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Immuno-therapeutic evaluation of *Withania somnifera* in bovine mastitis

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ABSTRACT

Mastitis is a severe problem of diary animals, and concept of non-antibiotic strategies for its prevention is gaining attention. An *in vitro* antibacterial evaluation of *Withania somnifera* against bovine mastitis pathogens, followed by its *in vivo* therapeutic trial was carried out in 20 HF × Sahiwal dairy cows with at least one specific subclinical mastitis quarter. *W. somnifera* root extract used in this study showed *in vitro* antibacterial activity of 78.19% and 55.67% against *Staphylococcus aureus* and *Escherichia coli* respectively. The treatment group was administered orally *W. somnifera* root powder @ 500 mg/kg body weight daily, divided into two doses for 7 days, and was evaluated for elimination of intramammary infections, subsiding of udder inflammation and immunomodulation. The therapy could eliminate 64.28% of intramammary infections ($c^2 = 4.14$), and resulted in a significant reduction in somatic cell count and ceruloplasmin concentration, therefore, subsiding udder inflammation and improving milk quality. Immunomodulation potential of the herb was evident from significant increase in phagocytic activity/phagocytic index along with enhanced lactoperoxidase and myeloperoxidase activities in the milk of treated cows.

Keywords: Immunomodulation, Mastitis, Therapy, *Withania somnifera*

Mastitis, the inflammation of udder, results in the reduction of milk yield and quality, thereby causing annual economic losses estimated to ₹ 7165.51 in India (Bansal and Gupta 2009). Mastitis is mainly caused by microbial pathogens, and their clearance from the bovine udder requires the effectiveness of the drug along with optimum functioning of the immune cells (Shafi *et al.* 2016), which depends largely till date on the use of antibiotics. Indiscriminate use of antibiotics leads to resistance in pathogens, impaired manufacture of dairy products and the development of hypersensitivity syndromes in human beings. Furthermore, the antibiotics used for the treatment of mastitis depress the activity of the polymorphonuclear cells (PMNs) that are considered primary cellular defences of the mammary gland (Hoeben *et al.* 1997). Also due to milk quality regulation to reduce the potential risk of milk contamination to the consumer, there is a need to reduce the frequency of antibiotic treatment of mastitis cases.

For this reason, the concept of using non-antibiotic strategies for controlling mastitis is gaining attention. One such strategy is based on enhancement of the animal's natural defence mechanism by use of non-specific immuno-modulators such as plant materials (Shafi *et al.* 2016). Medicinal plants (herbs) constitute a major source of alternative medicine and are used to treat diseases of man and animal since ancient times. The herbal medicine being organic in nature has gained importance due to their lesser toxicity or side effects and World Health Organization (WHO) has emphasized their use in comparison to the synthetic drugs. Roots, leaves and whole plant of *Withania somnifera* (Ashwagandha) are used for medicinal purposes in Ayurvedic and indigenous medicinal system because of its adaptogenic, astringent, antibacterial, anti-inflammatory, antioxidative, antistress, antitumor, hemopoietic and immunomodulatory properties (Mishra *et al.* 2000, El-Boushy *et al.* 2009). The data regarding its use as antibacterial and immuno-modulator in bovine mastitis is scanty. Therefore, the present study was planned to evaluate the *in vitro* and *in vivo* effectiveness of *W. somnifera* in the treatment of bovine specific mastitis and improvement of milk quality.

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MATERIALS AND METHODS

In vitro antibacterial activity of root extract against mastitis pathogens: *W. somnifera* (Ahswagandha) roots were collected locally and shade dried in the laboratory followed by drying in the incubator at 40°C to remove any excess moisture. The dried material was ground in a Willey Grinder (Arthur H. Thomas type) at room temperature, and the extraction was performed as discussed elsewhere (Shafi *et al.* 2018b). The percent recovery of extract obtained from the herb was recorded on dry weight (w/w) basis. The dilution representing herbal extract concentration of 500 mg/ml showed appreciable zone of inhibition and was chosen as test dilution for evaluating antibacterial activity of the herb. *Staphylococcus aureus,* coagulase negative

staphylococci (CoNS), *Corynebacterium* spp. *E. coli, Klebsiella* spp., *Pseudomonas* spp. *and Streptococcus* spp., isolated from natural cases of bovine subclinical mastitis were used for testing antibacterial activity of *W. somnifera*. The organisms were isolated as per standard microbial procedures of National Mastitis Council (2004). Five colonies of the freshly inoculated bacteria were emulsified in 5 ml of sterilized nutrient broth and incubated overnight at 37°C. The tubes showing marked turbidity represented stock inoculums and were kept in refrigerator at 4°C till further use. *In vitro* antibacterial activity was carried out as discussed elsewhere (Shafi *et al.* 2018b). Enrofloxacin showed good antibacterial activities against all the tested pathogens and was selected for comparing the antibacterial activity of the herbal extract. Eight replicates were prepared for each bacterial isolate. The antibacterial activity of herbal extract was determined as percent inhibition of bacterial growth calculated from the zones of inhibition produced by the herbal extract and standard antibiotic (enrofloxacin in this study) using the method suggested by Vidyasagar *et al.* (2002). The MIC of the extract was evaluated by the tube dilution method described earlier (Shafi *et al.* 2016). Serial dilutions of the extract were prepared starting from 250 mg/ml dilution with the help of tween-20. The confirmation of MIC was done by sub culturing of serial dilutions on nutrient agar petri plates as per Shafi *et al.* (2016).

In vivo therapeutic potential of herbs: Twenty cows (HF × Sahiwal crossbred; average weight 400 kg and day milk yield of 15–20 kg) found positive for specific mastitis (CMT score ≥ 1 or SCC > 400 ×10^3 cells/ml) in at least one quarter, in early to mid-lactation were included in trial as per the guidelines of International Dairy Federation.

The selected animals were divided randomly into two groups of 10 each and subjected to therapy as follows:

Group 1 - control (n = 10): administered with placebo treatment (wheat flour).

Group 2 - (n = 10): treated with *W. somnifera* crude root powder @ 500 mg/kg body weight, i.e. 200 g total dose orally, divided in two parts morning and evening × 7 days.

The dose of herb was calculated on the basis of following facts. The dose of crude powder was taken as four times the dose of herbs pure extract (Wynn and Fougere 2007). On the basis of pharmacokinetic studies, Wynn and Fougere (2007) recommended *W. somnifera* @ 125 mg/kg body weight (pure extract) for small ruminants. The same dose was taken in cows, and herb being used as crude powder was administered at 4 times, i.e. @ 500 mg/kg body weight (200 g total dose).

Sampling and parameters studied: To assess the quarter health status, milk quality and immune status of udder, sampling was done pre-treatment (d 0), and at d 7, 14 and 28 post-initiation of treatment. Quarter foremilk (QFM) and cow composite milk (CCM) were collected during the routine morning milking after proper cleanliness and sterilization of teats with a cotton swab soaked in 70% absolute alcohol. First few streaks of milk were discarded and about 10 mL of the individual QFM samples were collected in sterilized test tubes, followed by collection of about 80 mL of bucket milk (CCM) samples in clean disposable 100 ml plastic vials. The milk samples were immediately transported to the laboratory in ice box and analyzed for various parameters.

Analytical procedures used: Isolation and identification of bacteria was performed on QFM samples as per the standard microbial procedures of National Mastitis Council (2004). California Mastitis Test was conducted on CCM samples and interpreted as per standard method described by Pandit and Mehta (1969). The results were read as negative (-), trace, one plus (+), two plus (++) and three plus (+++) depending upon the degree of gel formation. The *p*H of milk was recorded with the help of digital pH meter (Systronics μpH System 361). The electrical conductivity (EC) was recorded with the help of Digital Conductivity Meter (Systronics Conductivity-TDS Meter 308). The results were expressed in milli Siemens per cm (mS/cm). The somatic cell count (SCC) in milk was analyzed by DeLaval Direct Cell Counter (DCC) and results were expressed as × 10^3 cells/ ml.

Phagocytic activity of milk neutrophils: The phagocytic activity of milk neutrophils (CCM) was evaluated using *Candida albicans* (ATCC 2091) following earlier described protocol (Shafi *et al.* 2016). The isolation of polymorphonuclear leukocytes (PMNs) from milk samples and their viability (Viable cell count = Average count per square × dilution factor × 10^4) was checked by Trypan blue exclusion technique. The cell concentration in cell suspension was adjusted to 1.0 × 10^6 cells/ml and Candidacidal assay was performed as described earlier (Shafi *et al.* 2016). Phagocytic activity was expressed by the % phagocytosed neutrophils in 100 cells and phagocytic index was determined from the unit of *C. albicans* ingested by single neutrophil, counted in 100 cells.

Myeloperoxidase (MPO), Lactoperoxidase (LPx) and Ceruloplasmin activities in milk: Estimation of MPO, LPx and ceruloplasmin level in milk (CCM) was done as per the method described elsewhere (Shafi *et al.* 2016).

Evaluation of therapy: The efficacy of herbal therapy in mastitis was evaluated in terms of elimination of intramammary infections, subsiding of udder inflammation, improvement of milk quality and immuno-potentiation of udder defence mechanisms.

Statistical analysis: All numerical data were processed via the statistical package for social science (SPSS version 16.0 for windows). Analysis of different numerical parameters was done using ANOVA, followed by Duncan's multiple range test. The effect of therapy on elimination of intramammary infections was analysed using Chi square test. Significance level was set at P≤0.05.

RESULTS AND DISCUSSION

The hydro-alcoholic (1:1) extract showed good antibacterial activity against different mastitis pathogens (Table 1), which agrees with the findings of earlier studies

Table 1. Antibacterial activity of hydro-alcoholic extract of *W. somnifera* against bovine mastitis pathogens (Mean±SE)

Pathogen	Zones of inhibition (mm)		% Activity	MIC (mg/ml)
	W. somnifera	Enro-floxacin		
Staphylococcus aureus	19.00±0.60	24.30±0.32	78.19	62.50
CoNS	19.25±0.53	27.70±0.34	69.49	31.50
Streptococcus spp.	15.63±0.50	27.40±0.37	50.03	15.75
E.coli	13.25±0.53	23.82±0.27	55.67	125
Corynebacterium spp.	11.75±0.59	26.90±0.33	43.68	31.50
Pseudomonas spp.	11.88±0.58	24.60±0.24	48.27	125
Klebsiella spp.	16.13±0.64	21.70±0.21	74.31	125

Table 2. Elimination of intramammary infections with *W. somnifera* therapy

Organism	Intramammary infection (IMI)			
	Control group		Treatment group	
	Present at 0 d	Eliminated at 14 d	Present at 0 d	Eliminated at 14 d
Staphylococci	9	3	4	4
Streptococci	1	0	3	2
Corynebacteria	5	1	7	3
Overall	15	4 (26.67)	14	9 (64.28)*

Figures in parentheses indicate percentage. Significant differences existed in elimination of IMI between treatment and control groups. *($\chi^2 = 4.14$; 01 df; $P<0.05$).

in which different types of extracts were tried against different bacterial pathogens (El-Boushy *et al*. 2009, Sundaram *et al.* 2011, Shafi *at al.* 2018b). Wathanolides, dehydrowithanolide, withasomniferin, withanone, witha-2,24-dienolide (steroid lactones), sitoindosides, betasitosterol (phytosterols) and ashwagandhine, cuscohygrine, anahygrine, anaferine, visamine (alkaloids) are main chemical constituents of *W. somnifera* root that are considered as most effective part of the plant and known to inhibit the growth of many pathogens (Rasool and Varalakshmi 2006). The results of MIC (Table 1) are in corroboration with Gupta (2014) who reported the MIC of hydro-alcoholic extract of *W. somnifera* roots to be 125 mg/ml both for *S. aureus* and *E. coli*, and 500 mg/ml for *S. agalactiae*. However, Rajendran and Ramkrishnan (2009) reported MIC of methanolic extract of *W. somnifera* roots at concentration of 100 mg/ml for *S. aureus* and *Streptococcus pyogenes,* and 60 mg/ml for *E. coli*.

Our findings on elimination of intramammary infections (Table 2) may not be comparable with the results obtained by other authors, due to differences in active compounds/ herbs chosen in different experiments. However, Shafi *et al*. (2018a) evaluated "Masticure®" a non-antibiotic preparation of phyto extracts, probiotics, minerals, amino acids and enzymes (containing *W. somnifera* as one of the constituents) as an adjunct therapy in the treatment of bovine mastitis and reported significant elimination of intramammary infections in the treated animals. Also, Shafi *et al.* (2016) studied the effect of oral feeding of *Ocimum sanctum* (Tulsi) leaf powder at a dose rate of 600 mg/kg body weight on a daily basis for 7 days and observed 69.23% elimination of intramammary infections. Giacinti *et al.* (2008) indicated beneficial effects of feeding herbal extracts evaluated for the control of bovine subclinical mastitis during lactation, and also commercially available herbal preparation "Mastilep gel" could eliminate 58.33% of intramammary infections as compared to 23.81% in control group at d 7 post-treatment (Bansal *et al.* 2013).

CMT point score showed a significant decline on d 14 and SCC, *p*H and EC on d 28, post initiation of treatment (Table 3). These findings are in agreement with other workers, whereby, the administration of the herb orally, or via intramammary routes produced a significant reduction in CMT and SCC, and other related parameters like TBC, EC and pH (De and Mukherjee 2013, Shafi *et al.* 2016, Shafi *et al.* 2018a). Somatic cells are mainly comprised of PMNs and their increase is reflective of udder inflammation and deterioration of milk quality. Present study also observed a significant reduction in ceruloplasmin, an acute phase protein on d 14 post treatment (Table 3). It seems that reduction of acute phase protein could be due to anti-inflammatory action of the herb that might have increased due to high permeability of blood milk barrier during episodes of mastitis (De and Mukherjee 2013, Shafi *et al.* 2016).

The present study proved immunomodulatory action of *W. somnifera* as evidenced from significant increase in phagocytic activity and phagocytic index of milk neutrophils (Table 3), and subsequent elimination of intramammary infections. Neutrophils are among the earliest leukocytes recruited to the site of infection and are important host defense mechanisms against invading pathogens in mastitis by virtue of their phagocytosis and intracellular killing. When there is any imbalance in their effective clearing of infective agents or reduction of some functional activities, infective agents over power and result in mastitis. Thus augmenting the migration of PMNs from the circulation to an inflamed udder, and also enhancing their phagocytic potential that helps in the removal of infective agents from the udder.

The activities of leukocytic enzymes (MPO and LPx) were also found significantly increased in animals treated with *W. somnifera* (Table 3). The MPO is a constituent of oxygen dependent antimicrobial activity of leukocytes, and it has been found that intramammary infection increases the number of myeloperoxidase positive macrophages and enhanced activity of MPO-H_2O_2 system in milk PMN cells (Ting *et al*. 2006). LPx, mainly synthesized by PMN leukocytes, a component of lactoperoxidase system along with thiocyanate and hydrogen peroxide serves as an effective antimicrobial weapon against many infective pathogens (Paape *et al.* 1981), and its activity is expected to increase with increase in SCC in milk (Shafi *et al.* 2016). Andrei *et al*. (2009) found increased LPx activity in

Table 3. Effect of *W. somnifera* therapy on inflammatory reaction of udder, phagocytosis and milk enzymes

Parameter	Group	Days after initiation of treatment (AT)			
		D 0	D 7	D 14	D 28
CMT point score	Control	$1.65\pm0.17^{1,b}$	$1.60\pm0.10^{1,ab}$	$1.50\pm0.13^{1,ab}$	$1.20\pm0.13^{2,a}$
	Treatment	$1.55\pm0.26^{1,b}$	$1.05\pm0.32^{1,ab}$	$0.85\pm0.30^{1,ab}$	$0.35\pm0.21^{1,a}$
SCC($\times10^3$ /ml)	Control	$807.80\pm84.96^{1,a}$	$782.40\pm33.72^{1,a}$	$771.90\pm47.13^{2,a}$	$750.40\pm44.37^{2,a}$
	Treatment	$764.10\pm122.22^{1,b}$	$576.10\pm120.59^{1,ab}$	$534.50\pm128.29^{1,ab}$	$378.30\pm85.45^{1,a}$
EC (mS /cm)	Control	$6.59\pm0.30^{1,a}$	$6.58\pm0.09^{2,a}$	$6.41\pm0.08^{1,a}$	$6.14\pm0.12^{2,a}$
	Treatment	$6.63\pm0.36^{1,b}$	$5.73\pm0.35^{1,ab}$	$5.72\pm0.30^{1,ab}$	$5.15\pm0.22^{1,a}$
pH	Control	$6.84\pm0.03^{1,b}$	$6.80\pm0.01^{1,ab}$	$6.79\pm0.02^{1,ab}$	$6.75\pm0.01^{1,a}$
	Treatment	$6.84\pm0.04^{1,b}$	$6.78\pm0.05^{1,ab}$	$6.78\pm0.04^{1,ab}$	$6.70\pm0.02^{1,a}$
Phagocytic activity (%)	Control	$15.30\pm0.70^{1,a}$	$16.30\pm0.47^{1,a}$	$16.50\pm0.67^{1,a}$	$16.80\pm0.66^{1,a}$
	Treatment	$16.80\pm0.49^{1,a}$	$32.90\pm2.77^{2,c}$	$28.50\pm0.67^{2,bc}$	$24.50\pm1.96^{2,b}$
Phagocytic index	Control	$1.07\pm0.02^{1,a}$	$1.09\pm0.03^{1,a}$	$1.10\pm0.03^{1,a}$	$1.10\pm0.03^{1,a}$
	Treatment	$1.07\pm0.03^{1,a}$	$1.56\pm0.08^{2,c}$	$1.25\pm0.02^{2,b}$	$1.22\pm0.02^{12,b}$
MPO (µmol/min)	Control	$437.325\pm16.75^{1,a}$	$423.15\pm9.95^{1,a}$	$412.28\pm7.09^{2,a}$	NE
	Treatment	$422.156\pm17.93^{1,b}$	$634.56\pm33.11^{2,c}$	$233.93\pm21.25^{1,a}$	NE
LPx (µmol/min)	Control	$0.300\pm0.01^{1,a}$	$0.293\pm0.01^{1,a}$	$0.287\pm0.01^{2,a}$	NE
	Treatment	$0.297\pm0.00^{1,b}$	$0.384\pm0.00^{2,c}$	$0.165\pm0.00^{1,a}$	NE
Ceruloplasmin (g/L)	Control	$0.139\pm0.01^{1,a}$	$0.137\pm0.01^{2,a}$	$0.135\pm0.01^{2,a}$	NE
	Treatment	$0.147\pm0.03^{1,b}$	$0.095\pm0.02^{12,ab}$	$0.064\pm0.02^{1,a}$	NE

The values having at least one same superscript (alphabets within row and numbers within column) do not differ significantly ($P<0.05$). NE, not estimated.

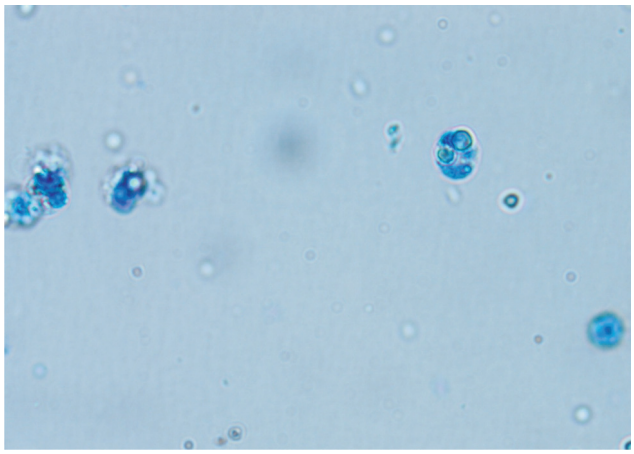

Fig. 2. Phagocytic activity of PMNs against *C. albicans.*

subclinical mastitis that was directly correlated with number of SCC.

Results obtained from the present study indicate beneficial effects of *W. somnifera* therapy against subclinical mastitis of lactating dairy cows. The positive effects of this remedy may be related to the presence of active constituents possessing anti-bacterial, anti-inflammatory and immunomodulation activities.

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