



Phenolic Compound Profile and Functionality of Aqueous Overripe Tempe Extracts

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Abstract

Tempe is a soy fermentation product and has been associated with many health benefits. It is usually consumed after 48 hours of fermentation; however extended fermentation is sometimes favoured. Since cooking involves water, it is of interest to study the health benefits of tempe water extract. In this study, we focused our study on total phenols, total flavonoids, DPPH free radical scavenging activity, cytotoxic activity, and identification of the main flavonoids. Our findings demonstrated that concentration of water-soluble flavonoids in tempe increased as fermentation time was prolonged. Fermentation for 60 hr resulted in extracts with very noticeable cytotoxic activity against MCF-7 cancer cell line (IC_{50} of 8.70 $\mu\text{g/ml}$). The cytotoxic activity of the extracts was fermentation time-dependent following non-linear relationship. The activity showed strong inverse correlation with total phenols and flavonoids in the extracts. LC-MS/MS data suggested that extracts from control and 24-hr fermented tempe demonstrated the highest responses for genistein, genistin, daidzein, daidzin, and glycitein, compared to those of other treatments and therefore suggested that excessive amount of the isoflavones, particularly genistein, induced cancer cell proliferation but lesser yet optimum concentration (observed at 60 hr-fermentation) contributed to otherwise effect.



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Introduction

Tempe is a fermented food originated from Indonesia and has gained popularity in many parts of the world. This food is prepared by exposing soybeans to solid state fermentation by mixed culture of *Rhizopus* spp.

Many studies suggested that tempe fermentation improved nutritional quality of soybeans. Isoflavones have mainly been the focus of the studies. During tempe fermentation, aglycone isoflavones, that possess higher functionality and bioavailability

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compared to their glycosidic counterparts,^{1, 2} increased in quantity as the results of the fungal metabolism.^{3, 4} Aglycone isoflavones, particularly genistein, have been known for its estrogen-like activity and cancer cell growth suppression.⁵ However, the effect is known to be dose-dependent.⁶ Besides isoflavones, other types of phenolic compounds are also present in soybean and tempe. However, many of the studies investigate the compounds that are extracted using semi-polar solvents.^{3, 7} While many known active phenolic compounds of tempe are semi-polar, it is of great importance to study the phenolic compound profiles derived from water extraction of tempe.

Water extraction is favored in this study because it reflects one type of tempe utilization in cuisines; it is used in the digestion/absorption process in the body; and water as the extraction solvent is safer for the environment. In addition, to the best of our knowledge, only limited number of studies utilized water for extraction while a considerable portion of phenols in soybeans are water-soluble.⁸ Moreover, semi-polar phenols, particularly isoflavones, may also be partially soluble in water. Besides phenols, water would potentially dissolve peptides, free amino acids, and water-soluble vitamins as well. During tempe fermentation, the cultures released proteolytic enzymes that helped hydrolyze proteins in soybeans into peptides and free amino acids.⁹⁻¹¹ Some tempe peptides and amino acids demonstrated biofunctionality, including antihypertensive, antidiabetic, antioxidative and antitumor activity.¹²⁻¹³ It was also reported that water extracts of tempe could reduce the severity of enterotoxigenic

Escherichia coli infection by preventing the bacteria attachment to intestinal wall cells.¹⁴

We were particularly interested in cytotoxicity against MCF-7 breast cancer cell line and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay of aqueous extracts of tempe fermented at varying duration. Moreover, total phenolic compounds and total flavonoids were explored, as well as isoflavone identification and intensity comparison using LC-MS/MS to observe correlation between the abovementioned activities and phenolic compound profiles. The fermentation time was modified in order to find the optimum duration for the cytotoxicity or antioxidant activity of the extracts.

Materials and Methods

Materials

Soybean/tempe samples were prepared and fermented (for 24 hours) in Rumah Tempe Indonesia (RTI, Bogor, Indonesia) while the inoculum was obtained from PT. Aneka Fermentasi Indonesia. Ultrapurified water was used as solvent. All chemicals were of analytical grade, except for hexane (CAS: 110-54-3) for defatting that was purified technical grade solvent. DMSO (102952, CAS: 67-68-5), methanol (822283, CAS: 67-56-1), Folin Ciocalteu (109001), sodium carbonate (106392, CAS: 497-19-8), and formic acid (822254, CAS: 64-18-6) were supplied by Merck Millipore (Burlington, Massachusetts, United States); RPMI (31800-022, LOT #1937557) and alamarBlue reagent (DAL1025, LOT #642297) were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, United States); DPPH (D4313, CAS: 1898-66-4) was from Tokyo Chemical Industry (Tokyo, Japan); while aluminium chloride (254134, CAS: 12125-02-9), quercetin (CAS: 6151-25-3) and gallic acid (CAS: 149-91-7) were from Sigma-Aldrich (St. Louis, Missouri, United States).

Methods

Sample Preparation

Tempe production was performed following some established procedure that has been cited in several publications.^{7, 12, 15} Soybeans were cleaned and then boiled (100°C, 30 min). After being kept for 22-24 hours in water containing lactic acid (pH 4.18, concentration of 1%), the soybeans were

Table 1: Time, flow rate, and composition of solvent A and B for gradient separation of isoflavones

Time (min)	Flow rate (ml/min)	Composition A (%)	Composition B (%)
0.00	0.300	90.0	10.0
1.00	0.300	90.0	10.0
3.00	0.300	70.0	30.0
11.00	0.300	0.0	100.0
13.00	0.300	0.0	100.0
15.00	0.300	90.0	10.0

separated from its coat with a dehulling machine. The dehulled soybeans were then added with boiling water and soaked for 15 min. Later, they were strained and allowed to reach room temperature prior to subsequent processing.

One kilogram of the soybeans was collected and later defined as control. Meanwhile, the rest was mixed with 2 g inoculum/kg soybean (Raprima, PT. Aneka Fermentasi Indonesia, Bandung, Indonesia). The mixture was then transferred into perforated polyethylene bags and placed in a room (30-37°C; 70-85% humidity) for 24 hours. Some samples were harvested immediately after 24 hour fermentation, while others were placed in a room with good air circulation to allow further fermentation. Other samples were collected when fermentation time reached 48, 60, 72, and 120 hours. Samples were quickly placed in a freezer (-18°C) to stop the fermentation process.

Samples were thawed in a refrigerator ($\pm 4^\circ\text{C}$) for approximately 24 hours. Afterward, samples were weighed, sliced, and then dried in an oven (Venticell, MMM Medcenter Einrichtungen GmbH, Germany) at 50°C for 24 hours until most moisture (55-59% of the initial weight) was removed. Dried samples were weighed and later ground using Tokebi grinder (Neo Tokebi Plus, HausElec Co., Ltd., South Korea) until they turned into fine powder. The powder was then sieved and kept in an airtight container until further treatment.

Defatting and Extraction

Samples (approximately 90 g) were transferred into flasks and hexane was added (with sample to hexane ratio was 1:10). The flasks were put in a shaking incubator (DS-310C2, Dasol Scientific Co. Ltd, South Korea) for 2 hours with orbital shaking speed of 150 rpm. Afterward, samples were filtrated using filter papers under vacuum condition. The residues were then exposed to an airy condition in order to evaporate the remaining hexane from the residues. Later, they were weighed and transferred into the flasks again.

Deionized water (with water to sample ratio of 10:1) was added into the defatted samples in the flasks. The flasks were then again placed in the shaking incubator to undergo extraction for 2 hours at orbital shaking speed of 150 rpm. After the period ended, samples were filtrated using filter papers under vacuum condition. The filtrate was then placed in a rotary evaporator (Eyela NE Series, Tokyo Rikakikai Co. Ltd, Japan), with water bath temperature at 73°C and orbital speed at 15 rpm, until most of the water evaporated. The extracts were then weighed and placed in oven (50°C, overnight) to remove the remaining water.

Determination of Total Phenols

This assay was conducted using spectrophotometric technique.¹⁶ Samples were solubilized in deionized water to obtain concentration of 2 mg/ml. Gallic acid was diluted so that its concentrations in the reaction

Table 2: Correlation between total phenols, total flavonoids, IC₅₀-antioxidant activity, and IC₅₀-cytotoxic activity

Parameter	IC ₅₀ – Cytotoxic	IC ₅₀ – Antioxidant Activity	Total phenols	Total flavonoids
IC ₅₀ – Cytotoxic Activity		-0.476	0,926	0,956
IC ₅₀ – Antioxidant Activity	-0,477		-0,432	-0,534
Total phenols	0,926	-0,432		0,865
Total flavonoids	0,956	-0,539	0,865	

mixture were 0, 10, 20, 30, and 40 ppm. Samples (40 μ l of sample solution with concentration of 2 mg/ml) were placed into each well. Deionized water was added to make the mixture volume reached 160 μ l. We added 10 μ l Folin Ciocalteu reagent and the mixture were incubated for 8 min. Finally, 30 μ l sodium carbonate (10% w/v) was added and the mixture was allowed to react for 2 hours. The absorbance (λ 765 nm) was determined by using Varioskan™ Flash Multimode Reader (Thermo Scientific, Massachusetts, United States).

Determination of Total Flavonoids

Similar to total phenol assay, total flavonoid analysis was also based on spectrophotometry.¹⁷ Prior to analysis, sample extracts were solubilized in deionized water to obtain concentration of 2 mg/ml. Quercetin was used as the standard. The chemical was solubilized in methanol to obtain concentration of 100 ppm. Quercetin solution (0, 5, 10, 15, 20, and 25 μ l) and samples (100 μ l) were added into each well. Deionized water was added so that the volume reached 108 μ l and followed by 6 μ l of NaNO₂ (5% w/v). After 5 min incubation period, 6 μ l of AlCl₃ (10% w/v) was added and the reaction mixture was allowed to react for 6 min. Later, 80 μ l NaOH (1 M) (100 μ l) was added. The absorbance was then read at wavelength of 510 nm using Varioskan™ Flash Multimode Reader (Thermo Scientific, Massachusetts, United States).

Determination of Dpph Free Radical Scavenging Activity

This antioxidant activity assay used radical 1, 1-diphenyl picrylhydrazyl (DPPH).¹⁸ Water extracts were solubilized in deionized water to obtain concentration of 2 mg/ml. From each extract solution, samples were taken with a set volume (12, 30, 62, and 125 μ l). Then, methanol was added until volume of methanol and extract mixture reached 200 μ l. Afterwards, 50 μ l of DPPH solution (from stock concentration of 40 ppm) was added. All of the samples were then placed at dark for 30 min. Later, the absorbance was measured at 515 nm using Varioskan™ Flash Multimode Reader (Thermo Scientific, Massachusetts, United States). Radical scavenging activity of extracts was expressed as inhibition concentration as follows:

$$\text{Inhibition concentration (\%)} = \frac{(A_b - A_s)}{A_b} \times 100\% \dots(1)$$

with A_b as absorbance of blank while A_s as absorbance of samples. Later, the quantification results were used to extract concentration values at 50% inhibition (IC₅₀) through extrapolation.

Determination of Cytotoxic Activity

Cytotoxic assay against MCF-7 cancer cells was determined using alamarBlue™ method.¹⁹ It measures viability of cancer cells by detecting molecular oxygen by addition of chromosphere

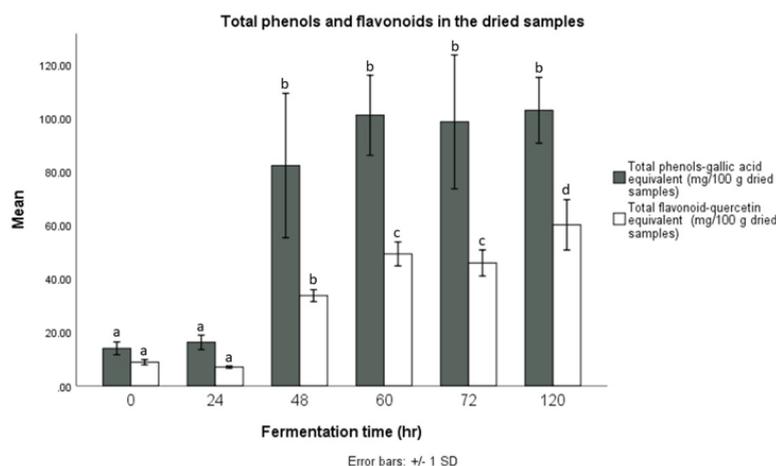


Fig.1: Level of total phenolic compounds-gallic acid equivalent and total flavonoids-quercetin equivalent (mg/100 g dried mass) from tempe fermented over varying time periods. Different letters on top of bars showed significant difference of means ($p < 0.05$)

resazurin that will transform to resafurin when it is reduced. 4 mg of samples were solubilized in 200 μ l of dimethyl sulfoxide (DMSO). This stock solution was then diluted to obtain a series of concentration (20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, and 0.039 mg/L). Breast cancer cells MCF-7 in Roswell Park Memorial Institute (RPMI) medium (1×10^4 cells/ml) were plated in a 96-well plate (100 μ l per well). The cells were kept for 24 hr and then the media was removed. Samples (5 μ l) with abovementioned concentrations were added. After re-adding RPMI and mixing, the cells were incubated for another 24 hr. Later, 10 μ l of alamarBlue reagent was added. The plates were then re-incubated for 4 hr at 37°C. Finally, the absorbance was measured at excitation wavelength of 560 nm and emission wavelength of 590 nm using Varioskan™ Flash Multimode Reader (Thermo Scientific, Massachusetts, United States). The data were then calculated as:

$$CV (\%) = \frac{Fl_{590} \text{ of sample} - Fl_{590} \text{ of negative control}}{Fl_{590} \text{ of positive control} - Fl_{590} \text{ of negative control}} \dots(2)$$

with CV as cell viability (%) and Fl_{590} referred to fluorescence intensity at 590 nm emission (560 nm excitation). Negative control was prepared by adding 100 μ l RPMI with 5 μ l RPMI and 10 μ l alamarBlue reagent. Positive control was prepared in a similar way, except for addition of 100 μ l cancer cells in RPMI, instead of RPMI only. Logarithmic values

of sample concentration were plotted as x-axis while percent viability was plotted as y-axis and inhibition concentration at 50% cell viability (IC_{50}) was determined.

Isoflavone Identification using LC-MS/MS

Detection of isoflavones were performed using LC-MS/MS consisting of a UPLC coupled to a quadrupole time of flight (QToF) (Waters Corporation, Milford, MA, United States). The samples were separated on an Acquity high strength silica (HSS) T3 column (1.8 μ m particle size, 100 mm x 2.1 mm i.d, Waters Corporation, Milford, MA, United States), with gradient separation using combination of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B), as described in Table 1. The mass spectrometer was operated in negative electrospray ionization mode using MSE function. The condition for detection by the mass spectrometry was as follows: capillary voltage 1.5 kV; source temperature 120°C; cone gas flow 50 L/h; cone at 30 V; desolvation temperature 500°C; and desolvation gas flow 1000 L/h.

Statistical Analysis

All assays were performed with duplicates from two different sample preparation, except for LC-MS/MS analysis that only used one replicate. Data were analyzed using one-way analysis of variance (ANOVA) then processed further for significance

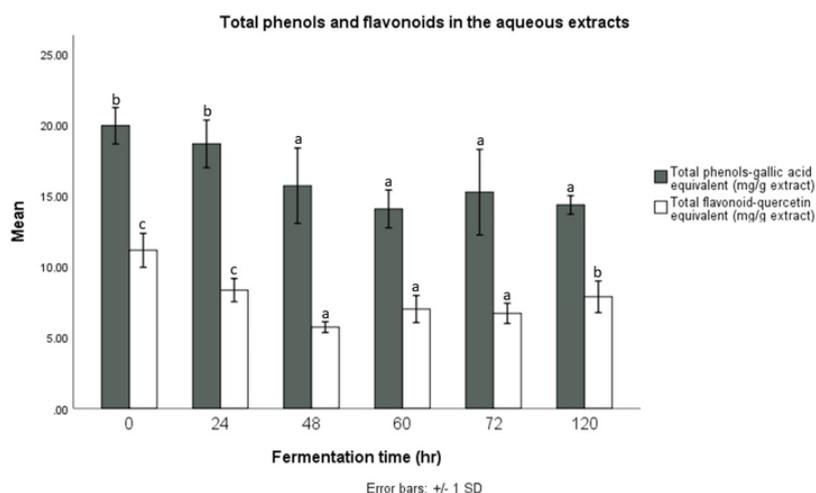


Fig. 2: Level of total phenolic compounds-gallic acid equivalent and total flavonoids-quercetin equivalent (mg/g extract) of aqueous extracts of tempe fermented over varying time periods. Different letters on top of bars showed significant difference of means ($p < 0.05$)

of differences among treatments using Duncan's multiple range test ($p < 0.05$). The statistical analysis was performed using IBM SPSS Statistics for Windows (IBM Corporation, Armonk, New York, United States).

Results

Determination of Total Phenols

Our data showed that concentrations of water-soluble phenols increased as soybean was fermented up to 60 hr. A very obvious increase was observed between 24 and 48 hours (Fig 1). From 48 to 120 hr, the concentrations were steady with slight decrease at 72-hr point. Meanwhile, for total phenols in the aqueous extracts of tempe, the concentrations decreased steadily as fermentation occurred between 0 and 60 hr. After 60 hr, an insignificant rise was detected but after 72 hr, the values declined again. The data also demonstrated that the maximum concentration of total phenols in the extracts was observed at control (Fig 2).

Determination of Total Flavonoids

Similar to total phenols, total flavonoids in the dried samples also tended to increase as fermentation was prolonged but in less obvious linear correlation as data at 24 and 72 hr disrupted the expected trend. The values decreased slightly between 0 and 24 hr and between 60 and 72 hr (Fig 1). Meanwhile, data of total flavonoids present in the extracts decreased as soybean was fermented up to 48 hr. Afterward, the data slightly increased when fermentation

was prolonged to 120 hr with an exception of an insignificant decrease between 60 and 72 hr (Fig 2).

Determination of DPPH Free Radical Scavenging Activity

DPPH antioxidant data did not show any straightforward relationship between fermentation time and IC_{50} values of the aqueous extracts. The lowest mean average for IC_{50} was observed at 24-hour fermentation (Fig 3), indicating that the treatment led to the highest DPPH free radical scavenging activity. A very notable increase in IC_{50} values was observed when fermentation time was extended from 60 to 72 hr. The maximum value was achieved at 72-hr period, showing low antioxidant activity. Further fermentation resulted in a sharp decline in IC_{50} values of the aqueous extracts.

Determination of Cytotoxic Activity

Breast cancer, which is prevalent among women, occurred as the result of abnormal cell growth that is affected by many factors including diet. Some food components may enhance the proliferation while others suppress it. The suppression or cytotoxic activity could be performed on culture cells. The cytotoxic activity was expressed as concentration that inhibited 50% of the proliferation of the cancer cells MCF-7 (IC_{50}). Lower IC_{50} values were commonly associated with higher cytotoxic activity of the extracts on the growth of the investigated cancer cell line which was MCF-7 for this study. Our data revealed a distinct relationship between fermentation

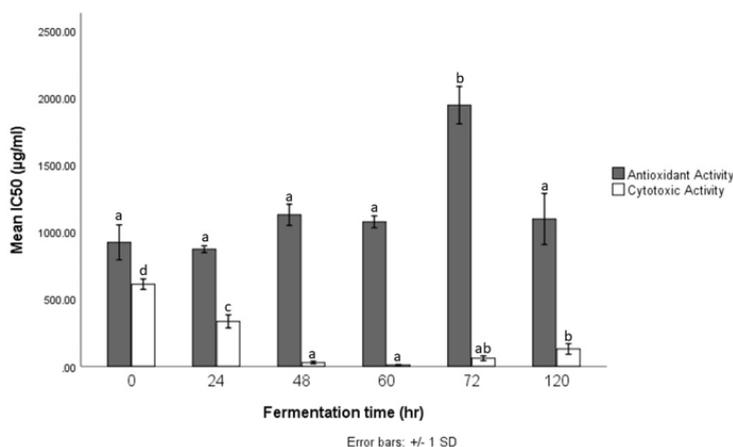


Fig. 3: DPPH free radical scavenging activity and cytotoxic activity of aqueous extracts of tempe fermented over varying time periods. Different letters on top of bars showed significant difference of means ($p < 0.05$)

time and cytotoxic activity. The IC_{50} values decreased as fermentation was performed from 0 (control) to 60 hr. After reaching the minimum value (8.7 ± 4.95 $\mu\text{g/ml}$) at 60 hr, the values increased slightly as fermentation was extended from 60 to 120 hr.

Isoflavone Identification using LC-MS/MS

Genistein was found at water extracts of non-fermented soybean and tempe fermented for 24 up to 120 hr. It was present at relatively high amount at non-fermented and 24-hr fermented tempe extracts. Extracts resulted from fermentation for 48, 60, 72, and 120 hr contained very minimum amount of genistein; however, there were slight increase in genistein content when fermentation time was extended from 48 to 60 hr but declined promptly when fermentation was further prolonged.

Similar to genistein, genistin, daidzein and daidzin were predominant at aqueous extracts of control (non-fermented soybean) and briefly (24 hr) fermented tempe (Fig 4). Afterward, the concentrations diminished remarkably, even to no response level for genistin and daidzin at 72 hr. Glycitein was observed at relatively low response for all treatments but somewhat higher at control and 24-hr fermented tempe. Meanwhile, glycitin was not detected in either control or tempe at all given fermentation durations.

The composition and percentage of each isoflavone was given in Fig 5. Aqueous extracts of control and 24-hour fermented tempe had very similar proportion

of the isoflavones. Composition of 48-hour tempe extract was also identical, but the percentages of both genistin and daidzin were larger while the proportion of genistein and daidzein declined. A notable change was observed after 48 hour fermentation. Portion of genistein and daidzein increased drastically while that of genistin and daidzin decreased. Similar finding was observed for 72-hr treatment.

Discussion

Our findings suggested that tempe fermentation altered total water-soluble phenolic compound and flavonoid profile of the products, as well as DPPH free radical scavenging activity, cytotoxicity and isoflavone profile of the extracts. Information regarding total phenolic compounds in food materials has been included in many studies on food functionality because this group of chemicals has been reported to show many health-promoting effects. Soybeans contained various phenolic compounds and water-soluble phenols comprised of approximately 68-81% of the total phenolic compounds.⁸ In general, the present study demonstrated that total water-soluble phenolic compounds of tempe increased as fermentation time was extended from 0 to 60 hr and afterward it tended to become steady. Total phenolic content in the dried unfermented soybean was much lower than that demonstrated by Kumar *et al.*,²⁰ and Alu'datt *et al.*,⁸ but it was expected considering the unfermented soybean in this present study had been clean, dehulled, soaked, boiled,

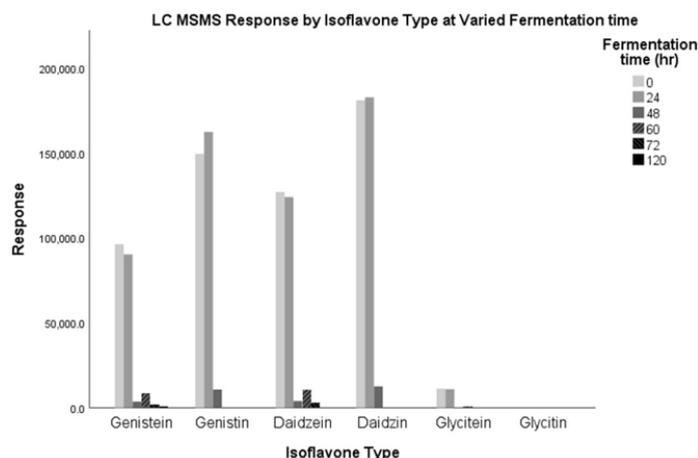


Fig. 4: Comparison of LC-MS/MS response of genistein, genistin, daidzein, daidzin, glycitein and glycitin of tempe fermented at varied fermentation time

and defatted as the steps were also performed on other treatments as preparation for fermentation and aqueous extraction. We predicted that some phenolic compounds were lost during the process. The most significant increase of total phenol content in the dried mass was observed between 48 and 60 hr fermentation, somewhat different with the findings of Kuligowski *et al.*,³ that showed the largest increase was between 24 and 48 hr fermentation. However, this dissimilarity was expected as it might be a consequence of different *Rhizopus* strains and fermentation condition. On the other hand, the relative steadiness of total phenolic content after 48 hr observation was similar to that of Kuligowski *et al.*,³ particularly of the *Rhizopus oligosporus* strain 2710.

Unfermented full fat soybean contained 10-40% of bound phenolic compounds.⁸ During fermentation, the mold *Rhizopus oligosporus* released hydrolytic enzymes that cleaved proteins, lipids, and starch¹¹ and consequently increased free water-soluble phenolic compounds as demonstrated in our findings and also confirmed by previous studies.^{3, 21-22}

Similar to total phenol in the dried mass of samples, our study showed that total flavonoids were inclined to increase as fermentation was prolonged up to 120 hr. Soybeans have been well known for its high flavonoid content that surpasses that of peanut, cowpea, mung bean, and lablab.²³ Total flavonoids of our samples, expressed as quercetin equivalent,

reached the highest points (62.59 mg/100 g dried mass) at 120-hr fermented tempe. This amount is somewhat lower than total flavonoids in soybeans (892.3-916.5 µg/g) as observed by Wang *et al.*²³ This inconsistency should be attributed to different sample tested (we used aqueous extracts while they used untreated soybeans and different analytical technique: they used HPLC analysis to quantify total of five flavonoids: myricetin, daidzein, quercetin, genistein and kaempferol while spectrophotometric method using quercetin as the standard was used in the present study. Besides, different soybean variety and weather condition where it was grown also might play a significant role.²⁴

Flavonoids and other phenolic compounds are often associated with health functionality. Flavonoids in plants consists of anthoxanthins (flavones and flavonols); flavanones; flavanonols; flavans (flavan-3-ols, flavan-4-ols, and flavan-3, 4-diols); anthocyanidins and isoflavonoids.²⁵⁻²⁶ While many compounds that belong to the group, for instance genistein; daidzein; quercetin; kaempferol, have been confirmed as antioxidants,²⁷ higher total phenols or flavonoids do not necessarily mean more intense antioxidant activity.^{20, 28} Our data also demonstrated low correlation between IC₅₀ DPPH free radical scavenging activity with either total phenols ($r=-0,432$) or total flavonoids ($r=0,534$) (Table 2). This could be a result of antagonistic effects of some compounds²⁹⁻³¹ or that the active compounds are not present in the amount at which they are

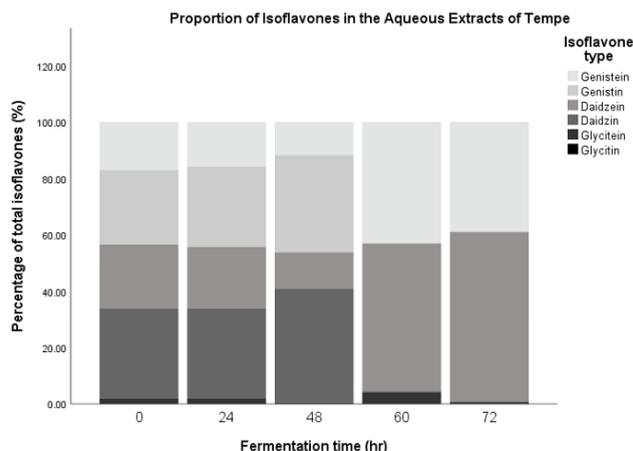


Fig.5 : Proportion of genistein, genistin, daidzein, daidzin, glycitein and glycitin in aqueous extracts of tempe

active as many functionality of the compounds are dose-dependent.^{6, 27}

A surprising fact was observed for relationship between cytotoxic activity and total phenols or flavonoids. Correlation coefficient of IC₅₀ cytotoxicity and total phenols and flavonoids are very positive and indicative of strong correlation: 0.926 and 0.956 for IC₅₀ cytotoxicity – total phenols and IC50 cytotoxicity – total flavonoids, respectively (Table 2). It signaled an inverse strong correlation between cytotoxic activity of the aqueous extracts of tempe against MCF-7 cancer cells and total phenols or flavonoids. This might be explained by negative effect of genistein when present at high concentration. LC-MS/MS responses showed that genistein was very intense at control and 24-hr fermented tempe extracts, and then drastically dropped to almost non-existent, except for a slight increase at 60-hr observation. Genistein had been reported to have both positive and negative effect in regards of cancer cell proliferation. It was believed to act as proliferation inhibitor at low concentration but induce the opposite effect as the concentration was increased.⁶

Our LC-MS/MS response for each isoflavone of aqueous extracts from tempe fermented at varying time demonstrated that both genistein and daidzein, two important isoflavone aglycones, were present at very high quantity at control and 24-hr tempe extracts, then the values dropped drastically to almost non-existent at 120-hr observation, with a slight increase at 60 hr. Mo *et al.*, argued that soybean variety, not fermentation, that largely contributed to concentration of isoflavone aglycones in soybean products because they found out that the level in tempe was not higher than that in tofu.⁷ Another possible explanation was that because water was not the best solvent for soybean isoflavone extraction,³² it could be that aglycone level increase during fermentation, as shown as increasing ratio of aglycones to their glycosides (Fig. 5), but only a portion was present in the water extracts.

Conclusion

We confirmed that tempe fermentation enhanced the release of water-soluble phenols, particularly flavonoids, and it was likely a consequence of hydrolysis promoted by the microorganisms. At several periods of fermentation, the extracts demonstrated cytotoxic activity, as expressed by noticeable reduction of MCF-7 cancer cell growth. In contrast, all extracts did not show any appreciable DPPH free radical scavenging activity. A deeper investigation on the extracts showed that the cytotoxic activity was inversely correlated with either total phenols or flavonoids and it was supported by LC-MS/MS data, suggesting that very high concentration of the compounds, particularly genistein, might trigger cancer cell proliferation. Prolonging fermentation up to 60 hr would result in low but optimum concentration of the isoflavones to achieve the intended cancer inhibition effect. To achieve a better understanding of this subject, the study should be expanded to investigation of isoflavone release during gastrointestinal digestion after tempe consumption in order to achieve recommended intake of the product.

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Conflict of Interest

We confirm that there are no known conflicts of interest associated with this study.

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