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

Qaiser Jabeen, PhD¹, Syed Ihtisham Haider, BS¹,², Awais Asif, PhD³, Rubina Rasheed, PhD³, Shaheen Gul, BSc¹, and Shafia Arshad, BSc⁴

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 ≤ .05) and 1000 mg/Kg (P ≤ .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 ≤ .001) in treated animals. TNF-α, interleukins, and COX-II mRNA expression were also significantly reduced at the doses of 300 (P ≤ .05), 500 (P ≤ .01) and 1000 mg/Kg (P ≤ .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 proteins⁴ triggering the activation of monocytes and migration of neutrophils and macrophages at the site of

¹ Department of Pharmacology, Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur, Pakistan
² Department of Pharmacology, Nawaz Sharif Medical College, University of Gujrat, Gujrat, Pakistan
³ Department of Biochemistry, Nawaz Sharif Medical College, University of Gujrat, Gujrat, Pakistan
⁴ University College of Conventional Medicines, Faculty of Medicine and Allied Health Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan

Received 11 March 2022; accepted 20 June 2022

Corresponding Author:
Shafia Arshad, University College of Conventional Medicines, Faculty of Medicine and Allied Health Sciences, The Islamia University of Bahawalpur, Khwaja Fareed Campus, Railway Road, Bahawalpur 63100, Pakistan.
Email: shafia.arshad@iub.edu.pk

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).
inflammation. Progressing inflammatory process results in an upsurge in the levels of TNF-α, interleukins and nuclear factor NF-κB. 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. 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.

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. 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. 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. Different studies have demonstrated the striking potential of crude extracts of plants to curtail the inflammation and bone damage in RA.

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. Geranium wallichianum is traditionally used to manage gonorrhea, arthritis, bone pain, gout, and sciatica. 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.
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 $(3.875 \pm .077)$ in arthritic control animals. IGE significantly curtailed the arthritic index at 1000 and 500 mg/Kg $(2.183 \pm .131$ and $2.383 \pm .060$, respectively) compared to the effect of piroxicam $(1.638 \pm .095)$. 

**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
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 \((36.225 \pm 1.125\) and \(34.832 \pm 1.683\), 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
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 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 \((11.234 ± .157\text{-folds})\). Treatment with IGE at 1000 \((2.619 ± .197\text{-fold})\), 500 \((3.900 ± .252\text{-fold})\), and 300 mg/Kg \((7.095 ± .655\text{-fold})\) and piroxicam \((2.467 ± .177\text{-fold})\) reduced this rise in NF-kB in arthritic rats (Figure 9). An exaggerated COX-2 expression \((P < .001)\) occurred in the arthritic control group \((11.504 ± .237\text{-fold})\). Treatment of arthritic rats with IGE at 1000 \((P < .001)\), 500 \((P < .01)\), and 300 mg/Kg \((P < .001)\) 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 \((9.488 ± .177\text{-fold})\) 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 \((14.655 ± .237\text{-fold})\).
± .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. 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 ≤ .001 vs normal control while *** = P ≤ .001, ** = P ≤ .01, * = P ≤ .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.\textsuperscript{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.\textsuperscript{19}  

Geranium wallichianum D. Don ex sweet belongs to the Geraniaceae family and is known as Ratanjot (also Srazela) in native people.\textsuperscript{14} This plant is traditionally used to manage
rheumatism, general weakness, gout, and sciatic pain. 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. Edema is produced due to tissue injury with subsequent migration of macrophages, leukocytes, mast cells, and extravasation of small blood vessels.

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. IL-6 is the key element in RA pathophysiology that is released systemically and is responsible for anemia, fatigue and acute phase reactions. 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. 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. 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.

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. This oxidative stress hurts gene transcription. There is a dropout 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. 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 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 ≤ .001 vs normal control while ### = P ≤ .001, ** = P ≤ .01, * = P ≤ .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, vanillic acid, p-coumaric acid, caffeic acid, kaempferol, syringic acid, and quercetin 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.

**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).

**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.

**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. A decrease in absorbance was determined at 517 nm using the NanoDrop spectrophotometer (Denovix Inc, USA). Per cent inhibition was calculated as under:

\[
\text{% Inhibition} = \left(\frac{Ac - As}{Ac}\right) \times 100
\]

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.

**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 autoanalyzer 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 of 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 freezeed at −20°C. After that, 5 μL of qPCR master mix (Simply Biosciles, 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 nuclelease-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 ΔACT 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 H2O2 was rapidly stopped by ammonium molybdate. The residual H2O2 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 was expressed in units/milliliters.29

Estimation of Malondialdehyde

Malondialdehyde in the catabolite of lipid peroxide reacted with thiobarbituric acid (TBA) and produced red compound
giving an absorption peak at 532 nm. Level of MDA was expressed as \( \mu \text{M/L}. \)

### 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.

### Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article. This is PhD student work so all the facilities and chemicals provided by Department of Pharmacology, Faculty of Pharmacy, The Islamia University

### 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.

### ORCID iD

Qaiser Jabeen [https://orcid.org/0000-0002-9838-2937](https://orcid.org/0000-0002-9838-2937)

### References

1. Hasan UH, Shahzad M, Jahan S, et al. (2019). Inhibitory effects of clematis orientalis aqueous ethanol extract and fractions on inflammatory markers in complete freund’s adjuvant-induced arthritis in sprague-dawley rats. *Inflammopharmacology*. 27: 781-797.
2. Lad H, Bhatnagar D. Amelioration of oxidative and inflammatory changes by swertia chirayita leaves in experimental arthritis. *Inflammopharmacology*. 2016;24:363-375.
3. Blin JA, Ali RM, Nurdin A, Abd Hamid R. Quinone-rich fraction of ardisia crispa (thunb.) a. Dc roots alters angiogenic cascade in collagen-induced arthritis. *Inflammopharmacology*. 2021;29:1-18.
4. Ahsan H, Irfan HM, Shahzad M, Asim MH, Akram M, Zafar MS. Anti-rheumatic activity of pseudoephedrine (a substituted phenethylamine) in complete freund’s adjuvant-induced arthritic rats by down regulating il-1\( \beta \), il-6 and tnf-\( \alpha \) as well as upregulating il-4 and il-10. *Inflammopharmacology*. 2021:1-10.
5. Shabbir A, Shahzad M, Ali A, Zia-Ur-Rehman M. Discovery of new benzothiazine derivative as modulator of pro-and anti-inflammatory cytokines in rheumatoid arthritis. *Inflammation*. 2016;39:1918-1929.
6. Bala A, Mondal C, Haldar PK, Khandelwal B. Oxidative stress in inflammatory cells of patient with rheumatoid arthritis: Clinical efficacy of dietary antioxidants. *Inflammopharmacology*. 2017;25:595-607.
7. Shabbir A, Shahzad M, Ali A, Zia-Ur-Rehman M. Anti-arthritic activity of n\(^{4}\)-(2, 4-dihydroxyphenyl) methylidine-2-(3, 4-dimethyl-5, 5-dioxidoypyrazolo [4, 3-c] [1, 2] benzothiazin-1 (4h)-yl) acetohydrazide. *European Journal of Pharmacology*. 2014;738:263-272.
8. Datta S, Kundu S, Ghosh P, De S, Ghosh A, Chatterjee M. Correlation of oxidant status with oxidative tissue damage in patients with rheumatoid arthritis. *Clinical Rheumatology*. 2014; 33:1557-1564.
9. Piloto A, Sancarlo D, Addante F, Scarcelli C, Franceschi M. Non-steroidal anti-inflammatory drug use in the elderly. *Surg Oncol*. 2010;19:167-172.

### Table 1. Sequence of Primers of Inflammatory Markers.

| S. No | Markers | Type | Sequence | Amplicon size | Annealing temperature | Gene Reference/ID |
|-------|---------|------|----------|---------------|-----------------------|------------------|
| 1     | COX-II  | Forward | TTAGGTCACTCGGTTGGAGG | 217 | 61.5°C | ENSRN0G0000002525 |
|       |         | Reverse | GAAAGTCGCTGTCATCCC  |              |                       |                  |
| 2     | IL-1\( \beta \) | Forward | AGTCTCAGACAGTTCCCAAC | 230 | 60.5°C | ENSRN0G0000004649 |
|       |         | Reverse | AGACCTGGACTGGGAGAAGG |              |                       |                  |
| 3     | IL-6    | Forward | TACCCCAACTCTCAATGCTC | 186 | 58.4°C | ENSRN0G0000010278 |
|       |         | Reverse | ACCACGGAGGGAATGTCCA  |              |                       |                  |
| 4     | NF-\( \kappa \)B | Forward | TCACCAAGCAAGGAGATGTG | 161 | 59.45°C | ENSRN0G0000023258 |
|       |         | Reverse | GATAAGGAGTGTGCCTTGC  |              |                       |                  |
| 5     | TNF-\( \alpha \) | Forward | CAGGCCTCGTGCCCTCTATA  | 170 | 60.5°C | ENSRN0G0000055156 |
|       |         | Reverse | AGAAGGCTCTGGGACAGA  |              |                       |                  |
10. Oyeleke SA, Ajayi AM, Umukoro S, Aderibigbe A, Ademowo OG. Anti-inflammatory activity of theobroma cacao l. Stem bark ethanol extract and its fractions in experimental models. *J Ethnopharmacol*. 2018;222:239-248.

11. Afsar T, Khan MR, Razak S, Ullah S, Mirza B. Antipyretic, anti-inflammatory and analgesic activity of acacia hydaspica r. Parker and its phytochemical analysis. *BMC Compl Alternative Med*. 2015;15:1-12.

12. Padmanabhan P, Jangle S. Evaluation of in-vitro anti-inflammatory activity of herbal preparation, a combination of four medicinal plants. *Int J Basic Appl Med*. 2012;2:109-116.

13. Perianayagam JB, Sharma S, Joseph A, Christina A. Evaluation of anti-pyretic and analgesic activity of emblica officinalis gaertn. *J Ethnopharmacol*. 2004;95:83-85.

14. Shaheen S, Bibi Y, Hussain M, et al. A review on geranium wallichianum d-don ex sweet: An endangered medicinal herb from Himalaya region. *Med Aromatic Plants*. 2017;6:2167-2412.

15. Abbasi BA, Iqbal J, Mahmood T, Ahmad R, Kanwal S, Afridi S. Plant-mediated synthesis of nickel oxide nanoparticles (nio) via geranium wallichianum: Characterization and different biological applications. *Materials Research Express*. 2019;6:0850a7.

16. Qureshi RA, Ghufran MA, Gilani SA, Yousaf Z, Abbas G, Batool A. Indigenous medicinal plants used by local women in southern himalayan regions of pakistan. *Pakistan J Bot*. 2009;41:19-25.

17. Shabbir A, Batool SA, Basheer MI, et al. Ziziphora clinopodioides ameliorated rheumatoid arthritis and inflammatory paw edema in different models of acute and chronic inflammation. *Biomed. Pharmacother*. 2018;97:1710-1721.

18. Foyet HS, Tsala DE, Bodo JZE, Carine AN, Heroyne LT, Oben EK. Anti-inflammatory and anti-arthritic activity of a methanol extract from vitellaria paradoxa stem bark. *Pharmacog Res*. 2015;7:367.

19. Anilkumar M. 10. Ethnomedicinal plants as anti-inflammatory and analgesic agents. *Ethnomedicine: A source of complementary therapeutics*. 2010:267-293.

20. Khan MA, Khan MA, Hussain M, Mujtaba G. Medicinal plants used in folk recipes by the inhabitants of himalayan region poonch valley azad kashmir (pakistan). *J Basic Appl Sci*. 2012;8:35-45.

21. Kim W, Park S, Choi C, et al. Evaluation of anti-inflammatory potential of the new ganghwaljetongyeum on adjuvant-induced inflammatory arthritis in rats. *Evidence-Based Complementary and Alternative Medicine*. 2016;1-10.

22. Allam R, Anders H-J. The role of innate immunity in autoimmune tissue injury. *Current opinion in rheumatology*. 2008;20:538-544.

23. Thite AT, Patil RR, Naik SR. Anti-arthritis activity profile of methanolic extract of ficus bengalensis: Comparison with some clinically effective drugs. *Biomed. Aging Pathol*. 2014;4:207-217.

24. Ogata A, Kato Y, Higa S, Yoshizaki K. Il-6 inhibitor for the treatment of rheumatoid arthritis: A comprehensive review. *Modern rheumatology*. 2019;29:258-267.

25. Kumar V, Verma A, Ahmed D, Sachan NK, Anwar F, Mujeeb M. Fostered antiarthritic upshot of moringa oleifera lam. Stem bark extract in diversely induced arthritis in wistar rats with plausible mechanism. *Int J Pharma Sci Res*. 2013;4:3894-3901.

26. Voon F-L, Sulaiman MR, Akhtar MN, et al. Cardamonin (2′, 4′-dihydroxy-6′-methoxyhalcone) isolated from boesenbergia rotunda (l) mansf. Inhibits cfa-induced rheumatoid arthritis in rats. *Eur. J. Pharmacol*. 2017;794:127-134.

27. Srirangan S, Choy EH. The role of interleukin 6 in the pathophysiology of rheumatoid arthritis. *Ther. Adv. Musculoskelet. Dis*. 2010;2:247-256.

28. Phull A-R, Nasir B, Ul Haq I, Kim SJ. Oxidative stress, consequences and ros mediated cellular signaling in rheumatoid arthritis. *Chemico-biological interactions*. 2018;281:121-136.

29. Akhtar MF, Khan K, Saleem A, Baig MMFA, Rasul A, Abdel-Daim MM. Chemical characterization and anti-arthritic appraisal of monotheca buxifolia methanolic extract in complete freund’s adjuvant-induced arthritis in wistar rats. *Inflammopharmacology*. 2021;29:393-408.

30. Saleem A, Saleem M, Akhtar MF, Shahzad M, Jahan S. Polysichum braunii extracts inhibit complete friend’s adjuvant-induced arthritis via upregulation of il-4, il-10, and il-12, downregulation of cox-2, pge2, il-1β, il-6, n-fkb, and tnf-α, and subsiding oxidative stress. *Inflammopharmacology*. 2020;28:1-16.

31. Chen D, Pan D, Tang S, et al. Administration of chlorogenic acid alleviates spinal cord injury via tlr4/nf-kb and p38 signaling pathway anti-inflammatory activity. *Molecular medicine reports*. 2018;17:1340-1346.

32. Ziadiou R, Barbero A, Martin I, et al. Anti-inflammatory and chondroprotective effects of vanillic acid and epimedin c in human osteoarthritic chondrocytes. *Biomolecules*. 2020;10:932.

33. Pragasam SJ, Venkatesan V, Rasool M. Immunomodulatory and anti-inflammatory effect of p-coumaric acid, a common dietary polyphenol on experimental inflammation in rats. *Inflammation*. 2013;36:169-176.

34. Choi HG, Tran PT, Lee J-H, Min BS, Kim JA. Anti-inflammatory activity of caffeic acid derivatives isolated from the roots of salvia miltiorrhiza bunge. *Archives of pharmacal research*. 2018;41:64-70.

35. Wang J, Fang X, Ge L, et al. Antitumor, antioxidant and anti-inflammatory activities of kaempferol and its corresponding glycosides and the enzymatic preparation of kaempferol. *PLoS One*. 2018;13:e0197563.

36. Ham JR, Lee H-I, Choi R-Y, Sim M-O, Seo K-I, Lee M-K. Anti-steatotic and anti-inflammatory roles of syringic acid in high-fat diet-induced obese mice. *Food & function*. 2016;7:689-697.

37. Rogerio A, Kanashiro A, Fontanari C, et al. Anti-inflammatory activity of quercetin and isoquercitrin in experimental murine inflammation in rats. *Food & function*. 2018;9:1340-1346.

38. Rasheed HMF, Rasheed F, Qureshi AW, Jabeen Q. Immunomodulatory and anti-inflammatory activities of theobroma cacao l. Stem bark. *BMC Compl Alternative Med*. 2016;186:244-250.
activities of ethanolic extract of indigofera argentea burm. F. J Ethnopharmacol. 2020;259:112966.

40. Akhtar M, Sharif A, Saleem M, et al. Genotoxic and cytotoxic potential of alternanthera bettzickiana, an important ethnomedicinal plant. Cellular and Molecular Biology. 2017;63:109-114.

41. Brand-Williams W, Cuvelier M-E, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT–Food Sci Technol. 1995;28:25-30.

42. Saleem M, Ali HA, Akhtar MF, Saleem U, Saleem A, Irshad I. Chemical characterisation and hepatoprotective potential of cosmos sulphureus cav. And cosmos bipinnatus cav. Nat Prod Res. 2019;33:897-900.

43. Saleem A, Saleem M, Akhtar MF. Antioxidant, anti-inflammatory and antiarthritic potential of moringa oleifera lam: An ethnomedicinal plant of moringaceae family. South Afr J Bot. 2020;128:246-256.

44. Uttra AM, Shahzad M, Shabbir A, Jahan S. Ephedra gerardiana aqueous ethanolic extract and fractions attenuate freund complete adjuvant induced arthritis in sprague dawley rats by downregulating pge2, cox2, il-1β, il-6, tnF-a, nf-kb and upregulating il-4 and il-10. J Ethnopharmacol. 2018;224:482-496.

45. Coura CO, Souza RB, Rodrigues Ja. G, et al. Mechanisms involved in the anti-inflammatory action of a polysulfated fraction from gracilaria cornea in rats. PLoS One. 2015;10:e0119319.

46. Ratheesh M, Shyni GL, Helen A. Methanolic extract of ruta graveolens l. Inhibits inflammation and oxidative stress in adjuvant induced model of arthritis in rats. Inflammopharmacology. 2009;17:100-105.