pure Aloe vera, its ethanol extract, and Ceftiofur against *Streptococcus dysgalactiae*.

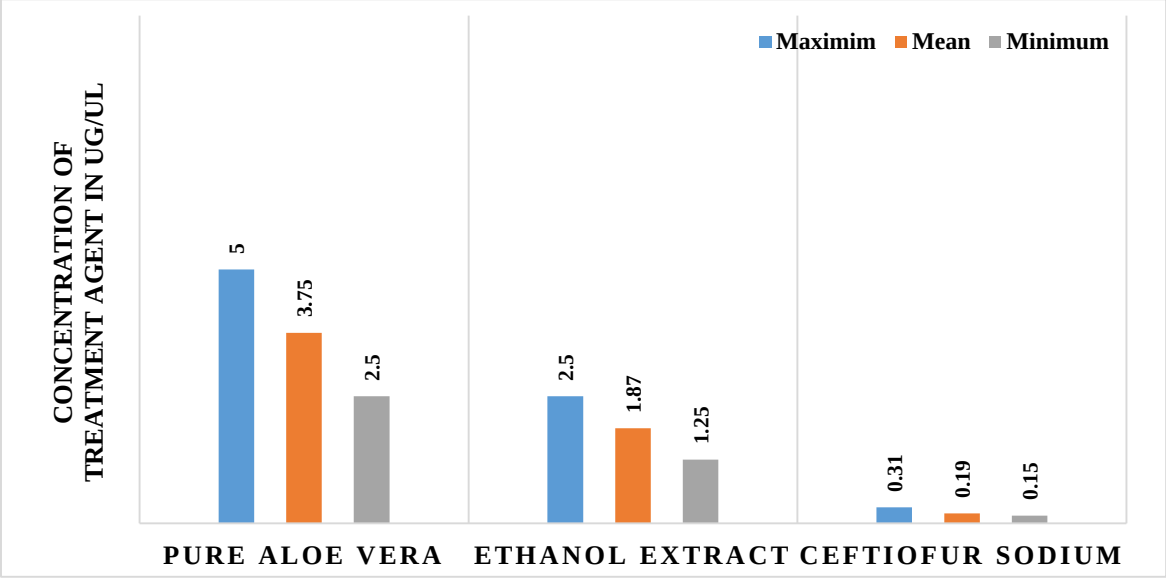

Figure 1: Inhibition of growth in *Streptococcus dysgalactiae* by pure Aloe vera, its ethanol extract, and Ceftiofur.

3. Susceptibility of *Streptococcus uberis*

Similarly, various concentrations of pure Aloe vera, its ethanol extract, and Ceftiofur were used to observe the susceptibility of *Streptococcus uberis*. The mean susceptibility values recorded were 7.5 µg/µl for pure Aloe vera, 3.75 µg/µl for the ethanol extract, and 0.4 µg/µl for Ceftiofur (Figure 2). The highest and lowest concentrations that inhibited the growth of *Streptococcus uberis* were 10, 5, and 0.62 µg/µl for pure Aloe vera, and 5, 2.5, and 0.31 µg/µl for its ethanol extract and Ceftiofur, respectively (Figure 2). These findings also showed

a significant difference ($P < 0.05$) among pure Aloe vera, its ethanol extract, and Ceftiofur against *Streptococcus uberis*.

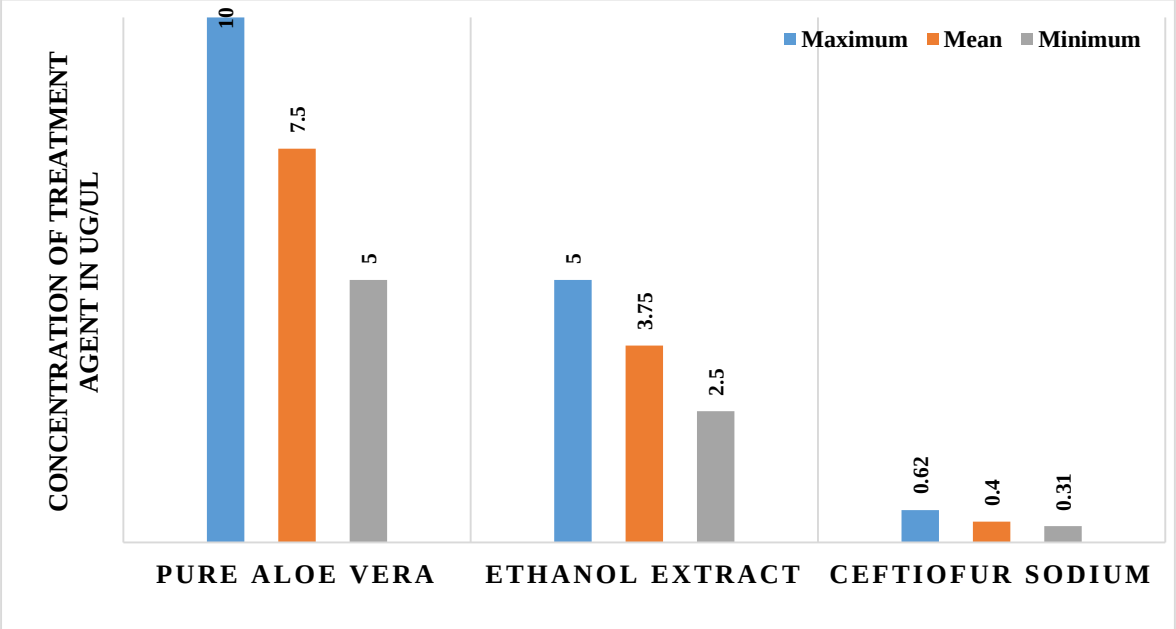

Figure 2: Inhibition of growth in *Streptococcus uberis* by pure Aloe vera, its ethanol extract, and Ceftiofur

4. Comparison of susceptibility among isolated organisms

The various concentrations of pure Aloe vera, its ethanol extract, and Ceftiofur were employed to examine the susceptibility of both *Streptococcus dysgalactiae* and *Streptococcus uberis*. The mean susceptibility values were 3.75, 1.87, and 0.19 µg/µl for *Streptococcus dysgalactiae*, and 7.5, 3.75, and 0.4 µg/µl for *Streptococcus uberis* with respect to pure Aloe vera, its ethanol extract, and Ceftiofur, respectively (Figure 3). The highest and lowest concentrations inhibiting the growth of *Streptococcus dysgalactiae* were found to be 5, 2.5, 0.31 µg/µl for pure Aloe vera, and 2.5, 1.25, 0.15 µg/µl for its ethanol extract and Ceftiofur, respectively. For *Streptococcus uberis*, the highest and lowest concentrations were 10, 5, 0.62 µg/µl for pure Aloe vera and 5, 2.5, 0.31 µg/µl for its ethanol extract and Ceftiofur, respectively (Figure 3). The results indicated significant differences ($P<0.05$) among pure Aloe vera, its ethanol extract, and Ceftiofur against both *Streptococcus dysgalactiae* and *Streptococcus uberis* isolated from mastitic milk of buffaloes.

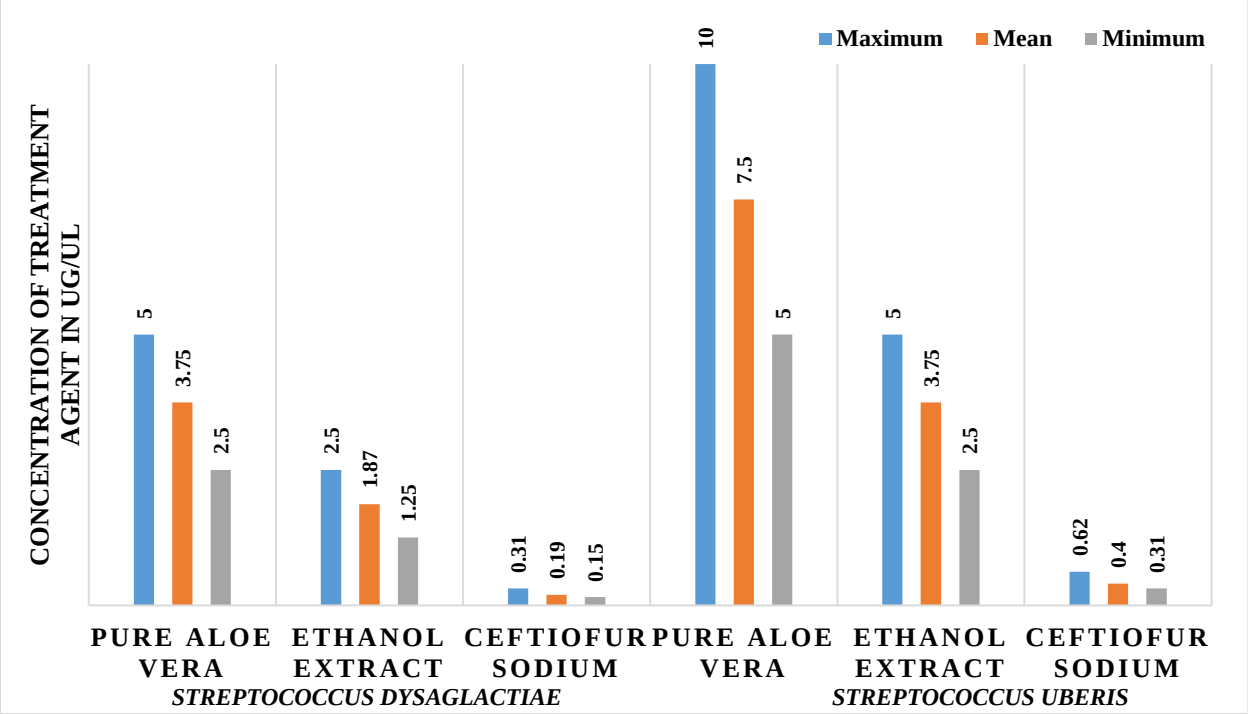

Figure 3: Comparative analysis of growth inhibition in response to pure Aloe vera, its ethanol extract, and Ceftiofur.

Discussion

The results of the present study align with previous findings that mastitis is caused by several organisms, including *Streptococcus uberis*, *Streptococcus dysgalactiae*, *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *Streptococcus agalactiae*, and *Streptococcus parauberis*. These bacteria are responsible for both clinical and subclinical mastitis (Soomro et al., 2022). The findings of the current study also conform to earlier research indicating that among environmental pathogens, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *E. coli*, and *Klebsiella pneumoniae* are responsible for clinical mastitis (Bradley et al., 2007). Additionally, this study corroborates previous findings that Staphylococcus spp. was the most prevalent organism, followed by *Streptococcus* spp., with percentages of 53.32% and 18.17%, respectively (Gomes & Henriques, 2016). Furthermore, *Streptococcus uberis* has been reported as a leading pathogen in an increasing number of dairy herds, causing both clinical and subclinical mastitis, which can be transmitted under unhygienic conditions (Kromker et al., 2014). It may also spread during milking or between milking sessions via milkers' hands and towels, which demonstrate poor milking hygiene.

The present study aligns with previous research indicating that a wide variety of pathogens cause mastitis; however, in Sweden, *Streptococcus uberis* and *Streptococcus dysgalactiae* rank as the third- and fourth-most common pathogens associated with clinical mastitis, accounting for 15.6% and 11.1%, respectively (Liaqat et al., 2016). Given the poor hygienic conditions in Pakistan and the inadequate implementation of strict hygiene measures, these organisms may gain entry via soil, handlers' hands, or other sources to the teat/udder, becoming primary agents responsible for mastitis.

In the current study, various concentrations of pure Aloe vera and its ethanol extract were used: 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019, and 0.009 µg/µl. The current study is consistent with previous research indicating that the growth of *Streptococcus* spp. and *Shigella* spp. was inhibited in media containing pure Aloe vera extract (Asa et al., 2014). Additionally, the findings of the current study are supported by earlier research identifying that the polysaccharides in Aloe vera gel possess direct antimicrobial activity by stimulating phagocytic leukocytes to produce a cidal effect against bacterial organisms (Chatterjee et al., 2015). This study also aligns with prior findings that the antimicrobial agent trans-cinnamaldehyde, derived from plant extract, exhibits antibacterial effects against mastitis-causing pathogens such as *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *S. aureus*, and *E. coli*, with MIC and MBC values of 0.1 and 0.45%, respectively, for *S. aureus*, *E. coli*, and *Streptococcus uberis*, and 0.5 and 0.4% for *Streptococcus agalactiae* and *Streptococcus dysgalactiae* (Lin et al., 2021).

It has also been reported that the antibacterial activity exhibited by Aloe vera and its ethanol extract is due to various active antibacterial chemical agents present in the extract. Aloe vera contains multiple biologically active ingredients, including carboxypeptidase, emodin, salicylate, magnesium lactate, anthrone, C-glucosyl chromone, dithranol, allantoin, and chrysarobin, as well as various enzymes and organic compounds (Pankaj et al., 2013). Furthermore, anthraquinone, an active ingredient in Aloe vera, exhibits antibacterial activity against various bacterial species. It functions similarly to tetracycline antibiotics by inhibiting bacterial protein synthesis. Specifically, it blocks the bacterial ribosomal unit at the acceptor site, preventing the transferase enzyme from acting on the peptide site; thus, its mode of action resembles that of tetracyclines (where aminoacylated tRNA enters). Consequently, bacterial organisms cannot grow in media containing Aloe vera extract (Zeb, 2012).

The current study corroborates previous findings indicating that the ethanol extract of Aloe vera outperformed methanol and aqueous preparations, demonstrating superior antibacterial activity against both Gram-positive and Gram-negative pathogens, including *Streptococcus uberis*, *Streptococcus dysgalactiae*, *E. coli*, *S. aureus*, *Candida albicans*, and *Klebsiella* spp. The results recorded for ethanol against these pathogens indicated zones of inhibition of 6, 5, and 4 mm using the agar well diffusion method (Mbajiuka et al., 2014).

Moreover, additional studies have shown that methanol and ethanol extracts of Aloe vera exhibited remarkable antibacterial activity compared to acetone extracts (Jothi et al., 2014). Similarly, results from previous research revealed that all Aloe vera extracts in various preparations, including hexane, petroleum ether, and ethanol, demonstrated antibacterial activity against *S. aureus*, *S. epidermidis*, *S. mutans*, *S. pyogenes*, *S. dysgalactiae*, *S. pneumoniae*, *M. luteus*, *Bacillus cereus*, *Bacillus subtilis*, and *Pseudomonas* (Alajaji & Alumzaini, 2025). It has been stated that Aloe vera contains anthraquinones, which are organic aromatic compounds. This compound is poorly soluble in water but readily soluble in organic solvents (Vogel, 2018). Therefore, it is likely that the antibacterial active ingredients in Aloe vera, including anthraquinones, were more effectively extracted with ethanol compared to aqueous extraction. Consequently, the concentration of anthraquinones in the ethanol extract may be higher than in the crude Aloe vera extract, resulting in a lower MIC for the ethanol extract.

In the current study, various concentrations of ceftiofur were used to examine the susceptibility of *Staphylococcus dysgalactiae* and *Streptococcus uberis*. The present results are consistent with previous findings that report ceftiofur's effectiveness against a wide range of contagious and environmental mastitic pathogens, including *Streptococcus uberis*, *Staphylococcus simulans*, *Staphylococcus aureus*, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, *E. coli*, *Streptococcus bovis*, and *Klebsiella* spp. (Dolhan et al., 2014). The findings of the present study are further supported by previous research indicating that ceftiofur, as a third-generation cephalosporin, is more resistant to β-lactamases produced by various bacteria than first-generation cephalosporins and penicillin antibiotics. Thus, it is more effective against various bacterial pathogens in the treatment of clinical mastitis (Schukken et al., 2013). Additionally, the present study corroborates previous research that reported

Streptococcus uberis has 100% sensitivity to ceftiofur (Idriss et al., 2014). Furthermore, it has been reported that intramammary therapy with ceftiofur remains effective for eliminating *Streptococcus uberis* in early lactating dairy cows (Lopez et al., 2013). The present study also aligns with previous studies demonstrating ceftiofur's effectiveness against several bacterial pathogens in vitro, including *Pasteurella multocida*, which showed high sensitivity to ceftiofur, with an MIC range of 0.625-2.5 µg/ml (Khalid, 2013).

In the current study, both isolated organisms were Gram-positive, with thick cell walls comprising approximately 50 to 100 layers and containing 80% peptidoglycan. Ceftiofur belongs to the cephalosporin group of antibiotics and works by blocking cell wall synthesis in growing bacteria. It acts on the peptidoglycan chain by inhibiting the transpeptidase enzyme responsible for the final stages of cell wall synthesis, resulting in immature peptidoglycan and cell death. Hence, this antibiotic is classified as bactericidal.

Conclusions

This study assessed the antibacterial efficacy of Aloe vera, its ethanol extract, and Ceftiofur against mastitic buffalo milk samples. The findings indicate that *Streptococcus dysgalactiae* is more prevalent than *Streptococcus uberis* in buffalo mastitis cases. Both pure Aloe vera and its ethanol extract shows promising antibacterial activity against these pathogens; however, Ceftiofur was more effective overall. Future research should focus on exploring different Aloe vera extracts and their active compounds, and on comparing them with those of other medicinal plants. Additionally, in vivo studies are warranted to evaluate the effectiveness of these treatments in clinical cases of mastitis.

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Author Contributions

Faisal Khan (conceptualization, design, investigation, writing), Shamsuddin Bughio and Rehana Buriro (conceptualization, supervision and co-supervision), Muhammad Bilawal (writing manuscript), Zainab Lanjar, Fayaz Ali Lighari, Muhammad Mubashir Farooq

(investigation, analysis), Arfa Gaffar (sample collection). All authors critically reviewed the manuscript and approved the final version.

Conflicts of interest

All other authors declare no conflicts of interest.

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