Cookies rich in iron (Fe), folic acid, cobalamin (vitamin B12), and antioxidants: a novel functional food potential for adolescent with anemia [version 1; peer review: awaiting peer review]

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Abstract

**Background:** This study aims to process porang flour, moringa leaves, and tempe into cookies that are high in iron (Fe), folic acid, cobalamin (vitamin B12), and antioxidants as potential functional food snacks for adolescents with anemia.

**Methods:** This study is experimental with a completely randomized trial design (CRD) with three treatments and triplicates. There are three comparison formulations of a combination of porang, moringa leaves, and black soybean-based tempe; respectively F1 (3:3:3)%, F2 (3:6:6)%, and F3 (3:10:10)%. The cookie sample was then analyzed for water content, ash, iron, folic acid, cobalt, and antioxidant activity. The differences between the samples were analyzed based on the activity of antioxidants, iron, folic acid, and vitamin B12 (cobalamin) in data obtained from triplicates using multivariate ANOVA analysis.

**Results:** The results of the ash and water content tests of all three cookie formulations showed values in accordance with the Indonesian National Standard (SNI) for cookie products. F3 has significantly higher iron, folic acid, and cobalt levels than F1 and F2 (p<0.05).
Antioxidant activity is highest in F3, but there is no meaningful difference between F2 and F3 (p>0.05).

Conclusions: The combination of porang’s tubers, moringa leaves, and tempe made from black soybeans, especially F3, has the potential to be used as a functional cookie processed product source of iron (Fe), folic acid, cobalamin, and antioxidants for adolescent anemia.

Keywords
Iron, Folic Acid, Cobalamin, Antioxidant, Anemia, Functional Food, Cookies
Introduction

Anemia is a serious public health problem faced by all countries in the world. According to the World Health Organization (WHO), 42% of children <5 years, 33% of women of productive age, and 40% of pregnant women in the world have anemia. In Indonesia, Basic Health Research (Riskesdas) shows an increase in the prevalence of anemia in adolescents aged 15-24 years. In 2007, the prevalence of adolescent anemia in Indonesia was only 6.90% (Riskesdas, 2007), which then increased to 18.40% in 2013 (Riskesdas, 2013) and 32% in 2018 (Riskesdas, 2018). The impact of anemia is complex, ranging from immunity (Hasan et al., 2016) to work productivity (Blakstad et al., 2020), growth (Soliman et al., 2009) to intelligence (Bahrami et al., 2020), and physical fitness (Tsai et al., 2019) to maternal, fetal and child health (Georgieff, 2020).

Various interventions need to be done to speed up the treatment of anemia. One of the Government of Indonesia's programs is the provision of iron and folic acid supplementation in the form of Blood Added Tablets (TTD) in school-aged teenagers. Although the program's scope is quite high, which is 76.2%, its effectiveness is relatively low because only 2.13% consume TTD as recommended ≥52 items per year (Riskesdas, 2016). Therefore, alternative efforts such as food fortification become important to do, especially in snacks that are mostly preferred by teenagers. In addition to promising results in terms of decreasing the problem of micronutrient deficiency (Dewi et al., 2021), fortification in snacks is also potentially effective because adolescents aged 18-25 years tend to consume snacks ≥3 times per day which contributes to 25% recommended dietary allowance (RDA) or average daily dietary intake level (USDA, 2012).

Many local Indonesian foodstuffs are of high nutritional value (Permatasari et al., 2021), for example, Porang tubers. Porang's tubers (Amorphophallus oncophyllus) can be used as potential glucomannan source flour in Indonesia (especially the original regency, Madiun, East Java, Indonesia) because of the high glucomannan content (Harmayani et al., 2014). The bulbs are cultivated in Indonesia as secondary plants under teak, mahogany, and sonokeling plantations (Yanuriati et al., 2017). As a source of glucomannan, people have many benefits as functional foodstuffs (Harmayani et al., 2014; Yanuriati et al., 2017). Glucomannan properties can bind to other components such as proteins, minerals, and active compounds such as antioxidants that form a matrix of tissues in the food developed from it (Herlina et al., 2011). Glucomannan has been widely used in the food industry as a thickener, gel-forming, texture, water binder, and emulsion stabilizer, including in cookie making (Imeson, 2009; Mikkonen et al., 2009; Zhang et al., 2005).

In addition to porang's tubers, another local foodstuff that is also known to have many health benefits is moringa leaves. *Moringa oleifera* is known as an excellent food source that is easy to digest and rich in protein (Fahey, 2005). According to Sultana and Anwar (2007), moringa leaves have many valuable compounds such as protein, vitamins, calcium, iron, folic acid, and antioxidants (carotenoids, flavonoids, and phenols) (Sultana & Anwar 2007). Various developing countries in the world feed their children with moringa (Kasolo et al., 2010). Busani et al. (2011) reported that the presence of various minerals and vitamins helps boost immunity against various diseases, especially moringa leaves contain various amino acids (Busani et al., 2011). In addition, the levels of iron, folic acid, and antioxidants contained in it also have the potential to become an anti-anemia agent (Mo et al., 2013; Kurtoglu et al., 2003).

Indonesia's third potential foodstuff, which has been known globally, is tempe. Tempe is made from soybeans fermented by *Rhizopus* spp mushrooms such as *R. oligosporus*, *R. stolonifer*, and *R. oryzae* with several characteristics such as white, compact texture, and mixed distinctive flavors of mushrooms and soy flavors (Astuti et al., 2013). Black soybean tempe has potential functional characteristics, as black soybeans contain phenolics, tannins, anthocyanins, cobalamin (B12), and isoflavones, as well as a higher antioxidant activity than yellow soybeans (Xu dan Chang, 2007). According to Nurrahman et al. (2011) black soybean tempe has the same level of fondness as yellow soybean tempe, and its antioxidant activity is higher (Nurrahman et al., 2011). In addition, Vitamin B12 (Cobalamin) in the tempe has the potential in the formation of red blood cells and its antioxidants to overcome red blood cell damage (Mo et al., 2013; Kurtoglu et al., 2003).

Therefore, the combination of porang’s tubers, moringa leaves, and tempe made from black soybeans can be used as processed products such as functional cookies as a snack for anemia adolescents. This study utilizes and formulates all three ingredients into functional cookies and determines levels of iron (Fe), folic acid, cobalamin (vitamin B12), and antioxidants.

Methods

This study is experimental with a completely randomized trial design (CRD) with three treatments and triplicates.
Cookies formulation

Cookie processing

Cookie making refers to Nugraha (2009), which consists of the preparation of materials, dough formation (cream formation and flour mixing), dough printing, roasting, refrigeration, and packaging. The preparation of materials starts from weighing the material according to treatment. The formation of the dough begins by mixing margarine, egg yolk, stevia sugar powder, and baking powder using a mixer so that cream is formed. Furthermore, we added flour, moringa leaf flour (freeze-drying methods), and tempe flour (made from black soybean), corresponding to the treatment. Then the dough is formed into sheets and printed using a molding tool. Cookies are then put into the oven at 140°C for 15-20 minutes. After the cookies are done cooking, the cookies are then cooled so that the cookies are perfectly served.

Ash levels and water content analysis

The procedure for determining ash levels was carried out using the Official Methods of Analysis of Association of Official Agricultural Chemists (AOAC) International 2005 (AOAC 2005). First, the cup was dried in the oven at a temperature of 105°C for one hour. Then, the cup was cooled for 15 minutes in a desiccator and weighed. A sample of 2 grams was put into a furnace with a temperature of 550°C for three hours. It was then cooled outside the furnace until the temperature was ± 120°C and put into a desiccator to remove purified water and crystals. Plates and ash were weighed so that constant weight was obtained.

The determination of the moisture content used was the AOAC method of drying (thermogravimetry). The principle of this method was based on the evaporation of water in materials by heating, then weighing to a constant weight. The weight loss that occurred is the water content contained in the material. The empty plate was heated in the oven at 105°C for 30 minutes, cooled in a desiccator for 15 minutes, then weighed (W0). A sample of 2 grams was then put in a cup that had a known weight, weighed (W1), then dried in the oven at a temperature of 105°C for three hours, cooled in a desiccator for 15-30 minutes, then the cup and its contents were weighed, re-dried for an hour, cooled in an extractor, and re-weighed (W2).

Iron (Fe) ortho-phenantroline analysis

The principle of determining total iron content by the method o-phenanthroline was the same as the method of α,α’ – the principle of iron (III) was reduced by hydroxyl amen to form iron (II). Iron (II), both present in foodstuffs and iron reduction (III), was then reacted with o-phenanthroline to form a red Fe-phenanthroline complex that can be measured with visible spectrophotometers at a wavelength of 510 nm.

Folic acid and B12 (cobalamin) analysis

This analysis used HPLC-UV with a gradient elevation system in which the composition of the phase of motion changes during the measurement process. The phase of motion used was dapar phosphate 10 mM pH 4, and the dapar phosphate mixture: acetonitrile, 7:3. As a stationary phase, the Atlantis column T3 C18 (4.6 mm × 150 mm), 5 μm was used. Oven temperature were 35 °C, with a flow rate of 0.5 mL/min and injection volume of 20 μL. UV detectors were set at wavelengths of 280 for folic acid and 275 nm for cobalt (vitamin B12). A raw solution of cobalt (vitamin B12) with a concentration of 2 μg/mL and folic acid of 0.4 μg/mL was injected into HPLC of 20 μL under appropriate analytical conditions.

Antioxidant activity assay

Antioxidant activity was determined using DPPH (2,2-diphenyl-1-pikrilhydrazil). Each sample consisting of 100 dL was placed in a microplate of 96 and added 100 L DPPH 0.3 mM, then incubated for 30 minutes in a dark room. The absorbance of the sample was measured using an ELISA reader (# Allsheng) at a wavelength of 517 nm.

Data management and analysis

Statistical analysis was done using SPPS 26.0 for apple's version of MacBook. The differences between the samples were analyzed based on the activity of antioxidants, iron, folic acid, and vitamin B12 (cobalamin). Data obtained from three repeats (triplicates) was analyzed with multivariate ANOVA at 95% CI (p < 0.05). The result was declared significant if the p-value < 0.05.
Results
Test of normality
Ash levels and water content analysis

The results of the ash and water content tests of the three cookie formulations above showed a value in accordance with the Indonesian National Standard for cookie products (SNI 01-2973-1992), which is a maximum ash content of 1.5% and water content of no more than 5%. Raw details of ash level and ash level test results are found in (Underlying Data) (Nurkolis & Bolang, 2021).

The results of the ANOVA multivariate test above showed that there was a significant difference (p < 0.05) between all treatments in each test result or variable (Fe, folic acid, and vitamin B12). However, in antioxidant activity, that has a significant difference only F2 & F3. While between F1 & F2 equal F1 & F3 there is no significant difference (p > 0.05). Details of raw data from laboratory tests on levels of antioxidant activity (%), iron (Fe), folic acid, and cobalamin (vitamin B12) can be seen in Underlying Data (Nurkolis & Bolang, 2021).

Table 1. Cookies formulation.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>F1 (%)</th>
<th>F2 (%)</th>
<th>F3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porang Flour</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Moringa Leaf Flour</td>
<td>3</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Tempe Flour (Black-Soybean)</td>
<td>3</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Stevia</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Skimmed Milk</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Egg Yolk</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Baking Powder</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Margarine</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2. Tests of normality.

<table>
<thead>
<tr>
<th>Formulasi</th>
<th>Kolmogorov-Smirnov*</th>
<th>Shapiro-Wilk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Statistic df Sig.</td>
<td>Statistic df Sig.</td>
</tr>
<tr>
<td>Fe (mg/100 g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>.322 3 .880 3 .324</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>.212 3 .990 3 .811</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>.382 3 .757 3 .015</td>
<td></td>
</tr>
<tr>
<td>Folate (mcg/100 g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>.270 3 .949 3 .565</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>.249 3 .967 3 .652</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>.358 3 .813 3 .146</td>
<td></td>
</tr>
<tr>
<td>B12 (mcg/100 g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>.212 3 .990 3 .813</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>.297 3 .917 3 .441</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>.227 3 .983 3 .749</td>
<td></td>
</tr>
<tr>
<td>Antioxidant activity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>.216 3 .988 3 .793</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>.283 3 .935 3 .506</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>.253 3 .964 3 .637</td>
<td></td>
</tr>
</tbody>
</table>

Based on the normality test results above, the data obtained is homogeneous (p > 0.05), followed by the Anova multivariate test. *Lilliefors significance correction.
Anemia is a severe public health problem faced by all countries globally, and various interventions need to be done to speed up the treatment of anemia. One of them is utilizing local natural ingredients as functional food sources of iron (Fe), folic acid, cobalamin (vitamin B12), and antioxidants for adolescent anemia. In this study, researchers utilized and formulated all three local natural ingredients (a combination of people’s tubers, moringa leaves, and black soy-based tempe) into functional cookies and determining levels of iron (Fe), folic acid, cobalamin (vitamin B12), and antioxidants. The selection of processing into cookie products is wrong as an alternative effort instant snack food that is majority preferred by teenagers without having to process it again (e.g., iron source chicken liver but must go through the cooking process, etc.). This research is a basic study, whose certainty of clinical benefits needs to be done further through pre-clinical studies and clinical trials in humans. However, the results of this study (cookie formulation) can be used as a

**Table 3. Results of ash levels and water content analysis.**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Ash</th>
<th>Moisture content</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.30 ± 0.35</td>
<td>3.42 ± 1.35</td>
</tr>
<tr>
<td>F2</td>
<td>1.37 ± 0.07</td>
<td>3.76 ± 1.26</td>
</tr>
<tr>
<td>F3</td>
<td>1.46 ± 0.16</td>
<td>3.73 ± 1.77</td>
</tr>
</tbody>
</table>

**Table 4. Multiple comparisons.**

<table>
<thead>
<tr>
<th>Tukey HSD</th>
<th>Dependent variable</th>
<th>(I) Formulasi</th>
<th>(J) Formulasi</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>Sig.</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F1</td>
<td>F2</td>
<td></td>
<td></td>
<td></td>
<td>Lower bound</td>
</tr>
<tr>
<td>Fe (mg/100 g)</td>
<td></td>
<td>F1</td>
<td>F2</td>
<td>1.81667*</td>
<td>.47005</td>
<td>.019</td>
<td>.3744</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F1</td>
<td>F3</td>
<td>−4.16667*</td>
<td>.47005</td>
<td>.000</td>
<td>−5.6089</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F2</td>
<td>F1</td>
<td>−1.81667*</td>
<td>.47005</td>
<td>.019</td>
<td>−3.2589</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F2</td>
<td>F3</td>
<td>−5.98333*</td>
<td>.47005</td>
<td>.000</td>
<td>−7.4256</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F3</td>
<td>F1</td>
<td>4.16667*</td>
<td>.47005</td>
<td>.000</td>
<td>2.7244</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F3</td>
<td>F2</td>
<td>5.98333*</td>
<td>.47005</td>
<td>.000</td>
<td>4.5411</td>
</tr>
<tr>
<td>Folate (mcg/100 g)</td>
<td></td>
<td>F1</td>
<td>F2</td>
<td>4.65333*</td>
<td>.71293</td>
<td>.002</td>
<td>2.4659</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F1</td>
<td>F3</td>
<td>−4.79000*</td>
<td>.71293</td>
<td>.001</td>
<td>−6.9775</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F2</td>
<td>F1</td>
<td>−4.65333*</td>
<td>.71293</td>
<td>.002</td>
<td>−6.8408</td>
</tr>
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<td></td>
<td></td>
<td>F2</td>
<td>F3</td>
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<td>.71293</td>
<td>.000</td>
<td>−11.6308</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F3</td>
<td>F1</td>
<td>4.79000*</td>
<td>.71293</td>
<td>.001</td>
<td>2.6025</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F3</td>
<td>F2</td>
<td>9.44333*</td>
<td>.71293</td>
<td>.000</td>
<td>7.2559</td>
</tr>
<tr>
<td>B12 (mcg/100 g)</td>
<td></td>
<td>F1</td>
<td>F2</td>
<td>5.39000*</td>
<td>1.02781</td>
<td>.005</td>
<td>2.2364</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F1</td>
<td>F3</td>
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<td>1.02781</td>
<td>.002</td>
<td>−9.4703</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F2</td>
<td>F1</td>
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<td>.005</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>F2</td>
<td>F3</td>
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<td>1.02781</td>
<td>.000</td>
<td>−14.8603</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F3</td>
<td>F1</td>
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<td>1.02781</td>
<td>.002</td>
<td>3.1631</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F3</td>
<td>F2</td>
<td>11.70667*</td>
<td>1.02781</td>
<td>.000</td>
<td>8.5531</td>
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<tr>
<td>Antioxidant activity (%)</td>
<td></td>
<td>F1</td>
<td>F2</td>
<td>3.46667</td>
<td>1.42911</td>
<td>.112</td>
<td>−.9182</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F1</td>
<td>F3</td>
<td>−2.14667</td>
<td>1.42911</td>
<td>.355</td>
<td>−6.5316</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F2</td>
<td>F1</td>
<td>−3.46667</td>
<td>1.42911</td>
<td>.112</td>
<td>−7.8516</td>
</tr>
<tr>
<td></td>
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<td>F2</td>
<td>F3</td>
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<td>.018</td>
<td>−9.9982</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F3</td>
<td>F1</td>
<td>2.14667</td>
<td>1.42911</td>
<td>.355</td>
<td>−2.2382</td>
</tr>
<tr>
<td></td>
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<td>5.61333*</td>
<td>1.42911</td>
<td>.018</td>
<td>1.2284</td>
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</table>
reference in the dosing of preclinical trials and future clinical trials. The results of the ash and water content tests of these three cookie formulations showed a value in accordance with the Indonesian National Standard for cookie products (SNI 01-2973-1992), which is a maximum ash content of 1.5% and water content of no more than 5% (Table 3).

Formulation 3 (F3) is a high iron cookie, amounting to 29.41 ± 0.65 mg / 100g, higher than F1 and F2 significantly (p < 0.05) (Table 4; Figure 1). In the scheme (Figure 5), iron is needed by the body for erythropoiesis or the process of red blood cell synthesis, precisely as the constituent material of hemoglobin (Singh, 2018). F3 cookies in this study have higher iron levels than the functional cookie of previous research 2020 which only has a level of 2.51 mg/100g (Rahmat et al., 2020). In line with AKG (Angka Kecukupan Gizi) Indonesia in 2019, iron adequacy rates for ages 10-18 years in men range from 8-11 mg, in women 8-15 mg per person per day. Seeing this, F3 cookies are potentially a food source of iron, especially for adolescents with anemia.

In addition to iron height, F3 cookies have a fairly high antioxidant content of 24.41 ± 1.31%, although there is no significant difference with F2 cookies (Table 4; Figure 4). In addition to needing iron food sources to avoid anemia is also

![Figure 1. F3 cookie has the highest Fe content.](image1)

In formulation 3 (F3) based on multivariate results, iron (Fe) levels were 29.41 ± 0.65 mg/100 g, significantly higher than F1 and F2 (p < 0.05).

![Figure 2. F3 cookie has the highest folic acid content.](image2)

In formulation 3 (F3) based on multivariate results, folic acid (vitamin B9) or folate levels were 308.24 ± 1.30 mcg/100 g, significantly higher than F1 and F2 (p < 0.05).
needed foods that have antioxidant abilities to protect red blood cell damage due to the occurrence of oxidative stress (Reactive Oxygen Species) (Lobo et al., 2010; Gwozdzinski et al., 2021).

Folic acid or vitamin B9 and cobalt (vitamin B12) also have an important role in minimizing the incidence of anemia. Folic acid collaborates with vitamin B12 in making red blood cells and helps iron function properly in the body (Figure 5) (Mahmood, 2014). Vitamin B9 works with vitamins B6 and B12 and other nutrients in controlling the amino acid homocysteine levels in increasing the effectiveness of red blood cell formation (Figure 5) (Mahmood, 2014). Formulation 3 (F3) is the best formulation when viewed from iron (Fe), folic acid (vitamin B9), cobalamin (vitamin B12), and antioxidant activity. F3 cookies have the highest levels of folic acid and cobalamin (308.24 ± 1.30 mcg/100 g and 109.76 ± 1.74 mcg/100 g) compared to F1 and F2 (Table 4, Figures 2 and 3). Cookie F3 has folic acid that is closest to AKG (Angka Kecukupan Gizi), which recommends 400 mcg per person per day (for men and women aged 10-18 years).

Cookie innovation made from a combination of porang’s tubers, moringa leaves, and tempe made from black soybeans has potential to be made a functional anti-anemia food or to minimize anemia comprehensively (see Figure 5). In addition,
the sugar or sweetener used in this formulation is stevia which is clinically safer (not hyperglycemia) (Ashwell, 2015). However, further studies are needed in both experimental animals and humans to find out more about the clinical benefits.

**Conclusion**

The combination of porang's tubers, moringa leaves and tempe made from black soybeans, especially F3 has the potential to be used as a functional cookie processed product source of iron (Fe), folic acid, cobalamin, and antioxidants for adolescent anemia.

**Data availability**

*Underlying data*


The project contains the following underlying data:

- Raw Data: Water and Ash Content, Iron (Fe), Folic Acid, Cobalamin, and Antioxidants.

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

**Acknowledgment**

We thank all contributors for their outstanding help in formatting the paper. I would also like to thank Prof. Ir. Hardsinyah, MS., Ph.D. (as President of the Federations of Asian Nutrition Societies; President of Food and Nutrition Society of Indonesia; and Chair of Southeast Asia Probiotics Scientific and Regulatory Experts Network), and Prof. Dr. Nurpudji A Taslim, M.D., MPH., Sp.GK(K) (Chairman of the Indonesian Association of Clinical Nutritionists), who has provided commentary, advice, and input in the research and writing of this paper, as well as the motivation that has been given to the author to keep the spirit of research during the pandemic. This research was supported by the Indonesia Endowment Fund for Education (LPDP: Lembaga Pengelola Dana Pendidikan) Ministry of Finance, Republic of Indonesia.
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