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The Cardioprotective Effects of *Sida rhombifolia* Leaf Extract in Cardiac Injury Rat Model

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Abstract

High-fat diets (HFD) and high-sucrose diets increase the risk of cardiometabolic diseases. The combination of the two diets reflects the Western dietary trend. The mortality due to cardiovascular disease is still high. Therefore, new treatment options are urgently needed to address the issue, such as through the exploration of medicinal plants. This study aimed to examine the effects of *Sida rhombifolia* (SR) leaf extract on IL-6 mRNA expression, histopathological findings, and troponin I level in the heart of rats induced by a high-fat-sucrose diet and carbon tetrachloride (CCl_4). Forty male Sprague Dawley rats were randomly divided into five equal groups: negative control (C-), positive control (C^+), and three groups given *SR* extract with the following doses: SRE1 (100mg/kgBW), SRE2 (200mg/kgBW), and SRE3 (400 mg/kgBW). The C+ and the extract groups were treated with the high-fat-sucrose diet (32.2% fat content, 10% sucrose) and CCl_4 injection (0.4 mg/kg body weight). All treatments were conducted for 12 weeks. The expression of IL-6 in the C+ was significantly lower than C-, and only the SRE2 expressed a significantly higher IL-6 than the C+. A higher degree of inflammation was also observed in the C^+ compared to the C^-, while the SRE2 had a significantly lower degree of inflammation than the C+ group. The myocardial tissue in the SRE2 and SRE3 groups also showed less fibrosis. There was no difference in plasma troponin I levels among groups. We collectively found that the *SR* extract provides cardioprotection by altering the degree of myocardial inflammation and IL-6 mRNA expression in rats with cardiac injuries.

Keywords: Cardiometabolic disease, inflammation, fibrosis, high-fat-sucrose diet, *Sida rhombifolia*

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of global deaths by non-communicable diseases, with a prevalence of 17.9 million deaths each year (31% of global mortality) [1]. The risk of CVD increases in patients with obesity, insulin resistance, and

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dyslipidemia, which can be referred to as cardiomyopathy-associated metabolic disorders [2]. In 2012, cardiometabolic diseases caused 702,308 adult deaths in the United States [3]. The most common causes of death from these metabolic diseases were coronary heart disease, heart failure, and atrial fibrillation [4].

High caloric contents from high-fat diets (HFD) can increase the risk of CVD, especially cardiometabolic diseases [5]. The high caloric dietary fats are known as risk factors for cardiometabolic diseases. Dietary fats and their metabolites increase cardiometabolic risks by modulating genetic regulation, organelle and cell membrane structure and function, ion channel activities, and electrophysiology [6]. There was no known direct effect of elevated serum lipids on cardiac function, apart from their effect on atherosclerosis. However, current findings suggest excess serum lipids can accumulate in the heart [7]. Triglycerides from HFD are broken down into free fatty acids for adenosine triphosphate production in mitochondria via fatty acid oxidation [8]. The imbalance between absorption and utilization of fatty acids can result in lipid accumulation in the heart that impairs normal cellular signaling and causes cardiomyocyte dysfunction and apoptosis, known as "lipotoxicity" [2, 9]. Inflammation is a component of lipotoxicity, and it has been reported that HFD can increase inflammatory markers, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) [10-11].
Other than HFD, the consumption of diets containing high levels of carbohydrates such as sucrose and fructose also causes inflammation and metabolic disturbances [8]. High carbohydrate diets can potentially increase the incidence of obesity, cardiovascular disease, metabolic syndrome, and type II diabetes [12]. Fructose can induce the production of lipids in cardiomyocytes and pro-inflammatory cytokines [13]. The combination of high-fat and high-carbohydrate diets reflects the current Western dietary trend [14]. This combination has been widely used to induce cardiometabolic disturbances in experimental animals [8, 15].
Experimental animal models can be helpful to assess the myocardial tissue damage caused by diet intervention. Interleukin-6 (IL-6) is an important inflammatory cytokine recently used as a marker of acute myocardial infarction and chronic heart failure [16]. In addition to IL-6, cardiac troponin I (cTnI) is also known as a marker of myocardial damage [17]. Previous studies showed that a 12-week HFD intervention in rats significantly increased the cTn-I serum levels [11, 18].

Currently, a high-fat, high-carbohydrate diet is the 3rd risk factor of all-cause mortality as well as CVD mortality worldwide [19]. Non-adherence to drugs was the most common problem in the management of CVD, causing high morbidity and mortality despite the advances in disease management and treatment [20]. Medicinal plants can be a potential alternative treatment for CVD since their acceptability is high among patients in particular countries, including Indonesia [21]. *Sida rhombifolia*, a widely grown and commonly used in Indonesia as a medicinal plant, possesses biological properties such as antimicrobial, antioxidant, anti-inflammatory, hepatoprotective, nephroprotective, antidiabetic, analgesic, and anti-cancer [22]. Studies showed the cardioprotective effects of *Sida rhombifolia* leaf in isoproterenol-induced myocardial necrosis in rats [23-24]. However, no studies have analyzed its effect on cardiac injury from HFD and chemical intoxication. Therefore, this study aimed to examine the impact of *S. rhombifolia* leaf extract on the expression of IL-6 mRNA in cardiac tissue, blood plasma levels of cardiac troponin I (cTn-I), and histopathological findings of the myocardial rats induced by a high-fat-sucrose diet and CCl_4.

2. Materials and Methods

2.1 Experimental Animals

The current study examined the effect of *S. rhombifolia* leaf extract on cardiac injury in rats induced by a combination of a high-fat-sucrose diet and CCl_4 injection. The study obtained ethical approval from the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (Reference No: KE/FK/0627/EC). Based on the sample size calculation and dropout rate consideration [25], forty male Sprague Dawley rats (*Rattus norvegicus*), aged 8-10 weeks with body weights between 160-210 grams, were obtained from the Integrated Research and Testing Laboratory, Universitas Gadjah Mada. The animals were randomly divided into five groups: (I) the negative control group/C- (given the standard diet), (II) positive control group/C+ (given HFD, 10% sucrose drink, CCl_4 injection), (III) treatment group with 0.5x dose of *S. rhombifolia* extract/SRE1 (given HFD, 10% sucrose drink, CCl_4 injection, and 100mg/kgBW extract), (IV) treatment group with 1x dose of *S. rhombifolia* extract/SRE2 (given HFD, 10% sucrose drink, CCl_4 injection, and 200mg/kgBW extract), and (V) treatment group with 2x dose of *S. rhombifolia* extract/SRE3 (given HFD, 10% sucrose drink, CCl_4 injection, and 400 mg/kg BW extract). Acclimatization of the experimental animals was carried out one week before interventions. The rats were kept in standard plastic cages with a temperature of 24-26°C, a humidity of 60-65%, and a twelve-hour light-dark cycle. Rats were given ad libitum access to food and drink in the cage.

2.2 Cardiac Injury Modeling and Sida rhombifolia Administration

The cardiac injury model was obtained from a combination of a high-fat-sucrose diet and CCl_4 administration. The standard diet used was RatBio® (Citra Ina Feedmill, Jakarta, Indonesia). This standard diet consists of 4% fat, 20% protein, 4% crude fiber, 12% calcium, and 12% water. Melted butter (2 mL) was used as the HFD, containing 32.2% fat. Sucrose was added to the drinking water with a concentration of 10%. CCl_4 (Merck, Germany) was administered at a dose of 0.4 mg/kg body weight by dissolving it in olive oil (ratio of 1:9) with the final volume of 0.5 ml per rat. The CCl_4 and olive oil mixture was injected intraperitoneally into the rats in C+, SRE1, SRE2, and SRE3 groups twice per week for 12 weeks. HFD was administered orally by gavage, and the 10% sucrose drink water was given daily for 12 weeks to the C+, SRE1, SRE2, and SRE3 groups.

Sida rhombifolia leaf extract was obtained using the maceration method using 96% ethanol. Based on a previous study, the effective dose of *S. rhombifolia* extract was 200 mg/BW [23]. In this study, *S. rhombifolia* extract was given to the SRE1, SRE2, and SRE3 groups with doses of 0.5x (100mg/BW), 1x (200mg/BW), and 2x (400mg/BW) of the effective dose respectively. The extract was administered daily through oral gavage for 12 weeks. Body weight data were collected once a week. All procedures were carried out following the published methods [26].

2.3 Blood Collection and cTnI Evaluation

The rats were anesthetized by intramuscular injection of 0.1 ml/kg body weight of ketamine. Blood samples were collected from the retroorbital sinus at week 0 (before the intervention) and week 12 (after the intervention). Blood samples were then stored in EDTA tubes and centrifuged for 10 minutes at 4000 rpm to collect the plasma. Blood plasma was temporarily stored at -80^{o}C before processing. The enzyme-linked immunosorbent assay/ELISA kit (Abbkine, Wuhan, China) was used to evaluate the cardiac troponin I level following the procedures from the kit.

2.4 Cardiac Tissue Collection and IL-6 mRNA Expression

At the end of week 12, the rats were euthanized using ketamine injection. The heart was taken and then divided in half, partly stored in RNA later solution to analyze the expression of IL-6 mRNA through real-time polymerase chain reaction (RT-PCR) using the SYBR Green method. The RNA extraction (Favorgen, Ping-Tung, Taiwan) and cDNA synthesis (Toyobo, Japan) were performed according to the manufacturer's protocol. The IL-6 primers were designed using the NCBI Primer-BLAST with the following sequences: (forward) 5'-CACCAGGAACGAAAGTCAACTC-3' and (reverse) 5'-GCAACTGGCTGGAAGTCTCT-3'. The relative mRNA expression of IL-6 was determined using Livak's $2^{-\Delta\Delta CT}$ algorithm [27]. We performed five biological and two technical replicates for each PCR reaction in this study, with GAPDH as the gene of reference. GAPDH was known to be the most stable reference gene in the rat cardiac tissue samples, particularly under different metabolic disturbances and hypoxia conditions [28-29].

2.5 Histopathological Evaluation

The other half of the heart was stored in neutral-buffered formalin for further histological processing of the cardiac tissue using the paraffin method. The histological slides were stained with Hematoxylin-Eosin (H&E) for inflammation observation and Sirius Red for fibrosis evaluation. The fibrosis was evaluated descriptively. The degree of cardiac inflammation was assessed under a light microscope (Olympus CX21®, Tokyo, Japan) with 200x magnification, using the inflammation score from previous studies [30]. The scoring system is divided into 6 categories: 0: no lesion; 1: <3 foci of inflammatory cell infiltration; 2: ≥3 foci of inflammatory cell infiltration; 3: mild diffuse inflammation; 4: moderate diffuse inflammation; 5: severe diffuse inflammation. The degree of inflammation of each group was obtained from the average score of 10 random fields of view of each histological slide. The histopathological evaluation was carried out by two blinded observers.

2.6 Statistical Analysis

All data were analyzed using IBM SPSS version 23.0. Numerical data were presented in mean ± SD or the median and quartiles. The Shapiro-Wilk test was used to analyze the normality of the data. Data from pre-intervention and post-intervention pairs (cTnI levels) in each group were analyzed using paired t-tests. The post-intervention data (IL-6 mRNA expression and inflammation score) were analyzed using ANOVA and Kruskal-Wallis test, followed by the Bonferroni post-hoc test. A *P*-value of <0.05 was considered significant.

3. Results

3.1 IL-6 mRNA Expression

The expression level of IL-6 mRNA for each group is illustrated in Figure 1. Based on the ANOVA test, there was a significant difference in IL-6 mRNA expression between groups (*P*=0.00). Bonferroni post-hoc test showed that the IL-6 expression in the C+ group was significantly lower than the C- group (*P*=0.006). Among the three groups that received SRE, only the SRE2 group showed higher IL-6 expression than the C+ group (*P*=0.000).

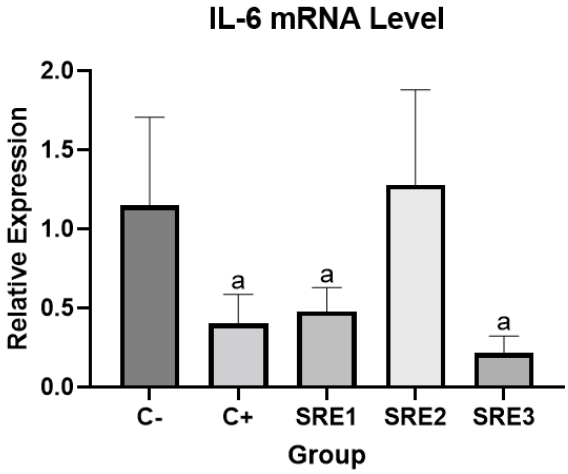

Figure 1: IL-6 mRNA expression. [a]p value <0.05 compared to C- and SRE2 groups, using the Bonfferoni post-hoc test; C-: negative control; C+: positive control; SRE1: 0.5x dose of *S. rhombifolia* extract; SRE2: 1x dose of *S. rhombifolia* extract; SRE3: 2x dose of *S. rhombifolia* extract.

3.2 Cardiac Inflammation Degree and Fibrosis

The cardiac inflammation degree data are presented in Table 1, and the representative histological images from all groups are shown in Figure 2. The data showed that there was a significant difference in cardiac inflammation degrees between all groups (*P*=0.003). The C+ higher showed a significantly higher degree of inflammation than the C- group (*P*=0.001). Among the three extract-treated groups, only the SRE2 group had a significantly lower degree of inflammation than the C+ group (*P*=0.013). The representative histological images of myocardial fibrosis of all groups are shown in Figure 3. The cardiac histological imaging of the C- group and SRE2 group appeared to be normal, with little to no fibrosis. More notable cardiac fibrosis was observed in the C+ group. In the SRE1 and SRE3 groups, the fibrosis was less extent than the C+ group.

Table 1: Cardiac inflammation degree.

Groups	n	Inflammation Degree (%)						Mean
		0	1	2	3	4	5	
C-	6	6 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0,0
C+	7	0 (0%)	7 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1,0[a]
SRE1	6	1 (16,7%)	5 (83,3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0,83[a]
SRE2	6	4 (66,7%)	2 (33,3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0,33[b]
SRE3	5	2 (40%)	3 (60%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0,60[a]

[a]p value <0.05 compared to the C- group, using Mann-Whitney test; [b]p value <0.05 compared to the C+ group, using Mann-Whitney test; C-: negative control; C+: positive control; SRE1: 0.5x dose of *S. rhombifolia* extract; SRE2: 1x dose of *S. rhombifolia* extract; SRE3: 2x dose of *S. rhombifolia* extract.

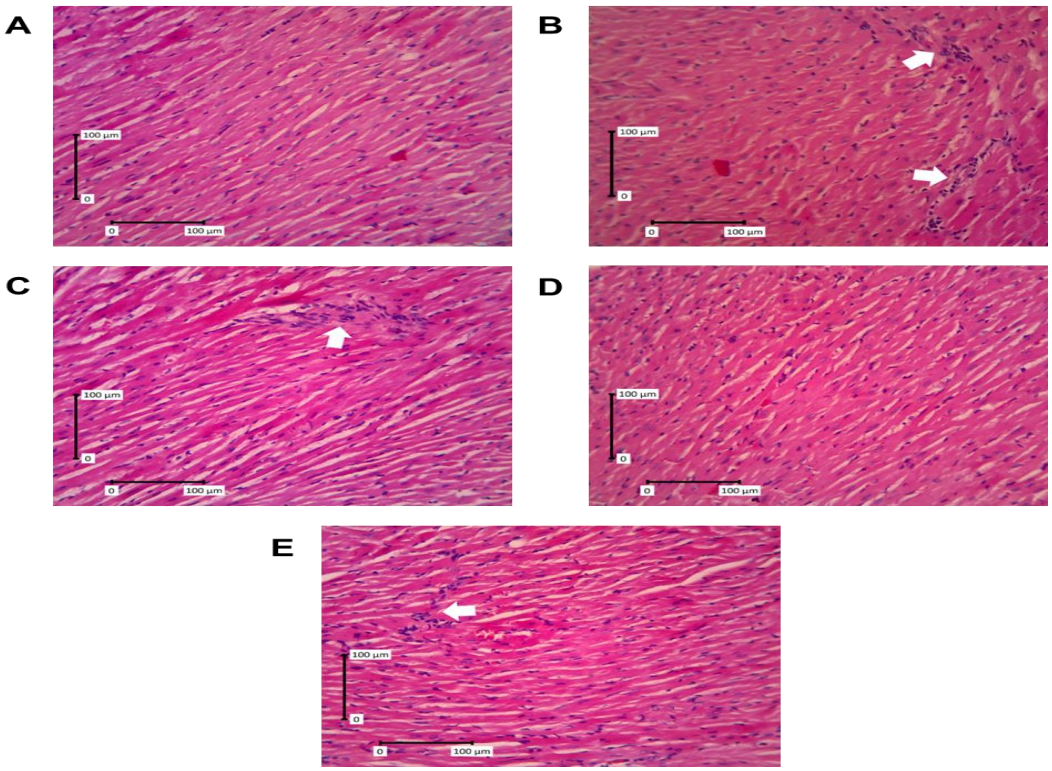

Figure 2. The histological images of cardiac tissue from all groups. HE staining with 200x magnification. (A) Normal histological appearance in the C- group; (B) There were several foci of inflammatory cell infiltration in the C+ group; (C) One focus of inflammation in the SRE1 group; (D) Normal histological features in the SRE2 group; (E) There was one focus of inflammation in the SRE3 group (white arrow: inflammation focus).

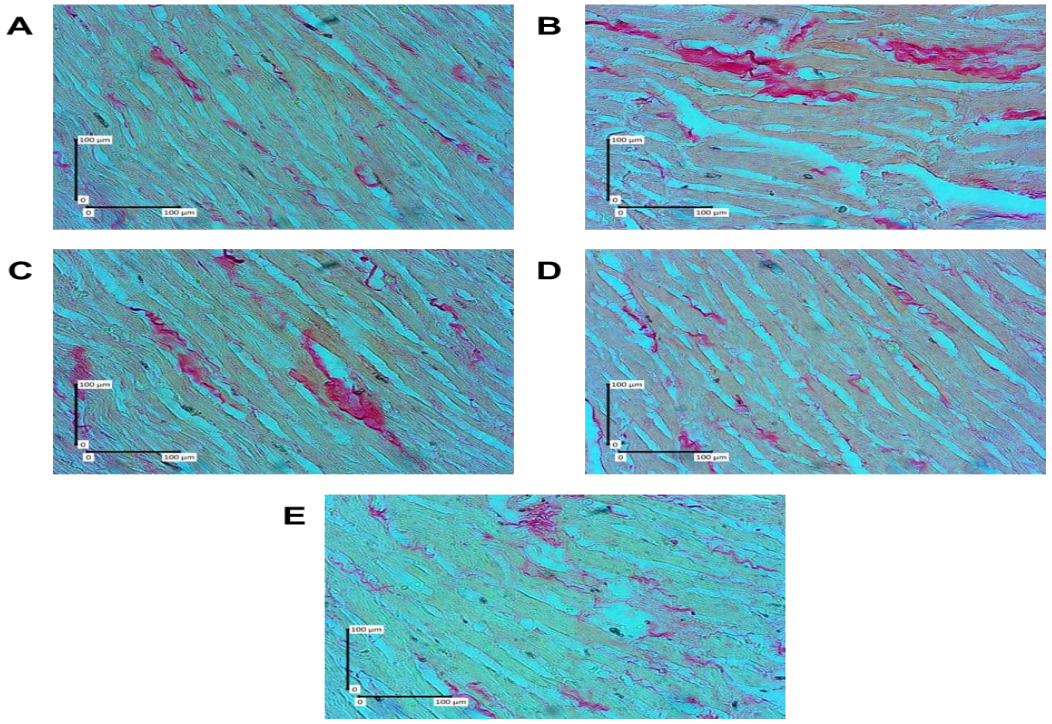

Figure 3: The histological imaging of fibrosis in the cardiac tissue from all groups. Sirius Red staining with 200x magnification. (A & D) Normal histological appearance in the C- and SRE2 group; (B) Notable fibrosis (red color) in the C+ group; (C & E) less fibrosis in the SRE1 and SRE3 group.

3.3 Plasma cTn-I Level

Figure 4 shows the cTn-I level of all groups in week 0 and week 12. There was no difference in cTn-I level among groups in week 0 (P=0.710) or week 12 (P=0.545). A slight increase in the cTn-I level from week 0 to week 12 was seen in the C+, SRE1, and SRE2 groups, but it was not significant (respectively, P=0.735, P=0.249, and P=0.713).

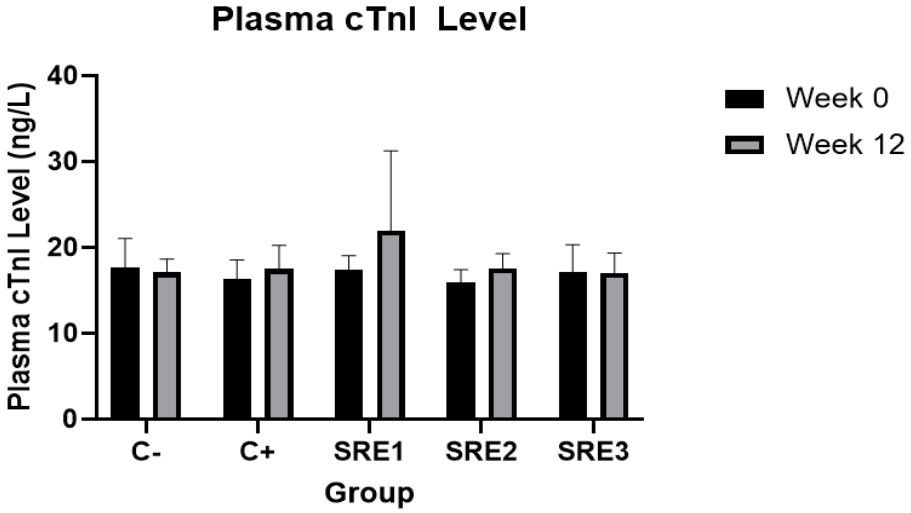

Figure 4: Plasma Cardiac Troponin I Level. C-: negative control; C+: positive control; SRE1: 0.5x dose of *S. rhombifolia* extract; SRE2: 1x dose of *S. rhombifolia* extract; SRE3: 2x dose of *S. rhombifolia* extract.

4. Discussion

This study aimed to analyze the effect of *S. rhombifolia* leaf extract on rat cardiac injury induced by a combination of a high-fat-sucrose diet and CCl_4 injection. The results showed that the IL-6 expression in the C+ group, which received a high-fat-sucrose diet and injection of CCl_4, was lower than C- group. This result was in contrast with previous findings. Rats that were given a high-fat-fructose diet (HFFD) for 16 weeks showed metabolic disturbances, structural and functional changes in the heart, and inflammation marked by significantly higher TNF-α and IL-6 plasma concentrations than the control group [31]. Another study analyzed the effect of HFD administration (60 kcal% fat) for 11 weeks in mice and found an increase in NF-κB/Nuclear factor-kappa B level and JNK/Jun N-terminal kinase phosphorylation, as well as a decrease in IκB level in the hearts of mice treated with HFD compared to the control group. The activation of NF-κB and JNK is associated with increased production of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β [32]. However, other studies also reported similar findings to our study. Chen [33] evaluated the role of IL-6 in HFD-induced cardiomyopathy using IL-6 knockout (IL-6 KO) mice fed with HFD (45% fat) for 14 weeks and found a decrease in IL-6 protein in the hearts of the wild-type mice given HFD. IL-6 KO mice hearts showed more hypertrophy, inflammation, and lipotoxicity. These results indicate that IL-6 deficiency also induces inflammation and damage to the heart. Overall, current evidence suggests that IL-6 disorders can lead to dyslipidemia, which predisposes to cardiac lipotoxicity, although it is still unclear whether excess or deficiency of IL-6 causes it [34]. These controversial findings suggest the complicated functions of IL-6 need to be further investigated to provide new insights into the role of IL-6 in obesity-induced cardiomyopathy, in addition to being a pro-inflammatory cytokine.

The degree of inflammation in the C+ group was significantly higher than in the C- group. In addition, notable cardiac fibrosis was observed in the C+ group. This showed that the diet

and CCl_4 intervention induced inflammation and fibrosis in the heart. These results were in line with a previous study that reported that the left ventricle in rats fed with HFD showed an increase in muscle fibers injury, karyolytic nuclei, muscle hypertrophy, and inflammatory cell infiltration [11]. Another study using rats given a high-fat diet (58.3% fat) for 16 weeks also showed histological changes in the cardiac tissue, such as myocyte hypertrophy, increased fat droplets, and accumulation of mononuclear cells associated with inflammation [35].
Cardiomyocyte injury can occur in several ways, with the main pathway involving disruption of cellular homeostasis resulting in cell death. The pathway can have a direct effect on the cell (mitochondrial injury) or involve some extrinsic changes, such as an increase in workload or an abnormal supply of energy substrates (oxygen, fatty acids, glucose) [36]. Histological development of cardiomyopathy in rats begins with degeneration and necrosis of cardiomyocytes with the loss of myofibers. These foci of degeneration and necrosis are then infiltrated by inflammatory cells. The proliferation of fibrous tissue will replace the damaged myocardium and leave areas of fibrosis [37]. The full spectrum of these three changes (necrosis, inflammatory cell infiltration, and fibrosis) can be seen in cases of repeated injury that may occur with chronic doses of a cardiotoxic compound or progressive cardiomyopathy [36].

In this study, there was no significant difference in the cTn-I level among all groups before and after the administration of the high-fat-sucrose diet and CCl_4. These results were in contrast with previous studies analyzing the effects of HFD administration for 12 and 14 weeks in rats which found that the serum troponin I level increased significantly [11, 18]. However, another study reported a similar finding to our result. Gumede [38] evaluated the myocardial damage in prediabetic rats induced by a high-fat-carbohydrate diet and drinking water containing fructose (15%) for 20 weeks. It was found that there was no significant difference in the average cTnI concentration in the prediabetic group. Another study investigated whether hyperlipidemia induced by a high-fat diet can cause heart damage and found no difference in serum troponin I levels between the treatment and control groups [39].
Troponin is a protein complex consisting of three units, namely troponin T, I, and C. Troponin I (cTnI) and T (cTnT) have specific isoforms for the heart. The main factor causing troponin release from damaged myocardial cells is the mismatch between the demand and supply of oxygen in the myocardium. In addition, inflammatory cytokine and reactive oxygen species can also cause direct damage to the myocardium due to the cytotoxic effects [17]. The insignificant increase of cTnI levels in the present study may be due to the less extensive and permanent cardiac damage. This hypothesis is also supported by the degree of inflammation data, in which all groups only had a degree of 0-1. In a rat model of doxorubicin-induced chronic myocardial injury, cTnI levels were found to be positively correlated with myocardial pathological injury [40]. In another study evaluating isoproterenol administration in Wistar rats, 6 rats showed minimal to mild myocardial damage with no increase in serum cTnI 8 to 24 hours after induction [41].

In this study, *S. rhombifolia* leaf extract at a dose of 200 mg/kg BW was the most effective in regulating the inflammatory process in rat cardiac injury induced by high-fat-sucrose diets and CCl_4. The dose significantly increased IL-6 production and reduced inflammation. Based on qualitative phytochemical analysis, *S. rhombifolia* leaf extract was found to be positive for various compounds such as flavonoids, saponins, alkaloids, phenolics, tannins, glycosides, and steroids. Flavanoid has been identified as the compound responsible for the anti-inflammatory properties in several plant extracts [42-44]. Flavonoids are known to inhibit the activity of NFκB transcription factors, so they can modulate the expression of pro-inflammatory-related genes, leading to inflammatory responses that underlie various pathologies of cardiovascular disease [45-46]. In this study, the flavonoid content in *S.*

rhombifolia may have played a role in inhibiting NFκB activation induced by a high-fat-sucrose diet and CCl_4, thus preventing the inflammatory process in the cardiac muscle cells. However, there are still controversial results regarding the functions of IL-6.

The main limitation of this study is that it only analyzed one inflammatory marker (IL-6). In cardiovascular disease, one biomarker cannot reflect all the pathogenesis processes involved in complex disorders, so it is recommended to also analyze other cytokines and biomarkers to generate more relevant biological information. In addition, this study only focused on structural abnormalities of the heart muscle by assessing the histopathological imaging and troponin level, which is a marker of cardiac muscle injury in the blood. However, this study could not yet analyze disturbances in cardiac functions. We also did not evaluate the biological components of the *S. rhombifolia* extract.

5. Conclusion

Sida rhombifolia extract provided cardioprotection by altering the degree of myocardial inflammation and mRNA expression of IL-6 in rat cardiac injury induced by a high-fat-sucrose diet and CCl_4. For future studies, other inflammatory biomarkers can be evaluated, such as TNFα and IL-1 as pro-inflammatory markers or IL-10 as anti-inflammatory markers. In addition, future studies can also evaluate the effect of *S. rhombifolia* extract on rat cardiac functions by examining the electrocardiogram/ECG, blood pressure, or echocardiography.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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