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Research Article

The Effect of *Carica pubescens* Seeds Ethanol Extract on Lymphocyte Count and Paw Edema in Male White Swiss Webster Mice

Fania Putri Luhurningtyas[1], Evi Novitasari[2]

[1]Agrotechnology Study Program, Tidar University, Magelang, Central Java, Indonesia
[2]Pharmacy Study Program, Ngudi Waluyo University, Semarang, Central Java, Indonesia
faniaputri@untidar.ac.id

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ABSTRACT

Inflammation is a natural tissue response to infection in the body. The potential anti-inflammatory effect of secondary metabolites in carica seeds has been overlooked. This study aimed to investigate and develop the anti-inflammatory activity of the ethanolic extract of carica seeds (*Carica pubescens*) against foot edema and lymphocyte count in male white mice. Bioactive carica seeds were extracted using the maceration method with 70 % ethanol solvent. The identification of secondary metabolites was conducted using the thin-layer chromatography method. Treatments were administered to six groups: the negative control group (sick), solvent control, positive control with Diclofenac Sodium, and three doses of the ethanol extract of carica seeds (cS) at 100, 200, and 400 mg/kg body weight (BW). Edema was induced using intraplantar 1 % carrageenan on the soles of mice, and lymphocyte count was determined by blood sampling from the lateral tail vein using a hematology analyzer. Carica seeds doses of 200 mg/kg BW and 400 mg/kg BW demonstrated anti-inflammatory activity comparable to the positive control Diclofenac Sodium ($p > 0.05$). Variations in CS doses influenced the lymphocyte count at the 3rd and 6th hours. In conclusion, the ethanolic extract of carica seeds exhibits anti-inflammatory effects in male white mice induced by 1 % carrageenan, reducing edema thickness and affecting leukocyte count.

Keywords: *Carica pubescens*; Lymphocyte; Paw edema; Seeds; Swiss Webster

INTRODUCTION

Inflammation is the body's attempt to remove antigens attacking from within, regulate tissue repair degrees, and eliminate irritant substances. One of the indicators in the inflammation process is the migration of lymphocytes (Zahra & Carolia, 2017). Lymphocytes are a type of white blood cell involved in the immune system in vertebrates. Consequently, the release of inflammatory mediators such as histamine, bradykinin, prostaglandin, and serotonin occurs. Increased mediator release causes increased capillary permeability leading to increased extravascular fluid accumulation known as edema (Balta et al., 2016).

Pharmacologically, inflammation aims to slow down or limit tissue damage in edematous areas. Commonly used modern drugs for inflammation are Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and corticosteroids (Buer, 2014). The use of herbal medicines as anti-inflammatory agents is based on the following reasons: the relatively lower cost of raw materials and the lower side effects compared to synthetic drugs. Diclofenac sodium, which is a non-steroidal anti-inflammatory drug (NSAID), has side effects such as digestive disorders, gastric ulcers, and gastrointestinal bleeding when used long-term or without proper supervision. Research results show that the adverse drug reaction profile for NSAID use in gout patients indicates that diclofenac sodium accounts

for 70 % of adverse drug reactions, while mefenamic acid accounts for 30 %. The most frequently reported adverse drug reaction is stomach pain, at 56.3 % (Permata & Azmi, 2023). Herbal medicines are generally considered safer for long-term use, although there is still a risk of side effects if consumed in very high doses or improperly.

One of the medicinal plants still under development is *Carica pubescens*, known as "carica". Carica is a typical flora of the Dieng Plateau and is better known for its processed candies. The utilization of carica is limited to its pulp only; its clustered and dense seeds are not very useful for the local community, resulting in a large amount of waste in the form of carica seeds in candy production (Sasongko et al., 2016). Carica belongs to the *Caricaceae* family and shares one genus with papaya (*Carica papaya).* Plants in the same family tend to have similar compound content (Bulla et al., 2020).

Recent research reported that the identification result of secondary metabolite content in carica seed extract is alkaloids, flavonoids of the quercetin group, and rutin glycosides (Luhurningtyas et al., 2020). The research results that the gel extract of carica seeds contains flavonoids that act as antioxidants. Antioxidant compounds can inhibit inflammation by capturing free radicals that cause tissue damage, triggering arachidonic acid biosynthesis and COX enzyme, thus inhibiting prostaglandin formation. The flavonoids contained in the ethanol extract of carica seeds have analgesic activity by inhibiting lymphocyte accumulation in inflamed areas.

Based on the background description above, the researchers tested the anti-inflammatory activity of the ethanol extract of carica seeds on the thickness of edema and the number of lymphocytes in male white mice induced by carrageenan. The objectives of this research are to evaluate the potential of carica seed extract as an effective anti-inflammatory agent and to provide scientific evidence supporting the use of carica seeds as an alternative herbal anti-inflammatory medication. By analyzing the impact on edema thickness and lymphocyte count, this study aims to validate the traditional use of carica seeds and contribute to the development of new herbal treatments for inflammation.

METHODS

Instruments and Materials

The instruments used were Memmert oven, Philips blender, glass maceration jar, rotary evaporator (RE-100 Pro), Whatman filter paper, Nescafe chamber, silica gel 60 F254 TLC plate (Merck), capillary pipes, UV lamps 254 and 366, Mitutoyo calipers, Rayto RT-7600 for Vet hematology analyzer, Monotes EDTA K3 microtubes, Braun surgical blade no.12, oral probe, 1 ml syringe (Terumo), mortar and pestle, glassware (Pyrex), porcelain cup, porcelain crucible, reaction tubes (Iwaki), glass funnel (Pyrex), analytical balance (Ohaus), water bath, muffle furnace (Thermolyne), moisture balance (Ohaus).

The materials used were carica seeds obtained from the "Exotic Carica" souvenir center in the Dieng highlands, 70 % ethanol (Technical Grade), reference standards for TLC namely quercetin (Sigma) and rutin (Sigma), butanol, glacial acetic acid, ethyl acetate, n-hexane, citroborate reagent, 0,9 % NaCl (Otsuka), distilled water, carrageenan, Na CMC, generic 50 mg Sodium Diclofenac tablets (PT First Medipharma).

Preparation of Ethanol Extract from Carica Seeds

Secondary metabolites from carika seeds were extracted using the maceration method. 250 grams of plant powder was macerated in 1750 mL of 70 % ethanol for three days. Maceration was carried out repeatedly with 750 mL of the same solvent for two days. The filtrate resulting from maceration is collected and concentrated using a rotary evaporator. The extract was then concentrated further using a water bath (Luhurningtyas et al., 2020).

Determination of Moisture Content

Water content was determined to determine the water content in plant material powder and ethanol extract of carika seeds. A total of 2-3 grams of sample was weighed into an aluminum cup that had been heated to 105 °C. The sample is then put into a drying chamber with a balanced humidity content. The sample is spread evenly in an aluminum cup.The samples are then dried in a drying chamber at a set temperature until the reading is automatic. Results the water content obtained if the value is <10 % is considered acceptable (Kementerian Kesehatan RI, 2017).

Determination of Ash Content

Two grams of plant material powder or ethanol extract of Carica seeds are weighed and placed in a porcelain cup that has been previously weighed (Kementerian Kesehatan RI, 2017). The sample is then heated gradually in a Muffle Furnace to around 600 °C for approximately 6 hours until the sample is judged to be carbon free (charred). After the heating process is complete, the sample is cooled and weighed again.

Chromatogram Profile of Carica Seeds Extract

Screening for secondary metabolites of carica seeds was carried out using the Thin Layer Chromatography (TLC) method. This method is used to identify flavonoid and alkaloid compounds (Luhurningtyas et al., 2020). The mobile phase for identifying flavonoid compounds is butanol, with a ratio of glacial acetic acid: water (4:1:5). The stationary phase used is silica gel 60 F254. Quercetin and Rutin were used as reference standards for flavonoid identification. Sitroborate spray reagent is used to detect compounds containing flavonoids. Visualization of stains at points that have been made on the TLC plate media uses visible light, with UV wavelengths of 254 and 366 nm.

Determination of Edema Reduction Effectiveness

Swiss Webster male white mice aged 2-3 months with body weights of 20-30 grams were used as test animals. The calculation of the required number of test animals was based on the Federer formula. The test animals were divided into six groups, each consisting of 4 randomly selected mice.

This study tested the anti-inflammatory activity using the edema formation method with 1 % carrageenan. This test has received approval from the Research Ethics Committee (REC) of Ngudi Waluyo University Ungaran with number 25/REC/EC/UNW/2022. In this study, there were several control groups to ensure the validity of the results. The negative control group serves to determine whether test animals that were only induced with carrageenan to cause inflammation will show a natural reduction in edema. Meanwhile, a solvent control group was used to evaluate whether the solvent given to the test animals had an effect in reducing edema. The positive control group was used to assess the effectiveness of the anti-inflammatory activity of test animals given conventional anti-inflammatory drugs, namely Diclofenac Natrium, according to the indications for use.

Before testing begins, the test animals are acclimatized for one week and fasted for approximately 18 hours, but are still given access to drinking water. Each mouse was marked with a marker on the edge of the left ankle for easy identification during measurements. The initial thickness (Co) of edema in the left leg of mice was measured using a caliper before induction with carrageenan. This study included all control groups to ensure that each variable tested could be measured precisely and that the results obtained were accurate and reliable.

Lymphocyte Test

Test animals that had been acclimatized and before being induced by carrageenan (at hour 0), had their blood taken via the lateral tail vein to determine the number of normal lymphocytes in mice. Next, blood was taken at 3 and 6 hours to observe the number of lymphocytes in the test animals after carrageenan induction. Each blood sample at each time point (t0, t3, and t6) was collected in EDTA-filled microtubes, with 1 mL of blood. The number of lymphocytes is checked using a hematology analyzer. blood was taken via the lateral vein in the tail of the test animal. The aim of taking blood was to determine the number of lymphocytes in test animals induced by carrageenan (Aldi et al., 2018).

Table 1. The grouping of test animals

No	Group	Treatment
1	Negative control (sick control)	Test animals were given 1 % carrageenan
2	Solvent control	Test animals were given 0.1 % Na CMC suspension
3	Positive control	Test animals were given 6.5 mg/kgBW sodium diclofenac suspension
4	Treatment dose 1	Test animals were given ethanol extract of carica seeds suspension at a dose of 100 mg/kg BW
5	Treatment dose 2	Test animals were given ethanol extract of carica seeds suspension at a dose of 200 mg/kg BW
6	Treatment dose 3	Test animals were given ethanol extract of carica seeds suspension at a dose of 400 mg/kg BW

RESULTS AND DISCUSSION

Characterization and Chromatographic Profile of Carica Seeds Extract

The herbal material utilized in this study is a part of the carica plant that is rarely utilized, namely its seeds. Seed samples were obtained as by-products from the production of carica fruit preserves at the Exotic Carica production facility in Wonosobo. The obtained carica underwent a determination process, confirming it to be *Carica pubescens*. The key identification features of the acquired carica plant are 1b-2b-3b-4b6b-7b-9b-10b-11b-12b-13b-14a-15a.

Secondary metabolite extraction from carica seeds was performed using the maceration method with 70 % ethanol as the solvent. The choice of maceration method and the use of 70 % ethanol were based on the research regarding the analgesic activity of carica seed extract (Sasongko et al., 2016).

a b

Figure 1. The carica plant (a) and its seeds (b).

The obtained yield percentage is 7.08 % w/v. The yield obtained is less than 10 %. The yield percentage may not necessarily estimate the quantitative amount of active compounds present in the extract, as well as the quality of the extract produced (Saifudin, 2014.) The determination of moisture content and ash content of the ethanol extract of carica seeds can be seen in Table 2.

The moisture content of the carica seed extract in the study does not exceed the predetermined moisture limit (<10 %). The purpose of determining the moisture content of the extract is to ascertain the water content in the extract, which serves as a medium for fungal growth. The total ash content in the carica seed extract is 7.5 %. The ash content is used to determine the extent of internal and external mineral content in the sample.

Table 2. Results of Moisture Content and Ash Content of Ethanol Extract of Carica Seeds

Parameter	Carica Seeds Extract (% w/w)
Moisture content	3.34
Total ash content	7.5

The chromatogram pattern of carica seed extract using Thin-Layer Chromatography method. The mobile phase used is the BAA mobile phase (4:1:5) to determine the flavonoid content and n-hexane: ethyl acetate mobile phase (1:9) to determine the alkaloid content. The chromatogram results in Figure 2 show positive ethanol extract separation of carica seed extract containing flavonoids as it produces five spots with successive Rf values of 0.36; 0.65; 0.75; 0.83; and 0.93. The Rf values generated meet the standard Rf for flavonoids, which is 0.31-0.98 (Harborne, 1987). The TLC plate (Figure 2) shows a single spot with a bluish-purple stain under UV 254 nm and bright blue fluorescence under UV 366 nm, with an Rf value of 0.87.

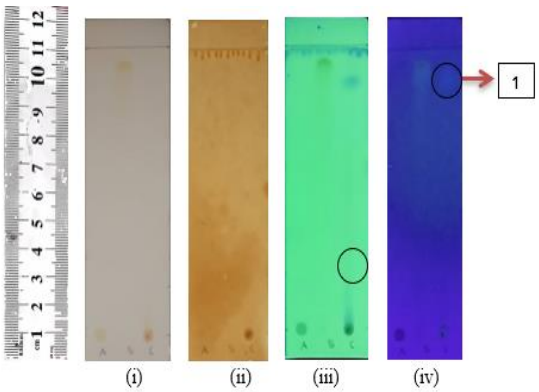

Figure 2. Separation and identification results of flavonoids using B:A: A solvent system. (A) Rutin standard (B) Quercetin standard (C) Ethanol extract of carica seeds (i) visible light (ii) UV light at 254 nm (iii) UV light at 366 nm without reagent (iv) UV light at 366 nm with reagent.

It is known that anti-inflammatory activity is influenced by the presence of secondary metabolites contained in the formula. The flavonoid content in this formula can act as an anti-inflammatory by inhibiting the release of arachidonic acid, secreting lysosomal enzymes from neutrophils and endothelial cells, and inhibiting the exudation and proliferation phases of the inflammatory process. Other research also reports that the flavonoid content in the Juniperus phoenicea plant of 11.33 mg EQ/g can prevent inflammation by reducing the number of white blood cells, platelets, and CRP (C-Reactive Protein) levels, as well as better fibrinogen formation. The research also wrote that compared to dexamethasone treatment when induced by carrageenan (Husna et al., 2022; Setyopuspito Pramitaningastuti, 2017). Based on a literature survey and in silico analysis, it contains alkaloids, including harmine, berberine, aloperine, oxymatrine, tetrandrine, sinomenine, tetrahydropalmatine, and galantamine. This compound has the potential as a lead compound which acts as an anti-inflammatory (Aryal et al., 2022).

Determining the Effectiveness of Edema Reduction

Anti-inflammatory activity testing was carried out using the edema formation method. The aim of the test was to see the sample's ability to prevent the formation of edema on the soles of mice's feet which was induced by intraplantar injection of 1 % carrageenan solution (Eze et al., 2019). Carrageenan solution is used to trigger edema/inflammation because carrageenan is a sulfated polysaccharide with large molecules which can cause tissue damage when induced in experimental animals (Sadeghi et al., 2013).

The tissue damage can disrupt cell membranes, triggering mast cells to release inflammatory mediators such as histamine and bradykinin in the early stages of inflammation, followed by the release of prostaglandins by cyclooxygenase (Collington et al., 2011). Additionally, carrageenan also triggers the production of free radicals such as Nitric Oxide (NO), which is an acute inflammatory mediator. These mediators increase capillary permeability, causing fluid to leak from blood vessels into the interstitial tissue, leading to an increase in extravascular fluid. As a result, protein-rich fluid accumulates, known as exudate, causing swelling (edema) (Abdulkhaleq et al., 2018).

The results of all treatment groups (Figure 3) showed thickening of the paw at one hour after intraplantar induction of 1 % carrageenan. This demonstrates that carrageenan is a substance capable of inducing edema formation, as evidenced by the observed peak in edema following carrageenan induction. The mechanism of edema formation by carrageenan involves a series of inflammatory reactions, wherein carrageenan stimulates the release of inflammatory mediators such as histamine and prostaglandins, which in turn increase blood vessel permeability and lead to fluid accumulation in tissues, which is characteristic of edema.

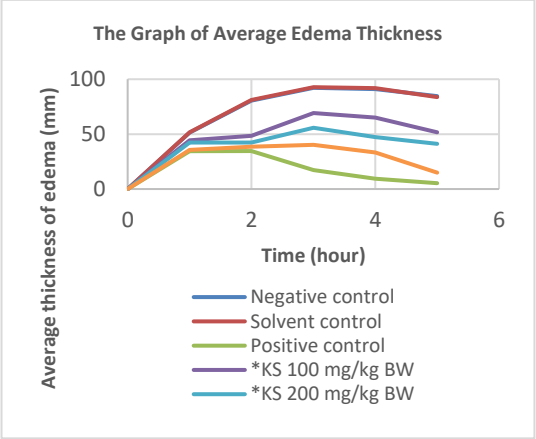

Figure 3. Observation results of the average thickness of edema in all treatment groups. CS: Carica seeds extract.

The largest edema thickening was observed in the negative control and solvent control groups, as they lacked anti-inflammatory activity. The graph indicates a decrease in edema at six hours for the negative control and solvent groups, reflecting the natural physiological response of the body to maintain and recover from the presence of foreign substances (Abdulkhaleq, 2018). The thickness of edema in the positive control group gradually decreased, starting at the 3rd hour. In the groups treated with ethanol extract of carica seeds at different doses, there was also a gradual reduction in edema thickness, starting from the 4th hour.

In the test, data analysis was conducted by calculating the AUC values of each group compared to the negative control (Table 3). The average total AUC value reflects the area under the curve. AUC data was obtained from the average difference in thickness of the soles of mice's feet which indicated the presence of edema at 0 to 6 hours. The anti-inflammatory activity of the ethanol extract of carica seeds is indicated by a decrease in the average total AUC value.

Table 3. Total AUC values and the percentage effectiveness of edema reduction (% PEER) of test groups.

Group	The total of AUC (mm.jam)	% PEER
Negative control	22.51 ± 2.58	0
Solvent control	22.94 ± 2.43	-1.90
Positive control	14.81 ± 1.42	34.20
CS 100 mg/kg BW	18.87 ± 2.26	16.19
CS 200 mg/kg BW	17.83 ± 1.83	20.81
CS 400 mg/kg BW	16.58 ± 1.77	26.33

The average total AUC value is inversely proportional to the anti-inflammatory percentage. The average total AUC value is inversely proportional to the percentage of anti-inflammatory power or percentage of edema reduction effectiveness (% PEER). The PEER percentage was calculated by comparing the AUC of the negative control with each treatment in mice. The smaller the AUC value, the greater the % PEER, and the greater the AUC value, the smaller the % PEER (Table 3). Increasing the dose of ethanol extract of carica seeds produces a smaller AUC value, indicating an increase in anti-inflammatory activity (Soemarie, 2016). In the positive control treatment with administration of diclofenac sodium, the AUC value was lowest with the highest % PEER of 34.20 %.

The AUC values of each test group, which are normally distributed and statistically homogenous, were then statistically analyzed using a One-way ANOVA test, namely the LSD test with a 95 % confidence level. Based on Table 4, the results of statistical analysis between the negative control and positive control, as well as the three groups of various doses of ethanol extract of carica seeds, showed significant differences ($p < 0.05$) between the groups given the extract and the positive control. These results indicate that there is an influence of administering various doses of extract and sodium diclofenac in reducing edema.

In this study, the administration of carica seeds extract at doses of 100 mg/kg BW, 200 mg/kg BW, and 400 mg/kg BW did not significantly differ ($p > 0.05$) in reducing edema thickness. This proves that the ethanol extract of carica seeds at a low dose of 100 mg/kg BW already has the potential as an anti-inflammatory, and increasing the dose does not show significant differences in anti-inflammatory effects. The administration of carica seeds extract at doses of 200 mg/kg BW and 400 mg/kg BW was not significantly different ($p < 0.05$) from the positive control. This indicates that doses of 200 mg/kg BW and 400 mg/kg BW have the same anti-inflammatory activity as the positive control (sodium diclofenac). The use of sodium diclofenac as a positive control is because this NSAID drug has a rapid effect in reducing inflammation (Fadlilaturrahmah et al., 2022).

Table 4. LSD Test Results for AUC Values of Various Groups

Group	Positive control	Negative control	Solvent control	*CS 100 mg/kg BW	*CS 200 mg/kg BW	*CS 400 mg/kg BW
Positive control	-	SD	SD	SD	NSD	NSD
Negative control	SD	-	NSD	SD	SD	SD
Solvent control	SD	NSD	-	SD	SD	SD
*CS 100 mg/kg BW	SD	SD	SD	-	NSD	NSD
*CS 200 mg/kg BW	NSD	SD	SD	NSD	-	NSD
*CS 400 mg/kg BW	NSD	SD	SD	NSD	NSD	-

Noted: *CS: Carica seeds extract, SD: Significantly different ($p < 0.05$), NSD: Not significantly different ($p > 0.05$).*

Table 5. Lymphocyte count in various groups

Group	Total of lymphocyte count (x10⁹/L)		
	t0	t3	t6
Positive control	3.9225 ± 1.37	4.8775 ± 0.71	2.9925 ± 0.43
Negative control	3.4425 ± 0.81	5.66 ± 0.52	4.92 ± 1.28
Solvent control	3.2475 ± 1.51	11.3225 ± 1.99	5.2825 ± 1.47
*CS 100 mg/kg BW	3.135 ± 1.03	7.115 ± 1.09	6.3225 ± 0.63
*CS 200 mg/kg BW	4.8975 ± 2.12	5.79 ± 0.56	5.295 ± 0.70
*CS 400 mg/kg BW	3.5375 ± 1.04	5.1325 ± 2.86	2.005 ± 0.19

Noted: *CS: Carica seeds extract.

Carica seeds extract at a dose of 100 mg/kg BW has lower anti-inflammatory activity compared to the positive control, as it significantly differs from the positive control ($p < 0.05$). This could be due to the lower concentration or amount of extract binding to receptors, thus not providing the same anti-inflammatory activity as sodium diclofenac. The administration of the solvent control and negative control (mice with edema without medication) showed non-significant results ($p > 0.05$), indicating that both controls were unable to inhibit edema formation. Secondary metabolites in carica seeds have pharmacological activity similar to sodium diclofenac, which can inhibit cyclooxygenase enzymes that decrease the biosynthesis process of prostaglandins, the cause of inflammation, fever, and pain, especially in peripheral tissues (Suryandari et al., 2021).

The effect of inflammation on lymphocyte count

The results seen in Table 5 indicate that the average lymphocyte count in the solvent control group experienced the highest increase at hour 3. The positive control group and the carica seeds extract dose of 400 mg/kg BW experienced an increase in lymphocyte count at three hours, but not as much as in the solvent control group.

The positive control group and carica seeds extract dose of 400 mg/kg BW were able to suppress the thickening of the edema due to the presence of active substances that produce anti-inflammatory effects. The ethanol extract of carica seeds at a dose of 100 mg/kg BW resulted in a higher decrease in lymphocytes compared to doses of 200 mg/kg BW and 400 mg/kg BW. Administration of carica seed extract results in a reduction of these lymphocytes. Lymphocytes respond to contact with foreign substances by eliciting an efficient and selective immune response that operates throughout the body to eliminate a foreign agent. These lymphocytes enter by increasing numbers, proliferate, transform into plasma cells, and produce antibodies to combat the entering foreign compounds (Suryandari et al., 2021).

The lymphocytes, part of the adaptive immune system, play a crucial role in defending against pathogens. In a similar study, Brazilian spinach leaf extract administration increased mice's lymphocyte count and spleen and thymus organ indices. The increase in spleen weight indicates an increase in the number of cells in the spleen, such as red blood cells, T lymphocytes, B lymphocytes, dendritic cells, and macrophages (Wuni et al., 2022).

CONCLUSION

The administration of carica seeds extract exhibits anti-inflammatory activity by reducing the thickness of edema on the paw and affecting the lymphocyte count in male white mice (Swiss Webster) induced with carrageenan. Doses of 200 mg/kg BW and 400 mg/kg BW of carica seeds extract showed similar anti-inflammatory effects as Diclofenac Sodium ($p > 0.05$). Additionally, all dosage variations affected lymphocyte count at the 3rd and 6th hours. The pharmacological activity is influenced by the presence of secondary metabolites in the carica seed, including flavonoids. Comparatively, both carica seeds extract and diclofenac sodium demonstrated comparable anti-inflammatory effects, suggesting that carica seeds extract could be as effective as Diclofenac Sodium in reducing inflammation induced by carrageenan. Additionally, both treatments affected lymphocyte count, indicating their immunomodulatory effects, albeit further research is needed to elucidate the specific pathways involved.

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CONFLICT OF INTEREST

All authors declared that there was no conflict of interest.

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