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Preferential cyclooxygenase inhibition by *Jasminum sambac*: A possible relationship with potent anti-arthritic activity☆

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1. Introduction

Rheumatoid arthritis (RA), a chronic autoimmune disorder, affects cellular aspects of articular region, tendon and cartilage while the immune system assaults vital joint spaces.[1] Activation of inflammatory reactivity in the synovial fluid and lining of joints is the characteristic feature of RA. Proinflammatory mediators such as interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), and prostaglandin E2 (PGE2) are elevated in the synovial fluid of the RA patients.[2] In western countries, the prevalence rate of RA is 1%, where females seem to be three to five times more susceptible to its advent, resulting in pronounced deterioration in the quality of life.[3] The frontline treatment for RA, Disease Modifying Anti-rheumatoid Drugs (DMARD) along with Non Steroidal Anti-inflammatory Drugs (NSAID), is aimed at reducing inflammation in the arthritic milieu maybe by nonselective cyclo-oxygenase (COX) enzyme inhibition. Although COX inhibition curtails PG synthesis, several patients undergoing treatment had elevated levels of PG in the synovial fluid.[4] NSAIDs, selective as well as nonselective, are known to develop potential risks ranging from gastrointestinal bleeding to cardiovascular toxicity.[5] Despite recent development of specific antibody-based therapy, the therapeutic spectrum of RA still presents a high unmet need in terms of safety and efficacy. Hence, a pleiotropic, nontoxic herb could be worth investigating for long term use without exhibiting major side effects.

Traditional medicinal herbs offer a better risk-benefit ratio and are used commonly for the treatment of inflammatory disorders. Ayurveda recommended the use of a combination of herbs or individual herb for the treatment of RA.[6] The practitioners of Ayurveda considered these ancient recipes safe and effective for centuries.[6,7] One of these herbs is Jasminum sambac Linn. (JS), an evergreen vine or shrub, belonging to family Oleaceae. A compendium of Indian medicinal plants mentioned the role of herbs from Jasminum genus in vitiated conditions of pitta, inflammations, rheumatism, and cephalalgia.[8] Ayurvedic texts referred to JS as a potent anti-inflammatory remedy along with its galactagogue potentials.[6,7] Similarly, Chinese and Thai folk medicine systems also use the oil of JS for the treatment of arthritis, conjunctivitis, and gastritis.[9]

Scientific evaluations of the root extract of JS exhibited anti-inflammatory, analgesic, and anti-pyretic activity[10] while its methanolic extract had free radical scavenging activity.[11] Major active constituent present in root and leaf extract like salicylic acid, compounds (+)-jasminoids A, B, C, and D are expected to have potent COX inhibitory activity.[12–14] Moreover, flower extract contain coumarin, cardiac glycoside, essential oil, flavonoid, phenol, saponin, and steroid responsible for its biological activities and are well tolerated at high doses in murine models.[14,15]

☆ The objective of this study is to investigate the anti-arthritic potential of *Jasminum sambac* (JS) in adjuvant induces arthritis model of rheumatoid arthritis (RA) in rats. *In-silico* docking and *in-vitro* assay demonstrated the effect on COX enzymes. Treatment of JS reduced oxidative stress, MPO activity, and damage to bones in rats exposed to RA. The demonstrated anti-arthritic activity of JS could be attributed to anti-inflammatory activity induced by COX inhibition, confirmed with *in-vitro* assay, along with the potent free radical scavenging activity.

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List of abbreviation:

AIA	Adjuvant induced arthritis
AIAcon	Arthritic control
ANOVA	Analysis of variance
COX	Cyclooxygenase
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CMC	Carboxymethyl cellulose
Con	Control
Diclo	Diclofenac
DMARDs	Disease modifying antirehumatic drugs
DTNB	5,5′-dithiobis-2-nitrobenzoic acid
ESR	Erythrocyte sedimentation rate
GSH	Glutathione
Hb	Haemoglobin
H&E	Hematoxylin and eosin
IL	Interleukin
JS	*Jasminum sambac*
JSE	Ethologic extract of JS
MDA	Malondialdehyde
MMPs	Matrix metalloproteinase
MAPK	Mitogen-activated protein kinases
MMP-1	Matrix metalloproteinase-1
MPO	Myeloperoxidase
NSAIDs	Nonsteroidal anti-inflammatory drugs
NF-kB	Nuclear factor-kappa B
PGI_2	Prostacyclin
PGs	Prostaglandins
PGE_2	Prostaglandin E_2
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SCW	Streptococcal cell wall
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TNF-α	Tumor necrosis factor-α

Besides, other species of the Jasminum genus have beneficial effects on chronic inflammation, indicating the role of the arachidonic acid pathway and endothelial vaso-relaxation in its therapeutic activity.[10,16] Although various parts of JS are used for the treatment of chronic inflammatory disorders, the systematic evaluation of its rich phytoconstituents in arthritis is still warranted.

Interestingly, the choice of suitable murine model is always critical for reproducible outcomes in preclinical evaluations. Adjuvant-induced arthritis (AIA) in Lewis rats with complete Freund's adjuvant (CFA) is a preferred immune-mediated arthritis model that mimics most of the pathological as well as clinical features of RA.[17,18] Acknowledging these facts, we designed this study to explore the crosstalk between COX, ROS, and the progression of arthritis. It aims at systematically investigating the mechanistic basis of anti-inflammatory activity of the JS concerning RA in CFA induced arthritis model using Lewis rats.

2. Materials and methods

2.1. Materials

Superoxide dismutase (SOD) and malondialdehyde (MDA) were purchased from Sigma Aldrich, USA. Reduced glutathione (GSH), 5, 5′-dithiobis-(2-nitrobenzoic acid (DTNB), thiobarbituric acid, and lipopolysaccharide (LPS) were procured from Merck Ltd., India. Dulbecco's Modified Eagle Medium (DMEM), Phosphate Buffer Saline (PBS), hexadecyl trimethyl ammonium bromide, orthodianisidine dihydrochloride, and Fetal Bovine Serum (FCS) were purchased from HiMedia Laboratories Pvt. Ltd. All other chemicals were of analytical grade.

2.2. Plant specimen and preparation of extraction

The leaves of JS collected from the herbal garden were identified (specimen 420/Bot/Safia/12) and authenticated. These leaves were shade dried and grounded to a coarse powder. This powdered material (500g), soaked in 95% ethanol for 3 days, was filtered with a muslin cloth and also with Whatman paper. The mixture distilled and concentrated under reduced pressure, using a rotary evaporator (Buchi, India), was stored in a desiccator. This leaf extract of JS (JSE), suspended in a carboxymethylcellulose (CMC), was administered to rats by oral route in a dose of 200 and 400 mg/kg according to body weight on each day of the study.

2.3. Phytochemical estimations

The total phenolic content of the extract was estimated by Folin-Ciocalteu reagent at 760 nm,[19] while the flavonoid content determined by ammonium trichloride at 415 nm using a spectrophotometer (Shimadzu- 1700).[20]

2.4. In-silico molecular docking analysis

A molecular docking study was conducted to correlate the anti-inflammatory activity of JS, where some of the active constituents were docked on the active sites of COX-1 and COX-2 enzyme. These active constituents were identified and selected for COX inhibition based on the available literature.

2.4.1. Preparation of ligands for docking

The structures of the active constituents of JS, namely isoquercetin, β-sitosterol, and linalool were drawn using ChemDraw ultra 10.0. These structures were converted from molfile to pdb format for docking analysis after an extensive review of the available literature.

2.4.2. In-silico molecular docking analysis

The prepared ligands were docked to the enzyme of interest, COX-2 using 'AutoDock 4.2'. Existing energies of the ligands were minimized using MMFF94 force field. Similarly, ligand atoms were added with Gasteiger partial charges while rotatable bonds were identified upon merging of the non-polar hydrogen atoms. AutoDock tools were also utilized to add essential hydrogen atoms and salvation parameters to the protein molecule which were used for docking calculations.[21] All protein-ligand interactions were observed using Discovery studio Visualizer 3.1 software.

2.5. In-vitro cyclooxygenase inhibition study

The *in-vitro* COX assay was conducted using murine macrophages isolated from Balb/c mice. In brief, the sterile PBS (20 mL), equilibrated at 37 $^{\circ C}$, was injected in the peritoneal cavity of the mice. The abdomen was massaged to collect peritoneal fluid under sterile conditions; the aspirate was washed and resuspended in DMEM supplemented with 10% FCS. The cells were counted and plated in a 96 well plate, having a concentration of 10000 cells per well. The plate was incubated at 37 °C with 5% CO2 for the

overnight period. Test samples 0.5 μg/mL, 5 μg/mL, and 50 μg/mL were added to the cells followed by stimulation with LPS (1 μg/mL). These cells were again incubated for 2 h and the cell supernatant was collected for centrifugation at 5000 rpm up to 10 min. The supernatant was analyzed for concentration of COX-1 and COX-2 using ELISA kits (Cayman's COX Assay Kit).

2.6. Animals

Eight to ten weeks old Lewis rats (n = 40) from the central animal housing facility of the institute were used for the study. Standard housing conditions; light and dark cycle (12/12 h) with optimum temperature (22 ± 2 °C) and humidity (40–70%) were maintained. The polypropylene cages housed two rats each; the rats had free access to food (standard rat pellets) and water *ad libitum*. The rats were randomized and grouped into five groups having 8 rats each. The experimental protocol was approved by the Institutional Animal Ethical Committee (PH/IAEC/VNS/2k12/18) of the organization and complies with the guidelines of Committee for the Purpose of Supervision of Experiments on Animals, Government of India.

2.7. Repeated dose treatment

The rats were administered either with a vehicle (0.1% CMC and 0.05% Tween 80 in deionized water) or a dose of JSE (200 and 400 mg/kg) once daily with oral gavage for the next 28 days, just before the beginning of the dark cycle. Diclofenac was used as a standard anti-inflammatory agent in the study. The change in body weight was recorded every day before the treatment of the rats; the dosing volume for each rat was maintained at 5 mL/kg of body weight.

2.8. Induction of arthritis in lewis rats

A single intradermal injection of 0.1 mL of CFA (heat-killed Mycobacterium butyricum, 3 mg/mL (Difco, Detroit, MI, USA) suspended in mineral oil), at the base of the tail of Lewis rat, was used to induce AIA. The day of CFA injection was considered to be day 0; all the groups received the treatments for the next 28 days of the study. A group of vehicle treated rats injected with mineral oil alone on day 0, was considered as Group I, vehicle control (Con) while all other groups of rats were injected with CFA. The group which received vehicle served as Group II, arthritic control (AIAcon); the group treated with diclofenac 2 mg/kg was Group III, positive control (Diclo); while other two groups, Group IV and Group V were treated with ethanolic extract of JS 200 mg/kg (JSE-200) and 400 mg/kg (JSE-400) respectively, once every day for next 28 days. At the end of the treatment, i.e. on the 29th day, all rats were sacrificed by profound inhalation of ether anesthesia; tissue and blood samples were collected for further estimation of various parameters.[22]

2.9. Assessment of paw edema and clinical arthritis score

The rats were observed for signs of arthritis on day 7, 14, 21 and 28 after induction of CFA. The arthritic score was assessed in every rat to grade the severity of the clinical signs. The grades were scored from 0 to 4 of each limb; grade 0 suggested no redness or swelling; 1 indicated erythema of one toe/finger joint; 2 signified redness and swelling of more than one toe/finger joints; 3 indicated the involvement of ankle and the joints, while grade 4 specified redness or swelling of the entire paw and distortion of joints. The sum of scores from all four limbs was considered as a total clinical arthritic score.[23] Similarly, edema in the hind paws was assessed by measurement of paw volumes using manual plethysmometer on day 0, 14, 21 and 28 of the study.

2.10. The ratio of spleen and thymus

The rats were sacrificed at the end of the study to isolate the thymus gland and spleen. The weight of both organs recorded and the percentage (%) wet weight versus body weight was determined to assess the immunological response of the rats to CFA or the treatment of JSE.

2.11. Estimation of erythrocyte sedimentation rate (ESR) and Haemoglobin (Hb)

Blood was withdrawn on day 29 from every rat through the retro-orbital plexus puncture into a tube containing anticoagulant to measure ESR and Hb. ESR was estimated using the method described by Eisen & Loveday[24] with few modifications. Briefly, blood was sucked into hematocrit tubes up to approximately 0 mm mark; these sealed tubes were made to stand stationary in an upright position without air bubbles away from vibration and direct sunlight. ESR was determined by reading the height of the column starting from 0 mm mark after 60 min and expressed as ESR in mm. The concentration of Hb in rats was estimated by using the cyanmethemoglobin method.[25]

2.12. Determination of free radical scavenging activity

The liver (1 g) was homogenized in ice-cold 10% trichloroacetic acid (TCA) using a tissue homogenizer. Malondialdehyde (MDA) levels were assayed as an index of lipid peroxidation by monitoring the formation of thiobarbituric acid-reactive substances and expressed as mmol MDA/mg of tissue protein.[26] Superoxide dismutase (SOD) activity was measured by the inhibition of pyrogallol autoxidation at 420 nm for 5 min.[27] One unit activity was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. Similarly, reduced glutathione (GSH) was determined using the method described by Moron[28] using DTNB reagent at 412 nm and expressed as μg of GSH/mg of protein.

2.13. Determination of myeloperoxidase activity

Myeloperoxidase was extracted and activity was measured using a method described by Bradley et al.[29] Briefly, plasma samples were homogenized in 50 mmol/L potassium phosphate buffer, pH 6, containing 0.5% hexadecyl trimethyl ammonium bromide. The homogenate was freeze-thawed three times and then centrifuged for 20 min at 11000 g and 4 °C. The supernatant (34 mL) was mixed with the same phosphate buffer (986 mL) containing 0.167 mg/mL ortho-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was recorded using a spectrophotometer. One unit of MPO activity was defined as that consuming 1 nmol peroxide/min at 22 °C. Results were expressed as unit/mg of tissue protein.

2.14. Protein estimation

Tissue protein content was estimated in aliquots of diluted membrane fractions using a colorimetric reaction with Folin's

phenol reagent. The color developed was measured at 640 nm.[30] Values were expressed as mg protein/g of wet tissue.

2.15. Radiographic analysis

Once sacrificed, the hind limbs of the rats were fixed in 10% formalin and limbs were imaged using the X-ray apparatus for radiographic assessment of joint tissues.

2.16. Histological studies

The hind limbs were removed up to the knees, immediately fixed in 10% formalin, decalcified with EDTA for 3 days and dehydrated. The 4 μm sections of sagittal slices of tissue were stained with hematoxylin and eosin (H & E); slides were evaluated for histological changes.

2.17. Statistical analysis

All the results were represented as mean ± SEM, analyzed with One Way Analysis of Variance (ANOVA) followed by Dunnett's test. Arthritic scores calculated as median values with the quartile range (25–75%) and analyzed with the Mann-Whitney *U* test. GraphPad Prism (GraphPad Software, USA) was used for statistical analysis and significance was considered as $p < 0.05$.

3. Results

3.1. Phytochemical screening

Screening of the ethanolic extract of JS revealed the presence of flavonoid content and phenolic constituents in the leaves of the plant (Supplementary data).

3.2. In-silico studies

The automated docking technique was utilized to determine the 'orientation' of iso-quercetin, β-sitosterol and linalool bounded with the active pockets of the COX-2 enzyme. The interaction of linalool with the active pockets of COX-2 was found to have significant fitness score and hydrogen bonding against COX-2. Isoquercetin and β-sitosterol demonstrated minimum binding energy with enzyme via non-covalent interaction. These *in-silico* observations corroborate in-vivo anti-arthritic activity of iso-quercetin, β-sitosterol and linalool as the major active constituents of JS (Table 1 and Fig. 1). There was no major change in COX-1 binding as compared to COX-2 binding of these constituents (data not shown).

3.3. In-vitro COX inhibition

The proinflammatory stimuli by LPS induced marked COX-2 activity in murine macrophages. This enhanced COX-2 activity was significantly inhibited by addition of 5 μg/mL and 50 μg/mL concentration of JSE in the wells having LPS stimulated cells. However, the 0.5 μg/mL concentration of JSE had no significant inhibition of LPS induced COX-2 activity. (Fig. 2; A).

3.4. Bodyweight change

Regular observation of body weight indicated a reduction in weight gain in AIA rats, however, on the establishment of arthritis the weight loss was stabilized in rats. There was a significant difference in the body weight of rats treated with JSE (400 mg/kg) as compared to vehicle treated AIA rats from 14 days till the end of study (Fig. 2; C).

3.5. Paw edema & arthritic score

Hind paw of rats exposed to AIA showed apparent swelling after 14 days; a significant increase in the volume of the hind paw was observed. The treatment with JSE did not reduce paw edema on day 7 however, on day 28, both doses, 200 and 400 mg/kg, of JSE, significantly restricted increase in hind paw volume in comparison with rats in the AIA group (Fig. 2; D and Fig. 6). After the 14th day, arthritic scores were prominently increased in vehicle-treated AIA rats while JSE treatment had significantly low arthritic scores on all observations (Fig. 3; A).

3.6. Thymus-spleen ratio

The ratio of thymus and spleen weight to body weight was determined at the end of the study. The AIA group had significantly higher spleen weight and decreased thymus weight as compared to normal control group. The treatment with both JSE 200 and 400 mg/kg significantly prevented the increase in spleen weight while the decrease in thymus weight was markedly arrested by the treatment when compared with AIA group on day 29th of the study (Table 2).

3.7. Hb and ESR

On day 29th of the study, a significant elevation in ESR and an apparent decrease in Hb count was observed in AIA group when compared with normal control group. Hb count was elevated in JSE-200 treated rats whereas ESR was significantly decreased by the treatment of JSE with both the doses (200 and 400 mg/kg) as compared AIA group of rats (Table 2).

3.8. Free radical scavenging activity

Induction and establishment of arthritis significantly elevated the level of lipid peroxidation in the liver of arthritic rats. On the contrary, SOD activity and the level of GSH was decreased as compared to the vehicle control group. Treatment with JSE

Table 1
Molecular docking of phytoconstituents on COX-2.

Parameters	Isoquercetin	β-sitosterol	Linalool	Diclofenac
Estimated Free Energy of Binding	−9.31 kcal/mol	−12.72 kcal/mol	−8.72 kcal/mol	−13.54 kcal/mol
Estimated Inhibition Constant	143.03 nM	473.55 pM	274.25 nM	3.16 nM
Final Intermolecular Energy	12.8 kcal/mol	−14.81 kcal/mol	−4.23 kcal/mol	−1.47 kcal/mol
vdW + Hbond + desolv Energy	−12.28 kcal/mol	−14.57 kcal/mol	−13.22 kcal/mol	−5.75 kcal/mol
Electrostatic Energy	−0.62 kcal/mol	−0.24 kcal/mol	−0.13 kcal/mol	−0.04 kcal/mol
Final Total Internal Energy	−5.15 kcal/mol	−1.04 kcal/mol	−1.11 kcal/mol	−1.08 kcal/mol
Torsional Free Energy	5.58 kcal/mol	2.09 kcal/mol	2.24 kcal/mol	1.05 kcal/mol
Unbound System's Energy	−5.15 kcal/mol	−1.04 kcal/mol	−1.55 kcal/mol	−0.15 kcal/mol

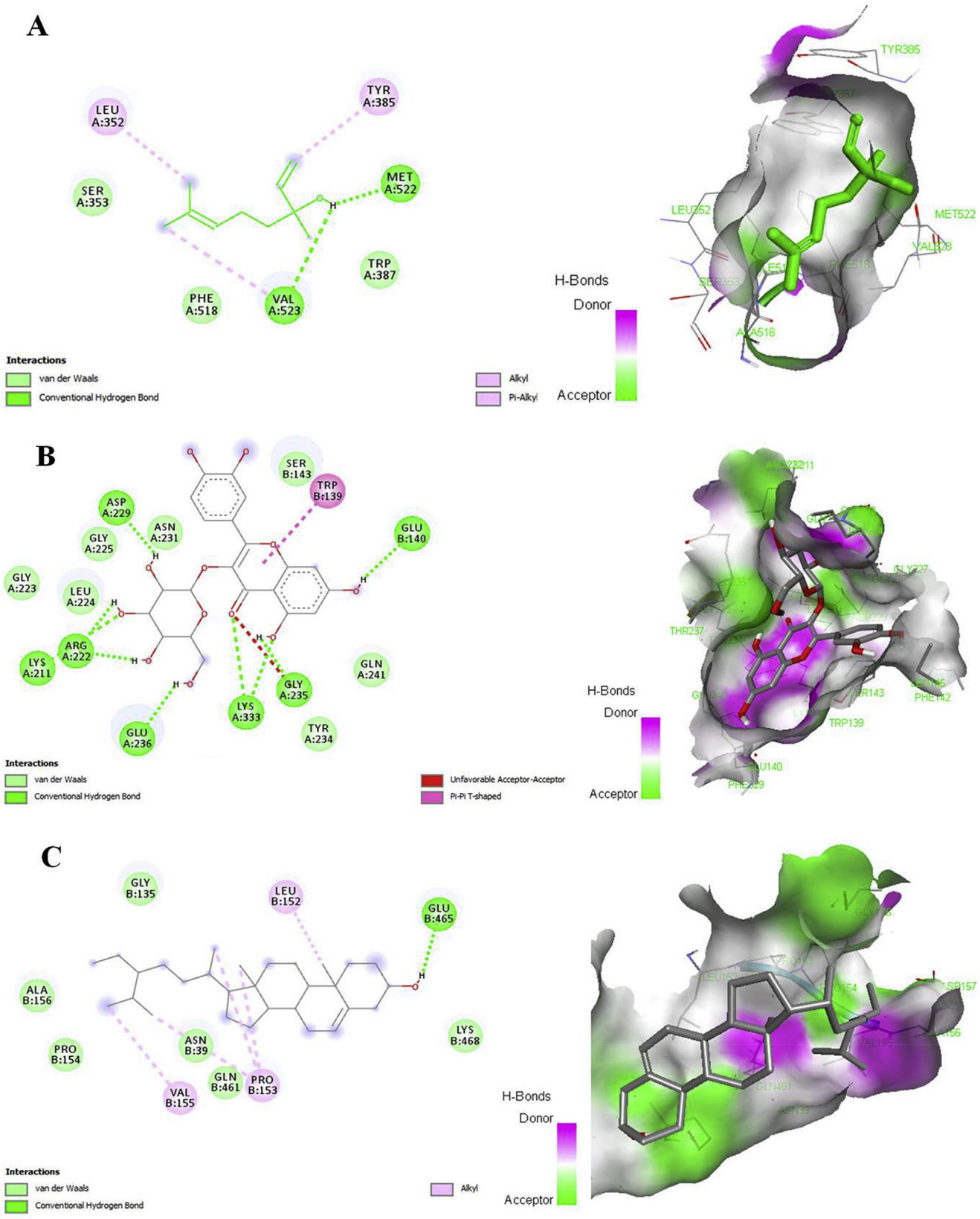

Fig. 1. Docking interaction of (A) linalool, (B) iso-quercetin and (C) beta sitosterol with COX-2.

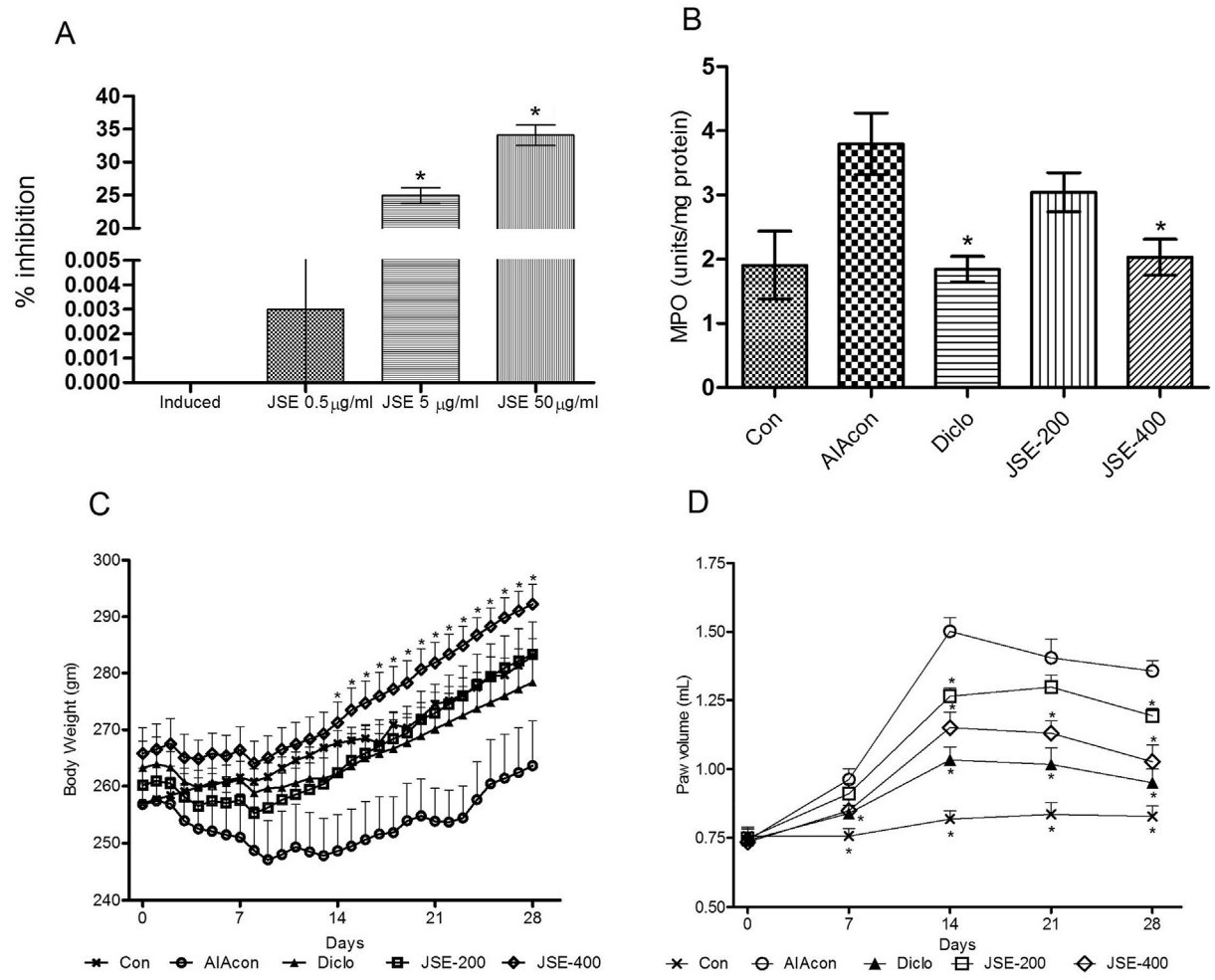

Fig. 2. Effect of JSE on (A) % COX-2 inhibition, *p < 0.05 against LPS induced COX activity; (B) Myeloperoxidase activity; (C) Body weight and (D) Paw volume. Values as mean ± SEM; *p < 0.05 against AIA control.

significantly elevated the activity of SOD as well as the level of GSH at the end of treatment. However, lipid peroxidation showed a marked decrease in the groups treated with JSE (200 and 400 mg/kg), this change was statistically significant as compared to the AIA group of rats (Fig. 3; B, C, & D).

3.9. Myeloperoxidase activity

Myeloperoxidase activity is a vital parameter for the inflammation. Treatment with JSE-400 significantly reduced MPO activity in the plasma samples of treated rats as compared to the AIA group of rats. However, JSE-200 treatment reduced MPO activity which was not significantly different to that of AIA group of rats (Fig. 2; B).

3.10. Radiographic and histological changes in joint tissue

Analysis of radiographs of the hind limbs in AIA group of rats revealed major distortion of the joints and partial destruction of the tibiotarsal joints. The JSE treated rats showed a decrease in swelling of soft tissue and the prevention of the distortion or destruction of the ankle joints (Fig. 4). This consistency in restriction of damage to structural aspects of the joints was also reflected in histological observations which showed reduced neutrophil infiltration and normal connective tissue in JSE treated arthritic rats. Whereas, AIA group of rats exhibited prominent edema of soft tissue, vascularity, distortion of normal structure in joint tissue, bone erosion, and infiltration of inflammatory cells (Fig. 5).

4. Discussion

The progression of RA must be viewed through the prism of intriguing mechanisms involving interplay between modulation of cytokines and T lymphocyte expressions. However, chronic inflammation accompanied by bone destruction, due to severe infiltration of inflammatory cells in synovium, is the cardinal features of RA.[31] In addition, elevated COX activity and overproduction of ROS also exacerbates inflammatory reactivity and endothelial damage.[32] Pulichino et al. emphasized the role of COX enzymes in RA by suggesting that inhibition of COX contributes to suppressing the signs of arthritis.[33] Hence, the exploration of the role of COX inhibition in the anti-arthritic activity of JS was at the core of the current investigation in Lewis rats.

Herein, we have demonstrated that phytoconstitutents of JS has the potential to modulate seminal mechanisms of AIA in Lewis rats. Dysregulation of balance between pro and anti-inflammatory mediators, locally and systemically, could be responsible for clinical advances of immune-mediated joint diseases. The disease progression exhibits acute inflammation much before the advent of visible structural damage.[32,34] Acute inflammation demonstrates edema, leukocyte infiltration, and fibrin extravasations in soft

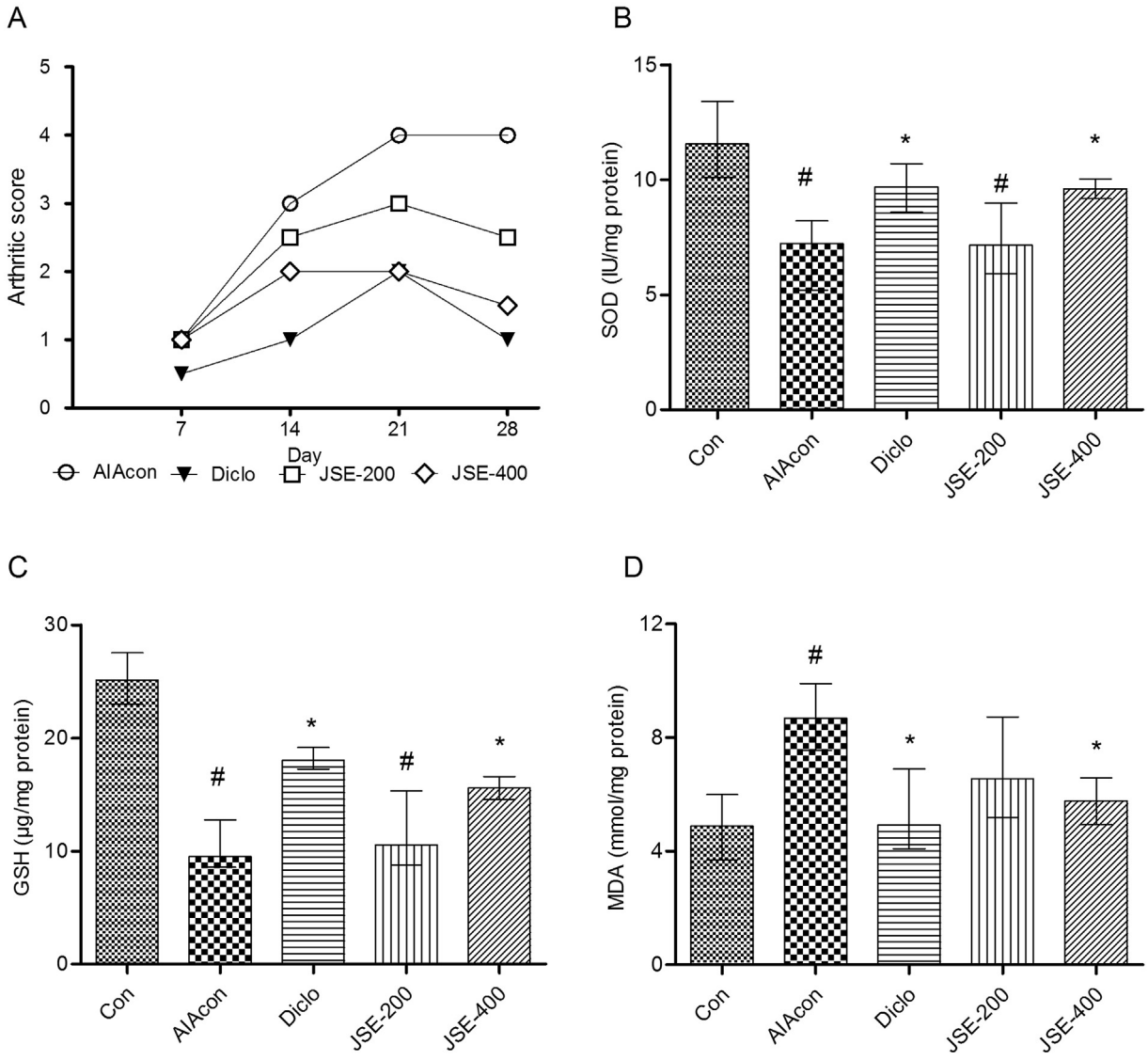

Fig. 3. Effect of JSE on (A) arthritic score, (B) superoxide dismutase (SOD), (C) glutathione (GSH) and (D) lipid peroxidation (MDA). Values as mean ± SEM; *p < 0.05 against AIA control.

Table 2
Tissue weight and hematological parameters.

Groups	Thymus weight (mg/100 g of body weight)	Spleen weight (mg/100 g of body weight)	Hb (g/dL)	ESR (mm)
Con	133.60±6.89*	184.80±7.44*	12.46±0.61	3.48±0.31*
AIAcon	81.80±4.84#	284.90±3.94#	10.29±0.64#	7.83±0.42#
Diclo	121.60±8.89*	200.00±7.72*	12.20±0.40	3.80±0.44*
JSE-200	99.68±5.45#	221.30±15.49*	11.14±0.50	3.87±0.35*
JSE-400	111.80±5.41*	207.20±5.69*	12.00±0.40	3.71±0.20*

Values as mean ± SEM.
*P < 0.05 against AIA control; #P < 0.05 against vehicle control.

tissues, whereas damage to joint displays cartilage matrix degeneration and skeletal erosion.[34] This elevated inflammatory reactivity results in enhanced local and systemic cytokine levels, accompanying exacerbated COX activity.[35] Treatment with JS, in the current study, significantly reduced the paw volume and clinical scores in AIA rats suggesting a decrease in acute inflammation. The initial phase of RA is characterized by acute inflammation, whereas structural damage of joints, reduced by the treatment, signifies the clinical phase of RA.[1,10] *In-vitro* COX inhibition, at various concentrations of JSE, also substantiates reduced inflammatory reactivity, indicating the role of the phytoconstituents of JS in anti-inflammation.[10,19] Since the advent of computational chemistry, virtual docking is a chosen technique for predicting pharmacological activity. Docking of iso-quercetin, β-sitosterol, and linalool (three of the active constituents in JS) with the active pocket of COX-2, was used as a tool to ascertain the role of COX enzyme in arthritis. All

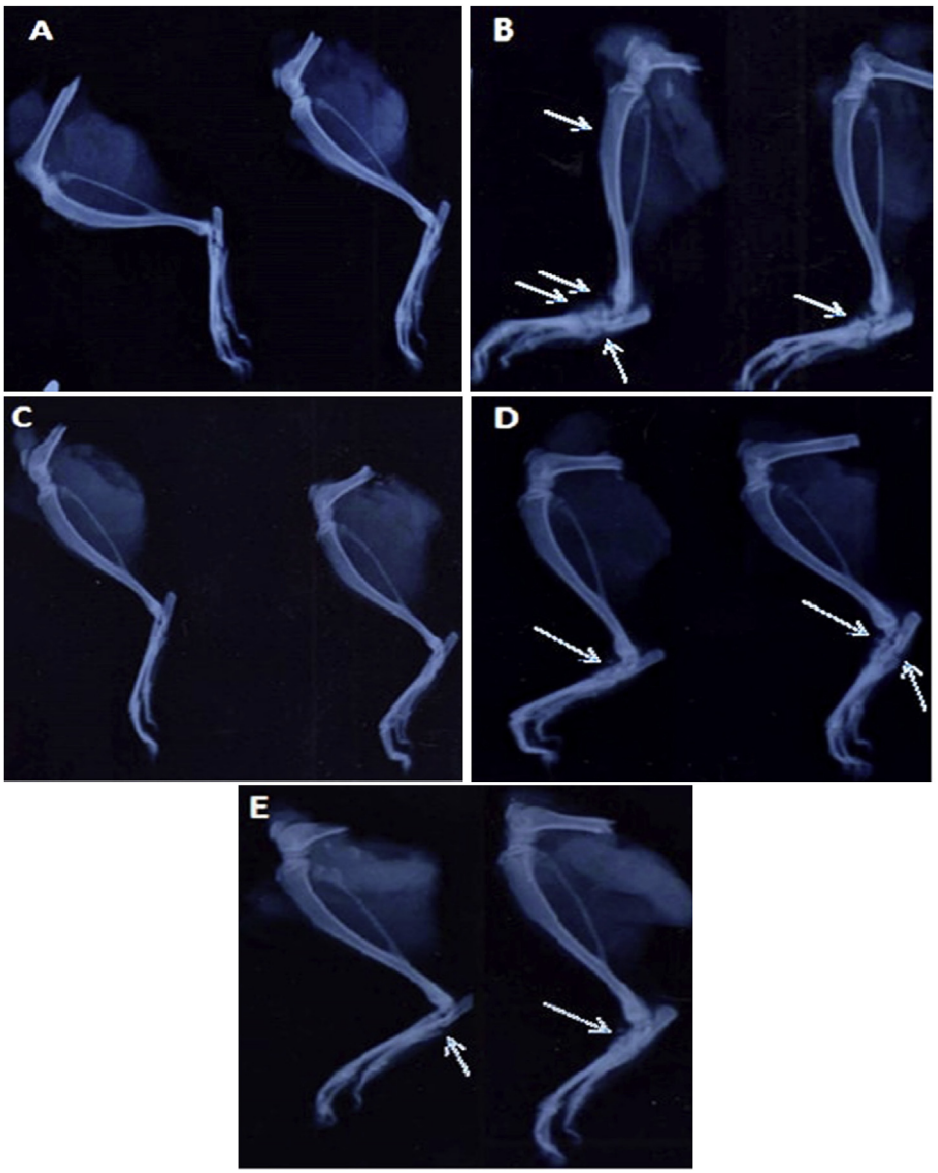

Fig. 4. Radiographic images of hind paws on day 28: (A) Control; (B) AIA Control: showed bone demineralization, joint space narrowing and bone erosion at the distal tibia, talus, calcaneus and metatarsus. (C) Diclo. (D) JSE-200 shows less destruction of bone as compare to AIA control (E) JSE-400 with reduced bone destruction.

three constituents have significant fitness score and demonstrated minimum binding energy with enzyme via non-covalent interaction. Further diclofenac, used as a standard drug, also had similar outcomes in docking studies. Our results suggest a specific correlation between the outcomes of *in-silico* studies and *in-vitro* assays.

Progression of secondary arthritic phase has a pronounced effect on body weight, which affects locomotor activity as well as muscle strength.[36] The observed rheumatoid cachexia, in the current study, was significantly arrested by JS treatment, suggesting an interconnect between reduced inflammation and improved body weight. In addition, the thymus-spleen ratio also served as an indicator of exacerbated immunological responses, where spleen may be enlarged due to splenomegaly while thymus gland shrinks as a consequence of enhanced inflammatory reactivity.[37] Splenomegaly is attributed to elevated extramedullary hematopoiesis induced by increased cytokine and PG release.[38] These cytokines and PGs, in arthritic milieu, are responsible for exaggerated inflammation and immunogenicity, resulting in cell destruction which leads to a decrease in thymus weight.[37,38] In the current study, treatment with JS showed improved thymus weight and reduced splenomegaly, suggesting immunomodulation and inhibition of inflammation. Besides, increased leukocyte count and lymphocyte infiltration in the synovium are closely associated with adjuvant-induced arthritis.[1,2] The stacking of erythrocytes, stimulated by protein synthesis during inflammation, increases the sedimentation rate of the erythrocytes (ESR count).[39] Similarly, low Hb levels observed in RA patients, signify reduced pain threshold, articular damage, and disability.[40] Thus, the reduction in ESR, restoration of Hb content, and improvement in the thymus-spleen index indicate modulation of immune response and reduced inflammation with the treatment, which is in agreement with earlier reports.[38]

In the present study, AIA stimulated an increase in ESR and splenomegaly, indicating plausible role of neutrophil infiltration to instigate activation of the oxidative cascade.[30,38,41] Generation of ROS like, superoxide anions, peroxides, and nitrates results in tissue

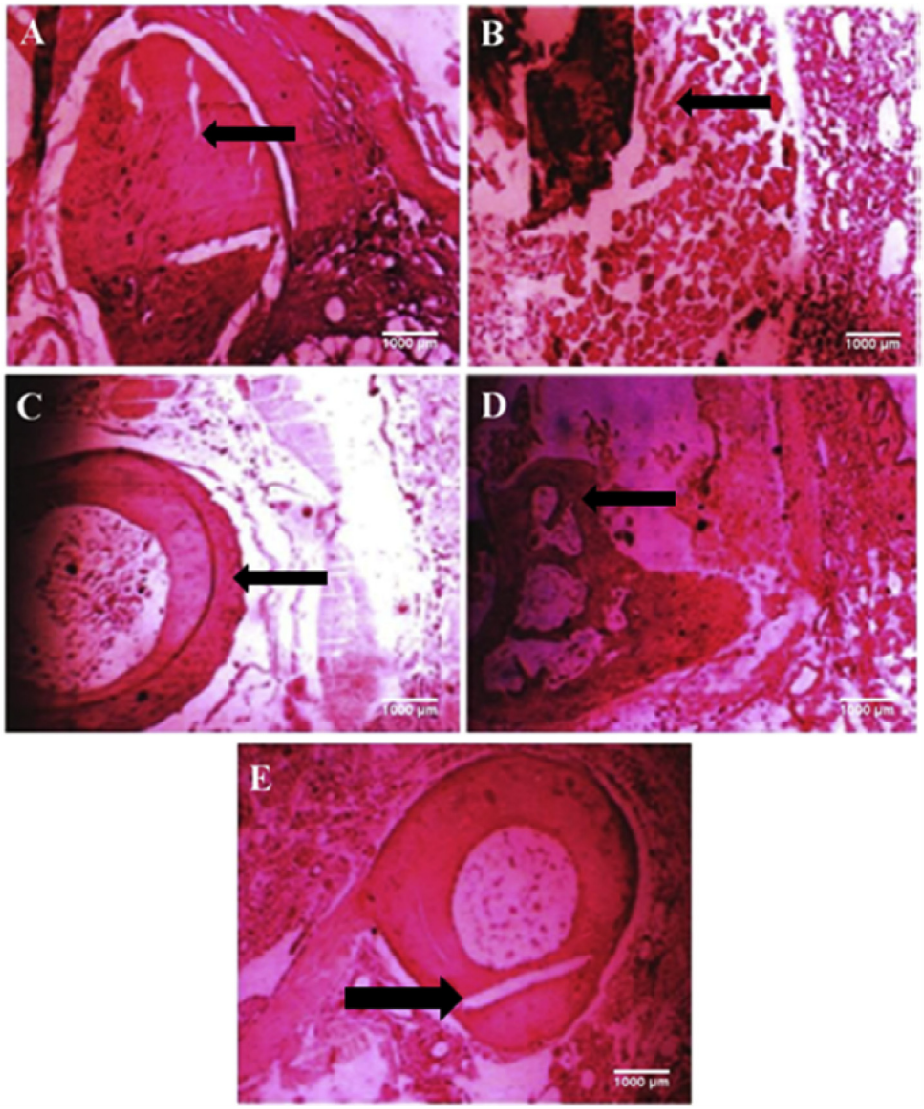

Fig. 5. Histology of rat paw joints: (A) Control-histology of normal ankle joint normal articular cartilage and absence of infiltrate in the synovum (B): AIA control-prominent abnormalities like edema formation, degeneration with partial erosion of the cartilage, destruction of bone marrow and extensive infiltration of inflammatory exudates in the articular surface (C) Diclo: normal bone marrow with less cellular infiltrates and reduced edema formation. (D) JSE-200: showed cellular infiltrates on the articular surface with less cartilage destruction (E) JSE-400: reduced inflammatory sign and absence of edema.

damage by multiple oxidative mechanims.[41,42] Our finding too suggests inter-relationship between oxidative stress and cartilage damage in arthritis.[43] Excessive generation of superoxide anions, increases the vulnerability of collagen in the joints which could catalyze the structural damage.[41,43] At the end of the treatment schedule, SOD activity was restored while lipid peroxidation was attenuated, while intracellular GSH, a thiol anti-oxidant responsible for the repair of the oxidative damage, was recuperated.[41,44] Activated neutrophil releases MPO in response to ROS generation[30]; MPO is a vital inflammatory marker in autoimmune disorders. Furthermore, RA patients showed accelerated neutrophil activation, ROS generation, and elevated MPO activity during disease progression.[45] Herein the treatment with JS significantly reduced plasma MPO activity in addition to histological observations; showed a decrease in neutrophil infiltration in the inflamed joints.

Thus, a decrease in MPO activity, ROS generation, and neutrophil activation contributed to the observed reduction of inflammatory reactivity and tissue injury in arthritic conditions.[46] Moreover, the presence of flavonoids, phenols, and sterols in the JS suggests possible crosstalk between its ROS scavenging ability and anti-inflammatory activity in chronic inflammation, corroborate with earlier reports.[10,46]

Histological observations in the joints of the arthritic rats exhibited a consistent accumulation of inflammatory cells. The aggressive infiltration of neutrophils in inflamed joints exacerbated the destruction of cartilage and bone in AIA rats.[44,45] Any decrease in chronic inflammation is associated with reduced edema, oxidative stress, and cartilage destruction.[44,47] It reinforces the role of JS to attenuate pro-inflammatory mediators and ROS generation responsible for swelling and bone erosion. Similarly, the radiographic analysis provides greater insight into the actual severity of joint destruction, which serves as a diagnostic tool for analyzing intensity and degree of late-stage structural damage like, bone erosion and narrowing of joint spaces.[47] Consistent with the histological observations, AIA rats had severe bone destruction and narrow joint spaces; these late phase arthritic features were alleviated after 28-day treatment with JS, which further substantiated

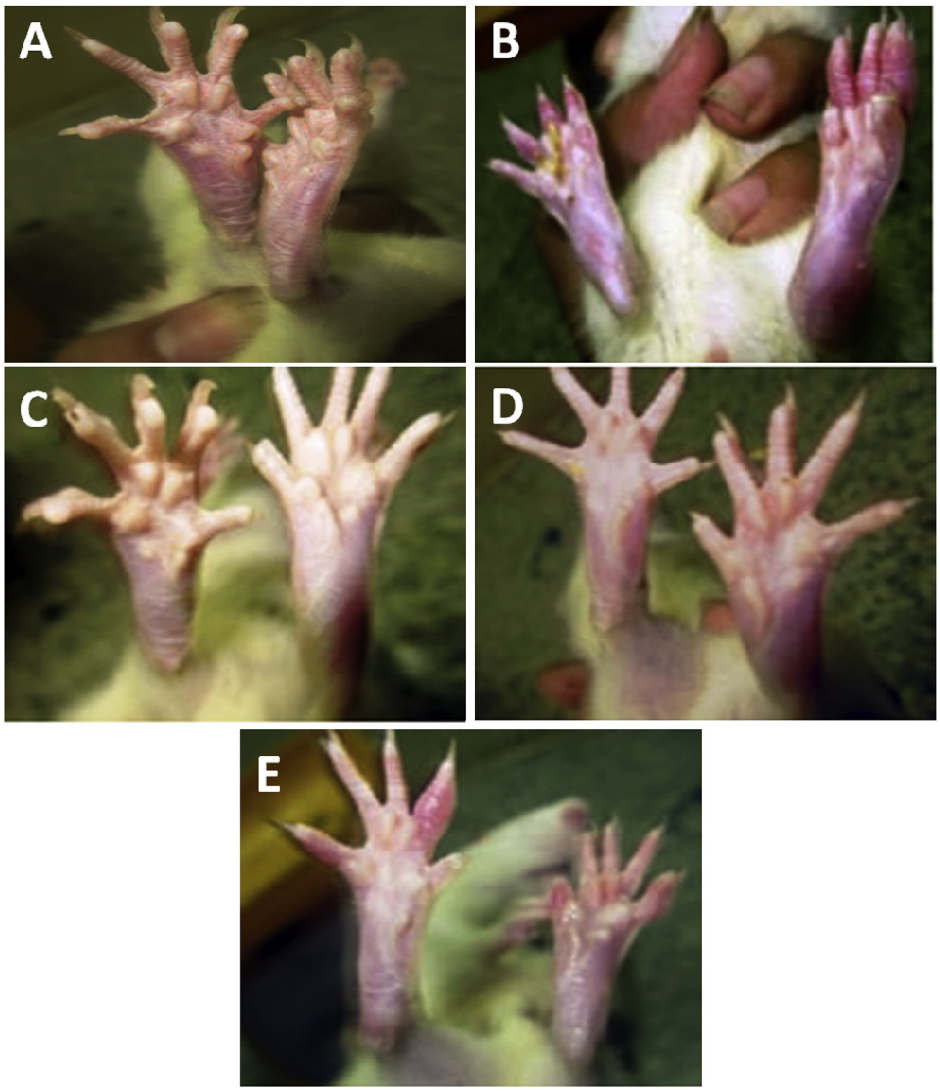

Fig. 6. Photographs of paw edema on day 28: (A) Control; (B) AIA Control; Prominent swelling on both paw (C) Diclo: reduced edema formation (D) JSE-200 and (E) JSE-400 with decreased edema and swelling of the tissue.

the other findings. Literature reveals that activation of the NF-kB and MAPK pathway by cytokines initiates a critical stage of destructive arthritis.[3,5,44] These intracellular pathways, stimulating IL-6, COX-2, and matrix metalloproteinase-1 protein, are considered to be at the center of structural destruction during the late phase of RA.[3,41] Since JS affects both early and late phases of arthritis, the possibility of inhibition of one or more proteins, expressed in the NF-kB or MAPK pathway, cannot be denied.

Taking cues from virtual docking of iso-quercetin, β sitosterol, and linalool on the COX-2 enzyme; the outcomes of the study underscore the significance of the anti-inflammatory activity exhibited by JS, probably due to its COX inhibition. Remarkably, the demonstrated minimum binding energy of linalool highlights the role of COX inhibition in arthritis because the expression of COX-2 is high in inflammatory and immune cells.[47,48] Hence, these in-silico observations corroborate the in-vivo anti-arthritic activity of JS, suggesting, reported earlier, a correlation between COX inhibition and anti-arthritic activity.[49,50] Thus, the demonstrated anti-arthritic activity of JS, against CFA induced AIA, could be attributed to attenuation of inflammation during both the stages of RA.

5. Conclusion

In conclusion, JS demonstrated anti-arthritic activity evident from a reduction in paw edema, improvement in ROS scavenging activity, and a decrease in structural damage. Although the vital mechanisms for the observed anti-arthritic activity of JS are COX inhibition and attenuation of oxidative stress, the exploration of the exact role of major signaling pathways is warranted. The completion of the ongoing COX selective in-vitro and in-vivo studies could add more insight into the activity of JS on various chronic inflammatory disorders.

Declaration of competing interest

The authors have no conflicts to declare.

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We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2020.04.002.

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