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FULL PAPER

The effects of tempe protein isolate from non-germinated and germinated soybean on oxidative stress in diabetic rats

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Abstract

Diabetes mellitus (DM) patients tend to be more susceptible to the COVID-19 virus. Evidence suggests that infection with the COVID-19 virus leads to increase reactive oxygen species (ROS) production. Moreover, hyperglycaemic conditions also produce excess ROS. Those can lead to oxidative stress in DM patients. This study aimed to prevent oxidative stress by using innovative functional food called tempe protein isolates, which were isolated from both non-germinated soybean (TPIN) and germinated soybeans (TPIG). TPIN, TPIG and commercial soy protein isolate (CSPI) were given 900 mg/kg BW to DM rats for 28 days. The DM rats were modelled by inducing an alloxan dose of 110 mg/kg BW intraperitoneally. Results showed there were no significant differences ($p>0.05$) in feed intake and blood profile. TPIN significantly prevented excessive weight loss and reduced blood glucose ($p<0.05$) better than TPIG and CSPI. TPIN and TPIG significantly ($p<0.05$) prevent the formation of MDA in the kidney, increase the activity of the SOD enzyme in both liver and kidney tissues and increase Cu,Zn-SOD antioxidant. This study concluded that the administration of TPIG and TPIN could prevent liver and kidney tissue damage by free radicals. Furthermore, TPIN has better potential than TPIG to prevent oxidative stress.

1. Introduction

The SARS-CoV-2 virus causes COVID-19, an acute respiratory disorder that has been declared a pandemic by the World Health Organization (WHO) due to the virus's rapid spread over the World. People with non-communicable diseases (comorbid), such as diabetes are more susceptible to COVID-19 infection (Onder *et al*., 2020; Yang *et al*., 2020). COVID-19 infection causes increased reactive oxygen species (ROS) production and hyperinflammation (Teuwen *et al*., 2020). At the same time, the hyperglycaemic conditions in people with diabetes mellitus (DM) can also increase the formation of excessive free radicals. These free radicals are generated through various mechanisms, such as glucose oxidation, lipid oxidation, sorbitol pathway, hexosamine pathway, NADPH oxidase, advanced glycation end products (AGEs), and others (Ighodaro, 2018).

The increased numbers of free radicals, along with the antioxidants' inability to neutralise them, results in oxidative stress (Ighodaro and Akinloye, 2018). These conditions can cause further complications or worsen the condition of DM itself. Measurements of biomarkers such as malondialdehyde (MDA) levels and superoxide dismutase (SOD) enzyme activity can reveal oxidative stress conditions (Astawan *et al*., 2012). The prevention of oxidative stress generation in DM patients can be done through antioxidant-rich food consumption (exogenous antioxidants) or increasing the antioxidant activity in the body (endogenous antioxidants). The health advantages of tempe, an indigenous Indonesian functional food, have been explored, both from nutritional and non-nutritive ingredients. The non-nutritive compounds in tempe consisted of flavonoid compounds, dietary fibre, saponins, and polyamines (Astawan, Rahmawati, Cahyani *et al*., 2020).

The germination process can alter the existing components in soybean, both nutritive and non-nutritive components. During the germination process, fats and carbohydrates are degraded to provide the energy for new protein synthesis (Shi *et al.,* 2010). Astawan

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Wresdiyati and Ichsan (2016) reported that tempe produced from germinated soybean had higher protein content, while fat and carbohydrate contents were decreased. In addition, isoflavones bioactive components, antioxidant capacity, vitamins and minerals (Ca, P, Fe and Zn) were also increased.

However, tempe has a relatively short shelf-life due to its high water and fat content (Astawan *et al*., 2016). Innovations by further processing tempe into tempe protein isolate can be used to improve the shelf life and usage of tempe. The tempe protein isolates generated are either tempe protein isolate from non-germinated soybeans (TPIN) or tempe protein isolate from germinated soybeans (TPIG). Protein isolate is the purest form of protein with a minimum protein content of 90%. The benefits of TPIN and TPIG had been studied both in terms of physicochemical, functional properties and hypoglycaemic ability (Astawan, Wresdiyati, Yoshari *et al.* 2020). Moreover, the application of TPIG and TPIN as food ingredients had been studied by Prayudani *et al.* (2020).

This study aimed to prove the potential of tempe protein isolates (TPIN and TPIG) to be better in preventing oxidative stress than commercial soybean protein isolates (CSPI). Research on the effect of IPTN and IPTG on oxidative stress was carried out on DM rats for 28 days. The effect was observed by measuring the parameters of body weight, blood glucose levels, haematological and biochemical serum profile MDA levels, SOD enzyme activity, as well as immunohistochemical profiles of Cu, Zn-SOD antioxidant content in the liver and kidneys.

2. Materials and methods

The primary materials used in tempe production were local Grobogan soybean variety (Grobogan, Central Java, Indonesia) and tempe mould (Raprima, PT. Aneka Fermentasi Industri, Bandung, Indonesia) obtained from KOPTI, Bogor, Indonesia. As a comparison, commercial soybean protein isolate (CSPI) was used.

2.1 Soybean preparation and germination process

Soybean germination process was carried out according to the instruction provided by Astawan, Wresdiyati, Subarna *et al.* (2020). Soybean seeds were sorted and soaked in water for 3 hrs. The aim was to increase soybeans' moisture content and shorten the boiling time to achieve a softer texture. For 24 hrs, the soybeans were kept in a perforated container in a dark environment. The soybeans were watered every 3 hrs until they sprouted radicles measuring 2.5–5.0 mm in length.

2.2 Tempe production

Tempe production was done according to the instruction provided by Astawan, Wresdiyati, Subarna *et al.* (2020). The soybeans were boiled at 100°C for 30 mins and then soaked for 12 hrs. The soaking process led to the growth of lactic acid bacteria, thereby lowering the water pH to 3.5–5.2. After that, soybeans were dehulled with a dehuller machine. In non-germinated soybeans tempe production, soybean shoots (hilum) were removed due to their bitter taste. Then, the soybeans were rinsed with hot water at 100°C, drained, cooled, and blow-dried. The dried soybeans were inoculated with tempe mould with 2 g/kg soybeans, packed in perforated polypropylene plastic with a distance of 2×2 cm. The tempe fermentation process was conducted at 28–30°C (RH 80%; 40 hrs).

2.3 Tempe flour defatting

Tempe flour was prepared from sliced tempe that was steam blanched and dried in an oven (60°C; 8 hrs). After drying, the tempe was ground in a disc mill and sorted through a 60 mesh. The tempe flour was mixed with organic solvent hexane (1:3 w/v) and homogenized for 2 hrs with a magnetic stirrer at room temperature according to the instruction presented by Puteri *et al*. (2018). A Buchner funnel was used to separate the mixture and the defatting process was repeated twice. The solids from the defatting process were dried in a drying oven (50°C, 2 hrs) and sieved again through a 60-mesh sieve.

2.4 Isolation of tempe protein

The tempe protein was isolated according to the instructions provided by Astawan, Wresdiyati, Yoshari *et al.* (2020). The extraction step was carried out by dissolving defatted tempe flour with distilled water (1:10 w/v) and then pH adjustment by adding 2N NaOH solution until it reached a pH of 11–12. The extraction process was carried out for 2 hrs with a magnetic stirrer. After that, the mixture was centrifuged at 3000×*g* (10 mins; 4°C), resulting in precipitate and filtrate. The protein precipitation step was carried out by adding 2N HCl solution into the filtrate until it reached pH 3–5, followed by centrifugation at 3000×*g* (10 mins; 4°C). After the precipitation process, the precipitate was washed with distilled water and neutralised with 2N NaOH solution until it reached pH 7. It was, lastly, freeze-dried to be used in the *in vivo* study.

2.5 In vivo study

This study used 30 white male Sprague-Dawley rats (*Rattus norvegicus*) with 2.5–3 weeks and a weight range of 180–250 g. Acclimatisation was carried out for seven

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days (casein as the protein source), and water was given *ad libitum*. The DM rats were modelled by inducing alloxan 110 mg/kg BW intraperitoneally (Wresdiyati *et al*., 2010). Rats that were classified as diabetic had blood glucose levels of >250 mg/dL. For this study, the rats will be divided into six groups, with each group consisting of 5 rats (Table 1).

The *in vivo* testing was carried out for 28 days. According to the instruction given by Paula *et al*. (2017) and El-Sayed *et al*. (2016), each group of rats received 900 mg/kg BW/day of TPIG, TPIN, and CSPI, respectively. The treatments and gliclazide were administered via oral gavage. All rats were given standard casein feed (AOAC, 2005), which was limited to 25 g/day and water *ad libitum*. On day 29, the rats were anaesthetized intraperitoneally with a mixture of ketamine (70 mg/kg BW) and xylazine (20 mg/kg BW). The observed biological samples were blood, liver, and kidney. For immunohistochemical staining, the livers and kidneys were immersed 24 hrs in Bouin's fixative solution before being transferred into 70% alcohol solution.

2.6 Measurement of the amount of consumed feed, body weight, and blood glucose levels

The amount of consumed feed was measured every day, body weight (BW) measurement was carried out every two days, and blood glucose levels were measured every four days.

2.7 Analysis of haematological and biochemical serum profile

The haematological profiles consisted of erythrocyte levels, haemoglobin, haematocrit percentage, total leukocytes and platelet levels. The blood serum analysis consisted of blood lipid profile, blood protein profile, uric acid level, aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

2.8 Measurement of SOD enzyme activity

Measurement of SOD enzyme activity was performed as describe by Misra and Fridovich (1972). Each group's kidneys and livers were weighed 0.5 g and crushed under cold conditions using a 1:10 ratio of phosphate buffer pH 7. After that, the extract was centrifuged at 3000 rpm (10 mins, 4°C). 1 mL of supernatant was mixed with 1.6 mL of 96% chloroform-ethanol (3:5) solution before being centrifuged (3000 rpm, 10 mins, 4°C). A total of 100 µL of the supernatant was taken and mixed with 2.8 mL of sodium carbonate buffer pH 10.2 and 100 µL of 0.003 M epinephrine solutions. At 0, 1, 2, 3, and 4 mins, the absorbance was measured at a wavelength of 480 nm.

2.9 Measurement of MDA levels

Measurement of MDA level was conducted by the method of Singh *et al*. (2002). The kidneys and livers from each group were weighed 0.5 g and then crushed with phosphate buffer saline (PBS) in a 1:5 ratio under cold conditions before centrifugation at 4000 rpm for 15 mins. A supernatant of 0.5 mL was mixed with 2 mL of cold 0.25 N HCl which contained 15% trichloroacetic acid (TCA), 0.38% thiobarbituric acid (TBA), and 0.5% butylated hydroxytoluene (BHT). The mixture was heated at 80°C for 1 hr before being centrifuged at 3500 rpm for 10 mins at 4°C. At a wavelength of 532 nm, the absorbance was measured. TEP (tetraoxypropane) was used as the standard with concentrations of 0, 5, 10, 20, 30, 40 nmol/mL.

2.10 Immunohistochemical staining of Cu,Zn-SOD on tissue

Organs that had been fixed were observed for tissue histology. Immunohistochemical staining was conducted by the procedure of Wresdiyati *et al.* (2020), initiated by deparaffinisation, rehydration and inactivation of endogenous peroxidase (methanol-H_2O_2 3%, 15 mins). After that, the tissue preparations were given with 10% normal goat serum (50 µL; incubation 37°C, 50 mins), background sniper (50 µL; incubated 37°C, 15 mins), and copper-zinc superoxide dismutase (Cu,Zn-SOD) (50 µL; incubated 4°C, 2×24 hrs) as the primary antibody.

Table 1. The group of treated rats

Group of Treatment	Number of rats	Types of rats	Treatment
C-	5	Non-DM	-
C+	5	DM	-
C+,D	5	DM	Gliclazide dose 3,6 mg/kg BB/ day
TPIG	5	DM	Tempe protein isolate from germinated soybean as much as 900 mg/kg BW/day
TPIN	5	DM	Tempe protein isolates from non-germinated soybean as much as 900 mg/kg BW/day.
CSPI	5	DM	Commercial soybean protein isolate 900 as much as 900 mg/kg BW/day

C-: negative control, C+: positive control, C+,D: gliclazide drug , TPIG: Tempe protein isolate from germinated soybean, TPIN: Tempe protein isolate from non-germinated soybean, CSPI: commercial soybean protein isolate, DM: Diabetes mellitus.

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At each stage, the slides were rinsed with PBS solution three times for 5 mins.

After that, the tissue preparations were given with Trekkie Universal Link (50 µL; incubated 37°C, 20 mins) and Trekk Avidin-HRP (50 µL; incubated 37°C, 10 mins). The results of the antigen-antibody reaction were visualised with 30% diaminobenzidine (DAB) substrate for 4 mins (until the core turned brown). Counterstaining was done with haematoxylin for 1-2 min. Then, the organ slides were dehydrated and cleared. The staining results were observed with a light microscope (Olympus CH-20) and documented using a camera microscope (Olympus CX31-CCD10 USB Camera).

2.11 Data analysis

The analysis of Cu-Zn-SOD content was calculated by the McMaster Biophotonics Image J software program. To establish the significance of the treatments, an analysis of variance (ANOVA) was done using SPSS 26 (IBM® SPSS Statistics 26). The treatments with significant results were analysed further using Duncan's Multiple Range Test (DMRT) to determine which treatments significantly differed at a 95% significance level.

3. Results and discussion

3.1 Feed consumption, body weight and blood glucose levels of experimental rats

The total feed consumption did not differ significantly ($p>0.05$) between the treatments. This outcome was due to the limited feed of 25 g/day for all groups of rats. A study by Johar *et al*. (2018) also reported that the diet comparison of alloxan-induced DM rats with normal rats was not significantly different. Although the total feed consumption was not significantly different, the statistical results showed significant weight loss between the treatments ($p<0.05$). TPIN and CSPI were shown to significantly prevent excessive weight loss in DM rats (positive control, C+).

In DM conditions, the body will look for new energy sources to meet its energy needs. The methods are carried out through lipid (lipolysis) and protein (proteolysis) breakdowns (Febrinda *et al*., 2014). Chung *et al*. (2015) explained an increase in gluconeogenesis in early type 2 diabetes patients. Figure 1 shows that the limited feed (25 g/rat/day) caused all treatments to lose weight. TPIN treatment reduced weight loss in diabetic rats during the 28 days of the *in vivo* study, compared to other treatments (Table 2).

Figure 1 shows the ability of TPIN to maintain body weight, which showed that there was no weight change fluctuation. A study by Park *et al*. (2019) reported that fluctuating weight changes could increase the risk of type 2 DM. The ability of TPIN to maintain body weight was suspected from the bioavailability of proteins or their amino acid components. Wu *et al*. (2014) identified arginine, glutamic acid, proline, leucine, isoleucine, and valine as amino acids that contribute to weight gain.

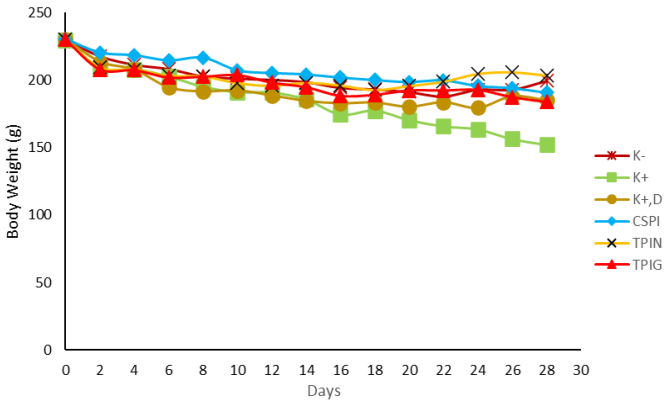

Figure 1. Changes in body weight of experimental rats for 28 days. C-: Normal rats (negative control), C+: Diabetic rats (positive control), C+,D: diabetic rat + gliclazide drug, CSPI: Diabetic rats + soy protein isolate, TPIN: Diabetic rats + non-germinated tempe protein isolate, TPIG: Diabetic rats + germinated tempe protein isolate

Figure 2 shows that the administration of TPIN significantly lowered the blood glucose level compared to CSPI and positive control (C+) ($p<0.05$). Additionally, the result in blood glucose levels were close to the negative control rats (C-) on day 28. This

Table 2. Total consumption of feed, weight loss and decrease in blood glucose of rats for 28 days

Group of Treatment	Total feed consumption (g)	Weight loss (g)	Decrease in blood glucose (g/dL)
C-	560.80±52.09	30.40±12.74^{a}	41.60±8.08^{a}
C+	491.60±59.07	77.40±25.85^{b}	205.40±67.77^{b}
C+,D	595.00±31.95	45.40±17.66^{ab}	407.20±88.25^{cd}
CSPI	499.20±59.76	39.80±36.24^{a}	298.00±136.53^{bc}
TPIN	547.80±83.17	26.60±12.74^{a}	434.20±82.48^{d}
TPIG	546.20±66.68	46.20±33.52^{ab}	380.60±132.13^{cd}

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different ($p<0.05$).

C-: negative control, C+: positive control, C+,D: gliclazide drug , TPIG: Tempe protein isolate from germinated soybean, TPIN: Tempe protein isolate from non-germinated soybean, CSPI: commercial soybean protein isolate, DM: Diabetes mellitus.

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result was superior to gliclazide administration at a dose of 3.6 mg/kg BW/day, which a medication used to treat type 2 diabetes. This medication is a sulfonylurea, which works by inhibiting K^+ channels on cells to increase insulin secretion (Newsholme *et al*., 2010).

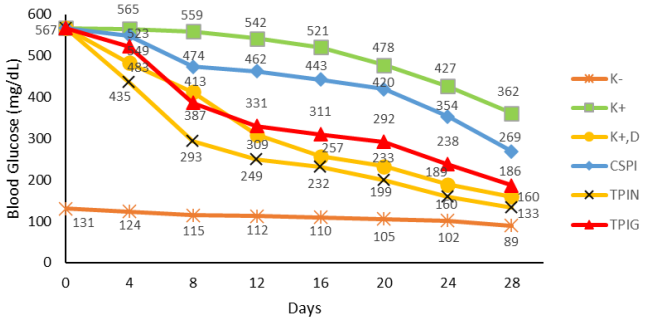

Figure 2. Graph of changes in blood glucose levels in experimental rats for 28 days. C-: Normal rats (negative control), C+: Diabetic rats (positive control), C+,D: diabetic rat + gliclazide drug, CSPI: Diabetic rats + soy protein isolate, TPIN: Diabetic rats + non-germinated tempe protein isolate, TPIG: Diabetic rats + germinated tempe protein isolate

The insulinotropic amino acid composition caused the ability of TPIN and TPIG in lowering blood glucose. These amino acids are consisting of alanine (Ala), arginine (Arg), phenylalanine (Phe), glutamine (Gln), isoleucine (Ile), leucine (Leu), and lysine (Lys) (Newsholme *et al*., 2007; Power *et al*., 2008; Ogawa *et al*., 2011). Amino acids increase insulin secretion via increasing the intracellular ATP/ADP ratio, which causes K+ channels to close. Closed K^+ channels cause Ca^{2+} channels to open, resulting in plasma membrane depolarization and insulin exocytosis (Singh *et al*., 2022).

In addition to amino acids, bioactive components such as bioactive peptides and isoflavones can act as anti -hyperglycaemic agents. Unfortunately, studies on bioactive peptides in tempe protein isolates (TPIG and TPIN) and CSPI has never been done before. However, bioactive peptides from tempe that have anti-hyperglycaemic abilities had been described by Tamam *et al*. (2019). Some of these peptides were Pro-Ser, Ser-Val, Ala-Glu, Ile-Ala-Lys, and others. The mechanism of the anti-hyperglycaemic peptide was through dipeptidyl peptidase IV (DPP IV) inhibition.

The mechanism of isoflavone aglycones in lowering blood glucose widely varies. In general, the mechanism was performed by modulating nuclear receptor activity and non-receptor signalling (Kuryłowicz, 2020). According to Rehman *et al*. (2019) administration of genistein to alloxan-induced diabetic rats could protect intestinal L-cells responsible for GLP-1 (glucagon-like peptide-1) secretion. Meanwhile, Srivastava *et al*. (2017) reported that daidzein could increase GLP-1 and GIP (glucose-dependent insulinotropic peptide) levels in STZ diabetic rats by inhibiting DPP IV.

3.2 Blood profile

Table 3 shows the haematological profile after 28 days of giving CSPI, TPIN, and TPIG. The erythrocyte and haemoglobin levels in DM rats (C+) were significantly ($p<0.05$) increased compared to normal rats (C-). According to Ferreira *et al*. (2019), insulin resistance was associated with increased RBC count and haematocrit in adults. Insulin resistance caused an increase in the production of insulin-like growth factor (IGF) and hypoxia-inducible factor-1 alpha (HIF-1α), which promoted the formation of erythropoietin that stimulated the increase in erythropoiesis (Guan *et al*., 2016; Ferreira *et al*., 2019). Further test results showed that the administration of TPIN was not significantly different ($p>0.05$) compared to other treatments. However, compared to CSPI and TPIG, TPIN was able to reduce erythrocyte and haemoglobin levels to their reference values and were close to levels in normal rats.

Increased erythrocyte levels are generally

Table 3. Haematological profile of rat after 28 days of the experiment

Group of Treatment	Haematology Profile				
	Erythrocytes ($\times10^6$/mm^3)	Haemoglobin (g/dL)	Haematocrit (%)	Leucocytes ($\times10^3$/mm^3)	Platelets ($\times10^3$/mm^3)
C-	6.80±0.67^a	12.54±0.96^a	31.82±2.64	4.68±0.45	482.60±106.67^b
C+	8.02±0.31^b	14.62±0.53^b	38.04±3.46	5.74±2.18	302.00±111.16^a
C+,D	7.66±0.91^{ab}	13.86±1.55^{ab}	35.60±4.57	6.20±1.61	571.00±106.48^b
CSPI	7.89±0.76^b	14.68±1.62^b	37.48±4.14	6.84±3.07	471.40±92.17^b
TPIN	7.23±0.25^{ab}	13.28±0.41^{ab}	33.34±1.71	5.06±1.32	542.80±71.90^b
TPIG	8.18±0.86^b	14.70±1.68^b	37.60±4.68	7.10±2.17	525.20±104.23^b
Reference value	6.4–8.0*	13.5–15.9*	33.0–50.0**	3.0–9.2*	370–1383***

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different ($p<0.05$).

C-: negative control, C+: positive control, C+,D: gliclazide drug , TPIG: Tempe protein isolate from germinated soybean, TPIN: Tempe protein isolate from non-germinated soybean, CSPI: commercial soybean protein isolate, DM: Diabetes mellitus.

*He *et al*. (2017);** Booth *et al*. (2010);***Petterino and Argentino-Storino (2006)

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accompanied by an increase in the percentage of haematocrit. The haematocrit and leukocyte levels in this study were not significantly different ($p>0.05$) between treatments, although several studies had shown the increase of haematocrit and leukocytes levels in DM patients (Guan *et al*., 2016; Narjis *et al.,* 2021). All treatments had leukocyte levels that fell into the reference value.

There were changes in platelet morphology and function in DM conditions, resulting in increased platelet activity or platelet hyperactivity (Buch *et al.*, 2020). Platelet levels in DM rats (C+) were significantly lower ($p<0.05$) than in normal rats and treated DM rats. These findings were consistent with a study by Buch *et al.* (2020), who observed that the platelet level was significantly lower ($p<0.05$) in DM conditions, whereas the platelet volume increased significantly. The ability of CSPI, TPIN and TPIG to increase platelet levels was influenced by isoflavones content, which could inhibit platelet aggregation through arachidonic acid and cyclooxygenase pathways (Faggio *et al.*, 2017).

For the serum fat profile, the treatment had no significant effect ($p>0.05$) (Table 4). These results are caused by different individual responses or the animal model used is not sensitive to changes in serum fat profile. The total protein and albumin levels of DM rats were found to be significantly lower ($p<0.05$) in this study's findings (Table 5). These findings were consistent with the study of Kondeti *et al.* (2010), who found a decrease in total protein in STZ-induced DM rats. Administration of CSPI, TPIN, TPIG, and gliclazide significantly increased total protein and albumin levels ($p<0.05$). This increase may be related to an increase in insulin secretion.

Blood urea and creatinine levels analysis can be used as an indicator of kidney function. In DM patients, hyperglycaemia causes kidney nephron damage, they are incapable of maintaining fluid and electrolyte homeostasis (Pandya, 2016). The administration of TPIG in DM rats caused a significant increase ($p<0.05$) in urea levels, and administration of CSPI and TPIG caused a significant increase in creatinine levels ($p<0.05$). The high level of urea in DM rats administered with TPIN and TPIG was due to the administration of the high dose (900 mg/Kg BW). Increased urea and creatinine in the diabetic rat's serum indicated decreased glomerular

Table 4. Profiles of rat lipid after 28 days of the experiment

Group of Treatment	Serum Lipid Profile			
	Total Cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
C-	68.00±6.93	55.80±29.52	36.40±5.18	20.40±8.02
C+	84.00±14.75	33.80±9.96	50.40±18.27	26.80±9.07
C+,D	60.00±32.46	62.60±32.14	45.40±12.44	17.40±9.74
CSPI	100.50±17.64	59.75±25.75	54.25±10.56	26.33±26.17
TPIN	83.00±25.43	72.60±30.81	51.20±16.10	17.20±6.83
TPIG	88.40±13.81	55.40±36.29	51.00±9.75	26.40±4.98
Reference value	24.4–78.5*	20.4–88.6*	40.0–68.3**	11.24-80.6**

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different ($p<0.05$).

C-: negative control, C+: positive control, C+,D: gliclazide drug , TPIG: Tempe protein isolate from germinated soybean, TPIN: Tempe protein isolate from non-germinated soybean, CSPI: commercial soybean protein isolate, DM: Diabetes mellitus.

*He *et al*. (2017), Ihedioha *et al.* (2013)

Table 5. Profiles of rat serum protein after 28 days of the experiment

Group of Treatment	Serum Protein Profiles			
	Total Protein (g/dL)	Albumin (g/dL)	Urea (mg/dL)	Creatinine (mg/dL)
C-	$4.88±0.40^{ab}$	$3.47±0.19^{b}$	$30.20±4.09^{a}$	$0.65±0.04^{ab}$
C+	$4.49±0.44^{a}$	$3.05±0.30^{a}$	$41.20±12.13^{a}$	$0.65±0.05^{ab}$
C+,D	$5.06±0.45^{b}$	$3.47±0.25^{b}$	$28.60±7.83^{a}$	$0.59±0.05^{a}$
CSPI	$5.12±0.19^{b}$	$3.48±0.12^{b}$	$39.25±9.88^{a}$	$0.80±0.12^{bc}$
TPIN	$5.39±0.44^{b}$	$3.65±0.16^{bc}$	$50.00±32.12^{ab}$	$0.71±0.16^{ab}$
TPIG	$5.32±0.31^{b}$	$3.82±0.21^{c}$	$74.60±33.46^{b}$	$0.87±0.16^{c}$
Reference value	5.1–6.5*	2.4–4.4**	19.7–63.6*	0.3–0.9**

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different ($p<0.05$).

C-: negative control, C+: positive control, C+,D: gliclazide drug , TPIG: Tempe protein isolate from germinated soybean, TPIN: Tempe protein isolate from non-germinated soybean, CSPI: commercial soybean protein isolate, DM: Diabetes mellitus.

*He *et al*. (2017), **Petterino and Argentino-Storino (2006).

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Table 6. Profiles of AST, ALT, liver weight and uric acid of rat after 28 days of the experiment

Group of Treatment	AST (U/L)	ALT (U/L)	Uric acid (mg/dL)
C-	96.80±8.79	46.20±5.80[a]	0.57±0.17
C+	137.80±48.49	82.40±28.84[b]	0.36±0.08
C+,D	107.00±47.71	47.00±12.73[a]	0.68±0.30
CSPI	88.75±30.58	47.25±23.21[a]	0.64±0.25
TPIN	91.80±20.87	49.40±5.32[a]	0.64±0.19
TPIG	83.20±27.12	53.80±21.72[a]	0.54±0.08
Reference value	60–144*	19–56*	1.50–3.00**

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different ($p<0.05$).

C-: negative control, C+: positive control, C+,D: gliclazide drug , TPIG: Tempe protein isolate from germinated soybean, TPIN: Tempe protein isolate from non-germinated soybean, CSPI: commercial soybean protein isolate, DM: Diabetes mellitus.

*He *et al*. (2017), ** Osada *et al*. (1993)

filtration rate (GFR) but did not indicate impaired kidney function.

Measurement of liver enzyme activity such as ALT and AST are essential in diagnosing and assessing liver function. Damage to the liver will cause an increase in AST and ALT activities which is measurable in serum. In DM patients, it is known that there is an increase in AST and ALT caused by insulin resistance. Insulin resistance will accumulate triglycerides in hepatocytes and cause lipid peroxidation. This condition can increase oxidative stress (Ghamar-Chehreh *et al*., 2012). However, this study (Table 6) showed that DM (C+) rats had a significant increase only ($p<0.05$) in ALT levels, whereas AST levels did not ($p>0.05$).

The administration of gliclazide, CSPI, TPIG and TPIN were able to reduce serum ALT levels. This finding suggests that Gliclazide, CSPI, TPIG and TPIN protect the liver in diabetic rats from oxidative stress-induced damage. The ability of CSPI, TPIG, and TPIN is caused by the isoflavones component in these ingredients. Isoflavones are known for their capacity to scavenge free radicals as antioxidants and prevent gluconeogenesis.

Uric acid can inhibit insulin by reducing the bioavailability of NO (nitric oxide), which is involved in glucose absorption (Choi *et al*., 2014). Insulin resistance causes an increase in uric acid levels by increasing uric acid absorption and lowering its excretion via the kidneys. However, this study revealed that there was no increase in DM rats or differences in results between treatments ($p>0.05$) (Table 6). The uricosuric impact of glucose in uric acid could explain the low uric acid content in serum, which affects uric acid excretion and reabsorption in the kidneys (Pavani *et al*., 2018).

3.3 MDA levels and SOD enzyme activity

Hyperglycaemic conditions cause an increase in the formation of free radicals and a decrease in antioxidant activity that leads to oxidative stress. Polyunsaturated fatty acids (PUFA) are the most abundant compounds in cell membranes and are very sensitive to oxidation (Pamplona *et al*., 2019). The results of PUFA oxidation form lipid hydroperoxides and aldehyde derivatives. MDA compounds are mutagenic aldehyde compounds widely produced during arachidonic acid peroxidation (C20:4). MDA compounds are generally used as indications/markers of oxidative stress (Barrera *et al*., 2018).

Many studies have shown that MDA concentrations increase rapidly in DM conditions, including in serum (Nakhjavani *et al*., 2010; Ghazizadeh *et al*., 2018), pancreas (Annadurai *et al*., 2012), liver (Johar *et al*., 2018), and kidney (Schmatz *et al*., 2012). TPIN and

Table 7. MDA levels and SOD enzyme activity in the liver and kidneys

Group of Treatment	MDA levels		SOD enzyme activity	
	Liver (nmol/g)	Kidneys (nmol/g)	Liver (U/mg)	Kidneys (U/mg)
C-	59.77±2.72	29.35±2.61[a]	25.43±2.73[c]	17.91±2.98[c]
C+	69.45±1.18	38.47±4.45[b]	14.13±1.78[a]	4.58±1.49[a]
C+,D	65.73±4.71	36.88±1.72[b]	15.32±2.06[ab]	15.31±3.70[bc]
CSPI	64.49±6.27	31.70±5.26[ab]	18.29±2.06[b]	12.06±2.98[b]
TPIN	63.25±5.40	29.01±5.44[a]	23.65±1.03[c]	16.61±0.98[bc]
TPIG	65.55±2.21	29.73±0.40[a]	22.46±1.03[c]	15.31±0.56[bc]

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different ($p<0.05$).

C-: negative control, C+: positive control, C+,D: gliclazide drug , TPIG: Tempe protein isolate from germinated soybean, TPIN: Tempe protein isolate from non-germinated soybean, CSPI: commercial soybean protein isolate, DM: Diabetes mellitus.

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TPIG treatments in DM animals showed a significant reduction of MDA levels ($p<0.05$) in kidney tissue but not ($p>0.05$) in liver tissue (Table 7). These results were caused by the higher expression of enzymatic antioxidant antigens in liver tissue than in kidney tissue (Lenzen, 1996). The high antioxidant activity in liver tissue is caused by liver detoxification, metabolism, and excretion processes. These roles made the liver more susceptible to oxidative damage.

Antioxidants can neutralise the effects of ROS in the body. An enzymatic antioxidant that acts as the first line in ROS defence is superoxide dismutase (SOD). This condition occurs from the role of SOD in superoxide dismutation, which is considered the initiator of free radical formation (Brewer, 2011). According to Hamden *et al*. (2011) under oxidative stress conditions, the activity of antioxidant superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) were decreased to 56, 77 and 78%, respectively.

The administration of TPIN and TPIG significantly increased ($p<0.05$) the antioxidant activity of SOD in liver and kidney tissue (Table 7). The antioxidant activity was close to the activity of normal rats (C). These results were caused by the isoflavones content in TPIN and TPIG. The antioxidant properties of isoflavones are related to their phenolic structure to maintain the redox balance of cells (Chadha *et al*., 2017). Suarsana *et al*. (2013) reported that the administration of Isoflavones at a dose of 1 mg/200 g BW/day reduced MDA levels and increased the SOD enzyme activity in the liver of rats that experienced oxidative stress condition.

Jia *et al.* (2018) reported that Nuclear respiratory factors (NRFs), which are key transcription factors for controlling antioxidant enzyme production, were activated by genistein treatment in the kidneys of STZ-induced DM rats. In addition, isoflavones could increase Mn-SOD gene expression via activating ERK1/2 and NFkβ (Borrás *et al.*, 2006). Lee (2006) discovered that giving STZ-induced diabetic rats genistein and daidzein increased the activity of antioxidant enzymes like SOD, CAT, and GPX in their liver tissue.

In addition to the isoflavones content, TPIN and TPIG's ability to suppress the formation of MDA was also influenced by other antioxidant compounds. These compounds include tocopherols, phytic acid, saponins, and phenolic components. Astawan, Wresdiyati, Subarna *et al*. (2020) performed *in vitro* analysis of the antioxidant capacity and total phenolic content of TPIN and TPIG. TPIG had the significantly higher antioxidant capacity and total phenolic than TPIN and CSPI, 171.91 mg AEAC/g and 1142.41 mg GAE/g for TPIG, respectively; 157.79 mg AEAC/g and 1020.39 mg GAE/g for TPIN; and 26.02 mg AEAC/g and 368.70 mg GAE/g for CSPI, respectively.

3.4 Profile of Cu, Zn-SOD antioxidant content in liver and kidney tissue

Mammalian tissue has three SOD isoforms: Cu,Zn-SOD (SOD1), Mn-SOD (SOD2), and extracellular SOD (SOD3). Cu,Zn-SOD is thought to be the most important of the three in the first line of antioxidant defences (Krishnamurthy and Wadhwani, 2012). This result could be explained by Cu,Zn-distribution, SOD's which were mostly in the cytoplasm with some in the nucleus, peroxisomes, and mitochondrial intermembrane. The inclusion of zinc (Zn) and copper (Cu) ions, followed by disulphide oxidation, resulted in the synthesis of enzymatically active homodimers, which led to the formation of SOD (Osredkar and Sustar, 2011).

The determination of the antioxidant contents of Cu, Zn-SOD was done by calculating the numbers of hepatocyte nuclei (liver) and renal tubules (kidneys) from immunohistochemical staining. The staining results were divided into three degrees of positive reactions based on the strength of the brown colour that appeared in the cell nucleus. Dark brown colour signified a strong positive (+++), brown colour indicated a medium positive (++), and light brown colour showed a weak positive (+). There was also a set of negative reactions (-), which were represented by the blue colour in the cell nucleus (Wresdiyati *et al*., 2010).

The antioxidant content of Cu,Zn-SOD in the liver and kidney tissues of rats administered with TPIN and TPIG were significantly increased ($p<0.05$) compared to DM (C+) rats (Table 8 and Table 9). Although the number of nuclei of liver cells that reacted positively (+++) could not match that of normal rats (C-), TPIN administration showed a significant increase in antioxidant content ($p<0.05$) compared to gliclazide (C+,D). In contrast to cells in the renal tubules, TPIN administration was not significantly different ($p>0.05$) from drug administration (C+,D).

These results imply that TPIN has the highest potential compared to other treatments in increasing Cu,Zn-SOD's antioxidant content and preventing damage to liver and kidney tissue. Meanwhile, TPIG has the potential to prevent damage only to liver tissue. This result is shown from the results of Cu,Zn-SOD's antioxidant content in the kidney tissue of DM rats administered with TPIG was not significantly different ($p>0.05$) with CSPI administration. More details can be seen in Figures 3 and 4.

The ability of TPIN and TPIG to increase the Cu,Zn-

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Table 8. The antioxidant content of Cu, Zn-SOD in the liver tissue of experimental rats

Group of Treatment	The number of nuclei in liver tissue cells at varied levels of Cu,Zn-SOD antioxidant content			
	+++	++	+	-
C-	51.13±5.66^{d}	49.47±10.10	63.60±8.83^{ab}	7.33±1.62^{a}
C+	4.53±1.42^{a}	42.67±6.24	80.60±14.37^{b}	76.60±6.80^{c}
C+,D	21.07±1.17^{b}	48.20±1.59	61.73±8.90^{a}	10.60±2.46^{a}
CSPI	23.73±3.58^{b}	41.00±3.83	73.53±11.83^{ab}	12.20±2.82^{a}
TPIN	31.73±2.42^{c}	44.67±4.69	59.13±4.77^{a}	9.33±1.80^{a}
TPIG	26.07±2.89^{bc}	48.00±6.35	98.20±5.56^{c}	18.87±2.66^{b}

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different ($p<0.05$).
C-: negative control, C+: positive control, C+,D: gliclazide drug , TPIG: Tempe protein isolate from germinated soybean, TPIN: Tempe protein isolate from non-germinated soybean, CSPI: commercial soybean protein isolate, DM: Diabetes mellitus.

Table 9. The antioxidant content of Cu, Zn-SOD in the kidney tissue of experimental rats

Group of Treatment	The number of nuclei in kidney tissue cells at varied levels of Cu,Zn-SOD antioxidant content			
	+++	++	+	-
C-	50.40±1.51^{e}	57.67±1.55^{d}	99.40±9.42	20.87±3.76^{a}
C+	16.60±3.12^{a}	40.40±2.82^{abc}	102.20±3.47	74.67±14.04^{c}
C+,D	31.93±4.11^{cd}	49.33±5.56^{cd}	100.87±4.88	42.73±5.44^{b}
CSPI	23.73±3.29^{b}	37.67±2.20^{ab}	106.33±9.32	38.40±7.11^{b}
TPIN	34.33±5.67^{d}	45.80±10.76^{bc}	104.07±5.35	28.93±5.64^{ab}
TPIG	27.07±2.04^{bc}	35.07±2.61^{a}	92.07±4.10	30.47±10.54^{ab}

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different ($p<0.05$).
C-: negative control, C+: positive control, C+,D: gliclazide drug , TPIG: Tempe protein isolate from germinated soybean, TPIN: Tempe protein isolate from non-germinated soybean, CSPI: commercial soybean protein isolate, DM: Diabetes mellitus.

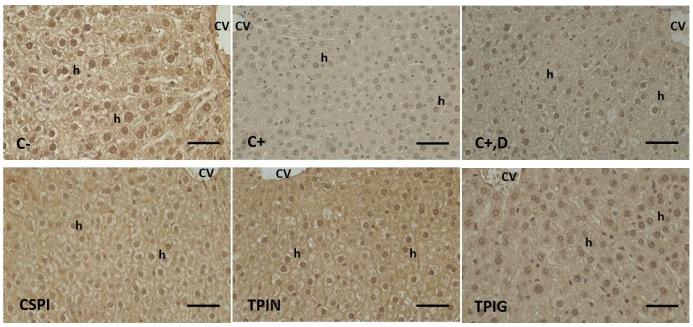

Figure 3. Photomicrograph of the antioxidant content of Cu,Zn-SOD in the liver tissue of experimental rats stained by immunohistochemical (400× magnification). CV: Central vein, h: hepatocytes, C-: Normal rats (negative control), C+: Diabetic rats (positive control), C+,D: diabetic rat + gliclazide drug, CSPI: Diabetic rats + soy protein isolate, TPIN: Diabetic rats + non-germinated tempe protein isolate, TPIG: Diabetic rats + germinated tempe protein isolate

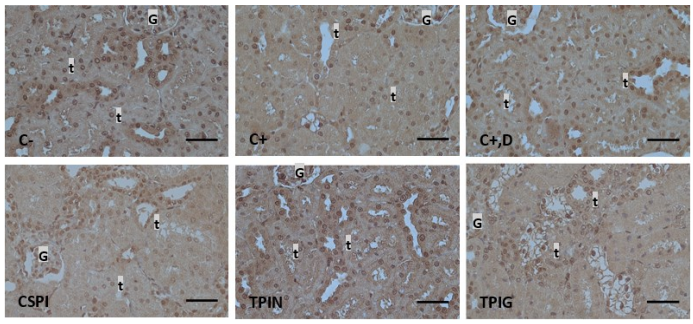

Figure 4. Photomicrograph of the antioxidant content of Cu,Zn-SOD in the kidney tissue of experimental rats stained by immunohistochemical (400× magnification). G: Glomerulus, t: renal tubule, C-: Normal rats (negative control), C+: Diabetic rats (positive control), C+,D: diabetic rat + gliclazide drug, CSPI: Diabetic rats + soy protein isolate, TPIN: Diabetic rats + non-germinated tempe protein isolate, TPIG: Diabetic rats + germinated tempe protein isolate

SOD content was due to the isoflavones content. Suarsana *et al*. (2013) reported that the administration of isoflavones could significantly increase the content of Cu,Zn-SOD ($p<0.05$) in liver tissue. Isoflavones helped the work of SOD antioxidants in neutralising ROS so that the SOD content in the tissue could be maintained.

Another factor that influences the formation of Cu,Zn-SOD is the presence of Cu and Zn minerals in the body. In DM conditions, there is a disturbance in the mineral balance, where Cu increases in the kidneys and liver while Zn decreases (Ozcelik *et al.,* 2011). Since the body does not have a particular storage system for these minerals, a sufficient daily intake of Cu and Zn is necessary to maintain Cu,Zn-SOD content. The germination process tends to reduce the levels of Mg and Zn due to the soaking and cleaning process (Márton *et al*., 2010). This factor may be the cause of TPIN having a better effect at increasing the Cu, Zn-SOD content than TPIG.

4. Conclusion

The administration of tempe protein isolate produced from non-germinated soybean (TPIN) and tempe protein isolate from germinated soybean (TPIG) was proved to be better than commercial soybean protein isolate (CSPI) in preventing oxidative stress in alloxan-induced DM

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rats. In general, the administration CSPI, TPIN and TPIG were able to maintain the value of the haematological and biochemical serum within the reference value range. Administration of TPIN showed the best potential in preventing oxidative stress in DM rats. TPIN had better effects in maintaining body weight and blood glucose fluctuations and increasing the Cu, Zn-SOD antioxidant content in the liver and kidneys.

Conflict of interest

The authors declare no conflict of interest.

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