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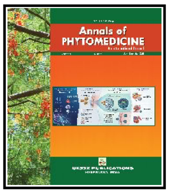

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Immunopathological changes and efficacy evaluation of *Hibiscus rosa-sinensis* L. extract in the rat mastitis model

Utpal S. Patel*, Jeetendra K. Raval*◆, Harshil A. Prajapati*, Priti D. Vihol*, Jatin H. Patel and Jaynudin H. Khorajiya***

**Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Navsari-396 450, Gujarat, India*

***Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Navsari-396 450, Gujarat, India*

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Abstract

The present study was carried out to evaluate effect of *Hibiscus rosa-sinensis* L. ethanolic extract in *Staphylococcus aureus* induced rat mastitis model following intra-mammary administration, based on *in vitro* antimicrobial susceptibility test. Rats were distributed into five groups. Group I (normal control), Group II (vehicle control), Group III (Mastitis control), Group IV (*S. aureus* induced mastitis rats treated with 10 µl (100 mg/ml) *H. rosa-sinensis* ethanolic extract) and Group V (*S. aureus* induced mastitis rats treated with 10 µl (10 µg/ml) Cefuroxime sodium). Rats in the mastitis control group displayed a number of clinical symptoms, including redness and hardness of the teat region, as well as tiredness, dullness, and decreased weight growth. Severe histopathological lesions and increased TNF-α antigen expression were seen in the mastitis control group. The oxidative stress value was significantly higher in the mastitis control group than in the normal control group. When applied intra-mammary, 10 µl of *H. rosa-sinensis* ethanolic extract (100 mg/ml) and 10 µl of Cefuroxime sodium (10 µg/ml) demonstrated a positive impact on experimental mastitis caused by *S. aureus*, as evidenced by less severe clinical symptoms, histopathological lesions, and a lower level of TNF-α antigen expression in comparison with the mastitis control group.

1. Introduction

Mastitis is the inflammation of the mammary gland categorized as clinical (severe) or subclinical (moderate) and is a major mammary gland complaint generally caused by bacteria and trauma. However, it leads to a serious trouble in dairy animal herds with commercial consequences, substantially affected by downfall in milk production (Seegers *et al*., 2003). In India, the frequency of subclinical mastitis in dairy cows was significantly greater, reaching up to 40-80% depending on breed and location, but the prevalence of clinical mastitis varied from 4.8% to 18.7% (Audarya *et al.,* 2021). According to estimates, mastitis alone is responsible for over 70% of all preventable losses that occur during the production of milk (Sadashiv *et al.,* 2013). Since most dairymen and farmers are still ignorant of the effects of subclinical mastitis, subclinical mastitis accounts for around 70% of economic losses in the dairy industry, which saw yearly losses of over 526 million dollars in India 2 billion dollars in the United States (Varshney and Naresh, 2004). Inflammation of the mammary gland in reaction to injury, to counteract aggressors, to promote healing and to restore physiological function is the typical characteristic of mastitis (Ciprian *et al.,* 2018). *S. aureus* is the primary bacterial agent that cause mastitis in dairy cows (Lundberg *et al*., 2016). Numerous pathogenic bacteria, including *S. aureus, Escherichia coli, Klebsiella, Streptococcus pneumoniae* and *Pseudomonas aeruginosa* have been the subject of significant research on the antibiotic activity of herbal plant extracts (Devi *et al.,* 2018). Although, several bacteria can beget mastitis but *S. aureus* is major etiological agents of mastitis in cattle, sheep and goat (Mork *et al.*, 2005). Dimitriadis *et al.* (2005) reported *S. aureus*-induced mastitis was related to the production of various pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α and generate oxidative stress through lipid peroxidation (Fox and Kelly, 2006). The hunt for new remedial alternatives for the control and treatment of bovine mastitis is demand in period of antibiotic resistance. In this context herbal constituents (like flavonoid, terpenoids, tanins alkaloid *etc*) are known for suppressing acute inflammatory storm by interacting with intermediary cellular reaction (Bala *et al.,* 2022). The *H. rosa-sinensis* plant belonging to the Hibisceae tribe of the Malvaceae family includes the red flower, sometimes referred to as the China rose which contains alkaloids, polyphenols, tannins, terpenoids etc (Bala *et al.,* 2022). *H. rosa-sinensis* petal (flowers) contains kaempferol and kaempferol-3-O-β-xylosylglucoside, as well as quercetin-3-di-O-β-D-glucoside, quercetin-3-7-di-O-β-D-glucoside and quercetin-3-O-β-D-sophorotrioside. Compared to the yellow or yellow-orange petals (flowers) of *H. rosa-sinensis*, the red petals were found to have a greater number of anthocyanin bands and contain cyanidin-3-sophoroside as the primary anthocyanin (Goutam *et al.,* 2018). These flavonoids, steroids and saponins of *H. rosa-sinensis* possess anti-inflammatory, antibacterial and antioxidant activities. In *in vitro* and *in vivo* analysis ethanolic extract made from *H. rosa-sinensis* showed anti-inflammatory, antipyretic and analgesic activities (Birari *et al.,*

Corresponding author: Dr. Jeetendra K. Raval
Assistant Professor, Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Navsari-396 450, Gujarat, India
E-mail: jkraval@kamdhenuuni.edu.in
Tel.: +91-9924823397

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2009; Raduan *et al.* 2013). The rat experimental model is suitable for research focusing on the pathogenesis and treatment of bovine intra-mammary infections. For exploring possibilities of herbal treatment in mastitis, this research was accepted to study the ameliorative effects of *H. rosa-sinensis* extract on *S. aureus*-induced mastitis with special reference to hematobiochemical changes, histopathology, immuno-histochemistry and oxidative stress.

2. Material and Methods

2.1 Plant authentication and ethical approval

The red flowers of *Hibiscus rosa-sinensis* L. were collected from the campus of Navsari Agricultural University, Navsari-396450, Gujrat, India. The plant species identification was confirmed by the Faculty of Horticulture, Navsari Agricultural University, Navsari-396450, Gujarat, India. A Voucher Specimen No. is 13933. Adult female lactating Wistar rats (No. 40) weighing 200-240 g were used for the experiment. The rats were procured from Aarsh Research and Development Centre, Daman-396210, India (CCSEA registration no. 2239/PO/RcBiBt/S/23/CCSEA). Ethical clearance was approved by IAEC vide Project No. 134-VCN-VPP-2023.

2.2 Preparation of *Staphylococcus aureus* inoculum

Pure culture of *Staphylococcus aureus* sub. spp. *aureus* (MTCC-737) was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh and stored at -20°C until use. The serial fold dilutions of culture in Phosphate Buffered Saline was made until they attained the concentration of 1.5×10^4 CFU/ml. Solution was infused 10 µl/teat (4th and 5th pair of mammary glands) of female rat by intra-mammary route (Zhong *et al.,* 2005; Cai *et al.,* 2020).

2.3 Extraction method of *Hibiscus rosa-sinensis* L.

The fresh petals of red flowers of *H. rosa-sinensis* (approx. 1 kg) were cleaned using running tap water and air dried for 3 days. Later, the flower was crushed to fine powder in a mixer grinder. The powder samples (1: 10) were time soaked and stirred in 95% ethanol. Later it was subjected to filtration and was evaporated by use of a rotary evaporator at 40ºC. The ethanolic extract was collected and kept at the refrigeration temperature (4°C) till further use (Raduan *et al.,* 2013). At the time of dosing, the ethanolic extract was dissolved in sterilized deionized water to make the final concentration of 100 mg/ml.

2.4 *In vitro* antibacterial assay

2.4.1 Cup borer method

The sterile petri dish plate was filled by Muller Hinton (MH) agar media to maintain the 3 to 4 mm depth in petri dish plate. The MH agar media was spread with 100 µl *S. aureus* inoculum (1.5×10^4 CFU/ml). Equally spaced cups were drilled into the agar plates using a sterile cork borer (4 mm) and hole bottom was sealed by one drop of sterile molten agar. The bored holes were filled with 100 microliters of different concentrations (50 mg/ml, 100 mg/ml, 200 mg/ml) of ethanolic extract of *H. rosa-sinensis* and subjected to incubation at 37ºC for 18 h. The zones of inhibition were interpreted using of zone scale.

2.4.2 Minimum inhibitory concentration (MIC)

Ethanolic extract of *H. rosa-sinensis* stock solution (800 mg/ml) was prepared in sterile distilled water. *S. aureus* inoculum was prepared and set to 1.5×10^4 CFU/ml as per 0.5 McFarland standard for serial dilution. 100 µl sterile Muller Hinton broth was added in each well except column no. 1, 9 and 10 of microtiter plate. *H. rosa-sinensis* stock solution (200 µl) was added in column no. 1 and serially diluted up to column no. 8. Cefuroxime stock sodium (100 µl, 10 µg/ml) was added in column no. 9 as positive control. Sterile water (100 µl) was added in column no. 10 as vehicle control. Sterile MH broth (200 µl) was added in column no. 12 as sterility control. Bacterial suspension (100 µl, 1.5×10^4 CFU/ml) was added in each column except column no. 12. The plate was subjected to incubation for 18 to 20 h at 37°C. After incubation, freshly prepared 30 µl of methylene blue dye (500 µg/ml) was added in every well. Plates were incubated once again about 30 min to observe reduction of colour from dark blue to light blue colour/colourless, to detect presence of living bacteria. Absence of colour change was noted as the minimum inhibitory concentration (MIC) of *H. rosa-sinensis* for *S. aureus*.

2.5 Experimental design

Following acclimatization, the rats were randomly assigned to five groups, each consisting of eight rats, in accordance with the experiment design. The groups were named as Groups I to V. Group I as a healthy/normal control in which no treatment was given. Before bacterial inoculation, the rats from Groups II, III, IV and V were anaesthetized using ketamine at dose rate of 22 to 25 mg/kg b.w. by intraperitoneal route and area of the mammary gland was made aseptic by use of 70% ethyl alcohol. Group II acted as the vehicle control group in which the sterile and deionized water at dose rate of 10 µl per teat was given in left fourth (L4), left fifth (L5), right fourth (R4) and right fifth (R5) teat (*i.e.,* intra-mammary route). Before initiating any treatments, *S. aureus* culture at dose rate of 10 µl (1.5×10^4 CFU/ml) per mammary gland was given *via* the intra-mammary route to induce the mastitis in Groups III, IV and V. Development of mastitis was confirmed by marked swelling, hardness and reddening at the teat area of the rats in Groups III, IV and V. After the development of mastitis, Group III was kept as mastitis control and further treatment was not given. After the development of mastitis, rats of Group IV were treated with 10 µl (100 mg/ml) *H. rosa-sinensis* ethanolic extract by intra-mammary route while rats of Group V were treated with Cefuroxime sodium infusion at dose rate of 10 µl (10 µg/ml concentration) by intra-mammary route for 5 days continuously. The above vehicle and test items were administered only in the left fourth (L4), left fifth (L5), right fourth (R4) and right fifth (R5) teat.

Clinical signs and mortality of the rats were observed daily. Weight of Rats was noted on the 0th day of an experiment and at the end (11th day) of the experiment. Blood samples were collected from the retro-orbital plexus of all rats using capillaries in K_3EDTA vials and clot activator vials for haematology and oxidative stress (Lipid peroxidation) analysis respectively at the end of the experiment. By use of malondialdehyde (MDA) production the membrane damage in erythrocytes was evaluated (Rehman, 1984). All the rats were sacrificed by an overdose of carbon dioxide at the end of the experiment. The detailed necropsies of all the rats were performed.

2.6 Histopathology

After a thorough gross examination, samples of mammary glands (About 2.5 sq. cm area and 0.5 cm thickness) were collected from sacrificed animals. Histopathological procedure was done according to Luna (1968). The paraffin-embedded tissue blocks were cut into thinner sections about 5-6 µm size by using of semi-automated rotary microtome (Yorko, YSI 122). The slides were stained by hematoxylin and eosin stain and observed under a light microscope. Significant and consistent lesions in different organs were categorized into normal, minimal, mild, moderate and severe.

2.7 Immuno-histochemistry (IHC)

Immuno-histochemistry (IHC) is a broadly used technique for detection of antigens (proteins) in tissue sections. When it comes to detecting tumor necrosis factor alpha (TNF-α), IHC can result in valuable information of its expression and localization within tissues. Fresh mammary gland tissues were collected. Tissues were trimmed and fixed. Tissues were hydrated and mounted onto poly-L-lysine glass slides. The slides were washed with TBS to reduce non-specific background staining. Heat induced antigen retrieval (HIAR) was performed by use of Microwave-oven for 20 min at 100°C. In container filled with antigen retrieval citrate buffer of 6.0 pH, tissue sections were placed and allowed to cool down to room temperature. Enzyme blocking was done by use of 3% H_2O_2.

2.7.1 Primary antibody reaction

Enough drop of diluted (1:100) primary antibody (TNF-α polyclonal antibody) were added to wrap the tissue sections and slides were incubated in humidity chamber for 60 min. Washing of slides was done by use of TBS for 10 min.

2.7.2 The secondary antibody reaction

Enough drop of diluted (1:500) the goat anti-rabbit IgG (H+L) HRP conjugated secondary antibody was added to cover the sections and incubated for 30 min in humidity chamber. Washing of slides was done by TBS for 15 min.

2.7.3 DAB chromogen

As per method freshly prepared DAB chromogen was used cover the tissue section and incubated for 5 to 10 min. Slides were washed with distilled water for 5 min.

2.7.4 Counter-staining

Enough drops of Gill's III hematoxylin were added to cover the section and kept 10 sec. The slides were rinsed in TBS for 1 min. Rinsing of slides was done in distilled water for 2 to 5 min. Slides were mounted by DPX mounting media and examined under microscope.

2.8 Scoring of TNF-α in immuno-histochemistry

$$\text{Labelling index} = \frac{\text{No. of positively stained cells}}{\text{Total number of cells}} \times 100$$

Tumor necrosis factor is a pro-inflammatory cytokine expressed by reaction of activated immune cells in reflection to a various pathogen (Villanacci *et al.,* 2019). The TNF-α can be ascertained by the majority of the inflammatory cells and their secretions. The analysis of the immuno-histochemistry stained cells involves the detection of the cells with high TNF content as they positively stained as brown colour. TNF-α was determined by counting an average number of TNF-α at higher magnification (× 400) in 10 microscopic fields of each section. The labelling index was derived as per below formula (Villanacci *et al.,* 2019; Urkude *et al.,* 2023).

2.9 Statistical analysis

Body weight, haematological and oxidative stress related data as well as immuno-histochemical results were analysed statistically by use of SPSS 20.0 statistical software (SPSS, Inc., 2009). One-way analysis of variance (ANOVA) with Duncan's test was performed.

3. Results

The *in vitro* antibacterial susceptibility test was carried out to estimate the lowest concentration of anti-bacterial drug and ethanolic extract that inhibits the growth of the *S. aureus* bacteria. In cup borer method, zones of inhibition observed were 13 mm in 100 mg/ml and 15 mm in 200 mg/ml concentration and no inhibitory zone was noted in 50 mg/ml concentration of *H. rosa-sinensis* ethanolic extract. In micro broth dilution technique, *S. aureus* growth was inhibited at lowest concentration of 100 mg/ml of concentration of *H. rosa-sinensis* ethanolic extract. The result suggested the susceptibility of *S. aureus* to 100 mg/ml *H. rosa-sinensis* ethanolic extract and same concentration was utilised for testing in mastitis model in rats.

3.1 Clinical signs and gross lesions

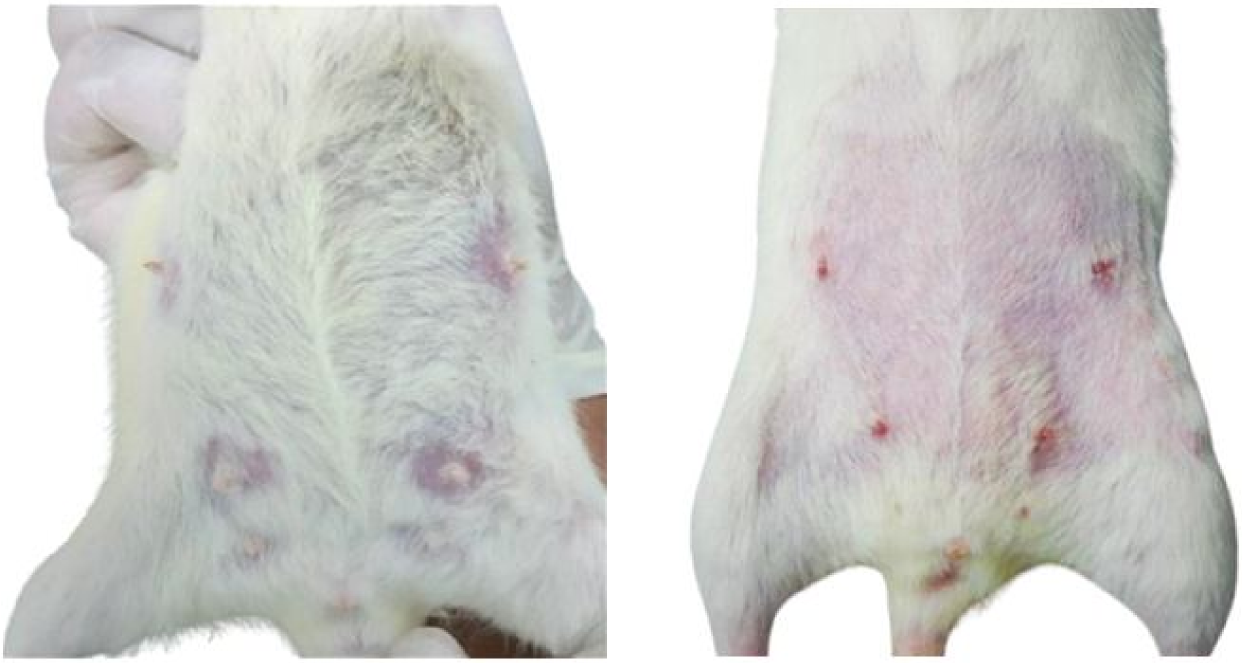
No mortality was recorded in present experiment. Group III (mastitis control) showed swelling (Figure 1A), redness (Figure 1B) and hardness of the teat area on day 6. These rats also exhibited moderate dullness, lethargy, weakness, and weight loss. However, Group IV (*S. aureus*-induced mastitis rats treated with *H. rosa-sinensis*) showed a reduction in the intensity of redness, swelling and hardness of the teat area on day 3 onwards and was found minimal at the end of the experiment and Group V (*S. aureus*-induced mastitis rats treated with Cefuroxime sodium) showed mild redness, swelling and hardness of the teat area. Group II (vehicle control) showed only minimal to mild swelling of the teat area. There were no clinical signs or gross lesions in Group I (normal control).

3.2 Body weight

At the beginning and end of the experimental period, the body weights of all the animals were recorded and data are depicted in Table 1. The experimental result showed increased body weight about 8.58% on the last day of the experiment in comparison to their weight on the first day in the normal control group. The mastitis control group (Group III) revealed lower weight gain (5.34%) as compared to normal control group, while Group IV and V revealed higher weight gain as compared to mastitis control group.

3.3 Haematological parameters

Samples of blood were collected from animals from all the groups at the 11th day of the experiment. The results are noted as mean and standard error of haematological values that are depicted in Table 2. In present study, significant elevation in TLC and downfall of lymphocyte count was reported in the mastitis control group in comparison to Group I and Group IV rats. Regardless of statistical consideration, it could be generally interpreted that the *H. rosa-sinensis* treated group showed decreased values of MCHC, TLC, neutrophils, eosinophils, monocytes and platelets and increased values of TEC, Hb, PCV, lymphocytes, MCV and MCH in comparison with the mastitis disease control group.



(A) (B)

Figure 1: Rats from mastitis control group. (A) Swelling of teat area and (B) Redness of teat area.

Table 1: Details of mean body weight (g) values of different groups

Days	Group I		Group II		Group III		Group IV		Group V	
	Mean ± SE	% change	Mean ± SE	% change	Mean ± SE	% change	Mean ± SE	% change	Mean ± SE	% change
Day 0	238.60^{a} ± 12.08	-	210.16^{a} ± 9.36	-	218.33^{a} ± 7.03	-	218.57^{a} ± 7.61	-	220.83^{a} ± 9.86	-
Day 11	261.00^{b} ± 11.00	8.58	232.33^{a} ± 7.68	9.54	230.66^{a} ± 6.85	5.34	242.00^{ab} ± 6.29	9.68	244.66^{ab} ± 8.84	9.74

Note: Statistical analysis was performed group wise and different superscripts in a row vary significantly (p<0.05).

Table 2: Details of haematological values of different groups

Parameter	Group I	Group II	Group III	Group IV	Group V
TEC (x 10^6/µl)	8.58^{b} ± 0.01	7.90^{a} ± 0.07	8.45^{b} ± 0.14	8.50^{b} ± 0.12	8.36^{b} ± 0.13
Hb (g/dl)	15.00^{b} ± 0.12	14.58^{b} ± 0.22	13.91^{a} ± 0.17	15.06^{b} ± 0.18	14.72^{b} ± 0.13
PCV (%)	43.82^{a} ± 0.55	47.35^{c} ± 0.22	44.61^{a} ± 0.24	46.16^{b} ± 0.10	46.08^{b} ± 0.06
MCV (f)	57.84^{ab} ± 0.63	55.06^{a} ± 0.82	60.10^{ab} ± 0.89	61.02^{b} ± 2.75	61.84^{b} ± 1.31
MCH (Pg)	20.23^{a} ± 0.24	19.59^{a} ± 0.18	19.19^{a} ± 0.17	21.77^{a} ± 1.59	19.94^{a} ± 0.23
MCHC (g/dl)	34.32^{bc} ± 0.25	35.28^{c} ± 0.30	33.01^{ab} ± 0.24	32.18^{a} ± 0.86	32.20^{a} ± 0.32
TLC (x 10^3 /µl)	9.44^{a} ± 0.41	9.55^{a} ± 0.63	12.21^{b} ± 0.45	9.78^{a} ± 0.38	9.68^{a} ± 0.45
Neutrophils (%)	32.42^{a} ± 1.88	32.46 ^{a} ± 1.34	34.93^{a} ± 0.52	31.81^{a} ± 0.63	32.84^{a} ± 0.73
Eosinophils (%)	1.50^{a} ± 0.22	1.16^{a} ± 0.30	1.57^{a} ± 0.20	1.42^{a} ± 0.20	1.33^{a} ± 0.09
Basophils (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocyte (%)	64.52^{ab} ± 1.88	63.81^{ab} ± 1.41	61.66^{a} ± 0.54	65.35^{b} ± 0.73	64.04^{ab} ± 0.69
Monocyte (%)	3.06^{ab} ± 0.06	3.71^{b} ± 0.18	3.40^{ab} ± 0.35	2.83^{a} ± 0.12	3.12^{ab} ± 0.11
PLT (x 10^3/µl)	1013.00^{a} ± 5.40	1130.83^{b} ± 14.78	1223.16^{bc} ± 14.14	1188.83^{cd} ± 17.72	1163.40^{d} ± 15.94

Note: Statistical analysis was performed group wise and different superscripts in a row vary significantly (p<0.05).

3.4 Oxidative stress parameters

In the present investigation, oxidative stress in the animals was studied from sera and whole blood by estimating the antioxidant enzyme, *i.e.,* superoxide dismutase (SOD) level and the lipid peroxidation (LPO). The values of LPO and SOD are depicted in Table 3. The value of lipid peroxidation (LPO) was expressed in respect of malondialdehyde (MDA) concentration. In the current study, the *S. aureus*-induced mastitis group showed a significantly (p<0.001) elevated level of LPO and a non-significantly decreased level of SOD.

Table 3: Details of oxidative stress parameters of different groups

Parameter studied	Group I Mean ± SE	Group II Mean ± SE	Group III Mean ± SE	Group IV Mean ± SE	Group V Mean ± SE
LPO (nM/ml)	13.13^{a} ± 0.65	11.71^{a} ± 1.40	19.33^{c} ± 0.17	15.92^{b} ± 0.35	16.36^{b} ± 0.5
SOD (ng/ml)	3.33^{a} ± 0.83	3.43^{a} ± 0.32	2.80^{a} ± 0.25	3.65^{a} ± 0.22	3.22^{a} ± 0.41

Note: Statistical analysis was performed group wise and different superscripts in a row vary significantly ($p \leq 0.05$).

3.5 Gross examination of subcutaneous area

In present study, gross examination of subcutaneous area was performed before post-mortem of the rats. In Group III (mastitis control), moderately dilated blood vessels were seen in subcutaneous area (Figure 2C) as compared to normal control (Figures 2A and 2B) and in Group IV and V minimal dilation of blood vessels were seen in subcutaneous area (Figures 2D and 2E) as compared to Group III (Figure 2C).

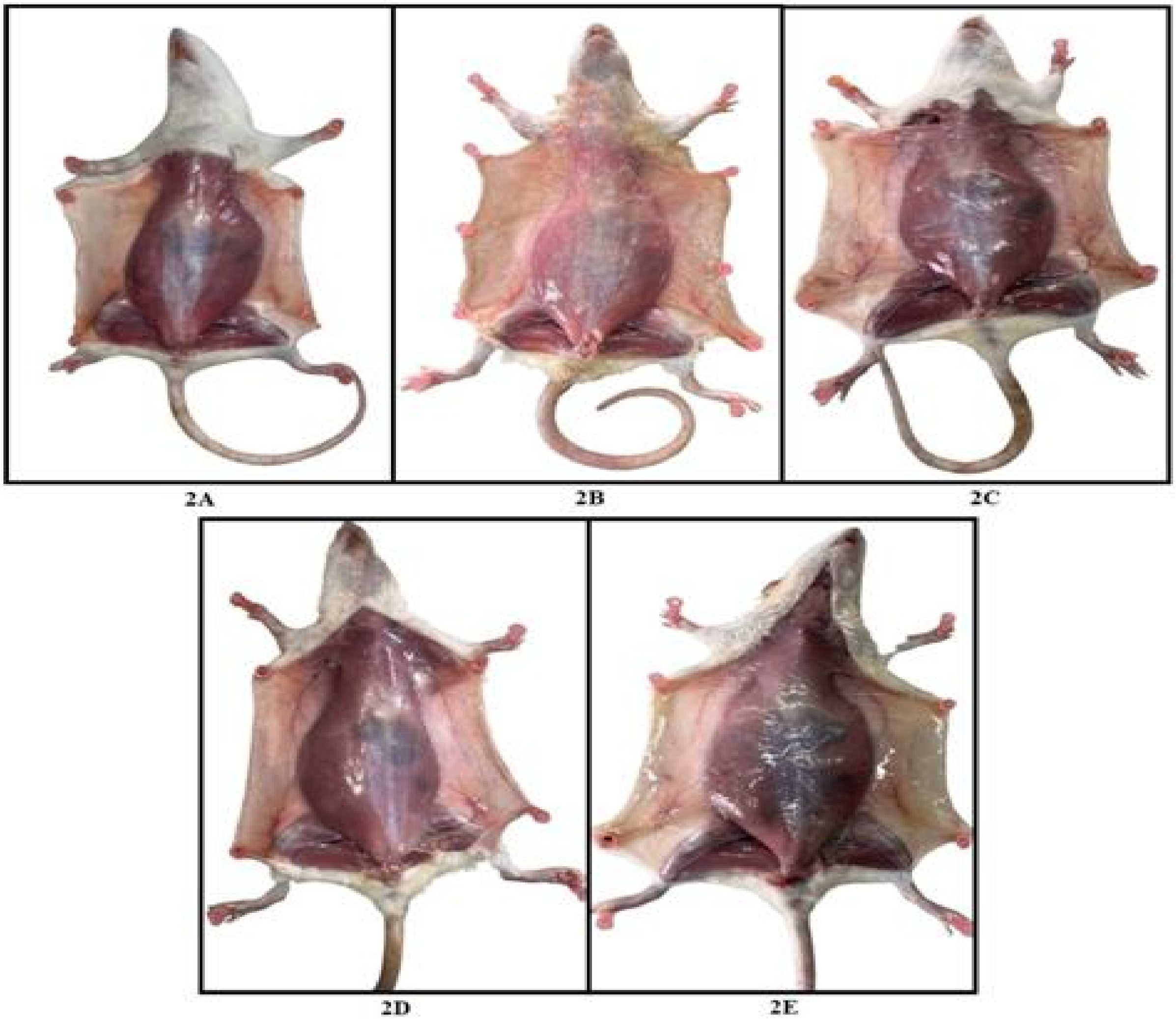


Figure 2: Gross examination of subcutaneous lesions. (A & B) No lesions (Group I & II). (C) Moderately dilated blood vessels (Group III) and (D & E) Minimal dilationo f blood vessels (Group IV & V).

3.6 Histopathology

Severities of the histopathological lesions if any were graded by a semi-quantitative scoring system. In the present study, Group III (mastitis control) exhibited severe histopathological lesions as inflammatory cells infiltration in mammary gland alveoli, inflammatory cells infiltration in the intra-lobular connective tissue, congestion in intra-lobular connective tissue and fat necrosis as compared to Group I (Normal healthy control group). Group IV (Mastitis-induced rats treated with 10 µl (100 mg/ml) *H. rosa-sinensis* extract) and Group V (mastitis-induced rats treated with cefuroxime sodium infusion) showed comparatively less severe histopathological changes as compared to Group III (mastitis control). These positively recovering histopathological changes may be attributed to the healing outcome of the *H. rosa-sinensis* extract and Cefuroxime sodium in *S. aureus*-induced mastitis.

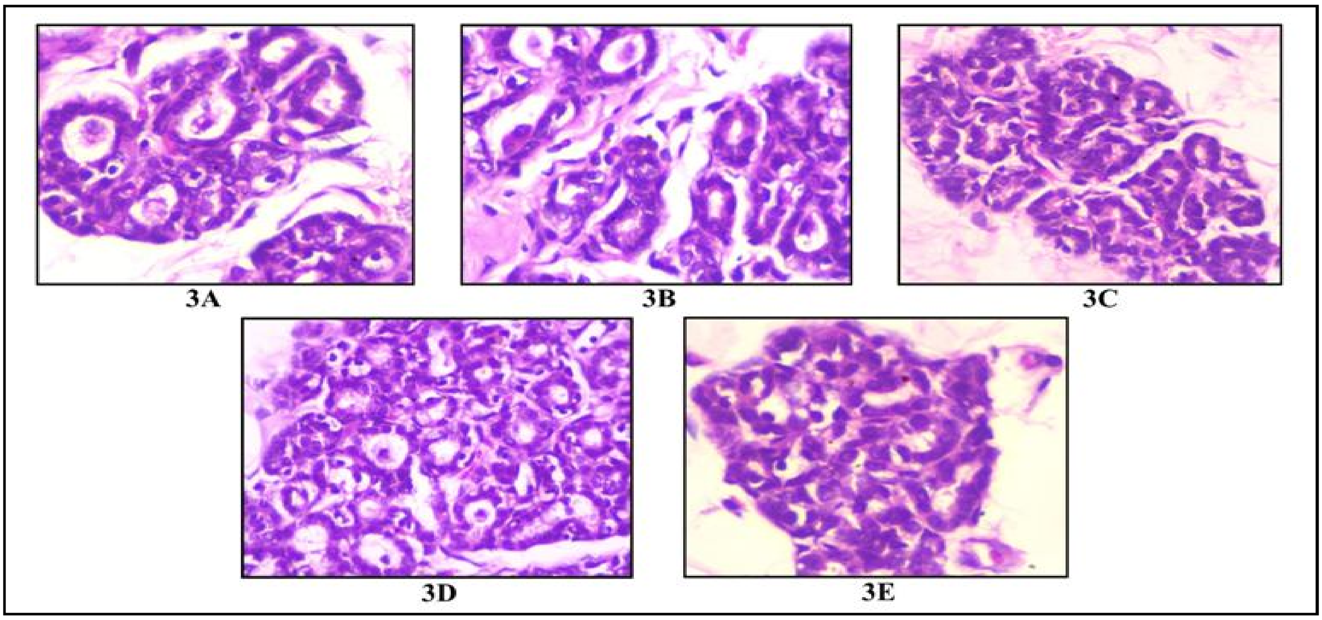


Figure 3: **Histopathology of mammary gland, H and E stain, 400X. (A) Normal mammary gland alveoli (Group I). (B) Normal mammary gland alveoli (Group II). (C) Severe inflammatory cell infiltration and destruction in mammary gland alveoli (Group III). (D) Mild inflammatory cell infiltration in mammary gland alveoli (Group IV). (E) Mild inflammatory cell infiltration in mammary gland alveoli (Group V).**

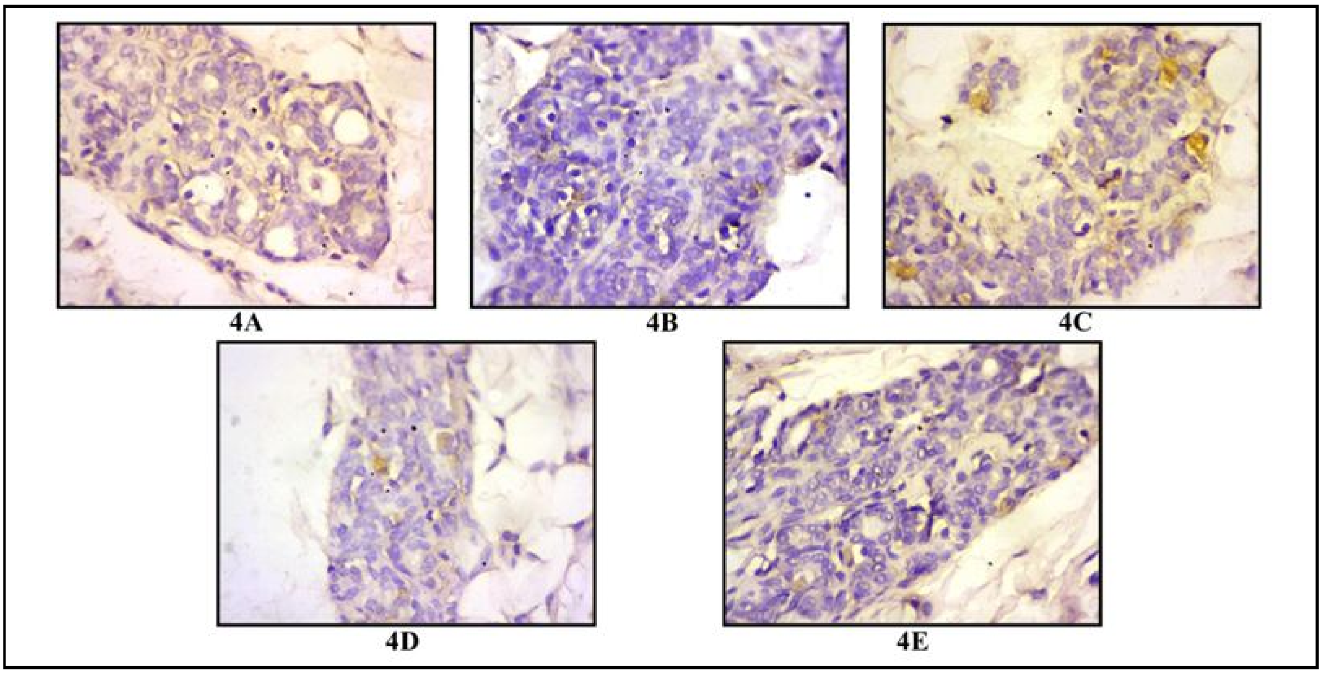


Figure 4: **Immuno-histochemistry (IHC) of mammary gland, IHC, 400X. (A) Revealing lower level of TNF-(Group I). (B) Revealing lower level of TNF-α (Group II). (C) Revealing higher level of TNF-(Group III). (D) Revealing lower level of TNF-α. (E) (Group V) Revealing lower level of TNF-(Group IV).**

Table 4: Details of immuno-histochemistry findings reported in different experimental groups

Parameter studied	Group I	Group II	Group III	Group IV	Group V
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
TNF-α (Labelling index)	$0.13^{a} \pm 0.03$	$0.20^{a} \pm 0.04$	$4.11^{c} \pm 0.07$	$1.82^{b} \pm 0.08$	$1.87^{b} \pm 0.03$

Note: Statistical analysis was performed group wise and different superscripts in a row vary significantly (p<0.05).

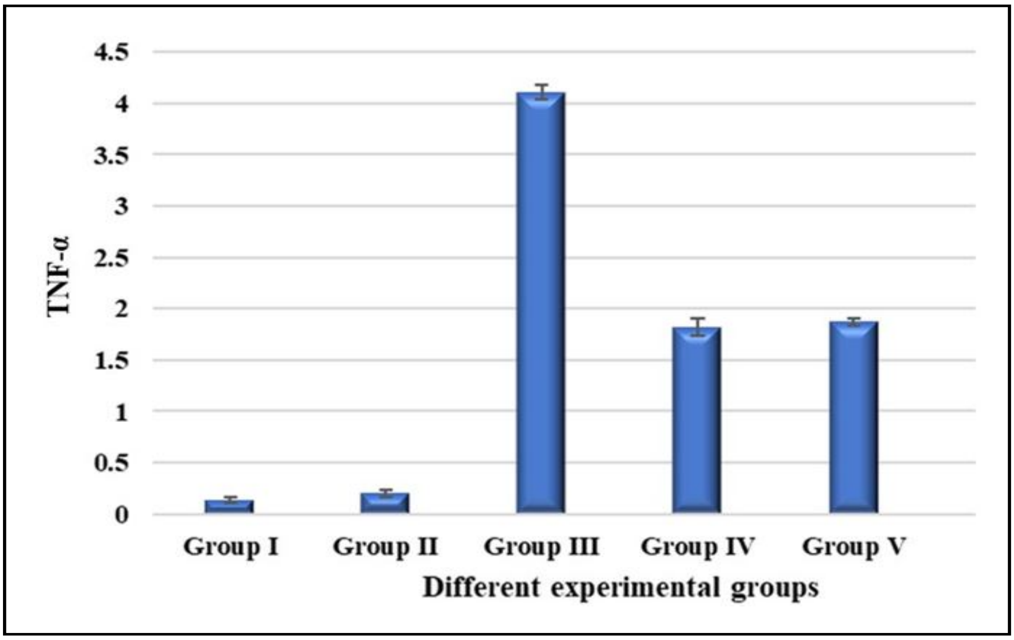


Figure 5: TNF-α level in different experiemntal groups at the end of experiment.

3.7 Immuno-histochemistry

In the present study, *S. aureus*-induced mastitis group showed a significantly ($p<0.001$) elevated level of TNF-α. The level of TNF-α was significantly ($p<0.001$) decreased in Group IV (Mastitis induced rats treated with *H. rosa-sinensis* extract) and Group V (Mastitis-induced rats treated with cefuroxime sodium infusion) as compared to the mastitis control group. However, there was a non-significant difference between Groups IV and V.

4. Discussion

The final interpretation was made by comparing *in vitro* cup borer and MIC methods results in present study. As evidenced by the 13 mm zone of inhibition in the *in vitro* cup borer method and inhibition of *S. aureus* bacteria at 100 mg/ml concentration in micro broth dilution method (*in vitro*), it was determined that 100 mg/ml concentration had enough anti-bacterial activity. A related study also documented the effectiveness of *H. rosa-sinensis* ethanolic extract, *i.e.,* a zone of 29 mm of inhibition against *S. aureus* and 25 µg/ml against *Staphylococcus mutans* (Uddin *et al*., 2010).

On day six of the current investigation, the teat area in Group III (mastitis control) displayed moderate to severe redness, swelling, and hardness. These rats also exhibited moderate dullness, lethargy, weakness and weight loss. This result agreed with observation of Taifa *et al*. (2022) and Cai *et al*. (2020), they found reddening and swelling of the teat area in rats of *S. aureus*-induced mastitis group.

In Group IV (*S. aureus*-induced mastitis rats treated with *H. rosa-sinensis*) showed a reduction in the intensity of redness, swelling and hardness of the teat area on day 3 onwards and minimum at the end of the study in comparison to Group III (mastitis control) rats. This anti-mastitis effect could be caused by substances found in flower petals, such as flavonoids (kaempferol glucoside and quercetin glucoside), tannins, ascorbic acid, citric acid, and anthocyanin (cyanidin-3-sophoroside) (Mukhopadhyay *et al*., 2018).

In comparison with normal control group (8.58%), mastitis control rats gained less weight (5.34%). It can be the result of oxidative stress brought on by an infection. Group IV rats gained approximately 9.68% more body weight than the mastitis control group (5.34%).

In present study, the mastitis control rats revealed significant rise in TLC count and decrease in lymphocyte count in comparison with treatment Group IV and normal control rats. In addition, haematology analysis showed that non-significant difference between treatment Group IV and normal control rats in RBC and WBC indices. Our findings are confirmed by the findings of other researchers (Jasim and Zgair, 2022; Prakoso and Wijayanti, 2022; Wu *et al*. 2017). Wu *et al*. (2017) reported elevated WBC count in infected mice compared with the normal control group. Jasim and Zgair (2022) noted that WBC, MCH, hemoglobin, granulocytes, monocytes, lymphocytes values increase and RBC, HCT, MCV, platelets values decrease in *S. aureus* infection group rats in relation to the normal rats. Prakoso and Wijayanti (2022) found that WBC, neutrophil and lymphocyte values increase and RBC, Hb, PCV, calculated indices (MCV, MCH, MCHC, HCT), platelet, monocyte values decrease in *S. aureus* infection group rats in contrast to normal rats. It can be generally interpreted that the *H. rosa-sinensis* treated group showed decreased values of MCHC, TLC, neutrophils, eosinophils, monocytes and platelets and increased values of TEC, Hb, PCV, calculated indices (MCV, MCH), lymphocytes in comparison with the mastitis control group. In addition, Mishra and Tandon (2012), presented significant increase in the count of the TEC and Hb content and a decline in platelet count in male Swiss albino mice by administration of aqueous extract *H. rosa-sinensis.*

In mastitis rats LPO level were significantly higher and a non-significantly lower level of SOD observed compared to normal rats. Result indicates that mastitis caused by *S. aureus* raises oxidative stress in the experimental mastitis. Furthermore, when compared to mastitis group rats (Group III), Group IV and V rats had a notable reduction in LPO, indicating the potential therapeutic benefits of *H. rosa-sinensis* extract. In accordance to present study, rats infected with *S. aureus* exhibited a substantial increase in plasma malondialdehyde (MDA) levels and a reduction in superoxide dismutase (SOD) expression levels (Soliman *et al.* 2015); Compared to the normal control rats, significant (p<0.001) decreases in SOD activity and significant (153.53%; p<0.001) increases in lipoperoxide (LPO) levels were seen in the liver of *S. aureus* infected rats (Prasad *et al.,* 2011). Furthermore, Pethe *et al.* (2017) reported that *H. rosa-sinensis* flowers were found to be manipulating SOD (increase) and LPO (decrease) in plasma, while Khatib *et al.* (2009) demonstrated in an *in vitro* experiment the antioxidant potentials of ethanolic extracts of flowers of *H. rosa-sinensis*, adding that this activity might be caused by phytochemicals such as flavonoids and phenols.

In the present study, Group III (mastitis control) showed moderately dilated blood vessels in subcutaneous area as compared to Group I (normal control). Group IV (Mastitis-induced rats treated with 10 µl (100 mg/ml) *H. rosa-sinensis* extract) and Group V (mastitis-induced rats treated with Cefuroxime sodium infusion) showed minimal dilation of blood vessels in subcutaneous area as compared to Group III. In support to present study El-Deeb *et al.* (2015) showed that blood vessels (subcutaneous) become inflamed and dilated, leads to increased blood flow to the inflamed area in mastitis. Rudmann *et al.* (2012) observed dilatation and congestion of blood vessels inside the mammary tissue, which may lead to increased blood flow and immune cell infiltration to the afflicted area in mastitis case.

Mastitis control rats exhibited severe histopathological lesions as inflammatory cells infiltration in the mammary gland alveoli, inflammatory cells infiltration and congestion in the intra-lobular connective tissue and fat necrosis in comparison with Group I (normal control). The findings were in accordance to various scientist and confirm the validation of *S. aureus*-induced mastitis model in rat for evaluation of compound. Boulanger *et al*. (2002) reported the accumulation of PMNs as an important factor in inducing damage to mammary parenchymal tissue in an *in vivo* study; Ahamed *et al.* (2010) opined that the *S. aureus* induced inflammation in the experimental murine mastitis model leads to pronounced pro-inflammatory cell infiltration and damage to epithelial cells; Liu *et al*. (2020) observed that *S. aureus* induced mastitis was specified by neutrophils and macrophages infiltrating the mammary gland alveoli, ducts, perivascular tissues and connective tissues and Taifa *et al.* (2022) reported that the induction of inflammation with *S. aureus* leads to lesions like severe congestion, swelling of the mammary glands, haemorrhagic congestion of blood vessels, infiltration of inflammatory cells and alveolar atrophy showing discontinuous epithelial in animals were observed.

Mastitis rats treated with 10 µl (100 mg/ml) *H. rosa-sinensis* ethanolic extract (Group IV) and mastitis rats treated with cefuroxime sodium infusion (Group V) showed comparatively less severe histopathological changes as compared to Group III (mastitis control). A positive recovery can be suggested form improved histopathological changes, likely due to the protective effects of *H. rosa-sinensis* extract and cefuroxime sodium in *S. aureus*-induced mastitis.

Inflammatory cells are the main producers of TNF-α, and macrophages, lymphocytes, plasma cells, dendritic cells, and mast cells are the main secretors (Horiuchi *et al.,* 2010). Endotoxemia causes tissue damage and pathological shock, which are mediated by TNF-α (*e.g.,* damage to the mammary glands) (Horiuchi *et al*., 2010). In the present study, Group I and Group II rats expressed minimal TNF-α (Labelling index). Difference observed in value of TNF-α between Group IV and V found to non-significant indicating similar ameliorative effect of *H. rosa-sinensis* and cefuroxime sodium on TNF-α antigens in *S. aureus* induced experimental mastitis. In our study, the reduced TNF-α level in the *H. rosa-sinensis* treated group may be due to the ameliorative effects of the subjected herb on experimental mastitis. TNF-α expression was significantly (p<0.001) elevated in Group III (mastitis control) in comparison with the normal rats and it is supported by finding of Wang *et al.* (2015), Zhang *et al.* (2019), Liu *et al.* (2020) and Zhang *et al.* (2022), *i.e.,* the expression levels of TNF-α to be significantly elevated after *S. aureus* infection in mammary glands of rat. Additionally, when compared with the mastitis control group, the TNF-α expression level was considerably lower (p<0.001) in Group IV (Mastitis induced rats treated with *H. rosa-sinensis* extract). This observation found to accordance to Kandhare *et al.* (2012), Gulati (2022) and Oluwamodupe *et al.* (2024), who observed the role of *H. rosa-sinensis* in expression level of TNF-α.

5. Conclusion

The experimental mastitis may be brought about in rats by injecting *S. aureus* (1.5 × 104 CFU/ml) into the mammary glands, which results in severe histopathological lesions and increased TNF-α antigen expression. *H. rosa-sinensis* ethanolic extract 10 µl (100 ug/ml) per mammary gland showed an ameliorative effect on the *S. aureus*-induced experimental mastitis as it protects haematology, oxidative stress, histopathological and immuno-histochemical alterations. In this study, only one solvent was used. In future studies, alternative solvents can be explored and the approach may be extended beyond the rodent model.

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Conflict of interest

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

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