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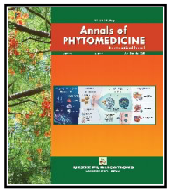

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Prophylactic potential of resveratrol, liposomal resveratrol, and Jamun (*Syzygium cumini* L.) seed powder in combination with cefquinome against *Staphylococcus aureus*-induced mastitis in goats

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Abstract

Mastitis and the associated mammary gland fibrosis in dairy animals, cause enormous economic losses globally. Literature suggests improvement in mastitis using therapies such as natural products, nanoparticles, antimicrobials, *etc*. Therefore, resveratrol, a plant-based polyphenol and Jamun (*Syzygium cumini* L.) plant, a native to India with numerous benefits along with a fourth-generation cephalosporin antibiotic, cefquinome were selected for the study. The present study aimed to assess the prophylactic effect of free resveratrol, liposomal resveratrol formulation and Jamun seed powder in combination with cefquinome in *Staphylococcus aureus* - induced mastitis and associated fibrosis in goats. Twenty-four lactating female Black Bengal goats (1.5-2 years age, 15-20 kg of body weight) were randomly divided into eight groups (Groups 1 to 8). Each group received different treatments except Groups 1 (Positive control) and 8 (Negative control). Characterization of resveratrol-containing liposomes was done by transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential estimation, which revealed spherical-shaped liposomes, around 200 nm size with zeta potential of -26.8 indicating good stability. Antioxidant parameters were found to increase and fibrosis biomarkers decreased in groups following Jamun or liposomal resveratrol administration in combination with cefquinome. The histopathological analysis revealed reduced mammary gland fibrosis in liposomal resveratrol/Jamun and cefquinome treated goats than others. Overall, the liposomal resveratrol formulation and the seed powder of Jamun along with cefquinome showed better prophylactic effect in preventing mastitis and associated fibrosis.

1. Introduction

Mastitis, an economic problem of utmost concern, affects dairy ruminants worldwide, including both developed and developing countries (Birhanu *et al.*, 2017; Guimarães *et al.*, 2017; Sharun *et al.*, 2021). In India, the economic losses due to mastitis amount to nearly Rs. 575 million per annum with a reduction in milk by 21% (Bardhan, 2013). Mammary gland fibrosis, a common pathological process associated with chronic mastitis, has a high prevalence exceeding 20-50% of cows in modern dairy herds (Pitkälä *et al.*, 2004). Fibrosis usually results from a sustained inflammatory response to chronic injury. It is characterized by the excessive growth of connective tissues with sclerosis and scars formation; attributed mainly to the accumulation of the extracellular matrix (ECM) on account of the deposition of extracellular matrix proteins, increase in cytokines, such as transforming growth factor-beta (TGF-β), basic fibroblast growth factor, platelet-derived growth factor and is associated with the process of epithelial-mesenchymal transition (EMT) (Chen *et al.*, 2017; Xu *et al.*, 2016).

In India, chronic mastitis is mostly caused by *Staphylococcus* species (nearly 55%) (Sharma *et al.*, 2012). *S. aureus* is mainly involved in the events such as increased TGF-β, *etc*., that lead to fibrosis and consequently decreased milk production (Bannerman *et al.*, 2006; Petersson-Wolfe *et al.*, 2010; Wu *et al.*, 2016; Zhao *et al.*, 2017). Although, there have been tremendous advancements in the therapeutic and management approaches in mastitis therapy, *viz*., antibacterial, herbal, and nanoparticle therapy (Algharib *et al.*, 2020; Sharun *et al.*, 2021), *in vivo* studies involving *Staphylococcal* mastitis, especially in ruminants, are scarcely available (Li *et al.,* 2023).

Cefquinome, a fourth-generation cephalosporin, was selected for the study since it has already been approved for mastitis treatment (El Badawy *et al.*, 2019) owing to its wide spectrum of antimicrobial activity and stability against chromosomally and plasmid-encoded beta-lactamase production (Limbert *et al.*, 1991). Natural products hold an important place in the drug discovery process and several

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herbal treatment strategies have been undertaken by researchers to control this chronic disease (Mushtaq *et al.*, 2018). Jamun (*Syzygium cumini* L.: Syn. *Eugenia jambolana* Lam. or *Syzygium jambolana* Dc. or *Eugenia cuminii* Druce.) belonging to the family Myrtaceae, is a large evergreen tree indigenous to India (Liu *et al.*, 2017) and its seeds are known to possess diverse bioactive phytochemicals such as resveratrol including jambosine, gallic acid, ellagic acid, quercetin, β-sitosterol, ferulic acid, guaiacol, resorcinol, p-coumaric acid, corilagin, catechin, epicatechin, tannic acid, *etc.*, with numerous biological benefits (Shrikanta *et al.*, 2015; Das *et al.,* 2023). Thus, on account of the frequent availability and ease of procurement by poor farmers as well as presence of the high amount of important phytochemical resveratrol (Shrikanta *et al.*, 2015), the seed powder of jamun was selected for the study.

Resveratrol, chemically known as 3,5,4'-trihydroxystilbene, is a naturally occurring polyphenolic antioxidant compound produced by a wide variety of plants and exhibits bioactivities such as anti-ageing, anti-inflammatory, antioxidant, antidiabetic, cardioprotective, neuroprotective and anticancer activity (Alanazi *et al.*, 2020; Elshaer *et al.*, 2018; Neves *et al.*, 2012; Salehi *et al.*, 2018; Xia *et al.*, 2017). There are reports indicating the antifibrotic effect of resveratrol (Chávez *et al.,* 2008*;* He *et al.,* 2017*;* Hessin *et al.,* 2017*;* Hong *et al.,* 2010*;* Wang *et al.*, 2018), but its use comes with certain limitations as evident from its pharmacokinetics that include low oral bioavailability, poor water solubility, less stability, extensive metabolism and short half-life (Amri *et al.*, 2012). To overcome such setbacks, resveratrol-loaded drug delivery systems to the target site, especially liposomes have been extensively studied by several researchers *(*Balanè *et al.*, 2015; Bonechi *et al.*, 2012; Isailoviæ *et al.*, 2013; Machado *et al.*, 2019). However, limited *in vivo* studies involving liposomes in mastitis condition have been performed (Balanè *et al.*, 2015; Bonechi *et al.*, 2012; Hsu *et al.*, 2017; Isailoviæ *et al.*, 2013). Reports on *in vivo* use of liposomal formulation in ruminants are still lacking.

Thus, in the present study, an attempt has been made to design a treatment protocol that can prevent the progression of mastitis towards the chronic fibrotic stage. Hence, an *in vivo* study involving a combination of antibacterial, herbal and nanoparticle therapy was carried out to determine their efficacy in mastitis of goats.

Considering the above, the aim of the present study was to evaluate the prophylactic efficacy of resveratrol and its liposomal formulation as well as powdered seed of Jamun (*Syzygium cumini* L.) in combination with cefquinome in *S. aureus* induced mammary gland fibrosis in Black Bengal goats.

2. Materials and Methods

2.1 Plant authentication

The plant under study, *i.e.*, Jamun (*Syzygium cumini* L.) was authenticated by Dr. Saswat Nayak, Head of the Department, Forest Products and Utilization, College of Forestry, Odisha University of Agriculture and Technology (O.U.A.T.), Bhubaneswar, India. The Plant Specimen Number (WBUAFS/SA-01) was kept at the Department of Veterinary Pharmacology and Toxicology, West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal, India.

2.2 Drugs used

Resveratrol (analytical grade, HPLC, purity ≥ 99%) and the transforming growth factor beta-2 (TGF-β2) ELISA kit were purchased from Sigma-Aldrich whereas the nuclear factor-kappa b (NF-κB p65) ELISA Kit was acquired from KINESISDx. Cefquinome sulfate (analytical grade, purity ≥ 90%) was used in the study. Resveratrol capsules (98% purity) were brought from Sharrets Nutritions LLP, India. All other chemicals and media preparation agents used in the present study were obtained from HiMedia, Sisco Research Laboratories Pvt. Ltd. (SRL) and Rankem.

2.3 Experimental design

The present study adheres to the internationally accepted guidelines, such as the 3Rs (Replacement, Reduction, and Refinement) principle for the welfare of animals. All institutional and national guidelines for the care and use of laboratory animals were followed. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) [No. IAEC/67/i(B) dt. 19.08.19], West Bengal University of Animal and Fishery Sciences, Kolkata, India and the Committee for Control and Supervision of Experiments on Animals (CCSEA), Department of Animal Husbandry and Dairying, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India [V-11011(13)/8/2022-CPCSEA-DADF dated 03.08.2022].

For the present experiment, 24 clinically healthy female lactating Black Bengal goats, 15-20 kg of body weight, aged 1.5-2 years were divided into eight groups (Groups 1-8) with each group containing 3 animals. A single intra-cisternal inoculation of *S. aureus* at 4000 CFU/ml in the teat of a single udder was done in animals of all groups (except Group 8) for mastitis induction. The group distribution was as follows:

Group 1: After *S. aureus* inoculation, the goats were left untreated and considered as Positive control group;

Group 2: After *S. aureus* inoculation, the goats received intra-venous (IV) dosing of cefquinome at 2 mg kg^{-1} body weight (b. wt.) once daily for 5 consecutive days;

Group 3: After *S. aureus* inoculation, the goats received liposomal resveratrol at 50 mg kg^{-1} b. wt. intra-peritoneally (IP) mixed in 1 ml glycofurol and an adequate amount of Normal saline as a carrier for 7 days along with cefquinome at 2 mg kg^{-1} b. wt. IV once daily for 5 consecutive days;

Group 4: After *S. aureus* inoculation, the goats received an oral dose of resveratrol at 500 mg kg^{-1} b. wt. mixed in 1ml of glycofurol and an adequate amount of Normal saline as a carrier for 7 days along with cefquinome at 2 mg kg^{-1} b. wt. IV once daily for 5 consecutive days;

Group 5: After *S. aureus* inoculation, the goats received resveratrol at 50 mg kg^{-1} b. wt. IP mixed in 1ml of glycofurol and an adequate amount of Normal saline as a carrier for 7 days along with cefquinome at 2 mg kg^{-1} b. wt. IV once daily for 5 consecutive days;

Group 6: After *S. aureus* inoculation, the goats received an oral dose (400 mg kg^{-1} b. wt.) of powdered seed of Jamun (*Syzygium cumini* L.) once daily for consecutive 28 days;

Group 7: After *S. aureus* inoculation, the goats received an oral dose (400 mg kg^{-1} b. wt.) of powdered seed of Jamun (*Syzygium cumini* L.) once daily for consecutive 28 days along with cefquinome at 2 mg kg^{-1} b. wt. IV once daily for 5 consecutive days; and

Group 8: Healthy lactating goats without any treatment or *S. aureus* inoculation were treated as Negative control and used for histopathological comparison.

2.4 Sample collection

Blood samples were collected at 0, 7, 14, and 28 days of the experimental period, whereas milk samples on 0, 7, and 14 days. They were used for enzymatic antioxidant parameters and fibrosis biomarker estimation. Mammary tissue samples of Groups 1 to 8 were collected using BARD® Disposable Core biopsy instrument after the application of local anaesthesia on the 28th day. A routine histopathological procedure was undertaken, followed by Van Gieson's staining for collagen fibers. The histopathological parameters of Groups 2 to 7 were compared against Group 1 (Positive control) and Group 8 (Negative control).

2.5 Estimation of antioxidant parameters and fibrosis biomarkers

The superoxide dismutase (SOD), reduced glutathione activity (GSH), and glutathione peroxidase (GPx) activities were estimated according to the methods described by Khan *et al.* (2012). The lactoperoxidase (LPO) activity was measured according to the methods described by Keesey (1987) and Putter and Becker (1983). The NF-κB p65 and TGF-β2 were quantitated using the standard ELISA Kit Protocol.

2.6 Formulation and characterization of liposomes containing resveratrol

2.6.1 Preparation of liposomes

A total amount of 4.3 g lecithin (80 mol%), 0.7 g cholesterol (20 mol%) and 1 g resveratrol were weighed in a 100 ml round bottom flask followed by addition of 20 ml 1:1 mixture of methanol and chloroform to it. The mixture was shaken vigorously and the organic solvent was removed by rotary evaporation to get a thin lipid film on the inside wall of the round bottom flask. The flask containing the thin film was subjected to high vacuum for 5 h to remove the residual organic solvent and kept overnight at 4°C. 1 ml glycofurol and 39 ml 20 mM phosphate buffer (pH 7.4, 255 mM dextrose in it) were added to it. The mixture was hydrated, vortexed vigorously at 37°C for ~4 h and subjected to bath sonication for 2 x 30 min (with an interval of 5 min in between). Next probe sonication was employed for 2 x 10 min (with an interval of 5 min in between) to get a more uniform distribution of vesicular morphology. Finally, the mixture was centrifuged for 30 min at a speed of 10000 rpm to remove the unwanted larger undissolved portion. The supernatant was the prepared liposomal formulation.

2.6.2 Characterization of liposomes

2.6.2.1 Dynamic light scattering (DLS)

It was recorded using a Malvern Zetasizer Nano ZS instrument (Malvern, UK) equipped with a 4 mV He–Ne laser operating at λ = 633 nm (scattering angle = 173°), an avalanche photodiode detector with high quantum efficiency, and an ALV/LSE-5003 multiple tau digital correlator electronics system.

2.6.2.2 Transmission electron microscopy (TEM)

A JEOL JEM-2100F instrument operational at 200 kV was used to record the morphologies of the liposomes. TEM samples were prepared by drop-casting a diluted formulation (500-fold dilution) on a carbon-coated copper grid and then dried for a few hours under vacuum at room temperature.

2.7 Statistical analysis

The data were analysed statistically using a general linear model with univariate data following Tukey's Honest Significant Difference Test in SPSS software (Version 21.0). Independent samples T-test was also used to compare between two groups where applicable. The results were expressed as Mean ± Standard error (S.E.). A value of $p<0.5$ was considered significant.

3. Results

3.1 Induction and confirmation of mastitis and associated fibrosis

In the present study, *S. aureus* was used for induction of fibrosis associated with chronic mastitis. Animals of all the groups showed the symptoms of anorexia and slightly increased temperature (103-104°F) during the first 7 days of intracisternal inoculation of *S. aureus*. From the 7th day, the goats of Group 1 showed slight pain sensation on touching the affected quarter as they were hot and swollen in comparison with the normal udder of 0-day. However, the swollen udder was not so prominent in goats of prophylactic treatment groups (Groups 2 to 7) on 7th day. The milk samples from goats of all groups collected on 0-day (before inoculation) did not show any growth of *S. aureus* colonies on mannitol salt agar media indicating the absence of any intramammary infection whereas the milk collected on 7th day post inoculation showed the bacterial colonies growth on the mannitol salt agar media (Figure 1a) indicating the confirmation of mastitis in the Group 1 to 7 goats. The inoculated half of the udder of Group 1 goats showed shrinkage of the gland with a marked hardness on the 28th day (Figure 1b). Interestingly, the shrinkage and hardening of the teat were less severe in the mastitis-induced goats of prophylactic treatment groups on 28th day (Figure 1c).

The BTB (Bromothymol blue) test and CMT (California Mastitis Test) did not show any significant reactions from milk collected on the 0-day (before bacterial inoculation) in goats of all groups. However, in the 7th day post-inoculation milk sample of Group 1 goat, the BTB paper test showed a dark green colour due to rising in pH of the milk whilst the prophylactic treatment groups showed a light green colour indicating that the intensity of infection was less compared to Group 1 goats. The mastitis was confirmed from CMT of the milk sample from the affected half of the udder collected on the 7th day post inoculation which showed viscous, severe clotting and flake formation especially in Group 1 goats. However, curdling of milk that varied in severity was noticed in the milk samples of the prophylactic treatment groups. Milk of the 28th day could not be collected as there was a complete cessation of milk yield due to the development of fibrosis condition.

Figure 1: Figure showing (a) Growth of *Staphylococcus aureus* colonies on mannitol salt agar media, (b) Shrunken mammary gland (characteristic of chronic mastitis) in the inoculated udder of Group 1 goat on 28th day post bacterial inoculation, and (c) Comparatively less shrunken mammary gland in prophylactic treatment groups on 28th day post bacterial inoculation.

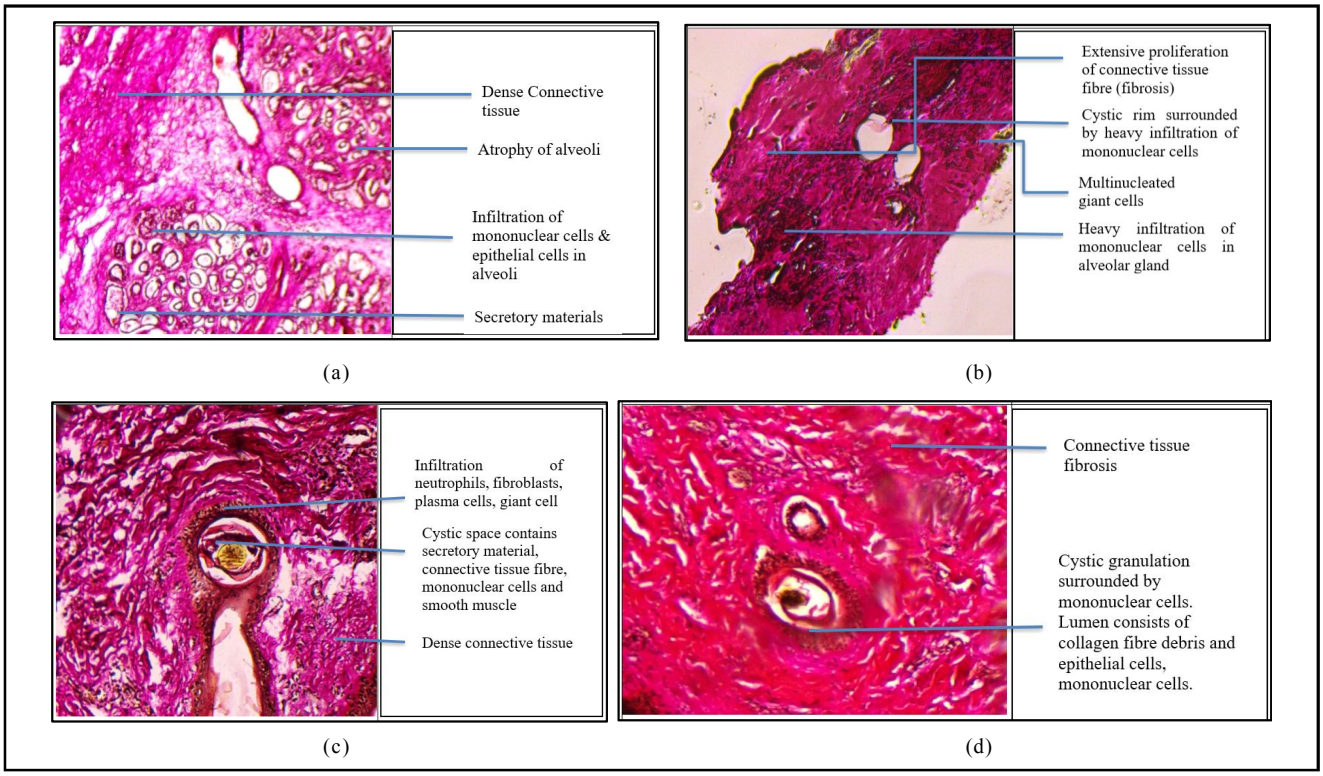

Figure 2: Microscopic view of mammary gland of Group 1 goats on 28th day post bacterial inoculation (Van Gieson's Stain (10X)) depicting, (a) infiltration of mononuclear cells and epithelial cells in alveoli with thickening of connective tissue and alveolar atrophy, (b) extensive proliferation of connective tissue with alveolar gland heavily infiltrated by mononuclear cells and presence of multinucleated giant cell, and (c and d) cystic granulation with PMNs infiltration and extensive connective tissue proliferation (fibrosis).

The histopathological examination of Group 1 goats is shown in Figures 2 (a-d) and of different treatment groups are depicted in Figures 3 (a-g). Group 1 (Positive control) goats showed fibrous mastitis with cyst formation. Mononuclear cells infiltrated both inter and intra lobular gland. There was also a desquamation of epithelial cells of acini into the lumen along with secretory materials. Furthermore, there was marked proliferation of connective tissue especially collagen fibres that is characteristic of chronic mastitis. In some cases, the proliferated connected tissue replaced other structure like the alveolar gland and there was entrapment of lipid cells.

In comparison, in the negative control group, *i.e.*, healthy animals without any bacterial inoculation (Group 8), there were no abnormal changes in the alveolar gland. The alveoli were properly arranged

with secretory epithelial cells. There was the absence of cyst formation and infiltration of inflammatory cells in the lumen. In the case of Jamun treatment (Group 6) and liposomal resveratrol treatment group (Group 3), there was comparatively less infiltration of mononuclear cells and proliferation of connective tissue compared to Group 1. Antibiotic and jamun-treated group (Group 7) too showed less inflammatory changes compared to Group 1 goats. However, groups treated with cefquinome only (Group 2), resveratrol administered IP (Group 5), and orally (Group 4) had moderate proliferation of connective tissue and infiltration of mononuclear cells.

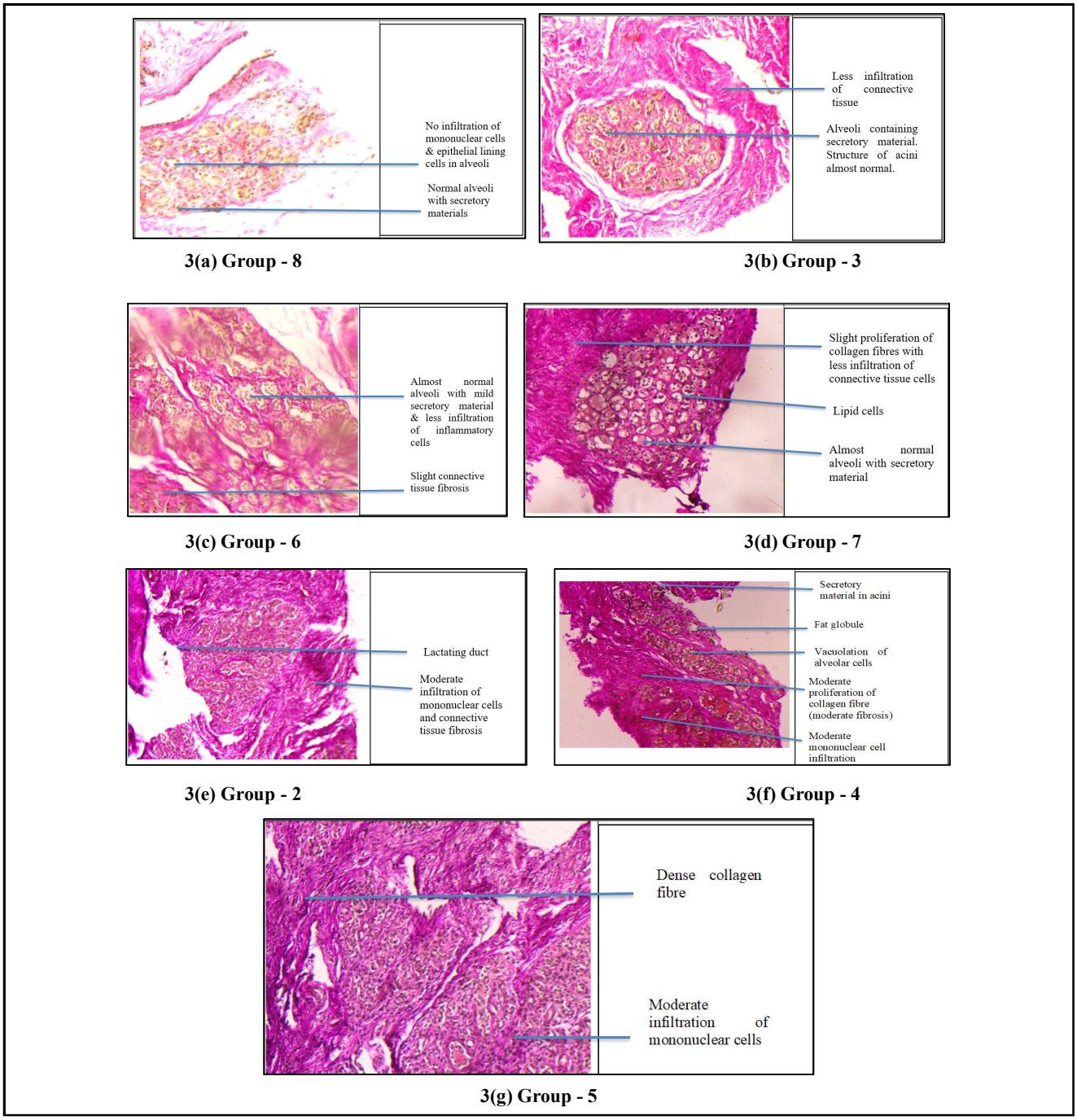

Figure 3: Microscopic view of mammary gland of goats on 28th day post bacterial inoculation (Van Gieson's Stain (10X)) depicting, (a) normal alveoli with properly arranged secretory epithelial cells and without any cellular infiltration in Group 8, (b) less infiltration of mononuclear cells and proliferation of connective tissue with almost normal structure of acini containing secretory material in Group 3, (c) less infiltration of mononuclear cells and proliferation of connective tissue with almost normal alveoli except mild secretory material in Group 6, (d) less mononuclear cell infiltration and proliferation of connective tissue in Group 7, and (e, f and g) moderate proliferation of connective tissue and more infiltration of mononuclear cells and secretory epithelial cell debris in the lumen in Group 2, 4 and 5, respectively.

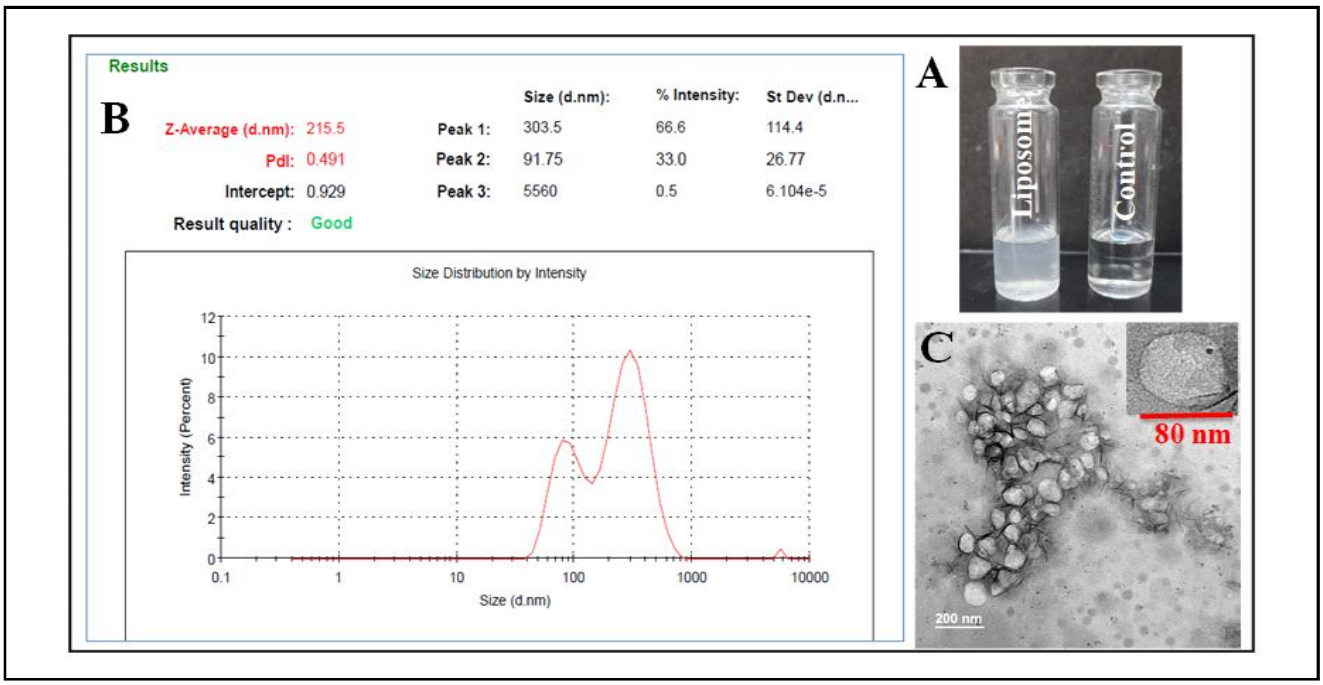

Figure 4: Characterization of prepared liposomal formulation by (a) naked eye visualization, (b) Dynamic Light Scattering (DLS), and (c) Transmission Electron Microscopy (TEM) analysis.

3.2 Characterization of the prepared liposome

Characterization of the prepared liposomal formulation is depicted in Figures 4 (a-c). The liposomal size (Z-average) was nearly 215 (d.nm.). This was in accordance with the findings of a study (Isailoviæ *et al.*, 2013) that reported liposomes that were subsequently reduced by sonication had an average diameter between 120 and 290 nm. The TEM images in the present study indicated a spherical shape of resveratrol-loaded liposomes and around 200 nm size similar to a study (Zu *et al.*, 2018). In general, particles with zeta potential values greater than +30 mV or lower than –30 mV possess long-lasting electrostatic stability (DeLuca *et al.*, 2006). Zeta potential of -26.8 was observed indicating good stability and a lower chance of aggregation for the liposomal resveratrol formulation as shown in (Figure 5).

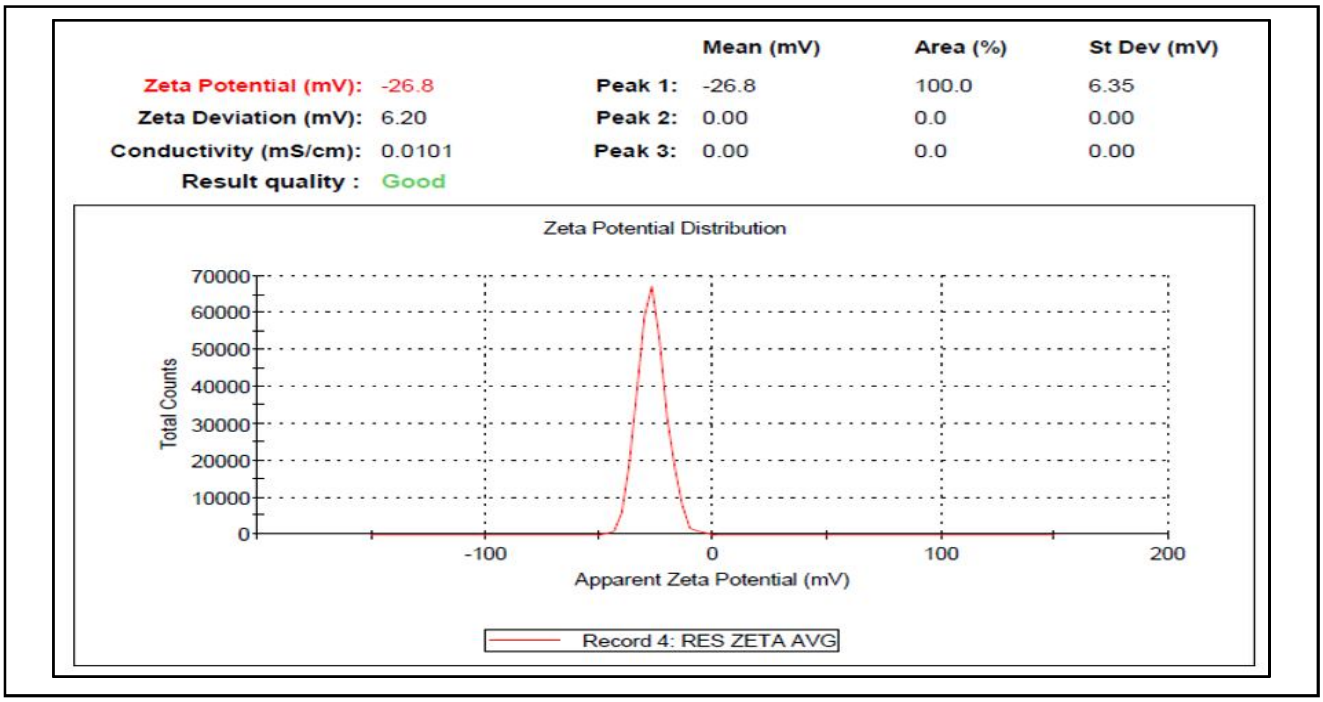

Figure 5: Estimation of zeta potential of the prepared liposomal formulation.

3.3 Estimation of antioxidant parameters and fibrosis biomarkers

The antioxidant parameters such as glutathione peroxidase (GPx), superoxide dismutase (SOD), reduced glutathione (GSH) and lactoperoxidase (LPO) activity have been incorporated in (Table 1, 2, 3, and 4), respectively. The fibrosis biomarkers such as nuclear factor kappa b p65 (NF-κB p65) and transforming growth factor beta-2 (TGF-β2) have been depicted in (Figure 6) and (Figure 7), respectively.

Table 1: Mean values with SE of glutathione peroxidase activity (n moles of NADPH consumed per mg protein per min) of RBC haemolysate following intracisternal inoculation of 4000 C.F.U/ml of *S. aureus* (n=3)

Group	Day 0	Day 7	Day 14	Day 28
1	$2.73^{c} \pm 0.11$	$1.74^{d} \pm 0.54$	$2.75^{b} \pm 0.49$	$3.73^{bc} \pm 0.21$
2	$2.51^{c} \pm 0.11$	$2.29^{cd} \pm 0.14$	$3.18^{ab} \pm 0.39$	$2.57^{bc} \pm 0.09$
3	$5.05^{a} \pm 0.76$	$3.60^{bc} \pm 0.23$	$4.52^{a} \pm 0.28$	$4.43^{ab} \pm 0.85$
4	$3.10^{bc} \pm 0.08$	$5.15^{a} \pm 0.14$	$3.22^{ab} \pm 0.13$	$3.21^{bc} \pm 0.10$
5	$4.57^{ab} \pm 0.19$	$4.57^{ab} \pm 0.13$	$3.66^{ab} \pm 0.23$	$3.14^{bc} \pm 0.48$
6	$2.23^{c} \pm 0.46$	$1.62^{d} \pm 0.26$	$2.69^{b} \pm 0.03$	$2.45^{cd} \pm 0.16$
7	$3.12^{bc} \pm 0.12$	$1.97^{d} \pm 0.21$	$2.40^{b} \pm 0.15$	$3.41^{bc} \pm 0.10$
Group	$p < 0.05$			
Days	$p = 0.243$			
Group × Days	$p < 0.05$			

Means bearing similar superscript (a, b, c) in a column does not vary significantly from each other. Means bearing similar superscript (x, y, z) in a row does not vary significantly from each other.

Table 2: Mean values with SE of superoxide dismutase activity (Units per mg of haemolysate protein) of RBC haemolysate following intracisternal inoculation of 4000 C.F.U/ml of *S. aureus* (n=3)

Group	Day 0	Day 7	Day 14	Day 28
1	$2.83^{ax} \pm 0.32$	$2.14^{abxy} \pm 0.30$	$1.51^{by} \pm 0.21$	$1.66^{axy} \pm 0.30$
2	$2.65^{ax} \pm 0.08$	$2.32^{axy} \pm 0.04$	$2.28^{axy} \pm 0.10$	$1.85^{ay} \pm 0.21$
3	$2.75^{ax} \pm 0.16$	$2.35^{ax} \pm 0.22$	$2.26^{abx} \pm 0.03$	$2.15^{ax} \pm 0.01$
4	$2.52^{ax} \pm 0.40$	$2.11^{abx} \pm 0.02$	$1.97^{abx} \pm 0.11$	$1.60^{ax} \pm 0.28$
5	$2.41^{ax} \pm 0.31$	$1.33^{bx} \pm 0.31$	$1.74^{abx} \pm 0.14$	$1.56^{ax} \pm 0.20$
6	$2.61^{ax} \pm 0.19$	$1.62^{aby} \pm 0.04$	$1.65^{aby} \pm 0.28$	$2.06^{axy} \pm 0.24$
7	$2.59^{ax} \pm 0.09$	$1.72^{aby} \pm 0.07$	$2.28^{ax} \pm 0.09$	$2.38^{ax} \pm 0.10$
Group	$p < 0.05$			
Days	$p < 0.05$			
Group × Days	$p = 0.097$			

Means bearing similar superscript (a, b, c) in a column does not vary significantly from each other. Means bearing similar superscript (x, y, z) in a row does not vary significantly from each other.

Table 3: Mean values with SE of reduced glutathione activity (n moles of TNB conjugate formed per min per mg of haemoglobin) of RBC haemolysate following intracisternal inoculation of 4000 C.F.U/ml of *S. aureus* (n=3)

Group	Day 0	Day 7	Day 14	Day 28
1	$0.15^{abx} \pm 0.01$	$0.11^{axy} \pm 0.01$	$0.08^{ay} \pm 0.01$	$0.11^{aby} \pm 0.004$
2	$0.17^{ax} \pm 0.01$	$0.07^{ay} \pm 0.01$	$0.06^{ay} \pm 0.01$	$0.08^{by} \pm 0.00$
3	$0.16^{abx} \pm 0.01$	$0.11^{ax} \pm 0.00$	$0.18^{ax} \pm 0.03$	$0.15^{ax} \pm 0.02$
4	$0.15^{abx} \pm 0.004$	$0.08^{ay} \pm 0.01$	$0.09^{ay} \pm 0.01$	$0.09^{by} \pm 0.02$
5	$0.14^{abx} \pm 0.01$	$0.08^{ax} \pm 0.02$	$0.14^{ax} \pm 0.04$	$0.09^{bx} \pm 0.003$
6	$0.13^{bx} \pm 0.01$	$0.07^{ay} \pm 0.01$	$0.07^{ay} \pm 0.01$	$0.14^{ax} \pm 0.01$
7	$0.17^{ax} \pm 0.01$	$0.11^{ax} \pm 0.04$	$0.09^{ax} \pm 0.01$	$0.17^{ax} \pm 0.01$
Group	$p<0.05$			
Days	$p<0.05$			
Group × Days	$p<0.05$			

Means bearing similar superscript (a, b, c) in a column does not vary significantly from each other. Means bearing similar superscript (x, y, z) in a row does not vary significantly from each other.

Table 4: Mean values with SE of milk lactoperoxidase activity (units per ml) of the affected half of the udder after intracisternal inoculation of 4000 C.F.U. ml of *S. aureus* (n=3)

Group	Day 0	Day 7	Day 14
1	$0.45^{abx} \pm 0.06$	$0.18^{ay} \pm 0.01$	$0.06^{aby} \pm 0.03$
2	$0.53^{abx} \pm 0.06$	$0.21^{ay} \pm 0.02$	$0.13^{aby} \pm 0.01$
3	$0.65^{abx} \pm 0.04$	$0.29^{ay} \pm 0.01$	$0.19^{ay} \pm 0.03$
4	$0.40^{bx} \pm 0.08$	$0.19^{axy} \pm 0.04$	$0.06^{aby} \pm 0.02$
5	$0.70^{ax} \pm 0.30$	$0.30^{ay} \pm 0.01$	$0.05^{bz} \pm 0.02$
6	$0.61^{abx} \pm 0.08$	$0.22^{ay} \pm 0.03$	$0.20^{ay} \pm 0.03$
7	$0.63^{abx} \pm 0.05$	$0.28^{ay} \pm 0.08$	$0.20^{ay} \pm 0.04$
Group		$p<0.05$	
Days		$p<0.05$	
Group × Days		$p=0.104$	

Means bearing similar superscript (a, b, c) in a column does not vary significantly from each other. Means bearing similar superscript (x, y, z) in a row does not vary significantly from each other.

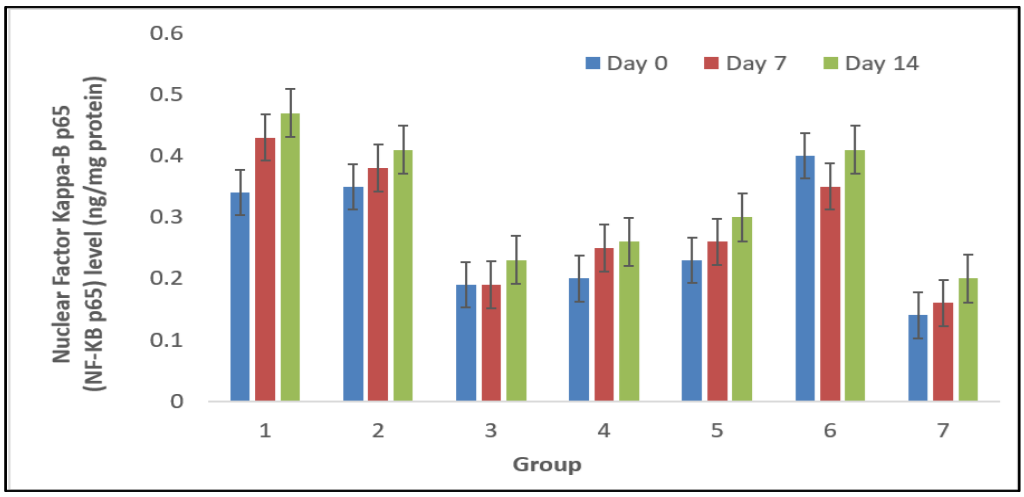

Figure 6: Mean values of blood nuclear factor kappa-B p65 (NF-κB p65) level (ng/mg protein) following intracisternal inoculation of 4000 C.F.U/ml of *S. aureus* (n=3).

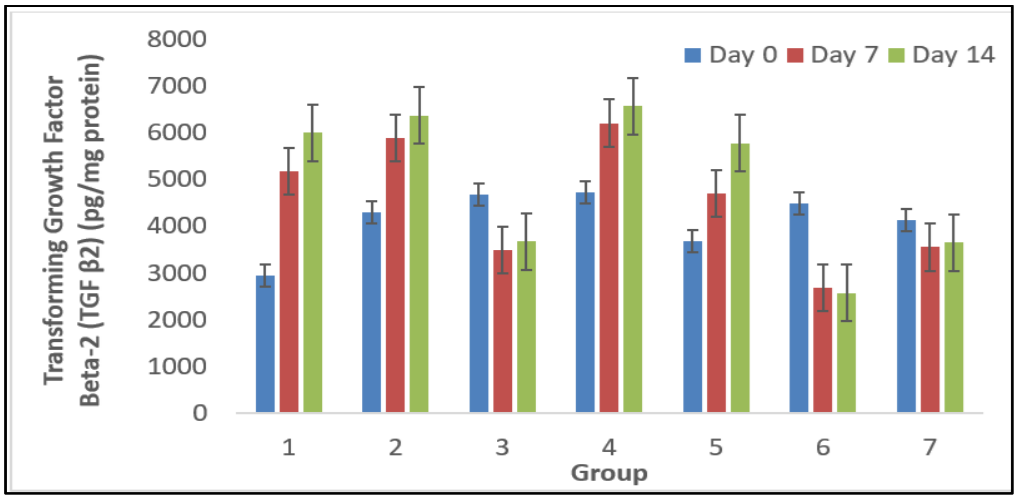

Figure 7: Mean values of milk transforming growth factor beta-2 (TGF β2) (pg/mg protein) following intracisternal inoculation of 4000 C.F.U/ml of *S. aureus* (n=3).

4. Discussion

The *S. aureus* inoculated half of the udder of Group 1 goats showed shrinkage of the gland with a marked hardness on the 28th day which was in accordance with (Singh *et al.*, 1994) who reported that from 20th to 40th days post-infection, the infected udder halves became progressively small and shrunken. There has been evidence of infiltration in goat's udder in experimental mastitis, composed mainly of neutrophils (Jing *et al.*, 2012; Lasagno *et al.*, 2012). A common observation in chronic mastitis such as the process of involution of parenchyma, shrinkage of alveoli, proliferation of connective tissues, and accumulation of cellular fragments in glandular alveoli as a consequence of the lysis of epithelial cells and PMN cells was also observed in enlarged pendulous caprine udder tissues (Alawa *et al.*, 2000; Dash *et al.*, 2016) which was in corroboration with the present study. An initial sharp fall in the milk yield was mainly due to severe damage to the acini leading to exfoliation of their epithelial cells. Later on, milk yield decreased due to progressive replacement of the secretory parenchyma by granulomas, cellular aggregates, and extensive fibrosis. Similar to the present study, reports of changes related to *Staphylococcal* mastitis have been reported in buffalo (Restucci *et al.*, 2019); in goats/ewes (Dash *et al.*, 2014; Sadiq *et al.*, 2019) and in mice (Chinchali and Kaliwal, 2014; Jiang *et al.*, 2017).

Polyphenols that are found abundantly in plants have been shown to exert antioxidant action through scavenging of a wide range of free radicals and inhibition of generation of reactive oxygen species, thereby preventing cell damage and resveratrol is one such potential polyphenol (Gutteridge and Halliwell, 2010; Mukherjee *et al.*, 2011). A report (Shrikanta *et al.*, 2015) showed Jamun seed having significantly higher polyphenols (55.54 mg GAE g^{-1}) compared to other fruit extracts and a higher amount of resveratrol content compared to Jamun skin and pulp. In the present study, the antioxidant activity of resveratrol and Jamun seed powder was evaluated by measuring levels of important antioxidant enzymes, *viz*., glutathione peroxidase, superoxide dismutase, reduced glutathione and lactoperoxidase.

Table 1 shows that there was a significant increase in the level of GPx activity in Group 3, Group 4 and Group 5 compared to Group 1 on 7th day post-inoculation. The GPx activity of Group 3 was also significantly increased on 14th day compared to that of Group 1.

It is evident from (Table 2) that the mean SOD activity was significantly increased on the 7th day in Group 3 goats compared to Group 5 goats indicating higher efficacy of liposomal resveratrol formulation over free resveratrol. On the 14th day post-inoculation, the mean SOD activity was significantly increased in Group 7 goats and in Group 2 goats compared to Group 1 goats. The mean SOD activity in Group 1 goats decreased significantly on the 14th day post-inoculation compared to the 0-day value. A similar trend was observed in Group 2 on the 28th day post- inoculation. However, the mean SOD activity did not decrease significantly in Group 3 after *S. aureus* inoculation on different days indicating the antioxidant potential of liposomal formulation of resveratrol. Albeit the mean SOD activity decreased significantly in Group 7 goats on 7th day post-inoculation compared to 0-day value but it increased significantly on 14th day post-inoculation when compared to 7th day value indicating antioxidant action of the combination therapy.

As shown in (Table 3), the mean GSH activity of Group 1 goats decreased significantly on 14th day and 28th day post-inoculation compared to the 0-day value. In Group 2 too, there was a significant decrease in the mean GSH activity on the 7th, 14th and 28th day post-inoculation compared to the 0-day value. However, in Group 3 and Group 7, no significant reduction in the mean GSH activity on different days post-inoculation when compared against their respective 0-day values was recorded. This indicates the potent antioxidative action of liposomal resveratrol formulation and the combination therapy of Jamun with antibiotics. In Group 6 too, albeit a significant reduction in the mean GSH activity on the 7th and 14th day post-inoculation compared to the 0-day values was found, but a significant increase in the mean GSH activity on the 28th day indicates the antioxidative potential of Jamun against oxidative stress.

In a study (Mukherjee *et al.*, 2011), pure resveratrol at the dose level of 8 mg/kg produced a significant increase in GSH, GPX, SOD and CAT activity ($p<0.01$) in doxorubicin-induced cardiotoxicity in rats. Similar findings were reported (Palsamy and Subramanian, 2010; Soufi *et al.*, 2012) where they found increased levels of antioxidant enzymes like SOD, GPx and GSH after resveratrol treatment in diabetic animal models. These findings were in accordance with the present study where there was an increase in the level of SOD, GSH and GPx in Groups 3 and 7 goats providing evidence for enhanced antioxidant enzyme activity in mastitis condition.

Varied mean LPO activity in goat milk have been reported by researchers (Koksal *et al.*, 2016; Özer, 2014) that ranged between 1.5-4.5 units/ml and between 0.04-9.28 units/ml. In the present study, the reduced amount of lactoperoxidase activity was noted in the mastitis affected goats that ranged between 0.05-0.70 units/ml. As evident from (Table 4), the LPO activity was found to be more in liposomal RSV, Jamun and combination of Jamun with antibiotic-treated groups in comparison to free RSV treated group given I/P indicating more antioxidant efficacy of the liposomal resveratrol formulation and Jamun seed powder compared to free resveratrol.

It is evident from (Figure 6) that the mean NF-κB p65 level in Group 1 goats increased significantly on the 14th day post *S. aureus* inoculation compared to their respective 0-day value. In Group 2 goats too the mean NF-κB p65 level increased significantly on 7th and 14th day post-inoculation compared to their 0-day value. However, the mean NF-κB p65 level of Groups 3 and 6 goats did not increase significantly indicating efficacy in preventing the rise in fibrosis biomarkers. In Group 7 goats, there was no significant increase in the level of NF-κB p65 on 7th day post-inoculation, however, on 14th day post-inoculation, there was a significant increase in the level compared to the 0-day value. The mean NF-κB p65 levels of Groups 3, 4, 5 and 7 goats were significantly less compared to the Group 1 goat values on 7th and 14th day post bacterial inoculation indicating the potent antifibrotic activity of Resveratrol and Jamun with an antibiotic combination.

The major TGF-β form in bovine milk and colostrum is TGF-β2, whereas the rest is TGF-β1 suggesting that TGF-βs might be involved in the regulation of the mammary gland development (Ginjala and Pakkanen, 1998). As evident from (Figure 7), there was a significant reduction in the mean TGF β_2 level of Group 6 goats on 7th and 14th day post-inoculation compared to the respective day values of Group 1 goats, indicating the antifibrotic potential of Jamun. In Groups 3 and 7 goats, the mean TGF β2 level was significantly lower on 14th

day post-inoculation compared to Group 1 goats. In Groups 1 and 2 goats, the mean TGF β2 level increased significantly on 7th and 14th day post-inoculation compared to their respective 0-day values. However, the mean TGF β2 level in Group 3 goats decreased significantly on 7th day post-inoculation but did not vary significantly on 14th day post-inoculation compared to their 0-day value.

Resveratrol has been found to have potential benefits in pathological diseases like cancer and fibrosis by preventing extracellular matrix (ECM) deposition and degradation. It blocks growth factor signalling pathways, particularly TGF-β, and suppresses the activation of the MAPK signalling pathway and NF-κB transcription factor (Zhang *et al.*, 2017), which in turn suppresses the transcription of profibrogenic TGF-β, COX-1/COX-2, and other pro-inflammatory mediators and cytokines. Numerous evidences (Elshaer *et al.*, 2018) suggest the anti-inflammatory and chemo preventive effects of resveratrol that involves inhibition of pro-carcinogen activation by downregulating cytochromes P450 and oxidative stress through Nrf2-dependent activation of antioxidant enzymes. It also inhibits EMT by downregulating pathways like TGF-β1/Smads, Wnt/β-catenin, PI3K/Akt/NF-κB, and Gli1. *Staphylococcus aureus* intramammary infection elicited increased production of Transforming growth factors like TGF-α, β1, and β2 in milk (Bannerman *et al.*, 2006). Similarly, in the present study, TGF-β2 levels in mastitis affected milk were estimated and found to increase in the Group 1 goats as well as in Group 2.

In a report (Chávez *et al.*, 2008) the antifibrogenic mechanism of resveratrol was elucidated whereby resveratrol prevented the activation of NF-κB and the increase of TGF-β on liver fibrosis. This finding was in accordance with the present study where Group 3, 6 and 7 goats showed decrease in NF-κB and TGF-β2 levels.

On the other hand, in several *in vivo* and *in vitro* studies (Kumar *et al.,* 2022; Das *et. al.,* 2023; Rizvi *et al.,* 2022), Jamun seed was reported to show numerous bioactivities such as antioxidant potential, anti-inflammatory, antimicrobial, anticancer, antidiabetic activity, antiamnesic activity, hepatoprotective, cardio and gastroprotective properties as well as activity against metabolic syndrome including hypertension, obesity, and hyperlipidaemia, *etc*. Thus, the antioxidant and antiproliferative activity of Jamun seed powder corroborates with the present study as evident from the antioxidant parameters and fibrosis biomarkers mentioned earlier. Although, the powdered Jamun seed contains resveratrol, it should be noted that other phytoconstituents have also been shown to exhibit these bioactivities, so it cannot be said that resveratrol alone is responsible for the effect that Jamun exhibits. According to Kumar *et al*. (2022), in the case of *in vivo* investigations, the concentration, form, and mode of administration of the active ingredient affect the biological activities of jamun seed extracts. The form of flavonoids and polyphenols, as well as how they interact, determine the antioxidative and antiproliferative properties of jamun seed extract. Therefore, more detailed research is needed to determine how each phytoconstituent of jamun contributes to each bioactivity.

5. Conclusion

Briefly, the findings of the present study indicate the better prophylactic effect of liposomal resveratrol formulation compared to free resveratrol on mammary gland fibrosis. The combination of antibiotic with Jamun provided a better prophylactic effect on mastitis and its associated fibrosis condition compared to antibiotics used alone. Thus, both liposomal resveratrol formulation and Jamun seed powder may have the potential to prevent mammary gland fibrosis associated with mastitis. However, since the present study was at a preliminary level and based on a limited number of animals per group, further large-scale studies are required to gain a detailed perspective in these areas.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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