RESEARCH ARTICLE

Effect of Polyherbal Teat Dip Solution on Somatic Cell Count, Standard Plate Count and Prevention of Bovine Sub-Clinical Mastitis

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Abstract

Mastitis is the most common and costly disease affecting dairy cattle worldwide. The long-term use of antimicrobial drugs in farm management poses serious health risks to humans. For pre-milking teat dipping, various teat cleaning disinfectants are currently used. Therefore, to find out its safer natural alternatives, different plant materials like leaves of *Ocimum sanctum, Azadirachta indica, Aloe barbadensis* and rhizome of *Curcuma longa* were collected to develop a polyherbal teat dip solution and to observe ABST against *E. coli, S. aureus* and *Streptococcus* spp., which were isolated from mastitic milk. The aqueous extract of these herbs showed antibacterial activity against all above pathogens in different concentrations (from 100 mg/mL to 700 mg/mL). According to the result of ABST of selected plant material, a polyherbal teat dip solution was prepared and used on 125 lactating cows divided into three groups, the control group (Gr-1, n=25), Gr-2 (polyherbal teat dip application, n=50) and Gr-3 (commercial teat dip application-Amoxicillin, n=50). The somatic cell count (SCC) and standard Plate count (SPC) were estimated on day 0, 15 and 30 post-applications. The SCC and SPC of milk showed a significant ($p<0.01$) decrease from day 0 to day 15 and 30 after the application of Gr-2 product and there was no significant difference between Gr-2 and Gr-3 on day 15 and 30. The application of polyherbal teat dips solution before and after milking decreased SCC and SPC with similar efficacy to commercial teat dip solution. Therefore, it can be concluded that polyherbal teat dip solution may be used as an alternative for the prevention of bovine mastitis.

Key words: Bovine, Mastitis, Polyherbal teat dip, Somatic cell count, Standard plate count.

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Introduction

Mastitis, an inflammatory condition of the udder due to bacterial infection, is the most common and costly disease affecting dairy cattle worldwide. Mastitis is caused by a variety of bacteria, *viz., Staphylococcus aureus, Streptococci, E. coli, Corynebacterium* spp., and *Klebsiella* spp., that can enter the udder, multiply, and produce hazardous substances that cause inflammation. It reduces cow productivity as well as milk quality, resulting in massive losses for breeders and, as a result, to the country's national income (Tewari, 2014). Somatic cells that composed of 75% leucocytes and 25% epithelial cells are indicators of both resistance and susceptibility of cows to mastitis and can be used to monitor the level or occurrence of subclinical mastitis in herds or individual cows (Sharma *et al.,* 2011).

Mastitis management is an essential component of the mastitis control programme. The long-term use of antimicrobials has led to the emergence of multidrug-resistant strains of several species of bacteria. A wide variety of teat dips containing several different active ingredients and chemicals are available in the market. However, the growing popularity of natural and herbal medications, easy availability of raw materials, cost-effectiveness and paucity of reported adverse reactions, prompted to formulate a polyherbal teat dip (Gonzalez *et al.,* 2011). India is rich with its plant biodiversity including medicinal plants. Mizoram, the land lock state of North eastern region of India is the home of more than 400 species of medicinal plants (Rai and Lalramnghinglova, 2010).

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Ocimum sanctum (Family Labiatae) is a small herb found throughout India and is commonly known as Vishnu-Priya or Tulsi in Hindi and India's Holy Basil in English. Antimicrobial properties of *Ocimum* extract have been discovered against variety of pathogens with significantly higher antibacterial activity against *Staphylococcus aureus* (Mishra and Mishra, 2011) for its essential oils containing carvacrol, methyl eugenol, and caryophyllene (Khosla, 1995). *Azadirachta indica,* is popularly known as Indian Neem (Margosa tree). Various parts of the neem tree have been used for millennia in traditional Indian medicine for their claimed multiple health benefit properties (Alzohairy, 2016). Curcumin, the principal active component or rhizome of turmeric *(Curcuma longa L.),* has previously been shown to have antimicrobial activity against several important pathogens (Singh and Jain, 2012). The aqueous extract of *Aloe vera* inhibited the growth and formation of biofilms against methicillin-resistant bacteria. Aloe-emodin is a natural anthraquinone derivative and an active ingredient that can be isolated from Aloe *vera,* inhibits *Staphylococcus aureus* biofilm formation by the inhibition of extracellular protein production (Xiang *et al.,* 2017). Considering different published documents regarding utility of these herbal plants, they were used in the present study to prepare a herbal teat dip solution and find out it's efficacy to prevent bovine mastitis.

Materials and Methods

The study was approved by the Institutional Animal Ethics Committee (Approval No. 770/ac/CPCSEA/FVSc/AAU/IAEC/15-16/337 dated 10.04.2015). The samples were collected as per standard procedure and no animals were harmed.

Bacterial Culture

Staphylococcus aureus (n=20)*, Streptococcus spp.* (n=20) and *E. coli* (n=20) isolated from milk samples of mastitis cows of Aizawl, Mizoram were collected and All the bacteria were characterized by standard bacteriological techniques as described by Ewing (1986) and further confirmed by BD Phoenix automated bacterial system. All the pure bacterial isolates were stored at -80°C in glycerol (25% V/V) for further use.

Collection of Plants and Preparation of Extract

The fresh leaves of *Ocimum sanctum, Azadirachta indica, Aloe barbadensis* and Rhizome of *Curcuma longa* were collected and thoroughly washed and air dried at room temperature for four weeks. The dry materials were ground into fine powder using a Corona manual grinding machine. Exactly 300 g each of the cursed fine powder of *Ocimum sanctum, Azadirachta indica, Aloe barbadensis* and Rhizome of *Curcuma longa* were soaked separately in 1 litre of distilled water for 24 h. The aqueous extractions were sieved using a muslin cloth and filtered using Whatman No. 1 (125 mm) filter paper followed by evaporation at 40°C under vacuum. The filtrate was concentrated in a rotary vacuum evaporator (IKA, RV10 digital, Germany) and the concentrated extracts were re-suspended in water to make the final concentration @ 700 mg/mL. All the extracts were then preserved in separate labelled glass vials at -20°C till further use.

Preparation of Impregnated Disc

Discs of 5 mm diameter were prepared using Whatman filter paper No.1. These were sterilized in the hot air oven at 160°C for 1 h. The discs were impregnated with 20 µL of aqueous extracts at different concentrations ranging from 100-700 mg/mL for four different plant extracts for 3-4 h to check their antimicrobial activity. The impregnated discs were dried in an incubator at 37°C for 18 to 24 h and immediately used for the sensitivity test.

Disc Diffusion Method

The disc diffusion method for antimicrobial susceptibility testing was carried out according to the standard method by Bauer *et al*. (1966). A bacteria culture (which was adjusted to 0.5 McFarland standard) was used to lawn Muller Hinton agar plates evenly using a sterile swab. The plates were dried for 15 min and then used for the sensitivity test using the discs impregnated with a series of plant extracts. Each test plate comprised five discs, one positive control, which was a standard commercial antibiotic disc (Amoxicillin 30 µg), and four treated herbal discs at different concentration viz. 700, 500, 300 and 100 mg/mL for sensitivity against *E. coli, S. aurous* and *Streptococcus* spp. and. The plates were then incubated at 37°C for 18 to 24 h depending on the species of bacteria used in the test, and then examined and measured the inhibition zone using Vernier calipers and recorded. The result was classified based on the diameter of the zone of inhibition as "not sensitive" for a diameter < 8 mm, "sensitive" between 9 and 14 mm, "very sensitive" between 15 and 19 mm and "extremely sensitive" for > 20 mm (Moreira *et al*., 2015). The tests were repeated three times to ensure reliability.

Preparation of Polyherbal Teat Dip

The aqueous extract of *Ocimum Sanctum*, *Azadirachta indica*, *Curcuma longa* and *Aloe barbadensis* were mixed in 700mg/mL concentrations with distilled water according to the zone of inhibition depicted by the major mastitis-causing pathogens. ABST of the polyherbal teat dip solution was also done against *Staphylococcus aureus*, *E.coli* and *Streptococcus* spp.

Clinical Evaluation of Polyherbal Teat Dip against SCM in Crossbred Dairy Cows

For the evaluation of the efficacy of polyherbal teat dip solution against subclinical mastitis (SCM), 125 crossbred lactating cows were screened by California mastitis test, and based on a CMT the cows with somatic cell count in the range of 2.0-3.5 lakhs/mL were considered for the study. The selected cows were in the mid-lactation and had a milk production of 7-10 lit/day. 25 cows were grouped as Gr-I kept as untreated control, 50 lactating cows each were grouped as

Gr-II & III, and were applied with prepared polyherbal teat dip and commercial chemical teat dip (Amoxicillin), respectively. The teat dipping was done twice daily, *i.e.,* morning and evening before and after milking for one month.

Evaluation of Post-Milking Teat Dip Application

The evaluation of pre- and post-milking teat dip application was judged by the changes in somatic cell count (SCC) adopting manual method described by Singh and Dang (2002) and standard plate count (SPC) by adopting procedure of Busta *et al.* (1984)

Statistical Analysis

All the data collected were stored on Microsoft (MS) Excel spreadsheet program and the quantitative data were analysed by two-way ANOVA as described by Snedecor and Cochran (1994) through the application of SPSS software. To test the significant difference between groups and between days the data were tested for normality using the Kolmogorov-Smirnova test.

Results and Discussions

In Vitro Antibacterial Activity of Plant Extracts against *Staphylococcus aureus, E.coli, and Streptococcus* spp.

In vitro antibacterial sensitivity assays of plant extracts of *Ocimum Sanctum* leaves, *Azadirachta indica* leaves, *Curcuma longa* rhizome and *Aloe barbadensis* miller leaves against *E. coli* , *Staphylococcus aureus,* and *Streptococcus* spp. are shown in Table 1. The highest antibacterial activity of the aqueous extract of *Ocimum sanctum, Azadirachta indica, Curcuma longa and Aloe barbadensis* against *E. coli* was observed at 700 mg/mL with a 16±0.01 16.5±0.01, 16±0.01 and 17±0.01 mm zone of inhibition, respectively, that was reduced a little at 500 mg/mL concentrations, and the lowest activity was observed at 300 mg/mL concentration of all 4 herbal extracts with a 14±0.02, 15±0.01, 12±0.01 and 10±0.02 mm zone of inhibition, respectively. The extracts of *Ocimum sanctum, Azadirachta indica* and *Curcuma longa* at 100 mg/mL concentration did not show any antibacterial activity and zone of inhibitions were negligible while *Aloe barbadensis* at the same 100 mg/mL concentration produced 5±0.01 mm zone of inhibition against *E.coli* (Fig. 1). Very similar patterns of antibacterial activity and zone of inhibitions were observed with different concentrations of four herbal extracts against *Staphylococcus aureus* and *Streptococcus* spp. also (Table 1).

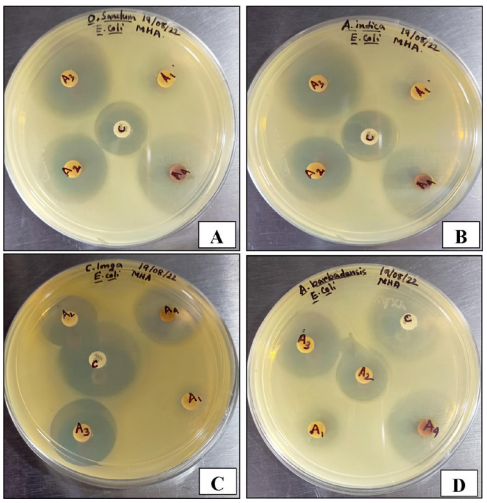

Fig. 1: Antibacterial sensitivity assay of different concentration (A1-700 mg, A2-500 mg, A3-300 mg & A4-100 mg) of aqueous extract of (A) *Ocimum sanctum*, (B) *Azadirachta indica*, (C) *Curcuma longa,* and (D) *Aloe barbadensis* against *E.coli.*

Table 1: Antibacterial activity of aqueous extract of *Ocimum sanctum* leaves, *Azadirachta indica* leaves, *Curcuma longa* rhizome and *Aloe barbadensis* miller leaves at different concentration against *E. coli, Staphylococcus aureus,* and *Streptococcus* spp.

Organism	Extract Concen- tration	Zone of inhibition (in mm)					
		Ocimum sanctum	*Azadirachta indica*	*Curcuma longa*	*Aloe barbadensis*	*Amoxicillin*	*Poly- herbal*
E. coli	700 mg/mL	16±0.01	16.5±0.01	16±0.01	17±0.01	18.5±0.02	17.5±0.02
	500 mg/mL	15±0.01	16±0.01	14±0.05	14±0.01		
	300 mg/mL	14±0.02	15±0.01	12±0.01	10±0.02		
	100 mg/mL	-	-	-	5±0.01		
S. aureus	700 mg/mL	17±0.05	18±0.05	18.5±0.01	17±0.01	27±0.03	19±0.01
	500 mg/mL	15±0.01	17±0.01	17±0.02	13.5±0.03		
	300 mg/mL	13±0.02	15±0.01	15.5±0.01	11.5±0.01		
	100 mg/mL	-	-	-	6±0.01		
Streptococcus Spp.	700 mg/mL	19±0.05	19±0.01	19±0.01	18±0.01	23±0.01	20±0.05
	500 mg/mL	17±0.03	17±0.01	18±0.01	17±0.01		
	300 mg/mL	15±0.01	15±0.01	16±0.02	16±0.05		
	100 mg/mL	-	-	-	13±0.01		

The control antibiotic disc, *i.e.* Amoxicillin showed the zone of inhibition against *E. coli*, *S. aureus* and *Streptococcus* spp. as 18.5±0.02 mm, 27±0.03 mm and 23±0.01 mm, respectively. The polyherbal solution also showed the zone of inhibition against *E.coli*, *S. aureus* and *Streptococcus* spp. as 17.5±0.02 mm, 19±0.01 mm and 20±0.05 mm, respectively. The critical analysis revealed that the leaves of *Ocimum sanctum, Azadirachta indica, Aloe barbadensis* miller and rhizome of *Curcuma longa* have antibacterial activity against *E. coli, Staphylococcus aureus* and *Streptococcus* spp. (Table 1).

The aqueous extracts of all four herbs showed antibacterial activity against *E. coli*. *S. aureus* and *streptococcus* spp. and concurred with the report of Saleem *et al*. (2018). Tulsi essential oil contains a valuable source of bioactive compounds such as camphor, eucalyptol, eugenol, alpha bisabolene, beta bisabolene, and beta caryophyllene. These compounds are proposed to be responsible for the antimicrobial properties of the leaf extracts of *Ocimum sanctum* (Yamani *et al.,* 2016). Mishra and Mishra (2011) observed the antibacterial activity of aqueous and chloroform extract of leaves of Tulsi against the bacteria, *i.e., E. coli, S. aureus, P. aeruginosa, S. Typhimurium.* The aqueous extracts of *Ocimum sanctum* (300-700 mg/mL) leaves showed antibacterial sensitivity against *E. coli, S. aureus* and *streptococcus* spp. A similar study was also reported by Solanki *et al*. (2022). Sikrodia *et al*. (2020) however reported that the extract of *Ocimum sanctum* showed a zone of inhibition against *S. aureus,* but resistance to *E. coli,* which is dissimilar from the present study. Bhattacharyya and Bishayee (2013) also reported that the aqueous extracts of *O. sanctum* leaves were more effective against pathogens as compared to methanolic extract. The present study found antibacterial activity against *E.coli* might be due to the use of higher concentrations.

The present findings of antibacterial activity of aqueous extract of *Azadirachta indica* (300-700 mg/mL) against *E.coli, S. aureus* and *Streptococcus* concurred with Sikrodia *et al*. (2020), who reported 16 mm and 19 mm zone of inhibition against *E. coli* and *S. aureus,* respectively, with Neem extract. The antibacterial activity of *A. indica* leaves is attributed to its bioactive compounds such as nimbin, nimbinin, and nimbidin, which act by disrupting the bacterial cell membrane and interfering with their metabolic processes (Wylie and Merrell, 2022). Sahrawat *et al.* (2018) reported that the extract of *Azadirachta indica* leaves contains many chemical components such as saponins, tannins, proteins, carbohydrates, alkaloids and phenols, which justified that the plant has biological activities such as antibacterial activity. Bhatt *et al.* (2013) reported antibacterial activity of *Curcuma longa* against *E. coli, S. aureus* and *Streptococcus* spp. as we found in the present study; while Sikrodia *et al.* (2020) found 14 mm zone of inhibition with *Curcuma longa* extract against *E. coli,* but resistance to *S. aureus*. This dissimilarity might be due to variation of solvent and the concentration of extract. The current findings of *in vitro* antibacterial activity of *Aloe barbadensis* leaf extract against *E. coli, S. aureus* and *streptococcus* spp. was in line with Forno-Bell *et al*. (2019) that *A. vera* gel extract disrupted the cell membrane causing lysis in 75% of *Staphylococcus aureus*, 88% of *E. coli*, in 97% of *Streptococcus* spp.

The *in vitro* antibacterial activities against *E. coli, S. aureus* and *Streptococcus* spp. by the crude extract could be due to presence of some active phytochemicals such as phenolic compounds, flavonoids, terpenoids, triterpene, tannins, essential oils, saponins, glycosides, carotenoid, and a number of other fixed substances. These phytochemicals are known to exhibit useful biological activities and a variety of medicinally important effects, including antibacterial activities and they may have acted alone or in combination to affect the bacterial organisms.

Evaluation of Polyherbal Teat Dips Solution on Somatic Cell Count (SCC) and Standard Plate Count (SPC) Before and After Application

The analysis of polyherbal teat dips solution on SCC and SPC before and after the application is depicted in Table 2. The result showed that there was no significant difference among the groups in terms of somatic cell count and standard plate count on day 0. However, the somatic cell count of group II and group III was significantly ($p \leq 0.01$) decreased on day 15 (152800±3876.04 and 148200±2849.85) in comparison to day 0 (254600±3576.04 and 258000±3168.04, respectively). Similarly on day 30 the somatic cell counts further significantly ($p \leq 0.01$) decreased in group II and group III (134400 ±4082.42 and 122200±2638.65 respectively) as compared to day 0. However, the standard plate counts of group II and group III were significantly ($p \leq 0.01$) decreased on

Table 2: Comparative evaluation of polyherbal teat dips solution and a commercial solution on somatic cell count (SCC) and standard plate count (SPC) before and after application

Parameter	Group	0 Day	15 days	30 days
SCC	Group I	250800^{a}±3508.09	252800^{Aa}±3508.09	258800^{Aa}±3508.09
	Group II	254600^{a}±3576.04	152800^{Bb}±3876.04	134400^{Bb}± 4082.42
	Group III	258000^{a}±3168.04	148200^{Bb} ±2849.85	122200^{Bb}±2638.65
SPC	Group I	535600^{b} ± 10536.90	572000^{aC} ± 10754.80	595200^{a} ± 13280.60
	Group II	532000^{b} ± 9655.28	504400^{abB} ± 9873.32	483600^{aB} ± 11189.5
	Group III	519800^{c} ± 9037.29	458400^{bA} ± 8667.84	386200^{aC} ± 7542.48

day 30 (483600±11189.5 and 386200±7542.48) as compared to day 0 (532000±9655.28 and 519800±9037.29, respectively).

In the present study, poly-herbel teat dipping effectively reduced somatic cell counts of milk in cows. The decrease in SCC was attributed to decline in total leucocyte counts including lymphocytes and neutrophils. Somatic cell count has been used extensively as an indicator of degree of intramammary infection. Somatic cell count plays a protective role against infection in bovine mammary gland as a normal part of defense mechanism. De and Mukherjee (2009) reported that the SCC of cows treated with hydro-methanolic extract of *A. indica* significantly ($p<0.05$) decreased to the extent of 30.66%, 63.99% and 77.37% on days 3, 7 and day 15, respectively, which is similar with the present study. Shafi *et al.* (2016) reported that treatment with *O. sanctum* leaf powder could eliminate 9/13 (69.23%) of intramammary infections as compared to 4/15 (26.67%) in the control group on day 14 after treatment. SCC of milk in the treatment group showed a significant ($p<0.05$) reduction on day 28 in comparison to the SCC on day 0. Similarly, Mukherjee *et al.* (2014) also stated that the SCC and total bacterial count (TBC) in cows treated with *Curcuma longa* and vitamin E + selenium significantly decreased ($p<0.05$) on day 7 and day 15. Kumar *et al.* (2018) also used polyherbal and found reduced somatic cell count comparable to standard antibiotic therapy. These findings indicated that the herbal teat dip helped in improving the increased SCC to normalcy within 30th day post-dipping, indicating its effectiveness. The constituent herbs of polyherbal teat dip solution, *viz., Ocimum sanctum*, *Azadirachta indica*, *Curcuma longa* and *Aloe barbadensis* are well known to possess antimicrobial, anti-inflammatory and immunomodulator activities (Biswas and Biswas, 2023). Pankey *et al.* (1987) reported that predipping reduced the SPC rate of intrammamary infection with major mastitis pathogens such as *Staphylococcus aureus, Streptococcus agalactiae* and coliforms. Gibson *et al.* (2008) concluded that most pre-milking teat cleaning treatments reduce the teat total bacterial count, but that cleaning effectiveness was influenced by the type of disinfectant and the application methods. These properties may be responsible for normalizing the SCC and SPC in subclinical mastitic animals.

Conclusion

The application of polyherbal teat dip solution (containing aqueous extracts of *Ocimum sanctum* leaves, *Azadirachta indica* leaves, *Curcuma longa* rhizhome and *Aloe barbadensis* miller power) before and after milking decreased somatic cell count and standard plate count, which was having similar efficacy to commercial teat dip solution (Amoxicillin). These findings indicated that the poly-herbal teat dip helped in improving the increased SCC & SPC to normalcy within the 30th day post-dipping, indicating its effectiveness. It can be concluded that poly-herbal teat dip solution may be used as an alternative for the prevention of bovine mastitis.

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