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Phenolic content and antioxidant activity of formulated biscuits with banana, tempeh and moringa flours

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Abstract

This research aimed to enhance the antioxidant activity, total phenolic content (TPC), and total flavonoid content (TFC) of formulated biscuits with moringa leaf, tempeh and banana flours. Four formulated biscuits have been developed with different percentages of moringa leaves, tempeh and banana flours, namely, F1, F2, F3, and F4. The antioxidant activity, TPC, and TFC of formulated biscuits as well as control biscuit with 100% of wheat flour were assessed by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), Folin-Ciocalteu, and aluminium chloride method, respectively. TPC, TFC, and antioxidant activity were significantly higher in all formulated biscuits as compared to the control biscuit. The best biscuit formulation containing 3% moringa leaf, 15% tempeh, and 20% banana flours showed significant and the highest TPC (0.98 ± 0.062) mg GAE/g, TFC (0.95 ± 0.095) mg QE/g, and antioxidant activity measured as IC50 value was 3.86 ± 0.091 mg/mL, compared to other biscuit formulas containing moringa leaf, tempeh, and banana flours.

Keywords: Total phenolics; Flavonoids; Antioxidant activity; Functional biscuit; Moringa; Tempeh.

Highlights

- Moringa, tempeh, banana increased antioxidant activity and total phenolics in biscuits
- 15% of tempeh flour showed the highest total phenolics and flavonoids
- Thermal process may damage bioactive compounds, especially phenolic acids

1 Introduction

Cellular metabolism normally produces oxygen free radicals (OFR), such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Shastri et al., 2016). A low concentration of ROS is typically needed to support normal physiological functions. Excessive amount of free radicals may lead to oxidative stress which contributes to the development of degenerative diseases, including autoimmune disorders, inflammatory diseases, cancer, cardiovascular diseases, as well as neurodegenerative diseases (Lobo et al., 2010; Shinde et al., 2012). Antioxidants, both endogenous and exogenous, are free radical scavengers that prevent and repair damages due to ROS and RNS, thus improving the immune system and lowering the risk of degenerative diseases (Pham-Huy et al., 2008; Shinde et al., 2012).

Plants have bioactive, non-nutrient compounds or phytochemicals. There are several types of phytochemicals, and two of them are phenolic and flavonoid compounds (Malta & Liu, 2014). Phenolic compounds could act as a defense mechanism against pathogens and parasites and have been reported to have various biological effects such as antioxidative activity, prevention of heart disease, and anti-inflammation properties. Flavonoids give huge benefits to human health, including antioxidant activity, anti-inflammation, antibacterial, and antiviral activities, as well as anti-allergic reaction, anticancer, and antimutagenic properties. Flavonoids were classified as flavones, flavonols, and isoflavonoids based on the difference in the generic structure of the heterocyclic C ring (Malta & Liu, 2014).

The development of functional foods with antioxidative properties might help to overcome non-communicable diseases related to oxidative stress. Bioactive compounds may function as antioxidants and help in reducing the risk of chronic illnesses and provide physiological health benefits (Ahmed et al., 2022). Several indigenous foods in Indonesia have potential as functional ingredients, including moringa, tempeh, and banana. Moringa (*Moringa oleifera* Lam.) is a plant that produces various secondary metabolites (carotenoid, polyphenol, flavonoid, glucosinolate) which exerts health benefits (Chelliah et al., 2017; Rani et al., 2018).

Tempeh is a fermented soybean cake from Indonesia, manufactured by fermenting cooked (boiled) soybean with *Rhizopus oligosporus* for about 48 hours. The white mycelia bind the soybean together, forming a dense and compact product. Tempeh is known as a source of bioactive compounds, especially isoflavones, while banana is a source of indigestible carbohydrates, especially resistant starch, and dietary fiber, and contains several antioxidant compounds (Surono, 2016; Khoozani et al., 2019; Khosravi & Razavi, 2021; Romulo & Surya, 2021).

Processing of tempeh, moringa leaf and banana into flours will allow them to be utilized as a composite flour, a functional ingredient for various food products, including biscuits; a ready-to-eat product that is widely consumed by various groups of people, especially children (Oyeyinka et al., 2014). Previous research has shown that biscuit incorporated with up to 10 % of moringa leaf flour increased antioxidant activity (Ajibola et al., 2015). Research conducted by Leite et al. (2013) showed that the incorporation of 50 % tempeh flour improved isoflavone aglycones (IFA) in crackers. The incorporation of 30% banana flour in cookies also increases its antioxidative properties (Amarasinghe et al., 2021). Therefore, it is interesting to formulate a functional biscuit with moringa leaf, tempeh, and banana flours to find out potential antioxidant activity, as well as the quantitative of bioactive compounds such as total phenolic content (TPC), and total flavonoid content (TFC). The research also aimed to find out the best functional biscuit formulation based on high phenolic, flavonoid contents and antioxidant activity.

2 Materials and methods

Formulation of biscuits was conducted at PT Garudafood Putra Putri Jaya Tbk, Jakarta, Indonesia. The assessment of antioxidant activity, TPC, and TFC were conducted at the Chemistry Laboratory, Department of Food Technology, Bina Nusantara University Alam Sutera Campus, Indonesia.

2.1 Chemicals and materials

Raw materials used in this research were obtained from several suppliers, including moringa leaf flour (CV. Samora Anugrah Moringa, Blora, Indonesia), tempeh flour (Indonesian Tempeh Movement Organization, Bogor, Indonesia), and banana flour (CV. Agro Nirmala Sejahtera, Cianjur, Indonesia). Ascorbic acid (Merck, Germany) and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) (Smart Lab, Indonesia) were used in the analysis of antioxidant activity. Several materials for total phenolic analysis were Folin-Ciocalteu reagent (Supelco, Germany), gallic acid (Merck, Germany), and sodium carbonate/Na_2CO_3 (Merck, Germany). While Quercetin (Kimia ARD, Indonesia), aluminium chloride/$AlCl_3$ (Merck, Germany), and sodium acetate/CH_3COONa (Merck, Germany) were used for total flavonoid analysis.

2.2 Biscuit formulation

There were four functional biscuit formulations (F1, F2, F3, and F4), a combination of moringa leaf, tempeh, and banana flours with different proportions besides wheat flour. There were two levels of proportion for each ingredient as follows: moringa leaf flour (3% and 5%), tempeh flour (5% and 15%), and banana flour (20% and 30%), substituting the wheat flour, and compared with control biscuit of 100 % wheat flour (F0). The biscuit formulas are listed in Table 1.

Table 1. Formulation of biscuits.

Ingredients	Ingredients in Each Formula (g)				
	F0	F1	F2	F3	F4
Wheat Flour (Low Protein)	100.0	62.0	62.0	60.0	60.0
Refined Sugar	34.4	34.4	34.4	34.4	34.4
Margarine	39.8	39.8	39.8	39.8	39.8
Glucose Syrup	3.0	3.0	3.0	3.0	3.0
Fructose Syrup	1.4	1.4	1.4	1.4	1.4
Skimmed Milk Powder	2.8	2.8	2.8	2.8	2.8
Sodium Bicarbonate	0.6	0.6	0.6	0.6	0.6
Salt	0.6	0.6	0.6	0.6	0.6
Vanilla Extract Powder	0.8	0.8	0.8	0.8	0.8
Flavor Enhancer	0.1493	0.1493	0.1493	0.1493	0.1493
Water	15.5	15.5	15.5	15.5	15.5
Moringa Leaf Flour	0	3.0	3.0	5.0	5.0
Tempeh Flour	0	15.0	5.0	15.0	5.0
Banana Flour	0	20.0	30.0	20.0	30.0
Lecithin	0.5507	0.5507	0.5507	0.5507	0.5507

2.3 Biscuit Manufacturing process

The production process of biscuits consists of several steps. First, all ingredients were weighed. The mixing process started with the creaming process, by mixing the fat (margarine) with sugar syrups, and lecithin, followed by the addition of sugar powder, milk powder, salt, vanilla extract powder, and flavor enhancer (from PT. Firmenich Aromatics Indonesia, Indonesia) for sweetness. The ingredients were mixed until the mixture was homogenous. Then, water was added and mixed until it formed a light and homogenous mixture. Moringa leaf, tempeh, and banana flours were added at once and mixed at medium speed. In the final step, sodium bicarbonate was mixed with wheat flour and added to the dough. The mixing process was conducted at medium speed for approximately 15-20 seconds until the dough came together and was moldable. The dough was sheeted with 4 mm thickness, molded (using a ring mold with 4 cm diameter), and baked at 150°C for 9 minutes in an electric convection oven. The biscuit samples were cooled down for approximately 10 minutes (until the texture became crispy), and the biscuits were packed.

2.4 Preparation of methanolic extract of biscuits

Extraction of the biscuits was prepared by maceration, using the method of Castillo-López et al. (2017) with slight modifications. In this research, biscuits were ground, and 80% methanol was used as the solvent. Fitriana et al. (2016) revealed that the methanol extract of *M. oleifera* showed the highest antioxidant activity in DPPH free radical scavenging assay, compared to other solvents. Centrifugation was conducted at 4200 rpm for 25 minutes, and the supernatant was filtered with Whatman filter paper No.5. Each stock sample extract was stored at 4°C until further analysis. Biscuits manufacturing was carried out in two experiments.

2.5 Total phenolic analysis

The methanolic extract of formulated biscuit was analyzed for TPC using the Folin-Ciocalteu method, based on the reduction of the Folin-Ciocalteu reagent by phenolic compound, which produces molybdenum-tungsten (has a blue color), to be measured spectrophotometrically (Malta & Liu, 2014). The total phenolic analysis was carried out based on Fapetu et al. (2022) with slight modifications. Each sample extract was diluted, to a concentration of 30 mg/mL. Gallic acid was used as standard. The absorbance was read with an Ultraviolet-visible (UV-Vis) Spectrophotometer at 725 nm, and the TPC of biscuit extracts was expressed in gallic acid equivalent (GAE) (mg GAE/g).

2.6 Total flavonoid analysis

The methanolic extract of biscuit samples was analyzed for TFC using the aluminium chloride method. The reaction of aluminium chloride and flavonoids (specifically the C-4 keto groups and C-3 or C-5 hydroxyl group of flavones and flavonols), produces yellow acid stable complex (Kumar et al., 2013; Nurlinda et al., 2021). TFC analysis was conducted based on Aminah et al. (2017) with slight modifications. In the analysis of TFC, each stock sample extract was diluted before analysis, to a concentration of 20 mg/mL. Quercetin was used as standard. The absorbance was measured with a UV-Vis Spectrophotometer at 431 nm, and the TFC of biscuit extracts was expressed in quercetin equivalent (mg QE/g).

2.7 Antioxidant (DPPH) analysis

The methanolic extract of formulated biscuit was analyzed for antioxidant activity using the DPPH method. The presence of antioxidant activity in formulated biscuit was indicated by a change in color of DPPH in methanol. The DPPH solution was originally dark purple, and the reaction between DPPH and antioxidant turned the color into yellow due to the attachment of hydrogen atom (from antioxidants) to radical form, hydrazine, which reduced conjugated double bonds in DPPH (Huong et al., 2019). Antioxidant activity is expressed as IC50 value, showing the concentration of antioxidant (sample) to reduce 50% of free radical activity. A lower IC50 value indicates higher antioxidant activity. The antioxidant activity analysis was carried out based on Sami & Rahimah (2016) with slight modifications. Functional biscuit samples (F1, F2, F3, F4) extracts were diluted to obtain a serial concentration, ranging from 2 mg/mL to 12 mg/mL, while the control biscuit sample (F0) extract was diluted to obtain a serial concentration, ranging from 2 mg/mL to 22 mg/mL. The DPPH blank (containing DPPH and methanol), sample blank (containing stock sample extract and methanol), and methanol blank (only methanol), were also prepared. The tubes were covered with aluminium foil. The absorbance was measured with UV-Vis Spectrophotometer at 515 nm.

2.8 Statistical analysis

Values of all experiments were represented as mean ± SD of two experiments and analyses were conducted in duplicate. The values were compared using one way Analysis of Variance (ANOVA) with post hoc Duncan using IBM SPSS Statistics version 25.0 (SPSS Inc., Chicago, Illinois, USA). The level of significance was set at $P < 0.05$.

3 Results and discussion

3.1 Total Phenolic Content (TPC) of biscuits

The TPC of each formulated biscuit is shown in Table 2.

Table 2. The total phenolic content of formulated biscuits.

Biscuit formulations [MLF : TF : BF]	Total Phenolic Content (mg GAE/g)
F0 [0% : 0% : 0%]	0.37 ± 0.121[c]
F1 [3% : 15% : 20%]	0.98 ± 0.062[ab]
F2 [3% : 5% : 30%]	0.83 ± 0.058[b]
F3 [5% : 15% : 20%]	1.16 ± 0.061[a]
F4 [5% : 5% : 30%]	0.95 ± 0.043[b]

Values are mean ± standard deviation. Analysis was conducted in duplicate. The value with the same letter in the same column indicates no significant difference at a significance level of 95% between the biscuits, while different letters under the same column indicate a significant difference. MLF: moringa leaf flour; TF: tempeh flour; BF: banana flour.

The TPC of all formulated biscuits containing moringa leaf flour, tempeh flour, and banana flour was in a range of (0.83 ± 0.058 to 1.16 ± 0.061) mg GAE/g, and found to be significantly higher ($P < 0.05$) than control biscuit, 0.37 ± 0.121 mg GAE/g, therefore it could be noted that moringa leaf, tempeh, and banana flours in formulated biscuits significantly increased the TPC of formulated biscuits. A higher percentage of tempeh flour at 15% significantly increased the TPC as compared to 5% tempeh flour.

Table 2 shows no significant difference in TPC between formulated biscuits containing 3% and 5% of moringa leaf flour at the same percentage of tempeh flour, and 3% of moringa leaf flour was adequate to give a significant and optimal increase of phenolic content, although samples with 5% of moringa leaf flour tend to have higher TPC compared to 3% of moringa leaf flour.

This result is in line with a previous study reported by Aishat et al. (2021), which showed no significant difference in the TPC of bread with 5% moringa leaf flour, compared to 3% moringa leaf flour. The soybean in tempeh contains a high concentration of polyphenols, especially isoflavones, while the main phenolic compounds in *M. oleifera* are phenolic acids, mainly gallic acid, chlorogenic acid (a phenolic ester), ellagic acid, and ferulic acid (Hashim et al., 2018; Khosravi & Razavi, 2021; Zhu et al., 2020).

Phenolic acids were also known to have anti-inflammatory activity by inhibiting human macrophage cytokine production, as well as antimutagenic and anticancer properties. Phenolic compounds are also known to act as primary antioxidants, due to their redox properties which inactivate lipid free radicals or prevent the decomposition of hydroperoxides into free radicals. Phenolic acids also have antidiabetic properties through their role in glucose homeostasis, by influencing the β-cell mass and function, and improving sensitivity in peripheral tissues (Vergara-Jimenez et al., 2017). Gallic acid is also known to have an inhibitory effect on cancer cell growth through the modulation of genes which responsible for encoding for cell cycle, metastasis, angiogenesis, and apoptosis (Verma et al., 2013).

In general, higher temperature resulted in a decline of total phenolic acids, since several phenolic acids were probably destroyed by heat treatment (Xu et al., 2007). The baking process of the biscuit in this study, at 150°C for 9 minutes may damage phenolic acids in moringa leaf powder, so increasing moringa leaf powder percentage did not cause a significant increase in TPC of the formulated biscuits. While isoflavones of soybeans were known to be heat stable (Otieno et al., 2006).

3.2 Total Flavonoid Content (TFC) of formulated biscuits

The TFC of each formulated biscuit is shown in Table 3.

Table 3. The total flavonoid content (TFC) of biscuits.

Samples [MLF : TF : BF]	Total Flavonoid Content (mgQE/g)
F0 [0% : 0% : 0%]	0.27 ± 0.050[c]
F1 [3% : 15% : 20%]	0.95 ± 0.095[a]
F2 [3% : 5% : 30%]	0.56 ± 0.095[b]
F3 [5% : 15% : 20%]	1.00 ± 0.015[a]
F4 [5% : 5% : 30%]	0.60 ± 0.068[b]

Values are mean ± standard deviation. Analysis was done in duplicate. Values with the same letter in the same column indicate no significant difference at a significance level of 95% between the biscuits, while different letters under the same column indicate a significant difference. MLF: moringa leaf flour; TF: tempeh flour; BF: banana flour.

The TFC of all formulated biscuits with moringa leaf, tempeh, and banana flours were in a range of (0.56 ± 0.095 to 1.00 ± 0.015) mgQE/g, significantly higher ($P < 0.05$) than control biscuit, 0.27 ± 0.050 mgQE/g. This means that moringa leaf, tempeh, and banana flours significantly increased the TFC of the biscuits. The presence of 5% tempeh and 3% moringa leaf flours significantly increased the TFC of formulated biscuits by two folds compared to the control, while 15% tempeh, and 3% moringa leaf flours significantly increased the TFC of formulated biscuits by more than three folds compared to control.

Higher percentage of tempeh flour at 15% resulted in a significant increase of TFC in formulated biscuit, as compared to lower percentage of tempeh flour at 5% (Table 3), showing that tempeh flour significantly increased the TFC in formulated biscuits, in line with the study reported by Leite et al. (2013), that incorporation of 50% tempeh flour in cookies significantly increased aglycone isoflavone, thus improving its nutritional and functional properties.

Increasing the percentage of moringa leaf flour from 3 % to 5 %, at the same percentage of tempeh flour showed no significant effect on the TFC in biscuit formulations, as shown in Table 3. The flavonoids in tempeh are mainly isoflavones, predominated by aglycone isoflavone, genistein, daidzein, and their glycosides, (Khosravi & Razavi, 2021). According to the study of heat stability as reported by Otieno et al. (2006), it could be noted that baking soy-enriched bread at 165 °C for 50 minutes resulted in stable total isoflavones.

In fact, *M. oleifera* leaves are rich in phenolic acids and flavonoids (Xu et al., 2019). Flavonoids in *M. oleifera* are mostly quercetin and kaempferol (Zhu et al., 2020). Heat treatment at 150 °C for 30 minutes, caused a significant decrease in quercetin and its glucoside content in several types of onion powder by oven drying (Sharma et al., 2015). The baking process of tiger nut enriched crackers at 165°C for 4 minutes, resulted in a reduction of kaempferol 3,7-dirhamnoside concentration (Abdel-Samie & Abdulla, 2016). Hence, increasing moringa leaf powder proportion did not give a significant increase in the TFC of formulated biscuits, presumably because part of the TFC in moringa, especially quercetin and kaempferol as the main flavonoid in moringa, was damaged due to baking of formulated biscuits at 150 °C for 9 minutes.

Flavonoids have been reported to have antioxidative properties. Quercetin as the main flavonoid in *M. oleifera* reported to have antibacterial and antiviral activities (Panche et al., 2016; Petrillo et al., 2022). Genistein and daidzein in tempeh are known as phytoestrogens. Oesterogens have a neuroprotective effect, and the potential for the treatment of various chronic diseases such as cardiovascular disorder, cancer, and osteoporosis (Panche et al., 2016). A study conducted by Ullah et al. (2020) revealed that quercetin was effective against colorectal cancer, through various mechanisms including replication of antioxidants, modulation of estrogen receptors (ERs), signaling pathways regulation, cell cycle arrest, increase in apoptosis, and inhibition of metastasis and angiogenesis, which shows the chemopreventive effects of quercetin. Flavonoids also have a cardio-protective effect, by decreasing ROS production and suppressing peroxide formation.

3.3 Antioxidant activity of formulated biscuits

The IC50 value of each formulated biscuit is shown in Table 4. The linear regression curve of ascorbic acid as standard is shown (Figure 1).

Table 4. The antioxidant activity (IC50) of formulated biscuits.

Samples [MLF : TF : BF]	IC50 Value (mg/mL)
F0 [0% : 0% : 0%]	12.20 ± 1.557^{a}
F1 [3% : 15% : 20%]	3.86 ± 0.091^{b}
F2 [3% : 5% : 30%]	5.25 ± 1.045^{b}
F3 [5% : 15% : 20%]	3.21 ± 0.248^{b}
F4 [5% : 5% : 30%]	4.26 ± 1.063^{b}

Values are mean ± standard deviation. Analysis was conducted in duplicate. Value with the same letter in the same column indicates no significant difference at a significance level of 95% between the biscuits, while different letters under the same column indicate a significant difference. MLF: moringa leaf flour; TF: tempeh flour; BF: banana flour.

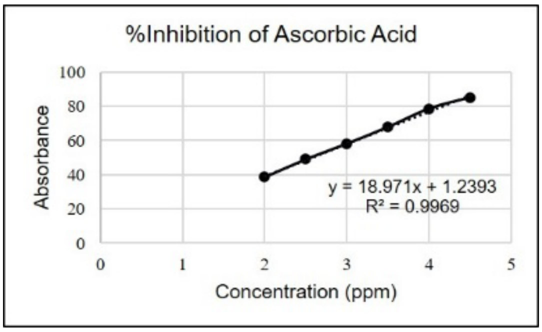

Figure 1. Linear regression curve of ascorbic acid.

Table 4 shows the IC50 value of formulated biscuits of different proportion of moringa leaf, tempeh, and banana flours were in a range of 3.21 ± 0.248 - 5.25 ± 1.045 mg/mL, significantly lower than control sample (F0) (12.20 ± 1.557 mg/mL), means, significantly higher antioxidant activity in formulated biscuits, due to the presence of bioactive compounds in moringa leaf and tempeh flours. However, there is no significant difference in antioxidant activity among all formulated biscuits, although there was a tendency to increase antioxidant activity at higher percentages of tempeh and moringa leaf flours. Figure 1 shows a linear regression curve of ascorbic acid with the IC50 value of 0.0025 mg/mL.

Some studies showed that an increase in TPC and TFC caused an increase in antioxidant activity due to their function as antioxidants (Gupta et al., 2018; Hashim et al., 2018; Nurhasnawati et al., 2019; Siskawardani et al., 2021). This study revealed a significant increase of TPC and TFC of the biscuits formulated with a higher percentage of tempeh flour at 15%, however, the antioxidant activity was not significant as compared to a lower percentage of tempeh flour at 5%. The sensory evaluation and nutritional value of formulated biscuits were also evaluated and will be reported elsewhere.

4 Conclusions

When mixing moringa leaf, tempeh, and banana flours, it could be observed a significant increase in the TPC, TFC, and antioxidant activity of formulated biscuits. The higher percentage of tempeh flour at 15% showed the most significant increase of TPC and TFC. Higher percentage of moringa leaf flour did not significantly increase the TPC, TFC, and antioxidant activity in formulated biscuits. Hence, formulation of 3% moringa leaf flour, 15% tempeh flour, and 20% banana flour was the best functional biscuit with TPC (0.98 ± 0.062) mg GAE/g, TFC (0.95 ± 0.095) mgQE/g, and antioxidant activity, with IC50 value of 3.86 ± 0.091 mg/mL.

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