Subchronic Toxicity of Cep-cepan (Castanopsis costata (Blume) A.DC.) Leaf Ethanol Extract on the Liver and Kidney Histopathology of Female White Mice (Mus musculus)

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Abstract

Castanopsis costata (cep-cepan) leaves are traditionally used in North Sumatra and have demonstrated antidiabetic, analgesic, anti-inflammatory, and antioxidant activities. However, their safety following repeated administration remains inadequately characterized, and comparative toxicological data within the Castanopsis genus and the broader Fagaceae family remain limited. This study aimed to evaluate the 28-day subchronic oral toxicity of C. costata leaf ethanol extract based on liver and kidney histopathological findings in female mice. An experimental post-test-only control group design was employed. Twenty female mice (n = 5 per group; four groups; total N = 20) were randomly allocated to a control group receiving 0.5% Na-CMC and three treatment groups receiving C. costata leaf ethanol extract at doses of 250, 500, and 1000 mg/kg body weight (BW) orally once daily for 28 days, following an adapted OECD Test Guideline 407. On day 29, the animals were euthanized; liver and kidney tissues were collected, processed using hematoxylin-eosin staining, and evaluated using a semiquantitative histopathological scoring system. Data were analyzed using the Shapiro-Wilk and Kruskal-Wallis tests. Phytochemical screening identified alkaloids, flavonoids, tannins, steroids/triterpenoids, and anthraquinone glycosides, whereas saponins were not detected. The extract yield was 11.39%. Kidney histopathology scores did not differ significantly among groups (H = 7.028; p = 0.071), and glomerular and tubular architecture remained preserved across all dose levels. In contrast, liver histopathology scores differed significantly among groups (H = 46.793; p < 0.001). Mild, non-diffuse hepatocellular degeneration and isolated apoptosis or necrosis lesions affecting less than 5% of the hepatic parenchyma increased with dose and were most pronounced at 1000 mg/kg BW, whereas steatosis and fibrosis were absent at all grouops. These field-level statistical findings should be considered preliminary pending reanalysis using the individual animal, rather than the microscopic field, as the unit of analysis. Under the conditions of this study, repeated administration of C. costata leaf ethanol extract for 28 days did not induce structural renal alterations but was associated with mild, dose-dependent hepatic histopathological changes that became evident at 500 mg/kg BW and were most pronounced at 1000 mg/kg BW. Doses up to 250 mg/kg BW showed no discernible histopathological changes in either organ, supporting the extract's relative safety at this dose for the parameters evaluated, whereas the 1000 mg/kg BW dose warrants caution pending biochemical confirmation. Further studies incorporating serum biochemical analyses, corrected animal-level statistical re-analysis, and longer-term toxicity assessments are recommended before prolonged use can be considered.

Keywords: Castanopsis costata, subacute toxicity, subchronic toxicity, hepatic histopathology, liver, kidney

INTRODUCTION

Indonesia is one of the world's most biodiverse countries, and medicinal plants have long played an important role in its traditional healthcare system. The increasing use of natural products necessitates robust scientific evidence of safety in addition to efficacy, particularly

for preparations intended for long-term consumption (Adane et al., 2023). Therefore, preclinical safety evaluation is an essential step in the development of natural products into standardized phytopharmaceuticals (Balkrishna et al., 2025).

Cep-cepan (*Castanopsis costata* (Blume) A.DC.; family Fagaceae) is a highland plant native to North Sumatra that has been traditionally used for the management of various ailments (Alkandahri et al., 2021). Several pharmacological activities of this species have been demonstrated experimentally, including dose-dependent antidiabetic effects in mice (Alkandahri et al., 2025), as well as the anti-inflammatory, antipyretic, analgesic, and antioxidant activities of its leaf fractions (Alkandahri et al., 2024). In addition, its analgesic activity has been associated with a high total phenolic and flavonoid content (Salim et al., 2017).

Toxicological data on the wider Castanopsis genus and Fagaceae family remain limited but provide a useful comparative context. A recent phytochemical and pharmacological review of the genus Castanopsis documented extensive anti-inflammatory, antioxidant, and antimicrobial activities across more than a dozen species, yet reported almost no repeated-dose safety data for any of them (Yang et al., 2024). This finding underscores the fact that the pharmacological potential demonstrated across the genus has rarely been accompanied by systematic toxicity evaluation. Notably, an ethanol extract of a related species, *Castanopsis sieboldii*, exhibited hepatoprotective rather than hepatotoxic effects in an acute carbon tetrachloride-induced liver injury model, reducing serum transaminase levels through the suppression of inflammasome-mediated pyroptosis (Kim et al., 2023). This observation makes the dose-dependent hepatic histopathological changes identified in the present study under repeated, rather than acute, exposure particularly important to characterize. It suggests that polyphenolic constituents within the genus may confer protection in acute injury models but exhibit different biological effects during cumulative subchronic exposure. Thus, this comparison highlights, rather than resolves, the existing research gap: although pharmacological activities within the genus are well documented, organ-level safety following repeated administration remains poorly understood, and the differential susceptibility of the liver and kidneys has not previously been investigated for *C. costata.*

These activities are closely linked to the plant's secondary metabolites. Phytochemical screening of *C. costata* leaf extract has consistently identified alkaloids, flavonoids, tannins, steroids/triterpenoids, and anthraquinone glycosides, along with notable antioxidant activity (Alkandahri et al., 2024). Polyphenolic compounds such as flavonoids and tannins are known to possess antioxidant properties that may contribute to hepatoprotective and nephroprotective

effects through free-radical scavenging and the enhancement of endogenous antioxidant defences (Gajender et al., 2023; Kukreti et al., 2023; Venmathi Maran et al., 2022). However, tannins and anthraquinone glycosides may also exhibit irritant or pro-oxidant effects at high concentrations. Tannins can precipitate mucosal and hepatocellular proteins and impair digestive enzyme activity, whereas anthraquinone glycosides are metabolized in the liver into reactive aglycones capable of generating oxidative stress when detoxification capacity is exceeded. This mechanism may provide a plausible explanation, although not a confirmed pathway in the present study, for the association between the extract's antioxidant-associated constituents and the hepatocellular stress observed at the highest dose.

Nevertheless, a promising pharmacological profile does not guarantee safety following repeated administration. Compounds that are beneficial at low doses may produce adverse effects at higher doses or after prolonged exposure, making repeated-dose toxicity testing essential (Balkrishna et al., 2025; Dossou-Agoin et al., 2023). Subchronic toxicity studies are designed to detect cumulative effects, biochemical changes, and histopathological lesions that may not be apparent in acute tests. Internationally, such studies are commonly conducted in accordance with OECD Test Guideline 407 for the repeated-dose 28-day oral toxicity study in rodents (OECD, 2025).

The liver and kidney are principal target organs in toxicity assessment because of their central roles in xenobiotic metabolism and excretion. Hepatocellular damage typically manifests as parenchymal degeneration, steatosis, sinusoidal congestion, and necrosis, whereas renal injury is characterized by tubular epithelial degeneration, tubular dilatation, and interstitial inflammatory infiltration (Loha et al., 2019; Tran, 2021). Histopathological examination of these organs is therefore a sensitive and reliable method for evaluating the safety of a test substance (Jannah & Budijastuti, 2022; Kusuma et al., 2020). In addition, changes in organ weight have been shown to correlate with, and may help predict, the presence of histopathological lesions in repeated-dose rodent studies (Mezencev et al., 2024).

To date, several pharmacological activities of *C. costata* have been reported; however, subchronic toxicity data, particularly regarding liver and kidney histopathology, remain scarce. Comparative toxicological evidence from related *Castanopsis* and other Fagaceae species is similarly limited, as discussed above. Therefore, this study aimed to evaluate the effect of 28-day administration of *C. costata* leaf ethanol extract on the liver and kidney histopathology of female white mice as a basis for preclinical safety assessment, with particular emphasis on whether these two organs differ in their susceptibility to toxicity. We hypothesized that repeated administration would produce a dose-dependent increase in hepatic histopathological

alterations because of the liver's first-pass exposure to absorbed metabolites, whereas renal histological structure would remain comparatively preserved across the tested dose range.

METHODS

Study design and ethics statement

This experimental laboratory study employed a post-test-only control group design based on a completely randomized design (CRD). Reporting follows the ARRIVE 2.0 guidelines for in vivo research (Percie du Sert et al., 2020).

Plant material and determination

Cep-cepan leaves were collected from Langkat Regency, North Sumatra, by purposive sampling and were authenticated at the Medanese Herbarium, Universitas Sumatera Utara (certificate no. 1265/MEDA/2023).

Extract preparation

The leaves were sorted, washed, chopped, dried, and ground into powder. A 312-g portion of the powdered material was macerated in 96% ethanol (1.5 L) for 24 h, and the residue was subsequently re-macerated twice using fresh solvent (750 mL each). The combined filtrates were concentrated to obtain 63.14 g of viscous extract, corresponding to a yield of 11.39%.

Phytochemical screening

Qualitative phytochemical screening was performed to identify alkaloids (Dragendorff, Bouchardat, and Mayer reagents), flavonoids, tannins, saponins, steroids/triterpenoids, and anthraquinone glycosides using standard procedures.

Animals

Twenty healthy female white mice (*Mus musculus*) weighing 20–30 g were used in this study. The animals were acclimatized for at least seven days, housed under room-temperature conditions with a natural light-dark cycle, and provided standard feed and water *ad libitum*. All animals were nulliparous and non-pregnant, in accordance with OECD Test Guideline 407 recommendations (OECD, 2025).

Grouping and dosing

The animals were randomly allocated to four groups (five each): a control group (Na-CMC 0.5%, selected because it is the vehicle used to suspend the extract and has no known pharmacological or histopathological effect of its own, thereby isolating the extract's effect from any vehicle effect) and three extract groups at 250, 500, and 1000 mg/kg BW. The dose range was selected to span the pharmacologically active doses reported in prior antidiabetic

and anti-inflammatory studies of this extract (Alkandahri et al., 2021, 2024) up to the internationally recognized limit dose of 1000 mg/kg BW (OECD, 2025). Preparations were administered orally by gavage once daily for 28 consecutive days following OECD TG 407 principles (OECD, 2025).

Necropsy and histopathological processing

On day 29, the animals were euthanized and subjected to necropsy. The liver and kidneys were harvested, fixed in 10% formalin, processed routinely for histopathological examination, sectioned, and stained with hematoxylin-eosin (HE). Tissue sections were examined under a light microscope to assess hepatic lobular architecture, hepatocytes, sinusoids, renal glomeruli, and renal tubules.

Scoring and statistical analysis

Histopathological lesions were assessed a semi-quantitative ordinal scoring system adapted from standard toxicologic-pathology practice (0 = no lesion; 1 = minimal (<5% of the examined field affected); 2 = mild (5–25%); 3 = moderate, (26–50%); 4 = marked (>50%). Scoring was performed separately for degeneration, necrosis/apoptosis, congestion, and inflammatory-cell infiltration.

Data normality was evaluated using the Shapiro-Wilk test. As the data did not satisfy normality assumptions, between-group comparisons were performed using the non-parametric Kruskal-Wallis test in SPSS version 25.0. Statistical significance was defined as $p < 0.05$.

Statistical unit of analysis methodological note

Two reviewers of this manuscript identified that the degrees of freedom reported in Tables 2 and 4 (ranging from 12–35) are far larger than the number of animals per group (n = 5), indicating that individual microscopic fields, rather than individual animals, were entered as the statistical unit. This is a form of pseudo-replication: because multiple fields from the same animal are correlated observations rather than independent biological replicates, treating them as independent inflates the effective sample size and can produce spuriously narrow confidence intervals and inflated significance (Eisner, 2021; Gibson-Corley et al., 2013).

This issue is acknowledged as a limitation of the present analysis. A more appropriate approach is to average (or otherwise summarize) each animal's field-level scores into a single composite score per animal before hypothesis testing, so that N = 5 per group (total N = 20) is the analytical sample size, consistent with the biological replicate. We were unable to perform this re-analysis for the present version of the manuscript because only the final SPSS output, not the field-level raw data, was available at the time of revision. Until that re-analysis is

completed, the H and p values reported below should be interpreted as preliminary, field-level (exploratory) statistics rather than confirmatory animal-level statistics.

RESULTS

Determination and extract yield

Determination confirmed the sample as *Castanopsis costata* (Blume) A.DC. Maceration yielded a thick extract with an 11.39% yield.

Phytochemical profile

Phytochemical screening (Table 1) showed the extract contained alkaloids, tannins, flavonoids, and steroids/triterpenoids, whereas saponins were not detected.

Table 1. Phytochemical screening of *Castanopsis costata* leaf ethanol extract

Compound class	Result
Alkaloid	+
Saponin	−
Tannin / Tannins	+
Flavonoid / Flavonoids	+
Steroid/triterpenoid	+
Anthraquinone glycosides	+

Note. (+) detected; (−) not detected.

Liver histopathology

Most liver sections showed hepatic lobules with hepatocytes arranged radially around the central vein and intact nuclei. The high-dose group showed non-diffuse mild apoptosis and necrosis, mild hydropic and parenchymatous degeneration (<5% of the examined parenchyma), and sinusoidal dilatation without severe congestion; steatosis and fibrosis were absent at all doses. The Shapiro-Wilk test (Table 2) indicated non-normal distribution ($p < 0.001$).

Table 2. Normality test (Shapiro-Wilk) of liver histopathology scores

Group	Statistic	df	Sig. (p)
Control (Dose 0)	—	24	—[a]
Dose 1 (250)	0.623	30	<0.001
Dose 2 (500)	0.751	24	<0.001
Dose 3 (1000)	0.465	12	<0.001

Note. [a] Not computable because all control values were identical (zero variance). Degrees of freedom reflect the number of microscopic fields, not animals; see the Statistical unit of analysis note in Methods regarding planned re-analysis with the animal as the unit of analysis.

The Kruskal-Wallis test (Table 3) showed a significant difference in liver histopathology scores among groups (H = 46.793; df = 3; p < 0.001 at the field level; see Methods), with lesion scores increasing with dose (a dose-response pattern).

Table 3. Kruskal-Wallis test of liver histopathology scores

Parameter	Value
Kruskal-Wallis H	46.793
Df	3
Asymp. Sig. (p)	<0.001

Field-level result pending animal-level re-analysis (see Methods).

Kidney histopathology

Renal glomerular and tubular structures were largely normal across all groups. Bowman's capsule and glomerular tufts retained their normal architecture, and the proximal and distal tubular epithelium showed intact brush borders and nuclei without evidence of degeneration. Findings were limited to mild vascular dilatation and minimal interstitial inflammatory infiltration; glomerulosclerosis, tubular epithelial degeneration, and calcification were absent at every dose, including 1000 mg/kg BW. The Shapiro-Wilk test (Table 4) indicated non-normal distribution (p < 0.001).

Table 4. Normality test (Shapiro-Wilk) of kidney histopathology scores

Group	Statistic	df	Sig. (p)
Control	0.188	28	<0.001
Dose 1 (250)	0.289	35	<0.001
Dose 2 (500)	0.286	28	<0.001
Dose 3 (1000)	0.616	14	<0.001

Note. Degrees of freedom reflect the number of microscopic fields, not animals; see the Statistical unit of analysis note in Methods.

Unlike the liver, the Kruskal-Wallis test of total kidney damage scores (Table 5) showed no significant difference among groups (H = 7.028; df = 3; p = 0.071).

Table 5. Kruskal-Wallis test of kidney histopathology scores

Parameter	Value
Kruskal-Wallis H	7.028
Df	3
Asymp. Sig. (p)	0.071

DISCUSSION

Phytochemical screening confirmed the presence of alkaloids, flavonoids, tannins, and steroids/triterpenoids in the extract, consistent with the metabolite profile of C. costata reported in previous studies (Alkandahri et al., 2024). Polyphenolics compounds, particularly flavonoids and tannins, are known to stabilize cellular membranes, enhance antioxidant defenses by increasing glutathione, superoxide dismutase, and catalase levels, and mitigate oxidative stress, which is a principal mechanism underlying the hepatoprotective and nephroprotective effects of many natural products (Gajender et al., 2023; Kukreti et al., 2023; Mansour et al., 2023; Putri et al., 2025).

In renal tissue, the preservation of glomerular and tubular integrity, together with the absence of a significant difference between groups ($p = 0.071$), indicates that filtration and re-absorptive functions were not structurally compromised across the tested doses. This finding is consistent with subchronic toxicity studies of other plant extracts that reported no significant microscopic renal alterations (Adane et al., 2023; Tran, 2021). The minimal interstitial inflammatory cell infiltration and mild vascular congestion observed are more likely to represent transient physiological response than evidence of nephrotoxicity (Kusuma et al., 2020).

In contrast, the liver histopathological analysis demonstrated a significant difference in histopathology scores between groups at the microscopic field level ($H = 46.793$; $p < 0.001$) with an apparent dose-response pattern. This finding warrants careful interpretation for two reasons. First, and most importantly, the reported statistical significance was derived using microscopic fields rather than individual animals as the analytical unit and should therefore be regarded as preliminary until reanalysis is performed using the animal as the unit of analysis. Nevertheless, the direction and consistency of the observed dose-response trend remain a potentially informative descriptive finding. Second, from a pathological perspective, the observed lesions were mild and focal, consisting predominantly of reversible cellular degeneration and isolated apoptotic cells without evidence of fibrosis or steatosis. Such findings are more consistent with an adaptive hepatocellular response to xenobiotic metabolic burden than with overt hepatotoxicity.

Mechanistically, hepatocytes bear the principal burden of phase I (cytochrome P450-mediated oxidation) and phase II (conjugation) biotransformation of absorbed plant metabolites, exposing the liver, unlike the kidney, which primarily handles the more hydrophilic conjugated end products, to reactive intermediates generated during first-pass metabolism. Anthraquinone glycosides, in particular, can be converted by phase I enzymes and

gut or hepatic microbia into aglycones capable of generating reactive oxygen species when local antioxidant defenses are exceeded. This mechanism provides a plausible, although unconfirmed, explanation for why the liver, rather than the kidney, exhibited a dose-related histopathological signal in the present study.

This pattern is consistent with a hormetic, dose-dependent shift in the biological activity of the extract's polyphenolic constituents. At low-to-moderate doses, flavonoids and tannins are expected to function predominantly as antioxidants, consistent with their previously reported hepatoprotective and nephroprotective effects (Gajender et al., 2023; Kukreti et al., 2023). At high doses, however, the same classes of compounds may undergo redox cycling, shifting their overall effect toward pro-oxidant activity and potentially overwhelming hepatic detoxification mechanisms, a phenomenon that has been increasingly recognized in recent reviews of polyphenol pharmacology (Vrankova et al., 2026). The mild, dose-dependent hepatic alterations observed at 500 and, particularly, 1000 mg/kg BW in the present study are consistent with the possibility that this hormetic threshold was being approached, rather than reflecting a simple linear toxic response.

A similar pattern, in which hepatic changes emerge at higher doses while lower doses remain relatively well tolerated, has been reported for other ethanol plant extracts (Abebe et al., 2021; Kanina et al., 2022; Rosidah et al., 2024). Furthermore, analyses of large repeated-dose toxicology databases have shown that changes in liver weight often co-occurs with, and may help predict, histopathological abnormalities (Kanan et al., 2024). This parameter was not evaluated in the present study and should therefore be incorporated into future investigations. Within the *Castanopsis* genus, the contrast with the findings of Kim et al., (2023), in which an extract from a related species demonstrated protection against acute chemically induced liver injury, highlights how the same broad phytochemicals may appear protective in an acute injury model yet produce measurable dose-related histological changes under repeated administration. This distinction is important and may be overlooked when evidence from acute pharmacological studies is generalized as proof of long-term safety.

Accordingly, safety cannot be inferred from the qualitative impression of 'largely normal' tissue alone but must account for the full statistical analysis, once that analysis has been corrected to the animal level. The antioxidant properties of flavonoids and tannins likely limited lesion severity but did not fully prevent hepatic histopathological change at high doses, so the therapeutic-window concept remains relevant to developing this extract (Shahat et al., 2018; Venmathi Maran et al., 2022).

From a translational and public-health perspective, these findings suggest that occasional or short-term traditional use of *cep-cepan* leaf preparations at levels broadly comparable to the low-to-moderate doses tested here (250 mg/kg BW) is unlikely, on histopathological grounds alone, to produce structural liver or kidney injury. However, we deliberately avoid proposing a specific numerical human-equivalent safety threshold, since this study evaluated histopathology only, in one sex, over 28 days, without biochemical confirmation or a recovery group, any translational dose recommendation would be premature until those parameters are added (see Limitations).

Limitations

Several limitations should be noted. First, and most importantly, the histopathological scores were analysed using microscopic fields as the statistical unit rather than individual animals, a pseudo-replication issue that inflated the effective degrees of freedom (Tables 2 and 4) and means the reported H and p values should be treated as preliminary until corrected (Eisner, 2021); we recommend this re-analysis, together with a Dunn's post hoc test if the corrected Kruskal-Wallis result remains significant, be completed before the next round of review. Second, assessment was confined to histopathology without serum biomarkers (ALT, AST, ALP, bilirubin, urea, creatinine) or relative organ weights, although OECD TG 407 recommends these (OECD, 2025) and organ-weight change has been shown to correlate with histopathological findings (Mezencev et al., 2024). Third, no recovery group was included to assess reversibility of hepatic lesions. Fourth, only female mice were used, and each dose group comprised only five animals, limiting statistical power and generalizability to males. Fifth, the scoring system, number of fields examined, and blinding status require the additional detail flagged in the Methods section to ensure objectivity and avoid pseudo-replication (Percie du Sert et al., 2020). Follow-up studies incorporating biochemical parameters, relative organ weights, a recovery group, both sexes, corrected animal-level statistics, and a 90-day duration are strongly recommended.

CONCLUSIONS

This study aimed to evaluate the effect of 28-day oral administration of *Castanopsis costata* leaf ethanol extract on liver and kidney histopathology in female white mice. Administration at 250, 500, and 1000 mg/kg BW for 28 days did not produce a statistically significant structural change in the kidney ($H = 7.028$; $p = 0.071$), whereas it was associated with a significant, dose-dependent pattern of mild hepatic histopathological change ($H = 46.793$; $p < 0.001$ at the field level), most evident at the highest dose. The sharp contrast

between these two organs, preserved renal architecture versus a graded hepatic response, indicates that, under the conditions tested, the liver was the more sensitive of the two target organs to repeated administration of this extract. Because the reported hepatic p-value reflects a field-level rather than an animal-level analysis (n = 5 per group; see Methods), this contrast should be regarded as a preliminary signal that requires confirmation with corrected, animal-level statistics before it is treated as definitive.

Within the histopathological parameters evaluated here, and with the sample-size (n = 5/group) and single-sex limitations noted above, doses up to 250 mg/kg BW showed no discernible adverse histological signal in either organ, while 1000 mg/kg BW warrants caution given the mild but consistent hepatic changes observed. This conclusion is intentionally limited to the histopathological endpoints assessed in this study and should not be read as a general statement about the extract's overall safety, which would additionally require biochemical confirmation.

Future research should prioritize, in order: (1) re-analysis of the existing histopathology data with the animal as the statistical unit, followed by a Dunn's post hoc test if warranted; (2) addition of serum hepatic (ALT, AST, ALP, bilirubin) and renal (urea, creatinine) biomarkers together with relative organ weights; (3) inclusion of a recovery group to assess lesion reversibility; (4) extension to a 90-day study duration; and (5) the inclusion of both male and female animals to assess sex-related differences in susceptibility.

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