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RESEARCH ARTICLE

An Economical Non-Antibiotic Alternative to Antibiotic Therapy for Subclinical Mastitis in Cows

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A B S T R A C T

The economic importance of mastitis and antibiotic resistance is dictating to search non-antibiotic alternatives for the therapy. Trisodium citrate (TSC) being buffer system of the glandular tissue and, vitamin C (Vit. C), zinc and copper being important ingredients required for functioning of immune system fancy chances for a suitable alternative mastitis therapy. The current study was planned to evaluate therapeutic efficacy and cost effectiveness of these ingredients in subclinical mastitis. For this purpose, 40 sub-clinically mastitis cows were randomly divided into 2 equal groups. Group T1 was treated orally with TSC, Vit. C, $ZnSo_4$ and $CuSo_4$, while group T2 was treated with standard antibiotic therapy. Milk pH significantly ($P<0.05$) differed between the two treatments till day 7th post-initiation of treatment when T1 restored the pH values within normal range earlier than T2. A non-significant ($P>0.05$) difference was observed in milk pH, fat, lactose, proteins, TS, SNFs, somatic cell counts and restoration of milk yield between the two treatments indicting comparable efficacy. A statistically significant ($P<0.05$) difference was observed in serum Cu and Zn levels indicating that the supplementation of Cu and Zn led to higher serum values in animals of T1. The use of non-antibiotic oral formulations as mastitis therapy resulted in a net profit of Rs. 457/animal/day. The oral non-antibiotic antibacterial formulation is a therapeutically and economically suitable alternative to rational antibiotic-based therapy to treat subclinical mastitis in dairy cows.

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INTRODUCTION

Mastitis is one of the leading reasons for using antibiotics in dairy cows (Lago *et al.*, 2011; Birhanu *et al.*, 2017). More than 80% antibiotics were used only for treating or preventing the disease in conventional dairy farming (Pol and Ruegg, 2007). Such injudicious use of antibiotics leads to many disastrous outcomes including antibiotic tainted milk, development of antibiotic resistance in pathogens and human health hazards (Ji *et al.*, 2020; Khan *et al.*, 2020; Shoaib *et al.*, 2020). Moreover, antibiotic tainted milk also affects the production and shelf life of processed milk and milk products (Kromker and Leimbach, 2017). The mastitis therapy based on antibiotic usage is not ideal due to limited success rate, drug residue issues, and the concerns of antibiotic resistance. One of the reasons for lower success rate is multiple etiology of the disease as more than one bacterium can cause the disease. The intracellular nature of *Staphylococcus aureus*, major mastitis causing pathogen, leads to its survival by escaping the effect of antibiotic even if the drug reaches in the udder. This necessitates the each of some suitable alternatives. The use of herbal and non-herbal non-antibiotic products having antibacterial potential has been targeted to find suitable non-antibiotic alternatives.

Many studies have reported that citrate is main constituent of buffer system of the udder and level of citrate decreases in mastitis infected milk. This decrease in milk citrate level leads to increased alkalinity of the udder which favors the growth of bacteria aggravating the condition (Manzoor *et al*., 2020). This justifies the use of TSC as major constituent of non-antibiotic mastitis therapy. A mastitis therapy based on TSC will not only restore milk citrate levels but also change the pH of milk to normal making the milieu unfavorable for bacterial growth and sustainability (Sarfraz *et al*., 2009; Dhillon and Singh, 2013). Moreover, decrease in milk citrate levels in the milk leads to clumping of calcium ions in milk which then appear as flakes in affected milk. This clumping of calcium ions increases the parenchymatic injury of the glandular tissue enhancing the inflammatory process. Thus, it can be assumed that replacing milk citrate contents will not only restore the pH values but will also help in reducing inflammatory process there by sequestrating free calcium (Dhillon and Singh, 2013).

Oxidative stress is major pathway for tissue damage resultant to infection causing mastitis. Vitamin C is an essential requirement of the body with proven antioxidant effects. Dairy animals affected with mastitis (sub-clinical or clinical) show a significant reduction in serum vitamin C (Vit. C) levels (Ranjan *et al*., 2005; Kleczkowski *et al*., 2005). Thus, reduction in the level of this potent antioxidant results in elevation of lipid hydroperoxide in red blood cells (RBCs) of the affected animals. Adirect correlation exists between lower vitamin C level in the serum and severity of clinical disease (Weiss *et al*., 2004). Similarly, Zn and Cu are trace minerals which are cofactors for many enzymes of the body which play important role in combating oxidative stress (Gaafar *et al*., 2010). Based on "anti-oxidant network theory" (Calvani *et al*., 2020) combining these antioxidants with TSC may prove beneficial in treating mastitis. In this scenario, the current study was planned to assess therapeutic efficacy and economic effectiveness of the combined TSC, Vitamin C, $ZnSo_4$ and $CuSo_4$ oral formulation as compared to standard antibiotic mastitis therapy.

MATERIALS AND METHODS

Experimental animals and treatment: Based on SFMT score (Muhammad *et al*, 2010), 40 cows suffering from subclinical mastitis were selected. These cows were divided randomly into two equal groups *viz.*
Treatment 1 (T1) = administered combination of TSC (40g), Vitamin C (10g), $ZnSO_4$ (3g), $CuSO_4$ (1g) administered orally (QD) for seven days and Treatment 2 (T2) = Injected Tylosin IM + I/Mammary infusion of amoxicillin & clavulanic acid (QD) for 5 days.

Evaluation parameters and sampling time points: The efficacy of the treatments was evaluated through milk pH, milk somatic cell count (SCC), milk quality parameters, restoration of milk yield and zinc and copper levels in serum. Milk pH was recorded using digital pH meter on daily basis for consecutive seven days and then on day 14^{th} and 28^{th} post-initiation of treatment. Milk SCC was recorded on day 7^{th}, 14^{th} and 28^{th} post-initiation of treatment by making milk film and staining it with Newman's Lampert stain (Schalm *et al.*, 1971). Milk fat contents were determined by Gerber's method **(**Aggarwala and Sharma, 1961). Fehling's solution titration method was used to estimate the lactose contents in milk **(**Egan *et al*., 1981). Determination of milk protein contents and calculation of milk solids not fat was carried out using Davide, (1977). The milk yield was recorded based on farm record or the subjective assessment of the owner/milker. Whereas determination of Cu and Zn in blood was carried out using spectrophotometric method at 576nm through commercial kits (Centronic GmbH, Germany). Cost benefit analysis of the treatments was carried out using formula devised by Young *et al*. (2012).
Net profit = (Additional returns + reduced cost) – (returns forgone + extra cost)

RESULTS

Milk pH: Milk pH is believed to be increased in mastitis and the same was observed in the present study wherein an elevated milk pH was observed in cows affected with subclinical mastitis (Table 1). Administration of respective regimen to animals of T1 led to a decrease in milk pH on day 1 post-initiation of treatment which was statistically significant ($P<0.05$) from baseline values. This trend was observed till day 3 post-initiation of treatment after which the decrease was statistically non-significant ($P<0.05$) from previous sampling days except at day 7, till the end of the study period. In antibiotic administered mastitic animals of T2, administration of the treatment resulted in increase ($P>0.05$) in milk pH till day 2 post-initiation of therapy then a decreasing trend in milk pH of animals of group T2 was observed throughout the study period being statistically significant ($P<0.05$) at day 4, 7 and 14 post initiation of treatment. Treatment T1 as compared to T2 led to a statistically significant ($P>0.05$) decrease in milk pH on day 1 after start of treatments which continued till day 7^{th}. On succeeding two sampling time points, the difference in pH values was statistically non-significant ($P>0.05$) between the two treatments (Fig 1).

Milk quality parameters: Milk Somatic Cell Count (SCC) in the cows were non-significantly ($P>0.05$) different between both groups before the treatments (Table 2). After provision of the allocated treatments, a statistically significant ($P<0.05$) decreasing trend was observed in SSC in both groups at 7^{th} day post-initiation of therapy than baseline values. A further decrease in SSC was observed in both the groups at day 14 post initiation of treatment which was significantly different ($P<0.05$) from the previous two time point. At day 28^{th}, the milk SCC decreased significantly ($P<0.05$) from the previous three time points in group T1 however in group T2 it did not differ significantly ($P<0.05$) from day 14 but did differ significantly ($P<0.05$) from day 7^{th} and baseline values. There was statistically non-significant difference ($P<0.05$) in milk fat, protein, lactose, solids not fat and total solid contents at different sampling points between the two groups except milk fat and lactose contents which increased significantly (Fig. 4 thru 8).

When compared between the groups, it was observed that there was statistically non-significant ($P>0.05$) difference in milk SCC values throughout the study period. A similar non-significant ($P>0.05$) difference was observed in milk fat, protein, lactose, solids not fat and total solid contents between the treatments throughout the study period.

Table 1: Effect (Mean ± SE) of oral non-antibiotic formulation *vis-à-vis* antibiotic therapy on milk pH at day 0-7, 14 and 28 of initiation of treatments in cows suffering from subclinical mastitis

Group	Baseline	Milk pH values at different sampling days post initiation of treatment								
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 14	Day 28
T1	7.24±0.061^{a}	7.01±0.039^{b}	6.87±0.037^{c}	6.71±0.045^{d}	6.70±0.033^{d}	6.68±0.028^{de}	6.70±0.033^{d}	6.59±0.020^{e}	6.61±0.017^{de}	6.60±0.019^{e}
T2	7.15±0.013^{a}	7.17±0.009^{a}	7.17±0.009^{a}	7.14±0.012^{a}	7.08±0.015^{b}	7.02±0.011^{c}	6.97±0.009^{d}	6.88±0.015^{e}	6.69±0.026^{f}	6.69±0.026^{f}

Means with identical small letters in rows are non-significant statistically (P>0.05).

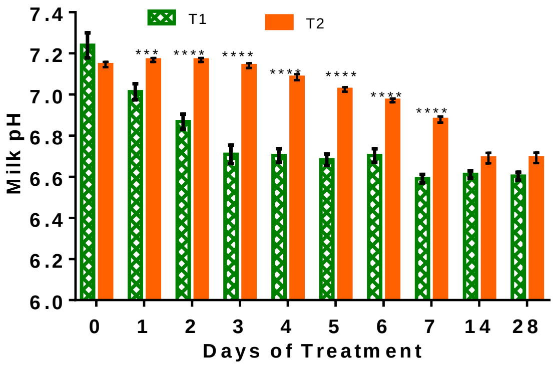

Fig. 1: Comparison of milk pH (Mean ± SE) values of cows treated with oral non-antibiotic formulation and rational mastitis therapy. Asterisks on the bars indicate significant difference.

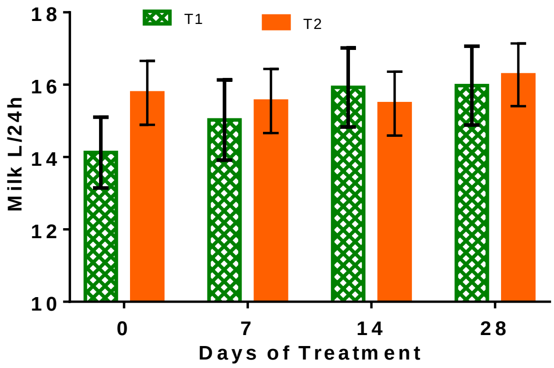

Fig. 2: Comparison of milk yield (Mean ± SE) of cows treated with oral non-antibiotic formulation and rational mastitis therapy.

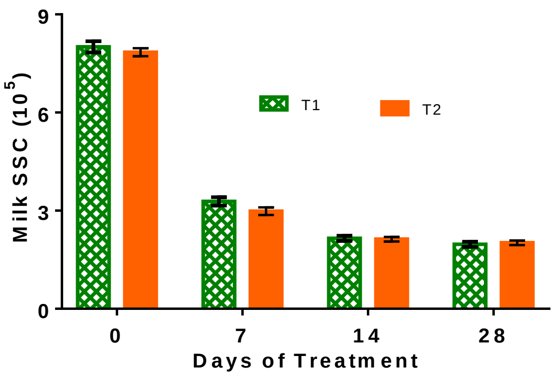

Fig. 3: Comparison of milk SCC (Mean ± SE) of cows treated with oral non-antibiotic formulation and rational mastitis therapy.

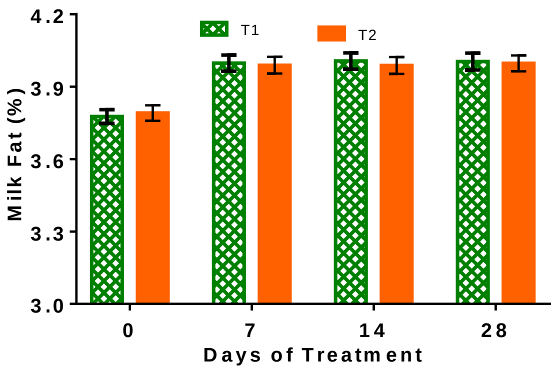

Fig. 4: Comparison of milk fat contents (Mean ± SE) of cows treated with oral non-antibiotic formulation and rational mastitis therapy.

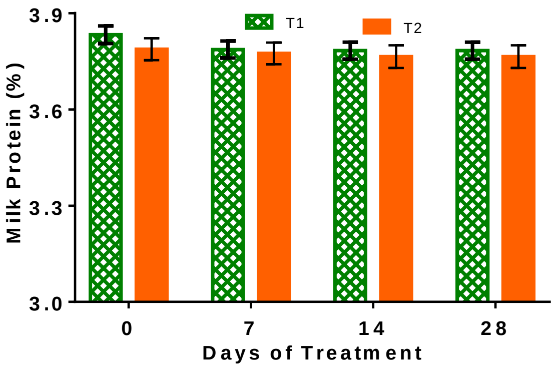

Fig. 5: Comparison of milk protein contents (Mean ± SE) of cows treated with oral non-antibiotic formulation and rational mastitis therapy.

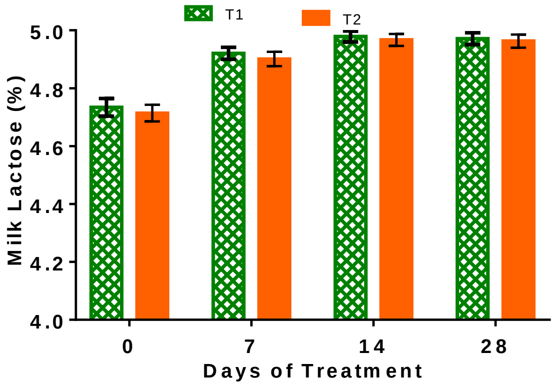

Fig. 6: Comparison of milk lactose contents (Mean ± SE) of cows treated with oral non-antibiotic formulation and rational mastitis therapy.

Restoration of milk yield: Provision of the oral formulation resulted in an increase in milk yield in the animals of the group at each sampling interval throughout the study period however the increase was not significantly (P>0.05) different among the sampling intervals or between T1 and T2. which was statistically non-significant (P>0.05) from previous study interval throughout the study period. (Table 3). The findings indicate that provision of T1 to the respective animals led to a 1.85L/24 h increase in milk yield compared to rational treatment (T2) where the increase in milk yield observed was 0.5L/24 h.

Serum Zinc and copper levels: Provision of serum and copper as component of oral formulation led to a significant increase (P>0.05) in serum copper and zinc levels of the animals of T1 from their counterparts in T2 (Fig. 10 and 11).

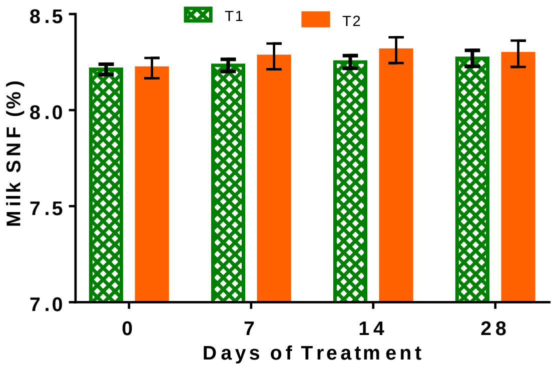

Fig. 7: Comparison of milk SNF contents (Mean ± SE) of cows treated with oral non-antibiotic formulation and rational mastitis therapy.

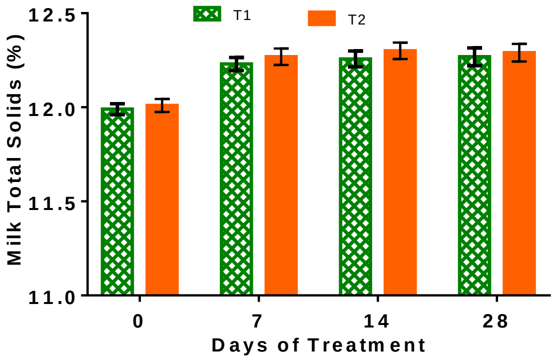

Fig. 8: Comparison of milk total solid contents (Mean ± SE) of cows treated with oral non-antibiotic formulation and rational mastitis therapy.

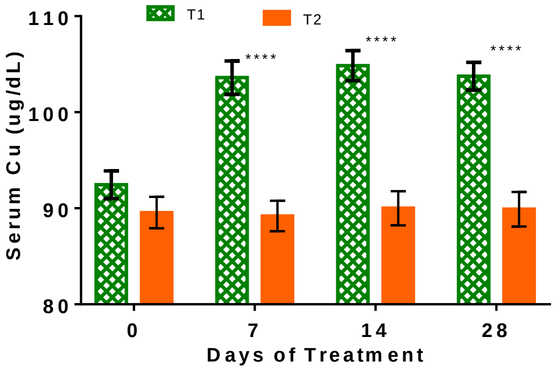

Fig. 9: Comparison of serum Cu levels (Mean ± SE) of cows treated with oral non-antibiotic formulation and rational mastitis therapy. Asterisks on the bars indicate significant difference.

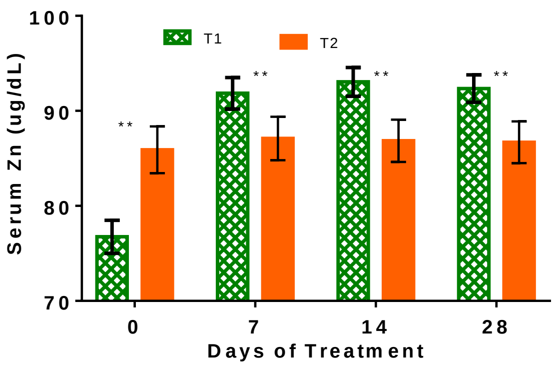

Fig. 10: Comparison of serum Zn levels (Mean ± SE) of cows treated with oral non-antibiotic formulation and rational mastitis therapy. Asterisks on the bars indicate significant difference.

Table 2: Effect (Mean ± SE; SCC x 10^5) of oral non-antibiotic formulation *vis-à-vis* antibiotic therapy on milk somatic cell counts at day 0, 7, 14 and 28 of initiation of treatments in cows suffering from subclinical mastitis

Group	Baseline	Milk somatic cell count (1 x 10^5) values at different sampling days post initiation of treatment		
		Day 7	Day 14	Day 28
T1	8.01 ± 0.172^{a}	3.28 ± 0.129^{b}	2.16 ± 0.077^{c}	1.97 ± 0.079^{d}
T2	7.85 ± 0.121^{a}	2.99 ± 0.116^{b}	2.13 ± 0.072^{c}	2.02 ± 0.070^{c}

Means with identical small letters in rows are non-significant statistically (P>0.05).

Table 3: Effect (Mean ± SE; L/24hrs) of oral non-antibiotic formulation *vis-à-vis* antibiotic therapy on restoration of milk yield on day 0, 7, 14 and 28 of initiation of treatments in cows suffering from subclinical mastitis

Group	Baseline	Days post initiation of treatment		
		Day 7	Day 14	Day 28
T1	14.13 ± 0.978^{a}	15.03 ± 1.109^{a}	15.93 ± 1.091^{a}	15.98 ± 1.089^{a}
T2	15.78 ± 0.885^{b}	15.55 ± 0.888^{b}	15.48 ± 0.883^{b}	16.28 ± 0.868^{b}

Means with identical small letters in rows are non-significant statistically (P>0.05).

Cost Benefit Analysis: Cost Benefit analysis of the treatments was carried out by Partial Budget Analysis through accepted formula described by Young *et al.* (2012). Net profit = (Additional returns + reduced cost) – (returns forgone + extra cost); Where additional returns mean increased milk received after administration of treatments. The milk yield increased from 14.13 to 15.98 L/24 h in T1, whereas corresponding values for group T2 were 15.78-16.28 L/24 h. Thus, the additional milk received in T1 was 1.35/L/animal/day. The cost of milk was Rs. 60/L, so: Additional returns= 1.35 x 60= Rs. 8. Reduced cost indicates here the difference in costs of two treatments (i.e., if the new drug is cheaper than old one or not). The cost of oral formulation for one animal per day was Rs. 14 whereas the rational treatment cost was Rs.390/animal/day, so:

Reduced cost= Cost of rational treatment- cost of trial drug = 390-14 = Rs. 376. Returns foregone means loss in milk production or any other losses due to treatment, whereas extra cost means if the trial drug is costly than the rational treatment. Both these were not true in the subject case, so:

Net profit = 81+ 376 – 0- 0 = Rs. 457/animal/day. The net profit gained by using the composite oral formulation as mastitis therapy instead of antibiotic therapy in sub-clinically mastitic cows was Rs. 457/animal/day.

DISCUSSION

Antibiotics-based mastitis therapy leaves a lot to be desired, including antibiotic resistance which necessitates efforts to search adjuncts or alternatives to reduce the colossal effects of this costly disease. A variety of remedial agents have been used either in replacement or as adjuncts to antibiotics in combating bovine and bubaline clinical and subclinical mastitis. Among these agents, non-antibiotic antibacterial have now been preferred to treat not only the intramammary infections but also the infections of other systems (Sarfraz *et al.*, 2009; Yousaf *et al.*, 2010; Dhillon and Singh, 2013;

Reddy *et al*., 2017). The non-antibiotics are supposed to reduce the udder infections without the concerns of bacterial resistance, eliminate milk and meat antibiotic residues and save the discarding of milk tainted with antibiotics. Moreover, the oxidative stress occurring in the mastitis will galvanize one to use antioxidants to stop the advent of oxidative stress. Using antioxidants will not only reduce the severity of disease but will also improve the cure rate (Gaafar *et al*., 2010; Latif *et al*., 2014; Yang and Li, 2015).

In the present study, milk pH was recorded to evaluate efficacy of treatments. The combination therapy significantly decreased the milk pH values. These findings are broadly concordant with those of previously reported studies (Sarfraz *et al.,* 2009; Dhillon and Singh, 2013; Prakash *et al.,* 2013; Mbonwanayo *et al*., 2016; Reddy *et al*., 2017). The increase in milk pH values is attributed to many factors including an increase in permeability of the glandular tissue to components of blood. Moreover, extracellular fluid and cellular components of blood along with inflammatory exudate get mixed with the milk resulting in an increase in milk pH values. This increase in milk pH is proportional to the extent of the damage or severity of the inflammatory process happening there (Bagri *et al*., 2018; Kandeel *et al*., 2019). Keeping in view this relationship, it can be assumed that returning of milk pH towards normal after administration of oral formulation indicates cessation of inflammatory process in the glandular tissue.

Restoration of the lost milk yield is probably the most cardinal measure of penultimate economic value of any mastitis therapy. Regarding restoration of milk yield, the values observed at baseline in the T1 group were 14.13±0.978 L/24 hrs, which increased to 15.98±1.089 L/24 hrs, due to the therapy. These findings are validated by the findings of Sarfraz *et al*. (2009), Prakash *et al.* (2013) and Reddy *et al*. (2017) who have reported an increase in milk yield in their studies after using trisodium citrate as mastitis therapy.

Milk somatic cell count (SCC) is one of the most used parameters of milk quality and determination of intramammary infections. Different researchers have used different cut-off SCC values to classify an udder quarter either infected or non-infected. National Mastitis Council recommends a count of < 2, 00, 000 cells per ml (Marzo *et al*., 2016) of milk to classify an udder quarter as uninfected. A significant reduction was observed in milk SCC in the animals after treating with combination T1 therapy. These findings are broadly in line with the findings of Mbonwanayo *et al*. (2016) who reported a significant reduction in milk SCC after treating mastitis with trisodium citrate. Somatic cells are inflammatory cells present in the glandular tissue/milk. When an inflammatory process is taking place in the glandular tissue of the udder these cells rush towards the site and due to increased permeability of glandular tissue pass into the milk. A significant increase in SCC is an indication of a strong inflammatory process going-on in the glandular tissue. A significant decrease in SCC after provision of oral formulation strongly indicates reduction in inflammatory process in the glandular tissue. Similarly, T1 improved other milk quality parameters (milk fat, SNF, TS, proteins and lactose contents) though non-significantly as compared to rational mastitis therapy, however it does indicate equal efficacy of the two treatments. Additionally, comparative increase in serum Cu and Zn levels also seems to be beneficial as both these minerals act as co-factors of many enzymes of immune system (Yang and Li, 2015).

Economic effectiveness is an added advantage of the oral non-antibiotic formulation as evident from the findings that it has produced statistically equivalent results to rational antibiotic mastitis therapy but being more economical. Farmer friendly administration of the formulation, no discarding of milk with the concern of drug residues and no antibiotic tainted milk for milk processing industry are other advantages which make the oral non-antibiotic formulation superior to rational antibiotic mastitis therapy.

Conclusions: The combined oral formulation of TSC, Vit. C, $ZnSo_4$ and $CuSo_4$helped to reduce inflammatory process during subclinical mastitis in the affected animals during. Elimination of tainted milk and concerns of antibiotic resistance and cost effectiveness of the non-antibiotic therapy make it suitable alternatives to antibiotic therapy to be used in cows suffering from subclinical mastitis on larger scale.

Authors contribution: MI and AM planned and executed main research trial. MTMUD, FH, ZMUD, MIS and HHK helped in collection and processing of samples. MA helped in serum chemical analysis while FK assisted in milk quality evaluation.

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