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*Original Research Article*

# Anti-Oxidative Stress and Immunosuppressive Effects of Ethanol Extract from Sacha Inchi Leaves in Mice with CFA-induced Rheumatoid Arthritis

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ABSTRACT

Sacha inchi (*Plukenetia volubilis* L.) is recognized for its antioxidative properties, making it a valuable herbal source for immune modulation and treating joint and muscle pain. This study investigated the antioxidative and immunosuppressive effects of the ethanol extract of Sacha inchi leaves (EESI) in a CFA-induced rheumatoid arthritis (RA) mouse model. Swiss albino mice were assigned to experimental groups receiving EESI at 100, 200, and 300 mg/kg doses, with mobic (0.2 mg/kg) serving as the standard drug. Key parameters assessed included malondialdehyde (MDA), hydroperoxide ($H_2O_2$), total glutathione (GSH), total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), white blood cell (WBC) count, phagocytic activity (PA), nitroblue tetrazolium (NBT) reduction, and total immunoglobulin (TI). Results indicated a significant increase in MDA and $H_2O_2$ levels in the CFA group ($p < 0.05$), reflecting oxidative stress due to RA. EESI treatment significantly reduced MDA and $H_2O_2$ levels in a dose-dependent manner ($p < 0.05$), with the 300 mg/kg dose achieving effects comparable to mobic ($p > 0.05$). EESI also prevented the depletion of GSH and TAC observed in CFA-induced mice ($p < 0.05$) and enhanced CAT, SOD, and GPx activities, suggesting improved antioxidant defenses ($p < 0.05$). Additionally, EESI normalized WBC count, PA, NBT reduction, and TI levels altered by CFA, particularly at the 300 mg/kg dose ($p > 0.05$). These findings demonstrate the potential of EESI as an effective antioxidative and immunosuppressive agent for RA management, supporting its use as an alternative therapeutic option.

***Keywords:*** Antioxidant effects, Immunosuppressive effects, Oxidative stress, *Plukenetia volubilis* L., Sacha inchi.

## Introduction

Oxidative stress results from an imbalance between reactive oxygen species (ROS) production and the body's antioxidant defenses, where ROS levels surpass the body's capacity to neutralize them. ROS, such as superoxide anion ($O^{2-}$), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxide, are oxygen-containing reactive species generated during oxygen reduction in organisms.[1] External factors can trigger oxidative stress, leading to excessive oxidation beyond the body's antioxidant capacity, causing tissue damage, oxidative DNA damage, lipid peroxidation, and protein oxidation.[2] In the context of rheumatoid arthritis induced by Complete Freund's Adjuvant (CFA), oxidative stress plays a pivotal role in the disease pathogenesis. The inflammatory response triggered by CFA leads to increased ROS production, exacerbating oxidative stress within the affected joints, causing damage to joint tissues, and contributing to the progression of arthritis.[3] Moreover, oxidative stress is associated with the modulation of immune responses in arthritis, affecting the function of immune cells, including macrophages, neutrophils, and T cells, by altering activation, proliferation, and cytokine production.

Dysregulation of immune function leads to immune suppression, where the body generates an effective immune response against pathogens or damaged abnormal cells. Conversely, immune suppression in arthritis can exacerbate oxidative stress. Dysregulated immune reactions lead to chronic inflammation, prolonged ROS production, and oxidative damage in the joints.[4] Oxidative stress, harmful to cells, is countered by antioxidants that neutralize reactive oxygen species (ROS), preventing damage. Glutathione peroxidase (GPx) is key, reducing oxidative stress by converting hydroperoxides using glutathione (GSH), and shielding cells from harm.[5] Superoxide dismutase (SOD) and catalase (CAT) are vital antioxidants, influence immunity.[6] Blood cells (WBC), nitroblue tetrazolium (NBT), phagocytic activity (PA), and total immunoglobulin (TI) regulate immune responses by detecting pathogens, measuring ROS generation, enhancing immune defense, and identifying pathogens, respectively.[7] The intricate interplay between antioxidants and immune factors necessitates precise adjustments to shield the body from oxidative stress and bolster the immune system. Glutathione (GSH) shields immune cells from oxidative damage, enhancing white blood cell (WBC) function and phagocytic activity. Nitroblue tetrazolium (NBT) assesses superoxide dismutase (SOD) activity, linked to GSH. Reduced SOD or GPx levels may diminish WBC phagocytosis.[8] Total antioxidant capacity (TAC) evaluates overall antioxidant synthesis, including SOD, CAT, and GPx. Elevated TAC fortifies antioxidant defenses, safeguarding immune cells and promoting WBC activity and immunoglobulin production. SOD generates hydro peroxide ($H_2O_2$), neutralized by CAT and GPx, mitigating oxidative stress and protecting immune cells. GPx crucially eliminates $H_2O_2$, reducing oxidative stress and impacting immune function.[9] Recent studies have highlighted plant extracts as emerging natural, effective, and safe antioxidants.[10] Sacha in chi (SI) (*Plukenetia volubilis* Linneo), is a perennial plant of the Europhorbiaceae family, originating from the rainforests of Peru and Northwestern Brazil. However, SI is now commercially grown in Asian countries such as Thailand, China, and Vietnam, as well as in Central and South America due to its exceptional nutritional value.[11] SI has long been traditionally used to treat joint issues and muscle pain. The leaves of SI contain numerous bioactive compounds, including alkaloids, tannins,

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flavonoids, leptin, saponins, and other bioactive compounds. Particularly, phenolic compounds, flavonoids, and terpenoids present in the leaves contribute to their antioxidant, anti-inflammatory, and anti-rheumatoid arthritis CFA-induced rheumatoid arthritis treatment properties.[12] SI seeds have also been used as a traditional remedy in the Amazon region to treat joint problems and muscle pain.[13] SI seeds are becoming increasingly popular due to their bioactive components, especially alpha-linolenic acid (ALA), which serves as a precursor to EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). ALA is incorporated into cell membranes, especially erythrocytes, retinal, renal, neutrophilic, and hepatic cells. Essential fatty acids generate several eicosanoid compounds such as prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs).[14] The extract from SI bark also provides a potential source of phenolics with antioxidant activity and is used to prevent lipid oxidation in the body.[15] *Plukenetia volubilis* L., one of the medicinal plants in Peru, is a source of immune-regulating drugs with potential usefulness against COVID-19.[16] Although SI exhibits notable pharmacological effects, its specific role and mechanisms in antioxidative stress and immune regulation remain unclear and warrant further investigation. This study aimed to investigate the antioxidative and immunosuppressive effects of the ethanol extract of Sacha inchi leaves (EESI) in a CFA-induced rheumatoid arthritis (RA) mouse model, highlighting its novel potential as an alternative therapeutic approach for RA management.

## Materials and Methods

*Chemicals and reagents*

The Complete Freund's Adjuvant (CFA) supplement was sourced from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The ELISA kits for quantifying inflammatory mediators and the chemicals for analyzing oxidative stress parameters and immune parameters were purchased from RayBiotech, Inc. (Norcross, GA) and imported through Nam Khoa Company (Vietnam). All reagents and experimental chemicals used were ensured to have high-quality analysis and purity, sourced from Sigma (St. Louis, MO).

*Collection of plant material and preparation of the extract*

Sacha inchi (SI) leaves were harvested in November 2023 from the farm of Eastern Agriculture Company, located in Buon Ma Thuot City, Daklak Province, Vietnam (Coordinates: 12°39'06.0"N 108°04'12.3"E). The reference sample (code PV201123VST) was meticulously preserved at the Department of Biotechnology, Industrial University of Ho Chi Minh City. Leaves were carefully collected from healthy plants at the appropriate growth stage and visually inspected to remove any damaged or diseased leaves. The selected leaves were washed to remove surface contaminants and air-dried to reduce moisture content. Subsequently, they were completely dried in a Memmert UN110 (Memmert, Germany) at 60°C until a constant weight was achieved. The dried leaves were then ground into a fine powder to increase the surface area for extraction. The powdered leaves were stored in moisture-proof bags at room temperature for subsequent experiments.

Five hundred grams of leaf powder were soaked in 2.5 liters of ethanol solvent for 72 hours, continuously stirring to enhance extraction efficiency. The extraction process was further assisted using a Cheersonic ultrasonic extractor CQ28 (Jiayuanda Technology, China) to improve yield. The resulting extract was filtered to remove solid impurities and concentrated under reduced pressure using a rotary evaporator RE301B-T at 50°C (Yamato, Japan) to obtain the ethanol extract (the name is EESI). The extract was stored in moisture-proof containers at 4°C, shielded from light, for subsequent experimental use.

*Screening and quantification of phytochemicals in extracts*

Phytochemical analysis was conducted qualitatively to identify bioactive compounds in the extract obtained from sacha inchi leaves extraction, following the established methods by Nhung and Quoc.[17]

To determine the total polyphenol content (TPC), the Folin-Ciocalteu colorimetric method was employed, following the study by Nhung and Quoc with some modifications.[18] Specifically, 0.3 mL of the extract was mixed with 2.25 mL of Folin-Ciocalteu phenol reagent. After incubating for 5 minutes, we added 2.25 mL of 6% sodium carbonate solution to the mixture and allowed it to stand at room temperature for 90 minutes. The absorbance of the solution was then measured at a wavelength of 725 nm using a UV5 spectrophotometer (Mettler Toledo, Switzerland). Following the same procedure, a standard curve for gallic acid was established in the concentration range of 0-200 µg/mL. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract.

The total flavonoid content was assessed using the aluminum chloride colorimetric method established by Nhung and Quoc with certain modifications.[19] Quercetin was used as the reference standard, and a calibration curve for quercetin was generated in the concentration range of 0-200 µg/mL. For this analysis, 0.5 mL of the extract and 0.5 mL of the standard solution were placed into separate test tubes. To each tube, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, 1.5 mL of 80% methanol, and 2.8 mL of distilled water were added and mixed thoroughly. A blank sample was prepared similarly, substituting 0.5 mL of distilled water for the sample or standard, and replacing aluminum chloride with distilled water. All tubes were incubated at room temperature for 30 minutes, after which the absorbance was measured at a wavelength of 415 nm using a UV5 spectrophotometer (Mettler Toledo, Switzerland).

The terpenoid content was evaluated using a method adapted from Nhung and Quoc.[20] Specifically, a 200 µL aliquot of the extract solution (0.1 mg/mL) was combined with 1 mL of perchloric acid and 300 µL of a 5% (w/v) vanillin/acetic acid solution. Then, 5 mL of glacial acetic acid was added to the mixture. The absorbance of the solution was then measured at 548 nm using a UV5 spectrophotometer (Mettler Toledo, Switzerland). Terpenoid quantification was conducted using a standard curve derived from various concentrations of ursolic acid, ranging from 0.0625 to 1 mg/mL.

*Experimental animals*

Swiss albino mice were procured from the Pasteur Institute in Ho Chi Minh City, Vietnam, and housed in the animal facility of the Eastern Agriculture and Food Organization, Ho Chi Minh City. The animals had *ad libitum* access to standard pellet diets and clean drinking water. Before experimentation, the mice were acclimated to laboratory conditions for one week in a temperature-controlled environment maintained at 25 ± 2°C with 55–60% relative humidity and a 12-hour light/dark cycle. Subsequently, 30 mice with an initial body weight of 30 ± 2 g were randomly assigned to six cages, with five mice per cage. Food and water intake, as well as body weight, were recorded weekly. All experimental procedures and animal handling adhered to the standards and guidelines outlined in the Basel Declaration on Animal Research.[21] All interventions, treatment protocols, safety procedures, and animal care practices were conducted under the guidelines of the Animal Research Ethics Committee at the Industrial University of Ho Chi Minh City, Vietnam (98/HD-DHCN).

*Experimental design*

For this study, six groups were established, each containing five mice. The groups were as follows: the normal control group, the negative control group (RA group), the positive control group (RA+Mobic group) at a dose of 0.2 mg/kg, and three experimental groups receiving ethanol extract of Sacha inchi (EESI) at doses of 100, 200, and 300 mg/kg (EESI100, EESI200, and EESI300 groups). A single dose of 1 mL complete Freund's adjuvant (CFA) (Sigma Aldrich, St. Louis, MO) was administered on day 0 to induce rheumatoid arthritis in the left hind paw of all groups except the normal control group. Treatment began on day 8 and continued until day 22. On day 23, 24 hours after the last treatment, all mice were sacrificed for subsequent analysis.[3]

*Assessment of oxidative stress*

Following euthanasia, the joint, liver, kidney, and spleen tissues of the mice were collected. Subsequently, the organs were dissected and immersed in pre-chilled 0.9% sodium chloride solution at ice temperature. Then, the tissues were homogenized in 50 mM phosphate buffer at pH 7.0, supplemented with 0.1 mM EDTA. The supernatant was collected after centrifugation of the 5% homogenate solution at 600 × g for 10 minutes at 4°C, which was utilized for subsequent analyses according to the method described by Nhung and Quoc.[4]

*Assessment of oxidative stress mediators*

***Malondialdehyde (MDA):*** A mixture comprising 2.5 mL of 10% trichloroacetic acid (TCA) solution and 0.5 mL of serum was heated to 90°C for 15 minutes in a centrifuge tube placed in a water bath. Following this, the mixture was cooled to room temperature and then centrifuged at 3000 rpm for 10 minutes. Subsequently, 2 mL of the supernatant was combined with 1 mL of 0.675% TBA solution in a test tube, and this mixture was reheated at 90°C for another 15 minutes in a water bath before being cooled to room temperature. The absorbance was then measured at a wavelength of 532 nm using a UV5 spectrophotometer (Mettler Toledo, Switzerland).[4]

***Hydroperoxide ($H_2O_2$):*** The sample was ground into a powder in 5% trichloroacetic acid (TCA) (2.5 mL per 0.5 g of powder) with 50 mg of activated charcoal at 0°C. The mixture was then centrifuged at 15,000 × g for 10 minutes. The supernatant was collected and neutralized to pH 3.6 using 4 N KOH. The reaction mixture consisted of 200 µL of the sample extract and 100 µL of 3.4 mM 3-methylbenzothiazolin hydrazone (MBTH). Subsequently, 500 µL of peroxidase solution (90 U per 100 mL) in 0.2 M sodium acetate buffer (pH 3.6) was added. After two minutes, 1400 µL of 1 N HCl was added, and the absorbance was measured at 630 nm using a UV5 spectrophotometer (Mettler Toledo, Switzerland) following a 15-minute incubation period.[22]

*Assessment of enzymes and antioxidants*

***Total glutathione (GSH):*** The method involved precipitating proteins using a 50% TCA solution, as described by Nhung and Quoc.[4] The mixture was centrifuged at 3000 rpm for 15 minutes. The supernatant was collected and mixed with 4.0 mL of 0.4 M Tris buffer (pH 8.0) and 0.1 mL of Ellman's reagent (DTNB). The reaction mixture was incubated for 10 minutes, followed by the addition of more DTNB and an additional 5-minute incubation. The absorbance was then measured at a wavelength of 412 nm using a UV5 spectrophotometer (Mettler Toledo, Switzerland).

***Total antioxidant capacity (TAC):*** The oxidation of Tween 80 was analyzed using a spectrophotometric method at a wavelength of 532 nm, as described by Cordeiro *et al*.[22] A 0.2 mL tissue sample was combined with 2 mL of 1% Tween 80 solution. For the blank sample, 0.1 mL of distilled water was used instead. The mixture was incubated at 37°C for 48 hours. Subsequently, 1 mL of 40% TCA solution was added, and the mixture was centrifuged at 3000 g for 10 minutes. The supernatant (2 mL) was collected and mixed with 2 mL of 0.25% TBA solution. This mixture was then heated in a water bath at 100°C for 15 minutes. The absorbance of the resulting solution was measured at 532 nm using a UV5 spectrophotometer (Mettler Toledo, Switzerland) and compared with the blank sample.

***Superoxide dismutase (SOD):*** The hemolysate obtained from a 5% erythrocyte blood sample was mixed with 50 µL of a solution containing 75 mM Tris-HCl buffer (pH 8.2), 30 mM EDTA, and 2 mM pyrogallol. The increase in absorbance was recorded spectrophotometrically at a wavelength of 420 nm using a UV5 spectrophotometer (Mettler Toledo, Switzerland) for 3 minutes.[4]

***Catalase (CAT):*** A 0.1 mL aliquot of the homogenized sample was mixed with 1 mL of 4% ammonium molybdate solution and 2 mL of 0.03% $H_2O_2$ solution. The resulting reaction mixture was then analyzed spectrophotometrically at a wavelength of 410 nm using a UV5 spectrophotometer (Mettler Toledo, Switzerland).[4]

***Glutathione peroxidase (GPx):*** The reaction mixture consisted of 500 µL phosphate buffer, 100 µL of 0.01 M reduced glutathione (GSH), 100 µL of 1.5 mM NADPH, and 100 µL of glutathione reductase (GR) (0.24 U). Subsequently, 100 µL of the homogenized tissue sample was added to the reaction mixture and incubated at 37°C for 10 minutes. Following this, 50 µL of t-butyl hydroperoxide was added to 450 µL of the reaction mixture. The absorbance was then measured at 340 nm over 180 seconds using a UV5 spectrophotometer (Mettler Toledo, Switzerland).[4]

*Assessment of immunological parameters*

***Blood cell (WBC):*** The procedure was conducted following the detailed protocol by Yonar *et al*.[23] White blood cells were fixed and stained using the Natt-Herrick solution. The count of white blood cells was calculated from the number of red blood cells using a Neubauer counting chamber (Brand GmbH + Co KG, Germany).

***Phagocytic activity (PA):*** A suspension containing $10^8$ cells/mL of *Staphylococcus* sp. in 0.1 mL phosphate buffer was added to 0.1 mL of blood sample on a Petri dish and incubated for 30 minutes. Subsequently, the mixture was thoroughly mixed in the well. The Petri dish was gently agitated, and 0.05 mL of this suspension was spread onto a glass slide. After air-drying, the smear was fixed with ethanol. The cell count and cell activity were observed under a microscope.[23]

***Nitroblue tetrazolium (NBT):*** NBT testing necessitates the addition of 0.1 mL of blood into individual wells of a microtiter plate. Subsequently, an equivalent volume of 0.2% NBT solution is added. After incubation for 30 minutes at room temperature, 0.05 mL of NBT-stained blood cells are extracted and transferred to glass tubes containing 1.0 mL of N,N-dimethylformamide. Following centrifugation, the absorbance of the sample is measured at a wavelength of 620 nm.[23]

***Total immunoglobulin (TI):*** The total serum protein concentration was quantified both before and after the precipitation of immunoglobulin molecules using a 12% polyethylene glycol solution. The difference in protein content reflects the total amount of immunoglobulins in the serum.[23]

*Statistical analysis*

Results were presented as mean ± standard deviation (SD). The differences among groups were determined by one-way analysis of variance ANOVA followed Fisher's least significant difference using the Stagraphics Centurion XVI software (Statpoint Technologies Inc., Warrenton, Virginia, USA), and the criterion of statistical significance was set as $p < 0.05$.

## Results and Discussion

*Phytochemical constituents of Sacha inchi extract*

Chemical analysis of ethanol extract from Sacha inchi leaves (EESI) revealed the presence of alkaloids, tannins, saponins, polyphenols, terpenoids, steroids, and flavonoids, while cardiac glycosides were not detected. Additionally, quantitative analysis of plant compounds indicated that the total flavonoid content was 39.52 ± 1.41 (mg QE/g), total terpenoid content was 68.87 ± 2.35 (mg TAE/g), and total polyphenol content was 66.29 ± 2.05 (mg GAE/g) (Table 1).

**Table 1:** Qualitative and quantitative phytochemical constituents of ethanol extract of Sacha inchi leaves

| Phytoconstituents | Qualitative | Quantitative |
|---|---|---|
| Tannins | + | NT |
| Flavonoids | + | 39.52 ± 1.41 (mg QE/g) |
| Terpenoids | + | 68.87 ± 2.35 (mg TAE/g) |
| Polyphenol | + | 66.29 ± 2.05 (mg GAE/g) |
| Saponins | + | NT |
| Steroids | + | NT |
| Cardiac glycosides | - | - |
| Alkaloids | + | NT |

Key: (+) present, (-) absent, GAE: Gallic acid equivalents, QE: Quercetin equivalents, TAE: Tannic acid equivalents, NT: Not tested.

Oxidative stress, characterized by an imbalance in oxidative agents, contributes to the development of various diseases. Counteracting oxidative agents is an effective strategy to minimize oxidative stress levels in organisms. Phytochemicals present in plant extracts play a pivotal role in preventing and treating diseases caused by oxidative stress by acting as antioxidants.[24] Particularly, polyphenols, flavonoids, and terpenoids exert antioxidant effects through a combination of directly scavenging free radicals, inhibiting oxidative damage to biological molecules, enhancing the activity of antioxidant enzymes, and regulating cell signaling pathways related to oxidative stress and inflammation. Polyphenols and terpenoids exhibit the ability to scavenge free radicals, inhibit ROS production, chelate transition metal

ions, and regulate enzyme activities associated with the oxidative stress pathway. They can also modulate the upregulation of antioxidant enzyme expression and inhibit pro-oxidant enzymes, thereby reducing oxidative damage to biological molecules such as lipids, proteins, and DNA. Furthermore, some terpenoids can regulate cell signaling pathways related to oxidative stress response and cell survival.[25] Flavonoids demonstrate antioxidative stress effects through various mechanisms, including scavenging free radicals, inhibiting lipid peroxidation, and enhancing the activity of endogenous antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Flavonoids also possess metal-chelating properties, aiding in the prevention of reactive oxygen species (ROS) formation by inhibiting metal-catalyzed oxidation reactions. Moreover, flavonoids regulate cell signaling pathways associated with oxidative stress and inflammation.[26] Immunomodulation involves stimulating, amplifying, or inhibiting certain stages of the immune response. Phytochemical substances also can regulate immunity by controlling leukocyte migration, inhibiting nitric oxide synthesis, lymphocyte proliferation, T-cell activation, cytokine stimulation, enhancing natural killer cell activity, and modulating NFκB, TNF, and apoptosis.[27] Polyphenols, flavonoids, and terpenoids can regulate the production and activity of cytokines, chemokines, and inflammatory mediators, modulate immune cell proliferation and differentiation, and enhance antigen presentation cell function. Additionally, they promote lymphocyte proliferation, regulate the activity of immune cells such as macrophages, dendritic cells, and T lymphocytes, and regulate immune signaling pathways, including NF-κB, MAPK, and JAK-STAT pathways.[28] The presence of phytochemicals, particularly polyphenols, flavonoids, and terpenoids in ethanol extract from Sacha inchi leaves (EESI), plays a crucial role in combating oxidative stress and immune modulation in mice with low-grade rheumatoid arthritis (RA) induced by CFA. The polyphenols, flavonoids, and terpenoids in EESI can prevent cellular damage caused by free radicals and other harmful substances resulting from inflammation and oxidative stress in the body. These compounds also regulate the production and secretion of cytokines and other immune chemicals, helping to balance inflammatory and immune responses. In this way, they may reduce joint damage and alleviate severe inflammatory symptoms in RA.

*Effect of Sacha inchi leaf ethanol extract on oxidative stress biomarker levels in experimental mice*

As shown in Table 2, MDA and $H_2O_2$ levels in the joints, liver, kidneys, and spleen significantly increased ($p < 0.05$) in the CFA group after CFA injection compared to the control group, indicating oxidative stress due to RA. Supplementation with EESI effectively inhibited the release of MDA and the excessive increase of $H_2O_2$ in the EESI-treated groups (100, 200, and 300 mg/kg) compared to the CFA group ($p < 0.05$). The $H_2O_2$ levels were significantly reduced in the groups pre-treated with 300 mg/kg EESI ($p < 0.05$), showing an effect comparable to that of mobic (0.2 mg/kg).

Significant oxidative stress in CFA-induced rheumatoid arthritis (RA) mice is indicated by elevated levels of malondialdehyde (MDA) and hydroperoxide ($H_2O_2$) in the joints, liver, kidneys, and spleen. This finding underscores the systemic nature of oxidative stress in RA, consistent with existing literature that reports similar oxidative stress in RA models.[29,30] Elevated levels of MDA and $H_2O_2$ indicate increased lipid peroxidation and reactive oxygen species (ROS) production, contributing to tissue damage and inflammation in RA.[31]

**Table 2:** The effect of ethanol extract of Sacha inchi leaves on the levels of malondialdehyde (MDA) and hydrogen peroxide ($H_2O_2$) in the tissues and organs of mice

| Group | Malondialdehyde (MDA) (nmol/mL tissue) | | | | Hydrogen peroxide ($H_2O_2$) (µmol/mL tissue) | | | |
|---|---|---|---|---|---|---|---|---|
| | Joints | Livers | Kidneys | Spleens | Joints | Livers | Kidneys | Spleens |
| Control group | $2.26 \pm 0.03^{a}$ | $2.42 \pm 0.05^{a}$ | $2.31 \pm 0.04^{a}$ | $2.35 \pm 0.06^{a}$ | $32.77 \pm 2.22^{a}$ | $35.08 \pm 1.52^{a}$ | $33.44 \pm 1.85^{a}$ | $34.09 \pm 1.54^{a}$ |
| CFA group | $4.07 \pm 0.04^{f}$ | $4.36 \pm 0.06^{f}$ | $4.16 \pm 0.02^{f}$ | $4.23 \pm 0.03^{f}$ | $58.58 \pm 2.01^{e}$ | $63.15 \pm 2.38^{e}$ | $60.18 \pm 2.61^{e}$ | $61.34 \pm 2.19^{e}$ |
| CFA+Mobic group | $2.49 \pm 0.03^{b}$ | $2.66 \pm 0.03^{b}$ | $2.54 \pm 0.06^{b}$ | $2.59 \pm 0.05^{b}$ | $36.05 \pm 1.45^{b}$ | $38.58 \pm 1.76^{b}$ | $36.77 \pm 1.56^{b}$ | $37.48 \pm 1.61^{b}$ |
| CFA+EESI100 group | $3.16 \pm 0.04^{e}$ | $3.38 \pm 0.02^{e}$ | $3.23 \pm 0.02^{e}$ | $3.29 \pm 0.04^{e}$ | $45.86 \pm 1.88^{d}$ | $49.11 \pm 1.96^{d}$ | $46.83 \pm 1.92^{d}$ | $47.72 \pm 1.89^{d}$ |
| CFA+EESI200 group | $2.94 \pm 0.03^{d}$ | $3.15 \pm 0.05^{d}$ | $3.01 \pm 0.04^{d}$ | $3.06 \pm 0.06^{d}$ | $42.54 \pm 1.66^{c}$ | $45.61 \pm 1.63^{c}$ | $43.46 \pm 1.85^{c}$ | $44.31 \pm 1.73^{c}$ |
| CFA+EESI300 group | $2.59 \pm 0.03^{c}$ | $2.78 \pm 0.05^{c}$ | $2.66 \pm 0.05^{c}$ | $2.71 \pm 0.04^{c}$ | $37.67 \pm 1.75^{b}$ | $49.11 \pm 1.54^{b}$ | $38.45 \pm 1.72^{b}$ | $39.19 \pm 1.42^{b}$ |

The values are expressed as Mean ± SD, where the letters (a, b, c, d, e, and f) indicate differences between treatments ($p < 0.05$).

The administration of ethanol extract from Sacha inchi leaves (EESI) effectively mitigated oxidative stress associated with RA. EESI treatment resulted in a marked reduction in MDA and $H_2O_2$ levels across all tested doses compared to the CFA group. This reduction supports recent studies highlighting the antioxidative potential of plant extracts in mitigating oxidative damage in inflammatory conditions.[32] Notably, the 300 mg/kg EESI dose significantly reduced $H_2O_2$ levels, demonstrating efficacy comparable to that of the standard anti-inflammatory drug, Mobic. This finding aligns with research indicating that natural antioxidants can offer comparable or superior effects to conventional medications in managing oxidative stress.[33,34] The ability of EESI to achieve results on par with a standard anti-inflammatory drug suggests its potential as a viable alternative or complementary therapy for managing oxidative stress in RA. These results support the hypothesis that EESI possesses significant antioxidative properties, which may contribute to improved clinical outcomes in RA management.

*Antioxidant effect of Sacha inchi leaf ethanol extract*

The utilization of EESI effectively prevents the significant decrease in glutathione (GSH) levels and total antioxidant capacity (TAC) observed in the joint, liver, kidney, and spleen tissues of CFA-induced mice (Figures 1 and 2). GSH and TAC levels markedly decreased in the CFA group compared to the control group ($p < 0.05$). However, this reduction is effectively counteracted following treatment with EESI (100, 200, and 300 mg/kg). GSH and TAC levels exhibit a substantial increase in the EESI100-300 groups compared to the CFA group ($p < 0.05$). Remarkably, the efficacy of EESI at a dose of 300 mg/kg is comparable to that of mobic ($p > 0.05$) in regulating TAC levels in the tissues.

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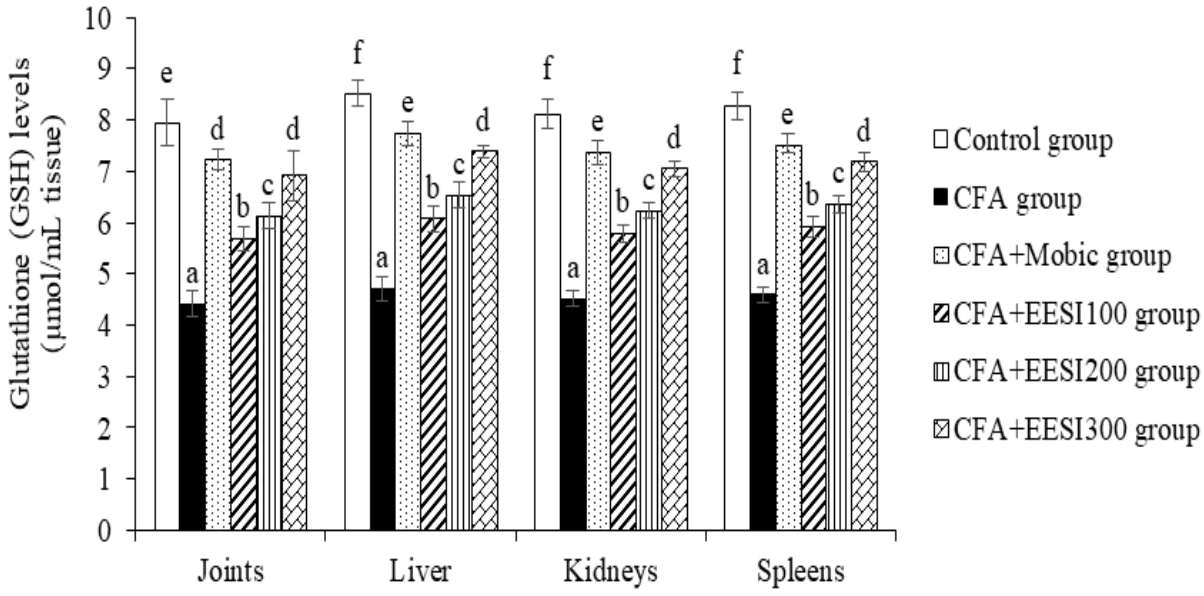


**Figure 1:** Effect of ethanol extract from Sacha inchi leaves on glutathione (GSH) levels
Results are expressed as Mean ± SD, with letters (a, b, c, d, e, and f) indicating statistically significant group differences ($p < 0.05$)

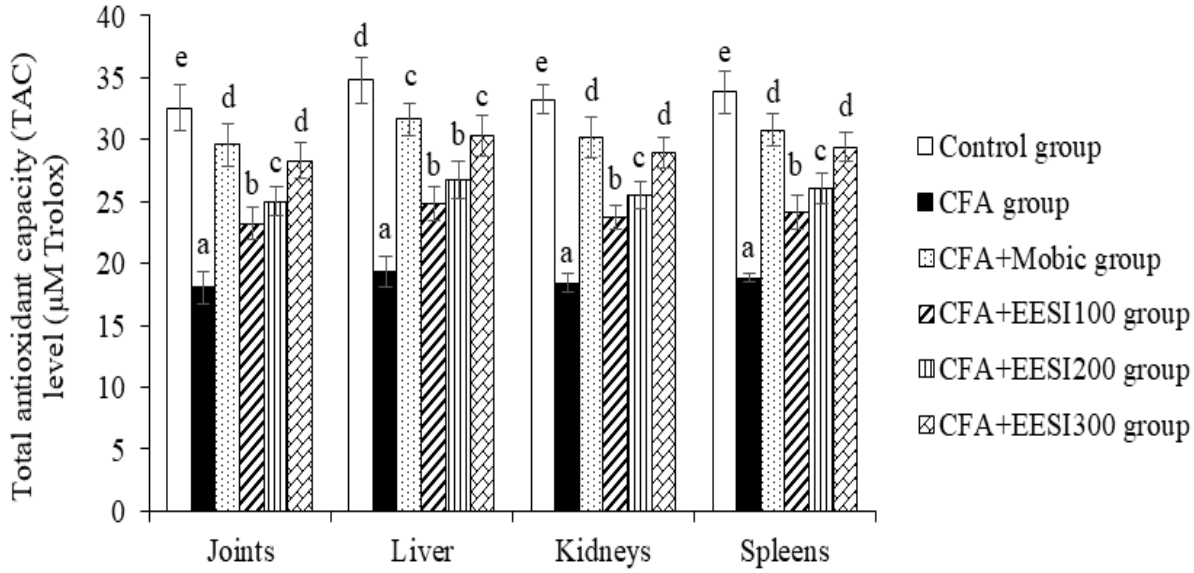


**Figure 2:** The effect of ethanol extract from Sacha inchi leaves on total antioxidant capacity (TAC) levels.
Results are expressed as Mean ± SD, with letters (a, b, c, d and e) indicating statistically significant group differences ($p < 0.05$)

The ethanol extract from Sacha inchi leaves (EESI) has demonstrated a significant capacity to mitigate oxidative damage induced by CFA in a rheumatoid arthritis (RA) mouse model. EESI was shown to effectively counteract the depletion of glutathione (GSH) and total antioxidant capacity (TAC) in the joints, liver, kidneys, and spleen caused by CFA. In the CFA-induced mice, a notable reduction in GSH and TAC levels indicated severe oxidative stress and impaired antioxidant defenses, a pattern consistent with previous studies linking RA to increased oxidative stress and diminished antioxidant levels.[35,36] EESI administration at various doses successfully prevented the reduction in these critical antioxidant markers, aligning with recent literature that emphasizes the potential of natural extracts to restore antioxidant balance and improve antioxidant capacity in inflammatory conditions.[37] The restoration of GSH and TAC levels following EESI treatment highlights its protective role against oxidative damage and its contribution to maintaining cellular redox homeostasis. Furthermore, the efficacy of EESI at higher doses in modulating TAC levels was found to be on par with conventional anti-inflammatory treatments, suggesting that EESI could offer comparable therapeutic benefits in enhancing antioxidant defenses. These observations are consistent with studies reporting that plant-based antioxidants can achieve similar or even superior outcomes compared to standard pharmacological interventions,[38] underscoring the potential of EESI as a promising antioxidant therapeutic agent for RA.

We also assessed the effects of ethanol extract from Sacha inchi leaves (EESI) on catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Table 3, Figure 3). EESI influenced the increase in GPx concentration dose-dependently.

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**Table 3:** The effect of ethanol extract from Sacha inchi leaves on the levels of superoxide dismutase (SOD) and catalase (CAT) in the tissues and organs of mice

| Group | Superoxide dismutase (SOD) (µmol/mL tissue) | | | | Catalase (CAT) (U/mL tissue) | | | |
|---|---|---|---|---|---|---|---|---|
| | Joints | Livers | Kidneys | Spleens | Joints | Livers | Kidneys | Spleens |
| Control group | 8.41 ± 0.14$^{a}$ | 8.99 ± 0.13$^{a}$ | 8.58 ± 0.14$^{a}$ | 8.75 ± 0.11$^{a}$ | 44.39 ± 1.65$^{a}$ | 47.49 ± 1.52$^{a}$ | 45.28 ± 1.18$^{a}$ | 46.17 ± 1.41$^{a}$ |
| CFA group | 4.67 ± 0.11$^{f}$ | 4.99 ± 0.19$^{f}$ | 4.77 ± 0.14$^{f}$ | 4.86 ± 0.14$^{f}$ | 24.66 ± 1.03$^{f}$ | 26.38 ± 1.38$^{f}$ | 25.16 ± 1.76$^{e}$ | 25.65 ± 1.31$^{f}$ |
| CFA+Mobic group | 7.65 ± 0.22$^{e}$ | 8.17 ± 0.11$^{e}$ | 7.81 ± 0.15$^{e}$ | 7.95 ± 0.14$^{e}$ | 40.35 ± 1.42$^{e}$ | 43.17 ± 1.19$^{e}$ | 41.16 ± 1.74$^{d}$ | 41.97 ± 1.33$^{e}$ |
| CFA+EESI100 group | 6.01 ± 0.15$^{b}$ | 6.42 ± 0.11$^{b}$ | 6.13 ± 0.13$^{b}$ | 6.25 ± 0.14$^{b}$ | 31.71 ± 1.38$^{b}$ | 33.92 ± 1.45$^{b}$ | 32.34 ± 1.23$^{b}$ | 32.98 ± 1.45$^{b}$ |
| CFA+EESI200 group | 6.47 ± 0.17$^{c}$ | 6.92 ± 0.11$^{c}$ | 6.61 ± 0.18$^{c}$ | 6.73 ± 0.13$^{c}$ | 34.15 ± 1.12$^{c}$ | 36.53 ± 1.16$^{c}$ | 34.83 ± 1.71$^{c}$ | 35.52 ± 1.35$^{c}$ |
| CFA+EESI300 group | 7.31± 0.14$^{d}$ | 7.82 ± 0.13$^{d}$ | 7.46 ± 0.17$^{d}$ | 7.61 ± 0.16$^{d}$ | 38.61 ± 1.14$^{d}$ | 41.32 ± 1.07$^{d}$ | 39.37 ± 1.96$^{d}$ | 40.15 ± 1.31$^{d}$ |

The values are expressed as Mean ± SD, where the letters (a, b, c, d, e, and f) indicate differences between treatments ($p < 0.05$).

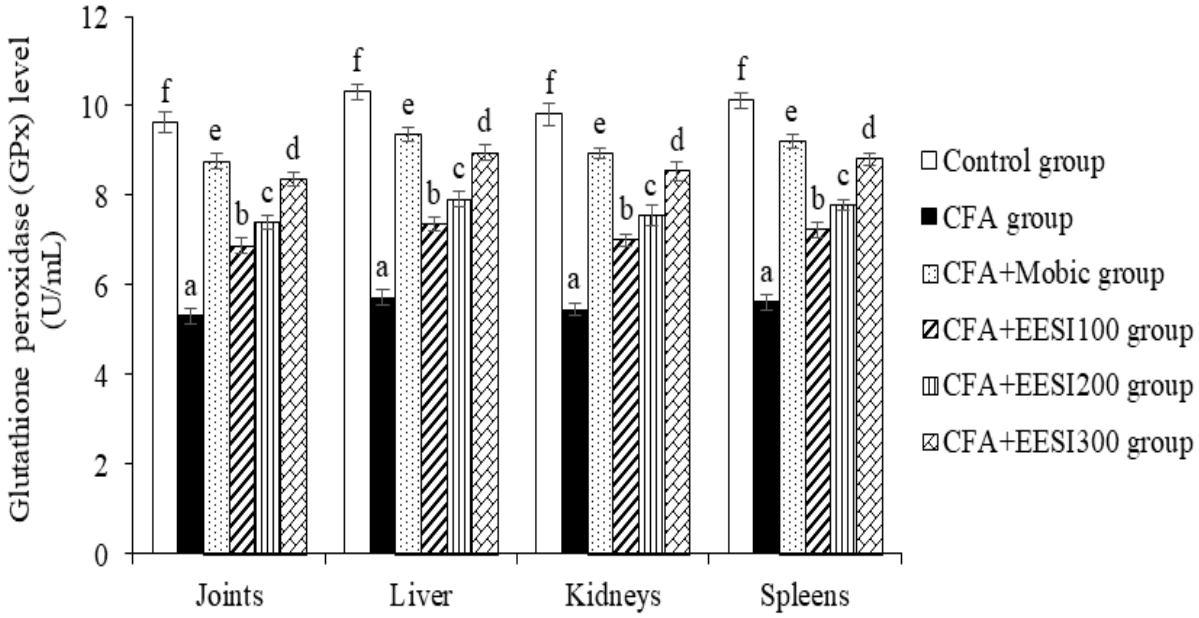


**Figure 3:** Effect of ethanol extract from Sacha inchi leaves on glutathione peroxidase (GPx) levels
Results are expressed as Mean ± SD, with letters (a, b, c, and d) indicating statistically significant group differences ($p < 0.05$)

However, among the experimental samples, EESI at 300 mg/kg recorded the highest GPx level in joint tissue at 8.37 ± 0.16 U/mL (Figure 3). EESI exhibited consistent increases in CAT activity dose-dependently. Among the experimental samples, the highest CAT activity was observed in the group treated with EESI at 300 mg/kg (Table 3). SOD activity increased dose-dependently with the EESI treatment used. Among the joint, liver, kidney, and spleen samples, EESI (100, 200, and 300 mg/kg) had the highest SOD levels at approximately 7.31 ± 0.14, 7.82 ± 0.13, 7.46 ± 0.17, and 7.61 ± 0.16 U/mL, respectively (Table 3). The ethanol extract from Sacha inchi leaves (EESI) exhibits a strong influence on key antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), within a CFA-induced rheumatoid arthritis (RA) mouse model. EESI effectively modulates these antioxidant defenses in a dose-dependent manner, highlighting its potent antioxidative properties. The treatment led to a significant enhancement in GPx levels, particularly at higher doses, with the highest concentrations observed in joint tissues. This aligns with established research emphasizing GPx's role in reducing oxidative stress by catalyzing the conversion of hydrogen peroxide into water, thereby protecting cells from oxidative damage.[39,40] The observed increase in GPx activity with EESI treatment reflects the growing evidence that natural extracts can upregulate GPx activity, thus strengthening cellular antioxidant defenses.[41] In parallel, EESI consistently elevated CAT activity, with the most pronounced effects at the highest dose. Catalase's critical function in decomposing hydrogen peroxide into water and oxygen, thereby alleviating oxidative stress, is well-documented, and the enhanced CAT activity with EESI treatment supports its relevance as a therapeutic strategy in managing oxidative

stress in inflammatory conditions.[42] The consistent increase in SOD activity across various doses of EESI further underscores its role in boosting antioxidant defenses.[43,44] The observed dose-dependent rise in SOD levels, particularly at optimal dosages, indicates EESI's efficacy in neutralizing superoxide radicals, key contributors to oxidative stress and inflammation. These observations align with current research underscoring the importance of SOD in mitigating oxidative stress,[45] supporting the hypothesis that EESI enhances cellular antioxidant defenses and offers therapeutic potential in conditions characterized by oxidative damage and inflammation.[46] The overall enhancement in GPx, CAT, and SOD activities highlights EESI's significant potential as an antioxidant therapeutic agent capable of effectively bolstering antioxidant defenses and mitigating oxidative stress in RA.

*Immunosuppressive effect Sacha inchi leaf ethanol extract*

Table 4 and Figure 4 illustrate a significant increase in white blood cell (WBC) count ($9.31 \pm 0.73 \times 10^3$ cells/mm$^3$) and a notable decrease in phagocytic activity (PA), nitroblue tetrazolium (NBT) reduction, and total immunoglobulin (TI) levels (PA 28.05 ± 1.08%, NBT 10.86 ± 0.91 mg/mL, and TI 8.47 ± 0.74 mg/mL, respectively) when stimulated by CFA compared to the control group (WBC $5.16 \pm 0.71 \times 10^3$ cells/mm$^3$, PA 50.48 ± 3.69%, NBT 19.54 ± 0.09 mg/mL, and TI 15.25 ± 0.83 mg/mL, respectively) ($p < 0.05$). However, the results were reversed after treatment with EESI (100, 200, and 300 mg/kg). In the EESI100-300 group, WBC count significantly decreased compared to the CFA group ($p < 0.05$), while PA, NBT, and TI levels increased significantly ($p < 0.05$). Notably, the levels of WBC, PA, NBT, and TI in the group treated with EESI at a concentration of 300 mg/kg (WBC $5.94 \pm 0.71 \times 10^3$ cells/mm$^3$, PA 43.91 ± 2.43%, NBT 16.99 ± 0.76 mg/mL, and TI 13.26 ± 0.88 mg/mL) yielded results comparable to the mobic group (WBC $5.68 \pm 0.72 \times 10^3$ cells/mm$^3$, PA 45.89 ± 3.32%, NBT 17.77 ± 0.98 mg/mL, and TI 13.87 ± 0.77 mg/mL) ($p > 0.05$).

**Table 4:** The effect of ethanol extract from Sacha inchi leaves on the levels of blood cell (WBC) and phagocytic activity (PA) of mice

| Parameters | Control group | CFA group | CFA+Mobic group | CFA+EESI100 group | CFA+EESI200 group | CFA+EESI300 group |
|---|---|---|---|---|---|---|
| Blood cell (WBC) ($\times 10^3$ cells/mm$^3$) | 5.16 ± 0.71$^a$ | 9.31 ± 0.73$^d$ | 5.68 ± 0.72$^a$ | 7.23 ± 0.72$^c$ | 6.71 ± 0.72$^{bc}$ | 5.94 ± 0.71$^{ab}$ |
| Phagocytic activity (PA) (%) | 50.48 ± 3.69$^d$ | 28.05 ± 1.08$^a$ | 45.89 ± 3.32$^c$ | 36.06 ± 1.42$^b$ | 38.83 ± 1.52$^b$ | 43.91 ± 2.43$^c$ |

The values are expressed as Mean ± SD, where the letters (a, b, c, and d) indicate group differences ($p < 0.05$)

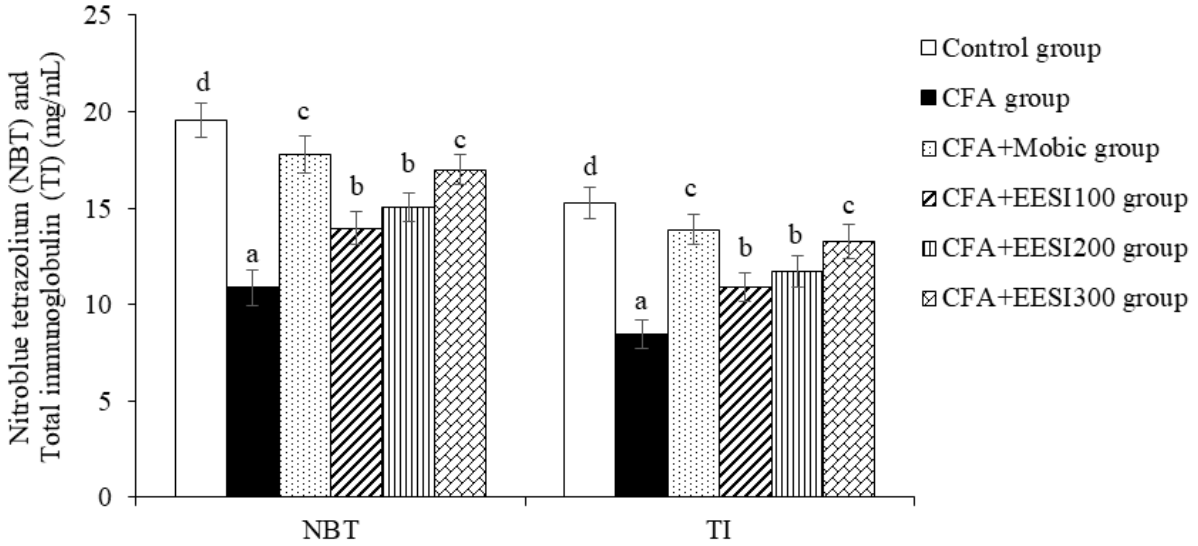


**Figure 4:** Effect of ethanol extract from Sacha inchi leaves on nitroblue tetrazolium (NBT) and total immunoglobulin (TI) levels
Results are expressed as Mean ± SD, with letters (a, b, c, and d) indicating statistically significant group differences ($p < 0.05$)

The ethanol extract from Sacha inchi leaves (EESI) has been evaluated for its impact on key immunological parameters, including white blood cell (WBC) count, phagocytic activity (PA), nitroblue tetrazolium (NBT) reduction, and total immunoglobulin (TI) levels, in a CFA-induced rheumatoid arthritis (RA) mouse model. The findings reveal EESI's potential to modulate immune responses in inflammatory conditions. The immune alterations observed in CFA-induced RA mice highlight the significant impact of the CFA-induced inflammatory response on immune function. An elevated white blood cell (WBC) count in CFA-induced mice reflects an acute inflammatory response, consistent with systemic inflammation and immune activation.[47] Additionally, substantial decreases in phagocytic activity (PA), nitroblue tetrazolium (NBT) reduction, and total immunoglobulin (TI) levels suggest compromised immune function, characterized by reduced phagocytic efficiency, diminished oxidative burst capacity, and decreased production of immune components.[48] These disruptions in immune homeostasis are indicative of the systemic immune dysfunction that characterizes the pathophysiology of rheumatoid arthritis, where chronic inflammation leads to widespread immune impairment.[49] EESI treatment effectively reversed these immune disruptions, underscoring its potential as a therapeutic agent for managing RA. Administration of EESI led to a significant reduction in white blood cell (WBC) counts, indicating a decrease in inflammatory cell infiltration, a key feature of RA pathogenesis. Moreover, EESI treatment markedly improved phagocytic activity (PA), nitroblue tetrazolium (NBT) reduction, and total immunoglobulin (TI) levels, critical indicators of immune

function. The most pronounced effects were observed at higher doses, where EESI's efficacy in normalizing these parameters was comparable to that of mobic, a well-established anti-inflammatory drug. These outcomes suggest that EESI exerts a significant anti-inflammatory effect by mitigating leukocyte infiltration and the associated inflammatory response. Additionally, the restoration of PA, NBT reduction, and TI levels highlights EESI's role in enhancing immune function, which is often impaired in RA. The observed improvements align with recent research suggesting that natural extracts can positively influence immune responses and immunological parameters in inflammatory models.[50,51] EESI, therefore, presents a promising alternative or complementary therapeutic strategy for RA, offering a potentially effective approach alongside or in place of conventional pharmacological treatments.

## Conclusion

This study highlights the antioxidant and immunosuppressive properties of Sacha inchi leaf ethanol extract (EESI) in a CFA-induced rheumatoid arthritis (RA) mouse model. At a dose of 300 mg/kg, EESI effectively reduced oxidative stress, increased the activities of key antioxidant enzymes (CAT, SOD, GPx), and improved immune function, demonstrating comparable efficacy to the standard treatment, mobic. Additionally, the extract reversed abnormal changes in white blood cell count and restored phagocytic activity. These findings suggest that EESI holds promise as a therapeutic agent for RA, offering potential benefits in reducing oxidative stress, enhancing antioxidant defenses, and modulating immune responses. Further research is needed to clarify the underlying molecular mechanisms, assess long-term efficacy and safety, and explore the potential synergy of EESI with other RA treatments.

## Conflict of Interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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