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# Safflower extract (*Carthamus tinctorius* Linn.) suppresses proinflammatory cytokines level in rheumatoid arthritis mice model stimulated by complete Freund's adjuvants

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**ABSTRACT**

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammation of the synovial membrane, and swelling, associated with excessive production of proinflammatory cytokines. In addition, anti-citrullinated protein antibodies is excessively high in RA. *Carthamus tinctorius* Linn. (*C. tinctorius*), commonly known as Safflower, is widely used as traditional medicine in Indonesia and has anti-inflammatory properties. This study aims to determine cell viability on HEK293 cells and the anti-RA activity of the ethanol extract of *C. tinctorius* on reducing edema in animal models of RA mice and to determine the anti-RA activity ethanol extract *C. tinctorius* against rheumatoid factor (RF) in RA mice. In this study, HEK293 cell line was treated with *C. tinctorius* extract at various concentrations. In addition, mice's paws were examined after being injected with complete Freund's adjuvant (CFA) as an intraplantar RA inducer and treated with 2.5 mg/kg body weight (BW) of methotrexate, and extract *C. tinctorius* at various concentrations orally once a day for 14 days. Then, the paw's thickness was measured using a gauge meter, arthritis index scoring was observed daily, and agglutination in the RF test was observed on the 17th and 31st days. The collected data was analyzed using one-way analysis of variance. Moreover, an In-Silico test was carried out by measuring the affinity and stability of *C.tintorius* extract as an anti-RA agent against the cytokines IL-1β and tumor necrosis factor-alpha. The test results showed *C. tinctorius* at various concentrations were safe to HEK293 cell lines and the administration of *C. tintorius* extract at 100, 200, and 400 mg/kg BW doses could reduce paw thickness, arthritis index scoring >1, and no agglutination occurred in the RF test. It can concluded that the C. tintorius extract is safe to HEK293 and has activity against CFA-induced RA.

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic disease with a prevalence of 0.5%–1% of the industrialized world's population. Smoking, gender (women are three times more susceptible to RA than men), obesity, age, and genetics are risk factors for RA [1,2]. RA is a multisystem autoimmune disease characterized by inflammation of the synovial membrane, swelling, and the production of anti-citrullinated protein antibodies, which is associated with excessive production of proinflammatory cytokines [3–5]. These cytokines are directly involved in many immune processes that correlate with the pathogenesis of RA [6]. Increased transcription factor Nuclear factor kappa-light-

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chain-enhancer of activated B cells (NF-kB, nuclear factor of activated T cells, activator protein-1 (AP-1), and other such as signal transducer and activator of transcription family of proteins, interferon regulatory factors (IRFs), Forkhead (Fox) family pro-teins, T-box transcription factor 21/T-boxexpressed in T cells (T-bet), the the CCAAT/enhancer-binding proteins (C/EBPs) family and the Ets transcription factor family) [7] and cytokine expression are part of the mechanism that causes joint degeneration in RA. Interleukins (ILs) and tumor necrosis factor (TNF)-α have been associated with the etiology of arthritis. TNF-α is the main cytokine that regulates the formation of other inflammatory mediators in synovial tissue and also destroys bone and cartilage by activating chondrocytes apoptosis and osteoclasts. IL-6 and IL-1β are other critical cytokines involved in the pathogenesis of RA. IL-6 involves various physiological processes, such as immune response, inflammation, and bone metabolism. IL-6 also regulates osteoclastogenesis in combination with TNF. Meanwhile, IL-1β plays an essential role in the development of RA, especially in production by monocytes and macrophage cells [3]. IL-1β involved in a variety of immunological functions such as proliferation, activation, and differentiation as well as in the recruitment of additional inflammatory cells [8]. Macrophages, which are the primary source of the proinflammatory cytokines, express HLA class II molecules. By virtue of their proximity to T cells, they may also function as antigen-presenting cells, thereby perpetuating immunological responses within the joint [9]. Natural killer cells, T cells, B cells, endothelial cells, synovial cells, and neutrophils also produce IL-1β. IL-1β activates monocytes/macrophages, thereby causing increased inflammation. In addition, IL-1β activates chondrocytes, causing cartilage damage, and activates osteoclasts that cause bone resorption [3]. These proinflammatory cytokines are also responsible for the formation of matrix-metalloproteases, inducible nitric oxide synthase, osteoclast differentiation, and expression of cell adhesion molecules [2]. As a result of the imbalance between proinflammatory and anti-inflammatory states, synovial membrane inflammation and joint injury occur [1]. If left untreated or not appropriately controlled, this disease can cause damage to cartilage and bones and reduce the sufferer's quality of life or even cause disability [5].

In RA therapy, pharmacological therapy consists of biological and non-biological disease-modifying anti-rheumatic drugs and anti-inflammatory therapy with non-steroidal anti-inflammatory drugs or glucocorticoids [10]. Anti-rheumatic drugs have a variety of significant benefits. However, its clinical use is limited for several reasons, including high cost and side effects. Hormonal irregularities, decreased immunity, digestive tract disorders, infections, osteoporosis, and cyclical vomiting syndrome difficulties have all been reported as side effects of RA drug use [1,11]. Glucocorticoid drugs have been reported to increase the risk of cardiovascular disease in RA patients due to their potential adverse effects on lipids, glucose tolerance, and the development of hypertension and obesity [6]. Based on this, many anti-RA products have been developed which are derived from natural ingredients. One of them was Kasumba Turate, more known as Safflower.

*Carthamus tinctorius* Linn., commonly known as Safflower, is widely used as traditional medicine in Indonesia. More than 200 compounds have been isolated from *C. tinctorius: flavonoids*, phenylethanol glycosides, coumarins, fatty acids, steroids, polysaccharides, and quinochalcones. Quinochalcones comprise almost all the red and yellow pigments in Safflower. The main component of yellow pigment is Hydroxysafflor yellow A (HSYA). Modern pharmacological studies show that Safflower has many beneficial bioactivities, such as anti-inflammatory, modulating the immune system, antioxidant, and antitumor effects [12–15]. Safflower has been a very effective treatment for RA [16]. The flavone luteolin and its glucopyranosides, such as luteolin 7-O-beta-Dglucopyranoside and luteolin-7-O-(6''-O acetyl)- beta-D-glucopyranoside have been reported to provide anti-inflammatory effects in vitro and *in vitro*. *In vivo* studies and several studies show that Safflower inhibits NF-κB activity at concentrations in the low micromolar range [16]. In another study, it was shown that ethanol extract from safflower leaves protects the LPS-lipopolysaccharide HaCaT cells by inhibiting the expression of iNOS, IL-6, and IL-1β and suppressing the phosphorylation of the p38, p65, phosphoactivated Jun N-terminal kinase via inactivation of mitogen-activated protein kinases/NF-κB signaling pathway [17].

Therefore, this study aims to determine the anti-RA activity of the ethanol extract of Safflower (*C. tintorius* Linn.) on reducing edema in animal models of RA mice and to determine the anti-RA activity of the ethanol extract of Safflower (*C. tinctorius*). Linn.) against rheumatoid factor (RF) in RA mice.

## MATERIALS AND METHODS

### Extraction

1 kg of dried Safflower (*C. tinctorius* Linn.) was obtained from the Safflower plantation in Waemppubu Village, Amali District, Bone Regency, South Sulawesi. Then, it was powdered and continued by macerating with ethanol for 3 × 24 hours. The filtrate collected was then evaporated to obtain a concentrated extract for further experiments.

### Cell viability (CV) assay

HEK293 (ATCC, USA) cell lines were maintained in Roswell Park Memorial Institute Medium 1640 medium (Gibco®, USA) and supplemented with 10% fetal bovine serum (Gibco®, New York, NY) and 1% penicllin/streptomycin (Gibco®, New York, NY). The cells were then incubated in an incubator at 37°C with 5% of $CO_2$. After a confluence of 80%, the HEK293 cells (4 × 105) were seeded in 96 well plates in the incubator. After 24 hours, the old medium was discarded, washed twice with phosphate buffer saline (PBS) pH 7.4 (Gibco®, USA), replaced with a new medium containing samples at various concentrations, and incubated for 24 hours. Then, the medium containing samples was discarded, washed with PBS pH 7.4 (2x), and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide solution for 4, followed by dissolving the formazan crystal by dimethyl sulfoxide.

Then, the absorbance was measured at λ570 nm. The %CV was calculated as follows: [18].

$$CV(\%) = \frac{\text{OD sample}}{\text{OD control}} \times 100$$

**RA modelling in experimental animals**

The study protocol was approved by the Institute of Research, Ethic Committee and Community Service, Universitas Halu Oleo with approval number (160/UN29.20.1.2/ PG/2023). Briefly, 30 Male mice (Mus musculus, BALB/c strain from Gold Mice Farm Mandai Maros, Makassar, Indonesia) weighing 20–30 g were used as experimenta l animals. The mice were acclimatized for seven days in a standard environment and accessed to food and water *ad libitum* in the Laboratory of the Faculty of Pharmacy, Halu Oleo University. After acclimatization, the mice were induced with 0.1 ml of complete Freund's adjuvant (CFA) intraplantarly, except the normal group. Then, they measured their paws' thickness (T0) and day 17 (Tt) using a gauge meter. The RF was also evaluated on day 17. In addition, the RA index was scored to determine whether the animals met the requirement as an RA mice model with a score > 1 was considered as experiencing RA [19,20]. RA index was scored in Table 1 below.

**Anti RA activity assay**

The RA mice model was then treated accordingly daily with one dose for 15 days of 0.5% of sodium carboxymethyl cellulose (Na-CMC) C(-) or negative control group, 2.5 mg/kg body weight (BW) of methotrexate for the positive group (C(+)), and with *Safflower* ethanolic extract at dose 100, 200, and 400 mg/kg BW, respectively (CTE100, CTE200, and CTE400) for 14 days. At day 31, their paws' thickness were re-measured (Tt). In addition, the normal group was a group which not receive CFA or any treatment. Then, the edema percentage (%edema) for each group was calculated as follows:

$$\%\text{Edema} = \frac{T_t - T_0}{T_0} \times 100 \quad (1)$$

Then, the inhibition percentage (% RA inhibition) was calculated as follows:

**Table 1.** The RA parameters.

| No | RA symptoms observed in mouse | Score |
|---|---|---|
| 1 | Swelling and redness at 1 of the mouse's toe | 0.25 |
| 2 | Swelling and redness in atleast 2 of the mouse 's toes | 0.50 |
| 3 | Swelling at mouse's feet | 0.75 |
| 4 | Swelling and redness with change of shape of mouse's feet | 1.00 |
| 5 | Swelling and redness at paws and pad of mouse's feet | 1.25 |
| 6 | Swelling and redness at paws and a bit of swelling at the pad and ankles of mouse's feet | 1.50 |
| 7 | Swelling and redness at paws, pad, and ankles of mouse | 1.75 |
| 8 | Swelling and redness at whole mouse's feet | 2.00 |

$$\%\text{RA inhibition} = \frac{\text{(edema volume of treatment group-edema volume of C- group)}}{\text{edema volume of C- group}} \times 100$$

After that, the mice were sacrificed, and blood was collected intracardially. The collected blood was put in an EDTA-containing sterile tube and centrifuged for 30 minutes at 3,000 rpm at 2°C. The plasma was then analyzed with the RF direct latex test to assay the agglutination of antigen, as well as anti-inflammatory cytokines by measuring its TNF-α, IL-1β, and IL-6 levels as per kit protocol's TNF-α, IL-1β, and IL-6 ELISA kit.

**Molecular docking studies**

The study utilized the molecular targets, namely IL 1β protein data bank (PDB code 5R8Q), IL-6 (PDB code 1ALU), and TNF-α (PDB code 7JRA). These targets were obtained from the RCSB website (https://www.rcsb.org/). After obtaining the targets, they were prepared by removing the bound ligands, solvent, and water molecules from the structures using BIOVIA Discovery Studio 2020.

Several compounds in *C. tinctorius* L. (Carthamine, HSYA, Anhydrosafflor yellow B, Safflor yellow A, and Safflor yellow B) were drawn, and their 3D structures were built using ChemDraw Ultra Professional 15.0 [21]. The geometrical structures of compounds were minimized and optimized using the semi-empirical AM1 method [22]. AutoDockTools 1.5.6 was used to prepare the targets and compounds before the docking process. Firstly, the proteins were protonated, and Kollman and Gasteiger charges were assigned to the target and compound structures [23].

Protein-ligand docking simulations were performed between several compounds in *C. tinctorius* L. and IL-1β, IL-6, and TNF-α with the assistance of AutoDock Vina [24]. The docking coordinates were set to the center positions of 1-methyl-N-{[(2S)-oxolan-2-yl]methyl}-1H-pyrazole-3-carboxamide (JGY) in IL-1β, Tartaric acid; VGY = 2-[5-(3-chloro-4-{[(1R)-1-(2-fluorophenyl)ethyl]amino}quinolin-6-yl)pyrimidin-2-yl] propan-2-ol (TLA) in IL-6, and VGY in TNF-α, with a grid area of 40 × 40 × 40 and 0.375 Å point spacing. The validated procedures identified a root mean square deviation of JGY, TLA, and VGY below 2 Å (Fig. 1). Other docking procedures were set to default. Finally, the simulation results were analyzed through visualization using BIOVIA Discovery Studio 2020.

**Statistical analysis**

Data collected was presented as mean ± SD and analyzed statistically using one-way analysis of variance with a significance of 0.05.

## RESULT AND DISCUSSION

**CV asssay**

Evaluation of *C. tinctorius extract* for its potential cytotoxicity is considered an essential step in evaluating its

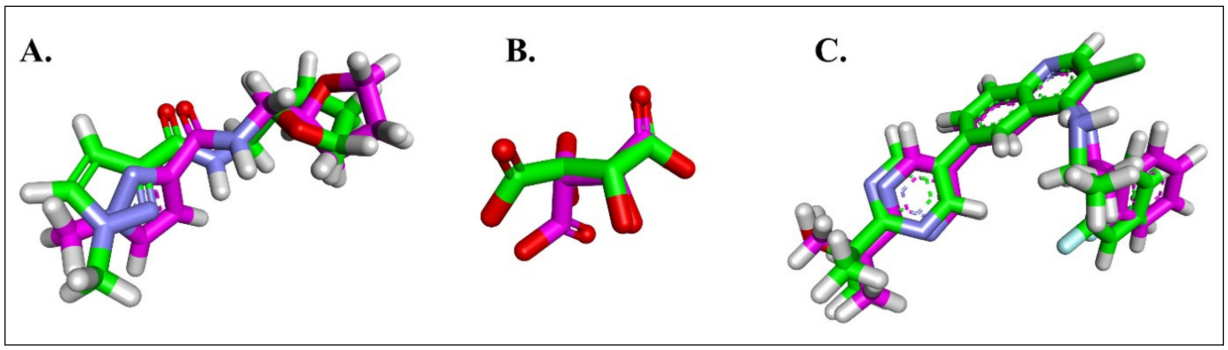


**Figure 1.** Overlaying the X-ray crystallography pose (in green) and the docked pose (in pink) of JGY with IL-1β (1.834 Å), TLA with IL-6 (1.813 Å), and VGY with TNF-α (1.179 Å)

**Table 2.** The HEK293 CV.

| Concentration (µg/ml) | Viability cells (%) |
|---|---|
| 7.5 | 94.95 ± 1.41 |
| 15.675 | 92.78 ± 1.98 |
| 31.25 | 92.12 ± 1.23 |
| 62.5 | 88.07 ± 1.27 |
| 125 | 88.17 ± 2.41 |
| 250 | 82.80 ± 0.57 |
| 500 | 74.89 ± 2.46 |
| 1,000 | 68.57 ± 2.27 |

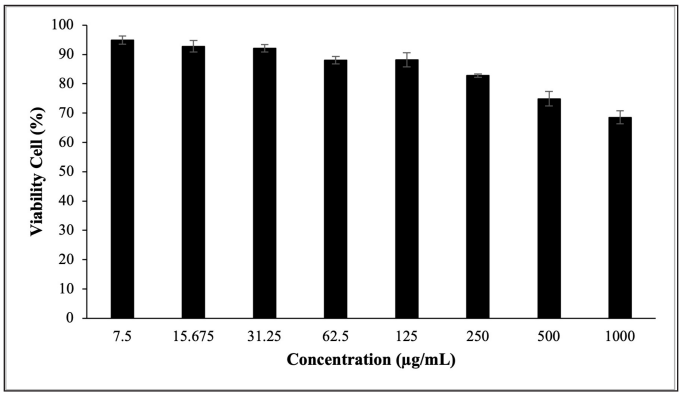


**Figure 2.** The CV (%) of HEK293 post treated with *C. tinctorius* at various concentrations.

suitability for further applications. The in vitro cytotoxicity test of *C. tinctorius* extract was performed in human embryonic kidney 293 (HEK293) cell lines to determine the CV whether it is safe or not to normal cells. The CV measure the percentage of number of living cell post treatment of samples [25,26].

It was found that *C. tinctorius* extract at various concentrations from 7.5 to 250 µg/ml were safe to HEK293 cells with %CV ≥ 80% (Table 2 and Fig. 2), while concentration 500 and 1,000 µg/ml were considered as weak toxicity. Hence, it can be concluded that *C. tinctorius* extract is safe against HEK293 cell lines [26].

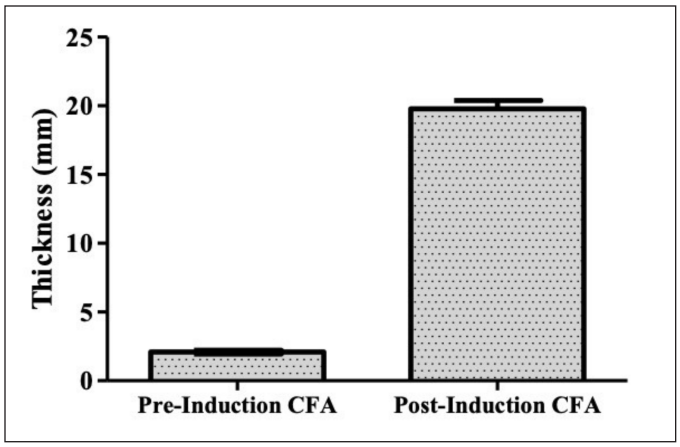


**Figure 3.** The effect of CFA injection on mice paw thickness. Data is presented as mean±SD.

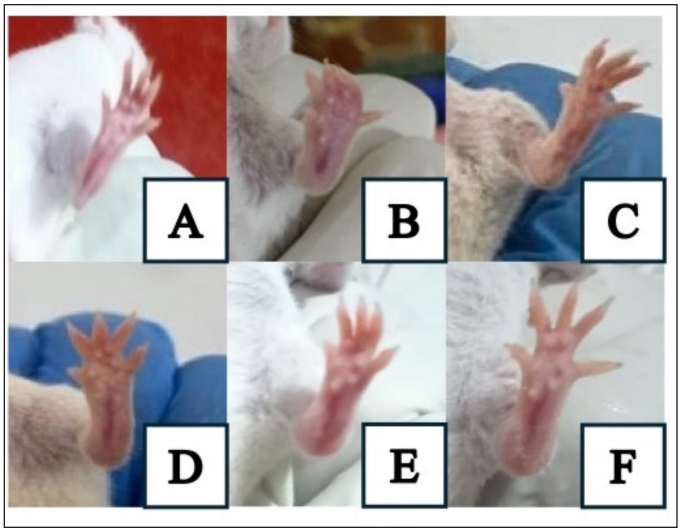


**Figure 4.** Representative pictures of RA model in mice, examined on day 30 after induced with CFA. (A) normal group. (B) C(-), which was induced with CFA and received no treatment. (C) C(+), which was induced with CFA and received 2.5 mg/kg BW of methotrexate. (D–F) treatment groups, which were induced with CFA and received *C. tinctorius* extract at concentration 100; 200; and 400 mg/kgbw (CTE100, CTE200, and CTE400).

**RA modelling in mice**

CFA-induced RA in mice is a test animal model commonly used for preclinical studies of arthritis due to its short test duration and easy measurements [27]. After administration of CFA, inflammation occurs caused by fluid exudation, neutrophil infiltration, and mast cell activity, including stimulating the phagocytosis and cytokine release such as IL-1β, IL-6, and TNF-α. The role of inflammatory mediators and serological and pathological changes in the CFA-induced arthritis model is similar to RA in humans [19,28,29].

Our result found that on day 17, Figure 3 exhibited the increased edema volume of mice's paws by measuring the thickness (in mm) of paws. The thickness of the mice's paw indicated the edema occurred post-inducing with CFA, causing RA. They significantly differed before and after inducing with CFA ($p$<0.05). In addition, the arthritis index scoring demonstrated that the index obtained was more than 1. It means that the mice were successfully modelled for the RA model. RF also proved that the administration of CFA was causing agglutination as a response in latex tests [19,30].

**Anti-RA activity**

The RA is shown in Figure 4. Inflammatory signs were characterized by swelling and redness (Fig. 4(B)). Figure 4(C–F) exhibit the improvement, indicated with decreased edema in mice's paw.

The anti-RA effect can be seen from the paw's thickness (Fig. 5(a)) and percentage of RA inhibition. The paw's thickness of mice were reduced after treatment with methotrexate, CTE100, CTE200, and CTE400. This measurement indicated the reduction of edema. The highest reduction was found in day 30, which ranking as follows: methotrexate > CTE400 > CTE200 > CTE100. After measuring the paw's thickness of mice, the RA inhibition was calculated. Based on the data in Figure 5(b), RA inhibition was calculated. The calculation of the percentage of RA inhibition starts from day 17 to day 30. The percentage of inhibition was calculated using the thickness of the mice paw edema at time t (Tt) subtracted by the thickness of the initial mice paw edema (T0). The percentage of inhibition of the paw edema of the normal mice group, CTE100. CTE200, and CTE400 were calculated and compared with the C(-). The RA inhibition according to treatment groups can be seen in Figure 5.

According to the result obtained, *C. tinctorius* provided an effect in a concentration-dependent manner, with the highest concentration used which was CTE400, followed by CTE200 and Group CTE100, with concentrations of 200 and 100 mg/Kg BW, respectively. The highest percentage of RA inhibition was showed with C(+) as a positive group, which was 98.88%, followed by CTE400, CTE200, and CTE100, which were 94.94%, 90.86%, and 86.99%, respectively. While C(-) did not provide anti-RA activity, as shown with %RA inhibition as 0%. Moreover, the normal group did not show any RA signs. CTE400 showed a good anti-RA result as a positive control, which was 2.5 mg/KgbBW of methotrexate ($p$ > 0.05).

CTEs decreased the TNF-α levels in a dependent-concentration manner, with CTE200 having higher activity compared to CTE100 and CTE50, respectively ($p$ < 0.05) compared to the RA model and C(-), yet not significantly different to C(+) used ($p$ > 0.05). It is illustrated at Figure 6. It means that CTE at a concentration of 200 mg/kg BW has similar potency in lowering TNF-α levels with control positive used, 2.5 mg/kg BW of methotrexate.

CTEs were lowering IL-1β in a dependent-concentration manner with higher concentration provided higher activity, starting from CTE200, CTE100, and CTE50, respectively ($p$ < 0.05), compared to the RA model, as illustrated at Figure 7.

On the other hand, CTEs also lowered IL-6 levels in a dependent concentration manner. CTE200 had a higher ability to lower IL-6 levels, followed by CTE100 and CTE50, respectively ($p$ < 0.05), compared to the RA model. It is presented in Figure 8.

In addition, *C. tinctorius* at a concentration of 100, 200, and 400 mg/kgbW showed no agglutination formation on the RF assay. RF is an immunoglobulin that can be detected in RA patients. RF potentiated antigen presentation to T cells through dendritic cell uptake of immune complexes with exogenous antigens and via B cells. RF plays a helpful role in diagnosis, providing information about prognosis, predicting patient subgroups, and predicting the onset of RA. In several studies, immunosuppressive treatment reduced RF serum levels by inhibiting the pro-inflammatory cytokines [31,32].

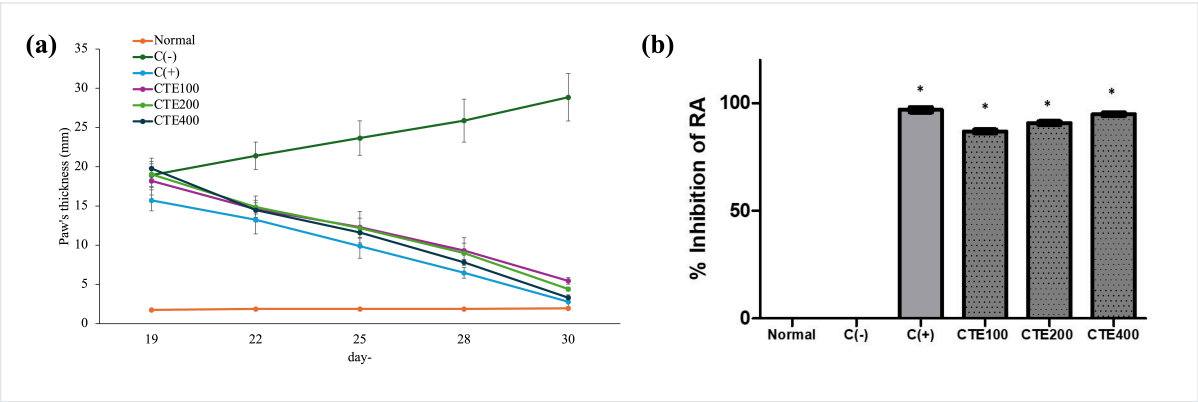


**Figure 5.** The % RA inhibition. Data is presented as mean ± SD (* indicates the significance difference to group I ($p$<0.05)).

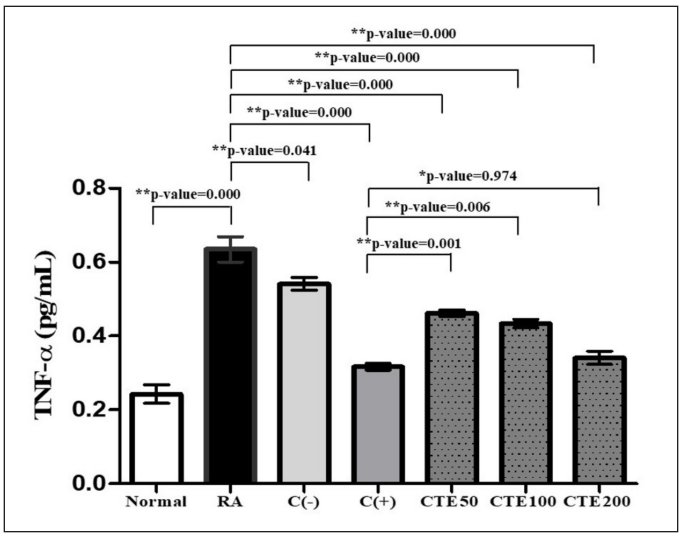


**Figure 6.** The effect of *C. tinctorius* extract on lowering TNF-α levels. Data is presented as mean ± SD.

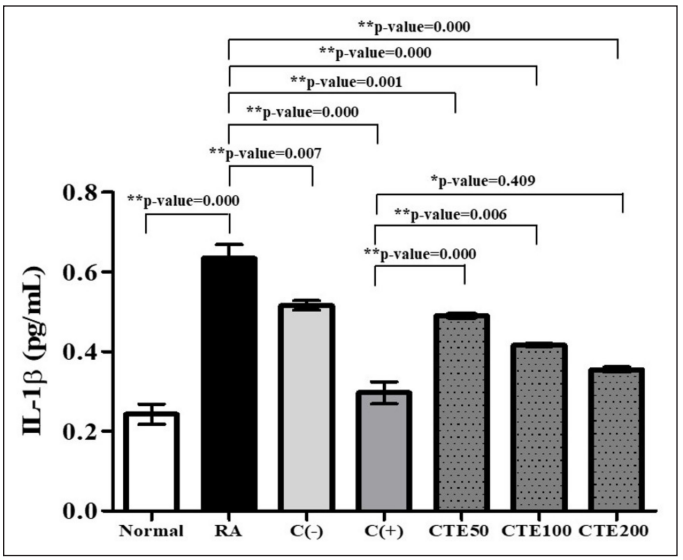


**Figure 7.** The effect of *C. tinctorius* extract on lowering IL-1β levels. Data is presented as mean ± SD.

The anti-RA activity of the ethanol extract of *C. tinctorius* flowers might be affected by the presence of alkaloids, saponins, terpenoids, flavonoids, tannins and anthraquinones and has been proven to have antipyretic, analgesic, antioxidant, anti-inflammatory [14], immunosuppressive activity [13].

### Molecular docking

In the IL-1β target, 5 compounds were identified to have had a satisfying affinity with a binding energy range of −6.9 to −7.9 kcal/mol (Table 2). Carthamine and Safflor Yellow B exhibited the most negative binding energy among these compounds. Interestingly, these two compounds showed different interactions when bound to the active site of IL-1β (Fig. 9; Table 4). Specifically, Carthamine was observed to

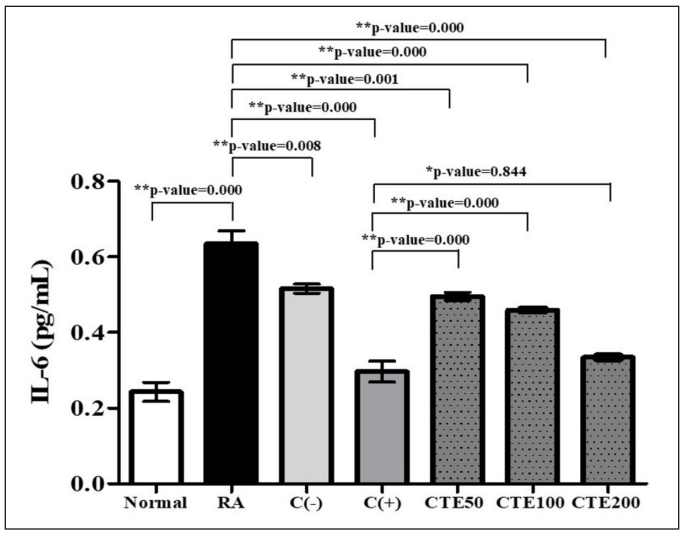


**Figure 8.** The effect of *C. tinctorius* extract on lowering IL-6 levels. Data is presented as mean ± SD.

**Table 3.** Summary of binding energies for several compounds in *C. tinctorius* L. against IL-1β, IL-6, and TNF-α.

| Compounds | Binding energy (kcal/mol) | | |
|---|---|---|---|
| | IL-1β | IL-6 | TNF-α |
| Carthamine | −7.9 | −6.2 | −5.4 |
| HSYA | −7.0 | −5.9 | −5.5 |
| Anhydrosafflor yellow B | −6.9 | −6.4 | −5.2 |
| Safflor yellow A | −7.4 | −6.1 | −5.7 |
| Safflor yellow B | −7.8 | −7.0 | −5.9 |

have formed four hydrogen bonds with the residues Val41, Lys65, Asn66, and Pro23, as well as two hydrophobic interactions with Val19 and Leu67. On the other hand, Safflor Yellow B had formed four hydrogen bonds with the residues Tyr24, Leu80, Leu82, and Val132, and one hydrophobic interaction with Lys77. A summary of these interactions can be seen in Table 4.

IL-1β interacts with the IL-1 receptor (IL-1R) to trigger an inflammatory response. The residues Lys65, Asn66, Leu80, and Leu82 are amino acids in the binding domain crucial for the activity of IL-1β [32]. The binding of IL-1β by Carthamine and Safflor Yellow B can disrupt or inhibit the interaction between IL-1β and IL-1R, potentially altering or halting the inflammatory response initiated by IL-1β [33].

In the IL-6 target, the identified compounds from *C. tinctorius* L. exhibited a binding energy range of −5.9 to −7.0 kcal/mol (Table 3). Safflor yellow B and Anhydrosafflor yellow B displayed better affinity than other compounds in this target. Safflor Yellow B was observed to have interacted with the residues Glu37, Pro47, Met49, Glu154, Ser151, Phe155, and Arg161 of IL-6 (Table 4), forming hydrogen bonds. Meanwhile, Anhydrosafflor Yellow B had only formed two hydrogen bonds with the residues Lys48 and Gln57 (Fig. 10; Table 4). Interestingly, all compounds exhibited the same

**Table 4.** Summary of molecular interactions for several compounds in *C. tinctorius* L. against IL-1β, IL-6, and TNF-α.

| Compounds | IL-1β | | IL-6 | | TNF-α | |
|---|---|---|---|---|---|---|
| | H-bond | Hydrophobic | H-bond | Hydrophobic | H-bond | Hydrophobic |
| Carthamine | Val41, Lys65, Asn66, Pro23 | Leu67, Val19 | Glu33, Ser35, Lys36, Arg150 | Phe56 | Tyr135, Tyr227 | Val89, Ile231 |
| HSYA | Met20, Ser21, Lys63, Val41 | Val40 | Met49, Glu51, Gln157 | Phe56 | His91, Tyr135, Tyr227, Ala232 | Tyr135 |
| Anhydrosafflor yellow B | Tyr24, Leu80, Leu134, Gly135 | Pro23 | Lys48, Gln57 | Phe56 | Ala90, His91, Tyr135, Gly224, Tyr227 | - |
| Safflor yellow A | Met20, Lys63, Lys65 | - | Lys48, Glu154, Gln157 | Phe56 | Arg82, Thr83, Ser85, Asn115, Tyr135 | Val89, Leu112 |
| Safflor yellow B | Tyr24, Leu80, Leu82, Val132 | Lys77 | Glu37, Pro47, Met49, Glu154, Ser151, Phe155, Arg161 | - | His91, Asn110, Gln137, Tyr195, Gly224, Tyr227 | Tyr135, Tyr195 |

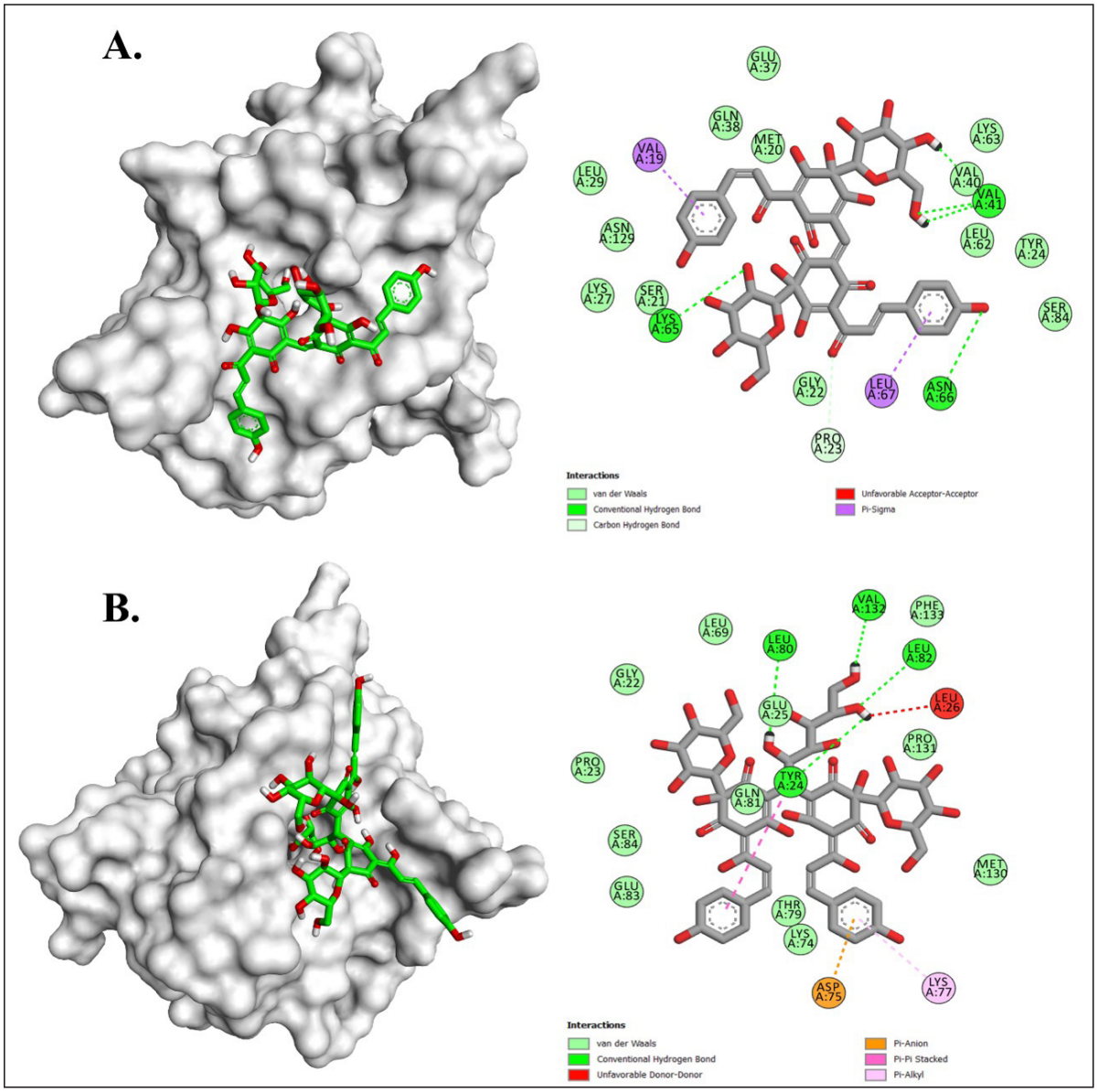


**Figure 9.** Molecular interactions of (A) Carthamine and (B) Safflor yellow B against IL-1β.

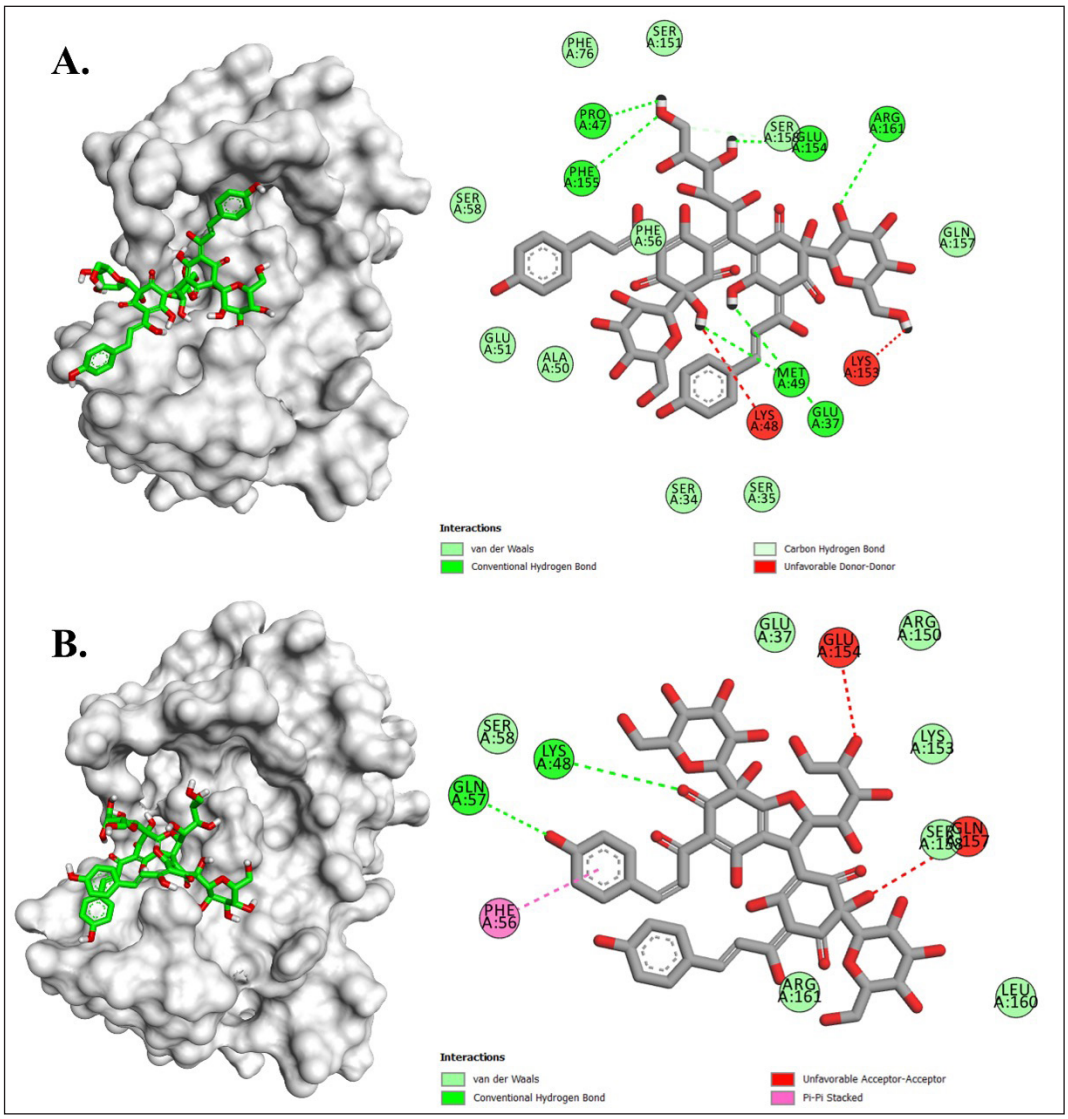


**Figure 10.** Molecular interactions of (A) Safflor yellow B and (B) Anhydrosafflor yellow B against IL-6.

hydrophobic interactions with Phe56 of IL-6. In IL-6, one of the domains involved in interactions with the receptor is the binding site loop domain, located in the amino acid sequence 30–60 in its three-dimensional structure [34]. The compounds Safflor Yellow B and Anhydrosafflor Yellow B are capable of interacting with this domain, and it is believed that they can disrupt the interaction of IL-6 with the IL-6 receptor to form homodimerization with gp130, thereby inhibiting the activation of the inflammatory signaling system [35].

In the TNF-α target, all compounds showed binding energies ranging from approximately −5.2 to −5.9 kcal/mol (Table 3). In this target, Safflor yellow A and B were estimated to have better affinity for TNF-α than other compounds. Safflor yellow A formed hydrogen bonds with residues Arg82, Thr83, Ser85, Asn115, and Tyr135, as well as hydrophobic interactions with Val89 and Leu112. Meanwhile, Safflor yellow B formed hydrogen bonds with His91, Asn110, Gln137, Tyr195, Gly224, and Tyr227, as well as hydrophobic interactions with Tyr135 and Tyr195 (Fig. 11; Table 4). Interestingly, the interactions with Tyr135, Tyr227, and Tyr195 resembled the interactions of the inhibitor VGY with TNF-α [36]. This study revealed the ability of Safflor yellow B to form hydrogen bonds and hydrophobic interactions with TNF-α, leading to a significant disruption of the TNF receptor binding site. Based on these results, it was reasonable to assume that the binding of Safflor Yellow B to TNF-α monomers prevented trimer formation, which was necessary for the signaling process [37,38].

This computational study highlights the potential of Safflor yellow B, a compound derived from *C. tinctorius* L., in playing a crucial role in anti-inflammatory effects, especially in RA. RA is a complex autoimmune disease characterized by chronic inflammation. Understanding interactions at the molecular level is highly beneficial in developing effective RA therapy. The ability of Safflor yellow B to tightly bind to IL-1β, IL-6, and TNF-α, which play a crucial role in the pathogenesis of

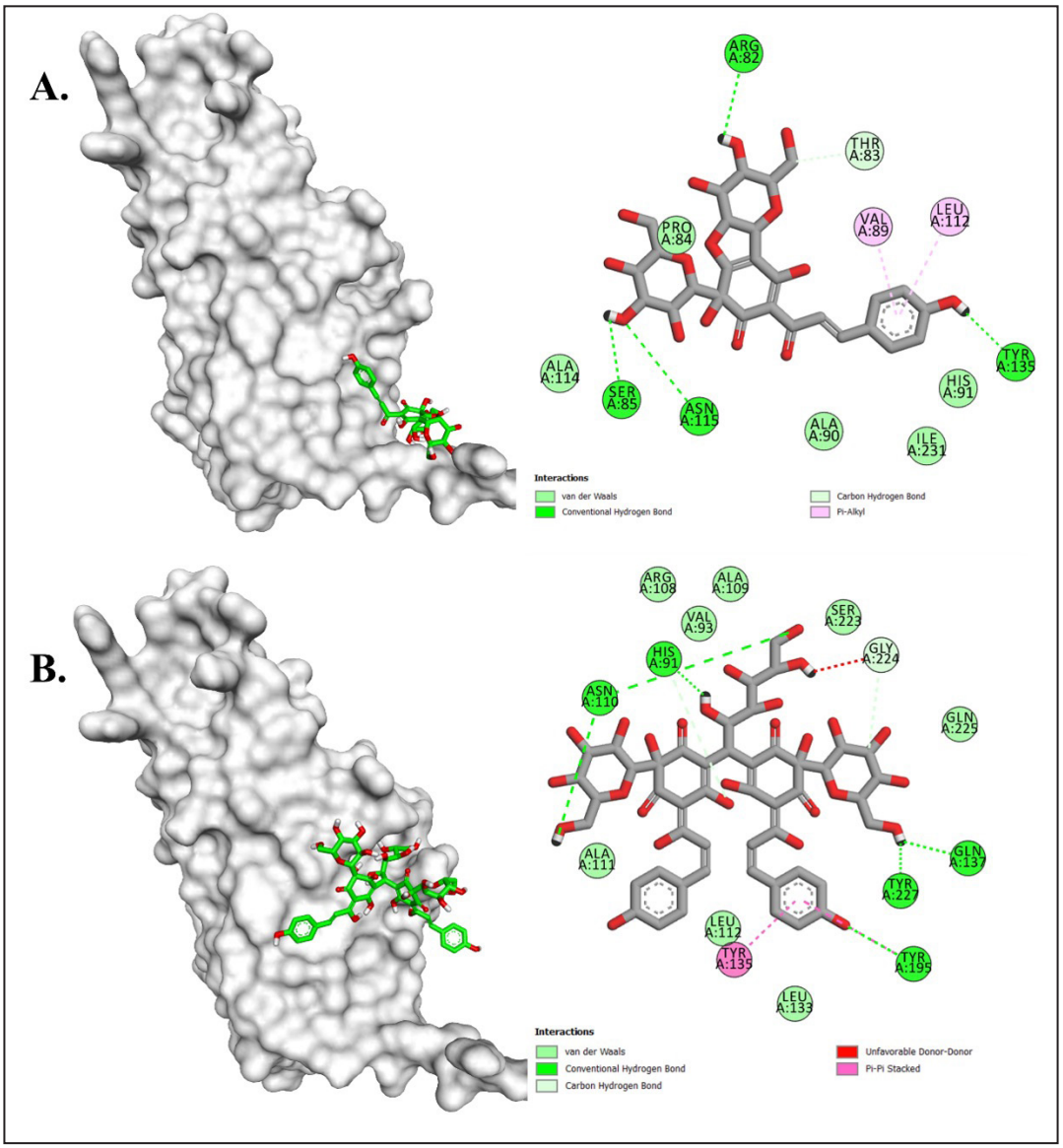


**Figure 11.** Molecular interactions of (A) Safflor yellow A and (B) Safflor yellow B against TNF-α.

RA, indicates that this compound could be a promising candidate for the development of anti-inflammatory drugs related to RA.

## CONCLUSION

Safflower (*C. tinctorius* Linn.) ethanolic extract is safe to HEK293 and has anti-RA activity by reducing paw edema in RA mice-model by inhibiting RF by not forming the agglutination of immunoglobulin, as markers for rheumatoid post treatment with *C. tinctorius* extract. It also can be concluded that by inhibiting the RF, the levels of pro-inflammatory cytokines serum such as TNF-α, IL-1β, and IL-6 will also be supressed . Moreover, In addition, 200 and 400 mg/kgBW were the most effective ($p < 0.05$) They reduced the paws edema and reducing the inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, as well the rheumatoid factor by not forming the agglutination of immunoglobulin, as markers for rheumatoid post treatment with *C. tinctorius* extract.

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## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all

aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest.

**ETHICAL APPROVALS**

The study protocol was approved by the Institute of Research, Ethic Committee and Community Service of Faculty of Pharmacy, Halu Oleo of University, Kendari, Indonesia (Approval no.: 160/UN29.20.1.2/ PG/2023).

**DATA AVAILABILITY**

All the data is available with the authors and shall be provided upon request.

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**USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY**

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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