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Antioxidant Activity of Tempeh Ethanolic Extract on Male Swiss Mouse Brain

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ABSTRACT

Tempeh is an Indonesian food made of soybean (*Glycine max* (L.) Merr.) by fermentation using *Rhizopus spp* mold. Soybean has a high content of protein, fatty acids, vitamins, and isoflavone glycosides. Isoflavone glycosides are poorly absorbed in the human intestine. Fermentation hydrolyzes isoflavone glycosides into isoflavone aglycones which are easily absorbed. Soybean has three main isoflavones, they are genistein, daidzein, and glycitein. They are good antioxidants and have estrogen-like activity. This research studies the antioxidant activity of tempeh ethanolic extract by determining the concentration of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the brain tissue of male Swiss mice. All test substances were diluted in 0.5% CMC-Na solution and administered orally once daily for 12 days. The 0.5 mL of 15% ethanol solution (16.67 mL/kg BW) was administered 1 hour before the other substances. Tempeh ethanolic extract (500 mg/kg BW) as the main treatment. Donepezil-HCl (1 mg/kg BW) and genistein (2.55 mg/kg BW) were used as the positive control groups. The mouse brain SOD and GPx concentration were determined on the 13th day. Results showed a significant depletion of SOD and GPx concentration by ethanol administration. Compared to the control group, mice with tempeh ethanolic extract showed no significant change in the GPx concentration but a little decrease in SOD. The SOD in the group of tempeh ethanolic extract was significantly higher than in the group of donepezil-HCl and genistein. Briefly concluded that tempeh ethanolic extract has better antioxidant activity than donepezil-HCl and genistein.

Keywords: Soybean; *Glycine max*; Tempeh; Antioxidant; SOD; GPx

INTRODUCTION

Tempeh (soybean tempeh) is well known as an original Indonesian fermented food, commonly made from soybean seeds (Hashim et al., 2018). Seeds of soybean (*Glycine max* (L.) Merr.) (Fabaceae) have very complex nutrition needs by the human body. Soybean contains proteins, fatty acids, carbohydrates, dietary fibers, vitamins, essential amino acids, flavonoids, and isoflavones. Especially, isoflavones concentration in soybean is high (~1–5 μg/g dry soybean) with many potential health properties. Isoflavones can act as antioxidants through the ability of hydrogen donors. Genistein, daidzein, and glycitein are the main isoflavones in soybean (Chen et al., 2019).

Based on the mechanisms of antioxidant activity, there are four categories of antioxidants (Ighodaro & Akinloye, 2017): (1) First-line defense antioxidants, that act to suppress or prevent the formation of free radicals or reactive species in cells. They act very fast in neutralizing any molecules with the potential of developing into free radicals or any free radicals with the ability to induce the production of other radicals. Three key enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). The others are metal-binding proteins like transferrin and caeruloplasmin. (2) Second-line defense antioxidants, often referred to as scavenging antioxidants, the activity of inhibit chain initiation and break chain propagation reactions. They neutralize or scavenge free radicals by donating electrons with the consequence that they become new free radicals but with lesser damaging effects. They can neutralize themselves with other antioxidants in this group. The antioxidants in this group are ascorbic acid, uric acid, glutathione, vitamin E, and ubiquinol. (3) Third-line defense antioxidants, only come into play after free radical damage has occurred. They are de novo enzymes that repair the damage caused by free radicals to

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biomolecules and reconstitute the damaged cell membrane. This group of enzymes can repair damaged DNA, protein, and lipids. They also recognize, break down and remove oxidized or damaged proteins, DNA, and lipids, to prevent their accumulation which can be toxic to body tissues. Examples of these enzymes are the DNA repair enzyme systems (polymerases, glycosylases, and nucleases), and proteolytic enzymes (proteinases, proteases, and peptidases). They are located both in the cytosol and mitochondria of mammalian cells. (4) Fourth-line defense antioxidants, with the action for an adaptation mechanism in which they utilize the signals required for free radicals production and reaction to prevent the formation or reaction of such free radicals. The signal generated from the free radical formed induces the formation and transport of an appropriate antioxidant to the right site.

Isoflavones act as antioxidants and fitoestrogen (Křížová et al., 2019), which can maintain and even improve cognitive ability (Cui et al., 2020). Isoflavones in soybean are mainly in the glycoside form that is difficult to be absorbed in the human digestive system. Fermentation causes the hydrolyze of the isoflavone glycosides to be free isoflavones, then rapidly absorbed (Křížová et al., 2019). Consumption of tempeh will enhance antioxidant activity in the body and brain of course. Antioxidant and phytoestrogen activity will have synergetic action to maintain and enhance cognitive ability (Cui et al., 2020; Křížová et al., 2019).

Normally, the human body needs free radicals to fight radicals, kill bacteria and regulate smooth muscle tone in organs and blood vessels. The human body produces free radicals through normal cell metabolism processes, inflammation, and nutritional deficiencies, as well as in response to gamma, and ultraviolet (UV) radiation, environmental pollution, and cigarette smoke. Trigger factors for the emergence of free radicals in the body include X-rays, car fumes, chemicals in food (preservatives, synthetic dyes, pesticide residues, and other food additives), and chemicals including drugs. Diet itself can also cause the formation of free radicals. The body can overcome oxidative damage through endogenous antioxidants such as catalase enzymes, glutathione peroxidase, superoxide dismutase, and glutathione S-transferase. However, if free radicals are present in excess or exceed the limit of cellular antioxidant protection capabilities, additional external antioxidants or exogenous antioxidants are needed to neutralize the radicals. Due to selenium deficiency, glutathione peroxidase activity is low in patients with hepatic necrosis and degenerative diseases. Meanwhile, in allergy sufferers, red blood cell glutathione peroxidase activity increased. The activity of this enzyme can also be induced by isoflavone as a secondary antioxidant (Sayuti & Yenrina, 2015).

Antioxidant activity in the mammalian brain can be detected by determining the amount of SOD dan GPx. Superoxide dismutase act as the first-line endogenic antioxidant enzyme. It will convert peroxide radicals ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2). The H_2O_2 is still harmful to the other substance, so the GPx act as a second-line endogenic antioxidant. The GPx and CAT will convert H_2O_2 to water (H_2O), a neutral and useful substance (Weydert & Cullen, 2009). With those enzymatic processes, molecular damage by free radicals can be minimized.

METHODOLOGY

Materials and Tools

Variety of Devon 1 soybean with certificate supplied by the Research Institute for Assorted Beans and Tubers ('BALITKABI: Balai Penelitian Tanaman Aneka Kacang dan Umbi'), Malang, East Java, Indonesia. Male Swiss mice 20-30 grams (3 months old) from Laboratorium Hayati at Faculty of Pharmacy Sanata Dharma University. Superoxide Dismutase (SOD) Activity Colorimetric Assay Kit (BioVision Inc., USA, Cat. #K335-100). Glutathione Peroxidase (GPx) Activity Assay Kit (BioVision Inc., USA, Cat. #762-100. Genistein (Sigma-Aldrich, USA), donepezil-hydrochloride (Merck & Co., Inc., Germany), ethanol (Merck & Co., Inc., Germany), aquadest. A microplate reader and 96-well plate were used to determine SOD and GPx. Dissecting tools for harvesting the mouse brain.

Methods

Tempeh extract

This research used the tempeh extract with total isoflavones content of 0,51% (w/w). Tempeh was produced with a variety of 'Devon 1' soybean since the previous work (Dwiatmaka et al., 2022). For extraction, 500 g of fresh tempeh was crushed and then macerated in 1500 mL of ethanol for about 24 hours. Extraction was carried out with two repetitions. All extracts were mixed and evaporated in a vacuum rotary evaporator, then reevaporated at 50 ^oC in a vacuum oven till constant weight. The total isoflavone content was then determined with Uv-Vis spectrophotometry.

Superoxide dismutase (SOD) activity

Mouse brain SOD activity was assayed using SOD Activity Colorimetric Assay Kit from BioVision (San Fransisco Bay, CA, USA) according to the instructions of the manufacturer. This SOD assay

kit utilizes WST-1, a tetrazolium salt, which produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase activity and is inhibited by SOD. The inhibition activity of SOD is determined by the colorimetric method with absorbance at 450 nm. The SOD activity is expressed as the percentage of inhibition of the WST-1 reduction rate (Andrés et al., 2008).

Glutathione Peroxidase (GPx) activity

The activity of GPx in mouse brain was assayed using the GPx assay kit from BioVision, by measuring the change of absorbance at 340 nm that follows NADPH consumption. The GPx reduces cumene hydroperoxide while oxidizing glutathione (GSH) to glutathione disulfide (GSSG). The generated GSSG is reduced to GSH with the consumption of NADPH by glutathione reductase (GR). Consumption of NADPH in the enzyme-coupled reactions was measured and expressed as U/mg protein (Mollica et al., 2017).

Animals

All experiments were carried out using 30 healthy male Swiss albino mice weighing about 20-30 g at 3 months of age, purchased from Laboratorium Hayati at the Faculty of Pharmacy Sanata Dharma University, Yogyakarta, Indonesia. The mice were housed in a 6-per-animal cage and placed under standard environmental conditions (24 ± 2 °C temperature, 70 ± 5% relative humidity) with automatic half daylight and dark cycle. Standard laboratory food and water were administered properly. The care and use of the animals according to the Guide for Laboratory Animals of the National Research Council (NRC) ("Guide For the Care and Use of Laboratory Animals," 2011). The protocol of the experiment (No. 00032/04/LPPT/VII/2019) was approved by the Ethical Clearance Commission for Preclinical Research, the Integrated Research Center Laboratory ('LPPT: Laboratorium Penelitian dan Pengujian Terpadu'), Universitas Gadjah Mada, Yogyakarta, Indonesia.

Administration of Drugs and Test Compounds

All drugs and tempeh extract solutions were prepared in 0.5% CMC-Na solution and administered orally to experimental mice.

Experimental Design

Drugs and extract solutions were administered daily at the time of 08.00 – 10.00 for about 12 days.

A count of 30 mice was randomly divided into 5 groups as follows: Group 1 (Ctrl): standard food and water; Group 2 (EtOH): 0.5 mL (16.67 mL/kg BW) of 15% ethanol; Group 3 (Don): 0.5 mL of 15% ethanol, after 1 hour followed by donepezil hydrochloride 1 mg/kg b.w; Group 4 (Gen): 0.5 mL of 15% ethanol, after 1 hour followed by genistein standard 2.55 mg/kg b.w; Group 5 (TE500): 0.5 mL of 15% ethanol, after 1 hour followed by tempeh extract 500 mg/kg b.w.

On the day 13th, mice from each group were terminated and the whole brain was harvested by brain tissue dissection to determine SOD and GPx.

Statistical analysis

Data are presented as the mean±standard deviation (SD). The statistical analysis was performed with Jamovi software (v1.6.23, https://www.jamovi.org). One-way analysis of variance, followed by the Tukey test was used for multiple comparisons. Differences between the groups were considered statistically significant at $p<0.05$.

RESULT AND DISCUSSION

Alcohol administration will increase ROS levels in the body cells (Wu & Cederbaum, 2003). Ethanol is predominantly metabolized to acetaldehyde through cytochrome P450 family 2 (CYP2E1). This process will produce ROS via electron leakage to oxygen to form superoxide ($O_2^{\bullet-}$) radical (Contreras-Zentella et al., 2022). Naturally, the body's cells will try to neutralize the free radicals that are triggered by the intake of alcohol. This will certainly reduce the amount of internal antioxidants such as SOD and GPx. Antioxidant enzymes in the brain such as SOD and GPX play an important role in reducing oxidative stress and improving cognition (Uddin et al., 2016).

The results of this study (Table I) showed that the administration of ethanol significantly reduced the levels of SOD and GPx in the brains of mice. Enzymes SOD and GPx rapidly convert ROS to harmless compounds. The results of this study also proved that the tempeh extract administration was able to increase the level of SOD and GPx. The administration of tempeh extract and genistein had the same ability to increase SOD levels and was higher than the administration of Donepezil-HCl. According to a previous study (Kawashiri et al., 2019), donepezil was able to reverse the decrease of SOD levels in cancer cell cultures due to oxaliplatin administration. Donepezil is also able to increase SOD and GPx levels in Alzheimer's patients (Zhang et al., 2018). This can also be seen from the results of this study that the levels of SOD

Table I. The concentration of SOD and GPX in the brain of mice.

Groups	SOD (%)	GPx (U/mg)
Ctrl	68.59 ± 5.12 [d]	58.90 ± 4.40 [b]
EtOH	18.65 ± 4.70 [a]	31.38 ± 2.88 [a]
Don	31.13 ± 4.68 [b]	57.75 ± 3.70 [b]
Gen	50.35 ± 4.12 [c]	57.62 ± 5.99 [b]
TE500	54.25 ± 4.98 [c]	62.89 ± 4.11 [b]

Means from six determinations ± standard deviation; followed by the different uppercase letters in the same column indicate a significantly different by the Tukey test ($P<0.05$)

and GPx in the Donepezil-HCl group were higher than in the ethanol group. Stress due to the administration of ethanol increases the level of peroxide in the mice's body cells. The cell's body will try to neutralize peroxide with SOD so that SOD levels fall. The amount of external antioxidants from the treatment (tempeh ethanolic extract, genistein, and donepezil-HCl) is suspected to be insufficient. The administration of tempeh extract increased GPx levels not significantly compared to the control group, and the levels were not significantly different between the donepezil-HCl and genistein groups (Table I). Tempeh extract contains enough isoflavones, thus results of this study are in line with previous studies (Sekikawa et al., 2022) that the administration of isoflavones can increase the activity of SOD and GPx. In other words, isoflavones can increase the body's antioxidant capacity, reduce nerve damage due to free radical attacks and effectively delay the degeneration (aging) and apoptosis of nerve cells (Sekikawa et al., 2022). Tempeh extract containing isoflavones in this study proved to be potential as an antioxidant by increasing the levels of SOD and GPx in the mouse brain. The SOD and GPx enzymes act as first-line internal antioxidants (Ighodaro & Akinloye, 2017). The SOD is a metalloenzyme group that has the main function of being antioxidant and anti-inflammatory. This enzyme protects cells that metabolize oxygen, helping to repair cellular damage from exposure to superoxide free radicals. The SOD enzyme can convert highly reactive and dangerous superoxide radicals ($O_2^{\bullet-}$) into relatively more stable and less dangerous compounds, namely H_2O_2 and O_2. Furthermore, GPx is in charge of converting H_2O_2 into H_2O, while CAT can convert it into H_2O and O_2. Under normal cell conditions, H_2O_2 is a harmful by-product, so it must be immediately converted to other harmless compounds (Uddin et al., 2016).

The administration of tempeh extract increased the production of the SOD enzyme (Figure 1), so the ability to neutralize the harmful superoxide radicals increased. GPx levels also increased after administration of tempeh extract, especially when compared to the group of mice that were given alcohol (Figure 2). This certainly makes the body's cells more able to suppress the effects of molecular and cellular damage due to the increased in free radicals.

In another mechanism, there are groups of compounds as free radical scavengers and known as second-line antioxidants (Ighodaro & Akinloye, 2017). Isoflavones can act as free radical scavengers by donating electrons from the hydroxyl group. Based on this study's results, the tempeh extract administration was able to increase the GPx levels in the mouse brain that had been given ethanol (Table I). It is possible that antioxidants from tempeh extract, especially isoflavones, play a very important role in reducing free radical levels in the body cells of mice and stimulating the formation of GPX.

Tempeh extract, donepezil-HCl, and genistein had relatively the same ability to increase GPx to normal conditions (Figure 2). For SOD, it turned out that only tempeh extract and genistein were able to significantly increase SOD levels, while donepezil-HCl did not (Figure 1). Actually, Donepezil-HCl can increase SOD and GPx levels (Zhang et al., 2018). However, the results of this study showed that the ability of Donepezil-HCl to increase GPx is greater than the increase in SOD. This phenomenon requires further study.

CONCLUSION

The administration of ethanol significantly reduces the level of SOD and GPx in the mouse brain. The administration of tempeh ethanolic extract was able to increase the level of SOD and

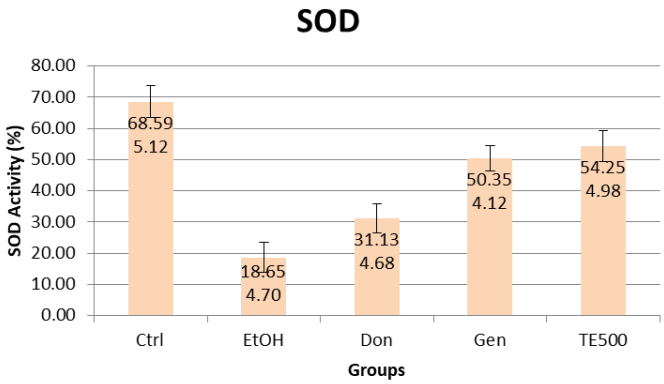

Figure 1. The activity of SOD in mouse brain tissue (mean ± sd; n=6).

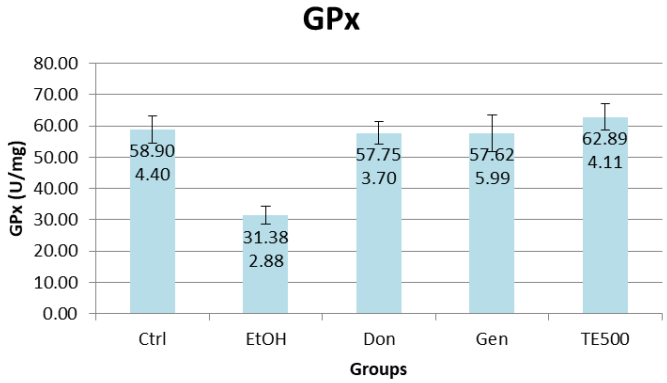

Figure 2. The activity of GPx in mouse brain tissue (mean ± sd; n=6).

GPx in the mouse brain. Tempeh ethanolic extract has significantly higher antioxidant activity than donepezil-HCl and genistein.

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