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# Isoflavones and Bioactivities in Over-fermented Tempeh Extracts

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## Abstract

Tempeh is nutritious food prepared through solid-state fermentation of cooked and dehulled soybeans with *Rhizopus* sp. for about 48 h. Fermentation beyond 48 h resulted in over-fermented tempeh. There may or may not have been similar research done before, especially related to its antioxidant and cytotoxicity. This study aims to determine the characteristics of fermented tempeh for up to 156 h. Samples were fermented at 0, 24, 48, 60, 72, 84, 96, 108, 120, and 156 h. Samples were dried, grounded, and extracted with acetone, followed by defatting with n-hexane. Extracts were dissolved in organic solvents for free radical scavenging activity (FRSA) and cytotoxicity assays. The 120-h tempeh extract, at the concentration of 1,000 µg/mL, demonstrated the highest FRSA (81.31% inhibition) against 100 µM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. Meanwhile, the 108-h tempeh extract at 1,000 µg/mL possessed the highest cytotoxicity ($IC_{50}$ of 2.54 µg/mL) against MCF-7 breast cancer cell lines. Liquid Chromatography-Mass Spectrometry/Mass Spectroscopy (LC-MS/MS) analysis revealed the presence of daidzin, genistin, daidzein, and genistein in all extracts. Extracts prepared from 108 h and 120 h tempeh stood out from other extracts in the Partial Least Square (PLS) bi-plot due to their high percentage of inhibition, low response of daidzin, and high responses of the other three isoflavones. The cytotoxicity assays of the standard isoflavones showed that genistein had the lowest $IC_{50}$ value at 4.82 ± 0.11 µg/mL. Standard genistein showed a low percentage of inhibition at 29.79 ± 9.10.

## 1. Introduction

Tempeh is a fermented soybean product and originally from Indonesia. It can be prepared from other pulses and leguminous seeds. Tempeh refers to those made from soybeans when it is mentioned with no further details in this article. Tempeh has gained popularity outside Indonesia as a meat substitute for vegetarians or vegans [1, 2, 3].

Tempeh is prepared from solid-state fermentation of pre-cooked soybeans using *Rhizopus* sp. Traditional inoculum is found in Indonesian Waru leaves (*Hibiscus tiliaceus*), predominantly consisting of *R. oligosporus* and *R. oryzae*. A commercial inoculum powder, a mixture of *Rhizopus* sp. and rice, is widely used by tempeh producers in Indonesia [3, 4, 5].

After 48 h of inoculation, tempeh is ready for harvest [6]. Even though the soybeans are pre-cooked, tempeh is usually further cooked and prepared as the main course, side dish, condiments, and beverage [1, 3]. Traditionally, consuming tempeh is believed to provide health benefits. The aforementioned has gained the interest of researchers and academicians. The health benefits of tempeh are diverse, including preventing flatulence, diarrhea, and cancer [3]. Those benefits are associated

with chemical changes during fermentation [2], which have been studied either as extracted or isolated compounds [7, 8, 9, 10].

Studies on tempeh expand to those who underwent fermentation for 48–72 h ("*tempeh semangit*" or slightly over-ripe tempeh) and more than 72 h ("*tempeh bosok*" or over-ripe tempeh). Traditionally, a small amount of either *tempeh semangit* or *tempeh bosok* is added to some dishes to impart flavor. This is due to the distinctive flavor, odor, and taste of the two types of tempeh, which are linked to the water-soluble components, including amino acids and peptides [11]. Besides its flavor, over-ripe tempeh was also studied for changes in its chemical contents and functionalities [7, 8, 9, 10]. For that purpose, over-ripe tempeh was extracted with various solvents, such as water [10], ethanol [9], and acetone [8]. Amongst the studies of over-ripe tempeh, most of them were prepared with distinct cultures. In contrast to Athaillah *et al.* [10] and Muzdalifah *et al.* [9] who used mixed culture that commercially available in Indonesia.

This study aimed to observe the bioactivities (cytotoxicity and FRSA) of the acetone extracts prepared from fermented tempeh, discover the type of isoflavones increasing the bioactivities, and classify the extracts based on their bioactivities and the drivers.

## 2. Methodology

The research process consisted of sample preparation, sample extraction, free radical scavenging activity (FRSA) assay, cytotoxicity assay, LC-MS/MS, and statistical analysis. The details of each step are explained below.

### 2.1. Materials, Tools, and Equipment

Soybeans were purchased from Rumah Tempeh Indonesia (RTI, Bogor, Indonesia). Tempeh inoculum RAPRIMA (PT Aneka Fermentasi Industri, Bandung, Jawa Barat, Indonesia) was used for fermentation. Genistin (#126M4188V, Sigma-Aldrich, St. Louis, MO, USA), Daidzin (#1400279V, Sigma-Aldrich, St. Louis, MO, USA), Genistein (#066M4075V, Sigma-Aldrich, St. Louis, MO, USA), and Daidzein (#WXBC1740V, Sigma-Aldrich, St. Louis, MO, USA) used as isoflavone standards.

Solvents for extraction (acetone and *n*-hexane) were technical grade and re-distilled for subsequent use. Solvents for FRSA assay were methanol (#822283, Merck Millipore, Burlington, MA, USA) and chloroform (#288306, Sigma-Aldrich, St. Louis, MO, USA).

An oven (Ecocell 22, MMM Medcenter Einrichtungen GmbH, Planegg, München, Germany) and a grinder (Neo Tokebi Plus, Buwon Electronic Co. Ltd., Dalseo-gu, Daegu, Korea) were used for sample preparations. A shaking incubator (DS-310C2, Dasol Scientific Co. Ltd., Hwaseong-si, Gyeonggi-do, Korea) and a centrifuge (Multi-purpose Centrifuge Combi 514R, with Rotor ABST-6, Hanil Scientific Inc., Gimpo-si, Gyeonggi-do, Korea) were used for sample extraction. A rotary evaporator (R-100, Rotavapor, BÜCHI Labortechnik AG, Postfach, Flawil, Switzerland) and a vacuum oven (VO400, Memmert GmbH + Co.KG, Schwabach, Bavaria, Germany) were used for solvent removal.

FRSA assay was done using a spectrophotometer (UV Vis Cary 60, Agilent Technologies, Santa Clara, CA, USA). The antioxidant property of extracts was determined using DPPH (#OF54G-GT, TCI, Tokyo, Tokyo Prefecture, Japan).

MCF-7 human breast cancer cell lines used in this assay were described in Jenie *et al.* [12]. The culture medium consisted of Roswell Park Memorial Institute (RPMI 1640, Lot #1937557, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA), sodium hydrogen carbonate (#K34791329526, Merck, Darmstadt, Hesse, Germany), Fetal Bovine Serum (FBS, #1907413, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA), Anti-Anti (#1924798, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA), and reversed osmosis (RO) water. The instruments used for cytotoxicity assay consisted of a $CO_2$ incubator (Thermo Fisher Scientific, Waltham, MA, USA) and a microplate reader (Varioskan™ Flash Multimode Reader, Thermo Fisher Scientific, Waltham, MA, USA). Dimethyl sulfoxide (DMSO, #30.06.08, Merck, Darmstadt, Hesse, Germany) and alamarBlue™ reagent (#767556, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) were used as reagents for cytotoxicity assay.

A Xevo G2-XS Quadrupole-Time of Flight (Q-TOF) Spectrometry (Waters Corporation, Milford, MA, USA) equipped with the Waters Acquity Ultra Performance Liquid Chromatography (UPLC) I-Class series autosampler, a binary pump and a thermostatted column compartment was used. LC separation was performed on a 100 mm × 2.1 mm ID 1.8 µm Acquity High Strength Silica (HSS) T3 column (Waters Corporation, Milford, MA, USA).

### 2.2. Sample Preparation

Tempe production was performed at RTI (Bogor, Indonesia) following the procedure of Tamam *et al.* [6] with some modifications (Figure 1). Before inoculation, one kilogram of the dehulled soybeans was collected as control and frozen before subsequent use. Tempe incubation was done at 30–37°C and 70–85% of humidity. Some samples were harvested immediately after 24 h fermentation. In contrast, others were transferred to a room with good air circulation for further fermentation and collected when the fermentation time reached 24, 48, 60, 72, 84, 96, 108, 120, and 156 h. In order to the fermentation process, samples were quickly placed in a freezer (-20°C) [13]. This chilling temperature was 50°C lower than the optimum temperature growth for *R. oryzae* [14]. Dehulled soybeans and tempeh samples were thawed at 4°C for 24 h and dried in an oven at 50°C for 24 h. Dried soybeans and dried temple samples were ground to a fine powder. The powder was then sieved, kept in an airtight container at 4°C until further analysis.

### 2.3. Sample Extraction

Extraction was performed with acetone (Figure 2). Powdered samples (100 g) were transferred into flasks and acetone was added with ratio of 1:10 w/v. The flasks were placed in a shaking incubator for 2 h at 45°C with the

orbital shaking speed of 175 rpm. After vacuum filtration, the acetone fraction was evaporated in a rotary evaporator with water bath temperature at 56°C to obtain crude acetone extract.

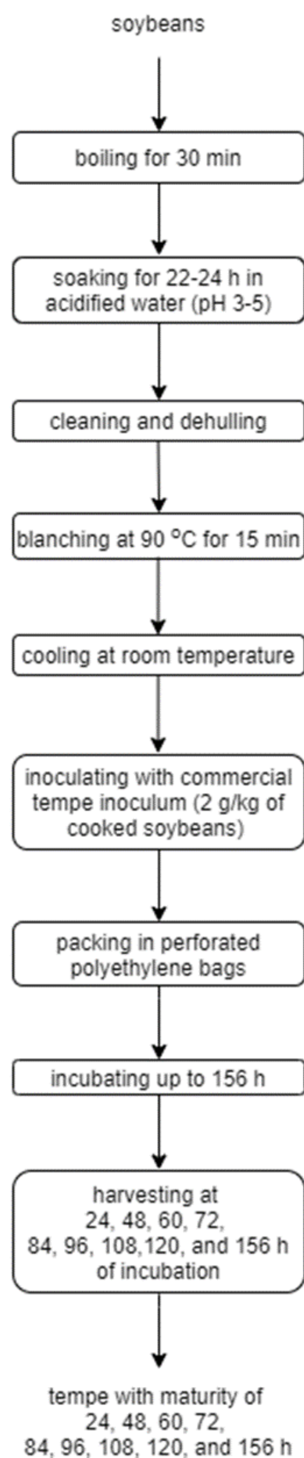


**Figure 1.** Scheme of tempeh production in RTI (Bogor, Indonesia) according to Tamam *et al.* [6] with modification to produce tempeh with various maturity

For defatting purposes, crude extract was mixed with 1:2 (v/v) *n*-hexane ratio in a centrifuge tube. The tube was turned upside down 25 times to allow good mixing. Separation was done using a centrifuge at 3,300×g for 15 minutes. The *n*-hexane phase, as the upper layer, was removed from the centrifuge tubes by pipetting. This defatting procedure was repeated two more times. The remaining bottom layer was then collected and evaporated using a rotary evaporator at 50°C. The evaporated samples were collected, transferred into wide neck glass jars, and placed in a vacuum oven at 30°C overnight to remove *n*-hexane residue. The lids of the glass jars containing tempeh acetone extracts were put on and the jars were stored in a freezer at -20°C until further analysis.

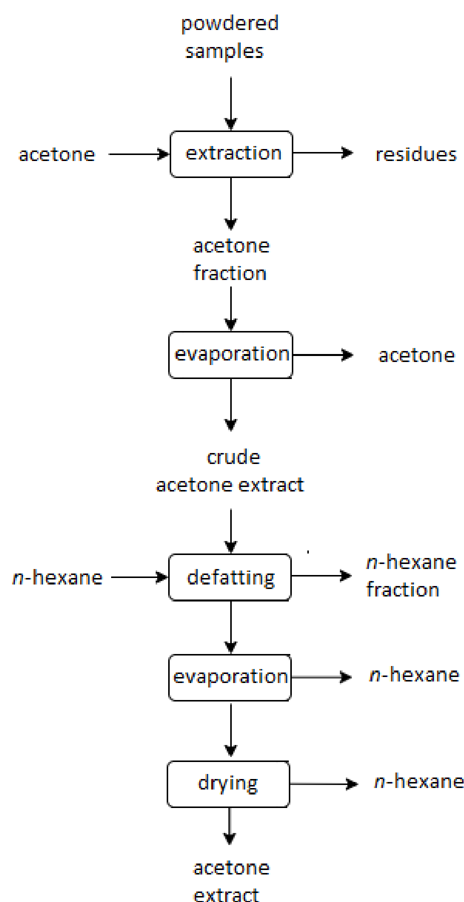


**Figure 2.** Scheme of tempeh extraction with acetone

### 2.4. FRSA Assay

The antioxidant property of each extract was determined based on the scavenging effect on DPPH as described by Dewijanti *et al.* [15] with some modifications. Extracts (10 mg) were dissolved in 8 mL of a mixture of chloroform and methanol (1:3). From each extract solution, 2 mL of samples were taken and added with 0.5 mL of 0.5 mM DPPH solution. Blank was prepared by adding 0.5 mL of 0.5 mM DPPH solution to 2 mL of solvent mixture (with chloroform to methanol ratio of 1:3). All samples and blank were then placed in the dark for 30 minutes at room temperature. The absorbance was immediately measured at 518 nm using a spectrophotometer. Analyses were performed in duplicates. Radical scavenging activity was expressed as inhibition concentration and calculated using the following equation:

$$Inhibiton\ concentration\ (\%) = \frac{(A_b - A_s)}{A_b}\ x\ 100\ \% \quad (1)$$

where $A_b$ was the absorbance of blank while $A_s$ was the absorbance of samples.

### 2.5. Cytotoxicity Assay

Cytotoxicity was determined by an anticancer assay using the alamarBlue™ method [16]. It measures cancer cells' viability by adding blue and virtually non-fluorescent resazurin that will transform to red and highly fluorescent resorufin when it is reduced by living cells.

MCF-7 cell lines were cultured in RPMI medium was prepared by diluting RPMI 1640 Medium and 2.2 grams of sodium hydrogen carbonate in 1 L of reverse osmosis (RO) water. The medium was supplemented with 10% FBS and 1% Anti-Anti. MCF-7 cell lines were cultured in a $CO_2$ incubator at 37°C and 5% $CO_2$.

Approximately 4 mg of extract were dissolved in 200 µL of DMSO. This stock solution was then diluted to obtain a series of concentration (20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.1562, 0.0781, and 0.0390 µg/mL).

MCF-7 in RPMI solution (1×104 cells/ml) was transferred into a 96-well plate (100 µL per well) and incubated in a $CO_2$ incubator at 37°C for 24 h. Samples (5 µL) with the serial dilution concentrations were added to the cells. The positive control was prepared in a similar way except that 5 µL of DMSO was added instead of sample solutions. The negative control consisted of 100 µL of RPMI and 5 µL of DMSO without any added cells (Table 1).

**Table 1.** The terminologies used during the cytotoxicity assay

| Terminologies | MCF-7 in RPMI solution | RPMI solution | Extract in DMSO | DMSO |
|---|---|---|---|---|
| Treated cells | 100 µL | 0 µL | 5 µL | 0 µL |
| Positive control | 100 µL | 0 µL | 0 µL | 5 µL |
| Negative control | 0 µL | 100 µL | 0 µL | 5 µL |

All treated cells, positive control and negative control, were incubated for another 24 h. Later, 10 µL of alamarBlue™ was added. The plates were then re-incubated for 4 h at 37°C. Finally, the absorbance was measured with a microplate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The data were then calculated as below:

$$\%\ \text{Cell viability} = \frac{FI_{590}\ \text{of treated cells} - FI_{590}\ \text{of negative control}}{FI_{590}\ \text{of positive control} - FI_{590}\ \text{of negative control}} \times 100$$

Where $FI_{590}$ referred to fluorescence intensity at 590 nm emission. Logarithmic sample concentration values 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 standards were diluted in DMSO the same way as tempeh extracts and subjected to cytotoxicity assay according to the protocols mentioned above.

### 2.6. LC-MS/MS

Tempeh extracts and isoflavone standards were solubilized in 10 mg/mL and 1 mg/mL methanol, respectively. The elution was performed at 0.3 mL/min using 0.1% (w/v) formic acid in water (solvent A) and 0.1% (w/v) formic acid in methanol (solvent B), with gradient elution as described in Table 2. The mass spectrometer was operated in positive electrospray ionization mode using the Mass Spectroscopy Engine (MSE) function. The condition of 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 1,000 L/h. The mass range detection started at 100.00 m/z and ended at 1,200.00 m/z.

**Table 2.** Gradient elution for isoflavone separation

| Time (min) | Flow rate (mL/min) | Composition of solvent A (%) | Composition of solvent B (%) |
|---|---|---|---|
| 0 | 0.3 | 90 | 10 |
| 1 | 0.3 | 90 | 10 |
| 3 | 0.3 | 70 | 30 |
| 11 | 0.3 | 0 | 100 |
| 13 | 0.3 | 0 | 100 |
| 15 | 0.3 | 90 | 10 |

The mass range detection started at 100.00 m/z and ended at 1,200.00 m/z. The LC-MS/MS data preprocessing and data extraction were performed using the UNIFI Scientific Information System from Waters Corporation.

### 2.7. Statistical Analysis

Analysis of variance (ANOVA) followed by Tukey (HSD) at 95% level of confidence and PLS analysis was conducted using XLSTAT version 2021 software (Addinsoft, New York, NY, USA), an add-on for Microsoft Excel.

## 3. Results and Discussion

### 3.1. FRSA

Tempe extracts displayed an increased percent of inhibition with increasing fermentation time (Figure 3). This was observed at a 1,000 µg/mL concentration against 100 µM DPPH. The highest percent of inhibition was achieved at 120 h fermentation. Nevertheless, fermentation within the period of 84–156 h did not result in any significant inhibition percentage changes.

Previously Chang *et al.* [7] studied FRSA of tempeh extracts, prepared from tempeh which underwent fermentation up to 10 days. Tempe was extracted orderly with various solvents, e.g., *n*-hexane, petroleum ether, ether, ethanol 95%, and water as the last serial solvent. In general, their tempeh extracts displayed increased FRSA against DPPH with increasing fermentation time. According to Chang *et al.* [7], the increased FRSA in tempeh compared to cooked soybeans was mainly due to the *R. oligosporus* fermentation. They proposed that diverse compounds with different polarities were formed or released during fermentation and exhibited high FRSA, particularly at 60–120 mg/mL of extract. They also concluded that optimum extraction was achieved using water and 95% ethanol.

Nevertheless, the increase in FRSA in the study conducted by Chang *et al.* [7] was observed at ≤60 mg/mL concentration against 125 µM DPPH. Their inhibition concentration was dramatically higher when compared to

the results of this study. However, with a slight difference in concentration of DPPH, the antioxidant activity of tempeh extracts was much higher than theirs. This difference possibly was due to different cultures, fermentation conditions, and extraction protocols.

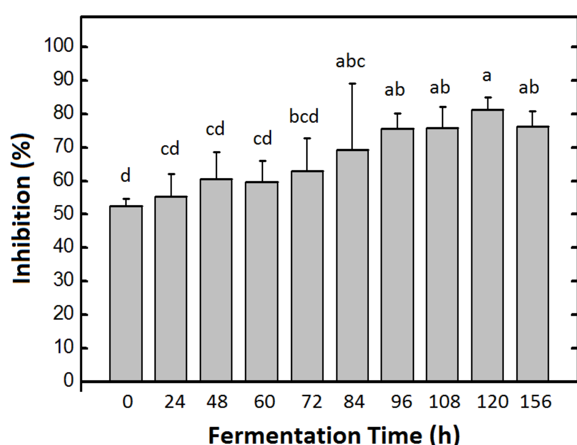


**Figure 3.** FRSA of tempeh extracts as expressed by percentage of inhibition, observed with increasing fermentation time (h)

A previous study on over-ripe tempeh showed that ethanol extract of 120 h tempeh at 1,000 µg/mL that had ~75% of inhibition against 100 µM DPPH [9] was slightly lower than the counterpart acetone tempeh extract (~80%). Even though the acetone extracts in this study showed similar antioxidant activity with ethanol extracts from a previous study by Muzdalifah *et al.* [9], the compounds responsible for that activity might differ. According to Yoshiara *et al.* [17], aglycone isoflavones were less polar than glycoside isoflavones. The empirical parameters of ethanol and solvent polarity (*ET*) of acetone were 51.9 kcal/mol and 42.2 kcal/mol, respectively [18]. Since a higher *ET* value refers to a more polar solvent while *n*-hexane had an *ET* value of 30.9 kcal/mol, acetone was less polar than ethanol.

Murakami *et al.* [19] showed that genistein and daidzein were the main isoflavones contributors to the antioxidant activity of methanol tempeh extract (prepared after 40 h of fermentation). They were transformed from their corresponding glycosides. Other compounds in tempeh that also displayed antioxidant activity of tempeh were peptides [6].

Aglycone isoflavone standards in this study were subjected to a DPPH assay. Genistein and daidzein at 1,000 µg/mL displayed inhibition of 29.79 ± 9.10 % and 29.06 ± 7.00 % against 100 µM of DPPH, respectively. This result showed the potency of aglycone isoflavones as the contributors to the overall FRSA of tempeh extracts. The LC-MS/MS chromatographs revealed their existence in the acetone extracts of over-ripe tempeh.

### 3.2. Cytotoxicity

A cytotoxicity test was performed on all acetone tempeh extracts (Figure 4). The lowest $IC_{50}$ was 2.54 ± 0.30 µg/mL, displayed by the extract from tempeh that underwent 108 h fermentation. Fermentation within 96–120 h did not significantly differ in $IC_{50}$. Prior to fermentation, the $IC_{50}$ of boiled and dehulled soybeans was 515.50 ± 36.06 µg/mL, indicating lower cytotoxic control activity than tempeh extracts. It is very likely that *Rhizopus* sp. releases various enzymes (proteases, lipases, carbohydrates, and phytases) during fermentation which degrade macromolecules of cooked soybeans into compounds with smaller molecular weights [2, 3], with a capacity to inhibit MCF-7 cancer cell growth.

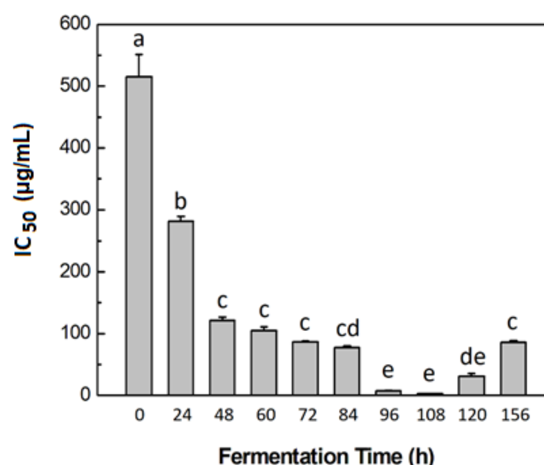


**Figure 4.** $IC_{50}$ (µg/mL) of acetone tempeh extracts, observed with increasing fermentation time (h).

Soy proteins have been indicated to have a significant role in lowering the risk of death and the occurrence of breast cancer in women. Among many components of compounds in soybeans, isoflavones are component that can decrease the risk of breast cancer [20]. Tempe was among the lists of soy products associated with a reduced risk of breast cancer [3]. The composition of isoflavones in tempeh is different from those in soybeans. Isoflavone glucosides (daidzin and genistin) in tempeh increased while the corresponding aglycones (daidzein and genistein) decreased [19].

In order to check the cytotoxicity of the presented acetone tempeh extracts were contributed by isoflavones, four isoflavone standards were tested (Table 3). Genistein which displayed an $IC_{50}$ below 5 µg/mL, indicated that this isoflavone was active as an anticancer agent. The acetone extracts most likely contained genistein since it was a less polar isoflavone.

Table 3. Cytotoxicity of isoflavone standards

| Standards | IC50 (µg/mL) |
|---|---|
| Daidzin | > 100 |
| Genistin | > 100 |
| Daidzein | > 100 |
| Genistein | 4.82 0.11 |

### 3.3. LC-MS/MS of Isoflavones

LC-MS/MS was performed on selected acetone extracts. The responses of four isoflavones (daidzin, genistin, daidzein, and genistein) were recorded. All four responses decreased during fermentation up to 48 h. Extending fermentation from 60 h to 120 h increased the responses of genistin, daidzein, and genistein; although the response of genistin was still considerably low

compared to those of aglycones (Figure 5). Acetone was possibly more appropriate to extract aglycone isoflavones rather than glycosidic isoflavone.

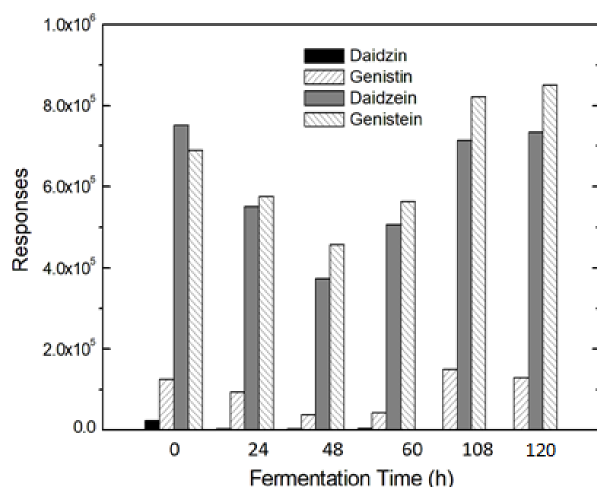


**Figure 5.** Responses of LC-MS/MS toward four isoflavones in acetone extracts prepared from tempeh with various fermentation times

The decrease of daidzin and genistin concentrations and increase of their corresponding aglycones during tempeh making (36 h of fermentation) was reported by Wuryani [21]. Depending on the used cultures, the extend of either increase or decrease varied from 2 to 9 folds. In this study, the reasons behind the changing responses of genistin, daidzein, and genistein against increasing fermentation time up to 120 h were not yet known (Figure 5). Extending fermentation time could incorporate other microorganisms other than *Rhizopus* sp. which affects isoflavones production.

### 3.4. PLS Analysis

PLS analysis is often used to relate the calculated field parameter properties and the observed biological activities [22]. The resultant bi-plot is later utilized to determine the drivers for the dependent variables. Sample identities are also anchored in the bi-plot so that their correlation with independent and dependent variables is described [23, 24]. In this study, the dependent variables (Ys) were FRSA (expressed as a percentage of inhibition) and cytotoxicity (expressed as $IC_{50}$), and the independent variables (Xs) were isoflavone data. The dependent variables were significantly ($p<0.05$) correlated with a Pearson correlation of -0.740. Thus, the dependent variables could be represented by either FRSA or cytotoxicity. In this study, FRSA was selected for subsequent PLS analysis. Both data sets were standardized to ZXs and ZY, resulting in a respective mean value of 0 and a standard deviation of 1 prior to PLS analysis.

The PLS regression was stopped after two components. It resulted in $Q^2$ cumulated index of 0.591. $R^2X$ cum and $R^2Y$ cum were and 0.858 and 0.977, respectively. Both $R^2ZX$ cum and $R^2ZY$ cum were close to 1 with two components. This indicated that the two components generated by PLS regression summarized both ZXs and ZY well.

As shown in Figure 6, The PLS bi-plot displayed the ZXs and the ZY variables far from the center. The correlation between the ZXs and the two components was good, as well as ZY. A strong correlation between genistein, genistin, and daidzein was observed.

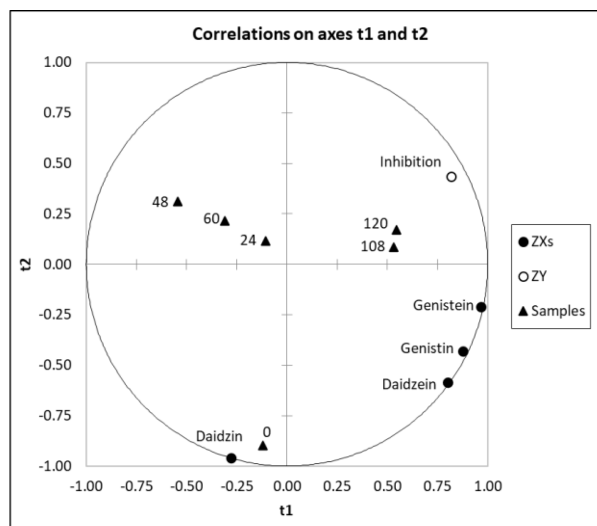


**Figure 6.** PLS bi-plot of the standardized isoflavones (ZXs) and bioactivity data (ZY) of the acetone extracts prepared from tempeh with various fermentation times, i.e., 0, 24, 48, 60, 108, and 120 h

The PLS bi-plot (Figure 6) showed that the samples were distinguished well. The extracts of 108 h and 120 h fermented tempeh formed a cluster. The inhibition was anchored relatively nearby, which means that both extracts stood out from the other samples due to the high percentage of inhibition. They appeared to be characterized by high responses of genistin, daidzein, and genistein, however slow response to daidzin.

In contrast, sample 0 h was anchored close to daidzin due to high daidzin response. This sample also displayed high responses of the other three isoflavones.

Another cluster on the right top of the bi-plot consisted of 24 h, 48 h, and 60 h samples. They showed moderate responses of daidzein, genistin, and genistein and slow response of daidzin. In addition, their percentage of inhibition displayed neither low nor high values.

From this analysis, it appeared that the presence of daidzin in the extracts reduced the percentage of inhibition. Daidzin is a glycoside. The bioactivities of isoflavone glycosides including antioxidant were lower than those of the corresponding isoflavone aglycones [25, 26].

## 4. Conclusion

Fermentation of cooked soybeans with commercial inoculum rich in *Rhizopus* sp. was done up to 156 h and samples were collected at several time points. Differences in FRSA activity against DPPH and cytotoxicity against MCF-7 breast cancer cell lines were observed in the acetone extracts at concentration of 1,000 µg/mL. FRSA increased with increasing fermentation times, with a maximum of ~80% inhibition, achieved at 120 h of fermentation. No significant differences in FRSA were

obtained for extracts prepared from 84–156 h tempeh. Meanwhile, the $IC_{50}$ study against MCF-7 breast cancer cell lines displayed the lowest value at 2.54 µg/mL after 108 h fermentation; and no significant ($p>0.05$) difference was observed in comparison to extracts prepared from 96–120 h fermentation. The LC-MS/MS observation revealed the presence of daidzin, genistin, daidzein, and genistein in all extracts even though no clear relationship between increasing fermentation time. The extracts prepared from 108 h and 120 h tempeh formed a cluster in the PLS bi-plot, which displayed a high percentage of inhibition. This cluster was characterized by the slow response of daidzin and high responses of the other isoflavones, particularly daidzein and genistein. In general, isoflavone aglycones had better bioactivities than the corresponding isoflavone glycosides. Nonetheless, the anticancer activity ($IC_{50} < 5$ µg/mL) was displayed only by genistein standard while the other isoflavone aglycone (daidzein) and both glycoside standards had $IC_{50} > 100$ µg/mL. At the same concentration of 1,000 µg/mL, both aglycone standards showed nearly 30% inhibition against DPPH 100 µM, that value much lower than those of tempeh extracts regardless of the fermentation time.

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