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Antimicrobial efficacy of different concentrations of Marigold (*Calendula officinalis*) leaf extract as a natural pre-milking udder sanitizer in crossbred cows

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Abstract

The present investigation evaluated the efficacy of different concentrations of Marigold (*Calendula officinalis*) leaf extract used as a pre-milking udder wash on the bacteriological quality of raw milk in crossbred cows. The study was conducted at the Dairy Farm of Sam Higginbottom University of Agriculture, Technology and Sciences (SHUATS), Naini, Prayagraj, Uttar Pradesh, India. Twelve healthy lactating crossbred cows were randomly allotted to four udder wash treatments, namely clean water wash as control, Marigold leaf extract at 20 g per litre of water, 40 g per litre of water and 60 g per litre of water. Milk samples were collected aseptically after milking and analyzed for total bacterial load and different groups of bacteria using standard microbiological techniques. The results revealed a significant reduction in bacterial counts in Marigold-treated groups compared to the control, with the highest reduction observed at the 60 g per litre concentration. Coliform bacteria were absent in all treatments, indicating satisfactory hygienic conditions. The study demonstrated that Marigold leaf extract can be effectively used as a natural, economical and eco-friendly alternative to chemical udder disinfectants for improving the bacteriological quality of raw milk under farm conditions.

Keywords: Marigold leaf extract, udder hygiene, raw milk quality, bacterial load, clean milk production, crossbred cows

Introduction

Milk is regarded as one of the most complete and nutritionally balanced foods for human consumption because of its rich composition of proteins, fats, lactose, minerals and vitamins. Owing to its high nutritive value, milk plays an important role in human nutrition and serves as an essential component of the daily diet across all age groups. However, the same nutrient-rich composition also makes milk highly susceptible to microbial contamination and rapid spoilage. The microbial quality of raw milk is therefore considered one of the most important factors influencing its shelf life, processing suitability, market acceptability and public health safety (Murphy and Boor, 2000) [6].

Microbial contamination of milk may occur during and after milking from several sources such as contaminated teat surfaces, udder skin, bedding materials, milking utensils, feed residues, water, air and milk handlers. Among these sources, the teat surface and udder skin act as major reservoirs of spoilage and pathogenic microorganisms that directly enter milk during milking operations (Rajorhia, 2001) [7]. Improper udder hygiene and poor milking practices significantly increase bacterial contamination in milk and adversely affect the keeping quality of dairy products.

The bacteriological quality of raw milk is commonly

evaluated through microbial indicators such as Standard Plate Count (SPC), Lactic Acid Bacteria Count (LABC), Proteolytic Bacteria Count (PBC), Lipolytic Bacteria Count (LBC) and Coliform Count. Standard Plate Count is widely considered an important indicator of overall hygienic quality and sanitary conditions during milk production and handling (Aneja, 1992) [1]. Elevated microbial counts in milk are generally associated with poor hygiene, reduced shelf life and deterioration in processing quality.

Lactic acid bacteria ferment lactose and produce lactic acid, leading to souring and coagulation of milk during storage. Although some lactic acid bacteria are beneficial in fermented dairy products, excessive growth in raw milk adversely affects milk quality and shelf life. Proteolytic bacteria produce extracellular enzymes that degrade milk proteins, resulting in bitterness, coagulation and reduced processing quality of milk and milk products. Similarly, lipolytic bacteria hydrolyze milk fat and cause rancidity along with development of undesirable off-flavours (Sharma and Sharma, 2001) [8]. Therefore, effective control of these spoilage microorganisms is essential for maintaining milk quality and improving the keeping properties of dairy products.

Pre-milking udder sanitation is considered one of the most effective farm-level interventions for improving the bacteriological quality of milk. Proper cleaning and

disinfection of the udder before milking substantially reduce microbial contamination originating from the teat surface. Conventionally, dairy farms use chemical disinfectants such as iodine compounds, chlorhexidine, potassium permanganate and quaternary ammonium compounds for teat sanitation. Although these chemical sanitizers are effective, prolonged and indiscriminate use may lead to higher cost, chemical residues in milk, skin irritation, environmental concerns and possible development of microbial resistance (Kaushal and Anand, 2000) ^[5].

In recent years, increasing consumer awareness regarding food safety, antibiotic residues and environmentally sustainable dairy farming has generated considerable interest in herbal and plant-based alternatives for dairy hygiene management. Herbal preparations possess antimicrobial, antioxidant and anti-inflammatory properties and are generally considered safer and more biodegradable than synthetic chemicals. Plant-based sanitizers are also comparatively economical, locally available and suitable for smallholder dairy production systems.

Marigold (*Calendula officinalis*) is an important medicinal plant widely recognized for its antimicrobial, antifungal, antioxidant and wound-healing properties. Different parts of the plant contain bioactive phytochemical constituents such as flavonoids, tannins, carotenoids, triterpenoids, saponins and phenolic compounds, which exhibit inhibitory activity against a broad range of microorganisms (Burtscher *et al.*, 2023) ^[3]. The antimicrobial activity of Marigold has mainly been attributed to its phenolic compounds and essential oils that interfere with bacterial cell membrane integrity and metabolic activity.

Several medicinal plants have been investigated for their antimicrobial potential in livestock production systems; however, scientific information regarding the practical application of Marigold leaf extract as a pre-milking udder sanitizer remains limited. Most available studies on *Calendula officinalis* have focused mainly on pharmaceutical and medicinal applications, while comparatively little attention has been given to its use in clean milk production and dairy hygiene management.

The concept of clean milk production has gained substantial importance in recent years because of increasing concern regarding microbial contamination, milk-borne diseases and maintenance of hygienic quality of dairy products. Adoption of herbal udder hygiene practices may contribute significantly toward sustainable dairy farming by reducing dependence on synthetic chemical disinfectants and promoting residue-free milk production systems.

Considering the antimicrobial potential of Marigold and the importance of udder hygiene in milk quality management, the present investigation was undertaken to evaluate the antimicrobial efficacy of different concentrations of Marigold (*Calendula officinalis*) leaf extract used as a pre-milking udder sanitizer in crossbred cows. The study was designed to assess its effectiveness in reducing various physiological groups of bacteria in raw milk and to explore its potential as a natural and eco-friendly alternative to conventional udder disinfectants used in dairy farms.

Objectives

1. To evaluate the antimicrobial efficacy of different concentrations of Marigold (*Calendula officinalis*) leaf

extract used as a pre-milking udder sanitizer in crossbred cows.

2. To study the effect of Marigold leaf extract on microbial load and different physiological groups of bacteria in raw milk.
3. To assess the suitability of Marigold leaf extract as a natural, economical and eco-friendly alternative to conventional udder disinfectants for clean milk production.

Materials and Methods

Experimental Site and Duration of Study

The present investigation was conducted at the Dairy Farm of Sam Higginbottom University of Agriculture, Technology and Sciences (SHUATS), Naini, Prayagraj, Uttar Pradesh, India, during the pre-monsoon season for a period of 30 consecutive days. Warm and humid environmental conditions prevailing during the pre-monsoon season generally favor microbial growth and increase the chances of milk contamination under farm conditions. Maintenance of proper udder hygiene during this season is therefore considered highly important for improving the bacteriological quality of milk (Rajorhia, 2001) ^[7].

Selection and Management of Experimental Animals

A total of twelve clinically healthy lactating crossbred cows in mid-lactation were selected for the study. Prior to commencement of the experiment, all animals were screened for both clinical and subclinical mastitis using the California Mastitis Test (CMT), and only mastitis-free animals were included in the investigation. Mastitis is known to significantly increase bacterial contamination in milk and may influence microbial counts and milk quality parameters (Murphy and Boor, 2000) ^[6].

The selected animals were maintained under uniform feeding, housing and management conditions throughout the experimental period. The cows were housed in well-ventilated sheds with proper sanitation and drainage facilities. Routine hygienic practices were followed regularly to minimize environmental contamination and maintain cleanliness of the dairy farm. The animals were fed according to standard dairy farm feeding schedules and had free access to clean drinking water throughout the experimental period.

Milking was performed twice daily during morning and evening hours under hygienic conditions. Uniform milking practices were maintained for all animals in order to minimize experimental variation.

Experimental Design and Treatment Details

The experiment was conducted using a Completely Randomized Design (CRD). Twelve lactating crossbred cows were randomly assigned to four treatment groups comprising three animals in each group.

The treatments were as follows:

- **T₀:** Clean water wash (Control)
- **T₁:** Marigold leaf extract prepared using 20 g fresh leaves per litre of water
- **T₂:** Marigold leaf extract prepared using 40 g fresh leaves per litre of water
- **T₃:** Marigold leaf extract prepared using 60 g fresh

leaves per litre of water

The objective of using different concentrations of Marigold leaf extract was to evaluate its antimicrobial efficacy as a natural pre-milking udder sanitizer.

Preparation of Marigold Leaf Extract

Fresh leaves of Marigold (*Calendula officinalis*) were collected from local sources and thoroughly washed with clean water to remove dust, dirt and foreign materials. The treatment concentrations were prepared on fresh weight basis using freshly collected Marigold leaves.

The required quantity of fresh leaves according to each treatment concentration was weighed accurately using an electronic balance. The leaves were crushed and boiled in one litre of water for approximately 10 minutes to facilitate extraction of bioactive compounds. After boiling, the extract was cooled to room temperature and filtered through a clean muslin cloth to obtain a uniform filtrate free from leaf particles and debris.

Fresh extract was prepared daily before milking in order to maintain maximum antimicrobial activity and prevent deterioration of phytochemical constituents. Marigold is known to contain flavonoids, phenolic compounds, tannins and saponins possessing antimicrobial activity against various spoilage microorganisms (Burtscher *et al.*, 2023) [3].

Pre-Milking Udder Wash Procedure

Prior to each milking, the udder and teats of experimental animals were washed thoroughly using the respective treatment solutions according to their treatment group. Adequate contact time of approximately one minute was provided to allow effective interaction between the treatment solution and microorganisms present on the teat surface.

Following washing, the udder and teats were dried using separate clean cloth towels for each animal in order to avoid cross-contamination among cows. Proper drying of teats after washing is considered essential for minimizing bacterial entry into milk during milking operations (Kaushal and Anand, 2000) [5].

Milk Sample Collection

Milk samples were collected aseptically immediately after milking in sterile screw-capped containers. Proper precautions were taken during sample collection to avoid contamination from external sources. The collected milk samples were properly labeled and immediately transported to the laboratory under refrigerated conditions for microbiological examination.

Repeated milk sampling was carried out throughout the experimental period in order to improve reliability and accuracy of observations. Samples collected during different sampling intervals were subjected to microbiological analysis, and average values were used for statistical evaluation.

Microbiological Analysis

The bacteriological quality of milk samples was evaluated using standard microbiological procedures described by Chalmers (1953) [4]. Appropriate culture media and incubation conditions recommended for dairy

microbiological analysis were used for enumeration of different microbial groups.

Standard Plate Count (SPC)

Standard Plate Count was determined to assess the overall microbial load and hygienic quality of milk samples. Serial dilution of milk samples was carried out under aseptic conditions followed by inoculation on standard plate count agar medium. After incubation, colonies were counted and results were expressed as colony forming units per millilitre (CFU/ml). SPC is widely regarded as an important indicator of sanitary quality and microbial contamination of milk (Aneja, 1992) [1].

Lactic Acid Bacteria Count (LABC)

Lactic Acid Bacteria Count was estimated to determine the population of lactose-fermenting microorganisms associated with milk souring and acid production. Excessive growth of lactic acid bacteria adversely affects the keeping quality of milk and dairy products (Sharma and Sharma, 2001) [8].

Proteolytic Bacteria Count (PBC)

Proteolytic bacteria count was determined to estimate the population of protein-degrading microorganisms capable of causing spoilage and deterioration of milk proteins. These bacteria produce extracellular proteolytic enzymes that reduce the shelf life and processing quality of milk.

Lipolytic Bacteria Count (LBC)

Lipolytic bacteria count was estimated to assess the population of fat-degrading microorganisms responsible for rancidity and development of undesirable off-flavours in milk and milk products.

Coliform Count

Coliform count was determined using selective culture media under standard incubation conditions to assess hygienic quality and possible fecal contamination of milk samples. Coliform organisms are generally considered important indicators of poor sanitary conditions and fecal contamination during milk production and handling.

Statistical Analysis

The experiment was conducted using a Completely Randomized Design (CRD) comprising four treatment groups with three animals in each treatment. Milk samples were collected repeatedly during the experimental period, and the average values obtained from repeated observations were used for statistical analysis.

The data generated during the investigation were analyzed using one-way Analysis of Variance (ANOVA) as described by Snedecor and Cochran (1994) [9]. Treatment means were compared using Duncan's Multiple Range Test (DMRT) at 5 percent level of significance.

The results were expressed as Mean $\pm$ Standard Error (SE), and differences among treatment means were considered significant at $p < 0.05$.

Results and Discussion

Standard Plate Count (SPC)

The Standard Plate Count (SPC) values of raw milk under different pre-milking udder wash treatments are presented in

Table 1. Statistical analysis revealed significant ($p < 0.05$) differences among the treatment groups. The control group (T_0) recorded the highest SPC value ($36.50 \pm 0.42 \times 10^3/\text{ml}$), whereas the lowest SPC was observed in the T_3 group ($31.70 \pm 0.42 \times 10^3/\text{ml}$), where Marigold leaf extract was used at the concentration of 60 g/L. Treatments T_1 and T_2 also showed significantly lower SPC values compared to the control group.

The reduction in SPC observed in Marigold-treated groups indicates the antimicrobial potential of Marigold leaf extract as a pre-milking udder sanitizer. The comparatively lower microbial counts observed in treated groups may be attributed to the presence of bioactive phytochemical constituents such as flavonoids, tannins, saponins and phenolic compounds present in Marigold leaves. These compounds are known to inhibit microbial growth by affecting bacterial cell membrane integrity and metabolic activity.

Among all treatments, T_3 showed the lowest SPC value and differed significantly ($p < 0.05$) from the control treatment, indicating comparatively better antimicrobial efficacy of the higher concentration treatment. Similar reductions in bacterial load following the use of herbal udder hygiene preparations have also been reported by Abdelfattah *et al.* (2018) [2] and Yadav *et al.* (2015) [10], who emphasized the importance of teat sanitation in improving the bacteriological quality of milk. The present findings are also in agreement with Murphy and Boor (2000) [6], who reported that improved udder hygiene practices significantly reduce microbial contamination in raw milk.

Lactic Acid Bacteria Count (LABC)

The Lactic Acid Bacteria Count (LABC) differed significantly ($p < 0.05$) among the treatment groups. The control group recorded the highest LABC value ($28.40 \pm 0.44 \times 10^2/\text{ml}$), while the lowest count was observed in the T_3 group ($26.30 \pm 0.44 \times 10^2/\text{ml}$). Treatments T_1 and T_2 showed intermediate values of $26.80 \pm 0.44 \times 10^2/\text{ml}$ and $27.10 \pm 0.44 \times 10^2/\text{ml}$, respectively.

Lactic acid bacteria are associated with lactose fermentation and acid production in milk, which may accelerate souring and coagulation during storage. Therefore, reduction in lactic acid bacteria may contribute toward improvement in the keeping quality and storage stability of milk.

The lower LABC values observed in Marigold-treated groups suggest inhibitory activity of Marigold phytochemicals against lactose-fermenting microorganisms. The antimicrobial activity may be associated with phenolic and flavonoid compounds present in Marigold leaves, which possess bacteriostatic and bactericidal properties.

Among the treatments, T_3 recorded the lowest LABC value and showed comparatively better antimicrobial effect than the other treatment groups. Similar antimicrobial effects of herbal extracts against spoilage microorganisms have also been reported in previous studies on dairy hygiene management.

Proteolytic Bacteria Count (PBC)

Proteolytic bacteria count (PBC) showed significant ($p < 0.05$) variation among treatment groups. The highest PBC value was recorded in the control group ($27.50 \pm 0.58 \times 10^2/\text{ml}$), whereas the lowest count was observed in the T_3

group ($21.40 \pm 0.58 \times 10^2/\text{ml}$). Treatments T_1 and T_2 recorded values of $23.60 \pm 0.58 \times 10^2/\text{ml}$ and $23.20 \pm 0.58 \times 10^2/\text{ml}$, respectively.

Proteolytic bacteria are capable of degrading milk proteins through production of extracellular proteolytic enzymes, resulting in bitterness, coagulation and deterioration of milk quality. Therefore, reduction in proteolytic bacterial population is important for improving shelf life and processing quality of milk and dairy products.

The lower PBC values observed in Marigold-treated groups indicate antimicrobial activity of Marigold leaf extract against protein-degrading microorganisms. The inhibitory effect may be due to phytochemical constituents present in Marigold leaves that interfere with bacterial growth and enzymatic activity.

Among all treatments, T_3 showed the greatest reduction in PBC and differed significantly ($p < 0.05$) from the control group, indicating comparatively better antimicrobial efficacy of higher concentration Marigold extract. Similar observations regarding reduction of spoilage microorganisms following improved udder hygiene management have also been reported by Rajorhia (2001) [7].

Lipolytic Bacteria Count (LBC)

Lipolytic bacteria count (LBC) also differed significantly ($p < 0.05$) among the treatment groups. The highest LBC value was observed in the control group ($30.20 \pm 0.33 \times 10^2/\text{ml}$), whereas the lowest count was recorded in the T_3 group ($26.60 \pm 0.33 \times 10^2/\text{ml}$). Treatments T_1 and T_2 recorded intermediate values of $28.30 \pm 0.33 \times 10^2/\text{ml}$ and $28.90 \pm 0.33 \times 10^2/\text{ml}$, respectively.

Lipolytic bacteria hydrolyze milk fat and are responsible for development of rancidity and undesirable off-flavours in milk and milk products. Therefore, reduction in lipolytic bacterial count is considered important for improving sensory quality and keeping properties of dairy products.

The lower LBC values observed in Marigold-treated groups indicate antimicrobial activity of Marigold leaf extract against fat-degrading microorganisms. The antimicrobial effect may be associated with the synergistic activity of flavonoids, tannins and phenolic compounds present in Marigold leaves.

Among all treatments, T_3 recorded the lowest LBC value and showed comparatively greater reduction in microbial count than the other treatment groups, indicating better antimicrobial efficacy of the higher concentration treatment.

Coliform Count

Coliform organisms were not detected in the analyzed milk samples during the experimental period. The absence of coliform bacteria may indicate satisfactory hygienic conditions maintained during milking and sample handling. Coliform bacteria are generally regarded as important indicators of fecal contamination and poor sanitary practices during milk production and handling. Overall, the findings of the present investigation suggest that Marigold leaf extract possesses antimicrobial potential when used as a pre-milking udder sanitizer in crossbred cows. Among all treatments, the higher concentration of Marigold leaf extract (60 g/L) showed comparatively greater reduction in microbial load and spoilage-associated bacterial populations in raw milk.

Table 1: Effect of Marigold Leaf Extract Udder Wash on Microbial Load of Raw Milk

Treatment	SPC ($\times 10^3/\text{ml}$)	LABC ($\times 10^2/\text{ml}$)	PBC ($\times 10^2/\text{ml}$)	LBC ($\times 10^2/\text{ml}$)	Coliform Count
T ₀	36.50 $\pm$ 0.42 ^a	28.40 $\pm$ 0.44 ^a	27.50 $\pm$ 0.58 ^a	30.20 $\pm$ 0.33 ^a	Nil
T ₁	33.00 $\pm$ 0.42 ^b	26.80 $\pm$ 0.44 ^b	23.60 $\pm$ 0.58 ^b	28.30 $\pm$ 0.33 ^b	Nil
T ₂	33.00 $\pm$ 0.42 ^b	27.10 $\pm$ 0.44 ^b	23.20 $\pm$ 0.58 ^b	28.90 $\pm$ 0.33 ^b	Nil
T ₃	31.70 $\pm$ 0.42 ^c	26.30 $\pm$ 0.44 ^c	21.40 $\pm$ 0.58 ^c	26.60 $\pm$ 0.33 ^c	Nil

Means bearing different superscripts within a column differ significantly ($p < 0.05$).

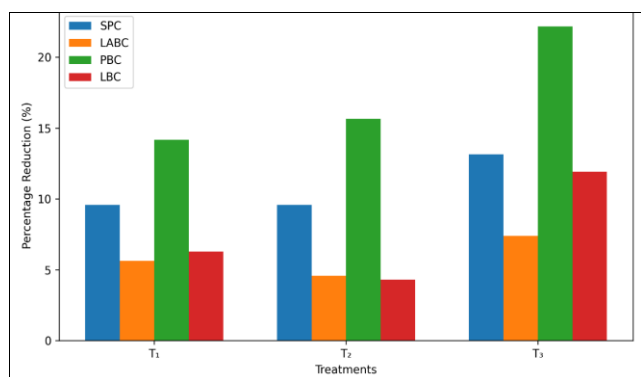
Table 2: Percentage Reduction in Microbial Counts over Control Following Marigold Leaf Extract Udder Wash Treatments

Treatment	SPC Reduction (%)	LABC Reduction (%)	PBC Reduction (%)	LBC Reduction (%)
T ₁	9.59	5.63	14.18	6.29
T ₂	9.59	4.58	15.64	4.30
T ₃	13.15	7.39	22.18	11.92

Percentage reduction was calculated using the following formula

$$\text{Reduction (\%)} = [(\text{Control} - \text{Treatment}) / \text{Control}] \times 100$$

Graphical representation of percentage reduction in microbial counts across treatment groups is shown below

**Fig 1:** Percentage reduction in microbial counts following different concentrations of Marigold (*Calendula officinalis*) leaf extract udder wash treatments in crossbred cows.

Conclusion

The present investigation demonstrated that Marigold (*Calendula officinalis*) leaf extract possesses antimicrobial potential when used as a pre-milking udder sanitizer in crossbred cows. Application of Marigold leaf extract reduced the microbial load of raw milk as evidenced by lower Standard Plate Count (SPC), Lactic Acid Bacteria Count (LABC), Proteolytic Bacteria Count (PBC) and Lipolytic Bacteria Count (LBC) in treated groups compared to the control.

Among the treatment groups, the higher concentration of Marigold leaf extract (60 g/L) showed comparatively greater reduction in microbial contamination and spoilage-associated bacterial populations. The antimicrobial activity observed in the present study may be attributed to the presence of bioactive phytochemical constituents such as flavonoids, tannins, phenolic compounds and saponins present in Marigold leaves.

Absence of coliform bacteria in milk samples may indicate satisfactory hygienic and sanitary conditions maintained

during the experimental period. The findings suggest that Marigold leaf extract has potential for use as a natural, economical and eco-friendly alternative for improving udder hygiene and clean milk production under dairy farm conditions.

However, further studies involving larger sample size, longer experimental duration and comparison with commercial chemical sanitizers are required before large-scale field recommendation.

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