"PHARMACOLOGICAL EVALUATION OF ACETYL-11-α-KETO-β-BOSWELLIC ACID MEDIATED NANO SILVER IN EXPERIMENTAL MURINE MASTITIS"

By

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Thesis Submitted to the

SRI VENKATESWARA VETERINARY UNIVERSITY

in partial fulfilment of the requirements
for the award of the Degree of

MASTER OF VETERINARY SCIENCE

IN THE FACULTY OF VETERINARY SCIENCE

(VETERINARY PHARMACOLOGY AND TOXICOLOGY)

DEPARTMENT OF PHARMACOLOGY & TOXICOLOGY
COLLEGE OF VETERINARY SCIENCE

TIRUPATI – 517 502 (A.P.) INDIA

MARCH 2013

CERTIFICATE

This is to certify that the thesis entitled **"PHARMACOLOGICAL EVALUATION OF ACETYL-11-α-KETO-β-BOSWELLIC ACID MEDIATED NANO SILVER IN EXPERIMENTAL MURINE MASTITIS"** submitted in partial fulfillment of the requirements for the degree of "MASTER OF VETERINARY SCIENCE" of Sri Venkateswara Veterinary University is a record of the bonafide research work carried out by Y.MURALIDHAR under my guidance and supervision. The student's Advisory committee has approved the subject of the thesis.

No part of the thesis has been submitted for any other degree or diploma. The author of the thesis has duly acknowledged all the assistance and help received during the course of investigation.

(Dr.K.ADILAXMAMMA)

Chairman of the Advisory committee

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DECLARATION

I**, Y.MURALIDHAR,** hereby declare that the thesis entitled **"PHARMACOLOGICAL EVALUATION OF ACETYL-11-α-KETO-β-BOSWELLIC ACID MEDIATED NANO SILVER IN EXPERIMENTAL MURINE MASTITIS"** submitted to Sri Venkateswara Veterinary University for the Degree of "**MASTER OF VETERINARY SCIENCE**" is a result of original research work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

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Place : Tirupati.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
β	:	Beta
%	:	Per cent
<	:	Less than
µg	:	Micro gram
^{0}C	:	Degrees Celsius
γ	:	Gamma
nm	:	Nanometer
µl	:	Microlitre
µm	:	Micrometer
mm^2	:	millimeter square
G	:	Gram(s)
Hr	:	Hour
L	:	Liter(s)
M	:	Molarity
mg	:	Milli gram
pH	:	-Log of hydrogen ion concentration
ml	:	Milli liter(s)
N	:	Normality
Kg	:	Kilo gram
ANOVA	:	Analysis of variance
β-BA	:	Beta boswellic acid
Aβ-BA	:	Alpha beta boswellic acid
KBA	:	Keto boswellic acid
AKBS	:	Acetyl-11-α-keto-β-boswellic acid
AKBANS	:	Acetyl-11-α-keto-β-boswellic acid Nano Silver
Cfu/ml	:	Colony forming units per milliliter
DLS	:	Dynamic light scattering
ppm	:	Parts per million

DTNB	:	5′dithiobis-2-nitrobenzoic acid
EDTA	:	Ethylene Diamine Tetra acetic acid
FT-IR	:	Fourier transform infrared spectroscopy
GSH	:	Reduced Glutathione
H & E	:	Hematoxylin and Eosin
MDA	:	Malondialdehyde
MIC	:	Minimum inhibitory concentration
MTT	:	(3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetra zolium bromide)
O.D.	:	Optical Density
ROS	:	Reactive oxygen species
RPMI	:	Roswell Park Memorial Institute medium
rpm	:	Revolutions per minute
SEM	:	Scanning electron microscope
SD	:	Standard Deviation
SOD	:	Superoxide dismutase
SPSS	:	Statistical Package for Social Sciences
TBARS	:	Thiobarbituric acid reacting substances
Cm^2	:	Centi meter square
Vs	:	Volt second

ACKNOWLEDGEMENTS

I deem it is a privilege to place on record my gratitude to my Chairman and Professor, Department of Pharmacology, ***Dr. K. Adilaxmamma****, for her unstinting generosity, moral support extended during the entire tenure of my study and untiring contribution to finalize this manuscript. I could never have embarked and started all of this without her prior teachings in Pharmacology. Honestly, no words can be in exaggeration for her timely help during crucial periods of this project. My inexplicable gratitude goes to her for invaluable suggestions and affectionate encouragement throughout the tenure of the study.*

I register my sincere sense of gratitude to ***Dr.G.Srinivasa Rao****, Professor, Department of Veterinary Pharmacology, for his constructive counsel and expertised opinion. His perpetual energy and enthusiasm in research had motivated all his advisees, including me. Besides, he sets an example of a world-class researcher for his rigor and passion on research.*

I am grateful from the bottom of my heart for having ***Dr.Ch.Srilatha****, Professor, Department of Veterinary Pathology, as a member in my advisory committee, who is always accessible and willing to help students with her research. Her constructive practical suggestions contributed a lot for my research.*

I gratefully acknowledge ***Dr. M.Alpha Raj,*** *Assistant Professor, Department of Veterinary Pharmacology for his advice, supervision, crucial contribution and incessant encouragement, which made him a backbone of this research. He has been my guide, my philosopher and above all my friend. His involvement with his originality has triggered and nourished my intellectual maturity that I will benefit from, for a long time to come. His ability to trouble shoot problems is amazing. His flawless planning, limitless support and encouraging guidance helped to sail through the work with lesser pains. His eagerness to help at every stage of my work is gratefully remembered. Thank you very much for everything, Sir.*

I would like to place on record my heartfelt thanks to ***Dr. T.V.N.K. Prasad,*** *Assistant Professor, Department of Soil Science, Acharya N. G. Ranga Agricultural University, for his generous help in synthesis and characterization of silver nanoparticles and co-operation extended throughout the study.*

I duly acknowledge ***Dr.P.Eswaraprasad,*** *Professor and* ***Dr.Padmaja,*** *Associate Professor, Department of Veterinary Biochemistry for their inspiration and co-operation during the course of study.*

I hereby thank ***Dr. P. Anandkumar****, Associate Professor and Head, Department of Veterinary Microbiology, Gannavaram, for providing the pure cultures to carry out my research.*

With a deep sense of gratitude I remember the teachings and help rendered by my teachers ***Dr. G. Venkatanaidu,*** *Professor, Veterinary Gynaecology and Obstetrics,* ***Dr. Jagapathiramayya,*** *Associate Professor, Veterinary Anatomy,* ***Dr. M. Srinivas,*** *Associate Professor, Veterinary Gynaecology and Obstetrics,* ***Dr. N. Lakshmirani,*** *Associate Professor, Veterinary Medicine,* ***Dr. D. Ashok kumar****, Assistant Professor, Department of Veterinary Biochemistry for their support during the study.*

I also hereby like to acknowledge, ***Dr.Kalyan****,* ***Dr.Amruth Kumar, Dr.Srikala, Dr.Devi*** *for the help extended by them in innumerable ways.*

I also like to acknowledge ***Mr. Ismail,*** *animalbiotechnology department, S. V. University for providing ELISA reader and help extended by him in many ways.*

I also extend my gratitude to my seniors ***Satish, Dilleswararao, Siva Prasad, Sai dilip gupta*** *for the everlasting and memorable company they provided.*

I warmly remember the co-operation and support of my colleagues ***Sreevani, Navtej, Aparna*** *during the course of my study.*

I owe a special boquet of thanks to my dear friends ***Lokesh, Rajesh, Santosh, Teja, Ramana, Nabeel, Vinod, Pulliah, D.N.Raju, Siviah, Amar*** *for their lovely company and support throughout my stay. I would like to mention the names of* ***Hemanth, Dinesh, Tejakalyan, Shafi, Sivanathredd****y among the* ***many juniors*** *for the great company they provided.*

T.V.Chaiyanya Kumar, *my friend, colleague, Dept of Veterinary Biochemistry has always been a wonderful colleague all along my work and for the ways he has helped me, a mere thanks would just not be sufficient.*

This piece of work is actually the reflection of my friendship with ***Sravanthi*** *and* ***Srividya****. They have helped me stay sane through these difficult days. Their friendship was like light house whenever I lose my way. Their support and care helped me overcome setbacks and stay focused on my postgraduate study. I greatly*

value their friendship and I deeply appreciate their belief in me. I have become a better person because of the mirror they hold up for me.

I find myself at loss of words to thank to my sister and brother-in-law ***Vandana & Kalyan,*** *for helping me get through the difficult times, and for all the emotional support, camaraderie, entertainment, and caring they provided.*

I sincerely acknowledge to my sisters and brother-in-laws, ***Sailaja & Naidu, Renu & Shankar.*** *Whatever I do, whatever I am, it is because of their Love, care and affection.*

I sincerely acknowledge to my aunt and uncle ***Devi & Ramana****. I have no suitable word that can fully describe their everlasting love to me. I remember their constant support when I encountered difficulties.*

No words can ever express my heartfelt gratitude to my fiancé ***Dr.Anusha,*** *whose constant and cheerful support helped me to overcome all the hurdles throughout the study.*

My deepest gratitude goes to my parents ***Sridhar*** *and* ***Suryalatha,*** *sister and brother-in-law* ***Sirisha & Suresh*** *for their unflagging love and support throughout my life; this thesis is simply impossible without them. They are the reason I did this; they are the reason I thrive to be better. They had always encouraged me to follow my heart and inquisitive mind in all my decisions. This thesis would certainly not have existed without their constant source of support.*

I also wish to thank the staff of the Department of Veterinary Pharmacology & Toxicology for their service. I also thank ***Hari****,* ***M/s. Bros Scientifics****, for timely providing the equipment throughout my degree programme.*

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Title of the Thesis	:	**"PHARMACOLOGICAL EVALUATION OF ACETYL-11-α-KETO-β-BOSWELLIC ACID MEDIATED NANO SILVER IN EXPERIMENTAL MURINE MASTITIS"**
Degree to which submitted	:	**MASTER OF VETERINARY SCIENCE**
Faculty	:	**VETERINARY SCIENCE**
Discipline	:	**VETERINARY PHARMACOLOGY & TOXICOLOGY**
Major Advisor	:	**Dr. K.ADILAXMAMMA**
University	:	**SRI VENKATESWARA VETERINARY UNIVERSITY**
Year of submission	:	**2013**

ABSTRACT

Acetyl-11-α-keto-β-Boswellic acid mediated nanosilver (AKBANS), was synthesized, characterized and evaluated in *Staphylococcus aureus* induced murine mastitis model. The characterization of AKBANS by UV visible absorption spectrum showed a maximum absorption around 200 nm and SEM images showed that AKBANS with irregular and spherical morphology of size 363 to 574 nm. The size of the particles measured by DLS technique was 262 nm. The results of FT-IR analysis for AKBANS showed the involvement of hydroxyl, carboxyl, amines, keto and nitrile groups in the synthesis of AKBANS. The MIC of the compound was found to be 3.6 ng/ml against *Staphylococcus aureus* showed an *in vitro* spleenocyte viability of more than 95% at the highest concentration of 87.5 ppm. No toxicity was found in the oral dose even at the limit dose.

A total of 40 female mice of 10-15 days post partum were utilized for the study. The animals were divided into five groups of eight animals each. Group I served as lactating control, groups II to V were inoculated with 20 µl of 24h broth culture of *S.aureus* containing 4.0 x 10^5 cfu/quarter (Log_{10} 5.60 cfu/quarter) under ketamine ananestheisa using 33G blunt hamilton needle. After 6h post inoculation, groups III and IV received 20 µl of AKBANS through intramammary and intra peritoneal routes respectively. Whereas, group V received Cefepime & tazobactum combination @ 1mg/kg body weight through intraperitoneal route. After 24 h post inoculation, 0.5ml of whole blood was collected from tail vein and serum was separated for the estimation of CRP. Later, the mice were euthanized and L4 mammary gland was collected under asceptic conditions into sterile eppendorf tubes. The mammary weights were recorded and tissue samples for histopathology and ultrastructural studies were collected in 10% neutral buffered formalin and 5% glutaraldehyde respectively. A 10% homogenate of the mammary glands was prepared in 0.5M phosphate buffer (pH 7.4) and was utilized for the evaluation of bacterial load and antioxidant parameters like TBARS, GSH, SOD and CAT.

All the groups inoculated with *S.aureus* showed significantly higher mammary weights, however, the bacterial load was found to be significantly higher in mastitis group II. Treatment with both AKBANS and antibiotic showed significantly reduced bacterial loads compared to mastitis control. Similarly, group II showed significantly elevated levels of CRP, which were significantly reduced in AKBANS treatment groups III and IV and antibiotic treated group V. However, AKBANS showed significant reduction compared to antibiotic treatment.

The malondialdehyde content measured as TBARS was significantly elevated in groups II and V compared to group I. Treatment with AKBANS in groups III and IV did not show any significant improvement compared to mastitis control. The levels of reduced glutathione and SOD activity showed no significantly difference among all groups. However, the activity of catalase, which was significantly increased in mastitis group II was reduced with AKBANS treatment in groups III and IV.

The histopathological examination of the mammary glands in mastitis group II showed heavy infiltration of PMN cells within and between acini along with desquamation and vacuolation of the acinar epithelium. Both the AKBANS treated groups III and IV showed significant reduction in the infiltration of PMN to the extent of 95% with very mild changes in the mammary tissue. The antibiotic treated group V showed a moderate reduction of infiltration of PMN to the extent of 40-50%.

Ultrastructure of mammary gland from AKBANS treatment groups III and IV indicated moderate progress in the architecture of the secretory epithelial cells showing reconstituting mitochondria and normal inter alveolar septa.

In conclusion, AKBANS was found to possess powerful antibacterial effect which was superior to Cefepime and Tazobactum combination and further, also possessed profound anti-inflammatory effect in staphylococcal mastitis. Hence, keeping in view the economics of production, safety and efficacy of the compound, AKBANS could provide a promising alternative to the use of traditional antibiotics for the treatment of bovine mastitis.

CHAPTER I

INTRODUCTION

Mastitis is defined as the inflammation of mammary gland resulting in reduction of the quantity and quality of milk produced (Brouillete and Malouin, 2005). Clinical mastitis was reported to be the most prevalent disease in dairy animals and the second most common cause for death and reason for culling worldwide (NAHMS, 2007). In the Indian context, subclinical mastitis was reported to be more important with a prevalence varying from 10-50% in cows and 5-20% in buffaloes compared to clinical mastitis (1-10%).

Bovine mastitis is considered as the major constraint for the growth of dairy industry both in India and abroad causing heavy losses in terms of reduced quality and quantity of milk besides incurring the cost of treatment (Ruegg, 2003). The annual economic loss due to mastitis was estimated to be Rs.16,702 million in India and $35 billion worldwide (Mubarack *et al*., 2011).

The most common microorganisms responsible for intramammary infections include Staphylococcus, Streptococcus and Coliforms. *Staphylococcus aureus* was reported to be the leading cause of mastitis worldwide (Reugg, 2003) and also for noscomial and community acquired infections. The annual economic loss caused by Staphylococcal bovine mastitis globally was estimated to be $2 billion (Schmelcher *et al*., 2012).

Further, *S.aureus* was reported to be the main reason for the use of antibiotics in dairy cows (Mitchell *et al*.,1998).

Staphylococcus aureus causes both clinical as well as sub-clinical mastitis, with the latter being more common, which can convert into chronic condition resulting in failure of conventional antimicrobials (Wilson *et al*., 1997, Sears and McCarthy,2003 and Zecconi *et al*., 2003). The pathogenesis of *S.aureus* mastitis was mainly attributed to the production of Staphylococcal Enterotoxin A (SEA) (Friedman *et al*., 2011). The major challenges encountered in *S.aureus* mastitis lies in the presence of structures which help in avoiding phagocytosis, aid in tissue penetration and immune cell recruitment (Dinges *et al*., 2000). The situation is further complicated with increasing number of multi-drug resistant (MDR) strains of *S.aureus* (Chen *et al*., 2012).

In this scenario, alternatives to traditional antimicrobial agents were suggested not only to overcome the pathogen but also to reduce antibiotic residues in food animals and for preventing emergence of MDR bacteria (Gruet *et al*., 2001) and countering the deleterious effect of antibiotics on immune cells (Hoeben *et al*., 1997).

Silver nanoparticles owing to the characteristics like reduced size and increased surface area (Yeo *et al*., 2003) provide enormous hope for use in biological systems by virtue of broad spectrum antimicrobial activity (Lok *et al*., 2006, Gogoi *et al*., 2006 and Sap-Iam *et al*., 2010) which includes bactericidal action against both Gram positive and Gram negative bacteria including MDR strains (Alt *et al*., 2004; Baker *et al*., 2005; Morones *et al*.,

2005 and Ip *et al*., 2006). Further, the development of bacterial resistance has not been reported against silver nanoparticles (Neu, 1992 and Stewart and Costerton, 2001) making it a good choice for therapeutic consideration in mastitis.

The additional advantage with silver nanoparticles resides in the fact that they are relatively non-toxic and safe antibacterial agents (Dowling *et al*., 2003) requiring much smaller concentrations for antimicrobial effect (Panacek, 2006 and Kvitek *et al*., 2009). Further, the biological synthesis of nanoparticles using plant sources (phytogenic) is advantageous over other methods of synthesis in being cost effective, practically non-toxic and eco friendly (Aymonier *et al.,* 2002 and Sun and Xia, 2002).

Hence, for the present study, nanosilver particles derived phytogenically from β-Boswellic acid were considered. β-Boswellic acid, the major constituent of Boswellia species was reported to have powerful anti-inflammatory effect (Ammon *et al*., 1991; Krieglstien *et al*., 2001 and Qurishi *et al*., 2010) besides the ability to decrease infiltration and migration of polymorpho-nuclear leukocytes (Sharma *et al*., 1988 and Sharma *et al*., 1989).

In the present study, the antimicrobial and anti-inflammatory effects of silver nanoparticles phytogenically derived by the reduction of silver nitrate using 11-α-keto- β-Boswellic acid were investigated in *Staphylococcus aureus* induced murine mastitis with the following objectives.

1. Development and standardization of murine mastitis model.
2. Synthesis and characterization of nano silver particles using acetyl 11α-keto-β-Boswellic acid (AKBA).
3. Evaluation of therapeutic potential of synthesized Nanosilver particles using acetyl-11α -keto-β-Boswellic Acid in murine mastitis model.

CHAPTER II

REVIEW OF LITERATURE

2.1 Staphylococcal Mastitis

Bovine mastitis is major disease affecting high yielding dairy cows and is an inflammation of the mammary gland frequently resulting from *Staphylococcus aureus* colonization (Sears and McCarthy, 2003). Despite emergence of drug resistant *S.aureus,* the pathogen is usually susceptible to many antibiotics *in vitro* (De Oliveira *et al.,* 2000; Werckenthin *et al.,* 2001; Erskine *et al.,* 2002). However, the major difficulty encountered with *S. aureus* is the persistence of the organism in mammary gland due to the ability to survive within host phagocytes and mammary epithelial cells (Hebert *et al.,* 2000).

Staphylococcus aureus can provoke clinical mastitis but more frequently causes subclinical infections that tend to become chronic and difficult to eradicate by conventional antimicrobial therapies (Sears and McCarthy., 2003) The frequent incapacity of both the immune response and antibiotics to overcome infection constitutes a major challenge to dairy producers in staphylococcal mastitis. The intracellular component of infection is believed to play a significant role in the persistence of *S. aureus* during antibiotic therapy for different types of infections, including bovine mastitis (Kerro *et al.,* 2002). Antimicrobial agents fail to counter the organism *in vivo* due to lack of penetration into host cells in adequate concentration (Sanchez

et al., 1988; Gruet *et al.,* 2001). Hence, mere *in vitro* evaluation of an antimicrobial agents can not suggest *in vivo* efficacy against *S.aureus*. This problem can be successfully solved by the use of murine mastitis model, as the organism is found within phagocytes and also in epithelial cells of the mouse tissue (Chandler *et al*., 1980; Brouillette *et al.,* 2003).

2.2 Murine mastitis model

A mouse model of infectious mastitis was first described by Chandler (1970) and was later utilized for assessing the physiopathology of *S.aureus* intra mammary infection (Reid *et al.,* 1976; Anderson, 1976 and 1978; Chandler *et al.,* 1980; Brouillette *et al.,* 2003), for understanding the role of potential bacterial virulence factors (Jonsson *et al.,* 1985; Brouillette *et al.,* 2003), for evaluation of protection conferred by immunization (Gomez *et al.,* 1998; Mamo and Froman 1994 and Mamo *et al.,* 2000) and evaluation of antibiotic administration (Anderson and Craven, 1984; Bramley and Foster, 1990; Sanchez *et al.,* 1994).

2.2.1 Murine mastitis model - advantages

Murine mastits model was reported to offer numerous advantages (Chandler, 1970 and Brouillette *et al.,* 2003, 2004 & 2005) as summerized below

- The mammary gland of mouse is anatomically and genetically similar to bovines with each gland being separate with single teat canal.
- Murine mastitis model offers cost effective alternative to research in cows or goats.

- Experimental mastitis in the mouse provides a useful model for fundamental studies and for screening of new antimicrobial agents.
- *Staphylococcus* and cell interactions observed consequent to bacterial inoculation in mouse mammary gland like PMN infiltration and tissue damage were reported to be similar to those found in the cow.
- Murine mastitis model also provides suitable environment for the growth of bacteria and hence helps in studying the organism.
- The experiments with staphylococci showed that mastitis could be produced readily and by a comparatively small number of organisms.
- The model is reported to be very suitable for detection of degrees of conferred artificial immunity and the protective or curative effects of pharmaceuticals.
- The appearance and histology of the affected mammary glands resembles mastitis produced in the bovine species by the respective bacterial species concerned.
- The size of the mouse mammary gland is also very convenient for histological and other studies requiring examination of the whole gland.
- Experimental mastitis in mouse, also proved as a convenient model for ultra-structural pathology studies. In addition, suitable specimens for electron microscopy could be obtained with ease, together with material for ancillary studies, and there appeared a good conformity and distributions of the lesion throughout the mammary gland.

- Uninoculated mammary glands examined at corresponding periods showed a variety of cell changes associated with involution and constituted an essential baseline or control in these studies.

- The small size of the mouse permits extensive screening of promising antimicrobial compounds, given that numerous animals can be infected. Moreover, relatively small quantities of compounds are necessary to carry out efficacy studies, and this is a major advantage over bigger animals, as limited supplies are available during the initial screening stages.

- Adherence of bacteria to the mammary gland cells was conferred by FnBPs, which prevent *S. aureus* from washing out during suckling. As opposed to *in vitro* adherence studies, the lactating mouse model of mastitis provides conditions that better represent the natural environment found during an infection (presence of serum and milk), incorporates physical factors such as suckling, and takes into account the complexity of inflammation and immune reactions that occur during infectious mastitis.

- Differential virulence expression of *S.aureus* grown in different media can be confirmed by murine mastitis model (Mamo *et al.,*1991) which strongly influences the pathogenesis. The knowledge is crucial for the development of new therapeutic treatments.

- The murine mastitis model provides a representative growth environment composed of milk and serum proteins, and it allows interaction of the pathogen with host mammary cells and with immune

components present or recruited during the infection. Such a complex environment is impossible to recreate *in vitro*, and accordingly, the mouse model of *S. aureus*-induced mastitis constitutes a unique alternative, which can be employed in any standard laboratory equipped with animal care facilities.

2.3 Pathology of *Staphylococcus aureus* induced murine mastitis

The pathology of *S.aureus* induced mastitis in mouse was described by Chandler and Anderson (1975). It was reported that gross cellular destruction, vacuolation of the cytoplasm of secretary epithelial cells, formation of cytosegresomes and increased numbers of densely staining cells with clefts in the cytoplasm were observed when a highly pathogenic strain was used. The characteristic features of a highly pathogenic strain was the appearance of areas of necrosis with complete loss of structure of the secretory epithelial cells. Whereas gross cellular destruction was not observed when a less pathogenic strain was inoculated. The mastitis produced by the less pathogenic strain of staphylococcus was not characterized by necrosis but the number of dense cells, often possessing several cytoplasmic clefts, and the number of cells with large intracytoplasmic vacuoles were observed was in greater number.

Phagocytosis of cocci by macrophages was observed along with milk protein because of which it was concluded that no special recognition for cocci exists for the macrophages. Cocci, of the more pathogenic strain tend to be confined to the acinar lumen, without any particular relationship to other structures.

The intracellular location of the staphylococci protects them against the action of the antibiotic (Chandler,1969). It was reported that the organisms enter intracellulary within 48 h post inoculation in mice (Anderson, 1977). The organisms were found in epithelial cells, macrophages and neutrophils of mammary glands and escaped the effects of antibiotics. The entry of staphylococci within epithelial cells seems to be part of the autophagocytic process of involution (Sekhri, *et al.,*1967) and the presence of staphylococci in neutrophils and macrophages was reported to be typical of a chronic reaction to infection (Cameron,1962).

2.4 Recent strategies against staphylococcal infections

Rowson *et al.,* (2011) studied the ability of a commercially available feed additive (OmniGen-AF) to reduce mammary infections caused by a single strain of mastitic pathogens (*Streptococcus uberis, Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae*) and to examine the effects of the additive on markers of mammary immunity. They reported that the additive increased the mammary inflammatory response and increased antigen presentation during a mammary infection.

Friedman *et al.,* (2011) evaluated the ability of the pure olive compound 4-hydroxytyrosol and a commercial olive powder called Hidrox-12, prepared by freeze drying olive juice, to inhibit *S. aureus* bacteria and SEA's biological activity. Two independent cell assays (BrdU incorporation into newly synthesized DNA and glycyl-phenylalanyl-aminofluorocoumarin proteolysis) demonstrated that the olive compound 4-hydroxytyrosol also inactivated the biological activity of SEA at concentrations that were not toxic to the spleen

cells. The results suggest that food-compatible and safe antitoxin olive compounds can be used to inactivate both pathogens and toxins produced by the pathogens.

Chen *et al.,* 2012 studied the protective effect of recombinant staphylococcal enterotoxin A entrapped in polylactic-co-glycolic acid microspheres against *Staphylococcus aureus* infection and reported that the recombinant staphylococcal enterotoxin A entrapped in polylactic-co-glycolic acid microspheres potentially be used as a vaccine against enterotoxigenic *S. aureus*.

Schmelcher *et al.,* 2012 evaluated two fusion proteins consisting of the streptococcal λSA2 endolysin endopeptidase domain fused to staphylococcal cell wall binding domains from either lysostaphin (λSA2-E-Lyso-SH3b) or the staphylococcal phage K endolysin, LysK (λSA2-E-LysK-SH3b) on mastitis causing *Staphylococcus aureus* in murine mastitis model. They reported that the killing of 16 different *S. aureus* mastitis isolates, including penicillin-resistant strains, by both constructs and in murine mastitis reduced gland wet weights and intramammary tumor necrosis factor alpha (TNF-α) concentrations, which serve as indicators of inflammation. The fusion peptidoglycan hydrolases have the potential as antimicrobials for the treatment of *S.aureus*-induced mastitis.

2.5 C-reactive Protein (CRP)

C-reactive protein (CRP) was identified in 1930 and was subsequently considered to be an “acute phase protein,” an early indicator of infectious or inflammatory conditions (Clyne and Olshaker 1999). Since its discovery, CRP has been studied as a screening device for inflammation, a marker for disease activity, and as a diagnostic adjunct. Further concentration of CRP may rise many folds in response to tissue injury or infection. C-reactive protein (CRP) estimation is used as a specific method for the early detection of infection (Gewurz *et al.,* 1982), it is increased dramatically during inflammatory processes occurring in the body. This increment is due to a rise in the plasma concentration of IL-6, which is produced predominantly by macrophages (Pepys and Hirschfield, 2003) and as well as adipocytes (Lau *et al.,* 2005).

Kruger and Neumann, (1999) reported that cows with mastitis caused by *Streptococcus uberis*, had significantly elevated serum CRP levels, that were much higher than those of healthy lactating cows.

2.6 Role of oxidative stress and inflammation in mastitis

Oxidative stress caused due to production of oxidizing agents (Suriyasathaporn and Vinikekumnen, 2006; Lykkesfeldt & Svendsen, 2007) and uncontrolled inflammation (Sordillo and Aitken, 2009) are implicated in the pathogenesis of numerous diseases including mastitis. Mastitis develops in oxidative stress due to modification of pro-inflammatory gene expression (Aitken *et al.,* 2009) and in inflammation by release of cytotoxic radicals and pro-inflammatory cytokines from phagocytic cells (Knaapen *et al.,* 1999). Earlier research demonstrated a decrease in ascorbate concentration

(Kleczkowski *et al.,* 2005; Ranjan *et al.,* 2005), GSH concentration (Kizil *et al.,*2007) and GSH-Px activity (Erskine *et al.,* 1987), increase in lipid peroxides (Suriyasathaporn and Vinikekumnen, 2006; Lykkesfeldt and Svendsen, 2007), level of some cytokines (TNF-α, lL-1b, IL-6, IL-8) and other molecules such as nitric oxide (NO) (Riollet *et al.,* 2000; Notebaert *et al.,* 2008), total oxidant capacity and somatic cell count (Atakisi *et al.,*2010) during intra-mammary infections.

Nitric oxide which is an important mediator of inflammation and is produced in significant quantities by macrophages and epithelial cells of the mammary gland during mastitis (Bouchard *et al.,* 1999). Nitric oxide owes its antimicrobial activity by virtue of peroxynitrite, formed by interaction of nitric oxide with superoxide anions (Beckman *et al.,* 1990). This peroxynitrite radical is implicated in the development of oxidative stress (Chaiyotwittayakun *et al.,* 2002) by way of causing oxidation of long chain fatty acids in cell membranes leading to lipid peroxidation and formation of free radicals (Al-Sa'doni and Ferro, 2000; Wang *et al.,* 2002). The increase in lipid peroxidation further causes a decrease in levels of antioxidant molecules leading to oxidative stress (Komine *et al.,* 2004; Weiss *et al.,* 2004).

2.7 Boswellic acid

2.7.1 Source

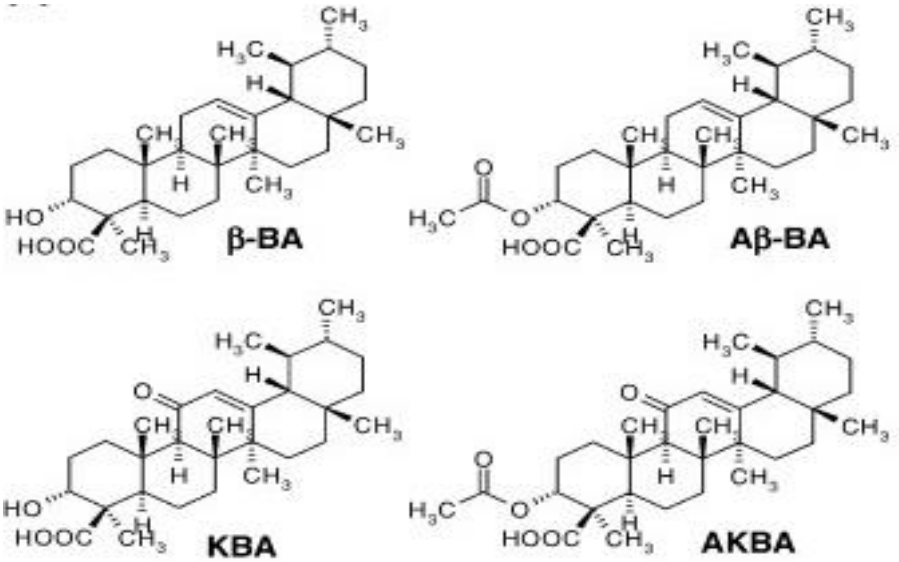
Boswellic acids (BAs), belonging to ursane group of triterpenoids (Kiela *et al.,* 2005) and are the major constituents of the gum derived from the plant *Boswellia serrata* Roxb. Ex Colebr. (family Burseraceae, Syn. *B. glabra*), commonly known by the names Salai guggal, white guggal, Indian olibanum

or dhup (Havel *et al.,* 2002).The presence of boswellic acids in almost all the species of *Boswellia* is a characteristic feature of the genus. The tri-terpenoids were synthesized through isopentenyl pyrophosphate pathway (IPP) from a squalene intermediate and are reported to possess diverse biological activities (Frank *et al.,* 2009) like antimicrobial activity (Farid *et al.,* 2003), anti-inflammatory effect (Safayhi *et al.,* 1992), anti-cancerous activity (Huang et al., 2000) and antiviral properties (Duan *et al.,* 2002).

Savithramma et. al., (2010) studied the phytochemical properties in stem bark and gum of *Boswellia ovalifoliolata* to improve the health states of local people and also to use in pharmaceutical and nutraceutical products of commercial importance.

2.7.2 Chemistry

The boswellic acids are organic acids, consisting of a pentacyclic triterpene structure with a carboxyl group and at least one other functional group. Boswellic acids occur in atleast six types. The alpha and beta forms of boswellic acid differ only in the triterpene structure but both have a general formula of $C_{30}H_{48}O_3$ and an additional hydroxyl group in common. In acetyl-alpha and acetyl-beta forms of boswelic acid, the hydroxyl group is replaced with an acetyl group and the general molecular formula is $C_{32}H_{50}O_4$. The other types of boswellic acids include the keto-boswellic acids and their acetyl counterparts. The structural requirements for boswellic acids indicated that of all the six acids, 3-O-acetyl-11-keto-β-boswellic acid (AKBA) showed most pronounced inhibitory activity against 5-LOX (Makare *et al.,* 2001). The structure of different forms of boswellic acids was depicted in fig 1.



2.7.4 Anti-inflammatory properties

Acetyl-boswellic acids also exhibit anti-inflammatory behaviour by inhibiting leukotriene synthesis (Reddy *et al.,* 1989, Safayhi *et al.,* 1992 and Ammon *et al.,* 1993). To be specific, it inhibits the activity of the enzyme 5-lipoxygenase (5-LOX) through a non-redox reaction. Boswellic acids do not affect 12-lipoxygenase and cyclooxygenase (COX) activities (Ammon *et al.,* 1993; Safayhi *et al.,* 1995; Safayhi *et al.,* 1997), the inhibition of which is known to result in multiple side effects like intestinal bleeding and impaired renal function (Fennerty, 2001 and Cuzzolin *et al.,* 2001). Hence, BA are superior over traditional NSAIDs. In addition, boswellic acids also inhibit leukocyte elastase, which may also contribute to the anti-inflammatory properties (Safayhi *et al.,* 1997; Kapil and Moza 1992).Clinical trials have investigated the effectiveness of boswellic acids in treating ulcerative colitis (Safayhi *et al.,* 1992; Ammon, 2002), on chemically induced colitis in mouse (Kiela *et al.,* 2005) and asthma (Gupta *et al.,* 1998).

Safayhi (1997) reporeted that boswellic acids inhibits human leukocyte elastase (HLE), which may be involved in the pathogenesis of emphysema. Human leukocyte elastase also stimulates mucus secretion and thus may play a role in cystic fibrosis, chronic bronchitis, and acute respiratory distress syndrome.

Ammon et al., (1991) tested boswellic acids *In vitro*, isolated from the gum resin of Boswellia, in a dose-dependent manner block the synthesis of proinflammatory 5-lipoxygenase products, including 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene B4 (LTB4).

Krieglstein et al., (2001) conducted a study to determine the efficacy of Boswellia extract and acetyl-11-keto-β-boswellic acid (AKBA), on leukocyte-endothelial cell interactions in inflammatory bowel disease. It was observed that Boswellia extract and both potencies of AKBA decreased rolling (up to 90%) and adherent leukocytes (up to 98%), attenuated tissue injury scores, and significantly reduced macroscopic and microscopic inflammation of the gut mucosa.

2.7.5 Antiproliferative or anticancer properties

Boswellic acids are also reported to have anticancer effects (Shao *et al.,* 1998; Glaser *et al.,* 1999; Jing *et al.,* 1999 and Winking *et al.,* 2000;). The boswellic acids were reported to be gaining prominence for their anti-proliferative effects (Safayhi *et al.,* 1992). Acetyl boswellic acids were found to be unique class of dual inhibitors of human topoisomerases I and II (Zhao *et al.,* 2003) and also produce immunomodulation (Syrovets *et al.,* 2000).

The anticancer effects of boswellic acids were reported to vary with the type of boswellic acid. Both keto and acetyl keto forms demonstrated significant effects on apoptosis where as boswellic acid alone is only effective in the inhibition of DNA biosynthesis. However, in terms of either anti-proliferative or apoptotic effects, the acetyl-keto form was reported to be more superior, followed by keto form and plain boswellic acid (Liu *et al.,* 2002).

Syrovets (2000) investigated the mechanism of action of acetyl-BA and show that these compounds were more potent catalytic inhibitors of human topoisomerases I and IIa than camptothecin, and amsacrine or etoposide, respectively.

Shao et al., (1998) Bhushan et al., (2007) and Huang et al., (2000) reported that Boswellic acids and triterpenoids from *Boswellia serrata* have an inhibitory and apoptotic effect against the cellular growth of leukemia HL-60 cells.

2.8 Nanosilver

Nanomaterials (such as nanotubes and nanorods) and nanoparticles are defined particles that having at least one dimension in the range of 1 to 100 nm (ASTM, 2006; Buzea, 2007). Nanomaterials are nanoparticles that have special physicochemical properties by virtue of their small size (Buzea, 2007).

2.8.1 History and Applications

Earlier, nanosilver or suspensions of nanosilver were referred to as colloidal silver and was used for the treatment many infections and illnesses (Nano Health Solutions, 2010).

2.8.2 Properties of nanosilver

Surface effects and quantum effects (Roduner, 2006) have great potential for medical applications.

2.8.2.1 Antibacterial properties

Nanosilver was reported to be effective killing agent against a broad spectrum of Gram-negative and Gram-positive bacteria (Burrell *et al.,* 1999; Wijnhoven *et al.,* 2009; Yin *et al.,* 1999), including antibiotic-resistant strains (Percival *et al.,* 2007; Wright *et al.,* 1998). Gram-negative bacteria include genera such as *Acinetobacter*, *Escherichia*, *Pseudomonas*, *Salmonella*, and *Vibrio*. *Acinetobacter* species are associated with nosocomial infections. Gram-positive bacteria include many well-known genera such as *Bacillus*, *Clostridium*, *Enterococcus*, *Listeria*, *Staphylococcus*, and *Streptococcus*. Antibiotic-resistant bacteria include strains such as methicillin-resistant and vancomycin-resistant *Staphylococcus aureus*, and *Enterococcus faecium*.

Silver nanoparticles are also known to enhance the antibacterial activity of various antibiotics (Shahverdi *et al.,* 2007). The antibacterial activities of penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin against *Staphylococcus aureus* and *Escherichia coli* increase in the presence of silver nanoparticles (Wijnhoven *et al.,* 2009). Size-dependent (diameter 1-450 nm) antimicrobial activity of silver nanoparticles has been

reported with Gram-negative bacteria (Baker *et al.,* 2005; Morones *et al.,* 2005 and Panacek*,* 2006) and Gram-positive bacteria (Panacek*,* 2006). Small nanoparticles with a large surface area to volume ratio provide a more efficient means for antibacterial activity even at very low concentration.

In addition to size and concentration, shape-dependent antimicrobial activity of silver nanoparticles has been shown with Gram-negative bacteria (Pal *et al.,* 2007). Silver nanoparticles of different shapes (spherical, rod-shaped, truncated triangular nanoplates) have been developed by synthetic routes. Truncated triangular silver nanoplates display the strongest antibacterial activity (Wijnhoven *et al.,* 2009). The top basal plane of truncated triangular silver nanoplates is a high-atom-density surface, i.e., a {111} facet. Generally, spherical silver nanoparticles (generally with cubo-octohedral, multiple-twinned decahedral, or quasi-spherical morphology) have {100} facets along with a small percentage of {111} facets, whereas rod-shaped silver nanoparticles (e.g., pentagonal rods) have side surfaces with {100} facets and end with {111} facets (Wijnhoven *et al.,* 2009 and Wiley *et al.,* 2005). Silver reactivity is favored by {111} facets (Hatchett & White, 1996).

Spherical silver nanoparticles with {111} facets attach directly to the bacterial surface of the cell membrane and are located inside bacteria (Morones *et al.,* 2005). The strong anti-bacterial activity of truncated triangular silver nanoplates could be due to their large surface area to volume ratios and their crystallographic surface structures.

2.8.2.2 Antibacterial mode of action

The antibacterial effect of nanosilver was reported to be due to the release of free silver ion (Nano letters). However, the exact bactericidal mechanism was only partially understood. Silver nanoparticles anchor to and penetrate the cell wall of Gram-negative bacteria (Sondi and Salopek-Sondi, 2004 and Morones *et al.,* 2005), leading to an increase in cell permeability, leading to an uncontrolled transport through the cytoplasmic membrane, and ultimately cell death.

Nanosilver, when in contact with bacteria and fungus, is known to adversely affect the cellular metabolism of the electron transfer systems, and the transport of substrate in the microbial cell membrane.It has also been proposed that the antibacterial mechanism of silver nanoparticles is related to the formation of free radicals and subsequent free radical-induced membrane damage (Danilczuk *et al.,* 2006 and Kim *et al.,* 2007).

Hwang *et al.* (2008) performed a study of stress-specific bioluminescent bacteria, based on a synergistic toxic effect of the silver nanoparticles and the silver ions that they produce. The ions move into the cells and lead to the production of reactive oxygen species. Because of the membrane damage caused by the nanoparticles, the cells cannot effectively extrude the silver ions and limit their effect. Based on the greater tendency of silver ions to strongly interact with thiol groups of vital enzymes and phosphorus-containing bases (Hatchett & White, 1996) and on the presence of silver nanoparticles inside the cells (Morones *et al.,* 2005), it is likely that further damage could be caused by interactions with compounds such as DNA. This interaction may prevent cell division and DNA replication from occurring, and also ultimately lead to cell death.

2.8.2.3 Anti-inflammatory properties

The anti-inflammatory effects of nanosilver dressings and nanosilver-derived solutions were reported by Nadworny *et al.* (2010). In animal models, it was demonstrated that nanosilver alters the expression of matrix metallo-proteinases (Kirsner *et al.,* 2001), suppresses the expression of tumor necrosis factor (TNF), interleukin (IL)-12, and IL-1, and induces apoptosis of inflammatory cells (Bhol & Schechter, 2005&2007). In addition, silver nanoparticles are known to modulate cytokines involved in wound healing (Tian *et al.,* 2007).

2.8.3 Green Synthesis of nanoparticles

Many techniques like physical and chemical methods are employed for the development of silver nanoparticles. However, the physical methods (Begum *et al.,* 2009) are highly expensive and chemical methods are harmful to the environment (Yang *et al.,* 2007). Besides, the chemical synthesis is also known to lead to the formation of agglomerations. Therefore, there is a growing need to develop eco-friendly nanoparticles that do not use toxic chemicals in the synthesis (Elumalai *et al.,* 2010). The recent reports include the synthesis of nanoparticles using microorganisms (Nair *et al.,* 2008), enzymes (Willner *et al.,* 2006), plant or plant extract (Shankar *et al.,* 2004) and medicinal plants (Mukunthan *et al.,* 2011 and Prasad *et al.,* 2011).

The green synthesis of AgNPs is characterized by three main steps (Raveendran *et al.,* 2003).

(1) Selection of solvent medium

(2) Selection of environmentally benign reducing agent, and

(3) Selection of nontoxic substances for the AgNPs stability

The reducing property of different plant constituents plays an important role in the reduction of silver ion to silver nanoparticles (Shankar *et al.,* 2003). There have been several reports on the synthesis of Ag-NPs using medicinal plants such as *Basella alba*, *Helianthus annus*, *Saccharum officinarum*, *Oryza sativa*, *Sorghum bicolour*, *Zea mays* (Leela and Vivekanandan, 2008), *Aloe vera (Chandran et al., 2006)*, *Medicago sativa* (Alfalfa) (Torresdey *et al.,* 2002), *Capsicum annuum* (Li *et al.,* 2007), *Magnolia kobus (Song et al., 2009)*, *Cinnamomum camphora* leaf (Huang *et al.,* 2007), and *Geranium* sp. (Shankar *et al.,* 2004) and from *Vitex negundo* L. leaf extract in water solution with heat treatment (Prabhu *et al.,* 2010).

2.9 Histopathology

Histopathological sections of mammary glands from the experimental groups has been evaluated to study the signs of development of inflammatory responses in mastitis groups and the extent to which the treatments were successful in reducing the inflammatory responses and the number of bacterial colonies.

Chandler, (1970 a) studied experimental induction of mastitis in mice using *Staphylococcus aureus, Streptococcus agalactiae, Corynebacteriurn pyogenes, and Escherichia coli.* Histopathological sections of mammary

glands induced with *S. aureus* showed acute mastitis characterised by neutrophil infiltration, early necrosis, and the presence of numerous cocci.

Bramley *et al*., (1989) evaluated the roles of alpha-toxin and beta-toxin in virulence of *S. aureus* for the mouse mammary gland. In his work the histopathological sections of mammary glands inoculated with *staphylococci* were associated with neutrophils which were predominately within alveoli. They also showed the secretory alveolar cells which were vacuolated and also distinguishable alveoli were observed.

Anderson *et al*., (1976) has studied mastitis, induced by *Mycoplasma* in murine model. The histopathological sections of the mastitis mammary gland showed degenerative changes in the epithelium of the alveoli and ducts, reduction in lactation tissue relative to normal lactating mammary glands, and infiltration of neutrophils showing pyknotic changes in larger alveolar ducts were observed. Few inflammatory cells were seen in the interalveolar fat and around the involuted alveoli.

2.11 Ultrastructure:

The ultrastructure of normal lactating gland of mice was first reported by (Sekhri et al., 1967) and the sections showed following features.

1) Secretory epithelial cells together with myoepithelial cells interspersed regularly at the base of the epithelium.

2) A basement membrane was discerned, below which the inter-alveolar tissue was rich in collagen fibrils, small blood vessels and fibroblasts.

3) Milk constituents were seen in many acini in the form of milk protein as dense particles, with characteristic mottled surface seen at high magnifications, and milk fat as larger, irregular, less densely stained masses with a boundary of cell membrane and sometimes associated with portions of cell cytoplasm.

4) The secretory epithelial cells showed differentiation into two types, one dense and one lighter in appearance. The dense type was rich in organelles and in many cases, protein secretory particles located either in individual vesicles or grouped in larger vesicles. The apical borders possessed microvilli and showed a close association with milk protein particles suggestive of secretory activity.

5) The lighter type of cell contained fewer organelles with occasional protein secretory particles and while the apical border when intact showed microvilli.

The ultrastructure of mammary gland inoculated with *staphylococci* was reported by Chandler, 1970 and the sections showed following features.

1) Gross cellular destruction with complete loss of normal architecture, nuclei and cell boundaries, together with major alteration of identifiable organelles such as mitochondria.

2) Vacuolation of epithelial cell cytoplasm, cytosegresomes containing mitochondria, large portions of cytoplasm in the lumens of acini and packing together of luminal milk protein particles suggestive of stasis or coagulation.

CHAPTER - III

MATERIALS & METHODS

An experiment was planned to study the efficacy of Acetyl-11-α-keto-β-bosweliic acid mediated phytogenic nanosilver particles in experimental murine mastitis and the following materials and methods were adopted**.**

3.1 INSTRUMENTS

- UV-Vis Spectrophotometer
- Laminar flow chamber
- BOD incubator
- Finn micropipettes
- Tissue homogenizer with speed regulator
- Laboratory centrifuge (Remi)
- Hamilton needles 33G

3.2 CHEMICALS

All chemicals were procured from SRL Pvt, Ltd, Mumbai and SD fine chemicals and were of analytical grade (AR).

Staphylococcus aureus culture was obtained from Department of Veterinary Microbiology, N.T.R. College of Veterinary Science, Gannavaram.

Cefepime and tazobactum (Epime - TZ Cefepime 1000 mg - Tazobactam 125 mg, United Biotech Private Limited, India).

3.3 Acetyl-11-α-keto-β-bosweliic acid (AKBA)

Acetyl-11-α-keto-β-bosweliic acid was obtained from Natural remedies, Pvt. Ltd, Bengaluru and was utilized for the preparation of nanosilver.

3.4 SYNTHESIS OF ACETYL-11-α-KETO-β-BOSWELLIC ACID MEDIATED SILVER NANOPARTICLES (AKBANS)

The Acetyl-11-α-keto-β-bosweliic acid (Natural remedies) was purchased and was stored in the refrigerator at 2-8^{o}c. Two percent Acetyl-11-α-keto-β-bosweliic acid solution was prepared with the dissolution of appropriate quantities in the distilled water and made up to 100 ml. 0.1M silver nitrate solution was prepared and 90 ml of which was added to 10 ml of 2 percent Acetyl-11-α-keto-β-bosweliic acid solution at 95^{0}c with vigorous stirring. Then change in colour of the solution was observed from pale yellow to brown which indicates the formation of the Acetyl-11-α-keto-β-bosweliic acid mediated silver nanoparticles (AKBANS). The prepared solution was cooled to the room temperature normally and finally particles were allowed to settle for 24 hrs. The solution was then changed to a plastic container for further characterization. Synthesis and characterization of Acetyl-11-α-keto-β-bosweliic acid mediated silver nanoparticles were carried out at Frontier Technology Labaratory, Regional Agricultural Research Station, Tirupati.

3.5 CHARACTERISATION OF SILVER NANOPARTICLES

3.5.1 UV-Visible Spectra Analysis

The reduction of pure Ag^{+} ions was monitored by measuring the UV-Visible spectrum of the reaction medium at 5 hr after diluting a small aliquot of

the sample into distilled water. UV-Visible spectral analysis was done by using UV-VIS spectrophotometer UV-2450 (Shimadzu).

3.5.2 SEM Analysis of Silver Nanoparticles

SEM (Scanning Electron Microscope) analysis was done using Hitachi S-4500 SEM machine. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid was allowed to dry by putting it under a mercury lamp for 5 minutes.

3.5.3 DLS Technique-Particle Size Measurement

DLS (Dynamic Light Scattering) analyzes the velocity distribution of particle movement by measuring dynamic fluctuations of light-scattering intensity caused by the brownian motion of the particle. This technique yields a hydrodynamic radius or diameter, which is calculated using the Stokes-Einstein equation from the above mentioned measurements. The measurements were carried out using the instrument Nanopartica SZ-100 (HORIBA).

3.5.4 Zeta potential

Zeta potential is a measure of the stability of the particles. It also indicate the interaction strength of a given particle with it's surroundings. Nanopartica SZ-100 (HORIBA) was used in zeta mode with an electrode voltage of 3.4V at temperature 25^0C to carryout zeta potential studies.

3.5.5 Fourier Transform Infrared Spectroscopy (FT-IR analysis) Measurements

FT-IR analysis was carried out on TENSOR-27 (BRUCKER) in the diffuse reflectance mode operated at a resolution of 4 cm^{-1} in the range of 400 to 4000 cm^{-1} to evaluate the functional groups that might be involved in nanoparticle formation.

3.6 *IN VITRO* EVALUATION OF NANOPARTICLES

3.6.1 Anti-Bacterial Activity

3.6.1.1 Minimum inhibitory concentration (MIC) (Geert Huys, 2002)

Tube dilution method was adopted to determine the MIC of the test compounds.

Turbidity standard for inoculum preparation

- A 0.5 ml aliquot of 1.175% (w/v) $BaCl_2$. $2H_2O$ is added to 99.5 ml of 0.18 mol/l H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.
- The correct density of the turbidity standard should be verified by using a spectrophotometer to determine the absorbance. The absorbance at 625 nm should be 0.08 to 0.10 for the 0.5 McFarland standards.

Preparation of inoculums

Staphylococcus aureus isolated from cases of bovine mastitis which was subjected to cultural, biochemical and molecular characterization at Department of Veterinary Microbilogy, N.T.R. College of Veterinary Science, Gannavaram was used for the study. Inoculum was prepared from cultures in nutrient broth and were incubated at 37^oC for 18 h and the count was

standardized to 0.5 McFarland unit.. This results in a suspension containing approximately 1 to 2 x 10^8 CFU / ml for *S.aureus.* This inoculum is used both for the determination of minimum inhibitory concentration (MIC) and for the induction of mastitis in mice.

Method

- 10 sterile test tubes were taken.
- 1ml of the test compound was added to first and second tubes and to the rest of tubes 1 ml of sterile normal saline was added.
- Two fold serial dilutions of the test compound was made from the second tube and 1ml was discarded from the last tube.
- 5 ml sterile nutrient broth was added to all the test tubes.
- Then 50 µl of standardized overnight broth culture was added to all the tubes.
- The tubes were incubated for 18 hrs at 37^oC.
- The end point was defined as the lowest concentration of the test compound at which there was no visible growth. The growth in the tubes was compared to that with positive and negative controls.
- The lowest concentration of the test compound inhibiting the growth of the organisms is recorded as MIC.

  Positive control – 1 ml culture + 5 ml broth

  Negative control – 1 ml sterile normal saline + 5 ml broth

  Compound control – 1 ml test compound + 5 ml broth

3.6.2 Cytotoxicity Study (MTT Assay) (Mosmann, 1983)

Principle

MTT is water soluble, yellow coloured tetrazolium salt that enters the cells and passes into mitochondria, where it is reduced to an insoluble purple formazan. The cells are then solubilized with an organic solvent and the released formazan is measured spectrophotometrically. Since the reduction of MTT occurs in metabolically active cells, the level of activity is a measure of cell viability.

Reagents

- RPMI – 1640 (Roswell Park Memorial Institute Medium)

  RPMI 1640 (with HEPES & L-Glutamine) – 16.4 g

  Sodium bicarbonate – 2.10 g

  Sodium pyruvate – 110.1 mg

  Penicillin – 61 mg

  Streptomycin – 100 mg

Dissolve the above ingredients in one litre of autoclaved and cooled triple glass distilled water. Adjust the p^H to 7.2 with the help of 1 N NaOH and sterilize using 0.22 µm millipore filter.

- Ammonium chloride lysing reagent

  Ammonium chloride – 8.30 g

  Potassium bicarbonate – 0.10 g

  EDTA – 0.03 g

Dissolve the above in about 800 ml of distilled water and make up the volume to one litre with distilled water. Adjust the p^H to 7.2 with the help of 1 N NaOH.

- MTT Solution

  Dissolve MTT at 5 mg/ml in RPMI-1640 and filter through 0.22 µm millipore filter.

Procedure

Isolation of spleenocytes from mice

- Collect the spleen from mice and add 2 ml of RPMI in a petridish.
- Remove the plunger from 2 ml syringe and with its black rubber end, mash the spleen and release the spleenocyte suspension into petridish.
- Transfer this suspension into 15 ml centrifuge tube. Wash the petridish several times for maximum recovery of spleenocytes and make up the full volume of the tube with RPMI.
- Centrifuge at 400 g for 10 minutes and discard the supernatant.
- Resuspend the cell pellet in 2 ml of RPMI. Add 10 ml of ammonium chloride lysing reagent and leave for 15 minutes at room temperature in a dark place.
- Centrifuge at 400 g for 5 minutes and discard the supernatant.
- Resuspend the cells completely and wash again with RPMI. Centrifuge at 400 g for 5 minutes and discard the supernatant.

- Resuspend the cells in RPMI. RPMI-1640 is supplemented with 10% fetal bovine serum.
- The viability of cells is assessed by using 0.4% trypan blue.
- Adjust the cell density to 1×10^7 cells/ ml using haemocytometer chamber. If necessary, dilute the cell suspension with RPMI.

Assessment of cell viability

- Add 100 µl of RPMI to the wells in 96 well ELISA plate.
- Add 100 µl of AKBANS to the first well.
- Make two fold serial dilution of the test compound.
- Add 100 µl of cell suspension to all the wells.
- Incubate the ELISA plate for 16 hr at 37ºC in a CO_2 incubator.
- Add 10 µl of MTT to all the wells 4 hr before the completion of incubation time.
- Centrifuge the plate at 1200 g for 10 minutes and discard the supernatant.
- Add 100 µl of DMSO to dissolve the formazan formed.
- After 10 minutes read the absorbance at 530 nm with an ELISA reader.

3.7 *IN VIVO* SAFETY EVALUATION

3.7.1 Acute Oral Toxicity Testing

Acute oral toxicity test was conducted by Up and Down procedure according as per OECD guidelines 425. Healthy adult albino wistar female

rats of 8-12 weeks age and weighing 200-250 g were used as experimental animals. The animals were caged in a solid bottom polypropylene cage and were managed as per OECD guidelines. The rats were fasted overnight prior to dosing of the compound. Acetyl-11-α-keto-β-bosweliic acid mediated nanosilver (AKBANS) was produced in a solution form with a concentration of 175 ppm. The maximum recommended oral dose volume of 20 ml/kg body weight, which equates to 3.5 mg/kg body weight, was taken as the upper bound dose. The drug was initially tested at this limit dose according to the guidelines.

3.8 EXPERIMENTAL ANIMALS

Female albino mice weighing 20-35 g were procured from the Department of Central Animal Facility, IISc, Bengaluru. The animals were transported to Tirupati on the same day in cool hours and subsequently housed in solid bottom poly propylene cages (Five animals in each) at an ambient temperature of 24 ± 2 °C and 45-55% relative humidity with 12-12 h light and dark cycle. The mice were kept on *ad libitum* feed and water. Animals of all groups were fed with standard pellet feed procured from Sri Venkateswara Enterprises, Bengaluru. Permission was obtained from the Institutional Animal Ethics Committee before the start of experiment. The experiment was conducted in Department of Veterinary Pharmacology, College of Veterinary Science, Tirupati.

3.9 EXPERIMENTAL DESIGN

Forty albino female mice between 10 -15 days of lactation were randomly divided into five groups (n=8).

S.No.	Experimental groups	Description	No. of Animals
1	GROUP I	Normal lactating group (0.9% sterile Normal saline)	8
2	GROUP II	Mastitis group	8
3	GROUP III	Mastitis mice treated with Acetyl-11-α-keto-β-boswellic acid mediated Nanosilver (AKBANS) @ 20µl (I/Mammary)	8
4	GROUP IV	Mastitis mice treated with Acetyl-11-α-keto-β-boswellic acid mediated Nanosilver (AKBANS) @ 20µl (I/Peritoneal)	8
5	GROUP V	Mastitis mice treated with Epime-TZ @ 1 mg/kg (I/Peritoneal)	8

For the animals of groups II, III, IV and V mastitis was induced by following method.

3.10 EXPERIMENTAL INDUCTION OF MASTITIS

Mastits was induced experimentally in mice as per Chandler, (1970) with slight modifications. Lactating female albino mice of 10–15 days post

partum typically weighing 35–40 g were used. The pups were removed 1–2 h before bacterial inoculation of mammary glands and a mixture of ketamine/xylazine at 87 and 13 mg/kg of weight I/M, respectively, was used for anesthesia. A 100 µl syringe with a 33-gauge hamilton blunt needle was used to inoculate both L4 and L5 abdominal mammary glands. Each udder canal was exposed by cutting the end of the teat under a binocular microscope and a total of 20 µl bacterial inoculum containing 4.0 x 10^4 organisms were injected through the orifice. The guidelines of the CPCSEA were followed during the procedure. Later the mice were given Butorphanal at the rate of 3-5mg/kg body weight I/M to prevent post inoculation trauma. Gross pictures of mastitic mammary gland from mice were presented in plate 1.

3.11 DRUG ADMINISTRATION

Six hours after inoculation, the treatment groups III and IV received 20µl of Acetyl-11-α-keto-β-bosweliic acid mediated nanosilver for each mice through intramammary and intra peritoneal routes respectively. Group V received Cefipime and tazobactum combination at a dose rate of 1mg/kg body weight through intra peritoneal route.

3.12 EVALUATION OF MASTITIC MAMMARY GLANDS

Animals were observed clinically and status of mastitis affected mammary glands was evaluated 18 hrs post drug treatment.

3.12.1 Physical Parameters

3.12.1.1 Weights of mammary glands

After the end of experimental period (18 hrs post treatment), animals were euthanized and mammary glands were aseptically dissected and collected in a pre-sterilized and pre-weighed eppendorf tubes. The weights of mammary glands were determined by the difference of weights of eppendorf tubes with and without mammary glands.

3.12.2 Enumeration of Bacterial Load

A one in 10 homogenate of each mammary gland was prepared by using sterile PBS followed by three 100 fold serial dilutions *viz.,* 10^{-3}, 10^{-5} and 10^{-7} were prepared from the homogenate in sterile PBS. A volume of 100 µl was plated on nutrient agar plates in triplicate for each dilution. The plates were incubated for 24 hours at 37^{0}C. The plates showing <300 colonies were counted using colony counter. The bacterial counts were later converted into $\log_{10}$ values for comparision.

3.12.3 Sero-Biochemical Parameters

Prior to the sacrifice, blood was collected by retro orbital puncture under light ether anesthesia using heparinised capillary tubes. A volume of 0.5 ml whole blood was collected in serum vacutainers and serum was separated after allowing for clotting followed by centrifugation at 3000 Rpm (Rotations per minute) for 15 minutes. The serum was utilized for the estimation of C-Reactive Protein (CRP).

3.12.3.1 C-Reactive protein (Marrack and Richards, 1971)

C - Reactive Protein was estimated using commercial kits supplied by ERBA Diagnostics, Mannheim, Pvt.Ltd, Mumbai.

3.12.4 Antioxidant Profile

Preparation of tissue homogenate

A 10% homogenate was prepared from the mammary gland using 0.2 M Phosphate buffer saline (pH 7.4). The homogenate was used for estimation of TBARS and reduced glutathione. The homogenate was precipitated with ethanol and chloroform and the supernatant was utilized for the estimation of SOD and Catalase. The precipitation procedure included addition of 0.5 ml of tissue homogenate with 0.25 ml of ethanol and 0.15 ml of chloroform and centrifuged at 3500 Rpm for 15 min.

3.12.4.1 Thiobarbituric acid reacting substances (TBARS) (Subramanian *et al*., 1988)

Principle

Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determinig the extent of peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red color absorbing light maximally at 535 nm.

Reagents

- 10% Trichloroacetic acid

  Dissolve 10 g of trichloroacetic acid in 100 ml distilled water.

- 0.67% Thiobarbituric acid

  Dissolve 0.67 g of thiobarbituric acid in 100 ml distilled water.

Method

- Take 500 µl of supernatant from the homogenate/100 µl plasma, 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid in a tightly stoppered tube.
- The tubes were heated to boiling temperature for 45 minutes and then cooled.
- After cooling the tubes, 2.5 ml of butanol was added and the contents were centrifuged.
- The supernatant was taken and read at 532 nm against the blank.
- The concentration of the test samples was calculated using molar extinction coefficient of MDA.

Units

Units of TBARS = mg of MDA / gm wt. of tissue.

3.12.4.2 Reduced glutathione (GSH) (Ellman, 1959)

Principle

The method is based on reaction of reduced glutathione (GSH) with 5-5’ dithiobis-2-nitrobenzoic acid (DTNB) to give a compound that absorbs light at 412 nm.

Reagents

- 5% TCA

  TCA-5 g

  Distilled water-100 ml

- Ellmans reagent

  19.8 mg of 5-5’ dithiobis-2-nitrobenzoic acid (DTNB) in 100 ml of 1% sodium citrate solution.

- Phosphate buffer – 0.2 M, pH 8.0

- Standard glutathione solution

  Reduced glutathione - 10 mg

  Distilled water - 100 ml

Procedure

A 0.5 ml of tissue homogenate was precipitated with 2 ml of 5% TCA solution. After centrifugation, to 1 ml of supernatant, 0.5 ml of Ellman’s reagent and 3 ml of phosphate buffer 0.2 M, pH 8.0 were added. The yellow colour developed was read at 412 nm against blank.

Calculation

$$\text{Glutathione (mg/g tissue)} = \frac{\text{Absorbance of the test sample}}{\text{Absorbance of the standard}} \times \text{conc. of the standard}$$

3.12.4.3 Superoxide dismutase (SOD) (Misra and Fridovich, 1972)

Reagents

- 50 mM sodium carbonate – bicarbonate buffer, p^H 9.8
- 0.1 mM EDTA

  Dissolve 3.72 mg of EDTA in 100 ml distilled water.
- 0.6 mM Adrenaline

  Dissolve 10.98 mg of epinephrine in 100 ml distilled water.

Procedure

The supernatant (500 µl) was added to 0.800 ml of carbonate buffer (100 mM, pH 10.2) and 100 µl of epinephrine (3 mM). The change in absorbance of each sample was then recorded at 480 nm in spectrophotometer for 2 min at an interval of 15 sec. Parallel blank and standard were run for determination of SOD activity.

One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of epinephrine auto oxidation.

Reagents	**Uninhibited (Standard)**	**Inhibited (Sample)**	**Blank**
Carbonate buffer	1.900 ml	1.900 ml	1.0 ml
Supernatant	……	0.1 ml	0.1ml dist water
EDTA	0.1 ml	0.5 ml	0.5ml
Epineprine		0.5ml	0.5ml

The reaction mixtures were diluted to 1/10 just before taking the readings in spectrophotometer.

Calculation:

$$\% \text{ Inhibition} = \frac{A_{480nm}/\text{min Uninhibited} - A_{480nm}/\text{min inhibited}}{A_{480nm}/\text{min Uninhibited} - A_{480nm}/\text{min Blank}} \times 100$$

$$\text{Units/ml enzyme} = \frac{\% \text{ Inhibition} \times V_t}{(50\%) \times V_s}$$

$$\text{Units/mg protein} = \frac{\text{Units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

3.12.4.4 Catalase *(CAT)* (Beer and Sizer, 1952)

Reagents

1**.** Phosphate buffer solution (50 mM)

A. Dissolved 6.81 gm of KH_2PO_4 in 1000 ml distilled water.

B. Dissolved 6.9 gm of Na_2HPO_4 in 1000 ml distilled water.

390 ml from solution (A) are mixed with 610 ml from solution (B), the pH is adjusted to 7.

2. Hydrogen peroxide (H_2O_2) 30 mM

Substrate Preparation - An approximately 5 X 10^{-3} M solution of hydrogen peroxide is prepared by diluting 0.15 ml. of superoxol (Merck) with 25 ml. of 0.05 M phosphate buffer, pH 7.0.

Method

The reagents were added to a quartz cuvette with 1 cm path length in a final volume of 3.0 ml. The assay mixture contained 100 µl of 10 % tissue homogenate, 1.9 ml of phosphate buffer, and 1 ml of H_2O_2. The change in absorbance was measured at 240 nm for 60 sec. Then ∆A / minute was calculated.

Reagents	Sample	Blank
Phosphate buffer solution	1.9 ml	2..9 ml
Supernatant	0.1 ml	0.1 ml
H_2O_2	1 ml	---------

The reaction occurs immediately after the addition of H_2O_2.

Solutions are mixed well and the first absorbance (A1) is read after 15 seconds (t1) and the second absorbance (A2) after 30 seconds (t2).The absorbance is read at wave length 240 nm.

Calculation

$$K = \frac{Vt}{Vs} \times \frac{2.3}{\Delta t} \times \text{Log} \frac{A1}{A2} \times 60$$

Where,

K= Rate constant of the reaction.

Δ t= (t2 – t1) =15 seconds.

A1= Absorbance after 15 seconds.

A2= Absorbance after 30 seconds.

Vt = Total volume (3 ml).

Vs = Volume of the sample (0.1ml).

3.13 HISTOPATHOLOGICAL STUDIES

The mammary glands from each group were collected for histopathological studies. The tissue was fixed in 10 % neutral buffered formalin until further analysis. The samples were processed and sections of 4-7 µm were cut and stained with heamatoxylin and eosin (H & E). The specimens were examined under light microscope (Singh & Sulochana, 1997).

3.14 ELECTRON MICROSCOPY (Bozzala and Russels, 1998)

Samples were fixed in 2.5% - 3% glutaraldehyde in 0.1 M phosphate buffer (P^H 7.2) for 24 hr at 4^0c and post fixed in 2% aqueous osmium tetroxide in the same buffer for 2 hr. Dehydrated in series of graded alcohols, infiltrated and embedded in araldite 6005 resin or spur resin (Spurr 1969). Ultra thin (50-70 nm) sections were made with a glass knife on ultra microtome (Leica Ultra cut UCT-GA-D/E-1/00), mounted on copper grids and stained with saturated aqueous uranyl acetate and counter stained with Reynolds lead citrate. Viewed under TEM (Model: Hitachi, H-7500 from JAPAN) at required magnifications as per the standard procedures at RUSKA Lab, College of Veterinary Sceinces, SVVU, Rajendranagar, Hyderabad, India.

3.15 STATISTICAL ANALYSIS

The data was analyzed by one way ANOVA followed by Tukey's post-hoc using statistical package for social sciences (SPSS 17.0 version).

CHAPTER IV

RESULTS

An experimental study was conducted on female albino mice to evaluate and compare the antibacterial properties of acetyl-11-α-keto-β-boswellic acid mediated nanosilver (AKBANS) with cefepime and tazobactum (Epime - TZ) combination in murine mastitis. Nanosilver was prepared using AKBA and characterized before conducting the study. The nanoparticles synthesized were characterized using UV-Vis spectrophotometer, SEM, DLS technique and FT-IR analysis. *In- vitro* anti-bacterial activity and MTT assay were conducted for the test compound prior to start of the experiment. A total of 40 female albino mice 10–15 days postpartum, weighing 35–40 g were divided into five groups. Group I served as lactating control, groups II to V were inoculated with 20 µl of 24 h broth culture of *S.aureus* containing 4.0 x 10^5 cfu/quarter (Log_{10} 5.60 cfu/quarter) under ketamine anaesthesia using 33g blunt hamilton needle. After 6 h post inoculation, groups III and IV received AKBA mediated nanosilver through intramammary and intra peritoneal routes respectively. Group V received antibiotic Cefepime and Tazobactum combination at a dose rate of 1 mg/kg body weight through intra peritoneal route. After 18 h post treatment, 0.5 ml of whole blood was collected through retro-orbital puncture and serum was separated for the estimation CRP levels. Animals were sacrificed under anesthesia and L4 mammary gland was collected aseptically. The mammary gland weights were

recorded and tissue samples for histopathology and ultrastructural studies were collected in 10% neutral buffered formalin and 5% glutaraldehyde respectively. A 10% homogenate of the mammary glands was prepared in 0.5 M phosphate buffer (pH 7.4) and was utilized for the evaluation of bacterial load and antioxidant parameters like TBARS, GSH, SOD and CAT. The tissue sections prepared from the mammary glands were used for histopathology and ultra-structural studies. The data for various parameters were analyzed using one way ANOVA. The results of the experiment are presented below.

4.1 SYNTHESIS AND CHARACTERISATION OF NANOPARTICLES

4.1.1 UV-Visible Spectra Analysis

UV Visible absorption spectrum of AKBANS is shown in Fig. 2. The spectra of AKBANS showed absorption maxima at 230 nm. Although the characteristic absorbance band of the silver falls around 400 nm, the prepared AKBANS are coated with the phytochemical, which leads to the lowering of the absorbance wavelength.

4.1.2 SEM Analysis of Silver Nanoparticles

The SEM image analysis of AKBANS depicted in Plate 2 showed that the particles are irregular and spherical in shape with uniform distribution. However, in most of the occasions agglomeration of the particles was observed. The measured sizes of the agglomerated nanoparticles are in the range of 363 to 574 nm. However the average size of an individual particle is estimated to be approximately 95 nm.

4.1.3 DLS Technique-Particle Size Measurement

The size of the AKBA mediated silver nanoparticles was measured using DLS technique and the size was recorded as 99.8 nm which is in good agreement with the SEM analysis (fig.3).

4.1.4 Zeta Potential

The measured value of zeta potential -27.5 mV indicates the higher stability of the formed AKBANS (fig.4). The conductivity 0.152mS/cm and electrophoretic mobility (mean), -0.000214 cm^2/Vs are also recorded.

4.1.5 FT-IR Analysis

The result of FT-IR analysis for AKBANS is depicted in Fig. 5. Spectra of AKBANS showed transmission peaks at 3356, 1635.93 and 1507.08 cm-1. The peak at 1635 cm-1 indicates primary amines, the peak at 3355 cm-1 corresponds to O-H, as also H- bonded phenols and alcohols in AKBANS while the peak at 1507 cm-1 in AKBANS corresponds to involvement of keto and acetyl groups.

4.2 *IN VITRO* EVALUATION OF NANOPARTICLES

4.2.1 Minimum Inhibitory Concentration (MIC)

The *in vitro* antibacterial activity of AKBA mediated nano silver and Citrate mediated nano silver was evaluated against *Staphylococcus aureus* isolated from a case of acute bovine mastitis and was depicted in plate 3. The minimum inhibitory concentration obtained by tube dilution method was 3.6 ng of AKBANS/ml. As minimum bactericidal concentration is four to five times

MIC and body water is roughly 70% of the body weight, a dose of 0.55 µg per 30 g mice was calculated. The dose corresponds to 18.33 µg/kg body weight. As the concentration of the given compound is 175 ppm, the dose can be equated to 104.74 µl of the preparation /kg body weight.

4.2.2 Safety Evaluation

4.2.2.1 *In vitro* evaluation

Safety evaluation of AKBANS was carried out by MTT assay on mouse spleenocytes *in vitro* and limit oral acute toxicity was carried out *in vivo* in mice and was presented in fig 6. In the MTT assay, the spleenocytes maintained a survivability of above 93% even at a concentration of 87.5 ppm of the compound and was above 95% at all lower concentrations.

4.2.2.2 *In vivo* evaluation

In the acute oral toxicity test conducted on rats, administration of the drug at the limit dose did not result in ether mortality or any symptoms of toxicity. Hence, this dose was considered as the stopping criteria as per the guidelines. The acute oral toxicity conducted on rats indicated that the compound was non toxic even at a dose of 20 ml/kg body weight which equates to 3.5 mg/kg body weight of AKBANS. The oral toxicity dose currently used was 60 times more than MIC.

4.3 Physical Parameters

4.3.1 Clinical Symptoms

During the experimental period, inoculated mammary glands of mice showed redness and swelling.

4.3.2 Mean Mammary Gland Weights

The mean ± S.D mammary gland weights (g) were depicted in table 1, fig.7. The mean ± S.D mammary gland weights (g) in groups I to V were 0.27±0.01, 0.45±0.02, 0.33±0.02, 0.35±0.01 and 0.39±0.04 respectively. A one way ANOVA (F=4.81 and Sig = 0.039) showed as significant difference among the groups. Upon conducting Tukey's *post hoc* test, the weights of mammary glands in groups II, III, IV and V were significantly higher compared normal lactating group I. There was no significant difference between mastitis group II and other treatment groups III, IV and V.

4.4 Microbiological Parameters

4.4.1 Mean Bacterial Load

The mean ± S.D bacterial load (Log_{10} cfu/g) were presented in plate 4, table 2, fig.8. The mean ± S.D bacterial load (Log_{10} cfu/g) in groups I to V were -- ± --, >9.48±--, 8.54±0.41, 8.62±0.52 and 9.09±0.27 respectively. A one way ANOVA (F=548.56 and Sig = 0.000) showed as significant difference among the groups. Upon conducting Tukey's *post hoc* test, the bacterial load in group II was significantly higher than the remaining groups. No significant difference was found between the treatment groups III, IV and V.

4.5 Serum Biochemical Profile

4.5.1 Mean C-Reactive Protein values (mg/dl)

The mean ± S.D C-Reactive Protein values (mg/dl) were presented in table 3, fig.9. The mean ± S.D C-Reactive Protein values (mg/dl) in groups I to V were 0.023 ± 0.003, 0.61±0.001, 0.14±0.002, 0.18±0.003 and 0.044±0.003 respectively. A one way ANOVA (F=5.477 and Sig = 0.006) showed as significant difference among the groups. Upon conducting Tukey's *post hoc* test, the C-Reactive Protein values in groups I, III and IV were significantly lower compared mastitis group II. There was no significant difference between antibiotic group V and groups I, III and IV.

4.6 Anti-Oxidant Parameters

4.6.1 Mean Malondialdehyde (µg/g)

The mean ± S.D Melondialdehyde (µg/g) were presented in table 4, fig.10. The mean ± S.D Melondialdehyde (µg/g) in groups I to V were 0.07 ± 0.11, 0.47±0.22, 0.32±0.19, 0.36±0.22 and 0.64±0.27 respectively. A one way ANOVA (F=7.302 and Sig = 0.003) showed as significant difference among the groups. Upon conducting Tukey's *post hoc* test, the Melondialdehyde values in mastitis and antibiotic groups were significantly higher compared to lactating group I. There was no significant difference between AKBANS (both treatments) and lactating group.

4.6.2 Mean Reduced Glutathione (mg/g)

The mean ± S.D reduced glutathione levels (mg/g) were presented in table 5, fig.11. The mean ± S.D reduced glutathione levels (mg/g) in groups I

to V were 0.12 ± 0.13, 0.11±0.13, 0.15±0.18, 0.14±0.02 and 0.09±0.03 respectively. A one way ANOVA (F=0.827 and Sig = 0.497) showed no significant difference among the groups.

4.6.3 Mean Superoxide Dismutase (U/mg protein)

The mean ± S.D Superoxide Desmutase (U/mg protein) were presented in table 6, fig.12. The mean ± S.D Superoxide Desmutase (U/mg protein) in groups I to V were 4.57 ± 1.32, 13.10±1.04, 8.84±0.58, 8.93±0.65 and 16.11±1.97 respectively. A one way ANOVA (F=0.827 and Sig = 0.497) showed no significant difference among the groups. However, the mean activity of SOD was found to be much reduced in AKBANS treated groups III and IV. Interestingly, the antibiotic group V showed higher activity of SOD than mastitis group II.

4.6.4 Mean Catalase values (U/mg protein)

The mean ± S.D Catalase (U/mg protein) were presented in table 7, fig.13. The mean ± S.D Catalase (U/mg protein) in groups I to V were 40.00 ± 2.96, 55.80±2.78, 12.80±1.04, 13.12±1.18 and 22.60±1.62 respectively. A one way ANOVA (F=3.967 and Sig = 0.027) showed as significant difference among the groups. Upon conducting Tukey's *post hoc* test, the Catalase values in AKBANS treatment groups III and IV were found to be significantly lower compared mastitis group II and normal lactating group I. The CAT activity was also reduced significantly in antibiotic group V compared to group I and ll. However, maximal reduction was observed in AKBANS treated groups.

4.7 HISTOPATHOLOGY

Histological sections from Group I animals were presented in plate 5a & 5b (H&E staining). The sections revealed normal structure of a mammary gland. The alveoli appeared to be normal with dilated sac like structure filled with milk contents and the alveolar epithelium is intact and appeared to be normal. The inter alveolar space is filled with myoepithelial cells. Sections from Group II animals were presented in plate 6a & 6b. The sections showed extensive infiltration of inflammatory cells in between and within the acini and majority of the cells are neutrophils. Alveoli and duct lumina were filled with cellular debris, sloughed off epithelial cells, bacterial rods and milk contents. Alveoli epithelium showed degenerative (vacuolation) changes. Areas of congestion are observed between the acini. Infiltration of inflammatory cells into adipose tissue (steatitis) is also observed. Histological sections of Group III and Group IV animals were presented in plate 7a, 7b and 8a, 8b respectively. The sections showed a marked reduction in all the inflammatory signs. There was a marked reduction of neutrophil infiltration in the animals treated by both intramammary (100%) and intraperitoneal (90%) routes with the test substance. The alveolar epithelium appeared to be normal. Alveolar cavity apperared clear without any bacterial rods in both the groups. Sections of Group V were presented in plate 9a & 9b. The sections showed mild infiltration of neutrophils in between and within the alveoli and moderate number of bacterial rods were observed in the alveolar and duct cavity.

4.8 ULTRASTRUCTURE

The ultrastructure images of mammary glands from groups I to V were presented in plates 10 to 14. The sections in normal lactating group 1 (plate10a & 10b) consisted secretory epithelial cells together with myoepithelial cells interspersed regularly at the base of the epithelium. The inter alveolar space appeared normal and the lumen of alveoli were filled with milk constituents (milk protein as dense particles, with characteristic mottled surface appearance). The sections of mastitis group II (plate11a & 11b) revealed areas of secretory epithelial cells showing gross cellular destruction with complete loss of architecture and devoid of nuclei and cell boundaries. Major alterations like condensed mitochondria, margination of chromatin and fibrosis of inter alveolar septa were also observed. Few sections in the same group revealed intra cellular dense colonies of *Staphylococci*.

In intramammary AKBANS treatment group III (plate 12), the section revealed secretory cells under repair with mitochondrial reconstitution and moderate progress in the repair of nuclear structures. Whereas in intraperitoneal AKBANS treatment group V (plate 13), the sections showed secretory epithelial cells with normal architecture and inter alveolar space. In both groups III & IV, no visible colonies of *Staphylococci* were observed.

In the antibiotic group V (plate 14a &14b), the sections depicted secretory epithelial cells with few visible colonies of *Staphyloccocci*, normal nuclear structure, but with disrupted chromatin. The inter-alveolar septa appeared to be normal. Few sections in the same group revealed degenerate cells and normal cells separated by normal inter alveolar septa.

CHAPTER V

DISCUSSION

The efficacy of Acetyi-11-α-keto-β-boswellic acid mediated nanosilver was investigated in *Staphylococcus aureus* induced murine mastitis model with the objective to evaluate the antibacterial and anti-inflammatory potential of the compound in comparison with Cefepime and tazobactum combination. The obtained results were tabulated, analysed and discussed as following.

Green synthesis of nanosilver using plant extracts was reported to be superior to chemical synthesis in that the former compounds offer better advantages as they are widely distributed, safe to handle, easily available with a range of metabolities (Kulkarni *et al.,* 2012). In the present study, a novel method for the synthesis of silver nanoparticles from silver nitrate reduction using phyto-compound Acetyi-11-α-keto-β-boswellic acid was carried out. The formed silver nanoparticles were characterized using UV-Visible spectroscopy, SEM, DLS technique and FT-IR analysis. The production of silver nanoparticles is demonstrated by the sharp peak around 200 nm for Acetyi-11-α-keto-β-boswellic acid mediated silver nanoparticles in UV-Vis spectrum, which indicate the availability of reducing biomolecules in Acetyi-11-α-keto-β-boswellic acid. Analysis of SEM image showed the formation of silver nanoparticles and exhibited the agglomerated appearance with irregular and spherical shape and size varying from 363 to 594 nm, the average size of an individual particle is estimated to be approximately 99.8 nm. However,

SEM micrograph of soapnut mediated silver nanoparticles showed that the particles are more or less spherical in shape with sizes in the range of 20 to 30 nm (Ramgopal *et al.*, 2011). The results of DLS technique used for the measurement of size of AKBANS in solution form showed a size of 99.8 nm which is in good agreement with the SEM analysis (70 nm). These findings are in agreement with Aparna, (2012) who reported that the particle size of nanosilver prepared to be of 78.8 nm with Boswellia extract mediated nano silver. With the results of the FT-IR studies indicated the involvement of keto, hydroxyl, acetyl and primary amine functional groups of Acetyi-11-α-keto-β-boswellic acid in the synthesis of silver nanoparticles. The Acetyl-11-α-keto-β-boswellic acid mediated nano silver was found to be practically non-toxic at the limit dose of 20mL/kg in rats ; the cell viability of spleenocytes was more than 95 % at a dose of 87.5 ppm.

Murine mastitis model was suggested to be an appropriate model for evaluating new antimicrobial compounds against microorganisms responsible for bovine mastitis (Chandler *et al*., 1980; Brouillette *et al.,* 2003; Anderson and Craven, 1984). The model was reported to simulate mastitis resembling bovine model with similar anatomical structure of the mammary gland as cow and each mammary gland of mice is separate with single teat canal and duct (Chandler, 1970 and Brouillette *et al.,* 2003, 2004 & 2005). Further, the *in vivo* infectious model was considered superior to *in vitro* evaluation as it facilitates the evaluation of antimicrobial activity against intracellular pathogens (Brouillette *et al.,* 2003).

The *Staphylococcus aureus* organisms offer particular difficulties for treatment with traditional antibiotics in that it is intracellular and many strains

were reported to be multi-drug resistant (Chen *et al*., 2012). Hence, for the present study, Staphylococcal murine mastitis model was chosen for evaluating Acetyi-11-α-keto-β-boswellic acid mediated nanosilver against Cefepime and Tazobactum combination.

The inoculation of Staphylococcal organisms into mammary gland of mice resulted in severe inflammation evidenced by increased mammary weights, elevated bacterial loads and elevated CRP levels in serum which is in accordance with Kruger and Neumann, 1999 who reported that cows with mastitis caused by *Streptococcus uberis*, had significantly elevated serum CRP levels, that were much higher than those of healthy, lactating cows. The induction of inflammation was further confirmed by histopathological picture of the mammary gland, which showed severe infiltration of PMN. Many researchers have earlier reported induction of inflammation in the mammary gland consequent to either natural or experimental inoculation of *Staphylococcus aureus* in the mammary gland of various species.

The mammary tissue containing milk provides suitable environment for the bacteria to multiply and further, invade the mammary tissue extensively. The Staphylococcal enterotoxin A released by the organisms was reported to be the primary factor responsible for induction of inflammation in *Staphylococcus aureus* mastitis (Friedman *et al*., 2011).

Administration of Acetyi-11-α-keto-β-boswellic acid mediated nanosilver resulted in a significant reduction of not only the bacterial load but was also able to arrest the ensuing inflammation. Despite both nanosilver and Acetyi-11-α-keto-β-boswellic acid were reported to have powerful antibacterial

effect, the anti-inflammatory effects of Acetyi-11-α-keto-β-boswellic acid mediated nanosilver could be attributed solely to the Acetyi-11-α-keto-β-boswellic acid component. Acetyi-11-α-keto-β-boswellic acid was shown to possess powerful anti-inflammatory effects in ulcerative colitis, chemically induced colitis in mouse and asthma models of inflammation in rats (Safayhi *et al.,* 1992; Ammon, 2002; Kiela *et al.,* 2005; Gupta *et al.,*1998). The mechanism of anti-inflammatory action of Acetyi-11-α-keto-β-boswellic acid was reported to be inhibiting leukotriene synthesis (Reddy *et al.,* 1989, Safayhi *et al.,* 1992 and Ammon *et al.,* 1993). Further, nanosilver was reported to exhibit antibacterial activity against wide range of gram -ve microorganisms like *Acinetobacter*, *Escherichia*, *Pseudomonas*, *Salmonella*, and *Vibrio* and gram +ve microorganisms like *Bacillus*, *Clostridium*, *Enterococcus*, *Listeria*, *Staphylococcus*, and *Streptococcus* and Antibiotic-resistant bacteria include strains such as methicillin-resistant (Wright *et al.,* 1998) and vancomycin-resistant *Staphylococcus aureus*, and *Enterococcus faecium* (Burrell *et al.,* 1999; Yin *et al.,* 1999; Percival *et al.,* 2007; Wijnhoven *et al.,* 2009) could have resulted in a superior antibacterial effect. The combination of Cefepime and Tazobactum showed mere antibacterial activity evidenced by significant reduction in bacterial counts, but there appears to be no anti-inflammatory effect. Hence, despite similar antibacterial effects of Acetyi-11-α-keto-β-boswellic acid mediated nanosilver and Cefepime combination, Acetyi-11-α-keto-β-boswellic acid mediated nanosilver could be considered much superior due to increased (36%) antibacterial effect and profound (95%) anti-inflammatory effect compared to the antibiotic.

The involvement of oxidative stress resulting from imbalance of free radical production was implicated in mastitis (Knaapen *et al.,* 1999). Excessive free radicals were reported to cause damage to lipids, proteins and DNA resulting in lipid peroxidation, protein oxidation and DNA fragmentation. Many endogenous antioxidants were reported to play an important role in overcoming oxidative stress which includes enzymes like SOD, CAT, GSHPx and non-enzymatic constituents like GSH, Vit C and Vit E. During oxidative stress, the activities of the enzymes were reported to increase to neutralize the excessively produced free radicals and the non-enzymatic constituents were reported to decreased.

In the present study, the levels of TBARS which is a measure of lipid peroxidation of lipids was found to be significantly increased in mastitis group along with increased activities of CAT suggesting the development of oxidative stress in the mammary tissue consequent to infection. Acetyl-11-α-keto-β-boswellic acid mediated nanosilver could significantly counter the oxidative stress as evidenced by significantly decreased CAT activities. The profound antibacterial effect and the antioxidant properties of Acetyl-11-α-keto-β-boswellic acid component of nanosilver could be attributed to the improved antioxidant status. Earlier, Acetyl-11-α-keto-β-boswellic acid was reported to reduce oxidative stress in chemically induced colitis (Kiela *et al.,* 2005). The antibiotic group despite possessing considerable antibacterial activity failed to counter the oxidative stress developed in the mammary tissue. However, both acetyi-11-α-keto-β-boswellic acid mediated nanosilver and antibiotic treatments could not decrease lipid peroxidation since the treatments were given 6 h post induction, by which time, the free radicals

generated already damaged the membrane lipids. Further, mammary glands are rich in adipose tissue making them more vulnerable to lipid peroxidation.

In conclusion, Acetyi-11-α-keto-β-boswellic acid mediated nanosilver was found to possess powerful antibacterial effect which was superior to Cefepime and Tazobactum combination and further, also possessed profound anti-inflammatory effect in staphylococcal mastitis. Hence, keeping in view the economics of production, safety and efficacy of the compound, AKBANS could provide a promising alternative to the use of traditional antibiotics for the treatment of bovine mastitis.

CHAPTER VI

SUMMARY

Phytogenically derived nanosilver, prepared by reduction of silver nitrate with AKBANS was evaluated against *Staphylococcus aureus* induced murine mastitis. A total of 40 female mice between 10-15 days of lactation were utilized for the study. The animals were divided into five groups of eight animals each. Group I served as lactating control, groups II to V were inoculated with 20 µl of 24 h broth culture of *S.aureus* containing 4.0 x 10^5 cfu/quarter (Log_{10} 5.60 cfu/quarter) under ketamine anaestheisa using 33g blunt hamilton needle. After 6 h post inoculation, groups III and IV received 20 µl of AKBANS through intramammary and intra peritoneal routes respectively. Group V received antibiotic Cefepime and Tazobactum (Epime – TZ, Cefepime 1000 mg - Tazobactam 125 mg, United Biotech Private Limited, India) @ 1mg/kg body weight through intraperitoneal route. After 24 h post inoculation, 0.5 ml of whole blood was collected through retro-orbital puncture and serum was separated for the estimation of CRP. Later, the mice were euthanized and L4 mammary gland was collected under asceptic conditions into sterile eppendorf tubes. The mammary gland weights were recorded and tissue samples for histopathology and ultrastructural studies were collected in 10% neutral buffered formalin and 5% glutaraldehyde respectively. A 10% homogenate of the mammary glands was prepared in 0.5 M phosphate buffer (pH 7.4) and was utilized for the evaluation of bacterial load and antioxidant parameters like TBARS, GSH, SOD and CAT.

The important findings of the study are summarized below

- Characterization of AKBANS by UV Visible absorption spectrum showed a maximum absorption around 200 nm for AKBANS. SEM images showed that AKBANS the particles are irregular and spherical in shape with uniform distribution of size 363 to 574 nm.
- The results of FT-IR analysis Spectra of AKBANS indicates the involvement of primary amines, O-H, H- bonded phenols and alcohols, keto and acetyl groups.
- The size of the particles measured by DLS technique was 99.8 nm.
- The compound showed an MIC of 0.0036 µg/ml against *Staphylococcus aureus* isolate obtained from a case of acute bovine mastitis.
- The compound showed an *in vitro* spleenocyte viability of more than 95% at the highest concentration of 87.5 ppm per well. The compound was also found to be safe at the limit dose in acute oral toxicity conducted in rats.
- The bacterial load was significantly higher in mastitis group II. All the treatments significantly reduced the bacterial load compared to mastitis control.
- All the groups inoculated with *S.aureus viz* groups II to V showed significantly higher mammary gland weights.
- The levels of C-Reactive Protein were significantly elevated ( ) in mastitis group II. AKBA treatment groups III and IV significantly reduced the elevated CRP in comparison with antibiotic administered group V.

- The malondialdehyde values in mastitis group II and antibiotic group V were significantly elevated ( ) compared lactating group I. AKBANS treatment groups III and IV showed no improvement.
- The reduced glutathione levels and SOD activities were not significantly affected either by the induction of mastitis or by the different treatments employed.
- The catalase values in AKBA treatment groups III and IV remained significantly lower ( ) compared mastitis group II and even normal lactating group I.
- The mastitis group II showed heavy infiltration of PMN cells within and between acini along with desquamation and vacuolation of the acinar epithelium. Both the AKBANS groups III and IV reduced the infiltration of PMN to the extent of 95% with very mild changes in the mammary tissue. Antibiotic group V showed a moderate reduction of infiltration of PMN to the extent of 40-50%.
- Ultrastucture in both routes of AKBANS treatment revealed improvement in the cellular architecture and other cellular organelles with no signs of inflammation. Whereas, antibiotic group showed moderate reduction in inflammatory signs.

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Table: 1` Mean Weights of Mammary gland (L4) (g) post treatment

Group		Mammary gland (l4) weight (g)		
I	Lactating	0.27^{a}	±	0.01
II	Mastitis	0.45^{b}	±	0.02
III	AKBS I/M	0.33^{b}	±	0.02
IV	AKBS I/P	0.35^{b}	±	0.01
V	Antibiotic	0.39^{b}	±	0.04
Df	(5,18)			
F	4.81			
Sig	0.039			
The results indicated that inoculation of *S.aureus* into mammary glands significantly increased the mammary weights.				

Values are Mean ± SD

One way ANOVA followed by Tukey's *post hoc* test using SPSS 17.0V

Means with different superscripts are significantly different ($p<0.05$)

Table: 2 Mean Bacterial Load (Log10 CFU/g of tissue) in mammary glands post

Group		Mean MBC Log10CFU/g		
I	Lactating	-	±	-
II	Mastitis	$>9.48^{b}$*	±	-
III	AKBS I/M	8.54^{a}	±	0.41
IV	AKBS I/P	8.62^{a}	±	0.52
V	Antibiotic	9.09^{a}	±	0.27
df	(3,10)			
F	548.56			
Sig	0.000			
The results indicated that both antibiotic and AKBS (both routes) could significantly reduced the bacterial load in the mammary gland 24h post induction of mastitis				

* The bacterial counts could not be performed in mastitis group due to formation of mat even at highest dilution of 10^{-7}. Hence the count is assumed to be $>300x10^{8}$ CFU/g.

Values are Mean ± SD

One way ANOVA followed by Tukey's *post hoc* test using SPSS 17.0V

Means with different superscripts are significantly different ($p<0.001$)

Table: 3 Mean C-Reactive Protein values (mg/dl) in serum post treatment

Group		Mean CRP (mg/dl)		
I	Lactating	0.023^{a}	±	0.003
II	Mastitis	0.061^{b}	±	0.001
III	AKBS I/M	0.014^{a}	±	0.002
IV	AKBS I/P	0.018^{a}	±	0.003
V	Antibiotic	0.044^{ab}	±	0.003
Df	(5,18)			
F	5.477			
Sig	0.006			
The mean CRP value was significantly increased in mastitis control group. AKBA administered by both route significantly reduced the CRP values in plasma				

Values are Mean ± SD

One way ANOVA followed by Tukey's *post hoc* test using SPSS 17.0V

Means with different superscripts are significantly different (p<0.01)

Table: 4 Mean Thibarbituric acid reactive substances (MDA µg/g) in mammary tissue post treatment

Group		Mean MDA (µg/g)		
I	Lactating	0.07^{a}	±	0.11
II	Mastitis	0.47^{b}	±	0.22
III	AKBS I/M	0.32^{ab}	±	0.19
IV	AKBS I/P	0.36^{ab}	±	0.22
V	Antibiotic	0.64^{b}	±	0.27
Df	(5,18)			
F	7.302			
Sig	0.003			
The mean TBARS measures as MDA (ug/g) was significantly increased in all groups in which mastitis was induced				

Values are Mean ± SD

One way ANOVA followed by Tukey's *post hoc* test using SPSS 17.0V

Means with different superscripts are significantly different (p<0.01)

Table: 5 Mean Reduced Glutathione level (mg/g) in mammary tissue post treatment

Group		Mean Glutathione (mg/g)		
I	Lactating	0.12	±	0.13
II	Mastitis	0.11	±	0.13
III	AKBS I/M	0.15	±	0.18
IV	AKBS I/P	0.14	±	0.02
V	Antibiotic	0.09	±	0.03
Df	(5,18)			
F	0.191			
Sig	0.901			
There was no significant difference in the GSH values among the treatment groups				

Values are Mean ± SD

One way ANOVA followed by Tukey's *post hoc* test using SPSS 17.0V

Means with different superscripts are significantly different ($p<0.05$)

Table: 6 Mean Superoxide Dismutase (SOD) (U/mg protein) in mammary tissue post treatment

Group		Mean SOD (U/mg protein)		
I	Lactating	04.57	±	1.32
II	Mastitis	13.10	±	1.04
III	AKBS I/M	08.84	±	0.58
IV	AKBS I/P	08.93	±	0.65
V	Antibiotic	16.11	±	1.97
Df	(5,18)			
F	0.827			
Sig	0.497			
There was no significant difference in the SOD activity among the treatment groups				

Values are Mean ± SD

One way ANOVA followed by Tukey's *post hoc* test using SPSS 17.0V

Means with different superscripts are significantly different ($p<0.05$)

Table: 7 Mean Catalase (CAT) (U/mg protein) in mammary tissue post treatment

Group		Mean CAT (U/mg protein)		
I	Lactating	40.00^{c}	±	2.96
II	Mastitis	55.80^{d}	±	2.78
III	AKBS I/M	12.80^{a}	±	1.04
IV	AKBS I/P	13.12^{a}	±	1.18
V	Antibiotic	22.60^{b}	±	1.62
Df	(5,18)			
F	3.967			
Sig	0.027			
Treatment with AKBA through both routes significantly reduced				

Values are Mean ± SD

One way ANOVA followed by Tukey's *post hoc* test using SPSS 17.0V

Means with different superscripts are significantly different ($p<0.05$)

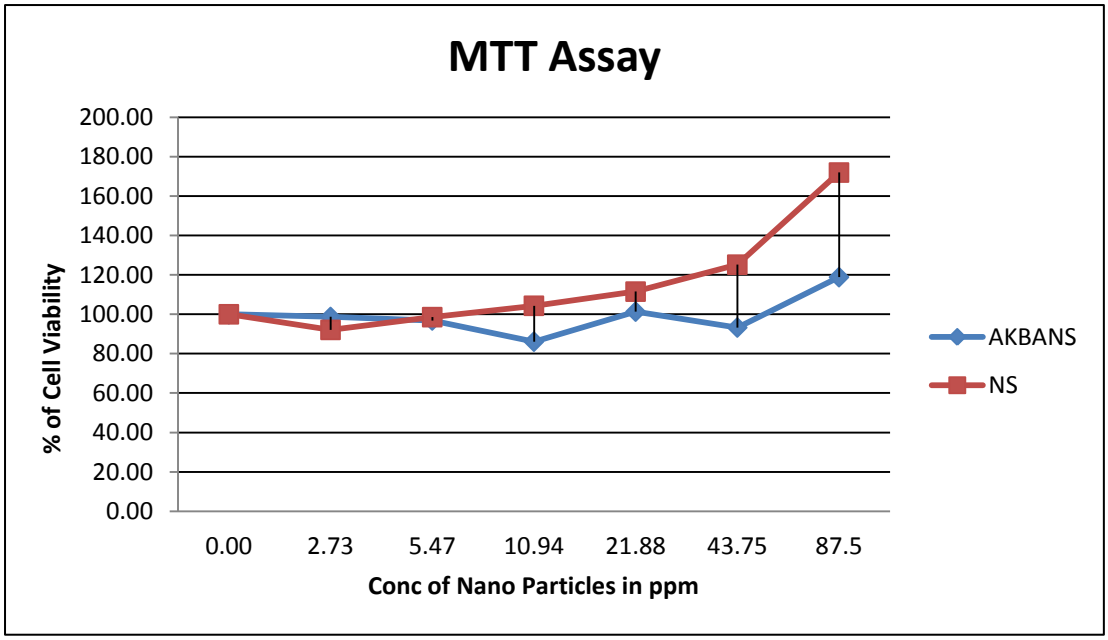


Fig.6: Invitro cytotoxic evaluation of AKBANS

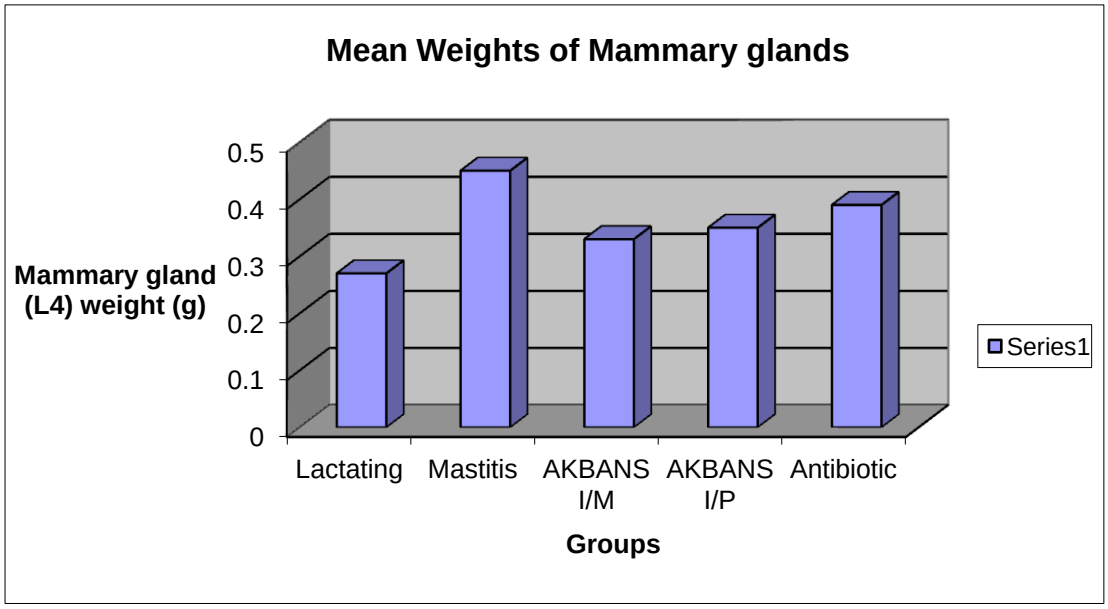


Fig.7: Mean mammary gland weights

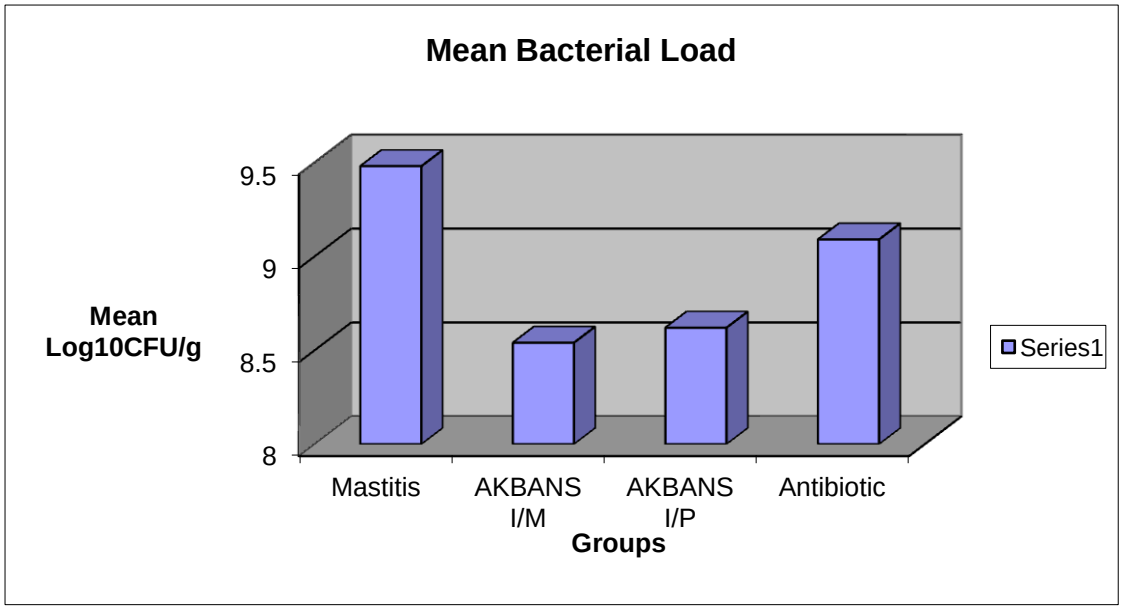


Fig.8: Mean bacterial load

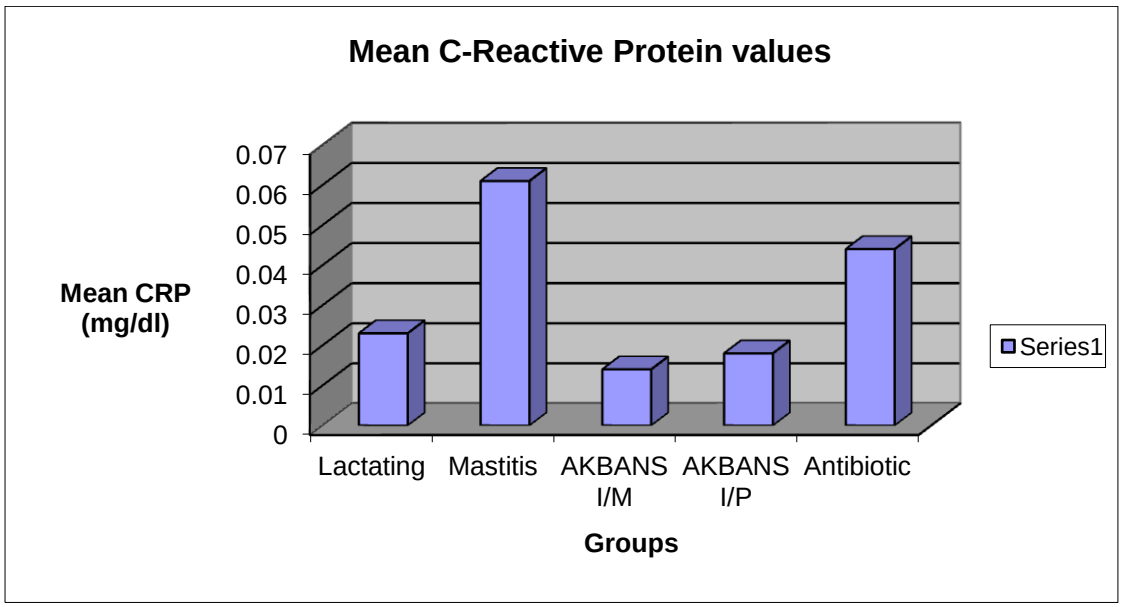


Fig.9: Mean C-Reactive Protein values

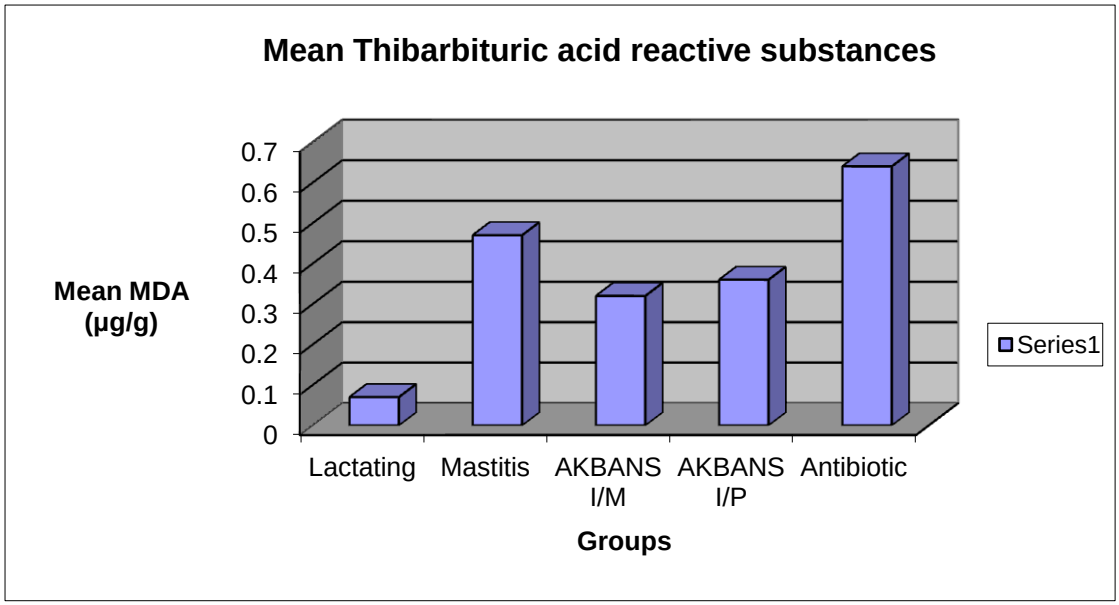


Fig.10: Mean malondialdehyde values

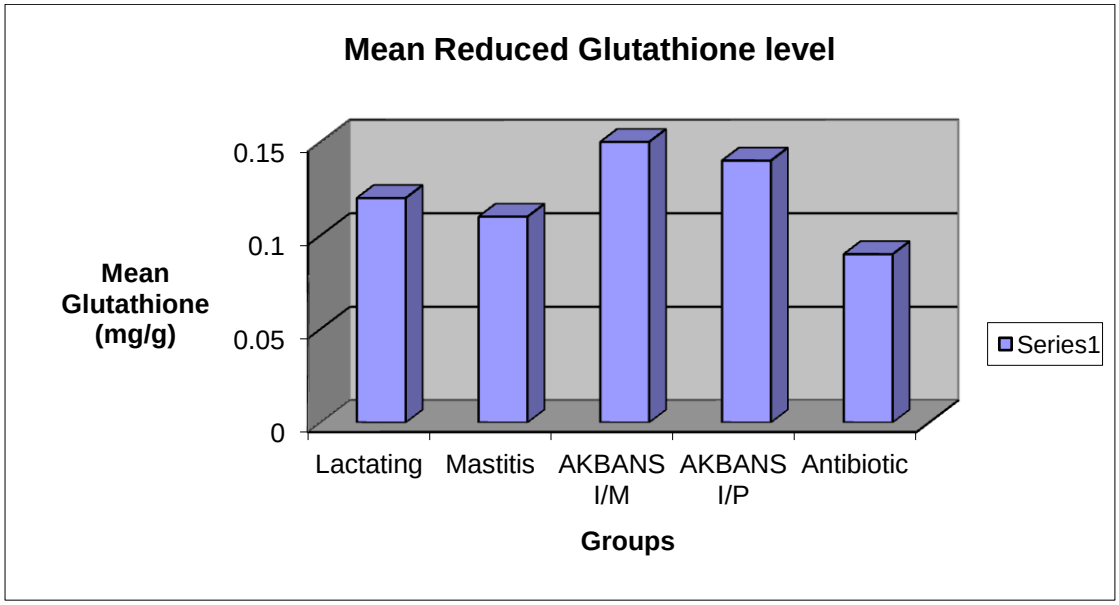


Fig.11: Mean reduced glutathione values

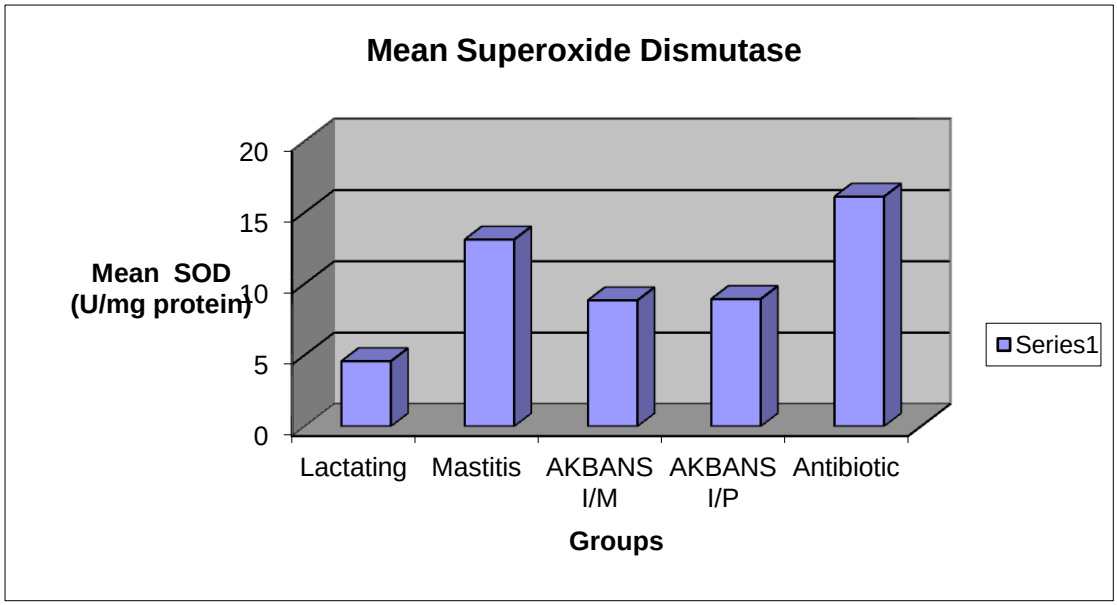


Fig.12: Mean Superoxide Dismutase values

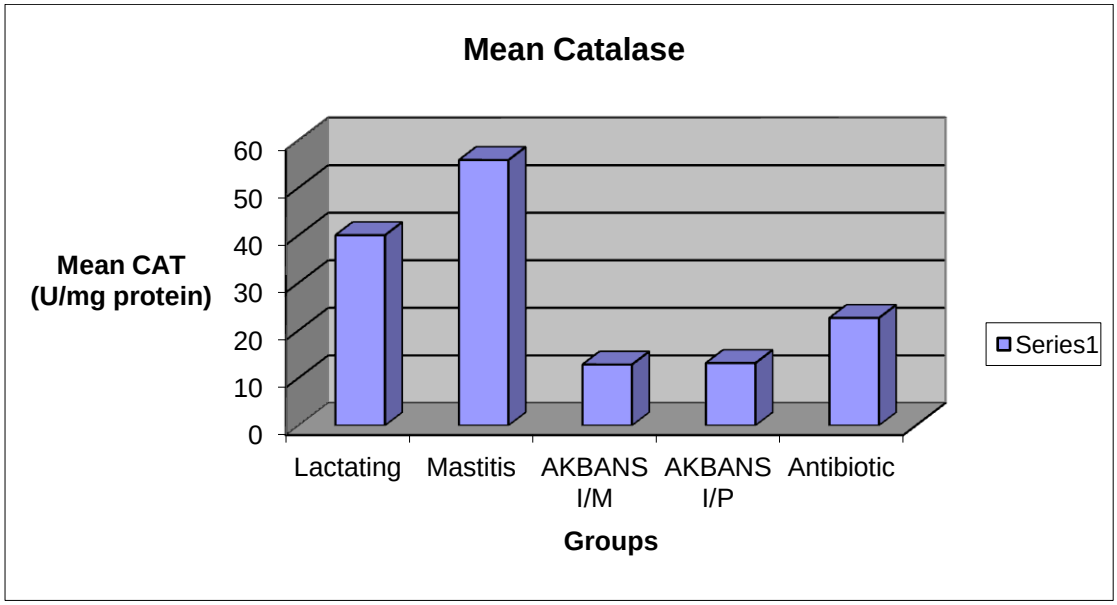


Fig.13: Mean Catalase values

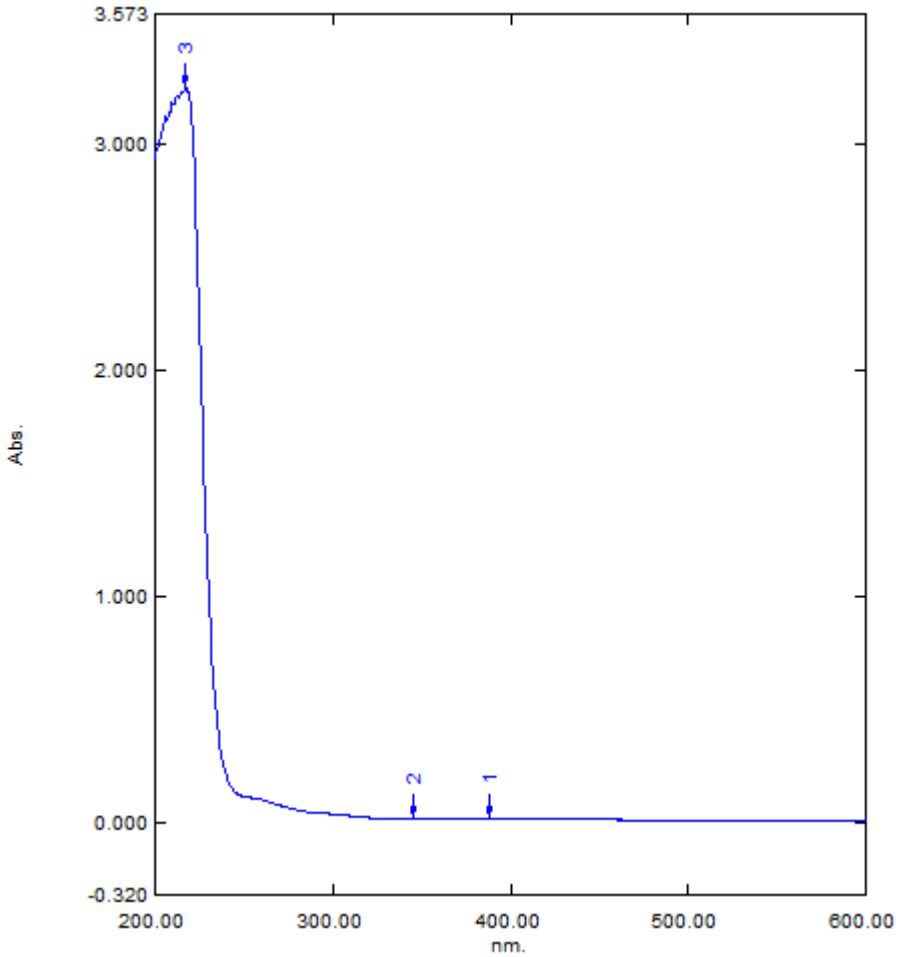


Fig.2: UV-Visible Spectra Analysis of AKBANS

201302151601005.nsz
Measurement Results

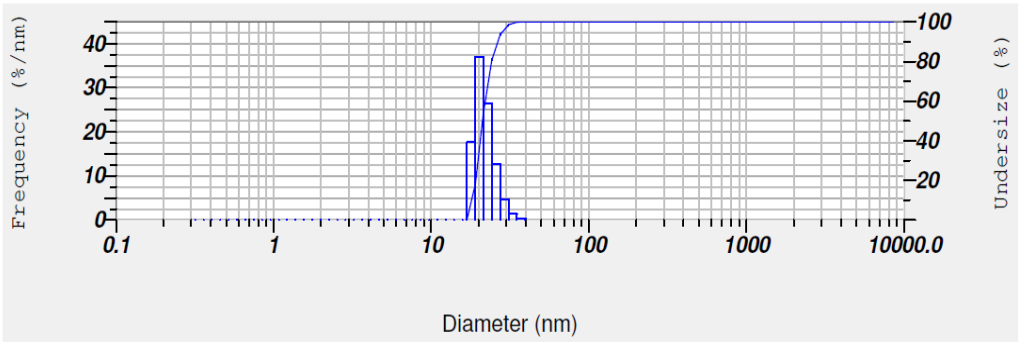
Date	:	Friday, February 15, 2013 4:01:52 PM
Measurement Type	:	Particle Size
Sample Name	:	ANS
Scattering Angle	:	90
Temperature of the holder	:	25.1 ℃
T% before meas.	:	26394
Viscosity of the dispersion medium	:	0.893 mPa·s
Form Of Distribution	:	Standard
Representation of result	:	Number
Count rate	:	385 kCPS

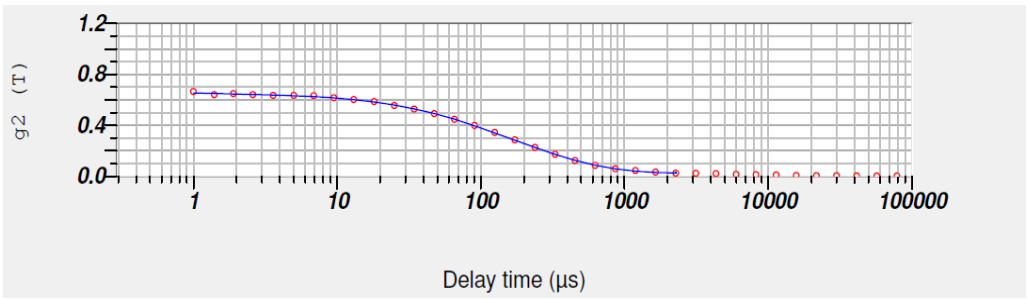
Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	21.9 nm	3.3 nm	20.5 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	21.9 nm	3.3 nm	20.5 nm

Cumulant Operations

Z-Average	:	99.8 nm
PI	:	0.486





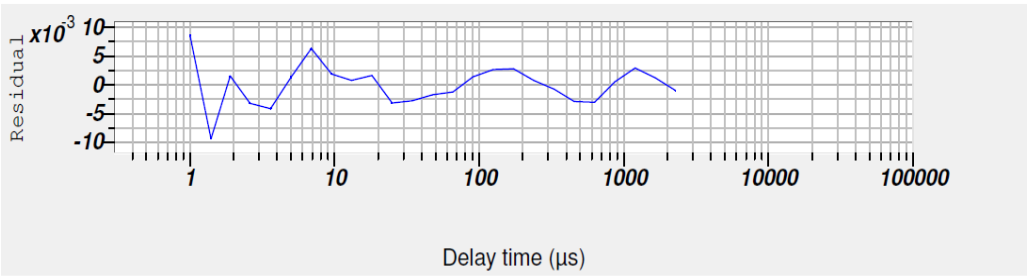


Fig.3: DLS Technique-Particle Size Measurement

Measurement Results

201302151533001.nzt
Measurement Results

Date	:	Friday, February 15, 2013 3:33:42 PM
Measurement Type	:	Zeta Potential
Sample Name	:	ANS
Temperature of the holder	:	25.1 ℃
Viscosity of the dispersion medium	:	0.893 mPa·s
Conductivity	:	0.152 mS/cm
Electrode Voltage	:	3.4 V

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-27.5 mV	-0.000214 cm2/Vs
2	--- mV	--- cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean)	:	-27.5 mV
Electrophoretic Mobility mean	:	-0.000214 cm^2/Vs

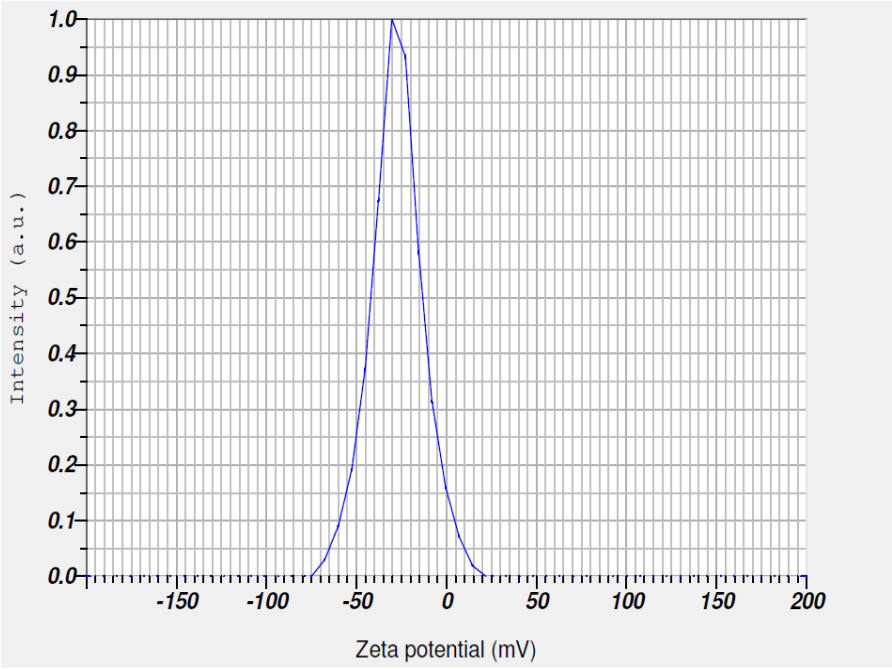


Fig.4: Zetapotential of AKBANS

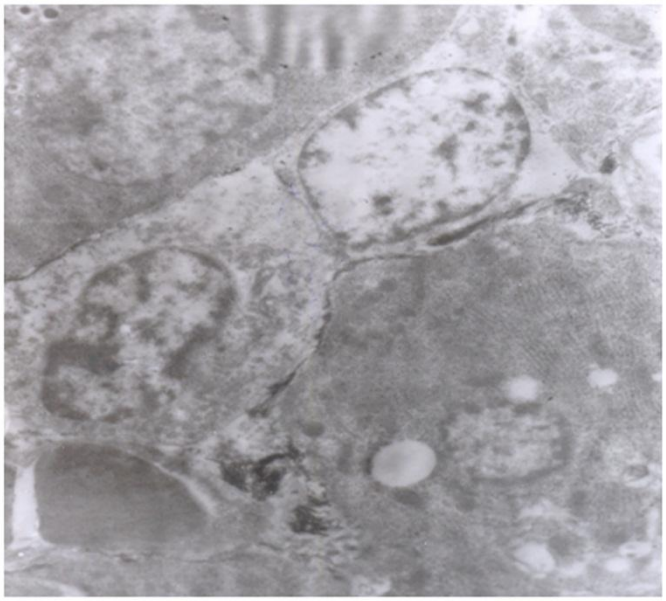


PLATE - 14a

Secretory epithelial cells with few visible colonies of Staphyloccocci, normal nuclear structure, but with disrupted chromatin

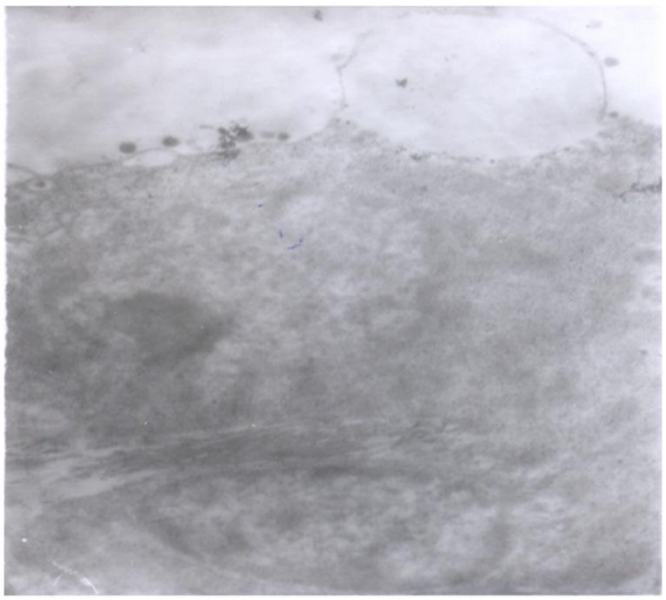


PLATE - 14b

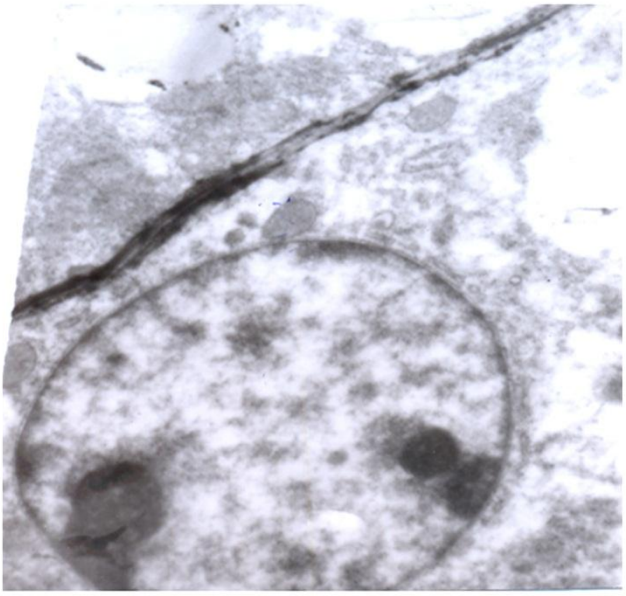


PLATE - 12

Secretory cells under repair with mitochondrial reconstitution and moderate progress in the repair of nuclear structures

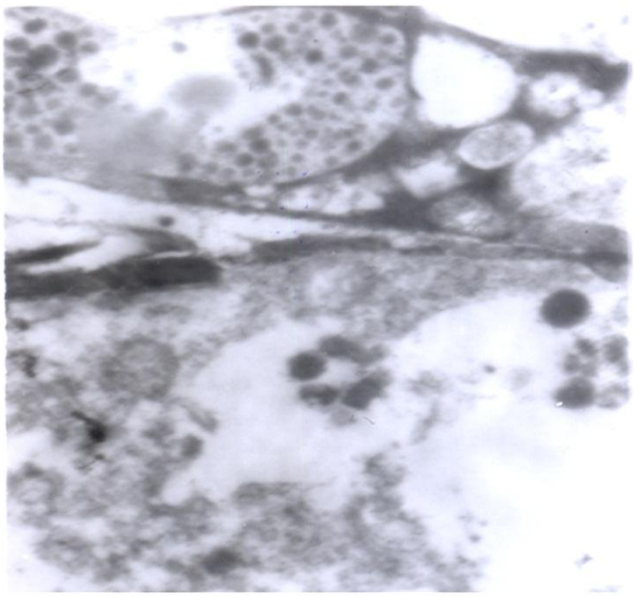


PLATE - 13

Secretory epithelial cells with normal architecture and inter alveolar space

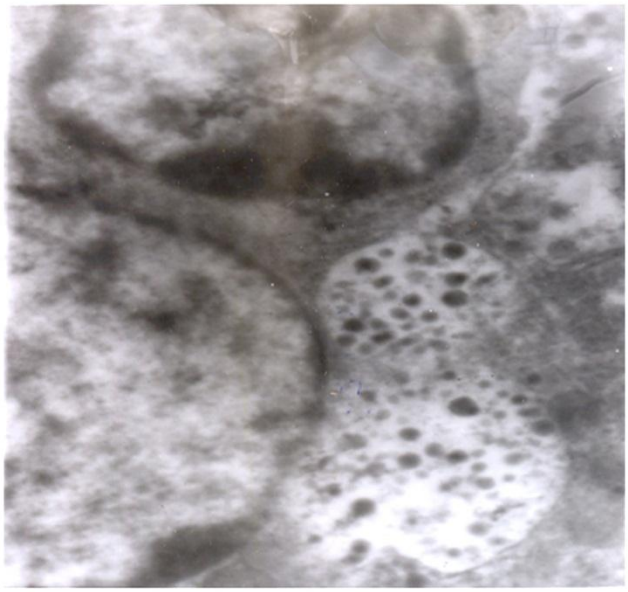


PLATE - 11a

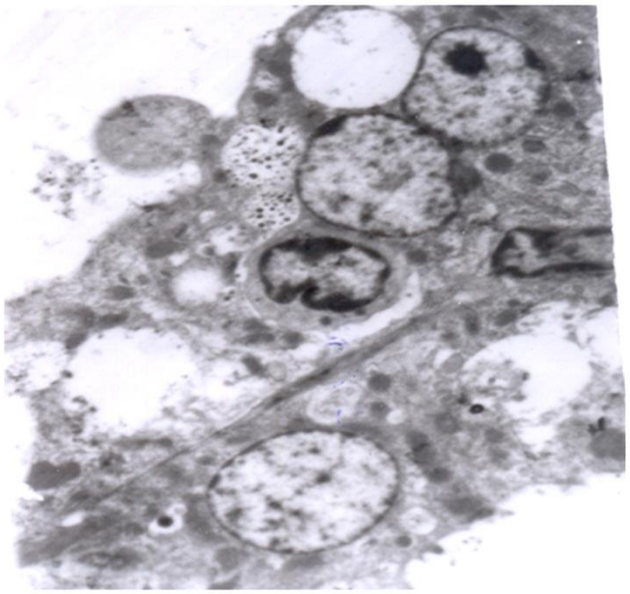


PLATE - 11b

Areas of secretory epithelial cells showing gross cellular destruction with complete loss of architecture and devoid of nuclei and cell boundarie

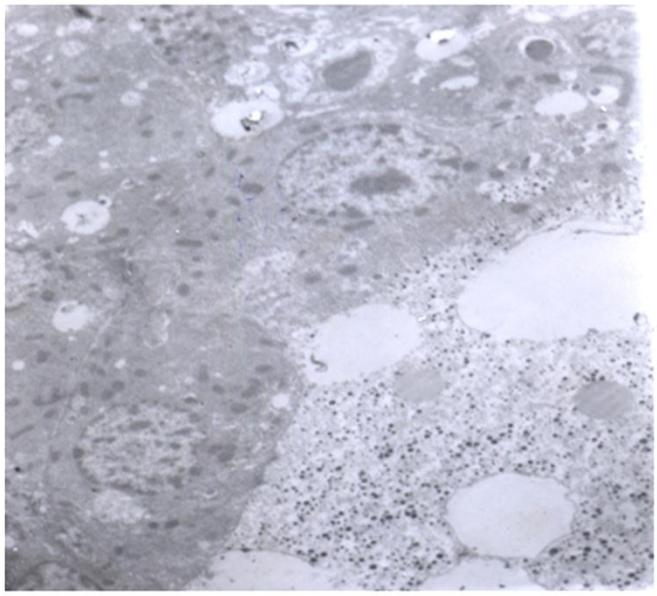


PLATE - 10a

Secretory epithelial cells together with myoepithelial cells interspersed regularly at the base of the epithelium.

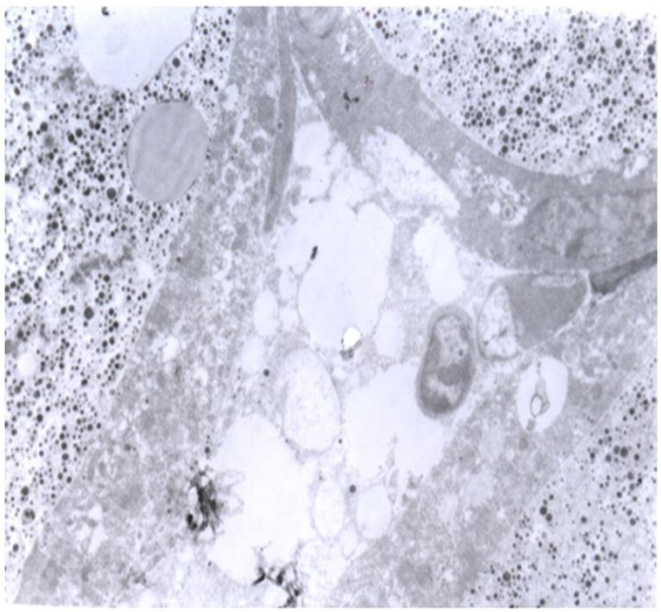


PLATE - 10b

Normal alveolar space and the lumen filled with milk constituents (milk protein as dense particles, with characteristic mottled surface appearance)

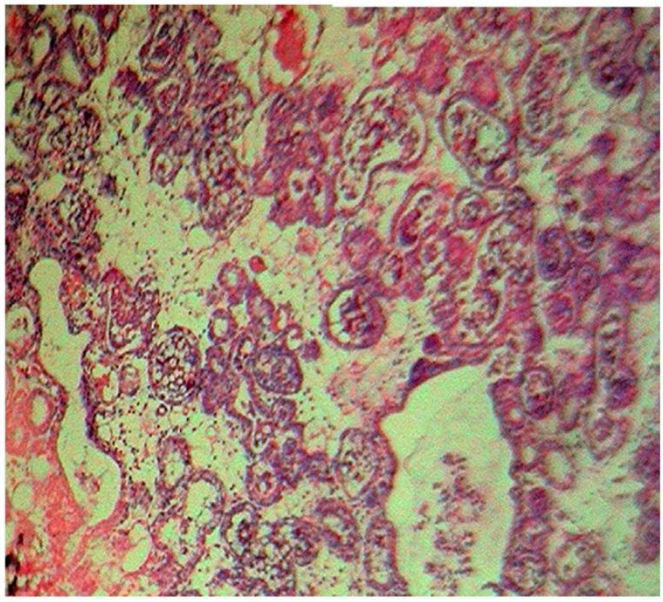


PLATE - 9a

Mild infiltration of neutrophils in between and within the alveoli with moderate number of bacterial rods

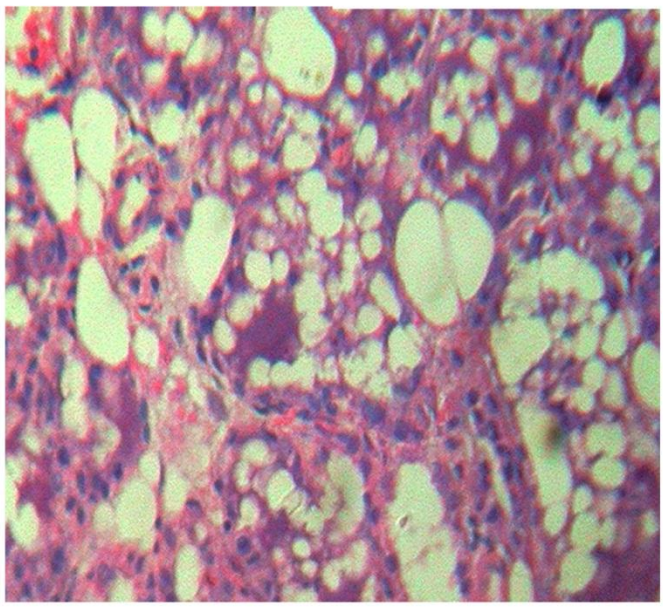


PLATE - 9b

Mild vacuolation of alveolar epithelium

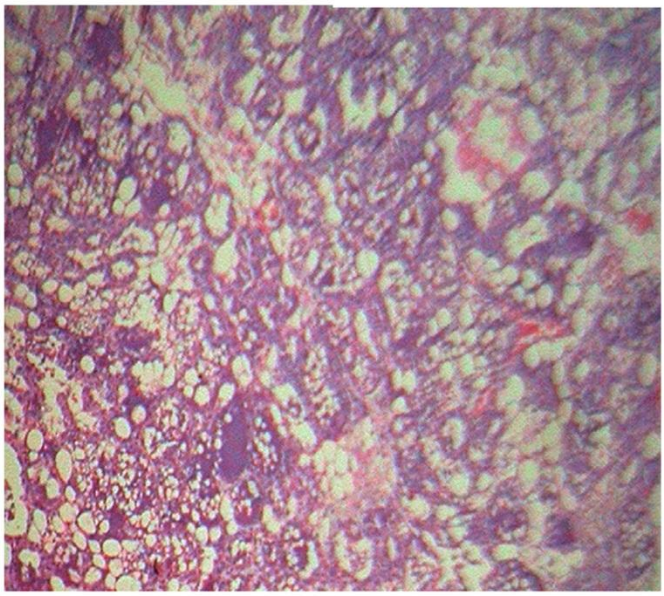


PLATE - 8a
Marked reduction in inflammatory cells

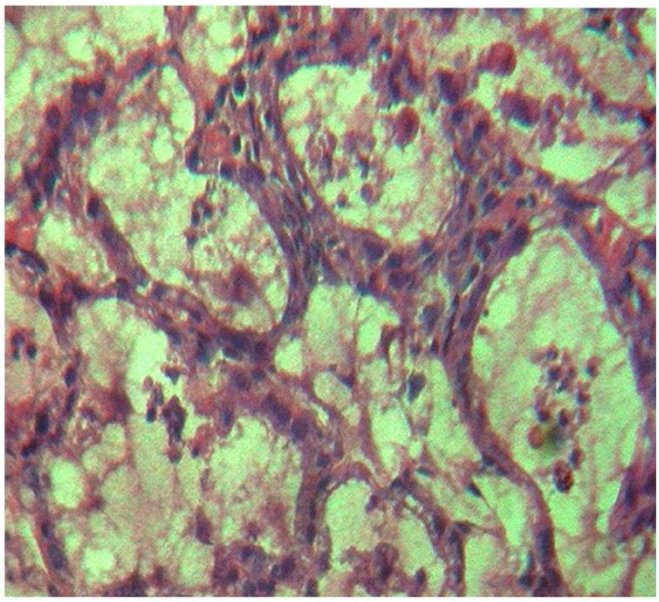


PLATE - 8b
Clear Alveolar cavity without without any bacterial rods

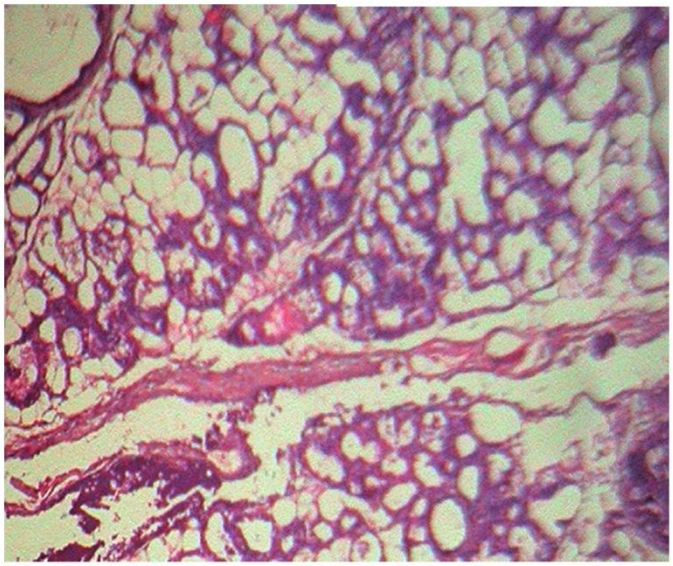


PLATE - 7a
Marked reduction in inflammatory cells

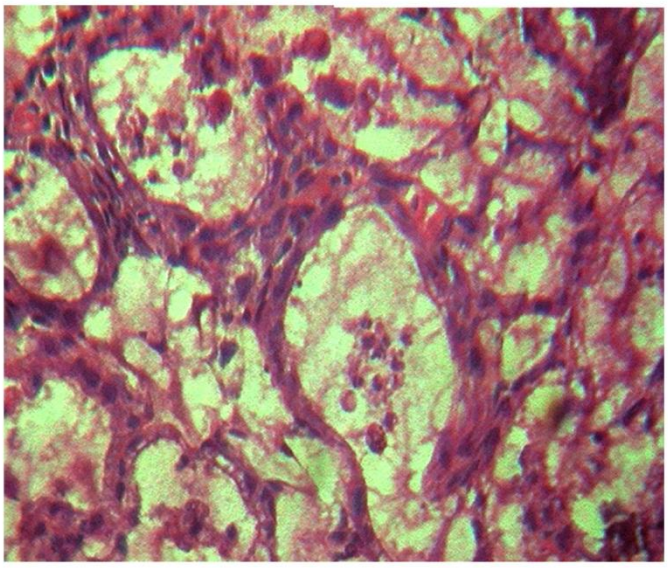


PLATE - 7b
Alveolar epithelium appeared to be normal

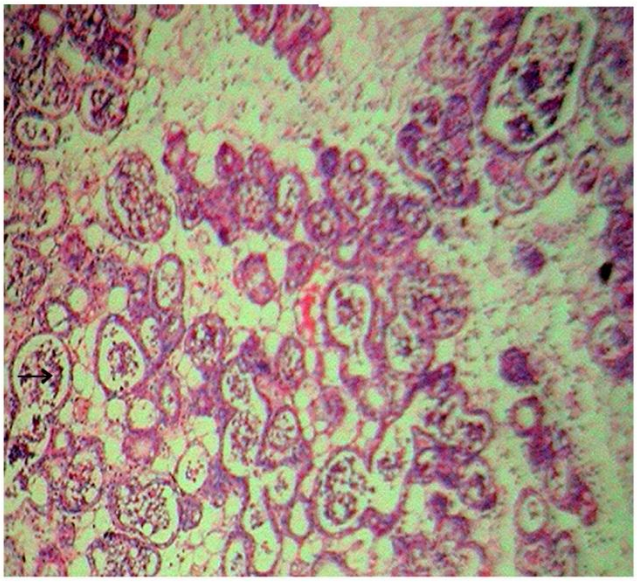


PLATE - 6a

Extensive infiltration of imflammatory cells in between and within the acini

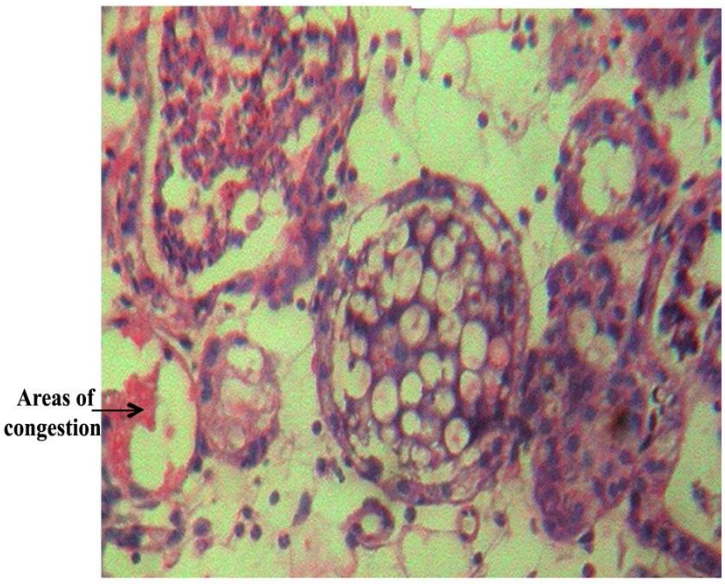


PLATE - 6b

Degenerative changes in alveoli epithelium and alveoli, duct lumina were filled with cellular debris, epithelial cells, bacterial rods and milk contents

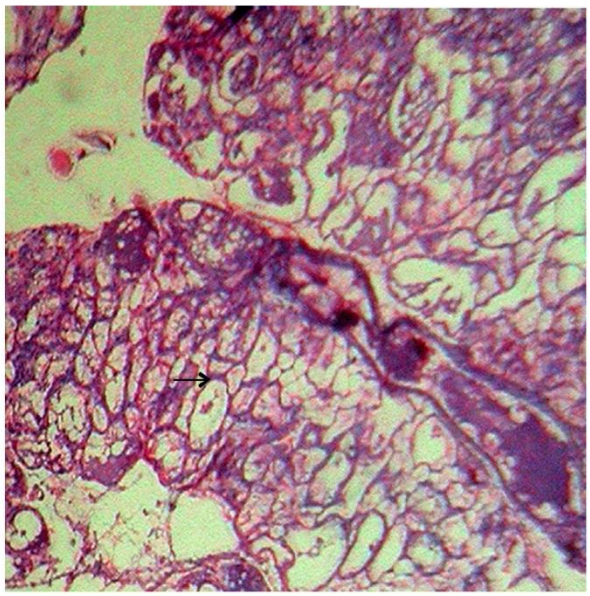


PLATE - 5a

Intact Alveoli epithelium and interalveolar space filled with myoepithelial cells

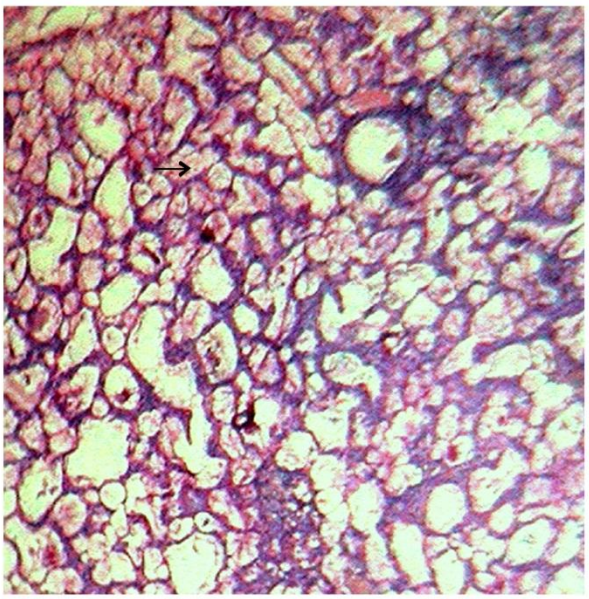
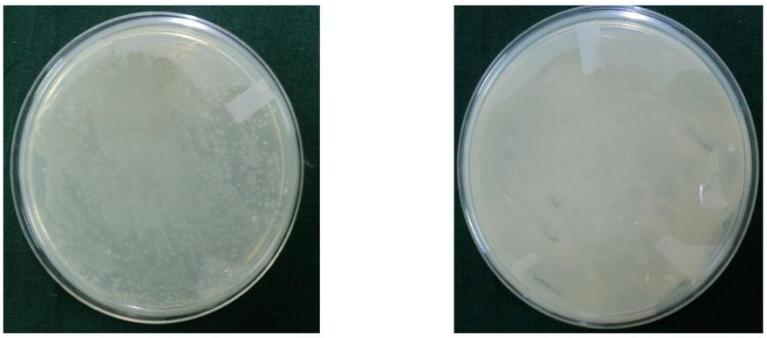


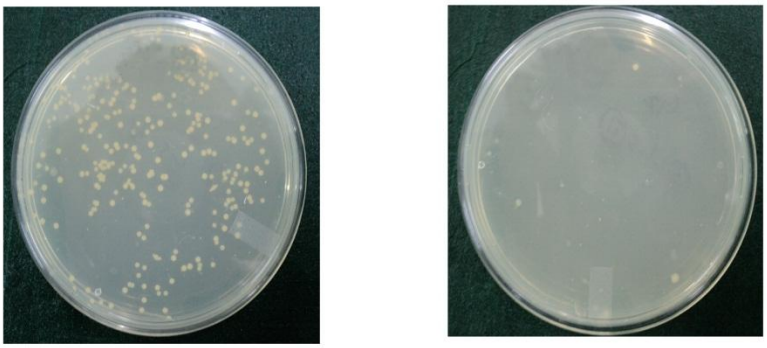
PLATE - 5b

Normal Alveoli with dilated sac like structure filled with milk contents

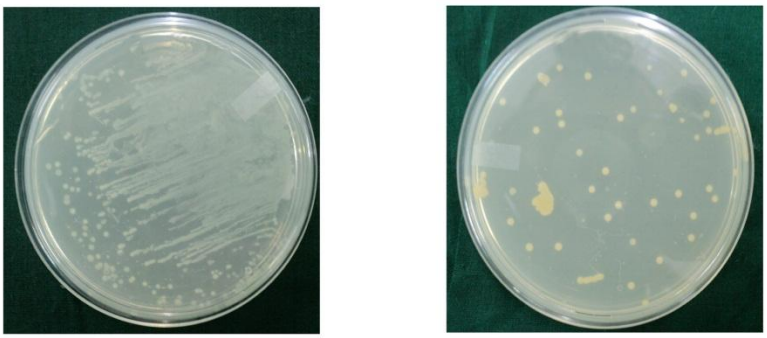
PLATE - 4



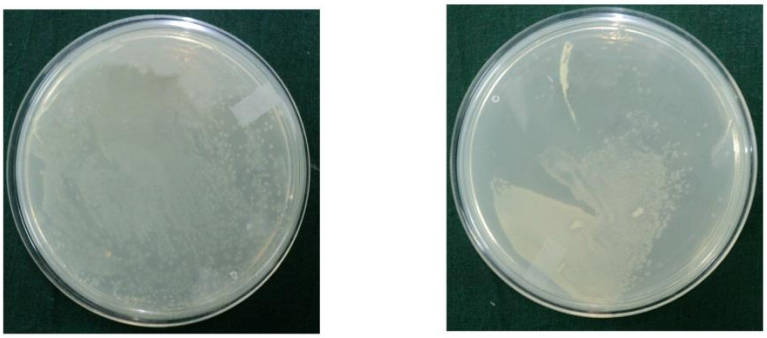
Mastitis Group



AKBANS I/m Group

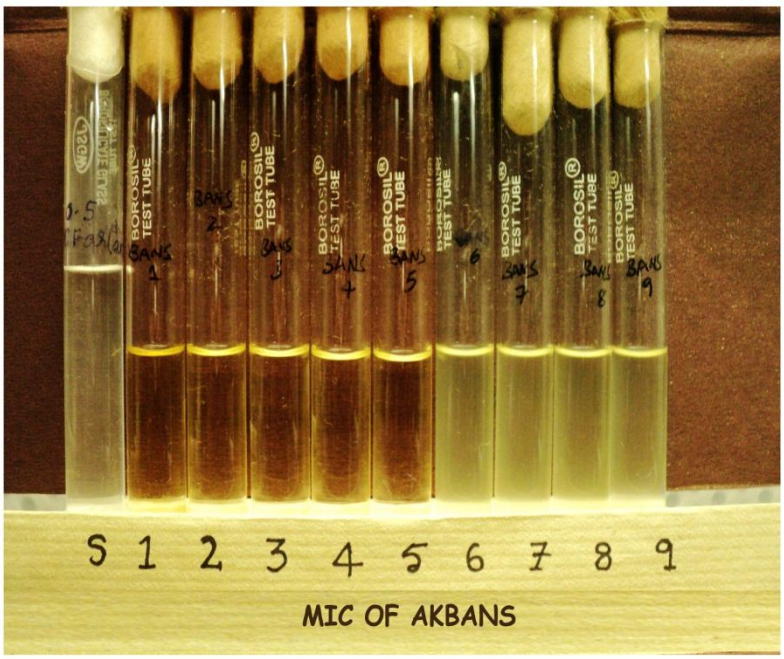


AKBANS I/p Group



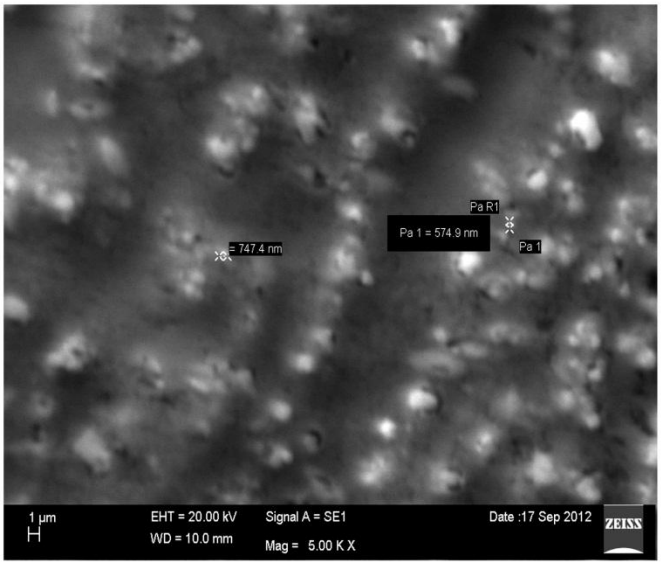
Antibiotic Group

PLATE - 3



Invitro **antibacterial activity**

PLATE - 2


Pa R1
Pa 1 = 574.9 nm
Pa 1
= 747.4 nm
1 µm
EHT = 20.00 kV
WD = 10.0 mm
Signal A = SE1
Mag = 5.00 K X
Date :17 Sep 2012
ZEISS

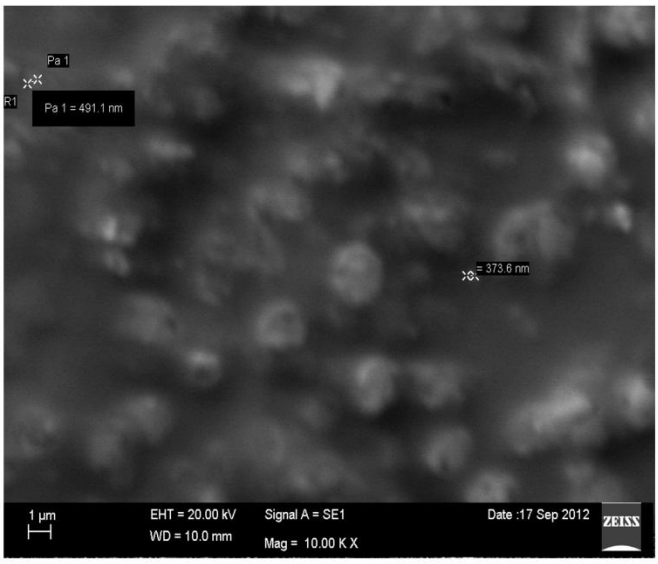
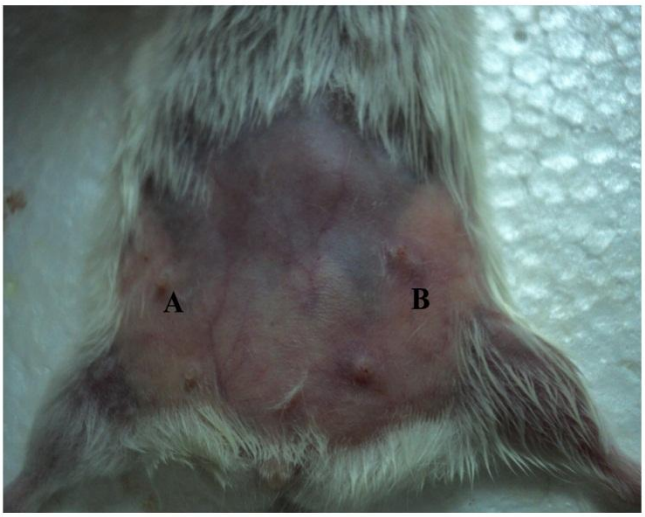

Pa 1
R1
Pa 1 = 491.1 nm
= 373.6 nm
1 µm
EHT = 20.00 kV
WD = 10.0 mm
Signal A = SE1
Mag = 10.00 K X
Date :17 Sep 2012
ZEISS

PLATE - 1



A. Normal Lactating mammary gland
B. Mastitis induced mammary gland