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Antioxidant Activity of *Zingiber cassumunar Rhizome, Guazuma ulmifolia* Leaves and Their Combination in High-Fat Diet-Fed Rats

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ABSTRACT

Oxidative stress is related to several metabolic diseases, including hyperlipidemia. Previous studies have shown the antioxidant properties of *Zingiber cassumunar* rhizomes and *Guazuma ulmifolia* leaves. The objective of this study was to examine the antioxidant activity of *Z. cassumunar* rhizomes, *G. ulmifolia* leaves extracts, and their combinations in high-fat diet fed (HFD) rat. *Z. cassumunar* rhizomes and *G. ulmifolia* leaves was extracted in a tiered pot for 30 min, then evaporated in a freeze dryer to obtain dry extracts. The Wistar rats were assigned into six groups at random: normal, positive, negative, *Z. cassumunar* rhizome extract at 400 mg/kg BW (ZC); *G. ulmifolia* leaf extract at 50 mg/kg BW (GU), and the combination of *Z. cassumunar* rhizome extract and *G. ulmifolia* leaf extract at (350+50) mg/kg BW (ZC+GU). The rats were fed HFD for 28 days, and the treatment groups also received the extracts for the last 14 days. The combination ZC+GU increased the endogenous antioxidant activity namely catalase (CAT), and glutathione peroxidase (GSH-Px) significantly ($p<0,05$), but the increasing of SOD activities (T-SOD, CuZn-SOD and Mn_SOD) are not significant ($p>0.05$). The metabolite oxidation, malondialdehyde (MDA) was found to decrease. The single treatments of ZC and GU give the better activities than the combinations. The conclusion of the study showed that ZC, GU, and ZC+GU could increase endogenous antioxidant activity to inhibiting oxidative stress, but the combination did not show the synergistic effect.

Keywords: Antihyperlipidemic, Antioxidant, High Fat Diet, *Guazuma ulmifolia, Zingiber cassumunar*

INTRODUCTION

Oxidative stress is a major contributor to the development of chronic disease, such as cardiovascular disease, cancer, and neurodegenerative disorders. Dietary factors, such as high-fat diet, can increase oxidative stress as a result of lipid peroxidation (Borza *et al*., 2013). Long term of high-fat diet stimulates the lipid oxidation and results the elevation of reactive oxygen species and was reported to contribute in metabolic syndrome (Lasker *et al*., 2019).

Low density lipoprotein (LDL) accumulation in the blood vessels due to a high-fat diet causes the modification of LDL to oxidized-LDL (Ox-LDL), resulting in oxidative stress (Amiya, 2016). Lipid peroxidation, protein oxidation, and DNA alterations are among the potential side effects of oxidative stress (Chen *et al*., 2012).

Lipid peroxidation has secondary products in the form of aldehyde compounds, including malondialdehyde (MDA). MDA is a marker for cellular free radicals induced cell damage. The more free radicals present, the more MDA is produced (Ayala *et al*., 2014). In addition, the presence of free radicals in the body results in decreased lipoprotein lipase (LPL) activity. Endogenous antioxidants including glutathione peroxidase, catalase, and superoxide dismutase

(SOD) can neutralize free radicals. (GSH-Px) (Lubos *et al*., 2011). They also reported the important role in pathogenesis some degenerative diseases (Khdair & Abdulridha, 2021).

Zingiber cassumunar rhizomes and *Guazuma ulmifolia* leaves have been widely studied for their antioxidant potentials. *Zingiber cassumunar* rhizome and *Guazuma ulmifolia* leaves are plants with strong antioxidant properties. Previous studies have shown that extracts of these plants can protect cells from oxidative damage (Silva *et al*., 2010) Previous research reported the increase of antioxidant activity of hyperlipidemic rats treated by *Z. cassumunar* rhizomes extract at 200 and 400 mg/kg BW. Following treatment, the SOD activity was found to increase (Sari *et al*., 2020). *Z. cassumunar* rhizome extract also exhibits an immunomodulatory activity by increasing the secretion of reactive oxygen intermediate (ROI) (Nurkhasanah *et al*., 2019). *G. ulmifolia* leaf extract at a dose of 125 mg/kg BW reduces lipid peroxide by increasing SOD activity (Berenguer *et al*., 2007).

The effectivity of polyherbal combination has been reported in some studies. The polyherbal combination was found to effective in treatment of metabolic syndrome (Palla *et al*., 2021). Clinically study also showed that polyherbal combination improve the glycemic control and lipid profile in hyperlipidaemic diabetes patiens (Shokoohi *et al*., 2017).The synergistic effect of polyherbal combination also was reported by enhancing therapeutic efficacy and minimizes side effects by reducing individual herb dosages in antihyperlipidemic and antihyperglicaemic animal models (Alhamhoom *et al*., 2023). The existing literature has shown that *Z. cassumunar* rhizomes and *G. ulmifolia* leaves have antioxidant potential. However, no study has investigated the combined antioxidant activity of these two extracts in a high-fat diet-induced rats model. The present study is the first study in investigating the combined antioxidant activity of *Z. cassumunar* rhizomes and *G. ulmifolia* leaves in a high-fat diet-induced rats model. The combination of both extracts was expected to give better results and benefits in inhibiting oxidative stress. The results of these study will provide valuable information on the potential use of these two extracts in the prevention and treatment of oxidative stress-related disease.

MATERIALS AND METHODS

Plant Material and Extract Preparations

Z. *cassumunar* rhizomes were obtained from a Yogyakarta market, while *G. ulmifolia* leaves were from Merapi Farma Herbal, Yogyakarta, Indonesia. Z. *cassumunar* rhizome and *G. ulmifolia* leaves were authenticated in the Biology Laboratory of Ahmad Dahlan University, Yogyakarta, with the identification number 239/Lab.Bio/B/X2020. *Z. cassumunar* rhizomes were rinsed with water and chopped into pieces. *G. ulmifolia* leaves were sorted and washed. Both samples were dried at 50°C in an oven (Sari *et al*., 2020), before being extracted using a distilled water solvent with a ratio of 1:5 (w/v) in a tiered pot for 30 min. The extract was concentrated after being evaporated in a vacuum rotary evaporator, followed by drying in a freeze dryer.

Animal

The experimental animals were 30 male Wistar rats at eight weeks old and weighing 150–250 g. Male rat was chosen to minimize hormonal impacts during the study. Animals were maintained in a room with good ventilation and a 12-hour cycle of light and dark and regulated humidity and room temperature. All experimental rats were acclimatized to the new habitat for seven days prior to treatment. The current study protocol has been authorized by Research Ethics Committee of Universitas Ahmad Dahlan with the approval reference 012009049.

Materials

The reagents for SOD, CAT, GSH-Px, and MDA measurements were obtained from Elabscience. All other materials used for analysis were analytical-grade and obtained from Merck

Induction of Hyperlipidemia in Rats

The experimental rats were fed with HFD, consisting of 300 g standard pellet (BR II), 20 grams of chicken egg yolk, 100 grams of butter, 10 grams of beef fat, and 0.05 grams of propylthiouracil (PTU). The mixture was molded into pellets and dried. The HFD inductions were given for 28 days; the experimental animals in all groups were fed 15 g/day with ad libitum access to water. For the first 14 days, all groups received HFD feed, and then for another 14 days, the treatment group's diet was combined with the tested extracts.

Experimental Design

The six groups of five rats each were randomly selected from among the thirty rats: Group 1: normal group, which was given standard feed; Group 2: negative control or HFD group, which was given HFD feed; Group 3: positive control,

which was given HFD and Nutrive Benecol at 1,8 mL/200g BW two times a day. The previous study have reported the effectivity of Nutrive Benecol in lowering cholesterol total and HDL-cholesterol in a clinical trial (Lestiani *et al*., 2018); Group 4: ZC group was given HFD feed and Z. *cassumunar* rhizome extract at 400 mg/kg BW; Group 5: GU group was given HFD feed and G. *ulmifolia* leaf extract at 50 mg/kg BW; Group 6: ZC+GU group was given HFD feed and a combination of Z. *cassumunar* rhizome extract and G. *ulmifolia* leaf extract (350:50 mg/kg BW).

The sample was dissolved in 0.5% Na-CMC and given orally using a probe. This study used a Nutrive Benecol as the positive control. A previous study showed that this beverage effectively reduced total cholesterol and LDL-cholesterol of subjects with hyper cholesterol after two weeks of treatment (Lestiani *et al*., 2018). During these 28-day treatments, the bodyweight of the rats was measured every week. Then, on Day 29, the animals were euthanized using CO_2 (Canadian Council of Animal Care, 2010) and dissected to collect the liver organ.

Preparation of the Liver Tissue Homogenate

The liver tissue was weighed, chopped into small pieces, then homogenized in PBS (0.01 M, pH 7.4) while it was chilled. The ratio of tissue weight (g) and PBS (mL) used was 1:9. After homogenizing the liver tissue, the mixture was centrifuged at 10,000 g for 10 min at 4°C. Following separation, the supernatant was utilized to measure the MDA level, SOD, CAT, and GSH-Px activity. To assess protein concentration in the homogenate, the Bradford assay was performed (Walker, 1996).

Measurement of SOD Activity

The assay kit (E-BC-K022-S) from Elabscience was used to measure the SOD activity in accordance with the manufacturer's instructions. The T-SOD (Total SOD) enzyme activity was evaluated in this test. T-SOD is cumulative total of CuZn-SOD and Mn-SOD. The SOD enzyme in this sample is specifically inhibited by superoxide anion (O_2^-), which is produced by the xanthine and xanthine oxidase systems and oxidizes hydroxylamine to produce nitrite. Following interaction with the chromogenic reagent, nitrite turns purplish. The difference between the absorbance values of the control and sample, which was measured at 550 nm, indicates the presence of SOD activity.

Measurement of Catalase Activity

The assay kit (E-BC-K031-S) from Elabscience was used to measure the CAT activity, as directed by the manufacturer. CAT enzyme functions by degrading H_2O_2 into H_2O. A yellowish complex is created by the interaction of the residual H2O2 with the ammonium molybdate. The CAT enzyme activity was determined by measuring the absorbance at 405 nm and comparing it to the control and sample.

Measurement of Glutathione Peroxidase (GSH-Px) Activity

The assay kit (E-BC-K096-S) from Elabscience was used to measure glutathione peroxidase (GSH-Px) activity as directed by the manufacturer's protocols. The 0.1 mL of the liver tissue homogenate and 0.1 mL of 1 mmol/L GSH were added. A tube containing the stock solution was heated in a water bath at 37°C for 5min. Following incubation, the solution was mixed with 0.05 mL of the heated stock solution and then reacted for five min in a 37 °C water bath. After that, a mixture of 1 mL of acid reagent was centrifuged at 3,100 g for 10 min. Following that, 0.5 ml of the supernatant was taken and combined with 0.5 ml of phosphate, 0.125 mL of dinitrobenzene (DTNB), and 0.025 ml of salt reagent in that order. The mixture was then mixed and let at room temperature for 15 min. The mixture's absorbance was then measured with a spectrophotometer at 412 nm.

Measurement of Malondialdehyde (MDA) Level

MDA level was measured colorimetrically using the Elabscience assay kit (E-BC-K025-S) in accordance with the directions from the manufacturer. First, 0.1 mL the liver homogenate, 0.1 mL thiobarbituric acid (TBA) clarivant, 3 mL acid reagent, and 1 mL of the chromogenic agent were mixed and heated at 95 °C in a water bath for 40 min. After cooling, the mixture was centrifuged at 3,100 g for 10 min. The supernatant was then taken and analyzed at 532 nm using a spectrophotometer.

Statistical Analysis

The statistical analysis of the MDA levels, SOD, CAT, and GSH-Px activities was performed using ANOVA, and the Least Significant Difference (LSD) test was used to compare the treatment outcomes between the groups (a significant level of 95%). The SPSS software was used for the statistical analysis (Version 22).

RESULTS AND DISCUSSION

Effects of *Z. cassumunar* and *G. ulmifolia* Extracts on Body Weight

All tested rats gained body weight during the experiment, but HFD induced rats experienced significantly higher weight gain per week than normal groups ($p<0.05$) (Table I). The high fat di*et al*so reported to increase the hyperlipidaemia as increase the lipid level significantly (Mahfudh *et al*., 2021). After receiving treatments in the last two weeks of the experiment, the groups receiving ZC, GU, ZC+GU, or Nutrive Benecol showed to reduce weight gain compared to HFD group as negative control, although there is not significant ($p>0.05$).

Table I. Effects of *Zingiber cassumunar* and *Guazuma ulmifolia* extracts on weight gain caused by high-fat diets.

Group	Initial body weight (g)	Final body weight (g)	Weight gain (g/week)
Normal	185.08±22.17	193.48±17.49	2.10±0.53
Positive control	178.14±9.94	199.82±11.23	5.42±2.55
HFD	175.80±19.18	210.50± 18.33	8.68±3.71[b]
ZC	155.78±5.64	178.33±15.25	5.64±2.83
GU	182.88±18.62	203.4±27.15	5.14±2.72
ZC + GU	182.82±18.80	201.28±17.06	4.62±2.88

[b] significant change with the normal group ($p<0.05$)

Effects of *Z. cassumunar* and *G. ulmifolia* on Endogenous Antioxidants

The HFD induction on Wistar rats decreases the endogenous antioxidant (SOD), including T-SOD, CuZn-SOD and Mn-SOD (Figure 1). Superoxide Dismutase (SOD) is one of the enzymatic antioxidants and metalloenzymes because its activity depends on the metal cofactors Cu, Fe, Zn and Mn. The activity of T-SOD is similar to the cumulative activities of CuZn-SOD and Mn-SOD which were found to increase in both of ZC and GU groups, however Mn-SOD only increase in ZC group, indicating that CuZn-SOD activities had a greater impact on increasing endogenous antioxidant activity. The T-SOD activity increased in the ZC and GU groups when compared to HFD control. The combination of GU and ZC resulted in an elevation of SOD activity, although this increase was not statistically significant. The SOD activity in ZC+GU group were found higher than GU, but lower than ZC group. This study found that combination of two herbals is not synergistic. The similar result were found in combination of Taxacum officinale (TU) and Momordica charantia (MC), which showed the activity of glucose uptake of combination lower than single of TU, even the combination of TU+MC increase the inhibition of α-amilase and α-glucosidase (Perumal *et al*., 2022).

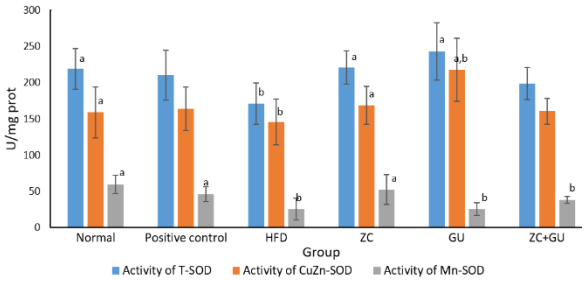

Figure 1. The Effect of *Z. cassumunar* and *G. ulmifolia* leaf extract treatment on high-fat died fed rat increase the T-SOD, CuZn-SOD and Mn-SOD activites, [a]demonstrated a significant change to negative control ($p<0.05$), [b]demonstrated a significant change to normal group ($p<0.05$).

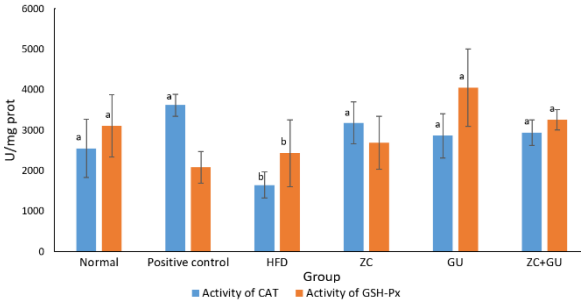

Figure 2. The Effect of *Z. cassumunar* and *G. ulmifolia* leaf extract treatment on high-fat died fed rat increase CAT and GSH-Px activities, [a]demonstrated a significant change to negative control ($p<0.05$), [b]demonstrated a significant change to normal group ($p<0.05$).

The activity of CAT, and GSH-Px endogenous antioxidant were also found to decrease significantly ($p<0.05$) following HFD induction. The previous study also found similar result which showed the correlation between the increasing of lipid and the decreasing of endogenous antioxidant activities (Mahfudh *et al*., 2022).The treatment of ZC, GU, and ZC+GU combination increases the CAT activity significantly ($p<0.05$). But the activity of GSH-Px were only found to increase significantly in GU group and GU+ZC combination. Increasing of GSH-Px activity in ZC group was found not significant. It is showed that ZC extract antioxidant activity was not involve in GSH-Px activity, meanwhile through the CAT and SOD activity (Figure 2).

The present study found that GU and ZC have different mechanism in antioxidant activity. Extract of ZC were found to increase CAT activity but did not affect the GSH-Px activity. The GU extract were found to increase the CAT and GSH-Px activities. The result of GU and ZC combinations were showed the additive effect of both.

A decrease in endogenous antioxidant activity was identified in HFD-fed rats, caused by the use of HFD and increased the amount of ROS produced. Hyperlipidemia increases reactive oxygen species (ROS) and leads to oxidative stress. The presence of high ROS can degrade polyunsaturated fats, which form MDA (Ayala *et al*., 2014). This study found that MDA levels in HFD-induced hyperlipidemic rats increased significantly than the normal group. Conversely, the ZC, GU extracts and the combination treatment could decrease the MDA level (Figure 3).

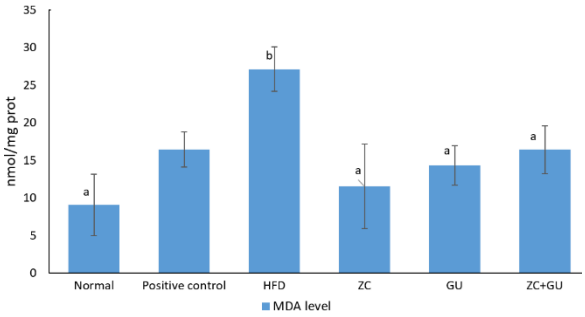

Figure 3. The Effect of *Z. cassumunar* and *G. ulmifolia* leaf extract treatment on high-fat died fed rat decrease MDA level, [a]demonstrated a significant change to negative control ($p<0.05$), [b]demonstrated a significant change to normal group ($p<0.05$)

This study found that extract of ZC and GU have antioxidant activity through increasing of endogenous antioxidant enzyme (SOD, CAT) and decrease the oxidative metabolite product (MDA). But the combination of both extract did not show better activities. The combination of two antioxidant did not give the synergistic effect.

A high lipid level in the body enhances fatty acid oxidation, increasing reactive oxygen species (ROS) levels (Sikder *et al*., 2018). The results have found that HFD increases the body weight of rats significantly ($p<0.05$) (Table I), which is followed by increasing in a high lipid profile in the blood. A high-fat diet have confirmed to increase the expression of HMG-CoA reductase in the liver organ and increase cholesterol synthesis (Thomas *et al*., 2012).

G. ulmifolia contains high flavonoid and phenolic levels (Duraiswamy *et al*., 2018; Rafi *et al*., 2020), potentially responsible for its antioxidant and antihypercholesterolemic properties. The phenolic contents, including tannin, can lower cholesterol levels because of their ability in micelles formation during the absorption process and reducing the absorption of cholesterol in the small intestine (De Jong *et al*., 2003; Acuff *et al*., 2007). Flavonoids suppress the formation of cholesterol in the liver through the inhibition of HMG-CoA reductase (Prahastuti *et al*., 2011) and lead to prevent the atherosclerosis (Lei & Yang, 2020). *G. ulmifolia* is also rich in saponins, which can inhibit the activity of pancreatic lipase enzymes and, thus, reduce fat absorption in the intestine (Han *et al*., 2000). Tannins are compounds that can lower cholesterol levels by binding and shrinking proteins through reactions with mucosal proteins and intestinal epithelial cells, inhibiting fat absorption (Sakaganta & Sukohar, 2021).

The active compounds of *Z. cassumunar* rhizome are phenylbutenoids, curcuminoids, and essential oils (Han *et al*., 2021). Curcumins can increase the expression of Cholesterol-7a-hydroxylase (CYP7A1), which turns cholesterol into bile acids before being excreted into the intestine (Kim & Kim, 2010). Excessive fatty acids in the blood and followed by accumulation in liver cells is a prerequisite for fatty degeneration (Dewi *et al*., 2019). Triglyceride accumulation increases TNFα and, subsequently, NADPH oxidase, which produces superoxide radicals that are extremely reactive to lipids, triggering lipid peroxidation and MDA formation (Ayala *et al*., 2014).

Fatty droplets and necrosis were still found in the group treated with *Z. cassumunar* rhizome extract. The flavonoids contained in *Z. cassumunar* rhizome may contribute to the inhibition of fat accumulation in the liver. Flavonoids can reduce lipids in the liver and increase antioxidant capacity through a mechanism related to inhibiting SREBP-1c and PPARγ activity in the liver (Wong-a-nan *et al*., 2018). *Z. cassumunar* rhizome extract plays a role in the activation of PPARγ in adipogenesis by suppressing the enzymes responsible for lipogenesis.

Meanwhile, the group receiving the combination treatment experienced less cellular damage compared to the negative control group.

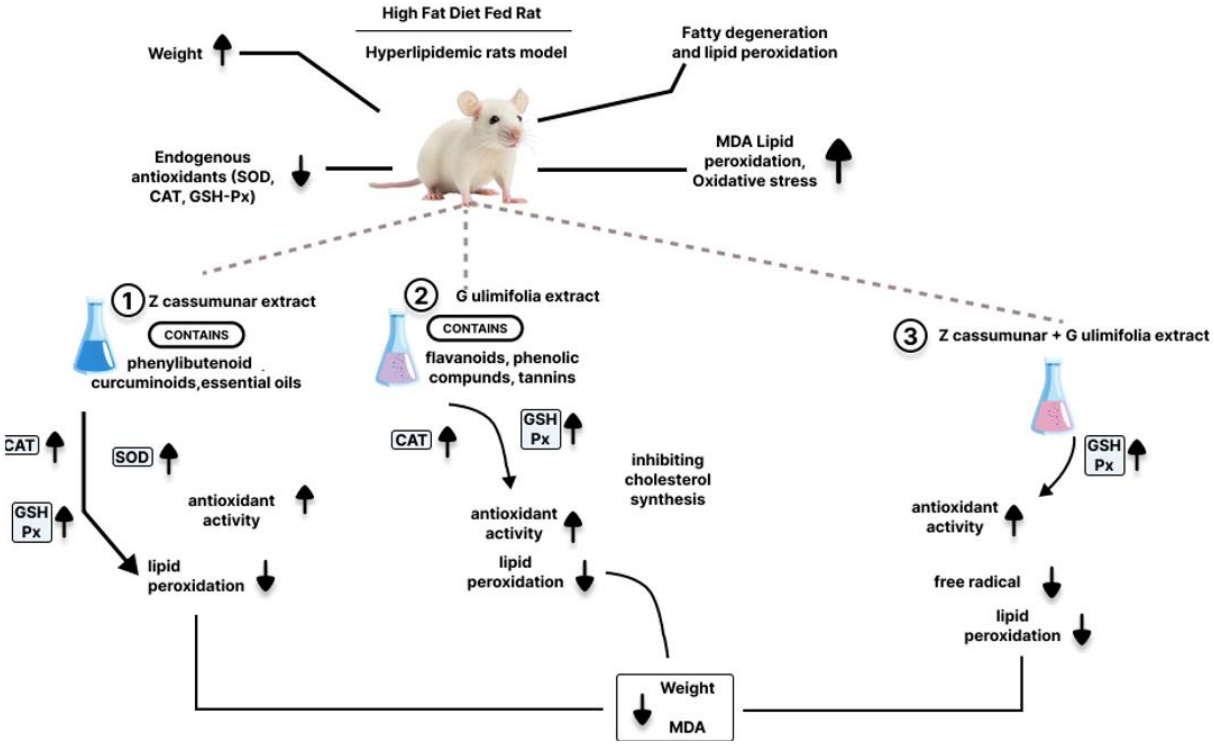

Figure 4. The flowchart of increasing endogenous antioxidant activity in high fat diet fed rat treated with Z. cassumunar and G ulmifolia extract

Combining the two antioxidants allows for a higher total antioxidant potent; this synergistic effect can properly reduce cell damage due to excessive free radicals. *G. ulmifolia* leaves contain flavonoids and tannins, potent antioxidant compounds (Prahastuti *et al*., 2020). Its combination with *Z. cassumunar* rhizome that contains other antioxidant compounds like kaempferol, phenylbutenoids, and curcuminoids (Rissanti *et al*., 2014), causes a synergistic effect in its antioxidant activity. The presence of a potent antioxidant in a combination can scavenge free radicals, suppress oxidation and lipid peroxidation, and increase antioxidant capacity (Sutaryono *et al*., 2016).

Endogenous antioxidants are the first-line defense that counters free radicals in the body. However, under the condition of excessive free radicals, they should be added with exogenous antioxidants (Zaetun *et al*., 2018). The treatment of ZC in HFD-induced hyperlipidemic rats increases the SOD, CAT, and GSH-Px activities significantly ($p<0.05$). The antioxidant activity of ZC was considered as high content of polyphenol compound (Marliani *et al*., 2014), phenylbutenoids, curcuminoids, and essential oils (Han *et al*., 2021).

SOD is the enzyme that decomposes superoxide radical (O_2^-) to hydrogen peroxide. The reaction will proceed by CAT and GSH-Px to decompose H_2O_2 into H_2O, which is fully nontoxic. SOD and its isoenzyme (Cu/Zn-SOD) and (Mn-SOD) were found to decrease in high fat diet animals (Skowron *et al*., 2018). The addition of antioxidative compounds in the ZC and GU extract could improve the enzymatic system.

Catalase (CAT) and Glutathione peroxidase (GSH-Px) are essential enzymes that catalyze the decomposition and conversion of hydrogen peroxide into hydroxyl radical groups, which will cause lipid peroxidation (Chen *et al*., 2012). High-fat food intake will increase hydrogen peroxide production in mitochondria (Rindler *et al*., 2013) and suppress the endogenous antioxidants. Treatment with ZC and GU extract in HFD-induced hyperlipidemic rats increases the antioxidative defense against oxidative stress resulting from lipid peroxidation in high fat diet rats.

Flavonoids found in *G. ulmifolia* leaves can scavenge the hydroxyl radical groups and inhibit the radical group from reacting with unsaturated lipids (Nuri *et al*., 2020). Daily ZC+GU intake

(*Z. Cassumunar* rhizome and *G. ulmifolia* leaf extracts at the dose ratio of 350:50 mg/kg BW) for 14 days after the HFD induction in rats has been found to increase GSH-Px activity significantly ($p<0.05$), compared with the HFD group. However, the resulting GSH-Px activity is lower than that of *G. ulmifolia* leaf extract. It means that the addition of *Z. cassumunar* rhizome extract has not significantly increased GSH-Px activities compared to the HFD group (Figure 4).

CONCLUSION

The extract of *Z.cassumunar* and *G. ulmifolia* inhibit the oxidative stress in high fat diet rats, however the combination of both extracts did not show the better effect. This finding show that combination of herbals should consider the mechanism of each component, to find the synergistic effect.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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