

## Original Research Article

### *In vitro* Herbal Sensitivity Test against Mastitis Milk of Large Animals

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#### ABSTRACT

##### Keywords

Sub clinical mastitis, California mastitis test (MCMT), total somatic cell count (TSCC), pH, electrical conductivity (EC)

The study was carried out at local veterinary hospitals and LRS, College of Veterinary and Animal Sciences, Bikaner where milk samples of 96 quarters from 24 apparently healthy Rathi cows were subjected to various tests *viz.* modified California mastitis test (MCMT), total somatic cell count (TSCC), pH, electrical conductivity (EC) and cultural examination for the diagnosis of sub-clinical mastitis. The aim of present investigation was to know the response of two medicinal plants *viz.* *Withania somnifera* (Ashwagandha) and *Ocimum sanctum* (Tulsi) which known to possess anti-inflammatory, immuno-modulatory and antibacterial properties in the therapy of subclinical mastitis *in vitro*. Thus, the results indicated that *O. sanctum* and *W. somnifera* have shown best response in mastitis milk.

#### Introduction

Mastitis is an economically important disease due to its high morbidity, loss of milk production, high cost of treatment (Blowey, 1986 and Beck *et al.*, 1992) and major adverse effects on the quality of by products made from contaminated milk (Rogers and Mitchell, 1989). It is one of the most costly diseases of dairy cattle resulting in the reduction of milk yield and quality (Atakisi *et al.*, 2010).

Bansal and Gupta (2009) have been estimated Rs. 4151.16 crore annual economic losses due to subclinical mastitis in India.

In subclinical mastitis, there are apparent abnormalities in the mammary gland but also presence of chemical and bacteriological changes in milk (Chakrabarti, 1996). Chahar (2001) reported that subclinical mastitis is caused by various factors such as bacteria, fungi, mycoplasma and yeast along with stress, reduced resistance, shape of udder and teats, inheritance of animal, environment including milking and feeding system. Although, mastitis can have an infectious or non-infectious etiology, the vast majority of bovine mastitis is of bacterial origin (Blowey and Edmondson, 1995). The major

contagious pathogens are *Staphylococcus aureus*, and *Streptococcus agalactiae* and the major environmental pathogens are *Enterobacteriaceae* and *Streptococcus uberis*.

The diagnosis of subclinical mastitis requires laboratory tests, which are largely indirect tests, depending upon the leukocyte count in the milk like California mastitis test (CMT) (Richter *et al.*, 1960). The other tests used for the diagnosis of subclinical mastitis are milk electrical conductivity, pH, Bromocresol purple (BCP), Bromothymol blue (BTB), Somatic Cell Count (SCC) and Hotis test (Radostits *et al.*, 1994).

Bacterial examination of milk samples is still a golden test for diagnosis of subclinical mastitis and selecting appropriate antibiotic according to sensitivity.

Appropriate clearance of the pathogens from the bovine udder requires, optimum functioning of the immune cells and effectiveness of the drug (Sordillo *et al.*, 1997), which largely depends on the use of antibiotics till date. However, antibiotic therapy of established mammary infection is moderately efficacious and requires prolonged milk withdrawal due to residues in milk (Daley and Hayes, 1992). Over and indiscriminate use of antibiotics has caused havoc by producing resistance in pathogens. The use of antimicrobials has increased the number of antimicrobial-resistant microbes globally (Williams, 2000). Another concern is their effect on the manufacture of dairy products and the development of hypersensitivity syndromes in human beings. Further, 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). Most developed countries tried to guarantee the food safety by the installation of drug

residue detection systems for antibiotic residues in milk and milk products. In addition to the application of milk quality regulation to reduce the potential risk of milk contamination to the consumer so it is 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. Medicinal plants or herbs constitute a major source of alternative medicine and are used to treat diseases of man and animal since ancient times. The herbal medicines have 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.

*Ocimum sanctum* (Tulsi) is a valuable herbal medicine being used in wide spectrum of animal diseases. The key constituents of *O. sanctum*, proposed by Chevallier (1996) are volatile oil (Eugenol 80%), flavonoids and triterpine (Ursolic acid). The herbal extract of *O. sanctum* possessed immuno-modulatory properties in addition to anti-inflammatory properties as evident in several experiments carried by Sadekar *et al.*, (1998). The therapeutic potential of *O. sanctum* is due to its active constituent eugenol (1-hydroxy-2-methoxy-4-allylbenzene) (Prakash and Gupta, 2005). Mukherjee *et al.*, (2005) reported that *O. sanctum* seed oil appears to modulate both humoral and cell-mediated immune

responsiveness and GABAergic pathways may mediate these immunomodulatory effects.

The root, leaf and whole plant of *Withania somnifera* (Ashwagandha) possesses anti-inflammatory, adaptogenic, antitumor, antistress, antibacterial, liver tonic, antioxidative, immunomodulatory, hemopoietic and astringent properties (Mishra *et al.*, 2000 and El-Boushy *et al.*, 2009). El-Boushy *et al.*, (2009) demonstrated the potential efficacy of both leaf and root extracts (alcoholic and aqueous solvent) of *W. somnifera* plant as having remarkable antibacterial activity *in vitro*.

### **Materials and Methods**

A total number 96 quarters of different lactation stage were screened from 24 Rathi cows suffering from subclinical mastitis. All the samples which were positive for subclinical mastitis parturated one month back. All 96 quarter milk samples were collected aseptically and subjected to various diagnostic tests like SCC, EC, CMT, pH and microbial cultural examination. The samples from affected quarters were collected aseptically for cultural and sensitivity examination by using standard procedure as per Cowan and Steel (1975).

### **Cultural examination**

Each apparently normal milk sample was screened for the presence of bacteria by cultivation, isolation and identification using standard procedures as per Cowan and Steel (1975).

Cultivation and isolation of bacteria and Identification of bacteria was done on the basis of morphology, motility, growth in air, Gram's reaction, spore formation, biochemical and metabolic reactions such as

catalase activity, oxidation reaction test, and oxidation and fermentation tests is done as per Hugh and Leifson, (1953).

### **Antibacterial sensitivity test of herbal extracts**

Antibacterial activity of the extracts was evaluated by using paper disc agar diffusion method. This is also known as Kirby-Bauer disk diffusion method (Bauer *et al.*, 1966).

### **Results and Discussion**

Out of 96 quarters of 24 Rathi cows, 29 (30.20 per cent) quarters were found positive for micro-organisms. *Staphylococcus* spp. were recorded in 12 (41.37 per cent) quarters, *E. coli* from 8 (27.58 per cent), *Streptococcus* spp. were recorded in 4 (13.79 per cent) and 5 (17.24 per cent) quarters showed mixed infection of various bacteria in different combination *viz.* *Staphylococcus* spp., *E. coli*, *Streptococcus* spp., *Enterobacter* spp. and *Klebsiella* spp. The organisms were identified up to genus only. The *Staphylococcus* spp. was the most frequent organism, accounting for 12 of the total 29 isolates (41.37 per cent) followed by *E. coli* 8 (27.59 per cent) and *Streptococcus* spp. 4 (13.79 per cent) (Table 1).

Mixed infection was recorded in 5 quarters. The organisms isolated in mixed infection were *Klebsiella* spp. and *Streptococcus* spp. recorded in 1 (20 per cent) quarter, combination of *Klebsiella* spp. and *Staphylococcus* spp. in 1 (20 per cent) quarter and *Staphylococcus* spp. and *E. coli* in 3 (60 per cent) quarters.

### **Group I**

In Group I, out of 7 quarters, *Staphylococcus* spp. was isolated from 4 quarters (57.14 per cent), and *E. coli*

infection was isolated from 2 quarters (28.57 per cent) and 1 quarter showed (14.29 per cent) mixed infection in combination of *Klebsiella* spp. and *Streptococcus* spp. (Table2)

## **Group II**

In Group II, Out of 7 quarters, *Staphylococcus* spp. was isolated from 3 quarters (42.85 per cent) and *E. coli* infection was isolated from 3 quarters (42.85 per cent) and 1 quarter showed (14.30 per cent) mixed infection of *Klebsiella* spp. and *Staphylococcus* spp. (Table3)

## **Group III**

In Group III, out of 6 quarters, *Staphylococcus* spp. was isolated from 3 quarters (50.00 per cent), *E. coli* isolated from 2 quarters (33.33 per cent) and 1 quarter showed (16.67 per cent) mixed infection of both bacterial species viz. *E. coli* and *Staphylococcus* spp. (Table 4)

## **Group IV**

In Group IV, out of 7 quarters, *Staphylococcus* spp. was isolated from 2 quarters (28.57 per cent), *Streptococcus* spp. was isolated from 2 quarters (28.57 per cent), *E. coli* infection was isolated from 1 quarter (14.29 per cent) and 2 quarter showed (28.57 per cent) mixed infection of both bacterial species viz. *E. coli* and *Staphylococcus* spp. (Table 5)

## ***In vitro* antibacterial activity sensitivity of herbal preparations**

The study was done to evaluate the *in vitro* antibacterial activity of *W. somnifera* (Ashawagandha) roots and *O. sanctum* (Tulsi) leaves against the common mastitis pathogens viz. *Staphylococcus* spp., *E. coli*,

*Klebsiella* spp. and *Enterobacter* spp. Antibacterial activity of the extracts was evaluated by using paper disc agar diffusion method. This is also known as Kirby-Bauer disk diffusion method (Bauer *et al.*, 1966).

## **Preparation of herbal extracts**

Two types of herbal preparations viz. powder was tried for *in vitro* sensitivity test.

Further, the preliminary testing of two herbal preparations for antibacterial activity revealed comparatively higher zones of growth inhibition for mastitis pathogens.

## **Inhibition of bacterial growth**

The antibacterial activity of the herb against particular mastitis pathogen was determined as proportion of zones of growth inhibition formed by the herbal preparations and the standard antibiotic i.e. cefotaxime depicted as percentage. The zones of inhibition produced by powdered *W. somnifera* and *O. sanctum*, and their antibacterial activity for different bacteria in terms of zones of inhibition formed by cefotaxime have been depicted in Table 6.

In this study, the average zone of inhibition shown by *W. somnifera* against *Staphylococcus* spp. was 14.20 mm and 13.10 against *E. coli*. Inhibitory zones formed by powder of *O. sanctum* (Tulsi) were found to be different for different bacteria, 12.60 mm against *Staphylococcus* spp. and 12.30 against *E. coli* during course of the present investigation.

The zones of inhibition produced by aqueous extract of *O. sanctum* and *W. somnifera* and their antibacterial activity for different bacteria in terms of zones of inhibition formed by cefotaxime have been depicted in Table 7.

**Table.1** Overall presence of organisms causing subclinical mastitis

S. No.	Bacteria isolated	No. of infected quarters	Per cent
1.	<i>Staphylococcus</i> spp.	12	41.37
2.	<i>E. coli</i>	8	27.59
3.	<i>Streptococcus</i> spp.	4	13.79
4.	Mixed infection	5	17.25
	Total	29	100

**Table.2** Quarter-wise presence of organisms causing subclinical mastitis in group I

S. No.	Bacteria isolated	No. of infected quarters (n=7)	Per cent
1.	<i>Staphylococcus</i> spp.	4	57.14
2.	<i>E. coli</i>	2	28.57
3.	Mixed Infections ( <i>Klebsiella</i> spp. and <i>Streptococcus</i> spp.)	1	14.29
	Total	7	100

**Table.3** Quarter-wise presence of organisms causing sub-clinical mastitis in group II

S. No.	Bacteria isolated	No. of infected quarters (n=7)	Per cent
1.	<i>Staphylococcus</i> spp.	3	42.85
2.	<i>E. coli</i>	3	42.85
3.	Mixed Infections ( <i>Klebsiella</i> spp. + <i>Staphylococcus</i> spp.)	1	14.30
	Total	7	100

**Table.4** Quarter-wise presence of organisms causing sub-clinical mastitis in group III

S. No.	Bacteria isolated	No. of infected quarters (n= 6)	Per cent
1.	<i>Staphylococcus</i> spp.	3	50.00
2.	<i>E. coli</i>	2	33.33
3.	Mixed Infections ( <i>E. coli</i> + <i>Staphylococcus</i> spp.)	1	16.67
	Total	6	100

**Table.5** Quarter-wise presence of organisms causing sub-clinical mastitis in group IV

S. No.	Bacteria isolated	No. of infected quarters (n=7)	Per cent
1.	<i>Staphylococcus</i> spp.	2	28.57
2.	<i>E. coli</i>	1	14.29
3.	<i>Streptococcus</i> spp.	2	28.57
4.	Mixed Infections ( <i>E. coli</i> + <i>Staphylococcus</i> spp.)	2	28.57
	Total	7	100

**Table.6** Mean  $\pm$  SE values of zones of inhibition (mm) of powder of root of *W. somnifera* and leaves of *O. sanctum* against subclinical mastitis pathogens of Group I and Group II of Rathi cows

Pathogen	Group I Average Zones of Inhibition (mm)		% Sensitivity	Group II Average Zones of Inhibition (mm)		% Sensitivity
	<i>W. somnifera</i>	Cefotaxime		<i>O. sanctum</i>	Cefotaxime	
<i>Staphylococcus</i> spp.	14.20	23.50	60.42	12.60	23.50	53.61
<i>E.coli</i>	13.10	24.00	54.58	12.30	24.00	51.25

**Table.7** Mean  $\pm$  SE values of zones of inhibition (mm) of aqueous extract of *W. somnifera* and leaves of *O. sanctum* against subclinical mastitis pathogens of Group III and Group IV of Rathi cows

Pathogen	Group III Average Zones of Inhibition (mm)		% sensitivity	Group IV Average Zones of Inhibition (mm)		% sensitivity
	<i>O. sanctum</i>	Cefotaxime		<i>W. somnifera</i>	Cefotaxime	
<i>Staphylo coccus</i> spp.	20.00	23.50	85.10	20.50	23.50	87.23
<i>E.coli</i>	18.00	24.00	75	19.00	24.00	79.16
<i>Streptococ cus</i> spp.	17.50	23.00	76.08	18.00	23.00	78.26

In this study, the average zone of inhibition shown by aqueous extract of *O. sanctum* against *Staphylococcus* spp. was 20.00 mm, 18.00 mm against *E. coli*. and 17.50 mm *Streptococcus* spp. and average zone of inhibition shown by aqueous extract of *W. somnifera* against *Staphylococcus* spp. was 20.50 mm, 19 mm against *E. coli*. and 18.00 mm *Streptococcus* spp. The zone of inhibition shown by control antibiotic cefotaxime against *Staphylococcus* spp. was 23.5 mm, 24 mm against *E. coli*. and 23.00 mm against *Streptococcus* spp.

The aqueous extract of *O. sanctum* and *W. somnifera* showed higher antibacterial activity against *Staphylococcus* spp. 85.10 per cent and 87.23 per cent, respectively and against *E.coli* 75 per cent and 79.16 per cent, respectively as compared to powder of

*O. sanctum* and *W. somnifera* which showed antibacterial activity against *Staphylococcus* spp. 53.87 per cent and 60.63 per cent and against *E. coli* 51.37 per cent and 54.79 per cent, respectively. Furthermore, aqueous extract of *W. somnifera* showed higher antibacterial activity against common mastitis pathogens viz. *Staphylococcus* spp. 87.23 per cent, *E. coli* 79.16 per cent and *Streptococcus* spp. 78.26 per cent as compared to *O. sanctum* which showed antibacterial activity against *Staphylococcus* spp. 85.10 per cent, *E. coli* 75 per cent and *Streptococcus* spp. 76.08 per cent.

In sub-clinical mastitic milk (mixed infection) in different quarter were also observed by Aydin *et al.*, (2009), Yuan Yuan *et al.*, (2012), Hanam *et al.*, (2015) and Mahenthiran *et al.*, (2016).

Shukla *et al.*, (1998) reported high prevalence of *Staphylococcus* spp. This may be due to the fact that *Staphylococcus* spp. survives better in the environment than other microorganisms. It is present in large numbers in different body sites, such as teat orifice and surfaces.

Further the prevalence of *Streptococcus* spp. in milk was found to be lower than *Staphylococcus* spp. as the *Streptococcus* spp. are unable to survive for longer periods in environment outside the body (Schalm *et al.*, 1971). *E. coli* is generally pathogenic for udder and causes some mild infections (Schalm and Lasmanis, 1968).

According to Michael *et al.*, (1991) the invasive property of *Staphylococcus* spp. enables it to survive in udder tissues and during cyclic infection, inefficient phagocytosis and killing contributes the relapse of infection. The low incidence of Gram negative rods may be due to their destruction by mammary glands (Waage *et al.*, 1998).

The antibacterial activities of crude extracts of *W. somnifera* against wide variety of pathogenic organisms such as *S. aureus*, *E. coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* have been reported by Sundaram *et al.*, (2011).

Similar to this study, El-Boushy *et al.*, (2009) demonstrated antibacterial activity of both aqueous root extracts of *W. somnifera* against pathogenic *E. coli in vitro*. Similarly, Tuteja *et al.*, (2005) found potent antibacterial activities of *W. somnifera* aqueous root extract against numerous pathogenic bacteria isolated from intramammary and skin infections of camel. Owais *et al.*, (2005) also found antibacterial activity of alcoholic extract of *W. somnifera* roots against *S. typhimurium*.

Bogdan (2001) attributed the antibacterial activity to the increased production of nitric oxide due to *W. somnifera* in mouse macrophages, in a concentration-dependent manner, to increase in inducible nitric oxide synthase, an enzyme generated in response to inflammatory mediators and known to inhibit the growth of many pathogens. Nitric oxide has been demonstrated to have a significant effect on macrophage cytotoxicity against microorganisms and tumor cells. 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 root that are considered as most effective part of the plant (Davis and Kuttan 2000, Arora *et al.*, 2004 and Rasool and Varalakshmi 2006).

Moreover, the extract did not induce lysis on incubation with human erythrocytes, advocating their safety to the living cells.

Mukherjee (2006) also recorded the similar zones of inhibition ranging from 12.5 to 18.3 mm for aqueous extract of *O. sanctum* leaves against *S. aureus*, *S. agalactiae* and *E. coli*. Ali and Dixit (2012) observed the zones of inhibition for flavanoids of *O. sanctum* as 20.12, 20.75, 20.95, 19.55 and 20.1 mm against *E. coli*, *Proteus*, *S. aureus*, *Staphylococcus cohni* and *Klebsiella pneumonia*, respectively.

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