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ORIGINAL ARTICLE

Hecogenin exhibits anti-arthritic activity in rats through suppression of pro-inflammatory cytokines in Complete Freund's adjuvant-induced arthritis

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ABSTRACT

Hecogenin is a steroidal sapogenin isolated from the leaves of *Agave genus* species that plays an important role in the treatment of a variety of inflammatory diseases. The aim of the present study was to evaluate the anti-arthritic activity of hecogenin in Complete Freund's adjuvant-induced arthritis in rats. The hecogenin (40 µl of 50 µg/kg, orally) and hecogenin + fluticasone (40 µl of 25 µg/kg, each, orally) was tested against Complete Freund's adjuvant-induced arthritis in rats by evaluating various parameters such as paw volume, arthritic score, joint diameter, spleen weight, thymus weight, haematological and biochemical parameters and pro-inflammatory cytokines. Histopathological and radiological analyzes of ankle joints were also carried out. Treatment of rats with hecogenin and its combination elicited significant reduction in paw edema, arthritic score and joint diameter. Hecogenin and its combination also inhibited joint destruction in histopathological and radiological analyzes of ankle joint. Hecogenin and its combination significantly increased the levels of red blood cells and hemoglobin but decreased the white blood cell count. The anti-arthritic activity was also confirmed with the change in biochemical parameters and myeloperoxidase assay. In the present investigation, hecogenin and its combination prevent destruction of cartilage and protect synovial membrane with improving health status through haematonic properties and down regulation of various cytokines. Hence, hecogenin may be a potential therapeutic candidate for the treatment of rheumatoid arthritis.

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Introduction

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune joint disorder characterized by synovial membrane inflammation, hyperplasia, cartilage and functional disability of joints[1]. Nearly 1% of the populace is affected by RA. Inflammatory mediators play an imperative role in the joint damage and inflammation process during the development of RA[2]. In the progression of RA, the risk of morbidity and mortality remains at a higher side in the last decade[3]. Though, the drug treatment of RA have been changed from conventional non-steroidal anti-inflammatory drugs (NSAIDS) including ibuprofen, aceclofenac and naproxen combined with prednisone hormones and or disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate, sulfasalazine and leflunomide to novel biological agents such as decoy TNF-α receptor and tumor necrosis factor-α (TNF-α) antibody[4]. But, these drug treatments are linked to many side effects such as gastrointestinal ulcerogenicity, hematologic toxicity, nephropathy, cardiovascular complication and to associated with increase in the cost of therapy. From various research reports, it has been observed that an imbalance between pro-inflammatory and anti-inflammatory cytokines leads to auto-immunity sensitization and chronic inflammation that to cause synovial membrane inflammation and joint damage[5]. Moreover, the anti-arthritic drugs that inhibits pro-inflammatory markers, such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12) and TNF-α shows severe side effects and restricting their clinical use[6]. Therefore, it is an urgent need to develop a novel, safer, efficient and economical agent for the treatment of RA.

Hecogenin (HG) is a steroidal sapogenin isolated from the leaves of *Agave genus* species such as *Agave sisalana*, *Agave cantala*, and *Agave aurea*[6]. The extracts obtained from these plants have been used for their cardioactive or larvicidal activity[7]. HG was reported to have a broad spectrum of pharmacological activity, including (anti-hypertensive) hypotensive, antifungal and anti-nociceptive effect[8]. It also showed an anti-inflammatory effect on ethanol-induced gastric mucosal inflammation in rats[9]. A research report showed that HG is a selective inhibitor of human UDP-glucuronosyl transferases enzymes responsible for the detoxification of several chemical toxins in the body[10]. HG was also found to have an anti-proliferative activity in human osteosarcoma cells[11]. The documented reports of Cerqueira et al. (2012) showed the effect of HG on oxidative stress, lipid peroxidation and myeloperoxidase, a biomarker of inflammation. Its gastric ulcer protective effect was also confirmed with histological and cyclooxygenase-2 (COX-2) immunohistochemistry studies[12].

Fluticasone (FC) propionate is a topically active corticosteroid molecule[13]. It is highly lipophilic in nature (three-fold

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more lipophilic than beclomethasone dipropionate and 300-fold more lipophilic than budesonide)[14]. FC has been shown to attenuate pulmonary inflammation in laboratory animals and human beings, and inhibit chemotaxis of neutrophil, endothelial cell adhesion molecule expression and cytokine production[15]. FC at high concentrations interact with DNA recognition sites to activate transcription through increased histone acetylation of anti-inflammatory genes and transcription of several genes linked to glucocorticoid side effects (*trans*-activation). Many physicians and health care providers have had a problem with the long term FC treatment because of their well-known adverse effects such as diabetes, metabolic disorders, gastro-intestinal irritation, ulcers, hypertension, glaucoma and bone marrow suppression associated with high doses[16]. FC also has post-transcriptional effects and then decreases stability of some pro-inflammatory mRNAs[17]. Several molecular mechanisms of glucocorticoid resistance have now been identified which involve phosphorylation and other post-translational modifications of GR.

The compounds isolated from herbal medicine serve as replacement for a plants extracts that have been studied for long time to establish newer, safer and more effective topical anti-inflammatory drug[18]. Hence, it is of utmost importance to search for less toxic steroidal drugs from plant origin (phytosteroids) that have been known for their anti-inflammatory effects[19]. Therefore, the present study was undertaken to evaluate the anti-arthritic effect of HG alone and in combination with FC. Further efforts were also made to elucidate the potential mechanism of HG and HG + FC in adjuvant-induced arthritis in rats.

Material and methods

Drugs and chemicals

HG was purchased from TCI, Hong Kong, China. Complete Freund's adjuvant (CFA), hexadecyltrimethylammonium bromide (HTAB) and *O*-dianisidine hydrochloride were procured from the Sigma-Aldrich Chemicals, St. Louis, MO. FC was obtained as a gift sample from Sun Pharma Advanced Research Company Ltd., Baroda, Gujarat, India. Cytokine ELISA kits for estimation of TNF-α, IL-6, IL-12 and thromboxane B_2 (TXB_2) were purchased from Krishgen Biosystem Private Ltd., Mumbai, India. Diagnostic kits for the assay of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were procured from Coral Laboratories, Goa, India. All other reagents and chemicals used in this experiment were of analytical grade and procured from Local suppliers.

Experimental animals

Healthy female Swiss albino mice (20–25 g) and Female Wistar albino rats (150–200 g) were procured from the National Institute of Biosciences (NIBS) and the National Toxicology Centre (NTC), Pune, Maharashtra, India, respectively and maintained in animal house standard conditions: temperature (24 ± 1 °C), relative humidity (45–50%), 12 h light/dark cycle, and fed with standard pellets diet and water *ad libitum*. All animals were acclimatized to animal house environment for at least 7 days prior to the start of experiments. All the experimental procedures and protocols were reviewed and approved by the Institutional Animal Ethics Committee constituted under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (SCOP/IAEC/2014–15/202). Ethical guidelines were strictly followed throughout the experiments.

Acute toxicity study

Acute oral toxicity was carried in female Swiss albino mice as per OECD-425 guidelines. The animals were fasted overnight, provided only with water. The animals were orally administered with a single dose of HG (2000 mg/kg). The mice were continuously observed for behavioral and autonomic parameters for 2 h and for any signs of toxicity or mortality up to 48 h[20].

Complete Freund's adjuvant-induced arthritis

Grouping of animals and induction of arthritis

The animals were divided into five groups ($n = 6$) as follows:

- Group I: Normal Control (NC); 40 µl of 1% Tween-80, Orally
- Group II: Arthritic Control (CFA); 0.1 ml, Intra-dermally
- Group III: Hecogenin (HG); 40 µl of 50 µg/kg, Orally
- Group VI: Fluticasone (FC); 40 µl of 50 µg/kg, Orally
- Group V: Hecogenin + Fluticasone (HG + FC); 40 µl of 25 µg/kg, each, Orally

Arthritis was induced in rats according to the method proposed by Omura et al.[21] and shown in Figure 1. Each rat was injected with 0.1 ml of CFA in to sub-plantar region of left hind paw on Day 1, under light ether anesthesia. Each milliliter consisting of 1 mg/ml *Mycobacterium* tuberculosis (H37Ra) in mineral oil after volume injected. The dosing of all the groups was started from Day 12 once daily, orally. The anti-arthritic activity of HG and it combination were evaluated using change in paw volume, joint diameter on Day 0, 4, 7, 10, 12, 14, 17, 21 and 28 and Arthritic score on Day 0, 4, 7, 14, 17, 21 and 28. On Day 28, blood was withdrawn by retro-orbital puncture for assessment of haematological (RBCs, Hb and WBCs), biochemical parameters (SGOT, SGPT and ALP) and cytokines estimations (TNF-α, IL-6, IL-12 and TXB_2). On Day 28, animals were sacrificed to study the joint histology and myeloperoxidase (MPO) assay. Body weights, paw weight, weight of immune organs (spleen and thymus gland) were taken after 28 days of arthritic study. On Day 28, animals were anesthetized and X-rays of the hind paw joints were taken under mild ether anesthesia for the assessment of bone, cartilage and other structural degenerative changes[21]. The COX-2 mRNA expression of hind paw joints were determined by using reverse-transcriptase polymerase chain reaction (RT-PCR) study.

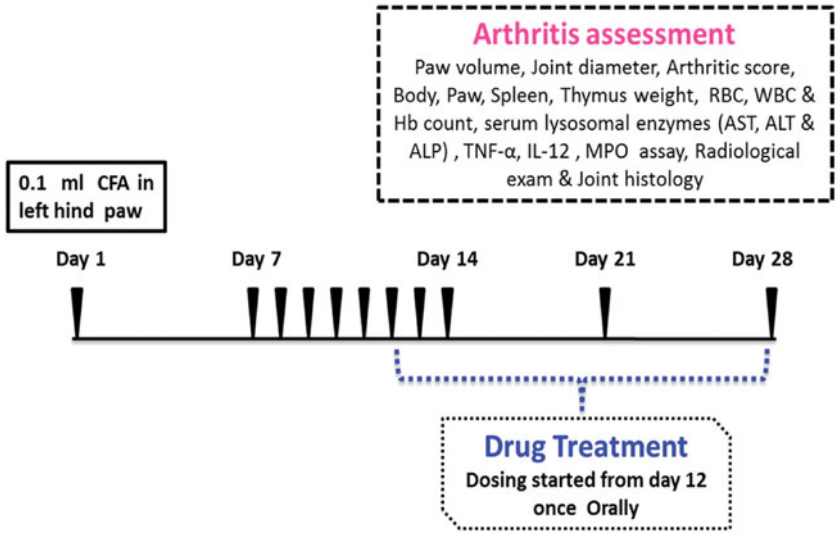

Figure 1. Schematic representation of CFA-induced RA in rats.

Measurement of paw volume

Paw volume was measured using a Plethysmometer (Orchid Scientific, Nashik, Pune, Maharashtra, India) on Day 0 before CFA injections and thereafter on Day 4, 7, 10, 12, 14, 17, 21 and 28. The change in paw volume was calculated as the difference between the final (28 day) and initial (0 day) paw volume[18].

Measurement of arthritic score

The morphological features of arthritis such as redness, swelling and erythema were determined by 5-point ordinal scale (0–4) scoring system as follows: normal paw = 0, mild swelling and erythema of digits = 1, swelling and erythema of the digits = 2, severe swelling and erythema = 3, gross deformity and inability to use the limb = 4 on respective days[22]. Thus, maximum score for both the paws was 8.

Measurement of joint diameter

The joint diameter was measured using a micrometer screw gauge (Vijay Surgicals, Pune, Maharashtra, India) on Day 0 before CFA injections and thereafter on Day 4, 7, 10, 12, 14, 17, 21 and 28. The change in joint diameter was calculated as the difference between the final (on 28 day) and initial (on 0 day) joint diameter[23].

Measurement of haematological parameters

On Day 28, haematological parameters such as red blood cell (RBC) count, hemoglobin (Hb) and white blood cell (WBC) count were determined by usual standardized laboratory method[24].

Measurement of biochemical parameters

On Day 28, serum biochemical parameters such as SGOT, SGPT and ALP levels were measured by standardized laboratory method[24].

Measurement of pro-inflammatory cytokines

The levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-12 and TXB_2) in serum were determined by using readymade ELISA kits (Krishgen Biosystem, Mumbai, India) according to manufacturer instructions.

Measurement of paw weight, spleen and thymus gland indices

On Day 28, rats were sacrificed and paw, thymus and spleen of all the rats were removed and weighed[25]. The thymus index and spleen index were expressed as the ratio (mg/g) of thymus and spleen wet weight versus body weight, respectively[26].

Radiological analysis of ankle joints

On Day 28, rats were anesthetized and radiographs of the ankle joint of hind paws were taken using an X-ray machine. Radiographs were analyzed by a radiologist who was blinded to the treatment groups. Radiographic analysis of ankle joint of hind paws were taken at 55 kV peak, 50 mA with exposure time of 5 s.

Histopathological analysis of ankle joints

The animals were scarified and ankle joints were separated from the hind paw. The joints were immersed in 10% buffered formalin for 24 h followed by decalcification in 5% formic acid, processed for paraffin embedding and sectioned at 5 μ thickness. The sections were stained with Haematoxylin & Eosin and evaluated under a light microscope at 10× magnification for the presence of inflammatory cells, hyperplasia of synovium and destruction of joint space[4].

Measurement of MPO activity

The ankle joint tissue samples were soon after removed and immediately frozen in liquid nitrogen. The frozen tissue sample were weighed, washed twice in phosphate buffer saline,

pH 6.0 at 4–8 °C, homogenized in a solution containing 0.5% HTAB dissolved in 50 mM K_2HPO_4 buffer (pH 6). The samples were then centrifuged at 10,000 rpm for 20 min at 4 °C. The samples were freeze-thawed three times and sonicated for 20 s. An aliquot of the supernatant (0.1 ml) or standard was then allowed to react with 2.9 ml solution of 50 mM K_2HPO_4 buffer at pH 6 containing 0.167 mg/ml of *O*-dianisidine hydrochloride and 0.0005% H_2O_2. After 5 min, the reaction was stopped with 0.1 ml of 1.2 M HCl. The change in absorbance was measured using a spectrophotometer at 460 nm. MPO activity was expressed in milli-unit per gram weight of wet tissue[27].

Determination of COX-2 mRNA expression using RT-PCR

The levels of COX-2 mRNA were analyzed in ankle joint tissue using RT-PCR approach as described previously[28]. Briefly, single-stranded cDNA was synthesized from 5 μg of total cellular RNA using reverse transcriptase (MP Biomedicals India Private Limited, Navi Mumbai, Maharashtra, India) as described previously[29]. The primer sequence for COX-2 was (forward: 5′-AAG ACT TGC CAG GCT GAA CT-3′ and reverse 5′-CTT CTG CAG TCC AGG TTC AA-3′ bp: 322) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was (forward: 5′-GAA CGG GAA GCT TGT CAT CAA-3′, reverse: 5′-CTA AGC AGT TGG TGG TGC AG-3′, bp: 764) provided by MP Biomedicals India Private Limited. GAPDH served as a control for sample loading and integrity. PCR products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml). The size of amplicons was confirmed using a 100-bp ladder as a standard size marker. The amplicons were visualized and images were captured using a gel documentation system (Alpha Innotech Inc., San Leandro, CA).

Statistical analysis

The data were expressed as mean ± standard error of mean (SEM), and statistical comparisons were carried out using analysis of variance (ANOVA). When differences were significant, Tukey's–Kramer *post hoc* test was used for multiple comparisons between groups. The data were analyzed by using Graph Pad Prism 5.0 software. p Values $<.05$ were considered significant.

Results

Acute oral toxicity study

Acute oral toxicity study of the HG in mice did not show any mortality and was found to be safe up to the dose of 2000 mg/kg. Therefore, the dose of 50 μg/mice was selected for further pharmacological study.

Effect of HG and Combination on paw volume

In the primary phase of arthritis (from Day 4 to 12), there was non-significant deceased in the paw volume was observed in CFA-treated rats. There was significant ($p < .001$) increase in the paw volume of the CFA-treated rats was observed as compared to NC rats. HG and Combination

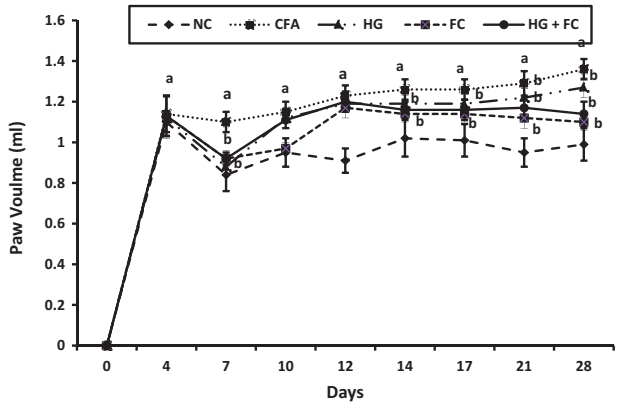

Figure 2. Effect of HG and Combination on paw volume in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. $^{a}p < .001$ represents the significance level compared to NC group; $^{b}p < .001$ compared to CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

treated rats has shown statistically significantly ($p < .001$) decrease in the paw volume from Day 14 to 28 days as compared to CFA group. The rats treated with FC (50 μg/kg) also showed statistically significantly decrease ($p < .001$) in paw volume from Day 14 to 28 as compared to CFA-treated rats (Figure 2).

Effect of HG and Combination on arthritic score

Sub-plantar administration of CFA resulted in significant increase ($p < .001$) in the arthritic score in the CFA-treated rats as compared to NC rats. This increase in the arthritic score showed a biphasic response. There was a significant decrease in the arthritic score from Day 4 to Day 7; however, this change was not statistically significant. The arthritic score of CFA-treated rats seems to be increase from Day 7 to Day 28 as compared to NC rats. Treatment of rats with HG and Combination showed significant decrease in arthritic score ($^{b}p < .05$, $^{c}p < .01$, $^{d}p < .001$, respectively) from Day 12 to Day 28 as compared to the CFA-treated rats. Rats treated with FC (50 μg/kg) showed statistically significant decrease ($p < .001$) in arthritic score from Day 12 to Day 28 as compared to the CFA-treated rats (Figure 3).

Effect of HG and Combination on joint diameter

There was a significant ($p < .001$) increase in the joint diameter of rats treated with CFA as compared to NC rats. The treatment of rats with HG and Combination significantly ($p < .01$ and $p < .001$, respectively) decreased the joint diameter from Day 14 to Day 28 as compared to CFA rats. The change in joint diameter of HG (2.1 ± 0.09) and Combination (1.58 ± 0.04) was observed as compared to CFA rats (3.75 ± 0.22) on Day 28 (Figure 4).

Effect of HG and Combination on haematological parameters

Decreased levels of RBC and Hb and increased levels of WBC were observed in CFA-treated rats as compared to NC rats.

These decreased levels of RBC, WBC and increased Hb levels were significantly ($^{b}p < .001$) elevated with the treatments of rats with HG, Combination and FC also (Table 1).

Effect of HG and Combination on biochemical parameters

The CFA-treated rats showed significant elevation of SGOT, SGPT and ALP levels. Treatment of rats with HG and Combination significantly inhibited the elevation in SGOT, SGPT and ALP levels. Treatment of rats with FC ($p < .05$; $p < .001$) elicited maximum inhibition of SGOT, SGPT and ALP levels (Figure 5).

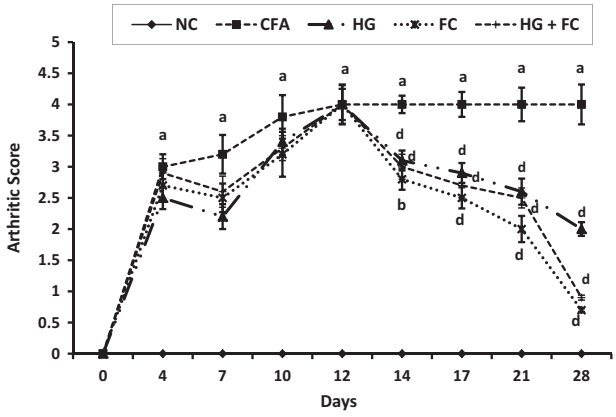

Figure 3. Effect of HG and Combination on Arthritic score in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. $^{a}p < .001$ represents the significance level compared to NC group; $^{b}p < .05$, $^{c}p < .01$, $^{d}p < .001$ as compared to CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

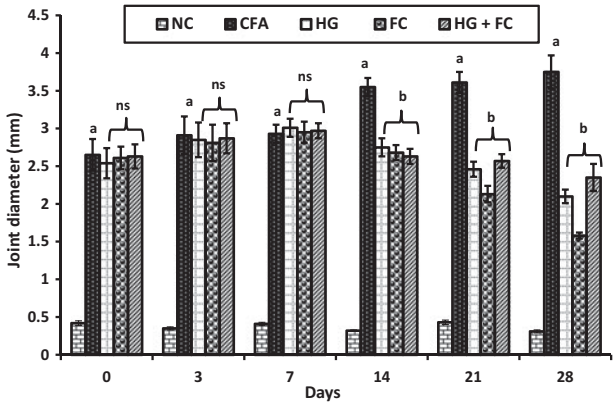

Figure 4. Effect of HG and Combination on Joint diameter in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. $^{a}p < .001$ represents the significance level compared to NC group; non-significant (ns) and $^{b}p < .001$ as compared to CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

Table 1. Effect of HG and Combination on RBCs, WBCs and hb in CFA-induced arthritis in rats.

Groups	RBC ($\times 10^6$/μl)	WBC ($\times 10^3$/μl)	Hb (g/dl)
NC	8.34 ± 0.63	9.06 ± 0.69	15.56 ± 1.02
CFA	5.89 ± 0.49[a]	19.33 ± 1.23[a]	5.30 ± 0.68[a]
HG	7.13 ± 0.51[b]	12.99 ± 0.98[b]	12.91 ± 0.98[b]
FC	7.89 ± 0.62[b]	5.76 ± 0.43[b]	12.14 ± 0.91[b]
HG + FC	7.59 ± 0.63[b]	12.48 ± 0.81[b]	13.01 ± 0.65[b]

Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test.
[a]$p < .05$ as compared to NC group.
[b]$p < .05$ as compared to CFA group.
NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

Effect of HG and Combination on pro-inflammatory cytokines

A significant elevation ($p < .001$) in serum TNF-α, IL-6, IL-12 and TXB_2 were observed in CFA-treated rats. Treatment of arthritic rats with HG (50 μg/kg) and Combination (25 μg/kg, each) and FC (50 μg/kg) significantly ($p < .01$) prevented the elevation of serum TNF-α, IL-6, IL-12 and TXB_2 levels (Figures 6–9).

Effect of HG and Combination on histopathological analysis of ankle joints

The histopathological analysis of ankle joint of NC rats showed normal connective tissue structure without inflammation, necrosis and intact synovial lining of bone (Figure 10(a)). Treatment of rats with CFA showed massive influx of

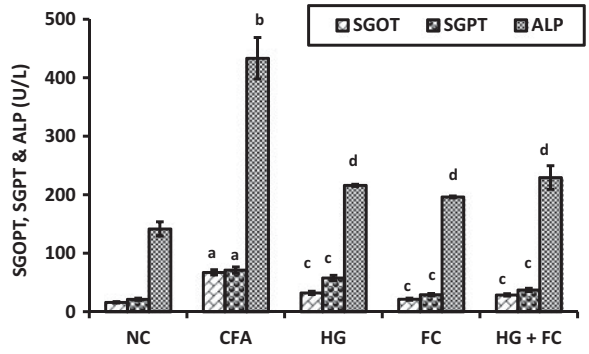

Figure 5. Effect of HG and Combination on SGOT, SGPT and ALP parameters in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. $^{a}p < .01$; $^{b}p < .001$ represents the significance level compared to NC group; $^{c}p < .05$ and $^{d}p < .001$ compared to CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

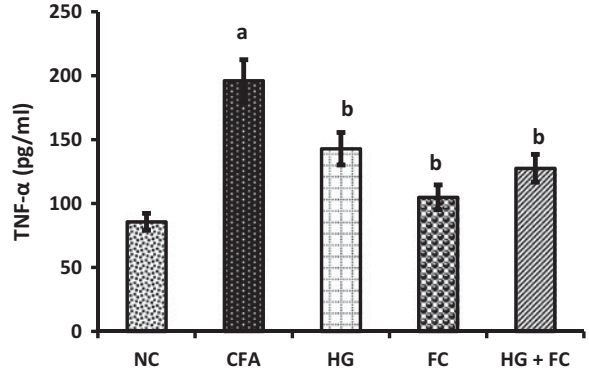

Figure 6. Effect of HG and Combination on serum TNF-α level in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. $^{a}p < .001$ represents the significance level compared with NC group, $^{b}p < .001$ when compared CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

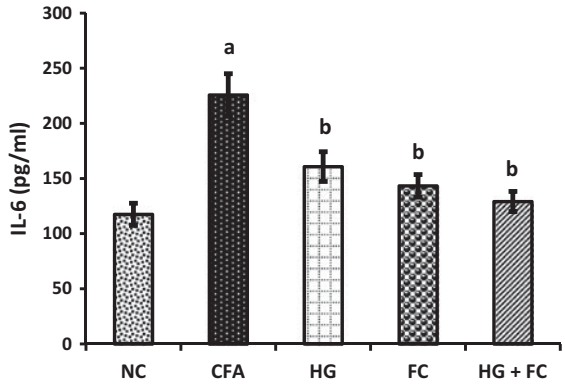

Figure 7. Effect of HG and Combination on serum IL-6 level in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. $^{a}p < .001$ represents the significance level when compared with NC group, $^{b}p < .001$ when compared with CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

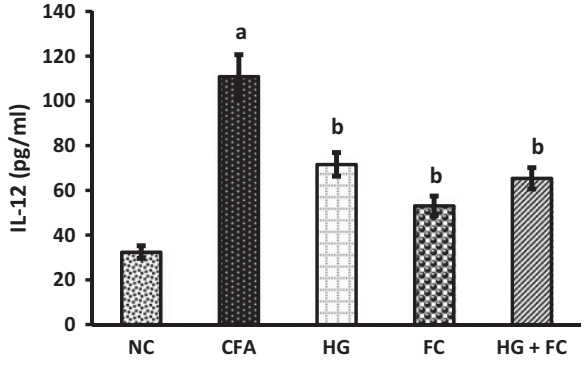

Figure 8. Effect of HG and Combination on serum IL-12 level in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. $^{a}p < .001$ represents the significance level when compared with NC group, $^{b}p < .001$ when compared with CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

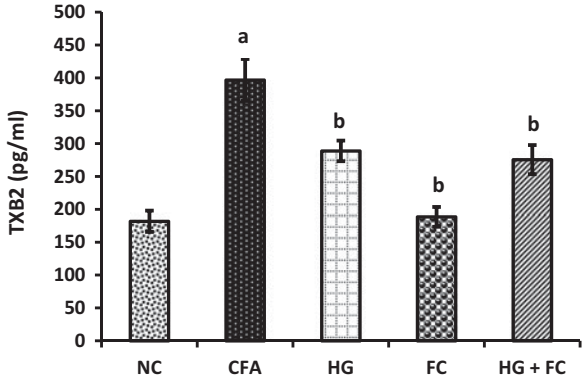

Figure 9. Effect of HG and Combination on TXB_2 level in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. $^{a}p < .001$ represents the significance level when compared with NC group, $^{b}p < .001$ when compared with CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

inflammatory cells, chronic inflammation, necrosis of synovial tissue, synovial hyperplasia and damaged synovial lining (Figure 10(b)). Whereas, the rats treated with HG and Combination showed significant protection comparable to normal structure of connective tissue, necrosis with low influx of inflammatory cells in the ankle joints (Figure 10(c,e)). Treatment of rats with FC (50 μg/kg) showed normal connective tissue structure with absence of massive influx of inflammatory cells and necrosis (Figure 10(d)).

Effect of HG and Combination on MPO activity

The MPO levels were significantly ($p < .001$) increased in the joint tissue of CFA-treated rats as compared to NC rats. The treatment of rats with HG ($p < .001$) and Combination ($p < .01$) significantly decreased the levels of MPO in joint tissue. The level of MPO in FC group also showed significant ($p < .001$) reduction when compared with NC rats (Figure 11).

Effect of HG and Combination on paw swelling

The treatment of rats treated with CFA exhibited a marked peripheral edema of the ipsilateral injected paw, observed 24 h after the CFA injection. As shown in Figure 12, after CFA immunization, the arthritis swelling of paw was significantly ($^{a}p < .001$) increased and maintained for 28 days as compared with NC group. However, the treatment of rats with HG and Combination significantly ($^{b}p < .001$) inhibited the paw swelling as compared to CFA group. FC ($^{b}p < .001$) also significantly inhibited the paw swelling in rats (Figure 13).

Effect of HG and Combination on spleen and thymus gland indices

As shown in Figures 14 and 15, the spleen and thymus gland indices were significantly ($^{a}p < .05$) decreased after CFA immunization in CFA rats compared with NC group rats. However, HG (50 μg/kg) and Combination (25 μg/kg, each) significantly ($^{b}p < .05$) improved the spleen and thymus gland indices as compared with CFA group. Whereas, the reduction in spleen and thymus gland indices was not observed in FC treated rats (50 μg/kg).

Effect of HG and Combination on radiographical analysis

The CFA-injected hind paw of rats had developed joint space narrowing; soft tissue swelling and extensive joint erosion of ankle joint (Figure 16). The treatment of rats with HG showed moderate effect on joint space narrowing and bone erosion. Whereas, treatment of rats with Combination and FC showed pronounced inhibition of joint space narrowing, soft tissue swelling and bone erosion of ankle joint.

Effect of HG and Combination on COX-2 mRNA expression

Increased expression of COX-2 mRNA was observed in the rats treated with CFA (Figure 17(h)), a low levels of COX-2 mRNA expression was detected in the HG (50 μg/kg) and Combination rats (25 μg/kg) groups (Figure 17(j,l)). Similarly, FC (50 μg/rat) also showed significant effect on COX-2 mRNA

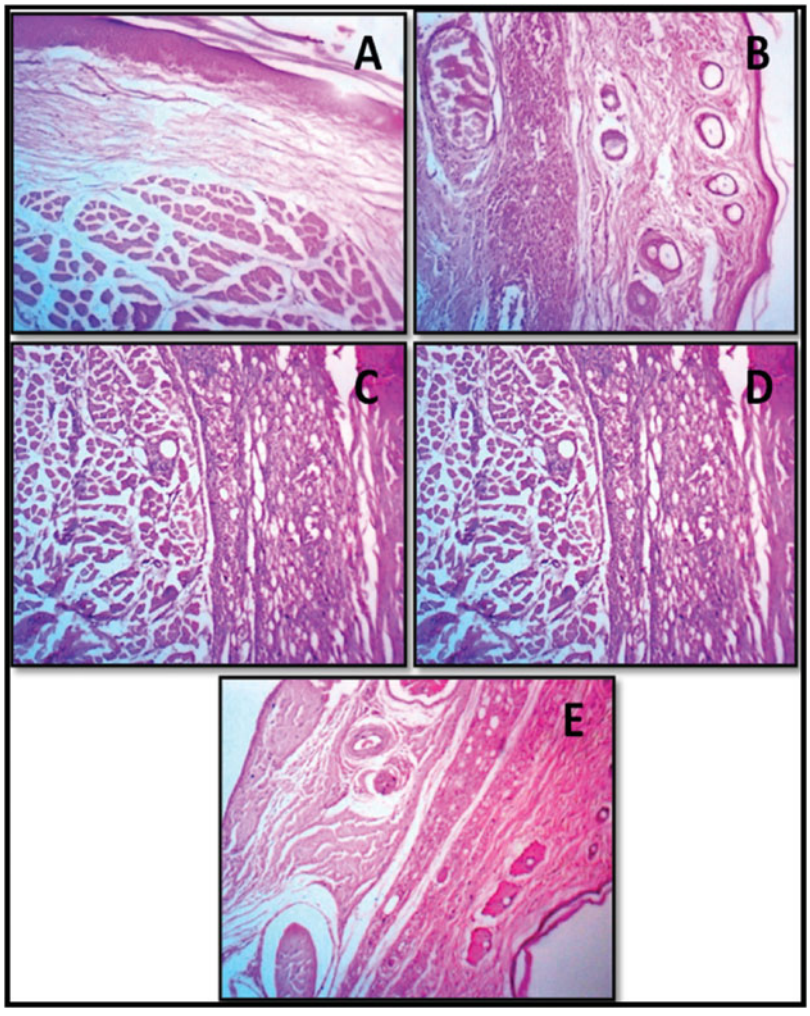

Figure 10. Photomicrograph of transverse sections of ankle joint tissue treated with CFA stained with H&E and examined under light microscopy (100×). Treatments: Acetone (A) CFA (0.1 ml) (B) HG (50 μg/rat) (C) FC (50 μg/rat) (D) HG + FC (25 μg/rat, each) (E). The shown sections are representative of six animals per group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

expression (Figure 17(k)). GAPDH expression was observed in all groups of animals (Figure 17(b–f)).

Discussion

In laboratory, animal arthritis is primarily induced with chemical (formaldehyde) or biological (*Mycobacterium*) agents and used for the screening of a range of anti-arthritic agents. Arthritis is not only a disease of joints, but also associated with immune, hepatic, renal and other organ systems damage that directly or indirectly affects the joints. Hence, it is required to determine the pathological and biochemical aspects of arthritis that are necessary in evaluating the activity of drugs[30]. Osteoarthritis and RA are two common types of arthritis characterized by joint inflammation, immune cell infiltration, synovial hyperplasia, joint pain and swelling that result in the destruction of joint integrity and function disability[31]. RA is a chronic inflammatory disease affecting about 1% of the population in developed countries[32]. Because of the similarities in clinical features, the adjuvant-induced arthritis in rat is extensively used for evaluating the efficacy of anti-inflammatory drugs in rheumatoid

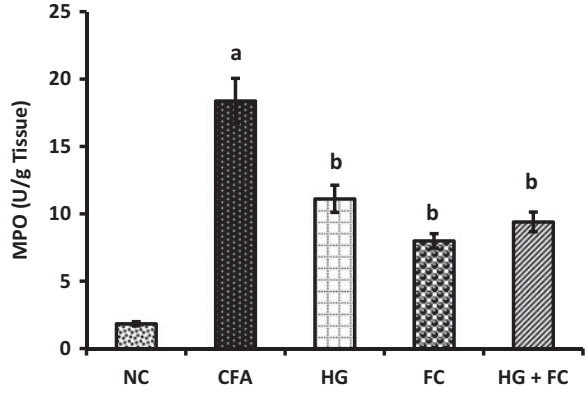

Figure 11. Effect of HG and Combination on MPO level in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. [a]$p < .001$ represents the significance level compared with NC group; [b]$p < .001$ compared with CFA. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

RA[33]. CFA-induced arthritis is well-recognized model and has been commonly used for the evaluation of anti-inflammatory and anti-arthritic potential of various agents[34]. The CFA-induced arthritis model was selected because

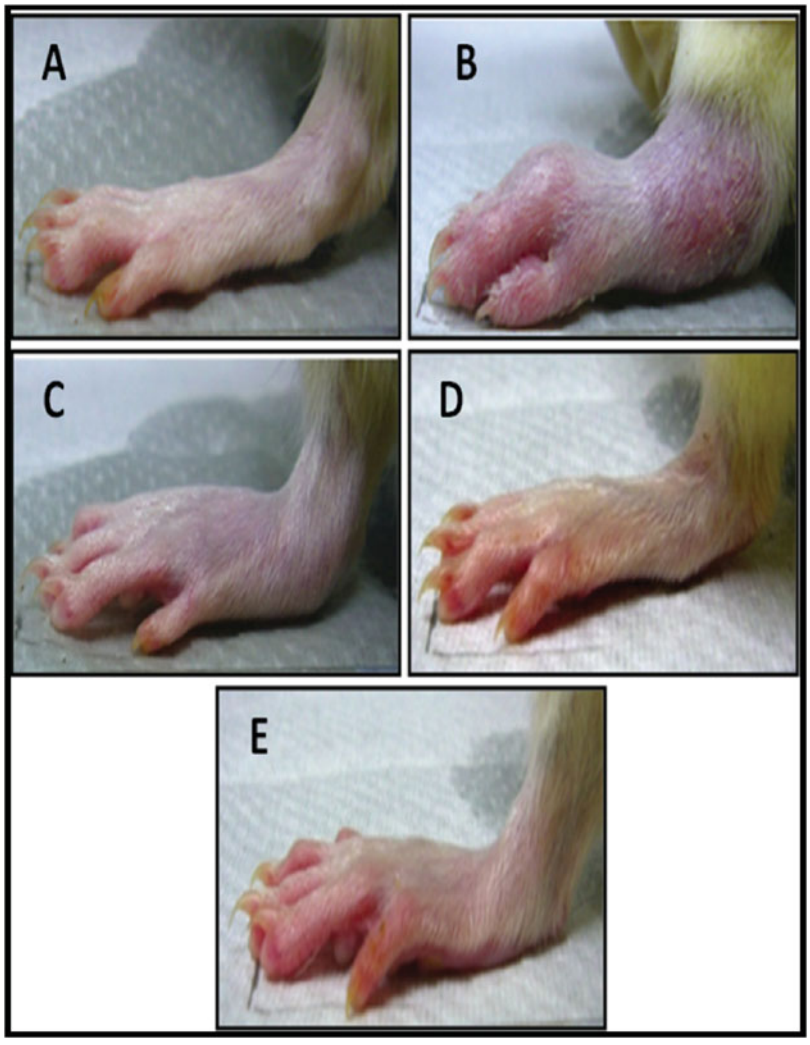

Figure 12. Representative photomicrographs showing effect of HG and Combination on paw swelling in CFA-induced arthritis in rats. A = NC, B = CFA, C = HG, D = FC and E = HG + FC. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

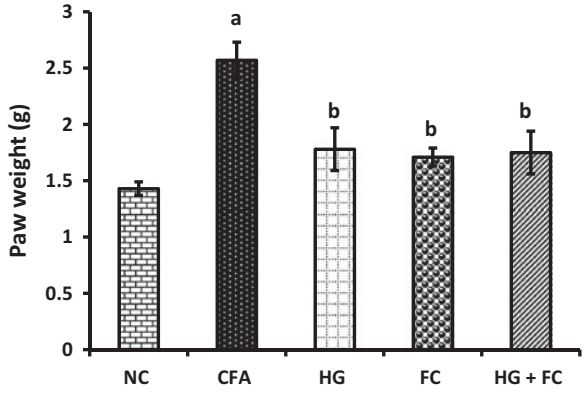

Figure 13. Effect of HG and Combination on Paw weight in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. [a]$p < .001$ represents the significance level compared to NC group; [b]$p < .001$ compared to CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

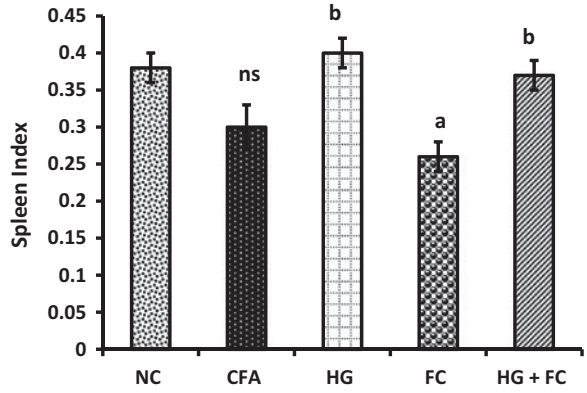

Figure 14. Effect of HG and Combination on Spleen gland indices in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. [a]$p < .05$ as compared to NC group and [b]$p < .05$ as compared to CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

rats develop the chronic swelling in the multiple joints with infiltration of inflammatory cells, destruction of joint cartilage and bone that to shows close similarities with human RA[35].

CFA is composed of mineral oil inactivated and dried mycobacteria, that contain different pathogen-associated molecular patterns including toll-like receptor 2, 4, and 9 agonists and responsible for stimulation of cell-mediated

immunity that increases the synthesis of certain immunoglobulins[36]. The intra-dermal administration of CFA into the paws of rats leads to reactivity to heat shock proteins, cartilage proteoglycans, and interactions with intestinal flora[37]. The CFA-induced arthritis follows a biphasic route an acute local inflammatory reaction that subsides after 3–4 days and a chronic systemic reaction that shows a relapsing–remitting course after initial 2 weeks and can persist for several months[38]. Further, release of diverse inflammatory mediators such as, cytokines, lysosomal enzymes, hydrolytic enzymes

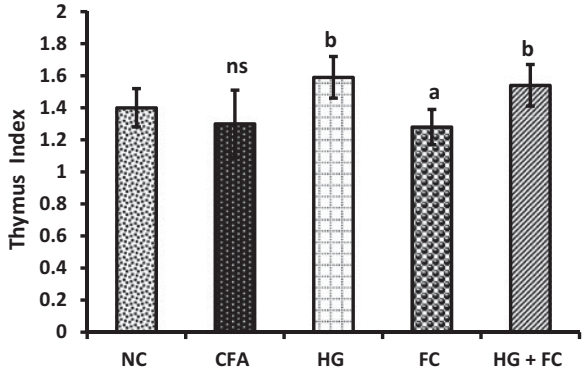

Figure 15. Effect of HG and Combination on thymus gland indices in CFA-induced arthritis in rats. Values expressed as mean ± SEM ($n = 6$) and analyzed by one-way ANOVA followed by Tukey's Kramer test. $^{a}p < .05$ as compared to NC group and $^{b}p < .05$ as compared to CFA group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

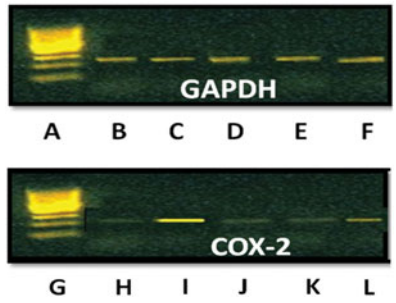

Figure 17. Effect of HG and Combination on COX-2 mRNA expression on joint tissue by using RT-PCR in CFA-induced arthritis in rats. GAPDH: B = NC, C = CFA, D = HG, E = FC, F = HG + FC; COX-2: H = NC, I = CFA, J = HG, K = FC, L = HG + FC. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

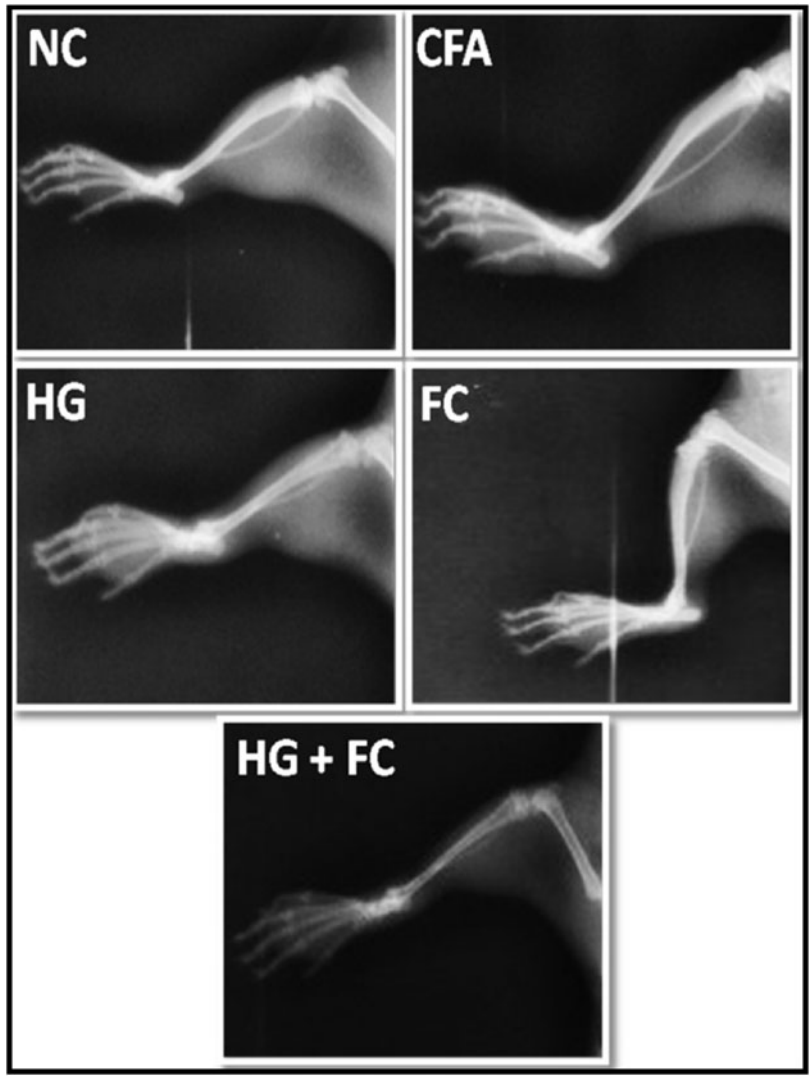

Figure 16. Effect of HG and Combination on Radiographic analysis of ankle joints in CFA-induced arthritis in rats. The shown radiographic films are representative of six animals per group. NC: Normal control; CFA: Complete Freund Adjuvant; HG: Hecogenin, FC: Fluticasone; HG + FC: Hecogenin + Fluticasone.

and prostaglandins (PGs) known to participate in the pathogenesis of RA[39,40].

The rationale behind such a research work was to minimize the dose of synthetic glucocorticoid by combining FC with plant-derived phytosteroid (HG). Hotchkiss et al. [41] studied the effect of FC on ozone-induced rhinitis and mucous cell metaplasia in rat nasal airway epithelium on intranasal administration of FC (50 μg/nasal passage) they showed that FC decreased the neutrophilic inflammation and cytokines expression (e.g., TNF-α, IL-6, IL-12 and TXB_2) which modulate airway mucin expression[41]. The dose of HG (50 μg/kg) in combination with FC (25 μg/kg, each) was selected on the basis of a pilot study. The dose finding study was performed by selecting various doses of HG for anti-arthritic effects in rats such as 50, 75 and 10 μg/kg and HG at all doses used exhibited significant effect, hence the smallest dose of 50 μg/kg was selected for anti-arthritic activity in rats. Thus, smallest dose of HG was preferred for further *in vivo* anti-arthritic activity i.e., 50 μg/kg.

In our previous study, we demonstrated the anti-inflammatory activity of HG and Combination in croton oil-induced ear edema, cotton pellet granuloma and trinitrobenzene sulfonic acid-induced colitis in rat that would help in predicting the mechanism of action of CFA-induced arthritis in rats[42].

Reduction in paw swelling is an index of measurement of anti-arthritic activity of various drugs[43]. The measurement of paw swelling is a simple, quick and sensitive method for evaluating intensity of inflammation[44]. RA presents with edema of periarticular tissues such as ligaments and joint capsules. The intensity of ligaments swelling and joint capsule swelling increases in the initial phase of inflammation. These changes in the paw volume are associated with an increase in the accumulation of granulocytes and monocytes in the joint tissue[45]. In the chronic inflammatory phase, the activation of macrophages results in the production of several cytokines such as IL-6 and TNF-α that have been associated with immune arthritis[46].

In the present study, HG (50 μg/kg), FC (50 μg/kg) and Combination (25 μg/kg, each) measured parameters revealed anti-arthritic effect. The significant increase in the paw thickness after sub-plantar administration of CFA is reflecting the phase of arthritis. In this model, arthritis score is an index of the joint inflammation after CFA immunization[47]. The oral treatment of HG (50 μg/kg), FC (50 μg/kg) and Combination (25 μg/kg, each) showed significant decrease in the paw thickness (Figure 2), arthritic score (Figure 3) and joint diameter (Figure 4) and by inhibiting the release of inflammatory mediators, indicating its anti-inflammatory potential in CFA-induced arthritis model.

Anaemia is frequently associated with chronic arthritis patients because of gastrointestinal blood loss due to arthritic medications and structural changes in the bone marrow which prevents the release of iron for incorporation into red blood cells[47]. In CFA-induced arthritis, the decreased levels of RBCs and Hb associated with the reduced levels of erythropoietin may be due to decreased bone marrow response to erythropoietin and destruction of premature RBCs. The treatment of rats with HG and Combination significantly restored the level of RBC and Hb. The elevated level of IL-1β results in increase in granulocyte and macrophages colony-stimulating factors that results in the elevation of WBC level in CFA-treated rats[39]. Treatment of rats with HG and Combination might play important roles in the inhibition of IL-1β inflammatory mediator release that may significantly decrease the WBC level in CFA-treated rats.

Among the patients of arthritis, involvement of liver has been reported only in RA. However, liver injury is not recognized as a significant extra-articular feature of RA. The abnormalities in liver tests were varying with the progression of disease, mainly raised ALP levels, have been reported in 18–50% of RA patients. Similarly, 65% of unselected RA patients had abnormal liver biopsies—mild portal chronic inflammatory infiltrate of the portal tract and small foci of necrosis and fatty liver[48]. In the present study, challenge of rats with 0.1 ml CFA significantly ($p < .001$) elevated the serum SGOT, SGPT and ALP level. Assessment of liver injury is done by determining the levels of the biomarkers SGOT, SGPT and ALP. Elevated serum levels of these enzymes indicate damage to hepatic architecture resulting in leaching of these enzymes into the systemic circulation. The estimation of serum biomarkers level provides good information about liver and kidney impairment indices that is also considered as an important feature of adjuvant arthritis[24]. So, in the present study significant rise in the aminotransferase level was observed in CFA-treated animals suggest that it might be released from the damaged liver cells[49]. The treatment of rats with HG and Combination significantly ($p < .001$) decreased the level of SGOT, SGPT and ALP that confirms the treatment have protective effects on liver and kidney function.

In animals the network of cytokines such as pro-inflammatory and anti-inflammatory mediators available in balanced state which is disturbed by a variety of factors such as infectious agent and environmental exposure in RA[50]. These cytokines to play essential roles in both initiation and maintenance of localized and systemic inflammatory changes[49]. These cytokines contribute to numerous features of RA, such as inflammation of synovial tissue, proliferation of synovial and cartilage tissue. The levels of inflammatory cytokines (TNF-α, IL-6 and IL-12) were found to be elevated in the early phase of arthritis[51]. However, TNF-α and IL-12 is associated with enhanced levels of inflammatory cells infiltration and cartilage destruction and joint swelling[52,53]. IL-6 is an immune-modulatory cytokines considered to be of great importance in the pathogenesis of RA in humans. This cytokine play a crucial role in the initiation and perpetuation of localized and systemic inflammatory changes[54]. TXB_2 is the key inflammatory mediator that is derived from arachidonic acid through the COX pathway in cell membranes of mammals and over-expressed in inflammatory conditions such as RA and cancers[55]. In the present study, levels of TNF-α, IL-6, IL-12 and TXB_2 were significantly increased in serum samples of CFA arthritic rats. In agreement with this, our results demonstrate that HG and Combination exerts an anti-arthritic effect by suppressing the production of cytokines such as, TNF-α, IL-6, IL-12 and TXB_2.

The histopathological analysis of ankle joint of NC rats showed normal connective tissue structure without

inflammation, necrosis and intact synovial lining of bone (Figure 10(a)). Treatment of rats with CFA showed massive inflammatory cells influx, chronic inflammation and necrosis of bone, synovial hyperplasia and damaged synovial lining (Figure 10(b)). Treatment of rats with FC showed normal connective tissue structure with absence of lymphocytic infiltration and necrosis (Figure 10(d)). Whereas, rats treated with HG and Combination showed significant protection against connective tissue necrosis with low influx of inflammatory cells in the ankle joints (Figure 10(c,e)).

The accumulation of neutrophils at the inflammation site is an important indicator of MPO assay. It produces destruction of lysosomes by increase in array of reactive oxygen species (ROS) that up regulates hydroxyl and hydrogen peroxide radicals leading to tissue membrane damage[56]. HG is a potent antioxidant and possesses free radical scavenging property[12]. Treatment with HG and Combination significantly decreases the elevated MPO level in CFA-treated rats (Figure 11). In the earlier studies, we have reported that, HG and Combination inhibited MPO in granular tissue of TNBS-induced colitis in rat due to antioxidant activity of HG[57].

The treatment of rats with CFA exhibited elevated thickness of the injected paw, subsequently observable 24 h after the injection (Figure 12). After CFA immunization, the arthritis swelling in the right hind paws of rats was significantly ($p < .001$) increased and maintained for 28 days compared with NC group. However, the treatment of rats with HG and Combination significantly ($p < .001$) inhibited the paw swelling as compared to CFA group. FC also significantly inhibited the paw swelling in rats (Figure 13).

Glucocorticoid produces effects such as reduction of spleen and thymus gland weight after long-term treatment. The spleen is an important organ and serves as a reservoir for the antibodies involved in the immune responses. The decrease in spleen weight and increase in thymus weight are correlated to a stimulatory effect on the immune system[58]. The sub-plantar administration of CFA significantly decreased the spleen and thymus gland indices that can be significantly increased by administration of HG and Combination as compared to CFA-treated rats (Figures 14 and 15). The significant increase in the spleen and thymus gland indices might be observed due to immune-stimulatory effect of HG[59].

Chronic inflammation involves the release of a number of mediators which are responsible for the pain, bone and cartilage destruction, which leads to severe disability[39]. PG's are formed in the inflammation process under the influence of two enzymes, COX-1 and COX-2. Enzyme COX-1 is responsible for the maintenance of gastric mucosa integrity, renal function and homeostasis through the release of PGs. Whereas, the expression of COX-2 enzyme is upregulated in inflamed tissue and may be responsible for increased production of PG's[57]. The increased expression of COX-2 mRNA was observed in the rats treated with CFA (Figure 17(h)), where as a low levels of expression was detected in the HG and Combination groups (Figure 17(j,l)). Similarly, FC also showed significant effect on COX-2 mRNA expression (Figure 17(k)). GAPDH expression was observed in all groups of animals (Figure 17(b–f)).

In view of the fact, synthetic drugs such as, non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, diclofenac and naproxen, and dexamethasone, as well as DMARDs, including cyclosporine, methotrexate have verified curative efficacy in RA. But, their side effects on gastrointestinal tract, haematological, cardiovascular complications and renal toxicity limit their usage in the treatment of RA[60]. This research has revealed the mechanisms of action of hecogenin through inhibition of COX mRNA enzyme and pro-inflammatory cytokines TNF-α, IL-6, IL-12 and TXB_2[61].

Conclusion

The above study revealed that HG (50 μg/kg) and Combination (25 μg/kg, each) possess promising anti-arthritic activity that was evaluated by their effects on haematological, biochemical, radiological and histopathological parameters. By evaluating various anti-arthritic parameters of HG makes it a good candidate for RA treatment.

Disclosure statement

No potential conflict of interest was reported by the authors.

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