



Comparative Evaluation of Antioxidant Properties and Isoflavones of Tempeh Fermented in Two Different Wrapping Materials

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Abstract

Tempeh is an Indonesian traditional fermented soybean food that is well known for its nutritional value and health benefit. This study aimed to evaluate the effects of two commonly used packaging materials in tempeh production, namely banana leaf and polyethylene bag, on the antioxidant properties. Isoflavones, include daidzein and genistein, of tempeh fermented in banana leaf (TBL) and polyethylene bag (TP) were analysed with high-performance liquid chromatography. Total phenolic content and antioxidant activities were evaluated using Folin-Ciocalteu reagent, DPPH radical scavenging and β -carotene-linoleate bleaching assays, respectively. The changes of antioxidant properties of tempeh after different fermentation periods in both wrapping materials were observed, on day 0 (unfermented), day 1, day 2 and day 4. Total phenolic content, daidzein, genistein and the antioxidant activities were significantly ($p < 0.05$) increased in both TBL and TP as the fermentation progressed. A higher total phenolic content was found in TBL on day 1 and day 2 but TP exhibited the highest total phenolic content on day 4. Daidzein was the highest in TBL on day 2 but it was reduced on day 4 while genistein was the highest in TP on day 4. Antioxidant activities were positively ($r = 0.272-0.969$, $p < 0.05$) correlated to total phenolic content, daidzein and genistein. Although tempeh wrapped in banana leaf is a traditional way for tempeh packaging, it provided better antioxidant properties on day 1 and day 2, which is within the best period of tempeh consumption. Hence, the current findings provided useful information to manufacturer and consumer in selecting the suitable wrapping material for tempeh with optimal phytonutrients.



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Introduction

Tempeh is a traditional Indonesian fermented soybean food that has been consumed for thousands of years particularly in the Asian countries^{1,2}. In Japan, such fermented soybean is called 'natto', while in China it is called 'douchi', and 'chung-kook-jong' in Korea³. The production of tempeh regardless of where it is produced involves these common steps; the raw soybeans are soaked in water to remove the skin and then cook before they are inoculated with *Rizophus oligosporus* (fungus). Once the fermentation has activated, the soybeans are packed into a compact and solid mass. The fermentation will later result in a product with a nutty flavour and a texture similar to a chewy mushroom¹.

In recent years, the consumption of tempeh has gained popularity among the Western countries for its nutritional values and metabolic regulatory functions⁴. This soybean product is not only natural and safe for consumption but also has shown by epidemiological studies to have various physiological activities³. Hence, this has prompted many ongoing research on tempeh especially its antioxidant properties. The antioxidative effect of tempeh is mainly due to the presence of polyphenolic compounds⁵. Extensive studies have been done on the role of fermented soybean food products in the prevention of chronic diseases². Kwon *et al.*, have presented many supporting scientific reports on the role of soy foods as preventive agents for insulin resistance and type 2 diabetes⁶. A study also reported that the supplementation of soy isoflavones to hypertensive rats had improved the systolic blood pressure and reduced the oxidative stress⁷.

Nonetheless, the scientific evidence still appears to be limited as most of them are derived from *in vitro* and animal studies. The main drawback when investigating the health effects of polyphenols is the existence of mixture compounds found in the food sample and thus yielding different biological activities⁸. Indeed, a continue study on the antioxidant properties of this available soybean product is important to provide a better view for scientist before conducting a human intervention. There are various studies that had covered on antioxidant capacity of tempeh and soybeans such as fermentation under

different conditions and periods⁹, different cultivars of soybean¹⁰ and different soybean parts¹¹. Traditionally, in Malaysia, the local people will utilise banana leaf as the wrapping material for tempeh. Polyethylene bag is then used in the production of tempeh due to their higher availability. However, limited study has been done on the effect of different wrapping materials on the antioxidant activity of tempeh. Thus, this study was prompted to evaluate the total phenolic content, isoflavones and antioxidant activities of tempeh wrapped in banana leaf and polyethylene bag as well as their levels after different fermentation periods. This study will provide information to the consumer and industry on the antioxidant properties of tempeh wrapping in different materials before reaching the consumer.

Materials and Methods

Chemicals and Reagents

Ascorbic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), β -carotene, linoleic acid, butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals namely Folin-Ciocalteu's phenol reagent (Merck, Darmstadt, Germany), sodium carbonate anhydrous, sodium bicarbonate (Fluka & Riedel-de Haën, Seelze, Germany), gallic acid purity at 98% (Acros Organics, Geel, Belgium), Tween 20, chloroform (Fisher Scientific, Loughborough, UK) and absolute ethanol (Scharlau Chemie S.A., Barcelona, Spain) were used.

Sample Collection and Preparation

Tempeh wrapped in banana leaf (TBL) and polyethylene bag (TP) were collected from the same manufacturer in Kajang, Selangor, Malaysia. The samples were collected at different fermentation periods as on day 1, day 2 and day 4 (Figure 1). Five packages of tempeh, approximately 100 g of each, were collected at the stated fermentation periods. Sample collected on day 0 (unfermented soybean) was used as a control. Each sample was cut and homogenised by a wet blending method. Subsequently, the tempeh paste was lyophilised and extracted with 70% ethanol for 1 h at 25°C. The extraction was assisted by an orbital shaker at 200 rpm (LM510, Yih Der Instrument Co. Ltd., Taipei, Taiwan). The solid-liquid ratio was 1:5. One gram of sample was added with 5 ml of 70% ethanol.

The mixture was then filtered and the residue was again subjected to the similar extraction procedure. The filtrate was pooled together and subjected to

lyophilisation. The tempeh extracts were kept at -30°C until further use.

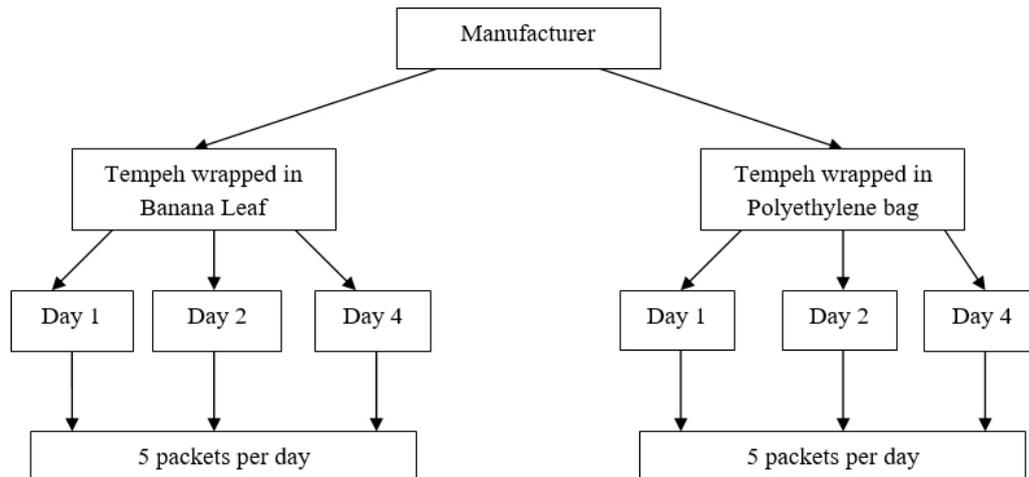


Fig. 1: Flowchart shows the sample collection of tempeh

Measurement of Total Phenolic Content

The appraisal of total phenolic content was done using Folin-Ciocalteu assay¹². Tempeh extract (200 μl) was added with 1.5 ml of Folin-Ciocalteu reagent (0.2 N). The mixture was vortexed for 1 min and let to react for 5 min at room temperature. After that, the reaction mixture was added with 1.5 ml of sodium bicarbonate solution (0.6 M) before subjected to vortexing. After allowed to react for 90 min, the absorbance was taken at 725 nm using a UV-visible spectrophotometer (UV-200-RS, LW Scientific, Georgia, USA). Gallic acid (0.04-0.15 mg/ml) was used to generate a standard calibration curve. Results were reported as mg gallic acid equivalent (GAE)/g of tempeh extract.

Determination of Daidzein and Genistein

The HPLC analysis was performed using an Agilent 1100 HPLC system consisted quaternary pump, autosampler and diode array detector (DAD). Daidzein and genistein were separated using extra-dense bonding (XDB) C18 column (250 mm x 4.6 mm x 5 μm ; Agilent, Walbronn, Germany). The mobile phase was 0.1% (v/v) acetic acid and methanol (52:48). The flowrate of the mobile phase was set at 1.0 ml/min. The temperature of the stationary phase was set at 30°C and the sample

was injected at 20 μl . The detection was performed at 254 nm. The analysis was done in triplicate. The chromatographic peaks of daidzein and genistein were analysed according to the retention time of the external standards. Standard calibration curves of daidzein ($y = 34.773x + 72.63$, $R^2=0.9978$) and genistein ($y = 76.24x + 125.08$, $R^2=0.9978$) were prepared at the range of 6.25-50 and 6.25-100 $\mu\text{g/ml}$, respectively. The results were reported as mg/g of tempeh extract.

Measurement of DPPH radical scavenging activity

The DPPH radical scavenging activity of the tempeh extract was determined as described by Othman *et al.*,¹³. Two hundred microlitres of tempeh extract (20-200 mg/ml) was added with 1 ml of DPPH solution (500 μM). The mixture was left to react in dark for 30 min. Following 30 min, the absorbance was read at 517 nm using a spectrophotometer. The analysis was done in triplicates and 70% ethanol was used as a blank. A control was prepared by replacing the sample with 200 μl of 70% ethanol. The percentage of DPPH scavenged was calculated according to the equation as follows;

$$\text{Scavenging effect (\%)} = 1 - \left[\frac{\text{Absorbance of sample or standard at 517 nm}}{\text{Absorbance of control at 517 nm}} \right] \times 100$$

A graph on scavenging effect against concentration was plotted and the EC_{50} of each tempeh extract was calculated. EC_{50} is the concentration of tempeh extract needed to inhibit 50% of DPPH radical.

Measurement of β -Carotene Bleaching Activity

The antioxidant activity of tempeh extract was determined by the method modified from Velioğlu *et al.*¹². In this assay, butylated hydroxytoluene (BHT) was used for comparison. One millilitre of β -carotene (0.2 mg/ml in chloroform) was aliquoted into a container which was pre-added with linoleic acid (0.02 ml) and Tween 20 (0.2 ml). Immediately, the mixture was dried at 40°C for 10 min under a vacuum evaporator (Büchi® Rotavapor, Flawil, Switzerland). After evaporation, 100 ml of ultra-pure water was added into the mixture and the mixture was shaken to produce emulsions. Then, the emulsion (5 ml) was aliquoted into each test tube with 0.2 ml of tempeh extract (100 mg/ml) or BHT (200 μ g/ml). The mixture was vortexed for a minute before incubating in the water bath at 45°C for 2 h. The absorbance at 470 nm was recorded at every 20 min interval for 120 min. All samples were determined in triplicates and 70% ethanol was used as blank. For control, the tempeh extract was replaced with 70% ethanol. Degradation rate (DR) of β -carotene was counted using the formula as shown;

$$DR_{\text{sample or standard}} = \ln(A_0/A_t) \times 1/t$$

\ln = natural log; A_0 = absorbance (470nm) at time 0; A_t = absorbance (470nm) at 20, 40, 60, 80, 100 or 120 min; t = time of absorbance reading. As for the antioxidant activity (AA), it was expressed as the percentage of inhibition comparative to the control. The following equation was used to calculate the antioxidant activity of the tempeh extract or standard;

$$AA(\%) = \left[\frac{DR_{\text{control}} - DR_{\text{sample or standard}}}{DR_{\text{control}}} \right] \times 100$$

Statistical Analysis

All results were reported as mean \pm standard deviation of triplicate. SPSS version 15 (SPSS Inc, Chicago, Illinois, USA) was employed for data analysis. Means among/between groups were compared using one-way analysis of variance (ANOVA) and independent t-test. Pearson correlation

analyses were applied to study the relationship between the variables. $P < 0.05$ was considered as significance.

Results and Discussion

Total Phenolic Content

In this study, the unfermented tempeh extract (day 0) had a lower ($p < 0.05$) total phenolic content (1.74 GAE mg/g of tempeh extract) as compared to their fermented counterparts (2.93 to 6.97 GAE in mg/g of tempeh extract) (Figure 2). The current findings were in agreement with previous study that also showed an increment in total phenolic content of soybean after fermentation. Juan and Chou reported that fermentation of black soybean at the solid state can improve the total phenolic content of the black soybean¹⁴. Another study had reported that isoflavone aglycones of Thai fermented soybean were higher than the unfermented one, but isoflavone aglycones were decreased at a longer fermentation period¹⁵. Hence, fermentation may cause the liberation of aglycones from its complexes via hydrolysis but this may expose the compounds to degradation when the time is prolonged¹⁶. Isoflavones are the derivatives of flavonoids which is one of the subgroups of polyphenols. Thus, total phenolic content of tempeh extract was higher than the initial unfermented tempeh extract.

The increments of total phenolic content were not significant ($p > 0.05$) in both TBL and TP on day 1 and day 2. On day 4 of fermentation, there was a rapid increment ($p < 0.05$) of total phenolic content in TP. Total phenolic content in TP on day 4 was about 6.97 GAE in mg/g of tempeh extract which was almost double of the total phenolic content of TBL on day 4, about 3.46 GAE in mg/g of tempeh extract. A study reported that black bean koji fermented by *Aspergillus awamori* also contained a higher total phenolic content as the fermentation period increased and reached its highest total phenolic content on day 4 of fermentation, but total phenolic content declined in the following days¹⁷. However, the extension of fermentation period was not done in the current study as it is not suitable for human consumption.

To the best of our knowledge, limited studies have been done on the effect of different wrapping materials on total phenolic content of fermented

soybean as well as their isoflavones compounds. Current results suggested that tempeh wrapped in polyethylene bag could enhance the polyphenolic components to release through the fermentation process. Cho *et al.*, suggested that isoflavones, flavanols and phenolic acids in the Korean whole soybean fermented food 'cheonggukjang' were augmented with the arising of enzymes activities like β -glycosidase and esterase, and later contributed to the remarkable increased in total phenolic content of the fermented soybean¹⁶. Additionally, aglycones are easy to be absorbed by enterocytes in human

small intestine as compared to their glycosides¹⁸. Despite the supposing increase in its nutritional values, tempeh wrapped in polyethylene bag was not in favour to be edible as it reached day 4 of fermentation. According to Liu, tempeh is commonly taken not more than 2 days after the fermentation started due to the off flavour, slimy texture, growth of contaminated bacteria and ammoniacal odour¹⁹. However, it will be useful for the food industry if the fermentation of the tempeh is purposely conducted for their bioactive components to produce functional products.

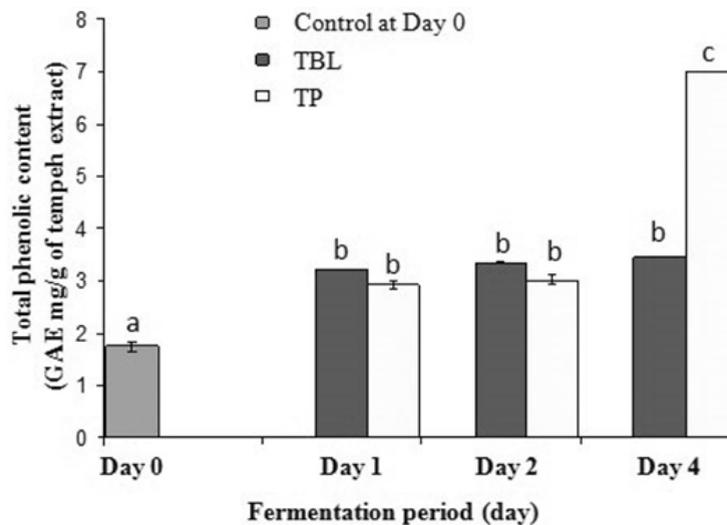


Fig. 2: Total phenolic content of the extracts of tempeh wrapped with banana leaf (TBL) and polyethylene bag (TP) under different fermentation periods. Results are mean \pm standard deviation. Data labelled with different characters are significantly different at $p < 0.05$

Daidzein and Genistein Contents

Separation and quantification of daidzein and genistein in the tempeh extracts were further done. Daidzein content was increased in both type of samples after fermentation, but TBL reached its highest daidzein content on day 2 (1.34 mg/g of tempeh extract) and started to decrease on day 4 (Figure 3). However, TP gave a consistent increment in daidzein content as the fermentation period increased and reached the highest daidzein content on day 4. The highest daidzein content was obtained in TBL on day 2 as compared to TP on day 4. However, the difference of daidzein content between TBL and TP was not significant ($p > 0.05$) on day 1.

A similar trend was observed in genistein content. It was found to be increased gradually from day 0 to day 1 for TBL and reached the highest level on day 2, which was 0.94 mg/g of tempeh extract (Figure 4). However, TBL on day 4 showed a slightly lower ($p < 0.05$) genistein content as compared to day 2. TP exhibited the highest genistein content on day 4, which is 96 h after the fermentation. Hence, the banana leaf may facilitate the production of daidzein and genistein using a shorter period, within 2 days. Chaiyasut *et al.*, demonstrated that daidzein content and genistein content in Thai fermented soybean were increased as the fermentation time increased¹⁵. In addition, Kwon *et al.*, reported that when the fermentation period increase, the bound

polyphenols 6-O-malonylglucoside content was declined and there was a concomitant increase in non-conjugated aglycones (genistein and daidzein)⁶.

Hence, a more complete hydrolysis of isoflavone glycosides may occur if the fermentation period is prolonged.

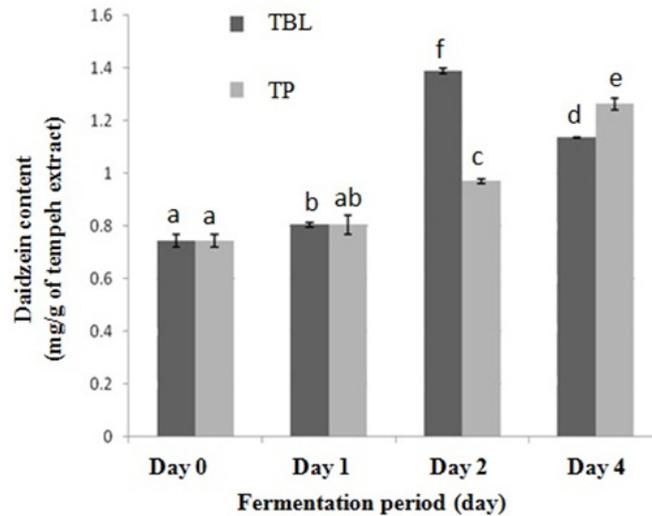


Fig. 3: Daidzein content of the extracts of tempeh wrapped with banana leaf (TBL) and polyethylene bag (TP) under different fermentation periods. Results are mean \pm standard deviation. Data labelled with different characters are significantly different at $p < 0.05$

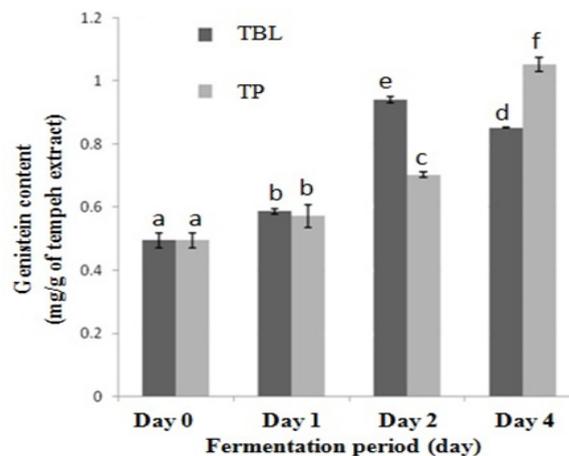


Fig. 4: Genistein content of extracts of tempeh wrapped with banana leaf (TBL) and polyethylene bag (TP) under different fermentation periods. Results are mean \pm standard deviation. Data labelled with different characters are significantly different at $p < 0.05$

However, the levels of daidzein and genistein of TBL were dropped ($p < 0.05$) on day 4, thus TBL is not suitable to be used for a longer fermentation period in order to prevent the degradation of these valuable

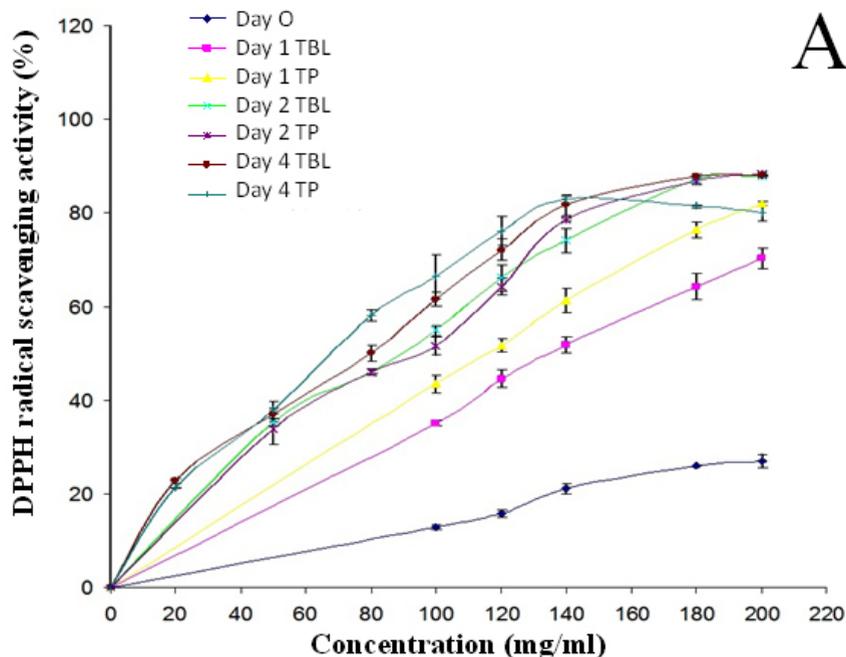
isoflavones. Generally, tempeh will be consumed by the consumers within day 1 or day 2 after purchased from the market. As similar to total phenolic content, TP gave a higher level of daidzein and genistein on

day 4 but it may be inedible. A study showed that when the fermented soybean was kept for a longer period at room temperature, a reduction of isoflavone content was found, including their aglycones and glucosides²⁰. Even though polyethylene bag is an easier way and considered as a modern material for wrapping the tempeh, it is less effective in providing an increment of isoflavone aglycones content via fermentation at the common period of local consumption as compared to those wrapped with banana leaf.

2,2-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The principle behind DPPH radical scavenging assay is to evaluate the reducing capacity of the sample tested²¹. The scavenging activities of the tempeh extracts of TBL and TP on DPPH radicals were increased as the fermentation period prolonged (Figure 5a). Besides that, DPPH radical scavenging activity was responded in the dose-dependent manner. The EC_{50} values were calculated and

shown in Figure 5b. A lower EC_{50} value indicates a better ability of the sample extract to scavenge DPPH radicals owing to least sample extract is needed to counteract with 50% of the DPPH radical. The lowest EC_{50} value was found in TP on day 4 which is 67.33 mg/ml. It indicates that as the fermentation period increased, the EC_{50} of TP was significantly ($p < 0.05$) decreased. Current results were agreed by Juan and Chou who also found that the EC_{50} value of DPPH radical scavenging activity obtained from fermented soybean was lower than the unfermented soybean¹⁴. It may be related to the release of the more active aglycones throughout the fermentation process and later increase the scavenging activities of the tempeh extract. This result was similar to Hu *et al.*, that fermented black soybean natto inoculated with *Bacillus natto* had a remarkably stronger DPPH free radical scavenging activity than the unfermented one³. At the same time, Hu and colleagues also demonstrated the radical scavenging activity was increased linearly with the duration of fermentation³.



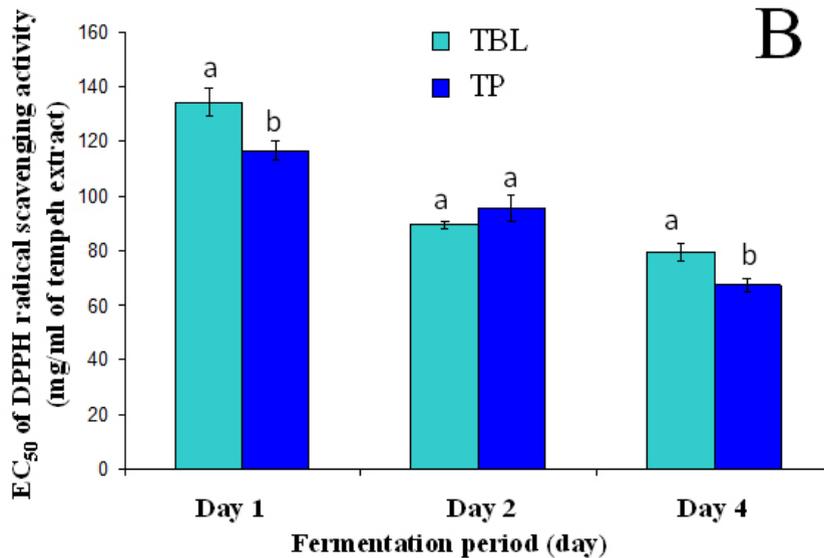


Fig. 5: DPPH radical scavenging activity (A) and EC₅₀ of DPPH radical scavenging activity (B) of the extracts of tempeh wrapped with banana leaf (TBL) and polyethylene bag (TP) under different fermentation periods. Results are mean ± standard deviation. Values at the similar fermentation day with different characters are significantly different at $p < 0.05$

As previously mentioned the fermentation increased the antioxidant components in both TBL and TP and hence contributed to the increment of DPPH radical scavenging activity. Correlation analysis revealed that there was a very significant positive relationship ($r = 0.969$, $p < 0.05$) between total phenolic content of TP and DPPH radical scavenging activity. However, TBL showed a weak relationship to DPPH radical scavenging activity ($r = 0.299$, $p < 0.05$). Total phenolic content was reported to be able to influence the DPPH radical-scavenging activity¹⁶. Both Folin-Ciocalteu assay for total phenolic content and DPPH radical scavenging assay are electron transfer based assays²¹. Hence, due to the similarity in the redox reaction of these assays, an excellent correlation between total phenolic content measured with Folin-Ciocalteu reagent and DPPH radical scavenging activity was seen in many studies²¹. Polyphenols are the reactive compounds that are susceptible to free radicals^{5,22}. Thus, the increment of total phenolic content can contribute to the increase radical scavenging activities. On the other hand, a significant but weaker correlation between total phenolic content of TBL and DPPH radical scavenging activity was observed. This may be due to other compounds that also contributed to the antioxidant activities.

Phytochemicals such as tocopherols (vitamin E) and soyasapogenol B (aglycone of soya saponins) are the good examples of DPPH scavenger that can also be found in soybean products².

β-Carotene linoleate bleaching

Due to the complexity of phytochemicals, antioxidant activity of a dietary extract is unable to be assessed with only one single method, as there is no single method that can clearly reflect the total antioxidant activity of a tested sample. Beta-carotene-linoleate bleaching assay is a hydrogen atom transfer based assay²¹. This assay involves the generation of hydroperoxides as free radicals from linoleic acid when incubated at 45°C. The colour of β-carotene (orange) will then undergo a rapid degradation without the protection of antioxidant.

Antioxidant activity of the tempeh extracts is shown in Figure 6. For TBL, no significant ($p > 0.05$) difference was found in antioxidant activity as the fermentation period prolonged but an ascending trend was observed. A similar trend was observed in TP. However, there was a significant ($p < 0.05$) increase in antioxidant activity from TP on day 1 of fermentation (28%) to day 2 of fermentation (51%).

The highest antioxidant activity was obtained from TP on day 2 of fermentation. The similar finding was reported by Chaiyasut *et al.*, that antioxidant activity of fermented soybean is increased during fermentation¹⁵. Kwak *et al.*, studied on antioxidant activity in chung-kook-jong, the fermented soybeans, also showed a better antioxidant activity than the unfermented steamed one, which is probably attributed to its improved contents of aglycone

isoflavone and malonylglycoside isoflavone after the fermentation²³. There was a positive correlation ($r = 0.272, p < 0.05$) between total phenolic content and antioxidant activity in TBL as well as total phenolic content and antioxidant activity in TP ($r = 0.635, p < 0.05$). Hence, polyphenolic compounds especially daidzein and genistein in the tempeh could somehow contribute to the prevention of linoleate oxidation.

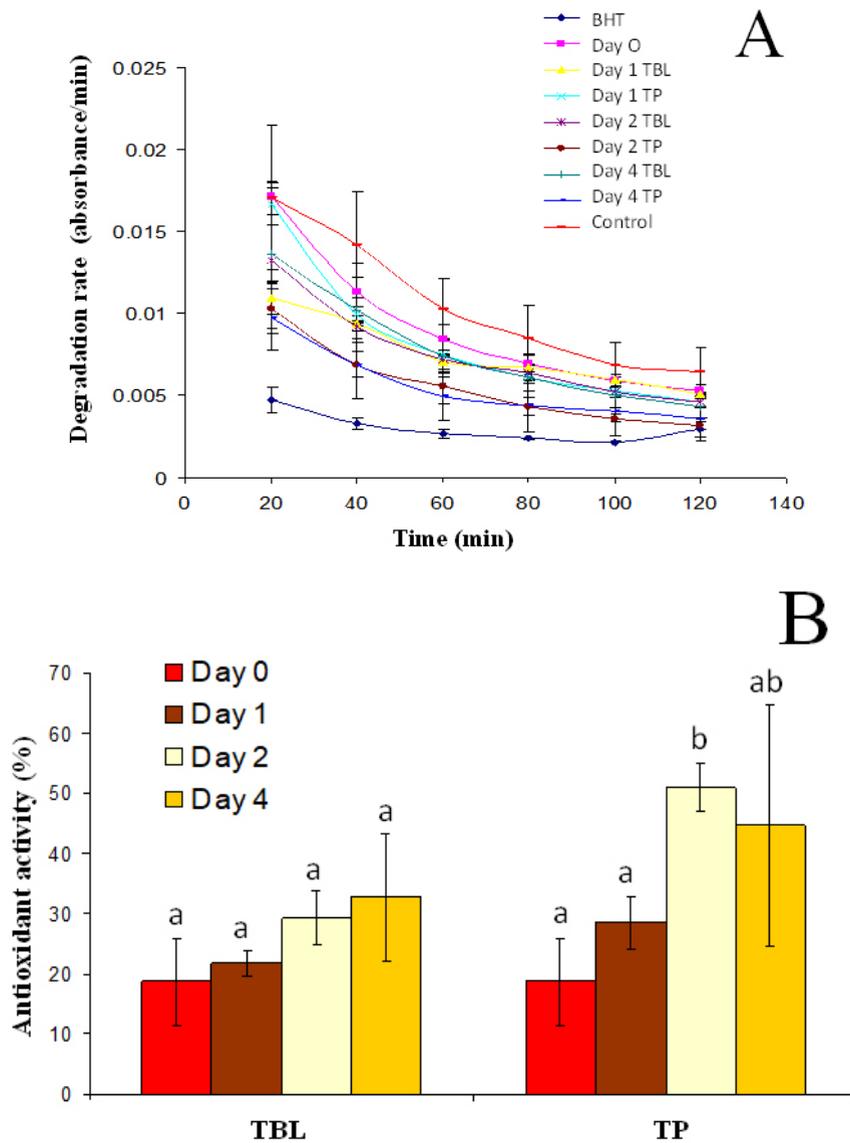


Fig. 6: Beta-carotene degradation rate (A) and antioxidant activity (B) of the extracts of tempeh wrapped with banana leaf (TBL) and polyethylene bag (TP) after different fermentation periods measured using β -carotene-linoleate bleaching method.

Conclusion

This study showed that total phenolic content, daidzein, genistein and antioxidant capacity were increased in both tempeh wrapped in banana leaf and polyethylene bag as the fermentation progressed. The antioxidant properties between tempeh wrapped in banana leaf and polyethylene bag were significantly increased on day 2 or after. However, the banana leaf was found to be a better material due to it provided a higher antioxidant content of tempeh on day 2 which is the best consumption period of this soybean product. Fermentation of tempeh using a polyethylene

material can be considered by the industry if the production of soybean-related products is mainly targeting for higher bioactive components. It is interesting for future research to develop a better wrapping material or method for tempeh production which can provide sufficient fermentation in a shorter period without compromising its nutritional values.

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