Formulation, Optimization and Pharmacological Evaluation of Lipid Based Therapeutic System for Bovine Mastitis

Thesis

Submitted for fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES

BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY

LUCKNOW
प्रज्ञा शील करुणा
ESTABLISHED 1996

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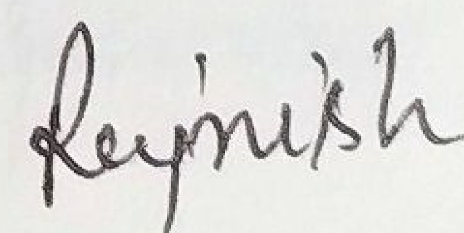
(2018)

DECLARATION

I hereby declare that the thesis entitled **"Formulation, Optimization and Pharmacological Evaluation of Lipid Based Therapeutic System for Bovine Mastitis"** has been prepared by me under the supervision of **Dr. Gaurav Kaithwas** at Department of Pharmaceutical Sciences, School for Biosciences and Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow (U.P.).

No part of this thesis has formed the basis for the award of my degree, diploma, or fellowship previously. I further declare that the material embodied in the present work is based on original research work and indebtedness to others has been duly acknowledged at relevant places. I hereby also declare that the thesis is essentially free from all kind of plagiarism.



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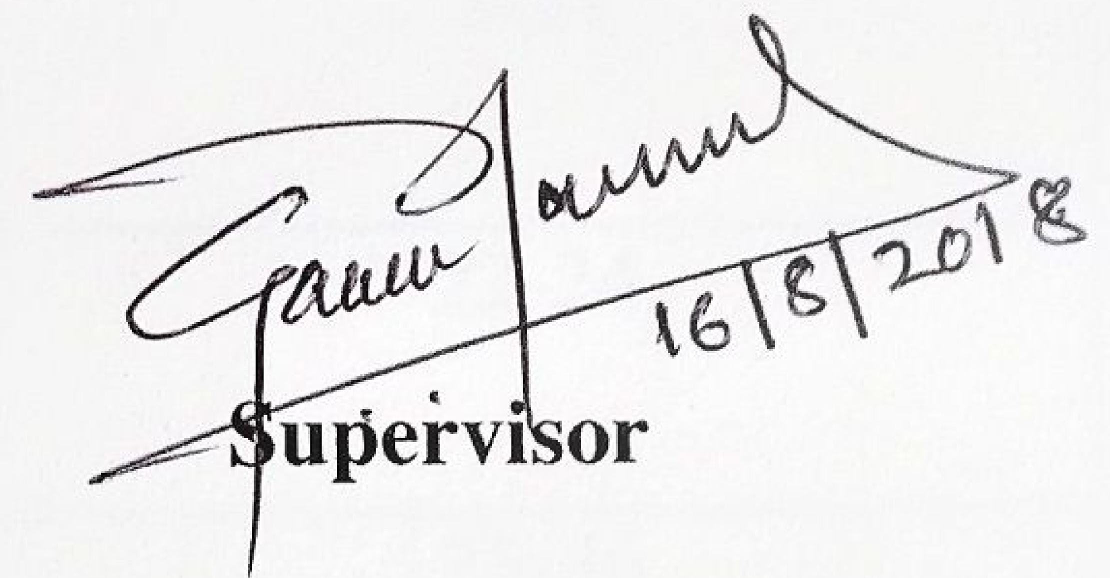

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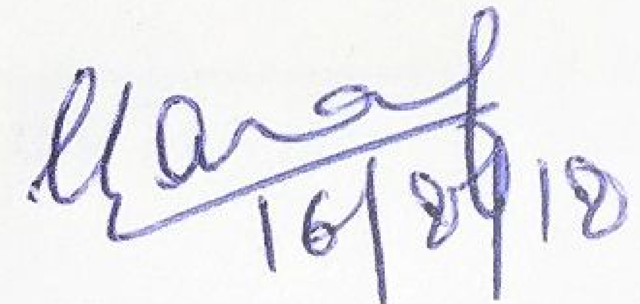

Head of the Department

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ACKNOWLEDGEMENT

I consider myself most lucky to work under the guidance of ***Dr. Gaurav Kaithwas, Associate Professor****, Babasaheb Bhimrao Ambedkar University, Lucknow (U.P) for his valuable support. His discipline, principles, simplicity, caring attitude, and provision of fearless work environment will be cherished in all walks of my life. I am very much grateful for his invaluable guidance and everlasting encouragement throughout my course.*

I express my heartily and humble thanks to ***Prof. Shubhini A. Saraf****, Professor, and Head of the Department, Babasaheb Bhimrao Ambedkar University, Lucknow (U.P) for making all facilities available, her timely help, encouragement, boosting my confidence in the progress of my dissertation work.*

I wish to offer my sincere thanks to ***Prof. R.C. Sobti****, Honourable Vice Chancellor of Babasaheb Bhimrao Ambedkar University, Lucknow for providing infrastructure facilities for carrying the project advices throughout my academic carrier.*

Besides my advisor, I would like to thank the rest of DRC committee members: ***Dr. V. Elangovan****,* ***Dr. N.K.S. More*** *and* ***Dr. Sudipta Saha*** *for their encouragement, insightful comments and meaningful quires.*

My sincere thanks also go out to Controller of examination and supportive staff for their guidance.

I also want to acknowledge the ***University Grant Commission****, Government of India for providing me fellowship to pursue the research work.*

I would like to express my sincere thanks to ***Dr. Sunil Goria*** *(Librarian),* ***Mr. O.P. Saini*** *(Assistant Librarian) BBAU for their kind support.*

*I must place on record very special thanks to my seniors and juniors, Mr. **Subhadeep Roy, Ms Manjari Singh, Ms. Swetlana, Mr. Lakhveer Singh, Mr. Jitendra Kumar Rawat** for their charming company, kind co-operation and encouragement throughout my doctoral study.*

*I wish to thank the staff members, **Mr. Ramesh, Mr. Anand Pandey, Mr. Amar** and **Mr. Bhandari** for providing all the needful official accessories.*

Finally, I acknowledge the people who mean a lot to me, my parents and my friends for showing faith in me and giving me liberty to choose what I desired. I wish to thank almighty God who keep his blessings on me every second and giving me the strength and patience to work through all these years so that today I can stand proudly with completion of my research work.

Rajnish Kumar Yadav

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List of Abbreviations

ALA- Alpha-linolenic acid

LA- Linoleic acid

IMI- Intramammary Infection

PMN- Poly-morphnuclear neutrophils

SCC- Somatic cell count

CMT- California mastitis test

TMC- Total microbial count

WST- White side test

NSIAD's- Non steroidal anti-inflammatory drugs

mMECs- Mouse mammary epithelial cells

Cox-2- Cyclooxygenase-2

iNOS- Inducible nitric oxide synthase

ERK- Extracellular signal-regulated protein kinase

JNK- c-Jun N-terminal kinase

NF-κB- nuclear factor-κB

LPS-Lipopolysaccharide

TNFα- Tumor necrosis factor

NEFA- Nonesterified fatty acids

BAEC-Bovine aortic endothelial cells

MIC- Minimum inhibitory concentration

PEG- Polyethylene glycol

PS- Particle size

ZP- Zeta potential

PDI-Polydispersity index

SV- Sedimenation volume

CFU- Colony-forming units

TBARs- Thiobarbituric acid reactive species

SOD- Superoxide dismutase

CAT-Catalase

GSH-Glutathione

PC-Protein carbonyl

NO-Nitric oxide

H_2S-Hydrogen sulphide

PVDF- Polyvinylidene difluoride

LOX-Lipoxygenase

HIF-1-Hyoxia inducible factor

PHD-2- Prolyl hydroxylase

FASN- Fatty acid synthase

SREBP-1c- Sterol regulatory element binding protein

BBT-Bromothymol blue test

CHAPTER-1

INTRODUCTION AND RESEARCH ENVISAGED

Introduction

Inflammation is characteristically defined as a response to microbial infection or cell/tissue injury. Consequently, the inflammatory circumstances are extremely heterogeneous in relations to the types of cells and molecular mediators convoluted in the mechanism. Inflammation also comes in diverse modalities that can be classified as acute/chronic inflammation and local/systemic inflammation. Likewise, microbial infection can cause acute/chronic and local/systemic inflammation on different type of tissues.

Mastitis

The term "mastitis" is imitated from the greek word “mastos” meaning breast and the suffix "itis" denotes inflammation. Thus, mastitis is illustrated as inflammation of the mammary gland (McDougall, 2002). According to international dairy federation, mastitis is an intra-mammary infection (IMI) of the parenchyma of mammary gland and that can be infectious, traumatic or toxic nature (International Dairy Federation, 1987).

Types of mastitis

Straightforward classification perceive mastitis as:

Clinical mastitis

Clinical mastitis is symbolized by the presence of inflammatory signs (swelling, heat, redness, and pain) along with anomalous texture and milk discoloration, flakes or clots in the milk, swelling, increased temperature and pain in the mammary gland (Philpot and Nickerson, 1991).

Sub-clinical mastitis

Sub-clinical mastitis is delineated by diversification in milk architecture with no signs of inflammation or visible milk abnormalities, but the somatic cell count (SCC) is

inflated with bacterial participation. Subclinical mastitis accounts the colossal financial loss to dairy farms through decreased milk production (Crist et al.1997). Nonetheless, diagnosis of the subclinical cases of mastitis has been an inquisitive task due to paucity of clinical correlation (Hiitiö et al., 2017).

Chronic mastitis

Chronic mastitis is an inflammatory proceeding that endures for months, and may prevail from one lactation to another lactation. Chronic mastitis, for the utmost part prevail as sub-clinical but may illustrate periodical flare ups. The execution of the diagnosis for clinical mastitis is analogously easy through clinical inter-relation(Boutet et al., 2003).

Pathophysiology of mastitis

Invasion of bacteria in the milk fertile tissues results in mastitis**.** Microorganisms causing mastitis can breach the udder by passing over the teat duct and augment inside the duct or by physical migration resulting from pressure placed on the teat end. The teat canal is the first line protective cover of the mammary gland. The keratin lining in the teat canal cater a physical and chemical impediment against microbial penetration (Capuco et al., 1992). Infection occurs when bacteria get admittance to the mammary gland and overthrow the anatomical defence. The main changes in the udder includes; increased permeability of the epithelial membrane leading to effusion of ions, proteins and enzymes from blood to milk along with invasion of phagocytic cells into the milk compartment and decrease in synthetic capacity of gland, resulting in tattered quality and quantity of milk (Korhonen et al., 1998).

Pathogenic invasion, commute diversified precipitation causing an influx of somatic cells, primarily polymorph nuclear neutrophils (PMN) into the mammary gland and upsurge in serum milk protease content (Zhao and Lacasse, 2008). During the early

phase of mastitis, about 90% leukocytes are constituted with neutrophils (Sordillo et al., 1987). Neutrophils exerts bactericidal activity by producing free radicals like oxygen and hydroxyl radicals. In addition, it is also a good source of defensins (antibacterial peptides) (Selsted et al., 1993). As a result of microbial infection in the mammary gland, the number of neutrophils leak into lumen of the alveoli from milk secreting cells resulting in increased leukocytes in milk. The upsurge in the number of leukocytes in milk is responsible for the increased number of SCC as well. This accumulation of leukocytes and blood clotting factors is accountable for the creation of clots, which may conceivably block the lacteal ducts and inhibit complete removal of milk, resulting in scar formation along with proliferation of connective tissue elements (Heinrichs et al., 2009).

Etiology

Penetration of pathogenic bacteria through the teat duct is common mode for IMI (Kennedy, 1993). The *Staphylococcus aureus, Streptococcus agalactiae,* coagulase negative Staphylococci species, *Escherichia coli,* Micrococcus species, Corynebacterium species, Bacillus species, Pasteurella species, Klebsiella species, Mycoplasma species and Nocardia species are the uttermost prevailing bacterial isolates pledged for mastitis (Anon, 2001). Notwithstanding, some viral infection like pseudocowpox, herpes mamillitis, cowpox, papilloma, foot and mouth disease and vesicular stomatitis affecting the epithelium of the teat orifice are quoted to result in or incline to mastitis (Mein et al., 2001).Compelling divergence is ascertained from country to country on the type of pathogens responsible for mastitis. Pathogens more ofently secluded from mastitis milk can be classified as contagious, environmental and opportunistic **(Table-1)**.

Table 1: Mastitis: infection, means of spread, and control measures

Bacteria	Source	Means of spread	Control measures
Contagious organisms			
Streptococcus agalactiae	Infected udders of other cows	Cow-to-cow, contaminated milking utensils	Dry teats with separate towels; teat dip; treat dry cows; use gloves
S*taphylococcus aureus*	Infected udder, contaminated milk	Cow-to-cow from contaminated udders, milking equipment	Dry teats with separate towels; teat dip; bedding etc.; treat dry cows; milking order; use gloves
Mycoplasma spp.	Various (inhabitant of respiratory tract, vagina, mucous membranes), infected udders	Cow-to cow from contaminated utensils/hands	No treatment. Use gloves/disinfectants. Between cows; milking order/clean clusters; teat dip; culling
Environmental organisms			
Non-agalactiae Streptococcus	Cow environment	Environment to cow by wet, dirty lots/bedding, milking wet cows, poor cow preparation,	Improve barn and lot sanitation; milk clean cows; avoid air leaks and liner slips; change, bedding frequently

		milking machine problems (reverse flow at teat end)	
Coliforms	Cow environment	Environment to cow by wet, dirty lots/bedding, milking wet cows, poor cow preparation, milking machine problems, teat injuries; hot humid weather	Improve barn and lot sanitation; milk clean, dry cows; keep cows standing 1-2 hours after milking; avoid air leaks and liner slips; change bedding frequently
S*taphylococcus* species	Normal inhabitants of skin, some bedding	Poor teat dip coverage, poor cow prep, soiled bedding	Teat dip; adequate cow prep; change bedding frequent

Contagious pathogens

Contagious bacteria disseminate from a cow with an infected udder to a healthy cow. Relocation of pathogenic bacteria between cows customarily transpire at milking time through hands, towels or the milking machine who acts as cistern for contagious bacteria. The organisms that fit into this category include: *Staphylococcus aureus* (coagulase positive staphylococci), *Streptococcus agalactiae* and the less common

causes of infection caused by *Corynebacterium bovis* and *Mycoplasma bovis* (Soltys and Quinn, 1999).

Additional mode of allocating the causative bacteria's is the major and minor pathogens. Pathogens which are culpable for more severe cases of mastitis are called major pathogens and the minor pathogens, are barely related with conspicuous leuckocytosis and other minor clinical manifestations (Rainard and Poutrel, 1988).

Environmental pathogens

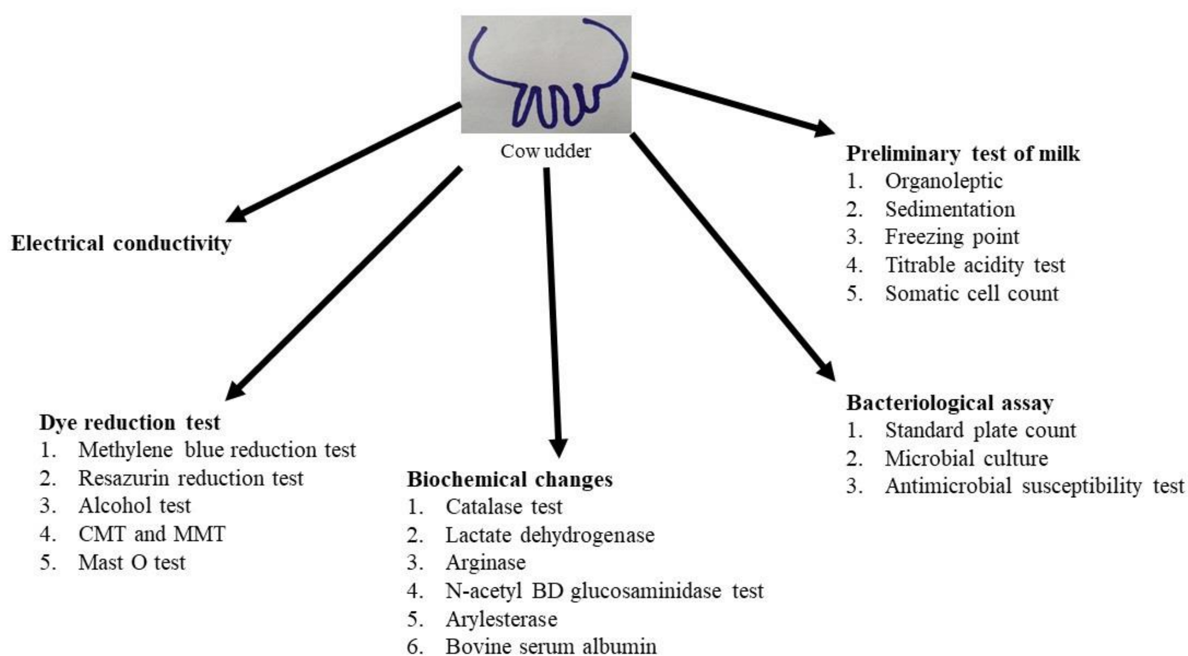
Mastitis caused by environmental organisms is essentially opportunistic in nature and becomes established if the immune system of the host is compromised or if sanitation and hygiene is not adequately practiced (Schukken et al., 2009).

Environmental pathogens are found in the immediate surroundings of the cow, such as the sawdust and bedding of housed cows, the manure of cattle, and the soil. Bacteria's include streptococcal strains other than *S. agalactiae,* such as *Streptococcus bovis*, *S. dysgalactiae*, *S. uberis, Enterococcus faecium* and *Enterococcus faecalis.* Other coliform bacteria such as *E. coli*, *Klebsiella pneumonia* and *Enterobacter aerogenes* are responsible for the infection (Schroeder, 2009).

Diagnosis

Tests more ofently engaged to regulate the trait of milk include dye-reduction (methylene blue reduction and resazurine reduction), alcohol test, standard plate count, coliform count, SCC, titrable acidity, and phosphatase tests. Scrutinizing the subclinical mastitis is a challenge due to lack of clinical interrelationship. Extensive number of field and laboratory based tests are available to detect the clinical and subclinical cases of bovine mastitis **(Figure-1)**.

Figure-1: Methods and test for detection of mastitis



The SCC and California mastitis test (CMT) are an important diagnostic tests for the identification of mastitis. The SCC is internationally perceived as a framework for appraising milk quality and udder heath (Loeffler et al., 1999). SCC is an unremarkable method to insure a high quality product. SCC levels are evaluated to ensure conformity with set milk quality standards. Today, utmost markets in developed countries pay a premium for low SCC, good quality milk. One can acknowledge the reasons, for paying a bonus for quality milk when the affiliation between mastitis (high SCC) and milk composition is presumed.

Somatic cells encompasses of white blood cells (WBC) and occasionally sloughed epithelial cells. Cells found in normal cattle milk from uninfected glands include neutrophils (1.1%), macrophages (66.68%), lymphocytes (10-27%) and epithelial cells (0-7%) (Taponen et al., 2006). When bacteria breaches and colonizes the mammary gland, the inflammatory response is initiated. SCC can be computed by a direct microscopic method on stained milk smears or by using automated devices. Fossomatic

cell counter is the more ofently used programmed device for exemplifying the SCC. The instrument stains cells with a fluorescent dye and then counts the number of fluorescent particles. A legitimate perceptible appraisal of udder health status is available through SCC data (Dohoo and Meek, 1982); (Radostits et al., 1994)**.** However, it is affirmed that SCC is a general indicator of udder health, subject to many factors including age, stage of lactation, season, stress, and management. Mean SCC decreases conspicuously soon after the commencement of lactation and increases during late lactation. The elemental pattern of changes over lactation remains the same in healthy or mastitic cows (Auldist and Hubble, 1998). Notwithstanding, it is pleaded that a conspicuous upsurge in SCC is a result of cells being captivated to the mammary tissue by virtue of direct mediators produced during a local infection (Harmon, 1994). To institute the specificity and sensitivity of SCC (four quarters, cow or bulk milk); manifold studies have been conducted. Larsen *et al*., proclaimed sensitivity ranging from 73-89% with corresponding specifications of 75-85% using a threshold of 200,000 cells/ml taking culture as "gold standard"(Larsen et al., 2002). Sensitivity and specificity are overwhelmed by threshold (cut point of intramammary infection). Emanuelson divulged a threshold of 200,000 cell/ml for the cow level to monitor herd mastitis in Sweden (Emanuelson, 1999). Dohoo and Meek reported 250,000 and 300, 000 cells /ml threshold to quarter and cow, proportionately (Dohoo and Meek, 1982). The inception for milk quality have no affiliation to the rationally of udder health categorization. At present a threshold of 100,000 cells/ml can be pretended an internationally endorsed definition of udder health (Hamann and Krömker, 1997).

CMT is one of the uttermost trust-worthy screening test for subclinical mastitis that can be easily used at the herd side. The CMT was developed to test milk from individual quarters, but also being used on composite and bulk milk samples. CMT is based upon

the chemical reaction between reagent and cellular DNA. Innumerable studies conceded that the SCC levels vary greatly, reaching a count up to 1.5 $x10^6$ cells / ml in a healthy udder. A CMT score of 1 is endorsed for the subclinical mastitis diagnosis, and the maximum score (+++) is advocated for the diagnosis of infectious mastitis (Pradieé et al., 2012).

The CMT involves mixing and swirling equal parts of bromocresol green reagent and milk in a plastic paddle with a compartment for each quarter (Lafi, 2006)**.** The test results are interpreted introspectively as either a negative, trace, 1+, 2+ or 3+ based on the viscosity of the gel formed by mixing the reagent with milk (Radostits et al., 1994). The degree of gel formation precisely corresponds to numbers of leukocytes present during mammary gland inflammation, which is characteristic feature of mastitis. Higher CMT score bank upon the gel formation (Kaithwas and Majumdar, 2010).

Fresh unrefrigerated milk can be tested using the CMT for up to 12 hrs. Reliable readings can be retrieved from refrigerated milk for up to 36 hrs. If stored milk is used, the milk must be comprehensively blend prior testing because the somatic cells tend to single out with milk fat. The CMT reaction must be scored within 15 sec of mixing because weak reactions will dissipate after that time. The degree of reaction between the detergent and the DNA of nuclei is a measure of the numbers of somatic cells in milk. The threshold for CMT score depends on the aspiration of the study. If it is used to downplay the rate of false negatives, the test should be read as negative versus positive with trace scores regarded as positive.

Economic loss

Mastitis is curse for dairy industry as it decreases the productivity and quality of milk, and increase the cost of herd management**.** In India, a recent study reported that annual economic loss due to mastitis was estimated approximately Rs. 7165.51 crores out of

which around Rs. 4151.16 crores has been credited to subclinical mastitis (Banal and Gupta, 2009).

Treatment strategy

Mastitis results in the destruction and disturbances of the mammary gland and affects milk production and productivity. Among the many actions that could be taken as treatment, the administration of antimicrobial agents is the most commonly used strategy. Pathogenic microorganisms are sensitive to one or more antimicrobial agents and at the same time are resistant to one or several conventional drugs (Tiwari et al., 2013).

The *in vitro* antibiogram studies of the bacterial isolates from mastitis milk revealed gentamicin to be most effective drug followed by enrofloxacin, ciprofloxacin, chloramphenicol, tetracycline, colistin, neomycin, cephalexin (Sumathi et al., 2008).The major obstacle in treating mastitis is antibiotic resistance. As per the reports by Tarfarosh et al (2007) efficacy of cloxacillin has decreased in the subsequent years from 1997-98 to 2002-03. Chloramphenicol, gentamycin, kanamicin, ciprofloxacin were highly to moderately sensitive but resistance was encountered with cloxacillin, ampicillin, erythromycin and norfloxacillin.

Followed by the antibiotic therapy, the anti-inflammatory drugs are also considered to treat acute clinical cases of mastitis. Most of the drugs currently in use, inhibit the metabolism of arachidonic acid (AA), thus synthesis of inflammatory eicosanoids. AA is an integral part of cell membrane of mammalian hosts and is precursor for eicosanoids synthesis by action of enzyme cyclooxygenase (COX) and lipoxygenase (LOX). NSAID's inhibit the AA metabolism, thus eicosanoids synthesis. NSAIDs have been used by dairy practitioners for the treatment of acute mastitis (DeGraves and Anderson, 1993). Currently, antibiotics either alone or in combination with NSAIDs are

most commonly prescribed for clinical management of bovine mastitis. However, long term use of antibiotics causes bacterial resistance and has negative impact on consumer health (Li et al., 2013). Therefore, alternative safer drugs with universal effectiveness, lasting benefits and fewer side effects is requisite in the area of mastitis management.

Polyunsaturated fatty acids (PUFAs)

Omega three (ω-3) fatty acids are probably one of the best examples of how diet may affect inflammation. ω- 3 fats exerts a remarkable variety of biological responses including inflammation and related clinical conditions (Yadav et al., 2018). α-Linolenic acid (ALA; ω- 3, 18:3) is a ω-3 (PUFA) and is transformed to class 3 and 5 eicosanoids through series of desaturation and elongation reactions. ω-3 fatty acids have a variety of anti-inflammatory and immune-modulating activity that may be of significance to atherosclerosis, arthritis, cancer, diabetes, liver and kidney inflammation. The other ω-3 fatty acids that appear to be most potent in the inflammatory conditions can be obtained from marine sources namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). A range of biologic activities of EPA and DHA have been validated using fish or fish oil supplements in humans and animals. These include effects on oxidative stress, pro and anti-inflammatory cytokines, and immune responses.

Previous study has affirmed the relationship between ALA (ω- 3, 18:3) rich flaxseed oil supplementation and anti-inflammatory effects (Anand and Kaithwas, 2014, Kaithwas and Majumdar, 2013). Moreover, a study also demonstrated *in-silico* and *in-vitro* inhibitory action of ALA (ω-3:18-3) against COX and LOX enzymes. ALA (ω-3:18-3) exhibited noteworthy *in-silico* binding efficacy towards COX and LOX enzymes. Flaxseed oil was additionally reported to have antiulcer and cytoprotective properties, which were attributed to inhibition of histaminergic receptors along with restoration of

sialic acid and collagen content in the esophageal tissue due to high amount of ω-3 fatty acids, in particular ALA (ω- 3, 18:3). It showed noteworthy down-regulation of tumour necrosis factor (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein (MCP-1), interferon-γ (INF-γ), and nuclear factor kappa-B (NF-κB); recommending that it diminishes the pro-inflammatory cytokines and vascular infiltration in rats. Many researchers additionally recommended flaxseed oil for protection against intestinal toxicity, hepato, and nephrotoxicity because of the presence of ω-3 fatty acids, in particular, ALA (ω-3:18-3).

Literature Review

Targeting treatment towards specific pathogens when possible is considered necessary for rational antimicrobial treatment of mastitis, as should be the case in all treatment of bacterial infections. Efficacy of supportive treatment such as non-steroidal anti-inflammatory agents, frequent milking, and fluid therapy in mastitis has been studied, in combination with antibiotic treatment. Use of NSAIDs has been shown to be beneficial at least in clinical mastitis. Herbal or homeopathic remedies have not been found to be effective for mastitis treatment in several independent and scientific studies. Some of the recent studies are enumerated below:

Rezamand et al. 2016: They reported that ALA (ω-3, 18:3) based diet on gene expression of systemic (blood) and local (mammary gland) inflammatory markers in Holstein dairy cows. Expression of pro-inflammatory (TNF-α) was linearly reduced (up to 40%) as ALA (ω-3, 18:3) increased in cow diet. Expression of other cytokines IL-1β, and IL-8 was also reduced after treatment.

Riefen et al. 2015: They investigated the effect of ALA (ω-3, 18:3) isolated from two different plant and animal sources on inflammatory bowel disease on rat models. They showed that colitic rats fed the sage oil diets had a decrease inflammatory response, better histological repair, and decrease necrotic damage in the colonic mucosa when compared to fish oil groups. IL-6, COX-2 and TNF-α were significantly decreased in rats fed fish and sage oils compared to control.

Kaithwas et al. 2014: They validated the anti-inflammatory activity of ALA (ω-3, 18:3) and linoleic acid (LA) using computational and experimental analysis. The binding affinity of ALA (ω-3, 18:3) was found better than LA for COX-1, COX-2, and 5-LOX. Furthermore, ALA (ω-3, 18:3) and LA showed good anti-inflammatory activity against different phologistic agents induced inflammation in rats.

Liang et al. 2014: They studied the anti-inflammatory effect of thymol in lipopolysaccharides (LPS) stimulated mouse mammary epithelial cells (mMECs). Study revealed that LPS increased the expression of COX-2, inducible nitric oxide synthase (iNOS), extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), NF-κB in mammary epithelial cells.

Arm et al. 2013: They studied the influence of botanical oils on PUFA metabolism and leukotriene generation in mild asthmatics and observed that Dietary supplementation with botanical oils that contain ω- 3 and ω- 6 fatty acids like eighteen carbon chain (18C)-PUFA such as γ linolenic acid (GLA, 18:3 n-6), stearidonic acid (SDA, 18:4n-3) ALA, (ω- 3, 18:3) have been shown to impact PUFA metabolism, alter inflammatory processes including AA metabolism and improve inflammatory disorders.

Cha et al, 2013: They investigated that the effect of a first and repeated cases of bacteria-specific clinical mastitis on the risk of mortality and culling in Holstein dairy cows. The pathogens they studied were *Streptococcus spp., Staphylococcus aureus, Staphylococcus spp., Escherichia coli, Klebsiella spp*.

Chen el al. 2013: They investigated the effect of alpinetin against LPS-induced mastitis in female rats and to elucidate the possible mechanism beneath. They observed that LPS increased the level of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6; with subsequent up regulation of the phosphorylation of IκB-α, NF-κB p65 and the expression of toll like receptor (TLR4).

Hanke et al. 2013: They observed that dietary administration of plant-based ω- 3 fatty acid, (ALA ω- 3, 18:3) modulating hepatic steatosis in rats. It also decreased the inflammation and oxidative stress markers in liver.

Williamson and Lacy-Hullbert; 2013: They determined the effects of disinfectant after pre-milking and post milking on the occurrence of new IMI, SCC and teat skin irregularities in cows. They reported that post-milking teat disinfection of cow had a decrease occurrence of new infection cases caused by *Staphylococcus aureus, Streptococcus uberis, Corynebacterium spp and coagulase negative staphylococci*, had decreased milk SCC during lactation, and had fewer teat skin irregularities compared with the non-disinfected cows ($p < 0.05$). Pre-milking teat disinfection, in addition to post-milking teat disinfection, did not decrease the incidence of new IMI for any pathogens and did not reduce SCC ($p > 0.05$).

Contreras et al, 2012: Recently, Contreras and his colleagues investigated the effect of nonesterified fatty acids (NEFA) on bovine aortic endothelial cells (BAEC). Treatment with NEFA mixtures altered the fatty acid profile of BAEC by increasing the concentration of stearic acid (C18:0) and decreasing the content of arachidonic acid (C20:4n) and other long-chain PUFAs in the phospholipid fraction. A significant decrease occurred in mRNA expression of cytokine and adhesion molecules that are associated with increased inflammatory responses during the transition period.

Wang et al, 2012: This study reports the antibacterial activity of tilmicosin by solid lipid nanoparticles (SLN) of tilmicosin loaded hydrogenated castor oil. Tilmicosin-SLN showed a sustained-release effect and enhanced the antibacterial activity in vitro. SLN significantly enhanced the therapeutic efficacy of tilmicosin determined by lower colony forming unit (CFU) counts and a decreased degree of inflammation.

Baravalle et al, 2011: The study enumerates the effects of a single intramammary infusion of *Panax ginseng* extract on the amount of pro-inflammatory cytokines and the number of monocytes/macrophages present in bovine mammary tissues at drying off. These results indicate the immunomodulator potential of the *Panax ginseng* extract.

Kaithwas et al, 2011: *In-vitro* antimicrobial activity of *L. usitatissimum* fixed oil against a number of microorganisms by disc diffusion method and minimum inhibitory concentration (MIC) determination was reported along with the efficacy against mastitis-affected cows. The animals were subjected to once-a-day intramammary infusion of oil, cefoperazone or an oil-cefoperazone combination for 7 days and by monitoring the CMT score, SCC and microbial count in milk samples. The results suggest possible therapeutic potential of *L. usitatissimum* fixed oil in bovine mastitis.

Mukherjee et al, 2010: SCC, total microbial count (TMC), phagocytic activity, and leukocyte lysosomal enzymes like myeloperoxidase and acid phosphatase activity and IL-8 level were evaluated after intramammary infusion of hydro-methanolic extract (stem) of *T. cordifolia* in diseased cows. The results suggest that the hydro-methanolic extract of *T. cordifolia* (stem) possesses antibacterial and immunomodulatory properties.

De and Mukherjee, 2009: The immuno therapeutic potential of hydromethanolic extract of *Azadirachta indica* was reported against bovine clinical mastitis. The results of the study indicate anti inflammatory, antibacterial and immunomodulatory potential of the herb, these activities could be due to the presence of bioactive principle in the extract.

Research Envisaged

ω-3 fats are probably one of the best examples of how diet may affect inflammation. ω-3 fats exert a remarkable variety of biological responses many of which affect inflammation and clinical conditions related to its presence. ALA (ω- 3, 18:3) is a ω- 3 PUFA and is transformed into anti-inflammatory eicosanoids through series of desaturation and elongation reactions **(Figure 2)**. Studies have also confirmed the relationship between ALA (ω- 3, 18:3) supplementation and anti-inflammatory effect (Paschos et al., 2004). It would be interesting to mention that the flaxseed oil, containing 57.38% of ALA (ω- 3, 18:3) has been found to exhibit anti-inflammatory and anti-arthritic activity. The same has been reported to exhibit significant antimicrobial activity against mastitis causing pathogens and efficacy against subclinical cases of bovine mastitis in a field trial (Kaithwas and Majumdar, 2010). The study reports that a 7-day intramammary fixed oil (2.5 ml) therapy, gives a cure rate comparable to that of the antimicrobial therapy of cefoperazone (a moderately beta-lactamase resistant cephalosporin). It is noteworthy that the flaxseed oil was already reported to have an anti-inflammatory effect in addition to antimicrobial property, which could be attributed towards the efficacy of the oil in bovine mastitis. This suggests that ALA (ω- 3, 18:3) could be an ideal candidate for its therapeutic evaluation against bovine mastitis. The efficacy and potency of ALA (ω- 3, 18:3) against bovine mastitis may be further strengthened by incorporating an antimicrobial agent to the ALA (ω- 3, 18:3) therapy and the same has been proposed in the present work.

The antibacterial, anti-inflammatory, and immunomodulatory activities of ALA (ω- 3, 18:3) facilitate the possibility of developing a therapeutic formulation with ALA (ω- 3, 18:3) as complimentary / alternate therapy to mastitis. ALA (ω- 3, 18:3) being a naturally derived product has remote chances for the development of resistance in

bacteria; in addition ALA (ω- 3, 18:3) has no known undesirable or unwanted effects as an anti-inflammatory agent. The present study is aimed to explore ALA (ω- 3, 18:3) as complimentary therapeutic agents and designed to provide a ready to use intra-mammary formulation (pre filled syringes) of ALA (ω- 3, 18:3) and cefotaxime nano-suspension for use against subclinical bovine mastitis. The present work is also undertaken to provide a ready to use intra-mammary therapeutic system of ALA (ω- 3, 18:3) and cefotaxime for treatment of clinical and subclinical cases of mastitis.

PUFA's get oxidized in three ways; firstly, free radical-mediated pathway, secondly non-free radical non-enzymatic metabolism, and lastly enzymatic degradation. The cellular components are surrounded by lipids, proteins, and carbohydrates that maintains the integrity and functions in a cell. Lipids are an easy target for attack by free radicals, leading cellular dysfunction. The generation of free radicals under certain circumstances initiate the free radical based lipid peroxidation mechanism. Free radicals-mediated lipid peroxidation can be elaborated with three steps, initiation, propagation, and termination. The free radical lipid peroxidation is responsible for the generation of to aldehyde mixture, short-chain fatty acids, keto, hydroxy or epoxy compounds; among which 4-hydroxy- 2-hexenal (HHE) (from ω-3) and 4 hydroxy-2-nonenal (HNE) (from ω-6) are

considered to be of prime importance. The autoxidation of HHE and HNE further replenishes short-chain fatty acids like malondialdehyde (MDA). The MDA can form adduct with amino acids and proteins, thereby producing a profound change in their biochemical behaviour. The HHE, HNE, and MDA are nowadays recognized as secondary markers for lipid peroxidation. HHE initiates numerous cytotoxic mechanisms within the cell. As reported previously, HHE treatment increased the expression of pro-apoptotic Bax, and decreased the expression of Bcl-2 protein in

YPEN-1 epithelial cells. It can also modulate the reactive oxygen species (ROS) system and peroxy-nitrite production. Furthermore, HHE alters the inflammatory pathway mediated by NF-κB with higher expression of nuclear p65 protein and decrease in cytosolic IκBα. HNE exerts its effects on the biological system through covalent modification of proteins, DNA, and phospholipids. It has been ascertained that LA and AA on the cellular membrane are more susceptible for production of HNE, due to breakdown of hydro-peroxide of these PUFA's at the sn-2 position of glycerolphospholipids.

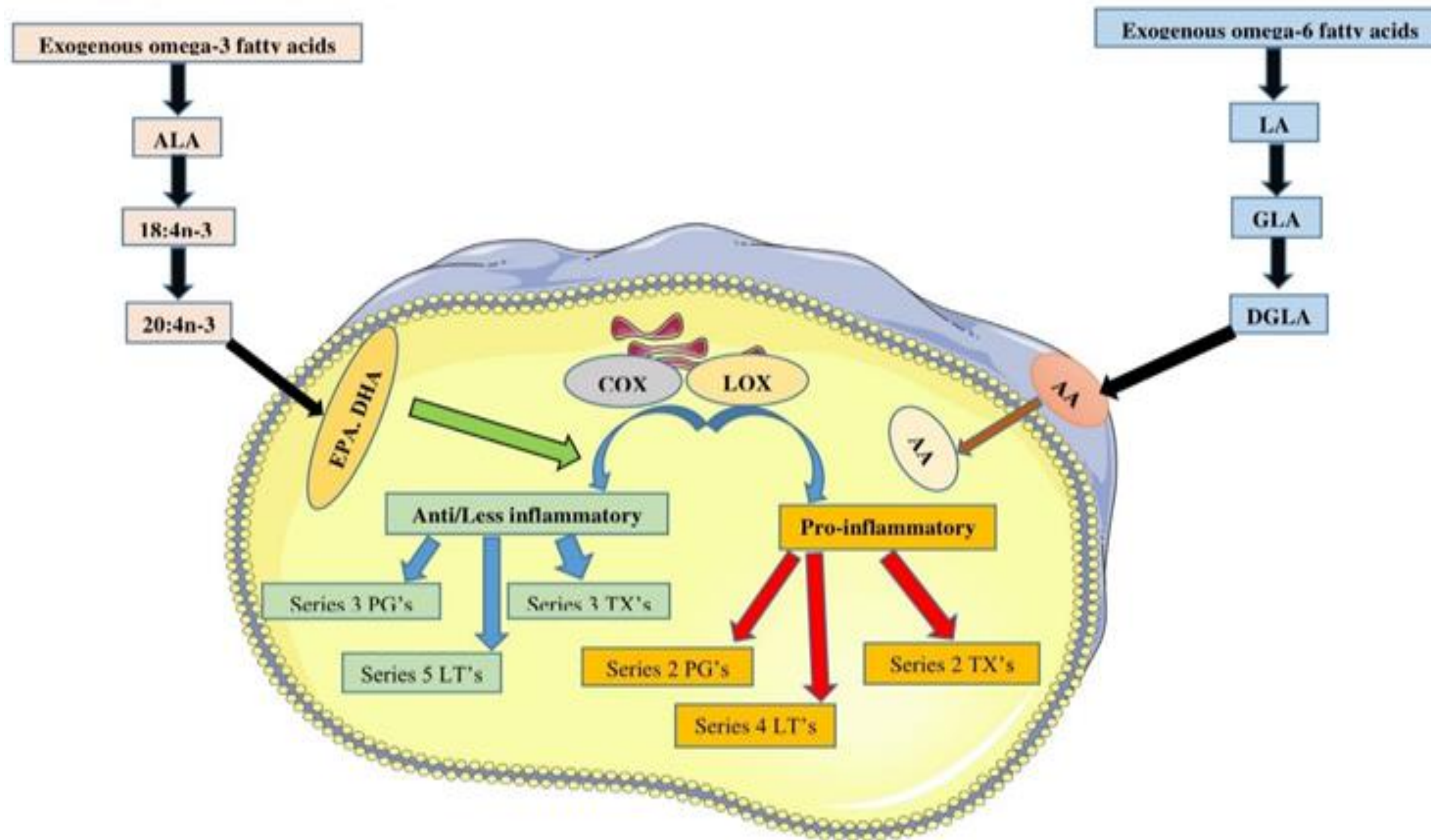
The non-enzymatic free radical-mediated autoxidation of PUFA's can likewise, create an extensive variety of cyclic peroxides and endoperoxides. Autoxidation of lipids forms the F1-phytosterone and F2-isoprostane. Researchers reported F1-phytoprostanes as immune-modulators and partial inhibitors of cytokine production by T helper cells type-1 (Th1) and type-2 (Th2). F2-isoprostanes level increases in oxidative damage of lipids and can be used as oxidative stress biomarker. Furthermore, studies also suggested the role of isoprostanes in obesity by directly correlating with body mass index, waist circumference, visceral fat area, and percent body fat.

ω-3 [ALA (ω-3:18-3)] and ω-6 (LA) fatty acids are metabolized by the same set of enzymes, but end products have contrasted biological activities. ALA/LA is metabolized to form EPA/AA. EPA is metabolized by COX and LOX to form eicosanoids, 3 series of prostaglandins (PGs), and 5 series of leukotrienes (LTs); whereas AA metabolism leads to the formation of 2 series of PGs and 4 series of LTs. Apart from eicosanoid production, metabolism of EPA generates biologically active compounds like E1 series resolvins, lipoxins, and hypoxins.

Considering the same the ω-3 fatty acids has gained the reputation to exert the anti-inflammatory action through dual inhibition of AA metabolism.

After elaborating all the possible mechanisms for peroxidation and biological activities of PUFA's, one could clearly say that the basic chemical structures of the byproducts of ω-3/ω-6 fatty acids are almost similar, but they perceive pro/anti-inflammatory and pro/antioxidant activity. The cell membrane phospholipids are majorly ω-6 fatty acids (AA). Henceforth, we hypothesize that, exogenous supplementation of either ω-3 (ALA) or ω-6 (LA) fatty acids render themselves as a target for the enzymatic, non-enzymatic and free radical mediated metabolism in comparison to the membrane bound AA and works as a cushion for the AA stored in the cell membrane **(Figure-2)**

Figure-2: Comparison between ω-3 and ω-6 fatty acids in cells.



CHAPTER-2
AIM AND OBJECTIVES

Aim and objectives

Aim: Formulation, Optimization and Pharmacological Evaluation of Lipid Based Therapeutic System for Bovine mastitis

Objectives

1. To formulate a ready to use intra-mammary lipid based therapeutic system using a polyunsaturated fatty acid and an antimicrobial agent.
2. Evaluation of *in-vitro* antibacterial activity of formulation against mastitis causing pathogens.
3. Evaluation of *in-vivo* efficacy of the formulation using lipopolysaccharides (LPS) induced mastitis in rat.
4. To evaluate the efficacy of the formulation against field based trial on the lactating cow suffering from subclinical mastitis.

Plan of work

1. Formulation of ALA based nano-suspension (ALA-NS).

    Particle size (PS)

    Polydispersity index (PDI)

    Zeta potential (ZP)

    Sedimentation volume (SV)

    Sterility test.

    Stability study

2. Antibacterial activity of ALA, cefotaxime, and ALA-NS on mastitis causing bacteria by using disc diffusion method and MIC.
3. *In-vivo* study of ALA-NS on LPS induced mastitis in rats.

Biochemical study

Superoxide dismutase (SOD)

Catalase,

Glutathione (GSH)

Thiobarbituric acids reactive species (TBARs)

Nitric oxide (NO),

Hydrogen sulphide (H_2S)

Histopathology study

Haematoxylin and Eosin (H&E) staining

Western blot analysis

Inflammatory markers [COX, LOX, NFkBp65, IFN-γ, ubiquitin carboxy-terminal hydrolase-L1 (UCHL-1)]

Hypoxia markers [sterol regulatory element binding protein (SREBP-1c, fatty acids synthase (FASN), hypoxia inducible factor (HIF-1), prolylhydroxylase-2 (PHD2)]

Apoptotic proteins (BAX, BAD, Bcl-2, Bcl-xl, Vdac, Apaf-1, cytochrome-c)

Serum Caspase-3 and 8.

Field based study of ALA-NS on mastitic cow

Field based test for confirmation of mastitis

Milk pH

Whiteside test (WST)

Bromothymol blue test (BBT)

CMT

TMC and SCC

Western blot analysis of milk cell

Inflammatory marker (NFkBp65), Fatty acids marker (SREBP-1c), Multidrug resistance marker (UCHL-1)

Result compilation and statistical analysis

CHAPTER-3

MATERIALS AND METHODS

Materials

Drugs and chemicals

ALA (463-40-1) was purchased from TCI Chemicals (India) Pvt. Ltd. Muller Hinton Agar purchased from Hi-Media, Mumbai., *E. coli* (LPS, O55:B5) was purchased from Sigma (St Louis, MO, USA), diluted in sterile pyrogen-free physiological saline, and adjusted to a concentration of 0.1 mg/mL, hematoxylin (Himedia, S058); eosin (Himedia, S007) (H&E); radioimmunoprecipitation assay (RIPA) lysis buffer (Amresco, N653); protein assay kit (Amresco, M173); bovine serum albumin (BSA) (Genetix, PG-2330); transfer buffer (Genetix, GX- 9411AR), caspase-3 (SC-4263) and caspase 8 (SC-4267) assay kits were procured from Santa Cruz Biotechnology Inc., California, Delaware. All others chemicals were of molecular biology grade and purchased from Genetix Biotech Asia Pvt. Ltd, New Delhi.

Equipment used

Table-2: Lists of equipment used

Sr. No.	Equipment	Manufacturer and model
1.	Homogenizer	Remi, Mumbai RQT-127A
2.	Weighting Balance	Sartorius, Mumbai BSA2245S-CW
3.	pH meter	Labman Scientific Instruments, Lucknow
4.	Vortex shaker	Remi, Mumbai CM101
5.	Cooling centrifuge	Eppendorf India Limited, Chennai 5418R
6.	Micropipette	Genetix Biotech Asia Pvt Ltd, New Delhi
7.	Refrigerator	Godrej India
8.	Deep freezer	Celfrost, BFS150, Lucknow

9.	Microvolume spectrophotometer	Agilent Technologies Mumbai
10.	Microplate reader	Bio-Rad Laboratories Inc, Model 680XR
11.	SDS-PAGE	Genetix Biotech Asia Pvt Ltd, New Delhi, GX-SCZ2
12.	Semidry transfer Unit	GY-ZY3, Genetix Biotech Asia Pvt Ltd, New Delhi

Methods

Formulation study

Solubility studies

The solubility of cefotaxime was determined by adding an excess amount of drug in phosphate buffer pH 6.8 and pH 7.4. The flasks were kept on a water bath shaker for 72 hrs at 37^0C. After 72 hrs, solutions were filtered through 0.45 µm membrane filter and aliquots were suitably diluted for estimation of cefotaxime spectrophotometrically at 249 nm.

Preparation of ALA-NS

ALA-NS was formulated using ALA (ω- 3, 18:3) as oil base and cefotaxime as model drug. The cefotaxime (10 mg) was suspended in tween-80 (350-750 µl) and polyethylene glycol (PEG-400) (100-450 µl). Subsequently, we added the ALA (ω- 3, 18:3) (750-1450 µl) in the mixture with continuous sonication. The formulation were prepared, evaluated, and optimized for the study.

PS, ZP, and PDI analysis

The PS, ZP, and PDI of the optimized formulations were observed using a Malvern particle size analyzer (Mastersizer 2000, UK). The particle size distribution of the

various batches were compared at D value of 0.9. Succeeding to the observation, the instrument was calibrated using latex standard to confirm the accuracy

Sedimentation study of optimized formulation

In sedimentation study, the suspension was transferred to a stoppered measuring cylinder and was stored at temperature (27±1^{0}C) for 72 hrs. The volume of sediment formed was noted at regular interval of time. The SV was calculated as the ratio of ultimate height (Hu) of the sediment to the final height (Ho) of the suspension.

Sterility test of formulation

Sterility test of formulation was performed using direct inoculation method with slight modification (Bharathi et al., 2007). Transfer the preparation (10 ml) directly into the culture medium fluid thioglycollate medium (100 ml) and incubated at 35^{0}C for 14 days. Then observed the microbial growth.

Stability study

Stability studies for ALA-NS conducted at different accelerating conditions 25^{0}C/60%, 30^{0}C/65% and 40^{0}C/75% for 3 months. Each one batch of ALA-NS were used for each storage condition. At periodic time intervals, the samples were withdrawn and analysed for PS.

Antimicrobial activity

Zone of inhibition

The antimicrobial activity of the cefotaxime, ALA (ω- 3, 18:3), and ALA-NS (F1 and F2) was evaluated against different microbial strains e.g., *Staphylococcus aureus* (ATCC-29737), *Streptococcus agalactiae* (ATCC-13813), *Staphylococcus epidermidis* (ATCC-12228), *Escherichia coli* (MTCC-118, ATCC-8739), *Lactobacillus sporogenes* (ATCC-31284), *Pseudomonas aeruginosa* (ATCC-25619) and *Candida albicans*

(ATCC-10231). Microorganisms were conserved on Muller Hinton agar slants. Antimicrobial activity was appraised by using paper disc diffusion method. Slants of microorganisms were wash away with 3 ml of sterile saline followed by centrifugation. The supernatant was pour out and the same process repeated thrice. After that, the cells were re-suspended in 3 ml sterile saline, and 0.1 ml of cell suspension was used to inoculate 100 ml of sterile Muller Hinton Agar culture media. Inoculated culture media (20 ml) was transferred into a sterile petri dish and settled to solidify. A sterile Whatman paper disc of 4 mm diameter was soaked in the cefotaxime (50 µg/ml), ALA (ω- 3, 18:3), and ALA-NS (F1 and F2). The petri dish was incubated at 37^0C. Zone of inhibition was measured in each petri dish after 24 hrs (Delignette-Muller and Flandrois, 1994).

MIC of formulation

The MIC of cefotaxime, ALA (ω- 3, 18:3), and ALA-NS (F1 and F2) was determined against all microbial strains. The cefotaxime was incorporated in molten media to a final concentration of 0.1, 0.2, and 0.3% (v/v). The medium was then shaken for 20 sec and cooled under running cold water with rotation until the temperature reaches about 45^0C. The resultant dispersion (20 ml) was poured aseptically into 9 cm sterile petri dishes. The petri dishes were dried for 3 hrs at 37^0C and then inoculated with microbial cultures. The inoculum cell suspension was prepared in sterile normal saline in such a way that 1 ml inoculum on plating into media gave 100 CFU. Plates were incubated at 37^0C for 24 hrs. The same procedure were also repeated for ALA (ω- 3, 18:3) and ALA-NS (F1 and F2). The MIC was defined as the lowest concentration of fixed oil required to inhibit the growth of 95% of the inoculated organism after incubation at 37^0C for 24 hrs (Singh et al., 2005).

LPS induced mastitis in rats

Animal

Male and female albino Wistar rats procured from the animal house were allowed for mating. Pregnant female (170-220 gm) was kept in individual breeding cages under the standard condition of temperature (25±1^{0}C) with 12 hrs light / 12 hrs dark cycle and had a free access to commercial diet and pellets *ad libitum*. All the experiments were performed according to the CPCSEA guidelines for laboratory Animals and Ethics, Department of Animal welfare, Government of India. Approval no. (SDCOP&VS/AH/CPCSEA/01/0039).

Experimental design

Total 30 animals were selected for the experiment after parturition and randomly divided in to the five groups (n=6). The group I (sham control, 0.9% normal saline, 3 ml/kg, L4 and R4 intra-mammary); group II (toxic control, LPS 10 µg/100 µl, L4 and R4 intra-mammary); group III (Cefotaxime 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary), group IV (ALA-NS; F-1, 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary); group V (ALA-NS; F-2, 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary). Seventy two hours after parturition, mastitis was induced by infusing with 10 µg LPS (dissolved in 100 µl physiology saline) in to the duct of inguinal left fourth glands (L4) and right fourth glands (R4) mammary glands followed by treatment with cefotaxime (group III) and ALA-NS (F1 and F2; group IV and V) for 3 days.

Table 3- Experimental design for the LPS induced mastitis in rat

Sr. No.	Groups	Treatment schedule
1	I (control)	0.9% normal saline, 3 ml/kg, L4 and R4 intra-mammary) (n=6)
2	II (Toxic control)	LPS 10 μg/100 μl, L4 and R4 intra-mammary (n=6)
3	III (Standard)	Cefotaxime 50 μl + LPS 10 μg/100 μl, L4 and R4 intra-mammary (n=6)
4	IV (F1)	Formulation-1, 50 μl + LPS 10 μg/100 μl, L4 and R4 intra-mammary (n=6)
5	V (F2)	Formulation-2, 50 μl + LPS 10 μg/100 μl, L4 and R4 intra-mammary (n=6)

The animals were anesthetized and the blood samples were collected under chloroform anaesthesia through retro orbital plexus in centrifugation tubes. The blood sample was incubated at 37^0C for 1 hrs and centrifuged at 10,000 rpm for 15 min at 4^0C to collect serum. The serum samples were stored at -20^0C till further use. Following the above, the animals were sacrificed by cervical dislocation followed by then isolated mammary gland tissue. Collected mammary gland tissues were evaluated on the prototypes of biochemical changes, inflammatory markers, histopathological changes, and western blot analysis.

Antioxidant markers

The mammary gland tissue homogenates (10% w/v) were prepared in 0.15M KCl and centrifuged at 10,000 rpm. The supernatants were evaluated for oxidative stress markers, including TBARs, PC, SOD, catalase, and GSH using the methods established in our laboratory (Kaithwas and Majumdar, 2012, Kaithwas et al., 2011a, Raj et al., 2014).

Determination of NO level in serum

Griess reagent (1% sulphanilamide, 0.1% N-(1-napthyl)-ethylenediamine dihydrochloride in 5% H_3PO_4) was used for the measuring nitrite accumulation in test serum sample. An equal quantity (500 µl) of serum and Griess reagent (500 µl) were mixed and incubated at 37^0C for 5 min. The mixture was subsequently read on UV-visible spectrophotometer (Cary 60, Agilent technologies, CA95051, US) at 540 nm using appropriate blank. Sodium nitrite was used for the standard curve preparation (Tiwari et al., 2016).

Determination of tissue H_2S level

The H_2S estimation was performed using the method described by our laboratory. Briefly, 500 µl of tissue added to the 400 µl premixed zinc acetate (1 % w/v) solution. The mixture was centrifuged and pellets from the tissue sample reconstituted 160 µl of Milli-Q water and mixed with 40 µl of dye (20 µl of 20 mM NNDP in 7.2 M HCl and 20 µl of 30 mM $FeCl_3$ in 1.2 M HCl). The mixture was incubated at 37^0C for 10 min and read spectrophotometrically at 670 nm (Carry 60, Agilent Technologies, CA, 95051, USA) (Al-Saeedan et al., 2018).

Histopathology of mammary gland tissue

A small piece of mammary gland tissue was fixed in 10% solution of formaldehyde and embedded in the wax. 5 µm sections were prepared using microtome followed by staining with H&E. The sections were visualized and photographed at 40X using digital biological microscope (N120, BR-Biochem Life Sciences, New Delhi, India).

Assay for caspase-3 and caspase-8

Caspase-3 and caspase-8 fluorometric assays were performed using the methods elaborated in the literature provided with the kits. The assay was carried out in amber colored 96-well plate. Equal volumes of serum sample from both control and experimental animals were diluted with reaction buffer. Dithiothriol (DTT) was added to a final concentration of 10 mM. To the reactant mixture 5 µl of IETD-AFC/DEVD-AFC substrate was added and incubated for 1 hrs at 37^0C. Free AFC levels formed were measured in a plate reader with a 400 nm excitation and a 505 nm emission. The results were expressed as fluorescence units/mg of protein.

Immunoblotting assay

The mammary gland tissue lysing in RIPA lysis buffer to extract total protein. The total protein content was measured using the Bradford reagent. According to the principles of Laemmli with slight modifications, proteins were separated on 12.5% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membrane (IPVH 00010 Millipore, Bedford, MA USA. Subsequently, the protein on PVDF membrane was blocked with 3% BSA and 3% skim milk in tris buffer saline-tween 20 (TBST) for 3 hrs and incubated overnight with primary antibody against, COX (MA5-14568), LOX (PA1-16953), IFN-γ (PA1-24782), Bcl-xl (MA5-15142), Bcl-2 (SC-7382), BAX (SC-23959), BAD (SC-8044), VDAC (SC-390996), cytochrome-c (SC-13561), Apaf-1 (SC-65891), NFκBp65 (MA5-1616), UCHL-1 (MA1- 83428), PHD2 (SC-67030), HIF-1α (SC-13515), FASN (SC-55580), SREBP-1c (SC-13551) at 4°C. β-actin (MA5-15739-HRP) was used as a standard reference. The membrane was washed with TBST thrice and incubated with the corresponding anti-rabbit (SC-2030), anti-goat (SC-2020), anti-mouse (31430, Pierce Thermo Scientific, USA) horseradish peroxidase (HRP) conjugated secondary antibody (1:5000 dilutions) at room temperature for 3 hrs. After

single wash with TBST, the PVDF membranes were developed using an enhanced chemiluminescence substrate (Western Bright ECL-HRP substrate, Advansta, Melanopark, California, US) in gel dock system. The quantification of protein was done through densitometry digital analysis of protein bands using Image J software (Roy et al., 2017).

Field based study on mastitic cow

Animals

Nine lactating mix breed cows were selected for the study from different places of Deoria (UP) with no other clinical illness. Groups were divided as, group 1: cefoperazone 2.5 ml/udder (intra-mammary), group 2: ALA-NS (F-1), 2.5 ml/udder (intra-mammary), group 3: ALA-NS (F-2), 2.5 ml/udder (intra-mammary). All the drugs were administered continuously once a day, for 10 days. The study was approved by Institutional Animal Ethics Committee (SDCOP&VS/AH/CPCSEA/01/0039).

Table 4- Experimental design for the mastitic cow

Sr. No.	Groups	Treatment schedule
1.	I	Cefoperazone 2.5 ml/udder (intramammary) (n=3)
2.	II	ALA-NS F1 2.5 ml/udder (intramammary) (n=3)
3.	III	ALA-NS F2 2.5 ml/udder (intramammary) (n=3)

Milk sampling

50 ml of milk from each cow was collected in sterile vials after cleaning the teat orifice with ethanol and discarding the first few streams of milk. Milk samples were collected on 1st, 5th, and 10th day, and stored in $4^{0}C$, till further estimations.

pH, consistency, and color of milk

pH of milk was measured by pen type pH meter (Hanna Instruments, HI 98107), and milk colour and consistency was visually identified.

WST

5 drops of milk were placed on glass plate (underside painted black) and mixed with 2 drops of NaOH (2%). The mixture was rapidly stirred with broomstick for 20-25 sec. The results were scored as, mixture remain particle free, -/0; fine dispersed particle on close inspection: +/1; a definite thickening and mixture separates into a milky whey and white particle; ++/2, formation of white flakes; +++/3 (Fthenakis, 1995).

BBT

BBT was performed by dividing whatman filter paper (7 cm diameter) in to four different quadrants marked as left fore (LF), right fore (RF), left hind (LH), and right hind (RH). A drop of bromothymol blue test solution was placed in each quadrant followed by a drop of milk. The changes in color were scored as follows: pale green : normal/ +/1, moderate green: ++/2, dark blue green: +++/3 (Marschke and Kitchen, 1985).

CMT

CMT was performed using the CMT reagent. The test was performed by mixing equal quantity (3 ml) of milk and CMT reagent (Kaithwas et al., 2011b). Both were mixed quickly and results were scored as:

(0) Slight or no precipitation which disappears with paddle movement: Normal.

(1) Distinct precipitation without gel formation: Suspicious.

(2) Distinct gelatinisation of the milk: Infected.

(3) Thick gel formation which adheres to the paddle: Severely infected.

SCC

SCC was evaluated by direct microscopy using methylene blue staining using the method described elsewhere (Kaithwas et al., 2011b).

TMC

A milk sample (50 µl) was mixed with 20 ml molten sterile muller hinton agar media and poured aseptically into a sterile petri dish. Inoculated plates were incubated at 37°C. Colonies were counted using a colony counter after 24 hrs, represented as CFU. (Kaithwas et al., 2011b).

Extraction of total protein from milk sample

Briefly, milk samples were centrifuged at 10000 rpm for 20 min. The cell pellet was re-suspended in HBSS buffer. The procedure was repeated three times. Protein was extracted by adding equal amount (100 µl) of PMSF and RIPA lysis buffer. The mixture was sonicated (10 sec) and centrifuged at 13000 rpm for 20 min. The supernatant was acetone precipitated and protein was quantified through Bradford method (Dang et al., 2013).

Western blot analysis of milk sample

Based on the principles of Laemmli with slight modifications, milk proteins were resolved on 12.5% SDS-PAGE gel and transferred to PVDF membrane (IPVH 00010 Millipore, Bedford, MA USA). Consequently, PVDF membrane was blocked with 5% BSA and skimmed milk in TBST for 3 hrs and incubated overnight with, NFκB-p65 (MA5-1616), UCHL-1 (MA1- 83428), and SREBP-1c (SC-13551) primary antibody at 4°C. β-actin (MA5-15739-HRP) was used as a standard reference. The PVDF membrane was washed three times with TBST and incubated with corresponding anti-rabbit (SC-2030), anti-goat (SC-2020) (Pierce Thermo Scientific, USA) HRP conjugated secondary antibody (1:5000 dilutions) at room temperature for 3 hrs. After

single TBST wash PVDF membranes were developed using an enhanced chemiluminescence substrate (Western Bright ECL HRP substrate, Advansta, California, US) in gel dock system. The quantification of protein was done through densitometry digital analysis of protein bands using Image J software (Roy et al., 2017).

For western blot analysis the protein was loaded according to following criteria lane-1 represented the 10th day of treatment sample of group-1, lane-2 as 1st day milk sample, lane-3 and 4, represented the 10th day of treatment sample of ALA-NS (F1, F2) respectively.

Statistical analysis

All the data are presented as mean ± SD and analysed by one way-ANOVA followed by Bonferroni multiple comparison test. Statistical analysis was carried out using Graph Pad Prism 3.0 (Graph Pad Software, San Diego, CA, USA).

CHAPTER-4

RESULTS AND DISCUSSION

Results

Formulation

Different formulation were prepared and characterized, the observed data was analysed by Design Expert 8.0.7.1 (trial version). Various parameters like (PS, ZP, and PDI) were used for analysis by software. First two formulation (F1 and F2) were observed best suitable for the further consideration and evaluation **(Table-5)**. For PS significant effect of interaction between tween-80 and PEG-400 was observed **(Figure-3A)** and the results expressed that there was no significant changes in PDI for the selected factors and their levels, the mean value for PDI was observed to be 0.3175. Similarly there was no significant effect on ZP was observed in the formulation. A linear relationship was observed between tween-80 and SV as shown in **Figure-3B**.

The overlay plot for ALA-NS (F1) obtained after applying constraint like (PS<2500; PDI<0.5; ZP>9, SV> 0.7) depicts the reason of desirable space for the selected response variables. The inset flag depicts the composition of optimized formulation [x1(Tween 80) =350, x2 (PEG-400) =100, C (ALA=1450)] and predicted value for response **(Figure-3C).**

The overlay plot for ALA-NS (F2) obtained after applying constraint like (PS<2500; PDI<0.5; ZP>9, SV> 0.7) depicts the reason of desirable space for the selected response variables. The inset flag depicts the composition of optimized formulation [x1(Tween 80) =450, x2 (PEG-400) =150, C (ALA=1350)] and predicted value for response **(Figure-3D)**.

Table-5: Formulation composition and response analysis by Design Expert 8.0.7.1 software

Formulation No.	Run	Factor 1 A:Tween-80 (µl)	Factor 2 B:PEG-400 (µl)	Factor 3 C:ALA (µl)	Response 1 PS (nm)	Response 2 PDI	Response 3 ZP (mv)	Response 4 SV
1	1	350	100	1450	1020	0.23	10.8	0.8
2	2	450	150	1350	1080	0.21	11.2	0.78
3	3	500	200	1250	2300	0.18	9.1	0.72
4	4	550	250	1150	3700	0.19	12.3	0.68
5	5	600	300	1050	3300	0.20	14.7	0.65
6	6	650	350	950	1800	0.45	15.09	0.62
7	7	700	400	850	1900	0.60	14.8	0.59
8	8	750	450	750	2400	0.48	15.9	0.55

Figure-3: Response surface analysis of optimized formulations by Design Expert 8.0.7.1 software

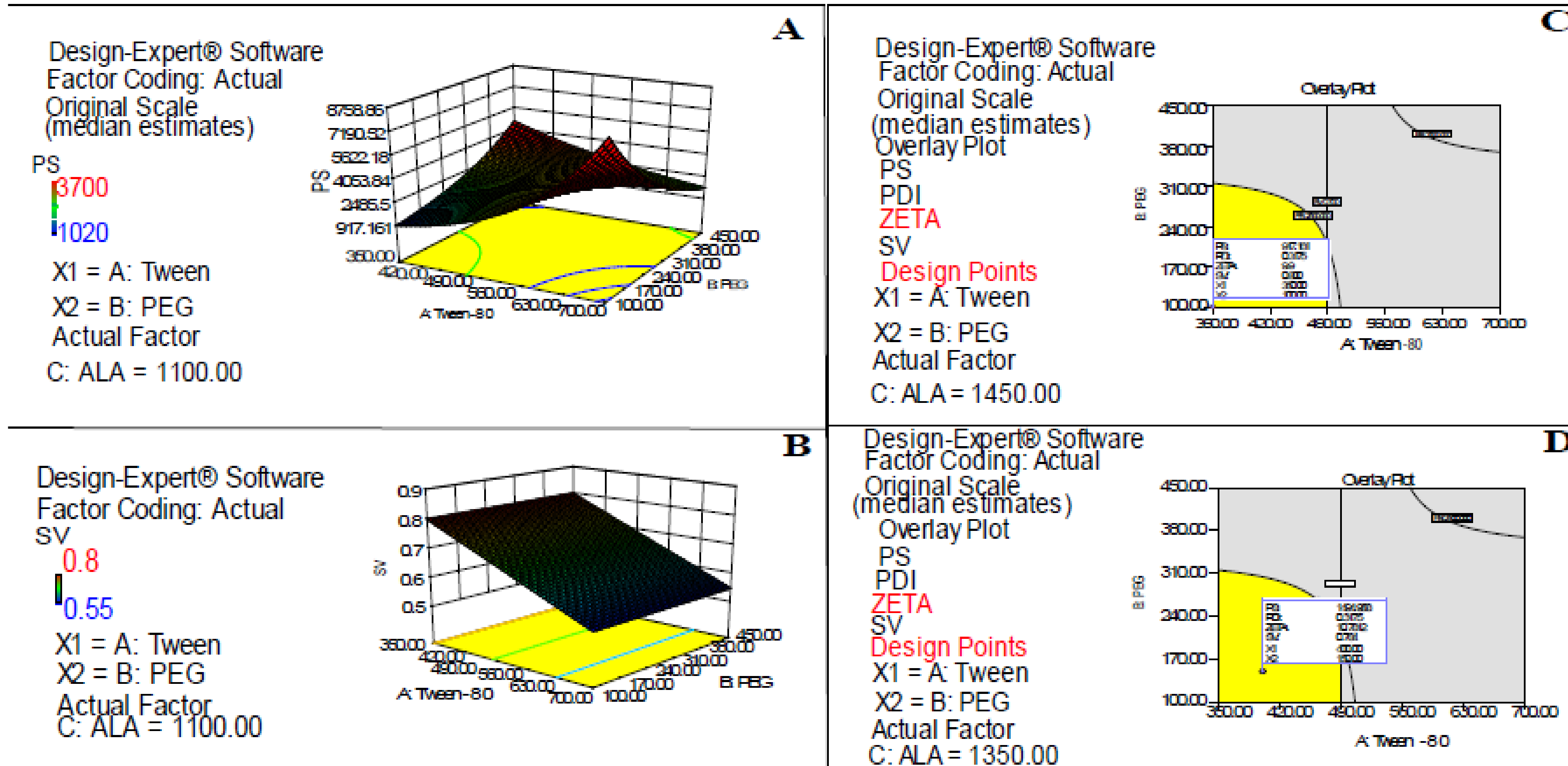


Table-6: Stability study of ALA-NS (F1 and F2)

Time Point	Storage (Temp/RH)	Particle size (ALA-NS F1) (nm)	Particle Size (ALA-NS F2) (nm)
30 days	40^0C/75%	1040	1090
	30^0C/65%	1022	1080
	25^0C/60%	1020	1080
60 days	40^0C/75%	1045	1095
	30^0C/65%	1030	1088
	25^0C/60%	1020	1080
90 days	40^0C/75%	1048	1130
	30^0C/65%	1030	1090
	25^0C/60%	1022	1084

Antimicrobial activity

Table-7: MIC of ALA and ALA-NS

Sr. no.	Microorganism	Strain no.	ALA (v/v)	ALA-NS F1 (v/v)	ALA-NS F2 (v/v)
1	*E.coli*	MTCC-118	0.4%	0.025%	0.01%
2	*Staphylococcus epidermidis*	ATCC-12228	0.7%	0.04%	0.03%
3	*Pseudomonas aeruginosa*	ATCC-25619	0.3%	0.02%	0.02%
4	*Staphylococcus aureus*	ATCC-29737	0.3%	0.01%	0.02%
5	*Streptococcus agalactiae*	ATCC-13813	0.6%	0.1%	0.2%
6	*Lactobacillus sporogenes*	ATCC-31284	0.1%	0.01%	0.03%
7	*Candida albicans*	ATCC-10231	0.4%	0.02%	0.02%

Antibacterial activity of ALA-NS:

It can be observed that all the microorganisms tested were susceptible to the action of formulation (F1 and F2), with a range of MIC values from 0.01% to 0.2% v/v **(Table-7)**.

The result suggested that both ALA-NS (F1 and F2) had potent antimicrobial activity against *S. aureus, S. agalactia, C. albicans, S. agalactiae, E.coli, S. epidermidis*, and *L. sporogenes* compared with the ALA and cefotaxime **(Table-8)**.

Table-8: Comparison of antimicrobial activity of ALA, cefotaxime, and ALA-NS

Sr. no.	Microorganism	Strain No.	Zone of inhibition (mm) (mean ± SD)			
			Cefotaxime	**ALA**	**F1**	**F2**
1	*Escherichia coli*	ATCC-8739	27.6±0.09	11.6±0.32	32.0±0.27	38.0±0.21
2	*Escherichia coli*	MTCC-118	11.3±0.17	7.2±0.41	12.7±0.97	13.1±0.68
3	*Staphylococcus epidermidis*	ATCC-12228	19.1 ±0.74	8.6±0.71	21.6±0.64	22.8±0.93
4	*Pseudomonas aeruginosa*	ATCC-25619	10.8±0.56	6.9±0.43	13.3±0.88	12.8±0.39
5	*Staphylococcus aureus*	ATCC-29737	19.3±0.77	10.7±0.57	25.6±0.41	21.4±0.91
6	*Streptococcus agalactiae*	ATCC-13813	12.5±0.30	6.8±0.54	11.4±0.39	13.8±0.22
7	*Lactobacillus sporogenes*	ATCC-31284	18.2±0.61	11.9±0.49	21.6±0.65	22.8±0.46
8	*Candida albicans*	ATCC-10231	21.2±0.76	12.4±0.88	24.6±0.72	17.2±0.87

Study on LPS induced mastitis in rats

Antioxidant activity

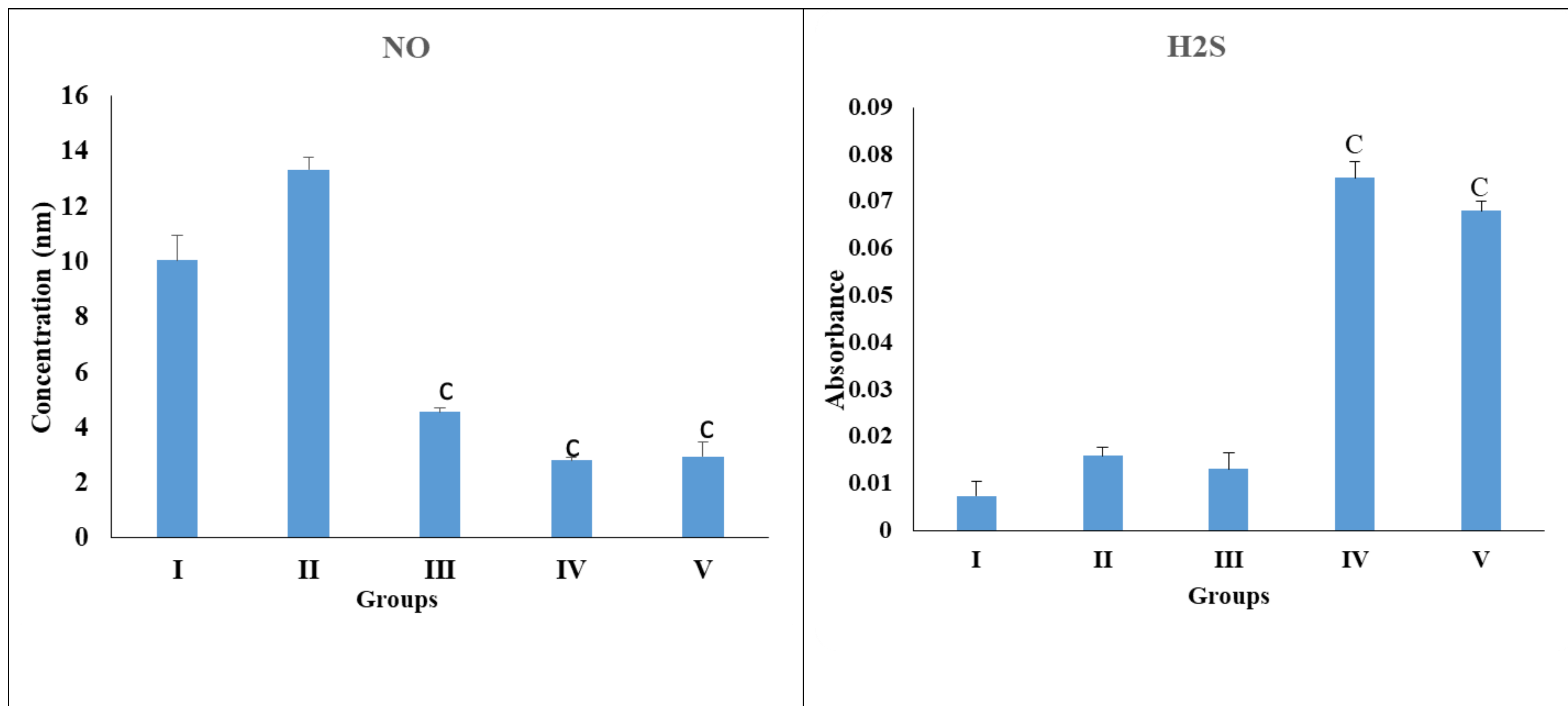
The antioxidant barriers were altered after the LPS treatment in female rats. The treatment with formulation was evident for the protective activity towards the lipid and protein peroxidation. ALA-NS (F1 and F2) significantly ($p<0.001$) decreased the TBARs level (0.14±0.05 nM of MDA/ µg of protein) and (0.17±0.04 nM of MDA/µg of protein) respectively, against the LPS (1.69±0.21 nM of MDA/µg of protein) treated rats. Furthermore, ALA-NS (F1 and F2) also significantly ($p<0.001$) reduced the PC level compared with the LPS treated rats. The level of SOD and catalase in LPS treated group (155.10±23.09 unit of SOD/µg of protein, 0.60±0.03 nM of H_2O_2 disappeared /min/µg of protein) was significantly ($p<0.001$) restored after treatment with both formulations. ALA-NS (F1 and F2) were also evident for significant ($p<0.001$) restoration of reduced GSH levels, when compared with the LPS treated rats **(Table-9)**.

Table-9: Effect of ALA-NS against biochemical markers of oxidative stress

Groups	**TBARS** (nM of MDA/µg of protein)	**Protein carbonyl** (nM /ml unit)	**SOD** (unit of SOD/µg of protein)	**Catalase** (nM of H_2O_2 disappeared /min/µg of protein)	**GSH*10^{-4}** (µg %)
I	0.17 ± 0.023[c]	63.73±5.26[c]	284.96±14.13[c]	1.03±0.02[b]	0.04±0.002[b]
II	1.69±0.21	137.72±6.86	155.10±23.09	0.60±0.03	0.02±0.001
III	0.35±0.07[c]	99.08±5.58[c]	264.79±77.20[b]	0.46±0.01[c]	0.0243±0.003
IV	0.14±0.05[c]	83.40±9.57[c]	622.12±28.34[c]	0.77±0.01[c]	0.04±0.002[c]
V	0.17±0.04[c]	86.98±11.93[c]	372.08±44.04[c]	0.77±0.04[c]	0.028±0.001[c]

(Values are Mean ± SD), each group contains six animals. Comparisons were made on the basis of the one-way Anova followed by Bonferroni test. All groups were compared to the toxic control group ([a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$).

Figure-4: Effect of ALA-NS on NO and H_2S level



(Values are Mean ± SD), each group contains six animals. Comparisons were made on the basis of the one-way Anova followed by Bonferroni test. All groups were compared to the toxic control group ($^a p<0.05$, $^b p<0.01$, $^c p<0.001$).

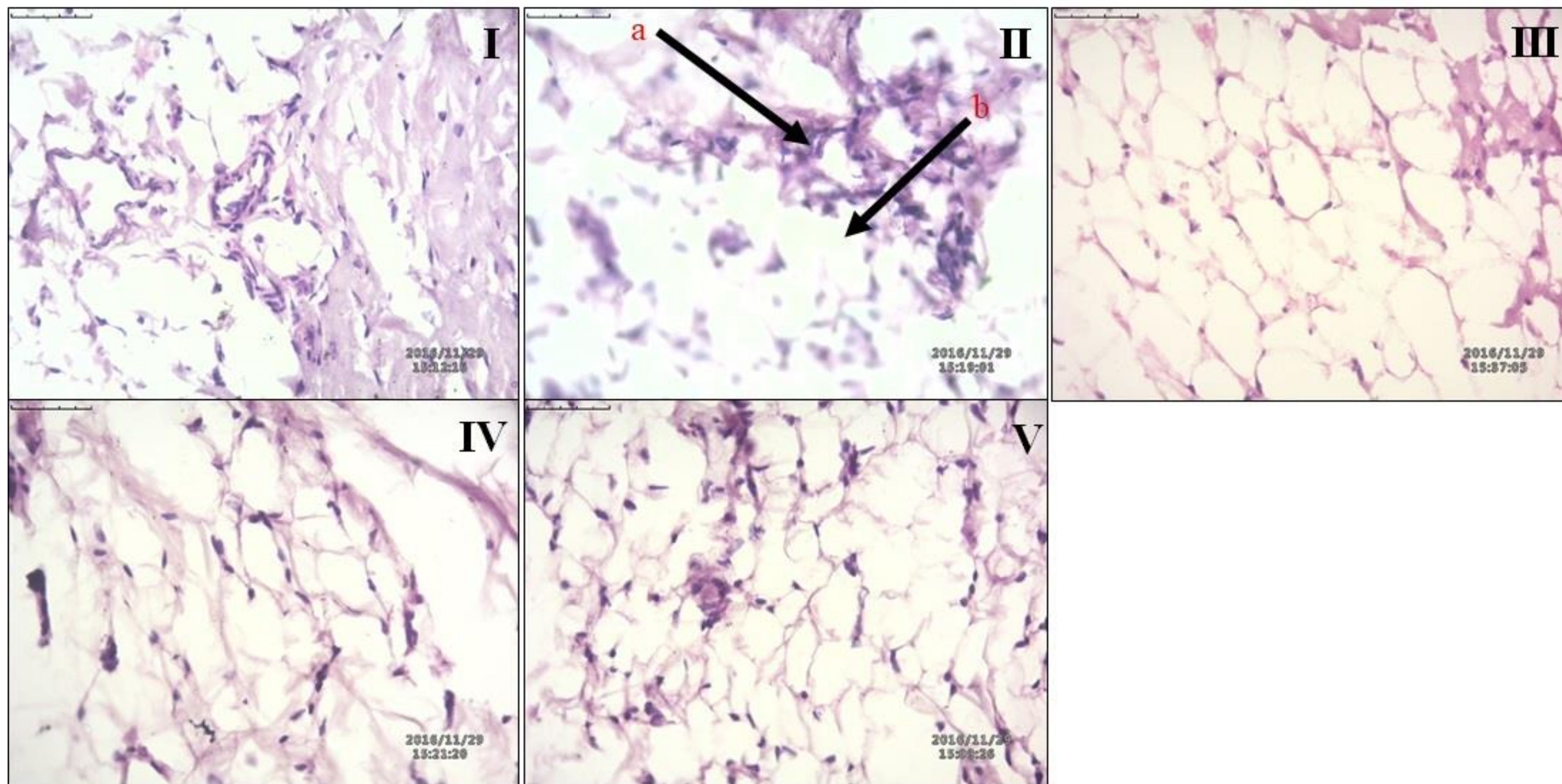
Determination of NO and H_2S level in serum

The LPS treatment upsurged the NO level in serum sample, furthermore rats treated with the ALA-NS (F1 and F2) significantly ($p<0.001$) downturned the levels of NO. But when scrutinized for the serum H_2S level both formulation significantly ($p<0.001$) up-regulated H_2S level **(Figure-4)**.

Histopathology of mammary gland tissue

The histological analysis of mammary gland depicted that the rat treated with the LPS experienced inflammatory cell infiltration, enhanced adipose tissue, and marked thickening of the alveolus walls in comparison to control. The treatment with the ALA-NS restored the morphology of mammary gland tissue **(Figure-5)**.

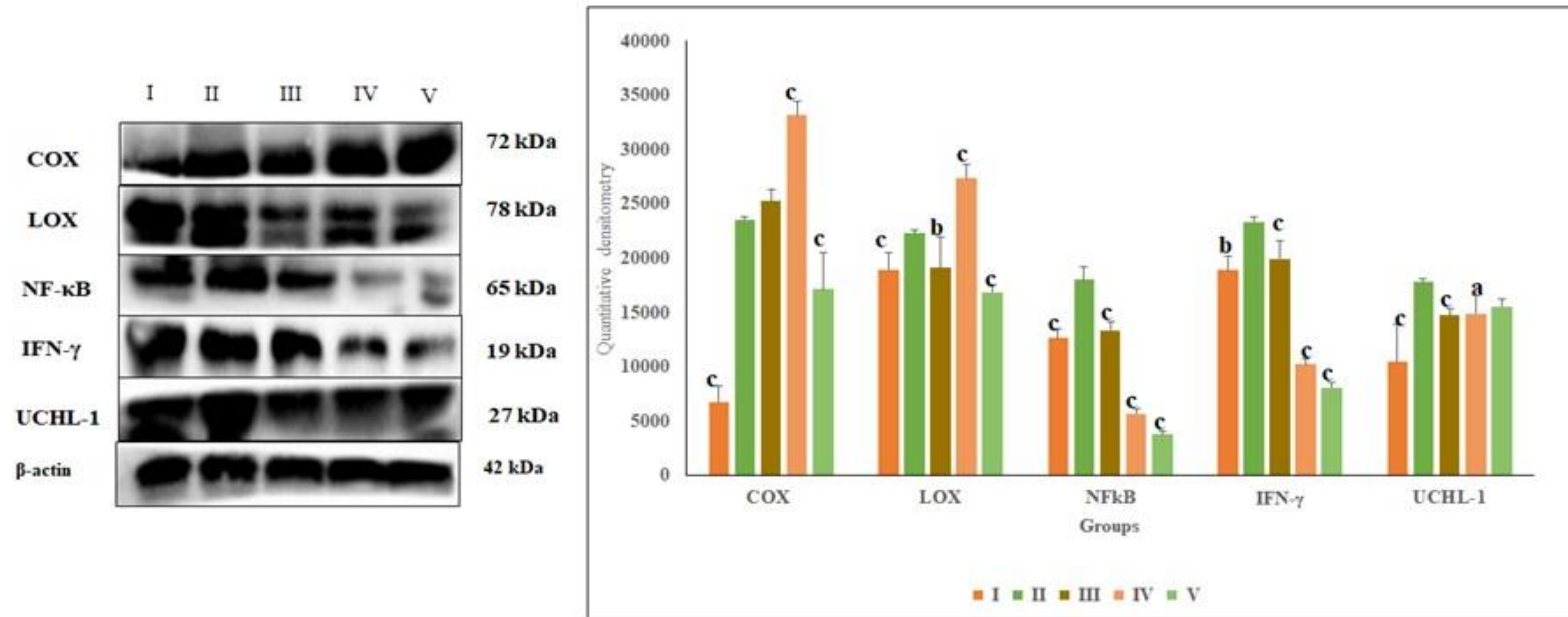
Figure-5: Histopathology study of mammary tissue after treatment with ALA-NS



Western blot analysis:

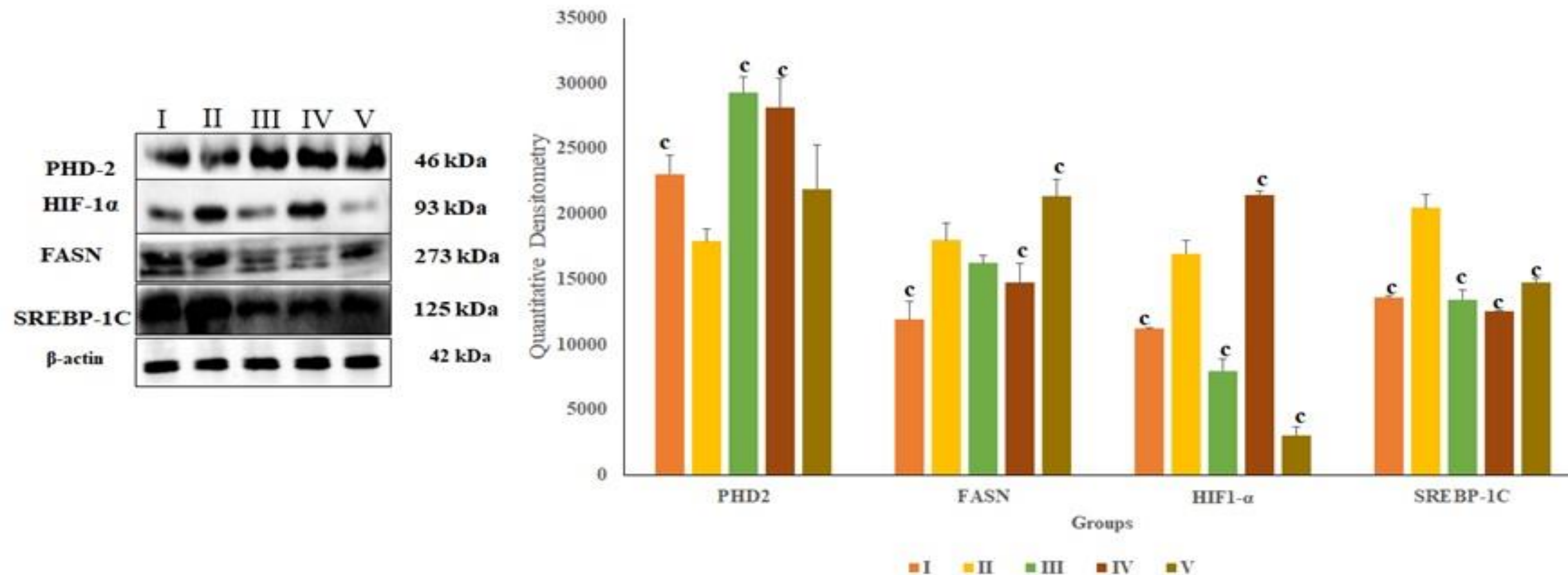
Both COX and LOX are essential enzymes involved in facilitating the inflammation process and LPS upregulated the expression of both COX and LOX which were ($p<0.001$) downregulated after the treatment with the ALA-NS (F1 and F2). Simultaneously, ALA-NS (F1 and F2) significantly ($p<0.001$) declined the expression of NFκBp65 and IFN-γ as compared with the LPS treated rats **(Figure-6)**. Furthermore, the protein involve in glycolytic and fatty acids synthesis were evaluated for their expression after LPS administration. The ALA-NS (F1 and F2) significantly decrease expression of HIF-1α, SREBP-1c, FASN, and UCHL-1; and increased the PHD-2 expression **(Figure-7)**. LPS administration increased the expression of anti-apoptotic proteins (Bcl-2 and Bcl-xl) and decreased the pro-apoptotic protein markers (BAD and BAX). Treatment with formulation helped to reinstate the pro-apoptotic and anti-apoptotic protein markers favourably suggesting apoptosis. When perceived through the downstream markers of mitochondrial mediated apoptosis (VADC, cytochrome-c, and Apaf-1), the LPS afforded increased expression of VDAC, and Apaf-1 along with curtailment of cytochrome-c expression. Treatment with ALA afforded marked regulation of apoptotic markers favoring apoptosis **(Figure-8, 9)**.

Figure-6: Effect of ALA-NS on inflammatory mediator proteins



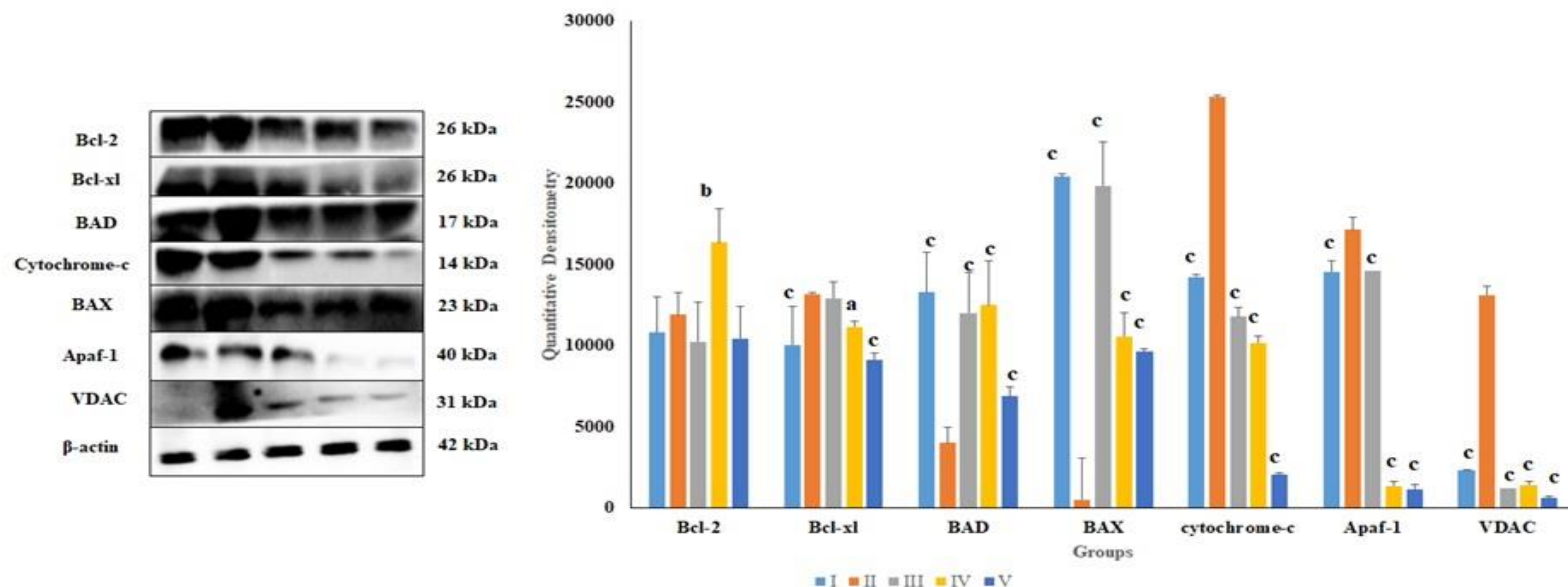
Western blot analysis of group I (sham control, 0.9% normal saline, 3 ml/kg, L4 and R4 intra-mammary); group II (toxic control, LPS 10 µg/100 µl, L4 and R4 intra-mammary); group III (Cefotaxime 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary), group IV (ALA-NS F1, 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary); group V (ALA-NS F2, 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary).
(Values are Mean ± SD), each group contains six animals; Comparisons were made on the basis of the one-way Anova followed by Bonferroni test; All groups were compared to the toxic control group ([a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$).

Figure-7: Effect of ALA-NS on hypoxia mediator and sterol synthesis pathway proteins



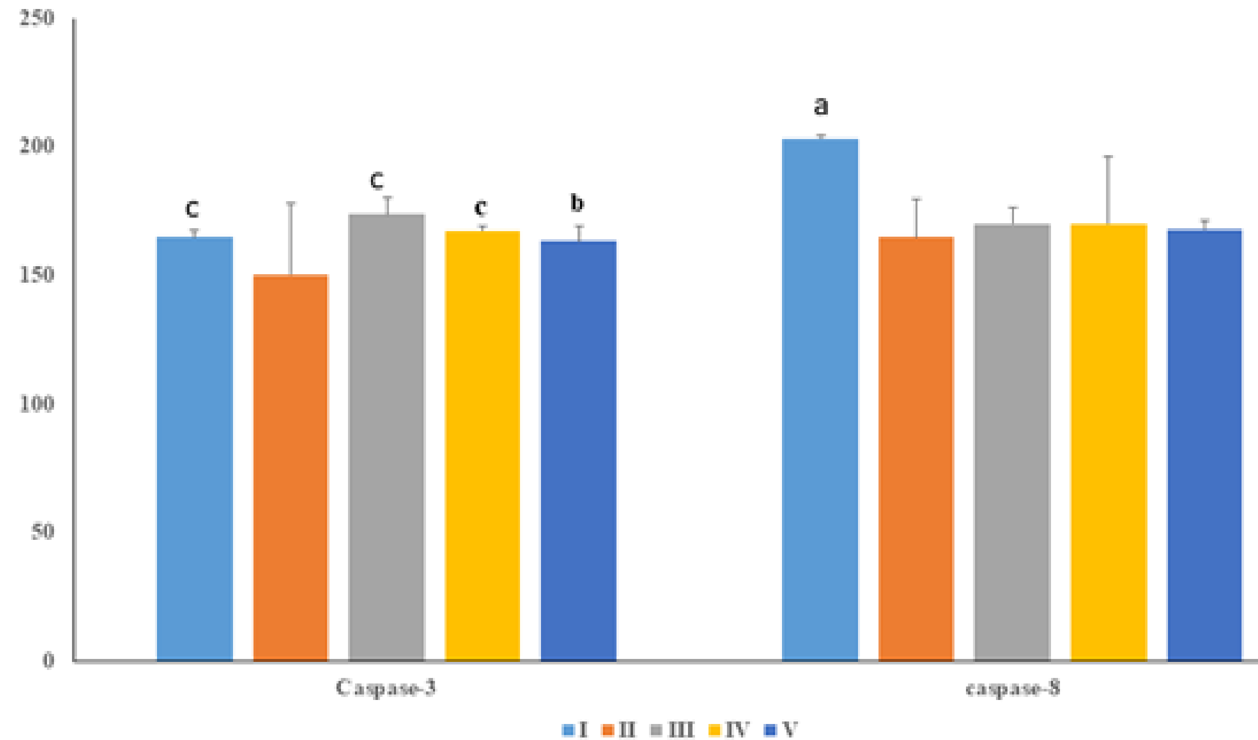
Western blot analysis of group I (sham control, 0.9% normal saline, 3 ml/kg, L4 and R4 intra-mammary); group II (toxic control, LPS 10 µg/100 µl, L4 and R4 intra-mammary); group III (Cefotaxime 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary), group IV (ALA-NS F-1, 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary); group V (ALA-NS F-2, 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary). (Values are Mean ± SD), each group contains six animals; Comparisons were made on the basis of the one-way Anova followed by Bonferroni test; All groups were compared to the toxic control group ($^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$).

Figure-8: Effect of ALA-NS on cell death mediated proteins



Western blot analysis of group I (sham control, 0.9% normal saline, 3 ml/kg, L4 and R4 intra-mammary); group II (toxic control, LPS 10 µg/100 µl, L4 and R4 intra-mammary); group III (Cefotaxime 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary), group IV (ALA-NS F-1, 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary); group V (ALA-NS F-2, 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary). (Values are Mean ± SD), each group contains six animals; Comparisons were made on the basis of the one-way Anova followed by Bonferroni test; All groups were compared to the toxic control group ([a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$).

Figure-9: Effect of ALA-NS on serum caspase-3 and caspase-8 level



Western blot analysis of group I (sham control, 0.9% normal saline, 3 ml/kg, L4 and R4 intra-mammary); group II (toxic control, LPS 10 µg/100 µl, L4 and R4 intra-mammary); group III (Cefotaxime 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary), group IV (ALA-NS F-1, 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary); group V (ALA-NS F-2, 50 µl + LPS 10 µg/100 µl, L4 and R4 intra-mammary). (Values are Mean ± SD), each group contains six animals; Comparisons were made on the basis of the one-way Anova followed by Bonferroni test; All groups were compared to the toxic control group ($^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$).

Field based study on mastitic cows

Color and consistency

Milk color changed from pale yellow to white, subsequently milk consistency changed from watery to thick whitish after treatment with ALA-NS (F1 and F2).

pH, CMT, WST, SCC, and BBT score

Milk pH was normalised significantly ($p<0.05$) on 10th day after intra-mammary treatment with both ALA -NS (F1 and F2). The inframammary injection of formulations significantly ($p<0.05$, and $p<0.001$) decreased the CMT score on 10th day (2±0.00 and 1.5±0.50) in comparison to 1st day of treatment (3±0.00) **(Table 10)**. Both ALA -NS significantly ($p<0.001$) reduced the WST and BBT score after 10 days of treatment **(Table 10)**. Similarly the SCC was also decreased near to normal by ALA-NS treatment with more favourable effect by F1 **(Figure-10)**.

TMC

The initial (1st day of treatment) TMC was 195±21.98, 173±46.10, and 218±29.56 CFU/50µl in group 1, 2, and 3, respectively. After completion of treatment (10th day of treatment) the TMC was reduced to normal level 37±11.4, 28±3.16, and 25±4.24 CFU/50 µl. The group 2 and 3 showed higher percentage inhibition (83.81 %, 88.53%), when compared with group 1 (81.05 %) **(Table-11)**.

Table-10: Effect of ALA-NS on field based parameters in bovine mastitis

	1st Day				5th days				10th days			
	pH	**CMT**	**WST**	**BBT**	**pH**	**CMT**	**WST**	**BBT**	**pH**	**CMT**	**WST**	**BBT**
Group 1	7.35±0.35	3±0.50	2±0.00	2±0.00	6.9±0.14	2±0.00^{b}	1±0.00^{c}	1±0.00^{c}	6.8±0.10	1.5±0.50^{b}	1±0.00^{c}	1±0.00^{c}
Group 2	7.45±0.21	3±0.00	2±0.00	2±0.00	7.1±0.00^{a}	2±0.00^{c}	1±0.00^{c}	1±0.00^{c}	6.9±0.28^{a}	2±0.00^{c}	1±0.00^{c}	1±0.00^{c}
Group 3	7.25±0..21	3±0.00	2±0.00	2±0.00	6.8±0.14^{a}	2±0.00^{b}	1±0.00^{c}	1±0.00^{c}	6.7±0.21^{a}	1.5±0.50^{a}	1±0.00^{c}	1±0.00^{c}

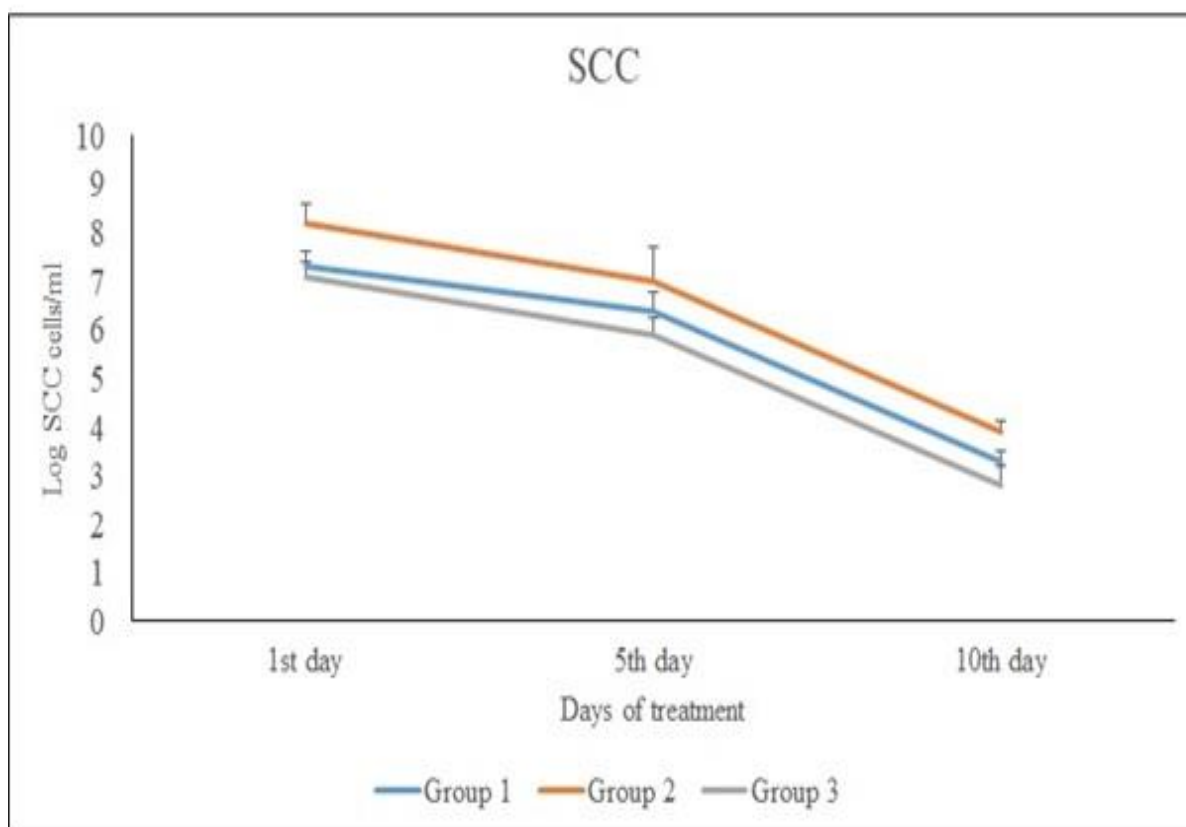
Values are represented in (mean ± SD) of three animals in each group. Values in parenthesis represent percentage inhibition. Statistical analysis has been compared with 1st day to others days as per Student's Newman-Keuls multiple comparison test. Comparison with the 1st day ($^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$). CMT- California mastitis test, WST- white side test, BBT- bromothymol blue test, TMC- total microbial count.

Table-11: Effect of ALA-NS on TMC of milk sample (50 µL) from infected udders

Groups	**TMC of milk sample (CFU/50 µL)**		
	1st Day	**5th Day**	**10th Day**
Group 1	195±21.98	118±13.35^{c} **(39.48 %)**	37±11.4^{c} **(81.05%)**
Group 2	173±46.10	121±39.81^{b} **(30.05%)**	28±3.16^{a} **(83.81%)**
Group 3	218±29.56	137±16.42^{c} **(37.15 %)**	25±4.24^{b} **(88.53%)**

Values in parenthesis represent percentage inhibition. Values of CFU (mean ± SD) of three animals in each group. Comparisons were made on the basis of the one-way Anova followed by Student's Newman-Keuls multiple comparison test. Comparison with the 1st day ($^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$). Group-1: cefaperazone intra-mammary injection (2.5 ml/udder) daily, group-2: ALA-NS (F1) intra-mammary injection (2.5 ml/udder) daily, group-3: ALA-NS (F2) intra-mammary injection (2.5 ml/udder) daily

Figure-10: Effect of ALA-NS on SCC of mastitic milk

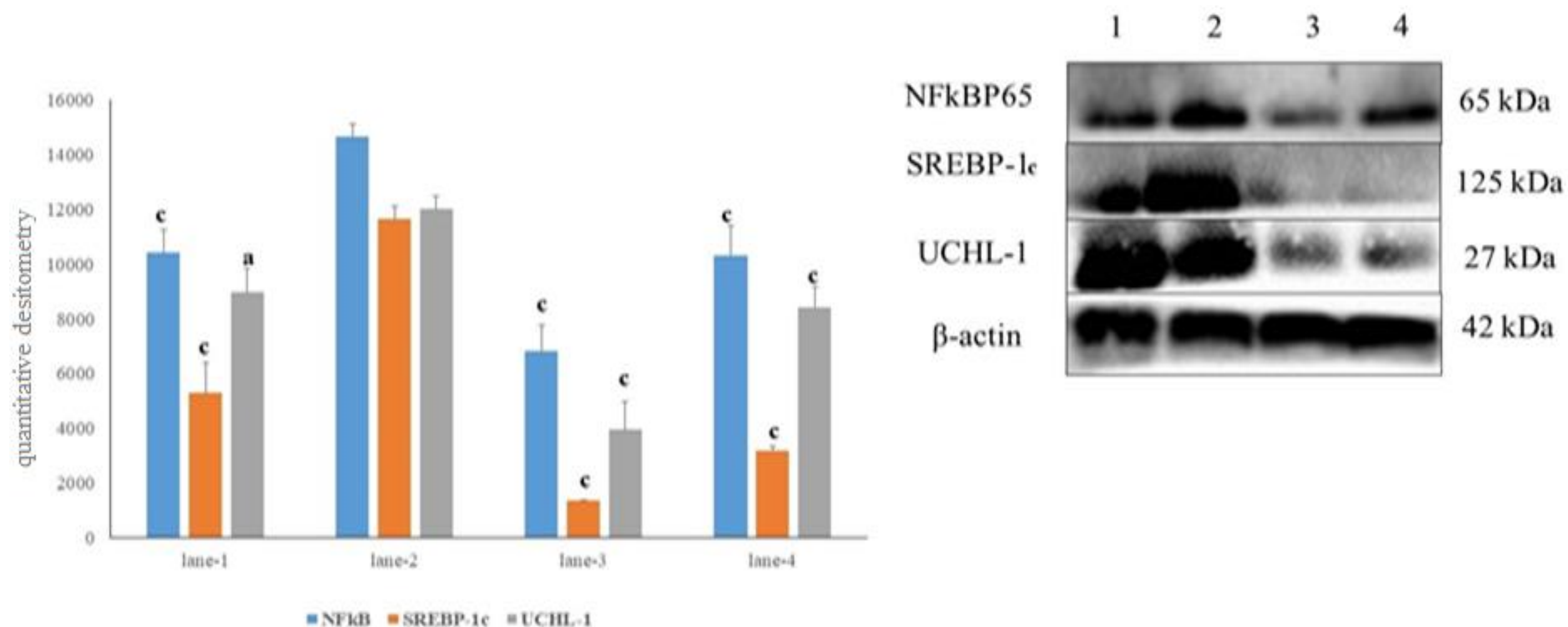


Values are presented as mean ± SD. Group-1: cefaperazone intra-mammary injection (2.5 ml/udder) daily, group-2: ALA-NS (F1) intra-mammary injection (2.5 ml/udder) daily, group-3: ALA-NS (F2) intra-mammary injection (2.5 ml/udder) daily

Western blot analysis of milk

The analysis of the blots revealed significant increased expression of NFκB-p65 and SREBP-1c on day 1, with diminished expression of the same on 10th day. The F1 was recorded to reduce the expression of NFκB-p65 and SREBP-1c more profoundly in comparison to F2 and cefoperazone. Subsequently, UCHL-1 expression was also significantly ($p<0.001$) diminished after the treatment with ALA -NS (F1 and F2) **(Figure 11)**.

Figure-11: Effect of ALA-NS on NFkBP65, SREBP-1c, and UCHL-1 protein isolated from mastitic milk.



(Lane 1, represented the 10th day of treatment sample of cefoperazone, lane 2 donated as 1st day milk sample, lane 3 and 4, represented the 10th day of treatment sample of ALA-NS (F1, F2) respectively.) β-actin was used as loading control. Each protein expression was performed in triplicate. All the data are presented as mean ± SD and analysed by one way-ANOVA followed by Student's Newman–Keuls multiple comparison test. Comparison with the 1st day ($^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$) was considered statistically significant

Discussion

PS analysis is an important factor for the evaluations of nanoformulations, since PS inversely correlated with the interfacial area, which is responsible for the better drug partitioning and absorption through the skin surface. The PS (1020 nm, and 1080 nm) and ZP (-10.8 and -11.2) was observed for both ALA-NS (F1 and F2) respectively. In the current study, it was observed that PS was increased with increasing the concentration of surfactant tween-80 and PEG-400. On contrast, the ZP was decreased with increasing the concentration of surfactant as (PEG-400 and Tween-80) as shown in **Table-5**. This could be attributed to the fact that Tween-80 has amphiphilic nature that settled down at the particle surface resulting in increment of PS. Moreover, it could shield surface charge of the nanosuspension leading to decrease in ZP. Similar result was also reported by Asasutjarit et al (Asasutjarit et al., 2013). In current study, the PDI value of all the formulation batches was observed between 0.23 to 0.48, indicating that all batches have uniform particle size distribution. It has been reported that monodispersed formulation with uniform particle size distribution have a PDI range from 0.1 to 0.7, whereas samples with broad size distribution have a PDI value 0.7 (Rajinikanth and Chellian, 2016).

Bacterial infection is responsible for the development of mastitis and the most common pathogenic bacteria are *E.coli*, and *S. aureus* (Kim et al., 2007). In the present study, ALA-NS (F1 and F2) showed good antimicrobial activity on different bacterial species. The result was in line with the previous report which hypothesized that the antibacterial activity of *Linum ussitassimum* seed oil against mastitis causing bacteria was due to significant amount of ALA (Kaithwas et al., 2011). Subsequently, ALA-NS exhibited much lower MIC value than ALA and cefotaxime alone against mastitis causing bacteria's.

In the instant study, LPS-induced mastitis model in rats was used to evaluate therapeutic effects of ALA-NS in anticipation that the same might help in the development of new therapeutic system for prophylaxis and treatment of bovine mastitis. LPS imitate the reactions perceived during natural mastitis deprived of the threats related with bacterial infection in rats (Chen et al., 2015). Many studies have indicated that LPS is able to induce pro-inflammatory responses and angiogenesis via activating the NFκB signalling pathways. Activation of pro-inflammatory agents causes increase in the oxidative stress due to generation of ROS (Li et al., 2013). Considering the same, we scrutinized the oxidative stress biomarkers predominantly depending upon inflammation. Antioxidant defence systems disturbed due to disease and/or aging leading in alteration of DNA, carbohydrates, proteins, and lipids (Raj et al., 2014). ROS augmented production of hydroxyl radical leads to the formation of lipid hydroperoxides that produces a family of α, β-unsaturated aldehydes. These reactive aldehydes are subject to Michael addition reactions with the side chains of amino acids (lysine, histidine, and cysteine), denoted as protein carbonylation (Rahman, 2012). The peroxidation of lipid results in production of MDA in the cells (Yadav et al., 2018). In the present study, we observed an upsurge level of both TBARs and PC in mammary gland of the LPS treated rats. The observation was in line with the previous reports which claim that LPS induces peroxidation of lipid and protein (Eslami et al., 2015, Çetin et al., 2005). ALA-NS restored the MDA and PC level to normal after the treatments with ALA-NS (F1 and F2). Moreover, antioxidant enzymes SOD, catalase, and GSH constitute the major supportive defense mechanism against oxidative stress. SOD scavenges the superoxide radicals to form hydrogen peroxide and catalase further dismutase hydrogen peroxide to H_2O and O_2 (Raj et al., 2014). The treatment with ALA-NS upsurged the antioxidant enzymes level in LPS treated rats. In addition, we also evaluated NO and H_2S levels in the mammary gland tissue.

NO is a reactive free radical that is synthesized from L-arginine by nitric oxide synthase (NOS), and an important messenger for biological responses (Moncada et al., 1991). Many reports suggested that overproduction of NO is responsible for the LPS induced inflammation (Onoda and Inano, 1998, Mukherjee, 2008, Bouchard et al., 1999). In the present study, NO level was augmented in the LPS treated rats and same was reduced after treatment with ALA-NS. Moreover, H_2S is a gaseous inflammatory mediator synthesized from cysteine amino acid by two important enzymes [cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase) (CBS)] (Li et al., 2011). LPS administration increased the H_2S level which was in line with the previously reported study (Whiteman et al., 2010). However, treatment with ALA-NS demarcated substantial increase in H_2S levels which possibly could be attributed to the feedback mechanism.

Lastly, mammary gland sections were then subjected to H&E staining. Histopathologic changes are the important parameter to observe the inflammatory reactions in tissue. In the current study, marked thickening of the alveolus walls and significant amount of PMN were observed in mammary gland tissue of LPS treated rats. Leukocytes transmigrated from circulating pool into the mammary gland was a striking feature of acute mastitis and result was in-line with the previous reports (Li et al., 2013, Hu et al., 2016). ALA-NS restored the normal structure of mammary gland after treatment.

Furthermore, the inflammatory modulation by ALA-NS was validated through more stringent protein biomarkers. LPS mediated ROS generation along with some other agents are responsible for the activation of NF-κB. The most widely considered form of NF-κB is a heterodimer of p50 and p65 subunits, responsible for potent activation of gene transcription. NF-κB regulates the expression of many genes and enzymes (COX, and LOX). The COX and LOX enzymes are responsible for the generation of 2 and 4 series of PGs and LTs receptively

by a membrane bound AA and are responsible for the pro-inflammatory responses in the cells. LPS treatment increased NFkB-P65, COX, and LOX protein expression in mastitic rats, the observation was in-line with the previous reports (Zhu et al., 2013, Hong et al., 2004). In the present study the ALA-NS markedly decreased the NFkB-P65, COX, and LOX protein expression in the LPS treated rats. Injection of LPS in rats also increased level of IFN-γ which further elevated the NO level in inflammation (Sohn et al., 2007). The treatment with ALA-NS decreased the IFN-γ level in rats. Considering the above, it can be suggested that the ALA-NS have the anti-inflammatory activity in LPS induced mastitis in rat.

Moreover, bacteria acquire resistance towards the antibiotics is a serious concern in the treatment for mastitis. A previous report exhibited that UCHL-1 is a the major elements in multi drug resistance (MDR) (Wang et al., 2016). The UCHL-1 belongs to the UCH protease family that deubiquitinates ubiquitin-protein conjugates in the ubiquitin-proteasome system (Rani et al., 2016). A previous study has suggested that the overexpression of UCHL-1 is responsible for the augmented activity of P-gp in cells which could be co-related with antibiotic resistance (Seral et al., 2003). In the current study, we observed that overexpression of UCHL-1 in LPS treated rats and treatment with ALA-NS decreased the UCHL-1 expression. Henceforth, authors can postulate that the formulation have additional advantage to combat the chances of MDR.

Recently, researchers revealed that HIF-1 played an important role in LPS treated rat (Chen et al., 2015, Tacchini et al., 2008). HIF-1 is a transcription factor composed of domain HIF-1α and HIF-1β. HIF-1α subtype is tightly controlled under normoxic conditions and degraded via normoxia-dependent PHD-2 (Singh et al., 2016). As a result, HIF-1α is stabilized and activated when its hydroxylation is inhibited under hypoxic conditions. Because the consumption of O_2 at inflammation site is elevated and the blood supply is interrupted, local

hypoxic microenvironments normally occur in pathogen-elicited inflammatory responses and result in the activation and accumulation of HIF-1α (Zeitouni et al., 2016). HIF-1α is also responsible for the alteration of glycolytic pathway, which upregulates the proteins responsible for the fatty acids synthesis (FASN and SREBP-1) in mammary gland (Furuta et al., 2008). SREBP-1 is considered as a significant controller of lipid homeostasis in mammals, including milk fat synthesis(Su et al., 2018). In the hypoxic condition the extracellular pH around the inflamed cells become acidic in nature due to expulsion of protons generated through the glycolytic, pentose phosphate pathway. Extracellular acidic pH and intracellular alkaline pH results in development of proton gradient across the plasma membrane of inflamed cell. Due to preceding mechanism, protons from extracellular site flows into intracellular site and reduces intracellular pH and activate endoplasmic protein SREBP-1c. Through activation, SREBP-1c translocate to the nucleus resulting in activation of sterol response element (SRE) which in turn enhance expression of protein involved in fatty acids synthesis like FASN and SCD (Furuta et al., 2008).The major role of SREBP1 in controlling milk fat synthesis has been demonstrated in bovine mammary epithelial cells. In the present study, ALA-NS decreased the HIF-1, SREBP-1c, and FASN with increased PHD2 expression in LPS treated rats demarcating regulation of hypoxia and milk fat synthesis by the same.

Moreover, the activation of NFkB, ROS, and HIF-1 altered the apoptotic pathway (Minet et al., 2000, Royds et al., 1998, Simon et al., 2000). To investigate the same, we performed the western blot analysis of the pro-apoptotic (BAX and BAD) and anti-apoptotic (Bcl-2 and Bcl-xl) regulators (Elmore, 2007). Treatment with ALA-NS reduced anti-apoptotic proteins, with visa-versa effect on the pro-apoptotic proteins. VDAC play a controversial role in the cell death mechanism. The mitochondrial apoptotic changes are further associated with

decreased expression of VDAC, with concomitant release of cytochrome c. The western blot analysis confirmed that the increased expression of VDAC with concomitant decreases in cytochrome-c expression after the ALA-NS treatment. Once released from mitochondria, cytochrome-c binds with Apaf-1 and procaspase-9 to form adduct termed, apoptosome. Apoptosome formation accounts for the decreased cytosolic levels of Apaf-1 and the same was evident after the treatment with ALA-NS. ALA-NS increased the level of caspase-3 and 8 and thereby accrediting apoptosis.

Subsequently the study was extended to investigate the effects of ALA-NS against the cases of subclinical mastitis. Previous studies have reported increase in milk pH in case of subclinical mastitis (Sena and Sahani, 2001). Additionally, field based tests including WST, BBT and CMT are also used to confirm subclinical mastitis. All the animals selected for the study were scored positive for subclinical mastitis on the basis of field based tests. Treatment with marketed cefaperazone and ALA-NS demarcated significant effect on milk pH along with WST, BBT, and CMT score.

Subclinical mastitis is an inflammatory condition of udder leading to leakage of cellular components in milk and exudation. Such exudation and cellular infiltration, increase SCC in milk which is an important indicator for mastitis (Viguier et al., 2009). Increased SCC is associated with reductions in milk protein casein, lactose; augmented enzymatic activity; and reduced quality and yield of dairy products (Forsbäck et al., 2010). A report suggested a positive correlation between SCC and CMT (Kaithwas et al., 2011). The CMT reagent reacts with leukocytes (somatic cells) infiltrated in milk after bacterial infection and forms a thick gel. The intensity of gel formation is directly proportional to number of leukocytes in milk. Most positive CMT reactions points towards abnormally high SCC. In the current study the SCC and CMT scores were in-line with previous reports and were normalized after treatment

with ALA-NS (Kaithwas et al., 2011). Treatment with ALA-NS reduced the TMC in milk suggesting reduced bacterial load and antimicrobial efficacy of the formulations in question. It would be important to put on records that ALA has been very well cited in literature as an antinflammatory, analgesic and antipyretic by peripheral actions. In addition, ALA has also been reported to curtail vascular leakage and cellular infiltration, which could have accounted for improved udder condition of the treated animals. In addition ALA has also been reported to have antibacterial properties particularly against the mastitis causing pathogens. All in all, NSAIDs like and antimicrobial properties of ALA could be the key players.

Encouraged with the above, authors considered it worth to study the effect of ALA-NS on milk quality and bacterial resistance using more stringent markers. Several bacteria (gram positive and gram negative) actuate the inflammatory reaction through the release of significant amount of pro-inflammatory cytokines (TNF-α, IL). Pro-inflammatory cytokines activate NF-κB in epithelial cells present at the site of infection. Several studies have correlated the role of NFκB-p65 homodimers in bacterial mediated chronic inflammation. In current study, higher expression of NFκB-p65 was observed in the milk of mastitis affected cows. This observation is in-line with a previous report, suggesting correlation between increased expressions of NF-κB in milk cells and development of mastitis (Boulanger et al., 2003). Consequently, it may be derived that raised NFκB-p65 activity detected in milk cells from mastitis-affected cows is due to direct and/or indirect invasion of these pro-inflammatory cells by pathogens. Treatment with ALA-NS (F1 and F2) decreased the NFκB-p65 expression more profoundly than cefaperazone. Furthermore, there are evidence that bacteria alters the milk fat synthesis in mastitis, by altering the stearoyl-CoA desaturase 1 (SCD1) (an important enzyme for the fat catabolism). The SCD1 expression is stimulated under lipid synthesis by transcription factors, notably SREBP-1 (Xu et al., 2016). The

SREBP-1 is synthesised and reserved as a membrane-bound precursor in the endoplasmic reticulum and stimulate gene transcription for lipid synthesis (Eberlé et al., 2004). In the present study, we observed upregulated SREBP-1c expression in the mastitic milk, suggesting increased lipid biogenesis through bacterial infection. However, treatment with ALA-NS curtailed the SREBP-1c expression in comparison to cefaperazone. All in all, ALA-NS demarcated a more favorable response upon inflammatory (NFκB-p65) and milk quality (SREBP-1c) markers in comparison to cefaperazone treatment, which is in-line with previous finding (Pyörälä, 2003).

MDR of antibiotics is an important concern contemplated with the therapy of mastitis. A previous study showed that proteins like UCHL-1 and P-glycoprotein (P-gp) play important role in MDR. The UCHL-1 belongs to the UCH protease family that deubiquitinates ubiquitin-protein conjugates in the ubiquitin-proteasome system (Wang et al., 2016) . Whereas, P-glycoprotein (P-gp) is one of the first members of the ATP-binding cassette (ABC) transporter which acts as a physiological barrier by extruding antibiotics and responsible for the suboptimum response (Sharom, 2011). P-gp inhibitors enhances the activity of antimicrobial agents like azithromycin, and erythromycin (Seral et al., 2003). A previous study has suggested that the overexpression of UCHL-1 is responsible for the augmented activity of P-gp in cells which could be co-related with MDR (Jin et al., 2015). In the current study, we observed overexpression of UCHL-1 in mastitis affected cow milk and treatment with ALA-NS decreased the UCHL-1 expression. Therefore, authors can postulate that the formulation have additional advantage to combat the chances of MDR.

CHAPTER-5

SUMMARY AND CONCLUSION

Bovine mastitis is an inflammatory condition of mammary gland of lactating animals, characterized by pain, oedema, swelling, and polymorph neutrophils (PMN) infiltration. Mastitis is curse for dairy industry as it decreases the productivity and quality of milk, and increase the cost of herd management. Currently, antibiotics either alone or in combination with nonsteroidal anti-inflammatory agents (NSAIDs) are most commonly prescribed for clinical management of bovine mastitis. However, long term use of antibiotics causes bacterial resistance and have negative impact on consumer health. Therefore, alternative safer drugs with universal effectiveness, lasting benefits and fewer side effects is requisite in the area of mastitis management.

Polyunsaturated fatty acids (PUFA's) are the set of fatty acids with vital pharmacological activities. Many characteristic seed oils and few nuts comprises large quantity of PUFA's. Linseed (flaxseed) oil normally comprises of 45–55% of ω-3 PUFA's as alpha linolenic acid [ALA (ω-3:18-3)], while soybean oil, rapeseed oil, and walnuts generally contain ~10% of PUFA's as ALA (ω-3:18-3). ω-3 and ω-6 PUFA's are biologically more important fatty acids.

PUFA's and corresponding metabolites have extensive variety of physiological activity, including cell membrane structure uniformity, signalling, and regulation of proteins expression. ω-3 fatty acids are perhaps one of the most suitable examples of how diet may affect inflammation. ω-3 fats exert a remarkable variety of biological responses many of which affect inflammation and clinical conditions related to its presence. α- Linolenic acid (ALA; 18:3) is a ω-3 PUFA and is transformed into anti-inflammatory eicosanoids through series of desaturation and elongation reactions. It would be interesting to comment that the *L. usitattissimum* fixed oil, containing

57.38% of ALA (ω-3:18-3) has been found to exhibit anti-inflammatory, analgesic, and antimicrobial activity. Furthermore, the same has been reported to exhibit significant efficacy against subclinical cases of bovine mastitis in a field based study. Considering the same, we selected the ALA as the lipid source for formulation with the cefotaxime. Total eight number of ALA based intra-mammary nanosuspension (ALA-NS) were formulated, optimized and evaluated. Only two formulation (ALA-NS; F1 and F2) were found stable after optimization. Furthermore, particle size, zeta potential, polydispersity index, sedimentation volume, stability and sterility testing were performed for both ALA-NS (F1 and F2) formulation.

Moreover, ALA-NS (F1 and F2) were evaluated for antimicrobial activity against mastitis causing pathogens like e.g., *Staphylococcus aureus* (ATCC-29737), *Streptococcus agalactiae* (ATCC-13813), *Staphylococcus epidermidis* (ATCC-12228), *Escherichia coli (*ATCC-8739, MTCC-118) *Lactobacillus sporogenes* (ATCC-31284), *Pseudomonas aeruginosa* (ATCC-25619) and *Candida albicans* (ATCC-10231). ALA-NS (F1 and F2) showed better antimicrobial activity and lower minimum inhibitory concentration (MIC) value than cefotaxime and ALA.

Considering the same, therapeutic efficacy of ALA-NS (F1 and F2) was analyzed against lipopolysaccharides (LPS) induced mastitis in female albino Wistar rats. ALA-NS (F1 and F2) were also evident for the regulation of oxidative markers like decreases the thiobarbituric acid reactive species (TBARs) and protein carbonyl level with restoration of superoxide dismutase (SOD), catalase and glutathione enzymes along with restoration of histological architecture by decreasing the inflammatory cells (leucocytes) infiltration, adipose tissue, and thickening the

alveolus walls in comparison to control. Subsequently, ALA-NS (F1 and F2) also restored altered nitric oxide and hydrogen sulphide level after LPS administration.

When perceived through more stringent biomarkers through western blot analysis. ALA-NS (F1 and F2) downregulated the inflammatory markers [nuclear factor kappa (NFκBp65), cyclooxygenase (COX), lipoxygenase (LOX), interferon- γ (IFN-γ), hypoxia and fatty acid synthesis markers [hypoxia-inducible factor-1 (HIF-1), prolyl hydroxylase-2 (PHD-2), sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase (FASN) in LPS induced mastitis in rats. Inflammation in mastitis leads to cell death and therefore we further evaluated the apoptotic marker in mammary gland tissue. ALA-NS (F1 and F2) favorably regulated the apoptotic markers and therefore endorsed apoptosis.

Furthermore, the ALA-NS (F1 and F2) was scrutinized through on field base study on subclinical mastitic cows. Nine mix-breed cows were divided into three groups and subjected to treatment for ALA-NS (F1 and F2) and cefoperzone intramammary suspension for 10 days. Subclinical mastitis on day 1 was confirmed through field based tests like pH, california mastitis test (CMT), white side test (WST), and bromothymol blue test score (BBT). The milk sample were collected on 1st, 5th and 10th days of study for further evaluation. The mastitic milk was also recorded for the increased somatic cell count (SCC) and total microbial count (TMC) on day 1. Subsequently, treatment with ALA-NS (F1 and F2) decreased the milk pH along with CMT, WST, and BBT score after 10th days of treatment.

Treatment with ALA-NS (F1 and F2) demarcated a significant effect on field based parameters along with curtailment of TMC, and SCC. The efficacy of ALA-NS (F1 and F2) was further affirmed using more stringent markers for inflammation (NFκB-

p65), milk quality (SREBP-1c) and bacterial resistance (UCHL-1) in milk samples. The treatment with ALA-NS decreased the expression of NFκB-p65, SREBP-1c, and UCHL-1 after 10 day of treatment.

To conclude, the treatment of mastitis majorly depends upon antibiotics and NSAIDs. However, antibiotics have several limitations like resistance and secreted in milk. The current study demonstrated that the intramammary injection of ALA-NS have anti-inflammatory and antibacterial effects with efficacy against sub-clinical mastitis. ALA being peripheral analgesic also subsides the pain, providing additional benefit. Apparently, anti-inflammatory, antibacterial, peripheral analgesic properties of ALA-NS could be accounted for the therapeutic efficacy of proposed regime.

CHAPTER-6

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Yadav, R. K., Singh, M., Roy, S., Ansari, M. N., Saeedan, A. S. & Kaithwas, G. 2018. Modulation of oxidative stress response by flaxseed oil: Role of lipid peroxidation and underlying mechanisms. *Prostaglandins & other lipid mediators*.

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ANNEXURE-I

ANIMAL APPROVAL CERTIFICATE

S. D. College of Pharmacy & Vocational Studies

(Approved By AICTE, PCI, New Delhi & Affiliated to U.P. Technical University, Lucknow)

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Ref. No. : SDCOP&VS/AH/CPCSEA/01/0039 Dated 06/05/2014

INSTITUTIONAL ANIMAL ETHICAL COMMITTEE (IAEC)

REG. No. 876/ac/05/CPCSEA Dated 13/09/2004 UNDER THE RULE 13 OF THE "BREEDING OF AND EXPERIMENTS ONANIMALS·(CONTROL AND SUPERVISION) RULE 1998"

DATE: 06/05/2014

Approval No.
SDCOP&VS/AH/CPCSEA/01/0039

CERTIFICATE

This is to certify that Mr./Ms./Mrs. **Rajnish Kumar Yadav** a student of M.Pharm / Ph.D. is permitted to carry out experiments for dissertation / thesis work entitled **"Formulation optimization and pharmacological evaluation of lipid based therapeutic system for bovine mastitis"** as per the details mentioned and after the observing the usual formalities laid down by IAEC as per the provisions made by CPCSEA.

MEMBER SECRETARY **CHAIRMAN**

ANNEXURE-II

PLAGIARISM REPORT

URKUND

Document	Rajnish Kumar Yadav.docx (D40402930)
Submitted	2018-06-27 14:04 (+05:0-30)
Submitted by	shodhganga.bbau@gmail.com
Receiver	gbl.bbau.bbau@analysis.urkund.com
Message	Rajnish Kumar Yadav, Department of Pharmaceutical Sciences, rajnishyadavpharma@gmail.com Show full message
	2% of this approx. 18 pages long document consists of text present in 6 sources.

1.1 Mastitis The term "mastitis" is imitated from the Greek word "mastos" meaning breast and the suffix "itis" denoting inflammation. Thus, mastitis is illustrated as inflammation of the mammary gland (McDougall, 2002). According to international dairy federation, mastitis is an intra-mammary infection (IMI) of the parenchyma of mammary gland and that can be of an infectious, traumatic or toxic nature (International Dairy Federation, 1987). 1.2 Types of mastitis Straightforward classification perceive mastitis as: 1.2.1 Clinical mastitis Clinical mastitis is symbolized by the presence of inflammatory signs (swelling, heat, redness, and pain) along with anomalous texture and milk discoloration, flakes or clots in the milk, swelling, increased temperature and pain in the mammary gland (Philpot and Nickerson, 1991). 1.2.2 Sub-clinical mastitis Sub-clinical mastitis is delineated by diversification in milk architecture with no signs of inflammation or milk abnormalities, but the somatic cell count (SCC) is inflated with bacterial participation. Subclinical mastitis accounts the colossal financial loss to dairy farms through submerged milk production (Crist et al.1997). Nonetheless, diagnosis of the subclinical cases of mastitis has been an inquisitive task due to paucity of clinical correlation (Hiitiö et al., 2017). 1.2.3 Chronic mastitis Chronic mastitis is an inflammatory proceeding that has endured for months, and may prevail from one lactation to another lactation. Chronic mastitis for the utmost part prevail as sub-clinical but may illustrate periodical flare ups. The execution of the diagnosis for clinical mastitis is analogously easy through clinical interrelation (Boutet et al., 2003). 1.3 Pathophysiology of mastitis Augmentation of bacteria in the milk fertile tissues results in mastitis. Microorganisms causing mastitis can breach the udder by passing over the teat duct and augment inside the duct, or by physical migration resulting from pressure placed on the teat end. The teat canal is the first line protective cover of the mammary gland. The keratin lining in the teat canal cater a physical and chemical impediment against microbial penetration (Capuco et al., 1992). Infection occurs when bacteria get admittance to the mammary gland and overthrow the anatomical defence. The main changes in the udder includes; increased permeability of the epithelial membrane leading to effusion of ions, proteins and enzymes from blood to milk along with invasion of phagocytic cells into the milk compartment

ANNEXURE-III

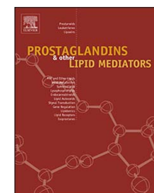
LIST OF PUBLICATION

Prostaglandins and Other Lipid Mediators 135 (2018) 21–26

Contents lists available at ScienceDirect

Prostaglandins and Other Lipid Mediators

journal homepage: www.elsevier.com/locate/prostaglandins



Review

Modulation of oxidative stress response by flaxseed oil: Role of lipid peroxidation and underlying mechanisms

Rajnish Kumar Yadav[a], Manjari Singh[a], Subhadeep Roy[a], Mohd. Nazam Ansari[b], Abdulaziz S. Saeedan[b], Gaurav Kaithwas[a,*]

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ARTICLE INFO

Keywords:
Polyunsaturated fatty acids
Lipid peroxidation
Inflammation
Enzymatic degradation
Cancer

ABSTRACT

Polyunsaturated fatty acids (PUFA's) are majorly classified as ω-3 and ω-6 fatty acids. The eicosapentaenoic acid (EPA, ω-3:20-5), docosahexaenoic acid (DHA, ω-3:22-6) and alpha-linolenic acid (ALA, ω-3:18-3) are known ω-3 fatty acids, extracted from animal (e.g fish oil) and plant sources (e.g flaxseed oil). Furthermore, linoleic acid (LA, ω-6:18-2) is recognized as ω-6 fatty acid and the most prominent biological fatty acid with a pro-inflammatory response. Flaxseed oil has variety of biological roles, due to the significant amount of ω-3/ω-6 fatty acids. Numerous studies have reported that ALA (ω-3:18-3) and LA (ω-6:18-2) has diverse pharmacological activities. The ALA (ω-3:18-3) and LA (ω-6:18-2) are recognised to be the pharmacological antagonist. For example, ALA (ω-3:18-3) is recognised as anti-inflammatory, whereas LA (ω-6:18-2) is considered to be pro-inflammatory. PUFA's get oxidized in three ways; firstly, free radical-mediated pathway, secondly non-free radical non-enzymatic metabolism, and lastly enzymatic degradation. The present report is an attempt to summarize various modes of PUFA's metabolism and elaborate biological effects of the associated metabolites concerning flaxseed oil.

1. Introduction

Polyunsaturated fatty acids (PUFA's) are the group of fatty acids with vital biological activities. Numerous distinctive seed oils and few nuts contain extensive quantity of PUFA's. Linseed (flaxseed) oil normally comprises of 45–55% of ω-3 PUFA's as alpha linolenic acid [ALA (ω-3:18-3)], while soybean oil, rapeseed oil, and walnuts generally contain ~10% of PUFA's as ALA (ω-3:18-3) [1,2]. Flaxseed oil is obtained from *Linum usitatissimum* seeds either through cold press method or by chemical extraction using petroleum ether. Along with ALA (ω-3:18-3), flaxseed oil contains ω-6 fatty acids [linoleic acid, (LA, ω-6:18-2)] (8–29%) and ω-9 fatty acids [oleic acid, (ω-9:18-1)] (12–30%) [3]. Other natural substances like lignans, cyanogenic glycosides, and cyclic peptides are also reported in flaxseed oil. However, the major part of the biological activities have been accredited to the constituents like ALA (ω-3:18-3) and LA (ω-6:18-2) [4].

PUFA's and respective metabolites have wide range of physiological roles, including cell membrane structure uniformity, signalling, and regulation of proteins expression [5]. PUFA's are susceptible to the oxidative reactions and can form by-products that can modulate the functions of biomolecules such as proteins, amino acids, and phospholipids to produce a diverse array of molecular adducts. It has been previously reported that lipid peroxidation of PUFA's causes alteration in integrity, fluidity, permeability, and generates potential toxic products. The peroxidation by-products of PUFA's have been studied for their carcinogenic and mutagenic potential [6,7]. Recent findings claimed that PUFA's by-products exhibit dual activity depending upon conditions, such as cytotoxic and cytoprotective, pro- and anti-inflammatory, pro- and anti-apoptotic, and pro- and anti-atherogenic and many more in line [8–10]. The pro and antioxidant effects of per-oxidation by-products have been arbitrated beneath dual activities. Considering the same, it was contemplated worth to elaborate the various biological activities of flaxseed oil/PUFA's in literature and elaborate the possible mechanism beneath the same. Efforts are also made to find possible reasons for the diverse biological responses and mechanism beneath the same.

2. Pharmacological activities of flaxseed oil

Flaxseed oil showed distinctive anti-inflammatory, analgesic, and

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https://doi.org/10.1016/j.prostaglandins.2018.02.003
Received 2 August 2017; Received in revised form 8 February 2018; Accepted 8 February 2018
Available online 13 February 2018
1098-8823/ © 2018 Elsevier Inc. All rights reserved.

XII

Synopsis on the Biological Activities of L. usitatissimum (Flaxseed/Linseed) Oil and Possible Mechanism Beneath the Same

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Abstract

L. usitatissimum (Flaxseed/Linseed) is an annual herb belonging to the family Linaceae. It contains imperative ω-3- fatty acids like alpha linolenic acid (ALA) and linoleic acid (LA). Seed oil possesses many important pharmacological activities such as anti-inflammatory, anti-arthritic, antipyretic, antidiabetic, anticancer, antioxidant properties in respective animal models. The oil also diminished the pro-inflammatory mediators in anti-arthritic model. Oil is reported to have significant efficacy against bovine mastitis in cow. Most of the pharmacological activities of LUFO have been accredited to the antioxidant/pro-oxidant along with anti-inflammatory potential of abundant polyunsaturated fatty acids (in particular alpha linolenic acid) present in seed oil. The polyunsaturated fatty acids/LUFO can exert their biological effects by three different mechanisms, including non-enzymatic free radical mediated degradation; non free radical enzymatic metabolism and lastly through non enzymatic non radical oxidation. The LUFO/ ALA get oxidised by free radicals in comparison to arachidonic acid, when supplemented exogenously and thereby imparting themselves

3 Biotech (2018) 8:240
https://doi.org/10.1007/s13205-018-1250-y

ORIGINAL ARTICLE

Antidiabetic activity of mefloquine via GLP-1 receptor modulation against STZ–NA-induced diabetes in albino wistar rats

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Received: 7 June 2017 / Accepted: 23 February 2018
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Abstract

Mefloquine was retrieved as a glucagon -like peptide-1 receptor agonist and, therefore, evaluated for its antidiabetic potential against non-insulin-dependent diabetes mellitus (NIDDM) in experimental animals. NIDDM was induced by single intraperitoneal injection of streptozotocin and nicotinamide (60 + 110 mg/kg) in albino wistar rats. The experimental animals were scrutinised for electrocardiographic (ECG) and heart rate variability (HRV) factors to study the autonomic dysfunction along with blood glucose, serum insulin, and liver glycogen levels for glycemic control. Simultaneously, antioxidant markers (TBARs, protein carbonyl, GSH, SOD, catalase) and inflammatory markers (COX, LOX, NO) were scrutinized as well. Oral administration of mefloquine normalised the heart rate with favourable regulation of time and frequency domain HRV parameters. Mefloquine restored the blood glucose, serum insulin, and liver glycogen levels favourably in diabetic rats. Treatment with mefloquine curtailed the antioxidant markers with favourable regulation of inflammatory signals. Mefloquine was also found to be less hepatotoxic in contrast to the standard metformin, providing an integrated advantage as an antidiabetic agent.

Keywords Streptozotocin · Nicotinamide · Non-insulin-dependent diabetes mellitus · Mefloquine · Heart rate variability · Electrocardiography

Introduction

The glucagon-like peptide-1 receptor (GLP-1R) gene resides on chromosome 6 and the protein encoding for this gene is a glucagon receptor family of G protein-coupled receptor (Dillon et al. 1993). The GLP-1R has an explicit binding affinity for GLP-1 and glucagon with the noteworthy appearance in pancreatic β cells (Hölscher 2014). The activation of GLP-1R leads to stimulation of adenyl cyclase (AC) pathway, which subsequently catalyses the conversion of adenosine tri-phosphate (ATP) to 3,5-cyclic AMP (cAMP). The cAMP appears as a regulatory signal via specific cAMP binding protein/transcription factor/enzyme (kinase) (Idevall-Hagren et al. 2010). GLP-1R can distress the glucose homeostasis in manifold ways; primarily, GLP-1 can facilitate the Ca^{2+}-dependent exocytosis of insulin through cAMP-dependent pathway intervened by protein kinase A (PKA), which phosphorylates the secretory granule-associated protein (Nakazaki et al. 2002). Moreover, GLP-1 can also modulate the cAMP-regulated guanine nucleotide exchange factor which subsequently promotes phosphatidyl inositol 4,5-bisphosphate (PIP2) hydrolysis and intracellular Ca^{2+} ion mobilisation (Seino and Shibasaki 2005; Garber 2011).

All in all, this research has demonstrated GLP-1 as an insulin secretagogue and in the same line class of drug has been developed called GLP-1R agonist/incretin mimetics. Recently, GLP-1R agonists are most prominently used for the treatment of type-2 diabetes due to low risk of hypoglycaemia in comparison with sulfonylureas and meglitinides (Sivertsen et al. 2012). However, currently GLP-1R agonist (e.g. exenatide, liraglutide) have been reported to have adverse effects such as increase in heart rate (HR), renal impairment/renal failure, acute pancreatitis, and trophic

Mohd Nazam Ansari and Abdulaziz S. Saeedan have contributed significantly towards the finalization of the manuscript.

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Published online: 04 May 2018

Other Publication during research work

Swetlana Gautam……**Rajnish Kumar Yadav**…..Gaurav Kaithwas, "DuCLOX-2/5 Inhibition Attenuates Inflammatory Response and Induces Mitochondrial Apoptosis for Mammary Gland Chemoprevention" Frontiers in pharmacology, 2018.

Subhadeep Roy…..**Rajnish Kumar Yadav**…..Gaurav Kaithwas, "Alpha-linolenic acid stabilize HIF-1 α and downregulate FASN to promote mitochondrial apoptosis for mammary gland chemoprevention" *Oncotarget*, 2018.

Swetlana Gautam……**Rajnish Kumar Yadav**….Gaurav Kaithwas "Rifaximin, a pregnane X receptor (PXR) activator regulates apoptosis in a murine model of breast cancer" *RSC advances*, 2018.

Subhadeep Roy…..**Rajnish Kumar Yadav**……Gaurav Kaithwas "GLA supplementation regulates PHD2 mediated hypoxia and mitochondrial apoptosis in DMBA induced mammary gland carcinoma" *The international journal of biochemistry & cell biology*, 2018.

Vidhata Rani…….**Rajnish Kumar Yadav**…….Gaurav Kaithwas "Effects of minocycline and doxycycline against terbutaline induced early postnatal autistic changes in albino rats" *Physiology & behaviour*, 2018, 183, 49-56.

Shreesh Raj Sammi……**Rajnish Kumar Yadav**…….Gaurav Kaithwas, "Galantamine attenuates N, N-dimethyl hydrazine induced neoplastic colon damage by inhibiting acetylcholinesterase and bimodal regulation of nicotinic cholinergic neurotransmission" European journal of pharmacology, 2018, 818, 174-183.

Abdulaziz S Al-Saeedan……**Rajnish Kumar Yadav**……Gaurav Kaithwas "Revisiting the systemic lipopolysaccharide mediated neuroinflammation: Appraising the effect of l-cysteine mediated hydrogen sulphide on it" *Saudi pharmaceutical journal*, 2018, 26(4), 520-527.

Saeedan AS……..**Yadav RK**…… Kaithwas G "Effect of early natal supplementation of paracetamol on attenuation of exotoxin/endotoxin induced pyrexia and precipitation of autistic like features in albino rats" *Inflammopharmacology,* 2018, 26(4):951-961.

Sneha Yadav…..**Rajnish Kumar Yadav**…..Gaurav Kaithwas. Comparative efficacy of alpha-linolenic acid and gamma-linolenic acid to attenuate valproic acid-induced autism-like features. *J Physiol Biochem,* 2016.

Uma Devi…..**Rajnish Kumar Yadav**…..Gaurav Kaithwas "Experimental Models for Autism Spectrum Disorder Follow-Up for the Validity" *Review Journal of Autism and Developmental Disorders*, 2016, 3:4, 358-376.

Rakesh K. Mishra….. **Rajnish Kumar Yadav**….. Gaurav Kaithwas "Palonosetron attenuates 1,2-dimethyl hydrazine induced preneoplastic colon damage through downregulating acetylcholinesterase expression and up-regulating synaptic acetylcholine concentration" *RSC Adv*.,2016,6,40527**.**

Chetan Manral……**Rajnish Kumar Yadav**……Gaurav Kaithwas **"**Effect of β-sitosterol against methyl nitrosourea-induced mammary gland carcinoma in albino rats" *BMC Complementary and Alternative Medicine,* 2016.

Virendra Tiwari…..**Rajnish Kumar Yadav**….Gaurav Kaithwas. Redefining the role of peripheral LPS as a neuroinflammatory agent and evaluating the role of hydrogen sulphide through metformin intervention. *Inflammopharmacology,* 2016 24(5):253-264.

Asha Rani,….., **Rajnish Kumar Yadav,** ……Gaurav Kaithwas "α-Chymotrypsin regulates free fatty acids and UCHL-1 to ameliorate N-methyl nitrosourea induced mammary gland carcinoma in albino wistar rats", *Inflammopharmacology*, 2016, 24:5, 277-286.

Book Chapter

Rajnish Kumar Yadav, Gaurav Kaithwas. "A Synopsis on the Biological activities and underlying mechanism for *L. usitassimum* (Flaxseed/Linseed) fixed oil" Book Chapter, *Springer*, 2018, Accepted.

Conference and Seminar

Presented scientific paper entitled "Hepatoprotective activity of the Ethanolic Root extract of *Tabernaemontana coronaria* in Albino Wistar rats" in 2nd Pharm. Tech IAPST International Conferences, Jadavpur University Kolkata, 2014.

Presented scientific paper at International seminar on "Nano formulations and Translational Research. Small getting Bigger in Oral presentation section. 2015.