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Isfahan University of Medical Sciences

Original Article

Pharmacological evaluation of anti-inflammatory, antipyretic, analgesic, and antioxidant activities of *Castanopsis costata* leaf fractions (water, ethyl acetate, and *n*-hexane fractions): the potential medicinal plants from North Sumatra, Indonesia

Maulana Yusuf Alkandahri[1,*], Asman Sadino[2], Barolym Tri Pamungkas[3], Zulpakor Oktoba[4], Maya Arfania[1], Nia Yuniarsih[5], Eko Sri Wahyuningsih[6], and Dea Eka Putri[7]

[1]*Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Buana Perjuangan Karawang, Karawang, West Java, Indonesia.*
[2]*Department of Pharmacy, Faculty of Mathematics and Natural Science, Universitas Garut, Garut, West Java, Indonesia.*
[3]*Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Mulawarman, Samarinda, East Kalimantan, Indonesia.*
[4]*Department of Pharmacy, Faculty of Medicine, Universitas Lampung, Bandar Lampung, Indonesia.*
[5]*Department of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Buana Perjuangan Karawang, Karawang, West Java, Indonesia.*
[6]*Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Buana Perjuangan Karawang, Karawang, West Java, Indonesia.*
[7]*Student of Pharmacy, Faculty of Pharmacy, Universitas Buana Perjuangan Karawang, Karawang, West Java, Indonesia.*

Abstract

Background and purpose: Inflammation, fever, and pain can be associated with several diseases, and the synthetic drugs used in the treatment of these conditions often have severe side effects. As a result, there is a need for effective, economical, and safe alternative drugs, such as those derived from medicinal plants. Therefore, this study aimed to evaluate the anti-inflammatory, antipyretic, analgesic, and antioxidant activities of *Castanopsis costata* leaf fractions (CcLF), as well as its acute toxicity.
Experimental approach: For anti-inflammatory, antipyretic, and analgesic tests, rats were given CcLF (WFCC, EAFCC, and *n*-HFCC) at 50 and 100 mg/kg, diclofenac sodium (10 mg/kg), paracetamol (150 mg/kg), aspirin (100 mg/kg), and tramadol (20 mg/kg). For the antioxidant activity test, various concentrations of CcLF were used ranging from 25 to 200 µg/mL. This study also looked into whether there could be any acute toxicity and histopathology of the liver, stomach, and kidneys in experimental animals.
Findings/Results: The administration of CcLF significantly inhibited the increase in foot edema volume, and CcLF (EAFCC at 100 mg/kg) considerably decreased rectal temperature and was proportional to the standard drug paracetamol, and significantly inhibited pain sensation in various models. Additionally, CcLF showed strong antioxidant activity, and its administration at a dose limit of 5000 mg/kg/day did not show any toxic effects or death in test animals.
Conclusions and implications: The results of the current confirmed that CcLF has demonstrated anti-inflammatory, antipyretic, analgesic, and antioxidant properties in experimental models, and is practically non-toxic.

Keywords*:* Acute toxicity; Analgesic; Anti-inflammatory; Antioxidant; Antipyretic; *Castanopsis costata*.

INTRODUCTION

Inflammation is a protective tissue response against endogenous and exogenous antigens through activation of the immune system (1,2). This inflammatory response leads to the release of several proinflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukins (IL-1, IL-6, and IL-8), bradykinin (BK), and histamine (3).

*Corresponding author: M.Y. Alkandahri
Tel: +62-82167757738, Fax: +02678403140
Email: alkandahri@gmail.com

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Meanwhile, some other pro-inflammatory mediators such as prostaglandins (PGs) and prostacyclins (PGI_2) are also produced during inflammation through the release and conversion of arachidonic acid from damaged cellular phospholipid membranes using cyclooxygenase enzymes (COX-1 and COX-2) (4).

PG (especially PGE_2) produced in this inflammatory response can directly affect the increase in thermoregulation setpoint in the hypothalamus and cause an increase in body temperature/fever (5). This response can also have harmful side effects such as excessive free radical production and a decrease in natural antioxidants in the body (1). Meanwhile, some other pro-inflammatory mediators, especially PGs, can also cause an increase in nociceptor sensitivity, causing pain and discomfort (6). Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the treatments that have long been used in the treatment of inflammation, fever, and pain because of their ability to inhibit COX-1 and/or COX-2 activities involved in PG production (7). However, long-term use of NSAIDs can increase the risk of gastrointestinal, liver, kidney, and cardiovascular disorders (8). Additionally, NSAIDs cannot neutralize the side effects of inflammation, such as excessive production of free radicals. Therefore, there is a need for alternative drugs with stronger anti-inflammatory, antipyretic, analgesic, and antioxidant effects as well as fewer side effects.

Castanopsis costata is one of the medicinal plants widely used in traditional medicine in North Sumatra. Leaf extract of this plant has been used empirically for the treatment of fever, digestive disorders, inflammation, wound healing, and pain relief (9). Meanwhile, several previous studies reported that *C. costata* leaf extract has pharmacological activity such as antimalarial (10), antidiabetic (11), anti-hypercholesterolemia (12), antioxidant (7), and antidiarrheal (13). Although there is an existing report on the traditional and scientific use of *C. costata* leaf extract, there are no reports in the literature on the anti-inflammatory, antipyretic, analgesic, and antioxidant activities of *C. costata* leaf fractions (CcLF). Therefore, this study aimed to phytochemical investigations, pharmacognostical evaluation, and investigate anti-inflammatory, antipyretic, analgesic, and antioxidant activities as well as the acute toxicity of CcLF.

MATERIALS AND METHODS

Chemicals and drugs

Sodium diclofenac (PT. Indofarma Tbk, Indonesia), paracetamol (Sigma Chemical Company, USA), aspirin (Bayer, Germany), tramadol (PT. Sanbe Farma Tbk, Indonesia), ascorbic acid (Sigma Chemical Company, USA), 0.9% sodium chloride (Otsuka®), diethyl ether (PT. Brataco, Indonesia), paraffin (PT. Kirana Mitra Abadi, Indonesia), 10% formalin solution (PT. Arjuna Utama Kimia, Indonesia), xylene (PT. Anugrah Putra Kencana, Indonesia), hematoxylin-eosin stains (H&E; HiMedia, USA), 1,1, dipheny-2-picrylhydrazyl (DPPH), acetic acid, 70% ethanol, 1% ferric chloride, 1% gelatin, hydrogen chloride, zink, magnesium, mercury (II) chloride, potassium iodide, bismuth subnitrate, acetic acid anhydride, sulfuric acid, aquadest, ethyl acetate, *n*-hexane, methanol, pulvis gummi arabicum, carrageenans, and peptone (EMSURE® ACS Merck, Darmstadt, Germany) of analytical grade.

Sample collection, determination, and fractionation of plants

About 15 kg of fresh *C. costata* leaves were obtained from the traditional market in Pancur Batu, North Sumatra, Indonesia in February 2022. *C. costata* was then identified at the Herbarium Medanense, Universitas Sumatera Utara, Indonesia (Voucher No. 183/MEDA/2022). Furthermore, the cleaned leaf was taken to the Pharmacognosy Laboratory, Universitas Buana Perjuangan Karawang for the fractionation process. About 5.0 kg of *C. costata* powder was macerated using 70% ethanol 3 times, 24 h each. The liquid extract was then collected and concentrated using a rotary evaporator (Eyela OSB-2100) at 50 °C (14). Subsequently, 100 g of dried *C. costata* extract was dissolved in a mixture of ethanol-water (1:3) and then fractionated using the liquid-liquid partition method with *n*-hexane (4 × 150 mL) and ethyl acetate (4 × 150 mL) as solvents. This procedure resulted in 3 fractions including *n*-hexane (*n*-HFCC; 20 g, 20%), ethyl acetate (EAFCC; 45 g, 45%), and water (WFCC; 30 g, 30%) fractions.

Preliminary phytochemical investigations

The phytochemical investigations of CcLF were performed to determine the presence of secondary metabolites such as polyphenols, saponins, flavonoids, tannins, alkaloids, triterpenoids, and steroids.

Determination of loss on drying

About 2 g of the CcLF powder was weighed and placed in a previously tared porcelain. The powder was spread into an even layer and put in an oven. The lid was opened and dried at 105 °C for 60 min until the constant weight. Before each drying, the closed crucible was left to reach room temperature in a desiccator (15). The percent of loss on drying (LOD) was calculated using equation (1):

$$\text{LOD (\%)} = \frac{\text{Initial weight of CcLF} - \text{Weight of CcLF after drying}}{\text{Initial weight of CcLF}} \times 100 \quad (1)$$

Determination of total ash

Three grams of the CcLF powder was accurately weighed in previously tared porcelain and then ignited with a meker burner for about 1 h. The ignition was completed by keeping it in a muffle furnace at 600 °C until grey ash and cooled into a desiccator, then weighed without delay (15). The percent of total ash (TA) was calculated using equation (2):

$$\text{TA (\%)} = \frac{\text{Ash weight}}{\text{Weight of CcLF}} \times 100 \quad (2)$$

Determination of acid-insoluble ash

TA was boiled with 25 mL 4 N hydrogen chloride for 5 min, covering the dish with a watch glass to prevent spattering; filtered through paper or previously ignited and weighed crucible and washed with hot water until a negative chloride test was obtained with silver NO_3 solution. Paper and content were returned to the crucible, dried at 100-102 °C, ignited for 30 min at 600 °C, covered with a watch glass, cooled in the desiccator, and weighed as soon as room temperature was attained (15). The percent of acid-insoluble ash (AIA) was calculated using equation (3):

$$\text{AIA (\%)} = \frac{\text{AIA weight}}{\text{Weight of CcLF}} \times 100 \quad (3)$$

Experimental animals

In this study, a total of 128 healthy male Wistar strain rats weighing 150 to 250 g were used for anti-inflammatory, antipyretic, and analgesic tests. The rats were collected from the Animal House, CV. Mitra Putra Animal, Bandung, Indonesia, and were well-maintained in the Pharmacology Laboratory, Universitas Buana Perjuangan Karawang under a 12/12-h light/dark cycle along with *ad libitum* access to standard pellets and water. The rats were kept in plastic cages with softwood shavings.

Treatment groups

CcLF (WFCC, EAFCC, and *n*-HFCC) was tested for anti-inflammatory (carrageenan-induced paw edema model) (16), antipyretic (peptone-induced pyrexia model) (17), analgesic (acetic acid-induced writhing test and hot plate model) (18,19), and antioxidant (DPPH method) (20) activities. This study also examined the potential for acute toxicity and histopathology of the liver, stomach, and kidneys in experimental animals. For anti-inflammatory, antipyretic, and analgesic tests, rats were given CcLF (WFCC, EAFCC, and *n*-HFCC) at 50 and 100 mg/kg orally, respectively. Meanwhile, for the antioxidant activity test, various concentrations of CcLF (WFCC, EAFCC, and *n*-HFCC) were used ranging from 25 to 200 µg/mL, and the acute toxicity test rats were given CcLF (WFCC, EAFCC, and *n*-HFCC) at 500, 1000, 2000, and 5000 mg/kg/day orally, respectively. The protocol and study design are presented in Fig. 1.

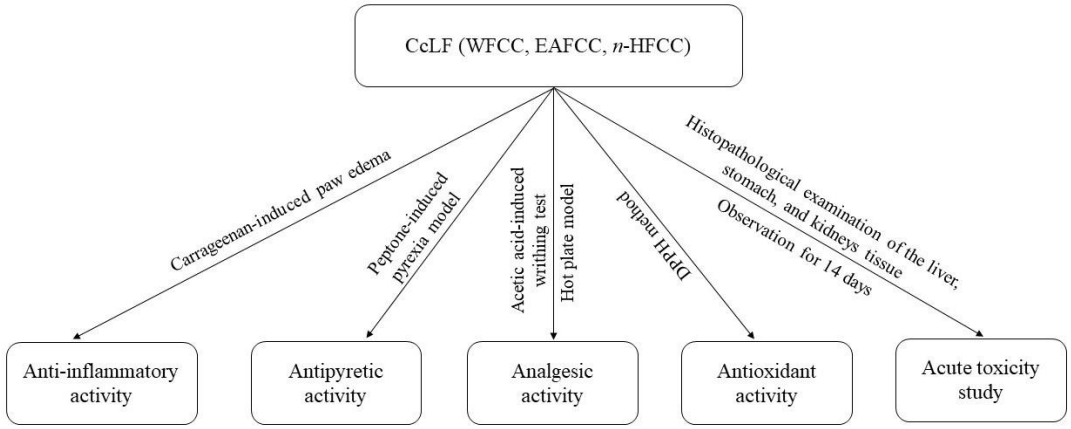

Fig. 1. Graphic schematic of the protocol and study design.

Anti-inflammatory activity

The anti-inflammatory activity test in this experiment was conducted using the carrageenan-induced paw edema model. The rats were divided into eight groups, 4 each. Subsequently, group I acted as a negative control and received a 1% w/v pulvis gummi arabicum (PGA) suspension, and group II acted as a positive control and received the standard drug (diclofenac sodium) at 10 mg/kg orally. Furthermore, groups III-VIII received WFCC, EAFCC, and *n*-HFCC at 50 and 100 mg/kg orally, respectively. After 30 min of therapy, 0.1 mL of carrageenan (1% w/v) in 0.9% sodium chloride was injected intradermally into the right hind paws of the rats. The edema volume was measured using a digital plethysmometer at 1, 3, and 6 h after the carrageenan injection, respectively (16). The paw edema volume of the rats and the percentage inhibition of edema were calculated using equations (4 and 5):

$$\text{Edema volume} = \text{PVAI} - \text{PVBI} \quad (4)$$

where PVBI stands for paw volume before carrageenan injection and PVAI for paw volume after carrageenan injection;

$$\text{Inhibition (\%)} = \frac{\text{EVNC} - \text{EVTD}}{\text{EVNC}} \times 100 \quad (5)$$

where EVNC stands for edema volume of negative control and EVTD for edema volume of test drugs.

Antipyretic activity (peptone-induced pyrexia model)

In this study, the antipyretic activity test was performed using a peptone-induced pyrexia model. The rats were randomly divided into eight groups, 4 each. Group I acted as a negative control and received a 1% w/v PGA suspension, while group II acted as a positive control and received the standard drug (paracetamol) at 150 mg/kg, orally. Furthermore, groups III-VIII were treated with WFCC, EAFCC, and *n*-HFCC at 50 and 100 mg/kg, orally, respectively. Before the experiment, the initial rectal temperature was measured using a rectal thermometer at a depth of 1.5 cm in the rectum of each rat. Furthermore, 0.5 mL of peptone (5% w/v) in aqua pro injection was injected into each experimental rat intraperitoneally. The fever induction was confirmed by an increase in rat temperature of more than 0.5 ℃ (17). Moreover, the rectal temperature of the rats was measured regularly at 1, 2, 3, and 4 h after the therapy administration. The % of fever reduction was calculated using equation (6):

$$\text{Inhibition (\%)} = \frac{\text{D} - \text{Xn}}{\text{D} - \text{E}} \times 100 \quad (6)$$

where, D stands for rats' rectal temperature after fever induction, Xn for rats' rectal temperature at 1, 2, 3, and 4 h after administration of therapy, and E for rats' rectal temperature before fever induction (normal temperature).

Analgesic activity

Acetic acid-induced writhing test

The peripheral analgesic effect in this experiment was evaluated by counting the acetic acid-induced torsional response in rats (18). About 10 mL/kg of 1% v/v acetic acid solution was injected intraperitoneally into each experimental rat, thereby inducing writhing (a condition where there is abdominal muscle contraction along with hind limb stretching) and the number of writhes was recorded. To evaluate analgesic activity in this model, rats were randomly divided into eight groups, each consisting of four rats. Group I received a negative control treatment of a 1% w/v PGA suspension, while group II received a positive control treatment of a standard drug (aspirin) at 100 mg/kg, orally. Meanwhile, groups III-VIII received treatment using WFCC, EAFCC, and *n*-HFCC at 50 and 100 mg/kg, orally, respectively, 30 min before acetic acid injection. The % of inhibition of writhing movement in rats was calculated using equation (7):

$$\text{Analgesia (\%)} = \frac{\text{WNC} - \text{WTD}}{\text{WNC}} \times 100 \quad (7)$$

where, WNC stands for the number of writhing in the negative control and WTD for the number of writhing in test drugs.

Hot plate model

In this model, the central analgesic effect was tested using the hot plate analgesia meter (PT. Andaru Analitika Sains, Bogor, Indonesia) (19). Before the experiment, the rats were pre-

tested by being placed on a hot plate maintained at a temperature of 55.00 ± 0.10 °C. Subsequently, rats with a lag time (the time the rats remained on the hot plate without licking their hind feet or kicking or jumping) during the pretest greater than 15 s were excluded from the experiment. Furthermore, the rats were randomly divided into eight groups, 4 each. Group I acted as a negative control and received a suspension of 1% w/v PGA, while group II acted as a positive control and received the standard drug (tramadol) at 20 mg/kg, orally. Meanwhile, groups III-VIII were treated with WFCC, EAFCC, and *n*-HFCC at 50 and 100 mg/kg, orally, respectively. After 30 min of therapy, the experimental rats were placed on a hot plate cylinder, and the latency time was recorded in seconds. A cut-off time of 30 s was set for all treatment groups to prevent tissue damage. For each treatment group, latency-time recordings were performed at 0, 30, 60, 90, and 120 min after drug administration. The analgesia percentage was calculated using equation (8):

$$\text{Analgesia (\%)} = \frac{\text{LT} - \text{CL}}{\text{COT} - \text{CL}} \times 100 \quad (8)$$

where, LT stands for latency test, CL for control latency, and COT for cut-off time.

Antioxidant activity

In this experiment, antioxidant activity was tested using the DPPH method (20). Furthermore, 5 mg of DPPH was dissolved in 100 mL of methanol to obtain a stock solution with a concentration of 50 µg/mL. About 250 mg of WFCC, EAFCC, and *n*-HFCC were dissolved in 25 mL of methanol, followed by dilution to obtain various test concentrations including 25, 50, 100, and 200 µg/mL. Subsequently, 2 mL of each solution was mixed with 2 mL of the DPPH stock solution until homogeneous and incubated at 30 °C for 30 min. The determination of antioxidant activity was performed using a UV-Vis spectrophotometer at a wavelength of 515.50 nm and repeated four times. Meanwhile, 1 mL of the DPPH solution was dissolved in 10 mL of methanol to obtain a blank solution, which was then measured at the same time and wavelength (Db). In this experiment, ascorbic acid with various concentrations (25, 50, 100, and 200 µg/mL) was used as the standard drug. The percent of DPPH radical scavenging activity was calculated using equation (9):

$$\text{Inhibition rate (\%)} = \frac{\text{Db}-\text{Ds}}{\text{Db}} \times 100 \quad (9)$$

where Db stands for absorbance of the blank and Ds for absorbance of the sample.

Acute toxicity study

In this experiment, an acute toxicity test was performed to evaluate the potential toxicity after administration of CcLF. The acute toxicity test was performed on Wistar strain rats of both sexes according to the guidelines of the OECD. The rats were divided into four treatment groups, each consisting of 10 rats. Each rat received WFCC, EAFCC, and *n*-HFCC at various doses including 500, 1000, 2000, and 5000 mg/kg, orally. Meanwhile, the control group rats were treated with the vehicle only (1% w/v PGA), and all treatment groups were monitored for 14 days. Any changes in behavior and signs of distress experienced by the test animals were carefully recorded (21).

Specimen collection

At the end of the 14-day experiment, on day 15, the rats in each treatment group were anesthetized with diethyl ether. Subsequently, all rats were euthanized by cervical dislocation. The abdominal cavity was opened to remove the liver, stomach, and kidneys from each rat, which were immediately wrapped in filter paper. The organs were rinsed with normal saline, and relevant parts of each organ were collected and placed in sample bottles for histopathological examination.

Histopathological examination

During the autopsy, tissue samples were collected and sent to the Histology Laboratory at the Department of Biology, Universitas Padjadjaran in Indonesia for histopathological examination. The liver, stomach, and kidney tissue samples were further processed through ethanol dehydration, followed by xylene clearing and paraffin infiltration. Subsequently, the paraffin blocks were cut into 3-4 µm thick sections, and the slides were deparaffinized in xylene and stained with H&E. The slides were observed using a light microscope (Olympus BX-51) equipped with a camera (Olympus Q

Color-5) connected to a computer. For further analysis, micrographs of liver, stomach, and kidney tissue were taken with a 100× objective. The micrographs from the treatment groups were compared to the control group. Changes were recorded using a standard nonlinear semiquantitative scoring system on a scale of 0-5 (22). The scoring criteria for tissue damage can be seen in Table 1.

Ethical approval

This study protocol was approved by the Research Ethics Commission, Universitas Padjadjaran, Indonesia, with the following numbers: 358/UN6.KEP/EC/2021 (for anti-inflammatory, antipyretic, and analgesic activity test) and 409/UN6.KEP/EC/2022 (for acute toxicity study).

Statistical analysis

In this study, all data obtained are expressed as mean ± SEM, and differences in the mean of the measured parameters were compared using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using GraphPad Prism version 8. *P*-values < 0.05 were considered statistically significant.

RESULTS

Phytochemical investigations

The phytochemical investigations of WFCC revealed the presence of chemical constituents such as polyphenols, saponins, flavonoids, and tannins, whereas the EAFCC contained polyphenols, saponins, flavonoids, tannins, and triterpenoids and steroids triterpenoids. Meanwhile, *n*-HFCC showed the presence of alkaloids, triterpenoids, and steroids. A summary of the phytochemical investigations of CcLF is presented in Table 2.

Table 1. Description of tissue damage scoring.

Scoring	Description
0	No change, with the usual morphology
1	< 10% of the affected tissue
2	< 20% of the affected tissue
3	More widespread changes that might be expected to be associated with changes in organ function or weight
4	Almost 75% of the affected tissue
5	The entire tissue being impacted by changes that might be functionally relevant

Table 2. Phytochemical investigations of *C. costata* leaf fractions.

Phytochemical compounds	Reagents	Results		
		WFCC	EAFCC	*n*-HFCC
Polyphenols	1% Ferric chloride	√	√	–
Saponins	Hot water + hydrogen chloride	√	√	–
Flavonoids	Zn + hydrogen chloride (p)	√	√	–
	Mg + hydrogen chloride (p)	√	√	
Tannins	1% Gelatin	√	√	–
Alkaloids	Mayer	–	–	√
	Dragendorff			
	Bouchardat			
Triterpenoids and steroids	Liebermann-Burchard	–	√	√

√, Contained; –, not contained; *C. costata*, *Castanopsis costata;* WFCC, water fraction of the *C. costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves.

Table 3. Phytochemical properties of *C. costata* leaf fractions. Data are presented as mean ± SEM of four replicates in each group.

C. costata leaf fractions	Loss on drying (%)	Total ash (%)	Acid-insoluble ash (%)
WFCC	8.11 ± 0.15[#]	7.44 ± 0.21[#]	0.58 ± 0.13[#]
EAFCC	7.65 ± 0.12[#]	8.66 ± 0.35[#]	0.77 ± 0.21[#]
***n*-HFCC**	9.52 ± 0.18[#]	8.71 ± 0.42[#]	1.37 ± 0.16[#]

[#], Meet the quality standards according to the Indonesian Herbal Pharmacopoeia-II; *C. costata*, *Castanopsis costata;* WFCC, water fraction of the *C. costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves.

Determination of LOD, TA, and AIA

LOD is residual substances after drying at 105 °C for 30 min or until the constant weight. LOD describes loss of water and volatile compounds (15). High water content in the CcLF will become a microbial growth medium during the storage as well as hydrolysis media which can cause decomposition of chemical compounds (23). Meanwhile, all CcLF in this study met the requirements of LOD (< 10%) (15,24). TA is designed to measure the total amount of material remaining after ignition. This includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological ash", which is the residue of the extraneous matter (*e.g.* sand and soil) adhering to the plant surface. Meanwhile, AIA is the residue obtained after boiling the TA with dilute hydrogen chloride and igniting the remaining insoluble matter. This measures the amount of silica present, especially sand and siliceous earth. The results showed that all CcLF in this study met the requirements of TA (< 10%) and AIA (< 3.4%) (15,24). LOD, TA, and AIA of WFCC, EAFCC, and *n*-HFCC are presented in Table 3.

Anti-inflammatory activity

Anti-inflammatory activity was assessed using a carrageenan-induced rat paw edema model. Based on the results obtained, administration of WFCC, *n*-HFCC, and EAFCC at 50 and 100 mg/kg, as well as the standard drug diclofenac sodium, significantly inhibited the increase in paw edema volume induced by carrageenan at 3 and 6 h. The highest percentage inhibition of edema was observed with EAFCC at 100 mg/kg after 6 h, which was 62.28%. This anti-inflammatory activity was not significantly different from the diclofenac sodium, which had an anti-edema effect of 63.40% (Table 4). These findings indicated that administration of WFCC, EAFCC, and *n*-HFCC can reduce carrageenan-induced paw edema in a dose and time-dependent manner.

Antipyretic activity

In this study, a model of peptone-induced fever in rats was used to determine antipyretic activity. The rectal temperature of the rats was measured using a digital thermometer immediately after 0 h (when the rats had reached a fever state). After the administration of the therapy, the rectal temperature of the rats was measured again after 1, 2, 3, and 4 h. Based on the results, EAFCC at 100 mg/kg and paracetamol significantly reduced the rectal temperature in a time-dependent manner (Table 5). Meanwhile, the maximum percentage of fever inhibition was shown with EAFCC at 100 mg/kg at 3 h and 4 h, as well as the standard drug paracetamol at 2, 3, and 4 h. Antipyretic activity of WFCC, EAFCC, and *n*-HFCC is presented in Fig. 2.

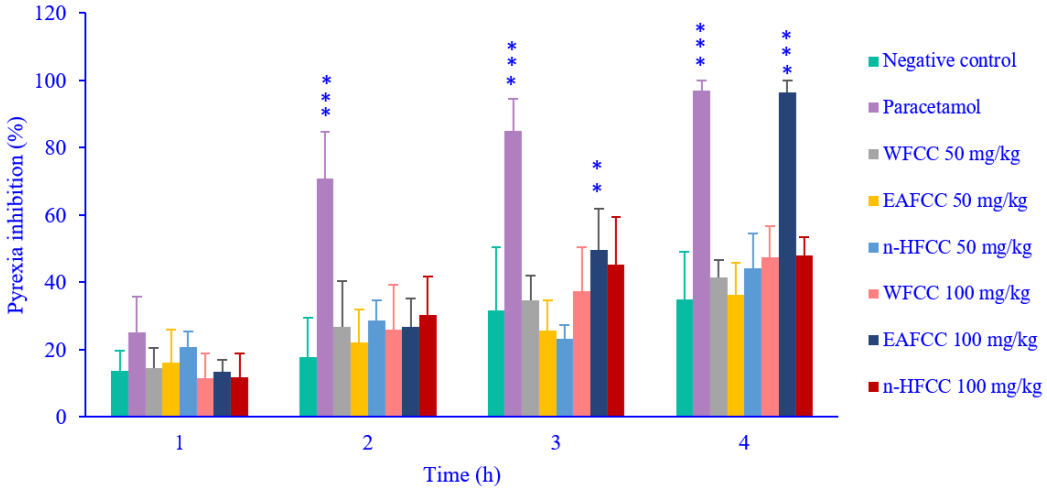

Fig. 2. Antipyretic activity of WFCC, EAFCC, and *n*-HFCC in rat model. rats were treated with for 1, 2, 3, and 4 h with paracetamol (150 mg/kg), WFCC, EAFCC, or *n*-HFCC. Data are presented as mean ± SEM of four animals in each group. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to the respective negative control. WFCC, Water fraction of the *Castanopsis costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves.

Table 4. Anti-inflammatory activity of WFCC, EAFCC, and *n*-HFCC on carrageenan-induced paw edema in rats. Data are presented as mean ± SEM of four replicates in each group. *$P < 0.05$ and **$P < 0.01$ indicate significant differences compared to the negative control.

Treatments	Dose (mg/kg)	PV (mL) before carrageenan injection	EV (mL)			% Inhibition		
			1 h	3 h	6 h	1 h	3 h	6 h
Negative control	1% PGA	1.01 ± 0.03	2.94 ± 0.02	3.51 ± 0.13	2.98 ± 0.05	-	-	-
Diclofenac sodium	10	1.05 ± 0.03	2.26 ± 0.07	1.44 ± 0.04**	1.09 ± 0.04**	23.11 ± 2.96	58.83 ± 1.25	63.40 ± 1.53
WFCC	50	1.09 ± 0.08	2.62 ± 0.06	2.01 ± 0.02*	1.52 ± 0.07**	10.98 ± 1.67	42.34 ± 2.34	49.08 ± 2.15
EAFCC	50	1.18 ± 0.05	2.61 ± 0.08	1.94 ± 0.04*	1.48 ± 0.06**	11.24 ± 2.29	44.42 ± 2.43	50.26 ± 1.94
***n*-HFCC**	50	1.14 ± 0.03	2.74 ± 0.06	2.14 ± 0.02*	1.56 ± 0.06**	6.98 ± 1.77	38.83 ± 1.93	44.07 ± 1.75
WFCC	100	1.12 ± 0.02	2.53 ± 0.08	1.88 ± 0.07*	1.36 ± 0.04**	14.04 ± 2.46	46.04 ± 3.10	54.29 ± 0.96
EAFCC	100	1.09 ± 0.02	2.37 ± 0.07	1.59 ± 0.06**	1.12 ± 0.01**	19.33 ± 2.81	54.64 ± 0.94	62.28 ± 0.70
***n*-HFCC**	100	1.12 ± 0.03	2.68 ± 0.06	1.98 ± 0.04*	1.43 ± 0.04**	8.77 ± 1.59	43.22 ± 2.90	52.19 ± 1.04

PV, Paw volume; EV, edema volume; PGA, pulvis gummi arabicum; WFCC, water fraction of the *Castanopsis costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves.

Table 5. Changes in rectal temperature after administration of WFCC, EAFCC, and *n*-HFCC. Data are presented as mean ± SEM of four replicates in each group. *$P < 0.05$ Indicates significant differences compared to the negative control.

Treatments	Dose (mg/kg)	Rectal temperature (°C)					
		Control (E)	0 h (D)	1 h (X1)	2 h (X2)	3 h (X3)	4 h (X4)
Negative control	1% PGA	36.68 ± 0.12	38.15 ± 0.16	37.95 ± 0.12	37.97 ± 0.27	37.82 ± 0.33	37.70 ± 0.31
Paracetamol	150	36.30 ± 0.10	38.00 ± 0.16	37.60 ± 0.10	36.80 ± 0.18*	36.57 ± 0.13*	36.37 ± 0.10*
WFCC	50	36.66 ± 0.11	38.00 ± 0.12	37.85 ± 0.10	37.70 ± 0.09	37.57 ± 0.13	37.47 ± 0.10
EAFCC	50	36.40 ± 0.10	37.97 ± 0.11	37.70 ± 0.11	37.60 ± 0.10	37.52 ± 0.10	37.35 ± 0.12
***n*-HFCC**	50	36.70 ± 0.20	38.00 ± 0.11	37.77 ± 0.11	37.70 ± 0.11	37.70 ± 0.10	37.50 ± 0.10
WFCC	100	36.30 ± 0.30	38.00 ± 0.10	37.85 ± 0.14	37.65 ± 0.15	37.45 ± 0.16	37.25 ± 0.15
EAFCC	100	36.30 ± 0.10	38.12 ± 0.12	37.90 ± 0.12	37.62 ± 0.11	37.22 ± 0.13	36.35 ± 0.12*
***n*-HFCC**	100	36.67 ± 0.11	37.97 ± 0.12	37.85 ± 0.14	37.60 ± 0.15	37.40 ± 0.13	37.37 ± 0.11

PGA, Pulvis gummi arabicum; WFCC, water fraction of the *Castanopsis costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves.

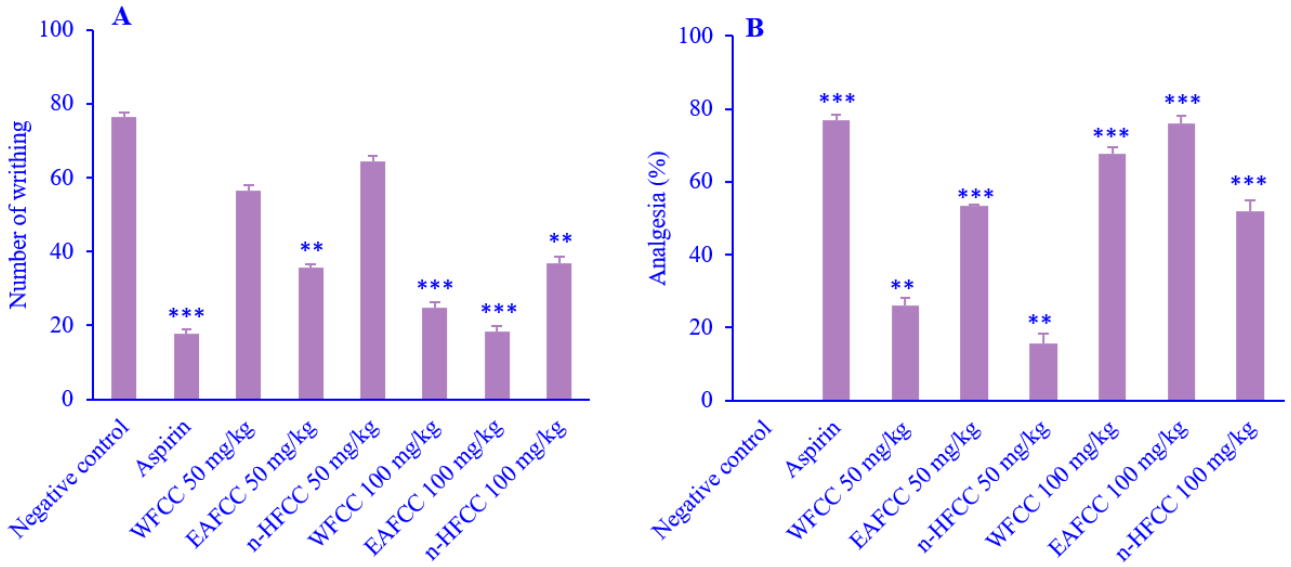

Fig. 3. Analgesic effect of WFCC, EAFCC, and *n*-HFCC on acetic acid-induced writhing test. (A) number of writhing and (B) % analgesia after the treatment with aspirin (100 mg/kg), WFCC, EAFCC, and *n*-HFCC. Data are presented as mean ± SEM of four animals in each group. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to the negative control. WFCC, Water fraction of the *Castanopsis costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves.

Analgesic activity

Acetic acid-induced writhing test (peripheral analgesic effect)

In this study, the evaluation of the peripheral analgesic effect was performed using the acetic acid-induced writhing model. The calculation of abdominal constrictions or writhing in rats was performed for 60 s for all treatment groups after 20 min-injection of 1% acetic acid injection. Based on the study, it can be observed that EAFCC at 50 and 100 mg/kg, as well as WFCC and *n*-HFCC at 100 mg/kg significantly reduced the number of abdominal constrictions or writhing in rats (Fig. 3A). The highest peripheral analgesic effect was observed with EAFCC at 100 mg/kg, which was 76.14%. This analgesic effect was not significantly different from the standard aspirin group, which had an analgesic effect of 76.79% (Fig. 3B). These findings indicated that the administration of WFCC, EAFCC, and *n*-HFCC can reduce the number of abdominal constrictions or writhing induced by acetic acid in a dose-dependent manner.

Hot plate model (central analgesic effect)

In this study, the central analgesic effect was evaluated using the hot-plate model. The calculation of latency time was performed for each rat in all treatment groups at 0, 30, 60, and 120 min. Based on the results obtained, WFCC, EAFCC, and *n*-HFCC at 50 and 100 mg/kg, significantly increased the latency time in the hot plate model compared to the negative control (Fig. 4A). The highest central analgesic effect was observed with EAFCC at 100 mg/kg, which was 76.04%. However, this analgesic effect was still below the standard drug tramadol, which had an analgesic effect of 85.42% (Fig. 4B). These findings indicated that the administration of WFCC, EAFCC, and *n*-HFCC can increase the latency time in the hot plate model in a dose and time-dependent manner.

Antioxidant activity

Antioxidant activity is quantitatively determined by testing with the DPPH method, with the results expressed as IC_{50}, which is the concentration required to inhibit 50% of DPPH free radicals (20). Various concentrations ranged from 25 to 200 µg/mL of each CcLF to test their antioxidant activity in the DPPH model. Based on the results obtained, WFCC, EAFCC, and *n*-HFCC were able to scavenge DPPH radicals in a concentration-dependent manner, and their respective IC_{50} values were 40.22, 39.46, and 44.20 mg/mL. These results indicated that WFCC, EAFCC, and *n*-HFCC possess strong antioxidant activity *in vitro*. Meanwhile, the ascorbic acid used as the standard drug had an IC_{50} of 20.88 µg/mL (Table 6).

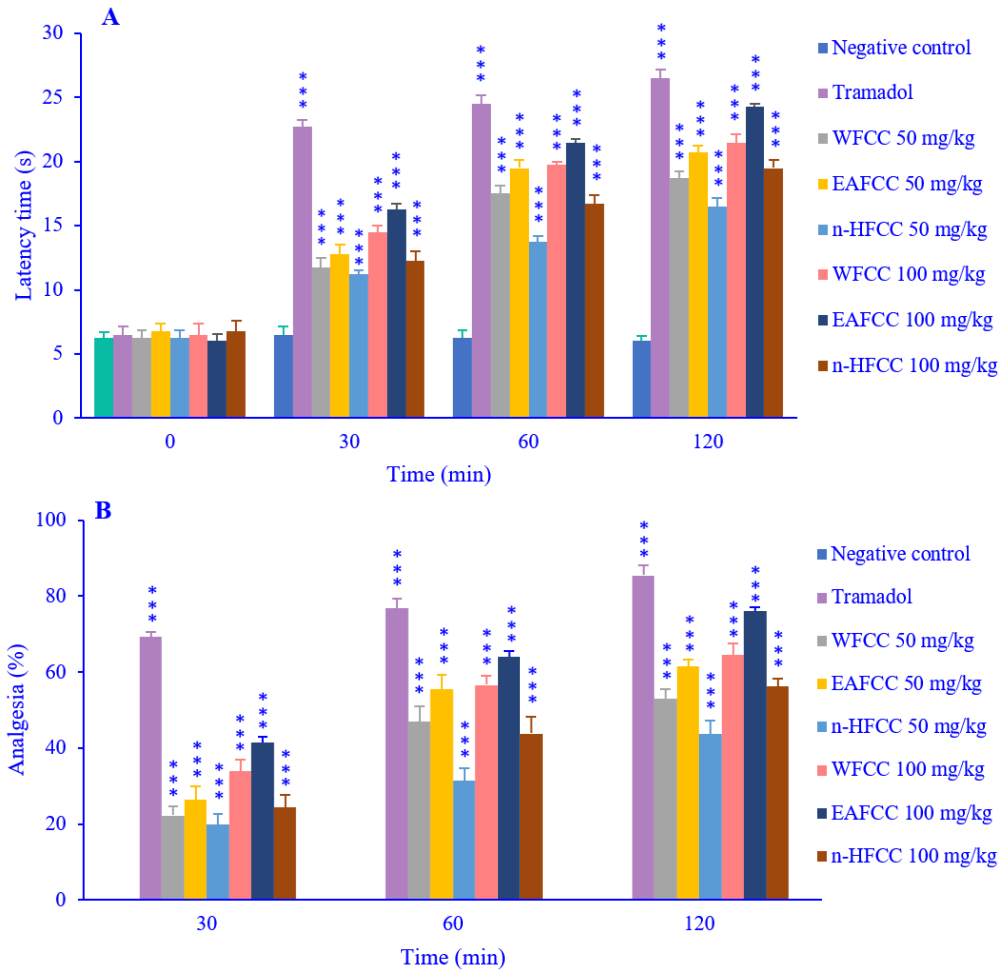

Fig. 4. Analgesic effect of WFCC, EAFCC, and *n*-HFCC in hot plate test. (A) Latency time and (B) percentage of analgesia after 30, 60, and 120 minutes of the treatment with tramadol (20 mg/kg) and WFCC, EAFCC, and *n*-HFCC (50 and 100 mg/kg). Data are presented as mean ± SEM of four animals in each group. ***$P < 0.001$ Indicates significant differences compared to the negative control. WFCC, Water fraction of the *Castanopsis costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves.

Table 6. IC_{50} value of ascorbic acid and WFCC, EAFCC, and *n*-HFCC. Data are presented as mean ± SEM of four replicates in each group.

Samples	IC_{50} (µg/mL)	Antioxidant activity (25)
Ascorbic acid	20.88 ± 1.58	Very strong
WFCC	40.22 ± 1.18	Very strong
EAFCC	39.46 ± 1.08	Very strong
***n*-HFCC**	44.20 ± 1.04	Very strong

WFCC, Water fraction of the *Castanopsis costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves.

Acute toxicity study

Based on the results of the acute toxicity test, p.o administration of WFCC, EAFCC, and *n*-HFCC in a limiting dose of 5000 mg/kg in rats showed normal behavior and no death or lethargy as well as any signs of toxic effects on any behavior pattern for up to 14 days. Meanwhile, the LD_{50} of WFCC, EAFCC, and *n*-HFCC was greater than 5000 mg/kg orally and appeared to be safe and non-toxic.

Histopathological examination

The effect of the administration of WFCC, EAFCC, and *n*-HFCC on the liver, stomach, and kidney organs of rats was determined by examining the histopathological changes of these organs compared to the control group. Based on the histopathological observations, the administration of WFCC, EAFCC, and *n*-HFCC up to a dose limit of 5000 mg/kg was reported to cause no damage to these organs (with a tissue damage score of 0 for each). The liver histopathological results showed polygonal-shaped hepatocyte plates with several nuclei. Additionally, the sinusoids between the hepatocyte plates and the portal/triad portal area (central vein, artery, bile duct) were visible and wide/large (Fig. 5).

Histopathological findings from the stomach revealed a clear histological structure characterized by mucosal, submucosa, and muscularis mucosa layers. In the area of the mucosa layer, gastric pits or foveolar gastrica were observed with single-layered cylindrical epithelial cells and small mucosal glands, and the gastric glands were visible in the lamina propria (Fig. 6).

Furthermore, the kidney histopathological results showed a clear histological structure of its parts. There were three renal corpuscles located in the glomerulus and Bowman's capsule. The proximal tubules appeared to be more abundant with relatively small, uneven, and darker lumens (brush border) as well as comprised of cuboidal-shaped cells. The distal tubules appeared in fewer numbers with larger and more regular lumens, and their cells were smaller and cuboidal and appeared brighter in color (Fig. 7).

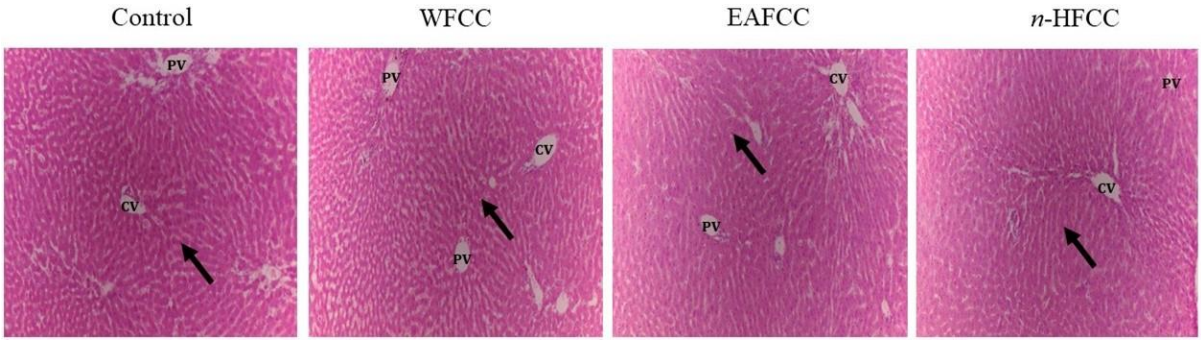

Fig. 5. The effect of WFCC, EAFCC, and *n*-HFCC on liver histopathology resealed using hematoxylin and eosin staining method; magnification of 100 ×. Black arrows show normal hepatocyte. WFCC, Water fraction of the *Castanopsis costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves; PV, portal vein; CV, central vein.

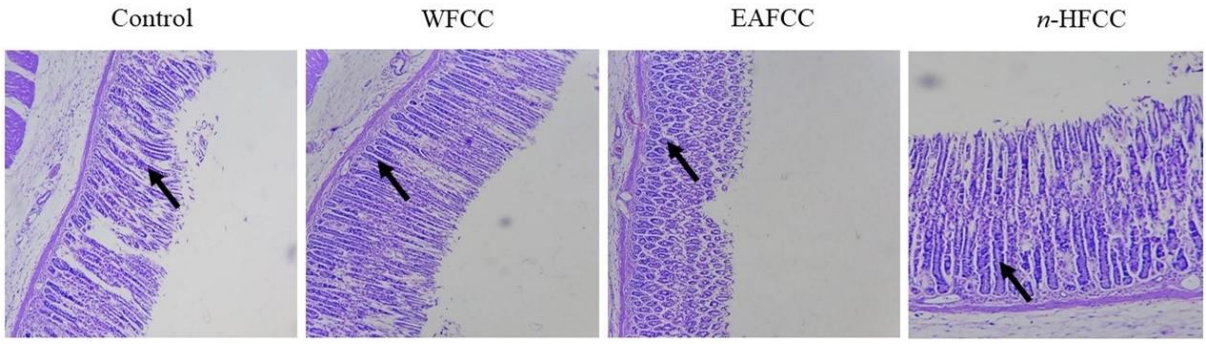

Fig. 6. The effect of WFCC, EAFCC, and *n*-HFCC on stomach histopathology revealed using hematoxylin and eosin staining method; magnification of 100 ×. Black arrows show normal cells with a basic structure of columnar epithelium. WFCC, Water fraction of the *Castanopsis costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves.

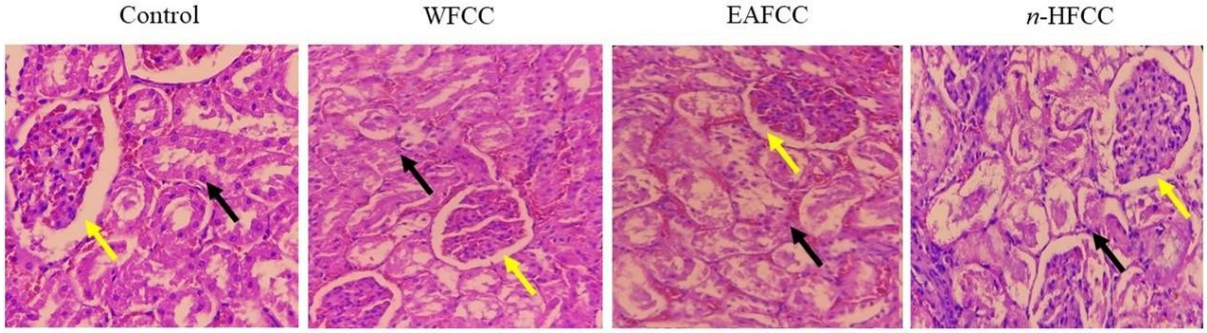

Fig. 7. Effect of WFCC, EAFCC, and *n*-HFCC on kidney histopathology revealed using hematoxylin and eosin staining method; magnification of 100 ×. Yellow arrows show the glomerulus; black arrows show normal cells. WFCC, Water fraction of the *Castanopsis costata* leaves; EAFCC, ethyl acetate fraction of the *C. costata* leaves; *n*-HFCC, *n*-hexane fraction of the *C. costata* leaves.

DISCUSSION

Generally, inflammation is known as the body's defensive response to various physiological conditions such as infection and thermal or physical injury (26). Inflammation aims to eliminate danger, induce tissue repair, and restore tissue homeostasis (27). Conceptually, the inflammation process is divided into 4 stages, consisting of the trigger system (danger), sensor mechanism (danger receptor), signal transmission, as well as mediator production, and cellular effector activation (28). Additionally, there are five major signs of inflammation such as pain, redness, heat, swelling, and loss of function (18). During inflammation, there is a significant increase in prostanoid biosynthesis in inflamed tissue. Several major prostanoids formed, such as PGE2 and PGI2, can increase vascular permeability, local blood flow, and leukocyte infiltration through activation of their respective receptors, namely IP and EP-2 (29). In this study, an anti-inflammatory activity test was performed using the carrageenan-induced paw edema model. Injecting carrageenan can lead to the release of many inflammatory mediators such as BK, PGs, serotonin, and histamine. Carrageenan-induced rat paw edema is a biphasic event in which the initial phase involves the release of histamine and serotonin, while the second phase involves the release of BK and PGs (30). The paw size is used as a parameter to measure inflammation in this model where the increase in rat paw size produced is directly proportional to edema. The results showed that the administration of EAFCC significantly inhibits the increase in paw edema volume induced by carrageenan at 3 and 6 h. Meanwhile, the administration of WFCC and n-HFCC significantly inhibited the increase in paw edema volume induced by carrageenan only at 6 h. Moreover, the results also revealed that the anti-inflammatory effect of EAFCC is not significantly different from the standard drug group diclofenac sodium.

Inflammation can also cause the release of IL-1β, IL-2, IL-6, IL-8, TNF-α, and interferon, whose role is to recruit inflammatory cells to the site of tissue injury (31). Additionally, the formation of these cytokines also increases the set point of thermoregulation in the hypothalamus. In the early phase, the thermoregulatory response to these cytokines is believed to be mediated by the release of ceramide in the preoptic area of the anterior hypothalamus (32). Meanwhile, in the slow response, the formation of PGE2 mediated by COX-2 and microsomal PGE synthase-1 (mPGES-1) occurs in the endothelium of blood vessels in the preoptic hypothalamus area (33). PGE2 can cross the blood-brain barrier and act on EP-1 and three receptors of temperature-sensitive neurons, causing the hypothalamus to increase body temperature by promoting heat production and reducing heat loss (34). In this study, an antipyretic activity test was carried out using peptone-induced fever rat models. Injection of peptone intraperitoneally can induce fever by increasing PG production (especially PGE2) (35). Previous studies have reported that *C. costata* extract has antipyretic activity in peptone-induced fever rats (7). Meanwhile, in this study, we evaluated the antipyretic activity of various fractions of *C. costata* leaves. The results showed that administration of EAFCC significantly reduces rectal temperature time-dependently. Meanwhile, the antipyretic effect of EAFCC is nearly equivalent to the standard drug paracetamol after 4 h of therapy.

Several inflammatory mediators such as histamine, BK, serotonin, PGs, and LTs released from non-neuronal cells during tissue injury cause an increase in the sensitivity of nociceptors (peripheral terminals of primary afferent fibers that sense pain) and improve pain perception (36). The pain response consists of two phases: in the first phase, there is nociceptor stimulation caused by the release of BK and substance P. In the second phase, there is inflammation caused by the production of serotonin, histamine, and PGs (37). Conversely, centrally active PGs can contribute to central sensitization by increasing stimulation of dorsal horn neurons in the spinal cord, leading to hyperalgesia (38). In this study, an analgesic activity test was conducted in 2 models, namely the acetic acid-induced writhing test (peripheral analgesic effect) and the hot plate model (central analgesic effect). The results indicated that the administration of

WFCC, EAFCC, and n-HFCC significantly reduces the number of writhing in rats and EAFCC has a peripheral analgesic effect almost equal to that of the standard drug aspirin. Meanwhile, based on the central analgesic effect test results, the administration of WFCC, EAFCC, and n-HFCC remarkably increased latency time. However, the central analgesic effect of these fractions is still below the standard drug tramadol.

In the case of inflammation, the immune cells consume a lot of oxygen for energy production, leading to an excess of free radicals produced by mitochondria (1,39). These free radical products are usually reactive oxygen species (ROS) and reactive nitrogen species, which are generated by cellular redox processes. At high concentrations, these free radicals can cause oxidative stress, which can damage all cell structures and lead to more severe diseases such as cancer, cardiovascular disease, aging, neurodegenerative disorders, and autoimmune disorders (39). Meanwhile, the human body is known to have several mechanisms to counteract oxidative stress by producing antioxidants (free radical scavengers), but during inflammation, the body's natural antioxidant production decreases (1), thereby requiring antioxidants from outside the body, such as those derived from food or herbal plants (40). In this study, a quantitative antioxidant activity test is conducted using the DPPH method. The results show that WFCC, EAFCC, and n-HFCC have a very strong *in-vitro* antioxidant activity. The results of this study are in line with the results of previous research, which reported that *C. costata* extract had a very strong antioxidant activity using the DPPH method (7).

It is believed that the anti-inflammatory, antipyretic, analgesic, and antioxidant effects of CcLF are due to the active ingredients it contains. Previous studies reported that the leaf of *C. costata* contains quercetin (12), which has anti-inflammatory, antipyretic, and analgesic activities by inhibiting the production of pro-inflammatory mediators such as NF-κB, TNF-α, AP-1, IL-1β, IL-6, IL-8, and COX, then stopping PGs synthesis (41-43). Furthermore, quercetin can reduce inflammatory hyperalgesia associated with hyperexcitability of nociceptive SpVc neurons by inhibiting peripheral COX-2 signaling cascades and voltage-gated ion channels (44). Meanwhile, quercetin can increase total GSH levels and reduce ROS levels, thereby reducing the excessive production of free radicals during inflammation (45).

Although natural, some studies have reported that phytoconstituents in plants may cause unexpected toxicity targeting certain organs such as the liver, stomach, and kidneys, and hence, a proper dose is needed for their use (46). Subsequently, as a preventive measure to avoid toxicity, further studies are needed to determine the effects of herbal administration on behavioral changes, signs of toxic effects, death of experimental animals, and histopathological changes in toxicity studies (47). The results of the current study indicated that the administration of WFCC, EAFCC, and n-HFCC at a single oral dose of about 5000 mg/kg does not show any signs of toxic effects and death in experimental rats. The administration of WFCC, EAFCC, and n-HFCC could also prevent histopathological changes in the liver, stomach, and kidneys. The findings of this study indicated that the oral administration of CcLF is safe and virtually non-toxic at doses of up to 5000 mg/kg/day. This suggests that CcLF could potentially be used as a safe treatment option for various conditions. Further research and clinical trials are needed to explore the full potential of CcLF as a therapeutic agent.

CONCLUSIONS

According to this study, CcLF exhibited anti-inflammatory, antipyretic, analgesic, and antioxidant activities. Additionally, the administration of CcLF up to 5000 mg/kg orally showed no signs of toxic effects and death in the experimental animals for 14 days. Histological observations indicated that there is no damage to the liver, stomach, and kidneys after the administration of CcLF. Based on these findings, it can be inferred that the traditional use of *C. costata* leaf for treating inflammation, fever, and pain is safe and poses minimal risk of toxicity.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

M.Y. Alkandahri and A. Sadino conceptualized the study. B.T. Pamungkas and Z. Oktoba contributed to the methodology. M. Arfania and N. Yuniarsih conducted the experiments. E.S. Wahyuningsih and D.E. Putri contributed to the data collection and analysis. All authors read and approved the finalized article.

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