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# Antioxidant and Cytotoxicity Properties of Tempe Oils from Various Fermentation Times

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The present study investigated the antioxidant and cytotoxic activities of tempe oils prepared from 0–156 h of fermentation. Extraction was performed using hexane, with or without prewashing with acetone or ethanol. Oils extracted with hexane only displayed lower antioxidant activity than that of corresponding oil collected with prewashing. The antioxidant activity of the former oils was not sinificantly ($p>0.05$) affected with fermentation time. Oils extracted with acetone prewashing showed increasing antioxidant activity as fermentation time continued whilst those collected ethanol prewashing did not display clear function with fermentation time. Oils from non-fermented soybean had IC-50 higher than 100 ppm regardless their methods of collection. Fermentation for 120 h reduced their IC-50 to less than 20 ppm. Improved antioxidant and cytotoxic properties of tempe oils was possibly due to formation of fat soluble antioxidants and other minor compunds during fermentation.

## Introduction

Tempe is nutritious food, originally from Indonesia, and usually made of soybeans. Boiled and dehulled soybeans are inoculated with *Rhizopus spp* moulds, and result in a solid cake with a whitish appearance. Mature tempe is usually ready for consumption or cooking after 48 h of fermentation. Although tempe is often kept to undergo further fermentation, i.e, for 72 h and 120 h, to produce *tempe semangit* and *tempe bosok*, respectively; both are used as flavour booster in Javanese traditional foods due to their umami taste (Owens et al., 2014).

Studies on health benefit studies of tempe have been started a half century ago (György, 1964) and became more intensive in the last two decades although the scientific publications are not as plentiful as those of other protein sources. Besides the nutritional values, health benefits of tempe is often associated with antioxidant (Owens et al., 2014), anticancer (Astuti et al., 2000), and antibacterial properties (Roubos-van den Hil et al., 2010), either as whole tempe (Astuti et al., 2000) or as non-oil extracts (Roubos-van den Hil et al., 2010).

The oil fraction of tempe has not been intensively studied yet, although the percentage of fat in tempe is significant (25% w/w dry basis) (Owens et al., 2014). An earlier study suggested that dried tempe was less prone to oxidation than dried soymeal and it was possibly due to tempe oil (György et al., 1974). A 10% addition of tempe oil to either lard or vegetable oils reduced oxidation up to 70% (Bégin et al., 1988) and later the claims were patented (György, 1973; 1974). Substances which are soluble in tempe oil were suggested to contribute to the antioxidative effect (Hoppe et al., 1997; Murakami et al., 1984). Studies on the antioxidant of tempe oil, however, have not progress much since then.

On the other hand, some studies on health potency of tempe showed that increased fermentation time induced more released of bioactives (Kuligowski et al., 2016; Chang et al., 2009). A study by Kuligowski et al., (2016) showed that acetone (70%) extract of over fermented tempe, *i.e.*, 5 day tempe, displayed significantly higher antioxidant strength, nearly 12 times, than those of unfermented soybeans (Kuligowski et al., 2016). Similarly, Chang et al., (2009) with various solvents showed that antioxidant activity of 10 day tempe extracts was two to four folds those of the corresponding unfermented soybean extracts. Chang et al., (2009) included hexane as one of the solvents, which implied that oil fraction of 10 day tempe was significantly higher in antioxidant strength than soybean oil. Previously, Murakami et al., (1984) presented that oil extracted from 40 h tempe hardly increased its peroxide value (PV) whilst oil from unfermented soybean experienced dramatic PV over 14 day incubation in the dark at 37 °C; it was believed that antioxidant compounds were behind tempe oil stability. To the best of our knowledge, other scientific publications on antioxidant potency of tempe oil as influenced by fermentation time are scarse.

Thus, our study aimed to investigate the effect of fermentation time on antioxidant activity of tempe oil. In addition, cytotoxicity of tempe oil is also studied since antioxidant activity is somehow related to anticancer activity.

## Materials and Methods

### Materials

Soybeans were purchased from Rumah Tempe Indonesia (RTI, Bogor, Indonesia). They were genetically modified type. Materials for analysis were pro-analys grade. Isoflavon standards were purchased from Sigma-Aldrich with this following specifications: Genistin (G0897, CAS: 529-59-9, Lot #126M4188V), Daidzin (30408, CAS: 552-66-9, Lot #1400279V), Genistein (G6776, CAS: 446-72-0, Lot #066M4075V), and Daidzein (16587, CAS: 486-66-8, Lot #WXBC1740V).

### Tempe production

Soybeans were cleaned from solid aggregates, washed, and then boiled (100 °C, 30 min). After soaking for 22–24 h in acidified water (pH 3–5), the soybeans were dehulled mechanically. The dehulled soybeans were then rinsed with boiling water for 15 min. Later, they were strained and allowed to reach room temperature prior to subsequent processing. One kilogram of the soybeans was collected as control. The rest was mixed with inoculum (Raprima, PT Aneka Fermentasi Indonesia, Bandung, Indonesia) with the ratio recommended by the manufacturer (2 g inoculum/kg soybean). The mixture was then packed in perforated polyethylene bags and placed in a room with controlled condition (30–37 °C; 70–85% humidity) for 24 h. Some samples were harvested immediately after 24 h fermentation, while others were transferred to a room with good air circulation for further fermentation. Other samples were collected when fermentation time reached 48, 60, 72, 84, 96, 108, 120, and 156 h. They were quickly placed in a freezer (–18 °C) to stop the fermentation.

### Tempe powder preparation

Samples were thawed in a refrigerator (4 °C) for approximately 24 h. Later, samples were weighed, sliced, and then dried in a microwave (Sharp Microwave Oven R-222Y (S), Sharp Corporation, Thailand). 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, kept in an airtight container at 4 °C until further analysis.

### Oil extraction

Powdered samples (approximately 40 g) were transferred into extraction flasks. Hexane, acetone, or ethanol was added to the samples with the ratio of 1:10. The flasks were put in a shaking incubator (DS-310C2, Dasol Scientific Co. Ltd, South Korea) for 2 hours, at 45 °C with orbital shaking speed of 175 rpm. Afterward, samples were filtrated using filter papers under vacuum condition. The filtrate was evaporated (Eyela NE Series, Tokyo Rikakikai Co. Ltd, Japan) at 50 °C and orbital speed at 15 rpm until most solvent evaporated. The concentrated filtrate were put in an (Venticell, MMM Medcenter Einrichtungen GmbH, München, Germany) at 50 °C for 30 min to remove the remaining solvent. Tempe oil was collected if the added solvent was hexane (Fig.1A). Acetone or ethanol extract was obtained when the added solvent was acetone or ethanol, respectively (Fig.1B and C).

Hexane was added at room temperature to each extract with the ratio of 1:2 in a 50 mL centrifuge tube. The tube was turned upside down for 25 times to allow oil extraction. Oil rich hexane fraction was separated using a centrifuge 3,000 rpm for 15 min (Multi Purpose Centrifuge Combi-514R, Rotor A50S-8, Hanil Science Industrial Inc., Gimpo, South Korea). This hexane extraction was performed in three stages. The first, the second, and the third filtrates were combined and evaporated at 50 °C and orbital speed at 15 rpm to remove most of hexane. The concentrated oil fractions were put in an oven at 50 °C for 30 min to remove the remaining hexane. Extraction method that was initially started with acetone was named as extraction with acetone prewashing (Fig.1B) whilst the other was called extraction with ethanol prewashing (Fig.1C). All resultant oils were transferred to bottles and kept at 4 °C in dark.

### Antioxidant assay

The antioxidant property of each oil was determined based on scavenging effect on free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Aluko and Monu (2003) with a modification. Extracts (10 mg) were solubilized in 2 mL of chloroform and shaked until dissolved. Later, 6 mL of methanol was added and the mixture was reshaked. From each extract solution, 2 mL 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 dark for 30 min at room temperature. The absorbance was immediately measured at 518 nm using Spectrophotometer UV Vis Cary 60 Agilent (Agilent Technologies, Santa Clara, United States). Analysis were performed in duplicates. Radical scavenging activity of extracts was expressed as inhibition concentration and calculated using the following equation:

$$\text{Inhibition concentration (\%)} = \frac{\quad}{\quad} \qquad (1)$$

Where $A_b$ was absorbance of blank while $A_s$ was absorbance of samples.

### Citotoxicity assay

Cytotoxicity was determined by anticancer assay using alamar BlueTM method (Forster et al., 2013). It measures viability of cancer cells by detecting molecular oxygen by addition of chromosphore resazurin that will transform to resafurin when it is reduced. Approximately 4 mg of samples were solubilised in 200 µL 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 ppm). Breast cancer cells MCF-7 in Roswell Park Memorial Institute (RPMI) solution (1x104 cells/ml) were transferred into a 96-well plate (100 µL per well). The cells were kept for 24 h. Samples (5 µL) with above mentioned concentrations were added. The cells were incubated for another 24 h. Later, 10 µL of Alamar Blue reagent was added. The plates were then re-incubated for 4 h 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 below:

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

Where $FI_{590}$ referred to fluorescence intensity at 590 nm emission. Negative control was prepared by adding 100 µL of RPMI with 5 µL of DMSO and 10 µL of alamar Blue™ reagent. Positive control was prepared in a similar way except for addition of 100 µL 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.

### Statistical analysis

Data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey (HSD) test were performed in the experiments with 95% level of confident.

## Results and Discussion

Three types of oils were obtained through different extraction schemes as explained in Fig.1. These oils look different in appearances (Fig.2).

Three types of tempe oils showed different trends of antioxidant activity as influenced by fermentation time. Oils that were collected through tempe extraction with hexane displayed no significant effect ($p$<0.05) of fermentation time on antioxidant activity (Fig.3).

A slight increase of antioxidant activity was observed at fermentation time ≥ 60 h although it was not significant. In general, their antioxidant activity were relatively low as none exceeded 15% inhibition at 1,000 ppm.

When oil extraction was preceeded by either acetone or ethanol prewashing, the resultant oils displayed higher scavenging effect on DPPH (Fig.4A and B, respectively) than those extracted using hexane only (Fig.3).

Daidzein and genistein standards were also subjected to DPPH assay at 1,000 ppm as a comparison. The percent inhibition of daidzein and genistein was 23.23±1.24 % and 34.62±0.86 %, respectively.

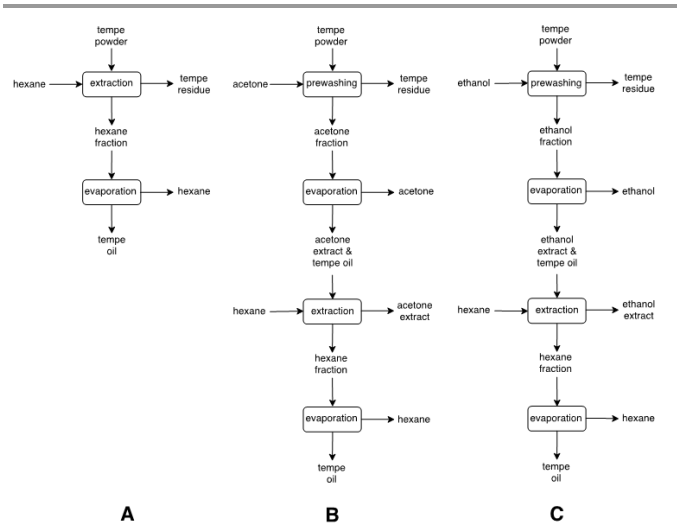


Fig. 1. Schematic diagram of tempe oil extraction, without prewashing (A) and with either acetone (B) or ethanol (C) prewashing.

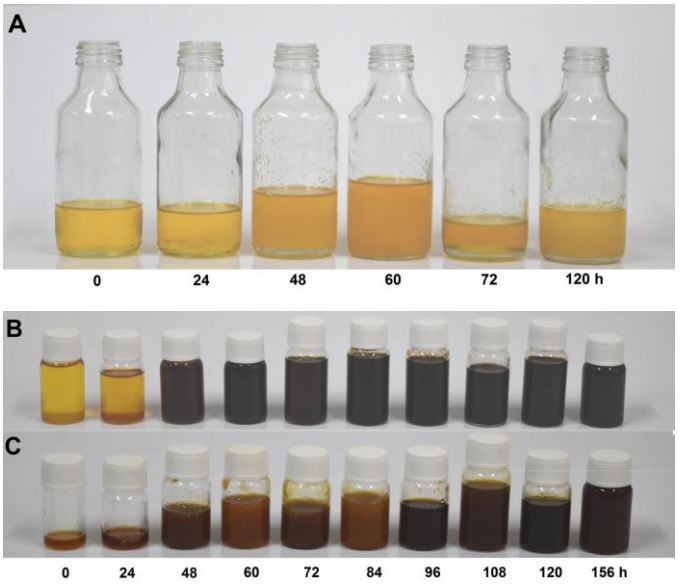


Fig. 2. Oils from tempe with various fermentation times, extracted with hexane, without (A) and with prewashing with etiher acetone (B) or ethanol (C) as illustrated by Fig.1A, B, and C, respectively.

Oils collected from 120 h tempe were selected for cytotoxicity test because this fermentation time was the maximum for tempe oils extracted with hexane only. Therefore, the other types of oils at the same maturity stage were selected for subsequent cytotoxicity test. It is interesting that oils from 120 h tempe which were prepared with acetone and ethanol washing showed much differences with regards to their DPPH scavening activity. The former displayed a high DPPH scavening activity (Fig.4A) whilst the later showed a low activity (Fig.4B). Thus, it was our interest to see their cytotoxicity. Oils from nonfermented soybean were also subjected to the same test for comparison as well as four isoflavone standards (Table 1). Three types of oils displayed smaller IC-50 after 120 h of fermentation.

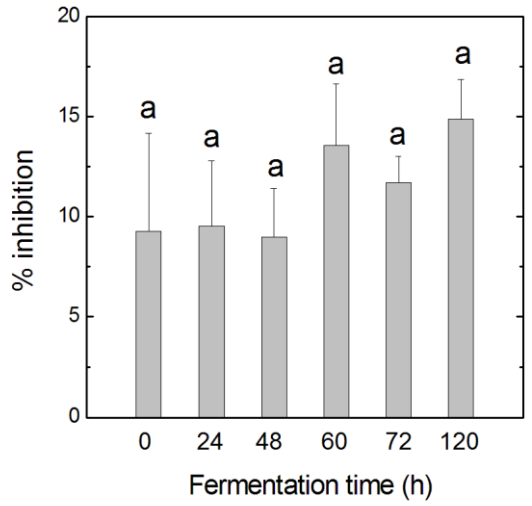


Fig. 3. Antioxidant activity of tempe oils at concentration of 1,000 ppm which were extracted with hexane only, as analysed using DPPH method, showed no significant effect ($p$<0.05) of fermentation time.

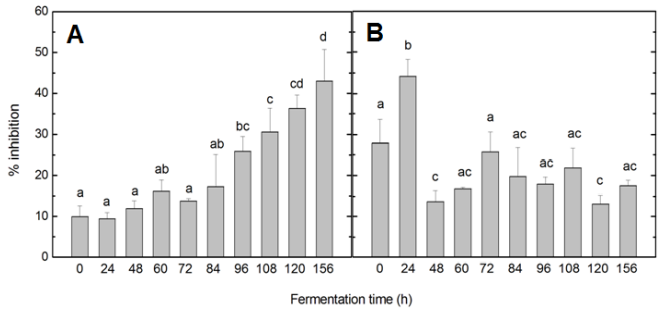


Fig. 4. Antioxidant activity of tempe oils at concentration of 1,000 ppm which were extracted with hexane after prewashing with either acetone (A) or ethanol (B), as illustrated in Fig.1B and C, respectively, as analysed using DPPH method, showed either a general increase of antioxidant activity (A) or inconclusive trend (B), respectively, as fermentation time was extended. Different indeces on top of the bars imply significant differences (p<0.05).

Soybean oil is considered as prone to oxidation compared to other vegetable oils, i.e., olive oil and corn oil (Naz et al., 2004). The antioxidative effect of nonfermented soybean oil might be due to its tocopherol content which is synergestic with phospholipid and other minor compounds (Jung and Min, 1990). The amount of tocopherol in tempe did not increase by fermentation up to 96 h (Denter et al., 1998). Extending fermentation to 7–30 days decreased tocopherol content and the resultant tempe did not have antioxidative property (Ishikawa et al., 1984); interestingly, the 30 day oil showed synergism with tocopherol in preventing auto-oxidation of lard whilst soybean oil did not display such synergism. It was suggested that microbial acitivity of *Rhizopus oligosporus* was responsible in forming the substances that synergestic with tocopherol in that oil. Thus, the slight increase of antioxidant activity of our tempe oil at ≥60 h of fermentation (Fig.3) was possibly due to the formation of compounds which were together with tocopherol displayed a synergims although only to a minor level (Ishikawa et al., 1984).

Table 1. Cytotoxicity of oils collected from 0 and 120 h tempe according to three different schemes as illustrated in Fig.1.

| Samples | Methods of collection | Tempe maturity (h) | IC-50 (ppm) |
|---|---|---|---|
| Tempe oils | Hexane only | 0 | > 100 |
| | | 120 | 15.66 ± 0.81 |
| | Hexane with acetone prewashing | 0 | > 100 |
| | | 120 | 3.96 ± 0.93 |
| | Hexane with ethanol prewashing | 0 | > 100 |
| | | 120 | 4.90 ± 1.50 |
| Daidzin standard | | | > 100 ppm |
| Genistin standard | | | > 100 ppm |
| Daidzein standar | | | > 100 ppm |
| Genistein standard | | | 4.82 ± 0.11 ppm |

It is likely that prewashing extracted compounds which had similar polarity to that acetone or ethanol, including oils which conjugated with semipolar and polar substances, respectively. The next hexane extraction divided these compounds, and thus compounds which conjugated with oils tended to be more soluble in hexane. Oils collected from tempe at the same maturity were different in term of their DPPH scavenging activity, for example tempe oil 120 h that were collected without, with acetone, and with ethanol prewashing displayed approximately 15, 36, and 13% of inhibition at 1,000 ppm, respectively. The difference in antioxidant activity between oils from all collection methods was alligned with a previous study by Hoppe et al., (1997). They observed the effect of different solvent blends in extracting bioactives from tempe and resulted in different antioxidant property.

The increasing antioxidant activity of tempe oils in Fig.4A implied increasing amount of fat soluble antioxidant compounds as fermentation time was extended. A study by György et al., (1974) indicated a formation of fat soluble antioxidants during tempe fermentation other than vitamin E. It is possible that a semipolar substances were collected from tempe during oil extraction since György et al., (1974) used a mixture of hexane and ethanol (with the ratio of 2:1) as solvent. Denter et al., (1998) reported increased betacarotene, a fat soluble pro-vitamin as well as antioxidant, in tempe as the fermentation time continued. They observed that particular *R. oligosporus* strains were able to form betacarotene in significant amount during tempe fermentation. For example, tempe oils which were collected after 34 and 96 h of tempe fermentation by *R. oligosporus* strain *Uju* displayed a significant increase of betacarotene amount from 0.4 to 3.0 µg/g (dry basis). Their finding indicated that antioxidant activity in oil fraction of tempe possibly increased as the fermentation time was extended as long as the culture strain used for fermentation was able to

form antioxidant compounds. Therefore, we proposed more formation of betacarotene as fermentation time was extended in our study. The indication was shown by more intensive yellow colour of oils that were collected from longer fermentation time (Fig.4A). Oils collected with ethanol prewashing also displayed higher antioxidant activity (Fig.4B) than corresponding oils extracted with hexane only (Fig.1). Their trend, however, is inconclusive. In addition, the changes of colour in Fig.4B are not as in order as those of Fig.4A.

Besides tocopherol and betacarotene, any fat soluble oxidant compounds might contribute to scavenging effect on DPPH of all types of observed tempe oil. Isoflavones have been known as the antioxidant in soybeans and peptides which were resulted through microbial proteolysis during fermentation could possibly contribute to antioxidant activity of tempe (Sheih et al., 2000). A study by Chang et al., (2009) showed that tempe extracts obtained from both polar and nonpolar solvents exhibited scavenging effect on DPPH; however, their study did not include identification of the compounds responsible for the antioxidant property. Previously, Murakami et al., (1984) concluded that the major isoflavones in tempe were genistein and daidzein, which were transformed from genistin and daidzin, respectively, by ß-glucosidase during *R. oligosporus* fermentation. In our present study, both genistein and daidzein displayed relatively good percent of inhibition at 1,000 ppm; 34.62±0.86 % for genistein and 23.23±1.24 % for daidzein. The percent of inhibition of our tempe oil samples regardless of the collection methods ranged from 9 to 45%. It is possible that isoflavone also played a role in the antioxidant properties of our tempe oil samples. Amongst the four isoflavone standards that subjected to cytotoxicity assay, only genistein that showed a low IC-50 value (<5 ppm) whilst the rest had IC-50 value above 100 ppm. This implies that genistein had a potential activity in reducing the population of MCF-7 breast cancer cells. Tempe oils were somehow affected by fermentation since there were reduction of IC-50 from above 100 ppm to less than 20 ppm after 120 h of tempe fermentation (Table 1). Oils collected from 120 h tempe with either acetone and ethanol prewashing displayed even lower IC-50 value, *i.e.*, less than 5 ppm although the antioxidant activity of the former oil was significantly higher than that of the later oil. This indicated that prewashing could help the extraction of bioactive compounds from tempe matrices which not only possess antioxidative but also cytotoxic properties though there were no direct correlation trend between those properties.

## Conclusions

Oils collected from tempe had different antioxidative and cytotoxic properties depending on the duration of tempe fermentation and/or the methods of extraction. Tempe oils extracted using hexane only did not show significant differences in antioxidant properties regardless the fermentation duration. On the contrary, those collected with either acetone or ethanol prewashing showed improved antioxidant activity. The effect of fermentation time could be seen from tempe oils collected with acetone prewashing; their antioxidative activity went up as fermentation time continued. Unfortunately, those collected with ethanol prewashing did not show a definite trend as fermentation time was extended. In all types of tempe oils, fermentation improved their cytotoxic activities, as shown by reduction of IC-50 from above 100 ppm to less than 20 ppm. Nonetheless, there is no clear correlation between their antioxidant activities with the cytotoxic activities. Fermentation with *R. oligosporus* possibly either transformed bioactive compounds in soybeans to be more active with regards to antioxidant and cytotoxic properties and facilitate them to conjugate with fatty acids, thus they could be extracted along with tempe oils.

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## Conflict of Interest

All the authors declare that they have no conflict of interest.

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