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Formation and Stability Improvement of Transparent Bi-continuous Microemulsion of Kyol Oil with Type-1 Collagen Synthesis Effect

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1. Introduction

Currently, the cosmetics industry is being transformed into cosmetics using natural resources [1-2]. In particular, water-soluble extracts derived from vegetable oil, vegetable oil, and high-purity powder are typically used [3,4]. Thousands of plant ingredients widely distributed in Africa, Southeast Asia, Russia, North America and South America are used in cosmetics for these ingredients obtained from nature [5]. Recently, oils obtained from various plants produced in the Amazon tropical region of South America have been applied to cosmetics, and plant ingredients based on Amazon are gaining popularity [6-9]. Kyol Oil is a fruit that is mass-produced in the Amazon and central Brazil and is known to have excellent efficacy in the cosmetics industry [10]. This Kyol oil has a large local raw material production volume and is recognized as a good raw material for antioxidant and anti-aging, so a more detailed study was attempted. Kyol oil is widely known as Pequi fruit oil [11]. This Kyol oil is the botanical name of the pequiziro tree, Caryocar brasiliense Camb. [12].

Fig. 1. Kyol Tree with Fruits from Brazil and seeds in fruits.

Fig. 2. Kyol oil from *Caryocar Brasiliense* Fruits extracting cold process, key ingredient: retinol.

It belongs to the family *Caryocaraceae* and is a typical fruit of the Brazilian Cerrado, which plays an economically important role for the population of the region. Cerrado Biome is the second largest plant formation in Brazil after the Amazon, with notable plant heterogeneity and containing many elements of the fauna and mainly plants [13]. Fig. 1 is a photograph of the kyol tree with fruits. Fig. 2 is a photograph

of the kyol oil with retinol of key ingredient. From high seradoes (trees about 20 m high), to more common savannas (with dense bushes and tree species of 8 to 10 m), to open pastures, each type of plant development is greatly influenced by the nature of the environment. In this study, Kyol oil native to Brazil was purified and highly purified as a cold process, and a double continuous microemulsion was formed to improve stability and to easy apply skin care formulas. The cytotoxicity of this nanoemulsion was tested by the WST-1 method. The protein expression level of type-1 collagen is measured. In addition, pure retinol contained in Kyol oil is quantitatively analyzed. The skin science mechanism and skin improvement effect were interpreted so that it could be widely applied to the cosmetics industry.

2. Materials and Methods

2.1. Materials

Pure Kyol oil used in this study was a raw material from Plantus Co. Ltd., Brazil. Succrose distearate (Sigma) and polyglyceryl-10 oleate (Sigma) were used for the bi-continuous microemulsion. As purified water, reagent-grade raw materials were used as they were. The raw materials used in the cytotoxicity and type-1 collagen synthesis rate test were purchased and used for reagent classification [14].

2.1. Device

To make a double continuous microemulsion, a dispensing homomixer (Model 2.5, TK, Japan), a water bath (WBE, Donglim Science, Korea), an activated carbon oil filter (KRD-G04238, Donglim Science, Korea), a collagen synthesis rate analyzer and a high-performance chromatography (Thermo Ultimate 3000 HPLC, Youngin, Korea), a thermometer, and a beaker were used.

Table 1. Prescription composition of bicontinental microemulsion of Kyol oil

Phase	Ingredient Name	Control Wt%	Remarks
A	Kyol Oil Tocopheryl Acetate	77.00 1.00	Emollient Stabilizer
B	Sucrose Distearate/ Polglyceryl-10 Oleate	20.00	Emulsifier
C	Water	2.00	Solvent
Total		100.00	

A: Oil phase, B: Surfactant mixtures, C: Solvent

2.3. Manufacturing method of bicontinuous microemulsion

Table 1 and Fig. 3 show the manufacturing method of the bicontinuous microemulsion of Kyol oil. As shown in Fig. 3, first, Kyol oil is mixed with tocopheryl acetate, and then sucrose distearate/polglyceryl-10 oleate is added and dissolved. It is stirred at 80-700 rpm for 10 minutes with a dispensing device. When water is added and stirred for 20 minutes, a transparent bicontinuous microemulsion is produced. If it is diluted in water to the desired concentration, it is in phase into a nanoemulsion.

2.4. Cytotoxicity test method

Safety can be evaluated through the cytotoxicity test of Kyol Oil, which was tested through WST-1 assay in this study. First, the animal cell culture medium is completely removed through vacuum. Trypsin-EDTA treatment was allowed to stand for 10 min. The trypsin-EDTA was then inactivated by

adding trypsin-EDTA at a 1:1 ratio to which DMEM medium was added. A 15 ml conventional tube was placed in a centrifuge and centrifuged at 1200 rpm for 10 minutes. After removing the supernatant except the pellet, the medium is added to carefully release the pellet. Put 10 µl in a Hemacytometer and count the cells to average them. Cultivate the cells in a microplate so that the final culture solution is 100 µl/well.

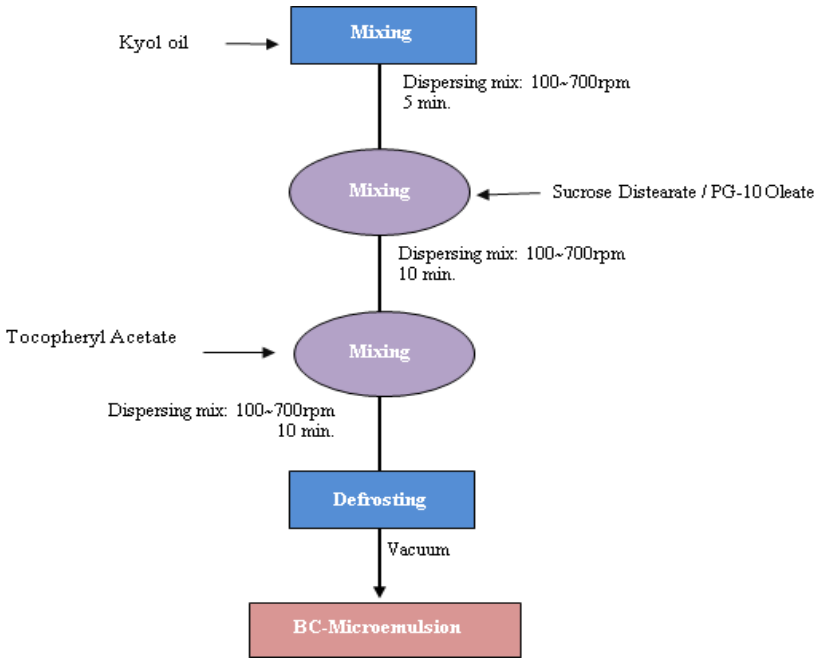

Fig. 3. Kyol oil from *Caryocar Brasiliense* Fruits extracting cold process.

The culturing time or cell concentration to be cultured depends on each experimental condition or the type of cell used. The cell concentration is 0.1~5 X10^4 cells/well, the culturing time is 24 hours. Chemical to be put into the well is diluted/manufactured by concentration, added to each well, and cultured for 1 to 3 days. After the cell culture is completed, 10 µl of premix WST-1 is added to each well, cultured under the same culture conditions for 0.5 to 4 hours, and the absorbance of the sample to the background control is measured using an ELISA reader. The wavelength for measuring the absorbance of Formazan products is between 420 and 480 nm depending on the filter of the ELISA reader (maximum wavelength is about 440 nm, Fig. 2), and the control wavelength is preferably 600 nm or more [15].

2.5. Test Method of Type-1 Collagen Protein Synthesis

The effect on type-1 collagen biosynthesis was tested using nanoemulsion Kyol oil as follows. HS68 cells used in the experiment were purchased from ATCC and used. 10% FBS and 1% phenicillin/streptomycin were added to the DMEM medium and used, and 37°C, 90% relative humidity, and 5% CO_2 incubator were cultured and used. The microemulsion of Kyol oil was experimented using the Procollagen Type I C-peptide (PIP) ELISA kit to confirm the biological performance of Type-1 Collagen. First, HS68 cells were dispensed into 24 well plates to be 5 x 10^4 cells/well and incubated in a 5% CO_2 incubator at 37°C for 24 hours. The microemulsion of Kyol oil was changed in concentration to treat TGF-β1 (10 ng/mL) for 24 hours. After 24 hours, the culture medium was collected and the

procollagen biological performance was measured using the procollagen type-I C-peptide (PIP) ELISA kit. The measurement method was measured according to the manufacturer's manual [16].

3. Results

3.1. Development of Bicontinuous Microemulsion of Kyol Oil

In order to generate Kyol oil's bicontinuous microemulsion, a ratio of surfactant, oil and water is important. The selection of a surfactant is most important. The region forming the bicontinuous microemulsion is shown in Fig. 4. When the concentration of the surfactant was mixed in the range of 20 to 50%, Kyol oil was 50 to 80%, and water concentration was 1 to 30%, a transparent microemulsion could be obtained. When a surfactant mixed with sucrose distearate and polyglyceryl-10 oleate was used as a vegetable surfactant, a transparent microemulsion could be made. When the microemulsion developed by this method was mixed with water, it was converted into a fine nano emulsion to quickly absorb the skin, and various effects could be obtained. The appearance of this nanoemulsion was a milky liquid, and the particle diameter distribution was 80 to 500 nm.

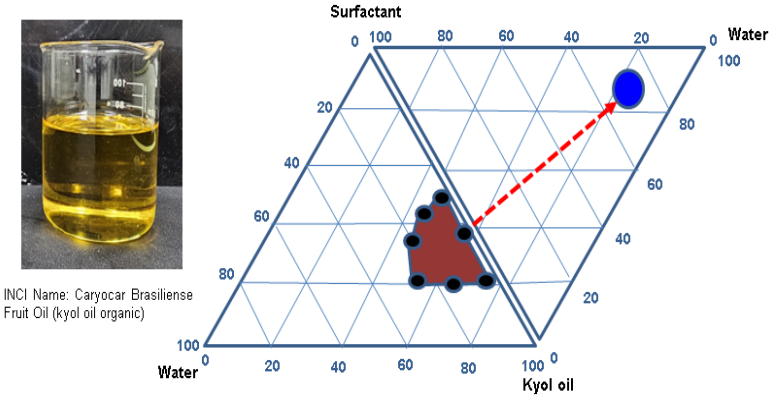

Fig. 4. The three-phase schematic diagram of the transparent microemulsion containing Kyol oil.

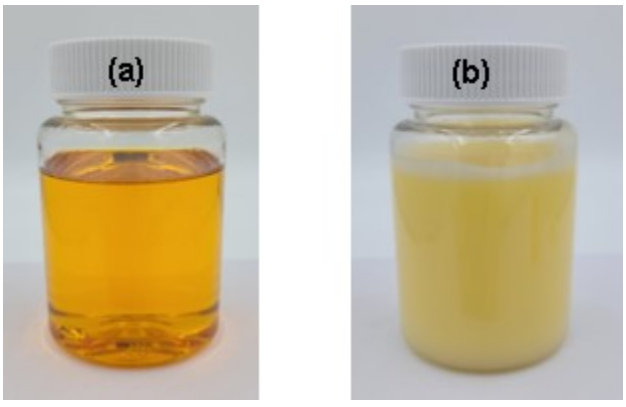

Fig. 5. Transparent bicontinuous microemulsion photograph of Kyol oil and phase-inversion emulsion (o/w) of nanoemulsion; (a): Bicontinuous microemulsion containing 77% of Kyol oil, (b): nano-emulsion containing 10% of Kyol oil.

Fig. 5 shows a photograph of a transparent bicontinuous microemulsion of Kyol oil and a photograph of an phase inversion micro-emulsion. (a) was a pale yellow transparent liquid phase, and (b) was a photograph of a o/w nicro-emulsion in which 10% was diluted. As shown in the picture, a transparent micro-emulsion was produced using a natural surfactant for convenience of use of natural oil, and all the bicomponent was used as a raw material derived from natural origin that does not contain chemical substances so that the inherent efficacy of oil could be maintained as it is.

3.2 Cytotoxicity of Nanoemulsion Containing Kyol Oil

The cytotoxicity test of the nanoemulsion containing Kyol oil is performed using WST-1 assay, and is shown in Fig. 6. The sample was treated with human dermal fibroblasts at a concentration of 0 to 1% (v/v) for 24 hours, and then WST-1 assay was performed, and the result obtained by measuring the cell viability. When the sample was treated at a concentration of 0.5% (w/v) or less (0.05, 0.1, 0.25, 0.5%, and w/v), the cell viability was 105.62%, 103.32%, 100.28%, and 98.87%, respectively, indicating that the sample was not cytotoxic, as compared to the negative control group. Since the cell viability at a concentration of 1% (w/v) of the study material was significantly reduced, it was confirmed that the sample was cytotoxic ($p<0.05$).

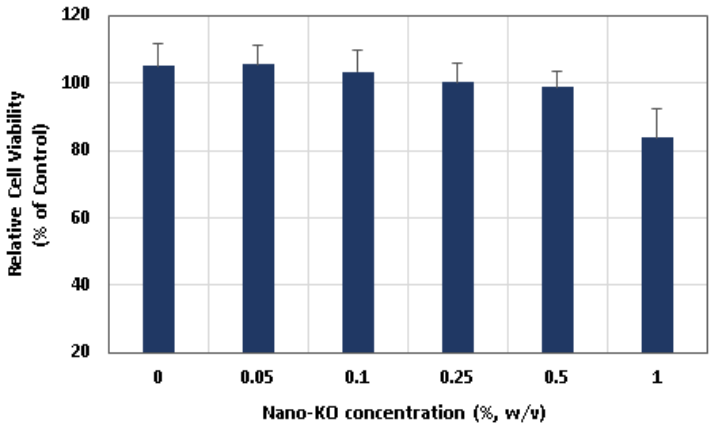

Fig. 6. Results of cytotoxicity measurement with increasing concentration of nano-emulsions containing Kyol oil.

3.3 Synthesis Rate of Type-1 Collagen Protein

Since Kyol oil contains abundant nutritional components, it is believed that it will have various effects. Mainly, oil contains ester oils, including oleic acid, and especially seeds and fruits contain polysaccharides and polyphenolic substances, so it is expected that it will have moisturizing or antioxidant properties. In this study, the results of testing whether the collagen synthesis rate contributes to wrinkle improvement are shown in Fig. 7. As shown in Fig. 7, when the concentration of COL1A1 mRNA expression level, which is a representative gene of type-1 collagen, was treated with 0.5% (w/v) or less (0.1, 0.25, 0.5%, w/v), the collagen protein expression level was increased to 14.19 ± 4.75%, 35.26 ± 3.96% and 43.66 ± 4.5%, respectively, compared to the negative control group ($p<0.05$). As a result of treatment with TGF-B (5 ng/mL), which was used as a positive control group, it was found that the expression of COL1A1 mRNA protein was increased by 73.25 ± 7.28% compared to the negative control group ($p<0.05$). The reason for this increase in collagen synthesis rate was considered to be the effect of retinol and tocopherol contained in Kyol oil. It was predicted that the factor that increases the activity of collagen is mainly because the retinol contained in kyol oil contributes to the synthesis of type-

1 collagen. Based on these results, it is expected that it will be applicable to the development of products having a fine wrinkle improvement effect in the prescription of skin care cosmetics.

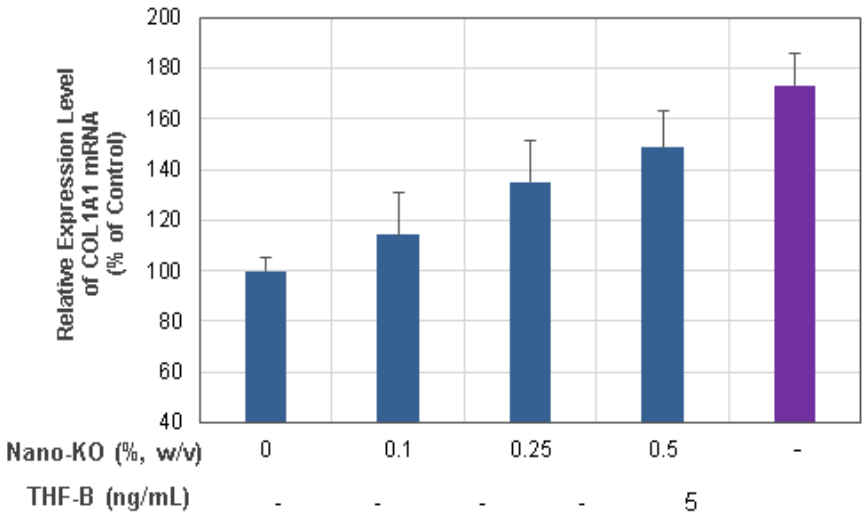

Fig. 7. The expression effect of type-1 collagen protein with increasing concentration of nanoemulsion containing Kyol oil.

3.4 HPLC Quantitative Analysis of Pure Retinol in Kyol Oil

Pequi or Kyol oil has a botanical name of *Caryocar brasiliense Camb*. Kyol oil obtained from seeds or fruits contains abundant nutritional ingredients. It is expected that ester oils including oleic acid and linoleic acid are contained, and especially seeds and fruits contain polysaccharides and polyphenolic substances. In this study, retinol components contained in oil extracted from seed fruits were quantitatively analyzed. This is the reason for the purpose of verifying whether retinol is involved in the protein synthesis of type-1 collagen according to the content of retinol [17].

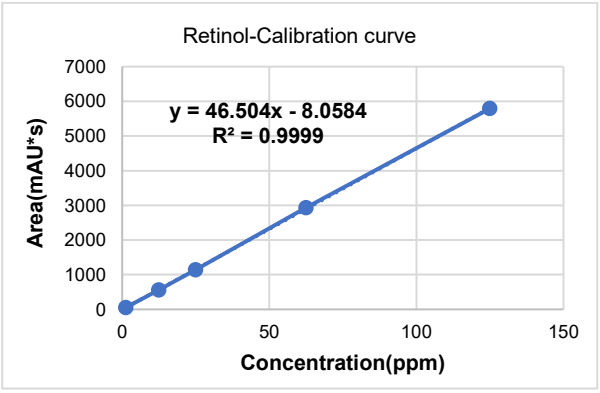

Fig. 8. Retinol calibration standard curve to measure HPLC determination.

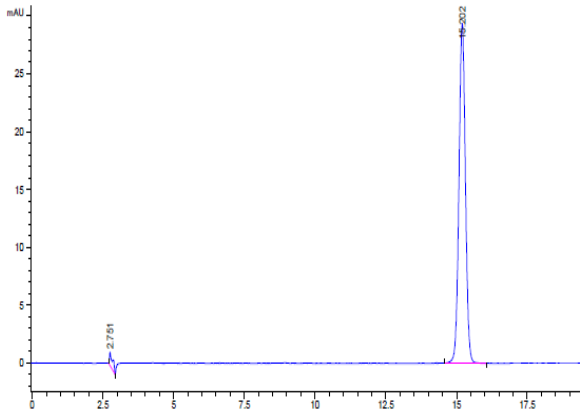

Fig. 9. Pure retinol content with in Kyol oil measured by HPLC analysis.

The standard curve for retinol of the standard product is shown in Fig. 8. As shown in the graph, it was found that the correlation coefficient R2 value was 0.9999, which had linearity. Based on this, it was found that retinol contained in Kyol oil was separated in the range of 15 to 16 minutes, and the content of retinol was measured by HPLC by converting the area of the curve, and it was found that about 1.54%

(16446 ppm) was contained (Fig. 9). This content was considered to be a very high sum and could be involved in the synthesis of collagen proteins.

4. Discussion

Kyol oil is other named Pequi oil, and it is a plant belonging to a fruit cultivated in the Amazon tropical region of South America. The method of making Kiol oil's nanoemulsion is a high-pressure Microfluidizer that passes through the nano-chamber to small particles, and since it uses a lot of heat and energy, its titer may decrease in the manufacturing process. In particular, organic oils are unstable at high temperatures and are difficult to store for a long time, so there are problems that need to be used quickly. In order to solve this problem, it is considered one of the smart solutions to add a stabilizer and make a nanoemulsion easier to use. The vegetable surfactant used in this study is a mixture of sucrose distearate and polyglyceryl-10 oleate and has hydrophilic surfactant properties. It was considered that this surfactant has strong hydrophilic properties because there are several -OH groups in the sucrose head group, and has structural characteristics that can create optimal conditions for forming a lamellar structure by connecting two staric acids with alkyl chains. In addition, polygkyceryl-10 oleate can be packed between the structures forming a lamellar structure to form a stable bicontinier structure, so it has the characteristic of supporting a high content of oil. Since Kyol oil contains a large amount of retinol, maintaining stability is an important part, so it was considered that stabilization using tocopheryl acetate was correct. This was considered to be because it has good miscibility with Kyol oil and has a function suitable for stabilizing unsaturated fatty acids. It is expected that this raw material will have not only the type-1 collagen synthesis rate but also the elastin synthesis rate, filaggrin protein synthesis, and moisturizing effect, and it is necessary to additionally widely test it.

5. Conclusion

This study was about the formation of a transparent duality microemulsion of Kyol oil having a type-1 collagen synthesis effect and the improvement of stability, and concluded as follows. First, Kyol oil (INCI name: *Caryocar Brasiliense* Fruit Oil) developed an organic oil extracted using a microbiome fermentation technology. Second, a transparent bicontinuous microemulsion containing Kyol oil could be made under conditions containing polyglyceryl-10 oleate, sucrose distearate, and Kyol oil and purified water. Third, stability was maintained when the Kyol oil contained tocopheryl acetate. Fourth, it was confirmed that the cytotoxicity of the nanoemulsion containing Kyol oil was not cytotoxic as a result of performing using WST-1 assay. Fifth, it was found that the mRNA protein expression level of type-1 collagen increased as the sample concentration increased. In addition, as a result of treatment with TGF-B (5 ng/mL) used as a positive control group, it was confirmed that the expression of the type-1 collagen protein mRNA was increased by 73.25 ± 7.28% compared to the negative control group. The reason for the increase in the collagen synthesis rate was considered to be due to the effect on the high content of retinol contained in Kyol oil. In the future, it is expected that it will be widely applied to various organic cosmetics and cosmetics that help improve fine wrinkles through more in-depth research.

Acknowledgement

This study reveals that the Biobeautech Co., Ltd. R&D Center in South Korea has jointly developed new nano-materials and prescription development by combining advanced interfacial chemical technology with fermented Kyol oil developed by Plantus Industry in Brazil.

Keywords: *Kyol oil, Pequi oil, bicontinuous oil, Collagen synthesis, Cosmetics*

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