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Memory Enhancement in Rats by Soybean and *Tempeh* Extracts is Associated with Improved Cholinergic and Reduced Neuroinflammatory Activities

(Peningkatan Daya Ingatan dalam Tikus oleh Ekstrak Soya dan Tempeh dikaitkan dengan Peningkatan Aktiviti Kolinergik dan Pengurangan Aktiviti Keradangan)

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ABSTRACT

The continued progression of neurodegeneration may result in dementia. The present study compared the neuroprotective activities between soybean and tempeh *extracts in rats. The extracts were administered orally at 10, 20 and 40 mg/kg for 15 days. Radial arm maze and elevated plus maze served as exteroceptive behavioural models for memory measuring. Brain cholinergic activities (acetylcholine and acetylcholinesterase) and neuroinflammatory related cytokines interleukin 1β and interleukin-10 were also tested. Soybean and* tempeh *extracts significantly improved memory, but overall 40 mg/kg* tempeh *showed better improvement (*$p<0.05$*). The* tempeh *extracts at 20 and 40 mg/kg exhibited a significant (*$p<0.05$*) increase and decrease in the level of acetylcholine and acetylcholinesterase activities, respectively.* Tempeh *extract (40 mg/kg) resulted in greater reduction (*$p<0.05$*) of inflammation than soybean extract. Altogether,* tempeh *extract may be beneficial in the management and prevention of dementia and Alzheimer's disease.*

Keywords: Cholinergic; memory; neuroinflammation; soybean; Tempeh

ABSTRAK

Neurodegradasi yang berlanjutan boleh menyebabkan penyakit demensia. Kajian ini membandingkan keupayaan ekstrak soya dan tempeh untuk melindungi otak menggunakan tikus. Ekstrak tersebut telah diberi secara oral pada 10, 20 dan 40 mg/kg selama 15 hari. Pengukuran daya ingatan dijalankan dengan menggunakan pagar sesat bentuk jejari dan pagar sesat bersilang tinggi yang merupakan model rangsangan persekitaran. Aktiviti kolinergik otak (asetilkolina dan asetilkolinesterase) dan sitokin yang berkaitan dengan keradangan otak IL-1β dan IL10 turut diuji. Ekstrak soya dan tempeh mampu meningkatkan daya ingatan, namun 40 mg/kg ekstrak tempeh menunjukkan peningkatan daya ingatan yang paling ketara ($p<0.05$*). Ekstrak tempeh (20 dan 40 mg/kg) menunjukkan peningkatan dan penurunan yang berkesan (*$p<0.05$*) dalam aktiviti asetilkolina (*$p<0.05$*) dan asetilkolinesterase. Ekstrak tempeh 40 mg/kg mengurangkan keradangan (*$p<0.05$*) yang lebih ketara berbanding dengan ekstrak soya. Sebagai kesimpulan, ekstrak tempeh mungkin berkesan mencegah demensia dan juga penyakit Alzheimer.*

Kata kunci: Daya ingatan; kolinergik; radang otak; soya; tempeh

INTRODUCTION

Neurodegeneration is a series of neuronal dysfunction due to the continual death of neurons. The loss of brain neurons leads to major age-related disease like dementia. The most deteriorating effect of neurodegeneration is memory loss. The primary mechanism involved in memory loss is the deficit of cholinergic neurons; where transmission of information is suppressed due to lack of neurotransmitters such as acetylcholine (ACh) (Auld et al. 2002). Apart from that, the regulation of acetylcholinesterase (AChE) is also very important in maintaining the degradation of acetylcholine. Excessive amounts of acetylcholinesterase however, results in low levels of acetylcholine, resulting in memory impairment (Pepeu & Giovannini 2010). Deterioration of cholinergic deficiency can be alleviated by inhibiting acetylcholinesterase. Studies by previous investigators showed that natural products such as *Emblica officinalis* and *Thesesia populnea* have the ability to inhibit acetylcholinesterase activity (Vasudevan & Parle 2007; 2006). In fact, most of the AD drugs such as tacrine, donepezil, galantamine and rivastigmine are anticholinesterase inhibitors (Samadi et al. 2011; Zheng et al. 2010).

Neurodegeneration via inflammatory pathway can also lead to memory loss. Proinflammatory cytokines and reactive oxygen species (ROS) produced by activated microglia cells and astrocytes may cause neuronal damage or neurotoxicity resulting in apoptosis and necrosis (Glass et al. 2010). Moreover, proinflammatory mediators released from microglial and astrocytes can activate each other to amplify inflammatory signals to neurons. The major proinflammatory cytokine which has been reported with elevation in AD is IL-1β (Pan et al. 2010). Other common anti-inflammatory cytokines include IL-4, IL-10, and TGF-β

(Akiyama et al. 2000). In neuroinflammation, the balance between pro- and anti- inflammatory mediators is very crucial.

Soybean (*Glycine max* L.) is a legume that is rich in indigenous isoflavones (Barnes 2010). Intake of soybean and its fermented products have been linked to many health benefits. Soybean has been shown to lower the incidence of cardiovascular diseases, risk of ischemic stroke and lower cholesterol levels reducing the incidence of atherosclerosis (Liang et al. 2009; Nagarajan et al. 2008; Pyo & Seong 2009; Reynolds et al. 2006). Efficacy of soybean phytochemicals has also been studied for its anticancer properties in various cancer types. Soybean intake was reported to reduce the risk of breast, prostate and also endometrial cancers (Goodman et al. 1997; Hwang et al. 2009; Korde et al. 2009). Besides, soybean has always been associated with lowering menopausal symptoms in women and in reducing the chances of diabetic type 21 (Pipe et al. 2009; Villa et al. 2009). Nonetheless, soybean is also well recognized to possess antioxidant activity (Devi et al. 2009). In recent years, interest on the health benefits of soybean in the prevention and management of AD has increased. Using different maze models, rats supplemented with soybean were found to improve memory and act as an acetylcholinesterase inhibitor (Bagheri et al. 2011; Ding et al. 2011; Yang et al. 2011).

Tempeh is soybean cake that is fermented with *Rhizopus oligosporus*, which originated from Indonesia and is a commonly available in Malaysia. *Tempeh* has been studied mostly for its antioxidant properties (Chang et al. 2009). The relationship between antioxidant and neuroprotection have been documented, however the neuroprotective potential with specific mechanisms of *tempeh* is rather scarce. In our previous studies we found tempeh to reverse scopolamine-induced amnesia, improve cholinergic activities and reduce neuroinflammaton in rats (Ahmad et al. 2014). In a more recent study we indicated that tempeh exhibited β-sectretase activity *in vitro* through bioactive aglycones (Ahmad et al. 2015). The present study is therefore, aimed to evaluate and compare the memory enhancing effects and neuroprotective potential of soybean and *tempeh* extracts in normal rats.

Materials and Methods

Soybean and Tempeh Extract Preparation

Soybeans (1 kg) were soaked overnight in tap water and the beans were dehulled followed by soaking for another 24 h. The dehulled soaked soybeans were boiled for 30 min. The beans were then cooled and the surface dried and either directly frozen at -80°C (500 g as a control without fermentation) or fermented with *Rhizophus* sp. after mixing with the fungus, the beans were incubated in air tight plastics at 28°C for 3 days. The fermented soybean cake, *tempeh* was then cut into small pieces and frozen at -80°C. After two days, both soybean and *tempeh* were lyophilised. The sample was coarsely grinded before being stored in air tight condition.

The extraction method was based on a previous standard protocol (Wei et al. 2004) but with slight modifications (Ahmad et al. 2015, 2014). 500 g of freeze-dried soybean and *tempeh* were weighed into separate conical flasks. 5 L of 80% methanol was added into the flasks. The flasks were shaken at 60°C for an hour. The solutions were centrifuged at 3800 g for 10 min. The upper layer of the solution was collected and evaporated to dryness. The dried material was dissolved in 2.5 L of 50% methanol and extracted 3 times with 2.5 L n-hexane. The methanol phase was concentrated and freeze-dried for storage. The collected extracts were standardized by the presence of major glycosides (daidzin and genistin) and aglycones (daidzein and genistein) using HPLC analysis (Ahmad et al. 2015).

Vehicles Used for Drug and Soybean or *Tempeh* Extracts

Piracetam (400 mg/kg) was diluted in normal saline. The freeze-dried soybean and *tempeh* extracts were suspended separately in 0.5% (w/v) carboxy methyl cellulose sodium (CMC) to concentrations of 10, 20 and 40 mg/kg. The extracts and piracetam were administered orally.

Experimental Details

All the experiments were carried out using male, Sprague-Dawley rats, which were purchased from the animal house at 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 (LAFAM), Faculty of Pharmacy, UiTM Puncak Alam, Selangor, Malaysia, of which the temperature and light were controlled at 26°C and a 12 h light cycle starting from 0700 to 1900. The animals had free access to standard laboratory food and water *ad libitum*. The Research Committee on the Ethical Use in Research (UiTM Care) Universiti Teknologi MARA, 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).

Acute Toxicity Studies

Acute toxicity studies were performed according to the Organization for economic co-operation and development (OECD423) guidelines (Ecobichon 1997). The animals were employed by random sampling technique. The animals were fasted for 4 h with access to water only. Soybean and *tempeh* extracts 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, only higher (50, 300 & 2000 mg/kg) doses of soybean and *tempeh* extracts were employed for further toxicity studies.

DRUG ADMINISTRATION

The rats were divided into 16 groups (6 animals per group). Control group was administered with normal saline while piracetam (400 mg/kg) acted as the reference drug group. Soybean (S10, S20 and S40) and *tempeh* (T10, T20 and T40) extract groups were administered orally at three concentrations (10, 20 and 40 mg/kg). Different sets of animals were used for radial arm maze (RAM) and elevated plus-maze (EPM) tasks.

RADIAL ARM MAZE

The radial arm maze was designed to measure spatial and learning memory of rats. It consists of eight arms made from black Plexiglas, numbered from 1 to 8 (48 × 12 cm), extending radially from a central area (32 cm in diameter), 50 cm above the floor and was surrounded by an extra maze (outside) visual cues placed on the same location during the study. The timeline during treatment is illustrated in Figure 1. Initially, the rats were acclimatized for 5 days to the laboratory environment. Since RAM is a food motivational task, prior to training, all rats were put on a restriction diet for 7 days (from 6th day) to maintain their weight at 85% of their free-feeding period (Foyet et al. 2011). On average, only 12 g of food pellet was given to each animal within the diet restriction period with water ad libitum. Every day, food was only given from 0900 to 1100 h and the animals were weighed. The treatment started after 7 days (from 13th day) of diet restriction. On the 15th day, each animal was exposed to 5 min training for 5 consecutive days (15 to 19th day) before the actual assessment began. The training was to adapt animals to the maze so they can explore, learn and consume food freely. The shaping of food location was important for the evaluation of animal learning and memory. On the first day, food was scattered all over the maze (Boast et al. 2000). On the second day, food was only available in all arms of the maze. On the third day, the food was available with the baited arms (1, 2, 4, 5 & 7) at the entrance of the arm. For the fourth and fifth day, food was only available at half way of the arm and finally at the end of the arm, in the cup, respectively. Along with training, the animals were given oral administration of extracts (S or T), control and standard drug. After 90 min of oral administration each animal was placed individually in the center of the maze. For memory assessment, the parameters were assessed for eight consecutive days from the 20th day of the experiment. Criterion performance was taken and it was defined as consumption of all 5 baits or until 5 min had elapsed. An arm entry was counted when all four limbs of the rat were within an arm. The parameters taken were working memory error (entering an arm containing food, but previously entered), reference memory error (entering an arm that was not baited) and time taken to consume all 5 baits (Foyet et al. 2011).

ELEVATED PLUS MAZE

The elevated plus maze for rat consists of two open (50 × 10 cm) and two enclosed (50 × 40 × 10 cm) arms. With the arms extended from the central platform (10 × 10 cm) and the maze is elevated 50 cm from the floor. The procedure used was as described by previous study (Mani et al. 2012). On the first day (14th day of treatment), each rat was placed at the end of the open arm, away from the central platform after an hour of oral administration according to the groups. Time taken for the rat to move from the open arm to either one closed arm was monitored. The duration is known as transfer latency (TL), with 90 s maximum time allowed for each animal in each trial. If the animal did not move within 90 s, the animal was gently pushed into the closed arm. The animal was allowed to explore the maze for another 2 min before returning to the cage. Retention of this learned-task memory was measured after 24 h of the first day trial (15th day, 24 h after the first dose). The reduction in TL of the retention indicated improvement in memory. The timeline for this task is illustrated in Figure 2.

COLLECTION OF BRAIN SAMPLES

At the end of the elevated plus maze experiment, animals were sacrificed by cervical decapitation under light

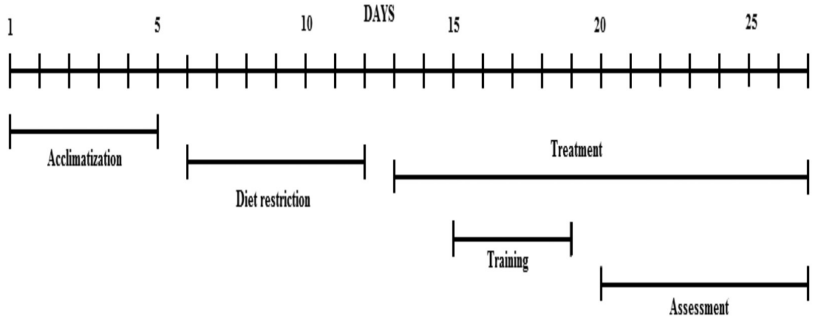


FIGURE 1. Experimental scheme for radial arm maze task

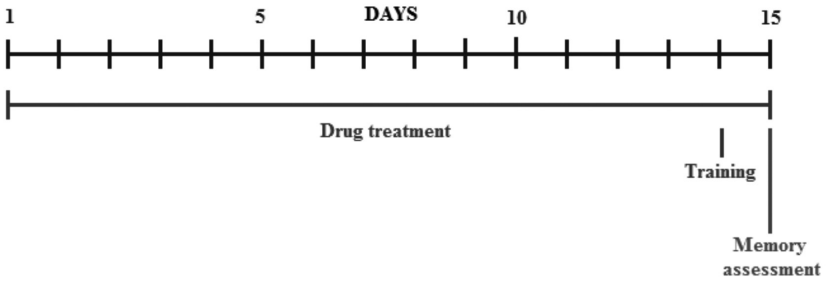


FIGURE 2. Experimental scheme for elevated plus maze

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 an ice bath after adding 10 volumes of normal saline solution. The homogenate was centrifuged at 3000 rpm for 10 min and the resultant cloudy supernatant liquid was used for estimation of brain acetylcholine, acetylcholinesterase and cytokines measurements.

ACETYLCHOLINE ASSAY

The brain acetylcholine level was measured in a 96 well plate reader, following the procedure of EnzyChrom assay kit (USA). The method was based on the hydrolysis of acetylcholine by acetylcholinesterase to choline, which is oxidized by choline oxidase to betaine and H_2O_2. The resulting H_2O_2 reacts with a specific dye to form a pink colored product. The colour intensity at 570 nm or fluorescence intensity (530/585 nm) is directly proportionate to the acetylcholine concentration in the sample (Vizi et al. 1985).

ACETYLCHOLINESTERASE ASSAY

The assay of acetylcholinesterase was based on an improved Ellman method on a 96 well plate reader using a QuantiChrome assay kit (USA) (Kovarik et al. 2003). The procedure is based on production of thiocholine by the action of acetylcholinesterase, which forms yellow color with 5,5'-dithiobis(2-nitrobenzoic acid). The intensity of the product colour, was measured at 412 nm and is proportionate to the enzyme activity in the sample.

INTERLEUKIN-1β (IL-1β) AND INTERLEUKIN-10 (IL-10) MEASUREMENT

The higher doses of both extracts (S40 and T40) were selected for cytokine measurements. It was performed by using Procarta® Immunoassay kit – Polystyrene beads from Affymetrix that combined with quantitative, multiplexed immunoassays based on the Luminex® technology. The kit was specifically designed with two interest cytokines, which were IL-1β and IL-10. Multiple-analyte profiling beads were used for the detection and quantification of the interest target protein in brain tissue simultaneously. The concentration of protein in the sample was calculated by plotting the expected concentration of the standards against the Multiplex Fluorescent Immunoassay (MFI) generated by each standard.

STATISTICAL ANALYSIS

All the results were expressed as mean ± Standard Error (SEM). Data was analyzed using one-way ANOVA followed by Dunnett's t-test test. The results were considered as statistically significant at $p<0.05$.

RESULTS

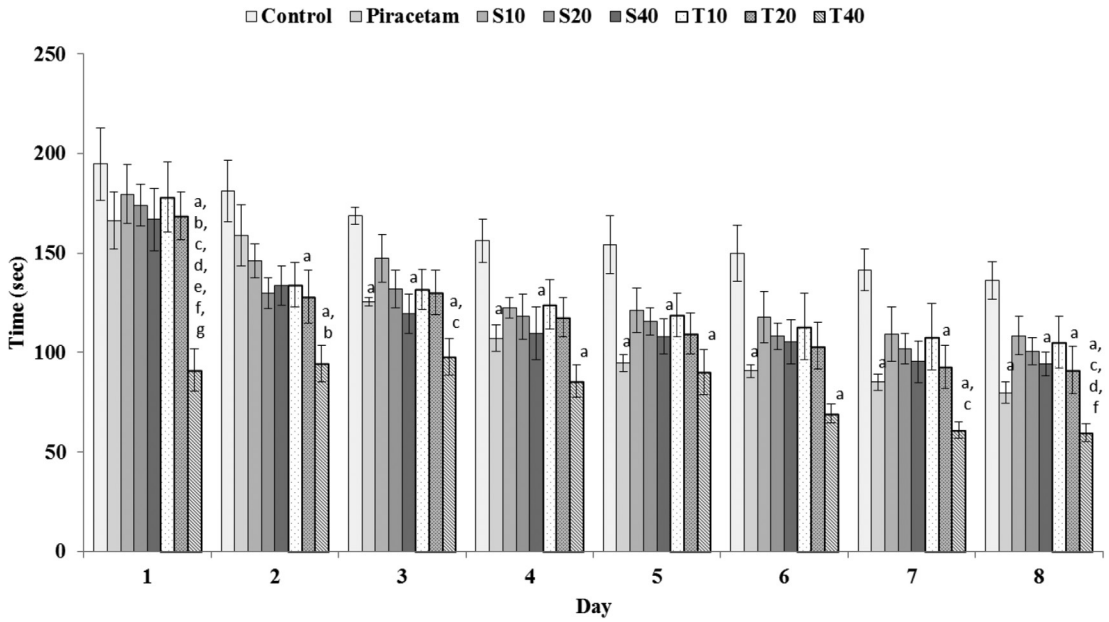
ACUTE TOXICITY STUDY

Since there was no sign of mortality when the animals were orally treated with 300 mg/kg of soybean and *tempeh* extracts, three doses were selected (10, 20 and 40 mg/kg) to be used in this experiment.

RADIAL ARM MAZE

Using radial arm maze, three parameters were observed; time taken to consume all baits, working memory errors and reference memory errors. These parameters represent the learning ability and memory capacity of the test animals.

On the first day of the memory assessment, only T40 showed a significant reduction ($p<0.05$) as compared to control, while other groups (S10, S20, S40, T10 & T20) including the standard drug showed no significant reduction in the time to finish all baits (Figure 3). However, a pattern of time reduction was observed in all groups within eight days of assessment. This same pattern was observed in the saline treated control group. For the piracetam group, significant reductions ($p<0.05$) were observed from day 3 onwards when compared to control. In soybean treated groups, only the S40 group showed significant ($p<0.05$) reduction on days 3, 4, 5 and 8 when compared to control group. These results indicated that higher concentrations of soybean extract (S40) exerted a better effect in memory improvement than lower concentrations (S10 and S20). The same pattern was also observed in the *tempeh* extracted groups. The



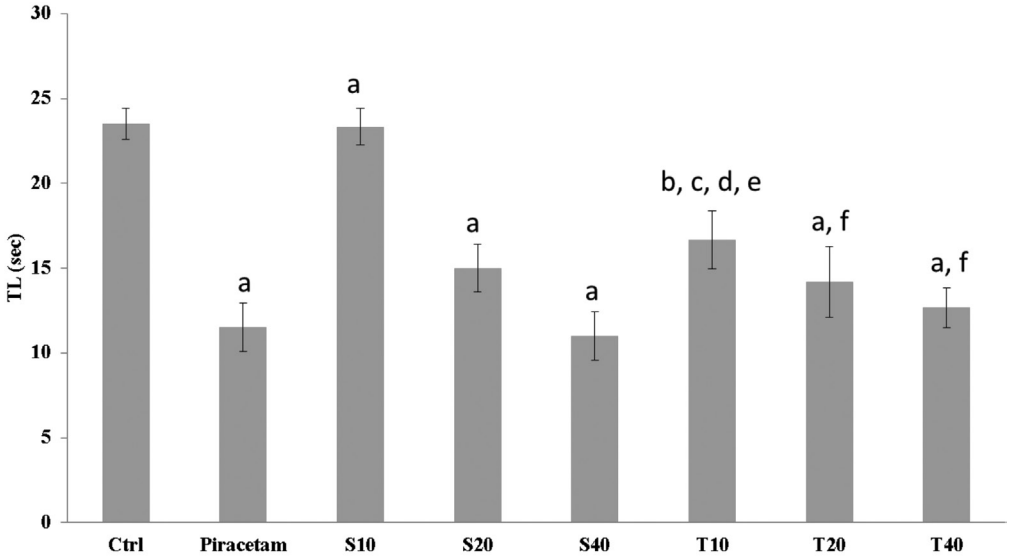
[a] significantly difference (p<0.05) as compared with control of respective day
[b] significantly difference (p<0.05) as compared with piracetam of respective day
[c] significantly difference (p<0.05) as compared with S10 of respective day
[d] significantly difference (p<0.05) as compared with S20 of respective day
[e] significantly difference (p<0.05) as compared with S40 of respective day
[f] significantly difference (p<0.05) as compared with T10 of respective day
[g] significantly difference (p<0.05) as compared with T20 of respective day

FIGURE 3. Effect of soybean (10, 20 and 40 mg/kg) and *tempeh* (10, 20 and 40 mg/kg) extracts administered orally for 15 days on time taken to consume all five baits during eight days of memory assessment in radial arm maze

high concentration of extract T40 exerted lesser time (p<0.05) to consume all baits from day 1 to day 8 when compared to control. The lower concentration of *tempeh* extract T20 only showed significant reductions (p<0.05) in the time taken to consume all five baits on days 7 and 8. The results also indicate that there was no significant difference between T40, S40 and piracetam after the third day onwards.

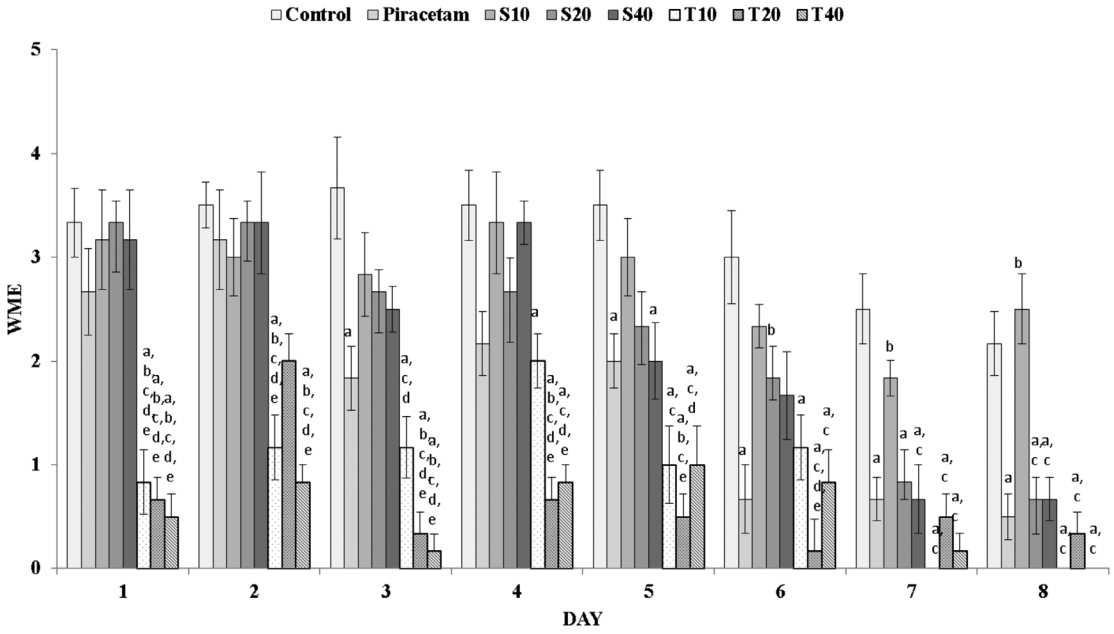
Short term memory enhancing effect was measured by a reduction of the number of working memory errors (WME). In piracetam group, reductions (p<0.01) in WME were observed from day 3 onwards (Figure 4). For the soybean extract group, no significant difference was observed in the lowest S10 treated group. In S20 group, significant reductions (p<0.05) when compared with the control were observed only towards the end, on days 7 and 8. The S40 group showed a significant reduction (p<0.05) at all days with no error observed at the final day when compared to control. In *tempeh* treated group, T10 showed a significant reduction (p<0.05) when compared to control group throughout the test period with no errors on the final day. The T20 group when compared to the control, exhibited significant reductions (p<0.05) throughout the experimental days except day 2. There were no significant differences in the WME among the soybean treated groups (S10, S20 and S40) except on day 8 where S20 and S40 showed significantly (p<0.05) lesser errors than S10. There were no significant differences between the tempeh treated groups on WME. Only T20 and T40 exhibited significantly (p<0.05) lesser errors than the S10 group, whilst T20 and T40 groups, were significantly better (p<0.05) than piracetam and the S40 groups in the first few days of the assessment.

Significant decrease in reference memory error (RME) indicates improvement in memory. In Figure 5, the control group showed the same RME pattern throughout the experimental period. The piracetam group showed significant (p<0.05) reductions in RME on days 6 to 8 when compared to the control. In the soybean treated group, no significant reductions were observed in S10 and S20 throughout the experiment but S40 group was better (p<0.01) than control on the final day. In the *tempeh* treated group, no significant (p<0.01) reduction was observed until day 8 in groups T10 and T20 and days 7 and 8 in group T40 when compared to control. The S40 group was significantly (p<0.05) improved when compared to S10 only on days 7 and 8, otherwise there was no significant differences among the soybean treated groups. Similar results were found among the *tempeh* treated groups only T40 was significantly (p<0.05) better on day 6 when compared to T10. The T40 group reduced the RME significantly (p<0.05) when compared with S10 and S20 at days 7 and 8. However there were no significant differences between *tempeh* and piracetam even at the highest dose, T40.



[a] significantly difference (p<0.05) as compared with control of respective day
[b] significantly difference (p<0.05) as compared with piracetam of respective day
[c] significantly difference (p<0.05) as compared with S10 of respective day
[d] significantly difference (p<0.05) as compared with S20 of respective day
[e] significantly difference (p<0.05) as compared with S40 of respective day

FIGURE 4. Effect of soybean (10, 20 and 40 mg/kg) and *tempeh* (10, 20 and 40 mg/kg) extracts administered orally for 15 days on the number of working memory errors during eight days of memory assessment in radial arm maze task



[a] significantly difference (p<0.05) as compared with control of respective day
[b] significantly difference (p<0.05) as compared with piracetam of respective day
[c] significantly difference (p<0.05) as compared with S10 of respective day
[d] significantly difference (p<0.05) as compared with S20 of respective day
[e] significantly difference (p<0.05) as compared with S40 of respective day
[f] significantly difference (p<0.05) as compared with T10 of respective day

FIGURE 5. Effect of soybean (10, 20 and 40 mg/kg) and *tempeh* (10, 20 and 40 mg/kg) extracts administered orally for 15 days on the number of reference memory errors during eight days of memory assessment in radial arm maze task

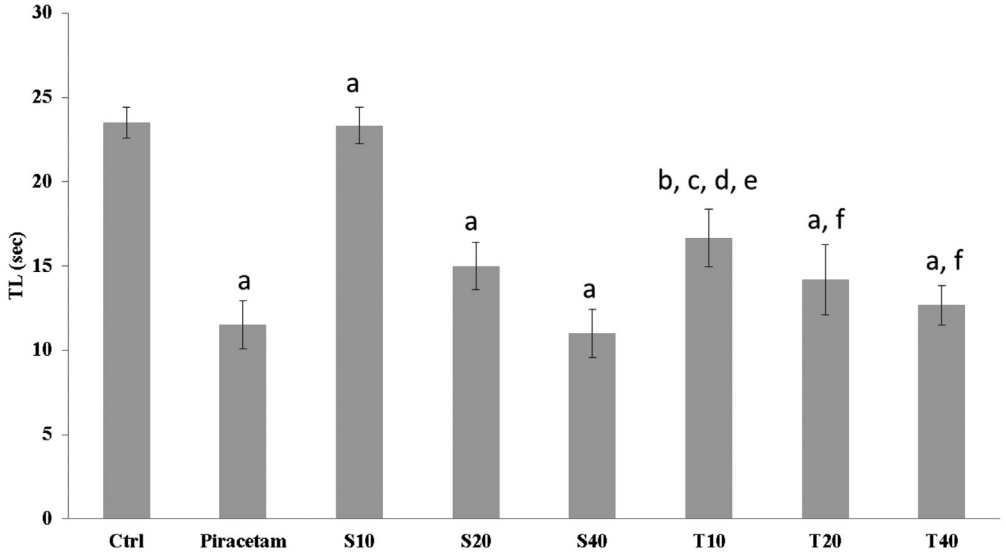
ELEVATED PLUS MAZE

A significant reduction in TL values (on 16th day) indicated improvement in memory (Boast et al. 2000) as shown in Figure 6. All the treatments including piracetam showed significant (p<0.05) reduction of TL as compared to the control group except T10. In fact T10 showed significantly

(p<0.05) poorer memory when compared to all the other treatments. No significant differences were found among the soybean treated groups S10, S20 or S40. There was also no significant differences between piracetam, T40 and S40.

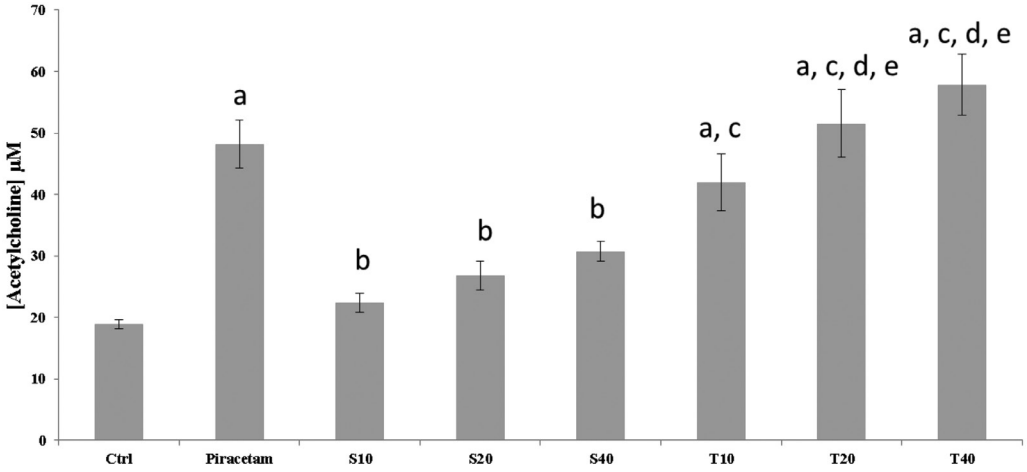
ACETYLCHOLINE LEVEL

The level of brain acetylcholine is shown in Figure 7. Piracetam (48.2 μM) the reference drug showed significant (p<0.05) increase of acetylcholine level when compared to control (18.88 μM). *Tempeh* extract treated groups also showed significant improvements (p<0.05) with concentrations at 42.0, 51.5 and 57.8 μM for doses of T10, T20 and T40, respectively. The soybean extract treated groups did not show any significant differences with the control group. Based on the results achieved, *tempeh* extract showed similar acetylcholine levels as compared to piracetam.



[a] significantly difference (p<0.05) as compared with control
[b] significantly difference (p<0.05) as compared with piracetam
[c] significantly difference (p<0.05) as compared with S10
[d] significantly difference (p<0.05) as compared with S20
[e] significantly difference (p<0.05) as compared with S40
[f] significantly difference (p<0.05) as compared with T10

FIGURE 6. Effect of soybean (10, 20 and 40 mg/kg) and *tempeh* (10, 20 and 40 mg/kg) extracts administered orally for 15 days on the transfer latency in elevated plus maze task



[a] significantly difference (p<0.05) as compared with control
[b] significantly difference (p<0.05) as compared with piracetam
[c] significantly difference (p<0.05) as compared with S10
[d] significantly difference (p<0.05) as compared with S20
[e] significantly difference (p<0.05) as compared with S40

FIGURE 7. Effect of soybean (10, 20 and 40 mg/kg) and *tempeh* (10, 20 and 40 mg/kg) extracts administered orally for 15 days on the level of brain acetylcholine levels

ACETYLCHOLINESTERASE ACTIVITY

The effect of soybean and tempeh extracts on the acetylcholinesterase activity is shown in Figure 8. The reference drug, piracetam (51.2 U/L) showed a reduction ($p<0.05$) in acetylcholinesterase activity as compared to control (122.8 U/L). Significant reductions ($p<0.05$) were also noticed in tempeh extract treated groups at concentrations of T20 (76.61 U/L) and T40 (57.61 U/L). Soybean extracts (S10, S20 and S40) however did not show any significant differences with the control and the acethylcholinesterase activity was much higher ($p<0.05$) than piracetam. In general T20 and T40 groups reduced acethycholinesterase similar to that of piracetam.

IL-1β AND IL-10 MEASUREMENT

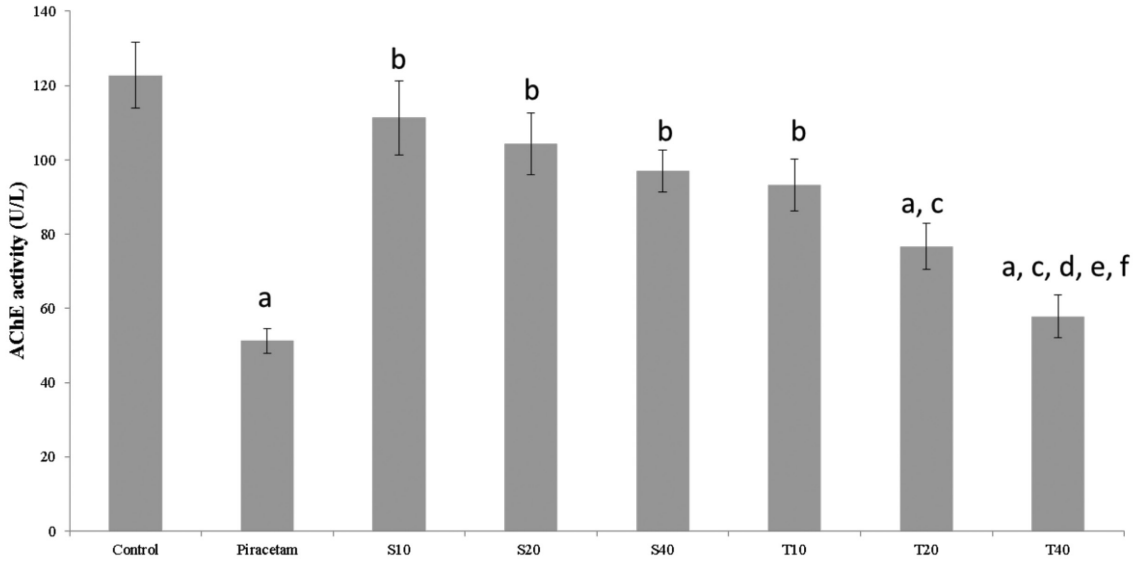
The effect of soybean and *tempeh* extracts (40 mg/kg) on the IL-1β level within the brain is shown in Figure 9. The results showed that piracetam (1.3 pg/mL) decreased the level of IL-1β significantly ($p<0.05$) as compared to control (3.9 pg/mL). The levels of IL-1β were reduced significantly ($p<0.05$) in S40 (2.25 pg/mL) and T40 (0.38 pg/mL) groups as compared to control. The results showed that T40 was able to reduce the IL-1β better than S40 and piracetam.

IL-10 within the brain homogenate of the piracetam and the tempeh T40 groups were significantly ($p<0.05$) increased up to 14.15 pg/mL and 11.12 pg/mL respectively as compared to control group (6.24 pg/mL) (Figure 10). No significant changes were observed in the S40 group.

DISCUSSION

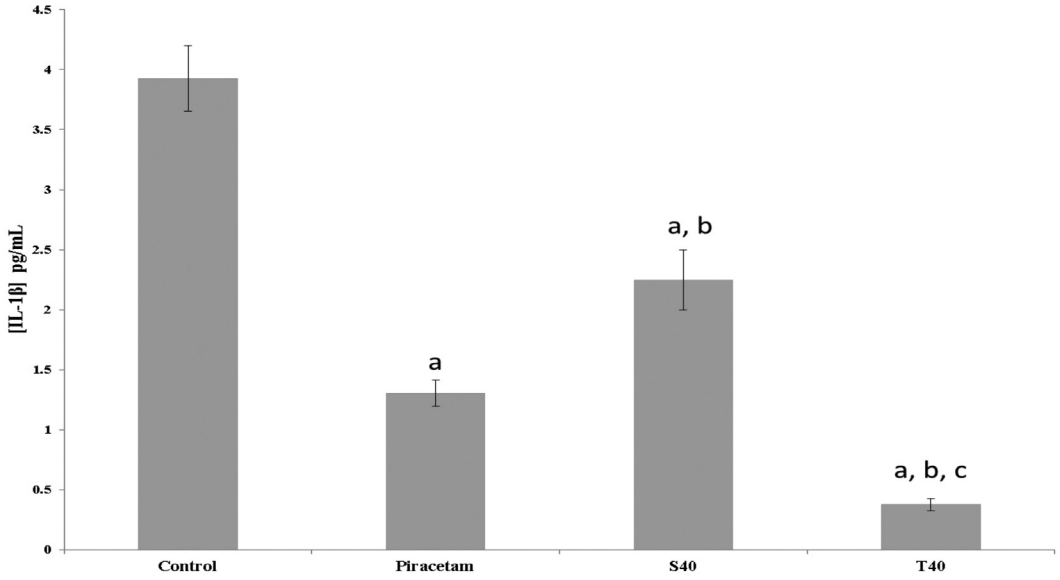
Learning is an adaptive behavior influenced by experience while memory is the neuronal activity of experience storage (Kovarik et al. 2003). Animal and human survive through the ability of learning and memory, which take part in the brain. Working memory is involved in temporarily maintaining cues of previously experienced events or it can be called short term memory. Reference memory means the performance based on well-learned responses in the presence of current stimulus or unmoved cues along the study. Reference memory can also be referred to as long term memory. Memory loss is one of the important features that advance with aging. However, memory loss is more common in neurodegenerative disease or due to injury to the brain. In the present study, we examined the effect of extracts from soybean and the fermented product *tempeh* on memory using radial arm maze and elevated plus maze. The results of these behavioral studies indicated that both the extracts have the ability to improve the cognitive performance in both the models. Both the extracts facilitated cholinergic activities and ameliorated the neuronal inflammation processes.

Radial arm maze (RAM) was used to evaluate the effects of soybean and *tempeh* extracts on the spatial learning and memory of the animals. In RAM, three parameters were chosen; the time taken to consume all baits, reference memory error (RME) and working memory error (WME). Reference memory and working memory are the two variables that report the physiological status of the



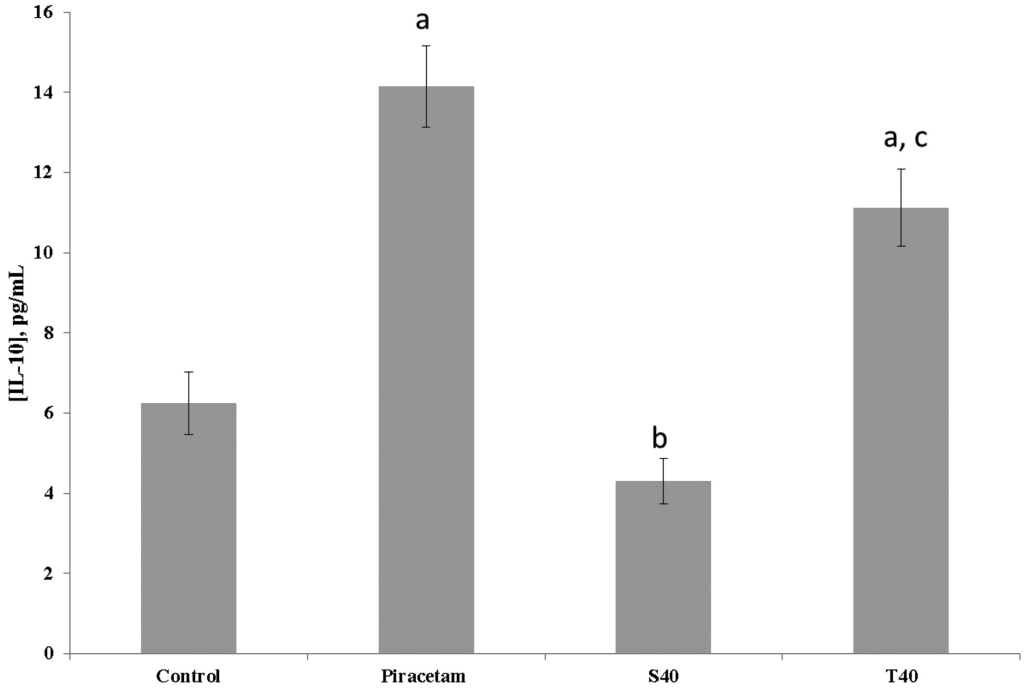
[a] significantly difference ($p<0.05$) as compared with control
[b] significantly difference ($p<0.05$) as compared with piracetam
[c] significantly difference ($p<0.05$) as compared with S10
[d] significantly difference ($p<0.05$) as compared with S20
[e] significantly difference ($p<0.05$) as compared with S40
[f] significantly difference ($p<0.05$) as compared with T10

FIGURE 8. Effect of soybean (10, 20 and 40 mg/kg) and *tempeh* (10, 20 and 40 mg/kg) extracts administered orally for 15 days on the activity of brain acetylcholinesterase activities



[a] significantly difference (p<0.05) as compared with control
[b] significantly difference (p<0.05) as compared with piracetam
[c] significantly difference (p<0.05) as compared with S40

FIGURE 9. Effect of soybean (40 mg/kg) and *tempeh* (40 mg/kg) extracts administered orally for 15 days on brain IL-1β levels. Values are in mean ± SEM (n=6)



[a] significantly difference (p<0.05) as compared with control
[b] significantly difference (p<0.05) as compared with piracetam
[c] significantly difference (p<0.05) as compared with S40

FIGURE 10. Effect of soybean (40 mg/kg) and *tempeh* (40 mg/kg) extracts administered orally for 15 days on brain IL-10 levels. Values are in mean ± SEM (n=6)

brain (Titus et al. 2007). The results showed that the time taken for the animal to consume all baits reduced during the eight days of assessment among the various groups. The reduction in time suggests the ability of the rats to recognize the maze and external cues based on experience. Short term memory improvement exerted by the soybean and *tempeh* extracts was represented by the significant reduction of WME in animal models. The improvement

of long term memory was represented by significant reduction in RME. The results obtained in the present study showed that in general *tempeh* extract improved memory better than soybean extract in the first few days of the assessment. Our previous results indicated that the amount of the most bioavailable aglycones such as daidzein and genistein was more abundant in the *tempeh* extract as compared to soybean extract (Ahmad et al. 2015). The higher aglycones in the *tempeh* extract may possibly enhance memory better than soybean extract. Reduction of transfer latency (TL) observed also indicates an improvement of memory in both soybean and *tempeh* extracts in the treated groups.

Cholinergic neurons of the brain play a vital role in the cognitive deficits related to aging and neurodegenerative diseases. In the brain of senile dementia of the Alzheimer's type, the common features observed included selective loss of cholinergic neurons, decrease in acetylcholine level and an increase of acetylcholinesterase activity (Auld et al. 2002). According to the cholinergic hypothesis, memory impairments in patients with senile dementia are due to a selective and irreversible deficiency of the brain cholinergic functions (Overk et al. 2010). Acetylcholinesterase involves the regulation of acetylcholine to proper levels. However, excessive acetylcholinesterase activity leads to constant acetylcholine deficiency and cognitive impairments (Pepeu & Giovannini 2010). According to the study, soybean isoflavones can influence the brain cholinergic system and cognitive function, by increasing the level of acetylcholine through the inhibition of acetylcholinesterase (Yang et al. 2011). In the present study, the *tempeh* extract down-regulated acetylcholinesterase activity and up-regulated the acetylcholine level even better than the soybean extract. The reduction of the brain acetylcholinesterase activities observed using both *tempeh* and soybean extracts may facilitate the improvement of memory in treated animals. Since the cholinergic system plays a vital role in the process of learning and memory, the inhibition of acetylcholinesterase is often targeted as therapeutics in AD.

Interleukin-1 (IL-1) is a pluripotent immunomodulatory cytokine that has an initiating role in cellular and humoral immunity in the periphery. IL-1α and IL-1β are partially homologous isoforms of IL-1 and both have a quite similar tertiary structure. The physiological function of interleukin-1 beta (IL-1β) has been known to initiate the immune response by playing a vital role in the trigger and the development of a complex hormonal and cellular inflammatory cascade (Rubio-Perez & Morillas-Ruiz 2012). It is detected in brain within a few hours after brain injury (Winter et al. 2002). Moreover, it was also believed that IL-1β would promote the progression of neurodegeneration. Nonetheless, previous study suggested that the IL-1β was high in the brain of AD transgenic mice (Patel et al. 2005). The IL-1β also relates to the pathology of AD through inducible NO production and a decline in the cholinergic functions through the increase of acetylcholinesterase levels, which in turn leads to the progressive neurodegeneration. The results from this study showed that the reduction of IL-1β levels by both extracts also correlated to the lower levels of acetylcholinesterase activities in this study. The reduction of IL-1β levels further suggests that these extracts have anti-inflammatory properties within the brain. The results were consistent with that obtained by a previous investigator (Danciu et al. 2012), of which soy isoflavones exhibited the ability to act as an anti-inflammatory agent. The present study shows that fermented soybean *tempeh* showed better results as a functional food as compared to soybean.

The IL-1β is a promoter to inflammatory activity, however, IL-10 is said to be a potent anti-inflammatory cytokine. This anti-inflammatory mediator is able to suppress the production of IL-1β, TNF and other chemokines that are pro-inflammatory mediators. In AD, it is hypothesised that Aβ plaques and tangles trigger a chronic inflammatory reaction to clear this debris (Town et al. 2005). Therefore, IL-10 might be crucial in dealing with brain injury due to inflammation. A clinical study found that various IL-10 polymorphisms may lower the risk of AD (Lio et al. 2003). In the present study, the level of IL-10 in the *tempeh* extract treated group improved, but no significant improvements were observed in the groups treated with soybean extract at the same dose levels. Improvement of soybean aglycones during the fermentation process might (Ahmad et al. 2015) play a crucial role in the elevation of anti-inflammatory cytokine IL-10 levels in the *tempeh* extract treated groups.

Conclusion

In the present study, using a radial arm maze and elevated plus maze we observed that both soybean and *tempeh* extracts, improved memory. Both extracts facilitated the central cholinergic activity by elevating the acetylcholine levels and inhibiting the cholinesterase enzyme activity in the brain. The levels of proinflammatory cytokine IL-1β was attenuated by both extracts but with *tempeh* being significantly better than the soybean extract. In fact, the anti-inflammatory cytokine IL-10 was elevated by *tempeh* extract only. The overall results highlighted that the *tempeh* extract exhibited better effects in most of the tested parameters as compared to soybean extract at the same dose levels and might be useful in the management as well as prevention of dementia and Alzheimer's disease.

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