Research Article

Chronic Toxicity Test of Secang Wood (*Caesalpinia sappan* L.) Extract on Heart Structure And Lipid Profile Of Male And Female Rats (*Rattus norvegicus*)

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Abstract | Chronic toxicity testing is a test to detect toxic effects that appear after repeated administration of the test preparation throughout the animal's life. Secang wood (*Caesalpinia sappan* L.) extract is widely recognized for its various potential health benefits, including its antioxidant, antibacterial, anticancer, and anti-inflammatory properties, and thus can be considered an herbal medicine. This study aimed to comprehensively assess the safety of secang wood extract as a herbal medicine through a chronic toxicity test, specifically focusing on cardiac structure and lipid profile. This study used laboratory experimental methods with rats (*Rattus norvegicus)*. This study employed a 6 × 2 factorial Completely Randomized Design (CRD), consisting of six doses of *Caesalpinia sappan* L. extract (0, 100, 200, 300, 400, and 600 mg/kg BW) and two sexes (male and female rats), resulting in 12 experimental groups, each group consisted of 5 rats (total 60). Research parameters included the number of necrosis and apoptosis in myocardial cells, measurement of fibrotic area in cardiac tissues, and examination of the lipid profile (total cholesterol, LDL cholesterol, and triglycerides). Data distribution normality was assessed, and if normal, analyzed using Two-Way ANOVA with a 95% confidence interval; significant differences were further analyzed by Duncan's test. The study results indicate that secang wood extract, administered within the 100 to 600 mg/kg BW dosage range, is safe for both cardiac structure and lipid profile in male and female rats.

Keywords | Histopathology, Heart, Sappan wood (*Caesalpinia sappan* L.), Lipid profile, Chronic toxicity, Antioxidant, Herbal medicine, Rats

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INTRODUCTION

Chronic toxicity testing is a crucial toxicological evaluation method that investigates the effects of long-term exposure to a substance on the survival, growth, reproduction, and organ function of test subjects (Li *et al*., 2017). This testing can be conducted through various primary routes of administration, such as oral, dermal, and inhalation. According to the established test protocol, the test substance is administered daily to the test animal groups for a minimum period of 12 months (Mhaske *et al*., 2023). Based on the OECD toxicity test protocol, the no-observed adverse effect level (NOAEL) is defined as the highest dose of a substance that does not cause adverse

effects leading to signs of toxicity in animal testing (Fitria *et al*., 2022).

Sappan wood is known to have significant benefits due to its active compounds, including flavonoids and brazilin. These compounds function as primary and secondary antioxidants, effectively neutralizing free radicals (Ramadhani, 2016). Sappan wood contains brazilin, a homoisoflavonoid compound responsible for its characteristic red pigment. Owing to its strong antioxidant properties, brazilin plays a critical role in protecting cells from oxidative damage (Hadi *et al*., 2023). Furthermore, secang wood extract has the potential to be used as an alternative iron chelator for thalassemia patients, where its administration has been proven to reduce iron levels in mice with iron overload (Maskoen *et al*., 2016).

The heart was chosen as one of the target organs in this study due to vulnerability to the toxic substances exposure. Damage to this vital organ can impair blood-pumping function, thereby disrupting the supply of oxygen and nutrients throughout the body (Kuncarli and Djunarko, 2014). Chronic toxicity testing also needs to be conducted on lipid profiles, as these levels can significantly affect heart function. Exposure to toxic substances can lead to lipid metabolism disorders, such as secondary dyslipidemia, which is characterized by increased triglyceride levels and small dense LDL concentrations (Ramadhani, 2016).

The pathological association between impaired lipid metabolism and cardiac damage is strongly supported by various studies in animal models. These models demonstrate that hyperlipidemia and increased expression of cardiac LDL receptors lead to myocardial lipid accumulation, fibrosis, diastolic dysfunction, arrhythmias, and preserved ejection fraction, even in the absence of other comorbidities such as hypertension or diabetes. In these models, histological analysis confirmed excessive myocardial lipid deposition and fibrosis, while functional studies showed evidence of diastolic dysfunction and arrhythmias (Williams *et al*., 2021, 2022). Mechanistically, oxidized LDL (ox-LDL) induces oxidative stress, mitochondrial dysfunction, and apoptosis in cardiomyocytes. These effects are associated with increased myocardial injury and dysfunction in both animal and cellular models (Li *et al*., 2022). Furthermore, chronic dyslipidemia and ox-LDL promote pro-inflammatory signaling, oxidative stress, and direct myocardial injury, supporting their pivotal role in the pathogenesis of fibrosis and cardiac dysfunction (Avagimyan *et al*., 2022; Vekic *et al*., 2023; Zhong *et al*., 2019).

This study, therefore, aimed to assess the chronic toxicity of sappan wood extract through evaluation of histopathological alterations in cardiac tissue (necrosis, apoptosis, and fibrosis) and changes in lipid profiles (total cholesterol, LDL cholesterol, and triglycerides) in male and female Rattus norvegicus. The novelty of this research lies in providing comprehensive evidence regarding the chronic cardiovascular toxicity and metabolic safety of sappan wood extract, which is crucial for its future development of sappan wood extract as a safe herbal medicine.

MATERIALS AND METHODS

The study employed an experimental method utilizing a Completely Randomized Design (CRD) with a 6×2 factorial arrangement. This design was modeled after a similar factorial approach conducted by Aswan and Nurmasari (2024) but modified to broaden the dosage scope. The first factor was the dose of *Caesalpinia sappan* L. extract, which consisted of one negative control group (distilled water) and five graded treatment groups (100, 200, 300, 400, and 600 mg/kg BW). The maximum dose of 600 mg/kg BW was selected based on results from a preliminary study, which was part of this research series (Safitri *et al*., 2017). In that preliminary study, doses up to 400 mg/kg BW did not show significant side effects. Therefore, the dose was increased to 600 mg/kg BW to expand the testing range, particularly to evaluate the safety profile and potential toxicity of the extract at a higher dose. The second factor, sex of the rats (*Rattus norvegicus*), was also incorporated into the design to identify potential variations in biological response between males and females. The combination of these two factors resulted in a total of 12 experimental groups, with five rats allocated to each group (total n=60). The test animals were randomly allocated to the groups using a random number generator. Furthermore, several parameters were observed, including the quantification of myocardial cell necrosis and apoptosis, measurement of fibrosis area in the tissue, and analysis of lipid profile levels, such as total cholesterol, LDL cholesterol, and triglycerides. This study was conducted in several laboratories, including the Biosystem Laboratory of the Biology Study Program, Eijkman Health Research Unit, Faculty of Medicine, Padjadjaran University, the Histology Laboratory of Hasan Sadikin Hospital, and the Bandung City Health Office Laboratory. All procedures were approved by the Research Ethics Committee of Padjadjaran University (No. 75/UN6.KEP/EC/2023) and were conducted according to animal welfare guidelines and the 3Rs principle (Replacement, Reduction, Refinement).

Test animals and treatment procedures

Healthy male and female *Rattus norvegicus* were acclimatized for 7 days before treatment. Acclimatization was conducted to allow rats to adapt in the laboratory environment. These rats were housed in a room with controlled temperature and a 12-hour light/dark cycle. The cages were plastic boxes

(40 × 30 × 15 cm) with iron grating covers and bedding made of rice husks, which was replaced daily. During acclimatization, rats were fed CP-551 feed and provided water *ad libitum*. BW was measured daily to determine the daily test substance volume, and grouped according to treatment categories (BPOM, 2014). Sapan wood extract was administered orally via gavage once daily for one year at doses of 100, 200, 300, 400, and 600 mg/kgBW. These doses were adjusted daily based on the individual body weight of each rat. Prior to treatment, the rats were subjected to a 16-hour fasting period. After each rat received its assigned dose of *Caesalpinia sappan* L. extract, a second 16-hour fast was implemented before the surgical procedure

Sample collection and laboratory analysis

After the treatment period, the heart was isolated and weighed to determine its absolute organ weight. The heart was then fixed in a 10% formalin solution for subsequent histopathological analysis. Blood samples (approximately 4 mL) were collected from the abdominal vein of the heart ventricle, centrifuged at 3000 rpm for 10 minutes to separate the serum, and stored at -20°C (BPOM, 2014). Lipid profile analysis was performed on the collected serum. Total cholesterol and LDL cholesterol were measured using the CHOD-PAP method with a Cobas C 311 Analyzer (Roche Diagnostics, 2016). Triglyceride levels were determined using the GPO-PAP method with a Sumifin C 1904 Semi Automatic Analyzer (DiaSys Diagnostic Systems, 2015).

Histopathological examination

Paraffin blocks of heart tissue were sectioned using a microtome into 5 µm thick slice. The sections were mounted on Meyer's albumin-coated slides and heated on a hotplate (40 °C) to remove residual paraffin. The sections were then stained with Hematoxylin-Eosin (H and E) to observe myocardial cell structure and with Trichrome to evaluate the area of fibrosis (Yulianti, 2017). Histological observation was performed microscopically. The percentage of necrosis and apoptosis in myocardial cells were quantified at 1000× magnification using a formula based on Dharmawan (2010). The area of tissue fibrosis was calculated using ImageJ software on 5 fields of view per group at 400× magnification before the data were statistically analyzed (Salam *et al*., 2016).

Statistical analysis

All data were analyzed using SPSS 25.0 for Windows (IBM Corp., Armonk, NY, USA). A Two-Way ANOVA was performed at a 95% confidence level (α=0.05). If a significant difference was detected, a Duncan's test was conducted as a post-hoc analysis. This data analysis procedure was based on the method used by Purnama *et al.* (2023) with some modifications.

RESULTS

Cardiac structure

Cardiac structure evaluation included measurements of relative heart weight and histological examination of myocardial tissue.

Relative organ weight (ROW) of the heart

Two-way analysis of variance (ANOVA) at a 95% confidence level indicated that varying doses of *Caesalpinia sappan* L. extract did not significantly affect the relative organ weight ($p>0.05$). Sex of the rats, however, showed a significant difference ($p<0.05$). Nevertheless, due to a significant interaction between dose and sex ($p<0.05$), the main effect of sex cannot be interpreted independently of the dose influence. The mean percentage data for the relative heart weight of male and female rats over one year of treatment, along with the results from Duncan's multiple range test, are presented in Table 1 and Figure 1.

Table 1: Mean percentage of relative heart weight in male and female rats.

Code	Treatment	Relative heart weight (%)	
		Male	Female
KN	Aquadest (control)	0.30 ± 0.07^{bc}	0.21 ± 0.05^{a}
P1	SWE 100 mg/kg BW	0.36 ± 0.06^{c}	0.22 ± 0.04^{a}
P2	SWE 200 mg/kg BW	0.25 ± 0.04^{ab}	0.23 ± 0.03^{ab}
P3	SWE 300 mg/kg BW	0.27 ± 0.03^{ab}	0.26 ± 0.04^{ab}
P4	SWE 400 mg/kg BW	0.25 ± 0.10^{ab}	0.24 ± 0.01^{a}
P5	SWE 600 mg/kg BW	0.28 ± 0.04^{ab}	0.26 ± 0.07^{ab}

Note: Data are presented as Mean ± Standard Deviation (SD). Letters above each bar indicate the results of the Duncan's post-hoc test. Different letters ($^{a, b, c}$) denote a statistically significant difference ($p<0.05$) between sexes and between treatments within the same sex.

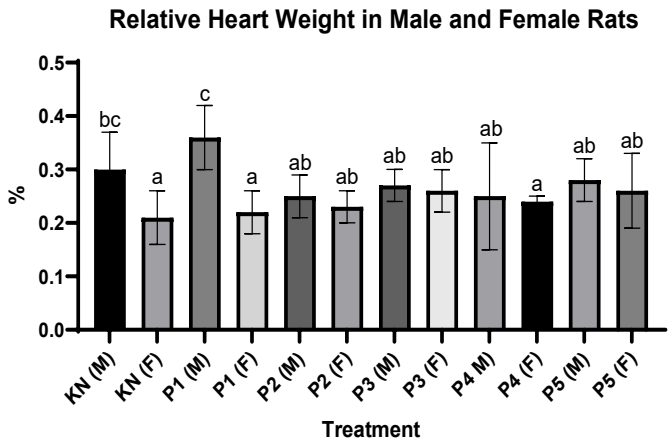

Figure 1: Cardiac relative organ weight (ROW) in male (M) and female (F) rats by treatment group.

Duncan's post-hoc test results showed that among male rats,

administration of the 100 mg/kg BW extract dose resulted in a significant difference compared to several other groups. In contrast, female rats exhibited no significant differences among the treatment groups. Consequently, the effect of *Caesalpinia sappan* L. extract on the relative heart weight was more pronounced in male rats.

Myocardial cell necrosis

Based on Two-way analysis of variance (ANOVA) at a 95% confidence level, neither the treatment factor (extract dose) nor the sex of the rats had a significant effect on the myocardial necrosis score ($p>0.05$). Furthermore, no significant interaction was observed between treatment and sex ($p>0.05$). Table 2 and Figure 2 displays the mean percentage of myocardial cell necrosis in male and female rats following one year of *Caesalpinia sappan* L. extract administration.

Table 2: Mean percentage of myocardial necrosis in male and female rats.

Code	Treatment	Necrosis (%)	
		Male	Female
KN	Aquadest (control)	21.6±1.0	20.2±1.1
P1	SWE100 mg/kg BW	21.2±5.3	19.6±2.0
P2	SWE 200 mg/kg BW	20.1±1.9	19.6±1.0
P3	SWE 300 mg/kg BW	22.6±2.5	21.9±1.7
P4	SWE 400 mg/kg BW	19.5±3.0	20.0±1.6
P5	SWE 600 mg/kg BW	20.5±2.1	20.2±2.1

Note: SWE (Sappan Wood Extract).

The data presented in Table 2 and Figure 2 demonstrate that the mean myocardial necrosis scores in both male and female groups were relatively similar across all treatments. Thus, the administration of *Caesalpinia sappan* L. extract within the dose range of 100 to 600 mg/kg BW did not induce an increase in myocardial cell necrosis.

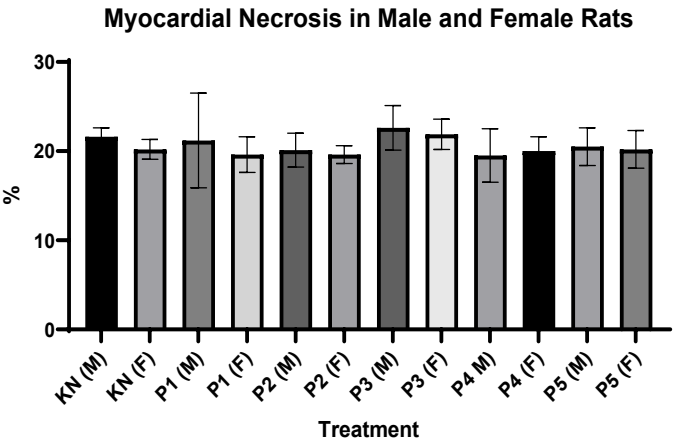

Figure 2: Myocardial necrosis in male (M) and female (F) rats by treatment group.

Myocardial cell apoptosis

Two-Way ANOVA analysis indicated that the varying doses of sappan wood extract had a significant effect on the average number of myocardial apoptosis ($p<0.05$). No significant effect was found for sex ($p>0.05$) or the interaction between dose and sex ($p>0.05$). A Duncan's post-hoc test was performed to determine the specific differences between the dose groups, with the results presented in Table 3.

Table 3: Mean percentage of myocardial apoptosis in male and female rats.

Code	Treatment	Apoptosis (%)	
		Male	Female
KN	Aquadest (control)	10.9 ± 2.3^{ab}	10.9 ± 1.5^{ab}
P1	SWE 100 mg/kg BW	9.8 ± 2.7^{a}	10.0 ± 0.9^{a}
P2	SWE 200 mg/kg BW	10.7 ± 1.0^{ab}	10.4 ± 0.7^{a}
P3	SWE 300 mg/kg BW	13.2 ± 2.5^{b}	12.3 ± 2.3^{ab}
P4	SWE 400 mg/kg BW	10.7 ± 1.7^{ab}	10.5 ± 1.6^{a}
P5	SWE 600 mg/kg BW	10.4 ± 1.7^{a}	9.7 ± 2.1^{a}

Note:Data are presented as Mean ± Standard Deviation (SD). Letters above each bar indicate the results of the Duncan's post-hoc test. Different letters ($^{a, b}$) denote a statistically significant difference ($p<0.05$) between sexes and between treatments within the same sex.

Duncan's post-hoc test results showed that although differences were observed among some treatment groups, no dose differed significantly from the control group (Table 3). This finding indicates that administration of *Caesalpinia sappan* L. extract up to a dose of 600 mg/kg BW did not induce a significant increase in myocardial apoptosis. This result is further supported by the histological findings in Figure 3, 4, where no massive increase in the number of apoptotic cells was observed in the treatment groups.

Myocardial fibrosis

Two-way analysis of variance (ANOVA) at a 95% confidence level indicated that varying doses of *Caesalpinia sappan* L. extract had a significant effect on the percentage of myocardial fibrosis area ($p<0.05$). Conversely, neither the sex factor nor the interaction between dose and sex showed a significant effect ($p>0.05$). The average percentage of the fibrosis area is presented in Table 4.

Table 4: Mean percentage of myocardial fibrosis area in male and female rats.

Code	Treatment	Fibrosis (%)	
		Male	Female
KN	Aquadest (control)	13.6 ± 3.0^{b}	$12.0\pm2.6^{ab.}$
P1	SWE 100 mg/kg BW	12.0 ± 1.5^{ab}	12.3 ± 3.5^{ab}
P2	SWE 200 mg/kg BW	13.3 ± 2.6^{b}	15.1 ± 3.4^{b}
P3	SWE 300 mg/kg BW	15.5 ± 2.6^{b}	15.7 ± 3.0^{b}
P4	SWE 400 mg/kg BW	11.9 ± 1.1^{ab}	8.7 ± 1.7^{a}
P5	SWE 600 mg/kg BW	15.5 ± 3.2^{b}	14.0 ± 3.4^{b}

Note: Superscript letters indicate significant differences between sexes and among treatments in the same sex ($p<0.05$). SWE: sappan wood extract.

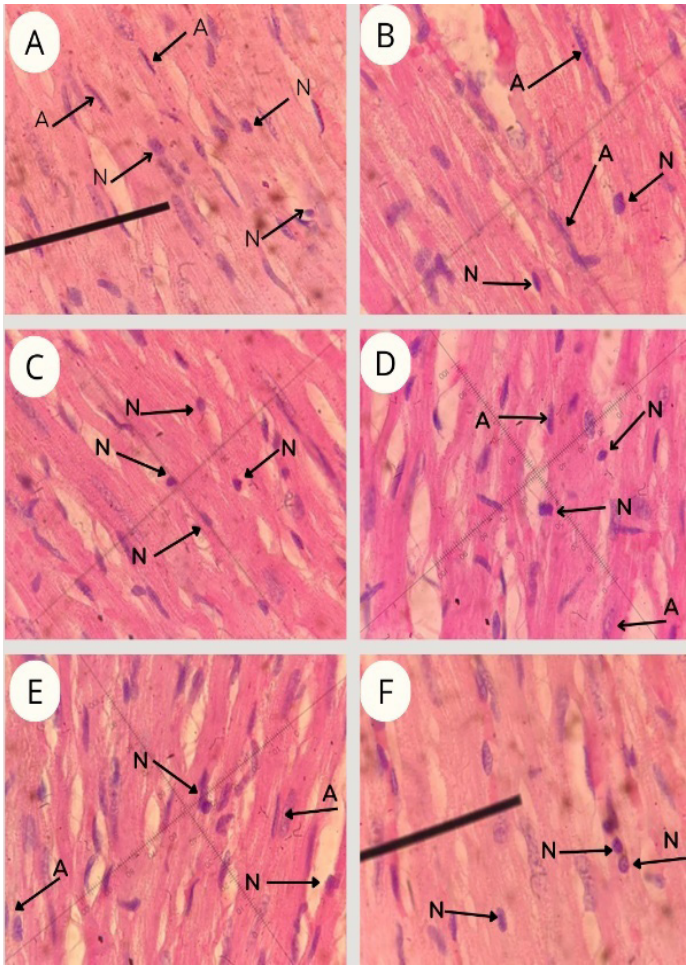

Figure 3: Histopathological features of rat myocardial cells in males treated with *Caesalpinia sappan* L. extract (Hematoxylin-Eosin Staining; 1000x Magnification). (A) KN (negative control); (B) P1 (*Caesalpinia sappan* L. extract dose 100 mg/kg BW); (C) P2 (*Caesalpinia sappan* L. extract dose 200 mg/kg BW); (D) P3 (*Caesalpinia sappan* L. extract dose 300 mg/kg BW); (E) P4 (*Caesalpinia sappan* L. extract dose 400 mg/kg BW); and (F) P5 (*Caesalpinia sappan* L. extract dose 600 mg/kg BW). Arrows indicate: N = Necrosis, and A = Apoptosis.

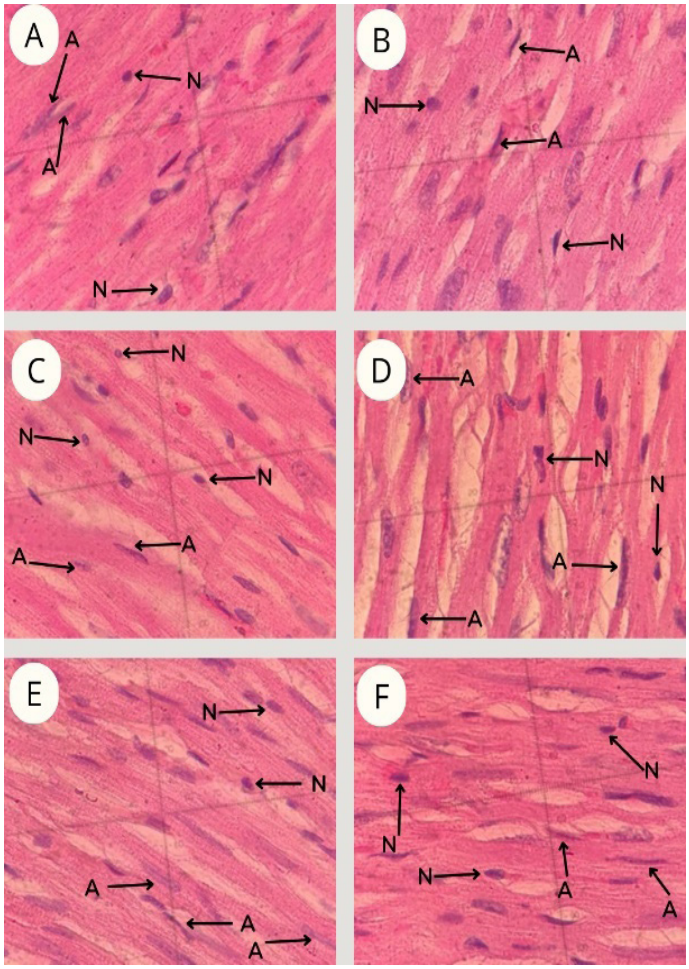

Figure 4: Histopathological features of rat myocardial cells in females treated with *Caesalpinia sappan* L. extract (Hematoxylin-eosin staining; 1000x magnification). (A) KN (negative control); (B) P1 (*Caesalpinia sappan* L. extract dose 100 mg/kg BW); (C) P2 (*Caesalpinia sappan* L. extract dose 200 mg/kg BW); (D) P3 (*Caesalpinia sappan* L. extract dose 300 mg/kg BW); (E) P4 (*Caesalpinia sappan* L. extract dose 400 mg/kg BW); and (F) P5 (*Caesalpinia sappan* L. extract dose 600 mg/kg BW). Arrows indicate: N = Necrosis, and A = Apoptosis.

Duncan's post-hoc test results revealed that for both male and female rats, the treatment groups did not differ significantly from the control group (Table 4). However, significant differences were found among several dose groups, particularly in female rats, where the 400 mg/kg BW dose (P4) resulted in a lower fibrosis area compared to the 200 mg/kg BW (P2), 300 mg/kg BW (P3), and 600 mg/kg BW (P5) doses. The histopathological description of myocardial fibrosis in male and female rats after one year of treatment with sappan wood extract, as visualized using Trichrome staining, is shown in Figures 5 and 6.

Lipid profile

Lipid profile represents blood lipid levels and serves as a reliable indicator for predicting coronary heart disease risk.

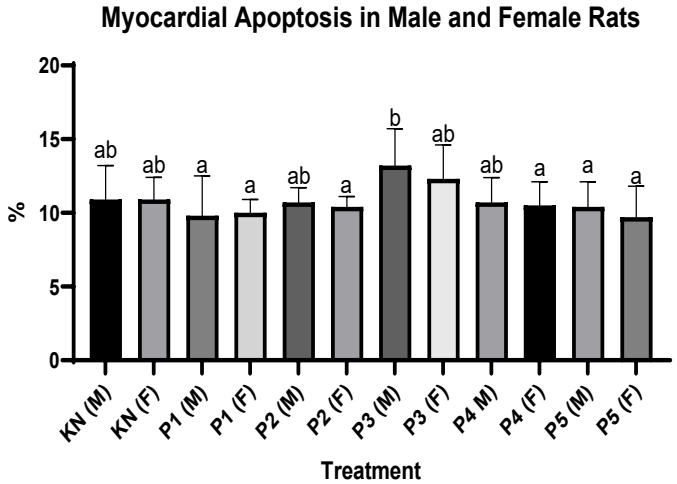

Figure 5: Myocardial apoptosis in male (M) and female (F) rats by treatment group.

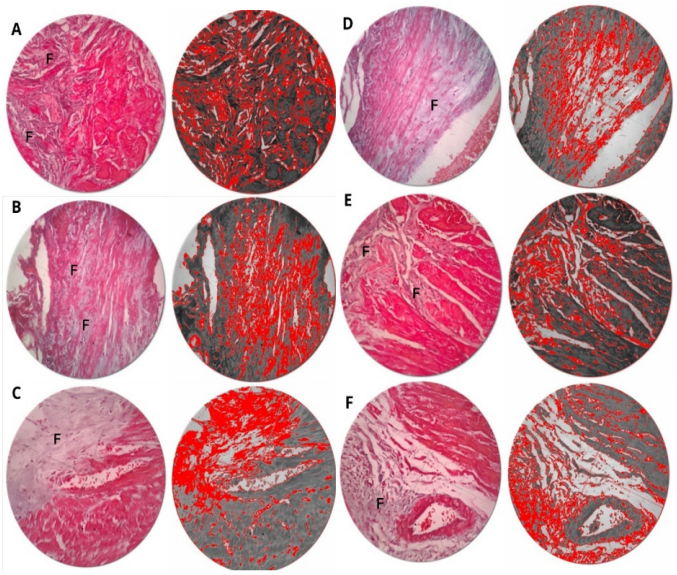

Figure 6: Histopathological features of myocardial fibrosis in male rats treated with *Caesalpinia sappan* L. extract (Trichrome Staining; 400x magnification). (A) KN (Negative Control); (B) P1 (*Caesalpinia sappan* L. extract dose 100 mg/kg BW); (C) P2 (*Caesalpinia sappan* L. extract dose 200 mg/kg BW); (D) P3 (*Caesalpinia sappan* L. extract dose 300 mg/kg BW); (E) P4 (*Caesalpinia sappan* L. extract dose 400 mg/kg BW); (F) P5 (*Caesalpinia sappan* L. extract dose 600 mg/kg BW). Note: The letter F (left panel) and the red color resulting from ImageJ quantification (right panel) indicate the area of fibrosis.

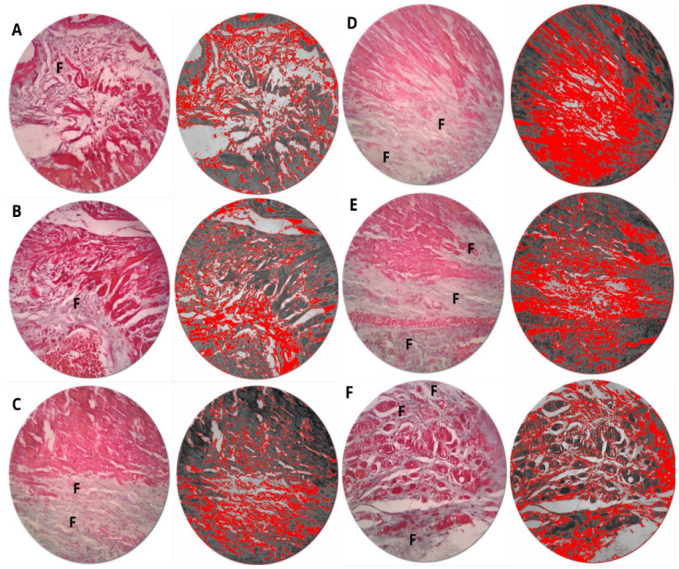

Figure 7: Histopathological features of myocardial fibrosis in female rats treated with *Caesalpinia sappan* L. extract (Trichrome Staining; 400x magnification). (A) KN (negative control); (B) P1 (*Caesalpinia sappan* L. extract dose 100 mg/kg BW); (C) P2 (*Caesalpinia sappan* L. extract dose 200 mg/kg BW); (D) P3 (*Caesalpinia sappan* L. extract dose 300 mg/kg BW); (E) P4 (*Caesalpinia sappan* L. extract dose 400 mg/kg BW); (F) P5 (*Caesalpinia sappan* L. extract dose 600 mg/kg BW). Note: The letter F (left panel) and the red color resulting from ImageJ quantification (right panel) indicate the area of fibrosis.

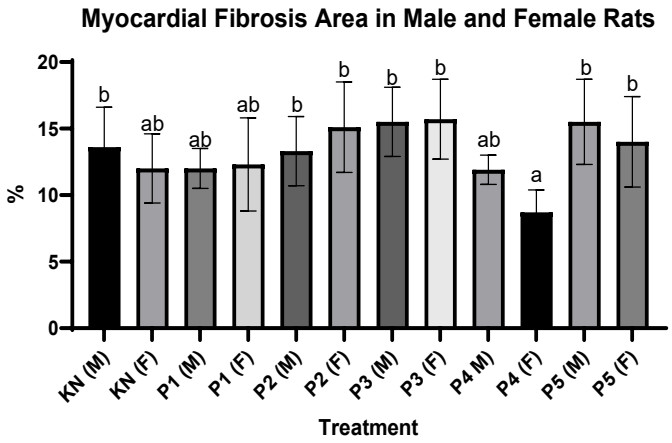

Figure 8: Myocardial fibrosis in male (M) and female (F) rats by treatment group.

Total cholesterol

Total cholesterol measurement is a supporting indicator for cardiac assessment and evaluating potential toxic effects of sappan wood extract. Based on Two-Way ANOVA at a 95% confidence level, treatment and sex did not significantly affect mean total cholesterol ($p>0.05$), and there was no interaction between these factors ($p>0.05$). Table 5 presents mean total cholesterol levels in male and female rats after one year of treatment.

Table 5: Mean total cholesterol levels in male and female rats.

Code	Treatment (one year)	Total cholesterol (mg/dL)	
		Male	Female
CN	Aquadest (control)	58.3±10.1	58.0±7.4
P1	SWE 100 mg/kg BW	44.4±9.4	48.4±5.9
P2	SWE 200 mg/kg BW	48.6±8.0	56.4±11.0
P3	SWE 300 mg/kg BW	49.6±5.9	58.0±10.8
P4	SWE400 mg/kg BW	85.2±9.3	47.6±6.0
P5	SWE 600 mg/kg BW	44.4±11.0	70.0±22.0

Note: SWE (Sappan Wood Extract).

LDL cholesterol

Two-way analysis of variance (ANOVA) at a 95% confidence level indicated that the varying doses of *Caesalpinia sappan* L. extract did not significantly affect the mean LDL cholesterol level of the rats ($p>0.05$). However, the sex factor exerted a significant effect ($p<0.05$), and a significant interaction was found between the treatment dose and sex ($p<0.05$). Consequently, a post-hoc analysis using Duncan's test was performed. The mean LDL cholesterol levels of male and female rats in each treatment group, along with the results of Duncan's test, are presented in Table 6. Despite the significant interaction detected by

the ANOVA, Duncan's test did not show a statistically significant difference between any treatment group and the control group. Therefore, the administration of *Caesalpinia sappan* L. extract was not consistently proven to increase the LDL cholesterol level in the rats.

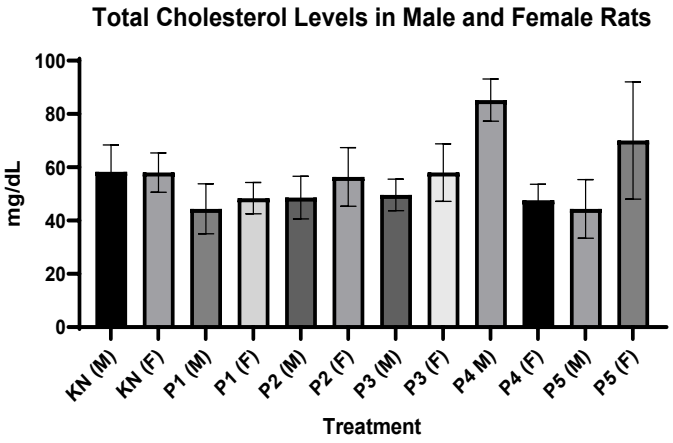

Figure 9: Total cholestrol in male (M) and female (F) rats by treatment group.

Table 6: Mean LDL cholesterol levels in male and female rats.

Code	Treatment (one year)	LDL Cholesterol (mg/dL)	
		Male	Female
CN	Aquadest (control)	11.5±2.3^{cd}	7.0±1.2^{ab}
P1	SWE100 mg/kg BW	10.8±3.4^{bcd}	7.2±2.9^{ab}
P2	SWE 200 mg/kg BW	13.8 ±2.2^{d}	6±1.4^{a}
P3	SWE 300 mg/kg BW	11.2 ± 2.4^{cd}	6.8±2.8^{ab}
P4	SWE 400 mg/kg BW	9.0 ± 1.9 ^{abc}	10.0±3.8^{abcd}
P5	SWE 600 mg/kg BW	11.4 ± 4.8^{cd}	6.8±2.4^{ab}

Note: Data are presented as Mean ± Standard Deviation (SD). Letters above each bar indicate the results of the Duncan's post-hoc test. Different letters $^{(a,b,c,d)}$ denote a statistically significant difference ($p<0.05$) between sexes and between treatments within the same sex.

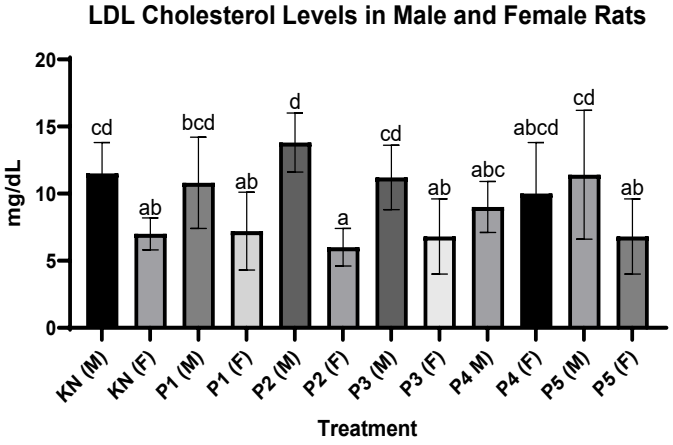

Figure 10: LDL cholesterol in male (M) and female (F) rats by treatment group.

Triglyceride

Two-way analysis of variance (ANOVA) at a 95% confidence level indicated that the varying doses of *Caesalpinia sappan* L. extract did not significantly affect the mean triglyceride level of the rats ($p=0.195$). However, the sex factor exerted a significant effect ($p=0.010$), and a significant interaction was found between the treatment dose and sex ($p=0.027$). Consequently, a post-hoc analysis using Duncan's test was performed. The mean triglyceride levels of male and female rats for each treatment, along with the results of Duncan's test, are presented in Table 7. Duncan's analysis showed that the administration of *Caesalpinia sappan* L. extract at doses of 100–600 mg/kg BW did not result in a significant effect on triglyceride levels in either male or female rats. Therefore, the *Caesalpinia sappan* L. extract treatment was not consistently proven to induce an increase in triglyceride levels.

Table 7: Mean triglyceride levels of male and female rats.

Code	Treatment	Triglyceride (mg/dL)	
		Male	Female
CN	Aquadest (control)	113.5±19.4^{abcd}	114.3±34.6^{abcd}
P1	SWE 100 mg/kg BW	101.8±40.0^{abc}	135.1±60.6^{bcd}
P2	SWE 200 mg/kg BW	137.0±39.6^{cd}	74.3 ±23.3^{a}
P3	SWE 300 mg/kg BW	105.7±13.8^{abc}	86.2±38.1^{ab}
P4	SWE 400 mg/kg BW	138.6±53.5^{cd}	95.8±15.7^{abc}
P5	SWE 600 mg/kg BW	160.3±11.2^{d}	110.5±15.8^{abc}

Note: Data are presented as Mean ± Standard Deviation (SD). Letters above each bar indicate the results of the Duncan's post-hoc test. Different letters $^{(a,b,c,d)}$ denote a statistically significant difference ($p<0.05$) between sexes and between treatments within the same sex.

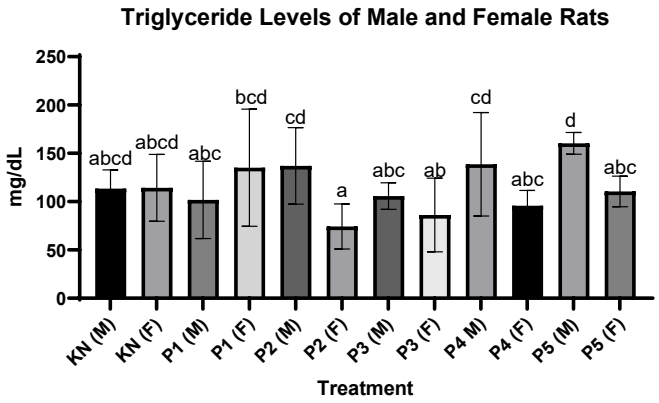

Figure 11: Triglyceride levels in male (M) and female (F) rats by treatment group

DISCUSSION

Cardiac structure

Throughout the study period, none of the test animals (rats) displayed significant clinical signs of toxicity, such as

changes in appetite or activity. Mortality was zero (0%), as no deaths were recorded. Furthermore, post-mortem (PM) examination did not reveal any significant macroscopic alterations in the organs observed. The absence of these clinical and macroscopic findings suggests that the extract doses administered were safe and did not cause acute toxic effects on the organs, particularly the heart.

Cardiac relative organ weight (ROW)

Duncan's post-hoc test results showed that among male rats, there was a significant difference in cardiac ROW between the group receiving the 100 mg/kg BW extract dose and several other treatment groups. Conversely, no significant difference in cardiac ROW was found among treatments in female rats. This finding indicates that the cardiac ROW response to *Caesalpinia sappan* L. extract is sex-dependent, with a more pronounced effect observed in male rats. Generally, the cardiac ROW of male rats is higher than that of female rats. Kusrohmaniah (2017) stated that the cardiac muscle of male rats is naturally larger and heavier than that of female rats. This difference is likely due to the inherent variations in muscle mass and total body weight between male and female rats.

An increase in cardiac ROW is often associated with heart abnormalities, particularly myocardial hypertrophy the enlargement of cardiac muscle fibers (myocardium) which increases the organ ratio (Sutrisni *et al*., 2019). Nugraheni and Saputri (2017) reported that the relative heart weight of rats treated with *Caesalpinia sappan* L. extract remained close to control values. This is attributed to the extract's anti-inflammatory ability to suppress heart muscle swelling caused by isoproterenol induction. Therefore, the absence of a significant increase in ROW in the rats in this study suggests that the administration of *Caesalpinia sappan* L. extract did not induce a cardiac abnormality in the form of myocardial hypertrophy.

Myocardial necrosis

The study results showed that administration of *Caesalpinia sappan* L. extract at doses up to 600 mg/kg BW did not cause a significant difference in the incidence of myocardial necrosis in either male or female rats. This finding suggests that the extract possesses a protective effect on myocardial cells against potential damage that might occur due to treatment.

The lack of increased myocardial necrosis is consistent with the known pharmacological mechanisms of *Caesalpinia sappan* L. extract. Mu'nisa *et al*. (2017) reported that the flavonoid and phenol content in the extract has high antioxidant activity. This antioxidant property is crucial because it can neutralize reactive oxygen species (free radicals) and protect cells from oxidative damage, thereby inhibiting myocardial cell death via necrosis (Nugraheni and Fadlina, 2017). Brazilin, the main compound found in *Caesalpinia sappan* L., has been proven to protect the heart from damage related to myocardial ischemia-reperfusion injury, a condition frequently leading to necrosis (cell death) in cardiac tissue. Brazilin reduces myocardial infarct size, enhances cell viability, and decreases the release of cardiac injury markers, such as creatine kinase-MB and lactate dehydrogenase, in both cell and animal models (Qi *et al*., 2021).

Myocardial apoptosis

Although the analysis of variance (ANOVA) results showed a significant effect of varying doses of *Caesalpinia sappan* L. extract on myocardial apoptosis, the Duncan's post-hoc test revealed that the significant differences occurred only between certain dose groups and that no dose differed significantly from the negative control group (Table 3). This finding suggests that the *Caesalpinia sappan* L. extract, within the tested dose range, did not trigger an increase in the rate of myocardial cell apoptosis compared to normal conditions. Visual observations (Figures 4 and 5) support this interpretation, as no massive increase in apoptotic cells was observed in the treatment groups.

The extract's cardioprotective effect in preventing apoptosis is highly likely related to its antioxidant capacity. This antioxidant capacity is influenced by the concentration of the ethanol solvent and correlates strongly with the phenolic content. This antioxidative mechanism allows the extract to protect myocardial cells from DNA damage induced by oxidative stress (free radicals) (Ameliana *et al*., 2024; Kongkham and Aylada, 2019)

Specifically, brazilin, the major compound in *Caesalpinia sappan* L., plays a central role in stabilizing myocardial cells and inhibiting apoptosis. Brazilin has proven cardioprotective activity that mechanically reduces stress-induced apoptosis, characterized by a decrease in the expression of cleaved caspase-3 (Qi *et al*., 2021). This protective effect is believed to be largely mediated through the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway. Brazilin facilitates the translocation of Nrf2 into the nucleus, which subsequently enhances the expression of target antioxidant genes (such as HO-1 and NQO1) that have an inhibitory effect on pro-inflammatory genes (Ahmed *et al*., 2017; Qi *et al*., 2021).

Furthermore, brazilin also demonstrates the ability to inhibit the expression of nuclear factor-κB (NF-κB), a crucial pro-inflammatory signaling pathway in the pathogenesis of cardiac damage. Another protective mechanism involves the inhibition of phosphodiesterase-1 (PDE-1), which indirectly increases cAMP levels, an important modulator in the fibrotic response (Delaunay *et*

al., 2020). Therefore, the combined dual action of Brazilin via Nrf2/antioxidants, pro-inflammatory inhibition (NF-κB), and fibrotic modulation (PDE-1) comprehensively explains how *Caesalpinia sappan* L. extract is able to protect cardiac cells from damage and inhibit programmed cell death (apoptosis).

Lipid Profile

Total cholesterol levels

The study results showed that the total cholesterol levels in the rats did not significantly increase following the administration of *Caesalpinia sappan* L. extract. This finding suggests that the extract, within the tested dose range of 100–600 mg/kg BW, is safe and does not induce negative effects on the total cholesterol parameter. The stability of cholesterol levels is consistent with the hypolipidemic potential reported for the active compounds in *Caesalpinia sappan* L., such as brazilin and alkaloids. These compounds are known to be capable of inhibiting cholesterol synthesis and increasing lipid excretion (Rahman *et al*., 2015; Artha *et al*., 2017). Mechanistically, the cholesterol-lowering effect is explained by the inhibition of the HMG-CoA reductase enzyme, a key enzyme in cholesterol biosynthesis. Molecular docking analysis indicates that major compounds like sappanone B and brazilin possess potential as competitive inhibitors of HMG-CoA reductase (Luhung *et al*., 2025). *In vivo* studies further support this by demonstrating that *Caesalpinia sappan* L. extract can reduce total cholesterol levels in alloxan-induced diabetic rat models and in obese rats subjected to a high-fat diet (Holidah *et al*., 2021; Mekala and Radha, 2016).

LDL cholesterol

Analysis of Low-Density Lipoprotein (LDL) Cholesterol levels showed that while there was a significant influence from the sex factor and extract dose interaction, the post-hoc test did not find a significant difference between any extract treatment group and the control group. Given that LDL cholesterol is a major risk factor for atherosclerosis and coronary heart disease (Dharma *et al*., 2013), the absence of a significant increase suggests that *Caesalpinia sappan* L. extract does not induce adverse effects related to cardiovascular risk. The observed difference in LDL levels based on sex is consistent with the influence of estrogen hormones in female rats, which are known to enhance the expression of LDL receptors in the liver, thereby lowering blood LDL levels (Stevani, 2017).

Beyond metabolic influence, the antioxidant potential of brazilin (Ramadhani, 2016) is hypothesized to act as a protective factor by preventing LDL oxidation, a critical step in the formation of atherosclerotic plaque. Literature also highlights the role of Proanthocyanidins (PrA) in the *Caesalpinia sappan* L. extract, which may reduce LDL levels by inhibiting hyperlipidemia activity (Kurniawan and Tukiran, 2021). Furthermore, several studies link *Caesalpinia sappan* L. extract to complex cellular mechanisms, such as the activation of the lysosomal pathway and autophagy mediation (Liu *et al*., 2022). This pathway assists myocardial cells in clearing excess intracellular lipids, which can enhance cellular function and improve lipid metabolism at the molecular level.

A broader implication of this extract lies in its potential to mitigate atherosclerosis itself. Research (He *et al*., 2025) found that *Caesalpinia sappan* L. extract works through the miR-126/VEGF signaling pathway. Specifically, the extract significantly increases miR-126 expression and decreases Vascular Endothelial Growth Factor (VEGF) expression, which is a primary trigger for angiogenesis (new blood vessel formation) supporting the growth of atherosclerotic plaques. Therefore, *Caesalpinia sappan* L. extract may inhibit atherosclerotic plaque formation by suppressing angiogenesis and promoting arterial endothelial health.

Triglycerides

For the triglyceride parameter, no significant effect was found from either the varying doses of *Caesalpinia sappan* L. extract or sex. This result further strengthens the evidence that *Caesalpinia sappan* L. extract administration does not disrupt lipid metabolism homeostasis. The lack of change in triglyceride levels is consistent with findings by Holidah *et al*. (2021) and Mekala and Radha (2016) who reported that the extract actually reduced triglycerides in dyslipidemia models (diabetes or obesity). The ability to maintain stable triglyceride levels (in the normal model) or reduce them (in dyslipidemia models) is linked to the multi-target content such as flavonoids, saponins, and phenolics (including rutin, quercetin, and gallic acid) within the extract, providing a strong scientific basis for its regulatory effects on lipid metabolism.

CONCLUSION

Within the dose range and statistical power of this study, the chronic administration of sappan wood (*Caesalpinia sappan* L.) extract (100–600 mg/kg BW for one year) showed no evidence of significant cardiotoxic effects in male and female rats. Specifically, the extract did not significantly affect relative heart weight, myocardial cell necrosis, or lipid profiles (total cholesterol, LDL cholesterol, or triglyceride levels). These findings collectively suggest that the extract is safe for long-term use within the tested dose range. However, the observed lack of cardiotoxicity is specific to this dose range and cannot be generalized to higher doses. Furthermore, extrapolation of these animal study results directly to humans is limited, underscoring the necessity for future

clinical trials. This research also did not investigate the specific molecular mechanisms of the extract's active compounds. Future studies should evaluate the extract's effects at higher doses to establish its toxicity threshold and explore its specific molecular mechanisms and therapeutic potential in relevant disease models, such as those with induced hyperlipidemia or cardiac damage.

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NOVELTY STATEMENT

The novelty of this research lies in providing comprehensive evidence regarding the chronic cardiovascular toxicity and metabolic safety of sappan wood extract, which is crucial for its future development of sappan wood extract as a safe herbal medicine.

AUTHOR'S CONTRIBUTION

RS: Conceptualization, funding acquisition, investigation, supervision, validation, writing-reviewand editing, project administration.
ZMK: Data curation, formal anlysis, visualization, wrting-original draft.
Yasmi Purnamasari Kuntana: Conceptualization, supervision, validation, writing-review and editing.
MRAAS: Conceptualization, methodology; validation, writing-review and editing.
RK: Writing original draft, writing-review and editing.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Ethics statement

The authors have nothing to report.

Generative AI and AI-assisted technology statement

Generative AI tools were used exclusively for editorial purposes (suggestions on clarity, grammar, and English editing). No scientific text, dataset, result, figure, table, or statistical analysis was generated by AI. All data processing and statistical analyses (Two-Way ANOVA and Duncan's test) were performed in [Sebutkan nama software statistikmu] and verified by the authors. AI outputs were critically reviewed and, where appropriate, adjusted by the team prior to inclusion. No AI models were used to design experiments, create scientific images, or interpret results. The final manuscript was thoroughly proofread and edited by the human authors to ensure its accuracy and integrity.

Conflict of interest

The authors have declared no conflict of interest.

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