EVALUATION OF THE ANTI-INFLAMMATORY AND ANTI-ARTHRITIC EFFECTS OF SYRINGIC ACID IN RATS

Shilpee Chanda, Sarayu A. Pai and Archana R. Juvekar*

Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Nathalal Parekh Marg, Matunga, Mumbai, Maharashtra 400019, India.

ABSTRACT
The aim of the present study is to evaluate the anti-inflammatory and anti-arthritic potential of syringic acid (SA) in carrageenan induced paw oedema and Freund’s complete adjuvant (FCA) arthritis in rats. The anti-inflammatory activity of SA was determined in carrageenan-induced paw oedema in rats. For evaluation of anti-arthritic activity, a single dose of FCA was administered in the paw of each rat and the paw volume, stair-climbing ability and arthritis index were measured. TNF-α and IL-6 were determined in the serum. The indices of the thymus and spleen were determined. The antioxidant parameters were estimated in the liver and paw. The histopathological examination of the paw was carried out. SA significantly reduced the paw volume and levels of TNF-α and IL-6 in both the models. SA reduced the arthritis index and improved the stair climbing ability of arthritic rats. FCA caused an increase in the thymus and spleen indices while SA reduced these. SA exerted antioxidant activity in the liver and paw of arthritic rats. The histopathological examination of the paw revealed reduction in the inflammatory cell infiltration and cartilage and bone damage score. SA may be a potential molecule for the treatment of inflammation and rheumatoid arthritis.

KEYWORDS: freund’s complete adjuvant, carrageenan, anti-inflammatory, anti-arthritic, syringic acid.
INTRODUCTION
Rheumatoid arthritis (RA) is an auto-immune disorder that is characterized by chronic inflammation and involves the infiltration of inflammatory cells, synovial tissue proliferation and destruction of knee joints. The RA affected joints are attacked by one’s own immune cells, thereby inducing the release of cytokines like tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). These cytokines further lead to the progression of RA via the activation of cyclo-oxygenase-2 (COX-2), which in turn increases the production of prostaglandin E2 (PGE2), matrix metalloproteinases (MMPs) levels and mediates synovial inflammation. These events lead to the formation of pannus that is brought about by angiogenesis and proliferation of synovial fibroblasts. The imbalance between the effects of pro-inflammatory and anti-inflammatory cytokines is responsible for the severity of RA. Therefore, an appropriate strategy for the treatment of RA could be the downregulation of pro-inflammatory cytokines.

Carrageenan induced paw oedema in rats is a widely used model to evaluate the acute anti-inflammatory activity of drugs. The sequential release of pro-inflammatory mediators and their amelioration by anti-inflammatory drugs can be studied well using this model. Freund’s complete adjuvant (FCA) model of arthritis in rats mimic the human pathophysiological condition of RA, which is characterized by the accumulation of inflammatory mediators, destruction of bones and erosion of joint cartilage. Hence, the model has been used to evaluate the activity of a number of potential anti-arthritic and anti-inflammatory agents.

Non-steroidal anti-inflammatory drugs (NSAIDs) alone or in combination with steroid hormones are effective in the management of pain and inflammation in RA. Other drugs used for the treatment of RA include disease-modifying anti-rheumatic drugs (DMARDs) as well as anti-cytokine therapies. However, these therapeutic agents can only ameliorate the symptoms, rather than significantly improving the condition of arthritis. The drawbacks of the currently available drugs for the treatment of RA include their potential to cause serious adverse drug reactions like gastrointestinal lesions, cardiovascular complications, hepatotoxicity, nephrotoxicity and reproductive toxicity. Owning to these shortcomings, the research interest lies in finding newer drugs which are safer and more effective than the current therapy.

Syringic acid (SA) (4-hydroxy-3,5-dimethoxybenzoic acid) is a phenolic compound naturally occurring in date palm, red wine, honey, marigold, salt cedar, chard, pumpkin, radish, olive, walnut, and Indian Sarsaparilla. SA has good bioavailability and is well-tolerated in animals. SA has been found to possess good antioxidant property. SA has demonstrated antimicrobial, anticancer, hepatoprotective, antidiabetic, anti-obesity, cardioprotective and neuroprotective activities, to name a few.\cite{10} SA has proved to downregulate the vascular endothelial growth factor (VEGF) mRNA expression, thereby providing evidence on the anti-angiogenic ability of SA in zebrafish embryo model.\cite{11} Significant anti-osteoporotic activity of SA has been noted in ovariectomized mice model.\cite{12}

Plant extracts containing SA have demonstrated anti-inflammatory activity \textit{in vitro}. However, no study till date has evaluated the effect of syringic acid \textit{per se} in the treatment of inflammation and rheumatoid arthritis \textit{in vivo}. The aim of the present study was to evaluate the anti-inflammatory and anti-arthritis potential of SA in carrageenan induced rat paw oedema and FCA induced arthritis in rats and to investigate the possible underlying mechanism of action of SA.

MATERIALS AND METHODS

General

Syringic acid (TCI Chemicals, India), indomethacin (S.D Fine Chemicals Ltd, India), λ-carrageenan (Sigma Aldrich, USA), Freund’s complete adjuvant (Sigma Aldrich, USA). TNF-α and IL-6 ELISA (enzyme-linked immunosorbent assay) kits were purchased from Krishgen Biosystems, India. All other reagents used were purchased from approved commercial sources and were of analytical grade.

Animals

Male Wistar rats (180-200 g) and female Wistar rats (130-150 g) were obtained from the National Institute of Biosciences, Pune, India. The animals were housed in solid bottom polypropylene cages, maintained at 25±2 °C, under a 12 h light/dark cycle, with relative humidity of 50±5%. Throughout the study, the animals had free access to standard chow pellets and water \textit{ad libitum}. The animals were acclimatized for a week prior to the start of the experiments. All the experimental protocols (ICT/IAEC/2017/P58 and ICT/IAEC/2017/P59) were approved by the Animal Ethics Committee of the Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, India.
Acute toxicity test
The acute toxicity study of SA was carried out in accordance with the Organisation for Economic Co-operation and Development (OECD) guidelines. The animals were fasted for 6 hours with free access to water, prior to the start of the experiment. Six female Wistar rats were orally administered 2000 mg/kg syringic acid suspended in 0.5% sodium carboxymethylcellulose (NaCMC) in distilled water (DW). The rats were critically observed for clinical and gross behavioural changes for the first 2 h after administration of SA. Thereafter, the animals were observed for 24 h for mortality and any treatment related toxicity for 14 days.

Carrageenan induced rat paw oedema
The paw edema was induced by using the method provided in the literature. The animals were divided into 5 groups with six rats as follows:
Normal control (NC)- received vehicle given orally;
Disease control (DC)-received 0.1 ml carrageenan by sub-plantar administration and vehicle by oral route;
SA 25-received syringic acid at 25 mg/kg given orally;
SA 50-received syringic acid at 50 mg/kg given orally;
Indo 10-received indomethacin 10 mg/kg given orally.

The normal control was injected by sub-plantar route with 0.1 ml of normal saline into the left paw while all other groups were given sub-plantar injection of 0.1 ml of 1% carrageenan into the left paw. SA (25 and 50 mg/kg), indomethacin and vehicle were administered orally 1 hr prior to the injection of carrageenan. The vehicle administered was 0.5% sodium carboxymethylcellulose (NaCMC) in distilled water. The selection of the doses of SA to be tested were based on the results of the acute toxicity study. As the dose of 2000 mg/kg showed no signs of toxicity and mortality, we decided to test the dose that was 40 and 80 times lower, to prevent any unnecessary exposure of the rats to a high dose.

Paw volume was measured using a plethysmometer (UGO Basile, Italy) before carrageenan injection and at 1, 2, 3 and 4 h post injection. The increase in paw volume was calculated as the difference between the final and initial paw volume. The blood was collected 3 h after the carrageenan injection by retro-orbital puncture. TNF-α and IL-6 levels in serum were estimated using the commercially available rat cytokine ELISA as per the instructions of the manufacturer.
Freund’s complete adjuvant induced arthritis in rats

Arthritis was induced as previously described in the literature.\textsuperscript{[14]} The animals were divided into 5 groups with six rats each as follows:

Normal control (NC): received vehicle;

Disease control (DC) received 0.1 ml of FCA by sub-plantar route and vehicle given orally;

SA 25- received syringic acid 25 mg/kg given orally;

SA 50- received syringic acid 50 mg/kg given orally;

Indo 1- received indomethacin 1 mg/kg given orally.

Except for the normal control groups that received a sub-plantar injection of 0.1 ml of normal saline, the induction of arthritis was done by a single sub-plantar injection of 0.1 ml of FCA into the right hind paw on day 0. All the treatment groups were orally administered with SA (25 and 50 mg/kg), indomethacin (1 mg/kg), while the normal control and disease control group were given equal volume of 0.5% NaCMC dissolved in distilled water by oral route. The dose of SA was selected on the basis of significant anti-inflammatory effect of SA at the two doses (25 and 50 mg/kg) exhibited in the carrageenan induced rat paw edema model. Daily oral administration of the above-mentioned doses was given after arthritis induction and continued until the end of the experiment (day 14).

Anti-arthritic activity of SA was evaluated on the basis of paw volume, arthritis index, motility score and stair-climbing ability on day 1, 7, 10 and 14. On day 15, blood was collected by retro-orbital puncture for the estimation of biochemical and haematological parameters.

Paw volume and arthritis index

The paw volumes of the injected paw of all the animals were measured using a plethysmometer (UGO Basile, Italy).\textsuperscript{[15]} The arthritis severity was scored as follows: 0-no sign of oedema or swelling; 1- slight oedema and limited erythema; 2- slight oedema and erythema extending from the ankle to the tarsal bone; 3-moderate oedema and erythema extending from the ankle to the tarsal bone; 4- oedema and erythema extending from the ankle to the entire leg. Scoring was done for all the 4 limbs for the calculation of the arthritic index, with the maximum score possible of 16 for each rat.\textsuperscript{[16]}
**Motility and staircase climbing test**

The motility of each rat was monitored for 5 mins and scored as follows: 0 - rats refrained from touching the feet to the ground; 1 - rats experienced difficulty in walking with the toe touching the ground; 2 - the rats walked with ease, showing no signs of difficulty. In staircase climbing ability testing, the rats were trained to climb a staircase for a period of 7 days prior to the start of the experiment. The staircase was designed in such a way that the steps were placed at 5, 10 and 15 cm from ground, with food pellets kept on the third step. The staircase climbing score ranged from 0 to 3 as follows: 0 = the rat did not manage to climb onto any step; 1 = the rat climbed the first step; 2 = the rat climbed the second step and 3 = the rat climbed the third step. [17]

**Thymus and spleen index**

The thymus and spleen were isolated from each animal after sacrifice on day 15 and immediately weighed. The thymus and spleen indices were expressed as the ratio of the wet weight of the thymus and spleen versus the body weight of the animal on the final day of the experiment, respectively. The unit of the index was expressed as mg/g. [18]

**Haematological parameters**

The blood was withdrawn by retro-orbital puncture and collected in EDTA-containing tubes. The parameters analysed were white blood cell (WBC), red blood cell (RBC) and haemoglobin (Hb) level, using the routine laboratory methods. Erythrocyte sedimentation rate (ESR) was also performed as mentioned in literature. [19]

**Biochemical analysis in liver and rat paw tissue**

On day 15, after sacrifice, parts of the liver and injected paw tissue of each rat were homogenised separately, the homogenates were centrifuged and the supernatant thus obtained was used to analyse the levels of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), malondialdehyde (MDA) and nitric oxide (NO) activities using the methods described previously. [18]

**Determination of TNF-α and IL-6 levels in serum**

Serum TNF-α and IL-6 concentrations were quantified using the commercially available rat cytokine ELISA kits, as per the protocol provided by the manufacturer.
**Histopathological studies**

The injected paw of the one rat from each group was removed for the histopathological examination. The paws were fixed in 10% phosphate buffered formalin, followed by decalcification in 10% EDTA for 30 days at 4 °C. The paws were then embedded in paraffin wax. Sections (5µm) were prepared and stained using haematoxylin and eosin (H&E) and observed using light microscope.

**Statistical analysis**

All the data are expressed in terms of mean ± standard error of the mean (SEM). Statistical comparisons between the different treatment groups were made using two-way ANOVA followed by Dunnett’s test. One-way ANOVA, followed by Dunnett’s test was used to analyse the data of biochemical estimations. The motility scores and staircase climbing ability scores are represented as median scores and comparison between the groups were made by Kruskal-Wallis test. Analysis was done using GraphPad Prism 7.05 software (GraphPad, San Diego, USA). Statistical significance was identified at $P < 0.05$.

**RESULTS**

**Acute toxicity test**

Acute toxicity study of syringic acid did not exhibit any clinical or behavioural signs of toxicity and did not produce death of any animal during the 14 days observation period. Therefore, it can be concluded that syringic acid is safe up to 2000 mg/kg.

**Carrageenan induced rat paw oedema**

*Effect of SA on paw volume*

Sub-plantar injection of carrageenan caused a marked increase in the paw volume when compared to the normal control group with the peak at 3 h post-injection and subsequent reduction at 4 h. Rats treated with SA (25 mg/kg and 50 mg/kg) showed significant ($P < 0.05$) decrease in the paw volume when compared to the disease control group from 1 h to 4 h. Indomethacin showed significant ($P < 0.001$) decrease in paw volume at 1, 2, 3 and 4-h post-injection (Table 1).
Table (1): Effect of SA on carrageenan induced rat paw edema model.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>0.37 ± 0.09</td>
<td>0.47 ± 0.08</td>
<td>0.55 ± 0.07</td>
<td>0.53 ± 0.04</td>
</tr>
<tr>
<td>SA 25</td>
<td>0.32 ± 0.07*</td>
<td>0.40 ± 0.03*</td>
<td>0.43 ± 0.07*</td>
<td>0.45 ± 0.04*</td>
</tr>
<tr>
<td>SA 50</td>
<td>0.27 ± 0.01**</td>
<td>0.31 ± 0.02***</td>
<td>0.29 ± 0.05***</td>
<td>0.28 ± 0.04***</td>
</tr>
<tr>
<td>Indo 1</td>
<td>0.19 ± 0.05***</td>
<td>0.16 ± 0.03***</td>
<td>0.17 ± 0.08***</td>
<td>0.15 ± 0.05***</td>
</tr>
</tbody>
</table>

Data are expressed in terms of mean ± SEM, n=6. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the disease control group by two-way ANOVA followed by Dunnett’s test. NC, normal control; DC, disease control; SA 25, syringic acid (25 mg/kg); SA 50, syringic acid (50 mg/kg); Indo 1, indomethacin (1 mg/kg).

**Effect of SA on TNF-α and IL-6 levels in serum**

Rats in the disease control group showed a significant increase in the serum TNF-α and IL-6 concentration at 3 hours after carrageenan injection (P < 0.001). Indomethacin and both the doses of SA caused a significant decrease in the serum TNF-α concentrations (P < 0.05), however only SA (50 mg/kg) and indomethacin caused a significant (P < 0.001) decrease in serum IL-6 (Fig. 1a, 1b).

![Fig. 1: Effect of SA on inflammatory cytokines a) TNF-α and b) IL-6 in carrageenan induced rat paw edema. Data are expressed in terms of mean ± SEM, n=6. *P< 0.05, **P < 0.01 and ***P < 0.001, compared to the negative control group, and # P < 0.05, ## P < 0.01 and ### P < 0.001, compared to the vehicle control group by one-way ANOVA followed by Dunnett’s test. NC, normal control; DC, Disease control SA 25, syringic acid (25mg/kg); SA 50, syringic acid (50mg/kg); Indo 1, Indomethacin (1mg/kg); TNF-α, tumour necrosis factor-alpha; IL-6, interleukin-6;](image-url)
Freund’s complete adjuvant induced arthritis in rats

*Effect of SA on paw volume and arthritis index*

FCA caused progressive increase in the paw oedema from the baseline to day 14. SA (25 and 50 mg/kg) demonstrated a significant ($P < 0.05$) decrease in the paw volume compared to the disease control rats. Indomethacin-treated rats elicited significant ($P < 0.01$) reduction in the paw volume (Fig. 2). Rats in the disease control group had the highest arthritic index amongst all the groups. SA (50 mg/kg) and indomethacin significantly suppressed the arthritis index of the rats from day 0 to day 14 ($P < 0.05$). However, SA (25 mg/kg) showed significant ($P < 0.01$) reduction in the arthritis index only on day 10 (Fig. 3).

![Effect of SA on the paw volume in FCA induced arthritis in rats. Data are expressed in terms of mean ± SEM, n=6. *$P<0.05$, **$P<0.01$ and ***$P<0.001$, compared to the negative control group, and # $P<0.05$, ## $P<0.01$ and ### $P<0.001$, compared to the vehicle control group by one-way ANOVA followed by Dunnett’s test. NC, normal control; DC, Disease control SA 25, syringic acid (25mg/kg); SA 50, syringic acid (50mg/kg); Indo 1, Indomethacin (1mg/kg);](image-url)
Fig. 3: Effect of SA on arthritis index in FCA induced arthritis in rats. Indo 1, indomethacin (1mg/kg); Data are expressed in terms of mean ± SEM, n=6. *P < 0.05, **P < 0.01 and ***P < 0.001, compared to the disease control group by one way-ANOVA, followed by Dunnett’s test. DC, disease control; SA 25, syringic acid (25mg/kg); SA 50, syringic acid (50mg/kg).

**Effect of SA on motility test and staircase climbing test**
SA at both the tested doses and indomethacin did not show any significant change in the motility scores of the rats, in comparison with the disease control group (Fig. 4a). SA (50 mg/kg) and indomethacin significantly (P < 0.05) improved the staircase climbing ability of the animals on day 7,10 and 14, when compared to the disease control group (Fig. 4b).

Fig. 4: Effect of SA on a) motility score and b) staircase climbing score. Data are illustrated in the form of box-plots, where the bold line at the centre represents the median values (n=6) and boxes denote the interquartile ranges (25th & 75th percentile). *P < 0.05 in comparison with the disease control group by Kruskal-Wallis test. DC,
disease control; SA 25, syringic acid (25mg/kg); SA 50, syringic acid (50mg/kg); Indo 1, indomethacin (1mg/kg).

**Effect of SA on thymus index and spleen index**

The thymus and spleen indices of the disease control group were significantly increased when compared to the normal control group ($P < 0.01$). SA (50 mg/kg) and indomethacin significantly lowered the thymus index of the arthritic rats ($P < 0.001$). The spleen index was significantly decreased in SA (50 mg/kg) and indomethacin group when compared to the disease control group ($P < 0.05$). SA (25 mg/kg) did not cause a statistically significant decrease in the thymus index and spleen index of the rats (Table 2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thymus index</th>
<th>Spleen index</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>0.42±0.0164</td>
<td>2.709±0.1453</td>
</tr>
<tr>
<td>DC</td>
<td>0.60±0.0208***</td>
<td>3.507±0.1106&quot;&quot;</td>
</tr>
<tr>
<td>SA 25</td>
<td>0.55±0.0171</td>
<td>3.09±0.1114</td>
</tr>
<tr>
<td>SA 50</td>
<td>0.47±0.0234***</td>
<td>2.858±0.2587&quot;&quot;</td>
</tr>
<tr>
<td>Indo 1</td>
<td>0.48±0.0009***</td>
<td>2.771±0.0997&quot;&quot;</td>
</tr>
</tbody>
</table>

Data are expressed in terms of mean ± SEM, $n=6$. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to the disease control group and #*$P < 0.05$, ###$P < 0.01$ and ####$P < 0.001$ compared to the normal control group by one-way ANOVA followed by Dunnett’s test. NC, normal control; DC, disease control; SA 25, syringic acid (25 mg/kg); SA 50, syringic acid (50 mg/kg); Indo 1, indomethacin (1 mg/kg);

**Effect of SA on haematological parameters**

Rats in the disease control group showed a significant decrease in the Hb levels and RBC count and an increase in the WBC count and ESR when compared to the normal control group ($P < 0.01$). Rats treated with SA (25 and 50 mg/kg) and indomethacin exhibited a significant decrease in the WBC count and ESR when compared to the disease control group ($P < 0.01$). Indomethacin treated rats showed a significant increase in Hb when compared to the disease control group ($P < 0.001$). None of the treatment groups showed any statistically significant differences in the RBC count (Table 3).
Table (3): Effects of SA on haematological parameters in FCA induced arthritic rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hb (g/dl)</th>
<th>RBC (millions/mm³)</th>
<th>WBC (thousand/mm³)</th>
<th>ESR (mm/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>20.98±0.3038</td>
<td>5.482±0.288</td>
<td>8.983±0.325</td>
<td>3.667±0.458</td>
</tr>
<tr>
<td>DC</td>
<td>7.667±0.2435***</td>
<td>3.513±0.2781***</td>
<td>16.44±0.2915***</td>
<td>9.5±0.4014***</td>
</tr>
<tr>
<td>SA 25</td>
<td>9.167±0.5</td>
<td>3.497±0.2756</td>
<td>10.86±0.331***</td>
<td>5.667±0.5426**</td>
</tr>
<tr>
<td>SA 50</td>
<td>8.667±0.3651</td>
<td>4.075±0.3401</td>
<td>12.73±0.5081**</td>
<td>7.333±0.4014</td>
</tr>
<tr>
<td>Indo 1</td>
<td>16.42±0.5123***</td>
<td>4.738±0.4136</td>
<td>10.35±0.2638**</td>
<td>4.667±0.3073***</td>
</tr>
</tbody>
</table>

Data are expressed in terms of mean ± SEM, n=6. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the disease control group and #P < 0.05, ##P < 0.01 and ###P < 0.001 compared to the normal control group by one-way ANOVA followed by Dunnett’s test. NC, normal control; DC, disease control; SA 25, syringic acid (25mg/kg); SA 50, syringic acid (50mg/kg); Indo 1, indomethacin (1mg/kg); Hb, haemoglobin; RBC, red blood cells; WBC, white blood cells; ESR, erythrocyte sedimentation rate.

Biochemical estimations in the liver and rat paw tissue homogenate

SOD, CAT, GSH, MDA and NO were evaluated in the liver and paw tissue homogenate (Table 4 and Table 5 respectively). In both the tissue homogenates, the levels of SOD, CAT and GSH were found to be significantly (P < 0.001) reduced, while level of MDA and NO was noted to increase significantly (P < 0.001) in the disease control group compared to the normal control group. Treatment with SA (25 and 50 mg/kg) and indomethacin significantly (P < 0.05) increased GSH, SOD and CAT while it reduced MDA and NO. Thus, SA exerted potential anti-oxidant effect.

Table (4): Effects of SA on the oxidative stress in the liver tissue of FCA induced arthritic rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SOD (U/ml/mg protein)</th>
<th>CAT (U/ml/mg protein)</th>
<th>GSH (µg/ml/mg protein)</th>
<th>MDA (nMol/ml/mg protein)</th>
<th>NO (µMol/ml/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>26.33±1.282</td>
<td>183.5±1.533</td>
<td>32 ±1.065</td>
<td>0.189±0.004</td>
<td>8.833±0.7923</td>
</tr>
<tr>
<td>DC</td>
<td>6.167±0.6009***</td>
<td>90±1.653***</td>
<td>9.333±1.358***</td>
<td>1.290±0.028***</td>
<td>3.383±1.905***</td>
</tr>
<tr>
<td>SA 25</td>
<td>7.667±0.5578</td>
<td>98.67±2.679</td>
<td>12.83±1.470</td>
<td>0.9077±0.1646</td>
<td>27.67±1.764</td>
</tr>
<tr>
<td>SA 50</td>
<td>18±0.9309**</td>
<td>146.1±1.461**</td>
<td>24.86±1.70**</td>
<td>0.5213±0.061**</td>
<td>18±2.082**</td>
</tr>
<tr>
<td>Indo 1</td>
<td>20.33±1.687***</td>
<td>153.3±2.186**</td>
<td>26.67±1.256**</td>
<td>0.4217±0.0553**</td>
<td>15.33±1.764**</td>
</tr>
</tbody>
</table>

Data are expressed in terms of mean ± SEM, n=6. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the disease control group and #P < 0.05, ##P < 0.01 and ###P < 0.001 compared to the normal control group by one-way ANOVA followed by Dunnett’s test. NC, normal
control; DC, disease control; SA 25, syringic acid (25mg/kg); SA 50, syringic acid (50mg/kg); Indo 1, indomethacin (1mg/kg); SOD, superoxide dismutase; CAT, catalase; GSH, glutathione reductase; MDA, malondialdehyde; NO, nitric oxide.

Table (5): Effects of SA on the oxidative stress in the paw tissue of FCA induced arthritic rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SOD (U/ml/mg protein)</th>
<th>CAT (U/ml/mg protein)</th>
<th>GSH (µg/ml/mg protein)</th>
<th>MDA (nMol/ml/mg protein)</th>
<th>NO (µMol/ml/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>32.33±1.726</td>
<td>206.3±4.185</td>
<td>24±2.129</td>
<td>0.152±0.0126</td>
<td>11.83±0.9058</td>
</tr>
<tr>
<td>DC</td>
<td>9.667±1.282***</td>
<td>105.5±6.454***</td>
<td>6±1.065***</td>
<td>1.453±0.0315***</td>
<td>23.17±1.701***</td>
</tr>
<tr>
<td>SA 25</td>
<td>8±1.291</td>
<td>126.2±4.700</td>
<td>9±1.528</td>
<td>1.253±0.049**</td>
<td>21.83±1.302</td>
</tr>
<tr>
<td>SA 50</td>
<td>27.17±1.108***</td>
<td>159.5±2.884***</td>
<td>16.83±1.515***</td>
<td>0.550±0.048***</td>
<td>16.5±1.057**</td>
</tr>
<tr>
<td>Indo 1</td>
<td>30.17±1.869***</td>
<td>172±3.679***</td>
<td>17.5±1.705***</td>
<td>0.433±0.053***</td>
<td>13±1.528***</td>
</tr>
</tbody>
</table>

Data are expressed in terms of mean ± SEM, n=6. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the disease control group and #P < 0.05, ##P < 0.01 and ###P < 0.001 compared to the normal control group by one-way ANOVA followed by Dunnett’s test. NC, normal control; DC, disease control; SA 25, syringic acid (25mg/kg); SA 50, syringic acid (50mg/kg); Indo 1, indomethacin (1mg/kg); SOD, superoxide dismutase; CAT, catalase; GSH, glutathione reductase; MDA, malondialdehyde; NO, nitric oxide.

Cytokine levels in the serum

FCA caused a significant increase in the levels of pro-inflammatory cytokines namely, TNF-α and IL-6 in the serum of disease control rats when compared to the rats in the normal control group (P < 0.01). SA (50 mg/kg) significantly (P < 0.001) decreased serum TNF-α and IL-6. Indomethacin significantly decreased serum TNF-α and IL-6 when compared to the disease control group (P < 0.001). SA (25mg/kg) did not cause any statistically significant reduction in the serum cytokine levels of arthritic rats (Fig. 5a, 5b).
Fig. 5: Effect of SA on serum inflammatory cytokines a) TNF-α and b) IL-6 in FCA induced arthritis in rats. NC, normal control; TNF-α, tumour necrosis factor-alpha; IL-6, interleukin-6; Data are expressed in terms of mean ± SEM, n=6. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the disease control group and #P < 0.05, ##P < 0.01 and ###P < 0.001, compared to the normal control by one-way ANOVA followed by Dunnett’s test. DC, disease control; SA 25, syringic acid (25mg/kg); SA 50, syringic acid (50mg/kg); Indo 1, indomethacin (1mg/kg).

**Histopathological examinations**

The representative images of the haematoxylin-eosin stained paw of the rats in the different groups is shown in Fig. 6. The histopathological examination of the paw from the normal control group showed normal histoarchitecture with no remarkable inflammation or degenerative changes (Fig. 6a). Macroscopic and histopathological observations of the rat paw tissue of the disease control group indicated severe infiltration of inflammatory cells, damage of cartilage and bone damage (Fig. 6b). Daily administration of SA (25 and 50 mg/kg) and indomethacin for 14 days showed a remarkable reduction in the inflammatory cell infiltration, cartilage damage and bone damage of the rat paw tissue when compared to the disease control group (Fig. 6c-6e).
DISCUSSION

Currently available drugs for the management of RA have limitations with respect to safety and cost. Thus, there is a need to develop drugs which are not only efficacious but also well-tolerated and cost-effective.\cite{20} SA is widely present in commonly consumed fruits and vegetables.\cite{21} SA has been investigated for the anti-inflammatory and anti-arthritis activity in carrageenan induced rat paw oedema and FCA-induced arthritis in rats, respectively. SA showed effects in both the models by reducing the paw volume, paw thickness and serum pro-inflammatory cytokines. SA also reduced the thymus and spleen indices, improved the haematological parameters, staircase climbing ability and histopathology of the paw in arthritic rats. SA could reduce the oxidative stress observed in the condition of rheumatoid arthritis.

The development of carrageenan induced paw oedema takes place in two major stages. The primary stage known as the acute phase (lasts for the initial 2 hours) is marked by the production and release of pro-inflammatory mediators like histamine, bradykinin, serotonin and platelet activating factor. The secondary phase (that takes place between 2 to 5 hours) is
characterized by infiltrating cells like neutrophils, release of prostanoids, protease, lysozyme and nitric oxide.\textsuperscript{[22-23]} SA showed a significant decrease in the paw volume and paw thickness after 3 hrs of carrageenan administration. Thus, SA exerts its effect on the secondary phase of carrageenan induced paw oedema.

FCA induced arthritis consists of two stages: the first and the second stage. The first stage is the inflammatory phase that involves the production of prostaglandins while the second stage is the immunological phase that generates auto-antibodies.\textsuperscript{[24]} SA exerted beneficial effects in both the stages of FCA-induced arthritis. The anti-inflammatory effect of SA as demonstrated in the carrageenan-induced paw oedema was corroborated in FCA induced arthritis in rats.

TNF-\(\alpha\) and IL-6 act as important mediators in RA.\textsuperscript{[25]} TNF-\(\alpha\) activates leukocytes, synovial fibroblast and induces matrix-metalloproteinases (MMPs), granulocyte-macrophage colony stimulating factor (GM-CSF) and collagenase proliferation. TNF-\(\alpha\) also inhibits the activity of regulatory T cells, while stimulating the differentiation and proliferation of B-lymphocytes, T-lymphocytes and natural killer cells and thereby contributes to the development of RA. IL-6 stimulates the reactive oxygen intermediates and the release of MMPs and other proteolytic enzymes. In synergism with TNF-\(\alpha\), IL-6 causes the production of vascular endothelial growth factor (VEGF), which plays a key role in the development of pannus.\textsuperscript{[26]} SA decreased the concentrations of TNF-\(\alpha\) and IL-6 in the serum of rats. This effect may be attributed to the downregulation of the expression of the pro-inflammatory gene called nuclear factor kappa B.\textsuperscript{[27]}

Hyperalgesia is one of the predominant features of clinical arthritis.\textsuperscript{[28]} The extent of hyperalgesia was determined by the evaluation of arthritis index, the motility and staircase climbing ability of arthritic rats. SA improved the arthritic index and staircase climbing ability of rats. These results suggest that SA does not only improve the inflammation associated with RA but may also be beneficial in improving the quality of life in patients suffering from RA.

The thymus gland is concerned with the cell-mediated immunity and is the site of T-cell maturation, while the spleen serves as a reservoir for immune cells that aid in the antibody formation.\textsuperscript{[29]} FCA causes an increase in the spleen and thymus indices. In our study, FCA caused a significant increase in the thymus index and spleen index in the rats in the disease control group which is consistent with the literature. SA caused a significant decrease in both
the thymus and spleen indices. The results highlight that SA may possess immunomodulatory effect which may be beneficial in RA.

Inflammatory conditions are characterized by an influx of leucocytes. Consequently, the WBC count and ESR were significantly increased in the rats of the disease control group when compared to the normal control group. SA by virtue of its anti-inflammatory effects could reduce the influx of leucocytes and thus there was a decrease in the WBC count and ESR in the rats treated with SA. Studies have revealed that the reduced Hb and RBC count in arthritic condition is due to the decreased erythropoietin levels, which is associated with the suppressed response of bone marrow erythropoietin and damage to the premature RBCs. SA did not have any significant effects on RBC count and Hb in arthritic rats.

Generation of oxidative stress serves as an essential feature in the induction of rheumatoid arthritis. Destruction of the joint structure occurs due to the downregulation of the antioxidant defence system, thus producing free radicals in the arthritic rats. Administration of SA led to the restoration of the free radical scavenging ability via the upregulation of SOD, CAT and GSH in the liver and rat paw tissue. The oxidative stress was predominantly noted by the marked increase in the MDA and NO activities in the disease control group. SA restored the levels of MDA and NO in both the liver and paw tissues. Hence, SA may protect the biological membranes from the oxidative damage by exerting a stabilizing effect.

The results of the histopathological examination of the paw of rats concurred with the biochemical changes. SA reduced the infiltration of inflammatory cells in the paw as well as improved the bone and cartilage damage. SA was found to be safe when administered at a dose of 2000 mg/kg body weight of rats. SA may thus be beneficial for the management of RA owing to its efficacy and safety.

CONCLUSION

Our study revealed that SA exerted significant anti-inflammatory and anti-arthritic activity in rats. It reduced the paw volume and paw thickness as well as reduced the concentration of pro-inflammatory cytokines. It reduced the arthritis index and improved the staircase climbing ability of rats and exerted antioxidant effects. It also improved the histology of the paw. Moreover, SA was well-tolerated in our study. Future studies could focus on
deciphering the molecular mechanisms responsible for the anti-arthritic effects of SA such as its effects on MMPs, collagenase and signalling pathways.

DECLARATIONS

Conflict of Interest

The authors have no conflict of interest to declare.

ACKNOWLEDGEMENT

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. The authors are grateful for the financial assistance provide by the All India Council for Technical Education (AICTE), New Delhi, India. The authors wish to thank Ms. Revati Dhayfule, Ms. Jyoti Batgire and Dr. Amrita Chowdhury for their valuable contribution to this study.

REFERENCES


33. Kothavade PS, Bulani VD, Deshpande PS, Chowdhury AS, Juekar AR. The petroleum ether fraction of Celastrus paniculatus Willd. seeds demonstrates anti-arthritic effect in

34. Sevgi K, Tepe B, Sarikurkcü C. Antioxidant and DNA damage protection potentials of selected phenolic acids. Food and Chemical Toxicology, 2015; 77: 12-21.