

**THERAPEUTIC EVALUATION OF *Emblica officinalis* AND *Citrus limon*  
FRUIT IN BOVINE SUB-CLINICAL MASTITIS**

**THESIS**

Submitted

In partial fulfilment of the requirements for the Degree of

**MASTER OF VETERINARY SCIENCE**

**IN**

**VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE**

**BY**

**CHAUNDE DIPAK SHIVAJI**

Enrolment No: V/15/045

**College of Veterinary and Animal Sciences, Parbhani-431 401**

**MAHARASHTRA ANIMAL AND FISHERY SCIENCES**

**UNIVERSITY, NAGPUR- 440 006.**

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**2023**

## DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled, “**THERAPEUTIC EVALUATION OF *Emblica officinalis* AND *Citrus limon* FRUIT IN BOVINE SUB-CLINICAL MASTITIS**” or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

Date:        /        /2023

**CHAUNDE DIPAK SHIVAJI**

Place: Parbhani

Enrolment No.V/15/045

**(Dr. T. A. Shafi)**

Chairman

Advisory Committee with date

## DECLARATION OF ADVISORY COMMITTEE

Mr. CHAUNDE DIPAK SHIVAJI has satisfactorily prosecuted his course of research for a period of not less than one semester and that the thesis entitled, “**THERAPEUTIC EVALUATION OF *Emblica officinalis* AND *Citrus limon* FRUIT IN BOVINE SUB-CLINICAL MASTITIS**” submitted by him is the result of original research work is sufficient to warrant its presentation to the examination in the subject of **VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE** for the award of **MASTER OF VETERINARY SCIENCE** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any other University.

Date:        /        /2023

(Dr. T.A.SHAFI)

Place: Parbhani

(Advisor/Guide)

<b>Advisory Committee</b>		
<b>Name and Designation</b>	<b>Signature</b>	
<b>1 Dr. M. P. Sakhare</b> Professor and Head Dept. of Veterinary Epidemiology and Preventive Medicine, COVAS, Parbhani	_____	
<b>2 Dr. M.F.M.F. Siddiqui</b> Assistant Professor Dept. of Veterinary Clinical Medicine, Ethics and Jurisprudence, COVAS, Parbhani	_____	
<b>3 Dr. P. R. Suryawanshi</b> Assistant Professor Dept. of Veterinary Microbiology COVAS, Parbhani	_____	
<b>4 Dr. S. P. Chaudhari</b> Professor and Head Dept. of Veterinary Public Health and Epidemiology, Nagpur Veterinary College, MAFSU, Nagpur	_____	

## CERTIFICATE

This is to certify that the thesis entitled, “**THERAPEUTIC EVALUATION OF *Emblica officinalis* AND *Citrus limon* FRUIT IN BOVINE SUB-CLINICAL MASTITIS**” submitted by **Mr. CHAUNDE DIPAK SHIVAJI** to the Maharashtra Animal and Fishery Sciences University in partial fulfilment of the requirement for the degree of **MASTER OF VETERINARY SCIENCE** has been approved by the student’s Advisory Committee after examination in collaboration with the External Examiner.

**Name and Signature of  
External Examiner**

**Dr. M. P. Sakhare**  
Professor and Head

**Dr. T. A. Shafi**  
Advisor/Guide

### Advisory Committee

	Name and Designation	Signature
1	<b>Dr. M. P. Sakhare</b> Professor and Head Dept. of Veterinary Epidemiology and Preventive Medicine, COVAS, Parbhani	_____
2	<b>Dr. M.F.M.F. Siddiqui</b> Assistant Professor Dept. of Veterinary Clinical Medicine, Ethics and Jurisprudence, COVAS, Parbhani	_____
3	<b>Dr. P. R. Suryawanshi</b> Assistant Professor Dept. of Veterinary Microbiology COVAS, Parbhani	_____
4	<b>Dr. S. P. Chaudhari</b> Professor and Head Dept. of Veterinary Public Health and Epidemiology, Nagpur Veterinary College, MAFSU, Nagpur	_____

**Associate Dean,  
COVAS, Parbhani**



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Place : Parbhani

Date :

(Chaunde Dipak Shivaji )

## TABLE OF CONTENTS

<b>Sr. No</b>	<b>Chapter</b>	<b>Page</b>
I	INTRODUCTION	1-4
II	REVIEW OF LITERATURE	5-21
III	MATERIALS AND METHODS	22-30
IV	RESULTS AND DISCUSSION	31-42
V	SUMMARY AND CONCLUSIONS	43-46
A)	BIBLIOGRAPHY	i-xiv
B)	APPENDIX	xv-xxii
C)	VITAE	

## LIST OF TABLES

Table	Title	Page No.
3.1	Serial dilution set up for finding MIC of whole fruit juice	25
3.2	The dosage regimen for therapy	27
3.3	Relationship between CMT score and somatic cell count	28
4.1	Zones of inhibition of aqueous extracts of <i>C. limon</i> fruit juice and <i>E. officinalis</i> fruit juice at 50/50 v/v	32
4.2	Zones of inhibition of aqueous extracts of <i>C. limon</i> fruit juice and <i>E. officinalis</i> fruit juice at 75/25 v/v	33
4.3	Zones of inhibition of aqueous extracts of <i>C. limon</i> fruit juice and <i>E. officinalis</i> fruit juice at 90/10 v/v	33
4.4	Comparison of MIC and MBC of <i>E.officinalis</i> and <i>C.limon</i> fruit juices	35
4.5	Effect of herbal therapy on level of intramammary infections (IMI)	37
4.6	Effect on inflammatory markers of the udder by <i>E.officinalis</i> (G2), <i>C.limon</i> (G3) therapy in comparison to control (G1)	39
4.7	Effect of herbal therapy on milk composition in control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3) groups	41
4.8	Effect of herbal therapy on DLC in control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3) groups	42

## LIST OF PLATES

Plate	Title	In between page
1	<i>Emblica officinalis</i> fruits	23-24
2	<i>Citrus limon</i> fruits	23-24
3	Bacterial culture of milk	27-28
4	MCMT test	27-28
5	pH of milk measured using digital pH mater	27-28
6	Electrical conductivity of milk measured using digital conductivity meter	27-28
7	Somatic Cell Count of milk	29-30
8	“Lactoscan” milk scan for biochemical analysis of milk	29-30
9	Differential Leucocyte Count of milk.	29-30
10	Antibacterial effect of <i>E.officinalis</i> fruit juice against <i>S.aureus</i>	32-33
11	Antibacterial effect of <i>E.officinalis</i> fruit juice against <i>E.coli</i>	32-33
12	Antibacterial effect of <i>C.limon</i> fruit juice against <i>S.aureus</i>	32-33
13	Antibacterial effect of <i>C.limon</i> fruit juice against <i>E.coli</i>	32-33

## LIST OF FIGURES

Figures	Title	In between page
4.1	CMT point score in milk of Control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3) groups	40-41
4.2	SCC in milk of Control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3) groups	40-41
4.3	EC in milk of Control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3)	40-41
4.4	pH in milk of Control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3)	40-41
4.5	Protein % in milk of Control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3) groups	42-43
4.6	Fat % in milk of Control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3) groups	42-43
4.7	SNF% in milk of Control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3) groups	42-43
4.8	Lactose % in milk of Control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3) groups	42-43
4.9	Neutrophil in milk of Control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3) groups	42-43
4.10	Lymphocyte in milk of Control (G1), <i>E.officinalis</i> (G2) and <i>C.limon</i> (G3) groups	42-43

## LIST OF ABBREVIATIONS

SCC	Somatic Cell Count
EC	Electrical conductivity
<sup>0</sup> C	Degree Celsius
<i>et al.</i>	Any Other
gm/ g	Gram
BID	Bis in die ; twice a day
SE	Standard Error
IMI	Intramammary Infection
CMT	California Mastitis Test
mL	Milli Litre
WF	Working Factor
P/O	Per Orally
viz.,	Namely
LF	Left Fore
LH	Left Hind
RF	Right Fore
RH	Right Hind
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
SID	sem´el in di´e; once a day
v/v	Volume By Volume
CTR	Ceftriaxone
Cip	Ciprofloxacin
Amc	Amoxicillin and Clavulanate
Df	Degrees of freedom
N	Number
BW	Body Weight
MI	Microliter
µm	Micrometer
DW	Distilled water
WFJ	Whole Fruit Juice
Mcg	Micro Gram
Hr	Hour
HF	Holstein Frisien
$\chi^2$	Chi square test
mŪ	Millimho
Cm	Centimetre
DLC	Differential Leucocyte Count
PBS	Phosphate buffered saline
ANOVA	Analysis of Variance



# Introduction



## CHAPTER I

### INTRODUCTION

Maharashtra state stands 5<sup>th</sup> in cattle population and 7<sup>th</sup> in livestock population and as per 20<sup>th</sup> Livestock Census conducted during 2019 total cattle population in the state was 13.9 million contributing 2354.32 lakh MT of milk (per capita availability of 394 grams/day). As per 2019 census total bovine population (Cattle, Buffalo, Mithun and Yak) of India was 302.79 million, including 192.49 million cattle mostly comprised of cows (145.12 million). The crossbred/exotic and non-descript/ indigenous cattle population in the country is 50.42 million and 142.11 million that has shown an increase of 26.9 % and 10% respectively as compared to previous census.

Mastitis a multi-etiological disease characterized by physical changes in the udder, pathological changes in glandular tissues, and mostly bacteriological changes in milk (Radostitis *et al.* 2007). Udder being a productive organ of dairy animals needs to be healthy and due to its anatomical position is subjected to outside influences that can lead to inflammatory and non-inflammatory conditions (Sharma and Vohra, 2011). Clinical mastitis usually an individual problem is characterized by inflammation of udder and gross abnormality in quantity and quality of milk, while as, sub-clinical mastitis is a herd problem, having no observable clinical signs or changes in milk quality/quantity. The occurrence of clinical mastitis is proportional to the prevalence of sub-clinical mastitis that may be incubating and also due to high risk of new infections (Zdunczyk *et al.* 2003). Subclinical mastitis 15 to 40 times more prevalent than the clinical mastitis affects milk quality/ quantity and is difficult to detect (Seegers *et al.* 2003).

Mastitis is globally most important disease of economic importance due to high morbidity, loss of milk production, milk rejection and high cost of treatment (Bardhan, 2013). Sub-clinical mastitis causes milk yield reduction with undesirable changes in the milk composition and increase cost burden in terms of management for its control (Halasa *et al.* 2009). Annual losses in India due to mastitis amount to Rs. 7165.51 crores, including Rs. 4151.16 crore (57.93%) attributed to subclinical mastitis (PDADMAS, 2011).

Due to huge economic importance of bovine mastitis, early diagnosis and treatment is of paramount importance. Mastitis treatment mainly involves use of antibiotics that poses a risk of development of resistance in the bacteria (Hoek *et al.* 2011) and residues in the milk thereby posing a threat to human health. Therefore, nowadays attention is being given to non-antibiotic remedies such as plant products that improve the natural defenses of the animal. The herbal medicine has attained significance due to lesser toxicity, lower side effects and non-requirement of milk withdrawal time. Due to their antibacterial and anti-inflammatory action and also not altering the milk quality herbal therapy has been found superior in many ways.

*Embilica officinalis* or *Phyllanthus emblica* (Syn: Amla, Indian Gooseberry) an evergreen tree highly prized in tropical Asia is natural to tropical Southeast Asia, particularly in Central and South India commonly cultivated in gardens and commercially as a medicinal fruit. Amongst the most important medicinal plants in the Ayurveda Materia Medica and widely used in Indian medicine for the treatment of various ailments (Poltanov *et al.* 2009). *E. officinalis* is known to possess potent antibacterial activity against *Staphylococcus aureus* (Dhale and Mogle, 2011; Varghese *et al.* 2013), *Proteus mirabilis*, *Klebsiella pneumoniae*, *K. ozaenae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *S. paratyphi A*, *S. paratyphi B*, *Serratia marcescens* and *Escherichia coli* (Saeed and Tariq, 2007). Amla is an excellent antioxidant and free radical scavenger (Hazara, 2010). Vitamin C in *E. officinalis* accounts for approximately 45-70% of the antioxidant activity (Scartezzini *et al.* 2006). Various investigators have reported that the fruits of *E. officinalis* have immunomodulatory (Srikumar *et al.* 2007), anti-inflammatory (Santosh Kumar *et al.* 2013; Golechha *et al.* 2014) and hepatoprotective effect (Pramyothin *et al.* 2006). Furthermore numerous other actions of amla have been reported by different researchers such as; analgesic (Perianayagam *et al.* 2004), anti-tussive (Nosalova *et al.* 2003), anti-atherogenic (Jeevangi *et al.* 2013), adaptogenic (Rege *et al.* 1999), gastroprotective (AI-Rehaily *et al.* 2002), nephro-protective (Yokozawa *et al.* 2007), memory enhancer (Vasudevan and Parle, 2007) and anti-carcinogenic (Madhuri and Pandey, 2008). Fresh amla fruit can be used as a feed additive in dairy animal diets for improving

antioxidant capacity, butter quality, protein efficiency and more desirable milk fatty acids (Tilahun *et al.* 2022). Limited studies have been conducted to explore the efficacy of *E. officinalis* against bovine mastitis and to explore its potential biological properties in the animal health sector.

*Citrus limon* an evergreen tree having yellow edible fruits belong to family Rutaceae and about 160 genera are cultivated in all over the continents (Kamal *et al.* 2013). Lemon, third most important citrus species after orange and mandarin has got strong commercial value for the fresh products market and food industry. *C. limon* is rich in ascorbic acid, citric acid, minerals, flavanoids and essential oils (Ekawati *et al.* 2019). A quarter cup of lemon juice is estimated to contain 31% of recommended vitamin C (Wagner *et al.* 2021). Lemon productive networks generate high amounts of wastes and by-products serving as a potential source for animal feed, manufactured foods, and health care (Molina *et al.* 2010). Two different tissues are found in what is colloquially called lemon peel, flavedo and albedo (Augusti, 2003). Flavedo (green to yellow) is the peel's outer layer, is a rich source of essential oils (Brat *et al.* 2001) which have been used since ancient times by the flavour and fragrance industry. Eriocitrin a flavanoid in lemon fruit had antioxidant activity (Miyake *et al.* 1997). Albedo is the major component of lemon peel that is a spongy and cellulosic layer laid under flavedo. Albedo has high dietary fiber content, and furthermore, the presence of associated bioactive compounds (flavonoids and Vitamin C) with antioxidant properties in fresh lemon. Albedo involves healthier benefits than other sources of dietary fiber. Many phytochemical compounds such as phenolic, flavonoids, alkaloids, saponins, tannins, steroids, glycosides and triterpenoids have been reported having antibacterial activity against gram positive and gram negative bacteria (Soetan *et al.* 2006; Dhale and Mogle., 2011). In addition, some of these components like phenols may interfere with the active transportation of substances across the cytoplasmic membrane (Davidson and Naidu, 2000), thereby causing bacterial death. Due to broad spectrum antibacterial activity *C. limon* is a better alternative for prevention of bovine mastitis caused by infectious agents.

Keeping in view the above facts the present study is designed with the following objectives:

## 11. OBJECTIVES:

- To evaluate *in vitro* antibacterial activity of *Emblica officinalis* and *Citrus limon* fruit against important bacteria of mastitis origin.
- To evaluate *in vivo* therapeutic efficacy of *Emblica officinalis* and *Citrus limon* fruit in sub-clinical mastitis



# **Review of Literature**



## CHAPTER II

### REVIEW OF LITERATURE

#### 2.1. Diagnosis

Bagri *et al.* (2018) collected and analyzed milk samples from 80 lactating dairy cows, and observed substantial changes in pH, fat, protein, lactose, solid not fat (SNF), total solids, and acidity ( $p > 0.05$ ) with mastitis intensity to the chemical properties of the milk samples. In clinical mastitis milk samples, pH and acidity increased significantly compared to normal milk samples, whereas in subclinical mastitis milk samples there were significant decreases in fat, protein, lactose, solid not fat (SNF) and total solids percent compared to normal milk samples from non-affected mastitis disease cows.

Bhat *et al.* (2017) evaluated accuracy of two indirect diagnostic tests for detection of subclinical mastitis in lactating dairy cattle. In the study, the percentage agreement of MCMT with bacteriological examination was found to be 77.25 % and accuracy of MCMT was found to be 87.77 %.

Kamal *et al.* (2013) studied correlation between some direct and indirect tests for detection of subclinical mastitis. One hundred random samples of cow's milk were collected from different dairy farms. SCC revealed 27 % of samples contained less than  $2 \times 10^5$  SCC/ml and were considered negative for presence of subclinical mastitis, while mastitis samples contained higher numbers of SCC exceeding  $2 \times 10^5$ /ml. CMT results revealed that 27.0 % of the examined cow's milk samples were negative and 73.0 % were positive samples. EC values of normal and mastitis cow milk samples were 4.08 and 7.42 mS/cm, respectively. Results showed significant correlation of these parameters in detection of subclinical mastitis.

Kandeel *et al.* (2018) evaluated association of CMT scores with intramammary infection status in lactating dairy cows admitted to a veterinary teaching hospital. For cows admitted without evidence of clinical mastitis, the sensitivity of a CMT scores trace in predicting an IMI on a quarter or cow basis was 0.45 and 0.68, respectively.

Kasikci *et al.* (2012) studied relation between electrical conductivity, SCC, CMT and some quality parameters in the diagnosis of subclinical mastitis in dairy cows. A total of 386 milk samples collected from quarters of 188 cows at 10 different farms were analyzed. It had been concluded that EC showed similarity with the CMT and the SCC in the detection of subclinical mastitis.

Reddy *et al.* (2014) studied comparative efficacy of commonly used diagnostic tests for detection of subclinical mastitis in dairy. Out of 135 quarter milk samples subjected to CMT, EC, SCC and cultural examination, the accuracies were found to be 73.33 per cent, 70.37 per cent and 71.00 per cent, respectively. The false positive reactions were more in CMT (24.60 %) followed by SCC (23.70 %) and EC (20.40 %) whereas the false negative reactions were highest in EC (34.90 %) followed by SCC (31.60 %) and CMT (28.60 %). It was found that EC had the highest specificity (84.84 %) and predictive value (79.59 %) with lowest sensitivity (56.62 %) than compared with the other diagnostic methods for diagnosis of subclinical mastitis in cows.

Swami *et al.* (2017) screened total 60 cows against subclinical mastitis and revealed the average pH value of subclinical mastitis milk was increased significantly ( $P < 0.01$ ) as compared the normal milk.

## **2.2. Bacterial Isolation**

Birhanu *et al.* (2017) studied bacteria causing sub-clinical mastitis among 170 cultured samples, and found 153 samples were positive for known subclinical mastitis pathogens. The dominant bacteria isolated were *Staphylococcus* species, among which *S. aureus* (44.9%) was the major one followed by *Streptococcus* spp. (25.3%) and among gram negative bacteria *E. coli* (8.8%) was the predominant.

Dereje *et al.* (2018) in a selected cows observed 70.43% had bovine mastitis, out of which 15.37% had clinical mastitis and 65.05% had subclinical mastitis. The different types of bacterial species isolated were *S. aureus* (30.93%), *Staphylococcus* other than *S. aureus* (14.43%), *S. agalactiae* (5.15%), *S. dysgalactiae* (5.15%), *S. uberis* (12.37%), *C. bovis* (13.40%), *E. coli* (6.18%), *P. aeruginosa* (10.3%) and *K. pneumonie* (2.05%).

Kabir *et al.* (2017) isolated bacteria from milk of mastitic cows such as *Staphylococcus*, *Streptococcus*, *E. coli* and *Bacillus*.

Sylejmani (2016) isolated total of 112 bacterial mastitogens from dairy cows. The most prevalent species were *S. aureus* (28.6%) followed by coagulase negative staphylococci (21.4%), *E. coli* (13.4%), *Streptococcus agalactiae* (14.3%), *S. dysgalactiae* (3.6%), *S. uberis* (6.2%), *Corynebacterium* spp. (8%), and *Bacillus* spp. (4.5%).

### **2.3. In vitro antibacterial activity of selected herbs**

#### **2.3.1. In vitro antibacterial activity of citrus lemon fruit**

Abdullah (2009) reported that the juice of *C. limon* has significant inhibition against *S. aureus* and *K. pneumoniae* with zones of inhibition 17.4 and 13.3 mm respectively, while as the juice of *C. limetta* showed no inhibition effect on these bacteria.

Amer and awwad (2020) studied the antimicrobial activity of aqueous extract of *C. limon* fruit and observed the zone of inhibition against *E. coli* and *S. aureus* were 4.5 mm and 2.2 mm respectively.

Deshwal and Kaur (2018) evaluated antimicrobial activity of *C. limon* juice on various pathogenic microorganisms and observed growth inhibition of *E.coli*, *Salmonella*, *P. aeruginosa*, *P. vulgaris*, *S. aureus* and *S. pyogenes*. The maximum antimicrobial activity was observed against *S. aureus* and lowest with *P. aeruginosa*.

Dhanavade *et al.* (2011) studied antimicrobial activity in terms of MIC of citrus lemon peel extract by using different solvents against microorganisms like *P. aeruginosa* NCIM 2036 for which MIC was 1:20 in presence of methanol, for *Salmonella typhimurium* NCIM 5021 the observed MIC was 1:20 in presence of acetone. In case of *Micrococcus aureus* NCIM 5021 the observed MIC was 1:20 when ethanol was used as solvent. The compounds like coumarin and tetrazene were identified by GC/MS of lemon peel extract.

Haraoui *et al.* (2020) studied the antimicrobial activity of citrus lemon juice and reported the zone of inhibition against *S. aureus* (26.33±1.53 mm), *S.*

*epidermis* (20±1.00 mm), *Bacillus subtilis* (21±1.00 mm) and *E. coli* (20.33±1.53 mm).

Hindi and Chabuck (2013) investigated antimicrobial activity of aqueous lemon extracts of peel and juice from fresh and dried citrus and sweet lemon against 6 Gram-positive and 8 Gram-negative bacterial isolates showing good effect in terms of zone of inhibition such as *S. aureus* (26mm), *S. epidermis* (no inhibition), *S. agalactiae* (20 mm), *Enterococcus faecalis* (28 mm), *S. Pneumoniae* (29 mm), *S. pyogens* (20 mm). The zones of inhibition against gram negative bacteria including *E. coli*, *E. aerogens*, *K. pneumoniae*, *Proteus* spp., *S. typhi*, *Acinetobacter* spp. were 10 mm, 20mm, 30mm, 20mm, 30mm and 20mm respectively.

Kadhim *et al.* (2013) investigated antimicrobial activity of different aqueous lemon extracts of peel and juice from fresh and dried citrus and sweet lemon against 6 Gram-positive and 8 Gram-negative bacterial and one yeast isolates, including *S. aureus*, *S. epidermidis*, *S. pyogenes*, *Enterococcus faecalis*, *S. pneumoniae*, *S. agalactiae*, *P. aeruginosa*, *Enterobacter aerogenes*, *K. pneumoniae*, *E. coli*, *Salmonella typhi*, *Proteus* spp., *Moraxella catarrhalis*, *Acinetobacter* spp. and *Candida albicans*. The water extracts of all the materials screened showed various inhibitory effects and the juice of *C. limon* has antimicrobial activities more than other types of extracts however *E. coli*, *S. epidermidis*, *S. agalactiae* and *C. albicans* showed the highest resistance to these extracts. The extract from the juice of *C. limon* presented the highest antimicrobial activities, as it inhibited most 13 isolates (out of 15 isolates used) with inhibition zone ranging from (10-30mm) except *S. epidermidis* and *P. aeruginosa* which showed no any susceptibility to this extract.

Kumari *et al.* (2016) studied antimicrobial property of *C. limon* juice against *E. coli* in terms of MIC and reported zone of inhibition as 3.6 cm.

Oikeh *et al.* (2016) studied the antimicrobial activity of citrus juice against two gram positive and three gram negative bacterial species with disc diffusion assay i-e, *S.aureus* (20 mm), *E. faecalis* (10 mm), *P. aeruginosa* (18mm), *E. coli* (12 mm) , *Salmonella* spp. (10 mm) tested by disc diffusion assay and noted the

minimum inhibitory and bacteriostatic concentrations of lemon juice against *S.aureus* (25 µg/ml, 50 µg/ml), *E. faecalis* (50 µg/ml, 100µg/ml), *P. aeruginosa* (12.5µg/ml, 50 µg/ml), *E. coli* (50µg/ml, 100 µg/ml ) and *Salmonella spp.*(25 µg/ml, 50 µg/ml).

Okeke *et al.* (2015) evaluated the antibacterial activity of *C. limon* fruit juice reconstituted in distilled water to a concentration of 100 mg / ml and observed zones of inhibition of 21 mm and 14 mm against *S. aureus* (ATCC 12600) and *E. coli* (clinical isolate) respectively.

Sajet *et al.* (2014) studied the antimicrobial activity of different dilutions with of *C. limon* juice diluted with distilled water (25%, 50%, 75%, 100% v/v) against methicillin resistant *S. aureus* and observed zones of inhibition of 21 mm,25 mm,28 mm,and 30 mm respectively.

Shafi *et al.* (2016) investigated *in vitro* antibacterial activity of hydro-ethanolic extract of *Ocimum sanctum* leaves against bovine mastitis pathogens. The MIC values for *E. coli* and *S. aureus* were 125 mg/ml and 62.5 mg/ml respectively and the percent inhibition when compared with enrofloxacin for *E. coli* and *S. aureus* were 72.95% and 58.71% respectively.

Shafi *et al.* (2020) studied *in vitro* antibacterial activity of hydro-ethanolic extract of *Withania somnifera* against bovine mastitis pathogens and observed the percent inhibition when compared with enrofloxacin of 78.19% and 55.67% against *S. aureus* and *E. coli* respectively.

Shakya *et al.* (2019) reported antibacterial activity of *C. limon* juice extract against *S. aureus* ATCC 25923, *S. aureus*, *E. coli* ATCC 25922, *E. coli* and zones of inhibition were 26.66 mm, 24 mm, 25 mm, 21.33 mm respectively.

Shuaib *et al.* (2021) investigated antimicrobial effects of *C. limon* fruit juice against *S. pyogenes* and *E. coli* bacterial isolates and observe zones of inhibition of 20 mm and 15mm respectively and concluded that *C. limon* juice can be used as a potential source for controlling infections caused by these microbial pathogens.

Solanki *et al.* (2022) observed antimicrobial activity of concentrated lemon juice was more against *P. vulgaris* followed by *E. coli* and *S. aureus* exhibiting zones of inhibition of 31 mm, 27 mm and 26 mm respectively.

Tawfik *et al.* (2010) studied the antibacterial activity of *C. limon* juice against *S. aureus*, *Proteus vulgaris*, *P. aeruginosa* at different concentrations and at 10% concentration observed zone of inhibition of 15 mm, 21 mm, 20 mm while as at 5% concentration zone of inhibition were 10 mm, 12 mm, 13 mm respectively.

Teke *et al.* (2019) studied *in vitro* antimicrobial activity of honey and lemon juice mixture proportions (90:10, 75:25, 50:50, 25:75 and 10:90% v/v respectively) against clinical isolates from patients with respiratory tract infections and observed *S. aureus* was most sensitive to mixture of 25/75% (v/v) producing a growth inhibitory diameter of 14 mm while as two test mixture proportions (75/25 and 90/10% v/v) were not able to inhibit the growth of *S. aureus* and *S. pyogenes*. The MIC for *S. aureus* and *S. pyogenes* were recorded as 60% concentration of test mixture and the MBC values for the test microorganisms were considered greater than 80% of test mixtures and they concluded that mixtures of honey and lemon juice (lemon 50-75%) could be a better alternative treatment against some bacterial infectious diseases.

### **2.3.2. *In vitro* antibacterial activity of *Emblica officinalis* fruit**

Abdulhamza (2013) studied antibacterial activity of *E. officinalis* (Amla) fruit extract against *S. aureus*, *K. pneumonia* and *P. aeruginosa* at different concentrations. The highest inhibition zone was recorded for *S. aureus* ranging from 16±0.57 to 33.3±0.3 at 10 mg/ml to 100 mg/ml concentrations while as zone of inhibition in case of *K. pneumonia* was 11.3±0.6 mm to 28±0.06 mm and for *P. aeruginosa* was 10.6±1.2 to 22.6±1.2 mm at same concentrations.

Badoni *et al.* (2016) evaluated antioxidant, antibacterial and toxic properties of *E. officinalis* and *Terminalia bellirica* fruit extracts. Antibacterial assay showed varying results for different bacterial strains such as, *E. officinalis* aqueous extract demonstrated antibacterial activity against *E. coli*, *P. aeruginosa* and *K. pneumonia* with zones of inhibition of 10±1, 12.5±0.5 and 13±2 mm

respectively, while as, *E. officinalis* methanolic extract exhibited maximum inhibition against *S. aureus* (11.5±0.5).

Dhale and Mogle (2011) studied the antimicrobial activities of the extracts of *E. officinalis* at different doses (10mg and 20mg/ml) against some common pathogenic bacteria using agar disc diffusion method and reported its superior activity against *S. aureus* at 20mg/ml forming 29 mm diameter zone of inhibition followed by 18 mm for *B. subtilis*, 15 mm for *P. aeruginosa* and for *E. coli* 12 mm.

Dharajiya *et al.* (2015) studied the antibacterial activity of *E. officinalis* fruits against different pathogenic bacteria (*E. coli*, *S. marcescens*, *P. aeruginosa* and *Bacillus cereus* and observed Maximum antibacterial activity for methanol extract against *E. coli* (ZOI = 17.0±1.0 mm and AI = 0.939) followed by aqueous extract (ZOI = 14.5±0.5 mm and AI = 0.801). The MIC value of methanol extract of *E. officinalis* fruit against *E. coli* was 6.25 mg/ml.

Javale and Sabnis (2010) evaluated antimicrobial of crude powder, aqueous as well methanolic extract of the fruit and leaf of *E. officinalis* against three commonly encountered respiratory pathogens viz. *S. aureus*, *K. pneumoniae*, *S. pyogenes* and observed fruit extract was more effective than the leaf extract in terms of the antimicrobial action of the phytochemicals from their respective extracts.

Kanthimathi and Soranam (2013) determined the antibacterial activity of aqueous crude extract of *E. officinalis* against five human bacterial pathogens using different concentrations of plant extracts (30µl, 60µl and 90µl) and noted the their zones of inhibition against *Bacillus* spp. (13,15,16 mm) , *Lactobacillus* spp. (16,16.5,17 mm), *Pseudomonas* spp. (0,12,13 mm), *Proteus* spp. (11,13,14.5 mm), and *Streptococcus* spp. (16,17,19 mm) respectively.

Kaushik *et al.* (2022) observed that the ethanol extract of *E. officinalis* fruit showed antibacterial activity with MIC of 6.25 µg/ml and 12.5 µg/ml against *S. aureus* and *S. typhi* respectively.

Kumar *et al.* (2011) studied antimicrobial potential of aqueous (infusions, decoctions) and methanolic extracts of *E. officinalis* against seven pathogenic

bacteria namely *S. aureus*, *S. saprophyticus*, *E. coli*, *E. faecalis*, *E. cloacae*, *P. vulgaris* and *K.pneumoniae* and observed its effectiveness against all the seven bacterial pathogens.

Mayachiew *et al.* (2008) studied antimicrobial and antioxidant activities of Indian gooseberry and Alpinia galangal extracts were investigated against *S. aureus*. The minimum inhibitory concentration (MIC) values of Indian gooseberry and galangal extracts were found to be 13.97 and 0.78mg/ml and the minimum biocidal concentration (MBC) values were 13.97 and 2.34 mg/ml, respectively.

Nadhom *et al.* (2018) evaluated antimicrobial effect of the phenolic extract of *E. officinalis* (10 to 100 mg/ml) against *S. aureus*, *E. coli*, *Proteus spp.*, and *Bacillus spp.* and observed zones of inhibition in the range of 16±0.57 to 33.3±0.3 mm, 10.6±1.2 to 22.6±1.2 mm, 8.2±0.1 to 20.3±0.57 mm 11.3±0.6 to 28±0.06 mm, respectively.

Rahman *et al.* (2015) evaluated antimicrobial properties of some locally available fruits like tamarind, Indian gooseberry (amla), star fruit, ambarella and shatkora against human pathogenic bacteria by disc diffusion method and reported MIC for aqueous extract of amla as 3.91 mg/ml.

Saeed and Tariq (2007) investigated antibacterial activity of aqueous infusions and aqueous decoctions of *E. officinalis* and *Coriandrum sativum* against 345 bacterial isolates belonging to 6 different genera of Gram negative bacterial population isolated from urine specimens and observed potent antibacterial activity against *E. coli*, *K. pneumoniae*, *K. ozaenae*, *P. mirabilis*, *P. aeruginosa*, *S. typhi*, *S. paratyphi A*, *S. paratyphi B* and *Serratia marcescens* however did not observe any antibacterial activity against Gram negative urinary pathogens.

Saxena *et al.* (2020) studied antibacterial activity of extracts of Amla pulp and observed that methanolic extract of Amla pulp produced zone of inhibition of 19 mm and 16 mm against *E. coli* and *S. aureus* respectively while as ethanolic extracts of Amla produced zone of inhibition of 15 mm and 14 mm against *S. aureus* and *E. coli* respectively and 20 and 22 mm against *K. pneumonia* and *S. pyogens* respectively.

Shah and Malik (2019) studied antibacterial activity of aqueous *Phyllanthus emblica* fruit extract against eight pathogenic cultures (*S. typhi*, *E. coli*, *S. aureus*, *Vibrio cholerae*, *S. paratyphi A*, *S. paratyphi B*, *Shigella spp.*, and *B. cereus*) using different concentrations of APE (25%, 50%, 75%, and 100%, v/v). The average zone of inhibition against the culture for the concentration of 25%, 50%, 75% and 100% (v/v), of APE were 12.2±1.48 mm, 15.4±1.6 mm, 17±1.7 mm and 19±2.0 mm respectively. MIC was observed in the range of 12.5% - 50% (v/v) and the MBC values indicated that a concentration of 50% (v/v) APE could kill 75% (6/8) test cultures.

Sudha *et al.* (2017) determined antimicrobial activities of aqueous extract of amla fruit and observed maximum activity against *K. pneumonia* (16.50±0.342 mm), followed by *E. coli* (14.33±0.33 mm), *S. aureus* (14.33±0.42 mm), *P. aeruginosa* (13.33±0.33 mm) and *C. albicans* (10.33±0.21mm).

Varghese *et al.* (2013) investigated the *in vitro* antibacterial activity of aqueous, ethanolic and acetone extracts of fruits of *E. officinalis* against *S. aureus* and *E. coli*. All the extracts exhibited significant antibacterial activity, more against *S. aureus* than *E. coli*. MIC for ethanol and aqueous extracts for *S. aureus* and *E. coli* were 0.3 and 1.0 µg and 1.5 and 3.75µg, respectively.

Yadav *et al.* (2020) studied *in vitro* efficacy of *E. officinalis* in terms of MIC MRSA isolated from Buffaloes Suffering from subclinical mastitis and observed zones of inhibition ranging between 10-13 mm for 200 and 225 mg/ml DMSO disc, followed by 12 mm for 175 and 150 mg/ml DMSO disc, 11 mm for 125 mg/ml DMSO disc and 10 mm in 100 mg/ml DMSO disc.

## **2.4. *In vivo* therapeutic potential of selected herbs in bovine mastitis**

### **2.4.1. Effect on level of intramammary infections (IMI)**

Gao *et al.* (2018) studied that *E. officinalis* fruit extract could effectively alter the oral microbiome and decrease the total bacterial count.

Keshamoni *et al.* (2021) investigated a paste preparation of lemon, aloe vera leaves 200 gm, turmeric powder 10 gm, castor oil 10 ml applied externally

over the udder daily for 7 days of affected buffalos and observed quarter wise recovery percentage was 64.29%.

Khan *et al.* (2018) compared the therapeutic efficacy of *E. officinalis* fruit extract and procaine penicillin in the treatment of subclinical mastitis in dairy buffaloes and observed quarter based bacteriological cure rate of 64.7% with amla fruit extract and concluded that amla fruit extract is an inexpensive alternative to procaine penicillin for treatment of subclinical mastitis in dairy animals.

Maramulla *et al.* (2019) studied efficacy of herbal preparations (*O. sanctum* dry powder, honey and lemon juice) in therapeutic management of sub clinical mastitis in cows administered as a bolus two times in a day for 7 days and observed animal wise cure percentage of 90 % (9 out of 10) and quarter wise cure percentage of quarters 93.75 % (15 out of 16 quarters).

Pandey *et al.* (2020) evaluated three different dosage regimens (@ 250 gram, 200 gram and 150 gm) of crushed deseeded fresh *E. officinalis* in cattle affected with subclinical mastitis and observed that supplementation significantly decreased total bacterial count as compared to non-supplemented group in which increase in somatic cell count and total bacterial count and decline in milk yield was recorded.

Rizwan *et al.* (2021) studied comparative therapeutic efficacy of procaine penicillin, *P. emblica* fruit extract and *Cocos nucifera* oil against subclinical mastitis and reported bacteriological cure rate of sub-clinically mastitic teats by *P. emblica* fruit extract as 40% and 50% at 7<sup>th</sup> and 14<sup>th</sup> day respectively.

Shafi *et al.* (2016) evaluated immunotherapeutic potential of *O. sanctum* against bovine subclinical mastitis animals that received 600 mg/kg body weight of *O. sanctum* leaf powder daily divided into two doses orally for 7 days. The treatment could remove 69.23% (9/13) of intramammary infections that was found to be statistically significant ( $P < 0.5$ ) with chi square values being 5.07 as compared to control group in which only 26.67 % (4/15) elimination was observed and also significantly reduced somatic cell count and udder inflammation, and improved the milk quality.

Shafi *et al.* (2018) evaluated efficacy of a composite formulation (masticure®) as an adjunct therapy in the treatment of bovine mastitis in terms of eliminating intramammary infections, relieving udder inflammation, and improving milk quality and quantity and reported therapy highly effective in eliminating intramammary infections, relieving udder inflammation and improvement in milk quality.

Shafi *et al.* (2020) evaluated *W. somnifera* root powder for elimination of intramammary infections @ 500 mg/kg body weight daily, divided into two doses for 7 days, in case of bovine mastitis and observed 64.28% elimination of intramammary infections ( $\chi^2 = 24.14$ ; 01 df;  $P < 0.05$ ) and also the therapy resulted in a significant reduction in somatic cell count, ceruloplasmin concentration, udder inflammation and improved milk quality.

Sharma *et al.* (2010) observed the effect of *P. emblica* and amoxicillin sulbactam combination on the bacterial growth of milk and observed no bacterial growth on day 30 of therapy as compared to heavy bacterial growth on day 0.

#### **12.4.2. Effect on inflammatory markers of the udder (SCC, CMT, pH and EC)**

Abbasi *et al.* (2013) reported that paste made from the fruit juice of *C. limon* and sugar when topically applied to the mammary glands for 10–15 days helped to cure mastitis in buffalo, cattle and goats.

Das *et al.* (2003) reported that polyherbal mixture containing *W. somnifera*, *Tinospora cordifolia* and *E. officinalis* has a potential in the prevention of mastitis as well as treatment.

Dutta *et al.* (2020) worked for indiscriminate use of antibiotics and drug residues in milk and evaluated the efficacy of ethno veterinary herbal preparations as an alternative approach for management of mastitis by using external application paste and 2 lemon fruits cutting into 2 halves were fed to the affected cattle twice daily for 3 days and observed a clinical success rate 92.09 %.

Ibrahim *et al.* (2016) observed that Vitamin E + Se and Garlic when used against subclinical mastitis showed significant reduction in milk pH and also

lemon @ 250 gm / animal orally BID, showed significant decrease in pH from  $6.8 \pm 0.03 - 6.5 \pm 0.02$ .

Keshamoni *et al.* (2021) investigated a paste preparation of lemon, aloe vera leaves 200 gm, turmeric powder 10 gm, castor oil 10 ml applied externally over the udder daily for 7 days of affected buffaloes and observed improvement in the value of pH from 6.92 at 0 day to 6.82 at day 7 after treatment and resulted in the reduction of SCC.

Khan *et al.* (2018) observed that aqueous fruit extract of *P. emblica* significantly decreased udder inflammation and ceruloplasmin concentration, an acute-phase protein in udder inflammation in bovine mastitis.

Maramulla *et al.* (2019) studied efficacy of herbal preparation (*O. sanctum* dry powder, honey and lemon juice) in therapeutic management of sub clinical mastitis in cows administered as a bolus two times in a day for 7 days and observed significant reduction in SCC, pH and increased fat content in milk.

Martinez *et al.* (2022) studied impact of a plant feed additive containing vitamin C on Holstein cows performance and observed composition of milk was not affected by the supplementation and mastitis problem reduced from 21.28 to 12.09%.

Pandey *et al.* (2020) evaluated three dosage regimens of crushed deseed fresh *E. officinalis* and observed that animals in A-250 group supplemented with amla @ 250 gm (P/O) for 15 days showed 15 % recovery, animals in A-200 (200g P/O, for 15 days) group showed 62.5 % recovery and animals in A-150 (150 g P/O, for 15 days) showed 25% recovery and also significant decrease in SCC, CMT, WST and MST score points was observed.

Patel and Gupta (2020) studied a non-antibiotic therapy (Magic-3) for specific subclinical mastitis in 24 HF × Sahiwal crossbred cows @ 100 ml P.O., b.i.d. x 7 days that resulted in significant reduction in the values of EC, CMT, SCC, NAGase enzyme activity and phagocytic activity ( $p < 0.05$ ) post-initiation of treatment.

Rathaur *et al.* (2020) studied the effects of *C. limon* fruit cut into pieces in subclinical mastitis affected animals @ 250g/animal /day for 10 days and observed significant reduction in CMT, pH, SCC and EC of milk and also 62.5% recovery percentage was noted.

Rizwan *et al.* (2021) studied comparative therapeutic efficacy of procaine penicillin, *P. emblica* fruit extract and *Cocos nucifera* oil against subclinical mastitis and reported bacteriological cure rate of sub- clinically mastitic teats by *P. emblica* fruit extract as 40% and 50% at 7<sup>th</sup> and 14<sup>th</sup> day respectively and also significant reduction in CMT score was observed.

Shafi *et al.* (2016) evaluated the immunotherapeutic potential of *O. sanctum* against bovine subclinical mastitis @ 600 mg/kg body weight daily divided into two doses orally for seven days and observed significant reduction ( $P < 0.05$ ) in CMT and SCC.

Shafi *et al.* (2018) evaluated efficacy of a composite formulation (masticure®) as an adjunct therapy in the treatment of bovine mastitis in terms of eliminating intramammary infections, relieving udder inflammation, and improving milk quality and quantity and reported therapy was found highly effective in combination with and without parental antibiotics in relieving udder inflammation in terms of significant decreases in CMT score, SCC score, pH, and EC.

Shafi *et al.* (2020) evaluated herbal therapy with *W. somnifera* root powder (@ 500 mg/kg body weight daily, for 7 days against bovine subclinical mastitis and observed significant decrease ( $P < 0.05$ ) in SCC, pH and EC on d 28 post initiation of treatment and significant reduction ( $P < 0.05$ ) in CMT point score on d 14.

Sharma *et al.* (2010) observed aqueous extract of *P. emblica* against bovine staphylococcal mastitis resulted in significant reduction in somatic cell count ( $P < 0.05$ ).

### 12.4.3. Effect on biochemical composition of milk

Daimi *et al.* (2006) evaluated aqueous extract of *A. indica* bark orally and *Curcumin longa* rhizome paste topically against sub clinical mastitis twice a day for 15 day and saw 30 % recovery and also biochemical composition of milk was improved.

Harjanti *et al.* (2019) evaluated effect of five Indonesian herbs on bacterial count and milk production in sub-clinical mastitis affected cows conducting a completely randomized design split plot (4 replications and 4 treatments) comprising of basal diet supplemented with 12.5 g/100kgBW of mixed-herbal preparation. Dietary treatments included T0 (without herbal additive); T1 (5% *Alpiniaga langa* 25% *Curcuma xanthorrhiza*, and 70% *Sauropus androgynus*), T2 (5% *Curcuma xanthorrhiza*, 25% *Curcuma domestica* and 70% *Piper betle*), and T3 (10% *Curcuma xanthorrhiza*, 10% *Curcuma domestica* and 10% *Alpiniaga langa*, 35% *Sauropus androgynus* and 35% *Piper betle*) given on days 0, 5 and 10. The results for T1 and T3 formulations indicated significant ( $P < 0.05$ ) increase in the milk production in Cows, for T1 treated significant ( $P < 0.05$ ) increase in the milk total solid and lactose contents and for T3 treated group highest ( $P < 0.05$ ) milk fat.

Pandey *et al.* (2020) evaluated three different dosage regimens (@ 250 gram, 200 gram and 150 gm) of crushed deseeded fresh *E. officinalis* in cattle affected with subclinical mastitis and observed that supplementation markedly increased fat, total solids and solid not fat content although the change was statistically non-significant.

Rathaur *et al.* (2020) studied the effects of *C. limon* fruits cut into pieces in subclinical mastitis affected animals @ 250g/animal /day for 10 days and observed effects on the percentage of mean  $\pm$  SEM of compositional changes on fat %, protein %, and lactose % before and after treatment that were  $3.30 \pm 0.02 - 3.48 \pm 0.03\%$ ,  $3.42 \pm 0.01 - 3.87 \pm 0.02\%$ ,  $4.32 \pm 0.03 - 4.56 \pm 0.01 \%$  respectively.

Rizwan *et al.* (2021) studied comparative therapeutic efficacy of procaine penicillin, *P. emblica* fruit extract and *Cocos nucifera oil* against subclinical mastitis and its effects on milk composition in A total of 30

lactating beetal goats, and divided into three groups (GA, GB and GC), each having 10 goats. The group receiving *P. emblica* fruit extracts showed marked increase in milk fat, protein, specific gravity as well as total milk yield increased with time from zero day to 14<sup>th</sup> day ( $P < 0.05$ ).

Shafi *et al.* (2018) studied efficacy of a composite formulation (masticure®) as an adjunct therapy in the treatment of mastitis in bovines in terms of eliminating intramammary infections, relieving udder inflammation, and improving milk quality and quantity and observed improved milk yield and biochemical constituents such as protein, SNF, and lactose in the milk.

Singla *et al.* (2021) studied that feeding of *E. officinalis* fruit pomace 20 g/kg of dry matter intake for 20 days to the 10 lactating buffaloes resulted improved milk yield, milk production efficiency, milk protein concentration ( $P=0.071$ ), solid not fat ( $P=0.011$ ) and somatic cell count ( $P=0.032$ ).

Tilahun *et al.* (2022) studied effect of feeding fresh amla fruits in lactating cows on milk fat, protein and lactose content of milk and observed animals receiving 600 g/d achieved higher milk yields than the animals receiving 200, 400 g/d and control and also animals receiving 400 g/d and 600 g/d led to greater milk protein yield ( $P < 0.01$ ), milk protein content ( $P < 0.01$ ), and milk nitrogen efficiency ( $P < 0.01$ ) compared to cows fed 200 g/d FAF or control.

Todaro *et al.* (2017) studied that the effect of feeding of fresh lemon pulp to lactating ewes with concentrate and ad libitum hay resulted in higher milk protein and casein percentage in group 2 (pulp dose -2kg /day) as compared to group 1 (pulp dose -1kg /day).

#### **12.4.4. Effect on milk differential leucocyte count**

Dhakal and Kapur (1992) reported that neutrophils are the predominant in mastitis milk followed by lymphocytes, epithelial cells and monocytes and observed increased percentage of neutrophils and decreased percentage of lymphocytes in infected animals in the milk leukocytes.

Gupta and Pachauri (2001) studied a Bonmilk (polyherbal mix) in the treatment of mastitis at 60 g orally, BID for 3 days followed by SID for 4 days

and observed 75 % cure rate, with quarters started producing normal milk after 6 days onwards having significantly decreased neutrophil percentage ( $P<0.01$ ) and significantly increased lymphocyte percentage ( $P<0.05$ ) and suggested the positive effect of polyherbal mix in improving the blood circulation to the udder that could strengthen the udder defenses by activating the leukocyte population of the udder.

Ibrahim *et al.* (2016) observed that Vitamin E + Se and herbal preparation (Garlic, Black pepper and Lemon) when used against subclinical mastitis showed significant immune-potentiating characteristics by increasing lymphocyte count and decreasing the overall WBCs count, and suggested that the effect could be correlated to the cure of subclinical mastitis by decreasing somatic cells.

Pragati *et al.* (2015) studied comparative efficacy of *E. officinalis* and *T. cordifolia* on hemato-biochemical profile of Murrah buffalo calves fed @ 250 mg/kg body weight once a day orally for 28 days and observed significant increases in total erythrocyte count, total leukocyte count and lymphocyte neutrophil ratio in calves fed *E. officinalis*; lymphocyte count was found significantly elevated in calves fed *E. officinalis* and *T. cordifolia* while as significantly decreased level of neutrophil and monocyte count calves fed *E. officinalis*.

Rathaur *et al.* (2020) studied the effects of *C. limon* fruits cut into pieces in subclinical mastitis affected animals @ 250g/animal /day for 10 days and observed significant effects ( $P<0.05$ ) on the haematological parameters after the treatment in crossbreed dairy cows.

Shafi *et al.* (2016) studied the immunotherapeutic potential of *O. sanctum* leaf powder against bovine subclinical mastitis affected animals receiving @ 600 mg/kg body weight daily divided into two doses orally for seven days and observed significant reduction ( $P<0.05$ ) in neutrophils and increase in lymphocytes from the of the milk of affected cows.

Shafi *et al.* (2018) evaluated efficacy of a composite formulation (masticure®) as an adjunct therapy in the treatment of bovine mastitis in in terms of eliminating intramammary infections, relieving udder inflammation, and

improving milk quality and quantity and observed significant reduction ( $P<0.05$ ) in neutrophil and increase in lymphocyte of the milk from mastitis affected cows.

Shafi *et al.* (2020) evaluated *W. somnifera* root powder @ 500 mg/kg body weight daily in therapeutic management of bovine mastitis resulted significant reduction ( $P<0.05$ ) in milk neutrophil and lymphocytes and increase in phagocytic activity by neutrophils.

Tola and Cabali (1985) concluded that SCC upto 0.3 million/ml of milk contained normal neutrophil percentage (10-12%), increased SCC (0.3-0.5 million/ml) leads to simultaneous increase of neutrophils (13-20%) is considered doubtful and SCC more than 0.5 million/ml consisting of more than 20% neutrophils in the milk leads to mastitis.



# **Materials and Methods**



## CHAPTER III

### MATERIALS AND METHODS

The present study entitled as “THERAPEUTIC EVALUATION OF *Emblica officinalis* and *Citrus limon* FRUIT IN BOVINE SUB-CLINICAL MASTITIS” was undertaken at Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Parbhani. The deseeded fruits of *E. officinalis* and *C. limon* were evaluated for antibacterial properties and also for anti-inflammatory and immunomodulatory activities in terms of therapeutic management against bovine sub-clinical mastitis.

#### **3. Methodology:**

##### **3.1. *In vitro* studies on antibacterial activities**

###### **3.1.1. Selection of herbal plants and preparation of whole fruit juice**

The fruits of the *E. officinalis* and *C. limon* were collected from the local market and were washed with tap water and vinegar and then allowed to air dry. Fruits were sterilized with 70% alcohol and cut using a knife sterilized with 70% alcohol followed by passing over flame. Whole fruit juice was prepared in the electric grinder after removing the seeds. The contents were squeezed through muslin cloth and filtered using whatman filter paper into the container.

###### **3.1.2. Preparation of different working dilutions**

For carrying out the antibacterial activity of the whole fruit juices different working dilutions were prepared using distilled water. Using a 1000 µl micropipette, five dilutions were prepared at different proportions of each substance to a final volume of five milliliters (5 ml). These working dilutions were filtered through a membrane filter of 25 µm pore size and stored in refrigerator at 4°C for further use.

10% (v/v) Whole Fruit juice + 90% (v/v) Distilled water (100 µl WFJ + 900 µl DW)

25% (v/v) Whole Fruit juice + 75% (v/v) Distilled water (250 µl WFJ + 750 µl DW)

50% (v/v) Whole Fruit juice + 50% (v/v) Distilled water (500 µl WFJ + 500 µl DW)

75% (v/v) Whole Fruit juice + 25% (v/v) Distilled water (750 µl WFJ + 250 µl DW)

90% (v/v) Whole Fruit juice + 10% (v/v) Distilled water (900 µl WFJ + 100 µl DW)

### **3.1.3. Preparation of antimicrobial discs from different working dilutions**

Antimicrobial discs of 6mm of diameter were made using whatman filter paper no. 3. These discs were sterilized under ultraviolet light for 2 hrs then submerged in different working dilutions of whole fruit juices (90/10, 75/25, 50/50, 25/75 and 10/90% v/v respectively) for 30 minutes such that each disc absorbed a quantity of about 20 µl of prepared mixture (Teke *et al.* 2019).

### **3.1.4. Preparation of stock inoculums**

The antibacterial activity of the different working dilutions of whole fruit juice were determined against *E. coli* and *S. aureus* isolated from sub-clinical mastitis positive animals. The organisms were isolated as per standard microbial procedures of National Mastitis Council (1987). In second stage organisms were subjected to preparation of stock inoculum. For this, microorganisms were taken and streaked on sterile agar plates in such a manner that individual colonies could develop. After incubation, 3 to 5 well isolated similar colonies of the test organisms from the pure cultures were emulsified in the sterile saline water in the labelled sterile plain tubes with each representing a particular isolate. Each microbial suspension in test tube were mixed vigorously, compared visually and adjusted to the turbidity standard of McFarland 0.5 and were kept in refrigerator at 4<sup>o</sup> C for further use (Cheesbrough, 2006).

### **3.1.5. Determination of antibacterial activity of whole fruit juices**

The antibacterial activities of the different working dilutions of whole fruit juice were determined using Agar disc diffusion test. A quantity of 100µl of suspension of the test microorganisms was spread on Muller Hinton agar plates. Prepared filter paper discs of whole fruit juices along with standard antibiotic



**Plate 1.** *Emblica officinalis* fruits



**Plate 2.** *Citrus limon* fruits

discs such as amoxicillin-clavulanate (10 mcg), ceftriaxone (30 mcg) and ciprofloxacin (5 mcg) were inoculated on the agar plates using sterile forceps. After 30 minutes of applying the discs, the culture plates were inverted and incubated aerobically at 35°C for 24 hours for bacterial growth. The *in vitro* antibacterial activity was determined as percent inhibition of bacterial growth calculated from the zones of inhibition (measured in millimeters) produced by the different concentrations of whole fruit juices and the standard antibiotics used as per the method suggested by Vidyasagar *et al.* (2002). Eight replicates for each bacterial isolate were made and the final values were taken as mean ± S.E of the recorded observations.

$$\% \text{ Inhibition} = \frac{\text{AIC} - \text{AIT}_x}{\text{AIC}} \times 100$$

Were,

AIC = Area of inhibition of standard antibiotic

AIT<sub>x</sub> = Area of inhibition of whole fruit juice

### 3.1.6. Determination of minimal inhibitory concentration (MIC)

MIC of the whole fruit juices (dilution showing max. antibacterial activity) was determined against *E. coli* and *S. aureus* isolated from mastitis positive animals using broth macrodilution technique (Barchiesi *et al.* 1994). Serial dilutions of the whole fruit juices in nutrient broth media were prepared as explained below:

- 11 clear, sterile, cotton plugged 5 ml capacity tubes were taken in duplicate and marked as 1 to 11.
- All the tubes were aseptically added 0.5 ml of sterile nutrient broth.
- 0.5 ml whole fruit juice was added to 1<sup>st</sup> and 2<sup>nd</sup> tubes.
- After properly mixing the contents in 2<sup>nd</sup> test tube, 0.5 ml of the mixture was taken out from it and transferred into third tube, and this procedure of 2-fold dilution was continued up to 10<sup>th</sup> tube. After mixing the contents of 10<sup>th</sup> tube, 0.5 ml of mixture from it was discarded.

- From tube 2<sup>nd</sup> to tube 11<sup>th</sup>, 50  $\mu$ L of freshly prepared bacterial stock inoculum was added.
- Tube 1<sup>st</sup> not received any inoculum, served as negative control and 11<sup>th</sup> tube not received any extract dilution served as positive control.
- All the tubes were incubated at 35<sup>0</sup> C for 24 hr and examined macroscopically for evidence of growth, if any.
- The lowest concentration of whole fruit juice where no turbidity occurred was considered MIC (Keizo *et al.* 2012).

**Table 3.1 : Serial dilution set up for finding MIC of whole fruit juice**

<b>Tube No.</b>	<b>Nutrient Broth added(ml)</b>	<b>Extract added (ml)</b>	<b>Inoculum added (ml)</b>	<b>Final Concentration (%)</b>
1	0.5	0.5 ml whole fruit juice	None	45%
2	0.5	0.5 ml whole fruit juice	50 $\mu$ l	45%
3	0.5	0.5 from tube No.2	50 $\mu$ l	22.5%
4	0.5	0.5 from tube No.2	50 $\mu$ l	11.25%
5	0.5	0.5 from tube No.2	50 $\mu$ l	5.62%
6	0.5	0.5 from tube No.2	50 $\mu$ l	2.81%
7	0.5	0.5 from tube No.2	50 $\mu$ l	1.40%
8	0.5	0.5 from tube No.2	50 $\mu$ l	0.70%
9	0.5	0.5 from tube No.2	50 $\mu$ l	0.35%
10	0.5	0.5 from tube No.2	50 $\mu$ l	0.17%
11	0.5	None	50 $\mu$ l	Zero

### **3.1.7. Determination of minimum bactericidal concentration (MBC)**

For the MBC determination, all the clear test tubes indicating no visible sign of microbial growth or turbidity in the MIC assay were further sub-cultured on sterile Muller Hinton agar plates by streak plate method. The plates were

incubated at 35°C for 24 hours for bacterial growth if any. The least concentration that did not show growth of test organism was considered as the MBC.

### **3.2. *In vivo* therapeutic efficacy**

#### **3.2.1. Selection of cows**

The study was carried out in HF × crossbred cows at an organized dairy farm following the farming practice of semi loose housing system. The cows showing any evidence of clinical mastitis (udder/ milk examination), and those having received antibiotic treatment during the last 21 days were excluded from the study as per the guidelines of International Dairy Federation. Based upon the milk culture and CMT score of individual quarter foremilk sample, the health status of quarters was defined as under:

<b>CMT score</b>	<b>Udder pathogen</b>	
	<b>Not detected</b>	<b>Detected</b>
<1	Healthy	Latent infection
≥ 1	Non-specific mastitis	Specific mastitis

The 18 cows found positive for specific mastitis in at least one quarter (CMT ≥ 1 and culture positive), showing an average weight of around 400 kg, with day milk yield of 7-12 kg per cow, in their early to mid-lactation were assigned randomly to three groups of 06 cows each and subjected to feeding of fresh fruits (amla and lemon; deseeded, cut into pieces) collected from the local market.

**Table 3.2 : The dosage regimen for therapy was as follows:**

<b>Group 1</b>	:	Animals administered with placebo (wheat bran) treatment (n=06, control)
<b>Group 2</b>	:	Animals receiving <i>E. officinalis</i> fruit (cut into pieces, deseeded) @ 250 gm total dose P.O., divided in two parts morning and evening × 5 days (n= 06, Treatment I)  (Pandey <i>et al.</i> , 2020)
<b>Group 3</b>	:	Animals receiving <i>C. limon</i> fruit (cut into pieces, deseeded) @ 250 gm total dose P.O., divided in two parts morning and evening × 5 days (n= 06, Treatment II)  (Bilal <i>et al.</i> 2009; Dilshad <i>et al.</i> 2010)

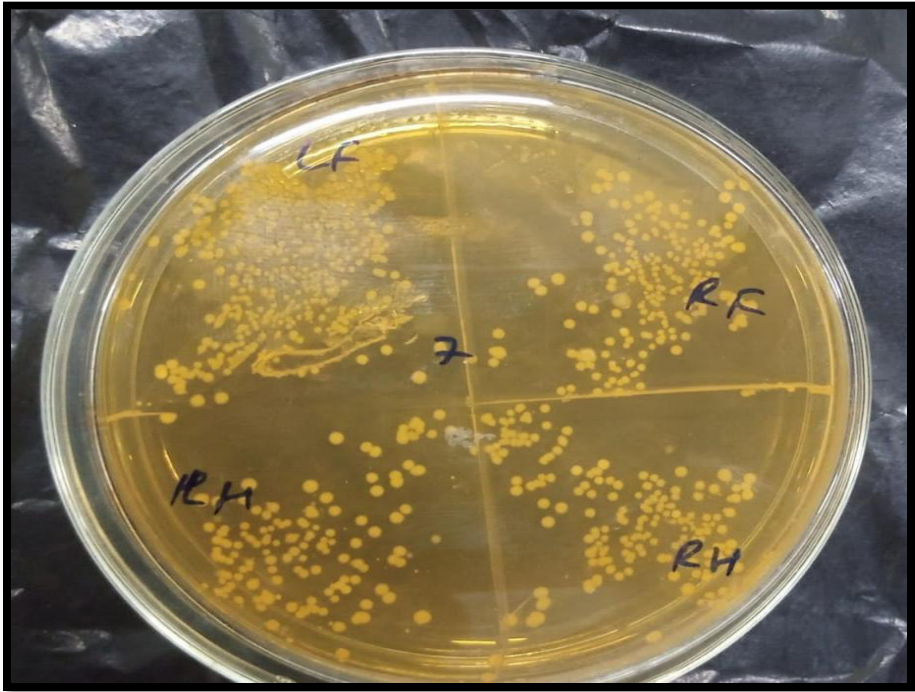
### **3.2.2. Collection of milk**

Quarter foremilk (10 mL) and cow composite milk (40 mL) samples were collected pre-treatment (d 0), and post-treatment (d 7) and (d 21). The udder and teats were properly cleaned and dried before collection of milk. The alcohol soaked cotton swab (spirit) was used for cleaning the teat orifice. The milk samples were packed in ice box and transferred immediately to the laboratory and analyzed.

### **3.2.3:-Parameters to be studied**

#### **a) Modified California Mastitis Test (MCMT).**

The MCMT was performed as per the method described by Pandit and Mehta (1969). The reagent was prepared by adding 2 ml stock solution – B (Bromocresol purple reagent) to make volume 100 ml by adding remaining volume of stock solution – A ( Sodium lauryl sulphate reagent ). The test was performed with a plastic paddle having four chambers in which 3 ml of milk from each quarter and approximately equal quantity of the reagent were added and mixed by circular movement of the paddle. After mixing the reaction was recorded and degree of gel formation was noted for grading the sub-clinical mastitis as shown in the table given below;



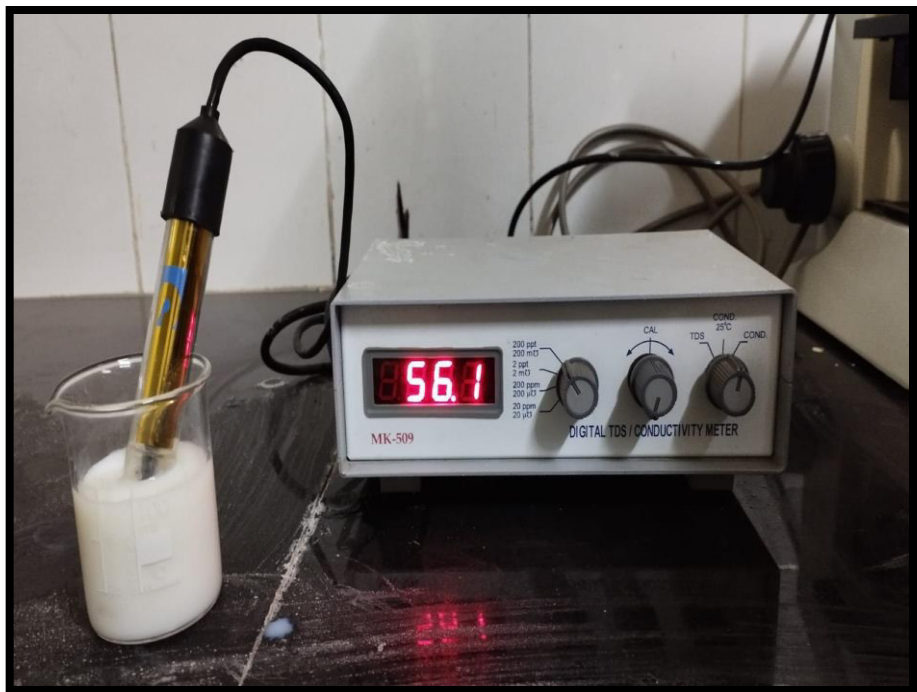
**Plate 3. Bacterial culture of milk**



**Plate 4. MCMT test**



**Plate 5. pH of milk measured using digital pH mater**



**Plate 6. Electrical conductivity of milk measured using digital conductivity meter**

**Table 3.3: Relationship between CMT score and somatic cell count**

<b>CMT infection</b>	<b>Score Somatic cell range</b>	<b>Interpretation</b>
<b>1 (Negative)</b>	<b>0 – 2,00,000</b>	<b>Healthy Quarter</b>
<b>2 (+)</b>	<b>150,000 – 500,000</b>	<b>Mild Subclinical Mastitis</b>
<b>3 (++)</b>	<b>400,000 – 1,500,000</b>	<b>Moderate Subclinical Mastitis</b>

**b) Milk pH**

The H<sup>+</sup> ion concentration of milk was determined by an electronically operated single electrode pen type digital pH meter. Before using, the instrument was calibrated against standard buffer solutions of known pH (pH 4, 7 and 9.2). The pH was recorded directly and there after the pH reading of the normal and mastitic milk samples were recorded. Recalibration of the pH meter was timely done as recommended by the supplier company.

**c) Electrical conductivity (EC) (m $\Omega$ )**

The EC of milk was measured by an MK-509 digital conductivity meter as per the instructions manual given by manufacturer. Before using, the instrument was calibrated against standard 0.1N KCl solutions. For calibration, potassium chloride 0.1491 gm was dissolved in 100 ml distilled water that gives a 1.413 mS/cm. Enough amount of milk, to cover the electrode was taken and the electrode was immersed into it. The electrical conductivity and temperature was indicated on the instrument display and reading was recorded directly. For better accuracy electrode was put in milk given sufficient time to stabilize the reading before noting it. Direct reading was recorded on digital screen in m $\Omega$ . The electrical conductivity meter was recalibrated timely as recommended by the supplier company.

**d) Somatic Cell Count (SCC) :**

The SCC of cow composite milk was determined as described by Schalm *et al.* (1971). Somatic cell count is a useful predictor of intramammary infection (IMI) that includes leucocytes (75%) (Neutrophils, Macrophages, Lymphocytes, Erythrocytes) and epithelial cells (25%). The measurement of somatic cells in

milk is known as somatic cell count. SCC can be used to monitor the level or occurrence of subclinical mastitis in herds or individual cows.

**i) Preparation of milk smears**

The test milk sample was thoroughly mixed by gentle shaking the vial and 10  $\mu$ l (0.01ml) was taken with a micropipette on to a pre drawn one square cm (1cm<sup>2</sup>) marked area over a clean, grease-free glass slide and uniformly smeared with a standard sterilized bacteriological platinum loop. The smear was allowed to air dry at room temperature while protecting from dust.

**ii) Staining of milk smears**

These dry milk film slides were stained by Newman-Lampart single dip stain kept inside a coplin jar for 1-2 minutes. The slides were gently washed in tap water and dried rapidly in air and then examined under the oil immersion lens of the light microscope.

**iii) Counting of cells**

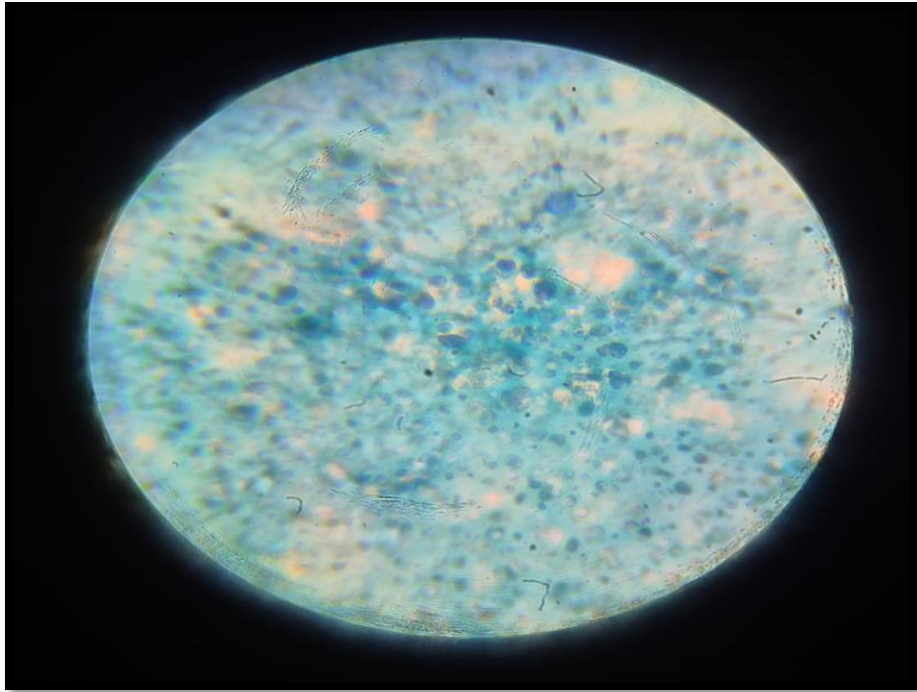
The counting of cells in 30 different field was done under oil immersion objective lens (100x). Total number of cells/ml of milk was estimated by multiplying total number of cells in 30 fields with working factor of the microscope (WF=21786.50).

**e) Biochemical composition of the milk**

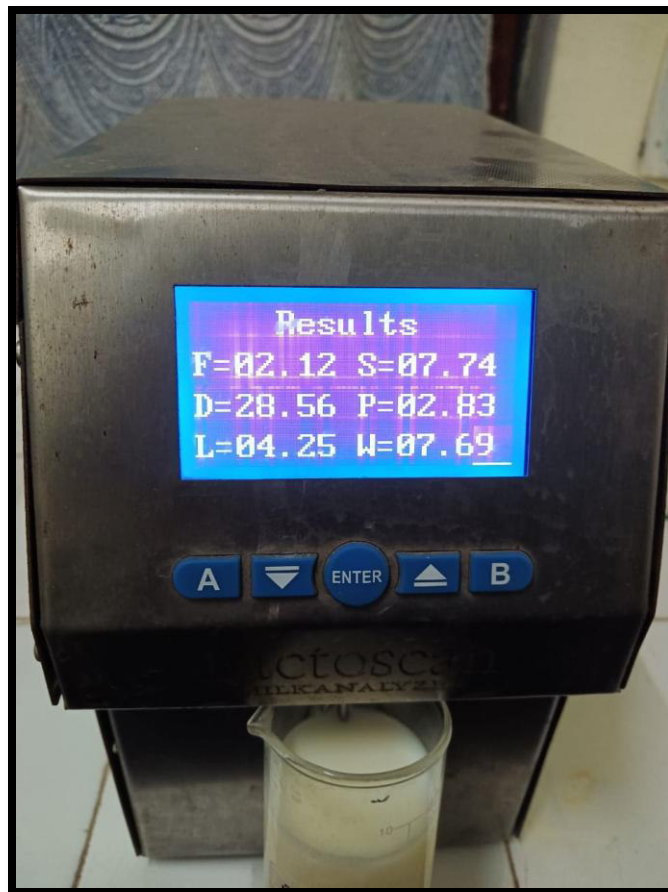
The fat, SNF, protein and lactose in the milk were analyzed using solar powered milk analyzer “Lactoscan”. The option of cattle milk was selected after the Lactoscan was turned ON. Milk sample was taken into a cup that was kept below the suction pipe of the Lactoscan and enter key was pressed. Milk composition results were displayed within 1 minute. Exit key was pressed for estimation of next sample and to remove residual milk from Lactoscan. At last thorough cleaning of Lactoscan with acidic and alkaline solution was done.

**f) Milk Differential leucocyte count (DLC):**

Differential leukocyte count in milk was performed as per the method described by Dulin et al. (1988). After thoroughly mixing cow composite milk



**Plate 7. Somatic Cell Count of milk**



**Plate 8. "Lactoscan" milk scan for biochemical analysis of milk**



**Plate 9. Differential Leucocyte Count of milk.**

samples, a drop of milk and a drop of sterile tryptic soya broth were taken on a clean, grease free micro slide, and mixed with a fine glass rod. A film was made with the help of another slide, air dried, fixed with methanol and stained with Giemsa stain for 40 minutes with a solution prepared with sterile PBS (pH 7.4) in the ratio of 2:3. The slide was washed in distilled water, dried and examined under oil immersion for neutrophils and lymphocytes per 100 cells that were expressed in percentage.

### **3.3 Statistical analysis**

The data obtained in the present study was analyzed using statistical package for social science (SPSS version 16.0 for windows). After analysis of mean value variance of different numerical parameters the subprogram ANOVA followed by Duncan's multiple range test was used. The effect of therapy on elimination of intramammary infection was analyzed using Chi square test. Significance level was set at  $P < 0.05$ .



# Results and Discussion



## CHAPTER IV

### RESULTS AND DISCUSSION

#### 4.1 *In vitro* antibacterial activity of selected herbs

The *in vitro* antibacterial activity of fruit juices of deseeded *E. officinalis* and *C. limon* fruit were recorded against *S. aureus* and *E. coli* isolated from the mastitis affected animals. The fruit juices were extracted aseptically and the antibacterial activity against *S. aureus* and *E. coli* was studied. The zones of inhibition and percent inhibition of *E. officinalis* and *C. limon* in comparison to ceftriaxone, ciprofloxacin and amoxicillin-clavulanate against *S. aureus* and *E. coli* are given in Table 4.1, Table 4.2 and Table 4.3. No marked antibacterial activity was noted for working dilutions 25/75 and 10/90% v/v of whole fruit juices against *S. aureus* and *E. coli* however marked antibacterial activity was noted for working dilutions of 90/10, 75/25 and 50/50 v/v (Plate no. 10, Plate no. 11, Plate no. 12 and Plate no. 13). In this study, average zone of inhibition shown by working dilution (90/10 v/v) of *E. officinalis* and *C. limon* against *S. aureus* and *E. coli* were  $19.7 \pm 0.05$  mm,  $18.81 \pm 0.07$  mm and  $16.43 \pm 0.04$  mm,  $11.94 \pm 0.04$  mm respectively.

**Table 4.1 : Zones of inhibition of aqueous extracts of *C. limon* fruit juice and *E. officinalis* fruit juice at 50/50 v/v**

Pathogen	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition			% of inhibition	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition
	<i>E.officinalis</i>	CTR		<i>E.officinalis</i>	Cip		<i>E.officinalis</i>	AMC		<i>C.limon</i>	CTR		<i>C.limon</i>	Cip		<i>C.limon</i>	AMC	
<i>S. aureus</i>	11.2± 0.10	23.1± 0.07	48.49%	11.2± 0.10	22.2± 0.10	50.46%	11.2± 0.10	Resistant	-	-	21.44 ±0.02	-	-	19.33 ±0.07	-	-	19.43 ±0.03	-
<i>E. coli</i>	-	28.11 ±0.04	-	-	21.2± 0.14	-	18.81± 0.07	Resistant	-	5.24± 0.08	19.23 ±0.05	27.25%	5.24± 0.08	17.98 ±0.06	29.1 5%	5.24± 0.08	17.75 ±0.04	29.53%

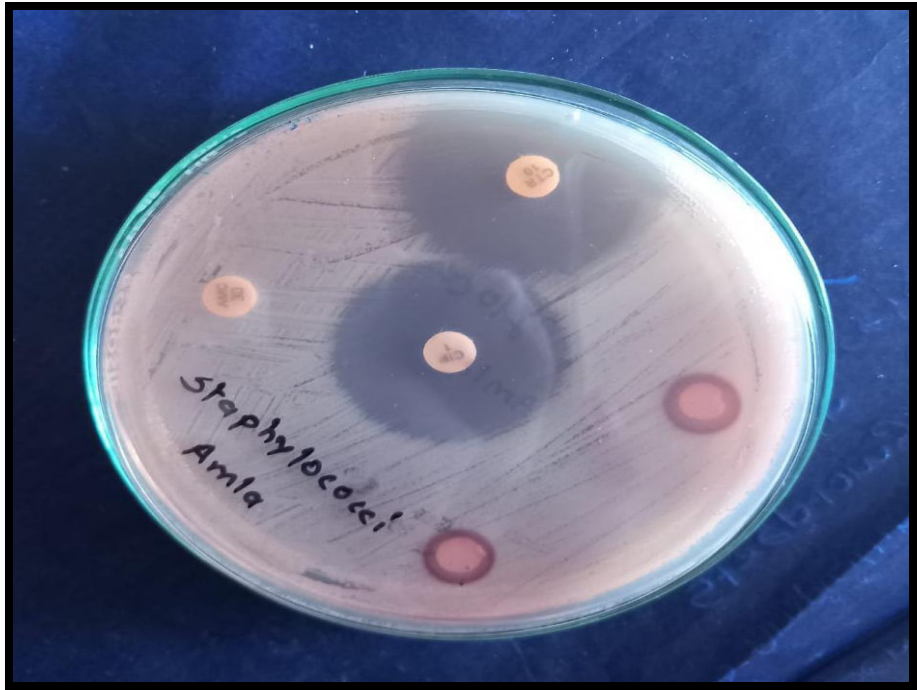


Plate 10. Antibacterial effect of *E. officinalis* fruit juice against *S. aureus*



Plate 11. Antibacterial effect of *E. officinalis* fruit juice against *E. coli*



**Plate 12. Antibacterial effect of *C. limon* fruit juice against *S. aureus***



Plate 13. Antibacterial effect of *C. limon* fruit juice against *E. coli*

**Table 4.2: Zones of inhibition of aqueous extracts of *C. limon* fruit juice and *E. officinalis* fruit juice at 75/25 v/v**

Pathogen	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition			% of inhibition	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition
	<i>E.officinalis</i>	CTR		<i>E.officinalis</i>	Cip		<i>E.officinalis</i>	AMC		<i>C.limon</i>	CTR		<i>C.limon</i>	Cip		<i>C.limon</i>	AMC	
<i>S. aureus</i>	16.50±0.08	23.1±0.07	71.43%	16.50±0.08	22.2±0.10	74.33%	16.50±0.08	Resistant	-	11.2±0.10	21.44±0.02	52.24%	11.2±0.10	19.33±0.07	57.95%	11.2±0.10	19.43±0.03	57.65%
<i>E. coli</i>	13.52±0.13	28.11±0.04	63.78%	13.52±0.13	21.2±0.14	63.78%	13.52±0.13	Resistant	-	9.38±0.09	19.23±0.05	48.78%	9.38±0.09	17.98±0.06	52.17%	9.38±0.09	17.75±0.04	52.85%

**Table 4.3 : Zones of inhibition of aqueous extracts of *C. limon* fruit juice and *E. officinalis* fruit juice at 90/10 v/v**

Pathogen	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition	Zones of inhibition		% of inhibition
	<i>E.officinalis</i>	CTR		<i>E.officinalis</i>	Cip		<i>E.officinalis</i>	AMC		<i>C.limon</i>	CTR		<i>C.limon</i>	Cip		<i>C.limon</i>	AMC	
<i>S. aureus</i>	19.7±0.05	23.1±0.07	85.29%	19.7±0.05	22.2±0.10	88.74%	19.7±0.05	Resistant	-	16.43±0.04	21.44±0.02	76.64%	16.43±0.04	19.33±0.07	85%	16.43±0.04	19.43±0.03	81.5%
<i>Escherichia coli</i>	18.81±0.07	28.11±0.04	66.92%	18.81±0.07	21.2±0.14	88.73%	18.81±0.07	Resistant	-	11.94±0.04	19.23±0.05	62.1%	11.94±0.04	17.98±0.06	66.41%	11.94±0.04	17.75±0.04	67.27%

Different researchers have studied the antibacterial activity of *E. officinalis* and *C. limon* and have found rewarding results (Kanthimathi and Soranam 2013, Varghese *et al.* 2013, Dharajiya *et al.* 2015, Shafi *et al.* 2016, Shafi *et al.* 2018, Haraoui *et al.* 2019, Shafi *et al.* 2020, Solanki *et al.* 2022). Sajet *et al.* (2014) evaluated the antimicrobial activity of different dilutions of *C. limon* juice diluted with distilled water (25%,50%,75%,100% v/v) against methicillin resistant *S. aureus* and observed zones of inhibition of 21 mm, 25 mm, 28 mm, and 30 mm respectively. Sudha *et al.* (2017) determined antimicrobial activities of aqueous extract of amla fruit and observed maximum activity against *K. pneumonia* (16.50±0.342 mm), followed by *E. coli* (14.33±0.33 mm), *S. aureus* (14.33±0.42 mm), *P. aeruginosa* (13.33±0.33 mm) and *C. albicans* (10.33±0.21 mm). Deshwal and Kaur (2018) evaluated antimicrobial activity of *C. limon* juice on various pathogenic microorganisms and observed growth inhibition of *E. coli*, *Salmonella*, *P. aeruginosa*, *P. vulgaris*, *S. aureus* and *S. pyogenes*. The maximum antimicrobial activity was observed against *S. aureus* and lowest against *P. aeruginosa*. Shah and Malik (2019) studied antibacterial activity of aqueous *P. emblica* fruit extract against eight pathogenic cultures (*S. typhi*, *E. coli*, *S. aureus*, *V. cholerae*, *S. paratyphi A*, *S. paratyphi B*, *Shigella spp.*, and *B. cereus*) using different concentrations of APE (25%, 50%, 75%, and 100%, v/v). The average zone of inhibition against the culture for the concentration of 25%, 50%, 75% and 100% (v/v) of APE were 12.2±1.48 mm, 15.4±1.6 mm, 17±1.7 mm and 19±2.0 mm respectively. Teke *et al.* (2019) studied *in vitro* activity of honey and lemon juice proportions (90:10, 75:25, 50:50, 25:75 and 10:90% v/v respectively) against clinical isolates from patients with respiratory tract infections and observed *S. aureus* was most sensitive to mixture of 25/75% (v/v) producing a growth inhibition diameter of 14 mm while as two test mixture proportions (75/25 and 90/10% v/v) were not able to inhibit the growth of *S. aureus* and *S. pyogenes*. Shuaib *et al.* (2021) investigated antimicrobial effects of *C. limon* fruit juice against *S. pyogenes* and *E. coli* bacterial isolates and observed zones of inhibition of 20 mm and 15 mm respectively and concluded that *C. limon* juice can be used as a potential source for controlling infections caused by these microbial pathogens. Amer and awwad (2020) studied the antimicrobial activity of aqueous

extract of *C. limon* fruit and reported zone of inhibition of 2.2 mm and 4.5 mm against *S. aureus* and *E. coli* respectively.

#### 4.1.3 Minimum inhibitory concentration (MIC) and Minimum bactericidal Concentration (MBC) of fruit juices.

The MIC and MBC of fruit juice of *E. officinalis* against *S. aureus* were observed to be 5.62 % and 11.25 % respectively and against *E. coli* were observed to be 22.5 % and 45 % respectively. MIC and MBC in case of *C. limon* fruit juice against *S. aureus* were 2.81 % and 5.62 % respectively and against *E. coli* were found to be 5.62 % and 11.25 % respectively.

**Table 4.4 MIC and MBC of *E. officinalis* and *C. limon* fruit juices**

Pathogens	<i>E. officinalis</i> fruit juice		<i>C. limon</i> fruit juice	
	MIC (%)	MBC (%)	MIC (%)	MBC (%)
<i>S.aureus</i>	5.62%	11.25%	2.81%	5.62%
<i>E.coli</i>	22.5%	45%	5.62%	11.25%

Teke *et al.* (2019) studied *in vitro* antimicrobial activity of honey and lemon juice mixtures (90:10, 75:25, 50:50, 25:75 and 10:90% v/v respectively) against clinical isolates from patients with respiratory tract infections. The MIC for *S. aureus* and *S. pyogenes* were recorded as 60% concentration of test mixture and the MBC values for the test microorganisms were considered greater than 80% of test mixtures and they concluded that mixtures of honey and lemon juice (lemon 50-75%) could be a better alternative treatment against some bacterial infectious diseases. Shah and Malik (2019) studied antibacterial activity of aqueous *P. emblica* fruit extract against eight pathogenic cultures (*S. typhi*, *E. coli*, *S. aureus*, *V. cholerae*, *S. paratyphi A*, *S. paratyphi B*, *Shigella spp.*, and *B. cereus*) using different concentrations of APE (25%, 50%, 75%, and 100%, v/v) and observed MIC in the range of 12.5% - 50% (v/v) and the MBC indicated that a concentration of 50% (v/v) APE could kill 75% (6/8) test cultures. Varghese *et al.* (2013) investigated the *in vitro* antibacterial activity of aqueous, ethanolic and acetone extracts of fruits of *E. officinalis* against *S. aureus* and *E. coli* and reported MIC for ethanol and aqueous extracts for *S. aureus* and *E. coli* as 0.3 and

1.0 µg and 1.5 and 3.75µg, respectively. Mayachiew *et al.* (2008) studied antimicrobial and antioxidant activities of Indian gooseberry and Alpinia galangal extracts against *S. aureus* and noted MIC of 13.97 and 0.78mg/ml and MBC of 13.97 and 2.34mg/ml, respectively.

#### **4.2 In vivo therapeutic potential of *E. officinalis* and *C. limon***

*In vivo* studies of *E. officinalis* and *C. limon* fruits involved 18 lactating cows (specific subclinical mastitic in at least one quarter) divided into 3 groups (n = 6). Group 1 (G1) served as control was administered placebo treatment (wheat bran) and Group 2 (G2) and Group 3 (G3) were treated with *E. officinalis* and *C. limon* deseeded fruits @ 250 gm total dose P.O., divided in two parts morning and evening for 5 days.

##### **4.2.1 Elimination of intramammary infection (IMI)**

In comparison to control group (5/19) the therapeutic management with *E. officinalis* and *C. limon* could eliminate 9/16 and 14/21 of intramammary infections at d21 post-treatment. The elimination of infections in *C. limon* treatment as compared to control group was found statistically significant ( $p < 0.05$ ) with  $\chi^2$  (01df, N=24) = 4.200,  $p=0.040$  (Table 4.5 and Plate no. 3) while as in comparison to control group elimination of infections with *E. officinalis* therapy were found non-significant ( $p > 0.05$ ) with  $\chi^2$  (01 df, N=24) = 0.974,  $p=0.324$ . Several researchers have studied different herbs/non antibiotic preparation against intra-mammary infections and observed beneficial effects of these therapies against bovine mastitis. Sharma *et al.* (2010) observed the effect of *P. emblica* and amoxicillin sulbactam combination on the bacterial growth of milk and observed no bacterial growth on day 30 of therapy as compared to heavy bacterial growth on day 0. Maramulla *et al.* (2019) studied efficacy of herbal preparations (*O. sanctum* dry powder, honey and lemon juice) in therapeutic management of sub clinical mastitis in cows administered as a bolus two times in a day for 7 days and observed animal wise cure percentage of 90 % (9 out of 10) and quarter wise cure percentage of 93.75 % (15 out of 16 quarters).

**Table 4.5 Effect of *E. officinalis* and *C. limon* therapy on level of intramammary infections (IMI)**

Infection status of individual quarters	Intramammary infections Vs. Treatment Groups					
	Control (G1)		<i>E. officinalis</i> (G2)		<i>C. limon</i> (G3)	
	Present at 0d	Eliminated at 21d	Present at 0d	Eliminated at 21d	Present at 0d	Eliminated at 21d
No. of quarters with IMI	19	5	16	9	22	14
Overall	19	5 (26.31%)	16	9 (56.25%)*	22	14 (63.63%)**

Figures in parentheses indicate percentage

Significant differences existed in elimination of IMI between *C. limon* treatment as compared to control, while as effect for *E. officinalis* was non-significant [ $*\chi^2 = 0.974$  (01df, N=24);  $**\chi^2 = 4.200$  (01df, N=24)]

Shafi *et al.* (2016) while evaluating *O. sanctum* for immunotherapeutic potential against bovine subclinical mastitis reported elimination of 69.23 % (9/13) of intramammary infections that was found to be statistically significant ( $P < 0.5$ ) with chi square value of 5.07 as compared to control group in which only 26.67 % (4/15) elimination was. Khan *et al.* (2018) compared *E. officinalis* fruit extract and procaine penicillin against subclinical mastitis in dairy buffaloes and observed quarter based bacteriological cure rate of 64.7% with amla fruit extract and concluded that amla fruit extract is an inexpensive alternative to procaine penicillin for treatment of subclinical mastitis in dairy animals. Gao *et al.* (2018) studied that *E. officinalis* fruit extract could effectively alter the oral microbiome and decrease the total bacterial count. Shafi *et al.* (2018) evaluated composite formulation (masticure®) as an adjunct therapy against bovine mastitis and reported therapy as highly effective in eliminating intramammary infections, relieving udder inflammation and improvement in milk quality. Pandey *et al.*, (2020) evaluated three different dosage regimens (@ 250 gram, 200 gram and 150 gm) of crushed deseeded fresh *E. officinalis* in cattle affected with subclinical mastitis and observed that supplementation significantly decreased total bacterial count as compared to non-supplemented group in which increase in somatic cell

count and total bacterial count and decline in milk yield was recorded. Shafi *et al.* (2020) evaluated root powder for elimination of intramammary infections @ 500 mg/kg body weight daily for 7 days and observed 64.28% elimination of intramammary infections (chi square =24.14; 01 df; P<0.05) and also the therapy resulted in a significant reduction in udder inflammation markers and improved milk quality. Keshamoni *et al.* (2021) investigated a paste preparation of lemon, aloe vera leaves 200 gm, turmeric powder 10 gm, castor oil 10 ml applied externally over the udder daily for 7 days of affected buffalos and observed quarter wise recovery percentage of 64.29%. Rizwan *et al.* (2021) evaluated efficacy of *P. emblica* fruit extract and reported bacteriological cure rate of subclinically mastitic teats as 40% and 50% at 7<sup>th</sup> and 14<sup>th</sup> day respectively.

#### **4.2.2 Effect on udder inflammation in terms SCC, CMT, pH and EC**

The values of SCC, CMT, pH and EC in different treatment groups are given in Table 4.6. The *E. officinalis* therapy resulted significant decline on day 21 for CMT score ( $0.33\pm 0.33$ ) and SCC ( $253.17\pm 59.53$ ) (Table 4.6, Fig.4.5 and Fig 4.2). Treatment with *C. limon* resulted significant decline on day 21 for CMT score ( $0.33\pm 0.21$ ) and SCC ( $299.50\pm 73.17$ ) (Table 4.6, Fig 4.1, Fig 4.2). The electrical conductivity on day 21 was found significantly decreased for the treatments with *E. officinalis* ( $46.03\pm 0.56$ ) (Table 4.6, Fig.4.3) and for *C. limon* significant decline was observed in EC on day 7 ( $51.13\pm 1.43$ ) and on day 21 ( $47.96\pm 1.27$ ) (Table 4.6, Fig 4.3). The treatments with *E. officinalis* and *C. limon* showed significant decrease in pH on day 7 with values of  $6.82\pm 0.05$  and ( $6.78\pm 0.04$ ) respectively and also significant decrease was observed on day 21 with values of  $6.68\pm 0.06$  and  $6.65\pm 0.05$  respectively (Table 4.6, Fig 4.4). Overall both the therapies showed significant improvement on day 21 with respect to improvement in the values of SCC, CMT, pH and EC thereby subsiding udder inflammation and improving the health of udder health and milk quality. Similarly various researchers have reported beneficial role of *E. officinalis* and *C. limon* therapy or other non-antibiotics herbal preparation in terms of reducing udder inflammation and improving milk parameters such as CMT, SCC, EC and pH (Das *et al.* 2003, Sharma *et al.* 2010, Ibrahim *et al.* 2016, Shafi *et al.* 2016, Shafi *et al.* 2018, Dutta *et al.* 2020, Pandey *et al.* 2020, Patel and Gupta 2020, Rathaur

*et al.* 2020, Shafi *et al.* 2020, Keshamoni *et al.* 2021, Siyal *et al.* 2021, Martinez *et al.* 2022). Abbasi *et al.* (2013) observed that paste made from the fruit juice of *C. limon* and sugar when topically applied to the mammary glands for 10–15 days helped to cure mastitis in buffalo, cattle and goats. Maramulla *et al.*(2019) studied efficacy of herbal preparations (*O. sanctum* dry powder, honey and lemon juice) in therapeutic management of sub clinical mastitis in cows administered as a bolus two times in a day for 7 day and observed significant reduction in SCC, pH, and increased fat content in milk. Rathaur *et al.* (2020) studied the effects of *C. limon* fruits cut into pieces in subclinical mastitis affected animals @ 250g/animal /day for 10 days and observed significant reduction in CMT, pH, SCC and EC of milk and also 62.5% recovery percentage was noted. Khan *et al.* (2021) observed that aqueous fruit extract of *P. emblica* significantly decreased udder inflammation and ceruloplasmin concentration, an acute-phase protein in udder inflammation in bovine mastitis. Rizwan *et al.* (2021) evaluated efficacy of *P. emblica* fruit extract and reported beneficial effect on SCC, CMT, pH and EC.

**Table 4.6 Effect of *E. officinalis* and *C. limon* therapy on inflammatory markers of the udder**

Parameters	Group	Days after initiation of treatment (AT)		
		Day 0	Day 7	Day 21
CMT point score	G1	2.17±0.30 <sup>1a</sup>	2.00±0.25 <sup>1a</sup>	1.67±0.21 <sup>1a</sup>
	G2	2.00±0.25 <sup>1a</sup>	1.33±0.33 <sup>1a</sup>	0.33±0.33 <sup>2b</sup>
	G3	2.33±0.21 <sup>1a</sup>	1.67±0.21 <sup>1b</sup>	0.33±0.21 <sup>2c</sup>
SCC ( x 10 <sup>3</sup> /ml)	G1	749.17±66.60 <sup>1a</sup>	628.83±60.68 <sup>1ab</sup>	518.67±29.55 <sup>1b</sup>
	G2	679.67±60.29 <sup>1a</sup>	495.83±84.28 <sup>1a</sup>	253.17±59.53 <sup>2b</sup>
	G3	803.17±62.69 <sup>1a</sup>	541.17±45.01 <sup>1b</sup>	299.50±73.17 <sup>2c</sup>
EC (mŪ)	G1	63.51±5.51 <sup>1a</sup>	60.76±0.95 <sup>1a</sup>	66.15±1.77 <sup>1a</sup>
	G2	54.16±1.05 <sup>1a</sup>	52.26±0.82 <sup>2a</sup>	46.03±0.56 <sup>2b</sup>
	G3	54.05±1.81 <sup>1a</sup>	51.13±1.43 <sup>2ab</sup>	47.96±1.27 <sup>2b</sup>
Ph	G1	7.02±0.07 <sup>1a</sup>	6.98±0.06 <sup>1a</sup>	7.0133±0.05 <sup>1a</sup>
	G2	6.95±0.06 <sup>1a</sup>	6.82±0.05 <sup>12ab</sup>	6.68±0.06 <sup>2b</sup>
	G3	7.02±0.07 <sup>1a</sup>	6.78±0.04 <sup>2b</sup>	6.65±0.05 <sup>2b</sup>

Superscripts in each row (a, b, c) and each coloumn (1, 2, 3) differ significantly (p < 0.05 )

### 4.2.3 Effect on biochemical composition of milk

Therapy with *E. officinalis* resulted in significant increase in lactose on day 7 ( $4.40 \pm 0.10$ ) and day 21 ( $4.56 \pm 0.15$ ) however non-significant effect on fat, SNF and protein was observed (Table 4.7, Fig 4.5, Fig4.6, Fig 4.7, Fig 4.8) while as milk protein, fat, SNF and lactose were found significantly increased on day 7 and day 21 with *C. limon* therapy (Table 4.7, Fig 4.5, Fig4.6, Fig 4.7, Fig 4.8). Milk composition in the terms of percentage of fat, SNF, protein and lactose were important parameters in measuring the milk quality and subclinical mastitis affected animals were seen with decreased percentage of these parameters, however, therapy with fruits of *E. officinalis* and *C. limon* showed beneficial effect on milk quality in terms of increasing the percentage of fat, SNF, lactose and protein. Also various researchers have reported beneficial role of *E. officinalis* and *C. limon* therapy or other non-antibiotics herbal preparations in terms having positive effect on fat, SNF, lactose and protein and thereby having potential of improving the milk quality (Shafi *et al.* 2016, Shafi *et al.* 2018, Harjanti *et al.* 2019, Pandey *et al.* 2020, Patel and Gupta 2020, Shafi *et al.* 2020, Singla *et al.* 2021). Daimi *et al.* (2006) while evaluating aqueous extract of *A. indica* bark via oral route and *Curcumin longa* rhizome paste topically against sub clinical mastitis daily twice for 15 day saw 30 % recovery and also improvement in the biochemical composition of milk. Tilahun *et al.* (2022) studied effect of feeding fresh amla fruits in lactating cows on milk fat, protein and lactose content of milk and observed animals receiving 600 g/d achieved higher milk yields than the animals receiving 200, 400 g/d and control and also animals receiving 400 g/d and 600 g/d led to greater milk protein yield ( $P < 0.01$ ), milk protein content ( $P < 0.01$ ), and milk nitrogen efficiency ( $P < 0.01$ ) compared to cows fed 200 g/d FAF or control. Rathaur *et al.* (2020) studied the effects of *C. limon* fruits cut into pieces in subclinical mastitis affected animals @ 250g/animal /day for 10 days and observed effects on the percentage of mean  $\pm$  SEM of compositional changes on fat %, protein %, and lactose % before and after treatment that changed from  $3.30 \pm 0.02$  -  $3.48 \pm 0.03\%$ ,  $3.42 \pm 0.01$  -  $3.87 \pm 0.02\%$ ,  $4.32 \pm 0.03$  -  $4.56 \pm 0.01$  % respectively. Todaro *et al.* (2017) studied that the effect of feeding of fresh lemon pulp to lactating ewes with concentrate and ad libitum hay resulted in higher milk

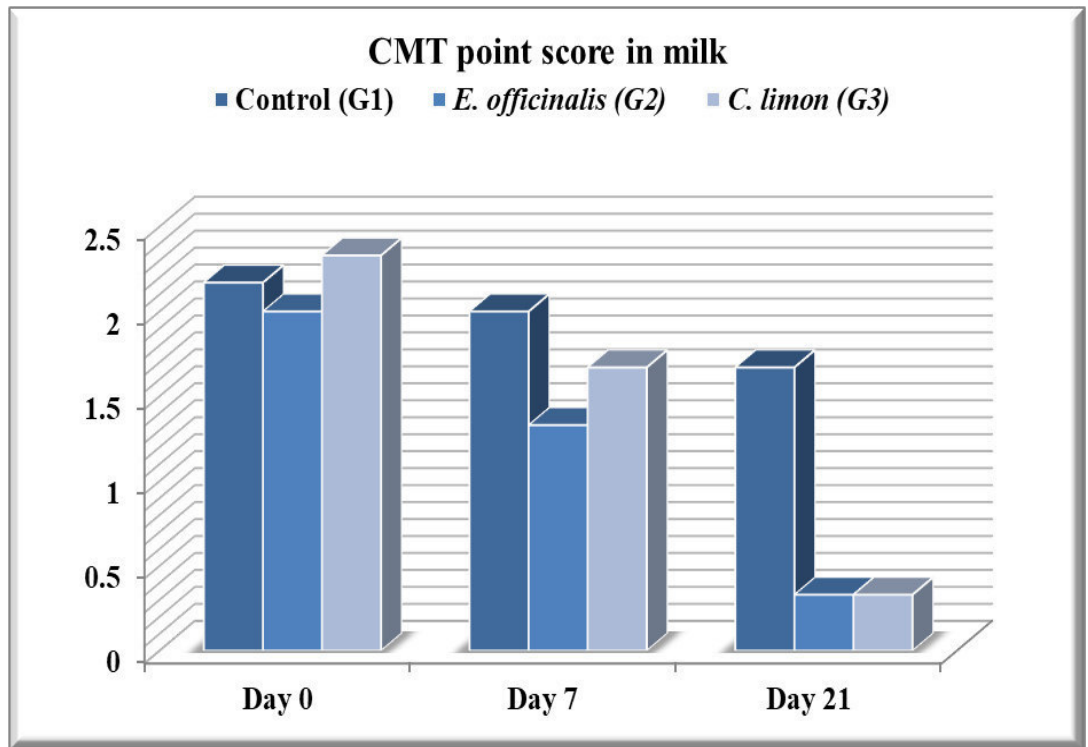


Fig 4.1 : CMT point score in milk of Control (G1), *E. officinalis* (G2) and *C. limon* (G3) groups

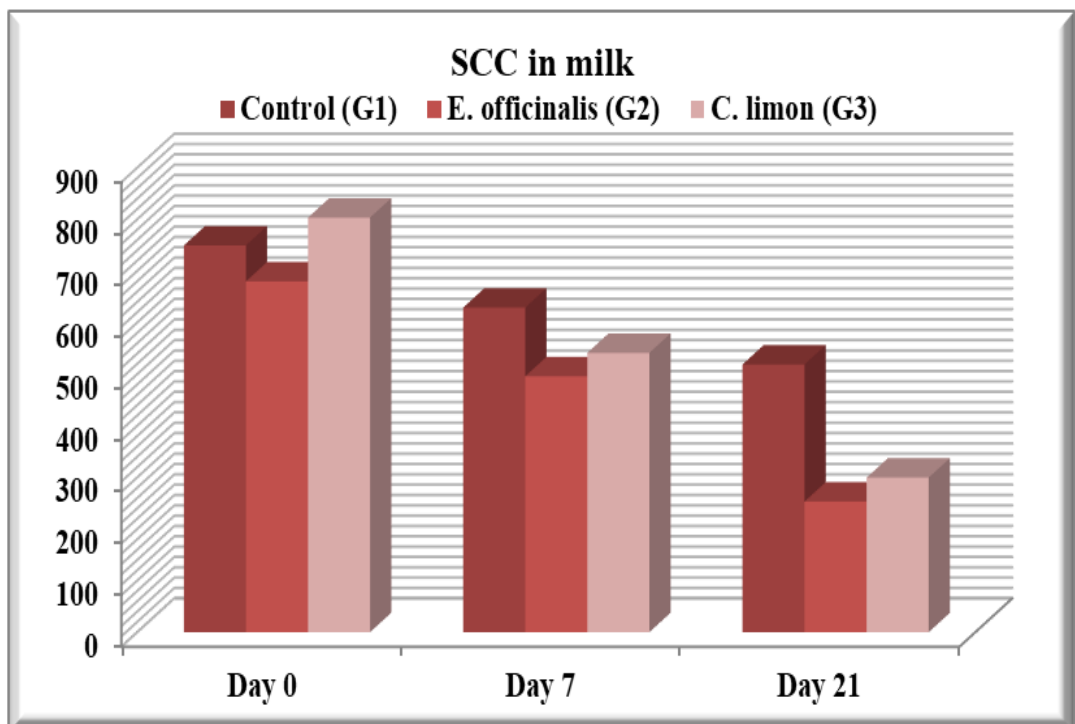


Fig 4.2 : SCC in milk of Control (G1), *E. officinalis* (G2) and *C. limon* (G3) groups

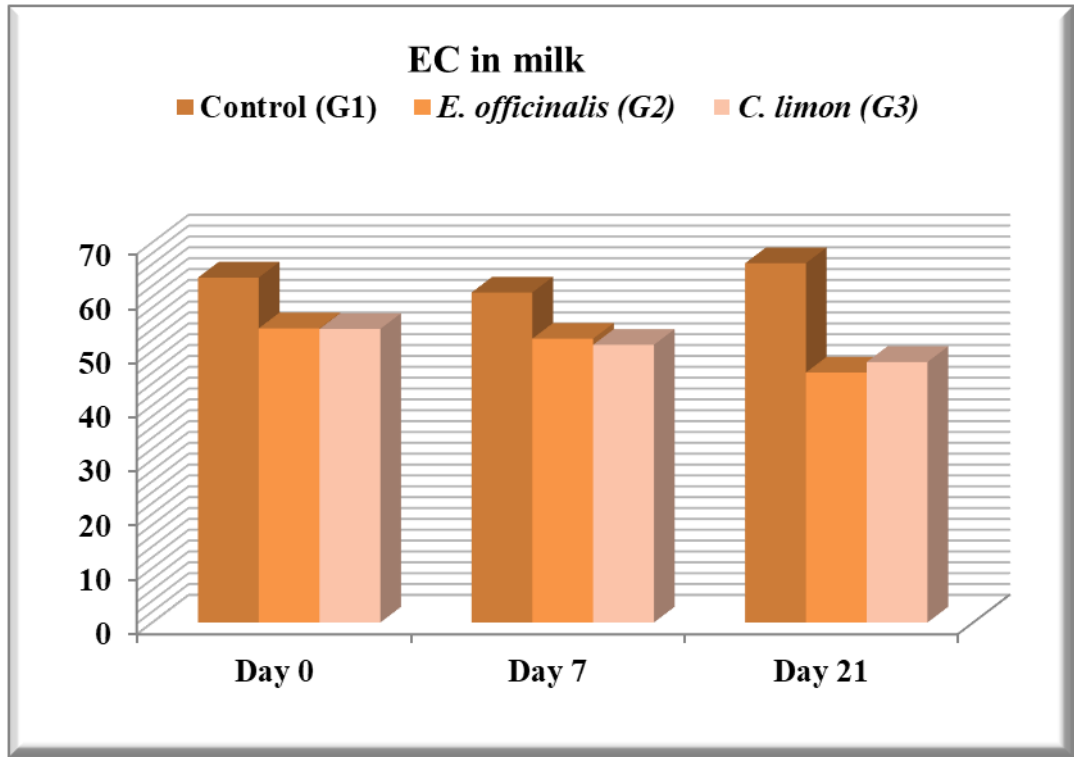


Fig 4.3: EC in milk of Control (G1), *E. officinalis* (G2) and *C. limon* (G3)

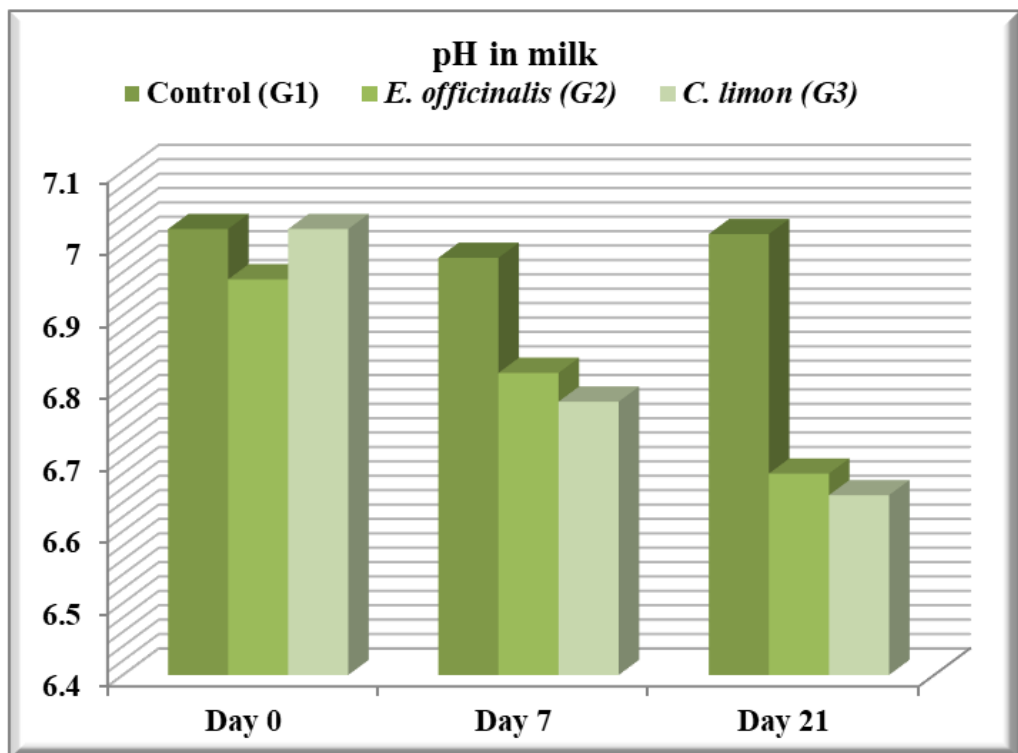


Fig 4.4 pH in milk of Control (G1), *E. officinalis* (G2) and *C. limon* (G3)

protein and casein percentage in group 2 (pulp dose -2kg /day) as compared to group 1 (pulp dose -1kg /day ). Rizwan *et al.* (2021) evaluated efficacy of *P. emblica* fruit extract and observed marked increase in milk fat, protein, specific gravity as well as total milk yield from zero day to 14<sup>th</sup> day ( $p < 0.05$ ).

**Table.4.7 Effect of *E. officinalis* and *C. limon* therapy on milk composition**

Parameters	Group	Days after initiation of treatment (AT)		
		Day 0	Day 7	Day 21
Protein (%)	G1	2.89±0.12 <sup>1a</sup>	2.75±0.15 <sup>1a</sup>	2.97±0.18 <sup>1a</sup>
	G2	2.79±0.05 <sup>1a</sup>	2.79±0.10 <sup>1a</sup>	2.98±0.05 <sup>1a</sup>
	G3	2.60 ±0.13 <sup>1a</sup>	2.99±0.05 <sup>1b</sup>	3.13±0.09 <sup>1b</sup>
Fat (%)	G1	2.70±0.48 <sup>1a</sup>	2.56±0.20 <sup>1a</sup>	2.52±0.21 <sup>1a</sup>
	G2	2.82±0.41 <sup>1a</sup>	2.90±0.32 <sup>1a</sup>	3.19±0.22 <sup>12a</sup>
	G3	2.33±0.23 <sup>1a</sup>	2.73±0.23 <sup>1a</sup>	3.55±0.30 <sup>2b</sup>
SNF (%)	G1	7.98±0.30 <sup>1a</sup>	7.65±0.23 <sup>1a</sup>	8.01±0.13 <sup>1a</sup>
	G2	7.59±0.30 <sup>1a</sup>	7.90±0.20 <sup>1a</sup>	7.97±0.31 <sup>1a</sup>
	G3	7.57±0.10 <sup>1a</sup>	7.79±0.12 <sup>1ab</sup>	8.09±0.14 <sup>1b</sup>
Lactose (%)	G1	3.48±0.62 <sup>12a</sup>	4.30±0.09 <sup>1a</sup>	4.41±0.09 <sup>1a</sup>
	G2	4.16±0.06 <sup>1a</sup>	4.40±0.10 <sup>1ab</sup>	4.56±0.15 <sup>1b</sup>
	G3	2.94±0.06 <sup>2a</sup>	3.25±0.10 <sup>2b</sup>	3.24±0.06 <sup>2b</sup>

Superscripts in each row (a, b, c) and each coloumn (1, 2, 3) differ significantly ( $p < 0.05$ ).

#### 4.2.4 Effect on milk differential leucocyte count

Therapy with *E. officinalis* fruit caused significant decline in percentage of mean neutrophil on day 7 (44.17±2.84) and also on day 21 (26.83±1.51) and significant improvement in the mean lymphocyte percentage on day 21 (19.50±0.76) (Table 4.8, Fig 4.9 and Fig 4.10). *C. limon* fruit therapy caused significant decline of mean neutrophil percentage on day 21 (30.67±2.89) and caused significant increase in mean lymphocyte percentage on day 21 (23.83±1.01) (Table 4.8, Fig 4.9 and Fig 4.10). Dhakal and Kapur (1992) reported mastitis milk contains neutrophils predominantly followed by lymphocytes, epithelial cells and monocytes and observed decreased percentage of lymphocytes while as increased percentage of neutrophils in the milk leukocytes of mastitis affected animals. Pragati *et al.* (2015) studied comparative efficacy of *E. officinalis* and *T. cordifolia* on hemato-biochemical profile of Murrah buffalo

calves fed @ 250 mg/kg body weight once a day orally for 28 days and observed significant increases in total erythrocyte count, total leukocyte count and lymphocyte neutrophil ratio in calves fed *E. officinalis*; lymphocyte count was found significantly elevated in calves fed *E. officinalis* and *T. cordifolia* while as significantly decreased level of neutrophil and monocyte count in calves fed *E. officinalis*. Ibrahim *et al.* (2016) observed that Vitamin E + Se and herbal preparation (Garlic, Black pepper and Lemon) when used against subclinical mastitis showed significant immuno-potentiality by enhancing lymphocytes and decreasing the overall WBCs count, and suggested that the effect could be correlated with reduction in the SCC thereby leading to cure of subclinical mastitis. Leukocytes recruited early in the infection due to their ability to phagocytose or eliminate intra mammary infections are vital for host innate immune response and due to this fact polymorphonuclear leukocytes predominate in the mammary secretions. The activation of polymorphonuclear leukocytes especially lymphocytes by herbal therapy is indication of immunomodulation necessary for elimination of intra-mammary infections that leads to subsiding of udder inflammation a finding also supported by various researchers (Gupta and Pachauri 2001, Acharya *et al.*, 2002, Mukherjee *et al.*, 2005, Shafi *et al.*, 2016, Shafi *et al.*, 2018 and Shafi *et al.*, 2020).

**Table 4.8 Effect of *E. officinalis* and *C. limon* therapy on DLC**

Parameters	Group	Days after initiation of treatment (AT)		
		Day 0	Day 7	Day 21
Neutrophil (%)	G1	55.33±2.56 <sup>1a</sup>	48.83±2.72 <sup>1a</sup>	49.00±2.43 <sup>1a</sup>
	G2	51.33±2.37 <sup>1a</sup>	44.17±2.84 <sup>1b</sup>	26.83±1.51 <sup>1c</sup>
	G3	56.00±2.46 <sup>1a</sup>	49.17±2.28 <sup>1a</sup>	30.67±2.89 <sup>2b</sup>
Lymphocyte (%)	G1	16.00±2.42 <sup>12a</sup>	15.17±1.07 <sup>1a</sup>	16.17±0.83 <sup>1a</sup>
	G2	14.17±0.47 <sup>1a</sup>	17.83±0.79 <sup>2b</sup>	19.50±0.76 <sup>2b</sup>
	G3	19.33±0.76 <sup>2a</sup>	19.83±0.65 <sup>2a</sup>	23.83±1.01 <sup>3b</sup>

Superscripts in each row (a, b, c) and each column (1, 2, 3) differ significantly (p<0.05).

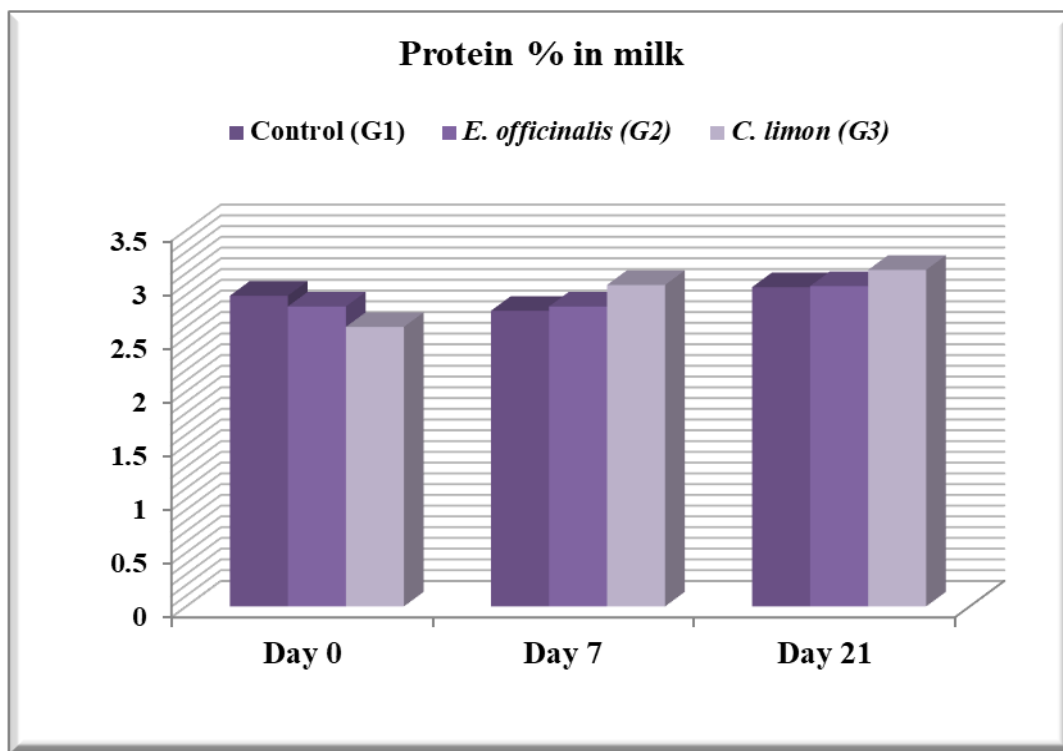


Fig 4.5: Protein % in milk of Control (G1), *E. officinalis* (G2) and *C. limon* (G3) groups

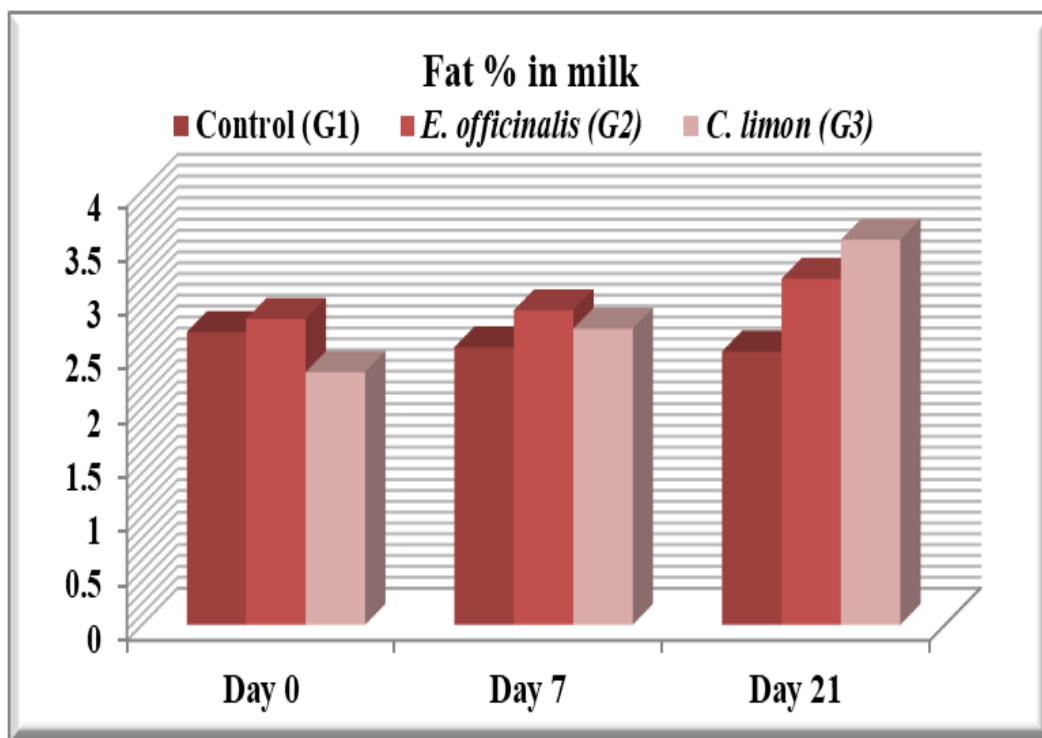


Fig 4.6: Fat % in milk of Control (G1), *E. officinalis* (G2) and *C. limon* (G3) groups

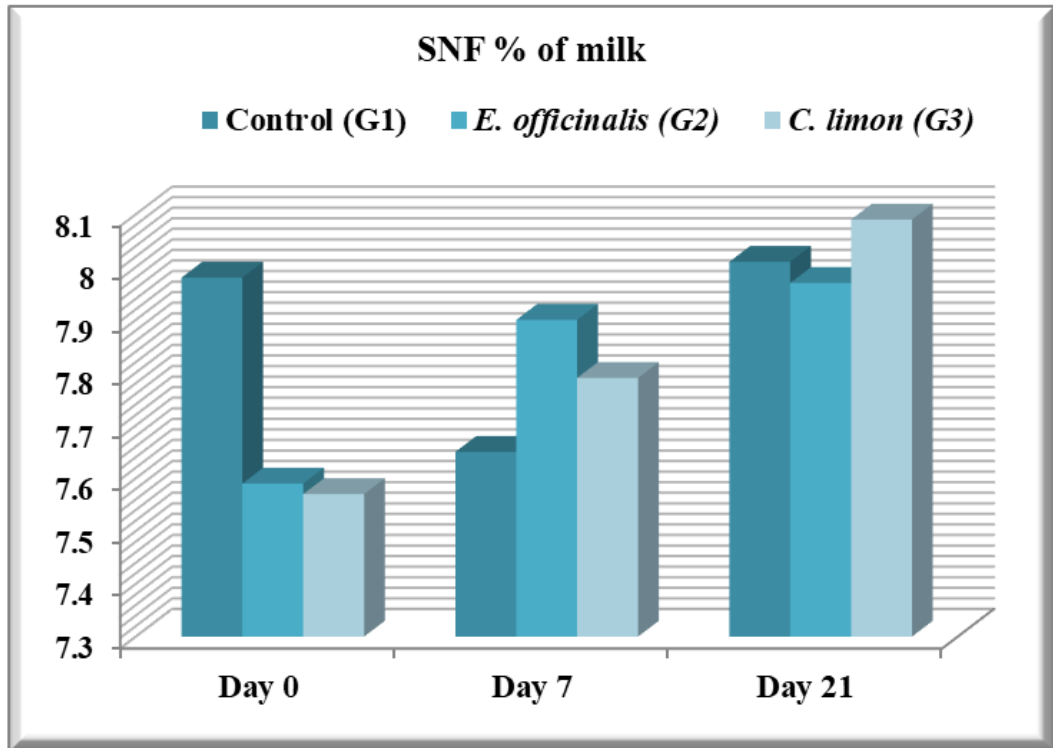


Fig 4.7: SNF% in milk of Control (G1), *E. officinalis* (G2) and *C. limon* (G3) groups

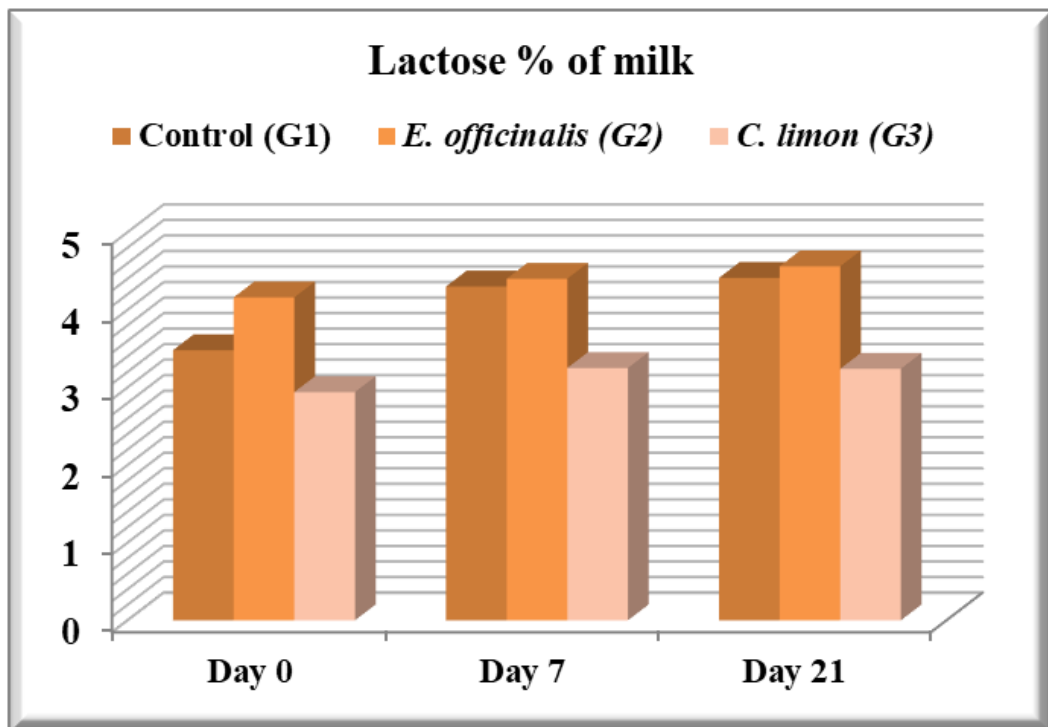
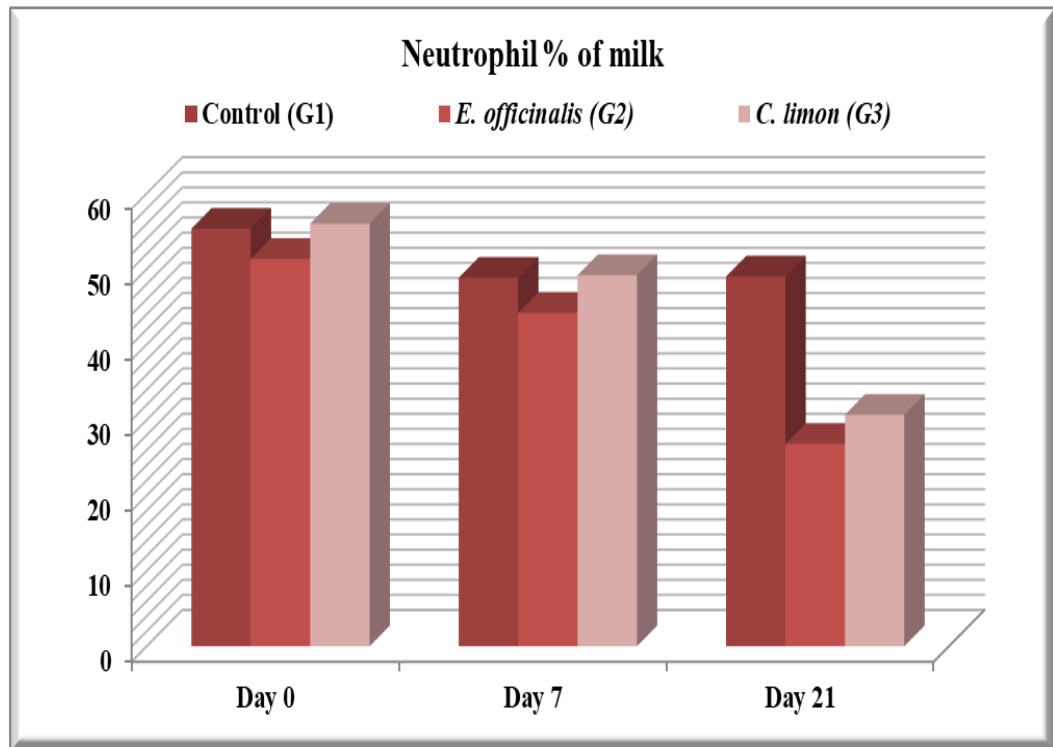
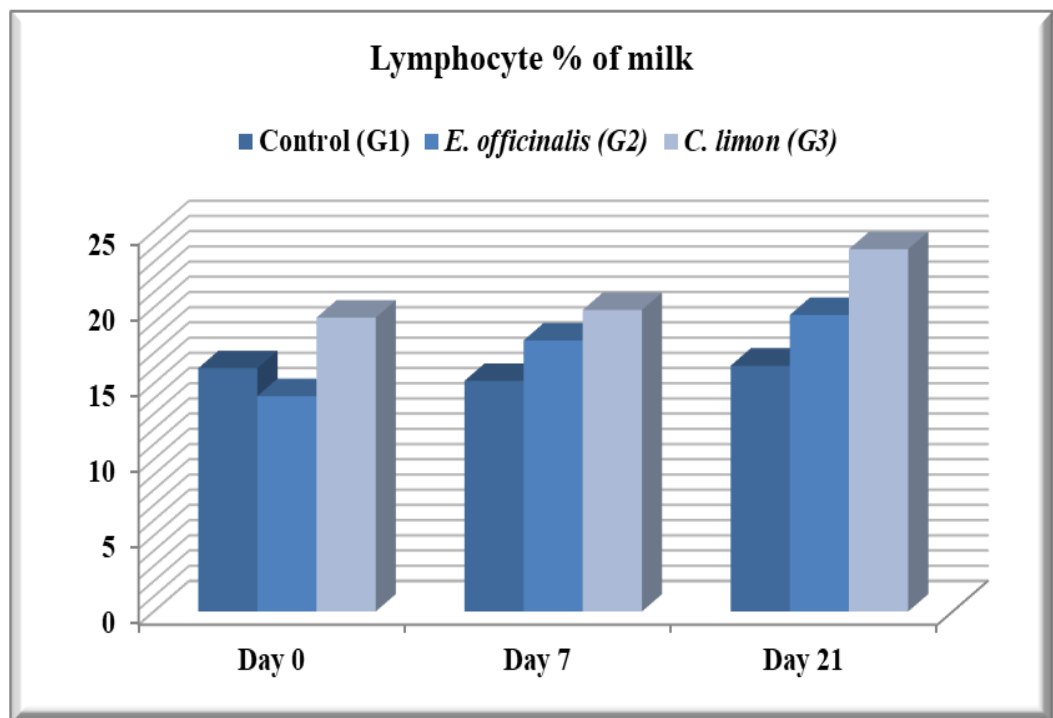


Fig 4.8: Lactose % in milk of Control (G1), *E. officinalis* (G2) and *C. limon* (G3) groups



**Fig 4.9: Neutrophil in milk of Control (G1), *E. officinalis* (G2) and *C. limon* (G3) groups**



**Fig 4.10: Lymphocyte in milk of Control (G1), *E. officinalis* (G2) and *C. limon* (G3) groups**



# Summary and Conclusions



## CHAPTER V

### SUMMARY AND CONCLUSIONS

The present study entitled “THERAPEUTIC EVALUATION OF *Emblica officinalis* and *Citrus limon* FRUIT IN BOVINE SUB-CLINICAL MASTITIS” was undertaken at Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Parbhani. The deseeded fruits of *E. officinalis* and *C. limon* were evaluated for antibacterial properties and also for anti-inflammatory and immunomodulatory activities in terms of therapeutic management against bovine sub-clinical mastitis.

The *in vitro* antibacterial activity of fruit juices of deseeded *E. officinalis* and *C. limon* fruit were recorded against *S. aureus* and *E. coli* isolated from the mastitis affected animals. The fruit juices were extracted aseptically and the antibacterial activity against *S. aureus* and *E. coli* was studied. No marked antibacterial activity was noted for working dilutions 25/75 and 10/90% v/v of whole fruit juices against *S. aureus* and *E. coli* however marked antibacterial activity was noted for working dilutions of 90/10, 75/25 and 50/50 v/v. In this study, average zone of inhibition shown by working dilution of (90/10 v/v) of *E. officinalis* and *C. limon* against *S. aureus* and *E. coli* were  $19.7 \pm 0.05$  mm,  $18.81 \pm 0.07$  mm and  $16.43 \pm 0.04$  mm,  $11.94 \pm 0.04$  mm respectively.

The MIC and MBC of fruit juice of *E. officinalis* against *S. aureus* were observed to be 5.62 % and 11.25 % respectively and against *E. coli* were observed to be 22.5 % and 45 % respectively. MIC and MBC in case of *C. limon* fruit juice against *S. aureus* were 2.81 % and 5.62 % respectively and against *E. coli* were found to be 5.62 % and 11.25 % respectively.

*In vivo* studies of *E. officinalis* and *C. limon* fruits involved 18 lactating cows (specific subclinical mastitis in at least one quarter) divided into 3 groups (n = 06). Group 1 (G1) served as control was administered placebo treatment (wheat bran) and Group 2 (G2) and Group 3 (G3) were treated with *E. officinalis* and *C. limon* deseeded fruits @ 250 gm total dose P.O., divided in two parts morning and evening for 5 days.

In comparison to control group (5/19) the therapeutic management with *E. officinalis* and *C. limon* could eliminate 9/16 and 14/21 of intramammary infections at d 21 post-treatment. The elimination of infections in *C. limon* treatment as compared to control group was found statistically significant ( $p < 0.05$ ) with  $\chi^2$  (01df, N=24) = 4.200,  $p=0.040$  while as in comparison to control group elimination of infections with *E. officinalis* therapy were found non-significant ( $p > 0.05$ ) with  $\chi^2$  (01 df, N=24) = 0.974,  $p= 0.324$ .

The *E. officinalis* therapy resulted significant decline in CMT score ( $0.33 \pm 0.33$ ) and SCC ( $253.17 \pm 59.53$ ) on day 21. Treatment with *C. limon* resulted significant decline in CMT score ( $0.33 \pm 0.21$ ) and SCC ( $299.50 \pm 73.17$ ) on day 21 for. The electrical conductivity on day 21 was found significantly decreased for the treatments with *E. officinalis* ( $46.03 \pm 0.56$ ) and for *C. limon* significant decline was observed in EC on day 7 ( $51.13 \pm 1.43$ ) and on day 21 ( $47.96 \pm 1.27$ ). The treatments with *E. officinalis* and *C. limon* showed significant decrease in pH on day 7 with values of  $6.82 \pm 0.05$  and ( $6.78 \pm 0.04$ ) respectively and also significant decrease was observed on day 21 with values of  $6.68 \pm 0.06$  and  $6.65 \pm 0.05$  respectively. Overall both the therapies showed significant improvement on day 21 with respect to improvement in the values of SCC, CMT, pH and EC thereby decreasing udder inflammation and improving the udder health and quality of milk.

Therapy with *E. officinalis* resulted in significant increase in lactose on day 7 ( $4.40 \pm 0.10$ ) and day 21 ( $4.56 \pm 0.15$ ) however non-significant effect on fat, SNF and protein was observed, while as milk protein, fat, SNF and lactose were found significantly increased on day 7 and day 21 with *C. limon* therapy. Milk composition in the terms of percentage of fat, SNF, protein and lactose are important parameters in measuring the milk quality and subclinical mastitis affected animals were seen with decreased percentage of these parameters, however, therapy with fruits of *E. officinalis* and *C. limon* showed beneficial effect on milk quality in terms of increasing the percentage of fat, SNF, lactose and protein.

Therapy with *E. officinalis* fruit caused significant decline in percentage of mean milk neutrophil on day 7 ( $44.17 \pm 2.84$ ) and also on day 21 ( $26.83 \pm 1.51$ ) and significant improvement in the mean milk lymphocyte percentage on day 21 ( $19.50 \pm 0.76$ ). *C. limon* fruit therapy caused significant decline of mean milk neutrophil percentage on day 21 ( $30.67 \pm 2.89$ ) and caused significant increase in mean milk lymphocyte percentage on day 21 ( $23.83 \pm 1.01$ ).

## CONCLUSIONS

1. The *in vitro* antibacterial activity in terms of zone of inhibition for 90/10 % v/v working dilution of *E. officinalis* and *C. limon* against *S. aureus* and *E. coli* were  $19.7 \pm 0.05$  mm,  $18.81 \pm 0.07$  mm and  $16.43 \pm 0.04$  mm,  $11.94 \pm 0.04$  mm respectively.
2. The MIC and MBC of fruit juice of *E. officinalis* against *S. aureus* were observed to be 5.62 % and 11.25 % respectively and against *E. coli* were observed to be 22.5 % and 45 % respectively. MIC and MBC in case of *C. limon* fruit juice against *S. aureus* were 2.81 % and 5.62 % respectively and against *E. coli* were found to be 5.62 % and 11.25 % respectively.
3. The elimination of infections in *C. limon* treatment as compared to control group were found statistically significant ( $p < 0.05$ ) with  $\chi^2$  (01df, N=24) = 4.200,  $p = 0.040$  while as in comparison to control group elimination of infections with *E. officinalis* therapy were found non-significant ( $p > 0.05$ ) with  $\chi^2$  (01 df, N=24) = 0.974,  $p = 0.324$ .
4. Therapies with *E. officinalis* and *C. limon* resulted in significant reduction in CMT score, SCC, EC, pH and milk neutrophils while as there was significant increase in milk lymphocytes observed.
5. Therapy with *E. officinalis* resulted in significant increase in lactose on day 7 ( $4.40 \pm 0.10$ ) and day 21 ( $4.56 \pm 0.15$ ) however non-significant effect on fat, SNF and protein was observed while as *C. limon* therapy resulted in significant increase in milk protein, fat, SNF and lactose on day 7 and day 21.

6. *C. limon* therapy showed significantly higher effect on elimination of intra mammary infections in comparison to control and *E. officinalis* groups but overall both the therapies resulted in significant improvement in terms of reduction in udder inflammatory markers, improved udder immune-modulation and improvement in the milk quality.



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# Appendix



## **APPENDIX I**

### **Mannitol Salt Agar M118B**

#### **Intended Use:**

Mannitol Salt Agar is used for selective isolation pathogenic staphylococci in accordance with British Pharmacopoeia.

#### **Composition**

<b>Ingredients</b>	<b>Gms/ Litre</b>
Peptic digest of animal tissue	5.000
Pancreatic digest of casein	5.000
Beef extract	1.000
Sodium chloride	75.000
D-Mannitol	10.000
Phenol red	0.025
pH after sterilization (at 25 <sup>0</sup> C)	7.4±0.2

\*\*Formula adjusted, standardized to suit performance parameters

#### **Directions**

Suspended 111.02 grams in 1000 ml purified / distilled water. Heat to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121<sup>0</sup> C) for 15 minutes or as per validated cycle.

## EMB Agar

### Intended Use:

Recommended for differential isolation of Gram-negative enteric bacilli from clinical and non-clinical specimens.

### Composition

Ingredients	Gms/Litre
Peptone	10.000
Dipotassium hydrogen phosphate	2.000
Lactose	5.000
Saccharose (Sucrose)	5.000
Eosin – Y	0.400
Methylene blue	0.065
Agar	13.500
Final pH (at 25°C)	7.2±0.2

\*\*Formula adjusted, standardized to suit performance parameters

### Directions

Suspended 35.96 grams in 1000 ml purified / distilled water. Mix until suspension is uniform. Heat to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. AVOID OVERHEATING. Cool to 45-50°C and shake the medium in order to oxidize the methylene blue (ie. to restore its blue colour) and to suspend the flocculent precipitate.(If EMB Agar is inoculated on the same day, it may be used without autoclave sterilization)

## MR-VP Medium (Glucose Phosphate Broth)

### Intended Use:

Recommended for the performance of the Methyl Red and Voges-Proskauer tests in differentiation of the coli-aerogenes group from clinical and non clinical samples.

### Composition

Ingredients	Gms/Litre
Buffered peptone	7.000
Dextrose (Glucose)	5.000
Dipotassium phosphate	5.000
Final pH (at 25°C)	6.9±0.2

\*\*Formula adjusted, standardized to suit performance parameters

### Directions

Suspended 17.0 grams in 1000 ml of purified / distilled water. Heat if necessary to dissolve the medium completely. Then Distributed in test tubes in 10 ml amounts and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

## Nutrient Agar

### Intended Use:

Nutrient Agar is used for the cultivation of bacteria and for the enumeration of organisms in milk, water and other materials.

### Composition

Ingredients	Gms / Litre
Peptone	5.000
Sodium chloride	5.000
HM peptone B	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25 <sup>0</sup> C)	7.4±0.2

\*\*Formula adjusted, standardized to suit performance parameters

### Directions

Suspended 28 grams in 1000 ml of purified / distilled water. Heat if necessary to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50<sup>0</sup> C.

## Nutrient Broth

### Intended Use:

Recommended for general purpose medium used for the cultivation of a wide variety of fastidious and non-fastidious microorganisms with non-existent nutritional requirements.

### Composition

Ingredients	Gms / Litre
Peptone	5.000
Sodium chloride	5.000
HM peptone B	1.500
Yeast extract	1.500
Final pH (at 25 <sup>0</sup> C)	7.4±0.2

\*\*Formula adjusted, standardized to suit performance parameters

### Directions

Suspended 13 grams in 1000 ml of purified / distilled water. Heat if necessary to dissolve the medium completely. Dispense into tubes or flasks as desired. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

## Mueller - Hinton Agar

### Intended Use:

Mueller-Hinton agar is commonly used for antibiotic susceptibility testing for the Kirby-Bauer disc diffusion method or standard antibiogram, it can therefore be used for clinical diagnosis.

### Composition

Ingredients	Gms / Litre
HM infusion B form #	300.000
Acicase ##	17.500
Starch	1.500
Agar	17.000
Final pH (at 25 <sup>0</sup> C)	7.3±0.2

\*\*Formula adjusted, standardized to suit performance parameters

### Directions

Suspended 38 grams in 1000 ml of purified / distilled water. Heat to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50<sup>0</sup> C.

## Mackonkey agar

### Intended Use:

MacConkey agar is a selective and differential culture medium for bacteria. It is designed to selectively isolate Gram-negative and enteric bacteria and differentiate them based on lactose fermentation.

### Composition

Ingredients	Gms / Litre
Peptone	17 gm
Proteose peptone	3 gm
Lactose monohydrate	10 gm
Bile salts	1.5 gm
Sodium chloride	5 gm
Neutral red	0.03 gm
Crystal Violet	0.001 g
Agar	13.5 gm
Distilled Water	Add to make 1 Liter
Final pH (at 25 <sup>0</sup> C)	7.3±0.2

\*\*Formula adjusted, standardized to suit performance parameters

### Directions

Suspend 49.53 grams of dehydrated medium in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well before pouring into sterile Petri plates.

## **Tryptic Soy Broth**

### **Intended Use:**

Tryptic Soy Broth is a general purpose liquid enrichment medium used in qualitative procedures for the differential leucocyte count test .

### **Composition**

<b>Ingredients</b>	<b>Gms / Litre</b>
Tryptone (Pancreatic Digest of Casein)	17.0 g
Soytone (Peptic Digest of Soybean)	3.0
Glucose (= Dextrose)	2.5
Sodium Chloride	5.0
Dipotassium Phosphate	2.5
Final pH (at 25 <sup>0</sup> C)	7.3±0.2

\*\*Formula adjusted, standardized to suit performance parameters

### **Directions**

Suspend 30 g of dehydrated media in 1 litre of purified filtered water. Sterilize at 121°C for 15 minutes. Cool to 45- 50°C. Mix gently and dispense into sterile Petri dishes or sterile culture tubes.



# Vitae



## VITAE

The author **Mr. Chaunde Dipak Shivaji** was born on **4<sup>th</sup> December ,1996** at A.P - Nandeshwar, Tq - Mangalwedha, Dist- Solapur, and State Maharashtra, India. He completed Secondary School Certificate (SSC) examination from English School, Mangalwedha and passed Higher Secondary Certificate (HSC) examination from Shri. Sant Damaji Mahavidyalay, Mangalwedha, Tq. – Mangalwedha, Dist - Solapur in 2013 and 2015 respectively.

With his affection towards animals and curiosity to know more about these speechless creatures of natures , he enrolled himself for B.V. Sc & A. H degree course in College of Veterinary and Animal Sciences, Udgir, under Maharashtra Animal And Fishery Sciences University (MAFSU), Nagpur.

Being interested in research and development related to animals he joined postgraduate (M.V.Sc.) studies in the discipline of Veterinary Epidemiology and Preventive Medicine in the College of Veterinary and Animal Sciences, MAFSU Parbhani and has completed course work and submitted his thesis.

He is active member of Veterinary Internal and Preventive Medicine Society . He actively participated in National Service scheme (NSS) unit of College of Veterinary and Animal Sciences, Udgir during his under graduation studies. In future, he wants himself to be one amongst the successful veterinary professionals.



# Thesis Abstract



## THESIS ABSTRACT

- a) Title of the thesis : **THERAPEUTIC EVALUATION OF *Emblica officinalis* AND *Citrus limon* FRUIT IN BOVINE SUB-CLINICAL MASTITIS**
- b) Full name of student : **Chaunde Dipak Shivaji**
- c) Name and address of Major Advisor : **Dr. T. A. Shafi**  
Department of Veterinary  
Epidemiology  
And Preventive Medicine, College  
of Veterinary and Animal  
Sciences, MAFSU, Parbhani –  
431 401 (M.S)
- d) Degree to be awarded. : M. V. Sc.
- e) Year of award of degree : 2023
- f) Major subject Medicine : Department of Veterinary  
Epidemiology and Preventive  
Medicine.
- g) Total number of Pages in the thesis : 46
- h) Number of words in the Abstract : 299
- i) Signature of Student :
- j) Signature, name and address of forwarding authority (HOD) :

**Dr. M. P. Sakhare**

## ABSTRACT

The study entitled “THERAPEUTIC EVALUATION OF *Emblica officinalis* AND *Citrus limon* FRUIT IN BOVINE SUB-CLINICAL MASTITIS” evaluated deseeded fruit juices of *E. officinalis* and *C. limon* for antibacterial properties and thereafter deseeded fruits were evaluated for therapeutic management against bovine sub-clinical mastitis. The fruit juices were extracted aseptically and the antibacterial activity against *S. aureus* and *E. coli* was studied revealing zone of inhibition of  $19.7 \pm 0.05$  mm and  $18.81 \pm 0.07$  mm by working dilution of *E. officinalis* (90/10 v/v) and  $16.43 \pm 0.04$  mm and  $11.94 \pm 0.04$  mm by working dilution of *C. limon* (90/10 v/v), respectively. The MIC and MBC of fruit juice of *E. officinalis* against *S. aureus* were observed to be 5.62 % and 11.25 % respectively and against *E. coli* were observed to be 22.5 % and 45 % , respectively. MIC and MBC in case of *C. limon* fruit juice against *S. aureus* were 2.81 % and 5.62 % , respectively and against *E. coli* were 5.62 % and 11.25 % , respectively. *In vivo* therapy with *C. limon* against specific subclinical mastitis of cows resulted significant elimination of infections ( $\chi^2$  (01df, N=24) = 4.200,  $p=0.040$ ) while as elimination of infections with *E. officinalis* treatment vs. control was non-significant ( $\chi^2$  (01df, N=24) = 0.974,  $p= 0.324$ ). The *E. officinalis* and *C. limon* therapy resulted significant decline on day 21 for CMT score, SCC, EC and pH. Therapy with *E. officinalis* resulted significant increase in lactose on day 7 and day 21 However non-significant effect on fat, SNF and protein was observed while as therapy with *C. limon* resulted significant increase in milk protein, fat, SNF and lactose on day 7 and day 21. Therapy with *E. officinalis* and *C. limon* caused significant decline in percentage of mean neutrophil and significant improvement in the mean lymphocyte percentage on day 21.

## प्रबंध सारांश

- a) प्रबंधाचे शीर्षक : गोवंशाच्या सुप्त स्तनदाहाच्या उपचारकरिता आवळा आणि लिंबुच्या फळांची उपयुक्तता अभ्यासणे"
- b) विद्यार्थ्यांचे पूर्ण नाव : दिपक शिवाजी चौंडे
- c) प्रमुख मार्गदर्शकाचे नाव व पत्ता : डॉ. तौ. अ. शफी  
सहाय्यक प्राध्यापक  
पशुवैद्यकीय साथरोगशास्त्र विज्ञान  
व रोगप्रतिबंधक औषधवैद्यकशास्त्र  
विभाग, पशुवैद्यक व पशु विज्ञान  
महाविद्यालय, मपमविवि, परभणी-  
४३१४०२ (महा).
- d) प्रदान करण्यात येणारी पदवी : एम. व्ही.एस्सी.
- e) पदवी प्रदान करण्याचे वर्ष : २०२३
- f) मुख्य विषय : पशुवैद्यकीय साथरोगशास्त्र विज्ञान  
व रोगप्रतिबंधक औषधवैद्यकशास्त्र
- g) प्रबंधातील पानांची एकूण संख्या : ४६
- h) सारांशामधील शब्दांची संख्या : २७६
- i) विद्यार्थ्यांची स्वाक्षरी :
- j) पाठविणाऱ्या अधिकाऱ्याची :  
स्वाक्षरी, नाव व पत्ता  
(विभागप्रमुख)

डॉ. मि. पुं. साखरे

## सारांश

“गोवंशाच्या सुप्त स्तनदाहाच्या उपचारकरिता आवळा आणि लिंबुच्या फळांची उपयुक्तता अभ्यासणे” या शीर्षकाच्या अभ्यासात जीवाणूंच्या वाढीस प्रतिबंध करणारा पदार्थ गुणधर्मासाठी आवळा आणि लिंबुच्या बिया काढून घेतलेल्या फळांच्या रसांचे मूल्यांकन केले गेले आणि त्यानंतर गोवंशाच्या सुप्त स्तनदाहाविरुद्ध उपचारात्मक व्यवस्थापनासाठी बिया काढून घेतलेल्या फळांचे मूल्यमापन केले गेले. फळांचे रस उत्तेजितपणे काढले गेले आणि स्टॅफायलोकोकस ऑरियस आणि ई-कोलाय जीवाणूंच्या वाढीस प्रतिबंध करणारा पदार्थ क्रियांचा अभ्यास केला गेला. आवळ्याच्या फळांच्या रसाची जीवाणूविरोधक क्षेत्र (९०/१० टक्के) प्रतिकारीता स्टॅफायलोकोकस ऑरियस व ईकोलायसाठी अनुक्रमे १९.७±०.०५ मिमी. व १८.८१±०.०७ मिमी. होती व लिंबुच्या फळांच्या रसाची जीवाणूविरोधक क्षेत्र (९०/१० टक्के) प्रतिकारीता या अनुक्रमे १६.४३ ±०.०४ मिमी. व ११.९४±०.०४ मिमी. होती. स्टॅफायलोकोकस ऑरियस विरुद्ध आवळ्याच्या फळांच्या रसाची किमान प्रतिकारक एकाग्रता आणि किमान जीवाणूनाशक एकाग्रता अनुक्रमे ५.६२ % आणि ११.२५ % आणि ई-कोलाय विरुद्ध अनुक्रमे २२.५ % आणि ४५ % आढळून आले. स्टॅफायलोकोकस ऑरियस विरुद्ध लिंबुच्या फळांच्या रसाची किमान प्रतिकारक एकाग्रता आणि किमान जीवाणूनाशक एकाग्रता अनुक्रमे २.८१ % आणि ५.६२ % आणि ई. कोलाय विरुद्ध अनुक्रमे ५.६२ % आणि ११.२५ % होते. लिंबुसोबत नैसर्गिक सजीव स्थितीत उपचारमध्ये गायींच्या विशिष्ट स्तनदाह विरुद्ध संक्रमणाचे लक्षणीय उच्चाटन झाले तर आवळ्याच्या उपचार वि. नियंत्रणासह संक्रमणाचे उच्चाटन लक्षणीय होते. आवळा आणि लिंबु उपचारात्मक पद्धतीमुळे २१ व्या दिवशी दुधाच्या कॅल्सिफोर्निया स्तनदाह चाचणीचा गुणांक , दुधातील सोमॅटिक पेशींची संख्या , विद्युतवहन क्षमतेत व दुधाच्या सामुत लक्षणीय घट दिसून आली . आवळ्याच्या उपचार पद्धतीमुळे ७ व्या दिवशी आणि २१ व्या दिवशी दुग्धशर्करामध्ये लक्षणीय वाढ झाली, परंतु दुधातील फॅट, एस.एन.एफ आणि प्रथिनांवर लक्षणीय प्रभाव दिसून आला नाही. लिंबुच्या उपचार पद्धतीमुळे ७ व्या दिवशी आणि २१व्या दिवशी दुधातील प्रथिने, फॅट, एस.एन.एफ आणि दुग्धशर्करामध्ये लक्षणीय वाढ झाली. आवळा आणि लिंबु उपचार पद्धतीमुळे २१ व्या दिवशी सरासरी न्यूट्रोफिलच्या टक्केवारीत लक्षणीय घट झाली आणि सरासरी लिम्फोसाइट टक्केवारीत लक्षणीय सुधारणा झाली.