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Research Article

Targeting the Sub-Clinical Mastitis through *Centratherum anthelminticum* in Buffalo

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ABSTRACT

Sub-Clinical Mastitis (SCM) is an inflammatory disorder affecting parenchymal cells of mammary gland, and is a major challenge in dairy business. In dairy-dependent countries like Pakistan, this is especially very difficult to diagnose, and treat SCM. This study examined the potential of *Centratherum anthelminticum* (*C. anthelminticum*) in SCM, and its potential to improve the quality of milk in buffalo. For this purpose, twenty animals were tested for SCM with California mastitis test (CMT), and fifteen were found CMT positive. These fifteen animals were divided into three groups (5 each group); G1 group were treated with Tylosin, and served as control group, while G2 were treated with combination Tylosin + *C. anthelminticum*, and G3 was treated with *C. anthelminticum* alone for 7 days, and served as experimental groups. The CMT results after treatment showed that, the experimental groups (G2&G3) became CMT negative like control group (G1). Standard plate count (SPC) were used to measure udder inflammation, and showed a significant decrease in G2, indicating a strong treatment effect. The results suggest that, milk solids not fat (SNF), lactose, milk fat, and milk protein levels found significant in the milk of SCM treated buffalo. White blood cell (WBC) count decreased significantly in both G1 and G2, while red blood cell (RBC) count increased significantly in G2. Another hand, the total antioxidant capacity (TAC) levels significantly decreased across all treatment groups, with group 2 showing a highly significant reduction, while total oxidant status (TOS) levels were initially high, but significantly declined by day 8 in all groups, indicating reduced oxidative stress. In conclusion, the combination of Tylosin and *C. anthelminticum* (G2) appeared to be most effective in treating SCM in buffaloes, but remained non-significant to improve milk quality. Further detail studies are suggested to investigate the potential of *C. anthelminticum* to improve the milk quality in SCM treated buffalo.

Keywords: *Centratherum anthelminticum*, Sub-Clinical Mastitis, Total Antioxidant Capacity, Total Antioxidant Status.



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INTRODUCTION

Mastitis is a complicated as well as economically significant disease in dairy animals that affects the milk production, quality and animal well-being. SCM presents unique challenges that delay early detection and treatment, because it usually exhibits no visible symptoms (Haider *et al.*, 2023). This particular kind of mastitis, which affects between 30 to 50% of the dairy cattle in a herd, decline milk yield, and increase the somatic cell counts (SCC), resulting in poor milk quality and reduced nutritional values (Tommasoni *et al.*, 2023).

The principal bacteria such as *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), responsible to induce inflammation of the udder tissue, which results in tissue fibrosis and necrosis, leading to the decrease in milk production (Cheng and Han, 2020; Khan, 2024)

Mastitis has traditionally been treated predominantly with antibiotics. However, through the use of antibiotics, concerns of antibiotic resistance (AMR) and drug residues in milk are raised, which is harmful to the animal and the consumer health (El-Sayed and Kamel, 2021). Due to such AMR concerns, people resort to alternative treatments approach especially those embedded from natural resource including ethno medicine. Ethno medicine involves using of plant to treat diseases and its future outlook in controlling disease such as inflammation and infection in cattle (Murtaza et al., 2024; Shah et al.)

C. anthelminticum, often known as black cumin, has long been utilized in Southeast Asia for a variety of medicinal purposes. With qualities such as anti-inflammatory, anti-bacterial, and anti-oxidant, this *C. anthelminticum* might be utilized to control SCM without the side effects of medications (Agnihotri, 2022). Numerous studies have examined the effectiveness of *C. anthelminticum* in curing bacterial infections brought on by its phytochemical composition, which includes phenolic compounds, alkaloids, and flavonoids (Mudassir et al., 2024). These bioactive substances may reduce inflammation and infection density in afflicted tissues by delaying the growth of bacteria and inflammatory cytokines including TNF- α and IL-6. The majority of these studies also show how efficient *C. anthelminticum* is against various bacteria, including *S. aureus* and *E. coli*, which are the primary bacteria that cause mastitis in buffalo (Misra et al., 2023). *C. anthelminticum* exhibits antioxidant qualities in addition to its antibacterial potential by stabilizing cell membranes and scavenging free radicals. These antioxidant benefits are especially helpful in controlling oxidative stress, which is a typical reaction to inflammation in SCM-affected mammary tissues (Avasthi et al., 2021). Additionally, by lowering SCC levels, antioxidant chemicals like the phenolic substances in *C. anthelminticum* have demonstrated promise in enhancing milk quality, which helps with improved shelf life, nutritional stability, and milk preservation (Al Saqr, 2019).

This study focuses on *C. anthelminticum*'s effectiveness as a treatment for buffalo's SCM through an analysis of its antibacterial, anti-inflammatory, and antioxidant properties, this research seeks to assess *C. anthelminticum* as a natural alternative remedy for SCM, lowering the need for antibiotics while improving milk quality and animal welfare. The results could help dairy farmers implement sustainable animal management techniques by reducing the health hazards associated with antibiotic residues and offering them natural, alternative for disease control. As the dairy sector moves toward safer and more efficient treatment choices, this strategy supports the growing interest around the world in creating herbal medicines to combat antibiotic resistance.

MATERIALS AND METHODS

Study area

In this study, buffaloes with SCM were identified and selected from a privately owned dairy farm in District Faisalabad.

Milk sample collection and analysis protocol

Milk samples were collected from each animal on days 0 (pre-treatment) and 8 (post-treatment), in accordance with National Mastitis Council guidelines (Al Saqr, 2019). To reduce contamination, the first few streams of milk were eliminated, and CMT samples were collected with a mastitis paddle. Sterile, sealed containers holding 15 mL of milk were used for collection, and teats were cleaned with ethyl alcohol before sampling. To avoid cross-contamination, different samples were taken from each animal.

Experimental design and treatment protocol

The purpose of this research was to analyze *C. anthelminticum*'s therapeutic potential for treating SCM in buffaloes, as well as its influence on milk quality. The 15 animals were divided into three groups (5 animals each). Tylosin was administered intramuscularly to control group 1 at a dose of 18 mg/kg every day for 8 days. The experimental group 2 was given 8 days combination of Tylosin (10 mg/kg intramuscular) and *C. anthelminticum* (120 mg/kg oral). The experimental group 3 was given solely *C. anthelminticum* orally at a dose of 120 mg/kg for seven days. Treatments with Tylosin and *C. anthelminticum* were given to all groups on days 0 and 8 to assess both immediate and long-term effects.

California mastitis test (CMT)

The CMT was used to determine SCM severity by visually examining milk samples in a paddle for gel-like forms. A score was assigned depending on the response seen, ranging from negative (no reaction) to very positive (dense gel

formation). The degree of thickness was associated with the presence of infection, allowing for a more exact assessment of mastitis severity and treatment success (Ferronato *et al.*, 2018).

Microbial load analysis (standard plate count)

Milk samples collected before treatment and after treatment were analyzed for microbial load using the standard plate count method. Each milk sample was thoroughly mixed, and portions were inoculated on agar plates in sterile conditions. Plates were then incubated to observe bacterial colony formation as an indicator of bacterial load in the milk (Makararpong *et al.*, 2024).

Milk quality analysis

Milk samples were analyzed for several quality indicators to assess the effects of treatment on milk composition. Parameters measured included, Solid-not-fat percentage (SNF), Fat content, Milk protein content, Lactose content, and Milk PH (Makararpong *et al.*, 2024). Analyses were conducted at the Dairy Tech Laboratory, National Institute of Food Science and Technology, University of Agriculture Faisalabad, using the Lactoscan Milk Analyzer.

Biochemical analysis (total antioxidant capacity (TAC) and total oxidative status (TOS) tests)

TAC and TOS tests were used in biochemical investigations to assess milk antioxidant and oxidative stress levels. The concentration of oxidative components in milk was determined using spectrophotometric techniques (BIOLAB-310) using hydrogen peroxide as the oxidizing agent, which assisted in determining oxidative damage in the milk sample (Deniz *et al.*, 2021). The TAC was determined using the Biosystem BTS-330 analyzer. The objectives of the current experiment focused on evaluating ROS scavenging by milk and effect of *C. anthelminticum* on antioxidant activity of milk. To assess the therapy efficacy concerning the TAC and TOS tests were conducted in GC University Faisalabad.

Hematological analysis

Blood samples (2-3 ml) were collected from each buffalo's jugular vein in EDTA vials are taken on ice and analyzed immediately for hematological parameters included hemoglobin (Hb), packed cell volume (PCV) and total leukocyte count. were used to determine systemic responses to therapy in animals with SCM (Lakshmi *et al.*, 2024).

Statistical analysis

Statistical analysis was carried out using two-way ANOVA and Tukey test (Minitab) so that p-values <0.05 were regarded as significant.

RESULTS

The study evaluated the effect of different treatment protocols for SCM in buffaloes with reference to *C. anthelminticum* and antibiotics. The obtained data of the different treatment groups are informative regarding the efficacy of treatments in relation to clinical as well as SCM indicators.

CMT-Based quarter cure rates

SCM in 35 buffaloes was treated and the effectiveness of treatment was assessed using the California Mastitis Test (CMT). First, 15 out of 20 tested animals were positive for mastitis and were randomly assigned into three groups. G1 was administered Tylosin, served as control group. The G2 was gauged with a combination of Tylosin and *C. anthelminticum* while G3 was given *C. anthelminticum* only, both G1 and G2 served as experimental groups. The results obtained from post-treatment CMT revealed that all the animals in G1 and G2 had reacted negatively to mastitis hence implying good treatment response. On the other hand, only one animal in G3 was negative, while the other two were still positive as shown in Figure 1(a). This lack of improvement in cure rates points to the potential benefits of integrating the use of antibiotic with that of herbal medicines by groups 1 and 2 recoveries.

Comparison of standard plate counts (SPC)

SPC serve as an important indicator of mastitis severity. The study compared SPC from day 0 (pretreatment) to day 8 (post-treatment) across the three groups. Group 1 showed a significant reduction in SPC. In group 2, SPC decreased significantly, demonstrating a strong treatment effect. Group 3 exhibited a reduction from 0.59 ± 0.02 to 0.11 ± 0.02 , although it lacked statistical significance. Collectively, these results indicate that while all treatment groups experienced a reduction in SPC, but the combination of Tylosin and *C. anthelminticum* (group 2) was particularly effective, reinforcing the importance of this treatment strategy as shown in Figure 1(b).

Milk quality analysis

Milk solids not fat (SNF)

The effects of the treatments on SNF percentages were evaluated, with the results showing comparison of SNF % non-significant across the between G1, G2, and G3. While the group 2 SNF increased slightly as shown in Figure 2

(a). Overall, these findings suggest that while there were fluctuations in SNF percentages post-treatment, but none of the changes reached statistical significance, indicating that the treatments employed did not significantly impact milk quality concerning SNF.

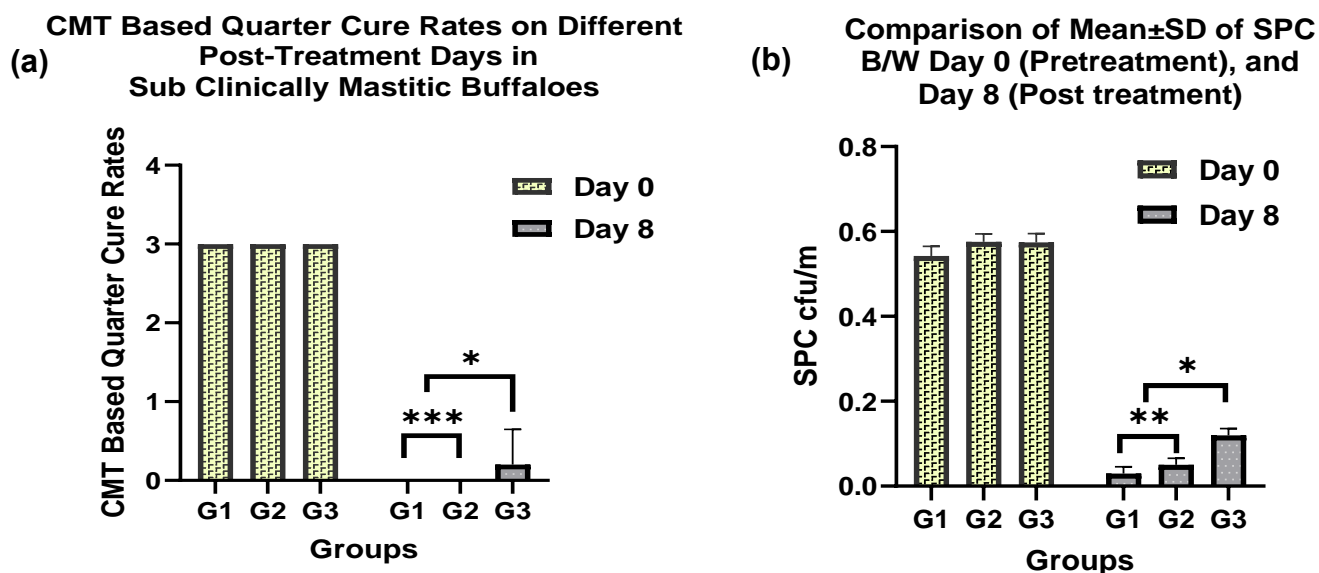


Figure 1. (a) Represents the CMT based quarter cure rates on different post-treatment days in SCM buffaloes (b) represents the comparison of SPC B/W day 0 (pretreatment), and day 8 (post treatment) in SCM buffalo.

Lactose levels

Lactose levels were also assessed in the study, with no significant changes observed in any group from day 0 to day 8. Group 1 and group II experienced a minor increase Lactose levels from day 0 to day 8. While there were observable increases in lactose percentages, the lack of statistical significance suggests that the treatments had minimal effect on lactose content, which is a crucial component of milk quality as shown in Figure 2 (b).

Milk fat percentage

The study examined the impact of *C. anthelminticum* and antibiotics on milk fat percentages. Group I showed no change, with a consistent value of across both days. Group 2 and group 3 exhibited a significant increase in milk fat indicating a positive treatment effect. These results suggest that *C. anthelminticum* may effectively enhance milk fat content in buffaloes with SCM as shown in Figure 2 (c).

Milk protein content

Milk protein percentages were analyzed, revealing increase across all groups, but these changes were statistically significant. The protein level in all groups was increase, but the protein level of G2 was highly significant. Despite the observed increase in protein level of G2, the results indicate that the *C. anthelminticum* have significant impact on milk protein levels as shown in Figure 2 (d).

Milk pH analysis

Milk pH was assessed across the treatment groups, with no significant changes observed between day 0, and day 8, but slight variations in milk PH was noted between the groups as shown in Figure 2 (e). The slight variations in milk pH indicate that the treatments did not significantly impact the acidity of the milk produced by the buffaloes.

Hematological analysis

White blood cell (WBC) counts

The analysis of WBCs highlighted significant findings, particularly in group 2. Group 1 showed a reduction in WBCs suggesting a lack of substantial change despite the decrease. Group 2 demonstrated a significant reduction in WBCs highlighting the effectiveness of the treatment in this group. Conversely, group 3 WBCs counts was without specific statistical significance. Overall, these findings indicate varying treatment responses among the groups, with group 2 showing the most significant reduction in inflammatory markers, which is critical in managing mastitis as shown in Figure 3 (a).

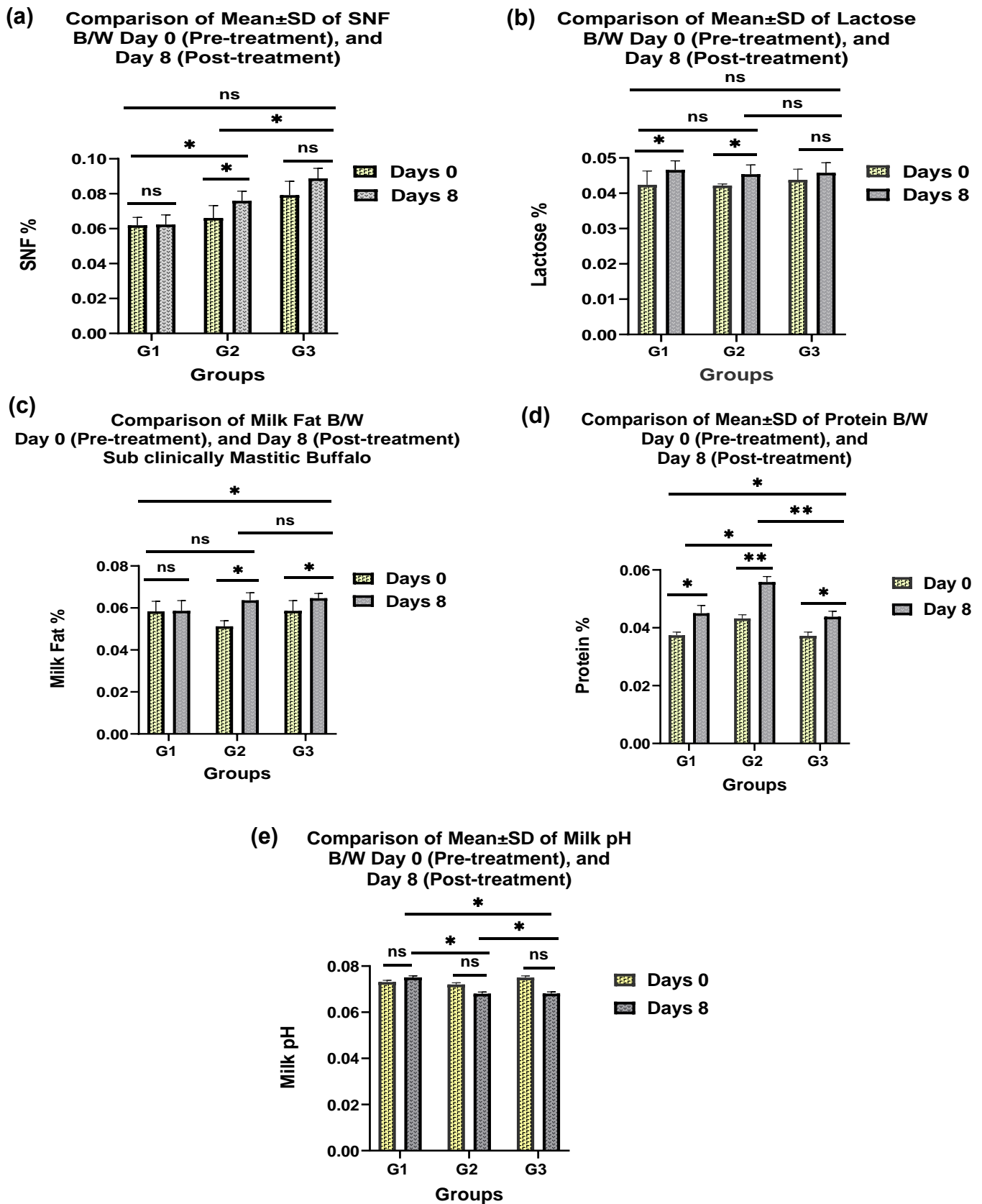


Figure 2. (a) Represents comparison of SNF b/w day 0 (pretreatment), and day 8 (post treatment) in SCM buffalo, (b) comparison of lactose b/w day 0 (pretreatment), and day 8 (post treatment) in SCM buffalo, (c) comparison of milk fat b/w day 0 (pretreatment), and day 8 (post treatment) SCM buffalo, (d) comparison of mean of protein b/w 0 and 8th day milk protein % in SCM buffalo, (e) comparison of milk pH b/w day 0 (pretreatment), and day 8 (post treatment) in SCM buffalo.

Red blood cell counts (RBCs)

Figure 3 (b) compares RBCs between day 0 (pretreatment) and day 8 (post-treatment) in SCM buffalo. In G1, the RBC count was increased but was not statistically significant. In G2 had an initial RBCs count was significantly, increased with a highly significant P-value, indicating a strong treatment effect. In G3, the RBCs count increased with specific statistical significance. Overall, the results indicate, that while G2 exhibited a significant increase in RBCs. These findings, suggest that the treatment effectively enhances RBC counts in buffalo affected by SCM, particularly in G2, warranting further exploration of the underlying mechanisms driving these changes.

Packed cell volume (PCV)

Figure 3 (c) presents the effects of *C. anthelminticum* alone and in combination with antibiotics on packed cell volume (PCV) in sub clinically mastitic buffalo, comparing data between day 0 (pretreatment) and day 8 (post-treatment). In G1, the PCV was increased by day 8, but this change, was not statistically significant. In G2, the initial PCV was significantly increased, showing a highly significant effect thereby highlighting the effectiveness of the treatment in this group, while G3 had specific statistical significance. Overall, the results indicate that while G2 demonstrated a significant improvement in PCV, while the comparison between G2 and G3 did not show statistically significant changes suggesting variability in treatment response among these groups. These findings emphasize the positive effect of the treatment on hematological parameters, particularly in G2 warranting further investigation into the underlying mechanisms involved.

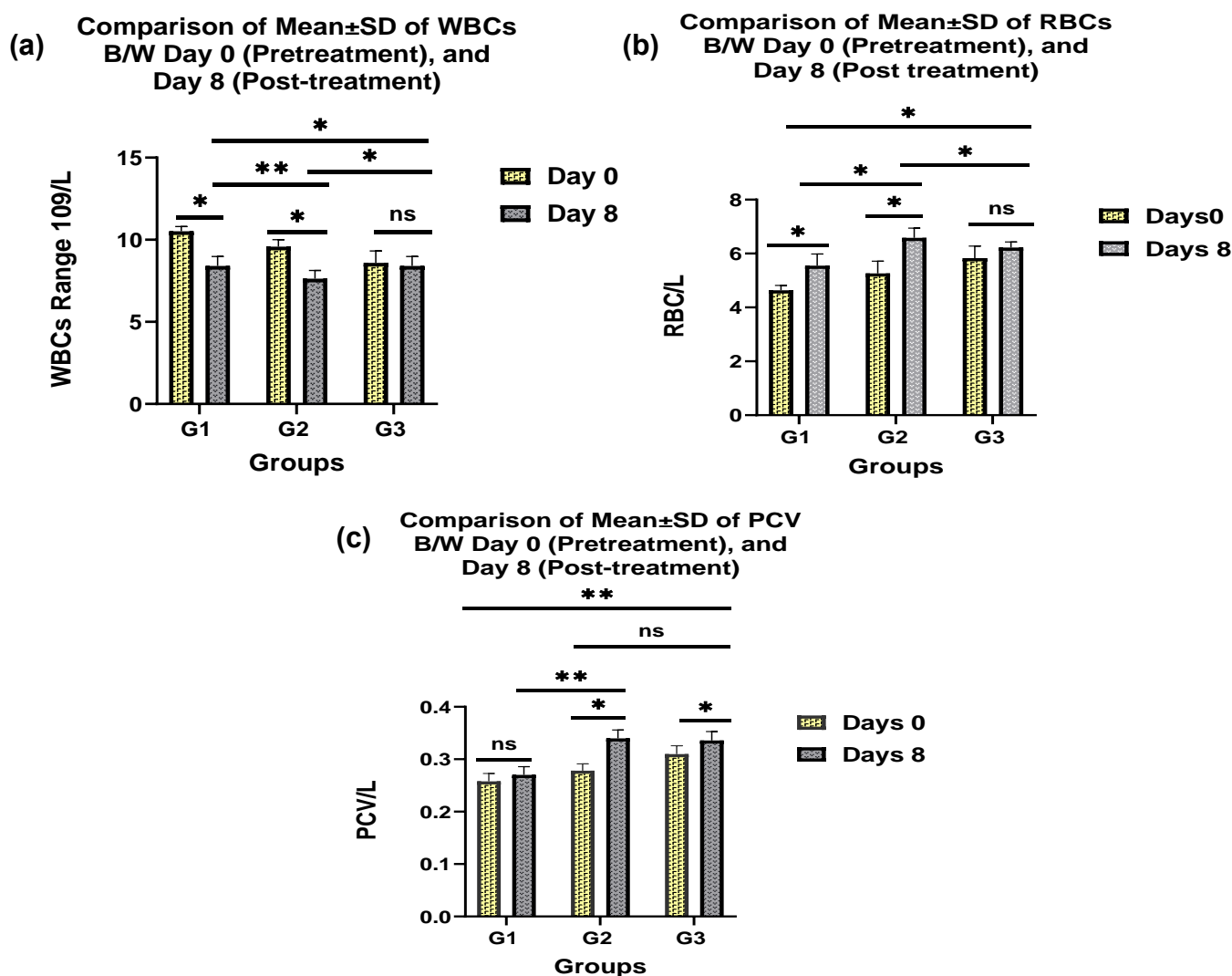


Figure 3. (a) Represent the comparison of WBCs b/w day 0 (pretreatment), and day 8 (Post treatment) in SCM buffalo, (b) Comparison of RBCs b/w Day 0 (Pretreatment), and day 8 (post treatment) in SCM buffalo, (c) comparison of Mean±SD of PCV b/w day 0 (pretreatment), and day 8 (post treatment) in SCM buffalo.

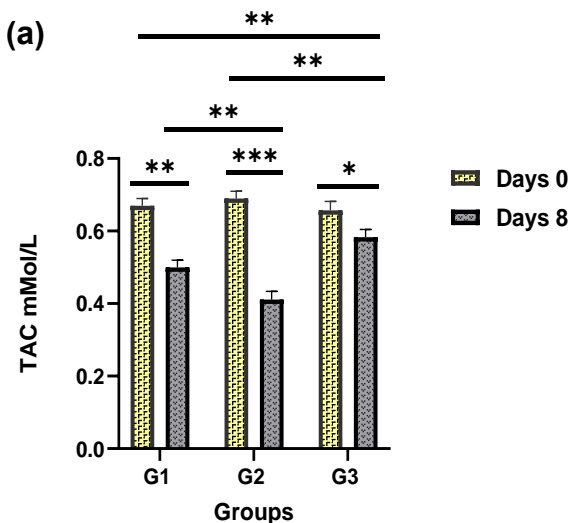
Evaluating the total antioxidant capacity (TAC) of *C. anthelminticum* in SCM in buffalo

In Figure 4 (a) the data presents a comparison of TAC between day 0 (pretreatment) and day 8 (post-treatment) across three groups. For group G1, the TAC value decrease showing significant effect, indicating statistically significant change in TAC over the treatment period. In group G2, the TAC also decreased, but this change was statistically highly significant suggesting a meaningful reduction in antioxidant capacity, following treatment. Group G3 exhibited a similar trend, with TAC decreasing. Overall, while G1, G2, and G3 demonstrated a statistically significant, decline in TAC, warranting further investigation into the treatment's impact on antioxidant levels.

Evaluating the total oxidant status (TOS) of *C. anthelminticum* in SCM in buffalo

In Figure 4 (b), the result showed the effect of *C. anthelminticum* alone and in combination with antibiotics on TOS over an 8 day period. At baseline (day 0), the TOS values were significantly higher across all groups, with G1 showing a mean of 4.70 ± 0.4 , G2 at 4.0 ± 0.20 , and G3 at 3.40 ± 0.26 . Notably, the P-value for day 0 comparisons was 0.008, indicating a statistically significant difference among the groups at baseline. By day 8, TOS levels significantly, decreased in G1 to 0.49 ± 0.005 ($p=0.002$), G2 to 0.31 ± 0.00 (also $P=0.002$), and G3 to 0.40 ± 0.001 . The P-value of 0.002 for day 8 suggests a statistically significant reduction, in TOS across the treatment groups, indicating that the intervention effectively reduced oxidative stress. These results highlight the potential of *C. anthelminticum* and its combinations to improve antioxidant status, warranting further investigation into their therapeutic applications.

Comparison of Mean \pm SD of TAC B/W Day 0 (Pre-treatment), and Day 8 (Post-treatment)



Comparison of TOS B/W Day 0 (Pre-treatment), and Day 8 (Post-treatment)

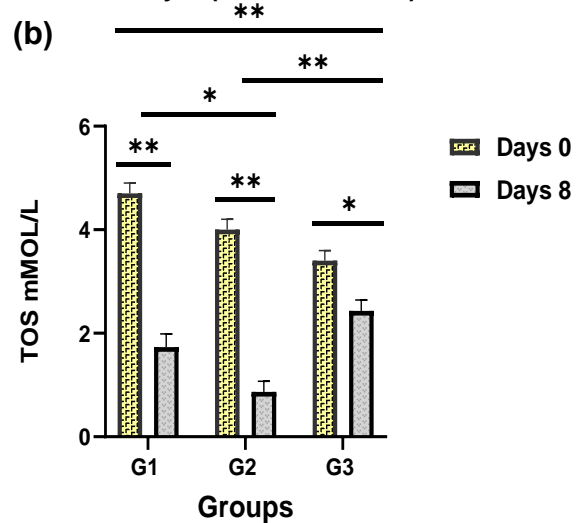


Figure 4. (a) Comparison of mean of TAC b/w day 0 and day 8 in SCM in buffalo, (b) comparison of TOS b/w day 0 (pretreatment), and day 8 (post treatment) in SCM in buffalo.

DISCUSSION

Mastitis, an inflammation of the mammary gland caused primarily by bacterial infections, remains one of the most challenging issues in dairy animal management. Not only does it reduce milk production, but it also causes significant economic losses for farmers and the livestock industry (Lakshmi *et al.*, 2024). Traditional treatments rely heavily on antibiotics, which, while effective, pose risks of antibiotic residues in milk and meat products. To mitigate these risks, alternative therapies such as plant-based supplements have been explored (Murtaza *et al.*, 2024). This study investigated the potential of *C. anthelminticum*, a plant extract, both independently and in combination with antibiotics, to manage SCM in lactating buffaloes.

This study successfully demonstrated that *C. anthelminticum* both independently and in combination with Tylosin in managing SCM in lactating buffaloes. According to subclinical phagocyte count, inflammation was significantly reduced in all groups, with a more pronounced reduction observed in group receiving combination Tylosin and *C. anthelminticum*. This synergistic effect demonstrates that the plant extract may enhance tylosin's anti-inflammatory action. Those in the group that received only *C. anthelminticum* their SPC was reduced which may indicate that the plant extract possesses anti-inflammatory properties. Further research is needed to understand more of the

mechanisms resulting in these effects on the immune cells in udder tissue. Nair *et al.* (2021) *C. anthelminticum* is known for its antibacterial, anti-inflammatory, and antioxidant characteristics, which make it beneficial in treating fowl pox, FMD, indigestion, and bloating. Shah *et al.* (2017) concluded that the methanolic extract of *C. anthelminticum* seeds demonstrates promising antiasthmatic properties. The potential antiasthmatic action of *C. anthelminticum* may be due to its ability to function as an. antihistaminic, stabilize mast cells, and inhibit the infiltration of inflammatory cells.

Milk composition data showed that SNF was generally slightly altered, thus suggesting that overall the treatments did not affect any of the components of milk apart from fat. But the combination of the treatment and the plant extract caused an increase in the milk fat content which was highly significantly different from the control. This fact gives the impression that *C. anthelminticum* is involved in milk fat synthesis and therefore its effect on the metabolism of the mammary gland needs further study.

Although milk protein levels declined somewhat in all groups, the difference was not statistically significant, indicating that neither Tylosin nor *C. anthelminticum* appreciably inhibited protein synthesis in mammary glands. Similarly, changes in milk pH were small and insignificant, with treatments producing slightly lower levels in all groups.

The white blood cell and red blood cell counts provide further information on the anti-inflammatory and general hematologic benefits of the patients' treatment. The group treated with a combination of Tylosin and *C. anthelminticum* exhibited a large decline in WBCs, signifying a strong anti-inflammatory response, as well as a considerable rise in RBC levels, indicating an increase in hematogenous. However, the impact of *C. anthelminticum* alone on WBC and RBC counts was not as substantial as in the control group. Furthermore, an increased PCV value after use of either Tylosin or *C. anthelminticum* indicated an improvement in blood health of the buffalo. Negi *et al.* (2014) stated mechanism of *C. anthelminticum*. anti-inflammatory action stems from its ability to control the Nrf-2/HO-1 and NF- κ B pathways, which has been thoroughly explored and confirmed in scientific studies.

Terminating with antioxidant capacity, all groups follow a decreased in the TOS, thus showing a decrease in oxidative stress. The decrease in TOS observed in this study in the combination therapy group suggesting that *C. anthelminticum* may possess antioxidant potential. The ability of *C. anthelminticum* may be attributable to its effect on the important source of TOS, inflammation. Ani and Naidu (2011) stated mechanism of antioxidant activity of *C. anthelminticum* is thought to be due to its ability to reduce lipid peroxidation and improve antioxidant indices. Mudassir *et al.* (2018) concluded that *C. anthelminticum* ethanolic seed extract has significant in-vitro antioxidant and antiglycation effects.

CONCLUSION

The study demonstrated that the combination of Tylosin and *C. anthelminticum* (G2) was the most effective treatment for SCM in buffaloes, showing significant reductions in CMT positivity, SPC, and inflammatory markers. While *C. anthelminticum* alone (G3) exhibited some improvement, its effectiveness was comparatively lower. Milk composition remained largely unaffected, but milk fat and protein levels increased significantly in G2. Hematological parameters, improved significantly in G2, indicating enhanced physiological recovery. Additionally, a significant reduction in TOS and TAC levels highlights the potential antioxidant benefits of the treatment, warranting further exploration of *C. anthelminticum* in mastitis management. Further research should be directed toward defining metabolic profiles that have an impact on the effects of *C. anthelminticum* moreover, its long-term effects deserve further research.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this research.

COMPETING OF INTEREST

The authors declare no competing interests.

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