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Geranium wallichianum D. Don Ex Sweet Ameliorates Rheumatoid Arthritis by Curtailing the Expression of COX-II and Inflammatory Cytokines as Well as by Alleviating the Oxidative Stress

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Abstract

Geranium wallichianum D. Don ex sweet traditionally been used as home remedy for backaches, joint pain, colic, and rheumatism. The objective of this study was to investigate the therapeutic benefits of plant in an adjuvant-induced arthritis paradigm. Immune-mediated rheumatoid arthritis was developed by injecting complete Freund's adjuvant (CFA) into the hind paws of rats and the aqueous methanolic crude extract was administered. The animals were physically monitored for changes in paw edema size and arthritic score. Hematological parameters and systemic inflammatory indicators evaluated. Genetic expressions of tumor necrosis factor (TNF-α), interleukins (IL-1β, IL-6), necrosis factor (NF-κB), and cyclooxygenase (COX-II) enzyme were studied using real-time qPCR. PGE2 levels in blood were quantified through Enzyme Linked Immunosorbent Assay (ELISA). On the 14th day, Immunoglobulin E (IGE) exhibited a substantial decline in paw edema and arthritic score. At the doses of 500 mg/Kg ($P \leq .05$) and 1000 mg/Kg ($P \leq .001$), IGE significantly reduced TNF-α, interleukins, and COX-II mRNA expression. IGE significantly lowered the MDA levels at the doses of 500 and 1000 mg/Kg (13.18 ± .70 and 9.04 ± .26 μM/L respectively) as compared to arthritic control (30.82 ± 1.12 μM/L) group. IGE significantly improved the antioxidant enzyme activities of CAT and SOD ($P \leq .001$) in treated animals. TNF-α, interleukins, and COX-II mRNA expression were also significantly reduced at the doses of 300 ($P \leq .05$), 500 ($P \leq .01$) and 1000 mg/Kg ($P \leq .001$) which were expressed as fold changes. This study shows that *Geranium wallichianum* D. Don ex sweet has a strong potential to alleviate immune-mediated arthritis by lowering oxidative stress and downregulating the proinflammatory cytokines signaling mechanisms.

Keywords

rheumatoid arthritis, complete Freund's adjuvant, tumor necrosis factor, ELISA, qPCR, superoxide dismutase

Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease that causes inflammation in smaller joints' synovium lining.[1] The primary stages of the illness are characterized by burning pain, edema, flush, and warmth.[2] There is synovial hyperplasia and pannus formation when disease gets worse. Subsequent damage to cartilage and bone leads to poor quality of life with compromised physical activity.[3] The etiology of RA comprises the production of antibodies against citrullinated proteins[4] triggering the activation of monocytes and migration of neutrophils and macrophages at the site of

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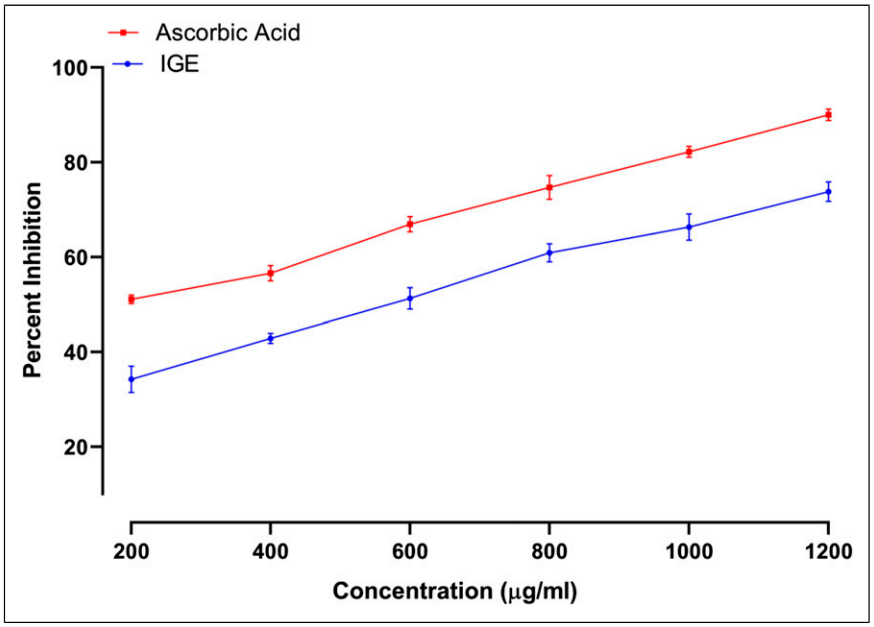

Figure 1. Dose-dependent antioxidant activity of aqueous methanolic extract of *Geranium wallichianum* D. Don. Ex Sweet (IGE) compared with that of ascorbic acid. All values (n = 3) are expressed as mean ± SEM.

inflammation. Progressing inflammatory process results in an upsurge in the levels of TNF-α, interleukins and nuclear factor NF-κB.[5] Activation of T-cells and B-cells in RA instigates the release of proinflammatory cytokines, prostaglandins especially prostaglandin E2 (PGE2), and several other mediators. Polymorphic neutrophils and lymphocytes trigger the bone degeneration and synovitis.[6,7] Synovial damage in RA is aggravated catastrophically by accompanied production of reactive oxygen species (ROS) which takes place in case of compromised compensatory capacity of the body thus resulting in damage to cellular components such as proteins, nucleic acids, lipids, and cell membranes.[8]

Anti-inflammatory and analgesic medications, such as non-steroidal anti-inflammatory drugs (NSAIDs), used in modern practice have a detrimental effect on the gastrointestinal tract, resulting in gastroesophageal reflux disease (GERD) and peptic ulcer disease.[9] The symptomatic therapy of rheumatoid arthritis involves disease-modifying anti-rheumatic drugs (DMARDs), biological agents, and glucocorticoids. These drugs are also hazardous to GI tract, liver, and kidney, posing major health risks.[10] Considering the lethal effects of conventional therapies, medicinal plants derived from natural flora may be a better and safer alternative for managing inflammation and pain in RA.[11] Different studies have demonstrated the striking potential of crude extracts of plants to curtail the inflammation and bone damage in RA.[12,13]

Geranium wallichianum D. Don ex sweet (common name Ratanjot) is a perennial herb that belongs to the family Geraniaceae and grows in Afghanistan, India, Pakistan, and another Himalaya region in East Asia. In Pakistan, it is abundantly found in Swat, Murree, Gilgit, and Azad Kashmir.[14] *Geranium wallichianum* is traditionally used to manage gonorrhea, arthritis,[15] bone pain, gout, and sciatica.[16] This study was designed to evaluate the potential of *Geranium wallichianum* to attenuate the adjuvant-induced arthritis in rat models with an emphasis to explore its effects on cytokine signaling mechanisms and reactive oxygen species (ROS).

Results

Antioxidant Activity of IGE by DPPH Method

Aqueous methanolic extract of *Geranium wallichianum* (IGE) showed a dose-dependent free radical scavenging effect with a maximum activity of 77.28% at 1200 µg/ml concentration as shown in Figure 1.

HPLC Analysis of IGE

High Performance Liquid Chromatography (HPLC) analysis of IGE reveals the presence of chlorogenic acid (Rt. 1.63), caffeic acid (Rt. 1.85), vanillic acid (Rt. 2.25), p-coumaric acid (Rt. 2.47), kaempferol (Rt. 28.05), syringic acid (Rt. 30.14) quercetin (Rt. 19.39), quercetin-rhamno-di-hexoside (Rt. 29.76), and quercetin-3-*O*-glucopyranoside (Rt. 33.03) where Rt indicates their retention times (Figure 2).

Effect of IGE on Paw Edema

Injection of CFA in subplantar tissues increased the inflammation in left paws resulting in marked swelling

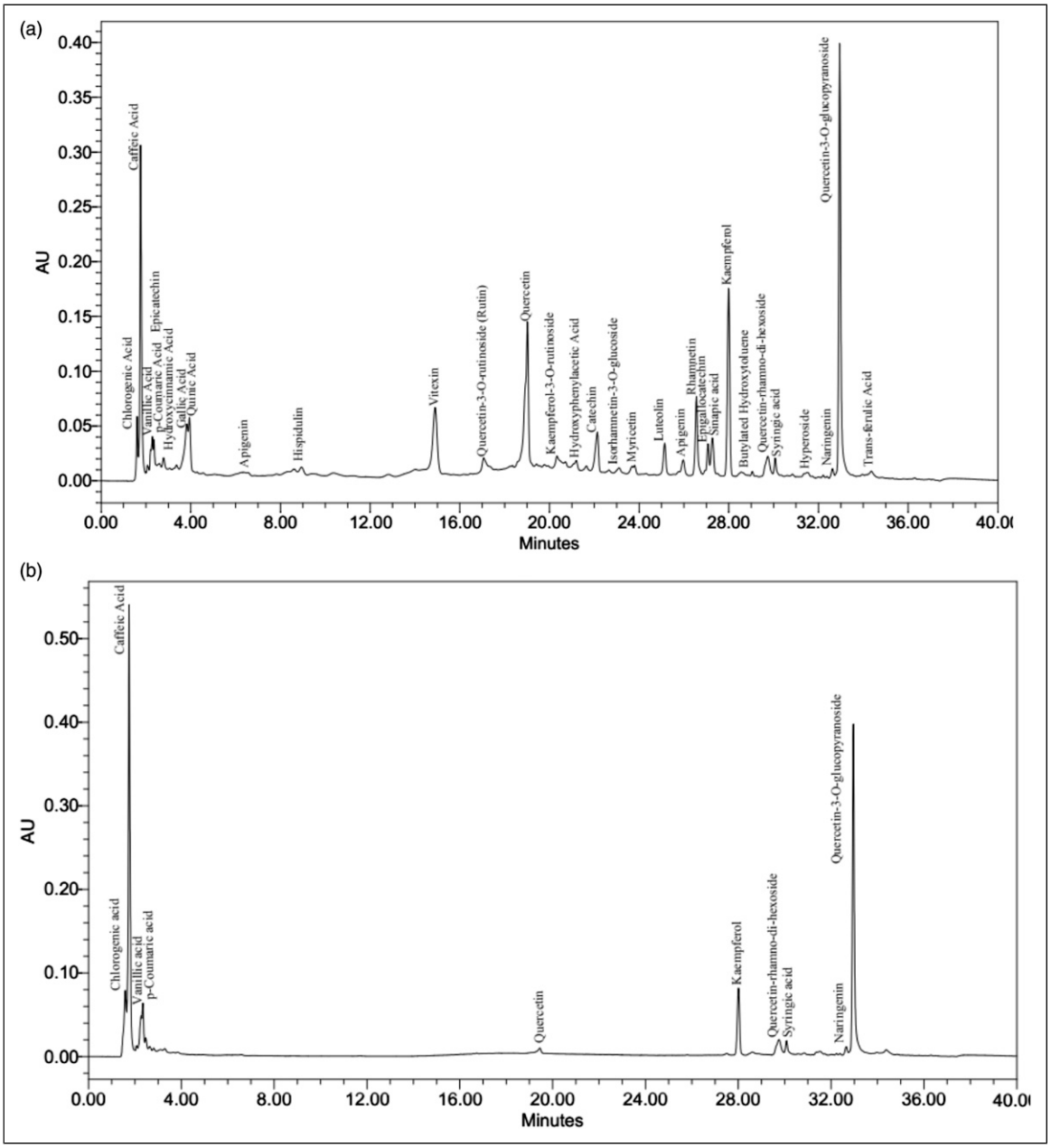

Figure 2. HPLC analysis of 70% methanolic extract (B) of IGE compared with standards (A).

observed on the 7th day. A significant enlargement in paw sizes in the arthritic control group was recorded from day 7 to 28 compared to the normal control animals. Treatment with IGE reduced paw diameter significantly compared to the arthritic control group at the doses of 1000 and 500 mg/Kg ($p < .001$ and $P < .01$, respectively) as shown in Figure 3A.

Effect of IGE on Arthritic Score

There was no paw inflammation in normal control group during the whole study period. The findings illustrated in Figure 3B exhibited the continuous rise in arthritic score (index of disease progression) in arthritic control group. Crude extract IGE commendably controlled the arthritic score in treatment groups at all doses. The extreme arthritic score observed on the 28th day was (3.875 ± .077) in arthritic control animals. IGE significantly curtailed the arthritic index at 1000 and 500 mg/Kg (2.183 ± .131 and 2.383 ± .060, respectively) compared to the effect of piroxicam (1.638 ± .095).

Effect of IGE on Weight of Animals

Body weight of arthritic control animals kept decreasing gradually from 14th day till the last day of study as shown by

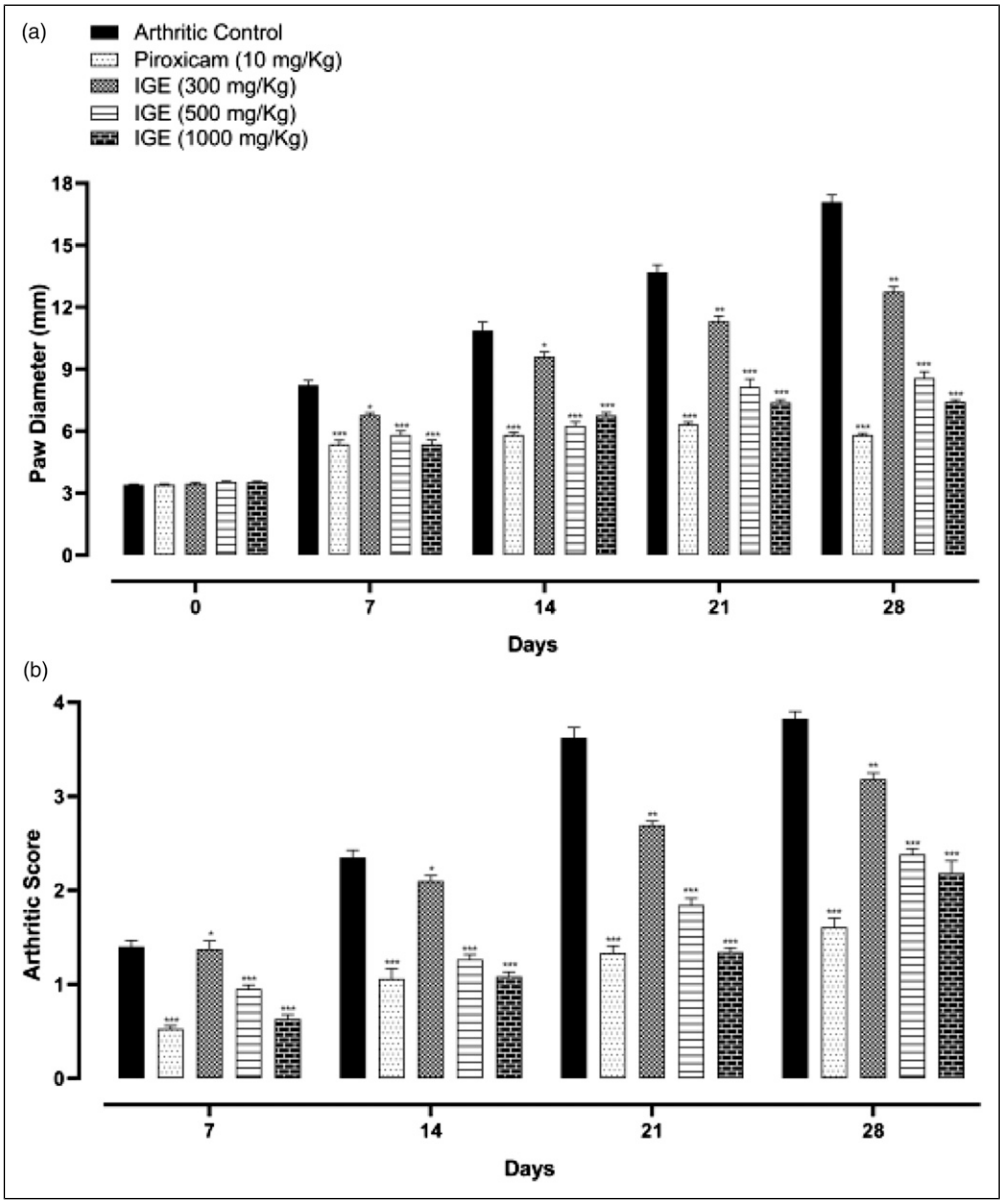

Figure 3. Effects of *Geranium wallichianum* aqueous methanolic extract (IGE) on rat paw edema (A) measured as diameter (mm) and on arthritic index measured as score (B) in CFA-induced arthritis. All values (n = 6) are expressed as Mean ± SEM using Two-way ANOVA followed by Tukey Test. *** = $P \leq .001$, ** = $P \leq .01$, * = $P \leq .05$ vs arthritic control.

the observations displayed in Figure 4 in comparison to that in normal control ($P < .001$) group. The crude extract IGE and piroxicam significantly maintained the body weight of treated animals when compared to arthritic control group ($P < .001$) from 14th to 28th day.

Effect of IGE on Hematological and Biochemical Parameters

It was observed that the induction of arthritis by Freund's adjuvant in rats ensued an up-rise in liver enzymes (ALP, ALT and AST), C-reactive protein (CRP), and rheumatic factor (RF) values. There was a decrease in Hb and RBCs in arthritic control rats; however, an increase in WBCs and platelets was seen contrary to normal control. Dose-dependent improvement on these hematological parameters was observed (Figure 5). Treatment with the IGE and piroxicam significantly normalized WBCs, ALP, ALT, AST, CRP, and RF ($p < .001$). The systemic biomarker as CRP and RF were substantially augmented (36.225 ± 1.125 and 34.832 ± 1.683, respectively) in arthritic control as shown in Figure 6. However, IGE significantly mitigated the CRP and RF at the doses of 500 ($p < .01$) and 1000 mg/Kg ($p < .001$) that was comparable to the effect of piroxicam. Instigation of

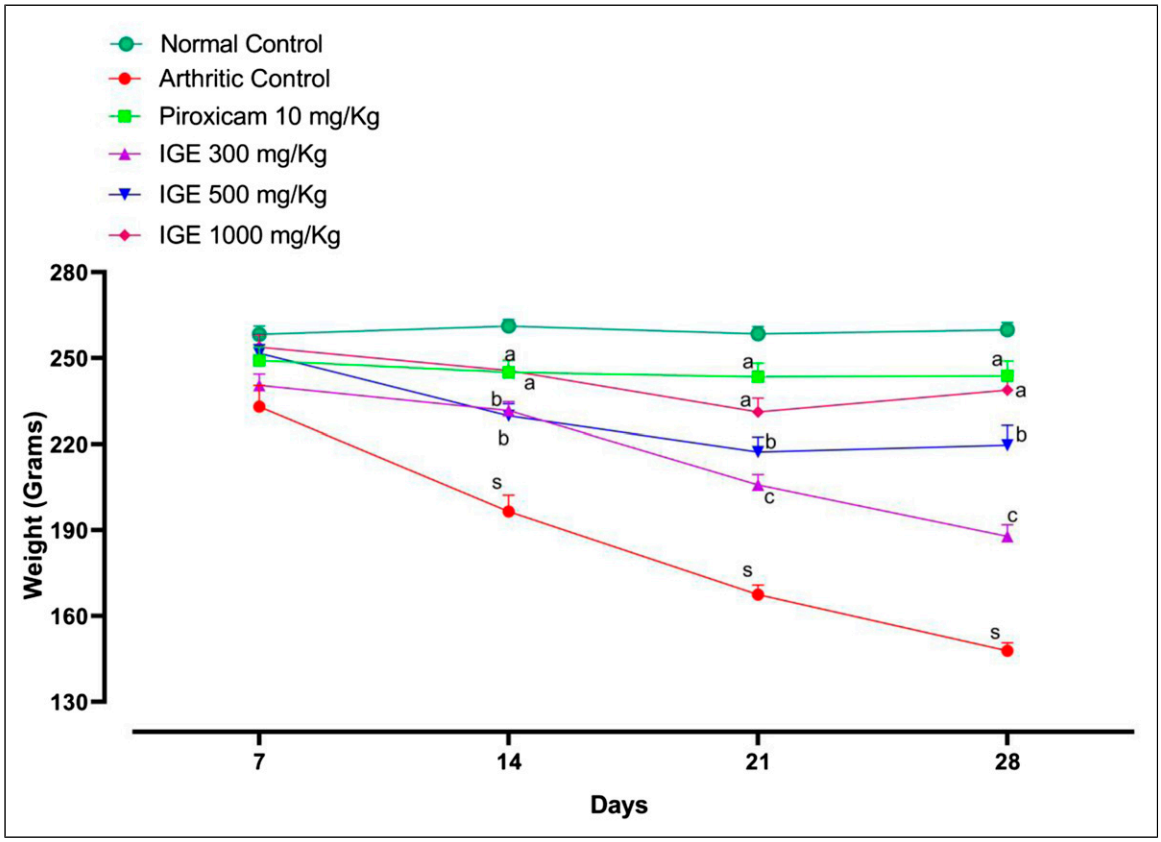

Figure 4. Effects of IGE on weight of animals in CFA-induced arthritis model. All values (n = 6) are expressed as mean ± SEM using two-way ANOVA followed by Tukey Test. s = $P \leq .001$ vs normal control while a = $P \leq .001$, b = $P \leq .01$, c = $P \leq .05$ vs arthritic control.

polyarthritis slightly affected the serum creatinine and blood urea nitrogen. In contrast, treatment with piroxicam had no significant effect on kidney function tests such as urea and creatinine. IGE significantly stabilized the BUN level at the dose of 1000 mg/Kg ($P < .001$) as reflected in Figure 7B.

Effect on Oxidative Stress Biomarkers

The findings of CFA-induced oxidative stress and ameliorating effects of IGE are presented in Figure 8. There was a significant decline ($P < .001$) of SOD (4.150 ± .114 U/ml) and CAT activities (35.905 ± 2.668 U/ml) in the arthritic control group in comparison to a normal control group (SOD: 11.655 ± .164 U/ml and CAT: 103.973 ± 5.003 U/ml). However, Piroxicam (10 mg/kg) and IGE reinstated ($P < .001$) the activities of CAT and SOD in arthritic rats, as shown in Figure 8A and B respectively. There was also an elevated level of MDA in the blood samples of arthritic control animals (32.022 ± 1.514 µM/L) as compared with the normal control group (8.305 ± .469 µM/L). The level of MDA was significantly reduced by treatment with piroxicam and IGE at various doses in arthritic rats, as shown in Figure 8C.

Effect of IGE on mRNA Expression Level of Proinflammatory Cytokines

The mRNA expression of pro inflammatory cytokines was evaluated following the 28 days of study in Wistar albino rats. It was found that mRNA expression of NF-κB ($P < .001$) was raised in the arthritic control group (11.234 ± .157-folds). Treatment with IGE at 1000 (2.619 ± .197-fold), 500 (3.900 ± .252-fold), and 300 mg/Kg (7.095 ± .655-fold) and piroxicam (2.467 ± .177-fold) reduced this rise in NF-κB in arthritic rats (Figure 9). An exaggerated COX-2 expression ($P < .001$) occurred in the arthritic control group (11.504 ± .237-fold). Treatment of arthritic rats with IGE at 1000 ($P < .001$), 500 ($P < .001$), and 300 mg/Kg ($P < .01$) and Piroxicam ($P < .001$) reduced the expression of COX-2 as compared with arthritic control group (Figure 9).

A significant upsurge ($P < .001$) in IL-6 expression was obvious in the arthritic control group (9.488 ± .177-fold) than the normal control group. However, IGE alleviated this elevation of IL-6 significantly at doses of 1000 ($P < .001$), 500 ($P < .01$) and 300 mg/Kg ($P < .05$) after 28 days of treatment. A significantly higher expression ($P < .001$) of TNF-α was demonstrated in arthritic control group (14.655

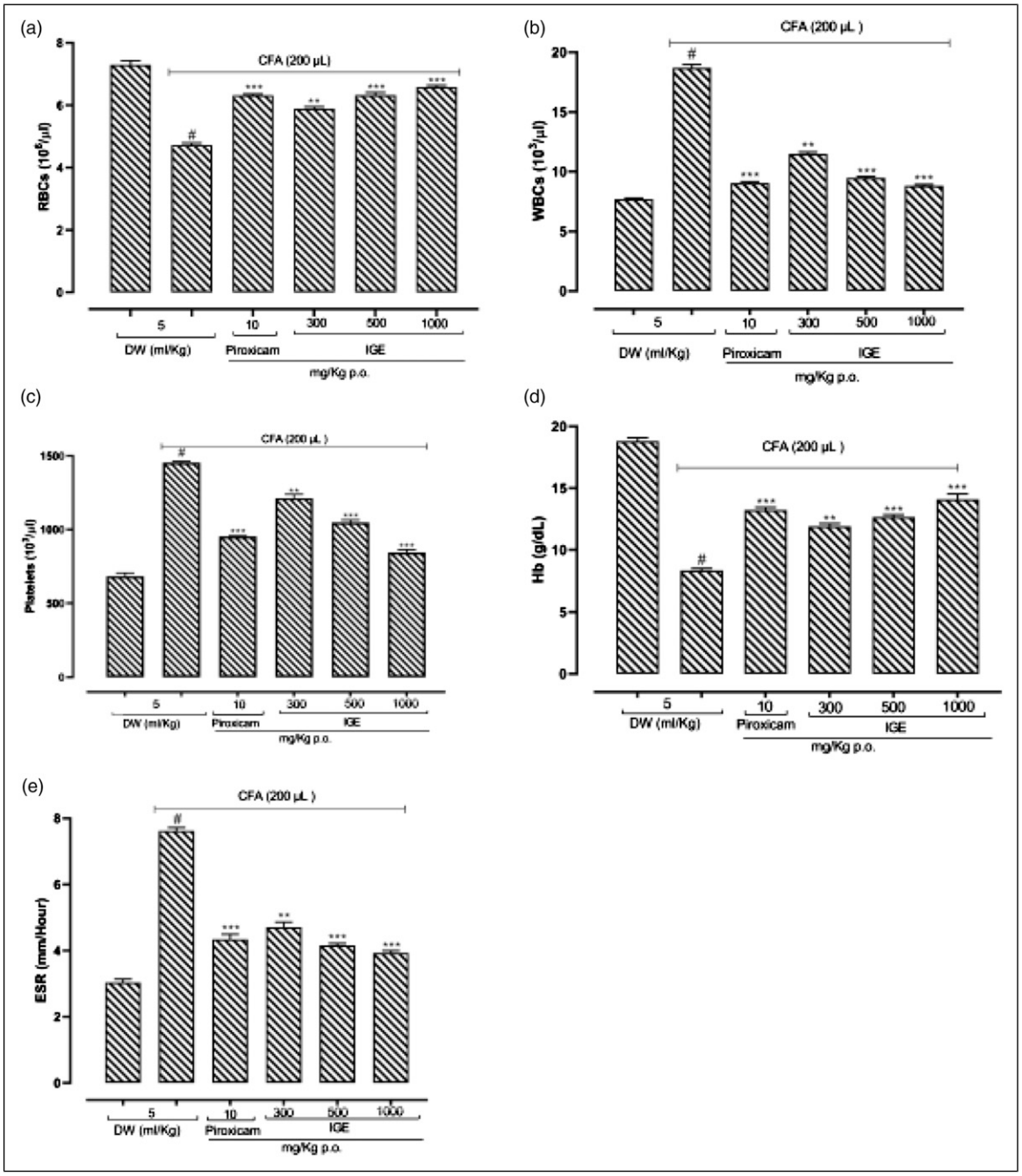

Figure 5. Effects of IGE on hematological parameters (A–E) in rats having CFA-induced arthritis. All values (n = 6) are expressed as mean ± SEM using two-way ANOVA Tukey's *post hoc* test. # = $P \leq .001$ vs normal control while *** = $P \leq .001$, ** = $P \leq .01$, * = $P \leq .05$ vs arthritic control.

± .339-fold) that was dropped in rats treated with IGE at 1000 (3.302 ± .234-fold), 500 (4.540 ± .429-fold) and 300 mg/kg (8.279 ± .426-fold). The mRNA expression of IL-1β notably augmented ($P < .001$) in the arthritic control group (11.451 ± .230-fold) than the normal control. The crude extract IGE significantly reduced the expression at doses of 1000 ($P < .001$) and 500 mg/Kg ($P < .01$) as shown in Figure 9.

Discussion

Suspension of heat-killed mycobacteria in mineral oil is termed as complete Freund's adjuvant (CFA). It is used to develop the arthritic model in rats which is a familiar method for preclinical studies as it has pathological similarities with rheumatoid arthritis in humans.[17] The CFA induces a biphasic joint inflammation in which the phase-1 is acute and continues

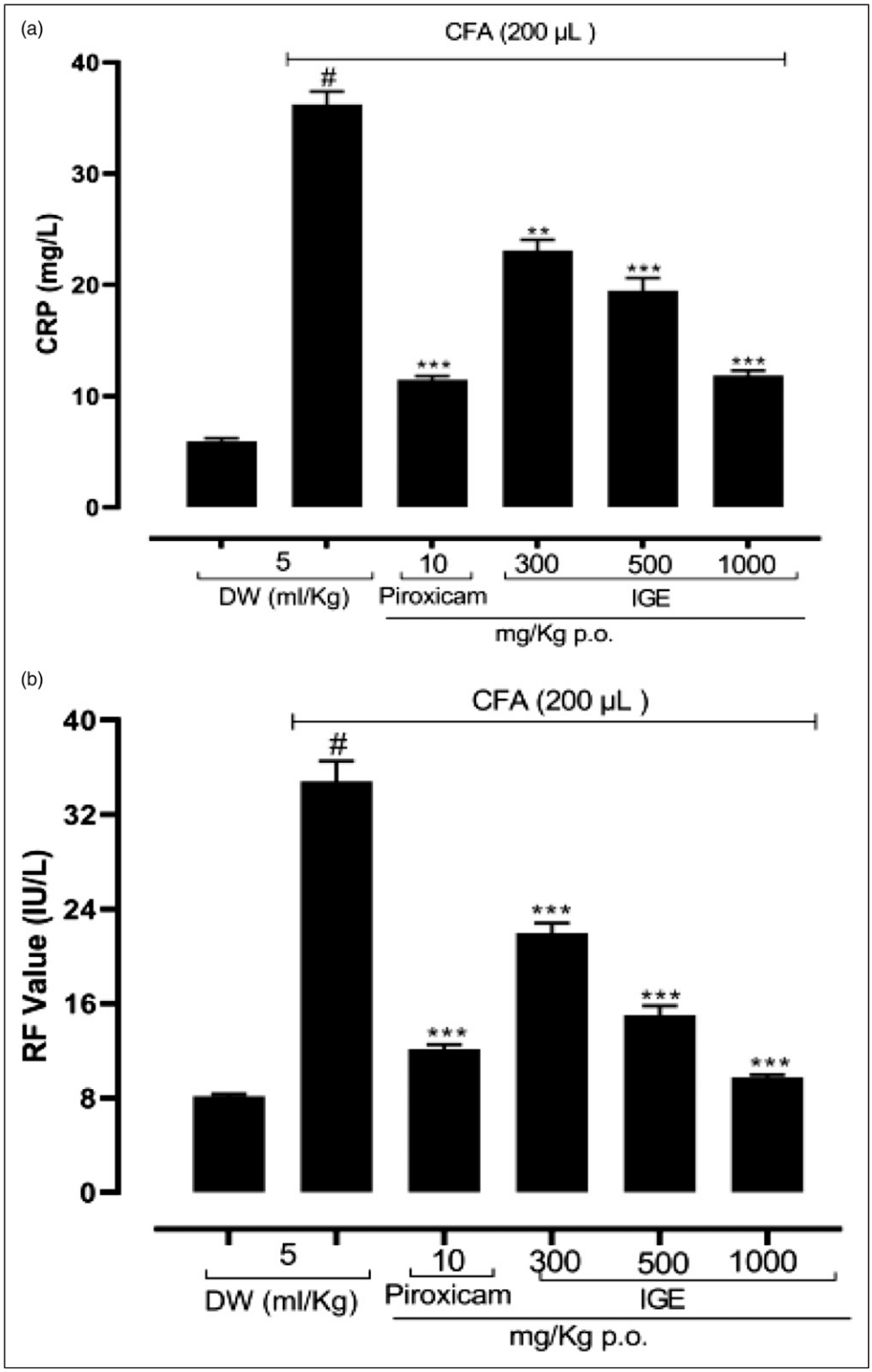

Figure 6. Effects of IGE on C-Reactive proteins levels (A) and rheumatic factor values (B) in rats with CFA-induced arthritis. All values ($n = 6$) are expressed as mean ± SEM using one-way ANOVA followed by Tukey's *post hoc* test. # = $P \leq .001$ vs normal control while *** = $P \leq .001$, ** = $P \leq .01$, * = $P \leq .05$ vs arthritic control.

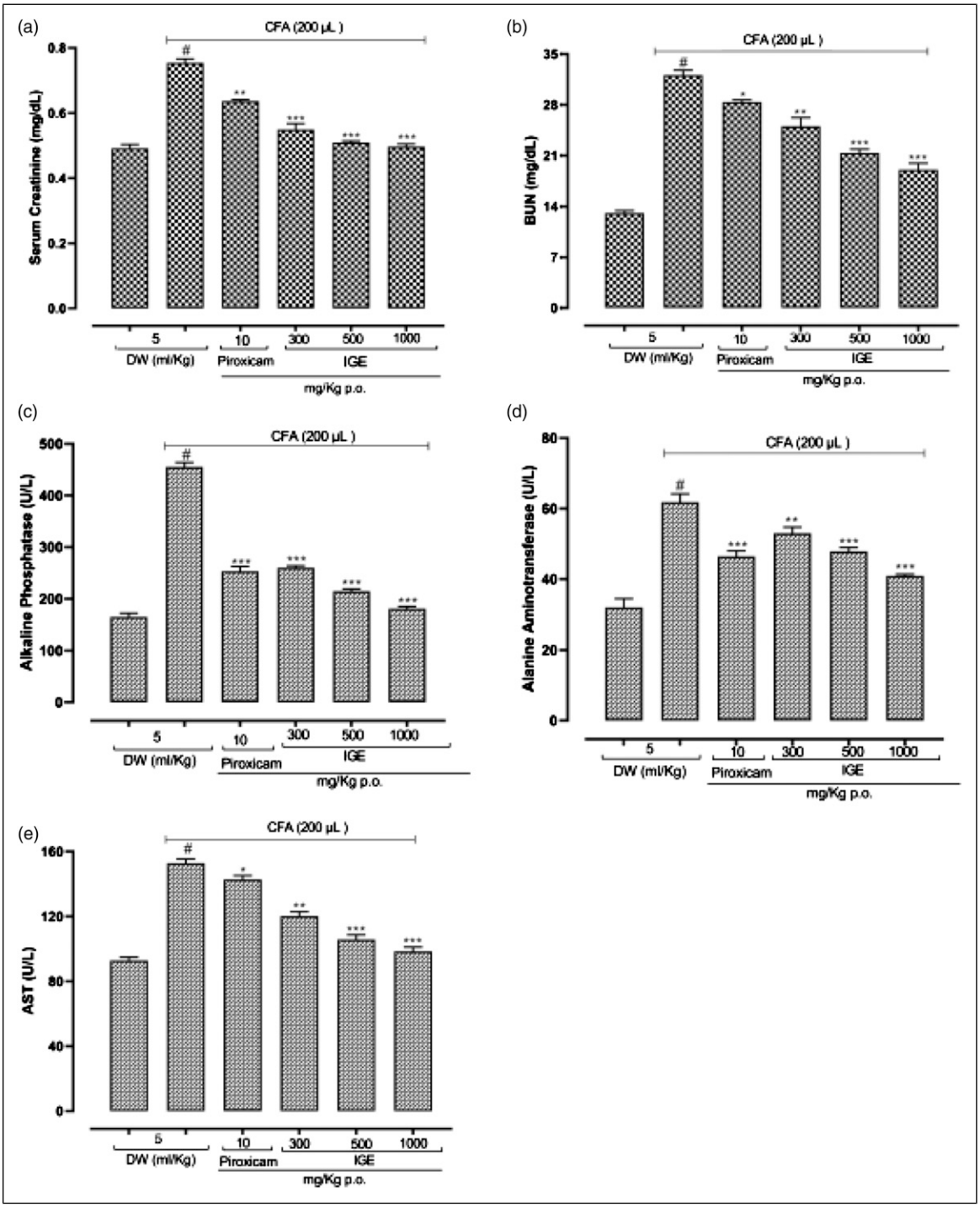

Figure 7. Effects of IGE on renal status (A–B) and hepatic enzyme levels (C–E) in rats with CFA-induced arthritis. All values (n = 6) are expressed as mean ± SEM using one-way ANOVA followed by Tukey's *post hoc* test. # = $P \leq .001$ vs normal control while *** = $P \leq .001$, ** = $P \leq .01$, * = $P \leq .05$ vs arthritic control.

for 10 days. This phase is commenced by emancipation prostaglandins, histamine, and serotonin from immune cells. Second phase is a prolonged phase enduring for 11–28 days.[18] Owing to the health hazards of existing therapeutic substances used to mitigate the arthritis, the medicinal plants are being explored worldwide to achieve innocuous and effective substitutes.[19]

Geranium wallichianum D. Don ex sweet belongs to the Geraniaceae family and is known as Ratanjot (also Srazela) in native people.[14] This plant is traditionally used to manage

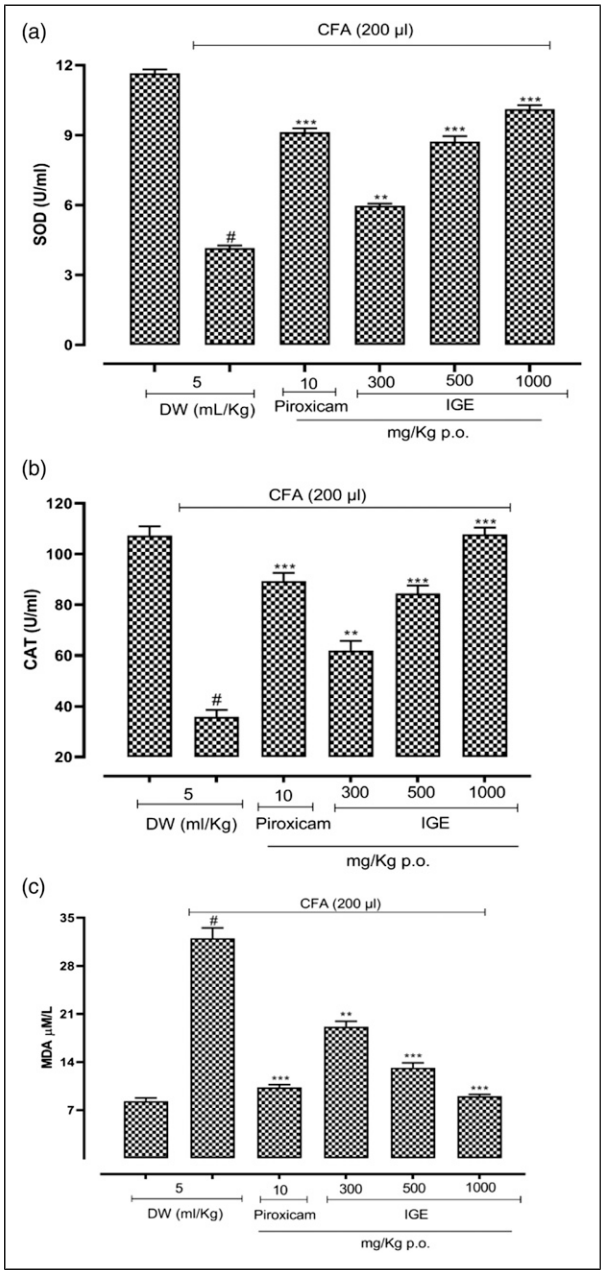

Figure 8. Effects of IGE on SOD (A), CAT (B) and MDA (C) in animals with CFA-induced arthritis. All values (n = 6) are expressed as mean ± SEM using one-way ANOVA followed by Tukey's *post hoc* test. # = $P \leq .001$ vs normal control while *** = $P \leq .001$, ** = $P \leq .01$, * = $P \leq .05$ vs arthritic control.

rheumatism, general weakness,[20] gout, and sciatic pain.[16] This study prepared an aqueous methanolic (70%) crude extract of *Geranium wallichianum* (IGE). In addition to phytochemical analysis, IGE was evaluated pharmacologically for its anti-arthritic and antioxidant activities. Alkaloids, phenols, tannins, sugars, glycosides, and flavonoids were identified in the screening test. The presence of several phytochemicals such as chlorogenic acid, vanillic acid, p-coumaric acid, caffeic acid, and quercetin was revealed by HPLC analysis of the crude extract.

There is a prominent upsurge in the expression of tumor necrosis factor-α (TNF-α), interleukins (IL-6 & IL-1β), cyclooxygenase enzyme, and nuclear factor (NF-Kb) in immune-mediated arthritis.[21] Edema is produced due to tissue injury with subsequent migration of macrophages, leukocytes, mast cells, and extravasation of small blood vessels.[22]

Anemia is one of the key clinical features of rheumatoid arthritis. Bone ruin is also correlated with an increase in liver enzymes and peri-articular osteoporosis.[23] IL-6 is the key element in RA pathophysiology that is released systemically and is responsible for anemia, fatigue and acute phase reactions.[24] IGE was found to restore the hemoglobin levels in the treated animals and normalize their hepatic function. Furthermore, elevated serum C-reactive proteins (CRP) and rheumatic factor (RF) reflect the systemic inflammation, indicating active inflammatory disease. Raised CRP and RF levels also indicate arthritic progression.[25] This work showed a reduction in systemic inflammation by the crude extract IGE as evidenced by low CRP and RF levels.

The elevated levels of prostaglandins, especially PGE2 in the early phase of disease are responsible for the edematous paw swelling in the RA paradigm. The crude extract IGE markedly decreased the serum levels of PGE2 in addition to a conspicuous waning in the expression of TNF-α, IL-1β, IL-6, and COX-II enzyme in contrast to the disease control group. Complete Freund's adjuvant (CFA) triggers the production and discharge of TNF-α, IL-6, and IL-1β from macrophages and monocytes. Subsequently, TNF-α stimulates the emancipation of additional inflammatory mediators such as IL-6 and IL-1β leading to an enhanced transport of leukocytes, infiltration, and vasodilation at the site of edema.[26] Likewise, these proinflammatory cytokines prompt the release of chemokines thus tempting the neutrophils and monocytes towards affected joints. Bone deterioration can be prevented by blocking the proinflammatory cytokines involved in gene expression of matrix metalloproteinases.[27]

Reactive oxygen species (ROS) play a role in the pathogenesis, progression, and worsening of various diseases such as pulmonary fibrosis, neurodegenerative disorders, and rheumatoid arthritis. An imbalance in the synthesis of oxidizing substances and antioxidant enzymes results in oxidative stress.[28] This oxidative stress hurts gene transcription.[29] There is a dropdown in the levels of superoxide dismutase (SOD) and catalase (CAT), whereas the production of malondialdehyde (MDA) is increased in RA. CFA is thought to boost the production of ROS thus provoking the immune cells to release more inflammatory cytokines and enzymes which cumulatively exacerbate the disease.[30] Therefore, it can be predicted that modulation in oxidative stress markers by IGE may be one of the strategic mechanisms to subdue the expression of genes inducing the synthesis of inflammatory cytokines and cyclooxygenase enzyme (COX-II) in RA.

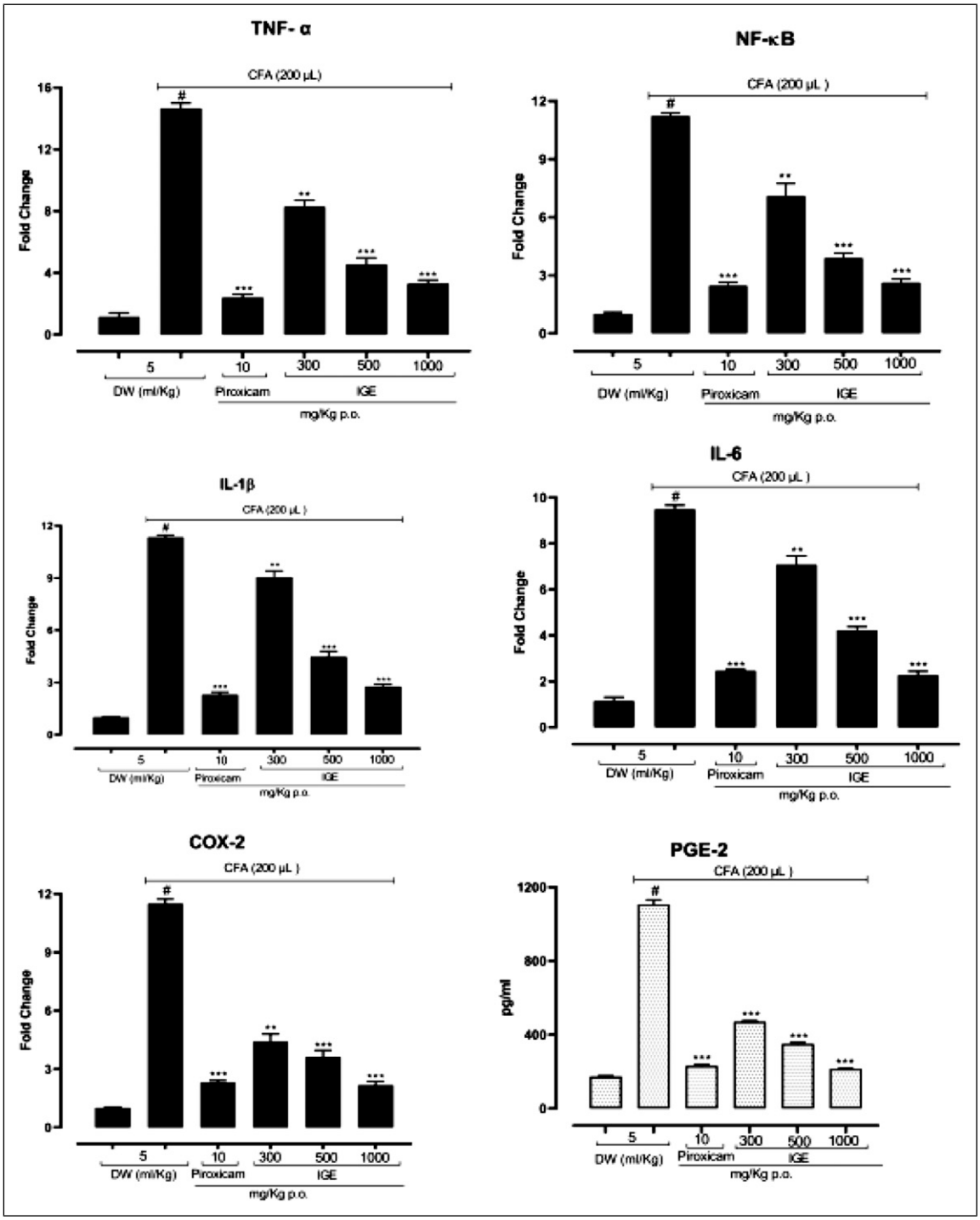

Figure 9. Effects of IGE on gene expression of COX-II, inflammatory cytokines and on serum levels of PGE2 in animals with CFA-induced arthritis. All values (n = 6) are expressed as mean ± SEM using one-way ANOVA followed by Tukey's post hoc test. # = $P \leq .001$ vs normal control while *** = $P \leq .001$, ** = $P \leq .01$, * = $P \leq .05$ vs arthritic control.

Primary phytochemical screening revealed the flavonoids, coumarins, phalabotanins, phenols, terpenes, and glycosides in aqueous methanolic extract of *Geranium wallichianum* (IGE). Previous studies have demonstrated the anti-inflammatory activities of chlorogenic acid,[31] vanillic acid,[32] p-coumaric acid,[33] caffeic acid,[34] kaempferol,[35] syringic acid,[36] and quercetin[37] against acute inflammation. The mitigating effects of IGE on joint inflammation, bone damage, and oxidative stress can be attributed to these phytochemical compounds as the HPLC

analysis confirms the presence of these compounds in crude extract.

Material and Methods

Plant Material

Plant material of *Geranium wallichianum* D. Don ex sweet was collected from hilly areas of Muzaffarabad and Neelam valley, Azad Jammu and Kashmir, Pakistan. After identification by a taxonomist (Department of Botany, University of Gujrat), the plant specimen was submitted in the herbarium of Department of Pharmacology, Faculty of Pharmacy, the Islamia University of Bahawalpur (voucher number: GW-WP-12-21-208).

Preparation of Extract

After shade drying, the plant material was ground into powder and macerated in a 70% aqueous methanolic solution for 3 days thrice. After filtration through a muslin cloth and then filter paper, the filtrate was subjected to vaporization under reduced pressure using rotary evaporator (Heidolph Laborota4000, Germany). A thick semisolid extract was prepared and was preserved at −20°C in an airtight container.[38]

Animals

In this study, either male or female Wistar albino rats (weighing 200 to 300 g) were employed. All of the animals were housed in a 12:12 light/dark cycle with appropriate temperature and humidity controls. The animals were given a standard feed and water *ad libitum*. They were acclimatized to the research Lab environment, one week prior to the start of the experiments. The study protocols were approved by the Pharmacy Animal Ethics Committee of the Islamia University of Bahawalpur (certificate no. PAEC/21/37) in accordance with NIH guidelines (NIH publications 85–23 updated in 2002).[39]

Phytochemical Analysis

Plants are rich source of numerous phytochemical constituents and secondary metabolites. In order to screen for phytochemicals, the aqueous methanolic extract of *Geranium wallichianum* (IGE) was examined through multiple assays. Alkaloids were detected using Mayer's and Wagner's tests while glycosides were recognized via Keller-Kiliani, Liebermann's, and Salkowski's tests. Flavonoids were confirmed by alkaline reagent and lead acetate tests. Ferric chloride and gelatin assays were employed to estimate tannin levels. The Fehling, Molisch, and Benedict assays were performed to evaluate the carbohydrate content. Amino acids and proteins were analyzed through the Ninhydrin and Xanthoproteic assays.[40]

In Vitro Antioxidant Activity by DPPH Assay

The free radical scavenging capacity of a stable 1, 1-diphenyl 2-picrylhydrazyl (DPPH) was used to determine the antioxidant potential of IGE, by following the methodology published by Brand-Williams et al with minor changes. 1 mL of .1 mM DPPH methanolic solution was mixed with 1 mL of IGE solution of varying concentrations (200, 400, 600, 800, 1000, and 1200 g/mL). L-ascorbic acid solution (same amounts) was utilized as a reference standard. A combination of 1 mL DPPH and 1 mL methanol was used as a control. The reaction was carried out 3 times, and each solution was kept in the dark for 30 minutes.[38,41] A decrease in absorbance was determined at 517 nm using the NanoDrop spectrophotometer (Denovix Inc, USA). Per cent inhibition was calculated as under:

$$\%\ Inhibition = \frac{(Ac - As)}{Ac} \times 100$$

Ac is the absorbance of control; whereas, As is the absorbance of the sample.

High Performance Liquid Chromatographic Analysis

The HPLC method was employed to probe the flavonoids and phenolic compounds qualitatively. Fresh solutions of both reference standard and IGE were made in methanol at 50 µg/ml and 10 mg/mL, respectively, and kept at 4°C throughout the experiment. The analysis was performed on a Shimadzu LC10-AT VP Liquid Chromatograph with SIL-20A auto sampler (Shimadzu Scientific Instruments, Kyoto, Japan) and SPD-10AV UV VIS Detector. A Shim-Pack CLC-ODS (C-18, 25 cm 4.6 mm, 5 m) was used for isolation at room temperature. The mobile phase consisted of a binary solvent system which contained solvent A (water: acetic acid-94:6, pH = 2.2) and solvent B (acetonitrile), with the following gradient elution: 0–15 min, 85% A:15% B (linear gradient, v/v), 15–30 min, 55% A:45% B (linear gradient, v/v), and 30–35 min, 0% A:100% B (linear gradient, v/(equilibration). The flow rate was 1.0 mL/min and the detecting wavelength was 280 nm. The retention periods of principal peaks produced by IGE and standard solutions were compared.[42]

Anti-Arthritic Activity of Geranium wallichianum Crude Extract

The animals were divided into several groups with six members in each group. Distilled water (5 mL/Kg p. o.) was given to the normal and arthritic control groups. Piroxicam (10 mg/Kg p. o.) and IGE (300, 500, and 1000 mg/Kg p. o.) were administered to the treatment groups. Rheumatoid arthritis model was developed by injecting (200 µL) the complete Freund's adjuvant (Sigma Aldrich, USA) in subplantar tissues of left paws in animals of all the groups except the

normal control group. Treatment was commenced from the first day of study which continued for 28 days consecutively.[43]

Assessment of Arthritis and Weight Variation

Paws of animals were measured with digital vernier calipers before intoxication and variations in paw size were recorded on days 7, 14, 21, and 28. On day 0, all the animals were weighed ensuing the measurement of weights at the given interval of days. Inflammation intensity was assessed by different symptoms, including heat, rubor, puffiness, tumor growth, and joint flexibility. Visual arthritic score technique was employed to classify the disease as 0, 1, 2, 3, and 4, where 0 represented no swelling or redness, 1 showed mild flush with swelling at metatarsophalangeal joints, 2 specified the flush, bulge, and warmth at interphalangeal joints, 3 indicated the swelling of ankle joints, and 4 reflected the swelling of whole paw with stiffness also affecting the contralateral paw.[4]

Screening of Hematological and Biochemical Parameters

The animals were anesthetized on the last day, and blood samples were drawn using heart puncture technique. An advanced hematology analyzer examined hematological parameters such as WBCs, RBCs, ESR, platelets, and Hb (Sysmex, USA). Hepatic enzymes, alanine transaminase (ALT), alkaline phosphatase (ALP), and aspartate transaminase (AST) as well as renal function markers (serum creatinine and blood urea nitrogen) were also appraised to determine the hepatic and renal status of animals. Systemic inflammatory markers, that is, C-reactive proteins and rheumatic factor (RF) levels were also evaluated by commercially available auto-analyzer kits (Selectra Pro M, France). All the tests were performed adopting the protocols of respective kits.[5]

Estimation of Serum Prostaglandin Level Through ELISA

Animal serum samples were evaluated for quantitative prostaglandin E2 (PGE2) using the enzyme-linked immunosorbent assay technique (E-EL-0034 ELISA kit). In the non-specific binding (NSB) and B0 wells, 100 µL diluent was added, respectively. On the other hand, 100 µL of standard solution and test samples were inserted into suitable wells. Phosphate buffer (50 µL) was introduced to NSB wells only, while all other wells, including NSB, B0, standard, and test samples, received PGE2 alkaline phosphatase solution (50 µL). PGE2 antibody (50 µL) was also added to the B0, standard, and test wells. All the wells reflected a yellow color except B0 wells. After that, the plates were covered and put into a shaking incubator (500 rpm for 2 h at room temperature). After that, the ELISA plates were washed twice using wash buffer. Prostaglandin E2 alkaline phosphatase (5 µL) was added to each activity well. In addition, 200 µL of reagent p-nitrophenyl phosphate was added as a substrate to each well and all the wells were retained at room temperature before adding the stop solution. At a wavelength of 450 nm, the optical density was recorded.[44]

Evaluation of mRNA Expression of Inflammatory Markers (TNF-α, IL-1β, IL-6, NF-kB, and COX-2)

TRIzol reagent was used to isolate RNA, and the yield of RNA was quantified using a NanoDrop spectrophotometer. Then, complementary deoxyribonucleic acid (cDNA) was prepared by reverse transcribing the RNA using cDNA synthesis kit (Vivantis Technologies® Malaysia) and freezed at −20°C. After that, 5 µL qPCR master mix rox (Simply Biologics, South Korea), cDNA (500 ng),0.3 µL gene specific forward, and 0.3 µL reverse primer of each marker were added followed by addition of nuclease-free water (up to 10 µL) in sterile PCR tubes. These tubes were placed in q-PCR and program was started to initiate the reaction. Reaction comprised of enzyme activation, denaturation and annealing. The reaction was completed in 45 cycles and ΔΔCT values were obtained. The comparative gene expression of TNF-α, IL-1β, IL-6, NF-kB, and cyclooxygenase enzyme (COX-II) was obtained estimated in terms of fold changes. The reference gene was GAPDH, and the primer sequences were as shown in Table 1.[4,45]

Determination of Oxidative Stress Biomarkers

Serum was separated from the blood samples drawn at the last day of study to estimate superoxide dismutase (SOD) and catalase (CAT) enzyme activities. Levels of malondialdehyde (MDA) were also quantified.

Activity of Superoxide Dismutase

Total SOD activity in serum was determined using xanthine oxidase method according to SOD kit protocol (E-BC-K020-M). The activity was expressed in units/milliliters.[29]

Activity of Catalase Enzyme

The reaction in which catalase (CAT) decomposed H_2O_2 was rapidly stopped by ammonium molybdate. The residual H_2O_2 reacted with ammonium molybdate and a yellowish complex appeared. CAT activity was calculated by generation the yellowish complex at 405 nm (E-BC-K031-M). The activity of CAT was expressed as units/ml.[30]

Estimation of Malondialdehyde

Malondialdehyde in the catabolite of lipid peroxide reacted with thiobarbituric acid (TBA) and produced red compound

Table 1. Sequence of Primers of Inflammatory Markers.

S. No	Markers	Type	Sequence	Amplicon size	Annealing temperature	Gene Reference/ID
1	COX-II	Forward Reverse	TTAGGTCATCGGTGGAGAGG GAACAGTCGCTCGTCATCCC	217	61.5°C	ENSRNOG00000002525
2	IL-1β	Forward Reverse	AGTCTGCACAGTTCCCCAAC AGACCTGACTTGGCAGAGGA	230	60.5°C	ENSRNOG00000004649
3	IL-6	Forward Reverse	TACCCCAACTTCCAATGCTC ACCACAGTGAGGAATGTCCA	186	58.4°C	ENSRNOG00000010278
4	NF-κB	Forward Reverse	TCACCAAGCAGGAAGATGTG GATAAGGAGTGCTGCCTTGC	161	59.45°C	ENSRNOG00000023258
5	TNF-α	Forward Reverse	CAGGTTCCGTCCCTCTCATA AGAAGAGGCTGAGGCACAGA	170	60.5°C	ENSRNOG00000055156

giving an absorption peak at 532 nm. Level of MDA was expressed as μM/L.[29,46]

Statistical Analysis

All data values were expressed as mean ± standard error mean (SEM). Difference between control and treatment groups was estimated at Graphpad Prism version 7.0 and comparison was performed using two-way and one-way analysis of variance (ANOVA) followed by Tukey's test.

Conclusion

This work substantiates the *Geranium wallichianum* D. Don ex sweet as a prospective drug to diminish the inflammation and burden of reactive oxygen species in immune-mediated arthritis, authenticating its use in rheumatism and joint disorders in the native populace.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

The study protocols were approved by Pharmacy Animal Ethics Committee of the Islamia University of Bahawalpur (certificate no. PAEC/21/37). The NC3Rs ARRIVE rules were followed while performing the animal research.

Availability of Original Data

All the data generated during this study is available and will be provided on reasonable request.

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