Comparison Between the Potential of Tempe Flour Made from Germinated and Nongerminated Soybeans in Preventing Diabetes Mellitus

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ARTICLE INFO

Received April 1, 2019
Received in revised form August 2, 2019
Accepted August 10, 2019

KEYWORDS: diabetes, tempe, antioxidant, isoflavones, α-amylase, α-glucosidase

1. Introduction

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia due to a defect in insulin production, insulin action, or both (American Diabetes Association 2010). Oxidative stress plays an important role in diabetes mellitus etiology and is responsible for damaging pancreatic β-cells. Therefore, controlling the blood glucose level, inhibiting oxidative stress (El-Kordy and Alshahrani 2015), and increasing body antioxidant defensive system (Shahidi et al. 2012) have been suggested to manage diabetes mellitus.

Plants high in antioxidants such as vitamin E, vitamin C, β-carotene, and polyphenols have been used as traditional medicine to treat diabetes in several regions. Antioxidants are compounds which actively bind reactive oxygen species (ROS) and protect pancreatic β-cells from oxidative stress (Firdaus et al. 2010). In addition to the ROS-binding mechanism, antioxidants can act as antidiabetic agents due to their ability to inhibit carbohydrate absorption. Inhibition of carbohydrate absorption occurs as antioxidants are able to inhibit α-glucosidase and α-amylase activities, inhibit gluconeogenesis, stimulate pancreas activity, and increase insulin sensitivity (Shahidi et al. 2012).

Acarbose is commonly used as an α–amylase and α-glucosidase inhibitor in diabetes treatment. Acarbose reduces the complex carbohydrate digestion rate which later decreases glucose absorption in the small intestines. However, acarbose has several gastrointestinal side effects, including flatulence, abdominal discomfort, and diarrhea (Setter et al. 2006). Phenolic extracts generally have slightly weak α-amylase inhibition but have a stronger ability in inhibiting α-glucosidase that does not give rise to significant side effects (Adefegha and Oboh 2016).

ABSTRACT

This study was aimed to compare the chemical characteristics of tempe flour made from nongerminated soybean (NST) and germinated soybean (GST), especially on their capacity in preventing diabetes mellitus (DM). Soybeans were germinated for 20 hours in the dark until 2.5-5.0 mm of the radicle emerged. The ungerminated soybeans and the germinated soybeans were then processed into tempe and tempe flour. The two types of tempe flour were subjected to proximate analysis, amino acid profiling, antioxidant capacity, total phenol content, isoflavone content, and α-amylase and α-glucosidase enzyme inhibition analyses. GST was superior in preventing DM in the protein content and antioxidant parameters, as these were significantly higher (p<0.05) than in NST. On the other hand, NST was superior in preventing diabetes in the isoflavon (daidzein, genistein, and total isoflavone) and α-amylase inhibition IC50 parameters which were significantly better (p<0.05) than in GST. On the contrary, the diabetes-preventing parameters total phenols, α-glucosidase inhibition IC50, and insulinotropic amino acids (arginine, alanine, phenilalanine, isoleucine, leucine, and lysine) were not different (p>0.05). Therefore, GST and NST both have potential in preventing diabetes through different mechanisms.

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Bioactive compounds that show antidiabetic activities in legumes are isoflavones, an α-amylase inhibitor, and an α-glucosidase inhibitor (Çeçek et al. 2014). A study regarding germination of soy demonstrated that it was able to increase the bioactive compounds (Puteri et al. 2018). During germination, the seeds undergo various biochemical and enzymatic reactions; therefore, increasing their nutritional value and digestibility compared to the seeds without germination process (Márton et al. 2010). Germination increases the amount of vitamin E, total isoflavones, antioxidant capacity, calcium, phosphorus, iron, and zinc content in germinated soy tempe flour compared to nongerminated soy tempe flour (Astawan et al. 2016a). In addition to the potential of germination soybean as antidiabetic has been evidenced by the presence of insulinotropic amino acids that play role in increasing insulin secretion such as alanine, arginine, phenylalanine, isoleucine, leucine, and lysine (Kanetro and Astuti 2013).

Tempe, a fermented soybean by Rhizopus spp mold originally from Indonesia, is an excellent protein source with high nutritional quality (Astawan et al. 2016b). Differences in the production process and unique fermentation condition in different regions result in varieties of tempe (Kadar et al. 2018). Tempe has higher nutritional values compared to soybeans because the fermentation process degrades carbohydrate, fat, and protein into their respective building blocks (Astawan et al. 2015). Aside from its increased digestibility, tempe contains higher aglycone isoflavones compared to soybeans (Sumi and Yatagai 2006). Fresh tempe is highly perishable and has a short shelf life. Processing tempe into flour is an alternative method to increase its shelf life (Astawan et al. 2017). This study was aimed to compare the characteristics of germinated soybean tempe flour (GST) and nongerminated soybean tempe flour (NST), especially the chemical compounds which act as antidiabetics.

2. Materials and Methods

2.1. Sample Preparation

The main ingredient used in this research was the local Grobogan variety soybean (Glycine max) collected from farmers in Grobogan, Central Java, Indonesia. The germination process was done by soaking the soybeans in water for 3 hours, then draining them, and incubating them for 20 hours in a dark condition. During the incubation process, the soybeans were sprayed with water every 4 hours to maintain their humidity (Puteri et al. 2018).

Tempe production was done according to Rumah Tempe Indonesia (Indonesian House of Tempe). Soybeans were soaked for 2–4 hours, boiled for 30 minutes, and soaked for 18–30 hours in clean water added with some of the boiled water used before. Through soaking, the pH would decrease (the target pH being 4.0–5.0). After that, the soybeans were ground, peeled, cleaned, rinsed with hot water, and drained at room temperature. After draining, the soybeans were inoculated with the commercial starter culture “RAPRIMA” produced by LIPI Bandung (2 grams per kilogram of dry soybeans) and then fermented for 48 hours. The first 24 hours was done at 30–34°C, while the second 24 hours at room temperature (below 30°C) (Astawan et al. 2017).

Tempe was further processed into flour according to the method by Astawan et al. (2015). Fresh tempe was sliced, steam blanched for 2 minutes, and then dried using an oven (GmbH 6072 Dreieich, West Germany) at 60°C for 8 hours. Dried tempe was ground using a disc-mill and sifted using a 60 mesh sieve. The flour was stored in a tightly sealed container. Tempe flour was used for chemical analysis consisting of proximate analysis, crude fiber, amino acids profile, isoflavones, and vitamin E analyses.

Tempe flour was further extracted with ethanol according to the Xu and Chang (2007) method with modifications. Flour was extracted with ethanol/water (70:30 v/v) at a ratio of 1:5 (b/v), shaken with a stirrer at room temperature for 3 hours, extracted for additional 12 hours in a dark condition, filtered through Whatman Paper No. 1, and followed by centrifugation (Centrifuge 5810R Eppendorf, Hamburg, Germany) 300 x g for 10 minutes. Ethanol was removed by a rotary evaporator (Rotavapor R-210 Buchi Labortechnik AG, Flawil, Switzerland) at 45°C. The extract was stored at 4°C in the dark until further analysis. This extract was used for antioxidant, total phenolics, and inhibition of α-amylase and α-glucosidase analyses.

2.2. Chemical Component Analysis

Moisture, ash, protein, fat, carbohydrate by difference, crude fiber, vitamin E, total isoflavone, and amino acids profile were determined using methods described by the Association of Official
Analytical Chemist (AOAC 2012). The total phenolics content was determined using the method described by Koh et al. (2012). A total of 1 ml tempe flour extract was placed in a test tube and mixed with 5 ml Folin-Ciocalteu reagent (1:1 with water). The mixture was allowed to stand at room temperature for 5 minutes. Then 4 ml of sodium carbonate solution (7.5% w/v) was added and mixed thoroughly. The tubes were placed in a dark condition for 2 hours and the absorbance was measured at 765 nm. Gallic acid (Sigma-Aldrich) was used as the standard and the amount of total phenolics content was calculated as a Gallic Acid Equivalent (GAE).

2.3. Antioxidant Capacity Analysis

The antioxidant capacity analysis was determined using method described by Hung and Nhi (2012). A total of 0.1 ml tempe flour extract was mixed with 3.9 ml of 0.075 mM DPPH (2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich) solution. The mixture was allowed to stand at room temperature in the dark for 30 minutes. The absorbance was measured at 517 nm. Ascorbic acid (Sigma-Aldrich) was used as the standard and the antioxidant capacity was expressed as an AEAC (Ascorbic Acid Equivalent Antioxidant Capacity).

2.4. Alpha-amylase Inhibition Assay

The alpha-amylase inhibition assay was conducted using method described by Thalapaneni et al. (2008). A total of 125 µl tempe flour extract was added to the same amount of sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing 1 U/ml α-amylase (from Aspergillus oryzae) solution (Sigma-Aldrich). The mixture was incubated at 37°C for 10 minutes. Then, 125 µl of 1% corn soluble starch (Sigma-Aldrich) solution was added and the mixture was incubated again at 37°C for 10 minutes. The reaction was terminated by adding 250 µl dinitrosalicylic acid (DNS) solution and incubated in a boiling water bath for 5 minutes. The reaction mixture was then diluted by adding 5 ml of distilled water. The absorbance was measured at 540 nm.

2.5. Alpha-glucosidase Inhibition Assay

The alpha-glucosidase inhibition assay was done using method described by Sancheti et al. (2009). A total of 10 µl tempe flour extract was mixed with 50 µl of 0.1 m potassium phosphate buffer (pH 7.0), 25 µl ρ-nitrophenyl-α-D-glucopyranoside (dissolved in 0.1 m potassium phosphate pH 7.0), and 25 µl of 0.04 U/ml α-glucosidase (from Saccharomyces cerevisiae) solution (Sigma-Aldrich). The mixture was incubated at 37°C for 30 minutes. The reaction was terminated by adding 100 µl of 0.2 m sodium carbonate. The mixture was then diluted by adding 1 ml of distilled water. The absorbance was measured at 405 nm.

3. Results

Soybean germination before processing into tempe flour changed the content of several components analyzed. The result of the proximate analysis and crude fiber analysis are presented in Table 1. The germination process increased moisture (p<0.01), protein and crude fiber contents (p<0.05) of the tempe flour yielded, while the carbohydrate content decreased (p<0.01). Fat, and ash contents demonstrated no change.

As shown in Table 2, most amino acids did not change significantly and only a few suffered a decrease. The germination process significantly decreased the aspartic acid level (p<0.01), followed by threonine, valine, and leucine (p<0.05). Among the essential amino acids, leucine was the highest in amount in both tempe flours, while the lowest in amount was methionine. In addition, the content of insulinotropic amino acids such as arginine, alanine, phenylalanine, isoleucine, and leucine tends to decline due to the germination process of soybeans.

Table 3 presents the result of the antioxidant analysis and the components which were expected to be involved in the antioxidant activity. The antioxidant capacity of GST was higher than NST (p<0.05). However, total isoflavones content in GST was decreased (p<0.01), while the total phenolics content did not show significant change.

Germination decreased the inhibition of α-amylase and α-glucosidase (p<0.05) that shown by the increased of their IC_{50} value. The IC_{50} value of α-amylase was significantly lower (74.8 mg/ml) in NST as compared to GST (92.0 mg/ml). The similar result was also observed in α-glucosidase, where NST had IC_{50} value significantly lower (85.3 mg/ml) as compared to GST (115.9 mg/ml), as shown in Figure 1.

4. Discussion

Various biochemical and enzymatic reactions occur during the germination process that breaks down macromolecules into their respective building blocks. Macromolecule breakdown increases the
However, a study in (Márton et al. 2010). The moisture content tends to increased after germination. Soybean absorb water during the germination process for its metabolism. Increased water absorption occurs due to the increasing number of cells to becoming hydrated seed. So that there were increased in the moisture content in tempe flour (Warle et al. 2015).

Germination tends to decrease ash content since the soaking and cleaning process leaches the mineral content to the water. The soaking process decreases Mg and Zn content (Márton et al. 2010). Ash content tends to decrease represents mineral loss during germination and washing, but some essential mineral such as Ca and Cu could rise during germination (Jiang et al. 2013). However, a study conducted by Astawan and Hazmi (2016) showed that the germination process can increase the ash content of soybean flour. The difference in results may be due to the calcium oxide solution added during the germination process in that study.

Carbohydrate and fat are hydrolyzed to provide the energy needed to synthesize protein (Shi et al. 2010). Decrease fat level is due to the use of fat deposits as an energy during the germination process. Decrease fat levels are also followed by a decrease in carbohydrate. Carbohydrate molecules will be broken down during the germination process to provide nitrogen absorption for the formation of amino acids (Joshi and Kanika 2016).

The increase in protein content may be caused by protein synthesis and reduction of other components from the leaching during boiling or they were used by the tempe mold for its growth (Astawan et al. 1994). Protein synthesis occurs during imbibition to produce enzymes, hormones, and other components needed for radicle growth. Despite the fact that throughout the germination process protein is broken down into amino acids, it seems that the protein synthesis exceeded the protein breakdown.

### Table 1. Proximate analysis and crude fiber content of tempe flours

| Parameter                  | Nongerminated soybean | Germinated soybean |
|----------------------------|-----------------------|--------------------|
| Moisture (% wb)            | 3.92±0.82             | 5.98±1.03          |
| Ash (% db)                 | 2.08±0.05             | 1.89±0.11          |
| Fat (% db)                 | 25.94±1.35            | 25.65±0.99         |
| Protein (% db)             | 50.69±0.53            | 52.98±0.55         |
| Carbohydrate (% db)        | 17.36±1.70            | 13.50±2.46         |
| Crude fiber (% db)         | 5.05±0.06             | 6.59±0.10          |

*Different superscripts in the same row means statistically different (p<0.05)

### Table 2. Amino acid profile of tempe flours

| Parameter (% db)   | Nongerminated soybean | Germinated soybean |
|--------------------|-----------------------|--------------------|
| Aspartic acid      | 6.14±0.06             | 5.64±0.05          |
| Glutamic acid      | 10.17±0.23            | 9.49±0.09          |
| Serine             | 2.76±0.03             | 2.48±0.15          |
| Histidine          | 1.18±0.01             | 1.18±0.08          |
| Glycine            | 1.93±0.03             | 1.97±0.06          |
| Threonine          | 2.15±0.03             | 1.97±0.04          |
| Arginine           | 3.70±0.08             | 3.42±0.04          |
| Alanine            | 2.34±0.04             | 2.15±0.01          |
| Tyrosine           | 1.86±0.01             | 1.70±0.03          |
| Methionine         | 0.58±0.05             | 0.61±0.04          |
| Valine             | 2.66±0.01             | 2.48±0.01          |
| Phenylalanine      | 2.89±0.06             | 2.75±0.02          |
| Isoleucine         | 2.66±0.03             | 2.50±0.00          |
| Leucine            | 4.21±0.07             | 3.94±0.06          |
| Lysine             | 2.60±0.58             | 3.39±0.08          |

*Different superscripts in the same row means statistically different (p<0.05)

### Table 3. Antioxidant analysis of tempe flours

| Parameter (per 100 g db) | Nongerminated soybean | Germinated soybean |
|--------------------------|-----------------------|--------------------|
| Antioxidant capacity (mg AEAC) | 186.819±17.34 | 249.79±5.06 |
| Daidzein (mg)            | 53.56±1.02            | 35.96±3.59         |
| Genistein (mg)           | 62.46±0.56            | 44.16±5.26         |
| Total isoflavones (mg)   | 116.02±1.58           | 80.13±8.86         |
| Total phenols (mg)       | 426.74±58.88          | 483.92±37.60       |
| Vitamin E (mg)**         | 4.75                  | 4.87               |

*Different superscripts in the same row means statistically different (p<0.05)

**Data were obtained from one analysis only

Figure 1. IC$_{50}$ value of α-amylase and α-glucosidase in tempe flours. NST=nongerminated soybean tempe flour; GST=germinated soybean tempe flour. Different superscripts means statistically different in each enzyme (p<0.05)
The crude fiber content was increased in tempe flour from germinated soybean (GST). In germinated soybean, the amount of crude fiber was contributed by the presence of seed layer, like epidermis, hypodermis, and parenchyma that contain fibers such as cellulose, pectin, galactomannan, and glycoprotein (Krisnawati and Adie 2008). Some studies have shown that intake of fibers especially water soluble fiber can also help to control glucose level. Fibers are able to absorb water and bind glucose, thereby reducing glucose availability. Insufficient dietary fiber also resulted in increased digestibility of carbohydrates (Santoso 2011).

Villegas et al. (2008) studied the relationship between soy consumption and the risk of type 2 diabetes mellitus in Chinese women aged 40-70 years. That study showed a higher consumption of soy decreased the occurrence of diabetes. The protective effect of soy was likely due to fiber, protein, polyphenols, and the low glycemic index of soy. It is suggested that the protective effect was not caused by a single component, but was due to the interaction of several components (Sugano 2006).

Soy protein inhibits body fat accumulation through the inhibition of insulin secretion, inhibition of lipogenesis, and enhancement of insulin sensitivity. These mechanisms are affected by the amino acid profile and isoflavones bound to the proteins. In experimental rats given soy protein, there was an increase in insulin sensitivity (Noriega-López et al. 2007). Reduction of body fat accumulation decreases the obesity risk which is one of the risk factors for diabetes mellitus.

Another study showed that soy protein and germinated soy protein were able to stimulate insulin secretion in diabetic rats. Germinated soy protein contains free amino acids which can stimulate insulin secretion better than non-germinated soy protein. Several amino acids show a stronger insulinotropic effect, such as arginine, leucine, and phenylalanine (van Loon et al. 2003). However, the germination process in this research did not increase the content of insulin-stimulating amino acids (Table 2). A decrease in leucine content was observed instead. The availability of amino acids is not necessarily a reference, but the bioavailability affects the effectiveness of the use of these insulinotropic amino acids.

The mechanism of several amino acids in stimulating insulin release are varied. The results of the study using alanine amino acid showed that the addition of 10 mmol/l alanine in basal glucose concentrations increased insulin secretion three times in cells β-BRIN-BD11 and 1.6 times on islet. Leucine amino acid stimulate the release of insulin in the cells of β pancreas through a process that includes increased mitochondrial metabolism through the activation of GDH, increased production of ATP through leucine transaminases into α-toxicity and subsequently enter the TCA cycle through acetyl CoA (Newsholmes et al. 2005).

Arginine affecting the wound healing, cause arginine as the one of the nitric oxide-forming materials (NO) that would help the collagen synthesis in the wound area. Another studies showed that NO will regulate the metabolism of glucose, fatty acids and amino acids, so that the consumption of arginine would decrease fat mass in the obese and diabetic rats. Nitric oxide also enhances glucose transport, lowers glucose, glycogen synthesis, stimulates release of insulin and inhibit the rate of pancreatic β-cell damage (Utari et al. 2011).

Studies about the amino acid profile in germinated and nongerminated soybeans demonstrated different results. Pomeranz et al. (1977) only found a little difference in amino acid composition between commercial soybean flour and germinated soybean flour. Different results were observed by Mostafa and Rahma (1987) where soybean germination relatively increased the essential and non-essential amino acid content. The highest increase was observed in leucine, tyrosine, phenylalanine, and glutamic acid, while methionine content decreased slightly. The different results obtained might be due to different germination conditions, different soybean varieties used, and different analysis methods.

The fermentation process also affected the amino acid profile of tempe flour. According to the study by Gibbs et al. (2004), fermentation of tempe for 24 hours decreased the content of aspartic acid, glutamic acid, lysine, tyrosine, and serine, while glycine, isoleucine, leucine, proline, and threonine increased. Some amino acids, for example, alanine, valine, arginine, histidine, phenylalanine, cysteine, methionine, and tryptophan tended not to change in content. This is affected by the peptidase activity of R. oligosporus.

Germination caused increase production of bioactive compounds with potent antioxidant properties. Studies demonstrated that germination can alter the level of bioactive compounds of soybean by
increasing luscin, isoflavone and saponins. Analysed soybeans after 48 h of germination, concluding that germination reduced tannins while increased total phenolics (Vernaza et al. 2012). The phenolics content of legumes tends to have a positive correlation with its antioxidant capacity. This current study showed that the increase in antioxidant capacity in GST was likely not due to the increase in phenolics as the phenolics content remained constant and the isoflavones decreased. Nevertheless, non-phenolics compounds, including ascorbic acid, tocopherols, phytic acid, carotenoids, and saponins, could also contribute to the antioxidant activity (Lee et al. 2011). The increase in antioxidant capacity may be affected by the increase in protein content in GST. Soy protein has been shown to potentially act as an antioxidant. Antioxidant amino acids and vitamin E work in synergy as antioxidants in the body.

Park et al. (2008) stated that several peptides and amino acids act as antioxidants in soy hydrolysates. The peptide with the highest antioxidant properties was found to have a low molecular weight (<3 kDa) and was mostly comprised of hydrophobic amino acids such as phenylalanine, alanine, and proline. Phenylalanine was the main component found and was believed to be the main active component. When exposed to hydroxyl radicals, phenylalanine is converted into tyrosine which has radical scavenging activity (Park et al. 2008). In Table 2, both tempe flours were observed to have no significantly different phenylalanine and tyrosine content (p>0.05). Proline, one of the abundant amino acids found in antioxidant peptides, was not analyzed here; therefore, the proline content was not known.

Yuan et al. (2009) has been reported that germination changed the distribution profile of isoflavone. The results showed that after germination, the glycoside contents decreased and the total aglycone content increased. In line with Shi et al. (2010) that malonyl genistin and malonyl daidzin are decreased, but daidzin, genistin, daidzen, and genistein are increased during germination. Zhu et al. (2005) studied the effect of germination on the isoflavone profile of two different soybean varieties, Hutcheson and Caviness. After soaking, the total isoflavone content of both varieties increased to the maximum amount (Hutcheson 2,491 mg/g db and Caviness 2,780 mg/g db). After this stage, a decrease in the isoflavone content was observed. Another possible cause for the decrease in the isoflavone content may be contributed to the conversion of isoflavones to other flavonoids.

Isoflavone conversion may happen not only during germination but also during fermentation. Soybean flour fermented with Aspergillus oryzae underwent a decrease of total isoflavones after 48 hours of fermentation. Various reactions such as hydroxylation, methylation, glycosylation, and acetylation can occur and change the structure of isoflavones (da Silva et al. 2011). Isoflavone structure is important in determining its antioxidant activity. The antioxidant activity of flavonoids is determined by the arrangement of the functional group bound to the structure such as the hydroxyl group, methyl, double bound, and carbohydrate moieties, and also the degree of polymerization (Heim et al. 2002).

Genistein was the most abundant isoflavone found in both tempe flours. Products high in aglycone isoflavones have higher efficiency in preventing chronic diseases because of their higher absorbability. El-Kordy and Alshahrani (2015) studied the effect of genistein on streptozotocin-induced diabetic rats. Administration of 20 mg/kg/day of genistein showed a protective effect on pancreatic β-cells against ROS.

The α-amylase and α-glucosidase is an enzyme that plays an important role in converting carbohydrates into glucose. Soybean flour known can significantly inhibit the work of enzymes by reducing the increase of postprandial blood glucose (Subramanian et al. 2008). The anti-diabetic actions of the plant is suggested by inhibiting α-amylase and α-glucosidase which could decrease postprandial blood glucose level, and also by repairing the damage of pancreatic beta cells, thus enhancing the insulin secretion directly (Febrinda et al. 2014). In this study, no improvement of inhibition activity on starch digestion enzymes was observed due to soy germination. In addition, a decrease in α-amylase inhibition (increase in IC_{50} value) was detected in GST. GST was expected to have lower glycosides due to its phenolics degradation during the germination process. Free phenolics were shown to have lower inhibition activity on α-amylase compared to their bound forms (Ademiluyi and Oboh 2013). Bound phenolics which exist mostly in glycosides are hydrophilic and since the enzymes work in an aqueous phase, the direct enzyme-inhibitor interaction is expected to be higher in NST. Compared to the study by Ademiluyi and Oboh (2013), the α-amylase and α-glucosidase inhibition of soybeans was higher than both tempe flours. This result may be likely because the tempe flours underwent various heating process.
Heating process decreases the enzyme inhibition property of soy (Gețek et al. 2014).

GST and NST were found to have several different compositions; therefore, they were expected to have different antidiabetic properties. With higher a protein content and antioxidant capacity, GST was expected to have a higher potential in increasing insulin sensitivity in a high-fat diet and in preventing pancreatic β-cell destruction. NST had a higher leucine content, hence it was expected to increase insulin secretion in a high blood glucose condition. In addition, NST might be more promising in inhibiting starch digestion since it had a higher α-amylase and α-glucosidase inhibition values (lower IC₅₀ values than GST). Further study to obtain the optimum germination conditions is needed to increase the bioactive content in germinated soybean tempe.

Conflict of Interest

The authors have no conflict of interest regarding the results of this research.

Acknowledgements

The authors would like to thank the Directorate of Research and Community Service, the Directorate General for Research and Development, Ministry of Research, Technology and Higher Education, Republic of Indonesia, through “Penelitian Berbasis Kompetensi 2019” scheme under the name of Made Astawan.

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