Indonesian Journal of Pharmacy

VOL 34 (3) 2023: 460–470 | RESEARCH ARTICLE

The Effects of Duration of Fermentation on Total Phenolic Content, Antioxidant Activity, and Isoflavones of The Germinated Jack Bean Tempeh (*Canavalia Ensiformis*)

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Article Info

Submitted: 02-02-2023
Revised: 07-05-2023
Accepted: 28-08-2023

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ABSTRACT

Tempeh is one of the food products from legumes often consumed by Indonesians as a side dish. Moreover, tempeh is a functional food that contains bioactive compounds, such as antioxidants and isoflavones. This study aims to determine the content of total phenolic compounds, antioxidant activity, and isoflavones content of germinated jack bean tempeh with a fermentation duration of 0-5 days. Isoflavones extract was obtained using the extraction method with 70% ethanol. The total content of phenolic compounds was measured using the method of Folin-Ciocalteau. Meanwhile, antioxidant activity was measured with DPPH, and isoflavones content was determined using high-performance liquid chromatography (HPLC). The total content of phenolic compounds (TPC), antioxidant activity, and isoflavones were analyzed using the ANOVA, and differences between treatments were compared to the Smallest Real Difference test with a significance level of 5%. The total phenolic compound content and the highest antioxidant activity during the tempeh fermentation process were obtained on the fifth day of the fermentation period of 10.70±0.31 (mg GAE/g) for the total phenolic and 457.04±151.91(%) for IC_{50} values. The intergroup test results show significant differences. The highest isoflavones deposits for all isoflavones, such as daidzein, glycitein, genistein, and factor-2, were also obtained at day 5 of tempeh fermentation duration. This study has discovered significant differences between treatment groups. The isoflavones content is 4.63±1.74 μg/g of daidzein, 5.45±2.29 μg/g of glycitein, 0.92±0.33 μg/g of genistein, and 0.46±0.21μg/g of factor-2. This study has discovered that the germination and fermentation process of jack bean tempeh changes the content of total phenolic compounds and antioxidant activity as well as increases and influences the isoflavones profile.

Keywords: germinated jack bean tempeh, total phenolic, antioxidant activity, isoflavones, duration of fermentation

INTRODUCTION

Indonesians' daily consumption pattern is very close to the consumption of side dishes made of legumes. The Central Agency on Statistics (BPS) of Indonesia records the average per capita consumption of side dishes from legumes, such as tofu and tempeh, in Indonesia is 0.304 kilograms (kg) per week in 2021; this number increased by 3.75% as the total consumption in the previous year is 0.293 kg per week. Tempeh has an average per capita consumption of 0.146 kg per week (BPS, 2021). Tempeh is one of the functional foods and is a superfood with bioactive compounds, such as antioxidants (Romulo & Surya, 2021). Antioxidants refer to compounds that can delay and prevent lipid oxidation processes. One of the bioactive compounds found in legume plants that act as antioxidants is isoflavones (Rigo *et al.*, 2015).

Specifically, isoflavones, with their phenolic groups, act as antioxidants through the mechanism of donating hydrogen ions and act as free radical scavengers directly (Irwan *et al.*, 2020). Isoflavones also results in health effects, such as anticancer (Basu & Maier, 2018; Boutas *et al.*, 2022), anti-obesity (Astawan *et al.*, 2018), anti-diabetic (Kuryłowicz, 2021), prevention of breast cancer (Finkeldey *et al.*, 2021; Wei *et al.*, 2020), and prostate cancer (Zhang *et al.*, 2016). Currently, jack bean (*Canavalia ensiformis*) has been used as a raw material for making tempeh in Indonesia, especially in the regions of Yogyakarta, Central Java, and West Java. Jack bean raw seed has a total protein content of up to 34.6%, dietary fiber of 1.2%, low total fat of 2.4%, ash of 2.8%, phospholipids of 0.1%, total free phenolic of 12.98 g, and catechin equivalent/100 g; moreover, Jack bean raw seed has a positive correlation with antioxidant activity (Hudiyanti *et al.*, 2015; Vadivel *et al.*, 2012). Another study has also reported that jack bean tempeh has an antioxidant activity of 68.63% and a content of isoflavones bioactive compounds of 0.78% (Istiani *et al.*, 2015). Jack bean also has bioactive peptides that can inhibit ACE activity (Puspitojati *et al.*, 2019).

Jack beans can be used as a source of isoflavones, such as soybean, because isoflavones are naturally and widely distributed across members of the leguminosae family (Prasad *et al.*, 2010). Isoflavones in plants have 4 forms: aglycon, β – glucoside, acetyl-β-glucoside, and malonyl-β-glucoside. These isoflavones can undergo structural changes, such as changes in glucoside isoflavones (daidzin, genistin, and glycitin) and aglycon forms (daidzein, genistein, and glycitein). These changes can occur during seed processing, such as soaking, boiling, and fermentation (Hsia *et al.*, 2016). During soaking and fermentation, microorganisms can also produce the β-glucosidase enzyme that hydrolyzes the glucose part of the conjugated isoflavones and increase their biological activity. This is due to the aglycone isoflavones are more fat-soluble and easily passed through the intestinal villi; thus, the bioavailability and bioactivity become greater than the glucoside form (Hsiao *et al.*, 2020). The activity of *Rhizopus* sp. can hydroxylize daidzein isoflavones and demethylation glycitein as well as convert daidzein and glycitein isoflavones into factor-2 isoflavones compounds (6,7,4'- trihydroxy isoflavones). Factor-2 has the highest antioxidant activity among genistein and daidzein (Barz *et al.*, 1991).

Factors of fermentation and germination can affect the nutritional content. Isoflavones as bioactive compounds are found higher in germinated soybean tempeh than in fresh tempeh (Puspitasari *et al.*, 2020). In addition to the duration of fermentation, germination can hydrolyze the sugar bond of isoflavones to form free aglycons. Other studies report that germination increased the aglycon isoflavones of glycitein and daidzein (Astawan *et al.*, 2016; Silva *et al.*, 2020), protein values by 2.26% in jack beans sprout flour, and antioxidant capacity, as well as change isoflavones profiles (Damayanti *et al.*, 2019; Huang *et al.*, 2014). This research studied jack bean sprouts with a germination period of 48 h and the potential for their use as raw materials for making tempeh. Additional benefits are also obtained. The scope of the discussion includes chemical properties, such as total phenolic content, antioxidant activity, and isoflavones content, and their profile characterization. This study aims to determine the effect of fermentation duration on total phenolic content, antioxidant activity, and isoflavones content as well as its profile on germinated jack bean tempeh. Tempeh, with the selected fermentation time, will have functional properties that are good for health; this aspect needs further study. It is related to the bioactive content of isoflavones obtained, the potential for enhancement, the bioactivity of antioxidants, and the bioactivity of total phenolic compounds.

MATERIALS AND METHODS

The main ingredient used in this study was the local jack bean (*Canavalia ensiformis*) obtained from jack bean farmers in Kulon Progo District, Yogyakarta Special Region, Indonesia. The ingredients needed for making sprouts were water and $NaHCO_3$. Meanwhile, the material for making tempeh was Raprima. Inoculum was obtained from PT. Aneka Farmasi Indonesia while polypropylene packaging plastics (pp) were purchased from a local market. The reagents and chemicals used were methanol p.a., ethanol 70%, Folin-Ciocalteau (Merck, Germany), DPPH solution (2,2- diphenyl-1-picrylhydrazyl), ascorbic acid, aquadest, quercetin, gallic acid, and isoflavones standards (genistein, daidzein, glycitein, and factor-2) with a purity of ≥ 98% (Sigma Chemical Co., United States). The tools used in this study were UV-VIS spectrophotometer (Optizen), high-performance liquid chromatography (Shimadzu), and a rotary evaporator (Büchi R-100).

Jack bean germination

The stage of jack bean germination was carried out with the initial stage, namely the sorting stage of jack bean to separate jack bean from impurities. Then, the jack bean was soaked for 24 h with initial soaking in water at the temperature of 50°C for 6 h. Afterward, $NaHCO_3$ (1% w/w) was added to the water. The water was changed every 6 h so that the jack bean was washed well and drained. The drained jack bean seeds are put into a plastic container and covered with a wet cloth to maintain their humidity. The germination process was carried out at room temperature of 20-25°C for 48h. During the germination process, the covering cloth was moistened once every 8 h. This method was modified from the germination procedure of Damayanti *et al*. (2019) and Kanetro *et al*. (2021).

Production of germinated jack bean tempeh

The tempeh was prepared following the procedures of Andriati *et al*. (2018) and Andriana *et al*. (2020) with modifications of the number of boiling processes (Andriati *et al*., 2018; Puspitasari *et al*., 2020). Firstly, the harvested jack bean sprouts (600 g) were manually removed from the seed coat from the cotyledon. Afterward, jack bean sprouts were washed thoroughly with running water. The next step was boiling the washed jack bean sprouts for 30 minutes at a temperature of 100^oC. Subsequently, the boiled jack bean sprouts were drained, sliced into 8-9 parts, and inoculated with 0.05 g yeast for every 100 g cooked jack bean sprouts. These jack bean sprouts were then packed into PP plastic bags with holes at a distance of 2 x 2 cm. Afterward, jack bean sprouts were incubated for 0, 24, 48, 72, 96, and 120 h at 28--30°C. Tempeh samples were dried in a freeze-dryer at -80°C for 24 h. Freeze-dried samples were mashed with a grinder, sifted with 60 mesh sievers, and kept at -20°C until the analysis process.

Sample Extraction

The powder of germinated jack beans tempeh (100 g) was put into a maceration container. Then 70% ethanol was added until the sample was submerged (1:8). Afterward, the container was closed, and stirring was carried out occasionally. The maceration process was carried out 3 x 24 h. The supernatants were collected and filtered, and the solvent was evaporated using a rotary evaporator with a temperature of 50°C until a concentrated extract was obtained.

Chemical analysis

Ethanol extract of germinated jack bean tempeh was chemically analyzed. The chemical analysis included total phenolics, the antioxidant activity of DPPH, and isoflavones levels of daidzein, glycitein, genistein, and factor-2.

Total Phenolic Content

The ethanol extracts of germinated jack bean tempeh were analyzed for TPC by the Folin-Ciocalteu assay following the theory of Mas and Heng (2019) with minor modifications from the theory of Johari and Khong (2019). Moreover, the analysis used gallic acid as a standard. The sample was prepared by dissolving 10 mg of extract in 10 mL of ethanol. Then, 100 μL of the extract was mixed with 0.75 mL Folin-Ciocalteu reagent in a tube. The mixture was allowed to stand for 5 minutes at room temperature of $20\text{-}25^0C$. After that, the mixture was added to 0.75 mL Na_2CO_3 and then mixed until homogenous. After 90 minutes, the absorbance was measured using a UV-VIS Spectrophotometer at 730 nm. The standard curve for the determination of the total phenolic content was made using gallic acid in the concentration range of 0-0.06 mg/mL. Total phenolic content was measured as mg gallic acid equivalent or mg GAE/g extract.

Antioxidant activity

The determination of IC_{50} from germinated jack beans tempeh and ascorbic acid (standard) was carried out by the free radical scavenging method using DPPH (1,1-diphenyl-2-picrylhydrazyl). The DPPH assay was conducted according to the method of Xu and Chang (2008) without modifications. A series of concentrations of the sample working solution in ethanol solvents were made with different concentrations: 100, 200, 400, and 800 ppm. Similarly, positive control (ascorbic acid) was made in four series of concentrations: 10, 20, 40, and 80 ppm. The concentration series of the created working solution was arranged in such a way so that the concentration value of the sample that can bind to 50% free radicals (DPPH) is in the created concentration series. The antioxidant activity assay was carried out by measuring 1.0 mL of each of the working solutions and put into a container (vial) covered with aluminum foil. Afterward, 4.0 ml of 40 ppm DPPH solution was added. The solution was shaken and then incubated for 30 minutes. The test indicator was the damping of DPPH as a result of absorbance on a *UV-Visible* Spectrophotometer at

517 nm. Negative control of the blank solution was prepared using ethanol solvent mixed with DPPH. Then, the percentages (% inhibition) of binding DPPH and the IC_{50} value of samples were calculated. The IC_{50} value was obtained from a linear regression graph; i.e. a plot between the sample concentration against the percentage of radical inhibition (Shahidi & Zhong, 2015; Xu & Chang, 2008).

$$\text{Antioxidant Activity (\%)} = \frac{\text{OD Blanko} - \text{OD Sample}}{\text{OD Blanko}} \times 100\ \%$$

Remark: OD = Optical Density

The criteria of antioxidant activity are as follows. If the IC_{50} value is < 50, the antioxidant activity is categorized as very strong. If the IC_{50} value is between 50-100, the antioxidant activity is categorized as strong. If the IC_{50} value is between 100-150, the antioxidant activity is categorized as medium. If the IC_{50} value is 151-200, the antioxidant activity is categorized as weak (Natsir *et al.*, 2019).

Isoflavones Analysis

Isoflavones analysis was carried out by extracting isoflavones from ethanol extract of germinated jack bean tempeh. The isoflavones content (daidzein, glycitein, genistein, and factor-2) was determined according to the method of Wang *et al.* (1990) and STIH (2015) (SITH ITB, 2018; Wang *et al.*, 1990). Initially, 1g of the sample was added with 10 ml of 70% (v/v) ethanol and 2 ml of HCL 2 N. The mixture was incubated in a water bath of 75°C for 2 h, was evaporated with ethanol at a temperature of 40°C and was analyzed for isoflavones content. The extract was dissolved in methanol until a volume of 10 ml. Subsequently, the extract was added with 1.5 ml of the solution and filtered with 0.45 micro PTFE. The supernatants were separated, and as much as 20 µL of supernatants were injected into the high-performance liquid chromatography (HPLC) column. Four reference isoflavones, namely daidzein, genistein, glycitein, and factor-2, were used as standards. The chromatographic separation was performed on the C-18 column with the following conditions: column type: Lichrosper(R) 100 RP-18 (nonpolar); mobile phase of methanol: acetic acid 0.02 (57.5%: 42.5%); injection volume: 20 µL; detector: UV light at 265 nm length; oven temperature: room temperature. Quantitative analysis of isoflavones genistein, daidzein, glycitein, and factor-2 was performed by calculating the area of the chromatogram.

Data Analysis

The data were processed using analysis of variance (ANOVA). If there was a significant difference ($p < 0.05$), further testing using the Duncan multiple range test (DMRT) at a significant level of 0.05 was conducted.

RESULTS AND DISCUSSION

Isoflavones Identification

HPLC is a tool that can identify the presence of a compound, including isoflavones compounds, by comparing the retention time of standard isoflavones compounds with the retention time of each sample. Identification can be seen from the presence of peaks with a relative retention time similar to the standard isoflavones compounds, such as daidzein, glycitein, genistein, and factor-2. These compounds indicate that the sample tested contains the same content of isoflavones compounds (Table I).

The test results in daidzein, glisitein, genistein, and factor-2 content in germinated jack bean tempeh have significance values of $p < 0.05$ (Table I). The result indicates a significant difference between treatment group T0 to group T5. A further test, DMRT, shows that glycitein and factor-2 have significant values of less than 0.05 (sig < 0.05) indicating a significant difference between treatments. Meanwhile, daidzein and genistein show significance values of more than 0.05 (sig > 0.05) indicating no significant difference between treatments. The highest average of daidzein (4.63±1.74 µg/g), glycitein (7.12±0.95 µg/g), genistein (0.92±0.33 µg/g), and factor-2 content (0.46±0.21 µg/g) was found in the T5 treatment. The lowest value was found in the T0 with the content of daidzein of 0.32±0.04 µg/g, glycitein of 0.57±0.55 µg/g, genistein of 0.02±0.01 µg/g, and factor-2 of 0.02±0.02 µg/g. According to Barz and Papendorf (1991), factor-2 is formed when the soaking process is carried out in the beans and activates the β-glucosidase. The subsequent fermentation process by the *Rhizopus oligosporus* also caused further bioconversion of isoflavones, daidzein, and glyceine into factor-2. Demethylation of glycitein could also occur by the bacteria *Brevibacterium epidermis* and *Micrococcus luteus* or through the hydroxylation reaction of daidzein (Barz *et al.*, 1991). Factor-2, as in this study, can be found in tempeh during the fermentation process.

Table I. Identification of Isoflavones from germinated jack bean tempeh

Group	Isoflavones Content (μg/g)			
	Daidzein Mean ± SD N=3	**Glycitein Mean±SD N=3**	**Genistein Mean±SD N=3**	**Factor-2 Mean±SD N=3**
T0	0.32 ±0.04[a]	0.57±0.55[a]	0.02±0.01[a]	0.02±0.02[a]
T1	0.58±0.10[a]	0.96±0.79[a]	0.10±0.15[a]	0.02±0.01[a]
T2	0.55±0.10[a]	0.69±0.59[a]	0.29±0.10[a]	0.03±0.02[a]
T3	0.79±0.45[a]	3.25±0.78[b]	0.43±0.26[a]	0.14±0.07[b]
T4	0.67±0.43[a]	4.99±0.54[c]	0.69±0.47[a]	0.12±0.02[b]
T5	4.63±1.74[a]	7.12±0.95[d]	0.92±0.33[a]	0.46±0.21[b]

Different notations indicate significant differences in the same column.
T0 is 0-day fermentation; T1 is 1 day of fermentation; T2 is 2 days of fermentation; T3 is 3 days of fermentation; T4 is 4 days of fermentation; T5 is 5 days of fermentation.

The increase in the number of isoflavones and changes in the isoflavone profile in the germinated jack bean tempeh is caused by the process of germination and fermentation. Germination and fermentation are processes that can increase isoflavone content and change isoflavone glycosides into isoflavone aglycones. aglycone isoflavone compounds increase because the β-glucosidase enzyme catalyzes the release of aglycones from the substrate. Isoflavone aglycones have a higher biological activity and can enhance their physiological health (Astawan *et al*., 2016). Huang *et al*. (2014) have discovered that one day of germination could increase soybean aglycone content by 84%. Germinated jack bean tempeh increased isoflavone content in the form of aglycone isoflavones along with the length of fermentation, which was extended from day 0 to day 5 and forming factor-2 isoflavones after fermentation using the activity of *Rhizopus* sp (Huang *et al*., 2014)

Germination changes the phenolic compounds and isoflavones profile and modifies the antioxidant activity (Huang *et al*., 2014; Tarzi *et al*., 2012). Isoflavones belong to the flavonoid class, a polyphenolic compound. Isoflavones are included in bioactive compounds, one of which acts as a natural antioxidant. The germination and fermentation process increases the number of isoflavones; this is related to the enzyme β-glucosidase activity that can hydrolyze isoflavone glycoside into isoflavone aglycon (Ribeiro *et al*., 2006)

The presence of isoflavones, either daidzein, glycitein, or factor-2, in all samples of germinated jack bean tempeh extracted from day 0 to day 5 is in line with the result of research on jack bean tempeh with whole bean and chopped treatment, which also contains isoflavones, namely genistein, daidzein, glycitein and factor-2 (Istiani *et al*., 2015). A longer fermentation time increases total isoflavones content. Differences between the number of isoflavones compounds and fermentation time show some fluctuating data. This can be caused by several factors, such as the characteristics of isoflavones compounds that are highly reactive, easily oxidized, and binding to other compounds to form new compounds. Another study states that isoflavones are easily deformed. The heating process with a temperature of 121°C for 30 minutes can convert malonylglycoside isoflavones into glycoside isoflavones. In this process, the total of isoflavones does not decrease but changes the chemical structure of isoflavones (Silva *et al*., 2020). In addition, the type or variety, harvest time, and planting location can affect the isoflavones content in legumes of each variety or genotype of legume plants (Wu *et al*., 2020). Isoflavones content is influenced by the environment and environmental conditions of plant growth, cultivation, and post-harvest handling. This research used the jack beans seeds from farmers in Kulon Progo. The jack beans from this region have the highest glycitein content among other isoflavones, such as daidzein and genistein (Table I). Through the methylation process, glycitein will resist greater hydroxyl radicals than daidzein and genistein do (Kim & Kim, 2020; Yoshiara *et al*., 2018). Other studies assert that germination followed by fermentation into tempe products can increase the nutritional value of soybean tempe (protein, vitamin B, C, and minerals), bioactive substances (quantities and types of isoflavones), and aglicon isoflavones .

The physical observation of tempeh from jack bean sprouts fermented on day 0 showed a yellow color, and mycelia did not yet form on the surface of the seeds. Fungal growth began with mycelia, which grew compactly and covered the surface of the seeds. Moreover, fungal growth began to form a specific flavor of tempeh starting from day 1 (24 h) to day 2 (48 h) when the specific flavor of tempeh reached its optimum result. On the third day of fermentation, the growth of the fungus tends to decrease. The fungal mycelium begins to light yellow with a slight ammoniacal odor. On day 4, fermented tempeh began to change its color to light brown, produced a sharper ammoniacal odor due to further protein degradation processes, and had a soft texture. Meanwhile, on day 5 of fermentation, tempeh began to experience decay with a dark brown color, contained much water, had a softer texture, and tended to smell bad.

The fermentation on day two resulted from germinated jack bean tempeh with the best physical appearance. Utami *et al.* (2016) state that tempeh, with the best physical appearance and flavor for consumption, is fermented for 24–48 h.

Antioxidant Activity

The antioxidant assay was carried out using the DPPH method through absorbance measurement using a UV-Vis spectrophotometer. IC_{50} values of germinated jack bean tempe fermentation on days 0, 1, 2, 3, 4, and 5 and ascorbic acid values (Table II).

Table II. Comparison of IC_{50} (ppm) germinated jack bean tempeh in different fermentation period

Group	IC_{50}(ppm) Mean±SD N=3
Control	29.72±0.25[a]
T0	3679.81±383.69[c]
T1	3018.37±980.32[bc]
T2	3436.64±215.90[c]
T3	3371.93±314.58[c]
T4	3180.62±228.58[c]
T5	457.04±151.91[b]

The results of significant differences are indicated by different notations in the same column. Control is ascorbic acid; T0 is 0 day of fermentation; T1 is 1 day of fermentation; T2 is 2 days of fermentation; T3 is 3 days of fermentation; T4 is 4 days of fermentation; T5 is 5 days of fermentation.

The DPPH method (*2,2 diphenyl-1-picril hydrazil)* was employed to test antioxidant activity in this study. Antioxidant compounds from natural ingredients would react with DPPH radicals through the hydrogen atom donation mechanism. Linear regression of the extract concentration range with % DPPH was used to determine the concentration of extracts that could decrease by 50% DPPH (IC_{50} value). Antioxidants are categorized into four: very strong, strong, medium, and weak categories (Natsir *et al.*, 2019). If the IC_{50} value is less than 50 ppm, the antioxidant activity is categorized as very strong). If the IC_{50} is 50-100 ppm, the antioxidant activity is categorized as strong. If IC_{50} is 100-150 ppm, the antioxidant activity is categorized as medium. If IC_{50} is 151-200 ppm, the antioxidant activity is categorized as weak.

The measurement of antioxidant activity has obtained the smallest IC_{50} of T5 (457.04±151.91 ppm) and the highest IC_{50} of T0 (3679.81±383.69 ppm). The experimental results show that the ethanol extract of germinated jack bean tempeh on T0-T5 has a weak antioxidant activity category because it has an IC_{50} value of > 100 ppm. There was a significant difference between the ethanol extract of germinated jack bean tempeh and ascorbic acid (IC_{50}=29.72±0.25 ppm). As a control, ascorbic acid has a strong antioxidant activity with IC_{50}<50 ppm. T5 ethanol extract shows better antioxidant activity. Antioxidant activity is influenced by the presence of antioxidant compounds contained in the ethanol extract of germinated jack bean tempeh, such as isoflavones and phenolic compounds. The compound's ability to capture DPPH free radicals is influenced by the OH group contained in phenolic compounds.

Isoflavones contain phenolic groups that can bind free radicals and can donate hydrogen ions (Yoon & Park 2014; Astuti *et al.*, 2009). Fermentation processing can increase antioxidant activity by elevating the number of substituted hydroxyl groups to increase the ability to capture free radicals (Yu Lin *et al.*, 2009). In the tempeh fermentation process, biotransformation of isoflavone glycosides and isoflavone aglycones also occur and are released from sugar compounds through hydrolysis of -o-glycosidic bonds. Aglycone isoflavone compound has a higher ability of antioxidative activity than isoflavone glycosides do. In the fermentation process of germinated jack bean tempeh, antioxidant factor-2 (6,7,4-trihydroxy isoflavone) is also formed and has the strongest antioxidant properties among other isoflavones (Fawwaz *et al.* 2017). The results of this study agree with Yu Lin *et al.* (2009) who have discovered that the total phenolic content and flavonoids increase along with the extended fermentation period and the strength of antioxidant activity in scavenging free radicals.

Tempeh fermentation could increase levels of isoflavones compounds in jack beans tempeh (Istiani *et al*., 2015). Germination treatment and continued fermentation also increase isoflavones levels along with the extended fermentation duration. Fermentation can produce primary metabolites and secondary metabolites in controlled environments with higher bioactivity, such as hydrolysis of isoflavones glycosides compounds; fermentation can also change primary metabolites and secondary metabolites into free isoflavones compounds (Barz *et al*., 1991). The increase in the content of isoflavones compounds affects the antioxidant activity of isoflavones compounds. This condition indicates that the IC_{50} value decreases with the length of the fermentation period. Therefore, the lowest IC_{50} value or the highest antioxidant activity was obtained on day 5. Glycitein are isoflavones with better antioxidant potential than genistein and daidzein isoflavones (Winarsi, 2005).

Total Phenolic Content (TPC)

The total phenolic content of germinated jack bean tempeh ethanol extract was determined based on Follin-ciocalteu's reagent and using gallic acid as the standard. The gallic acid standard curve was obtained by calculating the linear regression between the concentration of gallic acid as X and the absorbance of the gallic acid from the reaction with the folin reagent as Y. The obtained regression equation is $y = 8.1378x + 0.0422$ with $R^2 = 0.9971$. The equation was used to determine the total phenolic content of the sample. The TPC of extracts (Table III) shows that the T5 treatment has the highest level of 10.7008±0.3140 mg. GAE/g. Meanwhile, the T0 treatment has the lowest level of 2.4870±0.2510. The result of the ANOVA has a significance value of 0.000 (sig < 0.05) indicating a significant difference in the treatment group from T0 to T5. DMRT test also shows a significance value between treatments of less than 0.05 (sig < 0.05).

Total phenolic content of tempeh during the fermentation period increases along with the duration of fermentation (Table III). The highest total content of phenolic compounds is obtained on day 5 of the fermentation. Meanwhile, the lowest total phenolic compound is obtained on day 0 of the fermentation. During the fermentation period of tempeh, it is suspected that a transformation occurs in the compounds of tempeh. Transformation occurs in the compounds of tempeh; for example, the hydrolysis of glucosidic bonds produces higher phenolic monomers by breaking the glycosidic bonds and releasing biologically active aglycones as well as distort the hydroxyl groups in the phenolic structure to increase the amount of phenolic free radical (Salar *et al*., 2012). The number of phenolic compounds in the fermentation process can also increase due to synthesizing new bioactive compounds detected as phenolic compounds (Ayyash *et al*., 2018). When new compounds, which are phenolic groups, are formed, they will reincrease phenolic concentrations.

Table III. Total Phenolic Content (TPC) of Ethanol Extract of Germinated Jack Bean Tempeh

Group	TPC (mg.GAE/g) Mean±SD N=3
T0	2.4870±0.2510[a]
T1	3.9605±0.7897[b]
T2	7.0532±0.3956[c]
T3	7.9123±0.6986[c]
T4	8.7622±0.7601[d]
T5	10.7008±0.3140[e]

*Different letters indicate significant differences at $P < 0.05$. TO is 0 day of fermentation. T1 is 1 day of fermentation. T2 is 2 days of fermentation. T3 is 3 days of fermentation. T4 is 4 days of fermentation. T5 is 5 days of fermentation.

Correlation of antioxidant (IC_{50}) and total phenolic content

Correlation is a test to determine the relationship between two variables, namely phenolic and IC_{50}. The correlation test was performed using the Pearson correlation test. The correlation test results are declared significant if the significance value obtained is less than 0.05 (sig < 0.05). The results of the correlation analysis show that the two variables, total phenolic content (TPC) and IC_{50} value, have a significant relationship with a significance value of 0.006 < 0.05). The correlation coefficient value is -0.621. This result shows a strong relationship between TPC and IC_{50} with a negative relationship. Therefore, a high phenolic value would have a significant effect on the lower IC_{50} value. These results signify that total phenolics content (TPC) provides a high correlation to the antiradical activity of DPPH.

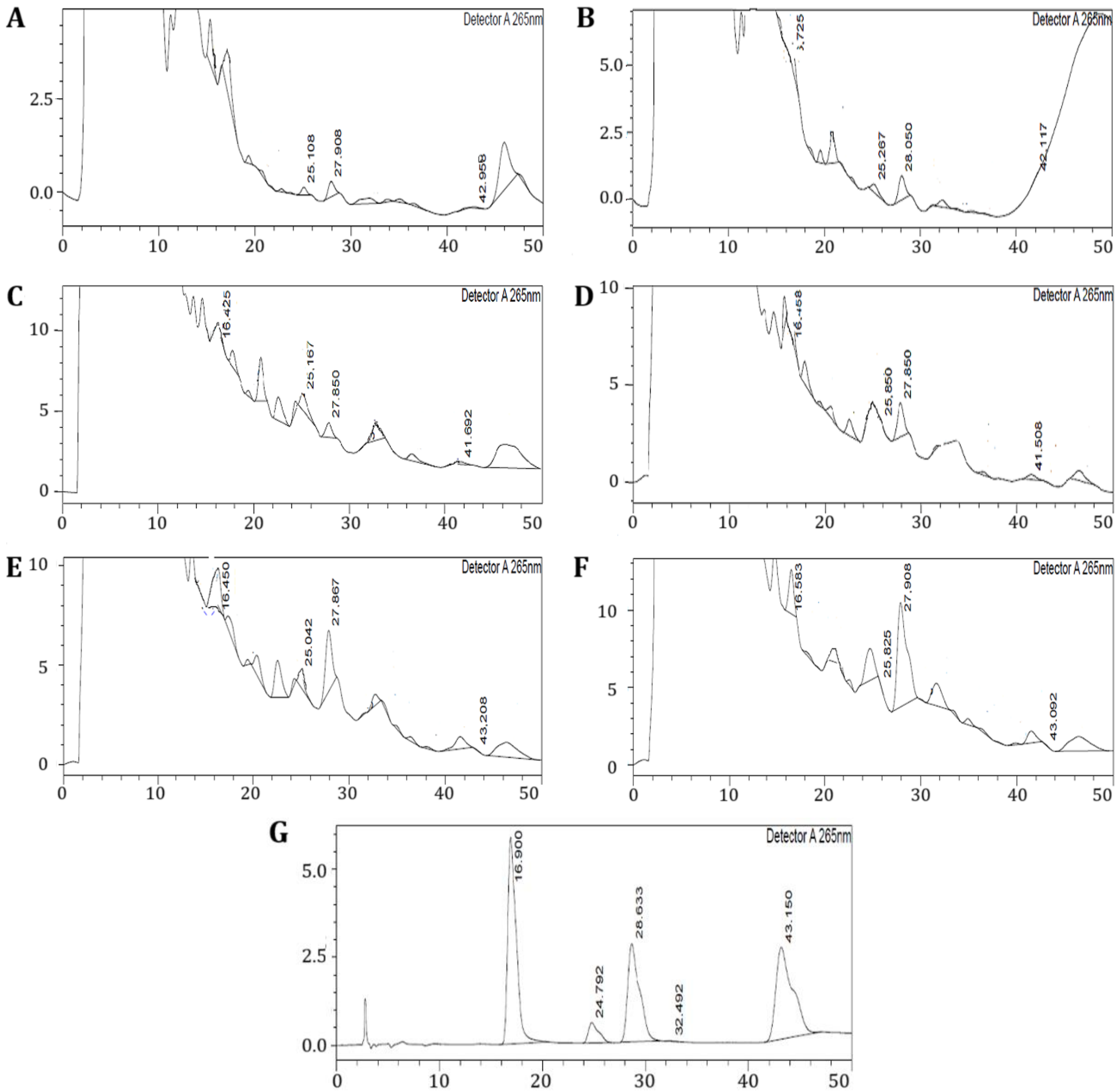

Figure 1. HPLC Chromatograms extract ethanol germinated jack bean tempeh 0 day – 5 days. RT, Retention Time; A, Extract 0 day; B, Extract 1 day; C, extract 2 days; D, extract 3 days; E, extract 4 days; F, extract 5 days; G, Standard isoflavones. RT Factor-2 16,583; Daidzein 24,717; Glycitein 27,908 and Genistein 43,092

CONCLUSION

Germination followed by fermentation can change the isoflavones profile and isoflavones levels in the germinated jack beans tempeh. The levels of phenolics and free radical scavenging activity increased when the fermentation time was extended. Further research is suggested to test the absorption of isoflavones extracts in vivo using animal models Because such a model can further investigate anticancer potentials.

ACKNOWLEDGMENTS

This study was supported by a Doctoral Dissertation Research Grant from the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia in 2022, with contract number: 033/E5/PG.02.00/2022

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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