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Soybean and tempeh total isoflavones improved antioxidant activities in normal and scopolamine-induced rat brain

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ABSTRACT

Objective: To highlight the comparative studies between total isoflavone extracts from soybean and tempeh on the neuronal oxidative stress and antioxidant activities.**Methods:** The total isoflavones were administered orally for 15 days with 3 selected doses (10, 20 and 40 mg/kg). Piracetam (400 mg/kg, *p.o.*) was used as a standard drug while scopolamine (1 mg/kg, *i.p.*) was used as a drug that promoted amnesia in selected groups. The oxidative markers (thiobarbituric acid reactive substances and nitric oxide) were measured in brain homogenate. The antioxidant activities evaluated were catalase, superoxide dismutase, glutathione reductase and glutathione.**Results:** Our results showed that soybean and tempeh isoflavones significantly improved the levels of catalase, superoxide dismutase, glutathione reductase and glutathione while decreased levels of thiobarbituric acid reactive substances and nitric oxide in both the brain of normal as well as scopolamine-induced animals.**Conclusions:** Our findings suggested that soybean and tempeh isoflavones could be useful in the management and prevention of age-related neurodegenerative changes including Alzheimer's disease through its antioxidant activities.

1. Introduction

Recent studies have gained much interest in free radicals or reactive oxygen species due to their role in the progression of cancer, diabetes, arteriosclerosis, arthritis and various neurodegenerative disorders[1,2]. Even though the brain comprises only 2% of the total body weight, it receives 15% of cardiac output and uses 20% of total body oxygen consumption. Therefore, the brain is easily susceptible to oxidative stress due to the high usage of oxygen, high level of polyunsaturated fatty acids and relatively low level of antioxidants[3]. Apart from this, the generation of action potential is also crucial in the brain. Hence, in order to maintain the ion gradients across the plasma membrane, neuronal cells consume large amount of energy. This intense energy requirement is continuous; even brief periods of oxygen or glucose deprivation can result in neuronal changes[4]. These suggested that excessive oxidation processes lead to the release of free radicals in the biological system.

Free radicals are released during normal metabolic and oxidation processes. The increased concentration in body can lead to oxidative damage to proteins, lipids, DNA and RNA[5]. The perpetual oxidative damages in turn may interfere with the activities in biological systems and weaken the defence mechanisms against any abnormal substances in animal tissues thus promoting neuronal death. In age-related neurodegenerative Alzheimer's disease (AD), the high level of free radicals escalates the risk of AD by increasing the production of short fragment of β -amyloid ($A\beta$). It has been reviewed that oxidizing agents increase the expression of ammonium polyphosphate, which results in high amount of potential-to-be- $A\beta$ ammonium polyphosphate protein. Increased β -secretase 1, which is the key enzyme in the production of $A\beta$ is also linked to oxidizing agents[6]. Over time, the aggregation of $A\beta$ plaques in the extracellular environment also further intensifies the oxidative stress to the brain.

Dietary interventions have been postulated to play a role in the prevention of oxidative stress and cognitive decline among the elderly. Soybean (*Glycine max* L.) is a legume that is rich in indigenous isoflavones. Intake of soybean and its fermented products has been linked to many health benefits mainly in lowering the incidences of cardiovascular disease, risk of ischemic stroke and cholesterol levels that in turn reduce the incidence of atherosclerosis[7,8]. Efficacy of soybean phytochemicals has also

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been demonstrated for its anticancer properties against breast, prostate and also endometrial cancers[9,10]. Besides that, soybean has always been associated with lowering menopausal symptoms in women and reducing the chances of diabetic type 2[11,12]. Although soybean is well known for its antioxidant activity, the effect of soybean and its fermented products on neuro-oxidative stress is not well documented. In recent years, interest on the health benefit of soybean as a neuroprotective nutrient in the management of AD has increased.

Tempeh is a fermented soybean (with *Rhizopus oligosporus*) food that is commonly found in Indonesia and Malaysia. Chang *et al.*, highlighted the antioxidant properties of tempeh through α , α -diphenyl- β -picryl-hydrazyl and superoxide-scavenging assays *in vitro*[13]. The present study is therefore aimed to evaluate and compare the neuronal antioxidant activities of total isoflavones from soybean and tempeh in rat brain.

2. Materials and methods

2.1. Soybean and tempeh isoflavones preparation

Soybeans (1 kg) were soaked overnight in tap water and the beans were dehulled by soaking for another 24 h. The dehulled beans were boiled for 30 min then cooled and divided into two parts. Part of the soybeans (500 g) was directly dried by placing at -80°C (soybean). The other part (500 g) was fermented with *Rhizopus* sp. and incubated in air tight plastic at 28°C for 3 days (tempeh). The tempeh was then cut into small pieces and stored at -80°C . After 2 days, both soybean and tempeh were lyophilized. The samples were grinded before being stored in air tight condition. The extraction method and standardization of both isoflavones were similar to that reported in our previous study[14].

2.2. Vehicles

Piracetam (400 mg/kg) and scopolamine (1 mg/kg) were diluted in normal saline. The freeze-dried soybean and tempeh total isoflavones (SI and TI) were suspended separately in 0.5% w/v carboxy methyl cellulose sodium to obtain concentrations of 10, 20 and 40 mg/kg. The isoflavones (SI and TI) and piracetam were administered orally. Scopolamine was injected intraperitoneally.

2.3. Animals

All the experiments were carried out using male Sprague-Dawley rats which were purchased from Institute of Medical Research, Kuala Lumpur, Malaysia. Young (3–4 months) rats weighing about (180 ± 20) g were used in the present study. The animals were kept at the Laboratory and Facility of Animal Management, Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Puncak Alam, Malaysia, of which the temperature and light were controlled at 26°C and a 12 h light cycle starting from 07:00 to 19:00. The animals had free access to standard laboratory food and water *ad libitum*. The Research Committee on the Ethical Use in Research (UiTM Care) UiTM, Malaysia, approved the experimental protocol [600-FF(PT.5/2)] and the care of laboratory animals was taken as per the guidelines of the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

2.4. Acute toxicity studies

Acute toxicity studies were performed according to the Organization for Economic Co-operation and Development (No. 423) guidelines[15]. The animals were employed by random sampling technique. The animals were fasted for 4 h with access to water only.

SI and TI were administered orally at a dose of 5 mg/kg initially and any signs of mortality were observed for 3 days. If mortality was observed in two out of three animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in only one out of three animals, then the same dose was repeated again to confirm the toxic effect. If no mortality was observed, then only higher (50, 300 and 2000 mg/kg) doses were employed for further toxicity studies.

2.5. Drug administration

The rats were totally divided into 16 groups (6 animals per group). Different sets of animals were used for normal and scopolamine-induced groups. Control group was administered continuously for 15 days with normal saline while piracetam (400 mg/kg) acted as the reference drug group. For normal groups, SI10, SI20 and SI40, and TI10, TI20 and TI40 were administered orally for 15 days at three concentrations (10, 20 and 40 mg/kg). Additionally, for scopolamine-induced groups, after 90 min of oral administration, the animals were injected with scopolamine (1 mg/kg, *i.p.*) in order to induce amnesia. On Day 15, after 60 min dose, the animals were sacrificed.

2.6. Collection of brain samples

At the end of the treatment, animals were sacrificed by cervical decapitation under light ether anaesthesia. Immediately after decapitation, the whole brain was carefully removed from the skull. For preparation of brain homogenate, the fresh whole brain was weighed and transferred into a glass homogenizer and homogenized using ice bath after adding 10 volumes of cold phosphate buffer saline (PBS) solution. The homogenate was centrifuged at 3000 r/min for 10 min and the resultant cloudy supernatant liquid was used for estimation of following parameters.

2.7. Catalase assay

The catalase assay was performed according to the manual using the catalase assay kit from Cayman Chemical Company, USA. In brief, brain tissue homogenate in cold PBS was centrifuged for 5 min at 8500 r/min at 4°C . The clear supernatant obtained was diluted to a concentration of 100 mg/mL for the assay. The sample was analyzed using spectrophotometer at 540 nm. Catalase activity was calculated by using equation as mentioned below and was expressed in $\mu\text{mol/L/min}$ in 100 mg/mL of brain homogenate.

$$\text{Formaldehyde } (\mu\text{mol/L}) = \left[\frac{\text{Sample absorbance} - (y - \text{intercept})}{\text{Slope}} \right] \times \frac{0.17 \text{ mL}}{0.02 \text{ mL}}$$

$$\text{Catalase activity } (\mu\text{mol/L/min}) = \frac{\text{Formaldehyde produced } (\mu\text{mol/L})}{20 \text{ min}}$$

2.8. Superoxide dismutase (SOD) assay

The SOD activity was measured by using SOD assay kit (Cayman Chemical Company, USA). The supernatant of brain homogenate was standardized to 100 mg/mL while the SOD standard ranged from 0.00 to 0.20 $\mu\text{L/mL}$. Then, 200 μL of tetrazolium salt solution (as a radical detector) was added to 10 μL of the sample and standard. The reaction was initiated by adding 20 μL of xanthine oxidase and the absorbance was read at 440 nm using a plate reader. For sample background, the same amount of buffer was added instead of xanthine oxidase. The SOD activity was calculated using the equation obtained from linear regression of the standard curve. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

$$\text{SOD (IU/mL)} = \left[\frac{\text{Sample linearized rate} - (y - \text{intercept})}{\text{Slope}} \right] \times \frac{0.23 \text{ mL}}{0.01 \text{ mL}} \times \text{Sample dilution}$$

2.9. Glutathione (GSH) assay

The GSH assay was performed according to the manual using the GSH assay kit from Cayman Chemical Company, USA. In brief, brain tissue homogenate in cold PBS was centrifuged for 5 min at 14000 r/min at 4 °C. The clear supernatant obtained was diluted to a concentration of 100 mg/mL for the assay. The sample was analyzed using spectrophotometer (Infinite M200, Tecan) at 405 nm. The GSH concentration of the sample was calculated in the following equation.

$$\text{GSH } (\mu\text{mol/L}) = \frac{\text{Absorbance} - (y - \text{intercept})}{\text{Slope}} \times 2$$

2.10. Glutathione reductase (GR) assay

The GR assay was performed according to the manual using the assay kit from Cayman Chemical Company, Michigan, USA. The supernatant of brain homogenates was standardized to 100 mg/mL each. For the non-enzymatic well, 120 μL of assay buffer (50 mmol/L potassium phosphate, pH 7.5 containing 1 mmol/L ethylene diamine tetraacetic acid) was added to 20 μL oxidized glutathione. In the positive control well, 100 μL assay buffer was added to 20 μL of oxidized glutathione, and 20 μL of GR, while in the sample wells, 20 μL of sample was added instead of GR. The reaction was initiated by adding 50 μL of nicotinamide adenine dinucleotide phosphate. The optical density was read once every minute to obtain at least 5 time points. Two points on the linear portion of the curve were selected and the GR activity was determined by using the equation mentioned below. The $\Delta\text{OD}/\text{min}$ at 340 nm was determined by the difference of the optical densities at two time points divided by the time difference in minutes.

$$\text{GR activity (nmol/min/mL)} = \frac{\Delta\text{OD}/\text{min}}{0.00373 \mu\text{mol}} \times \frac{0.19 \text{ mL}}{0.02 \text{ mL}} \times \text{Sample dilution}$$

2.11. Thiobarbituric acid reactive substances (TBARS) assay

Malondialdehyde (MDA) was measured according to the manual using the TBARS assay kit from Cayman Chemical Company, USA. Brain tissue homogenate in cold PBS was centrifuged for 5 min at 8500 r/min. The clear supernatant obtained was diluted to a concentration of 100 mg/mL for the assay. Each sample was assayed in triplicates. Using the assay kit, 100 μL of sodium dodecyl sulphate solution was added to 100 μL of MDA standards (ranging from 0 to 50 $\mu\text{mol/L}$) or sample. To these vials, 4 mL of colour reagent was added forcefully, and then the vials were capped and boiled for 1 h. The vials were removed and immediately placed on ice for 10 min. The vials were then centrifuged for 10 min. A volume of 150 μL from each vial was placed in a plate, and the absorbance at 540 nm was read using spectrophotometer. The MDA value of samples and standards were calculated as described in the following equation.

$$\text{MDA } (\mu\text{mol/L}) = \frac{(\text{Corrected absorbance}) - (y - \text{Intercept})}{\text{Slope}}$$

2.12. Nitric oxide (NO) assay

The NO was measured according to the manual using the nitrate/nitrite assay kit from Cayman Chemical Company, USA. In brief, brain tissue homogenate in cold PBS was centrifuged at 8500 r/min for 5 min at 4 °C. The clear supernatant obtained was diluted to a concentration of 100 mg/mL for the assay. The samples were analyzed using spectrophotometer (Infinite M200, Tecan) at 540 nm.

The NO concentration of the sample was expressed in $\mu\text{mol/L}$. The NO level was calculated according to the equation.

$$\text{NO} = \left(\frac{\text{Absorbance}_{540} - y - \text{Intercept}}{\text{Slope}} \right) \left(\frac{200 \mu\text{L}}{40 \mu\text{L}} \right)$$

2.13. Statistical analysis

All the results were expressed as mean \pm SEM. Data was analyzed using One-way ANOVA followed Dunnett's *t*-test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Tempeh isoflavone increased catalase level

Figure 1 shows that brain catalase activity was significantly increased ($P < 0.001$) in normal and animals administered with piracetam (57.02 nmol/min/mL), while control was 14.10 nmol/min/mL. The same significant increase was also observed in all the tempeh isoflavone treated groups (TI10, TI20 and TI40) as compared to the control group. The catalase levels were recorded as 31.92, 34.49 and 40.82 nmol/min/mL at TI10, TI20 and TI40 respectively. In contrast, no significant changes were observed in groups treated with soybean isoflavone in all three administered doses (SI10, SI20 and SI40) when compared to the control group. It is evident that tempeh isoflavone improved the catalase activity within the brain better than soybean. However, tempeh isoflavone was not able to improve the level of catalase as much as piracetam.

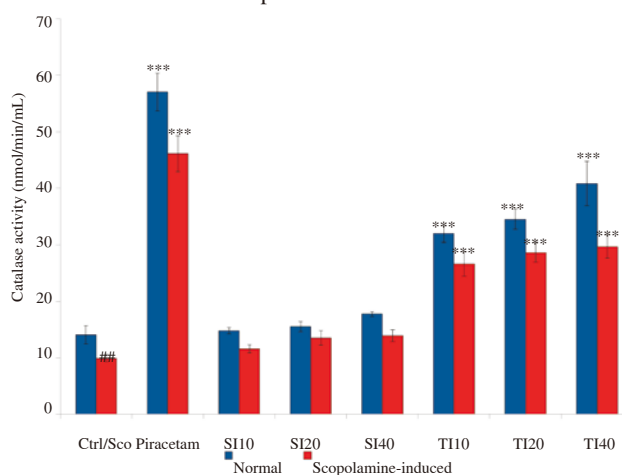


Figure 1. Effect of soybean (10, 20 and 40 mg/kg) and tempeh (10, 20 and 40 mg/kg) administered orally for 15 days on brain catalase activity in normal and scopolamine-induced groups.

Values were in mean \pm SEM ($n = 6$). ***: $P < 0.001$ as compared to control and scopolamine-induced groups respectively; ##: $P < 0.01$ was the significant value of scopolamine-induced group when compared to control. Ctrl: Control; Sco: Scopolamine.

A similar trend was observed in the scopolamine-induced animals as shown in Figure 1, which indicated that the brain catalase activity was also significantly improved ($P < 0.001$) after being treated with piracetam (46.08 nmol/min/mL), while scopolamine-induced group was 9.96 nmol/min/mL. Significant increase ($P < 0.001$) was also observed in animal groups treated with tempeh isoflavone at all concentrations (26.64, 28.70 and 29.73 nmol/min/mL in TI10, TI20 and TI40 respectively) when compared to scopolamine-induced group. No significant differences were observed in the soybean treated animals at any of the dose (10, 20 and 40 mg/kg). The tempeh isoflavone treated group exhibited higher catalase activity when compared to the soybean isoflavone group but lower than the piracetam group.

3.2. Soybean and tempeh isoflavones increased SOD level

The effect on SOD activities by soybean and tempeh isoflavones was shown in Figure 2. In normal animal model, piracetam treated group (11.05 IU/mL) showed significant increase ($P < 0.01$) in the SOD activity as compared to control group (8.99 IU/mL). Low and medium doses of soybean (SI10 and SI20) did not show any significant changes in the SOD activity. However, at a high dose of soybean (SI40 at 10.98 IU/mL) and low dose of tempeh isoflavone (TI10 at 11.30 IU/mL), the SOD activity was significantly increased ($P < 0.01$) when compared to control. Higher doses of tempeh (TI20, 11.96 IU/mL and TI40, 13.03 IU/mL) showed higher and significant ($P < 0.001$) increase of SOD activities as compared to control. The results also indicated that TI20 and TI40 significantly improved ($P < 0.001$) the catalase activity within the brain better than the piracetam group.

Figure 2 also shows the effect of soybean and tempeh isoflavones on SOD activity in scopolamine-induced animal model. Scopolamine significantly reduced ($P < 0.001$) SOD activity as compared to control group. In contrast, standard drug piracetam (10.66 IU/mL) showed significant ($P < 0.001$) elevation in SOD as compared to the scopolamine-induced group (6.68 IU/mL). It was observed that all doses of soybean (8.83–9.24 IU/mL) and tempeh isoflavones (9.51–9.96 IU/mL) showed significant increase in the SOD activities at $P < 0.001$. Tempeh isoflavone showed higher SOD activities as compared to soybean group. This further supported the ability of tempeh to reverse the memory impairment by scopolamine better than soybean[16].

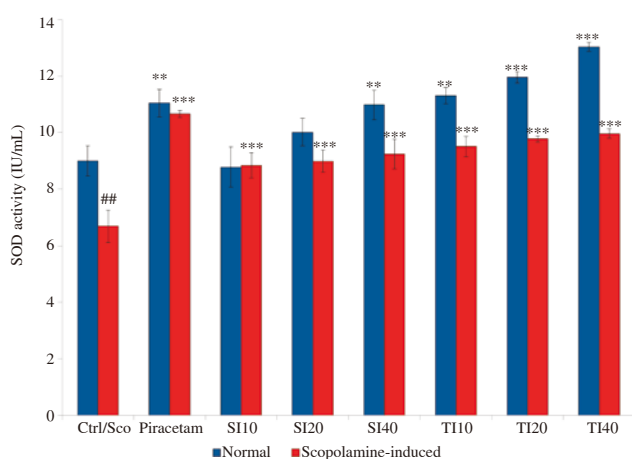


Figure 2. Effect of soybean (10, 20 and 40 mg/kg) and tempeh (10, 20 and 40 mg/kg) administered orally for 15 days on the activity of brain SOD in normal and scopolamine induced group.

Values were in mean \pm SEM ($n = 6$). **: $P < 0.01$ and ***: $P < 0.001$ as compared to control group and scopolamine-induced group respectively; ##: $P < 0.01$ was the significant value of scopolamine-induced group when compared to control. Ctrl: Control; Sco: Scopolamine.

3.3. Soybean and tempeh isoflavones increased GSH activity

The effect of soybean and tempeh isoflavones on the level of brain GSH was shown in Figure 3. In normal animal model, low doses of soybean isoflavone (SI10 and SI20) did not affect the brain GSH level. However, at a high dose of soybean isoflavone (SI40), significant increase ($P < 0.05$) in brain GSH level (5.84 $\mu\text{mol/L}$) was observed as compared to control group (3.73 $\mu\text{mol/L}$). In tempeh treated groups, all doses displayed a significant increase in the level of GSH (6.23, 6.86 and 6.99 $\mu\text{mol/L}$ in TI10, TI20 and TI40 respectively). In TI10 group, the increase was at the level of $P < 0.05$ whilst, TI20 and TI40 showed further significant increases at the level of $P < 0.01$ as compared to control. Piracetam (400 mg/kg) also showed a significant ($P < 0.05$)

elevation in brain GSH level (6.07 $\mu\text{mol/L}$). From the results, it can be concluded that TI20 and TI40 possessed better ability to improve the level of GSH within the brain as compared to the piracetam group. Tempeh isoflavone treated groups also had the ability to enhance GSH better than soybean treated groups and it showed that the low dose of tempeh isoflavone (TI10) increased the GSH level similar to that of the higher dose of soybean (SI40).

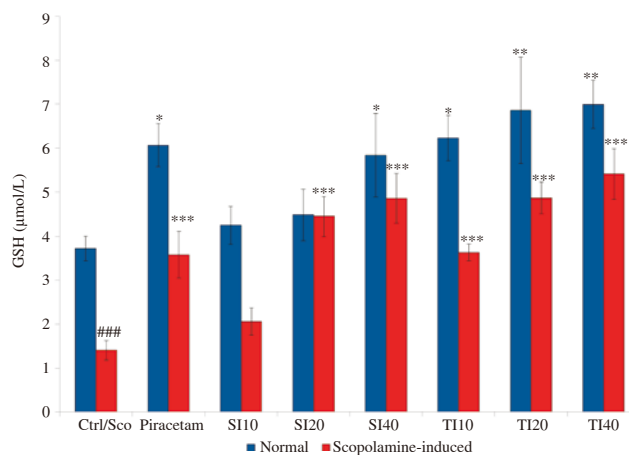


Figure 3. Effect of soybean (10, 20 and 40 mg/kg) and tempeh (10, 20 and 40 mg/kg) administered orally for 15 days on level of brain GSH in normal group and scopolamine induced group.

Values were in mean \pm SEM ($n = 6$). *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$ as compared to control and scopolamine-induced group for each model respectively; ####: $P < 0.001$ was the significant value of scopolamine-induced group when compared to control. Ctrl: Control; Sco: Scopolamine.

Scopolamine-induced group showed a significant decrease ($P < 0.001$) in the level of GSH (1.41 $\mu\text{mol/L}$) as compared to control group (Figure 3). The low dose (SI10) of soybean isoflavone did not show any effect in brain GSH but at higher doses of soybean isoflavone (4.45 and 4.86 $\mu\text{mol/L}$ in SI20 and SI40 respectively) significant increase ($P < 0.001$) was observed. For tempeh isoflavone, all doses showed significant increase ($P < 0.001$) of brain GSH (3.64, 4.87 and 5.41 $\mu\text{mol/L}$ in TI10, TI20 and TI40 respectively) as compared to scopolamine-induced group. Piracetam also showed a significant ($P < 0.001$) elevation in the level of brain GSH (3.58 $\mu\text{mol/L}$) when compared to scopolamine-induced group. Based on these results, the study suggested that tempeh isoflavone showed better significant improvement in the level of GSH as compared to soybean isoflavone. This suggested tempeh being more health beneficial than soybean.

3.4. Soybean and tempeh isoflavones increased GR level

Figure 4 shows the effect of soybean and tempeh isoflavones on the level of brain GR in normal animal model. In normal animal model, low and medium doses (SI10 and SI20) of soybean isoflavone did not affect the brain GR activity. At a high dose of 40 mg/kg of soybean isoflavone (32.74 nmol/min/mL in SI40), a significant increase ($P < 0.001$) in GR was documented when compared with control (24.03 nmol/min/mL). For tempeh isoflavone, at doses TI20 and TI40 (29.65 and 32.54 nmol/min/mL correspondingly) showed a significant increase in brain GR activities with values of $P < 0.05$ and $P < 0.001$ respectively. This suggested that tempeh treated groups showed better ability to improve the GR activity than soybean did. Piracetam also significantly increased ($P < 0.001$) the brain GR activity as compared to control.

Figure 4 shows that when the scopolamine-induced group was compared to control, significant decrease in the level of GR (17.60 nmol/min/mL) was observed at the level of $P < 0.01$. In scopolamine-induced group, only the highest concentration of soybean isoflavone (SI40) showed significant increase ($P < 0.001$) in the GR activity

(26.43 nmol/min/mL) while, SI10 and SI20 did not produce any significant changes. High concentration of tempeh isoflavone (TI40) showed significant increase ($P < 0.001$) in GR activity (29.72 nmol/min/mL). TI20 increased GR activity (26.39 nmol/min/mL) at the level of $P < 0.05$ while TI10 did not show any significant differences. Piracetam treated scopolamine induced rats showed improvement in the GR activity as compared to the control scopolamine-induced group ($P < 0.001$).

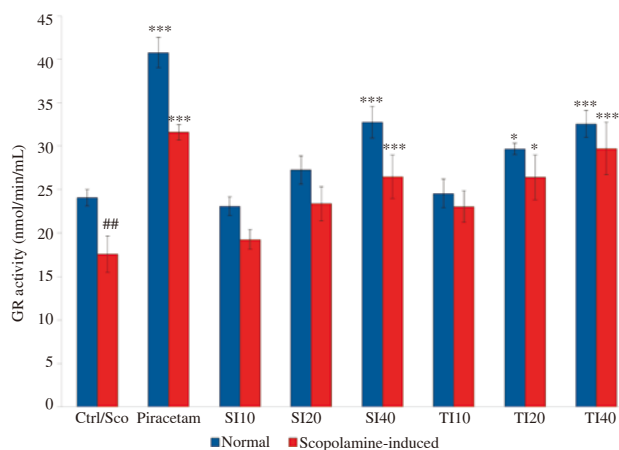


Figure 4. Effect of soybean (10, 20 and 40 mg/kg) and tempeh (10, 20 and 40 mg/kg) administered orally for 15 days on level of brain GR in normal group and scopolamine induced groups.

Values are in mean \pm SEM ($n = 6$). *: $P < 0.05$ and ***: $P < 0.001$ as compared to control and scopolamine-induced group for each model respectively; ##: $P < 0.01$ was the significant value of scopolamine-induced group when compared to control. Ctrl: Control; Sco: Scopolamine.

3.5. Soybean and tempeh isoflavones reduced TBARS level

In normal group, all doses (10, 20 and 40 mg/kg) of soybean and tempeh isoflavones exerted a significant reduction of MDA ($P < 0.001$) as compared to the control group (Figure 5). Soybean isoflavone reduced the MDA to 24.40 $\mu\text{mol/L}$, 24.12 $\mu\text{mol/L}$ and 23.82 $\mu\text{mol/L}$ (SI10, SI20 and SI40 respectively) as compared to control, 28.05 $\mu\text{mol/L}$. Tempeh isoflavone (16.83 $\mu\text{mol/L}$ –20.50 $\mu\text{mol/L}$) reduced the MDA even greater than soybean did. Piracetam (13.91 $\mu\text{mol/L}$) being the standard drug used also showed a significant decrease ($P < 0.001$) compared to control group.

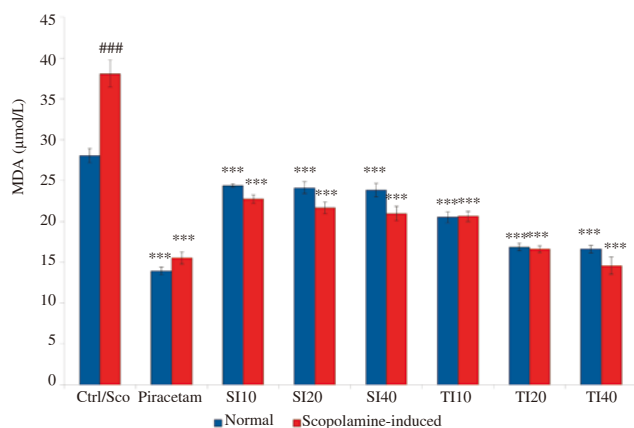


Figure 5. Effect of soybean (10, 20 and 40 mg/kg) and tempeh (10, 20 and 40 mg/kg) administered orally for 15 days on brain MDA levels in normal groups and scopolamine induced groups.

Values are in mean \pm SEM ($n = 6$). ***: $P < 0.001$ as compared to control and scopolamine-induced group for each model respectively; ###: $P < 0.001$ was the significant value of scopolamine-induced group when compared to control. Ctrl: Control; Sco: Scopolamine.

A similar trend (Figure 5) was observed in the scopolamine-induced

group, which exerted a significant reduction in the production of MDA ($P < 0.001$) at all doses of both soybean and tempeh isoflavones (10, 20 and 40 mg/kg). The level of MDA in scopolamine-induced group was higher (38.09 $\mu\text{mol/L}$) than control (28.05 $\mu\text{mol/L}$). Soybean isoflavone reduced the level of MDA to 22.73 $\mu\text{mol/L}$, 21.66 $\mu\text{mol/L}$ and 20.95 $\mu\text{mol/L}$ (SI10, SI20 and SI40 respectively) while tempeh isoflavone showed even greater reduction with the values of 20.59 $\mu\text{mol/L}$, 16.61 $\mu\text{mol/L}$ and 14.60 $\mu\text{mol/L}$ (TI10, TI20 and TI40 respectively) as compared to scopolamine-induced group. The scopolamine-induced animals treated with piracetam also showed a significant decrease ($P < 0.001$) in the level of MDA with value of 15.51 $\mu\text{mol/L}$ as compared to scopolamine-induced group.

3.6. Soybean and tempeh isoflavones reduced NO

The effect of soybean and tempeh isoflavones on NO level in brain was shown in Figure 6. In normal animal model, the level of NO was observed at 16.06 $\mu\text{mol/L}$. With treatment of piracetam at 400 mg/kg, significant decrease of NO level (11.29 $\mu\text{mol/L}$) was observed at the level of $P < 0.001$. The same significant level was also observed in SI40, TI20 and TI40 groups with values 13.03 $\mu\text{mol/L}$, 12.77 $\mu\text{mol/L}$ and 12.00 $\mu\text{mol/L}$ respectively. SI20 significantly reduced ($P < 0.01$) NO to 13.76 $\mu\text{mol/L}$. Low dose of tempeh isoflavone group, TI10 also showed significant reduction at the level of $P < 0.05$ with value of 14.35 $\mu\text{mol/L}$. Meanwhile, SI10 did not show significant reduction. All groups were statistically compared to control group.

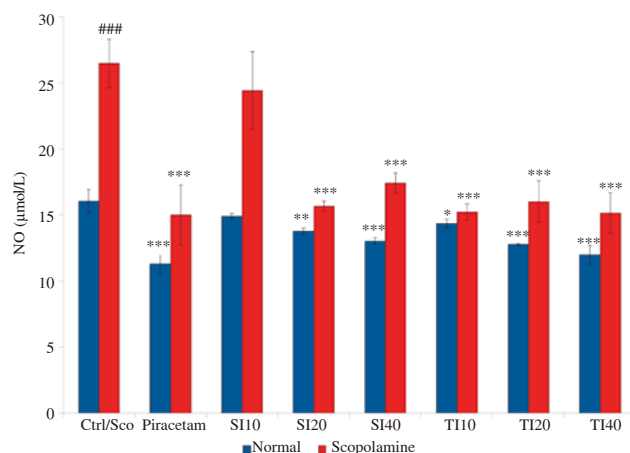


Figure 6. Effect of soybean (10, 20 and 40 mg/kg) and tempeh (10, 20 and 40 mg/kg) administered orally for 15 days on brain NO levels in normal groups and scopolamine induced groups.

Values are in mean \pm SEM ($n = 6$). *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$ as compared to control and scopolamine-induced group for each model respectively; ###: $P < 0.001$ was the significant value of scopolamine-induced group when compared to control. Ctrl: Control; Sco: Scopolamine.

Scopolamine-induced group (26.48 $\mu\text{mol/L}$) showed higher level of NO level as compared to normal control. Piracetam treated group (15.01 $\mu\text{mol/L}$) showed significant reduction ($P < 0.001$) in NO level as compared to scopolamine-induced group. In soybean treated groups, low dose, SI10 did not show any significant reduction as compared to scopolamine-treated group. However, medium and high doses of soybean isoflavones (SI20 and SI40) showed significant reduction ($P < 0.001$) as compared to scopolamine-induced group, with values of 15.66 $\mu\text{mol/L}$ and 17.42 $\mu\text{mol/L}$ respectively. Meanwhile, in tempeh treated groups, all doses (TI10, TI20 and TI40) showed significant reduction at the level of $P < 0.001$ as compared to scopolamine-induced group, with the values of 15.22 $\mu\text{mol/L}$, 16.02 $\mu\text{mol/L}$ and 15.15 $\mu\text{mol/L}$ respectively (Figure 6). From the results, it showed that tempeh isoflavone was able to reduce the level of NO better than soybean isoflavone. Tempeh isoflavones were able to reduce NO which often led to neurodegeneration which promoted AD.

4. Discussion

Oxidative stress can be very deleterious in the biological system especially in brain as it will lead to cell damage and death. Antioxidants, free radical scavengers and chelators are three agents that have been studied to directly suppress the formation of free radicals within the biological system. In contrast, free radical inhibitors that can be classified into antioxidant enzymes and other compounds may indirectly improve oxidative stress. The indirect reaction may be through the inhibition of the pro-oxidant enzymes or oxidative activity[17]. The free radical scavengers may react with free radical which produces inactive, stable radicals. The chelators act by chelating the active transition metals to form inactive complexes hence delay in oxidation.

Our *in vivo* experiments in the present study further confirm our previous *in vitro* study that tempeh isoflavone was a better free radical scavenger and ferrous ion chelator as compared to soybean isoflavone[14]. The tempeh isoflavone showed an elevated level of GSH and other antioxidant enzymes such as SOD, catalase and GR. Wang *et al.*[18] found that douchi, soybean fermented with *Aspergillus oryzae* increased the level of glutathione peroxidase, catalase and SOD in rats. Yet another study by Wu and Chu showed fermentation of soybean with *Bacillus subtilis* exerted an increment of SOD *in vitro*[19]. These suggest that the fermentation process of soybean increased the antioxidant enzymes both *in vitro* and *in vivo*.

SOD is an antioxidant enzyme that plays a vital role in regulation of free radical processes in biological systems. In general, SOD stabilises free radicals by donating its electron producing H_2O_2 . Theoretically, the dismutation of superoxide occurs at copper, manganese or iron centres of SOD isoenzymes CuZnSOD, MnSOD or FeSOD but only CuZnSOD and MnSOD are used by eukaryotes[17]. According to Deng *et al.*[20], superoxide activates cytokines, by which CuZnSOD may exhibit neuroprotective effect by suppressing the microglial activation by the cytokines. The MnSOD has a role in preventing the mitochondrial production of oxygen radicals. It is also believed that the overexpression of MnSOD in the brain prevents apoptosis induced by tumor necrosis factor- α [21]. Previous studies indicated that soybean is able to increase the SOD level within the brain[5,18,22,23]. In the present study, the results showed that in normal and scopolamine-induced animal models, tempeh isoflavones at all doses exhibited significant increase of SOD in the brain and better than soybean isoflavones due to the higher amount of aglycones after fermentation process[14]. This suggests that tempeh is a better antioxidant when compared to soybean. Tempeh was able to reverse the scopolamine-induced amnesia by increasing the level of SOD in scopolamine-induced animals. Thus, the risk of getting AD might be reduced by consuming soybean and its fermented product tempeh.

Catalase is known as a heme containing enzyme that decomposes H_2O_2 to oxygen and water. This reduces the deleterious effect of H_2O_2 such as cell damage and induction of apoptosis. Many recent studies supported that the increase of catalase in animals treated with soybean food products[5,18,22,23]. The H_2O_2 within the biological system should be balanced between the oxidation and repair mechanisms. The present study proved that soybean isoflavones significantly increased catalase within the brain. However, tempeh isoflavone treated animals showed a more significant increase of catalase in the brain as compared to soybean isoflavone treated animals. This suggested that tempeh possessed better antioxidant ability as compared to soybean. The consumption of tempeh may be more beneficial to health.

GSH is a tripeptide (γ -L-glutamyl-L-cysteinylglycine) which is the most abundant thiol antioxidant in mammalian biological systems. GSH is involved in metabolism, catalysis and the most important as an antioxidant. GSH is capable to act as free radical scavenger and electron donor in enzymatic redox cycle of glutathione peroxidase and GR catalyzing the reduction of peroxides[17]. As a water-soluble

antioxidant, GSH is very important to reduce the level of free radicals that prevent the cells from oxidative stress and cell damage in the brain. When the cells and neurons within the brain are depleted, brain loses its functions especially in memory, emotion, motor cognitive ability *etc.* These can be supported by a study which found that the depletion of GSH in the brain led to cytotoxicity, which correlated with the formation of excessive NO in the brain[24]. The NO has been well debated as one of the causes to oxidative stress whilst antioxidants have been reported to inhibit NO. Therefore, high levels of GSH as observed in this study may lead to the low NO level. The results showed that the tempeh isoflavone groups exhibited higher amount of GSH within the brain than soybean isoflavone groups did.

GR is important in GSH redox cycle. The GR is important in production of GSH as a result from reaction with glutathione disulfide, nicotinamide adenine dinucleotide phosphate and H^+ [25]. In this study, the results showed that tempeh isoflavone treated animals exhibited higher level of GR as compared to soybean isoflavone treated animals. This would suggest that the consumption of tempeh is more beneficial in preventing oxidative series within the brain that causes neurodegeneration and AD.

In this study, the measurement of MDA was performed to measure the effects of soybean and tempeh isoflavones on the magnitude of lipid peroxidation within the brain. MDA is a biomarker for oxidative stress as it is one of the end products of lipid peroxidation process[26]. Measurement of the MDA within the brain tissue represents the magnitude of the deleterious effect of lipid peroxidation in neurodegenerative diseases such as AD, Parkinson's disease and Huntington's disease[27]. Therefore, to overcome this process, it is crucial to inhibit the peroxidation that occurs within the brain. Much has been documented recently on food based products with health benefits especially in preventing oxidation. But to date, very few studies reported on the ability of tempeh as an anti-oxidative food. Based on the results obtained in this study, it showed that treatment with 40 mg/kg of soybean isoflavone reduced the MDA level significantly as compared to control group. In fact, even a low dose of 10 mg/kg of tempeh isoflavone showed more significant results as compared to 40 mg/kg of soybean isoflavone treatment. These results reflected that tempeh is rich with aglycones which are easily absorbed as compared to glycoside. Besides that, the results obtained in antioxidant studies also correlated with the result of MDA levels. Tempeh isoflavones facilitated the increasing level of GSH within the brain better than soybean isoflavone did. It suggested that antioxidant GSH would reduce the free radicals so that the peroxidation of lipids is weakened. A previous study showed that soybean attenuated oxidative stress by reducing TBARS formation and increasing GSH level within the rat brain[18]. While, another study found that soybean supplementation reversed the scopolamine-induced amnesia and significantly increased the GSH levels and lowered the levels of TBARS within the brain[22]. Both studies supported the results obtained from the present study, where soybean isoflavone decreased the level of lipid peroxidation. Tempeh however, has excellent ability to reduce lipid peroxidation as compared to soybean suggesting that tempeh has better health beneficial effects.

The NO is a vital molecule in signaling process of cells. However, imbalance in the metabolism can cause pathological conditions[28]. High levels of NO can lead to neurodegeneration through the combination with free radicals generated from mitochondria and/or highly toxic peroxynitrite that eventually causes neuronal cell death[29]. As high level of NO would promote neuronal cell death, the inhibition of NO is very important in preventing neurodegeneration. In this study, the results showed that both soybean and tempeh isoflavones have the ability to reduce the level of NO within the brain as compared to control group. Nonetheless, tempeh isoflavone treated groups showed more significant reduction as compared to soybean isoflavone treated groups of the same dose. The same results were also

observed in scopolamine-induced model, by which both soybean and tempeh isoflavones treated groups showed significant reduction in the level of NO as compared to scopolamine-induced group. Interestingly, tempeh isoflavone treated groups showed more significant reductions as compared to soybean isoflavone treated groups. Previous study also suggested that soybean genistein has the ability to alleviate the excessive NO level within biological system[29]. Besides that, high level of NO also can induce inflammation and tissue damage. Therefore, this study also correlated the significant reduction of NO by soybean and tempeh isoflavones.

From the SOD and catalase activities results, this study concluded that the increase in SOD activity was followed by the increase in catalase activity. The tempeh isoflavone showed an elevated level of GSH and other antioxidant enzymes such as SOD, catalase and GR. Also, both soybean and tempeh isoflavones showed significant reduction in the oxidative biomarkers, which are MDA and NO. Both isoflavones also reversed the scopolamine-induced effects, which also significantly reduced the NO production within the brain. However, tempeh isoflavone showed more significant results as compared to soybean isoflavone. The low level in these lipid peroxidation biomarkers would suggest that soybean and tempeh are beneficial to health especially in promoting anti-AD, with regards that tempeh is much more beneficial to health.

Conflict of interest statement

We declare that we have no conflict of interest.

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