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Increasing endogenous antioxidant activity in hyperlipidaemic rats treated with functional powdered beverage containing *Zingiber cassumunar* Roxb., *Cinnamomum burmanni* (Nees & T.Nees) Blume, and *Glycine max* (L.) Merr.

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Abstract

High blood lipid levels can lead to oxidative stress and contribute to various diseases. *Zingiber cassumunar*, *Cinnamomum burmanni*, and *Glycine max* have been reported to possess potential antioxidant properties capable of inhibiting oxidative stress. Developing these herbs into a drinkable form offers the advantage of convenience, thereby potentially enhancing their therapeutic effects. The objective of the present work was to formulate a functional powdered beverage (FPB) containing *Z. cassumunar*, *C. burmanni*, and *G. max* to prevent oxidative stress in rats fed a high-fat diet. The FPB consisted of *Z. cassumunar*, *G. max*, and *C. burmanni* powder in a ratio of 75:23:2. The present work utilised 30 Wistar rats divided into six groups: (1) normal control (standard diet, no treatment); (2) negative control (HFD induction followed by standard diet); (3 - 5) treatment groups induced by HFD followed by FPB administration at doses of 1,000, 1,500, and 2,000 mg/kg body weight (BW); and (6) positive control (HFD induction followed by commercial smoothie drink 1 [CSD1]). HFD induction was performed for 28 d, and FPB treatment was administered from day 15 to 28, right after HFD induction which was carried out 14 d prior. At the end of the experiment, rats were sacrificed, and liver homogenates were analysed for endogenous antioxidant activity. The results showed that superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activities were significantly increased in FPB-treated groups compared to the negative control ($p < 0.05$). FPB treatment also significantly reduced malondialdehyde (MDA) levels ($p < 0.05$). These findings indicated that FPB might inhibit oxidative stress in hyperlipidaemic conditions by enhancing endogenous antioxidant activity, and reducing lipid peroxidation. In conclusion, the formulation of *Z. cassumunar*, *C. burmanni*, and *G. max* into a functional powdered beverage demonstrated antioxidative activity and potential for preventing oxidative stress in hyperlipidaemic conditions.

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Introduction

Hyperlipidaemia is a condition characterised by abnormally elevated lipid levels in the blood (Abbasi *et al*., 2024). Excessive lipid levels can induce oxidative stress, a state that arises from increased metabolic reactions involving oxygen, disrupting the balance between pro-oxidant and antioxidant systems (Sarıkaya and Doğan, 2020). The resulting oxygen free radicals trigger lipid peroxidation in cell membranes, generating peroxide radicals and other reactive species (Masenga *et al*., 2023). An overproduction of free radicals leads to an imbalance between oxidants and antioxidants, commonly referred to as oxidative stress. Elevated levels of free radicals can cause tissue damage (Gusti *et al*., 2021). Oxidative stress can be assessed by measuring lipid peroxidation products, such as malondialdehyde (MDA), in biological systems.

Antioxidant compounds can inhibit oxidative stress by stabilising free radicals, and supplying the electrons needed to neutralise them. Inhibiting the formation of free radicals also prevents the initiation of chain reactions (Yang *et al*., 2019). Many natural antioxidant sources are found in plant-based foodstuffs, which generally exhibit antioxidant activity due to their content of bioactive compounds, including flavonoids, phenolics, tannins, and anthocyanins (Duraiswamy *et al*., 2018). Formulating several herbs into a beverage provides the advantage

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of convenience, potentially improving therapeutic efficacy.

Previous studies have reported that *Zingiber cassumunar* exhibits antioxidant activity by increasing superoxide dismutase (SOD) activity in hyperlipidaemic rats (Sari *et al*., 2020). This activity may be attributed to its curcuminoid and phenylbutanoid contents (Nagano *et al*., 1997; Ramadhan *et al*., 2020; Nakamura *et al*., 2022). Isoflavones in *Glycine max*, as major flavonoids, possess antioxidant potential by scavenging free radicals, and preventing chain reactions (Hu *et al*., 2020). *Cinnamomum burmanni* contains cinnamaldehyde, eugenol, cinnamic acid, catechin, epicatechin, and other polyphenolic compounds, all of which contribute to its antioxidant properties (Tisnadjaja *et al*., 2020).

The present work thus aimed to formulate three potential herbs (*Z. cassumunar*, *G. max*, and *Cinnamomum burmanni*) into a functional powdered beverage for convenient use, with the expectation of enhancing antioxidant activity. Studies on herbal combinations are still limited, and formulating them into a beverage may increase therapeutic effectiveness by improving compliance. The experiment was conducted in high-fat diet (HFD)-fed rats as a model of oxidative stress induced by high-fat consumption. Endogenous antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were measured, and malondialdehyde (MDA) levels were assessed as an indicator of lipid peroxidation.

Materials and methods

Plant collection

The *Z. cassumunar* rhizomes, *G. max* beans, and *Cinnamomum burmanni* barks were purchased from a local market in Yogyakarta, Indonesia. All plants were then identified at the Laboratory of Biology, Faculty of Applied Science and Technology, Universitas Ahmad Dahlan, with identification number of 189/Lab.Bio/B/VI/2021.

Preparation of functional powder beverage

Preparation of *Z. cassumunar* instant powder required 800 g of peeled rhizomes blended with 600 mL of water. The juice was then squeezed and filtered until a clear filtrate was obtained, free of starch deposits after settling for approximately 30 min. The filtrate was then crystallised by heating with 800 g of sugar at approximately 80°C.

Soybeans were dehulled, cleaned, and roasted to remove undesirable odours. Soybeans and cinnamon bark were separately blended and dried in an oven at 80°C for approximately 1 h. All powders were sieved through an 80-mesh screen.

The optimum formula for the functional powdered beverage was determined based on organoleptic evaluations by a sensory panel. The final composition consisted of 75% instant *Z. cassumunar* powder, 23% soybean powder, and 2% cinnamon powder.

Preparation of high-fat diet

High-fat diet (HFD) was prepared from 300 g of standard feed, 20 g of chicken egg yolk, 100 g of butter, 10 g of beef tallow, and 0.05% propylthiouracil (PTU). All ingredients were mixed, ground with a meat grinder, shaped into pellets comparable to standard feed, and dried in an oven at 50°C for 3 d (Mahfudh *et al*., 2022).

Animals

The research was carried out on 30 Wistar rats which were divided into six groups with each group consisting of five test animal subjects. The six treatment groups were as follows: (1) normal control group: standard feed; (2) negative control group: high-fat feed (HFD only); (3) positive control group: HFD plus a commercial smoothie marketed for cholesterol reduction; and (4) treatment groups (group iv-vi): HFD plus functional powdered beverage at doses of 1,000, 1,500, and 2,000 mg/kg BW.

Rats were acclimatised for 7 d prior to the experiment. HFD feeding was conducted for 28 d, with functional beverage or smoothie treatment administered from day 15 to 28. The treatment schedule is illustrated in Figure 1. All animal procedures were approved by the Research Ethics Committee of Universitas Ahmad Dahlan (approval no.: 012106034).

Preparation of liver tissue homogenates

At the end of the experiment, rats were euthanised by cervical dislocation. Livers were excised, rinsed with physiological saline, chopped into small pieces, and homogenised in phosphate-buffered saline (PBS) at a 9:1 ratio (mL PBS/g

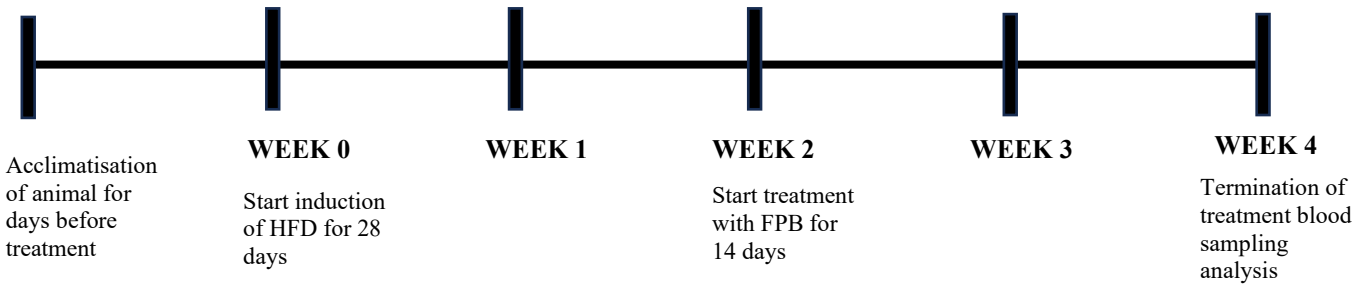

Figure 1. Schedule of animal treatment.

tissue). Homogenates were centrifuged at 3,000 rpm for 15 min, and the supernatant was collected for enzyme activity assays.

Protein concentration measurement

Protein concentrations were determined by the Bradford method (Walker, 2002). Briefly, 0.1 mL of supernatant was mixed with 5 mL of Bradford reagent, vortexed, and incubated at room temperature for 10 - 60 min. Absorbance was read at 595 nm.

Measurement of SOD, CAT, GPx activities, and MDA levels

The levels of SOD, CAT, GPx, and MDA in liver homogenates were quantified using commercial kits from Elabscience: E-BC-K022-S (SOD), E-BC-K031-S (CAT), E-BC-K096 (GPx), and E-BC-K025-S (MDA). Assays were performed following the manufacturer's instructions, and absorbance was measured using a Shimadzu 1900i UV spectrophotometer.

Statistical analysis

The data were analysed using SPSS. Normality and homogeneity were assessed with the Shapiro-Wilk test. Normally distributed, homogeneous data were analysed by One-way ANOVA at a 95% confidence level, followed by least significant difference (LSD) *post hoc* tests to determine differences between groups.

Results

The measurement of endogenous antioxidant activity was performed using liver homogenate sample. Protein concentration in the homogenates was determined to ensure accurate normalisation of enzymatic activity. The protein concentrations of the samples are presented in Table 1.

Table 1. Protein levels in liver homogenates of rats fed with high fat diet, and treated with functional beverage.

Group	Protein concentration (mg/mL)
Normal	0.6228 ± 0.0361
Negative	0.9758 ± 0.0287
Positive	0.7065 ± 0.0462
Dose of 1,000 mg/kg BW	0.699 ± 0.0621
Dose of 1,500 mg/kg BW	0.6908 ± 0.0415
Dose of 2,000 mg/kg BW	0.6876 ± 0.0636

Increased SOD activity

High-fat diet (HFD) consumption is known to trigger oxidative stress (Amiya, 2016). Consistent with this, the present work showed that SOD activity in the negative control group (HFD only) was significantly lower ($p < 0.05$) compared to the normal control group (Table 2). The SOD is a key enzyme responsible for maintaining redox homeostasis. Hyperlipidaemia resulting from HFD induction increases ROS production, which subsequently suppresses SOD activity (Cui *et al*., 2014). Treatment with the functional powdered beverage (FPB) significantly increased SOD activity compared to the negative control. Furthermore, the increase in enzyme activity was dose-dependent, with higher FPB doses showing greater effects (Table 2).

Increased CAT activity

As shown in Table 3, CAT activity was significantly reduced in the negative control (HFD group) compared to the normal control. The HFD induction promotes lipid peroxidation, and disrupts the balance between oxidants and antioxidants, thereby suppressing CAT activity (Skowron *et al*., 2018). The FPB treatment significantly restored CAT activity in a dose-dependent manner. CAT acts downstream of SOD by converting hydrogen

Table 2. Increased activity of SOD enzymes in rats fed with high fat diet, and treated with functional beverage.

Group	Activity of SOD ± SD (U/mg prot)
Normal	88.32 ± 10.29$^{\#}$
Negative	30.83 ± 11.18*
Positive	88.54 ± 12.18$^{\#}$
Dose of 1,000 mg/kg BW	91.08 ± 13.10$^{\#}$
Dose of 1,500 mg/kg BW	99.71 ± 11.15$^{\#}$
Dose of 2,000 mg/kg BW	106.67 ± 4.82*$^{\#\sim}$

*significant difference with normal group ($p < 0.05$); $^{\#}$significant difference with negative control group ($p < 0.05$); and ˜significant difference with positive control group ($p < 0.05$).

Table 3. Increased activity of catalase, enzymes in high fat diet rat treated with functional beverages.

Group	Activity of catalase ± SD (U/mg prot)
Normal	174.09 ± 23.68$^{\#}$
Negative control	59.62 ± 9.30*$^{\sim}$
Positive control	195.85 ± 12.27$^{\#}$
Dose of 1,000 mg/kg BW	93.64 ± 15.26$^{\#\sim}$
Dose of 1,500 mg/kg BW	136.61 ± 19.51$^{\#\sim}$
Dose of 2,000 mg/kg BW	180.77 ± 16.93$^{\#}$

*significant difference with normal group ($p < 0.05$); $^{\#}$significant difference with negative control group ($p < 0.05$); and ˜significant difference with positive control group ($p < 0.05$).

peroxide, a product of SOD activity, into water and oxygen (H_2O). Increased CAT activity in FPB-treated groups suggested that FPB supplementation helped mitigate oxidative stress by reducing hydrogen peroxide accumulation in HFD-induced rats.

Increased GPx activity

The GPx activity was also decreased in the negative control (HFD group) compared to the normal group (Table 4), indicating that hyperlipidaemia suppressed GPx activity. The FPB treatment for 14 d significantly increased GPx activity compared to the negative control group. Although GPx activity tended to increase with higher FPB doses, the differences between treatment doses were not statistically significant ($p > 0.05$).

Decreased MDA level

The present work demonstrated that HFD feeding markedly increased MDA levels in liver tissues, consistent with previous findings showing elevated MDA in cholesterol-fed animals compared to normal controls. Fat accumulation enhances free radical generation, and accelerates the oxidation of polyunsaturated fatty acids (PUFAs), leading to increased MDA formation (Gaweł *et al*., 2004). The FPB treatment significantly reduced MDA levels compared to the negative control ($p < 0.05$). Moreover, the reduction was dose-dependent, with the greatest decrease observed in the group treated with FPB at 2,000 mg/kg BW (Table 5).

Table 4. Increased activity of GPx enzymes in high fat diet rat treated with functional beverages.

Group	Activity of GPx ± SD (U/mg prot)
Normal	991.04 ± 89.54$^{\#\sim}$
Negative control	192.54 ± 90.34*$^{\sim}$
Positive control	661.79 ± 26.53*$^{\#}$
Dose of 1,000 mg/kg BW	507.17 ± 46.64*$^{\#\sim}$
Dose of 1,500 mg/kg BW	528.55 ± 60.95*$^{\#\sim}$
Dose of 2,000 mg/kg BW	587.44 ± 49.08*$^{\#\sim}$

*significant difference with normal group ($p < 0.05$); $^{\#}$significant difference with negative control group ($p < 0.05$); and ˜significant difference with positive control group ($p < 0.05$).

Table 5. MDA levels in liver tissue of rat fed a high fat diet and functional beverage.

Group	MDA level (nmol/mg prot)
Normal	2.28 ± 0.74$^{\#}$
Negative control	5.79 ± 0.38*$^{\sim}$
Positive control	2.69 ± 0.45$^{\#}$
Dose of 1000 mg/kg BW	4.40 ± 0.98*$^{\#\sim}$
Dose of 1500 mg/kg BW	3.50 ± 0.13*$^{\#\sim}$
Dose of 2000 mg/kg BW	2.74 ± 0.23$^{\#}$

*significant difference with normal group ($p < 0.05$); $^{\#}$significant difference with negative control group ($p < 0.05$); and ˜significant difference with positive control group ($p < 0.05$).

Discussion

The HFD induction in negative control group decreased the activity of SOD, CAT, and GPx. This decreased was caused by excessive fatty acid intake from HFD, which increased intestinal absorption, and elevated circulating lipid levels. The excessive transport of fatty acids to the liver led to lipid accumulation in hepatocytes. Moreover, large

amounts of unsaturated fatty acids in liver cell membranes contributed to the generation of free radicals. These free radicals initiate lipid peroxidation, which in turn produces more reactive species in a continuous cycle (Halder and Bhattacharyya, 2014; Sun *et al*., 2020). Increased free radical activity suppresses antioxidant enzyme activity, leading to oxidative stress.

Treatment with the FPB significantly increased antioxidant enzyme activities (SOD, CAT, and GPx), and decreased MDA levels compared to the negative control ($p < 0.05$). The highest efficacy was observed at the dose of 2,000 mg/kg BW, which produced the greatest increase in antioxidant enzyme activity, and the largest decrease in MDA levels, comparable to the effects of the positive control (commercial smoothie drink). These effects could have been due to the bioactive compounds in *Z. cassumunar*, *G. max*, and *C. burmanni*, which act as natural antioxidants. By scavenging free radicals and inhibiting lipid peroxidation, these compounds help restore redox balance, enhance endogenous antioxidant defences, and lower oxidative damage markers such as MDA. The similar result also was reported in formulation of *Z. cassumunar* into biscuit form in increasing SOD, and decreasing MDA level (Mahfudh *et al*., 2024).

Z. cassumunar contains curcuminoid and phenylpropenoid compounds with strong antioxidant and immunostimulant properties (Nagano *et al*., 1997; Sukatta *et al*., 2009). These compounds neutralise free radicals, suppress lipid peroxidation, and enhance overall antioxidant capacity (Ramadhan *et al*., 2020) . The presence of the ingredients is able to neutralise free radicals, can suppress oxidation processes and lipid peroxidation, and increase antioxidant capacity.

In addition, flavonoids and tannins which are contained in *Z. cassumunar* rhizomes may reduce triglyceride levels by enhancing lipoprotein lipase (LPL) activity, thereby lowering lipid peroxidation, and reducing fatty liver risk (Marliani *et al*., 2014; Musdja, 2021). Flavonoids protect against oxidative stress by stimulating SOD and CAT activities through hydrogen atom donation to lipid peroxyl radicals, thus halting chain reactions. Tannins, as polyphenolic compounds, also exert strong free radical scavenging activity (Duraiswamy *et al*., 2018).

Cinnamon contains active compounds in the form of cinnamic acid which can lower cholesterol by inhibiting cholesterol synthesis, and through cinnamaldehyde, which inhibits aldose reductase in the polyol pathway, thereby reducing oxidative stress (Pulungan and Pane, 2020; Nurisyah *et al*., 2021). *In vitro* studies have demonstrated high antioxidant activity of cinnamon using assays such as DPPH and ABTS (Gulcin *et al*., 2019).

Soybeans contribute both isoflavones and proteins. Isoflavones have been reported to have an antioxidant activity (Yoon and Park, 2014; Jang and Choi, 2019). Previous studies reported that soybeans at a dose of 2.12 g/200 g BW can increase SOD activity, and decrease MDA levels in hypercholesterolemic male Sprague Dawley rats (Setiawan *et al*., 2016). Isoflavones were also shown to lower lipid and triglyceride levels (Basharat *et al*., 2020). In addition, the high protein content of soybeans improves the organoleptic properties of the FPB.

Conclusion

Functional powdered beverages containing *Z. cassumunar* rhizomes, soybeans, and cinnamon demonstrated significant antioxidant effects, as indicated by increased activities of SOD, CAT, and GPx, and decreased MDA levels. Among the tested doses, 2,000 mg/kg BW was the most effective, producing antioxidant effects comparable to the positive control.

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