**Sarcococca saligna** Hydroalcoholic Extract Ameliorates Arthritis in Complete Freund’s Adjuvant-Induced Arthritic Rats via Modulation of Inflammatory Biomarkers and Suppression of Oxidative Stress Markers

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**ABSTRACT:** Traditionally, *Sarcococca saligna* has been used for the treatment of arthritis and many other inflammatory disorders. The current study was planned to give scientific evidence to this traditional use of *S. saligna*. Phytochemical profiling of SSME was carried out by using electrospray ionization mass spectrometry (ESI-MS/MS). Complete Freund’s adjuvant (CFA), 150 μL was injected in the subplantar region of the left hind paw to induce arthritis in rats. Aqueous methanolic extract of *S. saligna* (SSME) was administered orally at 250, 500, or 1000 mg/kg dose from the 7th day to the 28th day of the study to explore its anti-arthritic potential. Histopathological and radiographic assessment of joints and enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (RT-PCR) analyses were performed. Determination of oxidative stress biomarkers in the serum was also carried out. ESI-MS/MS identified ten such phytoconstituents which have reported strong anti-inflammatory and anti-arthritic activity. The SSME extract considerably reduced paw inflammation and arthritic index, subdued cachexia, and significantly improved biochemical and hematological changes. Oxidative stress decreased in SSME administered rats dose-dependently. Histopathological and radiographic evaluations also showed the anti-arthritic activity of SSME, which was associated with the downregulation of tumor necrosis factor (TNF)-α, nuclear factor (NF)-κB, COX-2, interleukin (IL)-6, and IL-1β and upregulation of I-kB, IL-4, and IL-10, in contrast to disease group rats. The outcomes of the study proposed that *S. saligna* have anti-arthritic potential, supporting its traditional use for rheumatoid arthritis treatment.

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune condition that leads to chronic inflammation of the synovium. This disease mainly disturbs various joints, especially little diarthrodial joints linked with big joints and their surrounding extra-articular tissues. All these little progressions eventually conclude in the clinical representation of disease in terms of pain, swelling, inflexibility in the joints of the knee and ankle, and difficulty in movement, due to modified bone structure at later disease stages. Internationally, the prevalence of RA is about 2−4%. This disorder has dynamic epidemiology and ethnicity throughout the world. Females are discriminatingly more vulnerable to this disorder compared to males. However, the definite etiology and associated risk factors accelerating the progression of RA are still elusive. RA is associated with a pathologically raised level of pro-inflammatory mediators like interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF-α), cyclooxygenases (COX), and lipo-oxygenase, with decreased level cytokines IL-10 and IL-4. Pro-inflammatory transcription factor, nuclear-factor-kappa B (NF-κB), principally participates in immunological reactions like swelling and cell propagation and markedly elevates in an RA patient’s serum. It is evident from previous findings that free radicals and reactive oxygen species (ROS) are also linked with the development of this disorder. RA is a chronic persistent disorder usually immedicable and treated symptomatically by nonsteroidal anti-inflammatory drugs (NSAIDS). The use of these medicines is generally linked with apparent adverse effects like ulceration, hypertension, and stroke. Therefore, safe alternative, natural treatment strategies are an unmet clinical need these days. Despite the progressive

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development in medical sciences and technology of the modern era, the global demand for plant-based therapeutics is on the rise.6

S. saligna is a member of the Buxaceae family; it is also called the sweet box or Christmas box, native to northern Pakistan.7 Whole plants of S. saligna and its various aerial portions are conventionally utilized for a variety of medical problems.8 In Pakistan, people use this plant for the treatment of hyperactive states of the gastrointestinal tract, syphilis, infections, fever, pain, liver diseases, inflammation, and rheumatism.9 It is also used for the treatment of malaria and many dermatological diseases.10 The leaves and twigs are simmered and applied in the form of a poultice on swollen joints or to relieve pain. The syrup is made from roots and used to treat gonorrhea.11

Usually, the leaves of S. saligna are applied as an aperient, a blood cleanser, and to treat myalgia. Some therapeutic effects of this plant also have been stated in previous literature, like cardiosuppressants, vasodilators, tracheal relaxants, antispasmodics, antiinflammatories, calcium antagonists, acetylcholinesterase inhibitors, and many others.12

Previous literature has reported the existence of multiple bioactive alkaloids, flavonoids, and polyphenolics. These bioactive compounds as preliminary constituents are responsible for the therapeutic potential. However, the S. saligna remedial activities against arthritis are not substantially reported. The current study was intended to investigate the mechanistic pathways targeted by S. saligna aqueous methanolic extract to mitigate arthritis caused by complete Freund adjuvant (CFA) in the rodent model.

2. RESULTS

Electrospray Ionization Mass Spectrometry (ESI/MS/MS) of S. saligna Aqueous Methanolic Extract. The SSME was run in the fusion mixture of chloroform and methanol in a 1:1 percentage. Sample injection was directly inserted. The complete picture information by using the negative mode of ionization is shown in Figure 1. Ten phytochemical compounds were detected in negative ion mode. These identified compounds were then arranged in the mass spectra of high resolution based on their accurate masses and isotopic arrangement. Then, search outcomes were confirmed further based on their MS/MS patterns. In the meantime, the MS/MS arrays were explored in suitable ESI mass spectral data libraries like Pub Chem MS library, NIST MS library, and human metabolome database (HMDB) along with the comparison with accessible literature data (Figure 1, Table 1).

In Vivo Evaluation of Anti-Arthritic Potential of SSME. Determination of Inflammation of Paw. Injection of the inducing agent in the subplantar region produced marked inflammation after 1 week. Paw volume and swelling in the disease group were persistently increased from day 8 to day 28. Treatment with aqueous methanolic extract of plant significantly decreased (p < 0.001) paw inflammation in rats in contrast to rats of the disease group. The best results were obtained with a 1000 mg/kg dose (Figure 2).

Determination of Body Weight. The decline in the values was determined to be caused by inflammation in the disease group from the first week until the fourth week, while SSME extract (dose-dependently) and diclofenac sodium considerably (p < 0.001) restored the weight of animals (Figure 3).

Arthritic Index. In the control group, swelling or inflammatory symptoms were not observed, while the consistent increase in the arthritic index in the disease group was noted (Figure 4). Administration of SSME and diclofenac sodium markedly (p < 0.001) reduced the values of the arthritic index in contrast to disease group animals. The highest value of arthritic index was observed on day 28 in disease group animals (5.80 ± 0.20), with decreased value by SSME at dose of 1000 mg/kg (1.00 ± 0.00), 500 mg/kg (1.20 ± 0.20), and 250 mg/kg (1.80 ± 0.20) and diclofenac sodium (2.20 ± 0.20) treatments.

Histopathological Evaluation of Joints. Histopathological assessment of joints revealed the significant pannus development and hyperplasia of synovial space. Cartilage annihilation, migration of inflammatory cells, and attrition of bones in disease group rats were noted compared to control group animals. SSME (250, 500, and 1000 mg/kg) treatment mitigated the pathological changes like pannus development, migration, or undefined movement of inflammatory cells, hyperplasia of synovial space, and erosion of bones compared with disease group animals (Figure 5). The effect of the highest dose of SSME (1000 mg/kg) on bone destruction,

Figure 1. ESI MS/MS spectrum of aqueous methanolic extract of S. saligna (SSME) in negative mode of ionization (mass range m/z 50–550).
| probable compound name | molecular formula | molecular mass (g/mol) | retention time (min) | observed precursor (m/z) | major fragmentation ms² (m/z) | pharmacological activities |
|-------------------------|-------------------|------------------------|---------------------|-------------------------|-----------------------------|--------------------------|
| Threonic acid           | C₄H₈O₅            | 135.20                 | 0.98                | 59, 71.08, 75, 87, 89, 98.92, 115, 117, 118.42, 135.08, 136.17, 161.08, 165.92 | 51.84, 59.01, 71, 73.02, 75, 87, 135, 91.05 | anti-arthritic activity, helps in early diagnosis of arthritis |
| Cinnamic acid           | C₉H₈O₂            | 147.16                 | 1.22                | 57.17, 60.25, 71, 75, 85.08, 88.25, 101, 103, 115, 117.17, 129, 130.83, 147, 151.33 | 146.8, 103 | Antioxidant activity, anti-arthritic activity |
| Caffeic acid            | C₉H₈O₄            | 179                    | 1.72                | 71.08, 75.08, 81.08, 87, 89, 99, 112.50, 120.92, 124.92, 135.08, 143, 156.17, 161.08, 163.92, 179.08, 188.17 | 178.9, 134.9, 149.2, 179.2, 223, 135, 181.1, 161, 137, 135.1 | Anti-inflammatory activity, antioxidant activity |
| Quercetin               | C₁₅H₁₀O₇          | 301.20                 | 2.47                | 98.83, 99.42, 121, 122.58, 139, 151.08, 154.75, 183.08, 201.08, 213.17, 215.17, 239.08, 241.25, 247, 257.08, 265, 268.08, 283.25, 286.17, 301, 303.08 | 106.97, 120.97, 150.92, 178.9, 192.94, 256.9, 272.92, 300.97, 110.9, 121.3, 151.1, 149.3, 163.3, 179.3, 201.1, 229.1, 301, 273.5, 184.9, 284.8, 257 | Anti-inflammatory activity, antioxidant activity |
| 5-p-coumaroyl quinic acid | C₁₆H₁₈O₈         | 337.20                 | 2.93                | 112.92, 129, 157, 175, 205.08, 218.83, 244.92, 265.25, 277.08, 301.17, 319.17, 337.08, 339.17 | 163, 173.1, 275.3, 319, 337, 298.8, 173.1 | Antioxidant activity, anti-inflammatory activity |
| p-Coumaric acid         | C₉H₈O₃            | 164.16                 | 6.88                | 57.08, 60.92, 70.92, 75, 82.92, 166.33, 87, 93, 101.08, 104.92, 116.92, 121, 129, 134.92, 147, 153, 164.92 | 117, 120.9, 163.5, 162 | Immunomodulatory and anti-inflammatory activity |
| Pachyaximine            | C₂₅H₄₁NO          | 359.6                  | 3.49                | 12.92, 135.25, 179.08, 187, 196.92, 209.08, 240.92, 255.17, 269.17, 297.17, 315.08, 323.25, 341.17, 359.25, 361.92 | 285, 447, 287.05, 288.05, 289.05 | Anti-inflammatory activity, antinociceptors |
| Kaempferol              | C₂₅H₂₁O₁₁         | 448.4                  | 4.35                | 133.83, 153.08, 163, 177, 192.92, 207.08, 219.33, 236.67, 267.08, 285, 297.17, 315.17, 327.17, 341.17, 357.25, 368.92, 403.25, 411.25, 429, 447.17 | 447.09, 285.03, 448.09, 192.92, 284.03, 286.04, 133.9, 284.8, 257 | Anti-inflammatory activity |
| Ferulic acid            | C₁₀H₁₀O₄          | 194.18                 | 7.26                | 129, 71, 75, 85, 97, 99, 110.92, 122.83, 147, 141, 159.08, 164.83, 177, 179.08, 195.08, 197 | 134.0, 178.02, 193.05, 149.05, 175.04, 135.03, 137.02 | Antioxidative and anti-inflammatory activity |
| Quercitrin              | C₂₅H₂₀O₁₁         | 447.30                 | 8.76                | 152, 177.08, 285.08, 315.17, 341.17, 403.25, 411.25, 429.25 | 300.02, 447.09, 301.03, 271.0, 243.03, 151.80 | Anti-inflammatory and anti-arthritic activity |
pannus development, and paw inflammation was comparable with diclofenac sodium.

**Radiographic Findings.** Radiographic examination (Figure 6) showed severe soft tissue swelling, obvious joint space congestion, stiffened connective tissue, rigorous bone attrition, joint abnormality, and resorption in the disease group in contrast to the control group. Diclofenac sodium administered rats exhibited the recovery of soft tissue inflammation, bone resorption and corrosion, congestion of joint spacing, and significant development of surrounding muscles of the joint.

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**Figure 2.** Effects of *S. saligna* aqueous methanolic extract on paw diameter of CFA-induced arthritic rats. Results are presented as mean ± SEM (n = 5) and analyzed by two-way ANOVA followed by Bonferroni’s post-test. “a” and “b” show the statistically significant difference (*p* < 0.05) as compared to disease and standard group, respectively.

**Figure 3.** Effects of *S. saligna* aqueous methanolic extract on body weight of CFA-induced arthritic rats. Results are presented as mean ± SEM (n = 5) and analyzed by two-way ANOVA followed by Bonferroni’s post-test. “a” and “b” show the statistically significant difference (*p* < 0.05) as compared to disease and standard group, respectively.
Similarly, changes observed in radiography evaluation were amended in SSME groups. SSME at 1000 mg/kg dose exhibited better results.

**Hematological and Biochemical Analysis.** The CFA injection markedly elevated the concentrations of ESR, platelets, aspartate aminotransferase (AST), blood urea nitrogen, urea, creatinine, ALP, ALT, CRP, and RF. SSME treatment considerably ($p < 0.001$) recovered the concentration of these parameters. The levels of RF and CRP variables were considerably raised ($37.6 \pm 3.04$ and $12.98 \pm 0.36$, respectively) in the disease group rats (Table 2), although SSME treatment decreased RF and CRP values in SSME (1000 mg/kg) treated rats ($21 \pm 1.81$ and $3.26 \pm 0.27$). Platelets, aspartate aminotransferase (AST), blood urea nitrogen, urea, creatinine, ALP, and ALT were significantly elevated in disease group rats. However, SSME significantly reduced the levels of these parameters in rats with SSM at 1000 mg/kg. RBCs, eosinophils and Hb levels were reduced in disease group rats in comparison with control group animals.

**Figure 4.** Effects of *S. saligna* aqueous methanolic extract on the arthritic index of CFA-induced arthritic rats. Results are presented as mean ± SEM ($n = 5$) and analyzed by two-way ANOVA followed by Bonferroni’s post-test. “a” and “b” show the statistically significant difference ($p < 0.05$) as compared to disease and standard group, respectively.

**Figure 5.** Effects of *S. saligna* aqueous methanolic extract on histopathological analysis of ankle joints in arthritic rats injected by complete Freund’s adjuvant at 40× and 20× magnification. (A) control; (B) disease; (C) rats treated with the standard drug; (D–F) paw histology of rats administered with aqueous methanolic extract at 250, 500, and 1000 mg/kg, respectively.
SSME dose-dependently ameliorated the abnormal hematological and biochemical parameters (Table 2).

**Effect on Serum Concentration of BCI2, HSP-70, IL-6, and TNF-α.** Marked elevation ($p < 0.001$) in the level of IL-6 ($728.68 \pm 16.50$ pg/mL) was observed in animals of the disease group, and decrease in values ($351.31 \pm 22.57$ pg/mL) were noted at SSME 1000 mg/kg. The raised level of BCI2 ($p < 0.001$) has been observed in the disease group ($27.28 \pm 1.28$ ng/mL), but it was considerably reduced in the 1000 mg/kg SSME treatment group ($14.99 \pm 0.95$ ng/mL). Similarly, a marked rise ($p < 0.001$) in the serum level of HSP-70 has been significantly reduced in the disease group ($1.09 \pm 0.12$ ng/mL); however, SSME 1000 mg/kg ($0.152 \pm 0.004$ ng/mL) and standard drug ($0.17 \pm 0.004$ ng/mL) significantly reduced HSP-70 level. The results showed an elevated serum concentration ($p < 0.001$) of TNF-α in the rats of the disease group ($521.56 \pm 8.39$ pg/mL) with a considerable decrease by SSME treatment dose (Figure 7).

**Effect of SSME on mRNA Expression of Inflammatory Biomarkers.** The gene expression study was performed after the completion of the experiment by taking blood samples. The mRNA expression levels of NF-κB, COX-2, IL-6, and IL-1β were significantly elevated ($p < 0.001$) in CFA treated animals (Figure 8). SSME treatment similar to diclofenac sodium considerably ameliorated the raised level of these inflammatory biomarkers, dose-dependently.

However, CFA treatment notably ($p < 0.001$) decreased the expression level of IL-4, IL-10 and IκB in the disease group. SSME treatment produced a dose-dependent effect and recovered the decline in these markers similar to diclofenac sodium in the treatment groups (Figure 8).

**Effect on Oxidative Stress.** Figure 9 depicted that CFA injection significantly increased oxidative stress ($p < 0.001$) by

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**Table 2. Effects of Aqueous Methanolic Extract of *S. saligna* on Hematological and Biochemical Parameters in CFA-Induced Arthritic Rats**

| parameters               | control group | disease group | standard group | SSME (250 mg/dL) | SSME (500 mg/dL) | SSME (1000 mg/dL) |
|--------------------------|---------------|---------------|----------------|------------------|------------------|-------------------|
| Hb (g/dL)                | 13.02 ± 0.34a | 10.8 ± 0.51b  | 13.94 ± 0.37a  | 11.3 ± 0.2b      | 12.6 ± 0.18a     | 14.875 ± 0.42a    |
| ESR (mm/first hour)      | 9 ± 0.54a     | 24.92 ± 0.55b | 15.8 ± 0.86a   | 18.2 ± 0.25a     | 7.8 ± 0.2a       | 7.8 ± 0.66a       |
| RBCs (x10^12/L)          | 7.46 ± 0.49a  | 4.55 ± 0.26b  | 8.57 ± 0.38a   | 5.274 ± 0.19b    | 5.622 ± 0.33b    | 8.18 ± 0.41232a   |
| Eosinophils (%)          | 3.2 ± 0.73a   | 6.8 ± 0.37a   | 3.4 ± 0.50a    | 3.8 ± 0.37a      | 3.4 ± 0.4a       | 3.2 ± 0.58a       |
| Platelets (x10^12/L)     | 658.2 ± 2.83b | 1876 ± 17.52b | 875.8 ± 15.81a | 1764.2 ± 45.55ab | 1568.6 ± 22.42ab | 1091.8 ± 21.74ab  |
| AST (U/L)                | 82.6 ± 0.87a  | 211.6 ± 0.87a | 113.6 ± 0.81a  | 129.6 ± 1.07a    | 121 ± 0.31a      | 115.8 ± 0.8a      |
| ALP (U/L)                | 226 ± 5.66b   | 414.8 ± 1.71a | 230.6 ± 13.13a | 318.6 ± 2.48ab   | 291.4 ± 6.18a    | 229.6 ± 4.37a     |
| ALT (U/L)                | 34 ± 1a       | 57.2 ± 0.26a  | 38 ± 1.87a     | 47.8 ± 1.82a     | 41.2 ± 1.15a     | 39.6 ± 1.50a      |
| Urea (mg/dL)             | 13 ± 0.44a    | 29.8 ± 0.86b  | 14.8 ± 0.37a   | 17.8 ± 1.06a     | 15.6 ± 0.24a     | 14.8 ± 0.37a      |
| Creatinine (mg/dL)       | 0.584 ± 0.06b | 1.93 ± 0.02b  | 0.364 ± 0.02a  | 0.834 ± 0.01b    | 0.698 ± 0.05ab   | 0.362 ± 0.02b     |
| CRP (mg/L)               | 5.8 ± 0.52a   | 12.98 ± 0.36b | 4.32 ± 0.22a   | 10.22 ± 0.06b    | 7.88 ± 1.74a     | 3.26 ± 0.27a      |
| RF (IU/mL)               | 15.76 ± 0.56a | 37.6 ± 3.04a  | 22.4 ± 1.50a   | 30.4 ± 1.43b     | 28.6 ± 0.74a     | 21 ± 1.81a        |
| BUN (mg/dL)              | 16.2 ± 0.37a  | 2.99 ± 0.70a  | 17.4 ± 0.24a   | 26.2 ± 0.2b      | 18.6 ± 0.24a     | 9.2 ± 0.2a        |

“Results are presented as mean ± SEM (n = 5) and analyzed by one-way ANOVA followed by Tukey’s Multiple Comparison Test. “a” and “b” shows the statistically significant difference ($p < 0.05$) as compared to disease and standard group, respectively. Hb: hemoglobin; ESR: erythrocyte sedimentation rate; RBCs: red blood cells; AST: aspartate aminotransferase; ALP: alkaline phosphatase; ALT: alanine aminotransferase; CRP: C reactive protein; RF: rheumatoid factor; BUN: blood urea nitrogen; HDL: high-density lipoprotein; LDL: low-density lipoprotein; SSME: *S. saligna* aqueous methanolic extract.”
decreasing the levels of SOD and CAT (18.22 ± 1.12 and 48.16 ± 2.0 U/mg protein) in the disease group, SSME, and diclofenac sodium treated groups. An increased level of MDA was also noticed in the rats of the disease group (0.56 ± 0.02 μM/g protein) at \( p < 0.0001 \) in contrast to the control group (0.13 ± 0.01 μM/g protein); however, the level of MDA was considerably decreased by standard drug, 500 mg/kg and 1000 mg/kg dose of SSME in arthritic animals.

3. DISCUSSION

Rheumatoid arthritis is clinically expressed through a series of persistent chronic progressions such as leukocyte infiltration, pannus development, and extreme mutilation of bone and cartilage. Conventional methods of RA treatment have improved this condition in terms of efficacy. Nonsteroidal anti-inflammatory drugs like etoricoxib; disease-modifying antirheumatic drugs such as hydroxychloroquine, leflunomide, sulphasalazine, and methotrexate; and corticosteroids like methylprednisolone and prednisolone have severe side effects.29 Traditional or herbal therapies are being used extensively by people in the whole world because they produce better results with fewer side effects.30

In the current study phytochemical analysis of S. saligna was done by using ESI-MS/MS. The anti-arthritis and anti-inflammatory potential of plant extracts probably linked with their phenolic compounds was also monitored. The extract was produced by using aqueous and acidified concentrations of methanol. MS displayed ten phenolics including threonic acid, kaempferol, quercetin, caffeic acid, 5-P-coumaroyl quinic acid, pachyoxime-A, p-cumaric acid, 5-P-coumaroyl quinic acid, and Pachyoxime-A decrease the pain by inhibiting the production of IL-6 and TNF-α.31 Ferulic acid, caffeic acid, and cinnamic acid have the potential of anti-inflammatory and antioxidant ability by delaying activation of NF-κB and expression of TNF-α.32 Quercetin acid has the potency to reduce the clinical presentation of inflammation. It prevents the production of NO, IL-6, and PGE2. It also reduces the level of CRP.33 Kaempherol belongs to flavonol, and it shows a potent anti-inflammatory effect.26

Previous animal studies have shown that rheumatoid arthritis occurs when protein denaturation starts at the site of administration of CFA which leads to the generation of auto-antigens and showed symptoms like paw edema, joint pain, alteration in joint structure, and reduction in weight.34 Arthritis produced by CFA is a dual-phase process; the first phase is called the acute phase or induction phase, which remains for 10 days, and in this state the release of prostaglandins G, serotonin, and histamine by the immune system occurs, but there is no validation of synovitis. Later, in the chronic phase, which remains for 11 to 28 days, the disturbance in pro-and anti-inflammatory mediators is expressed as infiltration, synovitis, hypertrophy, and bone erosion.35

Paw edema is related to vascular penetrability, cellular permeation, and increased fluid discharge in the affected area of inflammation.36 Assessment of paw diameter is a common tool for estimation of efficacy of anti-arthritis medicines.37 The reduction in paw diameter demonstrates reduced release of inflammatory mediators which is a key sign of anti-

Figure 7. Effect of S. saligna aqueous methanolic extract on inflammatory mediators: BCI2, HSP-70, IL-6, and TNF-α. Results are presented as mean ± SEM \((n = 5)\) and analyzed by Tukey's multiple comparison post-test and two-way ANOVA followed by Bonferroni's. “a” and “b” show the statistically significant difference \((p < 0.05)\) as compared to disease and standard group, respectively. SSME, S. saligna aqueous methanolic extract.
inflammatory action of a medicine or plant extract.\textsuperscript{38} In the disease group, inflammation was sustained for 4 weeks due to cellular incursion and persistent fluid and salt accumulation. SSME presented a prominent reduction in paw thickness compared to disease group animals. Results were compared with the previously performed studies where the use of \emph{B. calliobotrys} and \emph{P. amarus} extracts decreased the paw size.\textsuperscript{39} Similarly, reduced arthritic scoring (also called arthritic index)

\begin{figure}[h]
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\includegraphics[width=\linewidth]{figure8.png}
\caption{Effect of \emph{S. saligna} aqueous methanolic extract on mRNA expression: NF-kB, COX-2, IL-6, TNF-\(\alpha\), IL-1\(\beta\), IL-4, IL-10, and I-kB. Results are presented as mean ± SEM (\(n = 5\)) and analyzed by one-way ANOVA followed by Tukey’s post-test. “a” and “b” shows statistically significant differences (\(p < 0.05\)) as compared to disease and standard group, respectively; SSME, \emph{S. saligna} aqueous methanolic extract.}
\end{figure}
manifested its anti-inflammatory immunosuppressive activities. Our results of the arthritic index are similar to previously performed research work by Uttra et al., who determined that the maximum decrease in the scoring of arthritis was seen when aqueous ethanolic plant extract was used. RA is related to reduced body weight during the disease course which is called RA associated cachexia. Previous published data suggested that reduction in body weight is linked with swelling because it obstructs absorption through the gut, while treatment with drugs that lower the inflammation initiates better absorption. S. saligna extract improves body weight through downregulation of TNF-α and by decreasing swelling. The high levels of TNF-α, HSP-70, BCI2, and IL-6 were observed in the serum samples of disease group animals, while these mediators were considerably decreased in SSME and standard drug diclofenac sodium groups after treatment. Many studies have proposed that in the pathogenesis of RA HSP-70, BCI2 plays an important role. BCI2 is mainly involved in the apoptosis process of RA, while TNF-α, IL-1α, and IL-6 induce hsp70 expression during the disease.

Our outcomes of PCR are similar to the findings of Hassan et al., which showed significant downregulation of important inflammatory biomarkers by using different fractions and extract formulations of plant and piroxicam. Similarly, significantly decreased levels of IL-4, IL-10, and I-kB and significant upregulation of COX-2, TNF-α, IL-1β, and NF-kB were detected in disease group animals. Administration of methanol and aqueous extracts of M. rivae and piroxicam changed the concentrations of these mediators in favor of treatment of RA. IL-1β excites the production of enzymes like matrix metalloproteinase and osteoclast stimulation and causes bone damage. It also promotes the resorption of bone and the production of autoantibodies. Osteoclast stimulation is produced by NF-kB that provokes synovial apoptosis and erosion of bone. It also acts as an immunoregulator by regulating IL-6, IL-1β, and nitric oxide synthase. High levels of NF-kB intensify arthritic symptoms. The level of I-kB was reduced while there was an increase in the concentration of NF-kB in rats of the disease group; however, the reverse response was detected in SSME treated rats. NF-kB antagonists have curing activity against RA. Inflammatory mediators promote COX-2 and lead to high PGE2 levels at the synovium. SSME decreased angiogenesis and synovitis via the low level of COX-2 and PGE2. IL-4 antagonizes the production of Th1 and favors the development of the Th2 cell. IL-10 blocks Th1 cell-mediated cytokines and prevents the mRNA expression of IL-18. It is also involved in compromised functions of antigen-presenting cells and maintains the joint stability. SSME significantly decreased the levels of NF-kB, IL-6, TNF-α, IL-1β, and COX-2, but innocently upregulated I-kB, IL-10, and IL-4 concentrations.

Figure 9. Effect of S. saligna on oxidative stress biomarkers: SOD (superoxide dismutase), catalase, and MDA (malondialdehyde). Results are presented as mean ± SEM (n = 5) and analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. ‘a’ and ‘b’ show the statistically significant difference (p < 0.05) as compared to disease and standard group, respectively. SSME, S. saligna aqueous methanolic extract.
So, increased levels of immune-regulatory cytokines decreased concentrations of inflammatory mediators, and breakdown of arachidonic acid might be a mechanism liable for the SSME anti-arthritic activity.

Changes in biochemical and hematological parameters were also seen during this study. The low concentrations of Hb and RBC generally lead to anemia. The mechanism behind this is bone marrow defect, low concentration of erythropoietin, and erythrocyte loss produced by the increased level of IL-1/β in arthritic animals. S. saligna and the standard drug remarkably improved the levels of these parameters by reducing the levels of IL-1/β. Moreover, the increase in platelet level is due to stimulation of the immune process which is linked with the development of TNF-α and IL-1/β in the disease group. The reduction of these biomarkers in the SSME treatment groups showed the immunoregulatory function of S. saligna. Correspondingly, an increased level of CRP and RF was measured in the arthritic rats, while standard drug and S. saligna produced a reduction in these parameters, which showed the shielding effect of the extract.52 Previously performed research work has acknowledged that cytokines like IL-6, TNF-α, and IL-1/β stimulate the development of RF and CRP, while SSME decreased the concentration of these, leading to improvement in RA condition.53 Increased ALP, AST, and ALT concentrations are connected with raised bradykinin levels which leads to liver damage, and the role of ALP is related to the destruction of the bone and is a parameter to assess lysosomal integrity. SSME and diclofenac sodium significantly decreased the values of these parameters, which may enhance the integrity of lysosomes and inhibit bone annihilation, as shown by radiographic analysis in the present study. Increase in the levels of urea and creatinine was noticed which may enhance the integrity of lysosomes and inhibit bone annihilation.

Increase in the levels of SOD, CAT, and MDA in arthritic rats compared to the normal group. The standard laboratory conditions, i.e., temperature: 25 °C, humidity: 55%, and 12 h dark and light cycles, were maintained in the animal house. They had free access to food and water.

It was determined that CAT and SOD concentrations were reduced, while MDA level was increased in disease group rats. However, diclofenac sodium and SSME recovered (p < 0.001) the levels of SOD, CAT, and MDA in arthritic rats compared to arthritic rats. Similarly, a previously reported study presented that the level of these parameters was moved to normal levels in rats treated with extract of S. anacardium. Oxidative stress disturbs gene transcription. CFA enhances the level of inflammatory mediators and increases oxidative stress that leads to stimulation of immune cells and generates enzymes and cytokines to aggravate RA.56

The results of the CFA-induced arthritis model such as the decrease in paw diameter, arthritic index, improvement in hematological and biochemical factors, and histopathological and radiographic study along with the outcomes of ELISA and qRT-PCR are in favor of the plant’s anti-inflammatory and anti-arthritic activity.

5. MATERIALS AND METHODS

Plant Collection and Extraction. Fresh plant S. saligna was collected in September 2020 from the area of Abaspur, Azad Jammu and Kashmir, Pakistan. The plant was identified and authenticated by Dr Irfan Mehmood, associate professor at boys degree college Abaspur AJK. Voucher sample AJKH-21021 had been issued for the crude plant to the Azad Jammu Kashmir Medicinal & Aromatic Plants Herbarium for future reference. The aerial parts of the plant were rinsed with tap water and dried under shade for 1 week, and then ground to a fine powder.

Microwave extraction technology was used for the preparation of plant extract. This procedure has three main phases. The voltage of the microwave was set at 9000 W. In the first phase, 750 mL of aqueous methanol (20:80) was poured into a 1000 mL beaker that had 100 g of plant powder. The beaker was put into the oven for 2 min, and then for 30 s, its door was kept open. This process was repeated five times. Two more cycles were performed using the same procedure with 500 mL aqueous methanol (20:80) in the beaker. The extract was filtered first with the muslin cloth; then Whatman filter paper was used. Excess solvent was removed from the filtrate with rotary evaporation at 40 °C. The S. saligna aqueous methanolic extract (SSME) was stored in an amber-colored bottle until analysis.

Experimental Animals. Thirty healthy male and female Wistar rats (weighing 150 to 200 g) were kept in the animal house of the Govt. College University, Faisalabad-Pakistan. The standard laboratory conditions, i.e., temperature: 25 ± 2 °C, humidity: 55 ± 5%, and 12 h dark and light cycles, were maintained in the animal house. They had free access to food and water.

Ethical Approval. The experimental work was carried out after getting approval from the institutional review board, Government College University, Faisalabad (GCUF). The reference number issued was GCUF/ERC/2200. This study was conducted under the recommendations of the National Institute of Health regarding laboratory rodent ethics.

Electrospray Ionization Mass Spectrometry Study. ESI-MS/MS was carried out by using a Linear Ion Trap Mass Spectrometer (Thermo Scientific, USA) equipped with an electrospray ionization (ESI) machine. The test sample was investigated by direct injection into the syringe pump at a 13 μL/min flow rate in the negative mode of ionization of ESI source. The temperature of the capillary was set at 198 °C. A comprehensive scan of mass spectrum data was attained in the mass range of 50–2000 m/z. The final ionization produced was fragmented in the ion source and fragmented by collision-induced dissociation energies (CID) in the 16–32 range based on the constancy of the obvious forerunner ions identified for cyclic mass spectrometry.58

In Vivo Assessment of the Anti-Arthritic Potential of SSME against Complete Freund’s Adjuvant (CFA)-induced arthritis was performed under the guidelines of the National Institute of Health regarding laboratory rodent ethics.
Table 3. List of Primers Used in qRT-PCR

| biomarkers | forward/reverse | sequence | gene accession no. |
|------------|----------------|----------|--------------------|
| COX-2      | Forward        | 5-ATCAGGTCTACCTCGTGGAGACG-3′ | S67722.1 |
|            | Reverse        | 5-CTCGTCTATAGGATTAAGGAGGAGCTG-3′ |          |
| NF-kB      | Forward        | 5-AGATGTTGGTGAGGATCTGTAAG | NM_01276711.1 |
|            | Reverse        | 5-GAGTCCTGACATTCCATCGACGAGG-3′ |          |
| IL-1β      | Forward        | 5-AGTGGAGTTGAAGGAGGAGCTG-3′ | NM_031522.2 |
|            | Reverse        | 5-GAGGCCTGACATTCCATCGACGAGG-3′ |          |
| IL-6       | Forward        | 5-TCAGAGTGTTGAGGAGGAGCTGTAAG | M2674.1 |
|            | Reverse        | 5-GAGTCCTGACATTCCATCGACGAGG-3′ |          |
| IL-10      | Forward        | 5-CAGGGTTGGCTCTCCATAGTGA-3′ | L0926.1 |
|            | Reverse        | 5-AACATAGCGGATCCATCCAGGAGG-3′ |          |
| 1-κβ       | Forward        | 5-GAGTTGAGTTGAAGGAGGAGCTG-3′ | NM_030867.2 |
|            | Reverse        | 5-TAGCCTGACATTCCATCGACGAGG-3′ |          |
| IL-4       | Forward        | 5-AGCTATTTGAGGGTGCTACGAG-3′ | NM-201270.1 |
|            | Reverse        | 5-TGACCTGAGGTTCAAGGAGGAGCTG-3′ |          |
| TNF-α      | Forward        | 5-GGTTGCTTCTTACAGAGCCGAGGAGG-3′ | X66539.1 |
|            | Reverse        | 5-TCTCAGATTTGAGGGTGCTACGAGGAGG-3′ |          |
| GAPDH      | Forward        | 5-GCTCTCAGAGGAGGAAGACG-3′ | AF106860.2 |
|            | Reverse        | 5-GATGTTATTCGAGGAAGGAGG-3′ |          |

Induced Arthritis. Experimental Design for Anti-Arthritic Activity. Arthritis was induced by injecting 150 μL CFA in the subplantar region of the left hind paw in all the rat groups except the normal control group. Oral administration of the standard drug and the extract was started after the 7th day of CFA injection until the 28th day. LD₅₀ of SSME was greater than 1000 mg/kg.³⁹

Thirty rats were randomly divided into six groups (n = 5). Group I served as control and received only vehicle. Group II served as disease. Group III was designated as standard, and diclofenac sodium (10 mg/kg/day) was administered orally. Groups IV–VI were treatment groups, receiving aqueous methanolic plant extract at 250, 500, and 1000 mg/kg doses orally, respectively.

Evaluation of Arthritis by Physical Parameters. The weight of animals was noted before starting the study, on the 3rd day, and then weekly until the 28th day of study, and paw thickness was determined in terms of paw diameter by using a digital Vernier caliper and recorded on days 3, 7, 14, 21, and 28 after CFA injection.

Arthritis Index. The intensity of the disease was determined in the hind paw and scored from 0 to 4; i.e., 0 = no inflammation, while 1 to 4 are expressed as minimal swelling, swelling of the ankle and surrounding joints, intense swelling of ankle joints, and swelling of the entire paw.⁵⁰

Histopathological Analysis. For histopathological analysis, the ankle joints were excised and kept in 10% formalin.₆⁰

Radiography. The hind limbs of slaughtered animals had been detached from knee joints and were subjected to radiographic examination.₅¹

Haematological and Biochemical Analyses. After the completion of the study after 4 weeks, the rats were killed through slight chloroform anesthesia, and blood was collected through a cardiac puncture. An automatic hemocytometer was used to evaluate blood parameters like hemoglobin (Hb), white and red blood cells, erythrocyte sedimentation rate (ESR), and platelet count, whereas the biochemical parameters like Aspartate transaminase (AST), Alanine transaminase (ALT), urea, Alkaline phosphatase (ALP), creatinine, C-reactive protein (CRP), and Rheumatoid factor (RF) were determined by following the equipment manufacturer’s instructions of the auto analyzer.

Effect on Serum Concentration of BCl2, HSP, IL-6, and TNF-α. ELISA was performed for quantitative analysis of TNF-α (Elabscience, catalogue number E-EL-H0109), HSP-70 (Elabscience, catalogue number E-EL-R0479), BCl2 (Elabscience, catalogue number E-EL-R0096), and IL-6 (Elabscience, catalogue number E-EL-H0102) by using commercial relevant kits. ELISA reader (BI, 800TS-UV) was used to measure the absorbance at 450 nm.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Quantitative assessment of pro-inflammatory and anti-inflammatory mediators and metabolic enzymes in the blood samples of experimental rats was performed by qRT-PCR. Blood was taken from animals and kept in tubes containing EDTA, and the TRIzol process was applied to isolate RNA from samples of blood. The purity and final yield were determined by Nanodrop. The cDNA was developed via reverse transcription by applying the protocol of the kit manufacturer. For magnification and quantitative analysis, the Sybr green master mix (2X) kit standard procedure was used on qRT-PCR on the Bio-Rad machine. 1 μL each pair of primers, 7 μL nuclease-free water, 1 μL cDNA, and 10 μL Sybr mix were poured into the individual microplate well. Then, it was shifted to a machine called thermal cycler set at 45 cycles of denaturation at the temperature of 95 °C, annealing at 60 °C and termination at 72 °C. Housekeeping gene and primers were formulated by using primer 3, primer quest, and Genbank.⁶¹ The list of primers used in qRT-PCR is shown in Table 3.

Determination of Oxidative Stress Biomarkers. The blood was taken and serum was separated for the evaluation of the oxidative stress biomarkers by using suitable kits like CAT (Elabscience, catalogue number E-BC-K106), SOD (Elabscience, catalogue number E-BC-K020), and MDA (Elabscience, catalogue number E-EL-0060). ELISA reader model number BI, 800TS-UV was used to determine the absorbance.

Statistical Analysis. All results were expressed as mean ± SEM. Data were analyzed with GraphPad Prism version 5.00. One-way ANOVA followed by Tukey’s post-hoc test was applied to analyze inflammatory mediators measured by.
ELISA, the mRNA gene expression of inflammatory biomarkers, and oxidative stress biomarkers. Two-way ANOVA with Bonferroni posthoc test was applied to assess the paw diameter, scoring, body weight, and hematological and biochemical analysis of CFA induced arthritis.

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Author Contributions

US, BA, and MAS designed and supervised the study. MF, ZC, MM, MQ performed the experiments. MF, US, and MAS drafted the manuscript. IA, RHA, and GMS analyzed the results and reviewed and edited the final draft.

Notes

The authors declare no competing financial interest.

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