

Tyrosinase Inhibition Assay and Skin Whitening Cream Formulation of Edamame Extract (*Glycine Max*)

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ABSTRACT

Edamame or vegetable soybean (*Glycine max* L.) has very high protein content, and contains isoflavones which show many beneficial effects, including anticancer, antioxidant, and inhibiting tyrosinase activity. This study aimed to determine tyrosinase inhibitor activity of edamame extract, to formulate the extract into skin whitening cream, and to determine genistein content in extract and cream. The result showed that edamame extract had good tyrosinase inhibitor activity, and can be formulated into skin whitening cream with good cosmetological properties (pH, viscosity, and spreadability). Genistein content was retained in cream, indicating that the formulation process did not affect the genistein content in extract.

Keywords: Edamame, genistein, tyrosinase inhibitor, formulation, cream.

INTRODUCTION

Melanin is a heterogenous polyphenol like biopolymer with a complex structure and color varying from yellow to black¹. Melanocyte cells secrete melanin which is distributed to the basal layer of the dermis². Melanin has important role to protect skin from ultraviolet (UV) exposure³. It was known that tyrosinase catalyzes the initial step in the formation of the melanin in fungi and vertebrates. Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme which catalyze two distinct reactions of melanin synthesis: the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and the subsequent oxidation of this *o*-diphenol to the corresponding quinone, L-dopaquinone. This *o*-quinone is a highly reactive compound and competent to polymerize spontaneously to form melanin⁴. Although melanin plays major role in human skin protection against UV radiation, the production of abnormal pigmentation such as melasma, freckles, age spots, liver spots, and other forms of melanin, hyperpigmentation are undesirable, and can be a serious aesthetic problem. Therefore, inhibiting tyrosinase activity has become the subject of many studies recently⁵. Edamame or known as vegetable soybean had larger seeds compared to grain soybean, and had a sweet, nutty flavor, and better digestibility⁶. Edamame had very high protein content, and contained valuable phytochemicals, isoflavones. It was known that soybean's isoflavones had many beneficial effects, such as: anticancer, antioxidant, and as tyrosinase inhibitor⁷. The purposes of this study were to determine tyrosinase inhibition activity of Edamame extract, to formulate the extract into skin whitening cream, and to determine genistein content in both extract and the formulated

cream. To best of our knowledge, no studies were done regarding tyrosinase inhibition activity of Edamame extract and its formulation as skin whitening cream. In this study we chose vanishing cream base to formulate the extract, since the base had favorable characteristics such as transparent, easy to spread with cooling sensation, and easy to clean with water⁸. The genistein content in both extract and cream were also determined in this study, since genistein is major aglycone isoflavone in soybean⁹.

MATERIALS AND METHODS

Chemicals

Genistein was obtained from Tocris (Tocris Bioscience, Bristol-UK). Mushroom tyrosinase (EC 1.14.18.1) and L-tyrosine were obtained from Sigma Aldrich (Sigma Aldrich, Singapore). Toluene, ethyl acetate, acetone, formic acid, n-hexane, ethanol, and methanol were obtained from Smart Lab Indonesia. All of chemicals and solvents were analytical grade. Stearic acid, cetyl alcohol, stearyl alcohol, glycerin, triethanolamine, methyl paraben, propyl paraben, and propylene glycol were obtained from Brataco Chemika (PT. Brataco, Indonesia). Distilled water and jasmine oil were obtained from PT. Makmur Sejati, Indonesia. All of ointment ingredients were pharmaceutical grade.

Plant Materials

Edamame soybeans (*Glycine max* L.) were purchased from PT. Mitratani Dua Tudjuh, Jember Indonesia. The seeds were cut and air dried for 24 h without direct sun bathing. Afterward, plant fragments were grounded and sieved (80 mesh) to obtain seed powder prior to extraction process.

Table 1. Composition of the developed cream

No.	Ingredients	Uses	Quantity (%)
1	Edamame extract	Active ingredient	3.5
2	Stearic acid	Emulsifying agent	8.0
3	Cetyl alcohol	Stiffening agent	4.0
4	Lanolin	Emolient	2.0
5	Trietanolamine (TEA)	Emulsifying agent	0.5
6	Tween 80	Emulsifying agent	3.0
7	Propylene glycol	Humectant	10.0
8	Methyl paraben	Preservative agent	0.10
9	Propyl paraben	Preservative agent	0.05
10	Simetikon	Antifoaming agent	0,50
11	Jasmine oil	Corigen odoris	0.05
12	Water	Solvent	68.30

Table 2. Comparison the IC₅₀ value of edamame extract and references

Tested sample	IC ₅₀ (µg/mL)	Reference
Genistein	130.14	Present work
<i>Glycine max</i>	92.80	Present work
<i>Intsia palembanica</i>	14.80	Batubara <i>et al.</i> ¹²
<i>Durio zibethinus</i>	172.10	Batubara <i>et al.</i> ¹²
<i>Helminthostachys zeylanica</i>	128.80	Batubara <i>et al.</i> ¹²
<i>Smilax china</i>	5.10	Liang <i>et al.</i> ¹⁹
<i>Eugenia dysenterica</i>	11.88	Souza <i>et al.</i> ²⁰
<i>Pouteria torta</i>	30.01	Souza <i>et al.</i> ²⁰

Plant Extraction

About 150 g of plant powder was transferred into round bottom flask of soxhlet apparatus for lipids and waxes removal. Defatting process was carried out by hot extraction with *n*-hexane for 3 h¹⁰. The fluid (lipid-rich extract) was decanted and separated from plant residue. The wet residue was air dried for 15 m at room temperature to remove solvent residue. The dried plant residue (120 g) was extracted with 720 mL of 70% ethanol using ultrasonic washer (Elmasonic S 180 H, Elma Schmidbauer GmbH) at 25°C for 1 h¹¹. Afterward, the residue was separating from the fluid by filtration. Finally, the fluid (thin extract) was concentrated at 50°C in a rotary evaporator (Heidolph Laborota 4000, GmbH) to obtain dried extract.

Tyrosinase Inhibition Activity

Tyrosinase inhibition activity was determined using a method described by Batubara *et al.*¹² with slight modification. Dried extracts were dissolved in methanol and diluted with phosphate buffer (pH 6.5) to make a

Table 3. Comparison edamame cream and references

Active ingredient	Spreadability (g.cm/s)	Viscosity (dPa.s)	pH	Reference
Edamame extract	2.917	125.00	6.0	Present work
Various extract	3.333	270.25	6.1	Dhase <i>et al.</i> ²⁴
Diacerin	9.143	319.33	4.5	Kumar <i>et al.</i> ¹⁵

Table 4. Purity assay

Tested Sample	Track	Rf	r(s,m)	r(m,e)
Dried extract	Genistein standard	0,36	0,9979	0,9964
	Sample	0,35	0,9988	0,9985
Cream	Genistein standard	0,36	0,9978	0,9965
	Sample	0,35	0,9992	0,9983

Table 5. Identity assay

Tested Sample	Track	Rf	r(s,s)	r(s,a)
Dried extract	Genistein standard	0,36	0,9942	0,9955
	Sample	0,35	0,9942	0,9933
Cream	Genistein standard	0,36	0,9920	0,9972
	Sample	0,35	0,9920	0,9978

final concentration range at 10-500 µg/mL. In a 96 well plate, 70 µL of tested materials were transferred in each well and combined with 30 µL of tyrosinase (250 unit/mL in phosphate buffer). After incubation at 25°C for 5 m, 110 µL of substrat (1mM L-Tyrosine) was added to each well. The mixtures were incubated again at 25°C for 80 m. Afterward, absorbance in each well were read at 478 nm using microplate reader (Elx800 G, Dialab GmbH). Genistein was used as positive control. The percentage of tyrosinase inhibition was calculated as follows: % inhibition = [(A-B)/A] x 100, where A is the absorbance of mixture at 478 nm without inhibitor, B is the absorbance of mixture at 478 nm with inhibitor. The concentration of tested samples at which 50% enzyme activity was inhibited (IC₅₀) was obtained by linear curve fitting.

Formulation of Skin Whitening Cream

The cream formula was developed from previous study¹³ with slight modification. Accurate quantities of stearic acid, cetyl alcohol, stearyl alcohol, glycerin, and propyl paraben were weighed (Table 1) and melted at 70°C to form oil phase. Similarly, accurate quantity of edamame extract, TEA, propylene glycol, and methyl paraben were weighed and poured into corresponding water (Table 1) to form water phase. The water phase was stirred gently and heated up to 70°C. After the water phase reached the required temperature, the oil phase was slowly poured into the water with constant stirring until a smooth and uniform mixture of cream was obtained. The cream was cooling down at room temperature. Finally, accurate

Table 6. Comparison genistein content in edamame between present work with the references.

Tested sample	Genistein content ($\mu\text{g/g}$)	Reference
Dried extract (Ryokkoh genotype)	235.00	Present work
Edamame extract (Ryokkoh genotype)	19.79	Mebrahtu <i>et al.</i> ³¹
Soybean (Detam 1 genotype)	150.00	Hidayat <i>et al.</i> ³²
Fermented soybean (Wilis genotype)	576.00	Hidayat <i>et al.</i> ³²

quantity of jasmine oil was added into the cream and homogenized by gently stirring to obtain skin whitening cream.

Evaluation of Cream

The cosmetological characteristic such as: color, odor, spreadability, viscosity, pH, and type of emulsion were evaluated to determine cream properties. The spreadability of cream was evaluated using a method described by Garg *et al.*¹⁴. The consistency of cream was determined by viscotester (RION VT-04, Obsnap Instrument Malaysia).¹³ The pH of cream was observed by pH meter (UB-10, Denver Instrument, NY) as described by Kumar *et al.*¹⁵. The type of emulsion was determined using methylen blue dye method described by Lachman *et al.*¹³.

Preparation of Solutions for TLC Analysis

The dried extract (500 mg) was ultrasonically extracted with 10 ml methanol at 25°C for 1 h. Supernatant was taken TLC analysis. The cream (1.75 g) was ultrasonicated with 10 mL methanol at 25°C for 1 h. The combined supernatants were centrifuged at 3000 rpm for 30 m for the TLC analysis¹⁶. Genistein was dissolved in methanol to make a final concentration range at 20-100 $\mu\text{g/mL}$, and used as reference.

Determination of Genistein in Dried Extract and Cream

Densitometric analysis was applied for the determination of genistein in both dried extract and cream using TLC Scanner 3 (Camag, Mutten Switzerland). Extract solutions (6 μL) and reference solutions (2 μL) were spotted onto a GF₂₅₄ TLC plate (Merck, GmbH) at 25°C and 45% humidity. The plate was developed with eluent system of toluene-ethyl acetate-acetone-formic acid (20:4:2:1) in a TLC developing chamber (Camag)¹⁶. Spots detection was carried out under UV light at 254 nm. Cream solutions were spotted in another plate and developed with similar TLC analysis condition. The purity and identity assay of all tested samples were determined by absorbance scanning at 200-400 nm. Quantification of genistein spots of all tested samples were done at 266 nm. The identity check (rS,S and rS,A), purity check (rS,M and rM,E), and quantification of genistein were carried out using winCATS software version 1.4.1.8154 (Camag).

RESULTS and DISCUSSION

Tyrosinase Inhibition Activity

Edamame extract had lower IC₅₀ value than that of genistein as it can be seen in Table 2. It was indicated that

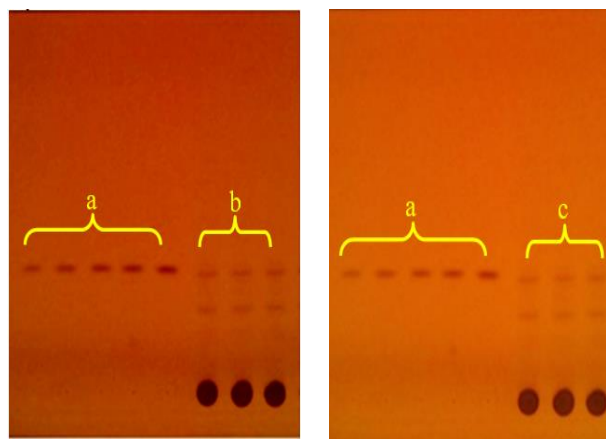


Figure 1. TLC profile of tested samples under UV-254 nm, genistein standard (a), genistein in dried extract (b), genistein in cream (c)

the extract exhibited higher tyrosinase inhibition activity than its corresponding pure compound. Edamame extract contained other aglycone isoflavone like daidzein and glycitein, and their corresponding glycosides like genistin and daidzin which also have tyrosinase inhibition activity^{5,17}. Although the tyrosinase inhibition activity of Edamame extract was lower than *Intsia palembanica*, the bioactivity was greater than that of other Indonesian medicinal plants, *Durio zibenthinus* and *Helminthosthachys zeylanica* (Table 2). These medicinal plants are traditionally used as skin whitening agent in Indonesia¹². The bioactivity of Edamame extract was lower than *Smilax china*, *Eugenia dysenterica*, and *Pouteria torta* respectively. However, according to Cuorto *et al.*¹⁸, any compounds which have IC₅₀ value less than 100 $\mu\text{g/mL}$ in tyrosinase inhibition activity were prospective to be developed as skin whitening agent. Hence, edamame extract could be a good candidate as natural skin whitening agent.

Cosmetological Properties of Cream

The main characteristic of vanishing cream base was its emulsifying property, which can be obtained by combining fatty acid with alkali²¹. In this regard, were used stearic acid and TEA. The cream formulation was targeting melanocytes which were located in basal epidermis. Genistein had log P value 3.04²², which indicated that this compound can across the epidermis. According to Rowe *et al.*²³, the excipients which were used for the cream shouldn't interfere the bioactivity of active ingredient. Hence, we carefully chose the excipients to ensure that the extract as the active compound retained its bioactivity. Glycerin was chosen as humectant, since it can give humidity for skin by water absorption from its surrounding environment. Humectant can also make stratum corneum layer to swell, and help the active ingredient crossing the layer to achieve target cells. Cethyl alcohol and stearyl alcohol were used as stiffening agent. However, cetyl alcohol gave smooth texture on cream, while stearyl alcohol gave good consistency. Methyl paraben and propyl paraben were used as synergistic preservative, since the former was distributed into aqueous phase, while the latter was in oil

phase¹³. The formulated cream had white-yellow color and jasmine fragrance. Spreadability of formulated cream was close to various extract cream (Table 3). Dhase *et al.*²⁴ reported that their cream was spread easily. However, the formulated cream was easily spreadable although its spreadability value was lower than that of diacerein cream (Table 3). Good topical cosmetic had viscosity ranging from 50-500 dPa.s²⁵. Therefore, the formulated cream had good consistency, although its viscosity value is the lowest among others, as it can be seen at Table 3. Moreover, according to SNI²⁵, the formulated cream had also good consistency as topical cosmetic. The formulations intended for application to the skin should have pH which close to the pH of skin (4.5-6.5) to prevent skin irritation²⁶. The pH of formulated cream was complied with the requirement, as depicted in Table 3. Hence, the formulated cream had good cosmetological properties.

Determination Genistein in Dried Extract and Cream

TLC profiles of all tested samples (dried extract and cream) were shown in Figure 1. Spots of genistein were clearly detected under UV-254 nm as black spots at Rf 0.35-0.36. Purity and identity tests for genistein spots were done for both dried extract and cream samples. It was known that genistein spots of all samples were pure and identical with genistein standard, since all of calculated $r(s,m)$, $r(m,e)$, $r(s,s)$, and $r(s,a)$ for all samples were higher than 0.99²⁷, as it can be seen Table 4 and Table 5. Hence, the analytical condition was suitable for the determination of genistein in both dried extract and cream samples. The genistein content in dried extract was higher than its corresponding genotype which was collected at Virginia, USA (Table 6). This difference content might be due to climate conditions²⁸. According to Tsukamoto *et al.*²⁹, the temperature had greater impact on the isoflavone content. Fermentation can change glycoside isoflavones (genistin, daidzin, and glycitin) into their corresponding aglycones (genistein, daidzein, and glycitein)³⁰. Consequently, genistein content in fermented soybean was higher than that of unfermented soybean, as it can be seen in Table 6. The recovery value of genistein content in cream was $98.75 \pm 0.60\%$. AOAC³³ mentioned range of recovery value was 90-107%. This result was fulfill the the spesification. Hence, the analytical condition was suitable for the determination of genistein in cream samples and formulation gave no effect on genistein content in cream.

CONCLUSION

Edamame extract was more potent as tyrosinase inhibitor than genistein as due to other aglycone isoflavones like daidzein and glycitein, and its corresponding glycosides like genistin and daidzin which also have tyrosinase inhibition activity. The formulated cream had good cosmetological properties include pH, viscosity, and spreadability. Additionally, preincubating the enzyme in the presence of 10 M of quercetin and in the absence of the substrate shows that quercetin did not directly inactivate the enzyme, since it did not significantly decrease the enzyme activity. The foregoing data confirm

that quercetin had a strong inhibitory effect on the action of mushroom tyrosinase (jeong)

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