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EFFECTS OF PARTICLE SIZE, EXTRACTION TIME, AND SOLVENT ON DAIDZEIN YIELD EXTRACTED FROM TEMPEH

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ABSTRACT

Daidzein, one of the isoflavone aglycones contained in tempeh, has several biological activities such as anti-inflammatory, anti-oxidant, anti-breast cancer, and suppression of expression of matrix metalloprotease-9. As a fermented product from soybeans, daidzein content in tempeh was found in higher concentration compared to the soybean raw material. It was important to optimize several factors affecting extraction process such as particle size of tempeh simplicia, extraction time, and solvent in order to develop an effective method for the daidzein isolation from the tempeh or other natural products. Evaluation of extraction factors was conducted by applying variations for each factor followed by quantitative analysis using HPLC methods. The optimization condition was performed by daidzein standard and achieved with the particle size of tempeh simplicia of 1.2 mm, extraction time of 360 minutes, and 70% ethanol was used as solvent. Furthermore, the optimized condition was applied for the daidzein isolation from tempeh, a soybean fermented product.

Keywords: daidzein; extraction time; particle size; solvent; soybean fermented product

INTRODUCTION

Daidzein, an isoflavone aglycone, was reported as the major compound contained in soybeans (Kuligowski *et al.* 2016). In recent years, pharmacological activities of daidzein were reported such as anti-breast cancer agent (Liu *et al.* 2012, Yuliani *et al.* 2016), cell growth inhibitor (Takaoka *et al.* 2018), anti-diabetic agent, (Park *et al.* 2013), anti-inflammatory agent, anti-oxidant agent (Peng *et al.* 2017), and matrix metalloproteinase-9 suppressor (Oh *et al.* 2013). Tempeh is a fermented product of soybean and commonly used as Indonesian traditional food with high isoflavones content (Yuliani *et al*, 2018). Fermentation process increases the isoflavone aglycone content in tempeh including daidzein due to the hydrolysis reaction that occurs on the glycoside bond of acetylglycoside and malonilglycoside (Hong *et al.* 2012).

Extraction can be a challenging step in the extraction of active compounds from natural products. There are several factors that can affect extraction process such as particle size, extraction time, solvent used, and temperature condition (Hernández *et al.* 2009, Zhu *et al.* 2011). Previous study reported that the longer the extraction time, the higher level of isoflavone aglycones extracted from simplicia (Jyoti *et al.* 2015). The particle size factor is related to the contact area between simplicia and solvent used in the extraction process. The smaller particle size, the bigger the contact area with solvent, and the higher the concentration of daidzein achieved from simplicia (Sapri *et al.* 2014). It is important to use appropriate solvents considering the physicochemical properties of target analyte. Ethanol was commonly used in isoflavone aglycones extraction due to the analyte solubility consideration (Lakshmi *et al.* 2013).

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Another study on isoflavones extraction reported that the addition of water up to 30% in the ethanol solvent will increase the concentration of genistein extracted from soybeans (Rostagno *et al.* 2009).

The aim of this study was to evaluate the effect of three factors on the extraction of daidzein i.e. particle size, extraction time, and solvent used toward daidzein yield from tempeh, a fermented product of soybeans.

METHODS

Materials

Reference standard daidzein (Sigma-Aldrich, Singapore) were used as external standard for this study. Other materials used were ethyl acetate, petroleum ether, methanol for liquid chromatography grade (E. Merck), and redistilled water from Organic Chemistry Laboratory, Faculty Pharmacy, Universitas Sanata Dharma, Yogyakarta. The tempeh used in this study was obtained from the traditional market in Yogyakarta. The selected tempeh "M" was controlled with the fermentation time of three days.

Instrumentation

The instrumentation used in this study were including: Shimadzu® LC-2010HT system with UV/Vis detector, Retsch® T460 ultrasonicator, ultramicro analytical balance RADWAG® UYA 2.3Y (max: 2.1 g, min 0.8 mg), membrane filter holder of Whatman®, organic solvent membrane filter of Whatman®; inorganic solvent membrane filter of Whatman®, Millipore syringe filter, and Socorex® micropipettes. Design Expert™ 10.0.6.0 software.

HPLC system

The HPLC used in this study was developed according to Yuliani *et al.* (2018). The Shimadzu LC-2010 CHT with Lab-Solution software and UV-VIS detector was used in quantitative analysis of daidzein. Analytical column used in this study was Luna Phenomenex® C_{18} column (250x4.6 mm, 5 µm). The mobile phase mixture containing methanol-water (70:30) and the flow rate of 0.6 ml/minute was applied in the isocratic reverse-phase HPLC system. The daidzein was measured at UV 261 nm.

Simplicia preparation

The tempeh "M" used in this study was obtained from the traditional market in Yogyakarta. The fresh tempeh was cut and dried in an oven at 50˚C for 24 hours. The small pieces of dried tempeh were ground into rough powder and then macerated using petroleum ether. About 100 ml petroleum ether were added into the 50 g rough powder tempeh in an erlenmeyer. The maceration process was conducted by shaking the mixture at 150 rpm for 24 hours. The suspended solid was separated from the solvent by centrifugation and dried in the oven at 50˚C for 24 hours. The dried mass obtained was called the tempeh simplicia.

Evaluation of Extraction Factors

Observations of the factors that affected the extraction were done to evaluate how the particle size, extraction time, and ethanol solvent affected the extraction process of the daidzein from tempeh.

Particle size

The tempeh simplicia was ground into four types of powder size i.e. 0.6; 0.85; 1.2; and 1.7 mm^2. Fifty-grams of the tempeh simplicia of each particle size group were mixed with 150 ml of 70% ethanol and macerated by shaking at 150 rpm for 270 minutes. After the filtering process, the yellow filtrate was separated from the suspended solid, then concentrated using rotary evaporator until 10% of initial volume was achieved.

Extraction time

Tempeh simplicia with certain particle size of 1.2 mm was used to evaluate the effect of extraction time. Fifty-grams of tempeh simplicia were mixed with 150 ml of 70% ethanol and macerated by shaking at 150 rpm for four extraction time i.e. 90, 180, 270, and 360 minutes, respectively. The yellow filtrate was separated from suspended solid then

concentrated using rotary evaporator until 10% of initial volume was achieved.

Solvent

The ethanol solvent concentrations used for optimization of extraction process were 50%, 60%, 70%, 80%, 90% and 96%. Fifty-grams of tempeh simplicia with the particle size of 1.2 mm were mixed with 150 ml of 70% ethanol and macerated by shaking at 150 rpm for 270 minutes. The yellow filtrate was separated from suspended solid and then concentrated using rotary evaporator until 10% of the initial volume obtained.

Sample Preparation

The liquid-liquid extraction method was performed to extract daidzein from the concentrated samples. One gram of concentrated sample was weighed and extracted by 30 ml ethyl acetate and water (50:50 v/v). The extraction process was repeated three times. The ethyl acetate fraction was collected and dried. A constant weight of the dried extract was transferred into a 10 ml volumetric flask followed by dilution to volume with methanol. Fifty microliters of the solution were transferred into a micro tube for dilution to 1.0 ml with methanol. All sample solutions were sonicated for 10 minutes, filtered using Millipore syringe filter, and transferred into HPLC vial before injection.

RESULTS AND DISCUSSION

Extraction could be one of the crucial steps in developing research on natural products. Several factors may contribute to the extraction efficiency of active compounds from the natural products (Rostagno *et al.* 2003; Zhang *et al.* 2007; Yoshiara *et al.* 2012). This study was conducted to evaluate the effect of three factors on extraction such as particle size, extraction time, and solvent used on the concentration of daidzein, one of an isoflavone aglycone extracted from tempeh, a fermented product of soybean. The highest daidzein concentration obtained among different variations for each factor was stated as the parameter for determining optimum condition.

Daidzein concentration was determined by HPLC from the previous study (Yuliani *et al*., 2018). The HPLC method obtained daidzein calibration curve equation y = 127910x – 179548 with r = 0.999 at the range of 5.07 – 17.75 µg/ml. The accuracy was within the required range of 80-115% (AOAC, 2012). Percentage of RSD for intraday and inter day as the precision parameter was 6%, lower than the maximum limit of AOAC requirements for RSD% (AOAC, 2012). The LOD and LOQ of the method were 0.796 and 2.653 µg/ml, respectively. This method indicated the successful result by a good separation of daidzein peak for further quantitative analysis achieved at retention time of 7.831 minutes (Figure 1).

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The effect of particle size of tempeh simplicia

Contact area between tempeh simplicia and the solvent used in extraction process was affected by the pacticle size factor. The higher the concentration of daidzein achieved from simplicia was related to the smaller particle size which leads to the bigger the contact area with solvent (Sapri *et al.* 2014). On the other hand, too small of particle size can cause disadvantages in the extraction process, such as the forming of particle aggregation which leads to the contact blocking between tempeh simplicia and the solvent (List and Schmidt, 1989).

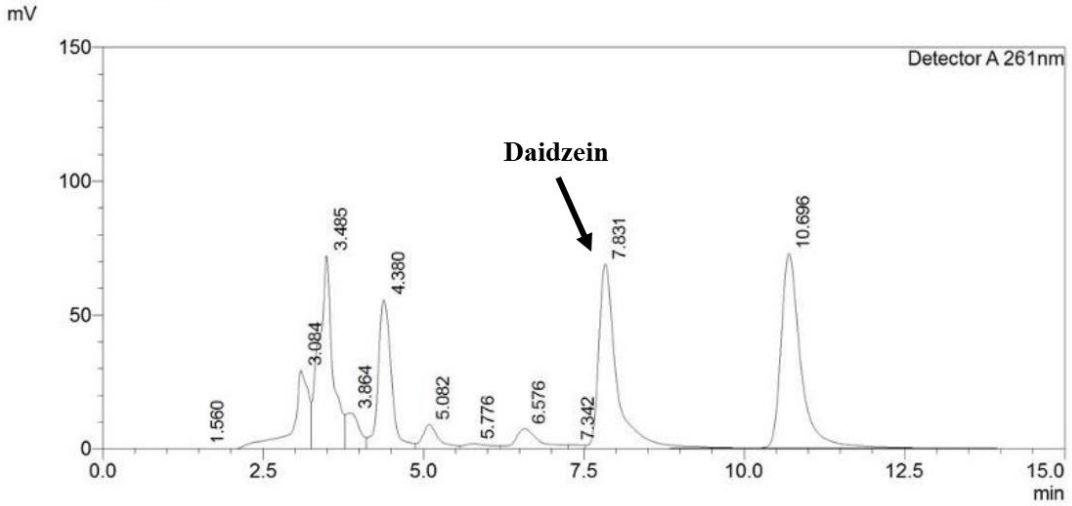

Figure 1. Representative chromatogram of tempeh simplicia containing daidzein. Column: Luna Phenomenex® C_{18} (250 x 4.6 mm, 5 µm). Mobile phase: methanol-water (70:30). Flow rate: 0.6 ml/min. Detection at 261 nm.

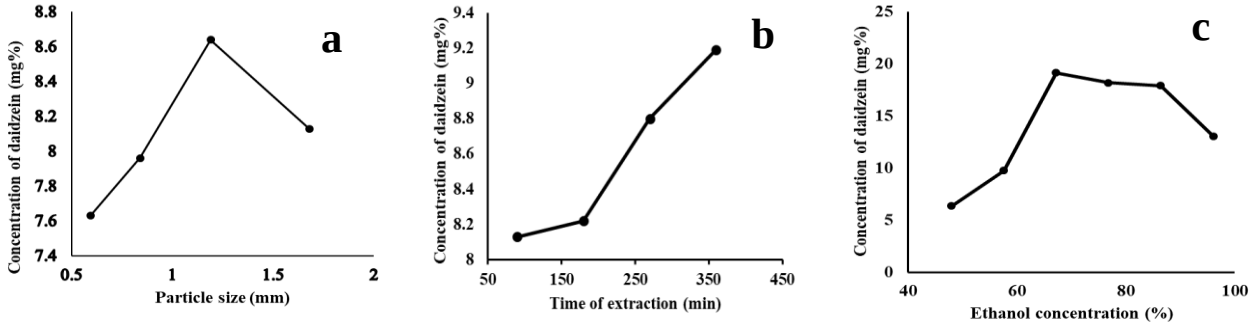

Figure 2. Graphic of effects from three factors on tempeh simplicia extraction such as particle size (a), extraction time (b), and ethanol concentration (c).

Selected particle sizes used in this study were 0.6; 0.85; 1.2; and 1.7 mm. The daidzein obtained from the tempeh simplicia for each particle size group were 7.63±1.1; 7.96±0.47; 8.64±0.85; and 8.13±0.38 mg%, respectively (Figure 2a). The particle size of 1.2 mm was the optimum for gaining the highest concentration there was 8.64 mg daidzein extracted from 100 g tempeh simplicia. The lower daidzein concentration extracted from tempeh simplicia with the particle size types smaller than 1.2 mm indicated that the particle aggregation may be formed in the particle size group of 0.6 and 0.85 mm.

The effect of extraction time

In general, longer extraction time in the extraction process lead to increases in the concentration of daidzein extracted from simplicia. It was related to the daidzein diffusion time into the surface of the simplicia before dissolving in the solven. Hence, increasing extraction time will enhance the extraction capacity. Nevertheless, too long extraction time could be ineffective for the extraction process because it is dependent on the equilibrium between the solute inside and outside the solid material (Zhang *et al*. 2018). As a result, it was important to optimize the extraction time in order to develop an effective extraction method with the highest analyte extracted from the simplicia.

Extraction time variations were 90, 180, 270, and 360 minutes. The daidzein concentration extracted from the tempeh simplicia for each extraction time were

8.13±1.00; 8.22±1.67; 8.80±0.81; and 9.19±0.50 mg%, respectively (Figure 2b). It could be concluded that the longer extraction time leads to the higher daidzein yield. The extraction time of 360 minutes has been recommended as the optimum extraction time.

The effect of solvent composition

In this research the effect of solvent composition was investigated. The solvent was prepared in the concentration series of 50%, 60%, 70%, 80%, 90% and 96% ethanol in water. The daidzein concentrations extracted from the tempeh simplicia by each ethanol concentration were 6.34±0.40; 9.73±0.57; 19.14±2.30; 18.19±4.90; 17.91±1,89 and 13.04±2.17 mg%, respectively (Figure 2c). The highest daidzein concentration was obtained by using 70% ethanol solvent since the molar solubility of daidzein in ethanol (3.2375 mol/L) was higher than its molar solubility in water (0.1695 mol/L) (Yang *et al.* 2013).

Based on its molar solubility, the highest concentration of daidzein should be obtained with the highest concentration of ethanol. However, tempeh simplicia used in this research were found in the form of dry powder. It was assumed that soybean cells underwent shrinkage during the drying process. The presence of water in the extraction steps enhanced the resizing cells into the normal size and resulted in a porous surface of simplicia. In addition, daidzein diffusion process was facilitated by the presence of pores on the surface of simplicia. Therefore solvent of 70% ethanol has been recommended as the optimum condition.

CONCLUSION

In summary, the results of this study showed the effect of particle size, extraction time, and solvent composition on the concentration of daidzein extracted from tempeh which was successfully evaluated in this study. The optimized condition was achieved with the particle size of 1.2 mm, extraction time of 360 minutes, and 70% ethanol used as solvent for maceration process.

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