Indian Journal of Animal Sciences **83** (9): 893–897, September 2013/Article

Activity of milk enzymes in response to *Withania somnifera* extract in bovine subclinical mastitis

UJJWAL K DE[1] and REENA MUKHERJEE[2]

Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

Received: 21 December 2011; Accepted: 19 March 2013

ABSTRACT

The present study was conducted to assess the anti-inflammatory and anti-oxidant potential of hydro-alcoholic extract of *Withania somnifera* root against bovine sub-clinical mastitis (SCM). Lactating cows (60) were divided in 4 equal groups. Normal healthy control (group 1) had cows that were clinically healthy, negative for California mastitis test (CMT) and having SCC lesser than 0.3 million cells/ml of milk sample while normal healthy drug control cows were in group 2. Fifteen cows in group 3 and 15 cows in group 4 positive for SCM, screened on the basis of CMT-positive reaction, SCC > 0.9 million cells/ml of milk and positive for intramammary infection, were taken for the drug trial. Sterile *W. somnifera* root extract (200 mg) was infused per teat by intramammary route in cows of groups 2 and 3, after diluting the drug in 5 ml sterile PBS, twice daily for consecutive 5 days, similarly 5 ml sterile PBS was infused by intramammary route in group 4 cows for 5 days. The results revealed that the herb extract treatment reduced the SCC, TBC and increased LPx and MPO activity significantly in post treatment period. The results suggested that the crude hydro-alcoholic root extract of *W. somnifera* possesses some biologically active principles that are anti-inflammatory, antibacterial and anti-oxidant in nature. In the present study, biological activity of *W. somnifera* extract at standardized dose against bovine subclinical mastitis is reported for the first time. Development of alternative therapy with medicinal plant is an opportunity for livestock farmers who are not able to use the allopathic drugs under certain farming system.

Key words: Bovine subclinical mastitis, Mastitis, Milk enzymes, Sub-clinical mastitis, *Withania somnifera* root

Subclinical mastitis (SCM), a problematic infectious disease of dairy animals across the world, spreads unrecognizably in a dairy herd. Mastitis is generally accompanied with huge infiltration of somatic cells into milk compartment and SCC (Somatin cell count) is considered as an important maker of inflammation of mammary gland (Sharma *et al.* 2011). Subclinical mastitis influences the activities of numerous enzymes in milk (De-Ying *et al.* 2009). Lactoperoxidase (LPx), an antioxidant enzyme, provides a potent non-specific bacteristatic or bactericidal system (Reiter *et al.* 1963, 1976). The LPx increases in mastitis milk is being established as a positive correlation between enzyme activity and number of SCC (Andrei *et al.* 2009). Myeloperoxidase (MPO), a lysosomal enzyme of neutrophilic granules, is a constituent of oxygen dependent antimicrobial activity of leukocytes and forms a system of defence against microbes with peroxide halide system (Cooray and Bjorek 1995). However, increased MPO activity during mastitis was observed (Mukherjee *et al.* 2004, 2010).

Present address: [1]Scientist (ujjwalde@gmail.com), [2]Principal Scientist (mukherjeereena@rediffmail.com), Division of Medicine.

Antibiotic is the choice of treatment for mastitis due to multiple bacterial etiologies; however chance of development of multidrug resistant bacteria strain and contamination of milk with antibiotic residue are the major problems in standard therapeutic management of mastitis. Researchers are constantly trying to develop plant based natural products against bovine mastitis as alternative therapy (Hu *et al.* 2001, Mukherjee *et al.* 2005, 2010). *Withania somnifera* or winter cherry, commonly known as *Ashwagandha*, possesses versatile medicinal properties (Gupta and Rana 2007). Traditionally *Withania somnifera* is an excellent natural herbal plant to treat scabies, sores and to enhance milk production in ruminants in ethnoveterinary practices (Williamson 2002). *Ashwagandha* root possesses antibacterial, anti-inflammatory, antioxidant and many other several biological activities (Mishra *et al.* 2000, Arora *et al.* 2004), however, clinical studies for validation of its therapeutic potential against bovine subclinical mastitis is required. The present study, therefore, is an effort to investigate the anti-inflammatory and anti-oxidant potential of hydro-alcoholic (1:1) extract of *W. somnifera* root against bovine subclinical mastitis.

MATERIALS AND METHODS

Fresh roots of *W. somnifera* were collected from the natural habitat around Bareilly, Uttar Pradesh and the plant material was identified and authenticated from the Department of Botany, Bareilly College, Bareilly, India. The roots were washed, dried in shade and pulverized by a mechanical grinder and the powdered roots of *W. somnifera* were extracted with 50% ethanol in soxlet apparatus up to 4 cycles. The extracted material was filtered through sterile muslin cloth and the filtrate obtained was evaporated to dryness in vacuum below 40ºC. The dried powder was weighed and reconstituted in sterile phosphate buffer saline (PBS, pH 7.4±0.1, 0.01 M) @ 200 mg of extract/5 ml of PBS. The dose of the extract was standardized in the pilot study, by taking 5 cows in 2 batches having sub-clinical mastitis. Finally, the filtrate was filtered through membrane filter (pore size 0.45 µm) and stored in refrigerator until used.

Crossbred lactating cows (60) were selected from institute's livestock research farm. These cows were maintained in the animal shed of the institute under identical environmental conditions and were divided in 4 equal groups. Cows of groups 1 and 2 (30) were clinically healthy, negative for California mastitis test (CMT) and those having SCC less than 0.3 million cells/ml of milk sample served as normal healthy control (group 1) and others were normal healthy drug control (group 2). Fifteen cows in group 3 and 15 cows in group 4 were positive for subclinical mastitis (SCM), screened on the basis of CMT-positive reaction, SCC > 0.9 million cells/ml of milk and positive for intramammary infection were taken for the drug trial. Sterile *W. somnifera* root extract (200 mg) was infused per teat by intramammary route in groups 2 and 3 cows, after diluting the drug in 5 ml sterile PBS, twice daily for consecutive 5 days, similarly 5 ml sterile PBS was infused by intramammary route in group 4 cows for 5 days. Observations were made up to 15 days post treatment. The untreated animals were given suitable treatments after completion of experiments.

SCC and TBC: The milk samples were collected from each animal before treatment (day 0) and thereafter on day 3, 7 and 15 after initiation of treatment (AIT) for SCC (Schalm *et al.* 1971) and TBC (Kolmer *et al.* 1951). The organisms were identified on the basis of colony morphology, characteristic hemolytic pattern and Gram's staining.

LPx assay: The LP in milk was performed as per Marshall *et al.* (1986).

MPO assay: The milk leukocytes were isolated from the milk samples and the cell suspension was adjusted to 1×10^6 cells/ml in PBS for MPO assay. The MPO was assayed by using o-dianisidine as electron donor and enzyme concentration was calculated by using molar extinction coefficient (20,040±400 $mol^{-1}dm^{-1}cm^{-1}$) for oxidized o-dianisidine (Bretz and Baggiolini 1974).

Statistical analysis: Data of the SCC and TBC was analyzed using repeated measurement model with cows as subject and period as repeated measurement. The data of LP and MPO were analyzed applying one-way analysis of variance to determine the level of significance between the groups, and Duncan's multiple range test was applied to determine the level of significance within the group at different time interval by using statistical software package (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

The results of SCC and TBC are presented in Table 1. Intramammary infusion of *W. somnifera* extract did not exhibit any significant change in milk SCC in group 2 cows. The SCC significantly (P<0.05) increased to 35.01% on day 3 AT, however, it reduced significantly (P<0.05) on day 7 and 15 PT in group 3 cows by intramammary infusion of the herbal extract. The cell count of milk did not change significantly in group 4 cows throughout the study period. Intramammary infusion of *W. somnifera* extract did not exhibit any significant change in TBC in group 2 cows. The bacterial count significantly (P<0.05) dropped on day 3, 7

Table 1. Comparison of SCC ($\times 10^5$ cells/ml on milk) and TBC ($\times 10^3$ cells/ml on milk) in different treatment groups

Groups	Days post treatment			
(N=15)	day 0	day 3	day 7	day 15
SCC				
Gr. 1	$2.788\pm0.196^{a,A}$	$2.760\pm0.187^{a,A}$	$2.820\pm0.133^{a,A}$	$2.980\pm0.109^{a,BC}$
Gr. 2	$2.528\pm0.180^{a,A}$	$2.808\pm0.146^{a,A}$	$2.801\pm0.229^{a,A}$	$2.542\pm0.119^{a,AB}$
Gr. 3	$9.204\pm0.169^{a,B}$	$12.427\pm0.530^{b,B}$	$8.580\pm0.201^{c,B}$	$4.554\pm0.129^{d,D}$
Gr. 4	$11.370\pm0.422^{a,C}$	$11.794\pm0.375^{a,B}$	$11.494\pm0.487^{a,E}$	$11.214\pm0.483^{a,E}$
TBC				
Gr.1	$0.443\pm0.052^{a,A}$	$0.397\pm0.051^{a,A}$	$0.495\pm0.046^{a,A}$	$0.474\pm0.060^{a,A}$
Gr. 2	$0.482\pm0.034^{a,A}$	$0.426\pm0.030^{a,A}$	$0.448\pm0.043^{a,A}$	$0.488\pm0.053^{a,A}$
Gr. 3	$4.406\pm0.272^{a,B}$	$2.725\pm0.280^{b,B}$	$1.594\pm0.070^{c,C}$	$0.420\pm0.047^{d,A}$
Gr. 4	$4.833\pm0.272^{a,BC}$	$4.952\pm0.288^{a,C}$	$4.909\pm0.287^{a,D}$	$4.908\pm0.253^{a,B}$

*Superscripts in each row (a, b, c, d) and each column (A, B, C, D, E) differ significantly (P<0.05).

and 15 AT in group 3 cows by intramammary infusion of the herbal extract. Statistically no significant changes in bacterial count were observed in group 4 cows throughout the study period.

Substantial infiltration of somatic cells from peripheral circulation and secretion of inflammatory mediators into udder are the common features in intramammary infection (Sharma *et al.* 2011). In this study, the mean SCC value enhanced on day 3 AIT in SCM infected group 3 animals treated with *W. somnifera* root extract. Initial enhancement of somatic cells in the mammary secretions with *W. somnifera* treatment indicated the chemoattractent property of the herb which could be due to presence of bioactive substances present in herb extract. Similar findings were also recorded form intramammary treatment with other herb extracts in bovine mastitis (Mukherjee *et al.* 2005, 2010). After the initial enhancement of SCC, significant reduction in cell count was observed on day 7 and day 15 AIT. Similarly, significant reduction in bacterial load was observed in group 3 cows treated with herb extract from day 3 onwards as compared with day 0 values. In the present study, drop of SCC and bacterial load could be due to anti-inflammatory and antibacterial activity. The extract of *W. somnifera* root is reported to contain chemical constituents like steroid lactones, phytosterols and alkaloids (Mishra *et al.* 2000, Williamson 2002, Gupta and Rana 2007). It was reported that naturally occurring steroidal content of *W. somnifera* root extract possesses a strong anti-inflammatory effect in various inflammatory conditions and antibacterial activities against numerous pathogenic bacteria causing intramammary and skin infections of camel (Begum and Sadique 1988, Owais *et al.* 2005, Tuteja *et al.* 2005, El-Boushy *et al.* 2009). However, the researchers affirmed that bioactive compounds of the herb extract inhibit cyclooxygenase activity which is one of the established mechanisms of anti-inflammatory action (Mishra *et al.* 2000, Agarwal *et al.* 2006). Sundaram *et al.* (2011) found that withanolides, the steroidal lactones extracted from *W. somnifera* in polar solvents (ethanol), are potent inhibitors of bacterial growth. In this study, reduction of SCC and drop of bacterial count in milk of group 3 animals at post treatment period might be due to presence of bioactive compounds in herb extract possessing anti-inflammatory and antibacterial action.

The LPx activity of milk in healthy animals ranged from 0.280±0.029 to 0.284±0.029 µ/ml and 0.275±0.025 to 0.292±0.024 µ/ml in group 1 and group 2 cows respectively. The MPO activity of milk in healthy cows ranged from 3.414±0.271 to 3.032±0.217 µmoles/1× 10^6 cells and 2.904±0.340 to 3.019±0.353 µmoles/1× 10^6 cells in groups 1 and 2 cows respectively. Intramammary infusion of *W. somnifera* extract did not change the LPx and MPO activities significantly in group 2 cows. However, the LPx and MPO activities increased significantly (P<0.05) to 70.67 and 62.62%, respectively, in group 3 cows on day 5 AT by intramammary infusion of the herbal extract. No statistically significant changes of these enzymes were observed in group 4 cows throughout the study period (Table 2).

During mastitis, the activity of indigenous milk enzymes such as myeloperoxidase and lactoperoxidase increases when SCC increases as these enzymes are mainly synthesized by polymorphonuclear leukocytes. The LPx, an antioxidant enzyme present in milk provides a potent non-specific bacteristatic or bactericidal system. LPx exhibits anti-oxidant activity through catalyzing the oxidation of thiocyanate and the reaction of hydrogen peroxide (Moatsou 2010). In this study, LPx activity increased significantly in group 3 cows upon intramammary infusion of the herbal extract. Mishra *et al.* (2000) reported that *W. somnifera* root extract has strong antioxidant property and it could be attributed to the presence of steroidal lactones which represent the main active component of the herb. Hamza *et al.* (2008) demonstrated the antioxidant effect of a purified root extract of *W. somnifera* against doxorubicin-induced oxidative stress in rats. Moreover, *W. somnifera* root extract treatment increase major free-radical scavenging antioxidant enzymes and prevent stress-induced lipid peroxidation in mice and rabbit models (Bhattacharya *et al.* 1997, Dhuley 1998). The MPO, a lysosomal enzyme of neutrophilic granules, is a constituent of oxygen dependent antimicrobial activity of leukocytes and forms a system of defence against microbes with peroxide halide system (Cooray and Bjorek 1995, Cooray *et al.* 1993). Myeloperoxidase with H_2O_2 and halide build potent antibacterial system against pathogen (Cooray *et al.* 1993). In this study, MPO activities increased significantly in group 3 cows in post treatment period by intramammary infusion of the herbal extract. Researchers reported an enhanced

Table 2. Changes of LPx activity in milk serum and MPO activity in milk leukocytes in different treatment groups

Groups	Lactoperoxidase (µ/ml)		Myeloperoxidase (µmoles/10^6 cells)	
(N=15)	day 0	day 5	day 0	day 5
Gr. 1	0.280±0.029^{a,A}	0.284±0.029^{a,A}	3.414±0.271^{a,A}	3.032±0.217^{a,A}
Gr. 2	0.275±0.025 ^{a,A}	0.292±0.024 ^{a,A}	2.904±0.340 ^{a,A}	3.019±0.353 ^{a,A}
Gr. 3	1.705±0.125^{a,B}	2.910±0.137^{b,B}	6.394±0.452^{a,B}	10.398±0.453^{b,B}
Gr. 4	1.719±0.144^{a,B}	1.606±0.093^{a,D}	6.624±0.416^{a,B}	6.503±0.338^{a,D}

* Superscripts in each row (a, b) and each column (A, B, C, D) differ significantly (P<0.05).

activity of phagocytic cells and lysosomal enzymes in myelosuppression in mice models with *W. somnifera* treatment (Ziauddin *et al.* 1996, Agarwal *et al.* 1999, Davis and Kuttan 1999). Whilst, enhanced MPO activity of milk PMN cells also observed by treatment of *Ocimum Sanctum* and *T. cordifolia* in bovine subclinical mastitis (Mukherjee *et al.* 2005, 2010). In this study, augmentation of MPO activity could be due to enhanced milk leukocyte activity by the possible bioactive compounds present in herb.

ACKNOWLEDGEMENTS

The authors thank the Director, IVRI, and Head, Division of Medicine, for providing necessary facilities. First author also thanks IVRI for granting Institute Research Scholarship during course work of his doctoral programme.

REFERENCES

Agarwal R, Diwanya S, Patki P and Patwardhan B. 1999. Studies on immunomodulatory activity of *Withania somnifera* (Ashwaganda) extracts in experimental immune inflammation. *Journal of Ethnopharmacology* **67**: 27–35.

Aggarwal B B, Ichikawa H, Garodia P, Weerasinghe P, Sethi G, Bhatt 1 D, Pandey M K and Shishodia S. 2006. From traditional Ayurvedic medicine to modern medicine: identification of therapeutic targets for suppression of inflammation and cancer. *Expert Opinion on Therapeutic Targets* **10**: 87–118

Andrei S, Pintea A, Groza 1, Bogdan L, Cipue S and Matei S. 2009. Milk antioxidant enzymes activity in cows with subclinical mastitis. *Lucrari-Stiintifice-Medicina,-University-de-Stiinte-Agricole-si-Medicina-Veterinara-"Ion-Ionescu-de-la-Brad"-Iasi,* **52**: 1–6.

Arora S, Dhillon S, Rani G and Nagapal A. 2004. The *in vitro* antibacterial/synergistic activities of *Withania somnifera* extracts. *Fitoterapia* **75**: 385–88.

Begum V H and Sadique J. 1988. Long term effect of herbal drug *Withania somnifera* on adjuvant induced arthritis in rats. *Indian Journal of Experimental Biology* **26**: 877–82.

Bhattacharya S K, Satyan K S and Chakrabarti A. 1997. Effect of Trasina, an Ayurvedic herbal formulation, on pancreatic islet superoxide dismutase activity in hyperglycaemic rats. *Indian Journal of Experimental Biology* **35**: 297–99.

Bretz U and Baggiolini M. 1974. Biochemical and morphological characterization of azurophilic and specific granules of human neutrophilic polymorphonuclear leucocytes. *Journal of Cell Biology* **63**: 251–69.

Cooray R A, Peterson C B G and Holmberg O. 1993. Isolation and purification of bovine myeloperoxidase from neutrophil granules. *Veterinary Immunology and Immunopathology* **38:** 261–72.

Cooray R A and Bjorek L. 1995. Bactericidal activity of the bovine myeloperoxidase system against bacteria associated with mastitis. *Veterinary Microbiology* **46**: 427–34.

Davis L and Kuttan G. 1999. Effect of *Withania somnifera* on cytokine production in normal and cyclophosphamide treated mice. *Immunopharmacology and Immunotoxicology* **21**: 695–703.

De Ying Y, XuePing Y, SuiZhong C, ShuMin Y, ChangSong L and LiXin D. 2009. *Journal of Nothwest A and F University Natural Science Edition* **37**: 43–46.

Dhuley J N. 1998. Effect of ashwagandha on lipid peroxidation in stress-induced animals. *Journal of Ethnopharmacology* **60**: 173–78.

El Boushy M E, Abdalla O M and Risha A F. 2009. Studies on antibacterial activity of *Withania somnifera* extract in guinea pigs experimentally infected with *E. coli*. *Mansoura Veterinary Medical Journal* **11**: 71 – 85.

Gupta G L and Rana A C. 2007. *Withania somnifera* (Ashwagandha): A Review. *Pharmacognosy Reviews* **1**: 129–36.

Hamza A, Amin A and Daoud S. 2008. The protective effect of a purified extract of *Withania somnifera* against doxorubicin-induced cardiac toxicity in rats. *Cellular Biology and Toxicology* **24**: 63–73

Hu S, Concha C, Johannison A, Meglia G and Waller K P. 2001. Effect of subcutaneous of ginseng on cows with subclinical *Staphylococcus aureus* mastitis. *Journal of Veterinary Medicine Series B* **48**: 519–28.

Kolmer J A, Spaulding E H and Robinson H W. 1951. Approved laboratory technic. *Methods for the bacteriologic examination of milk*. 5th Edition, Appleton-Century-Crofts Inc, New york, USA.

Marshall V M E, Cole W M and Bramley A J. 1986. Influence of lactoperoxidase system on susceptibility of the udder to *Streptococcus uberis* infection. *Journal of Dairy Research* **53**: 507–514.

Mishra L C, Singh B B and Dagenais S. 2000. Scientific basis for the therapeutic use of *Withania somnifera* (Ashwagandha): A Review. *Alternative Medicine Review* **5**: 334–46.

Moatsou G. 2010. Indigenous enzymatic activities in ovine and caprine milks. *International Journal of Dairy Technology* **63**:16–31.

Mukherjee R, Dash P K and Ram G C. 2005. Immunotherapeutic potential of *Ocimum sanctum* (L) in bovine subclinical mastitis. *Research in Veterinary Science* **79**: 37–43.

Mukherjee R, Ram G C, Dash P K and Goswami T. 2004. The activity of milk leukocytes in response to a water-soluble fraction of *Mycobacterium phlei* in bovine subclinical mastitis. *Veterinary Research Communication* **28**: 47–54.

Mukherjee R, De U K and Ram G C. 2010. Evaluation of mammary gland immunity and therapeutic potential of *Tinospora cordifolia* against bovine subclinical mastitis. *Tropical Animal Health and Production* **42**: 645–51.

Owais M, Sharad K S, Shehbaz A and Saleemuddin M. 2005. Antibacterial efficacy of *Withania somnifera* (ashwagandha) an indigenous medicinal plant against experimental murine salmonellosis. *Phytomedicine* **12**: 229–35.

Reiter B, Marshall V M, Bjock L and Rosen C G. 1976. Nonspecific bactericidal activity of the lactoperoxidase/thiocyanate/hydrogen peroxide system of milk against *Escherichia coli* and some some Gram-negative pathogens. *Infection and Immunity* **13**: 800–07.

Reiter B, Pickering A, Oram J D and Pope G S. 1963. Peroxidase-thiocynate inhibition of Streptococciin raw milk. *Journal of General Microbiology* **33**: 12.

Schalm O W, Carrol E J and Jain N C. 1971: *Bovine Mastitis.* Lea and Febiger, Philadelphia, USA.

Sharma N, Singh N K and Bhadwal M S. 2011. Relationship of somatic cell count and mastitis: an overview. *Asian-Australasian*

Journal of Animal Sciences **24**: 429–38.

Snedecor G W and Cochran W G. 1994. *Statistical Methods*, 8th ed. Iowa State University Press, New Delhi.

Sundaram S, Dwivedi P and Purwar S. 2011. *In vitro* evaluation of antibacterial activities of crude extracts of *Withania somnifera* (Ashwagandha) to bacterial pathogens. *Asian Journal of Biotechnology* **3**: 194–99.

Tuteja F C, Dixit S K and Pathak K M L. 2005. *In vitro* antibacterial activity of medicinal plants. *Indian Veterinary Journal* **86**: 898–99.

Williamson E M. 2002. *Major herbs of Ayurveda*. Dabur Research Foundation and Dabur Ayurvet Ltd, Churchill Livingstone, London.

Ziauddin M, Phansalkar N, Patki P, Diwanay S and Patwardhan B. 1996. Studies on the immunomodulatory effects of ashwagandha. *Journal of Ethnopharmacology* **50**: 69–76.