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ORIGINAL ARTICLE

The Study of Safety and Skin Whitening Efficacy of Melinjo (*Gnetum gnemon* L.) Seed Extract-Loaded Lipid Particle Gel

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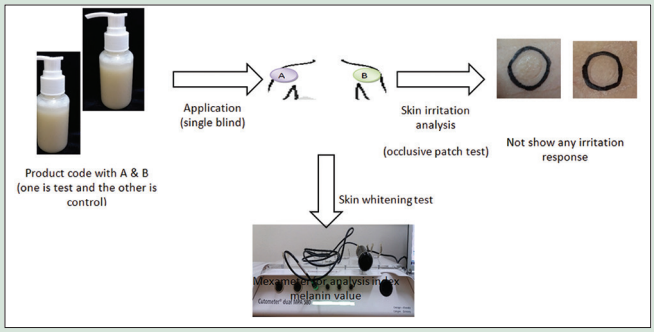
ABSTRACT

Background: Melinjo (*Gnetum gnemon* L.) seed extract (MSE) is potential as skin-whitening agent because it contains trans-resveratrol and its derivatives, to inhibit tyrosinase in melanogenesis process. Using MSE in cosmetic products will be challenging due to resveratrol chemical instability and bioavailability in the skin. Many cosmetic products have been developed using lipid particle technology to improve their limitation. The objective of this research was to examine the skin safety and whitening efficacy of MSE-loaded lipid particle gel in healthy human subjects. **Materials and Methods:** Single occlusive closed patch test for 24 h was used as the skin irritation analysis. Irritation responses were graded after patch removal and compared to the control for evaluation. The efficacy study was performed using Mexameter to measure skin melanin index on 25 female volunteers. **Results:** The result showed the test product did not induce skin irritation effect. The skin melanin index was statistically significant decreased ($P < 0.05$) after 28 days of application the test product, with the averaged by 3.50%, and skin melanin index changed by increase 0.75% in the control group. **Conclusion:** Application MSE-loaded lipid particle gel can brighten the skin, without cause irritation under normal conditions of use.

Key words: Gel, *Gnetum gnemon,* melinjo seed extract, resveratrol, safety, skin whitening

SUMMARY

- Melinjo seed extract (MSE) containing resveratrol has been shown a mechanism to inhibit tyrosinase in melanogenesis process, and thus, it would be potential for skin-whitening agent. The present study was designed to evaluate skin-whitening efficacy of MSEs that formulated into lipid particle gel on healthy female volunteer
- The skin irritation analysis of the product test, MSE-loaded lipid particle gel, was conducted in a single occlusive patch test for 24 h, and the irritation response is evaluated until 48 h after patch removal that did not show any irritation reaction compared with control
- The skin-whitening efficacy is analyzed using Mexameter to know skin melanin index, and after 28 days of use, the cosmetic product test showed that it is statistically potential as skin-whitening agent.



Abbreviations Used: α-MSH: α-melanocyte stimulating hormone; MITF: Microphthalmia-associated transcription factor; MSE: Melinjo seed extract.

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INTRODUCTION

Melanin contributing to human skin color, especially eumelanin, is the major pigment that has a primary function of protecting the skin from damage caused by ultraviolet (UV) exposure such as photoaging or photocarcinogenic.[1] Melanocytes cell in the basal layer of epidermis contains melanosome. The melanosome function is a synthesis, storage, and transfer of melanin to the outermost layer of skin (keratinocytes).[2] Melanogenesis could be induced by UV radiation through stimulated the secretion of a α-melanocyte-stimulating hormone that would be activated microphthalmia-associated transcription factor (MITF). MITF would lead to upregulation of tyrosinase, the primary enzyme in melanogenesis.[3]

Tyrosinase involved in two different oxidation reactions to melanin synthetic pathway. The first step is the hydroxylation of L-tyrosine to L-DOPA, and the second step is oxidation L-DOPA to dopaquinone. From dopaquinone, the melanogenesis pathway is divided. Dopaquinone spontaneous conversion to dopachrome and through the tyrosinase-related protein 1 and 2 turns dopachrome into eumelanin. In the other, dopaquinone also conjugated with cysteine or glutathione to form pheomelanin. Eumelanin is a black-brown melanin, whereas pheomelanin is a yellow-red melanin.[4]

UV exposure is one of the major causes of esthetic skin problem such as skin pigmentation. However, it causes abnormal activity of tyrosinase that involved excessive of melanin. Because some people prefer white skin, they always find cosmetics to reduce pigmentation. Thus, inhibition of tyrosinase and melanogenesis will be important as a target for skin-whitening agent.[5,6]

Gnetum gnemon L. belongs to the genus *Gnetum* in Gnetaceae family. This plant is widely cultivated in Southeast Asia, especially in Indonesia; melinjo is the familiar name for the plant. Melinjo seeds in ripe fruits are popular as a vegetable consumed in soup or made crackers (local name: emping) with a slightly bitter taste.[7]

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Resveratrol (a polyphenolic compound group of a stilbenoid) from melinjo seeds must be considered as one of a natural source of resveratrol with high bioavailability and safety. Melinjo seed extract (MSE) contains abundant in resveratrol derivatives, in the form of resveratrol monomer derivative, which is trans-resveratrol (3,5,4-trihydroxy-trans-stilbene), trans piceid, and isorhapontigenin; and resveratrol dimer derivative, which is gnetin C, gnemonoside A, gnemonoside D, gnemonoside C, and gnetin L.[8,9] Gnetin C, gnemonoside A, and gnemonoside D are major constituents, while trans-resveratrol is a minor constituent of MSE.[10]

Resveratrol derivatives in MSE revealed beneficial pharmacological effects. That is known as lipase and α-amylase inhibitors, antimicrobial,[10] antioxidant against free radicals,[11] immunostimulator,[12] inhibition on tumor angiogenesis,[13] decreased serum uric acid and increased high-density lipoprotein.[14] MSE has had cosmeceutical properties. Previously on the in vitro and in vivo study, trans-resveratrol potent inhibited of tyrosinase and suppress melanogenesis activity,[15,17] as good as the work of Gnetin C.[18] Therefore, MSE might be potential use as new skin-whitening agent.

Trans-resveratrol is restricted to formulate in general cosmetic product. Trans-resveratrol unstable because susceptible to degradation by the light,[19] which it would be changed trans-isomer resveratrol to cis-isomer, that less biologically active.[20] Moreover, resveratrol is poor solubility in water, which influences absorption and lowering penetration of resveratrol to the skin. Their limitation could be enhanced when resveratrol inclusion into lipid system.[21] Lipid particles in the topical cosmetic formulation have shown to be helpful to improve stability and biological activity by protecting effect, including water solubility to increasing penetration through stratum corneum by skin hydration that means to enhance the bioavailability of resveratrol in the skin.[22,23]

The aim of this current study was to examine the safety and whitening effect of MSE-loaded lipid particle gel in the human healthy skin.

MATERIALS AND METHODS

Materials

Cutina® glyceryl monostearate (GMS) V and Cremophor® A 6 (ceteareth-6 and stearyl alcohol) were provided by BASF GmBH (Germany), and Brij™ CS25 (ceteareth-25) was provided by Croda (United Kingdom) as a gift. Carbopol® Ultrez 20 polymer (Lubrizol), propylene glycol (Dow Chemical Ltd.), sodium metabisulfite, potassium sorbate, Na_2 EDTA (Akzo Nobel Chemical), and sodium hydroxide were used in the study. MSE contains trans-resveratrol of 3.3 mg/g.

Formulation of lipid particle gel

The formulation of MSE-loaded lipid particle system was GMS (10%), CS25 (4%), A6 (0.8%), MSE (10%), and water (75.2%). The lipid particle dispersion was prepared using the high-shear homogenization and hot-melted technique. After all the materials melted and mixed, added MSE, and then homogenized at 1250 rpm for 20 min and 30,000 rpm for 5 min (H04 Edmund Buhler GmBH, Germany). Characterization of MSE containing resveratrol-loaded lipid particles was determined by particle size, potential zeta, and entrapment efficiency. This method based on a previously reported method, with some modifications.[24,25]

The gel formulation was carbomer (0.4%), propylene glycol (10%), sodium metabisulfite (0.1%), potassium sorbate (0.15%), Na_2 EDTA (0.01%), 18% sodium hydroxide solution (0.5%), perfume (0.1%), MSE-loaded lipid particles (10%), and added water *ad* 100%. The gel was prepared with mechanically stirring by dispersed gel base into the water and added MSE-loaded lipid particles. Analysis amount of the MSE containing resveratrol in the product was performed using high-performance liquid chromatography (HPLC).

The products

The test product was MSE-loaded lipid particle gel as the active ingredient. The control product was gel base with the same formulation as the test product, without the active ingredient.

Human participants

All the women volunteers, who met the inclusion and exclusion criteria [Table 1], must sign an informed consent to participate in this study. The study was approved by the Ethical Committee, Faculty of Medicine Universitas Indonesia, and performed in accordance good clinical practice guidelines.

Skin irritation test

Skin irritation test of the products was performed using patch test. Human patch tests were conducted for 24 h with an occlusive patch. Thirty-eight women between 20 and 40 years old participated in this study. The products were dropping 20 μl in a Finn Chamber tape (SmartPractice, Arizona, United States). It was applied to the upper arm as a test side. The skin irritation response of patched side was observed and graded at 30 min, 24 h, and 48 h after patch removal by an experienced assessor.[26]

The grading criteria for human skin irritation are shown in Table 2. Skin responses were evaluated, and the score was calculated according to the formula.[27]

$$\text{Irritation Score (R)} = \frac{\sum(\text{grade} \times \text{number of responders})}{4 \times \text{total subjects}} \times 100 \times \frac{1}{2}$$

The score of irritation response was converted to human primary irritation index criteria for cosmetic products from human irritation patch test [Table 3].[27]

Skin-whitening test

The study was a single-blind clinical trial with the intersubject design. Twenty-five women between 20 and 40 years old were enrolled to

Table 1: Inclusion, exclusion, and dropout criteria for the participants

Inclusion criteria
1. Healthy women aged 20×clusion, and drop out criteria for the partic
2. Willing to signed informed consent to become a volunteer, after informed the purpose and any information of the study
3. Willingly stop used cosmetic products in the around test area, starting from 1 week before and during the test
4. Willing to cooperative during the study
Exclusion criteria
1. Pregnancy or breastfeeding
2. Around test area presence tattoos, scars, sunburn, or uneven skin tones, and excessive skin sunlight exposure
3. Allergy to any cosmetic products or has a history of skin allergic reaction
4. Anyone used immunosuppressive within 3 months, antihistamine, anti-inflammatory, retinoid, hormonal contraceptives, or laser therapy within 1 month
5. Anyone used any drug that affects skin reaction
6. Anyone with a chronic disease, infectious skin disease or any skin disease, atopic dermatitis, eczema, psoriasis that may interfere the study
7. Smoking, alcoholic, or drug user
Dropout criteria
1. Show of allergy reaction to the products during the test
2. No used the product
3. No came to the research center for examination
4. Resigns from the study due to illness

participate in this study. The participants received a test and a control product that was applied once daily at night before sleep to the left or right of the upper arms for 28 days. The side for applying the product was measuring 6 cm x 6 cm. Every participant received a diary for monitoring their compliance and recording adverse events during the test.

Participants came to the research center for measurement of melanin index on both sides of the upper arm. Their melanin index was analyzed using a Mexameter (Cutometer® dual MPA 580, Courage + Khazaka electronic GmbH, Germany). The evaluation was performed in the room, where the temperature was maintained at 22°C ± 2°C. The measurement was conducted at baseline before used the product and at treatment weeks 2 and 4. Each measurement is taken three times, and the average values were used for the analysis.

Statistical analysis

The statistical analyses were utilized using SPSS® software statistics Version 22.0 (IBM corp., New York, USA). The tests of normal distribution and homogeneity of variance were assessed using Shapiro–Wilk test and Levene statistic. The statistical significance of the differences was determined using the repeated measures ANOVA, with $P < 0.05$ which was considered to be statistically significant.

RESULTS

Melinjo seed extract-loaded lipid particle gel

Lipid particles system was prepared by the dispersed MSE into the melted GMS (lipid phase) and mixed with the hot water surfactant (water phase) to form the emulsion. Characterization of the MSE-loaded lipid particles showed that the higher average particle size was 794.6 ± 201.5 nm. The potential zeta value was 62.56 mV, which means that the lipid particle formula was stable when stored, and there is no tendency for aggregation or gelation phenomena. A good potential zeta value ranges from Z> +25 mv or Z <−25 mv.

The entrapment efficiency of MSE containing resveratrol in lipid particle system was evaluated to determine the amount of encapsulated resveratrol. Lipid system synthesis used centrifugation method. The concentration of resveratrol in the lipid system is as total resveratrol, and the filtrate of centrifugation result is as free resveratrol (not trapped in the system). They were measured using HPLC and calculated by the equation as below: Entrapment Efficiency = (total resveratrol - free resveratrol)/total resveratrol. The entrapment efficiency result was 89.46% ± 0.04%. The result was indicated MSE incorporation into lipid system as well. The good solubility of MSE in the lipid is one of the factors determining the high entrapment efficiency. GMS as a solid lipid matrix material is monoglyceride lipid that promotes enhanced MSE solubilization. Combination type of surfactant and lipid also would give a good result to enhanced MSE entrapment efficiency.

Furthermore, the gel was easily absorbed into the skin and did not leave a sticky impression when applied on the hand.

High-performance liquid chromatography

Final analysis of the resveratrol contained in products of MSE-loaded lipid particle gel was evaluated using HPLC. The HPLC system was isocratic, volume injection 20 µL. The stationary phase was a C-18 column (150 mm × 4.6 mm I. D., 5 µm) with temperature column 25°C. The mobile phase consisted of 75% water and 25% acetonitrile adjust pH 3. The flow rate was 1 mL/min. The UV detector was set at 306 nm. The final product contains 0.008% resveratrol (percent recovery: 155.10%). The HPLC profile is shown in Figure 1.

Safety study

The evaluation skin irritation potentials of the products were conducted using occlusive patch testing for 24 h. Every participant prohibited to wash or scrub the patches. Skin irritation was determined by a dermatologist to know skin clinical conditions. The results showed that neither of the products induced skin irritation in all of the volunteers. Irritation score and the human primary skin irritation index are shown in Table 4. The volunteers were also asked to check their skin condition when at home after patch removal. The result of self-report showed no sign of skin irritation potentials after 48 h (data not shown).

Table 2: Grading criteria for skin irritation

Grade	Clinical response
0	Negative reaction
1	Slight erythema, spotty, or diffuse
2	Moderate erythema
3	Intense erythema with edema
4	Intense erythema with edema and vesicles

Table 3: Human primary irritation index

Irritation score (*R*)	Criteria
$0.00 \leq R < 0.87$	No/slight irritation
$0.87 \leq R < 2.42$	Mild irritation
$2.42 \leq R < 3.44$	Moderate irritation
$R \geq 3.44$	Severe irritation

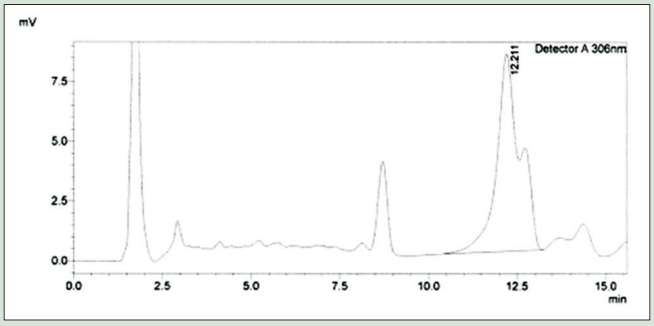

Figure 1: High-performance liquid chromatography chromatogram for resveratrol of the products used in this study

Table 4: Human primary irritation index of the products

Product	Number of responders of skin irritation response															Irritation score (*R*)				Primary irritation index
	30 min					24 h					48 h					30 min	24 h	48 h	Mean	
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4					
Test product	38	-	-	-	-	38	-	-	-	-	38	-	-	-	-	0.0	0.0	0.0	0.0	No/slight irritation
Control product	38	-	-	-	-	38	-	-	-	-	38	-	-	-	-	0.0	0.0	0.0	0.0	No/slight irritation

Efficacy study

The melanin index data for analyzed skin-whitening efficacy of the test product that contains MSE-loaded lipid particles and the control product that did not contain MSE-loaded lipid particles were determined using a Mexameter. All data used for analysis were homogeneous and normally distributed. The average intensity values of melanin index impairment for every volunteer after used the product on weeks 4 compared with the baseline values are shown in Table 5. The average before and after the test of all 25 participants was about +8.0 in the test product group and about -1.5 in the control group, that means the average of melanin index of the participant who used the test product decreased by 8 points, compared to controls that occurred an increase by 1.5 points on weeks 4 even though no alteration that occurred on weeks 2.

The statistic result showed that in Table 6, the melanin index of the test group on weeks 2 and 4 was significantly lower than the values before treatment ($P < 0.05$), with the change rates of melanin index for the test group on weeks 2 and 4 were 2.79% and 3.50%, respectively. In addition, the control group was not significantly different, before and after treatment. The change of melanin index in the test group was significantly greater than the control group in weeks 4 ($P < 0.05$), as shown in Table 7. The changes averaged of melanin index against time are shown in Figure 2.

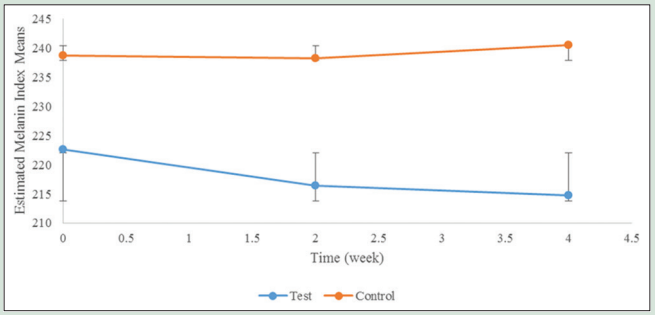

Figure 2: The changes average of melanin index on 4 consecutive weeks against the duration of treatment. Data are the means ± standard errors ($n = 25$)

DISCUSSION

Resveratrol is polyphenolic compound found in various plants, including *G. gnemon* L. On previous *in vitro* study to support these study result, resveratrol from melinjo seed has shown inhibit tyrosinase on melanin synthesis in murine.[18] Moreover, resveratrol also inhibits tyrosinase on human melanocytes,[15] and guinea pig skin induced pigmentation by UV-B radiation.[17] Resveratrol could act as a whitening agent due to inhibit melanogenesis activity through reduced MITF factor and tyrosinase promoter activities that would down-regulation tyrosinase, the effect on tyrosinase maturation and melanin synthesis.[28]

The resveratrol is known for poor water solubility, which is influence skin absorption. The way to improve skin penetration of resveratrol with lipid particles has been investigated by Sun *et al.*[21] Lipid particles are a suitable system for hydrophobic compound incorporation. The lipid particle, especially solid lipid matrix, could enhance the penetration into the skin. The amount of active compound is retained in the outer skin surface reduced. Meanwhile, the lipid particles have an occlusion effect, which could reduce transepidermal water loss and helps to increase the penetration of active ingredients into the skin. Thus, MSE containing resveratrol is lipophilic nature ingredient suggesting a preferential into particle lipid matrix to improve properties of resveratrol as an active.

In the cosmetic industry, the safety and efficacy study is important to the product. This study has been investigated skin irritation potentials of the products. And no one of the volunteers that has skin irritation after skin patch test during 48 h. The criteria for classification of primary irritation

Table 5: The average of difference of the melanin index value for every participant before and after treatment

No	Melanin index values					
	Test			Control		
	Before	After	Mean diff	Before	After	Mean diff
1	163	159	4	148	168	−20
2	182	174	8	177	173	4
3	136	132	4	159	158	1
4	179	190	−11	181	192	−11
5	183	179	4	190	206	−16
6	157	156	1	166	174	−8
7	176	162	14	214	210	4
8	188	181	7	216	219	−3
9	227	222	5	228	234	−6
10	212	208	4	262	269	−7
11	248	229	19	260	267	−7
12	259	251	8	280	287	−7
13	230	221	9	228	227	1
14	244	232	12	278	279	−1
15	265	249	16	263	267	−4
16	216	203	13	259	257	2
17	233	232	1	243	246	−3
18	252	233	19	287	283	4
19	224	226	−2	247	243	4
20	260	249	11	275	274	1
21	241	228	13	238	232	6
22	220	222	−2	255	243	12
23	231	226	5	248	244	4
24	304	287	17	286	284	2
25	336	320	16	379	377	2

Table 6: The changes of melanin index for 4 consecutive weeks after application the products

Product	Week	*N*	Mean	SD	SEM	*P**	Change rate (%)
Test	0	25	222.6	45.9	9.2	-	-
	2	25	216.4	44.5	8.9	0.030	-2.79
	4	25	214.8	42.2	8.4	0.000	-3.50
Control	0	25	238.7	50.8	10.2	-	-
	2	25	238.2	52.1	10.4	1.000	-0.21
	4	25	240.5	48.3	9.7	0.643	0.75

*Significantly different at $P<0.05$ compared with before treatment. SD: Standard devaition; SEM: Standard error of mean

Table 7: Analysis of differences in the average of melanin index between the test and control groups

Comparison	*n*	Week	Mean difference	SD	SEM	*P**
Test and	25	2	21.8	20.9	4.2	0.000
control	25	4	25.7	19.2	3.8	0.000

*Significantly different at $P<0.05$. SD: Standard deviation; SEM: Standard error of mean

index of human skin are used in the cosmetic products based on the actual usage. A cosmetic compound is a mixture of various chemical ingredients in the formula, which could be potential as an irritant and trigger skin irritation, depending on the length of exposure time and the amount of use.[27,29]

There has been no clinical trial study to examine the effects of MSE as topical skin whitening. Based on this study, the test product could give good result to support the claim of human skin-whitening effect of *G. gnemon* seed extract containing resveratrol. The application gel containing MSE-loaded lipid particles resulted in a significant reduction melanin index on the weeks 2 and toward lower melanin index afterward until weeks 4. This effect is probably due to the trans-resveratrol content and their derivates, such as gnetin. However, the lipid particles as a delivery system of an active ingredient may also help to improve biological activity and bioavailability by increasing skin penetration to the target side.

CONCLUSION

In conclusion, MSE which contains resveratrol has the potency as a cosmetic ingredient for the skin. Cosmeceuticals designed of MSE-loaded lipid particle gel exhibited a beneficial effect for skin whitening, without induced skin irritation.

Acknowledgments

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Nil.

Conflicts of interest

There are no conflicts of interest.

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