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Genistein Content and Tyrosinase Inhibitory Activity of Edamame (*Glycine max*) Extracts

Indah Yulia Ningsih, Lisa Kusuma Wardhani, Annisa Ragdha Eka Nuryuanda, Endah Puspitasari, Mochammad Amrun Hidayat*

Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Jember, Indonesia
*Corresponding author: amrun.farmasi@unej.ac.id

Abstract

The study aimed to determine genistein content and tyrosinase inhibition activity of 70% ethanolic and ethyl acetate extracts of edamame (*Glycine max*) with an extraction time of 15, 30, and 60 minutes. Extraction was performed using the ultrasonication method. Determination of genistein content was carried out using TLC-densitometry. Furthermore, all samples were tested for their tyrosinase inhibition activity using the spectrophotometric assay. Ethyl acetate extract with 60 min of extraction time exhibited the highest extraction yield (6.414% w/w), the highest genistein content (0.169 ± 0.007% w/w), and the highest tyrosinase inhibition activity (IC_{50} = 72.420 ± 0.550 µg/mL). It was known that the genistein content of 70% ethanolic and ethyl acetate extracts affected tyrosinase inhibition activity with correlation coefficient (r) values of 0.9973 and 0.9826, respectively. *G. max* was suggested as a tyrosinase inhibitor agent from natural sources for skin whitening product development due to its isoflavones content, mainly genistein.

Keywords: edamame, Glycine max, genistein, tyrosinase inhibitor

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1 Introduction

Generally, skin color depends on the amount of melanin pigments in the skin. It is synthesized in melanosomes, organelles of melanocytes located in the basal layer of the epidermis. If the amount of melanin is higher than in normal conditions, it causes a brown color to the skin indicating undesirable hyperpigmentation. This occurs because of the

overproduction of melanin through melanogenesis [1,2]. Hyperpigmentation can be a serious aesthetic problem, mainly for the woman. Moreover, abnormal production of melanin causes post-inflammatory and periorbital hyperpigmentation, maturational dyschromia, Riehl melanosis, melasma, exogenous ochronosis, ephelides, lentigines, etc. [3]. Therefore, it is necessary to use a depigmentation agent for pigmentation process inhibition. The main enzyme in melanogenesis is tyrosinase which catalyzes several steps of melanin biosynthesis. In fact, the enzyme affects considerably melanin quality and quantity. One of the mechanisms in preventing hyperpigmentation to whiten the skin is to reduce melanin formation through tyrosinase inhibition. It causes inhibition of hydroxylation of tyrosine to *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) and oxidation to DOPA-quinone. Consequently, the transformation to melanin is inhibited [1,4].

The skin whitening compound which is widely used for depigmentation is hydroquinone. The compound overcomes hyperpigmentation by inhibiting the tyrosinase enzyme, but it is cytotoxic for melanocyte cells in long-term use [5]. Therefore, it is necessary to explore alternative natural ingredients for skin whitening compounds. Furthermore, the demand for new natural whitening agents increases with the market development of whitening products [4].

Glycine max or vegetable soybean, more commonly known as edamame is one of the plants which is potentially developed as a tyrosinase inhibitor agent. It is rich in isoflavones, namely isoflavone aglycones (genistein, daidzein, and glycitein) and glucosides (genistin, daidzin, and glycitin) [6,7]. The isoflavone aglycones were known to have inhibitory activity against the tyrosinase enzyme [8]. The content of genistein, daidzein, and glycitein profiles was about 50%, 40%, and 10% of total isoflavone, respectively [9]. A previous study reported that fermented and non-fermented soy ethanol extract of *G. max* had antioxidant activity and whitened the skin by inhibiting the tyrosinase enzyme [10].

In natural product development, it is essential to optimize extraction parameters to obtain an effective and more economical method. Several extraction parameters which can affect the number of compounds dissolved in the extraction process are type and/or composition of the solvent, extraction time, pH, temperature, and the solid-to-liquid ratio [11–13]. The study aimed to determine genistein content and tyrosinase inhibition activity of 70% ethanolic and ethyl acetate extracts. Extraction times of 15, 30, and 60 minutes were applied to seek the extract with the highest content of genistein and the highest tyrosinase inhibition activity.

2 Experimental section

2.1 Materials

The seeds of *G. max* were purchased from PT. Mitra Tani Dua Tujuh, Jember district, East Java, Indonesia and authenticated by the Indonesian Institute of Sciences at Purwodadi Botanical Garden, East Java, Indonesia with voucher number 0693/IPH.04/HM/IV/2017. Tyrosinase enzyme from mushroom (EC 1.14.18.1) and *L*-tyrosin was supplied by Sigma-Aldrich (St. Louis, MO, USA). Genistein was purchased from Tocris Bioscience (Bristol, UK). Potassium phosphate monobasic and NaOH were obtained from Brataco Chemika (Jakarta, Indonesia). Toluene, formic acid, *n*-hexane, ethanol, methanol, ethyl acetate, and distilled water were obtained from Smart Lab (Jakarta, Indonesia). Silica gel GF_{254} plate was purchased from Merck (Darmstadt, Germany).

2.2 Preparation of extracts

The *G. max* seeds were separated from their pods, washed, and thinly sliced. After the oven-dried process, the seeds were ground into powder using a milling machine and passed through an 80-mesh sieve. The dried powder was defatted using a Soxhlet apparatus with *n*-hexane as solvent (1:5) for 3 hours. The residue was dried in an oven at 50 °C for 6 hours. The defatted powder of G. max seeds was extracted using an ultrasonicator (Elmasonic S180H, Germany) in two different solvents (i.e., 70% of ethanol and ethyl acetate (1:10)) for different periods (i.e., 15 minutes, 30 minutes and 60 minutes) at 25 °C for three times. The solid-liquid mixture was filtered using Whatman filter paper (No. 1) and the filtrate was concentrated at 50 °C using a rotary evaporator (Heidolph-

Laborota 400, Germany) to produce a thick concentrated extract.

2.3 Determination of genistein content

Genistein content was determined using the TLC-densitometric method. The mobile phase was toluene: ethyl acetate: acetone: formic acid (20:4:2:1) and the stationary phase was silica gel GF_{254} plate. Each extract was weighed and dissolved in methanol to obtain a concentration of 20,000 µg/mL Furthermore, 5 mg of genistein was dissolved in 10 mL of methanol using a volumetric flask and sonicated for 5 min for homogenizing it completely. The solution was diluted in methanol to obtain concentrations of 10, 20, 30, 40, 50, and 70 µg/mL.

The TLC plate was activated by placing it in an oven at 110º C for 20 min. The plate was spotted with 4 µL of extract solutions and 2 µL of standard solutions. It was eluted in the twin-trough TLC developing chamber (Camag, Switzerland) using the mobile phase, and dried at room temperature. The spots formed from elution were observed under UV light (254 nm) and scanned at 266 nm to read the area using a TLC densitometer (Camag TLC Scanner 3, Switzerland). Purity and identity tests were carried out before the determination of genistein content by absorbance scanning at 200-400 nm. These tests were carried out using winCATS software version 1.4.1.8154 (Camag, Switzerland). Calculation of genistein content was performed using an equation obtained from the calibration curve of the standard solution. Purity and identity tests were performed to measure the specificity of the analysis method. All samples were measured in triplicate analysis.

2.4 Determination of tyrosinase inhibition activity

The tyrosinase inhibition assay was carried out according to the previous method with minor modifications [14]. The concentrated extract was weighed and dissolved in phosphate buffer at pH 6.5 to obtain a concentration of 4,000 µg/mL. The solution was diluted to obtain concentrations ranging from 40 to 140 µg/mL. Moreover, genistein as a positive control was prepared by weighing 5 mg of genistein and dissolving it in 10.0 ml of methanol to obtain a concentration of 500 µg/mL. The solution was diluted in phosphate buffer at pH 6.5 to obtain concentrations ranging from 40 to 145 µg/mL. One millimolar of *L*-tyrosine solution in a phosphate buffer was used as substrate and the study was performed at pH 6.5. Meanwhile, the tyrosinase enzyme was dissolved in phosphate buffer at pH 6.5 to obtain a concentration of 2500 units/mL.

In a 96-well plate, 70 µL of each extract was added to 40 µL of enzyme solution in triplicate and incubated at room temperature for 5 minutes. Then, 110 µL of *L*-tyrosine was added to the mixed solutions and incubated for 110 minutes at room temperature. The absorbance of mixed solutions was measured using the microplate reader (Dialab ELx800, Austria) at 492 nm to determine the percentage of inhibition. The IC_{50} value was calculated from the linear regression curve equation between extract concentration and tyrosinase inhibition activity. The percentage of inhibition was calculated by comparing sample absorption without extract addition and sample absorption with extract addition using equation 1.

$$\% \text{ inhibition } = \frac{(A - B)}{A} \times 100\%$$

A = absorbance of the blank solution.
B = absorbance of sample solution.

(equation 1)

2.5 Data analysis

Data of genistein content and tyrosinase inhibition activity were expressed as mean ± standard deviation of triplicate measurements. The significant differences between groups were assessed using the one-way analysis of variance (ANOVA) followed by the Least Significance Different (LSD) method to test any significant differences among samples. Furthermore, data on genistein content and tyrosinase inhibition activity were analyzed using simple linear regression to evaluate its correlation.

3 Results and Discussion

3.1 Extraction yields

All samples were defatted before extraction to remove lipid contents which probably affected the analysis. From Table 1, it was known that extract with 60 min extraction time had more yield than other extracts with the same solvent. The longer the extraction process, the more yield of extracts was produced. The extraction process with a longer time resulted in more compounds dissolved into the solvent and more extracts were produced. Mohamad *et al*. [15] reported that enhancement of extraction time caused increasing extraction yields of *Eurycoma longifolia* with 45 minutes as the optimum extraction time. Furthermore, ethyl acetate solvent produced a larger amount of extracts than 70% ethanol. It was probably due to more semi-polar compounds contained in *G. max* than polar compounds. Hence, ethyl acetate solvent at 60 min extraction time produced the highest extraction yield (6.414% w/w).

Table 1. The yield of extracts, genistein content, and IC_{50} of 70% ethanolic and ethyl acetate extracts.

Samples	Yield of extracts (%w/w)	Genistein content (%w/w)	IC_{50} (µg/mL)
ET15	4.975	0.050 ± 0.003^{a}	121.320 ± 1.437^{a}
ET30	5.515	0.105 ± 0.004^{b}	99.509 ± 0.561^{b}
ET60	5.719	0.145 ± 0.004^{c}	79.027 ± 0.423^{c}
EA15	5.813	0.072 ± 0.005^{d}	108.787 ± 0.304^{d}
EA30	6.205	0.127 ± 0.007^{e}	94.071 ± 1.816^{e}
EA60	6.414	0.169 ± 0.007^{f}	72.420 ± 0.550^{f}

Data are the average of samples (mean) ± SD (n=3). The different superscript letters (in the same column) showed significant differences according to the LSD test (p<0.05). ET15: 70% ethanolic extract with 15 min of extraction time; ET30: 70% ethanolic extract with 30 min of extraction time; ET60: 70% ethanolic extract with 60 min of extraction time; EA15: ethyl acetate extract with 15 min of extraction time; EA30: ethyl acetate extract with 30 min of extraction time; EA60: ethyl acetate extract with 60 minutes of extraction time.

3.2 Determination of genistein content

From the elution process in the TLC method using toluene: ethyl acetate: acetone: formic acid at 20:4:2:1 ratio, the silica gel GF_{254} plate showed several spots for all samples under UV light (254 nm) (Figure 1). Based on a match in retention factor (Rf) of genistein between all samples and standard, it was known that all samples contained genistein (Rf = 0.39). A previous study using the same condition of TLC analysis exhibited a similar result. Semen Sojae Praeparatum derived from the fermented seed of *G. max* contained genistein with an Rf value of 0.38 [16].

In this study, purity and identity tests were carried out to observe the specificity of the analysis method. Therefore, it provided an exact result in the determination of analyte content [17]. The purity test aimed to ensure the purity of the genistein spot in all samples and to ensure that the genistein spots were not contaminated by other substances. Whereas an identity test was performed to ensure the identity of genistein in all samples. From Figure 2 and Figure 3, it was known that the major peak of all samples matched the genistein peak of the standard. Hence, 70% ethanolic and ethyl acetate extracts contained genistein.

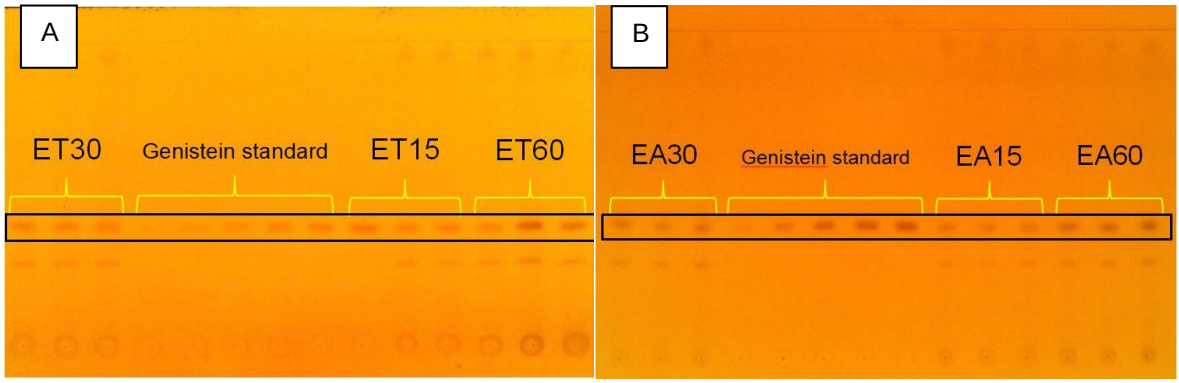

Figure 1. TLC plate of 70% ethanolic (A) and ethyl acetate (B) extract at 254 nm. Spots of samples in the box showed the same Rf value as the genistein standard. ET15: 70% ethanolic extract with 15 min of extraction time; ET30: 70% ethanolic extract with 30 min of extraction time; ET60: 70% ethanolic extract with 60 min of extraction time; EA15: ethyl acetate extract with 15 min of extraction time; EA30: ethyl acetate extract with 30 min of extraction time; EA60: ethyl acetate extract with 60 minutes of extraction time; Genistein standard: solution of genistein with concentrations ranging from 10 to 70 µg/mL.

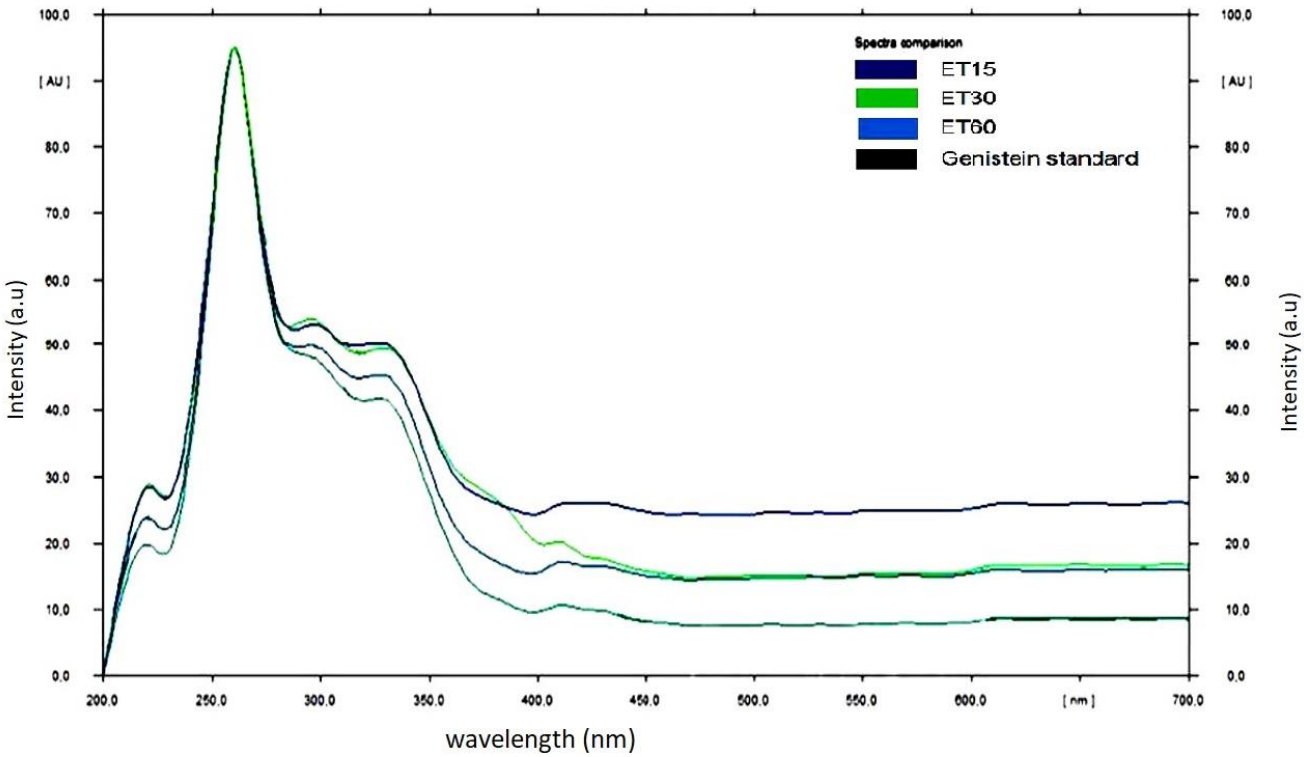

Figure 2. Spectra of identity and purity test of genistein in 70% ethanolic extracts compared with genistein standard. ET15: 70% ethanolic extract with 15 min of extraction time; ET30: 70% ethanolic extract with 30 min of extraction time; ET60: 70% ethanolic extract with 60 min of extraction time.

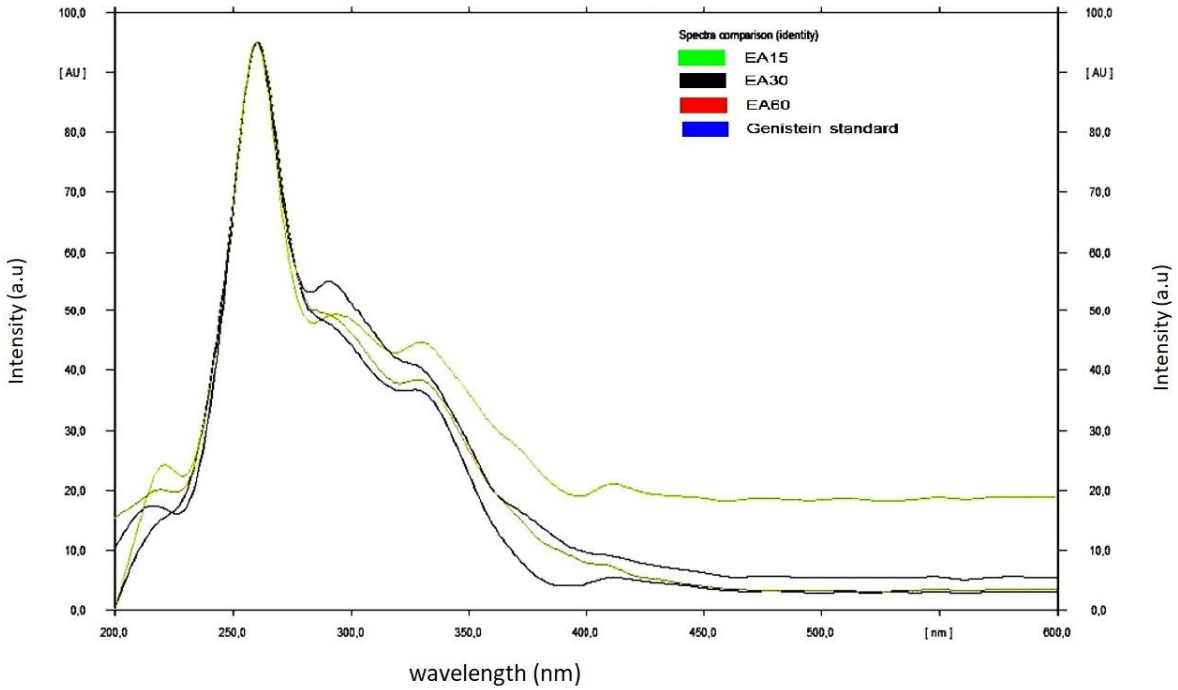

Figure 3. Spectra of identity and purity test of genistein in ethyl acetate extracts compared with genistein standard. EA15: ethyl acetate extract with 15 min of extraction time; EA30: ethyl acetate extract with 30 min of extraction time; EA60: ethyl acetate extract with 60 minutes of extraction time.

A purity test was carried out by comparing the spectra at three peak positions, namely initial position or start or s, maximum position or m, and end position or e. The purity of genistein in each sample was determined based on the values of correlation coefficient r (s,m) and r (m,e) indicating the correlation between the spectra taken at the initial position and maximum position, and the correlation between the spectra taken at a maximum position and end position. From Table 2 and Table 3, it was known that the value of r (s,m) and r (m,e) for 70% ethanolic and ethyl acetate extracts were higher than 0.990 which indicated that genistein contained in all samples complied with the parameter of purity.

The identity test was performed by comparing the value of r (s,s) and r (s, a). The value of r (s,s) indicated a spectral correlation between two standard tracks, while r (s,a) showed the correlation between the standard track and analyte track in the sample. The value of r (s,s) and r (s,a) of 70% ethanolic and ethyl acetate extracts were higher than 0.990, indicating that the analyte in the sample was considered to be identical to the genistein standard (Table 2 and Table 3). As purity and identity parameters have complied, hence, the analytical condition was suitable for the determination of genistein content.

From Table 1, it was known that the longer the extraction process, the higher the genistein content in the extracts. Extracts produced at 60 min extraction time had higher genistein content than other extracts with the same solvent. It was probably caused by the longer the extraction process, the more compounds dissolved in the solvent. A previous study showed that extraction time had a higher effect in extracting genistein from fermented *G. max* than ethanol concentration (as solvent) and particle size. Moreover, the increase in extraction time also increased the amount of genistein obtained from fermented *G. max* [18]. The study exhibited that extracts of ethyl acetate contained more genistein than 70% ethanolic extracts. Ethyl acetate extract with 60 min extraction time contained more genistein than the others (0.169 ± 0.007 %w/w). Genistein is one of the isoflavonoid aglycones with low polarity and low water-solubility [19]. Therefore, genistein was more soluble in ethyl acetate than 70% ethanol.

Table 2. Purity and identity test of 70% ethanolic extract.

Samples	r (s,m)	r (m,e)	r (s,s)	r (s,a)
Genistein	0.998341	0.997629	0.999224	-
ET15	0.998002	0.999042	0.999224	0.997321
ET30	0.998283	0.998201	0.999224	0.998626
ET60	0.998501	0.998323	0.999224	0.992460

ET15: 70% ethanolic extract with 15 min of extraction time; ET30: 70% ethanolic extract with 30 min of extraction time; ET60: 70% ethanolic extract with 60 min of extraction time.

Table 3. Purity and identity test of ethyl acetate extract.

Samples	r (s,m)	r (m,e)	r (s,s)	r (s,a)
Genistein	0.997729	0.998569	0.999216	-
EA15	0.999072	0.999903	0.999216	0.996265
EA30	0.999726	0.998710	0.999216	0.992154
EA60	0.999591	0.998763	0.999216	0.997930

EA15: ethyl acetate extract with 15 min of extraction time; EA30: ethyl acetate extract with 30 min of extraction time; EA60: ethyl acetate extract with 60 minutes of extraction time.

3.3 Determination of tyrosinase inhibition activity

The study exhibited that a longer extraction process results in a higher tyrosinase inhibitory activity. Extracts with 60 min extraction time showed higher capacity in tyrosinase inhibition (Table 1). Whereas extract of ethyl acetate resulted in higher tyrosinase inhibitory activity than 70% ethanolic extract. Its higher genistein content might cause its activity enhancement. Ethyl acetate extract with 60 min of extraction time exhibited the highest capacity in tyrosinase inhibition (IC_{50} = 72.420 ± 0.550 µg/mL). Its activity was greater than other Indonesian medicinal plants used as traditional skin whitening agents, namely *Durio zibethinus* and *Helminthosthachys zeylanica* with IC_{50} values of 172.10 µg/mL and 128.80 µg/mL [14]. The tyrosinase inhibition activity was also higher than genistein. The study showed that IC_{50} of genistein was 127.714 µg/mL. This finding was similar to a previous study which reported that IC_{50} of genistein was 130.14 µg/mL [20]. Cuorto *et al*. [21] reported that compounds with the IC_{50} value of less than 100 µg/mL could be a new candidate for skin whitening agents. Thus, *G. max* was highly potential to be developed as a natural resource of the skin whitening agent.

3.4 Correlation analysis

The correlation between genistein content and tyrosinase inhibitor activity of 70% ethanolic and ethyl acetate extracts was determined using regression analysis as shown in Figure 4. Both types of extract exhibited the same pattern of curves. The correlation coefficient (r) of 70% ethanolic and ethyl acetate extracts were 0.9973 and 0.9826 (>0.90), respectively. It indicated that there was a strong correlation between genistein content and tyrosinase inhibitor activity of 70% ethanolic and ethyl acetate extracts [22]. The capacity in tyrosinase inhibition of both types of the extract was affected by genistein content. The calibration curves also showed a negative correlation. An increase in genistein content led to a decrease in IC_{50} which indicated enhancement of tyrosinase inhibitor activity. However, as the tyrosinase inhibitor capacity of *G. max* extracts higher than genistein, it was expected that other compounds were also contributed to the activity. Isoflavones contained in *G. max* extracts, including its aglycones such as daidzein and glycitein, were also known as tyrosinase inhibitor agents [8,20,23,24].

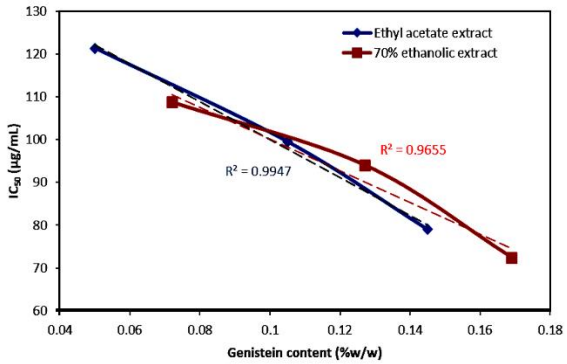

Figure 4. Linear correlation of genistein content and IC_{50} of 70% ethanolic and ethyl acetate extract.

4 Conclusions

Based on the study, it was known that *G. max* exhibited tyrosinase inhibitor capacity in the development of skin whitening products. The activity was expected due to its isoflavones content, mainly genistein. Ethyl acetate was the best extraction solvent, and 60 min was the best extraction time in dissolution genistein from *G. max* seeds.

5 Author Contribution

IYN: preparing the manuscript and analyzing the data; LKW and AREN: collecting and analyzing the data; EP: proofing the English language; MAH: analyzing the data and examining the final manuscript.

6 Conflicts of Interest

No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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