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ANTI-INFLAMMATORY ACTIVITY OF *CROCUS SATIVUS* EXTRACT IN EXPERIMENTAL ARTHRITIS

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ABSTRACT: Rheumatoid arthritis is one of the most serious medical problems leading to bone and cartilage destruction. The disease affects approximately 1% of the total world population. Mostly the women are affected. The pathogenesis of the disease is still not clearly understood. Free radicals and inflammatory molecules such as TNF-α, IL-1β etc have been recognized as major players in the progression of disease. Several herbal preparations have been reported to combat free radicals and relieve the sufferers of diseases such diabetes, atherosclerosis, asthma etc. *Crocus sativus* is considered as an anticonvulsant, antidepressant, antispasmodic and diaphoretic agent. Therefore, we planned this study to evaluate anti-inflammatory and antioxidant property of *Crocus sativus* extract (CSE) towards adjuvant induced arthritis. Arthritis was induced in mice by injecting Freund's complete adjuvant. Three different doses of *Crocus sativus* extract (25, 50, 100mg/kg b.w.) were orally administered to the adjuvant induced arthritic mice for 47 days. We observed significant ($p<0.05$) reduction in TNF-α and IL-1β levels in the mice of CSE-2 and 3 group as compared to arthritic mice, while non-significant change was observed in the CSE-1 group mice. We also recorded a significant ($p<0.05$) increase in SOD and GR activity in the mice of CSE-2 and 3 group as compared to arthritic mice, while non-significant change was observed in the CSE-1 group mice. We can conclude that CSE is effective in modulating the pro-inflammatory molecules and a good scavenger of free radicals, and hence was capable to reduce the inflammation and oxidative stress during disease.

INTRODUCTION: Rheumatoid arthritis (RA) is long lasting, systemic autoimmune disorder characterized by inflammation of the synovial joints and concomitant destruction of cartilage and bone [1]. It is generalized disease in which joint symptoms are merely the most prominent feature. About 1% of the world's population is affected by this disease, in which women are three times more affected than men.

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The disease onset is frequent between the ages of 40 and 70, but can influence people of any age [2]. Inflammation is central to progression of RA. Many inflammatory cytokines have been recognized to play pivotal role in the pathogenesis of the disease [3]. TNF-α and IL-1β are main cytokines which are abundant in an inflamed synovium [4]. Dysregulated expression of TNF-α in experimental arthritis has been reported to cause destructive arthritis [5].

The development of disease is markedly suppressed in IL-1β deficient experimentally induced arthritis [6]. These studies suggest the role of pro-inflammatory cytokines in arthritis and therefore can be targetted for therapeutical potential. Various

studies have been conducted and explored the strategies to treat arthritis include the blocking of TNF with high affinity antibodies [7, 8].

Several treatments are prescribed for RA. In recent years, several researchers have proved the possible role of reactive oxygen species (free radicals) produced during metabolic processes, in pathogenesis of RA [9, 10]. Further, many workers have reported the disturbed antioxidant levels during RA, and have proposed that antioxidants exert synergistic action in scavenging free radicals [11, 12]. Current treatment modalities for RA are mostly symptomatic. Although number of remedies like disease-modifying antirheumatic drugs (DMARDs) are present to limit the degree of irreversible joint damage [13] for a better health status of RA patients.

But the value of DMARDs for treating RA is limited by their side effects. The major side effects of NSAIDs are their propensity to cause stomach ulcers, GI bleeding and perforations [14]. So, there is still a need to look for therapeutic agents with lower side effects that can be used for long-term administration. In last decade, there has been increase in the popularity of alternative medicine in clinical practice [15]. Herbal preparations are mostly used therapy among alternative medicine for RA [16]. *Crocus sativus* L. *(Family*: *Iridaceae)* commonly known as saffron, is a perennial stemless herb which is cultivated in several countries like India, Iran and Greece.

In India, it is mainly cultivated in Kashmir an Uttaranchal. The dried red stigmas of the flower are mainly used for therapeutical purposes such as anticonvulsant, antidepressant and antitumor activities [17, 18, 19]. The stigmas of the plant are mainly used for therapeutic purposes. Saffron extract has also been shown to have protective effects on genotoxin induced oxidative stress in swiss albino mice [20]. We have taken up this study to assess the anti-inflammatory effect of saffron extract in adjuvant induced arthritic mice.

MATERIALS AND METHODS:

Preparation of *Crocus sativus* extract:

Crocus sativus L. stigmas were procured from Sigma, USA (Lot#031 M1486V). 4.0 gm. of stigmas were macerated in 70% ethanol for three days. The mixture was then filtered and collected. The solution thus obtained was lyophilized. As a result, the extract yield was 48.2%. The dried powder was then reconstituted to get desired concentration for experimentation i.e, 25, 50 and 100 mg/kg body weight.

Animals:

The experiment was carried out with female Swiss albino mice (12-14 weeks old) weighing 28-30 g. These animals were procured from NABL-compliant Institutional Animal facilities (Indian Institute of Toxicological Research, Lucknow) and maintained in the CPCSEA approved animal house of Era's Medical College and Hospital, Lucknow. The animals were kept in hygienic conditions and fed on the pellet diet (Lipton, India) and water *ad libitum*. Prior permission for the experiment was sought from the Institutional Animal Ethics Committee.

Induction of arthritis:

10 μL Freund's Complete Adjuvant (FCA) (Sigma, USA, Batch#SLBD8147V) containing heat killed Mycobacterium tuberculosis (H37Ra, ATCC, 25177) was injected in the sub planter surface of the right hind paw of mice to induce arthritis. A booster dose of 10μL FCA was given to animals in sub planter surface of the same hind paw on 12th day. Thus, adjuvant induced arthritis animals were prepared.

Treatment schedule:

All the animals were divided into 6 groups of 6 animals each. The group 1 comprised of normal mice, group 2 comprised of arthritic mice receiving distilled water, group 3, 4 and 5 comprised arthritic mice receiving *crocus sativus* extract (CSE) (25, 50, 100 mg/kg b.w.) daily till day 47. The *crocus sativus* extracts were administered orally. Group 6 comprise of arthritic mice receiving acetylsalicylic acid (ASA) (200 mg/kg bw). ASA is standard drug against arthritis. Daily oral dose was administered to animals using commercially available canula.

Blood collection:

Animals from each group were used for drawing blood on day 47. Blood was drawn from the retro-orbital sinus using capillary tube and divided in two

sterile tubes, one containing EDTA to obtain plasma and other was plain to obtain serum. Whole blood was kept at room temperature for 2 h. Serum was collected as supernatant after centrifugation at 2500 rpm for 5 minutes.

RBC Lysate preparation:
Plasma was separated from the blood collected in EDTA vials, by centrifugation at 2500 RPM for 10 minutes at room temperature. Plasma was then transferred to separate sterile tubes for biochemical analysis. The RBC pellet was found intact in the bottom of tube, which was then washed twice with normal saline to remove buffy coat. Further, chilled distilled water was added equal to the amount of plasma separated. Thereafter, it was centrifuged at 8000-10,000 rpm for 20 minutes and the supernatant (lysate) was collected and the pellet (cell debris) was discarded.

Preparation of Joint Homogenate:
Inflammatory site on the joints ranging from 4-5mm was dissected out, weighed and a 10% homogenate was prepared in ice cold phosphate buffered saline (PBS) containing 0.5% Tween-20. The homogenates was centrifuged at 2000g for 10 min and the supernatant was filtered using 0.2 μM Millipore filters and used for cytokine assay.

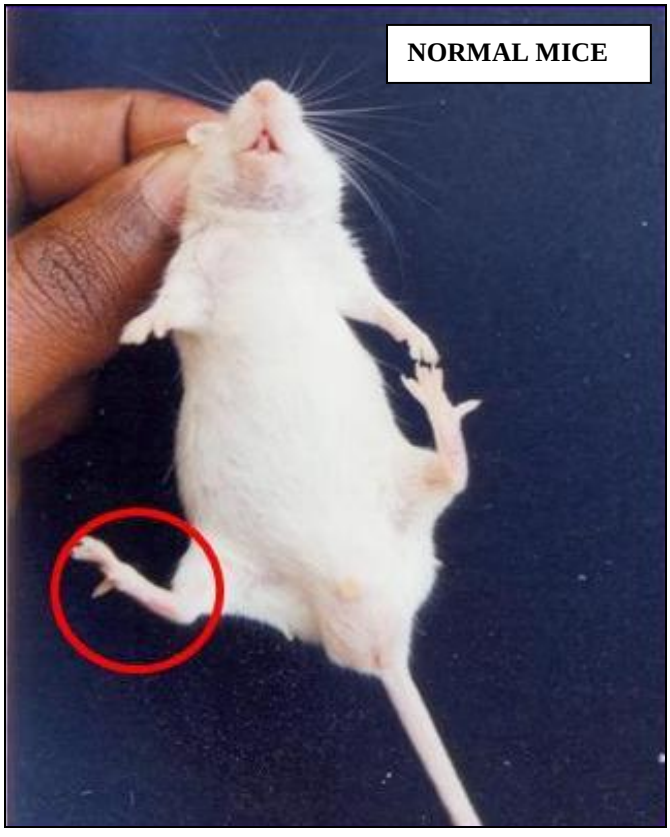
Quantitative determination of pro-inflammatory cytokines:
ELISA based cytokine analysis kits were procured from RayBiotech, Inc. USA (Lot#1004130408), and TNF-α and IL-1β were estimated, in the joint homogenates, using solid phase sandwich ELISA. The protocols laid in the technical bulletin of the manufacturers were followed. Plates were read on iMark microplate reader (Bio-Rad, CA).

Quantitative determination of biochemical parameters and antioxidant activity:
Total protein and uric acid levels were measured in the serum with the help of fully automated Biochemical Autoanalyser EM360 (Transasia, Germany) using Erba Manheim Diagnostic reagents. Technical bulletin supplied with the kit were followed. Superoxide dismutase [21] and Glutathione Reductase [22] activity were measured following the standard methodology.

Statistical Analysis:
Student 't' test was performed to evaluate the significance of the difference in the mean values of extract treated and untreated group. $P<0.05$ was considered as significant.

RESULTS AND DISCUSSION:
On day 2, we observed swelling in the paw of animals at the site of FCA injection. Further after the booster dose of FCA, we recorded a significant swelling and inflammation in the paw of animals on day 14th. The edema persists till day 47 as evidenced in figure 1. Oral administration of different doses of CSE (25, 50, 100 mg/kg b.w.) was started on day 1 in respective animal groups and continued till day 47. Following the oral dose of CSE, we observed a visible reduction in inflammation in the animals.

On day 47, we observed higher degree of swelling, redness and erythma in AIA group animals, while decreased swelling was recorded in CSE treated animals. Further, we collected the blood from all animals for biochemical and antioxidant enzyme estimation. The joints were surgically removed and processed to obtain joint homogenate.



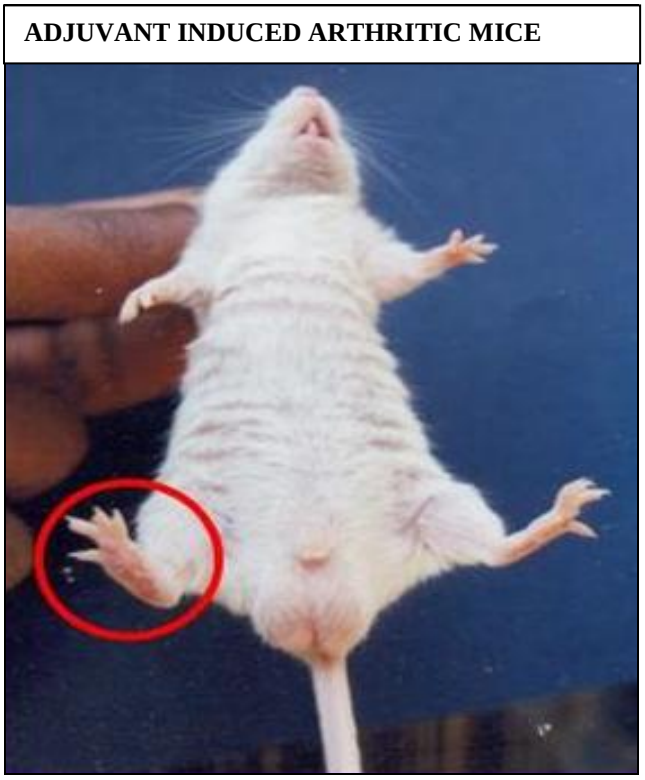


FIGURE 1: COMPARISON OF INFLAMMATION IN MICE PAW ON DAY 47, FOLLOWING FCA INJECTION

Figure 2. illustrates the results of TNF-α assay in joint homogenate of different study group animals. Cytokine assay in joint homogenate revealed significant (P<0.05) increase in TNF-α in adjuvant induced arthritic (AIA) (734.16 ± 109.66 pg/ml) mice as compared to the normal mice (257.66 ± 41.58 pg/ml).

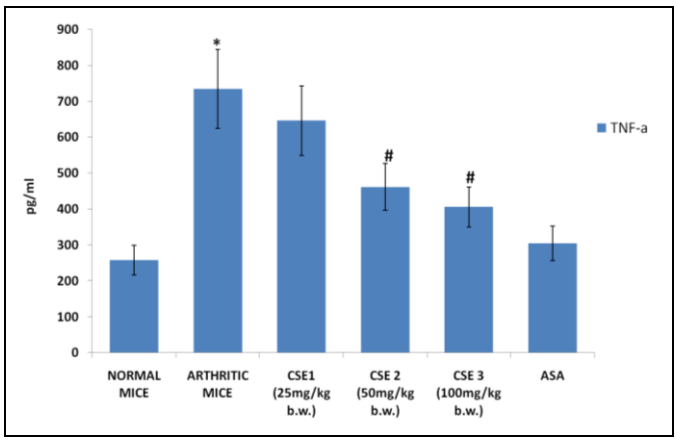


FIGURE 2: EFFECT OF *CROCUS SATIVUS* EXTRACTS ON TNF-α levels IN AIA MICE
* significant at p value <0.05 as compared to control
# significant at p value <0.05 as compared to AIA mice

The values are expressed as mean ± SD. Decrease in TNF-α levels was noted in all the CSE treated animal group. On one hand, non-significant decrease was noted in CSE-1 animals (645.66 ± 96.68 pg/ml), but a significant (P<0.05) decrease was noted in CSE-2 and 3 animal group (461.66 ± 65.31 and 405.66 ± 55.89pg/ml) as compared to AIA mice. Analysis of TNF-α levels among CSE treated animals and ASA treated animals reveal that the decrease in TNF-α levels by CSE3 is comparable to standard drug ASA.

Figure 3. illustrates the results of IL-1β assay in joint homogenate of different study group animals. IL-1β levels in joint homogenate of normal mice was recorded as 144.16 ± 49.74 pg/ml and in AIA mice as 463.33 ± 76.54 pg/ml. The values are expressed as mean ± SD. This remarkable increase in IL-1β levels is statistically significant (P<0.05) in AIA mice as compared to normal mice. Decrease in IL-1β levels was recorded in all the CSE treated animal groups. While, non-significant decrease was noted in CSE-1 animals (451.83 ± 43.73 pg/ml), a significant (P<0.05) decrease was noted in CSE-2 and 3 animal group (335.50 ± 49.19 and 293.66 ± 39.13pg/ml) as compared to AIA mice. Decrease in IL-1β levels by CSE3 administration is comparable to standard drug ASA.

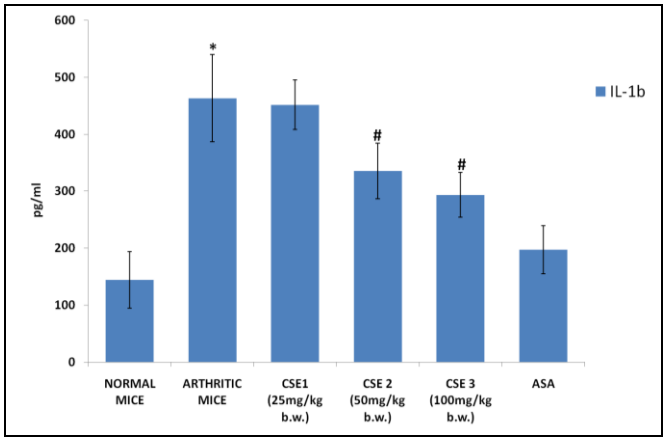


FIGURE 3: EFFECT OF *CROCUS SATIVUS* EXTRACTS ON IL-1β levels IN AIA MICE
* significant at p value <0.05 as compared to control
# significant at p value <0.05 as compared to AIA mice

Biochemical and antioxidant enzyme parameters are described in **Table 1**. The values are expressed as mean ± SD. On one hand, non-significant changes were recorded in protein levels in AIA mice as compared to normal mice, while on the other hand non-significant change was also recorded in CSE and ASA treated animal groups as

compared to AIA animals. On day 47, uric acid levels were found significantly (P<0.05) elevated in AIA mice as compared to normal mice. Among the CSE treated animal groups the significant (P<0.05) decrease was recorded in CSE2 and 3 group. Maximum decrease was recorded in CSE3 group animals. ASA treatment tried to lower the uric acid levels in AIA mice.

We also estimated the antioxidant enzyme activities and observed significant (P<0.05) decrease in glutathione reductase (GR) activity in AIA mice as compared to normal mice. Following the CSE treatment for 47 days, maximum improvement/increase in GR activity was recorded in CSE3 group animals as compared to AIA animals, this change was statistically significant (P<0.05). Significantly (P<0.05) decreased superoxide dismutase (SOD) activity was recorded in AIA mice as compared to normal mice. CSE treatment for 47 days tried to elevate the SOD activity in AIA mice. The maximum effect was recorded in CSE2 animal group.

Results of present study suggest that the saffron extracts have an overall protective effect against inflammation and oxidative damage. Various processes have been implicated in the pathogenesis of RA such as overproduction of pro-inflammatory cytokines [23, 24], increased production of reactive oxygen species and decreased antioxidant enzymes [25, 26]. We assessed the effect of saffron extract by studying its effect on pro-inflammatory cytokines and antioxidant enzymes. Under chronic disease condition, the balance between pro-inflammatory and anti-inflammatory cytokines, and balance between oxidant and antioxidant enzymes is disturbed [27].

Anti-inflammatory and antioxidant properties of saffron extract may be due to presence of various active constituents such as crocin, crocetin, safranal, flavonoids [28, 29, 30] etc. all these active constituents may have been quenching free radicals and exert protective effect against oxidative damage. The anti-inflammatory property of saffron extract may be attributed to synergistic action of these active constituents[31]. In this study, we can conclude that daily oral administration of saffron extract is capable of slowing the progression of RA.

TABLE 1: EFFECT OF *CROCUS SATIVUS* EXTRACTS ON BIOCHEMICAL AND ANTIOXIDANT PARAMETERS IN AIA MICE

	Control mice	AIA mice	CSE 1 treated AIA mice	CSE 2 treated AIA mice	CSE 3 treated AIA mice	ASA treated AIA mice
Protein (g/dl)	6.56 ± 0.71	6.85 ± 0.54	5.85 ±0.54	5.65 ± 0.75	5.98 ± 0.18	6.36 ± 0.67
Uric acid (mg/dl)	4.41 ± 1.08	8.06 ± 1.23*	7.48 ± 0.72	6.83 ± 0.61#	6.31 ± 0.66#	5.96 ± 0.31#
Glutathione reductase (nmole NADPH oxidised/min/mg protein)	55.29 ±4.36	35.78 ± 3.86*	40.07±6.80	44.01 ±7.54#	47.60 ±8.73#	52.21±11.78#
Superoxide dismutase (nmol of NBT reduced/min/mg protein)	36.10 ±2.78	24.03 ± 5.48*	29.04±4.87*	31.29± 4.13#	33.79 ±3.20#	34.77 ± 4.51#

The values are expressed as mean ± SD

* significant at p value <0.05 as compared to control

significant at p value <0.05 as compared to AIA mice

CONCLUSION: In the present study, we have observed that the *crocus sativus* extract is capable of reducing the pro-inflammatory cytokines such as TNF-α and IL-1β in adjuvant induced arthritic mice. This effectiveness of CSE reveals its anti-inflammatory action. On the other hand, CSE resulted in controlling the reduction of antioxidant enzymes such as glutathione reductase and superoxide dismutase in AIA mice. This finding led us to reveal that CSE also exerts antioxidant effect during the disease. We can thus conclude that CSE possess anti-inflammatory as well as antioxidant property due to presence of active constituents. Therefore, we can suggest that saffron may be a safer and effective choice of alternative medicine for the treatment of inflammatory disease such as RA.

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