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Formulation of *Zingiber cassumunar* Roxb.-purple sweet potato-based biscuit as antioxidant by decreasing malondialdehyde (MDA) level, and increasing superoxide dismutase (SOD) activity in high-fat-diet-fed rats

[1]*Mahfudh, N., [2]Solikah, W. Y., [1]Sulistiyani, N., [3]Kumalasari, I. D. and [4]Zakaria, Z. A.

[1]*Department of Analytical and Medicinal Chemistry, Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta 55164, Indonesia*
[2]*Pharmacy Study Program, Faculty of Health Sciences, College of Health Sciences Alma Ata, Yogyakarta 55184, Indonesia*
[3]*Faculty of Industrial Technology, Universitas Ahmad Dahlan, Yogyakarta 55164, Indonesia*
[4]*Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University of Malaysia Sabah, Sabah 88400, Malaysia*

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Abstract

Incorporating antioxidants in functional foods represents an excellent dietary approach to prevent oxidative stress. The present work aimed to create a novel formulation of biscuits using a combination of *Zingiber cassumunar* (ZC) and purple sweet potato (PSP) as the main ingredients, in producing a functional food product that possesses antioxidant properties. The present work involved conducting trials and implementing optimisation techniques in order to obtain three distinct biscuit formulations, namely F1 (ZC 0.75 g: PSP 5.25 g), F2 (ZC 0.45 g: PSP 5.55 g), and F3 (ZC 0.28 g: PSP 5.72 g). The proximate analysis of the three formulations met the Standard Nasional Indonesia (SNI) criteria for biscuits, which encompassed parameters such as moisture content, ash, fat, protein, crude fibre, and metal residue. The F3 biscuits were chosen as the test biscuits in subsequent experiments due to their superior quality. The administration of F3 biscuits at a dosage of 1.94 g per 200 g of body weight to rats fed with high-fat diet for 28 d resulted in a substantial reduction ($p < 0.05$) in malondialdehyde levels, with a mean value of 2.17 ± 0.22 nmol/mL. Additionally, this intervention improved superoxide dismutase activity, with a mean value of 72.95 ± 3.06%. The F3 biscuits demonstrated promising antioxidant potential. This could serve as a basis for further clinical trials in humans before its potential commercialisation. Further investigation into the formulation of the biscuit is necessary, especially concerning the moisture, ash, and crude fibre levels present.

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Introduction

Oxidative stress arises due to post-translational changes in proteins, lipids, and DNA induced by heightened levels of reactive oxygen species (ROS). These ROS cause detrimental effects on the antioxidant mechanisms (Kong *et al*., 2022; Bachheti *et al*., 2022). Elevated levels of oxidative stress have been identified as a prominent causative factor in developing several chronic diseases, including obesity, diabetes, cardiovascular diseases (CVD), and certain forms of cancer (Niemann *et al*., 2017; Robson *et al*., 2018). The excessive consumption of high-fat diets (HFD) poses severe threat to cardiovascular health, by elevating plasma cholesterol levels. Based on several studies conducted by Liu *et al*. (2020), Feng *et al*. (2022), and Ta *et al*. (2023), it has been observed that the consumption of HFD leads to the accumulation of adipose tissue in the dermis and perirenal adipose tissue, hence inducing oxidative stress.

Elevated levels of malondialdehyde (MDA) and reduced activity of superoxide dismutase (SOD) are recognised as two indicators of oxidative stress

*Corresponding author.
Email: nurkhasanah@pharm.uad.ac.id

resulting from an excessive cellular lipid peroxidation process (Hamed *et al*., 2020). The MDA is the final result of the process known as lipid peroxidation, and generated by the interaction of ROS with the fatty acid constituents of the cellular membrane (Tsikas, 2017). The SOD is a metalloenzyme that is an endogenous antioxidant in living organisms. It plays a crucial role in cellular defence by scavenging superoxide radicals, and catalysing the conversion of peroxides (H_2O_2) into water (H_2O) and oxygen (O_2). Simultaneously, H_2O_2 that permeates into the cytosol will undergo detoxification by the catalytic action of the enzyme glutathione peroxidase, as described by Song *et al*. (2019).

Including antioxidants in one's dietary intake has been found to contribute to overall health by enhancing immune function, mitigating the risk of chronic diseases, and reducing oxidative stress (Khalid *et al*., 2022). Antioxidants can scavenge free radicals, thereby exhibiting the potential to mitigate the occurrence of acute and chronic ailments, including, but not limited to, cancers, diabetes, liver diseases, cardiovascular diseases, and neurological disorders (Ayoka *et al*., 2022). Previous work showed that *Zingiber cassumunar* rhizome effectively enhanced antioxidant activity while mitigating the deleterious consequences of HFD. According to Sari *et al*. (2020), administering a particular substance resulted in increased SOD activity in mice. This effect was particularly pronounced when the substance was administered at 400 mg per kilogram of body weight. The observed changes in SOD activity were observed after four weeks. A recent study conducted by Herawati *et al*. (2020) demonstrated that consuming anthocyanin-rich foods, such as purple sweet potatoes (PSP), can potentially decrease urea and creatinine levels, thus improving renal function. Additionally, the study found that PSP consumption was associated with significantly lower blood levels of MDA, thus indicating potential antioxidant effects.

Zingiber cassumunar Roxb. (ZC), a plant of significant importance in medicine, has diverse bioactivities, and is widely recognised for its traditional medicinal use (Li *et al*., 2019). These herbs are utilised in gastronomy as condiments, conventional remedies, and aromatherapy (Bora *et al*., 2021). It possesses diverse attributes, including antioxidant. Three distinct antioxidants have been extracted from the rhizome of ZC which were curcuminoid derivatives. These antioxidants are called cassumunins A, B, and C, collectively called the "Three Cassumunins". Cassumunin, a recently discovered compound, belongs to a group of curcuminoids. Its chemical structure has been identified as S'-phenylbutenylated curcumins, as reported by Masuda *et al*. (1995).

The ZC exhibits potential as a viable resource for developing medicinal products and functional foods. The rising popularity of PSP can be attributed to their abundant presence of natural antioxidants which offer numerous health advantages (Toan *et al*., 2018). The utilisation of PSP in flour production has been found to offer advantages such as enhanced stability and reduced bulkiness (Sugri *et al*., 2017). Furthermore, including PSP flour in the national food system can contribute to the diversification and security of the food supply, hence reducing reliance on imported wheat flour (Ulfa *et al*., 2019). Prior research has demonstrated that including PSP in the formulation of biscuits resulted in elevated levels of polyphenols and notable antioxidant properties (Bakar *et al*., 2022).

There has been a notable rise in the popularity of functional foods. Functional meals have been shown to positively impact both physical and mental well-being, as well as a potential reduction in the likelihood of developing specific diseases such as cardiovascular diseases, cancers, high blood pressure, and high cholesterol (Gok and Ulu, 2019). Furthermore, these foods may also possess therapeutic properties for particular disorders. According to Lyu *et al*. (2017), functional food possesses several advantages, including its widespread accessibility, ease of preparation, and reduced adverse effects. These benefits are attributed to the necessary nutritional functions of functional food, which in turn contribute to various physiological advantages.

Biscuits, a popular non-fermented bread product, are consumed globally as a snack (Mounika and Maloo, 2018). Their extended shelf life can be attributed to their low moisture content (Sahni and Shere, 2016). According to Srivastava and Singh (2016) and Toan *et al*. (2018), there was a high probability of consumer acceptance and potential for enhancement in nutritional composition and health benefits of biscuits. Therefore, the present work aimed to enhance the antioxidant properties of biscuits by including a blend of ZC rhizome and PSP flour, transforming them into functional foods. A high-fat diet was employed to induce metabolic changes in rats over 28 days. The impact of this diet on the levels of MDA and SOD was assessed to evaluate the potential antioxidant benefits of the formulated biscuits.

Materials and methods

Materials

The rhizomes of ZC and tubers of PSP were acquired from a local farmer in Sleman, Yogyakarta, Indonesia. The materials underwent rinsing, then sliced into smaller pieces, and dried using a cabinet dryer. The ZC rhizomes were dried at 25°C, while the PSP tubers were dried at 40°C. Following drying, the ZC and PSP flour production involved grinding, sifting, and utilisation.

Chemicals

The chemicals employed in the measurement of MDA included 1,1,3,3-tetraethoxypropane (Sigma-Aldrich, USA), 2-thiobarbituric acid (Sigma-Aldrich, USA), and trichloroacetic acid (Merck, Germany). The measurement of SOD activity was conducted using a commercially available SOD Activity Assay kit (BioVision, USA) and a spectrophotometer (Optima, Japan).

Biscuit preparation

The inclusion of ZC rhizome powder was following the dosage conversion of ZC extract, which has been established at 200 mg/kg body weight in rats (Sari *et al*., 2020). According to Jokioja *et al*. (2020), the recommended upper limit for the daily intake of PSP powder is 160 g. Therefore, three biscuit formulations were developed to align with this dosage range. An optimisation trial achieved a good weight and taste for the biscuits. The selection of a superior formula for *in vivo* testing was based on the outcomes of the proximate evaluation conducted on the biscuits. The biscuit formula's design is outlined in Table 1.

The initial procedure involves measuring the quantities of all the biscuit materials under the prescribed formula. The egg yolk and margarine were thoroughly blended to achieve a homogeneous mixture. Subsequently, the inclusion of low-fat milk, refined sugar, and baking soda ensued, followed by thorough remixing. The dough was prepared by adding ZC and PSP flours to obtain a homogeneous consistency. Finally, the dough was moulded using a biscuit maker, and subjected to baking at 120°C for 50 min.

Table 1. Formula compositions of biscuits containing ZC and PSP flours.

Material	F1 (g)	F2 (g)	F3 (g)
ZC flour	7.5	4.5	2.8
PSP flour	52.5	55.5	57.2
Egg yolk	20	20	20
Margarine	20	20	20
Baking powder	2.5	2.5	2.5
Low-fat milk	5	5	5
Refined sugar	30	30	30
Total weight	**137.5**	**137.5**	**137.5**

*Each formula produces ten biscuits.

Quality evaluation of biscuits

The physical attributes of biscuits, encompassing organoleptic properties, weight, thickness, and hardness, were assessed. Following AOAC (1990), biscuit samples underwent proximate analysis to ascertain their moisture, ash, and crude fibre contents. The total fat content was determined using the Soxhlet method with the Weibull adjustment, as described by Suhardi and Sudarmadji (2007). The protein content was determined using the Kjeldahl technique, as described by Kjeldahl (1883). The difference method was employed to obtain the total carbohydrate content: Total Carbohydrate (%) = 100 – (% Ash + % Fat + % Moisture + % Protein). The chosen formula for *in vivo* experiments was determined based on the proximate analysis results.

High-fat diet preparation

The HFD consisted of 300 g of standard pellets, 20 g of chicken egg yolk, 100 g of butter, and 10 g of beef fat in pellet form. Additionally, the drinking water provided to the subjects was supplemented with 0.05% propylthiouracil (PTU), according to Sari *et al*. (2020).

Animals

Eight-week-old male Sprague-Dawley rats weighing 150 and 200 g were obtained from the Centre of Food and Nutrition at Universitas Gadjah Mada (UGM). The rats were then acclimated for 7 d in an animal housing facility. The diurnal pattern consisted of 12-h alternation between light and dark, accompanied by ambient temperature and humidity levels associated with wakefulness. Both pellets and water were readily accessible at all times. The present work received approval from the research ethics committee of Universitas Ahmad Dahlan (ref. no.: 012105028).

Experimental design

Twenty-four rats were divided into four groups. Group I: standard-fed group (standard control), Group II: HFD (negative control), Group III: HFD + Nutrive Benecol (3.6 mL per 200 g of body weight; positive control), and Group IV: HFD + F3 biscuit (1.94 g per 200 g of body weight; treatment group). The rats underwent 28-d treatment period in each experimental group. An unrestricted HFD was provided. The administration of Nutrive Benecol and biscuits occurred daily using a stomach probe. After the treatment, the rats underwent an overnight fasting period, and were subsequently euthanised by dislocating their necks, as described by Sari *et al*. (2020). The livers were isolated, rinsed with a saline solution containing 0.9% sodium chloride, and subsequently processed to produce a homogenate for subsequent analyses.

Preparation of sample homogenate

The liver homogenate was made by sectioning the liver into minute fragments weighing at least 0.1 g. These fragments were then homogenised in phosphate-buffered saline (PBS) at 0.01 M and pH 7.4. The ratio of liver to PBS was maintained at (g) = 9:1, based on the weight of the tissue. The tissue homogenate underwent centrifugation at 3,000 rpm for 15 min. The supernatant was then obtained for subsequent analyses (Assady *et al*., 2011).

Determination of liver MDA level

The concentration of MDA in the liver tissue was quantified by the thiobarbituric acid reactive substances (TBARs) assay, as described by Kei (1978) with slight modifications. The quantification of MDA was conducted by assessing the reaction between TBA and MDA, which forms red colour. This assay is known to have good sensitivity. Due to the inherent instability of MDA, tetra-ethoxypropane (TEP) was employed as a standard. One millilitre of a supernatant was added with 0.5 mL of a cold solution containing 20% trichloroacetic acid (TCA), and mixed vigorously for 1 min using a vortex mixer. Subsequently, the solution underwent centrifugation at 3,000 rpm for 10 min. The collected supernatant was transferred into a test tube, and added with 1 mL of 0.67% TBA solution. The mixture was homogenised using a vortex mixer. Subsequently, the tube was incubated at 95 - 100°C for 10 min. The reactant then underwent a cooling process, and was subjected to measurement using a spectrophotometer at 532 nm. The study's authors (Sunaryo *et al*., 2015) generated a calibration curve using the measured data. This curve was constructed by plotting the absorbance values (Y) against the standard solution concentrations (X), which were measured in nmol/mL.

Determination of liver SOD activity

The SOD assay kit was employed to assess its inhibitory activity using a colorimetric approach. Results were expressed using the percentage inhibition rate (%). The sensitive SOD assay kit uses the water-soluble tetrazolium salt (WST-1) to generate a water-soluble formazan dye upon reduction by superoxide anion. Sodium oxalate dihydrate (SOD) exhibits inhibitory effects on the enzyme xanthine oxidase (XO), resulting in a linear relationship between the rate of reduction, and the presence of a superoxide anion. In this method, four solutions were prepared: sample solution (20 µL sample + 200 µL WST working solution + 20 µL enzyme working solution); Blank 1 (20 µL ddH_2O + 200 µL WST working solution + 20 µL enzyme working solution); Blank 2 (20 µL sample + 200 µL WST working solution + 20 µL dilution buffer); and Blank 3 (20 µL ddH_2O + 200 µL WST working solution + 20 µL dilution buffer). The solution was measured using a spectrophotometer at 450 nm. The calculation of SOD activity (inhibition rate %) was performed using Eq. 1:

$$\text{SOD activity (inhibition rate \%)} = \frac{(Ablank1-Ablank3)-(Asample-Ablank2)}{(Ablank1-Ablank3)} \times 100 \quad \text{(Eq. 1)}$$

Statistical analysis

Results were expressed as mean ± standard deviation. The IBM SPSS program version 25 was used to conduct a difference test for the levels of MDA and the activities of SOD. Analysis of variance (ANOVA) was used to examine the data, and afterwards, the means were subjected to a Tukey multiple comparison test to identify any significant differences at a significance level of 0.05 or less ($p <$ 0.05).

Results and discussion

Quality evaluation of biscuit

Biscuits are highly stable commodities that offer numerous benefits, including cost-effectiveness during manufacturing, and long shelf life during storage. The physical characteristics of biscuits are contingent upon how the dough is shaped, and the specific variety of biscuits (Dogan, 2006). In the present work, the biscuit formulations underwent testing to assess their physical features against standards prescribed by the Standard Nasional Indonesia (SNI). Table 2 displays the physical attributes (including organoleptic properties, weight, thickness, and hardness) of the biscuit composites produced from ZC and PSP flours. The visual representation of the biscuits is shown in Figure 1.

In Table 2, it is evident that there was no statistically significant difference in the average weight of the biscuits among the three formulations, in addition to the dimensions and mechanical properties of the biscuit. According to SNI no. 01-2973-2011, which pertains to biscuits, no specific regulations are outlined on the organoleptic characteristics of biscuits, including limitations on thickness or hardness. Therefore, no comparison could be made on the different attributes of biscuits produced from ZC and PSP flours.

Table 3 presents the proximate analysis of the biscuits, and metal contamination. The moisture levels of the samples varied between 6.52 and 7.48%, suggesting a statistically significant variation in moisture content ($p < 0.05$) among the samples. The findings indicated that the biscuits produced using each formula exceeded the SNI quality standards of 5% (SNI, 2011). F3 had the lowest water content among all samples. This could have been due to the incorporation of ZC and PSP flours. It is noteworthy

Table 2. Physical characteristics of biscuits produced from ZC and PSP flours.

Sample	Organoleptic	Weight (g)	Thickness (mm)	Hardness (kg)
Formula 1	Round shape Dark brown colour ZC distinctive aroma Sweet and bitter taste	11.43 ± 0.34	0.61 ± 0.01	8.673 ± 0.09
Formula 2	Round shape Dark brown colour ZC distinctive aroma Sweet and slightly bitter taste	11.56 ± 0.25	0.62 ± 0.00	8.569 ± 0.05
Formula 3	Round shape Dark brown colour ZC distinctive aroma Sweet taste	11.63 ± 0.30	0.59 ± 0.01	8.946 ± 0.10

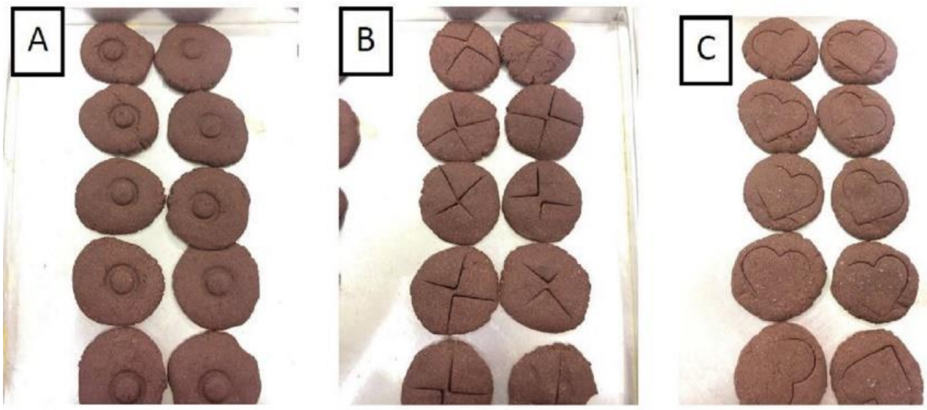

Figure 1. Appearance of biscuits produced from ZC and PSP flours for Formula 1 **(A)**, Formula 2 **(B)**, and Formula 3 **(C)**.

Table 3. Proximate chemical composition and metal contamination of biscuits produced from ZC and PSP flours.

Parameter	SNI	Formula 1	Formula 2	Formula 3
Moisture	< 5%	7.48 ± 0.07	7.04 ± 0.02	6.52 ± 0.01
Ash	< 1.6%	2.75 ± 0.05	3.14 ± 0.04	3.01 ± 0.06
Protein	> 5%	5.84 ± 0.09	6.00 ± 0.23	6.00 ± 0.17
Fat	> 9.5%	18.83 ± 0.13	18.23 ± 0.03	18.31 ± 0.14
Carbohydrate	> 70%	65.09 ± 0.07	65.55 ± 0.21	66.16 ± 0.26
Crude fibre	< 0.05%	1.70 ± 0.48	1.58 ± 0.29	1.37 ± 0.05
Metal residue				
Pd	< 0.5 mg/kg	0.09 ± 0.05	0.08 ± 0.31	0.08 ± 0.26
Cd	< 0.02 mg/kg	0.02 ± 0.13	0.02 ± 0.01	0.01 ± 0.12

that F3 comprised a maximum of 0.28 g of ZC flour and 5.72 g of PSP flour. The storage stability of a product can be enhanced by reducing its initial moisture content (Egwujeh *et al*., 2018). This implied that F3, characterised by its comparatively lower moisture content, was likely to have extended storage viability.

F1 had the highest concentration of ZC flour, thus resulting in the highest level of water content. This suggested that an increase in the amount of ZC flour resulted in a corresponding increase in the moisture content of the biscuits. This observation was consistent with existing literature which reports that ZC ginger has a short shelf life owing to its high moisture content of around 90%. Furthermore, the limited availability of ZC ginger throughout the year restricts its usage to specific seasons (Nimnuan and Nabnean, 2020). In a similar study, the incorporation of additional PSP flour will result in a decrease in the moisture content of the biscuits. The reduced water content in the biscuits can be attributed to the heightened enzymatic activity responsible for the hydrolysis of starch, resulting in a drop in water content. The decrease in carboxyl groups and in intermolecular and intramolecular hydrogen bonding subsequently led to a decrease in water retention capability of starch granules. According to Velly *et al*. (2022), starch granule drying results in the liberation and subsequent evaporation of water molecules bound to them.

The protein levels in F2 and F3 were similar, while that of F1 was the lowest. According to the Standard Nasional Indonesia (SNI, 2011), the protein content of biscuits should range from 5 to 9%. All formulations met this criterion. Therefore, it could be an important source of protein for low-income people in developing regions, since the majority of similar proteins come from meals with a vegetable origin (Olatunde *et al*., 2016).

F1 had the highest fat content, whereas F2 had the lowest. There was a lack of statistically significant variation in the fat contents among all formulations. This indicated that an increase in the quantity of PSP flour decreased the fat content. Fat content in biscuit indicates its caloric content. Lipids, including fats and oils, are present in virtually all food categories, exhibiting varying levels of composition (Pargiyanti, 2019). The minimum fat content of biscuits prescribed by SNI no. 01-2973-2011 is 9.5% (SNI, 2011). All formulations complied with this. According to Putri *et al*. (2018), high fat content in biscuits can significantly contribute to their overall energy value.

F3 had the highest carbohydrate content, and F1 the lowest. Based on SNI no. 01-2973-2011, the minimum carbohydrate content of biscuits should be 70% (SNI, 2011). All formulations did not comply with this. The variations in the addition of PSP flour could have contributed to the discrepancies in the carbohydrate contents. Based on Table 1, F3 had the highest proportion of PSP flour. This suggested that adding more PSP could increase the carbohydrate content of the biscuits. Previous research showed that a significant percentage of carbohydrates, precisely 80%, exhibited a low glycaemic index when PSP was the predominant macronutrient. Additional macro- and micronutrients included protein (6.05%), fat (0.76%), iron (6.7049 mg/kg), and zinc (8.5595 mg/kg), as reported by Fitri *et al*. (2023) and Legi *et al*., (2023). Sweet potato flour primarily consists of carbohydrates, rendering it a viable and efficient energy source. However, it is essential to acknowledge that in addition to raw materials, biscuits may contain other substances that contribute to their carbohydrate content, such as refined sugar and egg yolks.

Crude fibre refers to the indigestible food residue, similar to dregs, that acids or bases cannot break down. F1 had the highest concentration of crude fibre, whereas F3 had the lowest. This could have been due to an increase in proportion of ZC flour incorporated. In contrast, the incorporation of PSP flour decreased the crude fibre level of the biscuits. This contradicted Toan *et al*. (2018) that the inclusion of more significant quantities of PSP flour in the production of biscuits led to notable increase in the crude fibre content of the final product ($p < 0.05$).

Metal residue tests were conducted to determine the suitability of cookies or biscuits. Furthermore, assessments about impurities in these metals are incorporated into food product safety evaluations. Based on the specifications outlined by SNI no. 3451:2011, the permissible metal contents for biscuits are as follows: a maximum of 0.28 mg/kg for Cd (cadmium) and a maximum of 0.2 mg/kg for Pb (plumbum/lead). The findings indicated that all formulations met this criterion. Therefore, combining ZC and PSP flours to make biscuits was safe for consumption.

Biochemical analyses

A comprehensive assessment on the physical attributes of the biscuits, and a proximate evaluation determined the selection of the optimal biscuit for testing on Sprague-Dawley rats. In this case, F3 turned out as the preferred choice. The experiment was conducted *in vivo* for 28 d, during which the rats were subjected to various treatments. On the final day of the experiment, all the rats were euthanised to analyse the levels of MDA and SOD in their liver tissue. Table 4 displays the outcomes of the measurements conducted on MDA levels and SOD activities.

Table 4. MDA level and SOD enzyme activity of liver in rats after 28 days.

Group	**MDA level (nmol/mL)**	**SOD activity (%)**
A (Normal fed)	1.95 ± 0.21^a	82.51 ± 2.86^a
B (HFD fed)	9.91 ± 0.17^b	30.60 ± 4.48^b
C (HFD + Nutrive Benecol, 3.6 ml/200 g BW)	2.99 ± 0.21^c	66.39 ± 3.07^c
D (HFD + F3 biscuit, 1.94 g/200 g BW)	2.17 ± 0.22^a	72.95 ± 3.06^d

*Means in a column having different lowercase superscripts are significantly different ($p \leq 0.05$).

MDA level

Elevated levels of MDA are biomarkers for increased lipid peroxidation, a process associated with oxidative stress, and implicated in the pathogenesis of multiple diseases (Ito *et al*., 2019; Ghonimi *et al*., 2021; Pena *et al*., 2022). The lipid oxidation by-product can rapidly and directly interact with proteins, thereby expediting the occurrence of diverse biochemical reactions (Chen *et al*., 2021). Hence, the levels of MDA serve as an indirect indicator of both lipid peroxidation and oxidative stress, as demonstrated by Xia *et al*. (2020). The present work aimed to establish a causal relationship between the administration of HFD and the manifestation of oxidative stress in the hepatic tissue. It was seen that ingesting a diet high in fat led to a notable elevation in MDA levels compared to the control group. These findings were consistent with a previous investigation conducted by Qu *et al*. (2022), whereby it was seen that the consumption of HFD resulted in varying amounts of elevated nitric oxide (NO) and MDA, as well as reduced glutathione peroxidase (GSH-Px) activity in the liver, heart, and kidney. These alterations collectively indicated oxidative stress-induced damage, although to differing extents.

One product was officially recognised and certified as a functional food in Indonesia, serving as a designated positive control in the present work. The product incorporates plant stanol esters (PSE) which have been scientifically demonstrated to reduce cholesterol levels effectively. By including PSE in this product, a reduction in total cholesterol levels by 7 - 10% was observed following consistent intake over 2 - 3 weeks. Moreover, this consumption pattern has been found to mitigate the potential occurrence of coronary heart disease. In the present work, implementing a beneficial control intervention in conjunction with the consumption of HFD resulted in significant reduction in MDA levels ($p < 0.05$). However, the mean value differed from the control groups. For group D, the co-administration of HFD in conjunction with F3 biscuits yielded a noteworthy reduction ($p < 0.05$) in MDA levels. Furthermore, it is worth mentioning that there were no statistically significant differences observed in the mean MDA levels between group D and the control group receiving standard treatment. The findings suggested that the F3 biscuits significantly decreased oxidative stress in the rat liver by reducing the MDA levels.

F3 had 0.28 g of ZC flour and 5.72 g of PSP flour. Therefore, the decrease in MDA levels could have been influenced by the active constituent present in each type of flour. The inhibitory effect of oxidative stress is attributed to anthocyanin and polysaccharide compounds in PSP, which served as the primary active ingredients in the present work. Consistent with previous research, the application of a water-soluble polysaccharide (WPSPP-1) derived from PSP was shown to enhance the resolution of inflammatory lesions by upregulating interleukin-10 (IL-10) and SOD, while concurrently downregulating interleukin-1β (IL-1β), tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and MDA levels (Gou *et al*., 2019). The presence of anthocyanins in PSP was observed to effectively inhibit the activity of ROS by electron donation. This is achieved by the hydroxyl groups (-OH) attached to the phenolic rings of anthocyanins, which readily donate electrons to free radicals. The presence of these electrons will result in the stabilisation of free radicals, thus leading to their inactivation. The fundamental elementary processes that play a crucial role in free radical chain reactions consist of three stages: initiation, propagation, and termination. The main contributors to polymer breakdown produced by oxygen are highly active hydrogen radicals (•H), hydroxyl radicals (•OH), and associated hydrocarbon-oxygen free radicals (Sai *et al*., 2022). Conversely, the primary bioactive constituent found in ZC rhizome also serves a crucial function in reducing MDA levels. According to Jamir and Seshagirirao (2018), the cysteine protease glycoprotein known as ZCPG from *Zingiber montanum* has the potential to hinder the detrimental effects of reactive radical species on biomolecules inside biological and gastrointestinal systems. Consequently, it is worth investigating ZCPG as a potential natural antioxidant ingredient in developing functional foods.

SOD activity

Eliminating ROS is a crucial function performed by antioxidant enzymes, as Bodega *et al*. (2019) highlighted. SOD is an enzyme that facilitates the conversion of superoxide anions into H_2O_2. Subsequently, H_2O_2 can be efficiently eliminated from the system by the action of glutathione peroxidase or catalase, which serve as the principal enzymes responsible for detoxifying H_2O_2. Based on Table 4, the administration of HFD resulted in a considerable decrease in the rate of SOD inhibition in the liver of rats compared to the standard group. Significant changes were observed in the SOD measurement values across all treatment groups. Significant difference ($p < 0.05$) in the rate of SOD inhibition in the liver of rats after 28 d between the group that consumed HFD and the groups that were administered with F3 biscuits was observed. F3 had 0.28 g of ZC flour and 5.72 g of PSP flour. This was consistent with a previous study that suggested anthocyanins can potentially enhance the activity of enzymes associated with the endogenous antioxidant defence system. CAT, GPx, and SOD activities were increased in pancreatic, liver, and endothelial cells following *in vitro* exposure to extracts rich in anthocyanins and pure anthocyanins (Vinothiya and Ashokkumar, 2017; Wu *et al*., 2018; Huang *et al*., 2018; 2020; Garcia and Blesso, 2021).

ZC rhizome has exhibited pharmacological efficacy in both *in vitro* and *in vivo* settings, and in clinical studies. The effects of this substance encompass analgesic, antibacterial, anti-inflammatory, anticancer, and free radical scavenging properties (Verma, 2018). The study conducted by Nurkhasanah *et al*. (2019) demonstrated an augmentation in the levels of IL-10 and IL-14 after the administration of the ethyl acetate fraction derived from the ZC extract. In the context of experimental rats, it has been seen that the administration of ZC rhizome had significant impact on the enhancement of SOD activity. Furthermore, it has been found that ZC rhizome also mitigated the adverse effects of HFD, and improved antioxidant activity. According to Sari *et al*. (2020), applying anthocyanin extract in pre-treatment of PSP resulted in a significant decrease in AST, ALT, and MDA levels, hence protecting against CCl_4-induced damage. Furthermore, in other study, it was observed that the intervention resulted in the restoration of the antioxidant defence components, SOD and GSH, to their normal activity levels (Wang *et al*., 2017).

Research findings indicate that PSP flour exhibits a significant capacity in terms of its antioxidant properties. The antioxidant activity arises from the presence of active substances, namely anthocyanins and polysaccharides. The anthocyanin component in PSP facilitates the dispersion of the purple pigmentation from the outer skin to the inside meat of the tuber. The primary function of this substance is to act as an antioxidant. The compound in question functions as an antioxidant, potentially reducing the likelihood of hypercholesterolemia (Jawi *et al*., 2020). Additionally, it can avoid oxidative stress, and exhibit properties as a free radical scavenger, antimutagenic agent, and blood pressure reducer (Ginting *et al*., 2022). The predominant anthocyanins found in PSP are derivatives of cyanidin or peonidin, namely 3,5-glucoside derivatives. These derivatives are acylated with *p*-hydroxybenzoic, ferulic, or caffeic acids. Together, these compounds account for over 90% of the total anthocyanin content in PSP (Li *et al*., 2013). According to Liao *et al*. (2019), the comparative analysis of various domestic cooking methods revealed that the boiled, steamed, baked, and microwaved samples exhibited a higher concentration of anthocyanins than the fried, air-fried, and stir-fried samples. The present work involved the production of biscuits using PSP through the process of baking. Consequently, the biscuits exhibited a significant concentration of anthocyanins, which notably impacted their antioxidant capacity. A significant positive connection was seen between the overall anthocyanin concentration and its corresponding antioxidant activity.

Polysaccharides are an additional constituent that serve a crucial function as an antioxidant within PSP. The polysaccharide derived from PSP has numerous significant biological activities, and demonstrates exceptional antioxidant properties. Consequently, there is considerable importance in exploring its potential as a functional food (Yang and Huang, 2022). Furthermore, it has been demonstrated that the polysaccharides included in PSP exhibitED immune-enhancing properties (Tang *et al*., 2019). This tendency is supported by the presence of three types of polysaccharides, namely water-soluble polysaccharides (WSP), dilute alkali-soluble polysaccharides (DASP), and concentrated alkali-soluble polysaccharides (CASP). These polysaccharides have been observed to trigger immune responses in macrophages, and promote adaptive immunity in mice by increasing the production of immunoglobulins. A separate investigation demonstrated that the polysaccharide could block the growth of HT-29 cells by inducing apoptosis (Sun *et al*., 2020; Li *et al*., 2023; Meng *et al*., 2023).

Conclusion

The rats were fed with a high-fat diet and biscuits containing a blend of ZC and PSP flour for 28 d. This intervention resulted in significant decrease in MDA levels, and increase in SOD enzyme activities. Results indicated that the inclusion of ZC rhizome (0.28 g) and PSP (5.72 g) flour in a functional biscuit had a significant impact on mitigating oxidative stress in rats fed with a high-fat diet. The antioxidant capacity of the newly developed biscuit was evaluated through *in vivo* studies, indicating the need for human clinical trials before its potential commercialisation. However, further investigation of the biscuit formulation is necessary, especially concerning the moisture, ash, and crude fibre levels present.

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