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Original Research Article

Analgesic and Anti-inflammatory Activity of Ethanol Extract from Cocoa Pod Husk

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ABSTRACT

Phenolic compounds found in cocoa pod husk have the potential to inhibit pro-inflammatory immune responses, indicating their ability to reduce pain. Therefore, this study aims to evaluate the analgesic and anti-inflammatory properties of ethanol extract of cocoa pod husk (EECPH). Protein denaturation, membrane stability, and antiproteinase were used to evaluate the anti-inflammatory properties of cocoa pod husk, which was extracted using 96% ethanol. In vivo tests for analgesic and anti-inflammatory effects were carried out using formalin and Complete Freund's Adjuvant (CFA). EECPH was administered to mice at dosages of 250, 500, and 750 mg/kg body weight (BW), with diclofenac sodium serving as the positive control. The results indicated that the inhibitory activity of protein denaturation from EECPH (IC_{50} 16.542 ± 3.095 µg/mL) was comparable to diclofenac sodium. However, the proteinase inhibitory activity (IC50 100.691 ± 7.663 µg/mL) was lower compared to diclofenac sodium. In the membrane stabilization test using heat induction, the activity of EECPH (IC50 74.78 ± 5.53 µg/mL) was also lower than diclofenac sodium. Meanwhile, the hypotonicity test revealed that the IC_{50} (6.24 ± 0.37 µg/mL) was similar to diclofenac sodium. The results also showed that EECPH was effective in reducing pain and inflammation in mice. At a dosage of 500 mg/kg, it decreased the total leukocyte count and the percentages of neutrophils and lymphocytes in mice after CFA treatment. In conclusion, the ethanol extract of cocoa pod husk exhibited significant analgesic and anti-inflammatory properties by preventing protein denaturation, stabilizing cell membranes, and reducing proteinase activity.

Keywords: Inflammation, Protein Denaturation, Membrane Stabilization, Proteinase Inhibitor, CFA, Formalin.

Introduction

Pain is a common symptom associated with 90% of diseases, including those characterized by inflammation. According to previous studies, inflammation is the immune system's reaction to dangerous stimuli, including radiation, poisons, infections, or tissue damage. This reaction can manifest as heat, redness, discomfort, swelling, and compromised physiological function. In addition, inflammation is caused by immune cell migration and the production of inflammatory mediators from damaged tissues.[1,2] The process of inflammation also helps to stop harmful stimulation and initiate the healing process.[2] When tissues are damaged, inflammatory mediators, including prostaglandin E2 (PGE2), histamine, bradykinin, and serotonin, are released. Neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), are typically released when these mediators activate nociceptors.[3] Consequently, the mediators dilate blood vessels and increase permeability, which improves blood flow to the afflicted area. Leukocytes, specifically neutrophils, migrate into the wounded tissue, while protein-rich fluid builds up outside the blood vessels.[4] The process causes frequent pain, which can manifest as allodynia (pain from stimuli that do not ordinarily cause pain) or hyperalgesia (increased sensitivity to pain).[5]

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Several studies have recommended the use of non-steroidal anti-inflammatory medications (NSAIDs) to facilitate the healing of pain and inflammation. The primary mechanism through which NSAIDs work is by inhibiting the cyclooxygenase (COX) enzyme, which reduces the production of eicosanoids.[6] COX is essential for converting arachidonic acid into thromboxane, prostaglandins, and prostacyclin.[7] Despite their benefits, NSAIDs can have side effects, including gastrointestinal ulceration, thrombocytopenia, kidney complications, central nervous system (CNS) disorders, and allergic reactions.[8] In addition, other pain relievers, particularly those used for chronic pain management, such as gabapentin and amitriptyline, are reported to have lower efficacy and higher incidences of side effects.[9] Therefore, creating novel chemicals to effectively regulate inflammation and pain is essential. Exploring alternatives, such as cocoa pod husk, can help mitigate these side effects.

According to previous studies, cocoa pod husks contain phenolic substances in higher concentrations compared to cocoa beans.10 These phenolic compounds include flavonoids such as flavonols (including catechins and epicatechins), proanthocyanidins (specifically procyanidins B and C), and quercetin.[11] The compounds have the ability to inhibit pain-inducing, pro-inflammatory immune reactions, including the production of monocyte chemoattractant protein-1 (MCP-1), TNF-α, IL-6, PGE2, IL-2, and IL-1β.[12] Analyses using high-performance liquid chromatography (HPLC) have also shown that cocoa pod husk extract contains resveratrol, which is a non-flavonoid phenolic compound with anti-inflammatory properties.[13] Resveratrol works by blocking nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), and mitogen-activated protein kinases (MAPKs). This inhibition leads to a reduction in the expression of TNF-α, IL-1β, IL-6, and IL-8,[14]

which are crucial in regulating leukocyte infiltration.[15] By preventing the synthesis of several cytokines associated with the pathophysiology of acute inflammation, the phenolic compounds in cocoa pod husks can reduce pain and acute inflammation.

In vitro and in vivo animal models are essential in understanding the primary mechanisms underlying inflammatory responses. Numerous investigations evaluating anti-inflammatory effectiveness employ a range of techniques, including membrane stabilization, protein denaturation, and antiproteinase activity.[16] In experimental animal models, formalin is commonly used as a compound that induces acute inflammation. Formalin injections can cause edema as well as pain-related behavioral reactions, including biting or licking the afflicted area.[17] Complete Freund's Adjuvant (CFA) is frequently used to model chronic inflammation.[18] Inflammatory mediators, such as phosphorylated p65 (p-p65), phosphorylated extracellular signal-regulated kinases 1/2 (p-ERK1/2), cyclooxygenase-2 (COX-2), interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and PGE2 can accumulate after intraplantar subcutaneous injection of CFA. These mediators not only contribute to pain but also cause microvascular damage, increase capillary permeability resulting in edema, and promote the migration of leukocytes to the injured tissue.[19,20]

Materials and Methods

Plants collection and identification

The cocoa pod husk (mesocarp and endocarp), which was collected in April 2020, was 6 months old, was obtained from the Coffee and Cocoa Study Center. The Plant Laboratory at the Department of Agricultural Production, Politeknik Jember, identified the cocoa pod husk under reference No. 31/PL17.3.1.02/LL/2020. The cocoa pod husk was grinded into powder before used in the experiments.

Extraction Process

Cocoa pod powder was extracted using the maceration method. Initially, 0.2 g of cocoa pod shell powder was soaked in ethanol at a ratio of 1:4 for 2 days. After the initial stirring, the liquid was allowed to reach room temperature. After 2 days, the solvent was replaced, and the remaceration process was repeated three times. The remaining extracts from the maceration were collected and evaporated using a rotary evaporator. To determine the extract's yield, the concentrated extract was dried in an oven set to 50 °C until a thick extract was obtained. The percentage yield was then calculated using Equation 1.

$$\%\ yield = \frac{weight\ of\ extract}{weight\ of\ cocoa\ powder} x\ 100\% \quad \text{(Eq. 1)}$$

Anti-inflammatory Activity In Vitro

Heat-induced hemolysis

In this test, the ethanol extract of cocoa pod husk (EECPH) was used as the sample solution. Diclofenac sodium served as the positive control, while the negative control contained neither the extract nor diclofenac sodium. A 0.1 mL aliquot of a 10% red blood cell suspension was mixed with 5 mL of EECPH solution at varying concentrations. Following a 30-minute incubation at 56°C, the mixture was cooled using the water flow method. The solution was then centrifuged at 3,000 rpm for five minutes. The supernatant was carefully collected, and absorbance was measured at 540 nm using UV-Vis spectrophotometry. Hemolysis inhibition was determined with the appropriate formula after the test was conducted:[21]

$$\%\ inhibition = \frac{Abs\ negative\ control - Abs\ test\ samples}{Abs\ negative\ control} x\ 100\% \quad \text{(Eq. 2)}$$

Hypotonicity-induced hemolysis

A total of 0.04 mL of a 10% v/v red blood cell suspension was applied to each 1 mL solution of EECPH at different doses. After gently swirling the mixture for 30 minutes at 37°C, it was centrifuged for 10 minutes at 2000 rpm. Subsequently, the supernatant was collected, and UV-Vis spectrophotometry was used to determine absorbance at 577 nm. This test was carried out 3 times.[22] Equation 2 was used to determine the suppression of hemolysis, with diclofenac sodium acting as a positive control.

Protein denaturation assay

The EECPH was dissolved in methanol at several concentrations: 1, 2, 4, and 12 µg/mL. For the positive control, Diclofenac sodium was also dissolved in methanol at concentrations of 12, 14, 16, and 20 µg/mL. Furthermore, the negative control comprised only methanol. For each solution, 50 µL was combined with 0.2% BSA and brought to a total volume of 5 mL. After 30 minutes of incubation at 25 ºC, the mixture was heated for 5 minutes at 72 ºC in a water bath before being placed in a refrigerator. Subsequently, the solution was vortexed and UV-Vis spectrophotometry was utilized to quantify the absorbance at 660 nm.[23] Equation 2 was used to determine the percentage inhibition.

Analgesic Activity Test

To determine their baseline latency time, the animals were placed on a hotplate that was kept at 55 ± 1 ºC.[12] Mice with a latency duration of 15 seconds were used, and each group received different treatments. The latency time was calculated by measuring the interval between placing the mice on the hotplate and their response, which could be licking, lifting their feet, or jumping. To avoid tissue damage, a 15-second cutoff time was set. Periodically, latency times were recorded at 0, 0.5, 1, 1.5, 2, and 3-hour intervals, and peak times were used to calculate the percentage of analgesic action.

Antiproteinase assay

The EECPH was dissolved in 96% ethanol at concentrations of 40, 60, 80, and 100 µg/mL. For the positive control, diclofenac sodium was also dissolved in 96% ethanol at concentrations of 20, 40, 80, and 100 µg/mL. Furthermore, 1 mL of each solution was mixed with 2 mL of 0.06 mg trypsin and 1 mL of a 20 mM Tris-HCl buffer (pH 7.4) to create the mixes. Subsequently, the mixtures were incubated for 10 minutes at 37 ºC. A total of 1 mL of a 0.65% casein solution was then added, and the incubation continued for an additional 20 minutes. After incubation, the mixtures were centrifuged for 20 minutes at 5000 rpm following 2 mL of 70% perchloric acid. The absorbance of the resultant solution was determined at 280 nm using UV-Vis spectrophotometry.[24] Equation 2 was used then used to obtain the percentage inhibition.

Anti-inflammatory Activity In Vivo

Preparation of Test Animal

Mice were first acclimatized for a week in clean cages equipped with feeding and ventilation systems to ensure their health. Healthy mice were identified by their increased body weight and active movement. The University of Jember's Ethical Committee approved all animal procedures, with reference number 1104/UN25.8/KEPK/DL/2021.

Animal Grouping and Dosing

For the formalin induction experiment, mice were divided into 5 groups, where 3 groups received different doses of the extract, while the remaining 2 served as positive and negative controls. In the CFA induction experiment, the mice were assigned to 6 treatment groups, including 3 extract doses groups, a sham group, a negative control, and a positive control. The positive control group received gabapentin at 100 mg/kg body weight for the CFA induction and diclofenac potassium at 9.75 mg/kg body weight for the formalin induction. The sham and negative control groups were administered 1% CMC-Na. The extract groups were treated with EECPH at 250, 500, and 750 mg/kg body weight.

Formalin-induced acute inflammation

Each mouse was treated according to its assigned group and, one hour later, received an intraplantar injection of 50 µl of 1% formalin. During the procedures, 2 phases of the licking time observation were carried out, with the first and second lasting for 0–5 minutes and 20–30 minutes after injection, respectively.[25] the average licking time in each phase was computed using the following formula after the total licking duration for each phase was recorded. This allowed for the calculation of the percentage of pain inhibition:

$$\%\ inhibition = \frac{average\ licking\ time\ (negative-treatment)}{average\ licking\ time\ (negative)} x\ 100\% \quad \text{(Eq. 3)}$$

Edema which was measured as plantar thickness, was observed at intervals of 5, 10, 20, 30, 60, 120, and 180 minutes following the intraplantar injection of formalin. After 180 minutes, the proportion of edema was determined, and data analysis was carried out.[26] The average edema data from each phase was used to obtain the percentage of edema inhibition.

$$\%\ of\ edema\ inhibition = \frac{N-U}{N}\ x\ 100\% \quad \text{(Eq. 4)}$$

Where N was the average value of the plantar thickness of the negative control group before and after injection, U was the average value of the plantar thickness of the treatment group before and after injection.

Completed Freund's Adjuvants-induced chronic inflammation
Assessment of antihyperalgesic activity
A total of 40 µl of CFA was injected into the mice's left hind paws to create a rodent model of hyperalgesia caused by chronic inflammation. In comparison, the mice in the sham group received an injection of 40 µl of normal saline.[27] Each of these creatures was put on a heated plate that was set to 50 ± 0.5 °C. The latency time was measured from the moment the mice's feet touched the hot plate until it displayed pain behaviors, such as licking, lifting their legs, climbing the walls of the hot plate, standing, or walking backward. To avoid any potential nerve damage, a 30-second cut-off time was set.[28,29] Baseline latency time was measured on day 0, before the CFA injection, with subsequent measurements taken on days 1, 3, 5, 7, 8, 10, 12, and 14 following the CFA injection.[27] Using the following formula, the EECPH's antihyperalgesic activity was determined:[30]

$$\%\ antihyperalgesia = \frac{latency\ time\ (postest-pretest)}{latency\ time\ (baseline-prestest)}\ x\ 100\% \quad \text{(Eq. 5)}$$

Assessment of inflammatory activity
The edema was assessed by measuring the plantar diameter with a vernier caliper. On day 0, the mice were measured to determine their baseline plantar diameter. Following the CFA injection, measurements were taken on days 1, 3, 5, 7, 8, 10, 12, and 14. The previously defined techniques were utilized to obtain edema inhibition.[31]

Assessment of The Number of Leukocytes, Lymphocytes, Monocytes, and Neutrophils
On day 15, all mice were sacrificed, and blood was collected from the orbital eye socket. The number of leukocytes was counted using the Manual Improved Neubauer method. Additionally, blood was drawn from the tail to analyze the types of lymphocytes and neutrophils present. A 15% Giemsa stain was used for observation, and the percentages of total leukocyte count, lymphocytes, and neutrophils were determined by counting every 100 leukocyte cells.[32,33]

Statistical analysis
The standard error of the mean (S.E.M.) was used to express all data as means. An independent t-test was used to assess in vitro data sets, while in vivo data were analyzed using a one-way ANOVA. SPSS statistical analysis used a 95% confidence interval to determine significant differences.

Results and Discussion

The effect of EECPH on Heat-induced Hemolysis
The red blood cell membrane typically consists of a phospholipid bilayer. When exposed to heat, the bonds within the membrane components may break, leading to the formation of free radicals. These free radicals can react with membrane lipids and proteins, causing structural damage and resulting in the release of hemoglobin from red blood cells.[34] The inhibition percentages and IC_{50} values for diclofenac sodium and EECPH were shown in Table 1 and Figure 1. The findings showed that, as shown in Table 1, the inhibition of hemolysis rose with increasing concentrations. Diclofenac sodium exhibited a significantly greater inhibition effect compared to EECPH. Specifically, the IC_{50} values for diclofenac sodium and EECPH were 41.12 ± 5.53 µg/mL and 74.78 ± 2.49 µg/mL, respectively. A lower IC_{50} value signified a greater inhibitory capacity ($p < 0.05$), and this indicated that diclofenac sodium was more effective in inhibiting hemolysis than EECPH.

The Effect of EECPH on hypotonicity-induced hemolysis
Red blood cell membranes could be damaged by factors other than heat. A significant cause was exposure to hypotonic solutions, which had a hemolytic effect by promoting excess fluid accumulation within the cells, ultimately leading to membrane rupture.[35] In this study, a hyposaline solution with a tonicity of 0.3% was used, which demonstrated optimal effectiveness in damaging red blood cell membranes.[22] The results regarding the percentage of inhibition and the IC_{50} values for diclofenac sodium and EECPH were presented in Table 2 and Figure 2. Diclofenac sodium exhibited a higher inhibitory effect on hemolysis compared to EECPH. The IC_{50} value for diclofenac sodium was found to be 5.87 ± 0.77 µg/mL, which was lower than the IC_{50} for EECPH, measured at 6.24 ± 0.37 µg/mL. This difference was not statistically significant ($p>0.05$). This indicated that both diclofenac sodium and EECPH had comparable activities in inhibiting hemolysis.

Table 1: The effect of EECPH on heat-induced hemolysis

Sample	Concentration (µg/mL)	%inhibition of hemolysis (replication-)			Mean ± SEM
		1	2	3	
Diclofenac sodium	20	24.16	35.80	35.8	31.92 ± 6.72
	30	36.68	39.85	42.68	39.74 ± 3.00
	50	54.67	57.31	57.32	56.43 ± 1.53
	60	68.75	67.54	64.37	66.90 ± 2.27
	70	78.13	77.95	69.84	75.30 ± 4.74
	80	85.01	83.07	82.36	83.83 ± 1.37
EECPH	20	5.47	5.64	18.34	9.82 ± 7.38
	40	15.87	17.46	27.52	20.28 ± 6.31
	100	68.43	68.25	73.54	70.08 ± 3.01
	120	83.42	84.3	85.01	84.24 ± 0.80

Table 2: The effect of EECPH on hypotonicity-induced hemolysis

Sample	Concentration (µg/mL)	%inhibition of hemolysis (replication-)			Mean ± SEM
		1	**2**	**3**	
Diclofenac sodium	1	10.19	6.48	8.33	8.33 ± 1.07
	2	16.67	12.96	21.30	16.98 ± 2.41
	3	26.85	23.15	26.85	25.62 ± 1.23
	5	37.96	41.67	50.00	43.21 ± 3.56
EECPH	2	14.81	18.52	15.74	16.36 ± 1.11
	4	37.04	31.48	29.63	35.72 ± 2.23
	5	41.67	41.67	39.81	41.05 ± 0.62
	7	57.41	50.93	59.26	55.86 ± 2.52

The Effect of EECPH on Denaturation Protein

The BSA solution was prepared by mixing BSA powder with Tris Buffer Saline (TBS) at a pH of 6.2-6.5, reflective of the body's pathological pH.[24] Data on the percentages of protein denaturation inhibition and IC_{50} values for EECPH were shown in Table 3 and Figure 3.

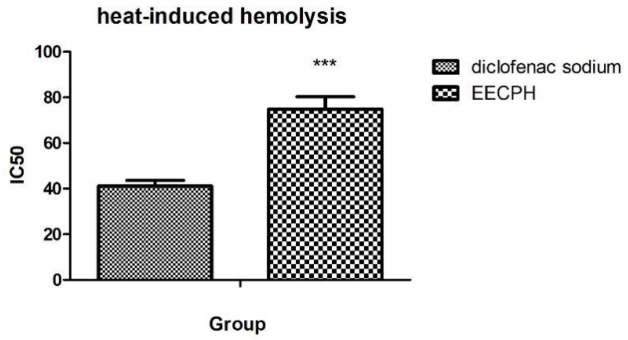

Figure 1: The IC_{50} of diclofenac sodium and EECPH on heat-induced hemolysis. *** showed significant differences between groups using unpaired t-test, 95%.

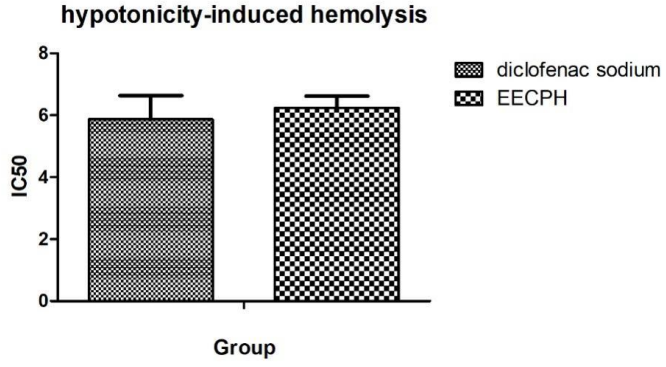

Figure 2: The IC_{50} of diclofenac sodium and EECPH on hypotonicity-induced hemolysis. There were no significant differences between groups using unpaired t-test, 95%.

Damaged endothelium and epithelial cells generated inflammatory chemicals during tissue damage, drawing neutrophils and monocytes. Neutrophils were the first responders, followed by monocytes, which were differentiated into macrophages.[2] These macrophages produced cytokines that triggered the release of arachidonic acid, which was converted into prostaglandins via the COX-2 pathway.[36] Prostaglandins led to vasodilation and increased blood vessel permeability, allowing inflammatory mediators to reach the site and causing edema.[37,38] Additionally, the buildup of inflammatory mediators could increase free radicals, resulting in protein denaturation and lysosomal damage.[39] Diclofenac sodium was found to inhibit protein denaturation more effectively than EECPH. The IC_{50} value for diclofenac sodium was 18.68 ± 3.32 µg/mL (Fig. 3), while EECPH had an IC_{50} of 16.54 ± 3.1 µg/mL. Both were equally effective in lowering protein denaturation, but the differences were not statistically significant ($p > 0.05$, Fig. 3).

The effect of EECPH on Proteinase

Table 4 and Figure 4 both displayed the proportion of antiproteinase inhibition. According to Table 4, diclofenac sodium exhibited a higher average inhibition rate compared to EECPH, indicating that diclofenac sodium was more effective at inhibiting proteinase activity than the extract. The IC_{50} results revealed that diclofenac sodium possessed a stronger potency, measuring 73.97 ± 4.44 µg/mL, which was significantly different from EECPH at 100.68 ± 7.66 µg/mL ($p < 0.05$).

The Effect of EECPH on Reducing Pain Behavior in Mice after Formalin Injection

This study indicated that giving 250 mg/kg body weight (BW) and 500 mg/kg BW of EECPH significantly reduced the licking time during the first and second phases ($p < 0.05$). However, when compared to the negative control, a dose of 750 mg/kg BW of the extract did not significantly decrease licking time during any phase ($p > 0.05$). A 500 mg/kg BW dose of EECPH produced the most notable curtailment of licking time in the first phase, leading to a 40.091% decrease. Nevertheless, this was still lower than the inhibition seen with diclofenac sodium in the positive control group, which had a reduction of 59.081% (Table 5).

The Effect of EECPH on Reducing Edema in Mice After Formalin Injection

Compared to the negative control, all EECPH doses (250 mg/kg, 500 mg/kg, and 750 mg/kg) significantly reduced edema levels at 180 minutes ($p < 0.05$). Additionally, at the same time point, a significant reduction in edema levels was observed following the administration of diclofenac potassium as the positive control ($p < 0.05$). The most notable percentage of edema inhibition was observed with diclofenac sodium, resulting in a 40.80% reduction (see Table 6).

The EECPH proved to be a potent inhibitor of protein denaturation and proteinase activity, rivaling the effects of diclofenac sodium. Rich in flavonoids, EECPH significantly reduced pro-inflammatory cytokine production.[40]

Table 3: The effect of EECPH on protein denaturation

Sample	Concentration (µg/mL)	%inhibition of hemolysis (replication-)			Mean ± SEM
		1	2	3	
Diclofenac sodium	12	32.65	35.88	28.44	32.32 ± 2.15
	14	39.12	43.51	33.99	38.87 ± 2.75
	16	45.47	48.98	37.76	44.07 ± 3.31
	20	56.90	65.38	44.86	55.71 ± 5.95
EECPH	1	20.69	23.01	32.65	25.45 ± 3.66
	2	24.14	24.60	34.06	27.60 ± 3.23
	4	26.94	26.42	36.23	29.86 ± 3.19
	12	37.82	44.42	46.84	43.03 ± 2.70

Table 4: The effect of EECPH on proteinase

Sample	Concentration (µg/mL)	%inhibition of proteinase (replication-)			Mean ± SD
		1	2	3	
Diclofenac sodium	20	20	17.78	18.10	18.63 ± 0.68
	40	31.76	30	27.59	29.78 ± 1.21
	80	55.29	54.44	52.59	54.11 ± 0.80
	100	68.24	66.67	60.34	65.08 ± 2.41
EECPH	40	9.41	15.56	27.59	17.52 ± 5.34
	60	21.18	26.67	35.21	27.68 ± 4.08
	80	34.12	37.78	44.83	38.91 ± 3.14
	100	45.88	47.78	53.45	49.04 ± 2.27

Antioxidants like epicatechins, catechins, and quercetin helped minimize free radical production, preserving protein structure and preventing denaturation. Quercetin also inhibited the COX-2 pathway, enhancing its anti-inflammatory properties.[24,41] Flavanols increased anti-inflammatory substances including IL-4 and TGF-β while decreasing TNF-α levels. Diclofenac sodium remained a powerful COX-2 inhibitor, renowned for blocking prostaglandin synthesis.[43] This study revealed that both diclofenac sodium and EECPH flavonoids were bound to albumin, stabilizing proteins under heat stress.[44] This highlighted EECPH's potential as a natural anti-inflammatory agent deserving of further investigation.

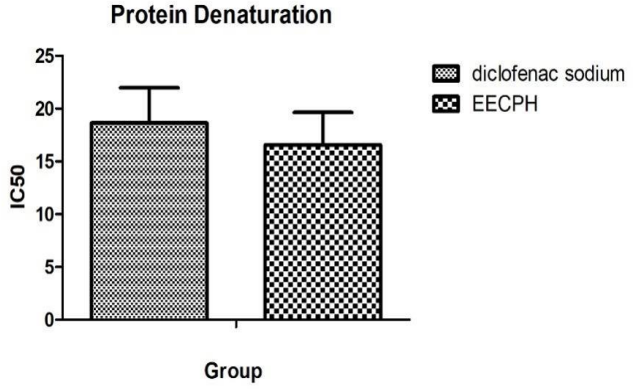

Figure 3: The IC_{50} of diclofenac sodium and EECPH on protein denaturation. There were no significant differences between groups using unpaired t-test, 95%.

The Effect of EECPH on Minimizing Hyperalgesia in Mice After CFA Injection

Figure 5 shows the mean delay time for both gabapentin and EECPH, which began treatment on the 8th day. The highest latency time was observed in the positive control and extract groups on the 14th day. As shown in Table 7, the maximum percentage of antihyperalgesic activity for EECPH was achieved at a dose of 750 mg/kg body weight (52.74 ± 9.41%). This effect did not differ significantly from that of the control group, which exhibited an activity of 62.63 ± 10.02% ($p > 0.05$).

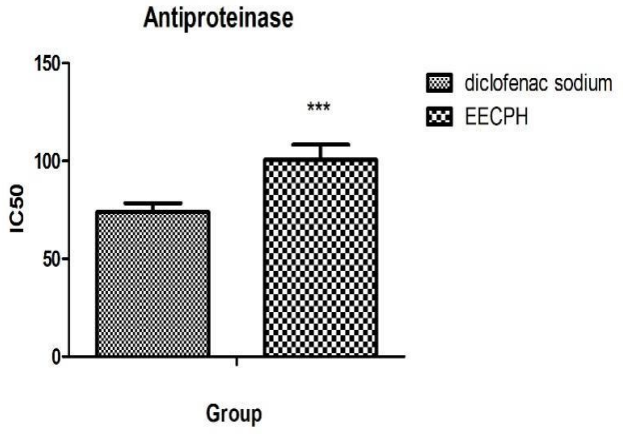

Figure 4: The IC_{50} of diclofenac sodium and EECPH on proteinase. *** showed significant differences between groups using unpaired t-test, 95%.

Table 5: The Effect of EECPH in Reducing Licking Time after Formalin-Induced Acute Inflammation

Group	Treatment	Mean of licking time (sec ± SD)		% inhibition	
		Phase 1	Phase 2	Phase 1	Phase 2
Negative control	CMC Na 1%	87.80 ± 6.30[a]	91.40 ± 15.00[a]	0	0
Positive control	Diclofenac potassium	56.40 ± 6.87[b]	28.20 ± 11.70[b]	34.624	69.147
EECPH	250 mg/kg BW	54.00 ± 9.41[b]	49.40 ± 11.78[b]	38.497	45.952
	500 mg/kg BW	52.60 ± 10.64[b]	37.40 ± 12.07[b]	40.091	59.081
	750 mg/kg BW	76.80 ± 5.79[a]	70.40 ± 4.50[a]	12.528	22.976

*Data were analyzed using One Way Anova and continued with the LSD Post Hoc test with a value of p <0.05, which indicated a significant difference; the same superscript letter indicated no significant difference in the LSD Post Hoc test. n = 5.

Table 6: The Effect of EECPH in Reducing Edema after Formalin-Induced Acute Inflammation

Group	Treatment	% edema in min-180 (mean ± SD)	% inhibition
Negative control	CMC Na 1%	73.55 ± 6.52[a]	0
Positive control	Diclofenac potassium	42.37 ± 1.38[b]	40.80
EECPH	250 mg/kg BW	50.03 ± 3.45[b]	32.76
	500 mg/kg BW	47.04 ± 2.77[b]	36.33
	750 mg/kg BW	51.65 ± 5.73[b]	29.88

*Data were analyzed using One Way Anova and continued with the LSD Post Hoc test with a value of p <0.05, which indicated a significant difference; the same superscript letter indicated no significant difference in the LSD Post Hoc test. *n = 5.*

The Effect of EECPH on Minimizing Edema in Mice After CFA Injection

Figure 6 illustrates that the plantar baseline diameter did not differ significantly between the CFA group (n = 20) and the sham group (n = 4; p > 0.05), indicating that both groups started under the same initial conditions. However, from day 1 to day 7, the plantar diameter in the CFA group increased compared to the sham group, suggesting that inflammation persisted until day 7.

Among the tested doses and gabapentin, EECPH at 750 mg/kg BW exhibited the lowest percentage of edema in relation to hyperalgesia activity (36.41 ± 5.06%; Table 8). This finding indicates that the 750 mg/kg BW dose of EECPH was the most effective in reducing edema compared to the other treatments (33.02 ± 9.31%; Table 8). Furthermore, the effect of EECPH did not differ significantly from that of gabapentin (p > 0.05), suggesting that its edema-reducing capability was comparable to that of the positive control.

The Effect of EECPH on The Number of Leukocytes, Lymphocytes, and Neutrophils in Mice After CFA Injection

Leukocyte, lymphocyte, and neutrophil counts in the positive control group were significantly different from those in the negative control group (p < 0.05, see Table 9).

Table 7: The Effect of EECPH on Minimizing Hyperalgesia after CFA Induction

Group	Treatment	% antihyperalgesia (mean ± SEM)
Negative control	CMC Na 1%	21.70 ± 10.72[a]
Positive control	Gabapentin 100 mg/kg BW	62.63 ± 10.02[b]
EECPH	250 mg/kg BW	40.29 ± 4.87[a]
	500 mg/kg BW	46.91 ± 7.45[a]
	750 mg/kg BW	52.74 ± 9.41[b]

However, no significant changes were observed (p > 0.05) between the positive control and the sham group. Gabapentin treatment resulted in a decrease in the number of leukocytes to 7.11 x 10^3 ± 7.82/mm^3, in contrast to the negative control group, which had 13.93 x 10^3 ± 3.25/mm^3, and an increase compared to the sham-treated group, which had 6.58 x 10^3 ± 1.66/mm^3. The proportion of lymphocytes in the positive group increased by 72.50% ± 7.59, compared to the negative group at 35.55% ± 11.71, and also increased compared to the sham group, which had 74.75% ± 4.99. Similarly, the proportion of neutrophils decreased in the positive group by 20.25% ± 8.54 compared to the negative group, which had 53.25% ± 13.79, but it showed an increase compared to the sham group by 14.25% ± 3.10.

When compared to the positive control and sham groups, there was no discernible difference (p > 0.05) in the number of leukocytes, lymphocytes, and neutrophils in the group given a dose of 500 mg/kg body weight (BW) of EECPH. This indicated that, in comparison to the doses of 250 mg/kg and 750 mg/kg, the EECPH at this level was more effective in increasing the percentage of lymphocytes while decreasing the percentages of leukocytes and neutrophils. Leukocyte counts and the percentages of lymphocytes and neutrophils were significantly different (p < 0.05) between the groups that received 500 mg/kg BW and those who received 250 mg/kg BW and 750 mg/kg BW. However, there was no significant change between the 250 mg/kg BW dose and the 750 mg/kg BW dose (p > 0.05). The administration of the extract (EECPH) at doses of 250 mg/kg to 500 mg/kg body weight significantly decreased licking time and edema in formalin-induced acute inflammation. In a chronic pain model induced by CFA, EECPH demonstrated a clear dose-dependent increase in antihyperalgesic effects and edema inhibition, extending latency times and reducing edema in CFA-induced mice. A reduction in pain and edema inhibition was observed at the maximum dosage of 750 mg/kg BW. This demonstrated the potential of EECPH for pain treatment by indicating that larger concentrations of active phytoconstituents with robust anti-inflammatory and pain-relieving qualities may be responsible for the enhanced efficacy at lower dosages.[45]

EECPH was a potent source of flavonoid molecules with anti-inflammatory properties, such as epicatechins, catechins, procyanidins, and quercetin.

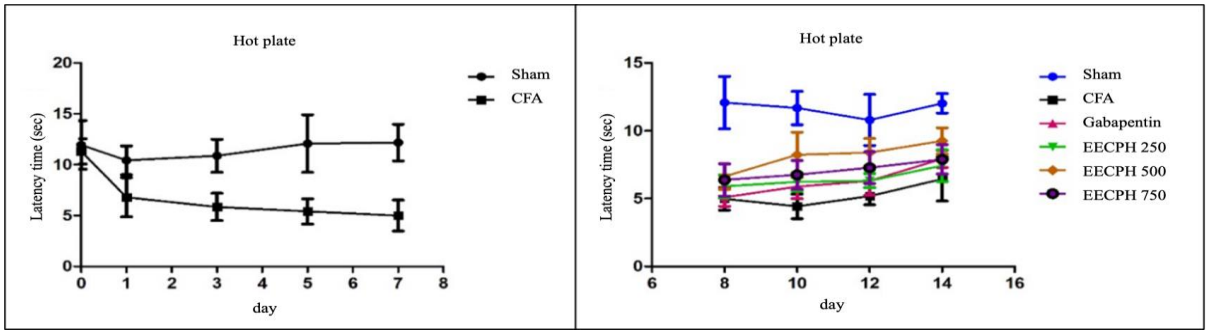

Figure 5: The Effect of EECPH in Latency Time Mice after CFA Injection

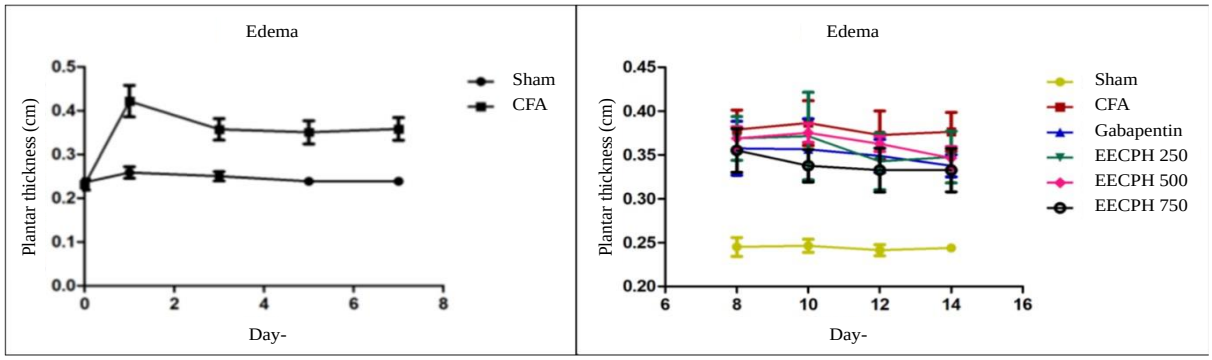

Figure 6: The Effect of EECPH on Minimizing Edema in Mice After CFA Injection

These compounds inhibited cytokines like TNF-α, IL-1, and IL-6, blocking the COX-2 enzyme responsible for pain-causing prostaglandins.[44,46,47] Flavonoids also provided significant analgesic effects by reducing inflammatory pain and activating the body's natural opioid mechanisms.[48] Procyanidin, a standout flavonoid from cocoa, blocked TNF-α from binding to its receptor, preventing the activation of NF-kB and lowering IL-8 levels.[46] EECPH also featured resveratrol, a potent polyphenol that inhibited ICAM-1, iNOS, and IL-1 mRNA expression.[11,13] This powerful compound reduced monocyte adhesion triggered by TNF-α and IL-6, further diminishing proinflammatory NF-kB levels.[49] With these powerful ingredients working together, EECPH was an excellent choice for alleviating inflammation and promoting overall health.

Table 8: The Effect of EECPH on Minimizing Edema after CFA Induction

Group	Treatment	% edema day-14 (mean ± SEM)	% edema inhibitory (mean ± SEM)
Negative control	CMC Na 1%	54.36 ± 4.54	0
Positive control	Gabapentin 100 mg/kg BW	38.46 ± 2.58	29.25 ± 4.75[a]
EECPH	250 mg/kg BW	42.56 ± 6.07	21.70 ± 11.16[a]
	500 mg/kg BW	42.05 ± 2.70	22.64 ± 4.96[a]
	750 mg/kg BW	36.41 ± 5.06	33.02 ± 9.31[a]

*Data were analyzed using One Way Anova and continued with the LSD Post Hoc test with a value of $p < 0.05$, which indicated a significant difference; the same superscript letter indicated no significant difference in the LSD Post Hoc test. *n = 4.*

Table 9: The Effect of EECPH on the Number of Leukocytes, Lymphocytes, and Neutrophils after CFA Induction

Group	Treatment	Leukocyte (x 10^3/mm^3 ± SEM)	Lymphocyte (% ± SEM)	Neutrophil (% ± SEM)
Sham	CMC Na 1%	6.58 ± 0.83[a]	74.75 ± 2.50[a]	14.25 ± 1.55[a]
Negative control	CMC Na 1%	13.93 ± 1.63[b]	35.50 ± 5.85[b]	53.25 ± 6.90[b]
Positive control	Gabapentin 100 mg/kg BW	7.11 ± 0.39[a]	72.50 ± 3.80[a]	20.25 ± 4.27[a]
EECPH	250 mg/kg BW	15.60 ± 2.02[b]	39.50 ± 3.80[b]	50.75 ± 4.99[b]
	500 mg/kg BW	7.49 ± 1.20[a]	70.25 ± 0.63[a]	24.50 ± 2.06[a]
	750 mg/kg BW	15.44 ± 3.58[b]	43.25 ± 4.40[b]	47.25 ± 4.23[b]

*Data were analyzed using One Way Anova and continued with the LSD Post Hoc test with a value of $p < 0.05$, which indicated a significant difference; the same superscript letter indicated no significant difference in the LSD Post Hoc test. *n = 4.*

The notable decrease in leukocytes counted in the chronic inflammation model underscored the strong anti-inflammatory effects of the secondary metabolites in EECPH, such as flavonoids, flavonols (like catechins and epicatechins), proanthocyanidins (procyanidin B and C), and flavonols (quercetin).[11] These compounds effectively inhibited harmful pro-inflammatory responses by lowering key mediators like MCP-1, TNF-α, IL-6, PGE2, and IL-1. The expression of the inflammatory markers TNF-α, IL-1, IL-6, and IL-8 was also decreased by resveratrol.[49] By targeting these pathways, the authors could significantly reduce leukocyte infiltration, decrease neutrophils, and increase beneficial lymphocytes, offering a promising strategy for managing chronic inflammation and enhancing overall health.[50-54]

Conclusion

In conclusion, these findings suggested that the EECPH was a promising source of analgesic and anti-inflammatory benefits for acute and chronic conditions. This effectiveness was linked to reduced protein denaturation and proteinase activity, alongside improved membrane stability during inflammation. However, more studies are still needed to investigate its mechanisms of action and therapeutic uses.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

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

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