# Evaluation of hepatoprotective and nephroprotective activities of *Castanopsis costata* extract in rats

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**Abstract.** The liver and kidneys are important organs for body homeostasis but susceptible to damage or injury caused by different factors. A number of medicinal plants, such as *Castanopsis costata* have been proven effective in protecting the liver and kidneys from damage. Therefore, the present study aimed to examine the effect of *C. costata* extract (CcE) on paracetamol-induced hepatotoxicity and gentamicin-induced nephrotoxicity in rat model. Each treatment group was given CcE at doses of 100, 200 and 400 mg/kg for 21 and 8 days for hepatoprotective tests and nephroprotective tests, respectively. To induce liver and kidney damage, rats were given paracetamol 1,000 mg/kg orally for 7 (15-21) and gentamicin 80 mg/kg intraperitoneally for 5 (4-8) days. To assess liver function, the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), total cholesterol (TC), total albumin (TA) and total protein (TP) were measured, as well as liver antioxidant enzymes. Meanwhile, to assess kidney function, the levels of serum creatinine (SCr), serum urea (SU) and uric acid (UA) were measured. TNF-α and IFN-γ were also measured with histopathology testing to assess the effects of liver and kidney organ damage in each experiment. The results showed that CcE reduced the levels of AST, ALT, ALP, TB and TC, increased TA, TP and liver antioxidant enzymes, as well as reducing SCr, SU and UA when compared with the pathological group. Additionally, CcE reduced the levels of TNF-α and IFN-γ, as well as improving the structure of liver and kidney tissue as confirmed by histopathology. CcE had hepatoprotective and nephroprotective effects on paracetamol-induced and gentamicin-induced rats, respectively.

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## Introduction

The most significant causes of different metabolic diseases and malnutrition are consuming fast and contaminated food (preservatives, pesticides, toxic metals), long-term use of drugs and alcohol, causing problems in liver and kidney (1,2). The liver is crucial for controlling the body's numerous physiological and biochemical functions, including metabolism, secretion, the delivery of nutrients and energy and vitamin storage (3,4). The liver can detoxify endogenous or exogenous substances. Therefore, it is very susceptible to exposure to toxic compounds from within and outside the body, which can cause metabolic and liver diseases (5,6).

The kidney is another vital organ with the main function in the process of excretion. This organ has several physiological functions, including maintaining homeostasis of body fluids by filtering metabolites and minerals from the blood, removing waste substances, playing a role in glucose metabolism, erythropoiesis and regulating blood pressure, as well as producing hormones and enzymes (7,8). The kidney filters ~180 liters of blood per day, equivalent to four times the amount passing

through other organs. Therefore, this organ is very susceptible to exposure to toxins in the blood that can damage the tissue and cause kidney disease (9,10).

According to the World Health Organization, liver disease accounts for >4% of global mortality (2 million mortalities annually) (11) and ~10% of the world's population (850 million individuals) suffer from kidney disease with 1.3 million mortalities each year (12). Current pharmacological treatment can alleviate various liver and kidney diseases according to the main causes and delay the occurrence of end-stage liver and renal failure. However, pharmacological treatment has not been able to treat or restore liver and kidney function completely. Most drugs cause liver and kidney damage to become severe and are considered risk factors for the organs (13). In this context, alternative treatments are needed to prevent or treat liver and kidney disease (14). Empirically, medicinal plants have long been used in a number of nations to cure and prevent a wide range of illnesses (15,16).

Indonesia is the second-largest biodiversity with 28,000 plant species, comprising 2,500 medicinal plants (17,18). In North Sumatra, traditional medicine frequently makes use of the medicinal herb *Castanopsis costata*. Empirically, *C. costata* leaves extract (CcE) is used to treat wounds, inflammation, fever and to act as an analgesic (19). Previous research reported that CcE has different pharmacological activities, such as antimalarial (20), antidiabetic (21), antioxidant (22), antipyretic (22), antihyperlipidemic (23), antidiarrheal (24) and anti-inflammatory (25).

Medicinal plants with antioxidant, anti-inflammatory and antihyperlipidemic activities have hepatoprotective effects, such as in liver fibrosis and non-alcoholic fatty liver disease (26-28). Antioxidant, anti-inflammatory and antidiabetic activities also have nephroprotective effects, such as in kidney fibrosis and diabetic nephropathy (29-31). Based on the results of previous research, CcE had antioxidant, anti-inflammatory, antidiabetic and antihyperlipidemic activities. This outcome validated the theory that the extract has hepatoprotective and nephroprotective properties. Therefore, the goal of the present study was to examine the hepatoprotective and nephroprotective activities of CcE against paracetamol-induced hepatotoxicity and gentamicin-induced nephrotoxicity in rat models.

## Materials and methods

*Chemicals and drugs.* Paracetamol, silymarin (MilliporeSigma), gentamicin (PT. Bernofarm Pharmaceutical Company), 0.9% sodium chloride (PT. Widatra Bhakti), diethyl ether (PT. Brataco), 10% formalin solution, xylene, paraffin, 70% ethanol, diethyl ether, hematoxylin-eosin stains, pulvis gummi arabicum and potassium chloride (EMSURE®; Merck KGaA) were of analytical grade. Kits for the estimation of total albumin (TA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), total cholesterol (TC), total protein (TP), serum creatinine (SCr), serum urea (SU), uric acid (UA) were from PT. Wacana Indo Mitra, tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) were from PT. Biolab Science Universal) and superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) were from Sigma-Aldrich (Merck KGaA).

*Sample collection, determination and extraction of plants.* A total of 10 kg of fresh *C. costata* leaves were bought from the Pancur Batu traditional market in North Sumatra, Indonesia in March 2022. The plant was identified at the Herbarium Medanense, Universitas Sumatera Utara, Indonesia (voucher number: 183/MEDA/2022). The cleaned *C. costata* leaves were brought to Pharmacognosy Laboratory, Universitas Buana Perjuangan Karawang for the extraction procedure. A total of 5 kg of *C. costata* powder was macerated in 70% ethanol three times in 24 h. The liquid extract was gathered and concentrated at 50˚C using a rotary evaporator (32).

*Fourier-transform infrared spectroscopy (FT-IR) analysis.* Potassium bromide (KBr) pellets were mixed with CcE and the results were evaluated with a Shimadzu IRPrestige-21 FT-IR Spectrophotometer (Shimadzu Corporation). At a resolution of 4 $cm^{-1}$, the spectra were collected in the 400-4,000 $cm^{-1}$ range.

*Randomization procedure and blinding.* For randomization, an identification number was first assigned to each rat and then randomization was performed, which generated random numbers and allocated rats to study groups. Randomization was performed using online software (https://www.graphpad.com/quickcalcs/randomize1/). Meanwhile, in blinding during the experiment, alphanumeric codes were used to identify vials and syringes and each rat was given a number. Then, each sample code was placed in a sealed envelope and revealed at the end of the experiment.

*Experimental animals.* A total of 44 male Wistar rats in good health, weighing 150-250 g and 8-12 weeks old, were employed in the hepatoprotective and nephroprotective research. Rats were acquired from CV. Mitra Putra Animal. The rats were kept at a 12-h light/dark cycle in the Pharmacology Laboratory at Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Buana Perjuangan Karawang with a temperature range of 20-26˚C and 30-70% humidity. In addition, the experimental animals had unrestricted access to drinking water and normal pellets. The human endpoints established for this study were deteriorating body condition, weight loss, the inability to rise or ambulate and the presence of labored respiration. No animal reached this stage.

*Protocol for hepatoprotective activity: Paracetamol-induced hepatotoxicity in rats.* A hepatotoxicity model produced by paracetamol was used to investigate hepatoprotective activity. The experimental rats were housed in six groups of four rats each. Group I, II and III served as normal, negative and positive control given 1% w/v pulvis gummi arabicum (PGA) suspension, paracetamol at a dose of 1,000 mg/kg and silymarin at a dose of 50 mg/kg, respectively. Groups IV, V and VI were each given CcE at doses of 100, 200 and 400 mg/kg orally. Determination of the CcE doses in this study refers to previously published research (21,23). The experimental treatments and group designs were:

Group I (normal control): For 21 days, rats were given 1% w/v PGA suspension orally (10 ml/kg/day).
Group II (negative control): For 7 days, rats received a dose of 1,000 mg/kg of paracetamol.
Group III (positive control): For 21 days, rats received 50 mg/kg of silymarin.
Group IV (CcE 100): CcE was administered to rats for 21 days at a dose of 100 mg/kg.
Group V (CcE 200): CcE was administered to rats for 21 days at a dose of 200 mg/kg.
Group VI (CcE 400): CcE was administered to rats for 21 days at a dose of 400 mg/kg.

Rats in groups II-VI were given paracetamol induction from days 15 to 21 at a dose of 1,000 mg/kg orally (33). Meanwhile, on the 22nd day, the treatment groups were anesthetized with diethyl ether at a dose of 4 ml. Diethyl ether was administered to rats by simple 'open-drop' methods using an ether-impregnated cotton ball in a bell jar for induction followed by inhalation via a simple face cone. The parameters monitored to ensure the animals were anesthetized after diethyl ether administration were ataxic, recumbent, with a steady, slow respiratory rate, immobile and loss of palpebral blink reflex. After a cardiac puncture, 2 ml of blood was extracted and placed in a tube holding heparin. The rats were euthanized by cervical dislocation. The liver was immediately removed and washed with cold 0.9% NaCl solution to remove the blood before weighing. For histological analysis, a portion of the liver's median lobe was preserved in a 10% formalin solution (fixation was carried out for 24 h at room temperature 20-22˚C) (34). Using a motor-driven Teflon pestle, homogenate was prepared for the liver antioxidant enzyme level test by combining one gram of wet tissue with 9 ml of 1.25% KCl. The homogenate was centrifuged for 10 min at 4˚C at 2,737 x g to extract the supernatant, which was then used to measure the levels of SOD, CAT, GPx and GSH (35).

*Protocol for nephroprotective activity: Gentamicin-induced nephrotoxicity in rats*. A nephrotoxicity model produced by gentamicin was used to perform the nephroprotective activity test. Random selection was used to choose five groups of four rats each from the experimental animals. Groups I and II as normal and negative controls were given a 1% w/v PGA suspension and gentamicin at a dose of 80 mg/kg, respectively. Meanwhile, groups III, IV and V were given treatment using CcE at 100, 200 and 400 mg/kg, respectively. The experimental treatments and group designs were as follows:

Group I (normal control): Rats were given 1% w/v PGA suspension orally (10 ml/kg/day) for 8 days.
Group II (negative control): Gentamicin was administered to rats for 5 days at a dose of 80 mg/kg.
Group III (CcE 100): For 8 days, rats received a 100 mg/kg dosage of CcE.
Group IV (CcE 200): For 8 days, rats received a 200 mg/kg dosage of CcE.
Group V (CcE 400): For 8 days, rats received a 400 mg/kg dosage of CcE.

Gentamicin induction was administered intraperitoneally to rats in groups II-V at a dose of 80 mg/kg from days 4-8 (36). On the ninth day, the rats in each treatment group were anesthetized using diethyl ether at a dose of 4 ml. Then 2 ml of blood was quickly collected into a heparin tube through a cardiac puncture and rats were euthanized by cervical dislocation. The kidney was cleaned with a cold 0.9% NaCl solution to remove blood and foreign tissue. This was followed by weighing and preserving the organs in 10% formalin solution for histopathological examination (fixation was carried out for 24 h at 20-22˚C) (37).

*Determination of liver and kidney serum biochemical parameters*. Fresh rat blood samples were centrifuged for 20 min at 503 x g and at 22˚C to produce blood serum. The serum was put in an Eppendorf tube and its levels of ALT, AST, TB, ALP, TC, TA, TP, SCr, SU and UA were promptly measured. In this procedure, commercial kits were used in accordance with the manufacturer's instructions [cat. nos. : ALT (32941-05121), AST (31335-05121), TB (3417012999-AL2-175423984), ALP (32918-05121), TC (3417012020-LAH-176618380), TA (3417012020-LAH-176587091), TP (3417012020-LAH-176626657), SCr (3417012020-LAH-176623909), SU (3417012020-BSS-211916981), UA (3417012999-LAB-205299812)] and a HumaLyzer 2000 photometer was used for measurement (PT. Sali Polapa Bersama).

*Determination of TNF-α and IFN-γ serum levels*. TNF-α and IFN-γ levels were measured in the present study using the ELISA technique. The collected serum was immediately analyzed using a commercially available ELISA kit [cat. nos. : TNF-α (MBS2707992) and IFN-γ (MBS2708210, PT. Biolab Science Universal] containing a microtiter plate coated with specific antibodies against TNF-α and IFN-γ standards as well as a washing buffer and horseradish peroxidase (HRP) conjugate. Meanwhile, an automatic microplate reader recorded optical density at 450 nm (ELx50; BioTek; Agilent Technologies, Inc.).

*Histopathological examination*. After being cleaned during the autopsy, the water content in liver and kidney tissue samples is removed using an alcohol dehydration process. Next, clearing was performed using xylene to remove alcohol and make the tissue transparent. Then, paraffin penetration was performed to make the tissue harden at room temperature and make it easier to cut using a microtome. Paraffin blocks were sectioned at 3.4-4.6 $\mu$m and the slides were deparaffinized in xylene, followed by H&E staining (at 30˚C: hematoxylin ~10 min, eosin 2 min). A 100x objective lens on a light microscope (BX-51; Olympus Corporation) with a connected camera (Olympus Q Color-5; Olympus Corporation) and computer connection was used to view the slides at a total magnification of x1,000. A pathologist assessed and rated the liver and kidney sections based on the degree of damage, somewhat modified from Zakaria *et al* (34).

*Statistical analysis*. The experimental results were shown using the mean ± standard error of the mean. One-way analysis of variance was used to examine the variations in the means of the variables that were measured. This was followed

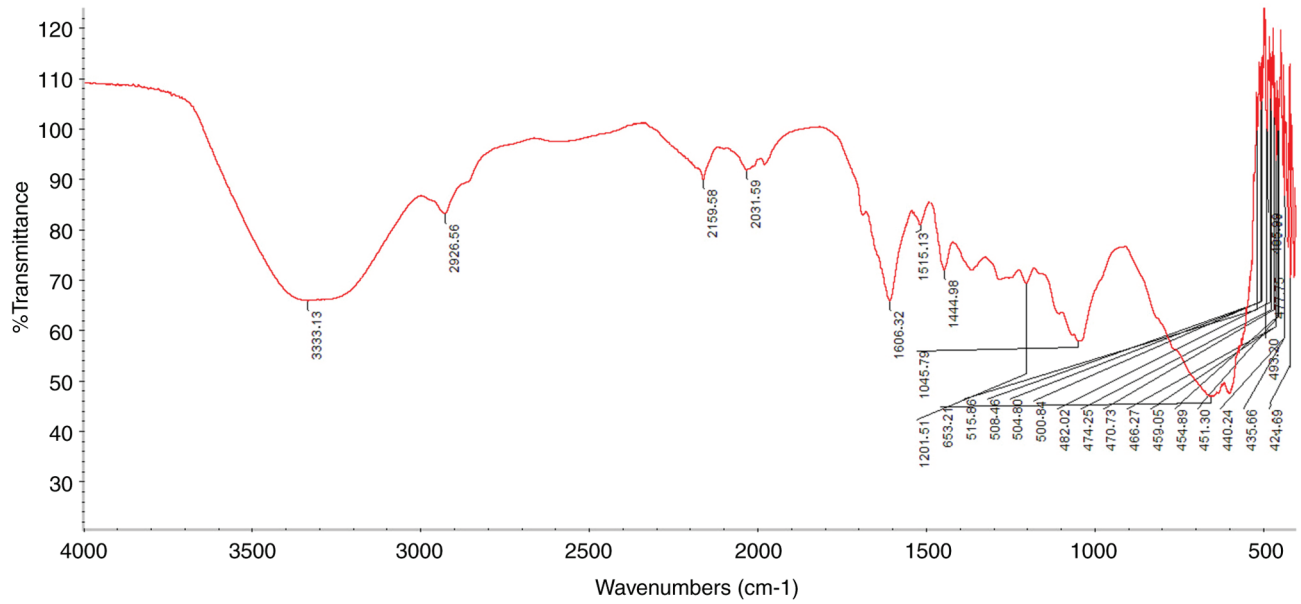


Figure 1. Fourier-transform infrared spectroscopy analysis of *Castanopsis costata* extract.

by Tukey's post hoc test using GraphPad Prism version 8 (Dotmatics). P<0.05 was considered to indicate a statistically significant difference. Sample size determination was based on Federer calculation formula, which is (t-1) (n-1) ≥15; where t is the number of the groups and n is the experimental animal per group. (6-1) (n-1) ≥15 -> n≥4, for the testing of hepatoprotective and (5-1) (n-1) ≥15 -> n≥4.75 for the testing nephroprotective properties. According to this calculation, the minimum sample size was four experimental animals in each treatment and control group.

## Results

*FT-IR analysis*. FT-IR revealed that there were several distinct functional groups by identifying 27 peaks for CcE. There were obvious peaks at 1,201.51, 1,444.98 and 1,515.13 $cm^{-1}$, showing C-O bending mode. This finding demonstrated the presence of a number of chemicals, including ethers, alcohols, esters and carboxylic acids. Furthermore, amines (N-H stretching), alcohol (O-H stretching), alkanes (C-H stretching), alkynes (C≡C stretching), carboxylic acid (C=O stretching), alkenes (C=C stretching) and imines (C=N) were among the functional groups found in a range of peaks that extended from 3,333.13 to 1,606.32 $cm^{-1}$. Fig. 1 shows the results of FT-IR analysis of CcE.

*Hepatoprotective activity of CcE against paracetamol-induced hepatotoxicity in rats: Effect of CcE on liver function parameters (AST, ALT, ALP, TB, TC, TA and TP) and liver weight*. Based on the present results, administration of paracetamol (1,000 mg/kg) to rats increased AST, ALT, ALP, TB and TC levels and also decreased TA and TP (P<0.001-<0.0001) when compared with normal controls. Pretreatment with CcE at all doses caused a significant decrease (P<0.05-<0.0001) in increasing AST, ALT, ALP, TB and TC, levels induced by paracetamol. Furthermore, the administration of CcE at all doses also caused a significant increase (P<0.01-<0.0001) in decreasing TA and TP levels induced by paracetamol. The pretreatment with silymarin (50 mg/kg) had an improved effect on changes in liver biochemical serum parameters compared with CcE. Additionally, compared with a normal control group, there was a statistically significant increase in liver weight (P<0.01) after the administration of paracetamol (1,000 mg/kg). Rats' liver weight significantly decreased (P<0.01) after receiving pretreatment with silymarin (50 mg/kg) or CcE at all dosages when compared with the paracetamol group. Table I shows the effects of pretreatment with CcE on liver function parameters and liver weight of rats.

*Effect of CcE on the levels of liver antioxidant enzymes*. The findings demonstrated that, in comparison with normal controls, the administration of paracetamol (1,000 mg/kg) significantly reduced the activities of SOD, CAT, GPx and GSH in liver tissue (P<0.001-<0.0001). The pretreatment with silymarin (50 mg/kg) and CcE at all doses showed a significant increase (P<0.05-<0.0001) in SOD, CAT, GPx and GSH activities when compared with paracetamol group. Therefore, CcE triggered hepatoprotective activity through the activation of endogenous enzymatic antioxidant systems. Fig. 2 shows the effects of the extracts on liver antioxidant enzymes.

*Effect of CcE on TNF-α and IFN-γ serum levels in paracetamol-induced hepatotoxicity rats*. Based on the present results, administration of paracetamol (1,000 mg/kg) induced a substantial increase in TNF-α and IFN-γ levels (P<0.0001) when compared with normal control. Pretreatment with CcE at all doses caused a significant decrease in increasing the levels of TNF-α (P<0.01-<0.0001) and IFN-γ (P<0.01-<0.001) induced by paracetamol. However, pretreatment with silymarin (50 mg/kg) had an improved effect on decreasing TNF-α levels (P<0.0001) and IFN-γ (P<0.001) than CcE. The effect of CcE on TNF-α and IFN-γ levels in rats with paracetamol-induced hepatotoxicity is depicted in Fig. 3.

Table I. Effect of CcE on paracetamol-induced liver injury in rats. For each group, the data are shown as the mean ± standard error of the mean of four replicates.

| Treatment | Dose (mg/kg) | AST (IU/l) | ALT (IU/l) | ALP (IU/l) | TB (mg/dl) | TC (mg/dl) | TA (g/dl) | TP (g/dl) | Liver weight (g) |
|---|---|---|---|---|---|---|---|---|---|
| NC | 1% PGA | 112.74±2.66 | 38.30±3.12 | 84.25±4.25 | 0.13±0.02 | 67.66±3.57 | 4.81±0.11 | 6.84±0.15 | 6.54±0.22 |
| PCT | 1,000 | 220.13±4.71$^{h}$ | 108.66±4.42$^{g}$ | 203.42±3.71$^{h}$ | 2.31±0.40$^{h}$ | 117.37±3.53$^{g}$ | 1.71±0.16$^{g}$ | 3.79±0.14$^{h}$ | 8.85±0.28$^{f}$ |
| SM | 50 | 117.74±3.58$^{d}$ | 45.82±3.27$^{c}$ | 105.83±3.74$^{d}$ | 0.27±0.04$^{d}$ | 75.29±4.15$^{c}$ | 4.70±0.46$^{c}$ | 6.31±0.19$^{d}$ | 6.66±0.17$^{b}$ |
| CcE | 100 | 191.96±3.73$^{a}$ | 85.62±4.03$^{a}$ | 186.44±3.92$^{a}$ | 1.51±0.06$^{a}$ | 98.54±4.16$^{a}$ | 4.37±0.19$^{c}$ | 5.36±0.15$^{b}$ | 6.83±0.16$^{b}$ |
| | 200 | 161.76±4.07$^{c}$ | 70.45±3.98$^{b}$ | 175.27±4.11$^{b}$ | 1.18±0.03$^{c}$ | 86.78±3.60$^{b}$ | 4.54±0.14$^{c}$ | 6.02±0.14$^{c}$ | 6.73±0.18$^{b}$ |
| | 400 | 135.69±3.57$^{d}$ | 54.81±4.20$^{c}$ | 112.26±3.67$^{d}$ | 0.68±0.03$^{d}$ | 79.27±4.84$^{c}$ | 4.61±0.18$^{c}$ | 6.21±0.12$^{d}$ | 6.65±0.22$^{b}$ |

$^{a}$P<0.05; $^{b}$P<0.01; $^{c}$P<0.001; $^{d}$P<0.0001 vs. paracetamol group. $^{f}$P<0.01; $^{g}$P<0.001; $^{h}$P<0.0001 vs. the normal group. CcE, *Castanopsis costata* extract; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TB, total bilirubin; TC, total cholesterol; TA, total albumin; TP, total protein; NC, normal control; PGA, pulvis gummi arabicum; PCT, paracetamol; SM, silymarin.

*Effect of CcE on histopathological analyses of liver of rats in paracetamol-induced hepatotoxicity*. Fig. 4A illustrated the cellular architecture with clear cells, sinusoidal gaps and central veins observed in the histopathological analysis of liver slices in the normal control group. However, the paracetamol group showed the most severe damage to cellular architecture, with centrilobular necrosis, hyperplasia, vascular and cellular degeneration, inflammation, polymorphonuclear aggregation, extensive lymphocyte infiltration and loss of cellular boundaries (Fig. 4B). Pretreatment with silymarin (50 mg/kg) showed complete improvement in cellular architecture, such as necrotic hepatocyte patches (Fig. 4C). In comparison with the paracetamol group, the pretreatment with CcE at all doses resulted in a lobular pattern that was nearly normal with mild degrees of necrosis and lymphocyte infiltration (Fig. 4D-F). Table II shows the histopathological scores of the changes.

*Nephroprotective activity of CcE against gentamicin-induced nephrotoxicity in rats: Effect of CcE on kidney function parameters (SCr, SU and UA) and kidney weight*. In comparison with normal controls, the administration of gentamicin (80 mg/kg) to rats resulted in a significant rise (P<0.001) in the levels of SCr, SU and UA. Gentamicin-induced levels of SCr, SU and UA were significantly (P<0.05-<0.01) reduced after pretreatment with CcE at all dosages. Rat kidney weight increased significantly (P<0.01) after receiving gentamicin (80 mg/kg) in comparison with the normal control group. Furthermore, in comparison with the gentamicin group, the pretreatment with CcE at all dosages resulted in a significant (P<0.01) drop in the kidney weight of the rats. Table III shows the effects of CcE pretreatment on kidney function parameters and kidney weight of rats.

*Effect of CcE on TNF-α and IFN-γ serum levels in gentamicin-induced nephrotoxicity in rats*. Compared with normal controls, the administration of 80 mg/kg of gentamicin resulted in a statistically significant rise (P<0.0001) in the levels of TNF-α and IFN-γ. The levels of TNF-α (P<0.01-<0.0001) and IFN-γ (P<0.01-<0.001) generated by gentamicin were significantly reduced following pretreatment with CcE at all dosages. Fig. 5 illustrates how CcE affects TNF-α and IFN-γ levels in rats that have gentamicin-induced nephrotoxicity.

*Effect of CcE on histopathological analyses of kidney of rats in gentamicin-induced nephrotoxicity*. When evaluated histopathologically, the kidney sections from the normal control group revealed normal tubules and glomeruli without any evident abnormalities (Fig. 6A). The gentamicin group showed severe acute glomerular and tubular necrosis, characterized by total obliteration of the tubular lumen, as well as intertubular hemorrhage and acute leukocyte infiltration (Fig. 6B). Meanwhile, pretreatment with CcE at all doses showed normal glomeruli, relatively normal tubular dilation, no interstitial edema and capillary congestion when compared with the gentamicin group (Fig. 6C-E). Table IV shows the histopathological scores of the changes.

## Discussion

Despite recent therapeutic advances and significant developments in modern medicine, liver and kidney diseases remain

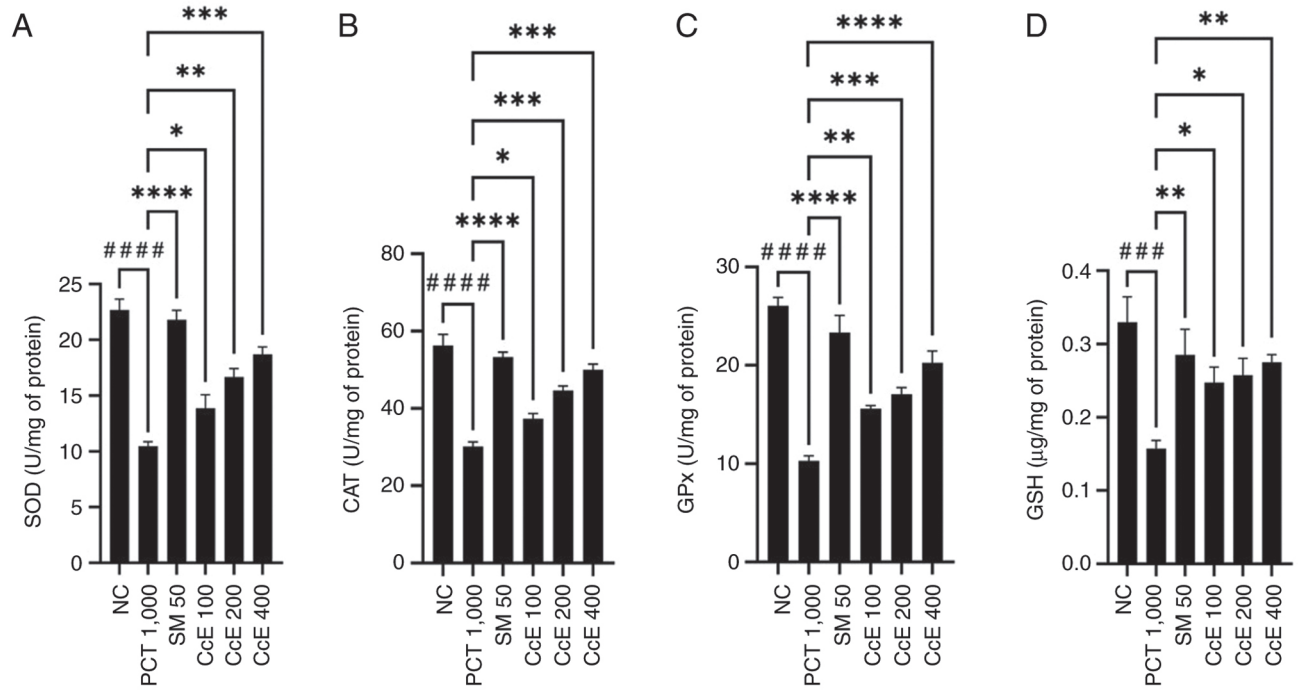


Figure 2. Effect of CcE on liver antioxidant enzymes in rats with hepatotoxicity induced by paracetamol. The data in each group is displayed as the mean ± standard error of the mean of four replicates showed significant differences $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001; $^{****}$P<0.0001 vs. paracetamol group. $^{###}$P<0.001 and $^{####}$P<0.0001 vs. normal group. (A) SOD levels. (B) CAT levels. (C) GPx levels. (D) GSH levels. CcE, *Castanopsis costata* extract; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione; NC, normal control; PCT, paracetamol; SM, silymarin.

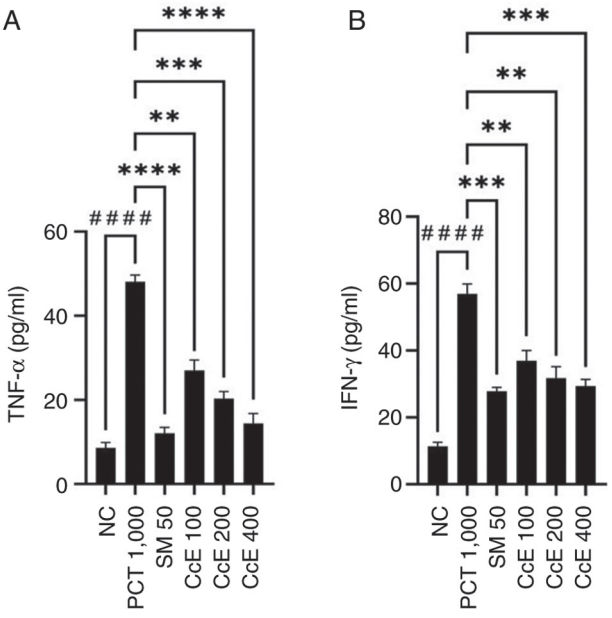


Figure 3. Effect of CcE on the levels of TNF-α and IFN-γ in paracetamol-induced hepatotoxicity rats. The data are displayed as the mean ± standard error of the mean of four replicates for each group. $^{**}$P<0.01; $^{***}$P<0.001; $^{****}$P<0.0001 vs. paracetamol group. $^{####}$P<0.0001 vs. the normal group. (A) TNF-α levels. (B) IFN-γ levels. CcE, *Castanopsis costata* extract; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; NC, normal control; PCT, paracetamol; SM, silymarin.

a global health problem (38,39). The most common cause of liver and kidney damage is long-term use of drugs [especially nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics and chemotherapy drugs]. This can cause the ability of these organs to regenerate to eventually become dysfunctional, leading to scarring and fibrosis (40,41). Currently, the conventional treatment focuses on symptom management and transplantation in severe cases of liver and kidney disease (42,43). However, there are no drugs used to increase the detoxification power of these organs (44). The quest to discover hepatoprotective and nephroprotective agents has become a significant challenge over the past decades (45,46). CcE has emerged as a promising alternative hepatoprotective and nephroprotective agent.

The hepatoprotective and nephroprotective properties of CcE were assessed in the present study on hepatotoxicity and nephrotoxicity caused by paracetamol and gentamicin, respectively. Several tests were performed to examine hepatoprotective effect of CcE, namely liver function tests, liver antioxidant enzyme levels, inflammatory cytokine levels and histopathology research in paracetamol-induced rats. Administration of high doses of paracetamol (1,000 mg/kg) is known to cause liver damage in rats (6). This happens as a result of the bioactivation of paracetamol, which creates the potentially dangerous reactive metabolite N-acetyl-p-benzoquinone imine. Specifically, CYP2E1 and CYP3A4 enzymes of the cytochrome P-450 (CYP) system produces these metabolite chemicals (47), which oxidize lipids or other significant sulfhydryl groups and bond covalently to tissue macromolecules (6). All treatment groups had their serum levels of AST, ALT and ALP tested in order to evaluate any changes in liver function parameters. Aspartic or alanine amino groups are transferred to ketoglutaric acid through the action of AST and ALT during the gluconeogenesis process, resulting in the production of pyruvic and oxaloacetate, respectively (48). Meanwhile, ALP functions

Table II. Effect of CcE on liver section histological score in rats with hepatotoxicity induced by paracetamol.

| Treatment | Dose (mg/kg) | Steatosis | Necrosis | Inflammation | Hemorrhage |
|---|---|---|---|---|---|
| NC | 1% PGA | - | - | - | - |
| PCT | 1,000 | + | +++ | ++ | ++ |
| SM | 50 | - | + | - | - |
| CcE | 100 | + | ++ | + | - |
| | 200 | - | + | - | - |
| | 400 | - | + | - | - |

The following scoring system was used to assess the severity of different liver damage features: - normal; + mild effect; ++ moderate effect; +++ severe effect. CcE, *Castanopsis costata* extract; NC, normal control; PGA, pulvis gummi arabicum; PCT, paracetamol; SM, silymarin.

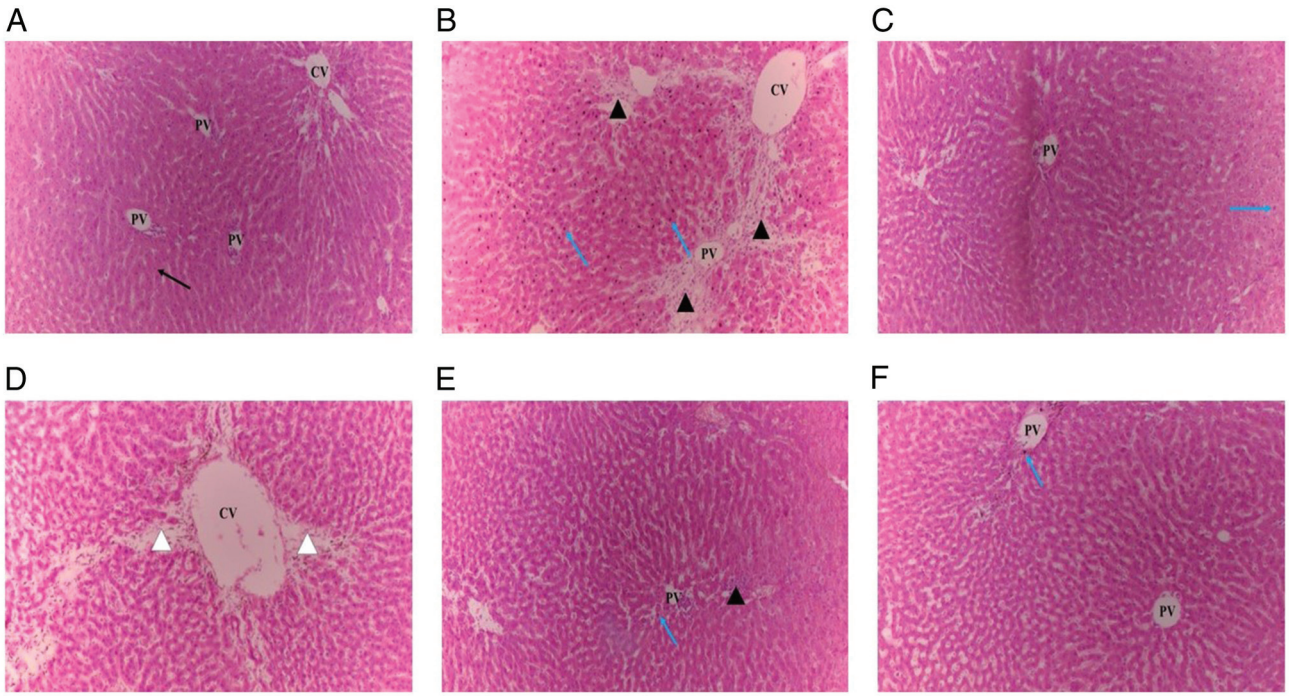


Figure 4. The effect of CcE on histopathological appearance of liver tissue. (A) Normal control. (B) Paracetamol 1,000 mg/kg. (C) Silymarin 50 mg/kg. (D) CcE 100 mg/kg. (E) CcE 200 mg/kg. (F) CcE 400 mg/kg. Black arrows (normal hepatocytes); Light blue arrows (necrotic hepatocytes); Black triangles (clustered necrotic cells); White triangles (single necrotic cells). Magnification, x100 and stained with hematoxylin and eosin. CcE, *Castanopsis costata* extract; PV, portal vein; CV, central vein.

to transport metabolites across cell membranes (49). AST, ALT and ALP were found in high concentrations when hepatopathy occurs. These enzymes leak into the bloodstream and are used as markers of hepatocyte damage (50). In the present study, the rats administered paracetamol had significantly higher serum levels of AST, ALT and ALP compared with the normal control group. It has been observed that pretreatment with CcE at different doses considerably lessens the rise in blood levels of AST, ALT and ALP in rats given paracetamol. This is due to the ability of CcE to prevent intracellular leakage of the enzymes by stabilizing the activity of hepatocyte membranes (35).

An additional method of evaluating liver function is to estimate serum levels of TB, TC, TA and TP (34,35). A class of enzymes known as uridine-diphosphoglucuronic glucuronosyltransferase transforms bilirubin, a byproduct of hemoglobin metabolism, into glucuronic acid in hepatocyte cells to increase the solubility in water. However, the bilirubin conjugation process is disrupted in the case of liver damage, resulting in hyperbilirubinemia (51). Previous research reports that liver damage affects the structure and function of membranes, as well as lipid metabolism, disrupting fluidity, permeability and the transport system (52,53). This condition decreases the number of hepatocytes and the capacity to synthesize protein and albumin (35).

The results of the present study showed that the rats given paracetamol had lower levels of TA and TP and greater levels of TB and TC compared with the normal control group. Pretreatment with CcE at all doses was reported to restore TB, TC, TA and TP levels, as shown in Table I. This was due to the antioxidant (22), antihyperlipidemic (23) and anti-inflammatory (25) effects of CcE. Consequently, there was an increase

Table III. Effect of CcE on renal damage caused by gentamicin in rats. For each group, the data are shown as the mean ± standard error of the mean of four replicates.

| Treatment | Dose (mg/kg) | SCr (mg/dl) | SU (mg/dl) | UA (mg/dl) | Kidney weight (g) |
|---|---|---|---|---|---|
| NC | 1% PGA | 0.51±0.11 | 19.66±0.99 | 3.83±0.23 | 1.02±0.04 |
| GM | 80 | 1.55±0.19[b] | 57.28±3.78[b] | 7.07±0.59[b] | 2.85±0.21[a] |
| CcE | 100 | 0.98±0.23[c] | 39.08±2.14[c] | 5.95±0.23[c] | 1.84±0.06[d] |
| | 200 | 0.83±0.14[c] | 33.11±2.09[c] | 5.42±0.55[c] | 1.76±0.03[d] |
| | 400 | 0.71±0.16[d] | 28.88±1.43[d] | 4.35±0.48[d] | 1.47±0.12[d] |

[a]P<0.01, [b]P<0.001 vs. normal group. [c]P<0.05, [d]P<0.01 vs. gentamicin group. CcE, *Castanopsis costata* extract; NC, normal control; PGA, pulvis gummi arabicum; GM, gentamicin.

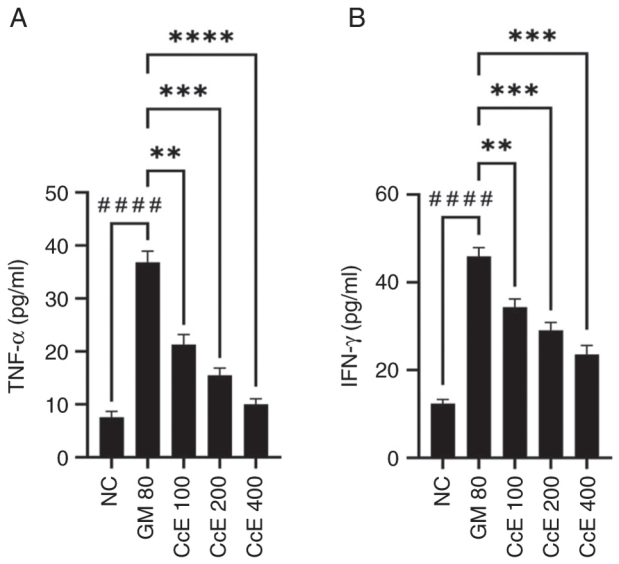


Figure 5. Effect of CcE on the levels of TNF-α and IFN-γ in gentamicin-induced nephrotoxicity in rats. The data are displayed as the mean ± standard error of the mean of four replicates for each group. **P<0.01, ***P<0.001, ****P<0.0001 vs. the gentamicin group. ####P<0.0001 vs. the normal group. (A) TNF-α levels. (B) IFN-γ levels. CcE, *Castanopsis costata* extract; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; NC, normal control; GM, gentamicin.

in hepatocyte count and liver function, as shown by the rise in TA and TP and fall in TB and TC levels.

The administration of high-dose paracetamol exacerbates oxidative stress, reactive oxygen species (ROS) and reactive nitrogen species, leading to alterations in the antioxidant enzyme system, a notable reduction in hepatic GSH and an upsurge in inflammatory cytokines (TNF-α and INF-γ) (54). Figs. 2 and 3 illustrate how pretreatment with CcE at all doses improved the activity of liver antioxidant enzymes and was found to reduce TNF-α and INF-γ levels. This was due to the strong antioxidant effect of CcE (22) which stimulated an increase in liver antioxidant enzyme levels. Therefore, pretreatment with CcE can metabolized ROS and neutralized free radicals, as well as non-radical oxidants to prevent or reduce oxidative damage (55). The reduction in TNF-α and INF-γ levels resulting from CcE was due to its anti-inflammatory properties (25). The present study found that CcE showed hepatoprotective action based on the data, which were corroborated by histological investigations. Thus, CcE was shown to enhance the architecture of liver tissue by lowering the degree of necrosis, enlarging cell borders and guarding against a significant infiltration of lymphocytes, as illustrated in Fig. 4.

However, based on the present results, pretreatment with silymarin (50 mg/kg) had an improved effect on changes in liver biochemical serum parameters, liver enzyme levels and cytokine levels compared with CcE. Silymarin is an active component of *Silybum marianum* L. which is a medicinal plant that has been used for centuries to treat various liver diseases (56). Silymarin administration prevents hepatic dysfunction and restored normal liver functionality in studies on hepatotoxicity in rats. Silymarin also functions as an antioxidant by reducing oxidative stress and preventing the loss of glutathione, increasing the regenerative ability of the liver cells by enhancing the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and inhibits elevated intrahepatic messenger RNA (mRNA) levels of interleukins (IL-2, IL-4), IFN-γ and TNF-α significantly (57,58).

Several tests were conducted to assess the nephroprotective effect of CcE including renal function, inflammatory cytokine level and renal histopathology analyses in gentamicin-induced rats. Administration of gentamicin (80 mg/kg) is reported to cause kidney organ damage in rats (37). It causes acute tubular necrosis, which is followed by renal failure, by inhibiting protein synthesis in the proximal tubules (59). Serum SCr, SU and UA levels were measured in all treatment groups to assess changes in renal functions due to gentamicin induction. SCr is a waste product derived from muscle breakdown and protein digestion. SU and UA are waste products produced by the liver when breaking down proteins and purines, respectively. These substances are filtered by the kidney and excreted in the urine (60). In a healthy kidney, SCr, SU and UA are found in urine, but filtering the substances is difficult during nephropathy (61). The rats exposed to gentamicin showed greater serum levels of SCr, SU and UA compared with the normal control group. Pretreatment with CcE at all doses significantly reduced serum levels of SCr, SU and UA in rats induced by gentamicin, as shown in Table III. This was due to the strong antioxidant effect of CcE (22), which prevented kidney damage caused by free radical exposure and quenched ROS formed due to gentamicin induction (62).

Table IV. Effect of CcE on histopathological scoring of kidney section in gentamicin-induced nephrotoxicity rats.

| Treatment | Dose (mg/kg) | Necrosis | Inflammation | Hemorrhage |
|---|---|---|---|---|
| NC | 1% PGA | - | - | - |
| GM | 80 | +++ | ++ | ++ |
| CcE | 100 | + | + | - |
| | 200 | - | - | - |
| | 400 | - | - | - |

The following scoring system was used to assess the severity of different kidney damage features: - normal, + mild effect, ++ moderate effect and +++ severe effect. CcE, *Castanopsis costata* extract; NC, normal control; PGA, pulvis gummi arabicum; GM, gentamicin.

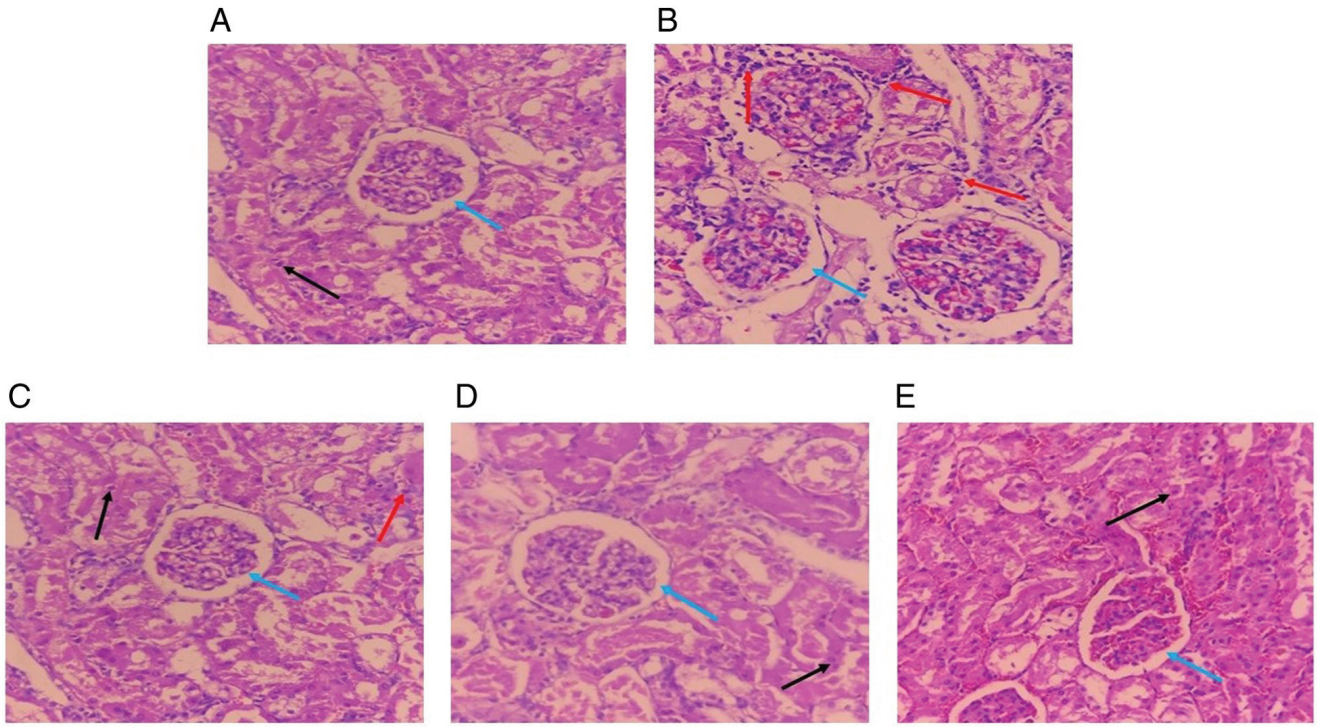


Figure 6. The effect of CcE on histopathological appearance of kidney tissue. (A) Normal control. (B) Gentamicin 80 mg/kg. (C) CcE 100 mg/kg. (D) CcE 200 mg/kg. (E) CcE 400 mg/kg. Black arrows (normal cells); Light blue arrows (glomerulus); and Red arrows (acute tubular necrosis). Magnification, x100 and stained with hematoxylin and eosin. CcE, *Castanopsis costata* extract.

Gentamicin is reported to cause tubular injury, triggering infiltrated renal epithelial cells to express proinflammatory cytokines in renal tissue (63). In the present study, pretreatment with CcE significantly reduced TNF-α and INF-γ, leading to an increase due to gentamicin induction (Fig. 5). The effect of reducing TNF-α and INF-γ levels by CcE was due to anti-inflammatory activity (25). According to histological analysis, CcE improved renal tissue architecture by averting capillary congestion, tubular necrosis, interstitial edema, glomerular congestion and interstitial with inflammatory cells (Fig. 6).

A limitation in the present study was that the sample size was too small, which had the potential to cause the loss of significant differences even if they exist in the population and may not be applicable to studies with larger populations. The authors suggest the use of freely downloadable software G Power for sample size calculation (64).

In conclusion, CcE had hepatoprotective activity in paracetamol-induced rats by improving liver function, increasing antioxidant enzyme levels, decreasing pro-inflammatory cytokine levels (TNF-α and IFN-γ) and enhancing liver tissue architecture. CcE also possessed nephroprotective activity in gentamicin-induced rats through improving kidney function, decreasing pro-inflammatory cytokine levels (TNF-α and IFN-γ) and enhancing renal tissue architecture. Thus, CcE served as a significant natural chemical source for the creation of novel hepatoprotective and nephroprotective medications. Further research *in vitro* was recommended to determine the exact mechanisms of hepatoprotective and nephroprotective effects of *C. costata* leaves.

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**Availability of data and materials**

The data generated in the present study are included in the figures and/or tables of this article.

**Author's contributions**

MYA designed the present study. AS, EA and FH contributed to the methodology. ZO, MFWS and PRAS wrote the manuscript and participated in the literature collection and evaluation. DW, NH and SWS confirm the authenticity of all the raw data. NAS, WI and SS contributed to the data collection and analysis. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The Research Ethics Committee of Universitas Padjadjaran in Bandung, Indonesia has accepted the present study protocol under the numbers 568/UN6.KEP/EC/2022 and 109/UN6.KEP/EC/2023 for the testing of hepatoprotective and nephroprotective properties, respectively, in compliance with ARRIVE guidelines.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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