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Ethnoveterinary Herbal Preparation Modulates Pro-inflammatory Cytokine Expression in Bovine Mastitis

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ABSTRACT

Bovine mastitis is a multi-etiological inflammatory condition of the udder in dairy cattle causing milk yield and economic loss worldwide. Bacteria are the common source of infection causing mastitis. The host response to infection involves release of pro-inflammatory cytokines IL-6, IL-8 and TNF-α. The abundant release of pro-inflammatory cytokines by the somatic cells results in tissue injury. Antibiotics are the common mode of treatment for bovine mastitis which largely target the pathogen but not the tissue microenvironment. The study was conducted to evaluate the effects of *Aloe vera* and *Curcuma longa* based fresh ethno-veterinary herbal formulation against mastitis (EVFM) on the expression of pro-inflammatory cytokines and bacterial load in the milk samples of mastitic cows. Crossbred Jersey / HF cows with clinical signs of mastitis were selected and the EVFM was applied topically on the udder for 5 days (six times per day). The qPCR analysis of cytokine expression in milk somatic cells revealed a significant ($p < 0.05$) reduction in IL-6 and IL-8 post EVFM treatment and a marked decrease in the TNF between day 0 and day 6 post treatment. The total Viable count was restored to normal from 1.3×10^7- 2.0×10^7 CFU/ml (Day 0) to 1.7×10^5-3.5×10^6 CFU/ml (Day 6). Our study provides evidence that EVFM possesses anti-inflammatory and anti-microbial effect in the treatment of clinical cases of mastitis.

HIGHLIGHTS

- Ethnoveterinary herbal formulation reduced mRNA levels of IL-6 and IL-8 in milk somatic cells.
- Ethnoveterinary herbal formulation reduced the total bacterial count in mastitis.

Keywords: Bovine, Mastitis, Ethnoveterinary, Cytokines

Bovine mastitis is an inflammatory response occurring in the mammary gland of dairy animals due to physical damage, chemical irritation or infection (Ruegg *et al*., 2014), leading to production and economic losses worldwide. The financial loss due to sub-clinical Mastitis in India was estimated to be INR 21,677 -INR 88,340 per cow per lactation (Rathod *et al*., 2017) and average drop in milk yield due to mastitis was estimated to be 3-4 kg per cow (Wani *et al*., 2022) . Mastitis is known to be predominately caused by bacterial invasion of the mammary gland, which can result in variety of clinical outcomes, ranging from acute and life-threatening to chronic and subclinical, which affect mammary tissue integrity and reduce the production performance of cows (Cobirka *et al*., 2020). Antibiotics remain an effective method for treating bovine mastitis, but their use is restricted due to growing problems of drug resistance and

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food safety (Krömker and Leimbach, 2017). Antibiotic use is associated with drug residues in milk and acquired drug resistance by the microbes (AMR), resulting in severe public health issues.

Cytokines are immune regulators pivotal in regulating the host's immune response against different infections. Cytokines secreted in response to infection and other cellular stresses trigger cellular activation, differentiation and cell recruitment. Interleukin- 6 (IL-6), Tumour Necrosis Factor (TNF-α) and IL- 8 are important pro-inflammatory cytokines (Kany *et al*., 2019). During intramammary infections, bacteria release toxins that trigger leucocytes and epithelial cells in the mammary gland to release cytokines (Vitenberga *et al*., 2022). The Rapid inflammatory response is characterized by the release of a large number of pro-inflammatory cytokines (IL-8, IL-1beta, IL-6 and TNF-α), which is beneficial for the attraction of circulating immune effector cells such as neutrophils to fight infection (Breyne *et al*., 2018). However, it was observed that an excessive inflammatory response cause injury to tissue and cells (Chen *et al*., 2018). Hence, the expression of pro-inflammatory cytokines needs to be regulated during an inflammatory response.

The use of natural products for the treatment of mastitis has gained a lot of interest. The search for natural substances and their active ingredients to prevent bovine mastitis have been conducted and reported, such as thymol isolated from time oregano tangerine peel (Wei *et al*., 2014) and curcumin from turmeric (Suresh *et al*., 2018). *Evernia prunastri*, *Artemisia absinthium*, and *Lavandula angustifolia* plant-derived products showed potent anti-microbial against 32 microorganisms isolated from milk (Pasca *et al*., 2017). Using mouse mastitis models, plant-derived metabolite curcumin was reported to exert anti-inflammatory effects in LPS-induced mastitis (Fu *et al*., 2014). The topical herbal gel has reduced bacterial load and cytokine expression in subclinical mastitis (Bhatt *et al*., 2014). Moringa extracts exerted anti-inflammatory effects in bovine mammary epithelial cells by attenuating the expression of COX-2 and deactivating NF kB upregulation of pro-inflammatory cytokines (Cheng *et al*., 2019). With this background, the present study aims to evaluate *Aloe vera* and *Curcuma longa* based fresh preparation of herbal formulation against mastitis for its effect on pro-inflammatory cytokine expression and bacterial load in milk samples of mastitic dairy animals.

MATERIALS AND METHODS

Animal selection, treatment and milk sample collection

Crossbred cows (Jersey/ Holstein Friesian) showing clinical signs of mastitis brought for treatment to the veterinary dispensary in Thanjavur district, Tamil Nadu, were chosen for the study. Clinical examination of the individual quarter of the affected animal was conducted with respect to the shape, size and consistency of the udder. Milk from each quarter was checked for gross abnormalities in the milk. Based on these examinations, animals having clinical mastitis were selected for the present investigation. About 250 g of *Aloe Vera* leaves, 100 g of turmeric powder (*Curcuma longa*) and 10 g of calcium hydroxide (Lime) were mixed and blended in an electric blender (quantity enough for six applications). All quarters of the udder in the affected animals were completely milked and washed with clean water, and about 50 g of the freshly prepared paste was diluted in 100 ml of clean water and applied externally over the affected udder with gentle pressure using hands. The process was repeated every one hour and continued six times per day. Milk samples (25-50 ml) were collected aseptically from the affected udder quarters at Day 0 (Before Treatment) and on Day 6 post-treatment to separate somatic cells to study cytokine expression. Milk samples meant for cytokine expression study were maintained in ice-cold conditions. Milk samples for microbiological studies were collected aseptically in sterile containers under ice-cold conditions and immediately processed.

Isolation of milk somatic cells and RNA extraction

Milk samples (25-50 ml) were diluted with an equal volume of ice-cold sterile DEPC treated 0.5 mM PBS-EDTA solution and centrifuged at 3000 rpm for 20 min at 4°C. The fat layer and supernatant were discarded the cell pellet was washed twice with ice-cold sterile PBS-EDTA by centrifugation at 3000 rpm for 20 min at 4°C. The cell pellet was transferred to fresh tube, further washed with PBS-EDTA, and processed for RNA isolation/ stored in RNA later solution at -20°C. Total RNA was extracted using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's protocol. RNA was eluted in 30 µl of RNase-free water and stored at -80°C until use. The purity of the extracted RNA was checked using

Denovix DS-II FX+ (D. A. I Scientific Equipment, USA) microspectrophotometer. The RNA samples with 260/280 ratio > 1.8 were considered as pure and used for further analysis.

Reverse transcription and qPCR

The total cDNA was synthesized using 400 ng of RNA in a 20 µl reaction using Quantitect RT kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The reaction was performed in an Insta Q96 (Himeda laboratories) qPCR Machine. Initially, genomic DNA wipeout buffer was added to the RNA sample, incubated at 42° C for 2 min, and then transferred to ice. Reverse transcription mix was added to the RNA template and incubated for 15 min at 42° C followed by incubation at 95° C for 3 min to inactivate the RT enzyme. RT reaction product (cDNA) was stored at -20° C until further use. Quantitative realtime PCR was carried out to study cytokine expression in the milk somatic cells using Quantitect SYBR Green PCR Kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's protocol. The primer sequences used for real-time PCR analysis are given in the Table 1.

The reaction was performed in a total volume of 50 µl in triplicate. Primers were used at a concentration of 0.25 µM in the final reaction volume. Primers were designed such that all of them have same annealing temperature. The real-time PCR programme consisted of an initial denaturation for 5 min at 95°C, and each of the 40 cycles included denaturation at 95°C for 10 s and annealing + extension at 60°C for 30 s. The melting curve was generated to check primer dimers and non-specific products using default settings in the qPCR machine. The relative expression level of each gene (IL-6, IL-8, TNF-α) was compared with GAPDH and measured with threshold cycle (Ct) values by using the comparative method of $2^{-\Delta\Delta CT}$. Experiments were conducted to verify the amplification of target genes (IL6, IL8, and TNF-α) and the reference genes (GAPDH) in the calibrator (control milk sample) and sample (Mastitic milk sample).

Microbiological analysis and Somatic Cell Count (SCC)

Total viable count in the milk

The aseptically collected milk samples were serially diluted and spread onto the tryptic soy agar (TSA) plates. The total colonies on the agar plate were counted after 24-48 h of incubation at 37°C. The number of colonies conunted was multiplied by the dilution factor, converted to the colony-forming unit and stated as the total viable count (TVC).

Somatic Cell count

Somatic Cell content in milk samples was evaluated using colour based TANUCHEK SCC kit (TANUVAS) as per the protocol advised in the kit (Fig. 1).

STATISTICAL ANALYSIS

All the results were analyzed by using Graph Pad Prism

Table 1: The primer sequences used in real-time PCR analysis

Sl. No.	Genes	Primer sequences	Accession Number
1	IL-6	F:TCATTAAGCGCATGGTCGACAAA	NM_173923
		R:TCAGCTTATTTTCTGCCAGTGTCT	
2	IL-8	F:CACTGTGAAAATTCAGAAATCATTGTTA	NM_173925
		R:CTTCACAAATACCTGCACAACCTTC	
3	TNF-α	F:TCTTCTCAAGCCTCAAGTAACAAGC	AF_011926
		R:CCATGAGGGCATTGGCATAC	
4	GAPDH	F:ATTGACCTTCACTACATGGTCTAC	AJ_00130
		R:TCCATTGATGACGAGCTTCC	

10 (Graph Pad Software, San Diego, CA, USA). The comparisons between the groups were made using one-way ANOVA. Bars in the figure indicate standard error (SE).

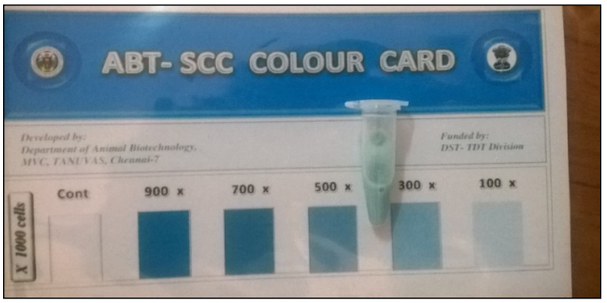

Fig. 1: Colour code reference to determine SCC (TANUCHEK SCC Kit)

RESULTS AND DISCUSSION

Expression of Milk Somatic cells Pro-inflammatory Cytokines reduced after EVFM treatment in bovine mastitis

Crossbred lactating dairy cows aged between 3.5 to 5 years (from I to IV lactation) having average milk yield of 8 litres/cow/day, exhibiting visible signs of mastitis viz., gross abnormalities in milk and shape, size and consistency of the udder were selected for this study (Fig. 2).

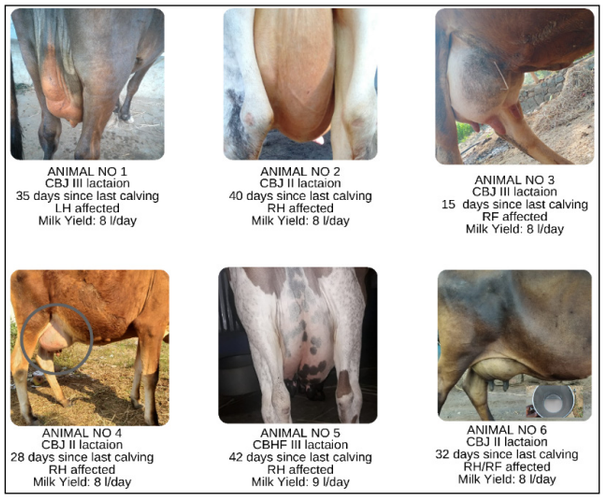

Fig. 2: Photographs of the affected animals selected for the study

The chosen animals were positive for at least a single quarter infection with clinical mastitis but without any systemic involvement.

Freshly prepared EVFM (Fig. 3) was applied over the affected udder six times per day for 5 days. The udder was completely washed with clean water and milk from all the four quarters was removed before every application of EVFM. The affected cows were also fed with two lemon fruits thrice daily for five days. The method of EVFM application was demonstrated to the farmers (Fig. 4) and they were advised to strictly adhere to the timings and frequency of application.

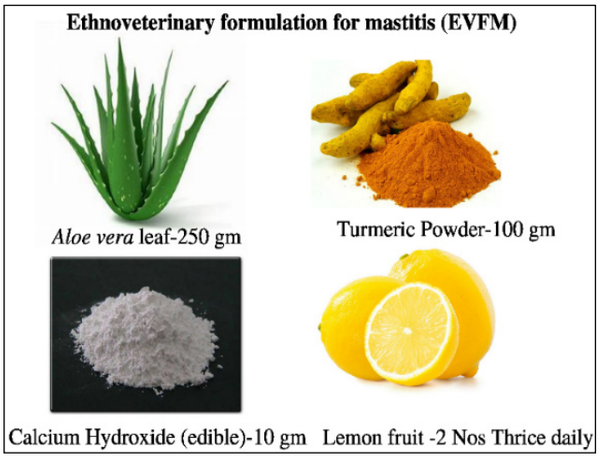

Fig. 3: Ingredients used in EVFM

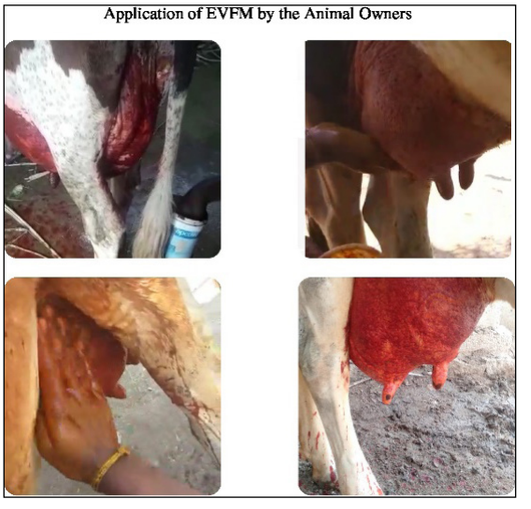

Fig. 4: EVFM application over the udder under field conditions

Out of the six animals, three animals (01, 05 and 06) recovered after 5 days of EVFM application, whereas the remaining three animals (02, 03 and 04) were provided additional three days of treatment for recovery. The curative effect was assessed based on morphological recovery and somatic cell count determined by colour based TANUCHEK SCC kit (TANUVAS) (Fig. 5).

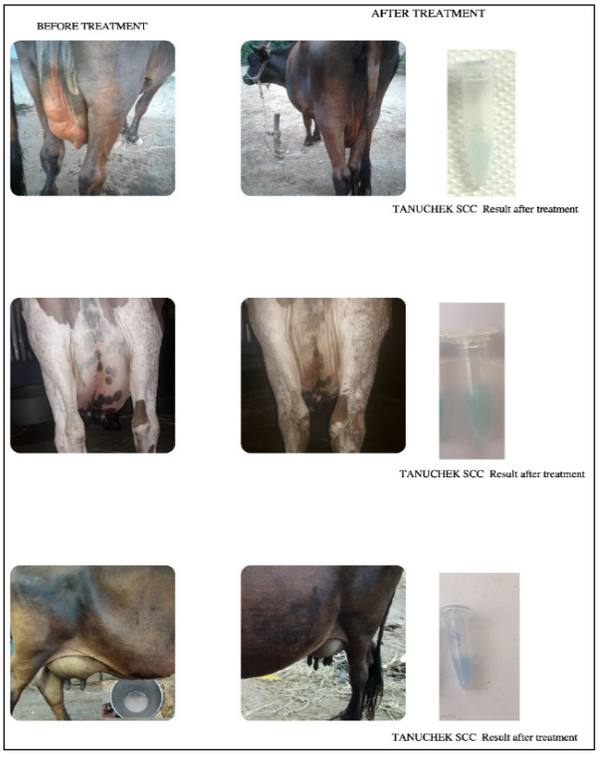

Fig. 5: Representative images of the affected animals before and after EVFM treatment

Pro-inflammatory cytokines are the indicators of inflammation exhibited as host immune response. We investigated, whether EVFM had any effect on the mRNA expression levels of IL6, IL8 and TNF α in milk somatic cells of the affected animals at Day 0 and Day 6 of treatment. The results of the experiment were shown in Fig. 6.

Expression of the pro-inflammatory cytokines IL-6, IL-8 and TNF- α mRNA levels was found to be significantly ($p<0.05$) higher in the affected animals compared to that of healthy controls (n=3). However, in the EVFM treated animals, IL-6 (Fig. 6 A) and IL-8 (Fig. 6 B) was reduced significantly ($p<0.05$) at day 6 compared to that at day 0, whereas TNF- α mRNA levels (Fig. 6 C) was found be decreased at day 6 compared to that at day 0, but not significant ($p<0.05$). Inflammatory responses of mastitis increases somatic cell counts (SCC) and the level of inflammatory cytokines IL-6, TNF-α and IL-8 in milk (Paduch and Kromker, 2011; Bannerman, 2009).

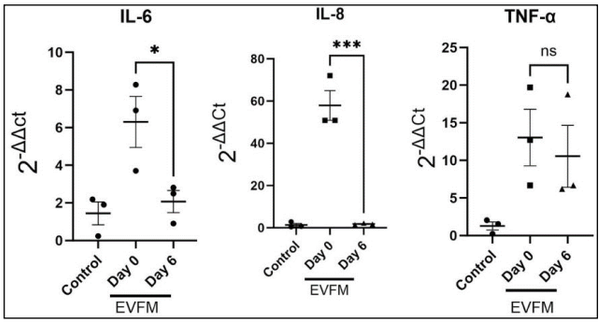

Fig. 6: IL-6, IL-8 and TNF-α expression in Control and EVFM Treated animals. qPCR analysis of IL-6, IL-8 and TNF-α expression in control and EVFM treated animals (Day 0 and Day 6). EVFM down regulates IL-8 expression at Day 6. The expression level of IL-6 normalized to GAPDH levels. The data represent fold change levels relative to control.*$p<0.05$ (n=3)

The affected animals in this study showed elevated levels of IL-6, IL-8 and TNF-α compared to healthy controls. Our results demonstrate that the level of these cytokines were reduced in the EVFM treated animals suggesting that EVFM have anti-inflammatory and immunomodulating activity. IL-8 and TNF-α play key roles in early inflammatory responses (Dinarello *et al*., 2011). The level of IL-6 can be used to assess the extent of tissue injury (Cronin *et al*., 2016). Many studies have reported the active principles in *Aloe vera* and curcumin to have anti-inflammatory and immune modulating roles. Aloin, the active ingredient in *Aloe vera* was reported to exert its anti-inflammatory effect through inhibition of JAK1/STAT1/3 signaling pathways (Ma *et al*., 2018). Aloe vera was shown to down regulate LPS induced inflammatory cytokine production through inhibition of phosphorylation of p 38, JNK and ERK molecules (Budai *et al*., 2013). Using mice mastitis model, Curcumin, the active ingredient in turmeric powder was shown to decrease synthesis of IL-6 and TNF-α in *S. aureus* induced mastitis (Xu *et al*., 2020). Calcium Hydroxide [$Ca(OH)_2$] was reported to have anti-microbial effect through

release of hydroxyl ions in aqueous conditions (Estrela *et al*., 2001). Also, $Ca(OH)_2$ inactivates endotoxins (Vianna *et al*., 2007) and play immunomodulatory role through decreasing TNF-α production in LPS stimulated macrophages (Pinar Karapinar *et al*., 2016). Our results demonstrate that combination of *Aloe vera*, turmeric powder and calcium hydroxide have protective effect against mastitis through reduction of pro-inflammatory cytokine production in milk somatic cells of affected animals. Citrate plays an important role in lactogenesis and udder health maintenance by means of ionic equilibration (Hyvonen *et al*., 2010). We propose through our results, that feeding lemon fruits may have the beneficial effects of citrate feeding for treating mastitis. In this study, we observed that topical application of EVFM and oral feeding of lemon fruits showed a significant reduction in the mRNA levels of IL-6 and IL-8 in milk somatic cells on day 6 post-treatment. Alternative therapy for mastitis using YXT, an intramammary infusion prepared from the extracts of *Angelica dahurica* and *Rheum officinale,* reported a significant reduction in the protein levels of IL-6 and IL-8 after three days of treatment (Yang *et al*., 2019). Another study using extracts of *Prosopis Juliflora* as an intramammary infusion reported a reduction in the mRNA levels of IL-6 and IL-8 28 days post-treatment (Shah *et al*., 2018). Our study differs from the above reports in the route of administration of the herbal formulation. In the present study, we adopted the topical application of the EVFM, which is the easiest method for animal owners to practice. Surprisingly, we could not detect a significant reduction in the TNF- α mRNA levels in this study except for a marked decrease in the mRNA levels. It was reported that IL-6 acts as the anti-inflammatory agent by inhibiting TNF- α (Xing *et al*., 1998). We suggest that the current finding on the TNF- α mRNA levels may partly be due to the absence of inhibitory effects of IL-6, which needs further investigation.

Total Viable count of bacteria reduced to normal after EVFM treatment

Total viable bacteria count was conducted in control animals and animals undergoing EVFM treatment for mastitis at Day 0 and Day 6 post-treatment. In the non-mastitis milk samples from the control animals, the TVC (CFU/ml) ranged from 1.7×10^5- 5.0×10^5, whereas in the mastitis-affected animals at Day 0 of the treatment ranged from 1.3×10^7- 2.0×10^7 CFU/ml. The TVC level significantly reduced at Day 6 post EVFM treatment with CFU/ml ranging from 1.7×10^5-3.5×10^6 (Fig. 7).

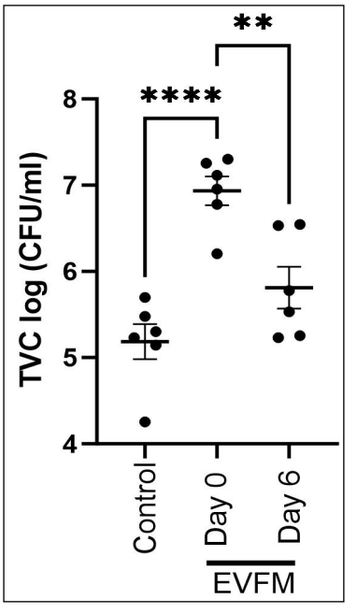

Fig. 7: Total viable count of bacteria (n=6) .**** <0.0001, ** <0.05

In a study using *Angelica dahurica* and *Rhetum officinale* extracts for mastitis treatment, the intramammary infusion of the extracts reduced the total bacterial count (Yang *et al*., 2019). The results from our study suggests that the herbal formulation contain antibacterial activity as described in the earlier reports. The antibacterial activity can be attributed to the compounds present in *Aloe vera*, *Curcuma longa* along with calcium hydroxide. *Aloe vera* gel was found to be 100% active against all gram-negative isolates and 75.3% active against gram positive pathogens (Bashir *et al*., 2011). Irshad *et al*. (2011) found that Aloe vera gel is effective against *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhi*, *Pseudomonas*, *Klebsiella epidermidis*. The effects of *Aloe vera* may be attributed to the active compounds such as Aloin and Aloe emodin which inhibit protein synthesis in the bacterial cells. In a study using mastitis causing organisms, *Aloe vera* gel extract was found to disrupt the cell membrane in 75% of *Staphylococcus aureus*, in 88% of *E. coli*, in 97% of *Streptococcus uberis*, and in 88% of Methicillin -Resistant *Staphylococcus aureus* (MRSA) (Forno-Bell *et al*., 2019).

The antibacterial effects of turmeric (*Curcuma Longa*) was attributed to the presence of phytochemicals, such as tannins, alkaloids, phenols, steroids, flavonoids, phlorotannin, cardiac glycosides, terpenoids, triterpenes and saponin (Oghenejobo *et al*., 2022). Aqueous extract of turmeric had inhibitory effect on *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* (Gul and Bakht, 2015; Pundir *et al*., 2010). Calcium hydroxide is an odourless white powder and act as strong base in contact with aqueous liquids with a pH of about 12.5 -12.8 (Ba-Hattab *et al*., 2016). Calcium hydroxide is commonly used in endodontic treatments as intracanal medicaments wherein it was reported to inhibit 90% of the bacteria (Varshini *et al*., 2019). Calcium hydroxide exerts its antimicrobial activity through hydroxyl ions which show extreme reactivity with biomolecules (Rehman *et al*., 1996). It damages the bacterial cell wall and develops strong alkaline environment which leads to protein denaturation and cell death.

Topical application of EVFM have advantage over the other oral or intramammary routes for the mastitis treatment. Oral route of drug administration demands large amount of herbal preparation depending on the weight of the animal, which will become costly and labor intensive. Intramammary route is technically demanding and may lead to infection.

CONCLUSION

In conclusion, our study demonstrates that a topical application containing *Aloe vera* leaf, turmeric powder, and calcium hydroxide paste diluted in water along with oral feeding of lemon fruits showed a significant reduction in the milk somatic cell mRNA levels of IL-6 and IL-8, at day 6 compared to that on day 0, and significantly reduced the total bacterial count on day 6 compared to that of day 0. Our study provides evidence that EVFM possesses anti-inflammatory and anti-microbial effect in the treatment of clinical cases of mastitis, providing the basis for the development of novel alternate therapy for mastitis.

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