

CONSUMPTION OF *Hibiscus sabdariffa* DRIED CALYX ETHANOL EXTRACT IMPROVED REDOX IMBALANCE AND GLUCOSE PLASMA IN VITAMIN B12 RESTRICTION DIET IN RATS

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ABSTRACT

This study aimed to evaluate the effect of *Hibiscus sabdariffa* L. dried calyx ethanolic (HSE) extract on the redox imbalance and glucose plasma of vitamin B12 restriction Sprague-Dawley rats. The rat was fed a Vitamin B12 Restriction Diet for 16 weeks and treated with HSE as the treatment group. One group was fed a restriction diet not treated with HSE as a positive control group, and another was fed a control chow diet not treated with HSE as a negative control group. Vitamin B12, MDA, SOD activity enzyme, and glucose levels were evaluated in this study. The extract had a significant antioxidant capacity in terms of reducing the production of free radical scavenging activity. The HSE can repair the skewed redox imbalance and oxidative damage generated by a vitamin B12-deficient diet in the rat liver. The result of fasting glucose plasma levels, in 16 weeks showed both significantly different in all groups. In Sprague-Dawley rats with vitamin B12 restriction treated with HSE, a decrease in the glucose plasma in week sixteen was observed, together with a reduction of hepatic redox imbalance. Treatment with HSE protected hepatocytes from oxidative damage caused by vitamin B12 deficiency.

Key words: Fasting glucose, MDA, rosella, SOD, Vitamin B12

INTRODUCTION

As part of the metabolic process, cells produce free radicals and reactive oxygen groups (reactive oxygen species/ROS). Malnutrition can produce oxidative stress, yet the molecular routes for this illness have not been thoroughly investigated. Vitamin B12 is a micronutrient that is infrequently investigated yet has a substantial impact on cellular damage.

Homocysteine is a key component in the methionine metabolism involving vitamin B12 (Kayhan *et al.*, 2020). The intracellular build-up of homocysteine occurs when methionine metabolism is impeded due to vitamin B12 deficiency, resulting in the rise in plasma homocysteine levels. Due to the toxic effect of homocysteine, vitamin B12 deficiency causes metabolic problems at the cellular level. Homocysteine is a hazardous amino acid that accumulates in cells due to the production of sulfhydryl groups, resulting in free radical products and protein misfolding (Pusparini *et al.*, 2020). Increased homocysteine is linked to oxidative stress, which occurs when the generation of ROS and antioxidant defenses are out of balance.

of ROS and antioxidant defenses are out of balance. This imbalance can cause biomolecules to oxidize, changing their molecular structure and function.

ROS is made up of superoxide anion, hydrogen peroxide, and hydrogen radicals, among other things. A tiny amount of ROS serves as a natural defensive mechanism against infections. However, an overabundance of ROS will disrupt cell function by causing cell damage through biomolecular interactions. When free radical production outnumbers cellular antioxidant defenses, oxidative stress can develop (Van Berkel *et al.*, 2021).

Malondialdehyde (MDA) is formed when free radicals combine with epithelial cell membrane phospholipids during lipid peroxidation. Antioxidant molecules in the body, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase, act to reduce MDA production (Zhang *et al.*, 2018). Vitamin B12 deficiency leads to an increase in intracellular homocysteine and also increases MDA levels. Endogenous antioxidants, such as SOD are primarily responsible to prevent MDA formation. Since the current research on vitamin B12 deficiency has not addressed the issue of toxicity

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to cells caused by ROS increase, particularly in liver tissue (Mathukumalli *et al.*, 2020), this study focused on the effect of an increase in homocysteine.

The use of natural plant substances to restore the various physiological impairments in the body caused by vitamin B12 deficiency is still a new topic of study. Plant compounds with antioxidant potential have also been shown to heal physiological damage induced by the increased ROS generation based on the scientific findings. Surprisingly, natural plant products are widely available in numerous places and it is cheap. However, there is still a lack of scientific evidence to back up the usefulness of these native plants and mixtures. The Malvaceae family, *Hibiscus sabdariffa* L., also known as roselle, is a succulent perennial herb that grows to a height of 0.5-3 meters and blooms throughout the year. The stems are red, round, upright, and woody. Single ovoid leaf with a finger-boned tip, jagged edges, and a grooved base. The leaves have 6-15 cm long and 5-8 cm wide. Local communities use roselle due to its therapeutic benefits in traditional medicine, such as relieving hypertension (Mojica *et al.*, 2012; Paramita *et al.*, 2020).

As previously demonstrated, roselle's petal extract contains a considerable phytochemical and nutritional composition. According to phytochemical analysis, roselle is high in antioxidants and organic acids. In this present work, the antioxidant activity of roselle petal extract against damage in rat liver was explored by investigating the therapeutic potential of this plant to give scientific support for its usage as a medicine. This study aimed to evaluate the effect of *H. sabdariffa* dried calyx ethanolic (HSE) extract on the redox imbalance and plasma glucose of vitamin B12 restriction.

MATERIALS AND METHODS

Extraction and purification

The sample was identified as roselle (*H. sabdariffa*) by the Laboratory of the Center for Biopharmaca Studies, IPB University, Bogor-Indonesia. The roselle's flower petals were cut into pieces, followed by the drying process, and ground into a simplicia powder. The extract was made using the maceration method from approximately 100.0 grams of Simplicia powder in 750 mL of 70% ethanol for five days under constant stirring and dark conditions. The maceration results were then thickened using a vacuum evaporator at 50-70°C to make a thick extract. As much as 1000 mL can be extracted. The juice was then covered and left in a cool and dark condition until a thick extract was received. The precipitate was then separated, concentrated, and evaporated by a rotary evaporator (Ulfah & Wahyuningrum, 2010).

Experimental animal

The study was an *in-vivo*, randomized

experimental study using male Sprague-Dawley rats aged 36-40 weeks. In this research, a total of 15 rats were divided into three groups. The 1st group was normal to control that received only a 58MI diet as a standard diet. The 2nd group was a positive control that received a 9GN8 diet, a vitamin B12 diet restriction. The 3rd group was a treatment group that received a 9GN8 diet (vitamin B12 restricted) 10 mg/100 gBW diet and roselle's extract 400 mg/kg BW/day orally using an intragastric tube. This dose was administered following previous research using roselle extract at 400 mg/kg/BW/day (Andraini & Yolanda, 2015) for 16 weeks. The 5G1R was a modification of TestDiet® AIN-93M semi-purified diet, 58M1, with 5% pectin (replacing some cellulose & cornstarch), and without vitamin B12. While the 58MI is the TestDiet® AIN-93M maintenance purified diet, which is the maintenance diet for rodents recommended by the American Institute of Nutrition. The 58MI was formulated to substitute for the previous version (AIN-76A) to improve animal performance.

All rats were kept in a temperature and light-controlled environment, housed in a ventilated micro insulator cage under 12 h light and dark cycle with free access to feed and water and *ad libitum* fasting. Animals were acclimatized to the cage for one week before treatments. All animals received human care and the change in body weight was measured weekly.

Organ harvesting and homogenization

The blood was taken from the supraorbital sinus at the given time after fasting at the 4th, 8th, 12th, and 16th weeks. Before homogenization, the frozen rat liver was resuspended in phosphate-buffered saline (PBS). 9 mL of PBS was mixed with 1 gram of liver tissue and homogenized on ice. The homogenate was then centrifuged for 5 min at 5000 g at 40 °C to obtain the supernatant.

Biochemical parameters

The MDA and SOD activity in the liver were measured in the homogenate. An ELISA standard kit (Rat vitamin B12 ELISA, FineTest, China) was used to determine the amount of Vitamin B12 in the liver homogenate using the procedure recommended by the kit. The thiobarbituric acid reactive substances (TBARS) were measured using spectrophotometry. As much as 1 mg of protein was added to 1.5 mL of the reaction mixture containing 250 mM mannitol, 6 mM α -ketoglutarate, 0.2 mM FeCl₃, 2 mM ADP, and 10 mM Hepes buffer (pH 7.2). The reaction mixture was incubated at 37 °C for 20 and 40 min in a shaker. Then 0.5 mL of this mixture was transferred into 2 mL of media containing 15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.25 mM HCl, and 0.01 % butylhydroxytoluene. This suspension was incubated for 15 min at 95 °C and then centrifuged for 10 min at 3000 rpm. TBARS in the supernatant

was measured by using spectrophotometry at 532 nm

The SOD activity was measured using the Ransod kit (Randox Labs. Paint. No. SD 125, Crumlin, UK). The procedure to determine the SOD activity followed the kit. Blood samples were taken from the tail vein of rats for blood glucose measurement. Blood glucose measurement was carried out using a portable glucometer (Accu-Check Advantage Performance, Roche Diagnostics, Germany) (Andraini & Yolanda, 2015).

Statistical analysis

The data of vitamin B12, MDA, SOD activity, and glucose levels are expressed in Standard Error of Means (SEM). The significance of the differences was analyzed with a Tukey's Post Hoc test or Kruskal-Wallis H test and Mann-Whitney test ($\alpha = 0.05$; 0.01; & 0.001). The glucose level was analyzed using two ways repeated ANOVA. The statistical analysis was performed using SPSS software (IBM).

RESULTS

Figure 1 shows the effect on liver vitamin B12 produced by daily administration of the ethanolic extract of *H. sabdariffa*. A significant difference occurred in the 16th week ($p < 0.01$), wherein the posthoc test showed a significant difference between the normal control and positive control group (13.4 ± 1.9 vs 7.6 ± 0.3 ng/dL; $p < 0.05$) and between positive control with treatment group (7.6 ± 0.3 vs 10.3 ± 0.5 ng/dL; $p < 0.01$).

The findings in Figure 2 demonstrated that rats fed with a vitamin B12-deficit diet had significantly higher MDA levels in their hepatocytes. MDA level was decreased in treatment with various doses of *H. sabdariffa* ethanolic extract. A significant difference in MDA levels occurred in the 16th week ($p < 0.001$), wherein the posthoc test showed a significant difference between the normal control and positive control group (1.9 ± 0.05 vs 3.5 ± 0.09 nmol/mL; $p < 0.001$) and between normal control and treatment group (3.5 ± 0.09 vs 2.3 ± 0.02 nmol/mL; $p < 0.001$).

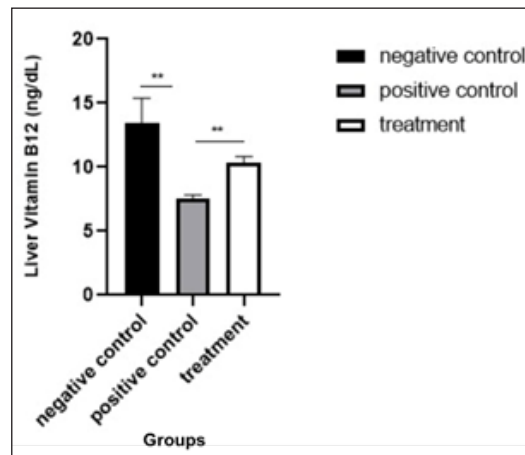


Fig. 1. The effect of HSE on liver Vitamin B12. Mean \pm SE ($n=5$); Normal control: 58M1 control diet; Positive control: 5GIR with restricted vitamin B12; Treatment: 5GIR diet with restricted vitamin B12 and HSE 400 mg/kg of weight. * $p < 0.05$, ** $p < 0.01$.

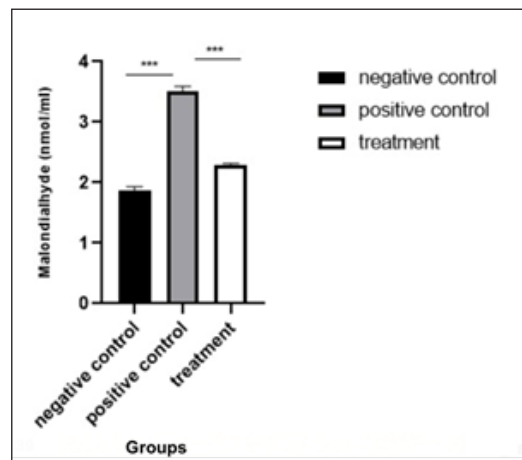


Fig. 2. The effect of HSE on liver malondialdehyde (MDA). Mean \pm SE ($n=5$); Normal Control: 58M1 control diet; Positive control: 5GIR with restricted vitamin B12; Treatment: 5GIR diet with restricted vitamin B12 and HSE 400 mg/kg of weight. ** $p < 0.01$, *** $p < 0.001$.

Figure 3 shows the effect on SOD activity in liver cells produced by daily administration of the HSE. SOD activity was increased after *H. sabdariffa* ethanolic extract treatment. A significant difference in SOD activity occurred in the 16th week ($p < 0.01$), wherein the posthoc test showed a significant difference between the normal control and positive control group (5.4 ± 0.2 vs 4.2 ± 0.2 U/mg protein; $p < 0.01$) and between normal control and treatment (4.2 ± 0.2 vs 6.1 ± 0.1 U/mg; $p < 0.001$).

Figure 4 shows the effect on plasma glucose levels produced by daily administration of HSE. The difference in baseline glycemia (day 0) was not significant between groups ($p > 0.05$). A diet restricted in vitamin B12 increased blood glucose levels starting from the 8th week, wherein the posthoc test showed

a significant difference between the normal control and positive control group (70.7 ± 1.4 vs 82.9 ± 1.4 mg/dL; $p < 0.000$). The HSE significantly reduced glycemia in vitamin B12 deficit rats in the 12th week wherein the posthoc test showed a significant difference between the positive control group and treatment group (80.8 ± 0.9 vs 65.4 ± 2.9 mg/dL; $p < 0.001$) and 16th week (72.8 ± 2.0 vs 60.7 ± 1.2 mg/dL; $p < 0.001$). HSE significantly reduced weight gain in vitamin B12 deficit rats in the 12th week wherein the posthoc test showed a significant difference between the positive control group and treatment group (355.4 ± 6.5 vs 293.8 ± 14.1 gram ($p < 0.01$)) and 16th week (346.2 ± 5.8 vs 296.2 ± 11 gram ($p < 0.01$)) (Figure 5). The results also showed no correlation between plasma glucose levels and body weight (Figure 6).

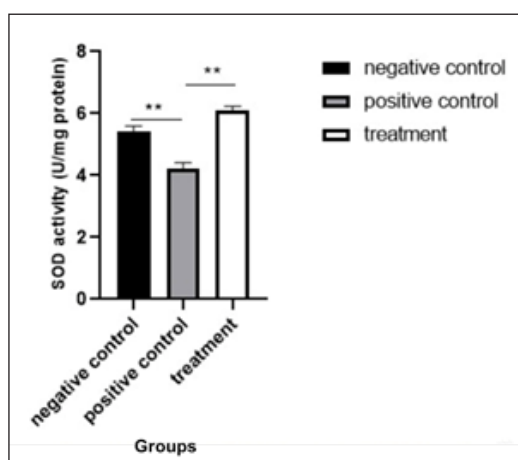


Fig. 3. The effect of HSE on SOD Activity. Mean \pm SE ($n=5$); Normal control: 58M1 control diet; Positive control: 5GIR with restricted vitamin B12; Treatment: 5GIR diet with restricted vitamin B12 and HSE 400 mg/kg of weight. ** $p < 0.01$, *** $p < 0.001$.

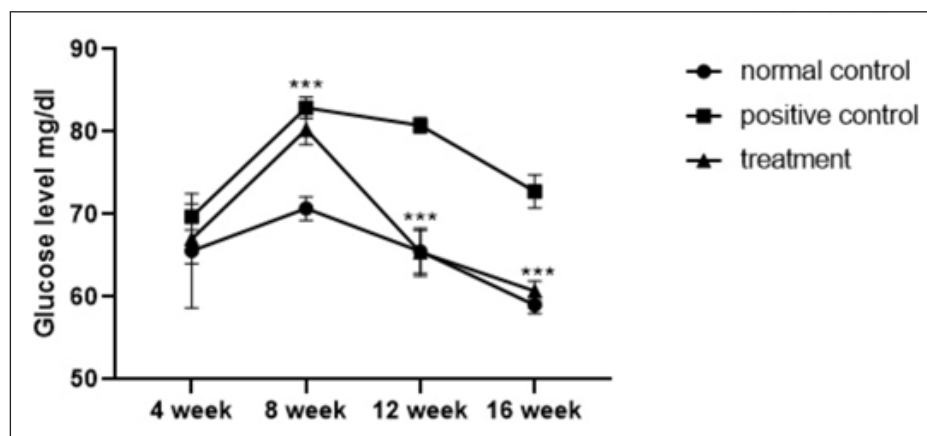


Fig. 4. The effect of HSE on plasma glucose levels. Mean \pm SE ($n=5$). Normal control: 58M1 control diet; Positive control: 5GIR with restricted vitamin B12; Treatment: 5GIR diet with restricted vitamin B12 and HSE 400 mg/kg of weight. ** $p < 0.01$, *** $p < 0.001$.

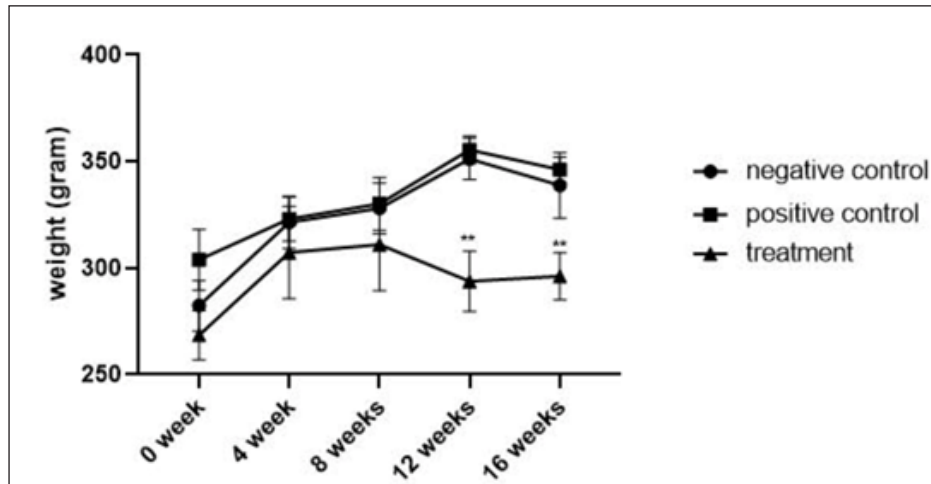


Fig. 5. The effect of HSE on body weight. Mean \pm SE ($n=5$). Normal control: 58M1 control diet; Positive control: 5GIR with restricted vitamin B12; Treatment: 5GIR diet with restricted vitamin B12 and HSE 400 mg/kg of weight. ** $p<0.01$, *** $p<0.001$.

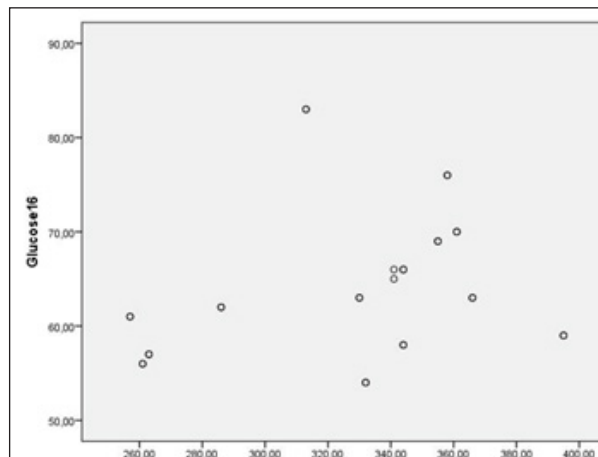


Fig. 6. The correlation between plasma glucose levels and body weight.

DISCUSSION

The antioxidant activity of *H. sabdariffa*'s ethanolic floral extract against oxidative damage caused by vitamin B12 deficiency in liver cells was studied in this work. The existence of bioactive components in the flower extract of *H. sabdariffa*, which are responsible for antioxidant capability, has been shown in various studies and varied results were observed. Numerous studies showed that *H. sabdariffa*'s flower petals are rich in polyphenols, such as anthocyanins, flavonoids, and phenolic acids (Chang *et al.*, 2014; Ojulari *et al.*, 2019). *H. sabdariffa* also contains gallic acid, chlorogenic acid, caffeic acid, quercetin, and tiliroside, which have been shown to reduce hyperglycemic dyslipidemia-related metabolic disorders (Ahn *et al.*, 2008; Cho *et al.*, 2010; Goto *et al.*, 2012).

The animals were fed a vitamin B12-deficit diet to induce oxidative stress *in vivo*. Humans are also susceptible to this illness, particularly those in

high-risk categories such as vegans, the elderly, or individuals who use metformin daily (Li *et al.*, 2020). Vitamin B12 is needed in the metabolic process of methionine change, where vitamin B12 acts as a cofactor for the conversion of homocysteine to methionine. If there is a deficiency of vitamin B12, it results in a buildup of intra-cell homocysteine. Homocysteine accumulation results in toxic conditions, one of which produces oxidation stress conditions (Mahalle *et al.*, 2013; Sianipar *et al.*, 2019). Homocysteine accumulates in the liver after entering the body's physiological system, disrupting the body's physiological equilibrium between pro-oxidant and antioxidant elements (Wolffenbuttel *et al.*, 2020). This serious condition results in an aberrant rise in pro-oxidant components, like MDA, and a consequent decrease in endogenous antioxidants like SOD. This generates a redox imbalance in the physiological system, resulting in oxidative damage to numerous body organs, including the

including the liver and kidney (Awoke *et al.*, 2020). Furthermore, the increase in the formation of free radicals and reactive oxygen species (ROS), the majority of which are unpaired electron species that target important biomolecules in their search for electrons, is triggered by the accumulation of homocysteine in the body. Although these ROS have certain positive functions, mostly as signaling molecules, their negative consequences are more common when they are created in large amounts (Gao *et al.*, 2021). Our findings demonstrate a redox imbalance in rats fed with a vitamin B12-deficit diet, advancing our understanding of the mechanism of oxidative damage in this condition. In rats with vitamin B12 deficiency, treatment of *H. sabdariffa* extract had the effect of regulating this redox imbalance state caused by the overproduction of ROS. Although numerous factors influence the redox imbalance in the biological system, such as inflammatory mediators and transcription factors, we established these in this work utilizing multiple *in vivo* antioxidant biomarkers (MDA, GSH, CAT, & SOD) in liver cells (Kim *et al.*, 2020; Al-Alawi *et al.*, 2021).

Excessive ROS generation in physiological systems causes lipid peroxidation, which leads to the development of MDA (Zhang *et al.*, 2018). As a result, MDA is a powerful signal of increased lipid oxidation as a result of increased amounts of free radicals in the form of ROS and oxidative stress in the body. It is a key indicator of oxidative stress in the body and it's been linked to several ailments (Delli Bovi *et al.*, 2021). Rats on a vitamin B12-deficit diet probably produced a large ROS in their physiological systems, leading to higher MDA levels as compared to the control group. On the other hand, the bioactive antioxidant components in the extract may have scavenged these ROS, restoring the redox balance and resulting in a considerable drop in MDA level and the restoration of liver dysfunction indicators in HSE-treated animals (Zuniga-Munoz *et al.*, 2013). The findings of this study are consistent with previous studies, which found that oxidative stress might cause a rise in MDA in experimental animals. HSE, on the other hand, can lower the MDA level in rats.

Superoxide dismutase, an endogenous enzymatic antioxidant that scavenges free radicals in the physiological environment, was also observed to increase. According to Ighodaro & Akinloye (2018), superoxide dismutase is the first-line antioxidant defense system that aids in the detoxification of endogenously produced hydrogen peroxide and superoxide in the body. The HSE-treated rat had lower activity of this antioxidant enzyme, which could increase the redox imbalance in hepatocyte cells according to our findings. The extract's apparent ameliorative activity against vitamin B12 deficiency circumstances reported in this study could be related to its action against different free radicals formed

in vivo as a result of the harmful process caused by homocysteine accumulation in the liver. The extract's bioactive components may quench free radicals and improve the effectiveness of the liver detoxification system by their antioxidant capacity.

Hyperglycemia was also discovered in rats fed a vitamin B12-deficit diet in this investigation. Oxidative stress is one of the factors that contribute to this occurrence. Insulin resistance in peripheral tissues is caused by ROS due to the homocysteine accumulation, which affects multiple sites in insulin receptor signal transduction, resulting in the decreased expression of the GLUT4 transporter in cellular membranes. Desensitization of cells to glucose has a systemic effect. The antioxidants in the *H. sabdariffa* extract can overcome this problem. The findings of this study are consistent with other studies, which found that oxidative stress might cause hyperglycemia in experimental animals (Wang *et al.*, 2021). HSE, on the other hand, can improve plasma glucose levels in rats. This was in line with research by Mohamed *et al.* (2012) who found that even the active components found in HSE content can help to heal the harm caused by the increased ROS production.

CONCLUSION

The ethanolic flower extract of *H. sabdariffa* contains important bioactive components, which may responsible for the strong antioxidant capacity. We also found that a 400 mg/kg extract dose reduced hepatic redox imbalance and protect hepatocytes from oxidative damage caused by vitamin B12 deficiency. HSE also decreased the glucose plasma in the 16th week of treatment. Further research into the extract's molecular and mechanistic foundation will improve the likelihood of it being employed to treat disorders caused by vitamin B12 deficiency in humans.

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ETHICAL STATEMENT

This research was approved by the Research Ethics Committee experimental procedures-Faculty of Medicine Universitas Indonesia/Cipto Mangunkusumo Hospital (FKUI/RSCM) with certificate number: KET-540/UN2.F1/ETIK/PPM.00.02/2020.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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