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Nareshi Meena
M.V.Sc., Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Jyoti Choudhary
Ph.D., Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

AP Singh
Professor and HOD, Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Corresponding Author
Nareshi Meena
M.V.Sc., Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Studies on therapeutic potential of *Withania somnifera* on subclinical mastitis in goat (*Capra hircus*)

Nareshi Meena, Jyoti Choudhary and AP Singh

Abstract
In the present investigation, milk samples of 192 quarters from 96 apparently healthy goats were collected from individual holdings and were subjected to modified California mastitis test (MCMT), electrical conductivity (EC), pH, somatic cell count (SCC) and microbial cultural examination for the diagnosis of subclinical mastitis. The samples were collected from goats after 1 month of parturition. The aim of the present investigation was to evaluate the therapeutic potential of one medicinal plant *viz. Withania somnifera* (Ashwagandha) known to possess anti-inflammatory, immuno-modulatory and antibacterial properties was selected for evaluation in the therapy of subclinical mastitis *in vitro* and *in vivo*. The 12 goats were divided into two groups having 6 goats in each *viz.* Group I (aqueous extract @150 mg/kg b.wt.) and Group II (alcoholic extract @125 mg/kg b.wt.). After 8th day post- treatment, there was no significant change (P>0.05) in electrical conductivity (EC) in both the two groups post-treatment. There was no significant change (P>0.05) in pH in both the two groups Post–treatment. Similarly, there was significant changes (P<0.05) in total somatic cell count (TSCC) in both the groups post-treatment. There was significant change (P<0.05) in milk constituents fat in both the two groups post-treatment.

Keywords: Diagnosis, goat, mastitis, *Withania somnifera*

1. Introduction
Mastitis has been recognized as the most important economical factor affecting the dairy industries worldwide (Ali *et al*., 2011) [1]. Singh (1994) [21] reported more than three times of economic losses are due to subclinical mastitis as compared to the clinical form of mastitis. The anatomical position of udder is outside which influences and make prone to both inflammatory and non-inflammatory conditions (Neelesh *et al.,* 2008) [11]. Subclinical mastitis is considered to be a herd problem, without observable clinical signs or no changes in milk grossly, which may be detected by the various indirect tests like modified California mastitis test (MCMT), total somatic cell count (TSCC), electrical conductivity (EC) of milk and cultural examination. The occurrence of clinical mastitis would be propositional to the prevalence of subclinical mastitis, because an existing subclinical phase of intra mammary infection. Subclinical mastitis denotes absence of apparent abnormalities in the mammary gland but also presence of chemical and bacteriological changes in the milk (Chakrabarti, 1996) [3]. Subclinical mastitis is caused by various factors such as bacteria, fungi, mycoplasma, yeast along with stress, reduced resistance, shape of udder and teats, inheritance of animal and environment including milking and feeding system (Chahar, 2001) [2]. The presence of significantly higher leukocyte counts in the milk from affected glands is to be considered as diagnostic test for clinical as well as subclinical mastitis cases. Criteria accepted by the Indian Dairy Federation for the diagnosis of subclinical mastitis is based on isolation of pathogen and cell counts more than 1000000/ml in the goat's milk. In Ayurveda these herbal medicines have been used since a time ago and now gained importance due to their less toxicity, lesser side effects and being organic in nature. The herbal therapy generally does not pollute the milk and hence there is no milk withdrawal period like in antibiotic use. Even, World Health Organization (WHO) has emphasized on the use of medicinal plants as these are considered safe and effective than the synthetic drugs. The root of *Withania somnifera* (ashwagandha) possesses anti-inflammatory, apoptogenic, antitumor, antistress, antibacterial, liver tonic, antioxidative, immunomodulatory, haemopoietic, and astringent properties (Mishra *et al*., 2000, El-Boushy *et al*., 2009) [10, 5] thus, it is an important herb in the Ayurvedic and indigenous medicinal system for over 3000 years.

2. Materials and Methods

a) Source of animals and milk

In the present investigation, milk samples of 192 udder halves from 96 apparently healthy goats were collected from individual holdings and were subjected to modified California mastitis test (MCMT), electrical conductivity (EC), pH, somatic cell count (SCC) and microbial cultural examination for the diagnosis of subclinical mastitis. The samples were collected from goats after 1 month of parturition.

b) Collection of milk sample

Sample for culture were collected before milking in the morning that was most convenient under the management conditions of the individual herd. Strict aseptic procedures were used while collecting the milk samples in order to prevent the contamination with microorganisms present on the skin of goat's flanks, udder, teats, hands of the sampler and in the barn environment. To collect the milk samples aseptically, udder and teats were washed with water and air-dried. Then each teat was wiped off by spirit swab. Two-three stripping of fore milk were discarded. Approximately 30 ml of fore milk from each teat was collected in sterilized test tube. Care was taken to avoid any type of contamination in the milk. All the samples of milk were brought to the laboratory and kept in refrigeration (4 ºC) until analyzed.

c) Diagnostic tests for the detection of subclinical mastitis

i) Modified California mastitis test

The principle of the test is the reactive reagent which reacts with the DNA of the somatic cell nuclei after the dissolution of their outer wall and the nucleus cell wall with the formation of filamentous mass which is proportional with the somatic cell count. A higher concentration in the somatic cell count leads to a higher CMT score. California mastitis test scores are directly related to the average somatic cell counts. In the present study tempol was used in place of pure allyl aryl sulphates or sulphonates of sodium or potassium as an anionic surface-active agent. Cresol red was replaced by bromo thymol blue. The test was carried out by following standard protocol of CMT and test reactions were scored according to Radostits *et al.* (2006) [15] as follows.

Table 1: California mastitis test (CMT) scoring system (Scandinavian scale) with interpretation and SCC range

Negative	Mixture remained liquid with no evidence of formation of a precipitate	0-2 lakh cells/ml
Trace	Slight precipitate that tends to disappear with continued movement of the paddle.	1.5- 5 lakh cells/ml
+/weak positive	Distinct precipitate but no tendency toward gel formation.	4-15 lakh cells/ml
++/Distinct positive	Mixture thickens immediately with gel formation on continued swirling, mass movement moved around the periphery leaving the bottom of the cup exposed.	8-50 lakh cells/ml
+++/Strong positive	Distinct gel forms that tend to adhere to the bottom of the paddle and during swirling a distinct-central peak forms.	More than 5 million cells/ ml

ii) Electrical conductivity

Electrical conductivity (EC) is a measure of the resistance of a particular material to an electric current. Because of the increased blood capillary permeability, mastitis causes a change in ion concentrations (increase in Na^+ and Cl^- ions) of milk and thus, the changes are found in EC (Ilie *et al.*, 2010). Measurement of electrical conductivity was done by passing AC voltage through the milk with conductivity cell. Electrical conductivity varies with the presence of dissolved solids in the solution. This was determined by Pen type EC-035 (ATC) Conductivity meter of ERMA instruments. For calibration, the meter was immersed in distilled water. Then, it was immersed in 12880 µS/cm (12.88 EC) calibration solution and was stirred gently. The battery compartment cap on the top of the meter was unscrewed. The reading was allowed to stabilize and with a small screwdriver the calibration trimmer was turned until the display showed "12.88 EC". The electrode was rinsed with distilled water and cleaned properly with filter paper.

iii) pH reaction

The pH may serve as the best indicator to assess the udder health status of the animal and food value of the milk. As the severity of mastitis increased, pH value also increased. H^+ concentration of the milk was determined immediately using single electrode Pen type digital pH meter (pH-035 (ATC) of ERMA instruments). The electrode of the instrument was immersed in the milk sample to be tested. The pH meter was calibrated at pH 5 and 9.2 with buffer before taking the pH of milk samples.

iv) Somatic cell count

Somatic cell count (SCC) is a useful predictor of the intramammary infection (IMI) that includes leucocytes (75%) i.e. neutrophils, macrophages, lymphocytes, erythrocytes and epithelial cells (25%). The measurement of the somatic cell in the milk is known as somatic cell count. Leucocytes increase in response to bacterial infection, tissue injury and stress. Somatic cells are the indicators of both resistance and susceptibility of goats to mastitis and can be used to monitor the level of occurrence of subclinical mastitis in herds or individual goats. The SCC counts were determined as described by Schalm *et al.* (1971) [20].

To prepare the milk smear, milk sample was mixed thoroughly so as to obtain uniform distribution of cells. The sample was allowed to stand for 2 to 5 minutes to permit air bubbles to rise and foam to disappear. Grease free glass slide was placed on a level area over a template to outline 1 square cm area. With the help of micropipette 10 µl of milk was withdrawn and spread evenly on a glass slide in 1 square cm area. The smear was dried in air. Thereafter, a few drops of xylene were poured over the milk smear and kept for 2 minutes to dissolve the fat globules of milk. The smear was then air dried and fixed with 99 per cent methanol for 2 minutes and washed with distilled water. After fixing smear, it was stained with Giemsa stain for 30 minutes. After staining, the smear was kept in phosphate buffer solution (pH 7.0) in coupling jar for 5 minutes and bloat dried. This smear was used for somatic cell count under oil immersion. Examination of milk smear was done at random. One square cm area of smear was divided into four equal parts by dividing it at the right angle. The smear was examined under oil immersion. Cells were counted in five fields from each divided area. Thus, the cells were counted in total 20 fields. The average number of cells per sq cm area was calculated. For counting of cells per ml of milk the average numbers of

cells per field were multiplied by microscopic factor.

V) Derivation of common microscopic factor

Common microscopic factor was determined as per Prescott and Breed (1910) [14] as under: Diameter of an oil immersion microscopic field of Nikon microscope used in study = 190 µ or 0.019 cm.

Radius of oil immersion microscopic factor = 0.0095 cm.

Area of oil immersion microscopic field = πr^2

$= 22/7 \times (0.0095)^2$ cm^2

$= 3.1428 \times 0.0095 \times 0.0095$ cm^2

$= 0.0002833 \times 10^{-5}$ cm^2

$= 28.33 \times 10^{-5}$ cm^2

$$\text{No. of fields in 1 cm}^2\text{ area} = \frac{1\text{cm}}{\text{Area of an oil immersion microscopic field}}$$

$$= \frac{1}{28.33 \times 10^{-5}}$$

$= 3529.82$

Number of cells counted in 20 fields = X

Then total numbers of cells in 1 cm^2 area are

$$= \frac{X}{20} \times 3529.82 \text{ in 0.01 ml of milk}$$

$$\text{So number of cells in 1 ml of milk} = \frac{X \times 3529.82 \times 100}{20}$$

$$= \frac{}{20} \times 352982 \text{ (multiplication factor)}$$

Here $\frac{X}{20}$ = Average No. of cells in 20 field of 1 cm^2 area.

Total number of cells in 1 ml of milk = Avg. No. of cells in 20 fields of 1 cm^2 area × 352982.

3. Results and Discussion

All the positive samples were subjected to laboratory for confirmative diagnosis of subclinical mastitis by electrical conductivity (EC), pH, somatic cell count (SCC) and microbial cultural examination. Out of 96 goats 12 goats suffering from subclinical mastitis were selected for evaluation of *in vivo* therapeutic potential of *W. somnifera* aqueous and alcoholic extracts. These 12 goats were categorized into 2 groups *viz.* Group I and Group II comprising of 6 goats in each group for 2 different therapeutic regimens. Out of 96 goats, 6 goats were selected as apparently healthy control which were negative for CMT.

3.1 Diagnostic tests

3.1.1 Modified California Mastitis Test (MCMT)

In the present study, out of 24 udder half milk samples, the results of modified California mastitis test of different groups of subclinical mastitis affected milk of goats have been presented in Table 2. The test reactions were scored as per Radostits *et al*. (2007). Twelve (50%) udder half milk samples were found positive for modified California mastitis test.

Table 2: Results of modified California mastitis test of udder half milk of Group I and Group II goats affected from subclinical mastitis

Group	Results of CMT		Total halves
	Right	Left	
Group I	-	++	1
	++	-	1
	++	-	1
	++	-	1
	-	++	1
	-	++	1
Group II	++	-	1
	-	++	1
	-	++	1
	++	-	1
	++	-	1
	-	++	1

(-) = Negative (++) = Distinctly positive

Similar findings were observed by Johri (2016) [6] and Savita (2017) [19] in cattle. The results of MCMT and SCC were compared and samples with MCMT results of weak positive (+) were considered as having total somatic cell count between 4,00,000/ml to 10,00,000/ml, while samples with MCMT results of distinct positive (++) were considered as having total somatic cell count between 10,00,000/ml to 50,00,000/ml Radostits *et al*. (2007). Obliged (1961) [13] stated that the normal milk may give positive and mastitis milk may give negative reaction to modified California mastitis test. Many managemental factors, such as ration, hygiene and storage of milk. Modified California mastitis test gives always strong reaction in the first week after calving or in the last stages of lactation as reported by Radostits *et al.* (2007). Reddy *et al*. (2014) [16] also reported more false positive reactions in CMT (24.60 per cent) than SCC (23.70 per cent).

3.2 Electrical conductivity

The mean ± SE values of electrical conductivity of apparently healthy and different groups of subclinical mastitis affected udder half milk of goats have been presented in Table 3. The mean ± SE value of electrical conductivity in normal milk of apparently healthy goat was 4.41 ± 0.20 mS/cm which ranged between 4.28-5.38 mS/cm. The mean ± SE values of electrical conductivity in sub clinically affected udder half milk in group I and group II were 3.85 ± 0.38 and 4.65 ± 0.29 mS/cm, respectively and ranged between 2.43-4.76 and 3.89-5.43

mS/cm, respectively.

Table 3: Mean ± SE values of electrical conductivity (EC) mS/cm of apparently healthy, Group I and Group II udder half milk affected from subclinical mastitis of goats

Group	Mean ± SE	Range
Apparently healthy	4.41 ± 0.20	4.28 - 5.38
Group I	3.85 ± 0.38	2.43 - 4.76
Group II	4.65 ± 0.29	3.89 - 5.43

The mean ± SE values of EC recorded in Group I and Group II showed no significant difference when compared to mean ± SE value of apparently healthy udder half milk of goats. The similar findings were also recorded by Milner *et al.* (1996) [8] in goats and Reddy *et al*. (2014) [16], Johri (2016) [6] and Savita (2017) [19] in cattle.

The EC of milk is mostly determined by the concentration of anions and cations. The most important cation and anion are Na^+, K^+, and Cl^-, respectively. The sodium pumps located on the baso-lateral membrane of the secretory cells of the mammary gland, pumping Na^+ into the extracellular fluid and K^+ into the cells, while Na^+ and K^+ are transported passively between the secretory cells and milk, across the apical membrane. In addition to it, a paracellular pathway is also present across the epithelium (tight junctions), where Na^+ and Cl^- are moving into the milk and K^+ and lactose are moving into the extracellular fluid. When an animal is getting infection to mammary gland resulting destruction of tight junctions and the active ion-pumping system. As a result of the cell damage, Na^+ and Cl^- leak into the lumen of the alveolus, and K^+ and lactose move together out of the milk. The EC of the milk increases due to an increased concentration of Na^+ and Cl^- in the milk. However, factors other than mastitis, like breed parity, lactation stage, milking interval and milk composition may affect EC of milk (Norberg *et al*., 2004) [12].

3.3 pH reaction

The mean ± SE values of pH of apparently healthy and different groups of subclinical mastitis affected udder half milk of goats have been presented in Table 4. The mean ± SE value of pH in normal milk of apparently healthy goats was 6.64 ± 0.08 which ranged between 6.45-6.98. The mean ± SE value of pH in sub clinically affected udder half milk in group I and group II were 6.82 ± 0.07 and 6.61 ± 0.03, respectively and ranged between 6.55-7.05 and 6.51-6.77, respectively.

Table 4: Mean ± SE values of pH of apparently healthy, Group I and Group II udder half milk affected from subclinical mastitis of goats

Group	Mean ± SE	Range
Apparently healthy	6.64 ± 0.08	6.45 - 6.98
Group I	6.82 ± 0.07	6.55 - 7.05
Group II	6.61 ± 0.03	6.51 - 6.77

The mean ± SE values of pH recorded in Group I and Group II showed no significant difference when compared to mean ± SE value of apparently healthy udder half milk of goats. The similar findings were also recorded by Coles (1986) [4] in goats and Kolte *et al.* (2001) in cattle. Alteration of pH of milk, depends on salt concentration in exudates. These exudates increase the alkalinity of milk by alkaline salt so that the test may give positive values. Further, the normal milk pH is 6.4-6.9, whereas pH of affected samples is abnormally alkaline, may be as high as 7.4. The degree of alkalinity depends on the severity of inflammation. The milk pH has less diagnostic value in detecting the existence of udder inflammation (Coels, 1986) [4].

3.4 Somatic cell count (SCC)

The mean ± SE values of somatic cell counts of apparently healthy and different groups of subclinical mastitis affected udder half milk of goats have been presented in Table 5. The mean± SE value of total somatic cell count in normal milk from apparently healthy goat was 7.24 ± 0.50 million/ml which ranged between 5.44-8.65 million/ml. The mean ± SE value of total somatic cell counts in sub clinically affected udder half milk in group I and group II were 17.2 ± 0.14 and 16.3 ± 0.12 million/ml, respectively and ranged between 12.4-21.6 and 11.8-20.4 million/ml, respectively. The threshold value for total somatic cell count to detect subclinical mastitis was considered 10, 00,000 cells/ml and above. All the sub clinically affected milk samples showed higher TSCC.

Table 5: Mean ± SE values of somatic cell count (SCC) million/ml of apparently healthy, Group I and Group II udder half milk affected from subclinical mastitis of goats

Group	Mean ± SE	Range
Apparently healthy	7.24^a ± 0.50	5.44 - 8.65
Group I	17.2^c ± 0.14	12.4 - 21.6
Group II	16.3^c ± 0.12	11.8 - 20.4

According to International Dairy Federation (IDF) recommendation the criteria for sub clinically mastitis having somatic cell counts more than 10, 00,000 cells/ml. The mean ± SE values of SCC recorded in Group I and Group II showed high ($P \leq 0.05$) significant difference when compared to mean ± SE value of healthy udder half milk of goats. Similar findings were also recorded by Tuteja *et al.* and Savita (2017) [19] in cattle. According to Salsberg *et al.* (1984) [18] season and type of pathogen influences somatic cell count. Robertson and Muller (2005) [17] reported that in the case of goat's milk, a considerable controversy exists as to the relationship between SCC and mastitis. The reason for this controversy is that milk secretion in the cow differs from that of the goat. In the cow, milk is squeezed out of the alveoli, while in the goat the alveoli actually burst open. Due to this apocrine secretion, large numbers of cytoplasmic particles occur in normal goat's milk. Due to the presence of these cells the total SCC in goat's milk does not correlate well with the leucocyte count in the milk. Min *et al.* (2007) [9] reported average SCC values ranging from $2,000\times10^3$ to $4,000\times10^3$ cells mL^{-1} in infected dairy goats and concluded that SCC in goat milk is not highly correlated to IMI.

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