

Estragole Ameliorates CFA Induced Rheumatoid Arthritis Symptoms in Wistar Rats by Inhibiting JAK-2/STAT-3 Pathway

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Summary

The present study was conducted to scrutinize the pharmacological effect of Estragole (ESG) against CFA-induced arthritis in rats. The rats underwent induction of arthritis using the administration of CFA and after that, the rats were randomly divided into five different groups, where three groups correspond to diverse dosages of ESG, and the other two were control and CFA-arthritic control. Results of the study suggested that ESG in a dose-dependent manner, improves body weight and arthritis score of rats as evidenced by reduction of hind-paw volume. ESG also improved the antioxidant status of rats by reducing MDA levels and enhancing the concentration of endogenous antioxidants SOD and GPx. The level of pro-inflammatory cytokines was also found to be reduced in the case of ESG treated group as compared to CFA-group. In a western blot analysis, ESG showed downregulation of p-JAK-2/STAT-3. The study provided concrete evidence for the protective effect of ESG against rheumatoid arthritis in rats.

Key words

Arthritis • Oxidative stress • Inflammation • Western blot analysis

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Introduction

Around the world, 18 million people had rheumatoid arthritis (RA) as of 2019 [1]. It is an autoimmune illness that affects the entire body and is distinguished by systemic chronicity. As a result of the damage to the lining of the joints, there may be excruciating swelling that may ultimately lead to bone erosion and joint deformity [2]. Although the pathophysiology of RA is still unknown, evidence points to the involvement of endocrine, immune, infectious, and hereditary variables in the development of the illness.

According to numerous documented epidemiological investigations performed across the Chinese mainland, the overall incidence of RA in China is estimated to be 0.2 to 0.93 % [3]. As a result, the burden of RA is among the top 10 main chronic illnesses in China.

Due to multiple etiological factors, the therapeutic management of RA has mostly focused on alleviating symptoms, including inflammation, preventing tissue damage, and eventually maintaining function. Analgesics, Non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, Disease-modifying antirheumatic drugs (DMARDs), and anti-cytokines, taken singly or in combination, are currently available treatment alternatives; nonetheless, they are insufficient to treat RA [4]. Therefore, finding an appropriate drug that may successfully treat RA symptoms is urgently needed.

Estragole (ESG) is a phenylpropanoid chemically known as methyl-chavicol and is considered to be an isomer of anethole, differing with respect to the location of the double bond. Although impure samples can appear yellow, it is a colorless liquid, found in various trees and plants, including turpentine (pine oil), anise, fennel, bay, tarragon, and basil. Previous studies reported that ESG showed significant antioxidants [5], and antilipase [6], and blocks neuronal excitability *via* inhibiting the Na⁺ channel [7]. It also showed an anti-inflammatory effect *via* inhibiting leukocyte migration and by stimulating macrophage phagocytosis [8]. It also showed anti-edematogenic effects in carrageenan-induced edema in Swiss mice [9]. But this study does not provide any detailed pharmacological benefit and mechanism underlying the anti-arthritic potential of ESG. Therefore, prompted by the above, the present study aimed to investigate the pharmacological benefit and possible mechanism of ESG in rheumatoid arthritis.

Methods

Chemicals

The chemicals including the Estragole (>98 %) was obtained from the Sigma Aldrich, USA unless otherwise stated.

Animals

Before the experiment, Healthy Sprague-Dawley male rats, 8-10 weeks old and weighing 150-200 g were taken from the institutional animal house and housed in a strictly sanitary environment with a 12/12 h light/dark cycle at room temperature and *ad libitum* access to food and drink.

CFA-induced arthritis in rats

The 0.1 ml of CFA (Complete Freund's adjuvant-heat killed Mycobacterium tuberculosis suspended in paraffin oil and mannide monooleate 1 mg/ml; Invivo Gen, USA) was subcutaneously administered to SD rats into the footpad of the left hind paw. The rats were further administered 0.1 ml of CFA on the same day, and the next day into the tail for a booster dose [10]. An increased immune response is triggered by CFA, which results in regional erythema and edema. Additionally, CFA generates an inflammatory reaction three hours after the injection, which can last for up to four weeks. The group that was given the vehicle received a single subcutaneous injection of (0.1 ml) PBS

in the left hind paw, but there was no inflammation-inducing substance in the solution. As an internal control for the inflammatory experiment, phosphate-buffered saline (PBS) was injected subcutaneously into the right hind footpads of each rat that was given an injection. The volume of the injection was 0.1 ml.

Treatment group

The groups will be as follows

- Group 1: Control (Normal Saline for 28 days)
- Group 2: CFA (administered with CFA with no treatment)
- Group 3: CFA + ESG (2 mg/kg for 28 days)
- Group 4: CFA + ESG (5 mg/kg for 28 days)
- Group 5: CFA + ESG (10 mg/kg for 28 days)

Determination of Hind Paw Volume (HPV)

An YLS-7A volume meter from the Shandong Academy of Medical Sciences Equipment Station in Shandong, China, was utilized in order to track the progression of the HPV in order to investigate the severity of the arthritis condition.

Estimation of oxidative stress biomarkers

The joint tissues were placed in cold saline (1:10, w/v), and then homogenized with a homogenizer machine. Next, the supernatant was obtained through centrifuging at 3000 rpm/min for detecting the concentration of malondialdehyde (MDA, No. S0131), superoxide dismutase (SOD, Kit No. S0101), and Glutathione Peroxidase (GPx, No. S0056) in joint tissues were estimated using the kits from Beyotime Biotechnology Co. (Shanghai, China).

Estimation of cytokines

The level of various cytokines tumor necrosis factor- α (TNF- α , R&D Systems, Minneapolis, MN, USA; Kit No. PMTA00B), interleukin-1 β (IL-1 β , RayBiotech, Peachtree Corners, GA, USA; Kit No. ELM-IL1b-1), and IL-6 (R&D Systems, Minneapolis, MN, USA; Kit No. PD6050) in joint tissues were recorded using as per the protocols supplied with the kits.

Western blot analysis

After the rats were euthanized, the synovium of knee joints was isolated and cut into pieces. The samples were snap-frozen in liquid nitrogen, homogenized, and lysed in ice-cold RIPA buffer (0.1 % phenylmethylsulfonyl fluoride) and then centrifuged at 12000 \times g for

20 min. The protein concentration was determined by the BCA kit (Beyotime Biotechnology, Nanjing, China) according to the manufacturer's instructions. The protein extracts were loaded in a 10 % SDS PAGE and then transferred onto a PVDF membrane. The membrane was blocked by skimmed milk, then incubated with primary antibody for overnight at 4 °C. The membrane was then washed with TBST, and incubated with a HRP conjugated secondary antibody (1:10,000) for 1 h at room temperature. The antibody-reactive bands were visualized using the ECL-chemiluminescence system.

Docking analysis

The docking of ESG was performed with JAK2 (PDB:2B7A), using the default setting of the CB-Dock tool. It is designed to perform blind docking only at the places that have been predicted, rather than throughout the full surface of a protein. Consequently, the initial step is to locate potential binding sites, often known as cavity detection. Cavity sorting is the process by which we choose a few top cavities for further research based on cavity size. This is done because ligand binding sites are often larger cavities. After that, we calculate the docking center and make any necessary adjustments to the size of the docking box. The molecular docking process with AutoDock Vina necessitates the inclusion of these parameters (Center and Size). The docking process is finished, and then the bound poses are reranked according to the docking score (this process is referred to as Dock and Rerank). It has been determined that the first conformation offers the best binding pose and that the site that corresponds to it is the most effective binding site for the query ligand.

Statistical analysis

Data are expressed as means \pm standard error of the mean (SEM) and evaluated using one-way ANCOVA. Data considered significant at a $P < 0.05$.

Results

Effect of ESG on the HPV of the CFA rats

In the beginning, the antiarthritic impact of ESG was determined using the hind paw volume of the rats in the CFA-induced arthritis model. As seen in Figure 1, the CFA-treated rats had HPV levels whose levels were found significantly higher than the control group. In addition, the paw edema brought on by CFA was noticeably decreased by ESG at the selected dosages (2, 5, and 10 mg/kg). ESG may have had a dose-dependent anti-arthritis effect in

rats, according to this theory.

Effect of ESG on the Body-weight of the CFA rats

In the next study, rat body weight was measured after a seven-day delay between the commencement of the experiment and its conclusion to further support the preventive effect of ESG against CFA-induced arthritis. Until the end of the trial, or after 28 days, the CFA-treated rats' weights had significantly decreased, as seen in Figure 2. However, the body weight among the rats treated with ESG improved dose-dependently, reaching its peak in the 10 mg/kg treatment group. Therefore, it has been hypothesized that ESG significantly reduced arthritis in CFA rats.

Effect of ESG on the arthritis score in CFA rats

The arthritic index was determined for each animal in several treatment groups, and a score was given to each group in order to determine the severity of CFA-induced arthritis, Figure 3. To determine their arthritic index, nodules and inflammation on their paws, noses, tails, and ears were examined. The CFA rats showed the greatest arthritic index and inflammation, both of which were indicated by a score of more than 4. The involvement of paws other than the one that received the CFA injection is indicated by an arthritic index greater than 4.

Effect of ESG on the pro-inflammatory cytokines in CFA rats

In this part of the study, we aimed to investigate the effect of ESG on the various pro-inflammatory cytokines (e.g. TNF- α , IL-1 β , IL-6) in the serum of rats. As shown the Figure 4, the CFA rats showed significantly higher levels of these above cytokines as compared to the control. However, the ESG-treated rats showed a significant reduction in the serum level of these cytokines as compared to the arthritic control CFA group. This finding highlights the anti-inflammatory effect of ESG on CFA rats possibly *via* reduction of pro-inflammatory cytokines.

Effect of ESG on the Oxidative stress biomarkers in CFA rats

The CFA rats were used to measure the impact of ESG on oxidative stress in rats, and the results are presented in Figure 5. When compared to rats treated with control, the CFA-treated rats experienced lower levels of GSH and SOD in addition to higher levels of MDA. Furthermore, after taking ESG, it was discovered that the level of these investigated biomarkers had

returned to nearly normal, confirming the antioxidant action of ESG.

Effect of ESG on the JAK2 using Western blot analysis

Western blot analysis was conducted to scrutinize the effect of ESG on the levels of p-JAK2 and p-STAT3. The results have been shown in Figure 6. It has been found that the level of these two tested genes was found to abruptly elevated in the CFA group. However, the level of these genes was found significantly restored near to normal in the ESG-treated group. This restoration was found dose-dependent, with maximum activity, was achieved in the case of 10 mg/kg treated group.

Docking of ESG with JAK2

The last part of the study aimed to study the interaction of ESG with JAK2 (PDB: 2B7A), and the results have been enumerated in Table 1. Whereas, Figure 7, and 8 depicted 3D and 2D interaction diagrams of ESG with JAK2. It has been found that ESG showed an excellent docking score with JAK as confirmed by

Vina score of -6.0. It also showed binding with critical amino acid residues, such as LEU983, VAL863, ALA880, TYR931, LEU855, LEU932, and MET929.

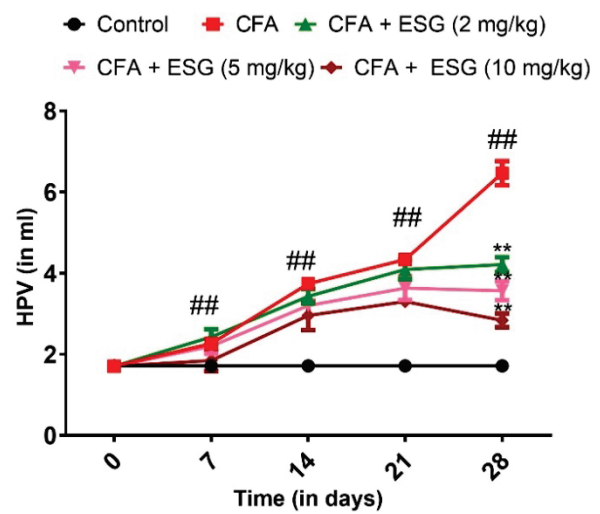


Fig. 1. Effect of ESG on the hind paw volume (HPV) of Wistar rats. ##P<0.05 vs. control and **P<0.01 vs. CFA group. Data are presented as means ± SEM.

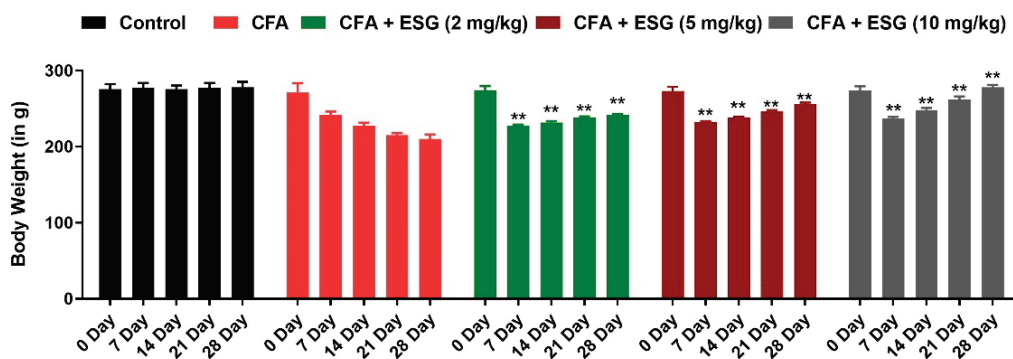


Fig. 2. Effect of ESG on body weight of Wistar Rats. **P<0.01, vs. CFA group.

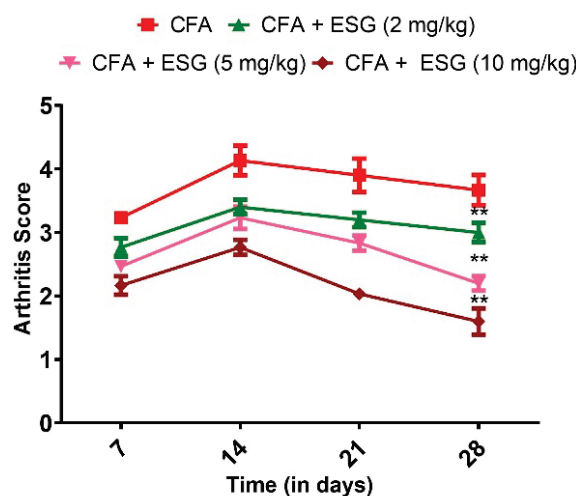


Fig. 3. Effect of ESG on arthritis score of the Wistar rats. ##P<0.05 vs. control and **P<0.01 vs. CFA group. Data are presented as means ± SEM.

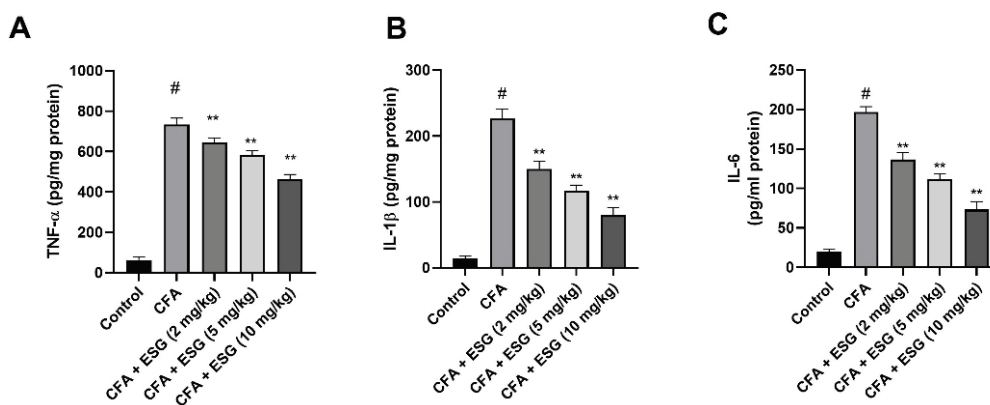


Fig. 4. Effect of ESG on the pro-inflammatory cytokines. [#]P<0.05 vs. control and ^{**}P<0.01 vs. CFA group. Data are presented as means \pm SEM.

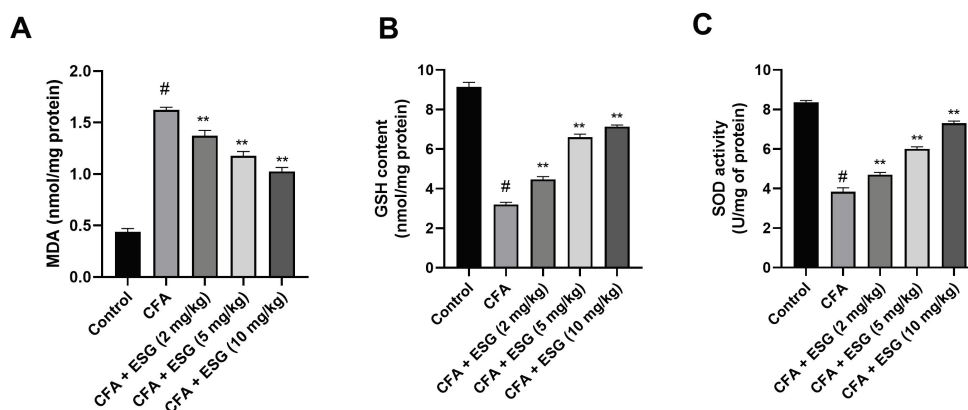


Fig. 5. Effect of ESG on the oxidative stress biomarkers. [#]P<0.05 vs. control and ^{**}P<0.01 vs. CFA group. Data are presented as means \pm SEM.

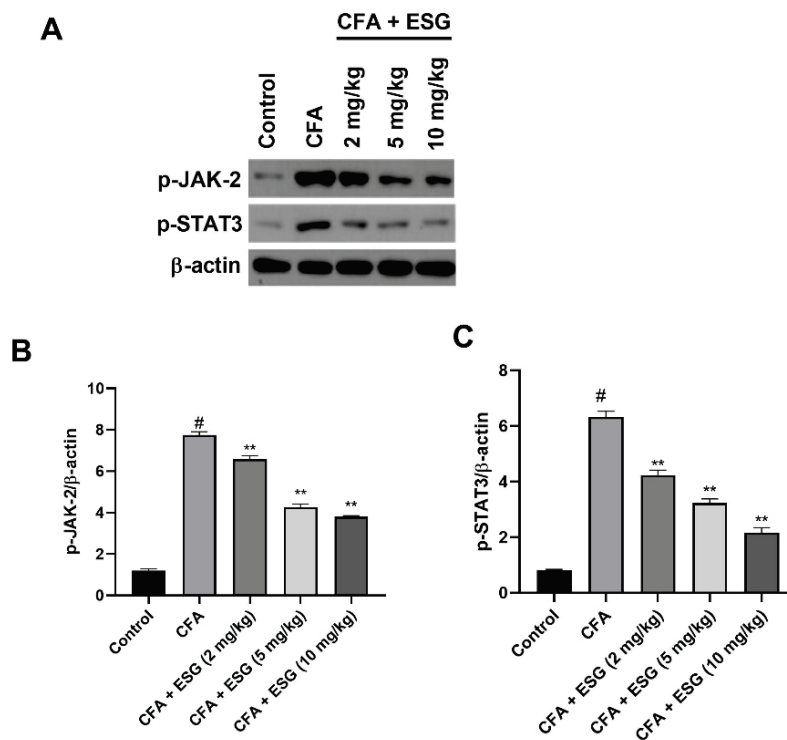


Fig. 6. Effect of ESG on the p-JAK2 and p-STAT3 levels. [#]P<0.05 vs. control and ^{**}P<0.01 vs. CFA group. Data are presented as means \pm SEM.

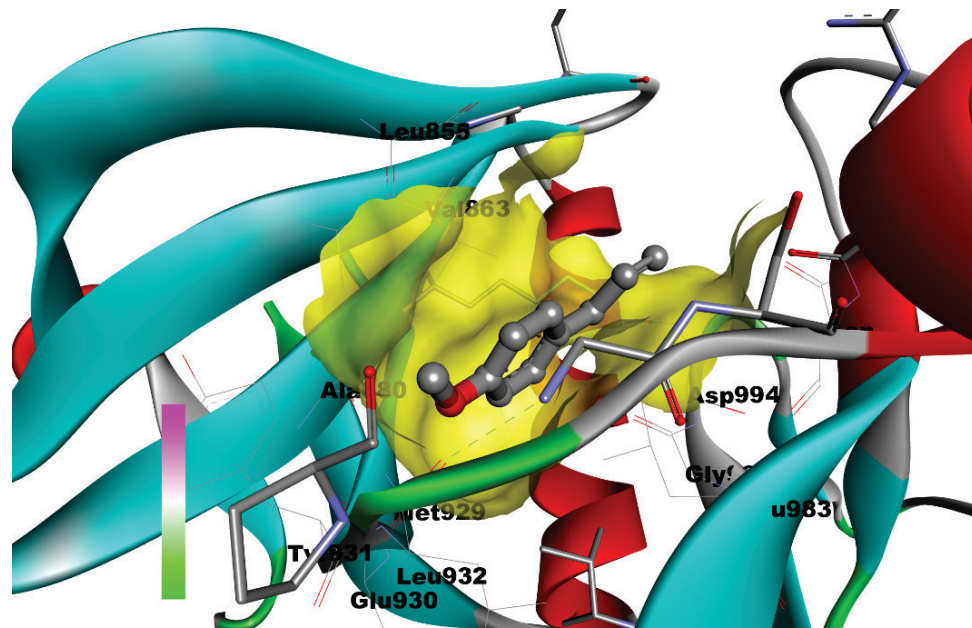


Fig. 7. 3D Docking interaction of ESG with JAK2 using CB-Dock.

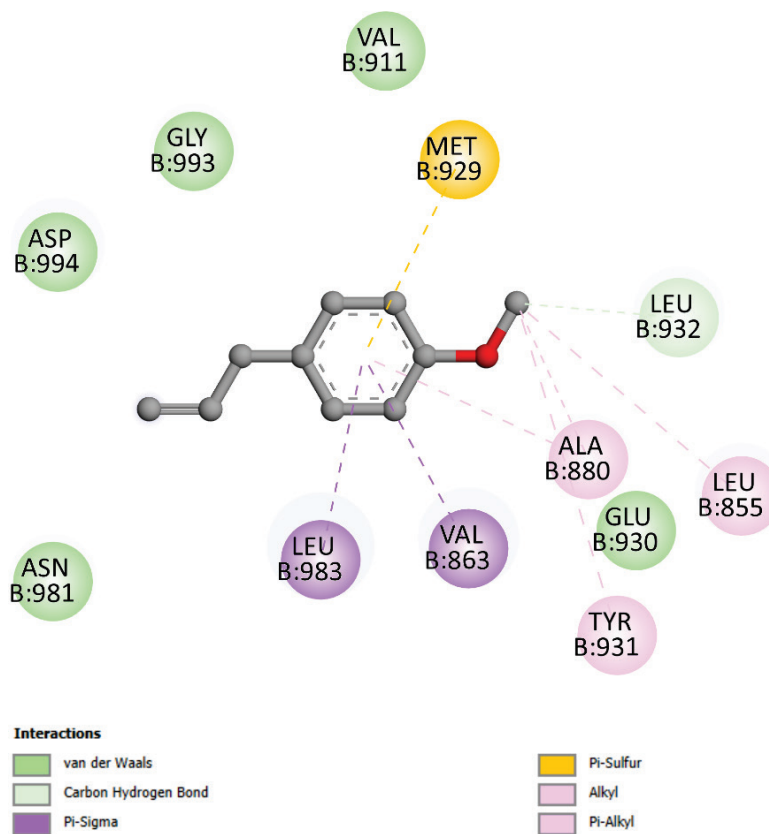


Fig. 8. 2D interaction of ESG with JAK2 using CB-Dock.

Table 1. Details of Interacting residues of ESG with JAK2.

Name	Residues	Vina Score
<i>Estragole</i>	LEU855, VAL863, ALA880, VAL911, MET929, GLU930, TYR931, LEU932, PRO933, ARG980, ASN981, LEU983, GLY993, ASP994	-6.0

Discussion

Rheumatoid arthritis makes daily life challenging. It can affect one's quality of life and result in multiple negative health and social effects. Decreased mobility, weakened muscles, and joint discomfort and swelling are potential consequences [2]. Additional common signs include generalized weariness, sleep disturbances, and fatigue. Another explanation is that it might be challenging to foresee symptoms in advance: It's challenging to determine whether they will become better or worse the next day [11]. The current clinical modality to manage RA is greatly dependent on the use of aggressive treatment plans and strong pharmacological regimens which has its own serious side effects. In the last decade, we have also witnessed the surge of immunotherapy as a prospective option against RA, however, its clinical significance has been not fully understood. Thus, the discovery and development of natural products against RA have shown tremendous success due to the multi-factorial approach [12]. Various studies showed the potential benefit of natural products like, Curcumin, Quercetin, Genistein, Luteolin, Kaempferol, Myricetin and etc. in various *in vitro* and animal studies.

ESG is also known as methyl chavicol or p-allyl anisole, belonging to the class of phenylpropanoids found in essential oils of medicinal and food plants. It showed a wide range of pharmacological actions [5-8]. Given the outstanding pharmacological activity of ESG in earlier research, we have shown its pharmacological advantage and likely method of action against a rat model of arthritis caused by CFA in this investigation. Due to its resemblance to the clinical characteristics of human arthritis, this model is one of the most favored and frequently employed approaches for simulating RA in animal models [13].

The defining feature of RA is the onset of edema brought on by inflammation. It is also thought that RA is associated with a decrease in body weight [14]. So, first, we recorded how ESG affected the rats' HPV, body weight, and arthritis-related inflammation. The CFA rats had reduced body weight, and noticeably increased paw redness, swelling, and deformed joints. However, ESG-treated rats substantially and dose-dependently inhibited the elevated arthritis score, paw edema, and swelling, and improved body weight in the treated RA rats [15]. Therefore, it was assumed that ESG, due to its anti-inflammatory activity, lowers the inflammation of

animal paws.

In order to further ascertain the anti-inflammatory effect of ESG, we have also documented the effect of ESG on the levels of pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6. Various basic research and clinical studies have documented the role of these pro-inflammatory cytokines in the pathogenesis of RA [16,17]. They are prominently involved in arthritis and bone and cartilage erosion. This led to the development of anti-cytokine therapy that targets numerous pathways/cytokines involved in the pathogenesis of RA [18]. In the present study, ESG showed a significant reduction of these pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) in a dose-dependent manner.

According to studies, severe oxidative stress causes persistent inflammation, which lowers the antioxidant capacity of cells. Free radicals that are created in excess interact with the fatty acids and proteins in cell membranes, irreversibly affecting their function. It can also cause DNA damage and mutation in age-related disorders, such as RA [16-22]. Therefore, reducing the level of oxidative stress has a considerable anti-RA protective impact and anti-inflammatory action. Several biomarkers, including MDA, SOD, and GPx, can be used to gauge the level of oxidative stress in people. In the present study, ESG causes a reduction of MDA, a biomarker related to lipid peroxidation which was found excessively elevated in the disease state due to oxidative stress. The level of natural antioxidant biomarkers, SID and GPx was found significantly elevated in ESG treated group as compared to the CFA group.

An important step in the pathophysiology and development of rheumatoid arthritis is the activation of the Janus kinase/signal transducers and activators of the transcription (JAK-2/STAT-3) signal transduction pathway by pro-inflammatory cytokines [23-27]. The suppressor of cytokine signaling and the protein inhibitor of activated STAT are two examples of negative regulators of JAK-2/STAT-3 that are active when circumstances are normal. Both of these regulators, nevertheless, are not working properly in rheumatoid arthritis (RA). Therefore, the effect of ESG was elucidated on the JAK/STAT-3 pathway using western blot analysis. It has been found that ESG in a dose-dependent manner downregulated the level of phosphorylated-JAK2 and STAT-3. This inhibition by ESG possibly reduces matrix metalloproteinase gene expression and thereby prevents cartilage destruction.

In recent years, both academic and professional contexts have made extensive use of molecular docking as a quick and affordable procedure. It examines how molecules behave within a macromolecular target's binding location (referred to as the "pose" collectively) [28-30]. Poses that could be taken are generated by search algorithms, and scoring systems rank them [31,32]. Therefore, in the present study, we have examined the docking interaction of ESG with JAK2 using CB-Dock [33,34]. This docking method utilizes AutoDock Vina to execute molecular docking after automatically determining the binding sites, determining the size and center, and customizing the docking box size for the query ligands. The results of the study suggested that ESG efficiently binds to JAK2 and interacts with critical amino residues necessary for biological activity.

Conclusions

This study reveals that ESG has a protective effect against CFA-induced arthritis in rats. The suppression of the JAK-2/STAT-3 pathway, along with the prevention of oxidative stress and inflammation, led to a reduction in the release of pro-inflammatory cytokines and an attenuation of the inflammatory response, hence preventing damage to the tissues and articular cartilages. However, the specific antioxidative mechanism on arthritic illnesses needs to be explored in further research before ESG may be used as a therapeutic medication for inflammatory and arthritic conditions.

Conflict of Interest

There is no conflict of interest.

References

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