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Effect of *Ocimum sanctum* essential oil as post milking teat spray in sub clinical mastitis affected cross bred dairy cattle

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Abstract
Subclinical mastitis (SCM) derails nearly 10-20% milk production. Essential oils exhibit anti-bacterial activity thereby, an alternate to antibiotics. A study was designed to evaluate the effect of *Ocimum sanctum* oil as post milking teat spray upon the Total Bacterial Count (TBC) of teat surface and milk, milk pH of SCM affected dairy cows. The study was carried out for one lactation period spread over three different seasons in Thiruvallur district of Tamil Nadu. To the positively detected SCM cases commercially available *Ocimum sanctum* essential oil was applied as post milking teat spray using pressure spray bottle. The results discernibly showed that the TBC of teat surface and milk samples evidenced no significant difference on the 0th day but depicted a significant difference (p<0.05) on the 7th and 15th day post application. Therefore, post-milking teat spraying was effective in preventing the invasion of pathogenic microorganisms into the udder.

Keywords: Essential oil, post teat dip, total bacterial count, sub clinical mastitis

Introduction
Tamil Nadu produces around 7234.64 tonnes of milk from cross bred dairy cows (2018-19) and is one among the progressive milk producing states in the country (BAHS 2019). However the individual production margins of cross bred dairy cattle is 8.55 Kg/day/Animal which is restricted due to shortfalls in management especially udder health and hygiene. Exclaimed that subclinical mastitis has a negative influence on the performance of dairy cows and buffaloes. It has a significant impact on production, nearly 10-20% decrease in milk production, as it causes an undesirable effect on the milk constituents, which affects its nutritional value and renders it unfit for processing and consumption (Iraguha *et al.*, 2015) [7]. It includes reduced milk production, changes in milk quality and treatment cost. Rathod *et al.*, (2017) [15] estimated the economic losses due to subclinical form of mastitis were approximately in the range of INR 21,677 to INR 88,340 per animal for a lactation period. It has been reported that SCM causes threefold more production losses as compared to clinical mastitis leading to substantial economic losses of 60-70% all due to mastitis (De Vliegher et al. 2012; Sinha et al. 2014) [5, 19]. Aiemsaard *et al.*, (2020) [1] opined that essential oils contain various chemical constituents such as monoterpenes, sesquiterpenes, diterpenes and other aromatic or aliphatic compounds which exhibit anti-bacterial activity. Hence these essential oils could be promising post-milking teat dip especially as an alternate to antibiotic treatment for mastitis which poses a public health concern (Oliver and Murinda, 2012) [12]. Sharma (2018) [18] envisaged that *Ocimum sanctum* possesses immunomodulatory and anti-inflammatory properties attributed to its active constituents such as volatile oil (eugenol, 80%), flavonoids, and triterpene, which are largely responsible for its therapeutic potential. Therefore, this study was designed to evaluate the effect of Tulsi (*Ocimum sanctum*) oil as post milking teat spray upon the Total Bacterial Count (TBC) of teat surface and milk following changes in milk pH of treated animals and non-teat-dipped animals to determine the impact of post milk teat spraying strategies on udder health.

Materials and Methods

The present study met the ethical committee considerations.

Study area and season

The study was carried out for one lactation period spread over three different seasons as described by Rajalakshmi *et al.*, (2013) [13] *viz* winter months (January and February), Summer months (March, April and May), South west monsoon (June to September) and North East monsoon (October to December).

The study was carried out in the Thiruvallur district which is a part of north eastern zone of agro climatic zone of Tamil Nadu. It lies between 12º 55'N to 13º 34'N Latitude, 79º 17'E to 80º 21'E Longitude and has an aerial extent of 3550 sq.km. For this purpose three types of flooring systems were which are commonly prevalent in semi urban dairy farms *viz*., Cement floor, stone slab floor and mud floor were selectively chosen.

In Thiruvallur district the following blocks were selected

- Avadi
- Thirumullaivoyil
- Kasva Pakkam
- Annanur

Dairy farm selection criteria

- Dairy farms with not less than 5 adult cattle holdings
- Cross bred cattle holdings
- The daily average yield of each cattle head had not less than 5 liters per day

Detection of sub clinical mastitis

To have a preliminary study CMT (Californian Mastitis Test) was carried where a plastic paddle with four chambers of shallow cups was used to perform the test. About 3.0 ml of milk sample from each quarter was stripped directly into the cups. To this equal volume of California mastitis test reagent was added and mixed through gentle swirling the paddle in a circular motion for few seconds. Based on the thickness of the gel formed by CMT reagent-milk mixture, test results were scored as 0 (negative/ trace), +1 (weak positive), +2 (distinct positive), and +3 (strong positive) as described by Sangwa *et al.,* (2025). The animals which were detected positive were further confirmed with TANUCHEK SCC kit developed by Tamil Nadu Veterinary and Animal Sciences University, Chennai as suggested by Ramya and Madanmohan (2021) [14]. The teats were washed properly and first two streams of milk were discarded. The teat sample was collected from each teat was transferred to a clean container. From that, a drop of milk sample was taken using a Pasteur pipette and added in a SCC tube which was provided in the kit. Following, 3 drops of the enhancer was added with uniform mixing and kept at room temperature for 30 minutes. The blue colour developed is compared with the TANUCHEK SCC colour card. The value obtained by matching the colour card was multiplied by 1000 (100x1000 cells, 300x1000 cells, 500x1000 cells, 700x1000 cells, 900x1000 cells) to evaluate the SCC per ml of milk. The mentioned test was carried out to check the prevalence of SCM post application of the essential oil teat spay.

Tailoring the aforesaid, a total of 120 numbers of positive cases of SCM affected cross bred dairy cattle were purposively sampled for the study during one season. Hence total of 360 numbers of SCM cases were used for the trial.

Calculation of accuracy of the test result

The percentage accuracy of the tests, sensitivity, specificity, and the predictive values of the TANUCHEKK SCC kit results, were calculated in comparison with the gold standard bacterial culture results as described by Sharma *et al.,* (2010) [17]. The Kappa statistics was used for analysis and is tabulated in Table 2.

Application of teat spray

To the positively detected SCM cases commercially available *Ocimum sanctum* essential oil was applied as post milking teat spray using pressure spray bottle. A split udder design (Fitzpatrick *et al*., 2021) [6] was used wherein the fore quarters are maintained as control (T_1) and hind quarters for receiving the teat disinfectant protocol (T_2). The fore quarters were washed with water for sampling purpose. The teat spray was applied continuously for a period of 15 days after milking the animals.

Teat swab sampling method

Sample swabs would be taken drawing the swabs which were dipped in sterile normal saline, across the teat orifice and down the side of each teat avoiding contact with the udder hair or cows flank at all times. Swabs were taken after 15min of post teat spray application as suggested by Yadav *et al.,* (2022) [21]. For this purpose sterile cotton tipped swabs (Hi-Media) were used. Three swabs as post teat spray were taken.

Collection of milk samples

The milk samples collected during milking process in sterile sampling bottles (TARSONS) were capped, labelled, transported aseptically to the Department of Livestock Production Management in Madras Veterinary College, Chennai, Tamil Nadu in an ice box, and stored at 4 °C. Bacteriological culturing work was conducted under aseptic condition in laminar air flow cabinet within 24 hours after sampling.

- **Total bacterial count (TBC) of milk samples:** The total bacterial count as opined by Miles and Mishra (1938) wherein serial dilution up to $1:10^{-12}$ in triplicate were prepared. The test-tubes and the petri dishes were marked appropriately. Following, 0.5 ml of fresh alliquot is taken out and mixed in 4.5ml of sterile normal saline. The inoculum from each dilution is deposited as a drop onto the surface of a plate count agar from a calibrated dropping pipette. Each 20 microlitre drop (0.02ml) is allowed to fall from a height of 2.5 cm onto the surface of the well-dried growth medium, where it spreads over 1.5-2cm. The plated sample were allowed to solidify and then incubated at 37 degree Celsius for 24 hours. Colony counts were made in the drop areas showing the largest number of discrete colonies that are not confluent.
- **Total bacterial count (TBC) of teat samples:** The teat swabs were immersed in sterile normal saline and vortexed for uniform mixing. The procedure of Miles and Mishra (1938) as mentioned above was followed and diluted up to $1:10^{-8}$ in triplicate samples.
- **pH of milk**: Milk pH was tested with pH strips.

Statistical analysis

The statistical analysis was performed using SPSS software (version 25.0). The comparison of TBC and pH values was delegated through student ‘t’ test.

Results and Discussion

The chemical composition of *Ocimum sanctum* oil as analyzed through GC-MS in SAIF, IIT Madras is shown in Table 1.

The Mean ± SE of TBC of teat skin surface, TBC of milk and pH of milk is shown in Table. 3. It is discernible that the TBC of teat surface and milk samples showed no significant difference on the 0th day but depicted a significant difference (p<0.05) on the 7th and 15th day post teat spray application. The mean variation observed between treatment groups followed similar pattern in all seasons for treatment groups. It could be noted that the essential oil had decreasing effect on TBC of teat surface and milk samples invariable of the seasons. The lower levels of TBC in the treatment groups clearly indicate that post-milking teat spraying was effective in preventing the invasion of pathogenic microorganisms into the mammary gland and thereby resulted in improving udder health. Reported that raw milk samples contained an average total bacterial count of 5.84 log cfu/ml attributing the cause should be due to inadequate sanitary conditions during milking, collection and transport. Shafi *et al.*, (2016) [16] opined that *Ocimum sanctum* eliminated 69.23% of intramammary infections ($\chi 2$ = 5.07; $p \leq 0.5$) and resulted in a significant reduction in somatic cell count in mastitis infected HF × Sahiwal cows elucidating the immunotherapeutic potential of an herb.

Johri *et al.*, (2018) [8] ascertained the findings by showing that aqueous extract of *O. sanctum* showed higher antibacterial activity against *Staphylococcus* by 85.10 per cent, and against *E. coli* 75 per cent. The results are supported by Tiwari *et al.*, (2024) [20] who explained that increased TBC readings indicate udder illness, lower milk production, changes in milk composition, higher production costs and lower profit. Through the teat canal, bacteria enter the mammary gland and multiply in the milk. The mammary epithelium's ability to function is directly impacted by bacterial toxins, enzymes and cell wall components. Envisaging the results, the antibacterial, anti-inflammatory and immunological modulatory properties of the essential oil has influenced the udder's defence system and improved udder health, explaining the decline in the TBC of teat skin and milk. Teat disinfectants offer a broad spectrum of efficacy and kill swiftly while leaving a persistent film on the teat skin that forms a physical and chemical barrier that provides long-term protection.

From the table 3 it could be noted that means of pH also exhibited significant variation (p<0.05) on 7th and 15th day post application, irrespective of season, suggesting that the essential oil had modulatory effect on the pH of milk. The possible reason explained by Mukherjee *et al.*, (2005) [11] is that *O. sanctum* (leaf) possesses some biologically active principles that are antibacterial and immunomodulatory in nature, thereby increasing the lysosomal enzymes contents of the milk, inducing acidity changes. The findings are akin to Bagri *et al.*, (2018) [2] who explained that the average pH of normal and subclinical mastitis milk was 6.37 and 6.69, respectively such that the average pH values of subclinical mastitis milk were increased than normal milk pH values but non-significantly.

Table 1: Chemical composition of *Ocimum sanctum* oil as analyzed through GC-MS

Compounds	Peak	Peak area percentage (%)
Camphene	3.089	0.32
o-Cymene	4.090	0.62
D-Limonene	4.158	1.06
Eucalyptol	4.216	4.216
Linalool	5.377	6.93
Isoborneol	6.601	1.17
Estragole	7.533	27.17
Geraniol	8.759	0.64
α-Cubebene	11.074	0.93
Phenol, 2-methoxy-3-(2-propenyl)-	11.382	15.40
Copaene	11.748	11.59
Methyleugenol	12.470	10.28
Caryophyllene	12.865	14.63
Humulene	13.576	2.57
1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene	15.205	3.72
Caryophyllene oxide	16.574	2.43

Table 2: Kappa statistics for TANUCHEKK SCC kit used for the detection of sub clinical mastitis. Data are presented as number (percentage)

Test name	TANUCHEKK SCC kit
Samples examined	356
Positive samples	186
True positive	170 (91.39)
False positive	6 (3.22)
True negative	162 (87.09)
False negative	18 (9.67)
Accuracy	93.25%
Sensitivity	96.59%
Specificity	90%
Chi square value	267.74**
Kappa	0.8653 (Perfect agreement)

Table 3: Mean ± SE of Total Bacterial Count (TBC) and pH of milk of treatment groups during different seasons

Parameters	T_1	T_2	T-Value
Summer season			
TBC of teat surface on 0th day	1.7 x 10^8±0.048	1.6 x10^8±0.055	0.541 ^{NS}
TBC of teat surface on 7th day	1.1 x10^8±0.065	8.3 x10^7±0.069	3.6157 **
TBC of teat surface on 15th day	1.2 x10^8±0.071	5.7 x10^7±0.099	5.416**
TBC of milk on 0th day	1.3 x10^{12}±0.096	1.09 x10^{12}±0.077	-1.491^{NS}
TBC of milk on 7th day	1.2 x10^{12}±0.077	9.2 x10^{11}±0.056	3.153**
TBC of milk on 15th day	1.09 x10^{12}±0.063	3.6 x10^{11}±0.084	10.499**
pH of milk on 0th day	6.70±0.021	6.64±0.025	1.777**
pH of milk on 7th day	6.63±0.023	6.46±0.016	-0.927^{NS}
pH of milk on 15th day	6.68±0.016	6.47±0.004	12.493**
Rainy season			
TBC of teat surface on 0th day	1.9x 10^8±0.056	1.8 x10^8±0.050	0.590 ^{NS}
TBC of teat surface on 7th day	1.4x 10^8±0.0453	9.0 x10^7±0.057	6.499**
TBC of teat surface on 15th day	1.3x 10^8 0.050	6.3x 10^7±0.070	9.002**
TBC of milk on 0th day	1.6 x10^{12} 0.0438	1.4 x10^{12}±0.257	1.670 ^{NS}
TBC of milk on 7th day	1.3 x10^{12} 0.081	8.6 x10^{11}±0.074	4.219**
TBC of milk on 15th day	1.3 x10^{12} 0.061	3.9 x10^{11}±0.102	9.599**
pH of milk on 0th day	6.70±0.021	6.67±0.022	0.736^{NS}
pH of milk on 7th day	6.65±0.0237	6.38±0.014	-4.160**
pH of milk on 15th day	6.72±0.021	6.48±0.009	-2.707**
Winter season			
TBC of teat surface on 0th day	1.9 x10^8±0.046	1.7 x10^8±0.039	1.617^{NS}
TBC of teat surface on 7th day	1.7x 10^8±0.032	9.8 x10^7±0.099	5.220**
TBC of teat surface on 15th day	1.7 x10^8±0.0961	6.3 x10^7±0.070	8.541**
TBC of milk on 0th day	1.7 x10^{12}±0.0417	1.6 x10^{12}±0.032	0.0608^{NS}
TBC of milk on 7th day	1.3 x10^{12}±0.081	8.6 x10^{11}±0.074	4.218**
TBC of milk on 15th day	1.4 x10^{12}±0.037	3.9x 10^{11}±0.102	12.142**
pH of milk on 0th day	6.78±0.023	6.73±0.022	1.422 ^{NS}
pH of milk on 7th day	6.66±0.016	6.48±0.0108	7.496**
pH of milk on 15th day	6.68±0.11	6.56±0.188	2.443**

Conclusions

A study was conducted to assess the efficiency of *Ocimum sanctum* oil as post milking teat spray trying it as an alternative to antibiotics in sub clinical mastitis affected cows. The Total bacterial load skewed down on the 7th and 15th day thus preventing the invasion of pathogenic microorganisms into the mammary gland and thereby resulted in improving udder health. Moreover the spray formulations are convenient to use at small farmers farm. Because, the spraying device does not need to come in direct contact with the teats, which could help to reduce the transmission of infectious agents between animals. Second, using a spray ensures that the concentration of disinfectant is consistent across the area being sprayed.

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