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An *in vivo* assessment of a non-antibiotic therapy for specific subclinical mastitis in dairy cows

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Abstract
Dairy sector is growing exponentially and the biggest challenge to it is subclinical form of mastitis, which causes huge economic losses. The present study aims at controlling subclinical mastitis in dairy cows in a holistic way. The study was carried out in 24 HF × Sahiwal crossbred cows at an organized dairy farm. The selected animals were divided randomly into two groups of 12 cows each. The cows in Group 1 served as control. The cows in Group 2 were treated with commercial non-antibiotic preparation (Magic-3) i.e. 100 ml P.O., b.i.d. × 7 days. The culture examination of milk was done in quarter foremilk samples on d 0, 15 and 30 while Electrical Conductivity (EC), California Mastitis Test (CMT), Somatic Cell Count (SCC), NAGase enzyme activity and phagocytic activity were studied in cow composite milk samples on d 0, and d 7, 15, 30, 60 and 90 post initiation of the treatment. There was 70.73% and 80.49% elimination on d 15 and d 30, respectively post-treatment with commercial herbal preparation. The values of EC, CMT, SCC, NAGase enzyme activity and phagocytic activity declined significantly (p<0.05) post-initiation of treatment and remained within normal range. Hence, the findings of present study indicate therapeutic and protective nature of herbal medicine.

Keywords: Herb, non-antibiotic, cow, subclinical, mastitis

Introduction
Mastitis, inflammation of parenchyma of mammary glands, is generally classified into clinical and subclinical forms. Subclinical mastitis is the most serious form as the infected animal exhibits no obvious symptoms. Milk, being apparently normal for a long period serves as source of infection in the herd [1]. Subclinical mastitis is 3–40 times more common than clinical mastitis and causes the greatest overall losses in most dairy herds [2]. Annual economic losses due to subclinical and clinical mastitis in India have been estimated to the tune of Rs. 4151.16 and Rs. 3014.35 crores, respectively with a total of Rs. 7165.51 crores [3]. Indian Dairy Association has declared "Knowledge of routine physical examination of udder and diagnostic screening tests for early detection of mastitis and proper treatment of affected animal is of paramount importance in order to minimize losses encountered due to sub clinical as well as clinical mastitis." Most clinical cases start as subclinical; thus, controlling subclinical mastitis is the best way to reduce the clinical cases. The incidence level of subclinical mastitis in various parts of the country ranged from 11.51 to 23.55%, 3.94 to 17.25% [4] in crossbred cows, local cows, respectively. The only therapy for subclinical mastitis at present is antibiotic treatment. The overuse and/or indiscriminate use of such antibiotics have caused havoc by producing resistance in the pathogens [5]. A serious outcome of the use of antibiotics in milk is their effect on the manufacture of dairy products and the development of sensitivity syndromes in human beings. A total of 60% violations with respect to drug residues in milk take place due to mastitis treatment and the relative risk of drug residues in milk increases to 7.1 fold for SCC > 700, 000 cells/ml.
To overcome this issue, the concept of using non-antibiotic strategies for controlling mastitis is gaining more attention. One possible approach to control mastitis involves manipulation of host defense mechanism. Hence, current strategies targeting to improve the immunity of the diseased udder during immunosuppressive stages would certainly give impetus to resistance ability of the animal against pathogens. World Health Organization (WHO) has also emphasized on the use of medicinal plants, as they are cheaper, safer and effective than the synthetic drugs. Therefore, the present study was planned to evaluate the *in vivo* effectiveness of a commercial herbal product in the treatment and prevention of mastitis in dairy cows.

Materials and Methods

The study was carried out in HF × Sahiwal crossbred cows at an organized dairy farm near Talwandi Khurd, Ludhiana. The farm followed the practice of semi loose housing system. The lactating cows were screened for any evidence of clinical mastitis (udder/ milk examination), and a complete history, particularly with respect to antibiotic treatment of animal if any during the last 21 days, was noted. The cows found positive in at least one quarter for specific mastitis (California Mastitis Test (CMT) score of ≥1 representing >200 × 10^3somatic 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 of 12 cows each. The cows in Group 1 (n = 12) were kept as control. The cows in Group 2 (n = 12) were treated with commercial non-antibiotic preparation (Magic-3* comprising of *Glycyrrhiza glabra, Curcuma longa, Tinospora cordifolia, Psoralea Corylifolia, Argemone Mexicana, Asperagus racemosus, Vermonia anthemintica, Emblica officinalis,* neem ext., citric acid, Vitamin A, Vit. D_3) i.e. 100 ml P.O., b.i.d. × 7 days.

Sampling was done pre treatment (day 0) and at days 7, 15, 30, 60 and 90 post initiation of treatment during the routine morning milking hours to assess the quarter health status, milk quality, and immune status of the udder. 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, analyzed for milk culture and California mastitis test (CMT). Cow composite samples (about80 ml) were collected in clean disposable plastic vials following cow milking which were analyzed for Somatic cell count (SCC), CMT, EC, pH, NAGase enzyme activity and phagocytic activity of milk polymorphonuclear (PMN) cells. The milk samples were packed in an ice box, transferred immediately to the laboratory, and analyzed for various parameters. The culture examination of milk was done on d 0, 15 and 30 while other parameters were studied on d 0, and d 7, 15, 30, 60 and 90 post initiation of the treatment.

Isolation and identification of bacteria was performed as per the standard microbial procedures [6]. The CMT was conducted and interpreted as per the method described by Pandit and Mehta [7]. The results were read as negative (-), trace, one plus (+), two plus(++), and three plus (+++) depending upon the degree of gel formation. The electrical conductivity was recorded using Digital Conductivity Meter (Mettler Toledo, Five Easy Plus). The results were expressed in milli Siemens per cm (mS/cm). The analysis of milk samples for SCC was done using milk somatic cell counter from DELTA Instrument, BV Kelvinlaan 3, 9207 JB Drachtenand results were expressed in ×10^3cells/ml. The NAGase enzyme activity in milk was analyzed by fluorometric microplate reader (Fluoroscan Ascent FL, Thermo scientific make) and results were expressed in nMoles/ml/min [8]. Phagocytic activity of milk PMNs was carried out using the same method as described elsewhere [9].

All numerical data were processed via SPSS 20.0. 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 $P \leq 0.05$.

Results and Discussion

Effect of therapy on intramammary infections (IMI)

The effect of herbal therapy on quarter infection level with commercial herbal preparation as compared to control is presented in Table 1.Thetherapy with commercial preparation could eliminate, in overall, 29/41 (70.73%) of IMI at d15 and 33/41 (80.49%) at d30 post-treatment. The corresponding elimination of infection in control group was 12/43 (27.90%) and 13/43 (30.23%) at d15 and d30, respectively. The variation in the elimination of intramammary infections in treatment group was observed to be statistically significant ($p<0.05$) in commercial preparation on d15 and d 30 (Table 1). The present findings are in line with Acharya *et al.* [10] who found that Immu-21 alone was found to be effective in 60% of subclinical mastitis cases and it was effective in 100% of the cases when used with antibiotics. A recovery in 75% of mastitis cases was observed using Bonmilk, a polyherbal mix. Most of the quarters started producing normal milk after 6 days onwards post-therapy [11]. Use of herbal spray in subclinical mastitis cases reported elimination of 58.33% of intramammary infections as compared to 23.81% in control group at d 7 post-treatment [12]. Similarly, therapy of specific subclinical mastitis with *Ocimum sanctum* leaf powder at 600 mg/kg body weight daily divided into two doses orally for 7 days could eliminate 69.23% of intramammary infections ($\chi 2 = 5.07$; $P \leq 0.05$) [9]. Cows treated with 5 gr. of a standardized fluid extract of *Spirea ulmaria* L. and 6 gr. of standardized extract of *Astragalus* for the control of bovine subclinical mastitis during lactation showed a significant reduction in infected quarters from d 0 to d 56 as 32.7 to16.7% in treated group as compared to 35.4 to 30.2% in control group [13].

Effect of therapy on inflammatory reaction of udder

Therapy with commercial herbal preparation significantly reduced electrical conductivity (EC) on d 15 in comparison to d 0. The values of EC remained within normal range upto d 90. Therapy exhibited a significant ($p<0.05$) decline in CMT score on d 7 (0.5±0.19, $P<0.05$) of treatment in comparison to the CMT score on d 0 (Table 2). The SCC of milk in this group showed significant ($p<0.05$) decline on day 7 (156.92±34.19 x 10^3 cells/ml) in comparison to the SCC on day 0 (589.33±65.85× 10^3 cells/ml). The present findings are in agreement with Sharma (2010) who reported a significant reduction in CMT and SCC post treatment in subclinical mastitis-affected animals treated with oral administration of herbal powder mix. Similar study found that poly herbal supplementation reduced the incidence of subclinical and clinical mastitis and improved udder health, milk yield, and milk quality [14].Gupta [15] observed a significant reduction in CMT, SCC post treatment in subclinical mastitis-affected animals treated with oral administration of herbal powder mix containing *O. sanctum* and *W. somnifera*. A significant reduction in SCC and consequently enhanced milk yield after treatment with oral supplementation of phytobiotics-rich herbal mixtures was observed in subclinical mastitis affected animals [16].

A significant decline in NAGaseenzyme activity was observed from d 7 (130.65±20.85, $P<0.05$) to d 90 (73.2±0.28) of treatment in comparison to d 0 (Table 2). No literature was found in support of our study, however, Pyorala [17] found that NAGaseenzyme is one of the indigenous enzymes which increase in milk during inflammation and

originates from phagocytes. NAGase activity was very low in the milk of healthy quarters, increased in subclinical mastitis and was highest in quarters with clinical mastitis [18]. During mastitis, NAGase, a member of enzymes stemming from phagocytes increases exponentially and its activity was found to be reliable for the detection of mastitis pathogen induced IMI [19]. The levels of EC, CMT, SCC and NAGase enzyme activity remained within normal range even after 90 days of the treatment which indicate preventive nature of herbal therapy.

Phagocytic activity and Phagocytic Index (PI)

Phagocytic activity was expressed by the % phagocytosed neutrophil in 100 cells and the phagocytic index was determined by the unit of *C. albicans* ingested by single neutrophil, counted in 100 cells. The mean phagocytic activity in G1 (control) did not differ significantly throughout the course of study (Table2). In G2 (commercial preparation treated) cows, a significant increase ($P<0.05$) was observed in phagocytic activity at d7 of treatment, and it remained significantly elevated throughout the course of study as compared to d0. The phagocytic index (PI) in G1 (control) did not vary statistically during the period of study. The treatment with commercial preparation showed a significant increase in the phagocytic index ($P<0.05$) between d7 to d90 of treatment in comparison to d0. The phagocytic index was seen somewhat decreased at d30 and d90 as compared to d7 but still it was a significant change as compared to d0. No significant differences were seen in PI of milk leukocytes between control (G1) and treatment (G2) groups in pre-treatment phase (d0). But, a significantly higher index ($P<0.05$) was observed in the treatment group as compared to that in control group at d7 to d90 of treatment. Similarly, treatment of subclinical mastitis with dried stem powder of *Tinospora cordifolia* (100 mg/ kg BW) showed a significant increase ($P<0.05$) in the values of total serum immunoglobulin and mean phagocytic index [20]. Increased lymphocyte count and augmented phagocytic activity of milk PMNs was reported after treatment with sterile 250 mg of a polysaccharide fraction of *T. cordifolia* via intramammary route twice daily for five consecutive days in animals affected with subclinical mastitis [21]. The ameliorative efficacy of *T. cordifolia* in subclinical mastitis could be attributed to the increased migration and phagocytic activity of PMNs in the udder [22]. An increase in immune modulation and microbicidal activity was seen in bovine mastitis using the herbal product (Immu-21) containing *T. cordiofolia* [23]. Oil extract of *Ocimum sanctum* with *Azardichta indica* and aqueous stem extract of *Tinospora cordiofolia*, as an intramammary infusion in subclinical mastitis showed immune potentiating activity signified by enhanced milk PMN cell phagocytosis [24].

Table 1: Elimination of intramammary infections with herbal therapy

Organism	Control (G1)			Commercial preparation (G2)		
	d 0	d 15	d 30	d 0	d 15	d 30
Coagulase-positive staphylococci	30	8	8	32	22	25
Coagulase-negative staphylococci	7	3	3	4	3	3
Corynebacteria spp.	6	1	2	2	2	2
Bacilli spp.	_	_		3	2	3
Overall	43	12 (27.90)	13 (30.23)	41	29 (70.73)*	26 (63.41)#

Figures in parentheses indicate percentage

Significant differences existed in elimination of IMI between treatment and control groups * ($\chi2 = 15.40$; 01df; $p<0.01$),#($\chi2 = 9.29$; 01df; $p<0.01$)

Table 2: Changes in inflammatory markers of udder infection, phagocytic activity and index post-administration of treatment

Parameters	Group	Days post-administration of treatment					
		d 0	d 7	d 15	d 30	d 60	d 90
EC (mS/cm)	G1	6.25 ± 0.26^{a}	6.34 ± 0.21^{b}	6.31 ± 0.24^{c}	5.97 ± 0.33^{d}	5.83 ± 0.31^{e}	5.74 ± 0.30^{f}
	G2	6.79 ± 0.23^{a}	$6.13\pm0.07^{b,c}$	$4.75\pm0.14^{a,c,d}$	$4.19\pm0.17^{a,c,d,e}$	$4.1\pm0.22^{a,c,d,e,f}$	$4.06\pm0.21^{a,c,d,e,f}$
CMT point score	G1	2.25 ± 0.13^{a}	1.83 ± 0.17^{b}	1.83 ± 0.21^{c}	1.58 ± 0.31^{d}	1.58 ± 0.31^{e}	1.75 ± 0.35^{f}
	G2	2.42 ± 0.15^{a}	$0.5\pm0.19^{b,c}$	$0\pm0^{a,c,d}$	$0\pm0^{a,c,d,e}$	$0\pm0^{a,c,d,e,f}$	$0\pm0^{a,c,d,e,f}$
SCC (×103/ml)	G1	794.33 ± 60.35^{a} (503-1075)	712.92 ± 64.41^{b} (390-940)	663.92 ± 76.9^{c} (247-1125)	624.58 ± 96.6^{d} (200-1167)	604.17 ± 83.16^{e} (141-981)	624.08 ± 84.9^{f} (161-1028)
	G2	589.33 ± 65.85^{a} (225-897)	$156.92\pm34.19^{b,c}$ (17-396)	$67.92\pm18.18^{a,c,d}$ (18-244)	$63.33\pm19.54^{a,c,d,e}$ (19-261)	87.42±26.35 (19-319)	$97.75\pm27.42^{a,c,d,e,f}$ (19-339)
NAGase(nMoles/ml/min)	G1	298.89 ± 48.07^{a}	218.72 ± 35.35^{a}	159.94 ± 20.16^{a}	174.94 ± 21.9^{a}	156.59 ± 17.54^{a}	185.5 ± 23.65^{a}
	G2	148.78 ± 17.22^{a}	$82.21\pm7.16^{a,b}$	$75\pm0.59^{a,b,c}$	$75.06\pm0.74^{a,b,c,d}$	$75.29\pm0.72^{a,b,c,d,e}$	$74.91\pm0.76^{a,b,c,d,e}$
Phagocytic Activity (%)	G1	8.5 ± 0.86^{a}	10.5 ± 0.96^{a}	10.83 ± 1.11^{a}	10 ± 0.9^{a}	9.75 ± 0.92^{a}	10.5 ± 1.49^{a}
	G2	12.42 ± 2.09^{a}	$27.17\pm2.66^{a,b}$	$33.58\pm2.11^{a,b,c}$	$31.42\pm2.17^{a,b,c,d}$	$27.67\pm1.86^{a,b,c,d,e}$	$26.08\pm3.22^{a,b,c,d,e}$
Phagocytic Index	G1	1.07 ± 0.03^{a}	1.03 ± 0.01^{a}	1.07 ± 0.02^{a}	1.08 ± 0.02^{a}	1.06 ± 0.03^{a}	1.08 ± 0.04^{a}
	G2	1.01 ± 0.01^{a}	$1.48\pm0.05^{a,b}$	$1.31\pm0.03^{a,b,c}$	$1.26\pm0.03^{a,b,c,d}$	$1.22\pm0.03^{a,b,c,d,e}$	$1.18\pm0.02^{a,b,c,d,e}$

The values having at least one same superscript (alphabets within row) differ significantly ($p<0.05$)

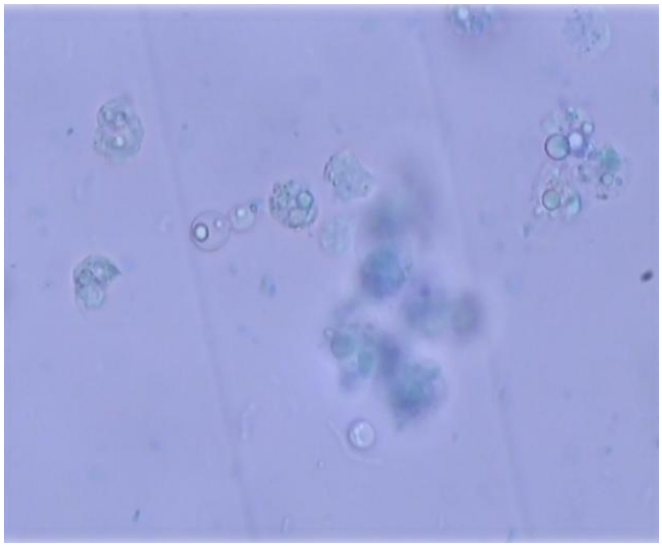

Fig 1: Phagocytic activity of milk leucocytes against *C. albican* (100X Oil emulsion)

Conclusion

The findings of present study indicate therapeutic and protective nature of herbal medicine in contrast to antibiotic treatment. The positive effects of this therapy may be attributed to the presence of lipophilic constituents of herb with anti-bacterial and anti-inflammatory action. Immuno-therapeutic potential of this therapy is substantiated by elimination of intramammary infection, decrease of milk EC, SCC and NAGase enzyme activity and enhanced phagocytic activity of milk PMNs in the present study. These alternative medicines which are cheaper, safer and effective can be of much importance in managing subclinical mastitis in dairy animals. Besides, they can also be tried in clinical mastitis to reduce the days in treatment with antibiotics and ill effects of the treatment.

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