RESEARCH ARTICLE

Ethanol Extract of *Cyclea barbata* Miers Exhibited A Favorable Safety Profile at Moderate Doses in Wistar Rats

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Abstract

BACKGROUND: *Cyclea barbata* Miers has been identified as a phytoestrogenic candidate with comparable estrogen receptors (ER)α binding activity with 17β estradiol. Though phytoestrogens are reported to modulate reproductive and hormonal physiology, but no systematic *in vivo* toxicological evaluation of *C. barbata* extract has been reported. Therefore, this study was conducted to evaluate the acute and subacute oral toxicity of the ethanol extract of *C. barbata* (EECB) in Wistar rats.

METHODS: For acute toxicity, rats received single oral doses of 2,000 or 5,000 mg/kg BW and were observed for 14 days. For subacute toxicity, EECB was administered orally at doses of 100–500 mg/kg BW/day for 28 consecutive days. Body weight, relative organ weights, haematological and biochemical parameters, and histopathological changes in major organs were evaluated.

RESULTS: No mortality occurred at 2,000 mg/kg BW in either sex, whereas mortality was observed only in female rats at 5,000 mg/kg BW (20%). EECB was therefore classified as GHS Category 5 or unclassified (LD_{50} >2,000 mg/kg BW), indicating low acute oral toxicity. Repeated administration for 28 days caused no significant changes in body weight, organ weights, serum biochemical markers, or histopathological findings. However, a significant reduction in white blood cell counts was observed at doses ≥400 mg/kg BW/day (p<0.05). The subacute no-observed-adverse-effect-level (NOAEL) and lowest-observed-adverse-effect-level (LOAEL) were established at 300 and 400 mg/kg BW/day, respectively.

CONCLUSION: Based on the parameters assessed, the acute NOAEL for EECB was 2,000 mg/kg BW, while the subacute NOAEL and LOAEL for EECB were 300 and 400 mg/kg BW/day, respectively. These findings indicate that EECB might be safe at moderate doses, providing *in vivo* evidence supporting its further development as a phytoestrogenic agent.

KEYWORDS: *Cyclea barbata* Miers, ethanol extract, acute toxicity, subacute toxicity, haematological parameters

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Introduction

The integration of medicinal plants into healthcare systems continues to expand worldwide, driven by cultural traditions and increasing public interest in natural alternatives. In developing countries, plant-based remedies often constitute the primary source of healthcare for large segments of the population.(1-3) Despite this widespread use, the safety of many herbal preparations has not been systematically evaluated.(4) The assumption that natural origins confer inherent safety is scientifically unfounded, as numerous plant-derived compounds exert potent biological effects that can be harmful at inappropriate doses or in vulnerable

populations.(5) Rigorous preclinical toxicological evaluation is therefore an indispensable prerequisite for the responsible development of any phytotherapy.

Green grass jelly (*Cyclea barbata* Miers) is a plant endemic to Southeast Asia and widely cultivated in Indonesia.(6) The leaves are traditionally processed into a gelatinous food product valued for its perceived cooling and anti-inflammatory properties.(7) *C. barbata* has been used for various health conditions, including fever reduction, management of hypertension, and as a general health tonic. Its antihypertensive potential is supported by a recent clinical study reporting significant reductions in systolic and diastolic blood pressure following administration of green grass jelly preparations in hypertensive patients, an effect attributed to its alkaloid and flavonoid content acting as vasodilators.(8) Phytochemical investigations have identified a diverse array of bioactive secondary metabolites in *C. barbata* leaves, including flavonoids, alkaloids, tannins, and saponins, which underpin its reported pharmacological activities encompassing antioxidant, anti-inflammatory, and antimicrobial properties.(7,9)

Of particular contemporary significance, an emerging body of research has demonstrated that the ethanol extract of *C. barbata* possesses estrogenic activity. *C. barbata* ethanol extract and its marker compound, coclaurine, a benzylisoquinoline alkaloid with binding affinity to estrogen receptor alpha (ERα) at −8.3 kcal/mol, stimulate follicular development and induce ERα expression in *in silico* and *in vitro* experimental models.(10) A subsequent study further demonstrated that *C. barbata* extract increases 17β-estradiol (E2) levels and upregulates luteinizing hormone receptor (LHR) expression.(11) These findings open a compelling avenue for the development of *C. barbata* as a phytoestrogenic therapeutic agent, with potential applications in conditions characterized by estrogen deficiency or disrupted reproductive function. However, phytoestrogens are not pharmacologically inert compounds; their estrogenic receptor activity can modulate reproductive physiology, alter hormonal homeostasis, and potentially affect reproductive organ morphology in a dose-dependent and sex-specific manner.(12)

Compared with other medicinal plants reported to exhibit phytoestrogenic potential, *C. barbata* offers distinct translational advantages that it has a long history of dietary consumption without documented adverse effects, is widely and sustainably cultivated in Indonesia, and possesses a well-characterized marker compound, coclaurine, with confirmed ERα binding affinity.(9) These attributes provide a stronger rationale for prioritizing *C. barbata* over other candidate species with comparatively less-defined phytochemical or pharmacological profiles. Notwithstanding the growing pharmacological evidence base, comprehensive *in vivo* toxicological characterization of *C. barbata* ethanol extract has not been reported in the peer-reviewed literature. Existing studies have focused on phytochemical profiling and specific *in vitro* pharmacological activities (13-16), without conducting the systematic *in vivo* toxicological assessments required by international regulatory guidelines. This gap is particularly consequential given the estrogenic properties of the extract, which necessitate evaluation not only of conventional safety endpoints, hepatic and renal function markers, and haematological parameters, but also of reproductive organ integrity. The Organisation for Economic Co-operation and Development (OECD) guidelines provide the internationally recognized framework for preclinical toxicological evaluation. OECD Test Guideline 423 enables determination of the acute lethal dose range and GHS toxicity classification following single-dose administration.(15) OECD Test Guideline 407 facilitates identification of target organs and no-observed-adverse-effect levels (NOAEL) and lowest-observed-adverse-effect level (LOAEL) following repeated subacute exposure (17), an approach that has also been applied to characterize subacute toxicity in recent rodent models (18).

The present study was therefore performed to provide an *in vivo* toxicological evaluation of ethanol extract of *C. barbata* (EECB) in male and female Wistar rats, encompassing both acute (OECD 423) and subacute (OECD 407) toxicity assessments. By including biochemical, haematological, and organ histopathology endpoints, this study addresses the critical intersection between pharmacological activity and safety assessment, which is essential for the responsible advancement of *C. barbata* as a phytoestrogenic therapeutic candidate.

Methods

Plant Material and Extraction

Fresh *Cyclea barbata* Miers leaves were collected from Manna, South Bengkulu, Indonesia and identified at Laboratory of Forestry, Universitas Bengkulu, with a voucher specimen deposited at the Herbarium of Universitas Bengkulu (No. KSC002). Leaves were cleaned, dried at 30°C, and ground (369.28 g powder from ~437 g fresh leaves). The powder was macerated in 70% ethanol (1:10 w/v, 3×24 h), filtered, and concentrated by rotary evaporation (50°C) to yield 86.57 g extract (23.44%

w/w). EECB was stored at −18°C and freshly suspended in 0.5% CMC-Na before each administration. Qualitative phytochemical screening followed standard colorimetric and precipitation methods (19): alkaloids (Dragendorff's/ Mayer's reagents), flavonoids (Shinoda test), terpenoids (Liebermann–Burchard test), tannins (1% $FeCl_3$), saponins (foam stability test, ≥10 min), and anthraquinones (Bornträger's test). All tests were performed in triplicate. Standardization of EECB in this study was limited to qualitative phytochemical screening and tentative marker-compound identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS); quantitative determination of marker-compound content and chromatographic fingerprinting were not performed.

LC-MS/MS Analysis

Identification of the marker compound in EECB was based on LC-MS/MS characterization of the extract performed in a prior analysis by the authors' research group using the same extraction protocol, using a Phenomenex® HPLC column (C8, 5 µm, 150 × 2 mm i.d.) (Phenomenex, Torrance, CA, USA) in positive ion mode (ESI+). A full mass scan was conducted over an m/z range of up to 1200 at a source temperature of 140°C. Prior to injection, samples (0.5 g) were diluted in methanol and filtered through a 0.22 µm membrane filter. Nitrogen was used as the drying gas (350°C, flow rate 6 mL/min) and nebulizing gas (25 psi). Compound identification was based on molecular ion mass and fragmentation pattern in comparison with previously reported spectral data for coclaurine; as an authentic reference standard was not used for confirmation, this identification was regarded as tentative.

Experimental Animals

Healthy adult male and female Wistar rats (6–8 weeks old, 150–200 g) were obtained from PT Biofarma, Bandung, and acclimatized for 7 days under standard conditions (22±2°C, 50–60% humidity, 12-h light/dark, food and water *ad libitum*). Animals were housed 3 per cage and fed a standard pelleted rodent diet (Ratbio; PT Japfa Comfeed Indonesia Tbk, Jakarta, Indonesia). Rats were randomly allocated to treatment groups by simple randomization. The minimal sample size (n=10/sex/group) was determined in accordance with OECD Test Guideline recommendations.

The end-point criteria was when subjects' body weight loss exceeding 20% of initial body weight. Estrous cycle monitoring in female rats was not performed in this study, due to the potential influence of hormonal cycling on estrogen-related end-points. All animal treatments were performed following the ethically approved protocol and in accordance with institutional animal welfare protocols.

Acute Oral Toxicity Study

The acute toxicity study was adapted from OECD Test Guideline 423.(15) Rather than the sequential 3-animal stepwise design specified in the guideline, an expanded parallel design of 10 rats/sex/group was used to improve statistical power and enable detection of sex-specific responses, while GHS classification criteria were retained. Rats that have been fasted for 12 h received a single oral dose by gavage (≤10 mL/kg) of either 0.5% CMC-Na (Control group), 2,000 mg/kg BW EECB (EECB2000 group), or 5,000 mg/kg BW EECB (EECB5000 group) (n=10/sex/ group). Clinical signs were observed continuously for 1 h, at 2 and 4 h, then daily for 14 days; body weight was recorded on day-0, -1, -3, -7, and -14. On day-14, surviving rats were euthanized by CO_2, necropsied, and major organs weighed. GHS toxicity category was assigned per OECD 423 criteria.

Subacute Oral Toxicity Study

This subacute toxicity study was adapted from OECD Test Guideline 407.(16) A total of 120 rats (60 males, 60 females) were assigned to six groups (n=10/sex/group) that received either 0.5% CMC-Na (Control group) or EECB at 100, 200, 300, 400, and 500 mg/kg BW/day (EECB100, EECB200, EECB300, EECB400, and EECB500 groups, respectively), administered by daily gavage for 28 days (volume ≤10 mL/kg, recalculated weekly). Clinical signs and mortality were monitored daily; body weight was recorded weekly. Food consumption, urinalysis, differential leukocyte counts, neurological observations, and reproductive/endocrine endpoints specified in the full OECD 407 battery were not evaluated in this study.

Blood Collection and Laboratory Analyses

On day-29, overnight-fasted rats were anesthetized (ketamine 75 mg/kg + xylazine 10 mg/kg, IM), euthanized, and blood was collected by cardiac puncture into EDTA (haematology) and plain (biochemistry) tubes. Haematological parameters, including white blood cell (WBC), red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width–coefficient of variation (RDWc), and platelet (PLT), were measured using an automated analyzer (Mindray BC-2800; Shenzhen Mindray Bio-Medical Electronics, Shenzhen, China). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT),

fasting glucose, albumin, urea, and creatinine were measured by UV-Vis spectrophotometry (Shimadzu Vision Lite; Shimadzu Corporation, Kyoto, Japan) using commercial reagent kits.

Relative Organ Weight

Liver, heart, kidneys, and spleen were excised, trimmed, blotted, and weighed. Relative organ weight (%) was calculated as (absolute organ weight (g) / final body weight (g))×100. Macroscopic abnormalities in color, texture, size, or surface morphology were recorded.

Histopathological Examination

Liver, heart, kidney, and spleen samples were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, sectioned (4–5 µm), and stained with haematoxylin and eosin (HE). Slides were examined at 100× and 400× magnification by a pathologist and graded as normal, minimal (+1), mild (+2), moderate (+3), or severe (+4).

Statistical Analysis

Data (mean±SD) were tested for normality (Shapiro–Wilk) and variance homogeneity (Levene's test). Parametric data were analyzed by one-way ANOVA with Dunnett post hoc test; non-parametric data by Kruskal–Wallis with Dunn's correction. A p-value<0.05 was considered significant. Analyses were performed in SPSS v25.0 (IBM Corportion, Armonk, NY, USA).

Results

Extraction Yield and Phytochemical Screening

Maceration of 369.28 g of dried *Cyclea barbata* leaf powder in 70% ethanol followed by concentration under reduced pressure yielded 86.57 g of a dark green viscous extract, corresponding to an extraction yield of 23.44% (w/w). Qualitative phytochemical screening of EECB revealed the presence of six classes of secondary metabolites (Table 1). Alkaloids, flavonoids, terpenoids, tannins, saponins, and anthraquinones were all detected. The presence of alkaloids was of particular pharmacological relevance, as prior LC-MS/MS characterization of this extract identified a peak consistent with coclaurine as the key marker compound, based on a molecular ion at m/z 286.1 $(M+H)^+$ and characteristic fragmentation ions at m/z 171.15, 149.02, and 82.54 (Figure 1); this identification was regarded as tentative in the absence of an authentic reference standard.

Acute Oral Toxicity

No mortality or signs of toxicity were observed in either male or female rats receiving the vehicle control throughout the 14-day observation period. All animals in the control group maintained normal activity, posture, respiration, salivation, coat appearance, and defecation pattern, with no mortality recorded (Table 2). At a dose of 2,000 mg/kg BW, no mortality was recorded in either sex. However, a mild reduction in spontaneous activity was observed in female rats during the first 24 hours post-administration, characterized by a more passive behavioral appearance. No abnormalities were detected in posture, respiration, salivation, coat condition, or fecal output. All animals in this group recovered fully and survived to day-14.

Body Weight Changes

Single oral administration of EECB up to 5,000 mg/kg BW did not significantly alter body weight gain in surviving female (Figure 2A) or male (Figure 2B) Wistar rats compared to control over the 14-day observation period (p>0.05). Two-way repeated measures ANOVA confirmed a significant main effect of time (p<0.0001), reflecting normal physiological growth, with no significant treatment effect (p>0.05) or time-by-treatment interaction (p>0.05).

Table 1. Qualitative phytochemical screening of EECB leaves.

Phytochemical Constituent	Test Method	Observation	Result
Alkaloids	Dragendorff & Mayer reagent	Orange/red precipitate formed	+
Flavonoids	Mg + HCl (Shinoda test)	Red-orange coloration observed	+
Terpenoids	Liebermann–Burchard test	Reddish-brown ring at interface	+
Tannins	$FeCl_3$ solution (1%)	Dark blue-green coloration observed	+
Saponins	Foam stability test	Stable foam persisted ≥10 min	+
Anthraquinones	Bornträger's test (KOH)	Pink-red coloration in alkaline layer	+

(+): present; (−): absent. All tests were performed in triplicate.

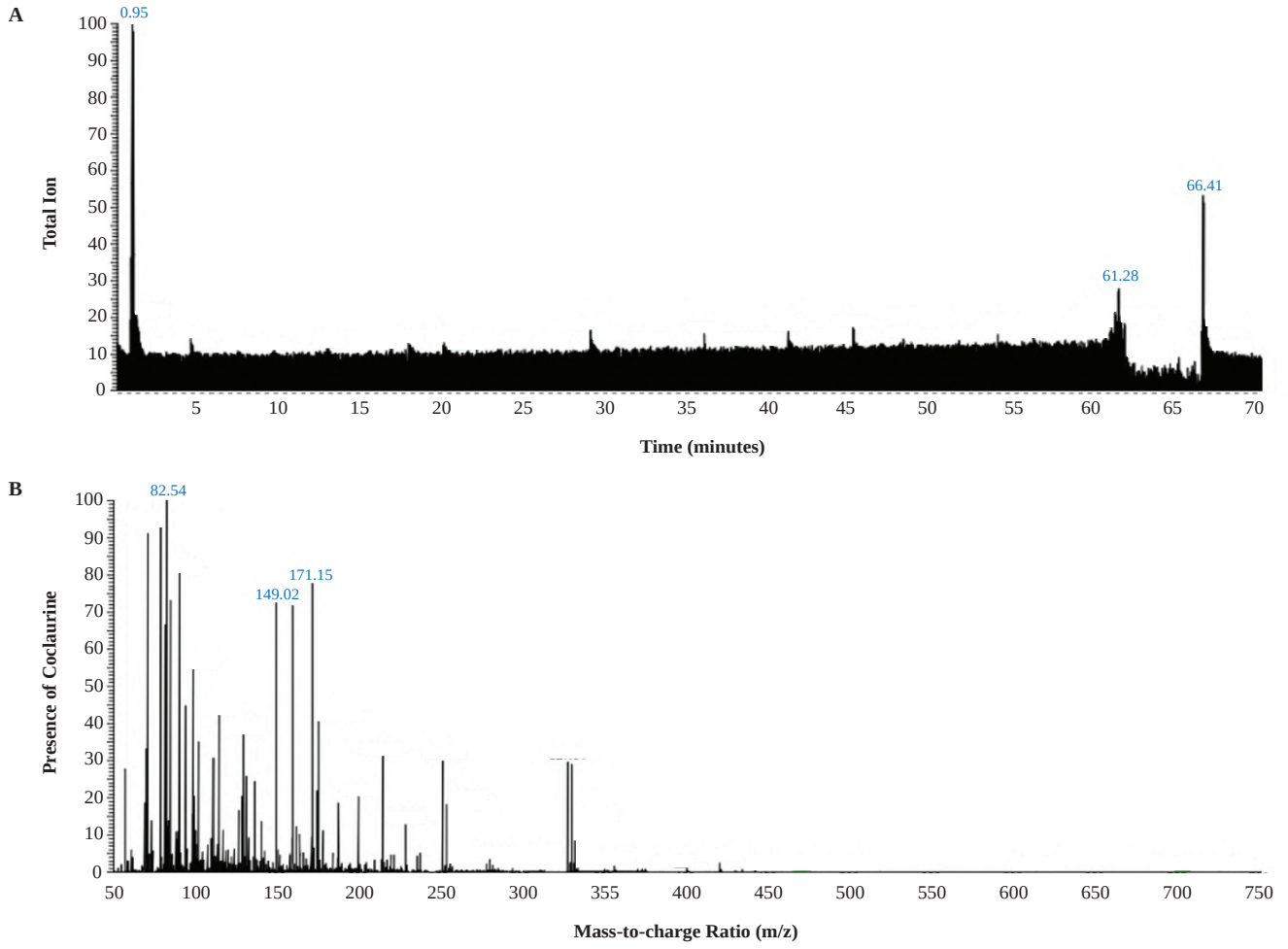

Figure 1. LC-MS/MS chromatographic profile of EECB leaves in positive ion mode (ESI+). A: Total Ion Chromatogram (TIC) showing the overall chemical complexity of EECB across a retention time of 0–70 minutes, with a dominant peak at RT 66.41 min. B: Mass spectrum at RT 0.01 min confirming the presence of coclaurine with base peak at m/z 82.54 and characteristic fragment ions at m/z 171.15, 149.02, and 79.02 (M+H).

Relative Organ Weights

The relative weights of the liver, kidneys, spleen, and heart following single oral administration of EECB are presented in Figure 3. In female rats (Figure 3A), a significant increase in the relative weights of the liver, kidneys, and spleen was observed in the 2,000 mg/kg BW group compared to the control ($p<0.05$), whereas the 5,000 mg/kg BW group showed no significant differences from the control ($p>0.05$); relative heart weight remained unchanged across all groups ($p>0.05$). In male rats (Figure 3B), EECB administration up

Table 2. Acute oral toxicity observations of EECB in male and female Wistar rats following single oral administration.

Group	Sex	D/T	Latency	Toxic Symptoms
Control	Male	0/10	—	None
	Female	0/10	—	None
EECB2000	Male	0/10	—	None
	Female	0/10	—	Mild reduction in spontaneous activity within 24 h; full recovery
EECB5000	Male	0/10	—	Reduced spontaneous activity within 24 h; full recovery by day-7
	Female	2/10	day-3, day-5	Reduced spontaneous activity; mortality on day-3 and -5

D/T: number of dead animals/number of animals treated. (-): No mortality recorded. All surviving animals completed a 14-day observation.

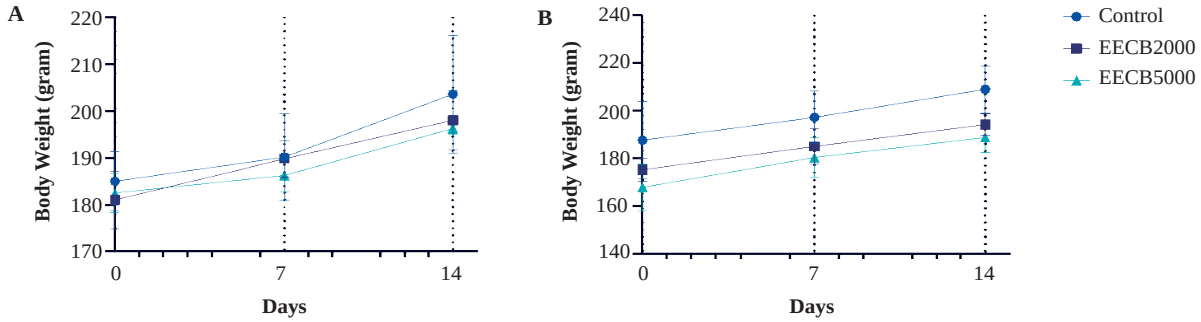

Figure 2. Body weight changes in female(A) and male (B) Wistar rats during 14 days of acute oral toxicity study of EECB. Data are expressed as mean±SD (n=10 per sex per group). No statistically significant differences were observed between treatment groups across time points, nor was there a significant group-by-time interaction effect (two-way repeated measures ANOVA, $p>0.05$).

to 5,000 mg/kg BW produced no significant differences in any evaluated organ weights compared to the control group ($p>0.05$).

At a dose of 5,000 mg/kg BW, reduced spontaneous activity was observed in both male and female rats within the first 24 hours, with female rats appearing notably more passive. Mortality was recorded exclusively in female rats: one animal died on day-3 and one on day-5 post-administration, yielding a mortality rate of 20% (2/10) in females at this dose level. No mortality was observed in male rats at 5,000 mg/kg BW. All surviving animals completed the 14 days observation period without further adverse events. Based on these findings, the LD_{50} of EECB administered orally could not be precisely determined, as mortality was observed only at 5,000 mg/kg BW and exclusively in female rats. Based on the absence of mortality at 2,000 mg/kg BW in either sex, EECB would be provisionally consistent with GHS Category 5 or unclassified (LD_{50} >2,000 mg/kg BW); however, as the parallel-group design used in this study departs from the sequential dosing procedure specified by OECD TG 423 for formal GHS classification, this categorization should be regarded as provisional rather than definitive. The NOAEL for acute oral toxicity was established at 2,000 mg/kg BW for male rats and could not be definitively established for female rats at the doses tested, given the mortality observed at the next tested dose level.

Subacute Oral Toxicity

Body Weight Changes

Changes in body weight in male and female rats during the 28 days of treatment period were illustrated in Figure 4. In female rats, no significant differences in body weight were observed between the control group and any of the EECB-treated groups at any time point ($p>0.05$). Similarly, in male rats, no significant differences in body weight were detected between the control and treatment groups throughout the 28 days period ($p>0.05$). No lethality was observed in any group during the course of the 28 days subacute treatment at doses up to 500 mg/kg BW.

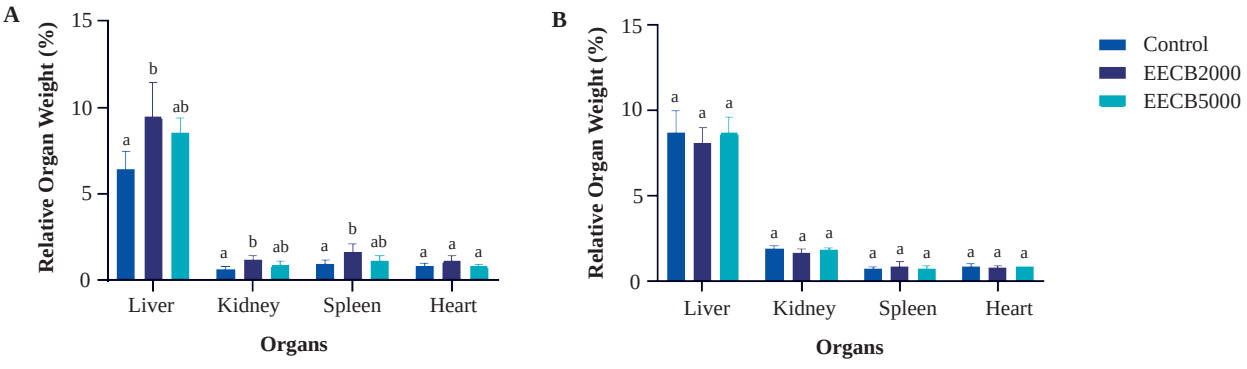

Figure 3. Relative organ weights of female (A) and male (B) Wistar rats following 14 days of acute oral administration of EECB. Data are expressed as mean±SD (n=10 per sex per group). Different letters above bars within the same organ denote statistically significant differences between groups (One-way ANOVA with Dunnett post hoc test, $p<0.05$).

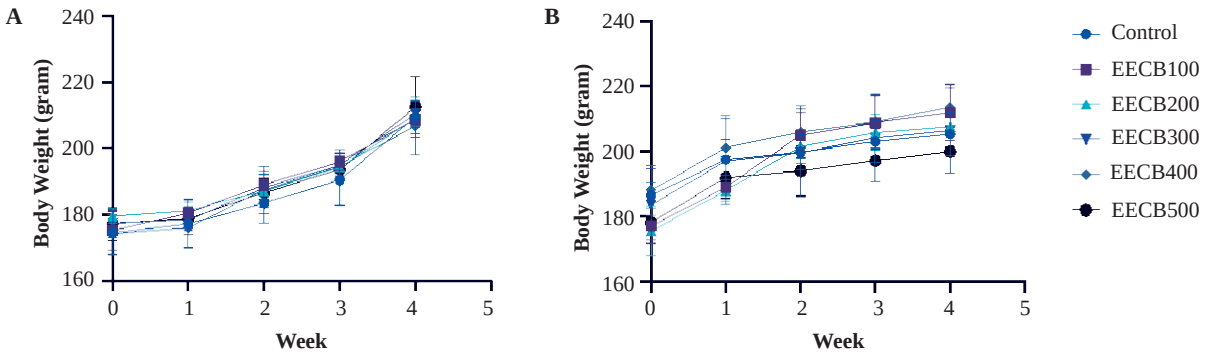

Figure 4. Body weight changes in female (A) and male (B) Wistar rats during 28 days of subacute oral administration of EECB. Data are expressed as mean±SD (n=10 per sex per group). No statistically significant differences were observed between any treatment group and the control at any time point (two-way ANOVA, $p>0.05$).

Relative Organ Weights

Relative weights of the liver, kidney, spleen, and heart in female and male rats following 28 days of EECB administration were presented in Figure 3. In female rats, no significant differences in relative organ weights were observed between the control group and any treatment group for all organs evaluated (liver: $p=0.140$; kidney: $p=0.545$; spleen: $p=0.818$; heart: $p=0.816$). In male rats, a statistically significant difference in relative liver weight was detected across groups ($p=0.021$); however, post hoc analysis revealed that this difference was attributable to a borderline significant difference between EECB400 and EECB 500 groups ($p=0.050$), with no individual treatment group differing significantly from the control (all $p>0.05$). Relative weights of the kidney, spleen, and heart in male rats showed no significant differences across all groups (kidney: $p=0.502$; spleen: $p=0.662$; heart: $p=0.407$).

Hematological Parameters

The effect of subacute oral administration of EECB on hematological parameters was summarized in Table 3. In female rats, a statistically significant reduction in WBC count was observed in the EECB400 group compared with the control (−42.8%, $p<0.05$). No significant reduction in WBC was observed in the EECB500 group in female rats ($p=0.051$). In male rats, significant reductions in WBC count were observed in both the EECB400 and EECB500 groups compared with the control (−47.4%, $p<0.05$ for both). No significant differences were observed in RBC, hemoglobin, hematocrit, MCV, MCH, MCHC, RDWc, or platelet count in either sex across all dose levels ($p>0.05$).

Serum Biochemical Parameters

The effect of subacute oral administration of EECB on serum biochemical parameters was summarized in Table 4. In female rats, post hoc analysis revealed a statistically significant difference in AST levels between the EECB200 and EECB400 groups ($p=0.036$), with EECB200 group showing higher AST values relative to EECB400. No treatment group differed significantly from the control for AST in female rats (all $p>0.05$). A similar pattern was observed in male rats, where AST levels in the EECB200

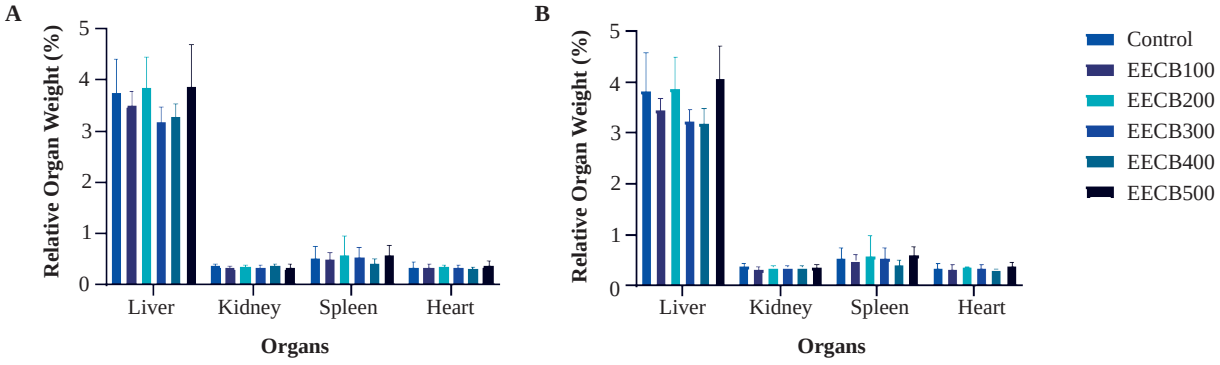

Figure 5. Relative organ weights of female (A) and male (B) Wistar rats following 28 days of subacute oral administration of EECB. Data are expressed as mean±SD (n=10 per sex per group). No significant differences in relative organ weights were observed between any treatment group and the control (one-way ANOVA followed by Dunnett post hoc test, $p>0.05$).

Table 3. The effect of subacute oral administration of the EECB on hematological parameters in rats.

Parameters	Control	EECB100	EECB200	EECB300	EECB400	EECB500
Female						
WBC (10^3/µL)	14.02±3.61^{b}	10.70±2.63^{ab}	9.37±4.40^{ab}	10.45±3.21^{ab}	8.02±1.84^{a}	8.58±2.15^{ab}
RBC (/µL)	8.03±0.59^{a}	7.75±0.64^{a}	6.73±0.67^{a}	7.35±1.53^{a}	8.03±0.57^{a}	7.34±1.25^{a}
HB (g/dL)	15.48±0.90^{a}	14.32±1.85^{a}	13.58±1.36^{a}	13.83±2.75^{a}	15.08±1.31^{a}	14.25±2.31^{a}
HCT (%)	46.87±2.59^{a}	43.47±4.15^{a}	39.30±3.90^{a}	41.95±7.46^{a}	44.73±3.35^{a}	42.47±6.46^{a}
MCV (fl)	58.53±2.35^{a}	56.12±2.37^{a}	58.52±2.09^{a}	57.48±2.43^{a}	55.82±1.74^{a}	55.03±1.59^{a}
MCH (pg)	19.27±1.03^{ab}	18.35±1.16^{a}	21.70±3.37^{b}	18.80±0.86^{ab}	18.73±0.86^{ab}	19.17±2.21^{ab}
MCHC (g/dL)	33.00±0.79^{a}	32.82±1.30^{a}	34.67±3.74^{a}	32.85±1.17^{a}	33.67±0.75^{a}	33.47±0.75^{a}
RDWc (%)	14.93±1.38^{a}	14.53±0.83^{a}	15.37±1.96^{a}	14.75±1.70^{a}	13.28±0.44^{a}	14.10±2.06^{a}
PLT (×10^3/µL)	570.67±209.46^{a}	690.67±143.35^{a}	490.00±230.15^{a}	608.67±97.02^{a}	527.83±152.68^{a}	422.00±149.26^{a}
Male						
WBC (10^3/µL)	15.18±6.99^{b}	9.33±2.56^{ab}	10.17±4.15^{ab}	9.78±3.07^{ab}	7.98±2.89^{a}	7.98±1.91^{a}
RBC (/µL)	6.95±0.49^{a}	7.62±0.46^{a}	6.43±0.40^{a}	7.03±1.39^{a}	7.23±1.07^{a}	6.67±1.71^{a}
HB (g/dL)	14.67±1.17^{a}	15.47±1.61^{a}	13.07±1.06^{a}	13.68±2.58^{a}	14.58±1.18^{a}	13.18±3.02^{a}
HCT (%)	44.70±5.48^{a}	46.73±3.99^{a}	39.13±2.69^{a}	42.53±7.91^{a}	44.53±3.46^{a}	39.50±8.99^{a}
MCV (fl)	64.32±5.69^{a}	56.57±13.22^{a}	60.93±3.32^{a}	60.75±4.50^{a}	62.35±6.82^{a}	59.73±4.60^{a}
MCH (pg)	21.07±0.77^{a}	20.15±2.37^{a}	20.27±1.05^{a}	19.47±1.52^{a}	20.32±1.87^{a}	19.88±1.63^{a}
MCHC (g/dL)	33.05±3.94^{a}	33.00±0.84^{a}	33.32±0.85^{a}	32.12±0.45^{a}	32.72±0.64^{a}	33.32±0.47^{a}
RDWc (%)	15.93±2.52^{a}	15.25±0.96^{a}	16.20±1.12^{a}	15.72±1.82^{a}	15.67±1.61^{a}	14.65±1.21^{a}
PLT (×10^3/µL)	696.33±204.82^{a}	576.67±186.13^{a}	390.33±162.72^{a}	538.17±45.20^{a}	672.33±290.49^{a}	406.67±133.49^{a}

Data are presented as mean±SD (n=10 per sex per group). Different annotation indicates significant difference $p<0.05$.

group were significantly higher than those in the EECB400 group ($p=0.036$), while neither group differed significantly from the control (all $p>0.05$). No significant differences were observed between any EECB-treated group and the control for ALT, glucose, albumin, urea, or creatinine in either sex (all $p>0.05$).

Table 4. The effect of sub-acute oral administration of the EECB on biochemical serum parameters in rats.

Parameters	Control	EECB100	EECB200	EECB300	EECB400	EECB500
Female						
AST (U/L)	77.91±7.32^{ab}	76.09±9.88^{ab}	84.19±7.28^{b}	71.36±15.16^{ab}	65.10±11.68^{a}	70.67±8.79^{ab}
ALT (U/L)	53.70±14.87^{a}	43.15±11.70^{a}	57.93±18.37^{a}	48.28±13.87^{a}	45.00±11.65^{a}	50.76±8.96^{a}
Glucose (mg/dL)	94.94±25.23^{a}	114.66±20.86^{a}	102.57±16.71^{a}	107.29±14.80^{a}	127.75±23.66^{a}	116.62±39.55^{a}
Albumin (g/dL)	5.29±0.52^{a}	4.59±0.60^{a}	4.44±0.47^{a}	4.53±1.59^{a}	4.65±0.73^{a}	4.28±1.58^{a}
Urea (mg/dL)	56.17±6.59^{a}	55.67±5.82^{a}	56.83±7.28^{a}	65.33±6.71^{a}	59.17±8.59^{a}	60.17±3.82^{a}
Creatinine (mg/dL)	1.76±0.59^{a}	1.36±0.20^{a}	1.75±0.54^{a}	1.49±0.44^{a}	1.71±0.39^{a}	1.75±0.77^{a}
Male						
AST (U/L)	69.92±6.59^{ab}	68.28±8.89^{ab}	75.57±6.56^{b}	64.03±13.65^{ab}	58.39±10.52^{a}	63.40±7.91^{ab}
ALT (U/L)	48.13±13.38^{a}	38.63±10.53^{a}	51.94±16.53^{a}	43.25±12.48^{a}	40.30±10.48^{a}	45.49±8.06^{a}
Glucose (mg/dL)	85.25±22.71^{a}	102.99±18.78^{a}	92.12±15.03^{a}	96.36±13.32^{a}	114.77±21.29^{a}	104.76±35.59^{a}
Albumin (g/dL)	4.56±0.47^{a}	3.93±0.54^{a}	3.80±0.42^{a}	3.87±1.43^{a}	3.99±0.65^{a}	3.65±1.42^{a}
Urea (mg/dL)	50.35±5.93^{a}	49.90±5.24^{a}	50.95±6.55^{a}	58.60±6.04^{a}	53.05±7.73^{a}	53.95±3.43^{a}
Creatinine (mg/dL)	1.39±0.53^{a}	1.03±0.18^{a}	1.38±0.49^{a}	1.14±0.40^{a}	1.34±0.35^{a}	1.38±0.69^{a}

Data are presented as mean±SD (n=10 per sex per group). Different annotation indicates significant difference $p<0.05$.

Histopathological Findings

The results of gross necropsy performed on day-29 showed that there were no macroscopic abnormalities in color, texture, size, or surface morphology of the liver (Figure 6), kidney (Figure 7), spleen (Figure 8), or heart (Figure 9) in either sex across all treatment groups. Histopathological examination of these organs confirmed the absence of degenerative, necrotic, inflammatory, or hyperplastic lesions at any dose level in both male and female rats. Cellular architecture of the hepatic lobules, renal cortex and medulla, splenic white and red pulp, and cardiac myofibers remained intact and comparable to the vehicle control group across all EECB-treated groups. These histopathological findings were consistent with the absence of significant alterations in hepatic and renal function markers across all treatment groups.

Discussion

The present study provides the first *in vivo* toxicological evaluation of EECB in Wistar rats following acute and subacute oral administration in accordance with OECD Guidelines 423 and 407, respectively. The findings

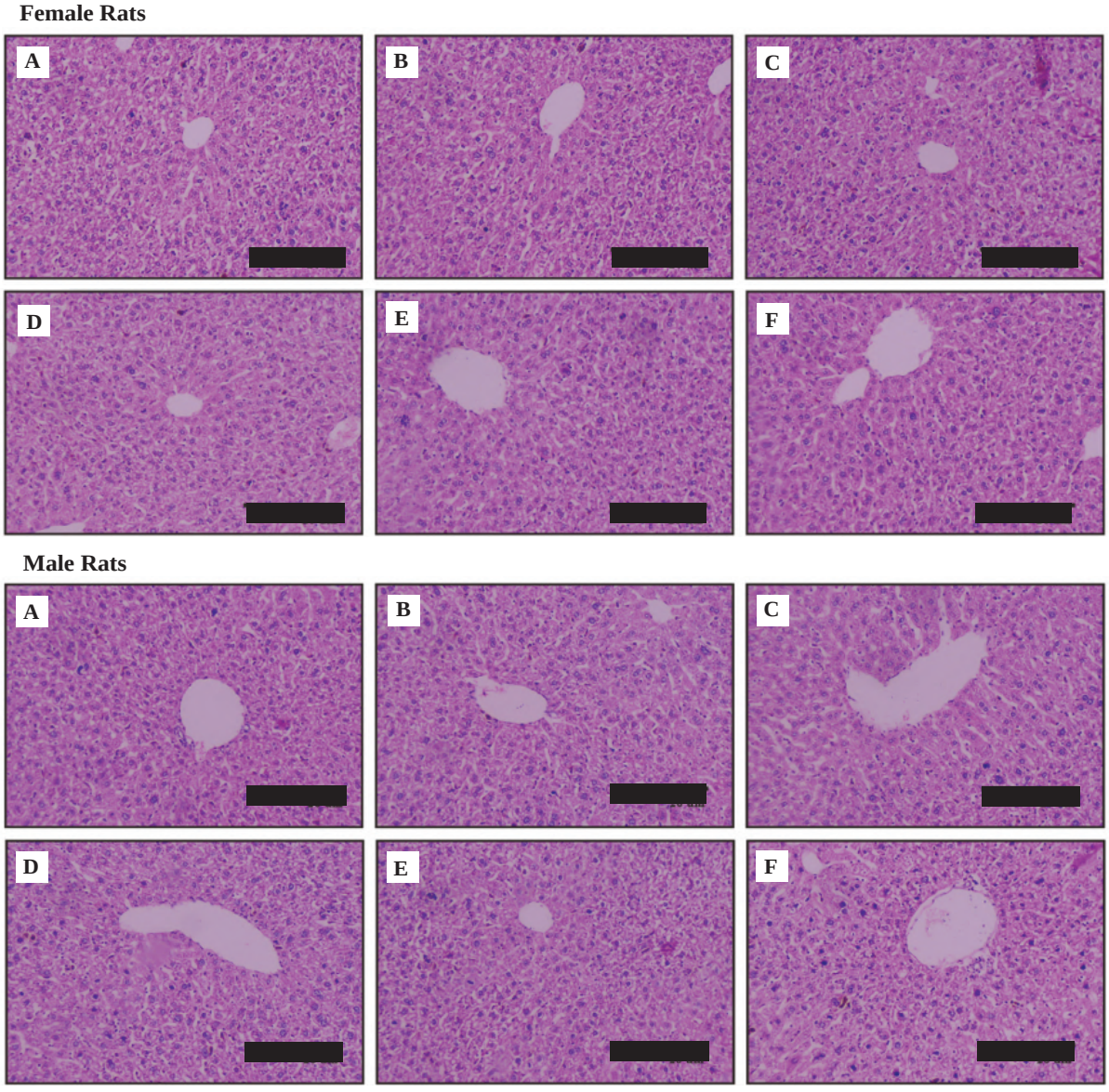

Figure 6. Histological examination result of the liver in female and male Wistar rats following 28 days of subacute oral administration of EECB. A: Control; B: EECB100; C: EECB200; D: EECB300; E: EECB400; F: EECB500. Black bar: 20 µm. No histopathological abnormalities were observed in any treatment group.

collectively indicate that EECB exhibits low acute oral toxicity and does not induce overt adverse effects on body weight, organ weights, serum biochemical parameters, or histopathological architecture of vital organs following 28 days of repeated oral administration at doses up to 500 mg/kg BW. The estrogenic potential of EECB is attributable to its marker compound, coclaurine, which has been reported to bind the ERα ligand-binding domain at the same site as 17β-estradiol (docking scores −8.3 and −10.9 kcal/mol, respectively), sharing key interacting residues (ARG394, GLU353, LEU387, LEU525, MET388, and PHE404).(9) An *in silico* ADMET profiling of coclaurine has further indicated compliance with Lipinski's rule of five, low predicted human intestinal absorption, negative blood–brain barrier penetration, and an inactive hERG blocker classification (20), supporting a plausible oral bioavailability and low predicted cardiotoxic liability. These pharmacological properties underscore the rationale for the present toxicological evaluation, which was designed to empirically establish the *in vivo* safety profile of EECB rather than to characterize its receptor-binding pharmacology.

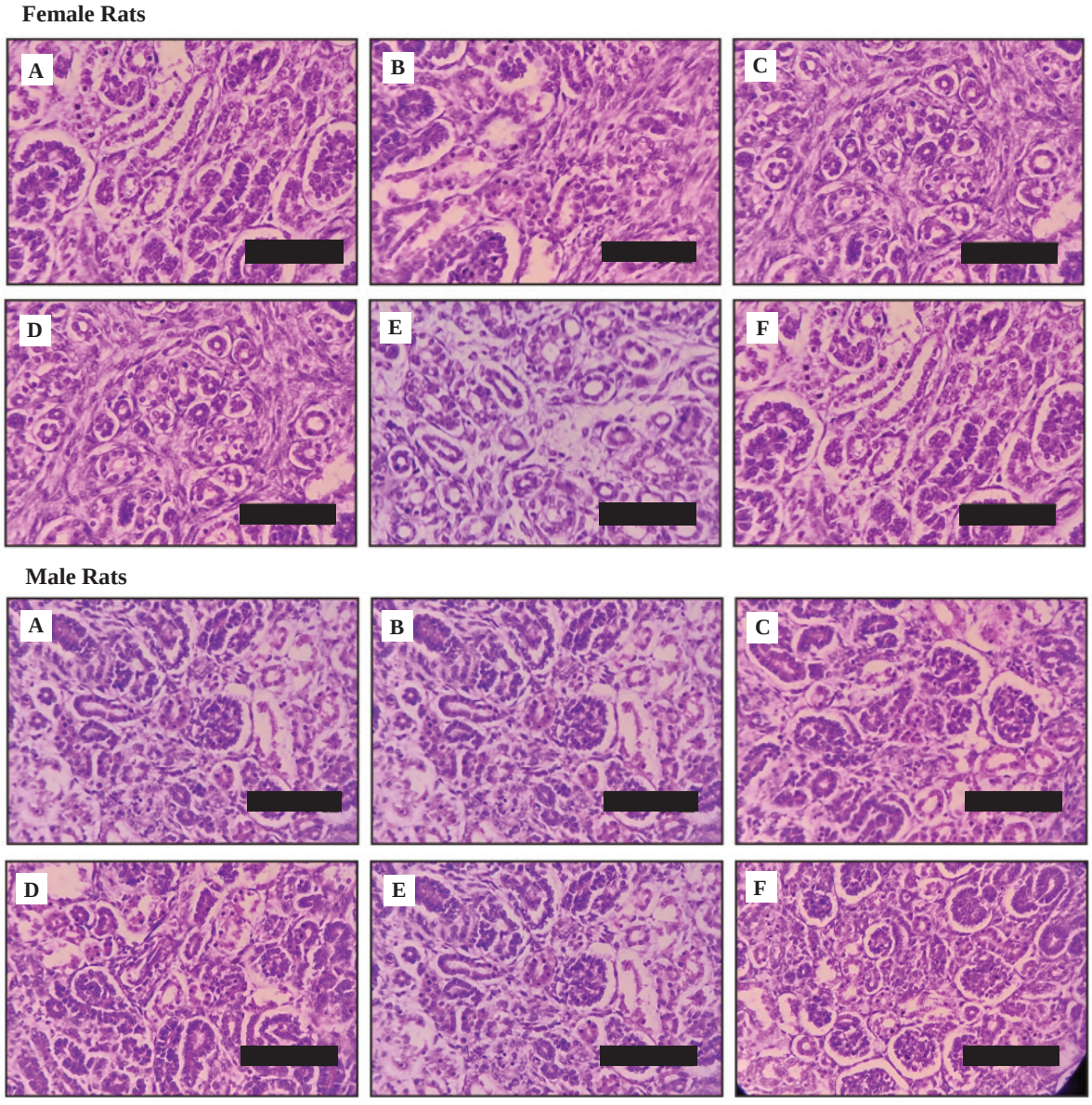

Figure 7. Histological examination result of the kidney in female and male Wistar rats following 28 days of subacute oral administration of EECB. A: Control; B: EECB100; C: EECB200; D: EECB300; E: EECB400; F: EECB500. Black bar: 20 µm. No histopathological abnormalities were observed in any treatment group.

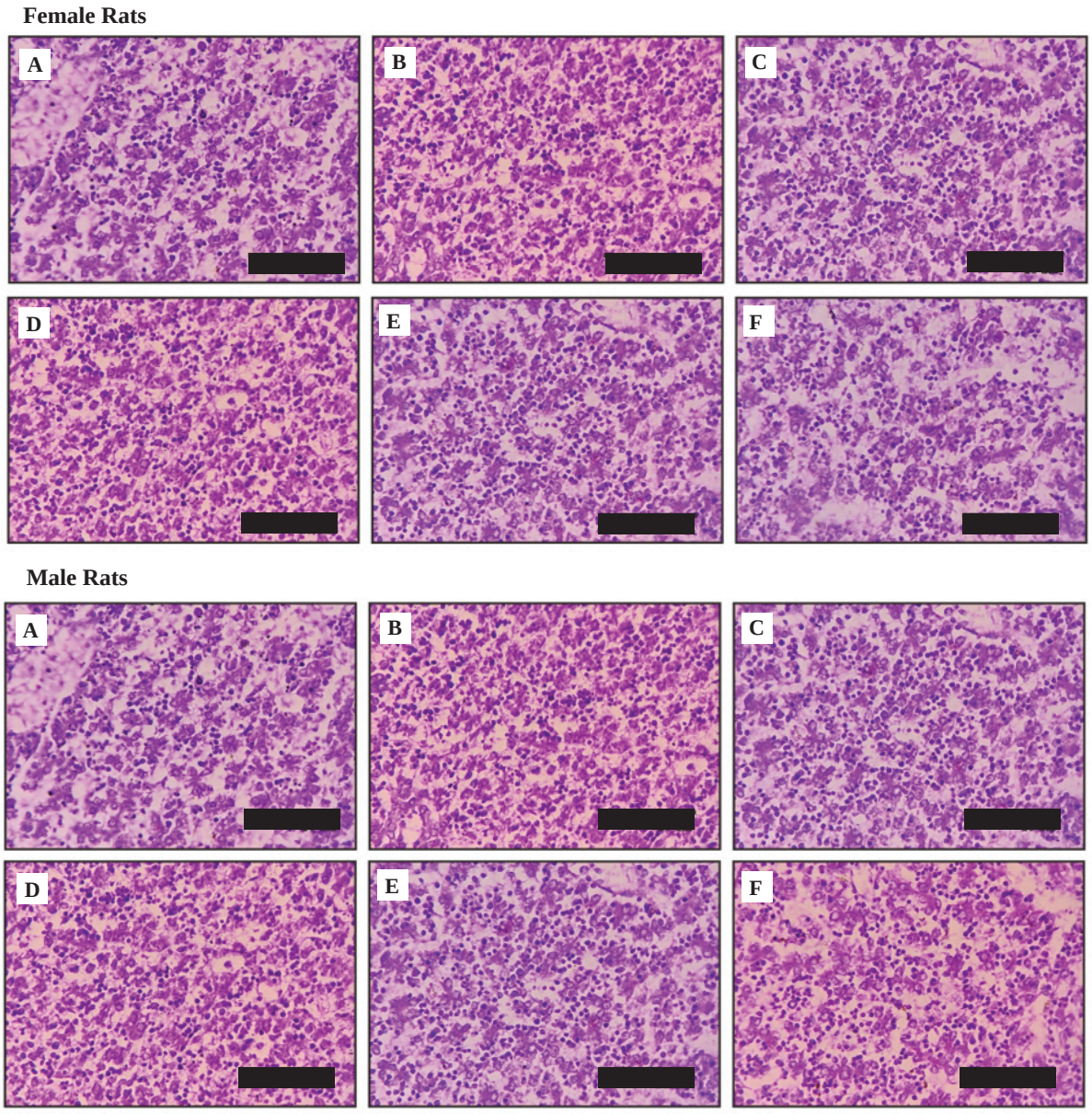

Figure 8. Histological examination result of the spleen in female and male Wistar rats following 28 days of subacute oral administration of EECB. A: Control; B: EECB100; C: EECB200; D: EECB300; E: EECB400; F: EECB500. Black bar: 20 µm. No histopathological abnormalities were observed in any treatment group.

The qualitative phytochemical screening of EECB confirmed the presence of alkaloids, flavonoids, terpenoids, tannins, saponins, and anthraquinones, consistent with previous phytochemical reports on *C. barbata* leaves. (7,9) Some particular pharmacological relevance is the identification of coclaurine as the key alkaloid marker, a benzylisoquinoline alkaloid with reported binding affinity to estrogen receptor alpha (ERα) at −8.3 kcal/mol, which underlies the reported estrogenic activity of EECB.(12,20) The exact cause of the mortality observed exclusively in females at the limit dose of 5,000 mg/kg BW remains undetermined, as toxicokinetic profiling and target-organ histopathology at this lethal dose level were not performed. Sex-dependent variations in acute susceptibility are well-documented in rodent safety studies; however, any proposed pharmacokinetic or metabolic explanation for this finding remains speculative in absence of direct biological evidence.

No significant changes in body weight or feed consumption were observed in either sex throughout the 28-day subacute treatment period, indicating an absence of severe systemic toxicity or overt metabolic disturbance. (10) Similar findings have been reported in subacute

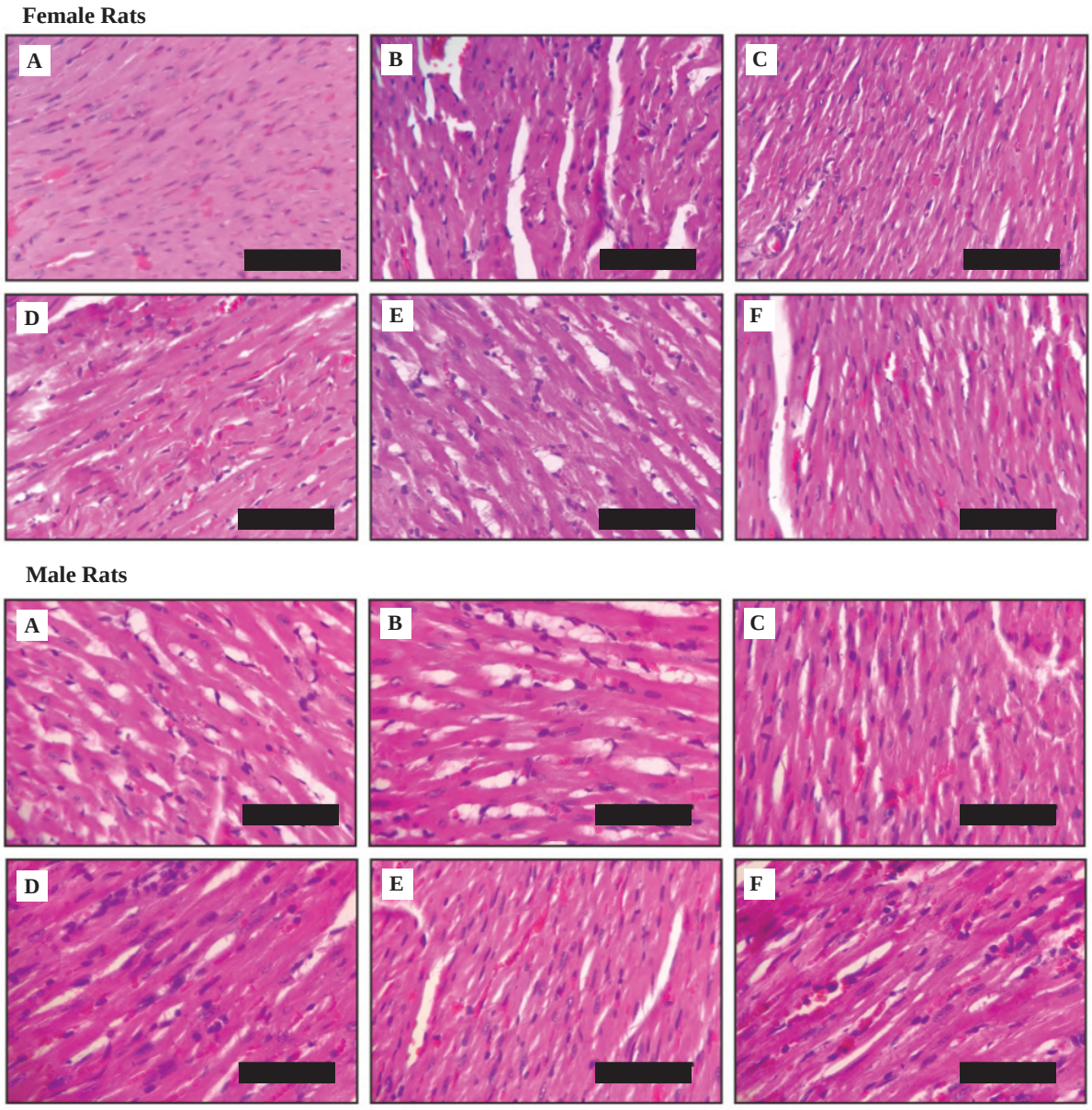

Figure 9. Histological examination result of the heart in female and male Wistar rats following 28 days of subacute oral administration of EECB. A: Control; B: EECB100; C: EECB200; D: EECB300; E: EECB400; F: EECB500. Black bar: 20 µm. No histopathological abnormalities were observed in any treatment group.

toxicity studies of other plant extracts, including *Ferula communis* aqueous extract in mice (21) and *Erodium guttatum* in rodents (5), as well as ethanol extract of Syzygium polyanthum in Wistar rats (22), where repeated oral administration at comparable dose ranges did not significantly alter body weight. Relative organ weight evaluations revealed no significant alterations in female rats across all groups. In male rats, although a statistically significant difference in relative liver weight was detected across groups (p=0.036), no individual treatment group differed significantly from the control group. In the absence of corresponding histopathological alterations or elevations in hepatocellular enzymes (AST/ALT), this minor inter-group variation is unlikely to represent a toxicologically meaningful lesion, as changes in organ weight are generally considered adverse only when accompanied by structural or functional impairment.(23) This underscores that hepatic responses to botanical extracts require integrated histological and biochemical evaluation rather than reliance on organ weight alone.

The most notable finding in the 28 days study was a statistically significant reduction in total WBC count in

female rats at 400 mg/kg BW/day (–42.8%, $p<0.05$) and in male rats at 400 and 500 mg/kg BW/day (–47.4%, $p<0.05$). Notably, the WBC reduction in females at 500 mg/kg BW/day did not achieve statistical significance ($p=0.051$), reflecting a non-linear dose-response pattern in females. All other erythroid parameters (RBC, hemoglobin, hematocrit, MCV, MCH, MCHC, RDWc) and platelet counts remained within normal physiological ranges across all groups ($p>0.05$). However, normal erythroid and platelet parameters alone do not fully exclude selective leukocyte toxicity or lineage-specific bone marrow suppression. Furthermore, because differential leukocyte counts, bone marrow cytology, and functional immunological assays were not performed, the precise cellular mechanism underlying this leukopenic effect remains unknown. Comparative subacute toxicity studies on other medicinal plant extracts rich in secondary metabolites such as *Salvia przewalskii* (15) and *Peronema canescens* (18) have similarly reported transient reductions in circulating leukocytes following repeated exposure under OECD 407 protocols, highlighting the hematopoietic system as a sensitive indicator for botanical safety assessments.

Serum biochemical evaluation revealed no treatment-related hepatotoxicity or nephrotoxicity. Parameters including ALT, AST, glucose, albumin, urea, and creatinine showed no significant differences compared to the vehicle control ($p>0.05$). The isolated statistical difference in AST observed between the EECB200 and EECB400 groups ($p=0.036$) lacks toxicological relevance, as neither group differed significantly from the control, ALT levels remained baseline, and concurrent elevation of both transaminases is required to diagnose hepatocellular injury.(24,25) Histopathological examination of the liver, kidneys, spleen, and heart confirmed intact cellular architecture without degenerative, inflammatory, or necrotic changes in any treatment group.

Based on the integrated evaluation of all safety endpoints, a provisional NOAEL for repeated oral administration of EECB is set at 300 mg/kg BW/day, with a provisional LOAEL at 400 mg/kg BW/day, based on the statistically significant decrease in circulating WBC count. These values must be regarded as provisional until further studies incorporating differential leukocyte counts, historical control comparison, and a post-treatment recovery period can verify the reversibility and lineage specificity of the observed leukopenia. Given the identified estrogenic potential of EECB and its marker compound coclaurine, subsequent stages of this research roadmap must incorporate comprehensive reproductive toxicity, endocrine disruption, and extended 90-day subacute toxicity evaluations (OECD TG 408) to establish a definitive human safety profile prior to clinical development.

Conclusion

Based on the assessed parameters, a provisional acute NOAEL is set at 2,000 mg/kg BW for male rats, while the provisional subacute NOAEL and LOAEL are established at 300 and 400 mg/kg BW/day, respectively. These preliminary findings delineate the basic safety limits of EECB under repeated moderate-dose exposure, suggesting *in vivo* evidence supporting its further development as a phytoestrogenic agent.

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Authors Contribution

N was involved in the conceptualization, study design, and protocol development. RN and IA performed experimental execution, data collection and processing. EK was involved in the animal study supervision, as well as clinical and laboratory data interpretation. N and JNP performed the formal analysis and wrote the original draft of the manuscript. N was involved in the funding acquisition. All authors were involved with the manuscript reviewing and editing, and had approved the final version of the manuscript.

Ethical Statement

The protocol of this study was approved by the Institutional Animal Ethics Committee of Universitas Jenderal Achmad Yani (Ethical Clearance No. 136/KEPK/FITKes-Unjani/II/2026).

Conflict of Interest

The authors declare no conflicts of interest.

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