J. Soc. Cosmet. Scientists Korea
Vol. 30, No. 3, November 2004, 409-414

코직산 유도체의 합성과 미백효과

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Synthesis of Novel Kojic Acid Derivative and Its Anti-pigmentation Effect

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요 약: 코직산은 티로시나제의 억제에 의한 미백효능을 가지는 물질로 널리 알려져 있으나, 낮은 안정성으로 인하여 화장품 원료로의 사용에 제약을 갖고 있다. 이에 본 연구에서는 유기합성적 방법에 의해 안정성과 미백효과를 가지는 코직산 유도체를 고수율로 합성하였다. *O*-pentaacetyl-β-D-glucose를 루이스산과 유기염기를 이용하여 위치 선택적이고, 입체 선택적으로 코직산의 6번 위치에 도입시켜 kojic acid 6-*O*-2′,3′,4′,6′-tetraacetyl-β-D-glucopyranoside (KTGP, 80%)를 합성한 후, 가수분해하여 kojic acid 6-*O*-β-D-glucopyranoside (KGP, 70%)를 수득하였다. ^{1}H-NMR과 ^{13}C-NMR 분석으로 구조를 확인하였다. KGP를 가지고 티로시나제 활성저해, 프리라디칼소거능과 멜라닌합성저해 실험을 실시하였고, 그 결과 티로시나제 활성저해와 프리라디칼소거능에서 코직산보다 높거나 비슷한 활성을 보여주었다. 코직산 수준의 미백효능을 확인하였기에 유기 화학적으로 합성된 KGP의 미백원료로서 사용을 기대할 수 있다.

Abstract: Kojic acid is well known for its anti-pigmentation effect with tyrosinase inhibition activity. However, kojic acid is a unstable compound. In order to improve stability, kojic acid derivative, kojic acid 6-*O*-2′,3′,4′,6′-tetraacetyl-β-D-glucopyranoside (KTGP), was synthesized with *O*-pentaacetyl-β-D-glucose through the *regio*- and *stereo*-selective glycosylation of 6-OH group of kojic acid. High yield (80%) was obtained by the use of Lewis acid and organic base in nonpolar solvent. Hydrolysis of KTGP with the aid of sodium methoxide in methanol afforded kojic acid 6-*O*-β-D-glucopyranoside (KGP), which was confirmed by ^{1}H-NMR and ^{13}C-NMR. KGP is freely soluble in water and soluble in methanol and ethanol. Inhibition activity of KGP for tyrosinase was investigated by measuring the activity of mushroom tyrosinase compared with those of ascorbic acid, kojic acid, and arbutin. The free radical-scavenger activity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. In toxicity assay, KGP was much less toxic than kojic acid and arbutin, Therefore, glycosylation of kojic acid may be useful for the development of stable kojic acid derivatives.

Keywords: *kojic acid, kojic acid 6-O-β-D-glucopyranoside, anti-pigmentation, glycosylation, tyrosinase*

1. Introduction

The color of skin highly depends on the amount of melanin in the skin. Melanin is produced by melanocytes, normally found in the epidermal basal layer. Within the melanocytes, melanin is bound to a protein matrix to form melanosomes. In the melanosomes, tyrosinase converts tyrosine to eumelanin or pheomelanin. By blocking at the various points of the pathways, skin lightening agents can inhibit or can also be used to treat local hyperpigmention or spots which are caused by a local increase in melanin synthesis or uneven distribution[1-3].

Arbutin, kojic acid, vitamin C, and its derivatives were all tyrosinase inhibitors[4,5]. Among them, kojic acid (2-hydroxymethyl-5-hydroxy-γ-pyrone) isolated from *Aspergillus oryzae* is well known inhibitor of tyrosinase responsible for melanin biosynthesis in the melanocytes, which conferred the cosmetic skin whitening effect of kojic acid[6,7]. Kojic acid inactivates tyrosinase

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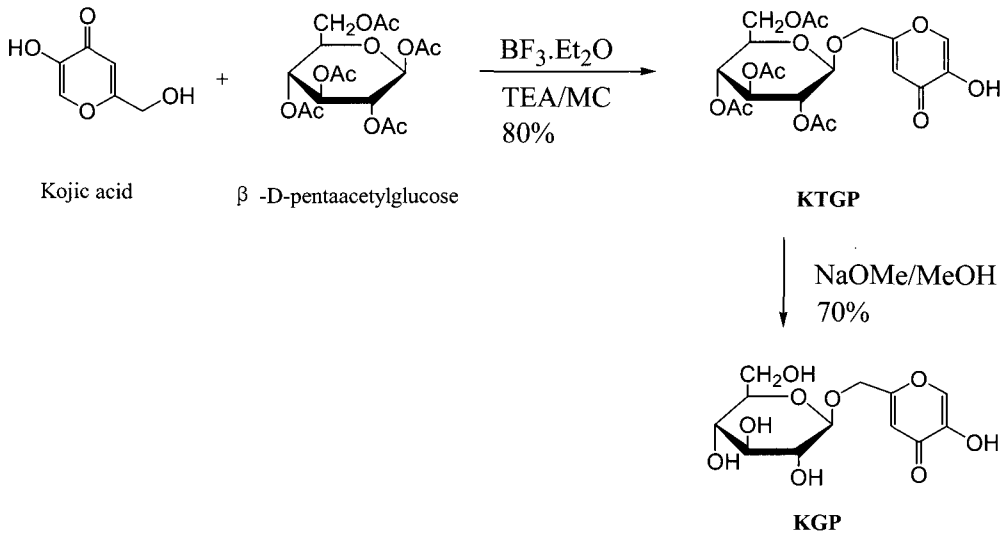

Kojic acid β -D-pentaacetylglucose **KTGP** **KGP**

Scheme 1. Synthesis of kojic acid 6-*O*-*β*-D-glucopyranoside.

by chelating with its vital copper ion and suppressing the tautomerization from dopachome to DHICA (5,6-dihydroxyindole-2-carboxylic acid). However, kojic acid is too unstable to light, heat, and pH to make various formulation for cosmeceutics. The color of kojic acid usually turns into yellowish brown with the lapse of time in the final product due to chelating with many metal ions, such as iron ion (Fe^{3+}) to give colored complex, slowly oxidized by contact with air. To overcome these demerits, many attempts has been tried until now, for example, amino acid derivatives of kojic acid were developed, which exhibited enhanced inhibitory activity relative to kojic acid, but their synthetic yield was generally very low[8,9].

Glycosylation is an important structural modification method of a wide variety of exogenous compounds and widely used, resulting in the conversion of unstable and water-insoluble compounds to more stable and water-soluble ones by usually microbe and cell culture[10-12].

The major drawback of these methods is mainly due to low yield, providing a mixture of *α*- and *β*- isomer. Therefore, we have tried to find the method to give stable kojic acid derivative with high yield and *stereo*-seletivity.

Herein, we report the synthesis of novel kojic acid derivative, kojic acid 6-*O*-*β*-D-glucopyranoside (KGP) though the *regio*- and *stereo*-selective glycosylation of kojic acid with *O*-pentaacetyl-*β*-D-glucose (Scheme 1), and their whitening effect, such as tyrosinase activity inhibition assay, free radical scavenger assay and inhibition of melanogenesis. this kojic acid derivative showed good activities and negligible cytotoxicity, suggesting their safety in the application to cosmetic ingredient.

2. Materials and Methods

2.1. Materials and Instruments

Chemicals and organic solvents were purchased from Sigma-Aldrich (St. Louis, MI, USA) and reagent grade unless otherwise indicated. Solvents were purified in common methods before use. The reactions were routinely carried out under inert atmosphere. All culture media were purchased from GIBCO-BRL (Grandland, NY, USA). Melting point was measured using an MEL-TEMP® (Laboratory Devices inc. USA). ^{1}H and ^{13}C NMR spectra were recorded on Varian-gemini 200 spectrometer. Absorbance at 480 was read by ELISA reader (Tecan A-5082, Austria).

2.2. Synthesis of Kojic Acid 6-*O*-2′,3′,4′,6′-Tetraacetyl-*β*-D-Glucopyranoside (KTGP)

Under the stream of nitrogen, to a 300 mL round bottom flask were added kojic acid (10 g, 0.07 mol), *O*-pentaacetyl-*β*-D-glucose (27.45 g, 0.07 mol), anhydrous methylene chloride (50 mL) and triethylamine (14.16 g, 0.14 mol). Borontrifluoride etherate (39.9 g, 0.28 mol) was dropwise added to the mixture for 30 min with stirring. After stirring for 48 h at 30℃ the re-

action was terminated and 70 mL of water was added and the organic layer was separated. The separated organic layer was washed with 50 mL of water, dried over anhydrous $MgSO_4$, filtered, and concentrated to yield a crude product. The crude product was recrystallized with toluene to give kojic acid 7-*O*-2′,3′,4′,6′-tetraacetyl-β-D-glucopyranoside (33 g).: Yield 80%, mp: 130℃, ^{1}H-NMR (200 MHz, $CDCl_3$, ppm) 2.04 (d, 6H), 2.1 (d, 6H), 3.70~3.77 (m, 1H), 4.18~4.30 (m, 2H), 4.44~4.69 (m, 3H), 5.03~5.28 (m, 3H), 6.49 (s, 1H), 7.84 (s, 1H).

2.3. Synthesis of Kojic Acid 6-*O*-β-D-Glucopyranoside (KGP)

Under the stream of nitrogen, to 100 mL round bottom flask were added KTGP (2 g, 4.23 mmol), dried methanol (10 mL) and sodium methoxide (0.24 g, 4.4 mmol) in methanol (5 mL) solution. The mixture was refluxed for 12 h. After the reaction was terminated, 0.5 g of cation-exchange resin was added and stirring for 30 min, filtered. The solution was evaporated under the vacuum to yield a crude product. The product was recrystallized with ethanol to give kojic acid 6-*O*-β-D-glucopyranoside (0.9 g).: Yield 70%. mp: 194~196 ℃. ^{1}H-NMR (200 MHz, DMSO. ppm) 3.66~3.75 (m, 2H), 4.25 (d, 1H), 4.47~4.69 (m, 3H), 4.98 (d, 1H), 5.04 (d, 1H), 5.30 (d, 1H), 6.61 (s, 1H), 8.09 (s, 1H), 9.17 (s, 1H). ^{13}C-NMR (50MHz, DMSO, ppm) 60.00, 64.18, 68.95, 72.33, 75.54, 75.96, 101.10, 110.32, 138.28, 144.78, 163.04, 172.79.

2.4. Tyrosinase Activity Inhibition Assay

Inhibition of tyrosinase activity is determined by spectrophotometry. The tyrosinase activity inhibition assay was carried out as previously described by Vanni *et al.* [13]. The reaction mixture consists of 1 mL of 0.1 M phosphate buffer (pH 6.8), 1 mL of L-tyrosine solution (0.3 mg/mL) and 0.9 mL of sample solution at each concentrations. After the pre-incubation for 20 min in water-bath at 37℃, 0.1 mL of 1250 U/mushroom tyrosinase (Sigma) was added to reaction mixture and incubated for another 10 min at 37℃. The optical density at 480 nm was measured by a spectrophotometer. The percentage of inhibition was calculated as:

$$\text{Inhibition (\%)} = 100 - [(A-B)/A \times 100]$$

where, A is the absorbance at 480 nm without sample and B is the absorbance at 480 nm in the presence of sample.

2.5. Free Radical-Scavenger Activity Assay

The measurement of scavenging effect against free radical generation was carried out as described by Fugita *et al.*[14]. The sample solution (1 mL) was added to 1 mL of μM 1,1-diphenyl-2-picrylhydrazine (DPPH) radical ethanol solution and kept at room temperature for 10 min. The absorbance was measured at 520 nm.

2.6. Measurement of Melanin Amounts Using B16 Melanoma

2.6.1. B16 Melanoma Cell Culture

B16 melanoma cells were purchased from Korean Cell line bank and cultured in DMEM (Dulbecco's modified eagle's medium, sigma, D-2902, St. Louis, MO 63178 USA) supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), and 10% fetal bovine serum (Fetal bovine serum, Gibco, 26140-079, Invitrogen Co.) at 37℃ in an incubator flushed continuously with 5% CO_2.

2.6.2. Measurment of Melanin Amounts

B16 melanoma cells were seeded at 2×10^5 cell/mL on 12-well plates (Nunc). After one day, media were replaced and 0.34 g/mL of α-MSH (α-Melanocyte stimulating hormone, sigma, M-4135) was treated. The desired concentration of samples (kojic acid, KGP, arbutin, ascorbic acid) was added to each well. The cells were incubated at 37℃ in a humidified incubator for four days and media of each well was transferred in 15 mL tube. After washing with PBS (Phosphate buffered saline, 136.89 mM NaCl, 2.68 mM KCl, 8.06 mM Na_2HPO_4 $7H_2O$, 1.47 mM KH_2PO_4, pH 7.4), 100 L of Typsin-EDTA (Gibco, 25300-054) was treated in each well and collected cells were transferred into previous 15 mL tube. 500 L of PBS was treated in each well in order to collect remained cells of well. Cells and media were centrifuged 3000 rpm 30 min and supernatants were discarded. 200 L of 1 N NaOH were treated and the pellet was sonicated for 20 min. Free Melanin was transferred 96 well plates (Nunc) and measured by ELISA reader (Tecan A-5082, Austria) at 480 nm.

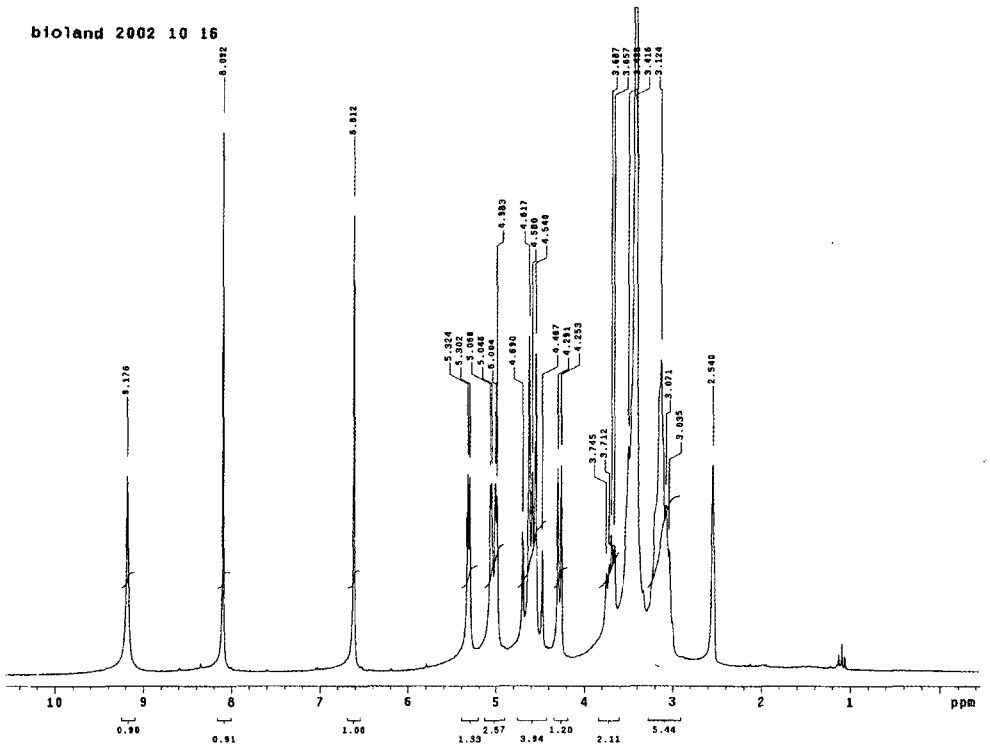

Figure 1. ^{1}H-NMR spectrum of KGP in DMSO-d_6.

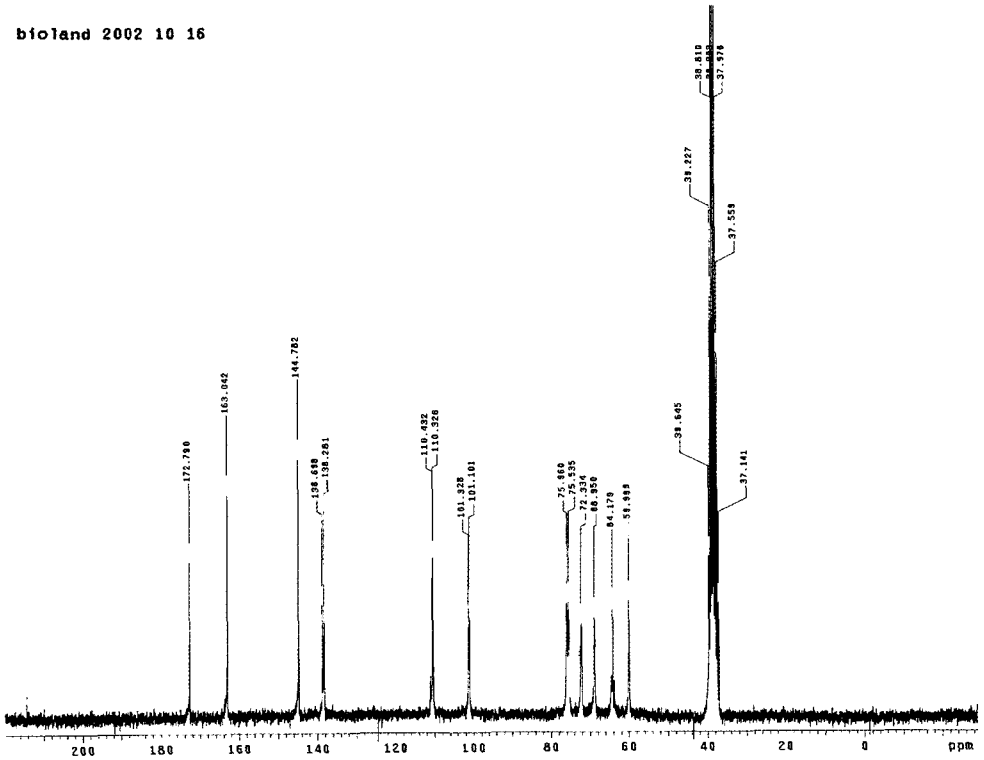

Figure 2. ^{13}C-NMR spectrum of KGP in DMSO-d_6.

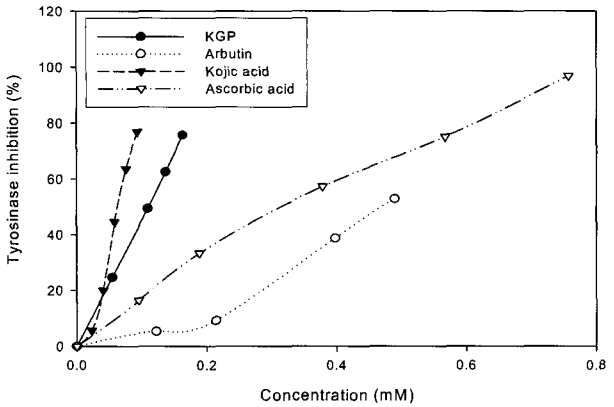

Figure 3. Inhibitory activity of KGP on mushroom tyrosinase.

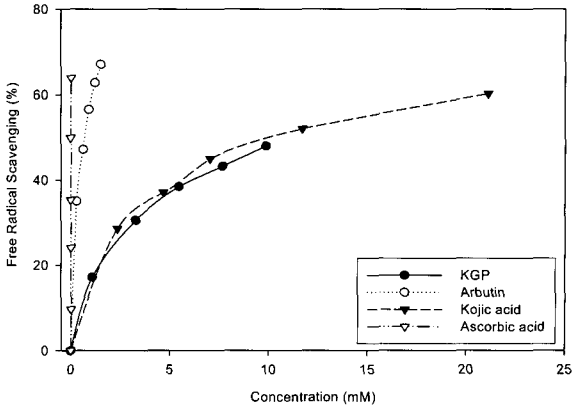

Figure 4. Free radical-scavenger activity of KGP.

3. Results and Discussion

3.1. Synthesis

3.1.1. Kojic Acid 6-*O*-2′,3′,4′,6′-Tetraacetyl-*β*-D-Glucopyranoside (KTGP)

KTGP was obtained through the reaction of kojic acid and *O*-pentaacetyl-*β*-D-glucose with the aid of a Lewis acid BF_3. It was characterized with NMR analysis supporting that the *β*-anomeric hydroxyl group on glucose was substituted by 6-OH of kojic acid, due to the so-called 'neighboring group participation reaction' which was that acetyl group of glucose linkage in 2′-position help 6-OH of kojic acid to substitute 1′-OH of glucose. Moreover, it was also found that *O*-pentaacetyl-*β*-D-glucose was only bonded to 6-OH group of kojic acid without bonding to 5-OH of kojic acid.

3.1.2. Kojic Acid 6-*O*-*β*-D-Glucopyranoside (KGP)

Reaction of KTGP with sodium methoxide in anhydrous MeOH at 25℃ afforded KGP, whose structure was supported by the presence of the peak for *β*-anomeric proton (H^1) at 5.3 ppm in the ^{1}H NMR spectrum and by the chemical shift change of the peak for $C^{1'}$ from 92.9 to 101.1 ppm and for C^7 from 59.5 to 68.9 ppm in the ^{13}C NMR spectrum (Figure 1, 2). These results was quite similar to that of literature, previously reported by N. Nakajima *et al.*[12].

The production yield of glycosylation using the cultured cells is usually low and in the ranging from 10 ~40%. In the case of kojic acid 6-*O*-*β*-D-glucopyranoside, pure *β*-amomer was obtained with very low yield (below 4%) as describe by N. Nakajima *et al.*[12].

We achieved high regio- and stereo-selectivity (6-*O*-*β*-D-glucopyranoside) with high yield by the chemical reaction (~56% in two steps).

3.2. Tyrosinase Activity Inhibition Assay

Tyrosinase is the rate-limiting enzyme in melanin synthesis. Some melanin production-inhibiting agents such as arbutin, ascorbic acid and kojic acid are known to inhibit the tyrosinase activity. To determine the effect of KGP on tyrosinase activity *in vitro*. Compared with arbutin, ascorbic acid and kojic acid, KGP showed higher effect than ascorbic acid and arbutin (Figure 3).

3.3. Free Radical-Scavenger Activity Assay

In order to investigate anti-oxidant efficiency, the scavenging effect for free radical generation was carried out. Although KGP showed lower efficiency than well-known whitening compounds such as arbutin of ascorbic acid, but it still showed the comparable degree of radical scavenging activity with that of kojic acid, which was suggesting anti-oxidation activity (Figure 4).

3.4. Effect of KGP on B16 Melanoma Cell Test *in vitro*.

KGP showed little inhibition on melanin synthesis effect compared with kojic acid, ascorbic acid and arbutin. The glycosylation at 6-position in kojic acid results in the increase of molecular weight and hydrophilicity of kojic acid. Therefore, we conjectured that these increased molecular weight and hydrophilicity cause the little inhibition due to the reduced perme-

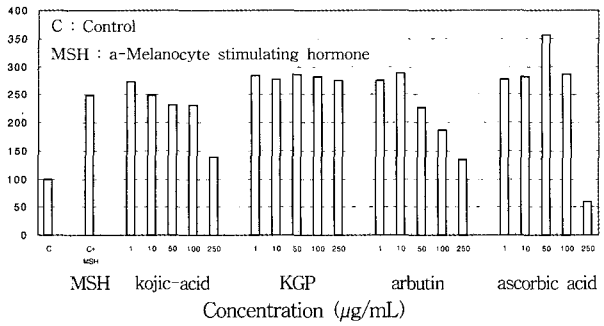

Figure 5. Inhibitory activity of melanogenesis on B16 melanoma cell.

ability to cell-membrane (Figure 5).

4. Conclusion

We snythesized kojic acid 6-*O*-β-D-gulcopyranoside (KGP) though the *regio*- and *stereo*-selective glycosylation of 6-OH group of kojic acid with *O*-pentaacetyl-β-D-glucose. to increase the stability of kojic acid. High yield (80%) was obtained by the use of Lewis acid and organic base in nonpolar solvent. KGP showed higher tyrosinase inhibition activity, measured by mushroom tyrosinase, than those of ascorbic acid, kojic acid, and arbutin. The results of *in vitro* free radical-scavenger activity assay was quite similar to that of tyrosinase inhibition activity assay. In toxicity assay, KGP was much less toxic than kojic acid and arbutin, Therefore, Glycosylation of kojic acid may be useful for the development of a stable kojic acid derivatives and KGP may be used as a safe and effective ingredient for the whitening cosmetic ingredient.

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