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Evaluation of immunotherapeutic potential of *Ocimum sanctum* in bovine subclinical mastitis

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Abstract: Mastitis, the inflammation of mammary glands, is caused by a wide range of contagious and environmental microbes, whose clearance from the udder is generally dependent on the use of antibiotics. However, antibiotic therapy may result in development of drug-resistant microbes and depress the activity of polymorphonuclear cells. The present study was therefore undertaken to explore the immunotherapeutic potential of an herb, *Ocimum sanctum*, in mastitis in dairy cows. The study involved twenty HF × Sahiwal lactating dairy cows, each identified with at least one specific subclinical mastitis quarter as per International Dairy Federation criteria. The cows were divided into two equal groups: a control group and a group administered *O. sanctum* leaf powder at 600 mg/kg body weight daily divided into two doses orally for 7 days. The treatment could eliminate 69.23% of intramammary infections ($\chi^2 = 5.07$; $P \leq 0.5$) and resulted in a significant reduction in somatic cell count and ceruloplasmin concentration, thus subsiding udder inflammation and improving milk quality. The herb was also found to possess immunomodulation potential, as evidenced by the significant increase in phagocytic activity of milk neutrophils and enhanced lactoperoxidase and myeloperoxidase activities. Thus, the results indicated the immunotherapeutic potential of *O. sanctum* in treating bovine-specific subclinical mastitis.

Key words: Dairy cattle, mastitis, *Ocimum sanctum*, therapy, immunomodulation

1. Introduction

Mastitis, the inflammation of the udder, is caused by various contagious and environmental pathogens. Excessive and indiscriminate use of antibiotics for the clearance of these pathogens from the udder results in emergence of drug-resistant pathogens and also depresses the activity of polymorphonuclear cells, the primary cellular defenses of the udder (1). Antibiotic therapy is also disliked as it is only moderately efficacious against established mammary infections and requires prolonged milk withdrawal time (2), producing hypersensitivity syndromes in humans along with effects on the manufacturing of dairy products. To overcome the adverse effects of antibiotic use and enhancement of the animal's natural defense mechanism by use of nonspecific immunomodulators, the concept of alternative antibiotic strategies for controlling mastitis is gaining attention (3). Medicinal plants (herbs) constitute a major source of alternative medicine and are used to treat diseases of humans and animals since ancient times. Herbal medicines have gained importance due to having lower toxicity, fewer side effects, and no residues in the milk. For similar reasons, the World Health Organization has emphasized the use of medicinal plants.

Ocimum sanctum (known as tulsi) is a valuable herbal medicine being used in a wide spectrum of animal diseases. It possesses immunomodulatory and antiinflammatory properties attributed to its active constituents such as volatile oil (eugenol, 80%), flavonoids, and triterpene, which are largely responsible for its therapeutic potential (4). Singh et al. (5) observed *O. sanctum* fixed oil, alone or in combination with cloxacillin, a beta-lactamase-resistant penicillin, to be beneficial in treating bovine mastitis. Mukherjee et al. (6) reported that *O. sanctum* seed oil appeared to modulate both humoral and cell-mediated immune responsiveness, and GABAergic pathways may mediate these immunomodulatory effects. Though some information is available on the beneficial activities of *O. sanctum* in human and animal medicine, data regarding its use as an antibacterial and immunomodulator in bovine mastitis are scanty. Therefore, the present study was planned to evaluate the in vivo effectiveness of *O. sanctum* leaf powder in immunomodulation of the udder and therapy of mastitis in dairy cows.

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2. Materials and methods

2.1. Design and animals allocated

Twenty HF × Sahiwal crossbred cows kept at an organized dairy farm, found positive in at least one quarter for specific mastitis (California Mastitis Test (CMT) score of ≥1 representing >200 × 10^3 somatic cells/mL and positive for culture as per International Dairy Federation criteria), in early to middle lactation with average weight of over 400 kg and daily milk yield of above 15–20 kg, were included in the trial. The selected animals were divided randomly into two groups: a control group (n = 10) administered with placebo treatment wheat flour and a treatment group (n = 10) administered with *O. sanctum* leaf powder at 600 mg/kg body weight daily (240 g total dose) divided into two doses orally for 7 days. The dose of herb was calculated on the following observations: 1) when administered as a crude powder, the herb's dose was taken as four times the dose of herbal pure extract (7); 2) for safety reasons, the maximum dose of a chemical should not be more than 1/10 of the 50% lethal dose (LD_{50}) dose of that particular chemical; 3) the LD_{50} dose of *O. sanctum* (eugenol) pure extract was found to be 2 g/kg body weight in rats (8). Thus, to be on the safe side, its dose was taken as 1/13 of the LD_{50}, i.e. 150 mg/kg body weight. Since the herb was fed as a raw powder, it was administered at 4 times the dose of pure extract i.e. 600 mg/kg body weight × 400 kg body weight (240 g total dose). The calculated dose was given separately from the animals' daily diet and the treatment did not show any adverse effects on the health of the cows.

2.2. Sampling and parameters studied

To assess the quarter health status, milk quality, and immune status of the udder, sampling was done before treatment (day 0) and at days 7, 14, and 28 after initiation of treatment during the routine morning milking hours. Two types of milk samples, quarter foremilk and cow composite milk, were collected. During collection of milk samples, proper cleanliness and dryness of the udder were ensured. Quarter foremilk samples (about 10 mL) were collected in sterilized test tubes, and cow composite (about 80 mL) samples were collected in clean disposable plastic vials following cow milking. The milk samples were packed in an ice box, transferred immediately to the laboratory, and analyzed for various parameters as shown in Table 1.

2. 3. Analytical procedures used

2.3.1 Isolation and identification of bacteria and CMT, pH, EC, SCC, and DLC analyses

Isolation and identification of bacteria was performed as per the standard microbial procedures of the National Mastitis Council (9). The CMT was conducted and interpreted as per the method described by Pandit and Mehta (10). The results were read as negative (-), trace, one plus (+), two plus (++), and three plus (+++) depending upon the degree of gel formation. The pH of milk was recorded with the help of a digital pH meter (Systronics μpH System 361). Electrical conductivity (EC) was recorded with the help of a digital conductivity meter (Systronics Conductivity-TDS Meter 308) and the results were expressed in mS/cm. The milk somatic cell count (SCC) in milk was analyzed by direct cell counter (DeLaval) and results were expressed in ×10^3 cells/mL. Differential leukocyte count in milk was assessed as per Dulin et al. (11).

2.3.2 Phagocytic activity of milk neutrophils

2.3.2.1. Preparation of milk leukocytes and adjustment of viable cell count

The isolation of polymorphonuclear cells (PMNs) from milk was carried out as per the method of Daley et al. (12). In brief, 50 mL of milk was passed through cheese cloth. The milk was then poured into 50-mL conical tubes and centrifuged at 1200 × *g* or 2942 rpm for 30 min at 4 °C (Heraeus Biofuge Primo R Centrifuge). The fat was removed and the skim milk was poured off and discarded. The milk leukocyte pellet was washed with Hanks' balanced salt solution (HBSS, pH 7.2) and resuspended in HBSS with final cell concentration adjusted to 5 × 10^6 cells/mL. The viability of milk leukocytes was checked by Trypan blue exclusion technique (13), involving the mixture of 20 μL each of cell suspension and 0.1% Trypan blue that was kept at room temperature for 2 min. A drop of mixture was then loaded on a hemocytometer and the viable (unstained) and dead (stained) cells were counted in four WBC counting chambers. The viable cell count was expressed as:

Viable cell count = Average count per square × dilution factor × 10^4

The cell concentration in cell suspension was adjusted to 1.0 × 10^6 cells/mL following the method of Gentle and Thompson (14).

$$\text{X (mL) of HBSS added} = \frac{\text{No. of viable cells/mL}}{1.0 \times 10^6}$$

Thus, (X – 1) mL of HBSS was added to PMN suspension to get a concentration of 1.0 × 10^6 cells/mL.

2.3.2.2. Preparation of working broth of *Candida albicans*

A lyophilized culture of *C. albicans* (ATCC 2091) was revived on Sabouraud agar and a colony of it was inoculated in Sabouraud broth (6 mL) followed by overnight incubation at 37 °C. The culture suspension was then centrifuged at 400 × *g* or 1699 rpm for 5–6 min to yield a pellet that was washed twice and resuspended in HBSS to a final concentration of 10^7 organisms/mL. The viability of organisms was checked by 2 × 10^{-4} mol methylene blue.

Table 1. Parameters studied in quarter foremilk and cow composite milk.

Type of milk	Sampling days	Parameters studied
QFM	0, 7, 14, and 28	Bacterial culture, California Mastitis Test (CMT), pH, electrical conductivity (EC)
CCM	0, 7, 14, and 28	Phagocytic activity, phagocytic index, somatic cell count (SCC), differential leukocyte count, CMT, pH, EC
CCM	0, 7, and 14	Myeloperoxidase, lactoperoxidase, ceruloplasmin

QFM: Quarter foremilk, CCM: cow composite milk.

2.3.2.3. Candidacidal assay

Candidacidal assay was performed as mentioned earlier (15). The assay involves the adding of 0.2 mL each of milk leukocytes (10^6 cells), *C. albicans* (2×10^6 cells), and HBSS and 0.15 mL of fresh autologous pooled bovine serum in a test tube. This suspension was then incubated at 37 °C for 65 min with intermittent shaking, followed by the adding of 0.25 mL of methylene blue (2×10^{-4} mol) and further incubation for another 10 min. A drop from the final incubation mixture was placed on the hemocytometer to assess the phagocytic activity of milk neutrophils. The phagocytic activity was expressed by the percentage of phagocytosed neutrophils in 100 cells, and the phagocytic index was determined on the basis of units of *C. albicans* ingested by a single neutrophil, counted in 100 cells.

2.3.3 Myeloperoxidase (MPO), lactoperoxidase (LPx), and ceruloplasmin activity

MPO activity in milk leukocytes was assayed according to the method described by Bretz and Baggiolini (16). For this substrate solution was prepared by adding citric acid sodium citrate buffer (0.1 M, pH 5.5) in the presence of hydrogen peroxide (0.08 M), o-dianisidine (0.32 mM), and Triton X-100 (0.05%). The test was then initiated by adding 0.2 mL of leukocyte suspension to 2.0 mL of substrate solution (incubated at room temperature for 1.0 min) and the reaction was terminated by adding 2.0 mL of 35% perchloric acid. Absorbance was read at 560 nm in a spectrophotometer (PerkinElmer Lambda 25). The molar extinction coefficient for oxidized o-dianisidine is 20,040 ± 400.

LPx in milk was measured by the method of Marshall et al. (17). In brief, milk was diluted five times in 0.1 M acetate buffer (pH 4.5), and from this 30 µL was rapidly added to 2.95 mL of 1.0 mM ABTS in an acetic buffer in a cuvette. The baseline absorbance at 412 nm was adjusted to zero before addition of 30 µL of 10 mM hydrogen peroxide solution in acetate buffer in a spectrophotometer (PerkinElmer Lambda 25). The increase in extinction was followed for 5 min and enzyme activity was expressed as the amount of enzyme required to oxidize 1 mol ABTS/ min. The molar extinction coefficient of ABTS is 32,400 $\times 10^3$.

The estimation of ceruloplasmin level in milk was conducted by the method of Sunderman and Nomoto (18). The assay involves the adding of 0.1 mL of milk in both reaction and blank tubes containing 2 mL of acetate buffer that were then placed in a water bath (37 °C) along with a flask containing buffered PPD. After thermal equilibrium was achieved 1 mL of prewarmed buffered PPD solution was added to both the tubes and they were again placed in the water bath after proper mixing. After 5 min, 50 µL of sodium azide solution was added to the blank that was placed in the water bath after mixing the contents properly. After 30 min, 50 µL of sodium azide solution was added to the reaction tube and the contents were mixed properly. The absorbance of both tubes was read at 530 nm in a spectrophotometer (PerkinElmer Lambda 25) and calculations were made as follows: ceruloplasmin (g/L) = 0.752 ($A_R - A_B$), where A_R and A_B are absorbance of the reaction and blank, respectively.

2.3.4 Statistical analysis

All numerical data were processed via SPSS 16.0 for Windows. Analysis of parametric data was conducted by using ANOVA, and when the main effect was significant then Duncan's multiple range test was performed. The effect of treatment on elimination of intramammary infections was analyzed using the chi-square test. Significance level was set at $P \leq 0.05$.

3. Results

The effects of herbal therapy are detailed in Tables 2–6. Treatment with *O. sanctum* leaf powder could eliminate 9/13 (69.23%) of intramammary infections as compared to 4/15 (26.67%) in the control group at day 14 after treatment, a significant difference ($\chi^2 = 5.07$; $P < 0.05$; Table 2). Treatment showed a significant ($P < 0.05$) decline in CMT scores on day 14 and day 28 of treatment in comparison to the CMT score on day 0 (Table 3.). The SCC of milk in the treatment group showed significant ($P < 0.05$) reduction on day 28 in comparison to the SCC on

Table 2. Elimination of intramammary infections with herbal therapy.

Organism	Intramammary infections (IMIs)			
	Control (C)		*O. sanctum* (T)	
	Present at day 0	Eliminated at day 14	Present at day 0	Eliminated at day 14
Staphylococci	9	3	9	6
Streptococci	1	0	2	2
Corynebacterium	5	1	2	1
Overall	15	4 (26.67)	13	9 (69.23)*

C: Control group, T: treatment group.
Values in parentheses are percentages.
Significant differences existed in elimination of IMIs between treatment and control groups. *: $\chi^2 = 5.07$; 01 df; $p < 0.05$.

Table 3. Effect of therapy on inflammatory reaction of udder.

Parameter	Group	Days after initiation of treatment			
		0	7	14	28
CMT score	C	$1.65 \pm 0.17^{1,b}$	$1.60 \pm 0.10^{1,ab}$	$1.50 \pm 0.13^{1,ab}$	$1.20 \pm 0.13^{2,a}$
	T	$1.50 \pm 0.15^{1,b}$	$1.00 \pm 0.21^{1,ab}$	$0.80 \pm 0.28^{1,a}$	$0.40 \pm 0.23^{1,a}$
SCC ($\times 10^3$/mL)	C	$807.80 \pm 84.96^{1,a}$	$782.40 \pm 33.72^{1,a}$	$771.90 \pm 47.13^{2,a}$	$750.40 \pm 44.37^{2,a}$
	T	$762.60 \pm 89.28^{1,b}$	$564.10 \pm 95.09^{1,ab}$	$556.90 \pm 102.36^{1,ab}$	$394.30 \pm 83.91^{1,a}$
EC (mS/cm)	C	$6.59 \pm 0.30^{1,a}$	$6.58 \pm 0.09^{2,a}$	$6.41 \pm 0.08^{1,a}$	$6.14 \pm 0.12^{2,a}$
	T	$6.42 \pm 0.30^{1,b}$	$6.03 \pm 0.23^{12,ab}$	$5.87 \pm 0.25^{1,ab}$	$5.44 \pm 0.28^{1,a}$
pH	C	$6.84 \pm 0.03^{1,b}$	$6.80 \pm 0.01^{1,ab}$	$6.79 \pm 0.02^{1,ab}$	$6.75 \pm 0.01^{1,a}$
	T	$6.84 \pm 0.03^{1,b}$	$6.76 \pm 0.02^{1,a}$	$6.74 \pm 0.02^{1,a}$	$6.72 \pm 0.02^{1,a}$

C: Control group, T: treatment group.
Values having at least one common superscript (letters within rows and numbers within columns) do not differ significantly at $P < 0.05$.

Table 4. Differential leukocyte count in control (C) and treatment groups (T).

Parameter	Group	Days after initiation of treatment			
		Day 0	Day 7	Day 14	Day 28
Neutrophils (%)	C	$51.30 \pm 2.79^{1,a}$	$53.60 \pm 2.87^{1,a}$	$51.30 \pm 3.00^{2,a}$	$44.50 \pm 3.09^{2,a}$
	T	$50.30 \pm 2.57^{1,c}$	$55.70 \pm 2.54^{1,c}$	$38.40 \pm 2.35^{1,b}$	$24.10 \pm 2.72^{1,a}$
Lymphocytes (%)	C	$15.20 \pm 0.42^{1,a}$	$15.30 \pm 0.30^{1,a}$	$16.20 \pm 0.66^{1,ab}$	$17.30 \pm 0.70^{1,b}$
	T	$16.20 \pm 0.61^{1,a}$	$15.40 \pm 0.85^{1,a}$	$16.80 \pm 0.53^{1,ab}$	$18.30 \pm 0.72^{1,b}$

Values having at least one common superscript (letters within rows and numbers within columns) do not differ significantly at $P < 0.05$.

Table 5. Phagocytic activity and phagocytic index in control (C) and treatment groups (T).

Parameters	Group	Days after initiation of treatment			
		Day 0	Day 7	Day 14	Day 28
Phagocytic activity (%)	C	$15.30 \pm 0.70^{1,a}$	$16.30 \pm 0.47^{1,a}$	$16.50 \pm 0.67^{1,a}$	$16.80 \pm 0.66^{1,a}$
	T	$17.10 \pm 0.64^{1,a}$	$29.10 \pm 1.62^{2,c}$	$28.80 \pm 1.20^{2,c}$	$24.00 \pm 1.34^{2,b}$
Phagocytic index	C	$1.07 \pm 0.02^{1,a}$	$1.09 \pm 0.03^{1,a}$	$1.10 \pm 0.03^{1,a}$	$1.10 \pm 0.03^{1,a}$
	T	$1.10 \pm 0.03^{1,a}$	$1.58 \pm 0.09^{2,c}$	$1.22 \pm 0.04^{2,ab}$	$1.31 \pm 0.07^{2,b}$

Values having at least one common superscript (letters within rows and numbers within columns) do not differ significantly at $P < 0.05$.

Table 6. Myeloperoxidase (MPO), lactoperoxidase (LPx), and ceruloplasmin levels in control (C) and treatment groups (T).

Parameters	Group	Days after initiation of treatment		
		Day 0	Day 7	Day 14
MPO (µmol/min)	C	$437.325 \pm 16.75^{1,a}$	$423.15 \pm 9.95^{1,a}$	$412.28 \pm 7.09^{2,a}$
	T	$452.910 \pm 11.89^{1,b}$	$717.4 \pm 50.06^{2,c}$	$228.91 \pm 25.53^{1,a}$
LPx (µmol/min)	C	$0.300 \pm 0.01^{1,a}$	$0.293 \pm 0.01^{1,a}$	$0.287 \pm 0.01^{2,a}$
	T	$0.294 \pm 0.01^{1,b}$	$0.378 \pm 0.00^{2,c}$	$0.173 \pm 0.00^{1,a}$
Ceruloplasmin	C	$0.139 \pm 0.01^{1,a}$	$0.137 \pm 0.01^{2,a}$	$0.135 \pm 0.01^{2,a}$
(g/L)	T	$0.137 \pm 0.01^{1,b}$	$0.061 \pm 0.01^{1,a}$	$0.065 \pm 0.01^{1,a}$

Values having at least one common superscript (letters within rows and numbers within columns) do not differ significantly at $P < 0.05$.

day 0, as presented in Table 3. A similar trend was also observed with pH and EC values in the treatment group. The ceruloplasmin concentration in the treatment group decreased significantly ($P < 0.05$) to 0.061 ± 0.01 on day 7 from 0.137 ± 0.01 on day 0 (Table 6). During the course of investigation, i.e. day 0 to day 28, the mean neutrophil percentage remained high, although it decreased from $51.30 \pm 2.79\%$ to $44.50 \pm 3.09\%$ and the lymphocyte percentage increased from $15.20 \pm 0.42\%$ to $17.30 \pm 0.70\%$ in the control group, indicating the presence of some subclinical mastitis in the herd (Table 4).

In *O. sanctum*-treated cows, a significant ($P < 0.05$) increase was observed in phagocytic activity (Figure) at day 7 of treatment, and it remained significantly elevated throughout the course of the study as compared to day 0, although it showed some decreasing trends from day 14 to day 28 of treatment (Table 5).The phagocytic index was seen to be decreased at day 14 and day 28 as compared to day 7, but still it was a significant change as compared to day 0.

The MPO and LPx activities were significantly ($P < 0.05$) augmented in the treatment group at day 7 and decreased significantly ($P < 0.05$) at day 14 as compared to day 0 (Table 6).

4. Discussion

Mastitis is one of the most costly diseases resulting in huge global economic losses, including in India. The use of antibiotics as the mainstay of treating this problem instigates public health hazards due to the persistence of antibiotic residues in the milk and development of drug-resistant bacterial pathogens. To lessen the adverse effects of antibiotics, a different approach of preventing mastitis, i.e. *O. sanctum* administration, was evaluated in vivo for its therapeutic potential in specific subclinical mastitis of dairy cows.

The differences in elimination of intramammary infections in the treatment vs. the control group were observed to be statistically significant ($P < 0.05$) with a chi-square value of 5.07 (Table 2). Several studies have evaluated different herbs and indicated beneficial effects of herbal remedies against bovine mastitis (19,20). Baskaran et al. (21) evaluated the antimicrobial effect of plant-derived antimicrobials, including carvacrol, eugenol,

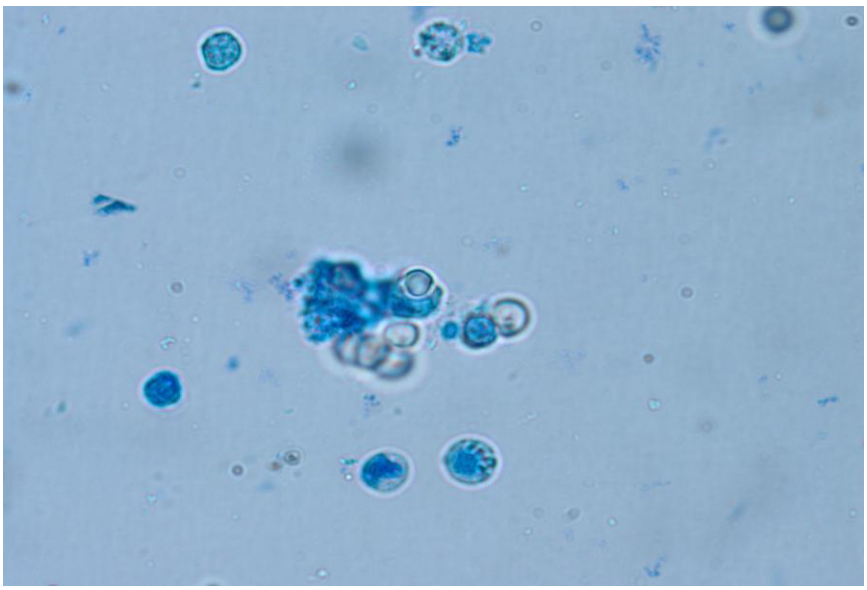

Figure. Phagocytosis of *C. albicans* (arrow) by PMN cell. Under oil immersion, 100× magnification, staining with methylene blue.

thymol, and *trans*-cinnamaldehyde, and concluded that all 4 ingredients exhibited antimicrobial activity against the 5 mastitis pathogens tested. However, due to the differences in active compounds of different herbs, the efficacies may differ.

There was a significant reduction in SCC, CMT score, and acute phase protein (ceruloplasmin), suggesting the antiinflammatory action of the herb. Mastitis results in increased somatic cells, which are reflective of udder inflammation, and there is also an increase in acute phase protein due to the high permeability of the blood–milk barrier due to inflammatory changes in the udder (22). The significant decline in CMT, SCC, pH, and EC in the present study are in agreement with Gupta (23), who administered oral herbal powder mix containing *O. sanctum* to animals affected with subclinical mastitis. Mukherjee et al. (6) also reported that intramammary use of aqueous extract of *O. sanctum* leaves in bovine subclinical mastitis resulted in significant decreases in CMT and SCC.

Neutrophils, the first line of defense against invading pathogens, are recruited earliest among other leukocytes to the site of infection and are also important host defense mechanisms in mastitis by virtue of their phagocytosis and intracellular killing (24). Any imbalance in their effective functioning can result in the flourishing of pathogens in the udder, resulting in mastitis. This imbalance can be kept in check by enhancing phagocytic potential and migration of PMNs from the circulation to an inflamed udder that helps in removal of infective agents from the udder. In the present study, the significant increases in phagocytic activity/phagocytic index and subsequent elimination of intramammary infection suggest that the herb has immunomodulatory action, a finding in agreement with Rastogi et al. (25).

MPO, a constituent of oxygen-dependent antimicrobial activity of leukocytes, and LPx, a component of the lactoperoxidase system along with thiocyanate and hydrogen peroxide, serve as effective antimicrobial weapons against many infective pathogens. Their activity is expected to increase with an increase in SCC in milk and is directly correlated with the level of the SCC (26). The activity of both MPO and LPx was significantly increased in the present study, suggesting the positive effect of *O. sanctum* on the activity of leukocytes, which is also supported by the results of significant elimination of infection and decrease in SCC. Our findings are in conformity with the findings of Rastogi et al. (25), who observed that aqueous extract of *O. sanctum* in the case of mastitis enhanced the lysosomal enzyme contents of the milk PMNs. The results of enhanced phagocytosis, LPx, and MPO activities could be attributed to antioxidant properties, enhanced milk leukocyte activity (i.e. hydrogen peroxide production), and increased activity of the lysosomal enzymes conferred by the immunomodulatory properties of *O. sanctum*.

In conclusion, the data obtained from the present study indicate beneficial effects of herbal therapy against subclinical mastitis in lactating dairy cows. The positive effects of these remedies may be related to the presence of lipophilic constituents in herbs that have antibacterial, antiinflammatory, and immunomodulation potential as substantiated by elimination of intramammary infections,

decrease of milk SCC and ceruloplasmin activity, and enhanced phagocytosis, LPx, and MPO activities of milk leukocytes in the present study.

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