PURIFICATION OF FLAVONOIDS FROM GYNOSTEMMA PENTAPHYLLUM AND ITS ANTI-AGING EFFECTS

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ABSTRACT

Recently, natural plant sources have been developed as cosmeceutical ingredients that may be involved in anti-aging and anti-wrinkle care. *Gynostemma pentaphyllum* has been used as a traditional herbal drug in Northeast Asia including China, Japan and Korea and believed to be effective on lowering cholesterol, preventing cancer and chronic bronchitis. We separated flavonoid-rich fraction from its crude ethanol extract by highly porous polymer column chromatography to examine its anti-aging and UV-blocking effect. The purification yield of flavonoid-rich fraction from *Gynostemma pentaphyllum* (FFGP) was 1.5%. The main compounds of FFGP were quercetin-3-O-rutinoside, kaempferol-3-O-rutinoside, quercetin-4′, 7-dimethoxy-3-O-rutinoside and kaempferol. At 10, 50 and 100μg/mL of FFGP, its procollagen synthesis stimulating effect was 13, 15 and 20%, respectively, compared with control on human dermal fibroblast after 48 hours cultivation. The UV-blocking effect was observed at the minimum dose of 0.06% FFGP after 1.5hr UV radiation which means that FFGP possess UV absorbance capacity. Therefore, these results demonstrate that FFGP could be a quite effective cosmeceutical material and might provide new possibility for finding novel and powerful anti-aging compound.

INTRODUCTION

Skin aging mainly occurs through two factors, intrinsic aging and extrinsic aging. Intrinsic aging describes irreversible physiological processes, while extrinsic aging is mainly a consequence of environmental damage. The primal environmental factor of extrinsic aging is constant exposure to UV rays which generates free radicals and reactive oxygen species (ROS) [1]. By these free radicals and ROS, the extracellular matrix (ECM) which consisted of collagen, elastin and hyaluronic acid, maintaining resilience of skin, could be degraded and wrinkles can appear. The most abundant structural protein in skin connective tissue (dermis) is type-1 collagen. Collagen is synthesized primarily by dermal fibroblasts and is responsible for retaining strength and resiliency to skin. It is synthesized as a soluble precursor, procollagen, which is secreted from fibroblasts and proteolytically processed to form insoluble collagen fibers. The synthesis and degradation of ECM constituents are being controlled properly by human homeostasis, but with age the ECM synthesis is declined and wrinkle occurrences could be promoted by MMPs (matrix metalloproteinase) [2]. So, the inhibition of collagenase, elastase or MMPs and their expression could be the best way to suppress the wrinkle formation and to delay aging. Many chemical ingredients such as ascorbic acid, alpha-tocopherol, retinol and SOD (super oxide dismutase) have been used to inhibit wrinkle formation, but the real biological activities effect is being exerted so far because of their physico-chemical instability and high prices. Therefore, herbal extracts such as noni fruit extract and green tea extract which is relatively cheap and stable are increasingly being utilized.

Gynostemma pentaphyllum is a well-known edible medicinal plant in oriental countries. *G. pentaphyllum* is known as 'Dolwoe' or 'Jiao-Gu-Lan' and has been clinically used for depressing cholesterol levels, regulating blood pressure, strengthening the immune system, treating chronic bronchitis and gastritis, and reducing inflammation, and has been described as having minimal toxicity [3]. In this study, we show that flavonoids from *G. pentaphyllum* could be used as a functional anti-wrinkle cosmetic ingredient.

MATERIALS AND METHODS

Plant Material. *G. pentaphyllum* were collected from Ulleungdo, Gyeongsangbuk-do Province, Korea.

Extraction and Purification. The dried aerial part of *G. pentaphyllum* (1.00 kg) were ground and extracted by agitating in 70% EtOH at 80°C for 4 hours, then filtered. The process was repeated twice, and the filtrates were then combined and evaporated under vacuum to dryness to give a brown residue. The crude EtOH extract was dissolved in 15% EtOH and subjected to DIAION^{TM} HP20 column chromatography using EtOH and water as the mobile phase with gradient elution. The flavonoid-rich fraction from *G. pentaphyllum* (FFPG) was eluted with 35% EtOH.

Analytical. HPLC was performed on Agilent 1260 Infinity series (UV detector = 260 nm), using CAPCELL PAK C18, 4.6 x 250mm, 5μm. Water and Acetonitrile were used as Mobile phase with formic acid in the gradient binary pump system. The operation condition was summarized in Table 1. Quercetin-3-O-rutinoside (Rutin), kaempferol-3-O-rutinoside and quercetin-4′, 7-dimethoxy-3-O-rutinoside (Ombuoside) were identified from FFGP by comparing retention time and UV spectrum with each standards.

Table 1. HPLC operation conditions for determination of flavonoids

System		Agilent 1260 Infinity
Column		CAPCELL PAK C18 (4.6 mm x 250 mm, 5 m)
Mobile Phase	A	0.05% Formic acid in Water
	B	0.05% Formic acid in Acetonitrile
Elution Speed		1.0 ml·min-1
Column Temperature		30°C

Injection Volume	20 μl
Detector Wavelength	260 nm

Cell and Cell Culture. Human dermal fibroblasts (HDF, Cascade Co.) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic–antimycotic at 37°C in a humidified 5% CO_2 atmosphere, and all experiments were conducted in DMEM with 0.5% heat-inactivated FBS and 1% antibiotic–antimycotic.

Procollagen type I C-peptide assay. The assay was performed using commercial kit as suggested by the instructions. Briefly, 5×10^4 cells were plated on a 24-well microplate. After 24 h of incubation, the medium was exchanged with fresh DMEM without FBS and incubated for 24 h again. DMEM with several concentrations of FFGP dissolved in a final concentration of 0.1% DMSO was added. After incubation for 48 h, medium was collected and procollagen contents were measured using procollagen type I C-peptide enzyme immunoassay kit (Takara Bio). Values were standardized to total cell protein concentrations measured, and all analyses were performed on sets of 3 wells.

Measurement of UV-Protection Capacity.

To evaluate UV-protection activity of FFGP, UV absorption indicator (UVLABEL®, NIGK Co., JAPAN) was used which changes its color from white to yellow by ultraviolet light irradiation. 0.2mL of FFGP solution and O/W emulsion which contain 0.01~ 0.1% of FFGP were spread on the cover glass which was covered on the UV absorption indicator. After storing for 1.5 under the UV rays (200~400nm), the discoloration of UV absorption indicator was observed. The commercial sun spray (SPF 30, PA +++) was applied as positive control.

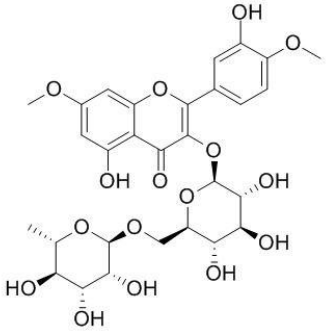
Preparation of Cosmetic Formulae and Assessment of Stability.

To estimate the stability of main ingredient (ombuoside) in FFGP, 3 cosmetic formulas were prepared, 2 types of O/W emulsion with high and low viscosity and 1 type of solubilized formula. O/W emulsions mainly contain humectant, oil, carbomer and preservatives while solubilized formula contain humectant, polymer, solubilizer and preservatives. Each formula contains 0.2% of FFGP powder. Cosmetic samples were kept at 4°C, 30°C, 45°C and sunlight, the recovery rate (%) of ombuoside, changes of color and odor were checked for 12 weeks.

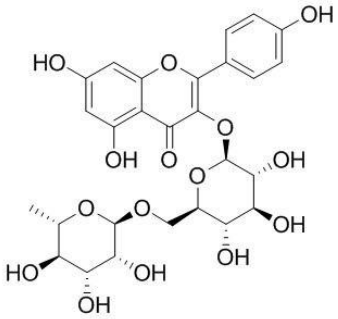
RESULTS AND DISCUSSION

The purification yield of flavonoid-rich fraction from the dried *G. pentaphyllum* was about 1.5%. To identify the main flavonoids in FFGP, we conducted the analysis using HPLC and **(1)** Quercetin-3-O-rutinoside (Rutin), **(2)** kaempferol-3-O-rutinoside, **(3)** quercetin-4′, 7-dimethoxy-3-O-rutinoside (Ombuoside) which were identified as main components of FFGP. The contents of each flavonoid were 13.4 mg/100mg, 5.9 mg/100mg and 1.5mg/100mg respectively. (Table 2)

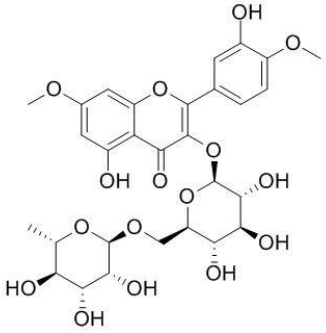
Figure 1. The chemical structure of main flavoniods from FFGP.



Quercetin-3-O-rutinoside **(1)**



Kaempferol-3-O-rutinoside **(2)**



Quercetin-4′, 7-dimethoxy-3-O-rutinoside **(3)**

Figure 2. The HPLC chromatogram of FFGP.

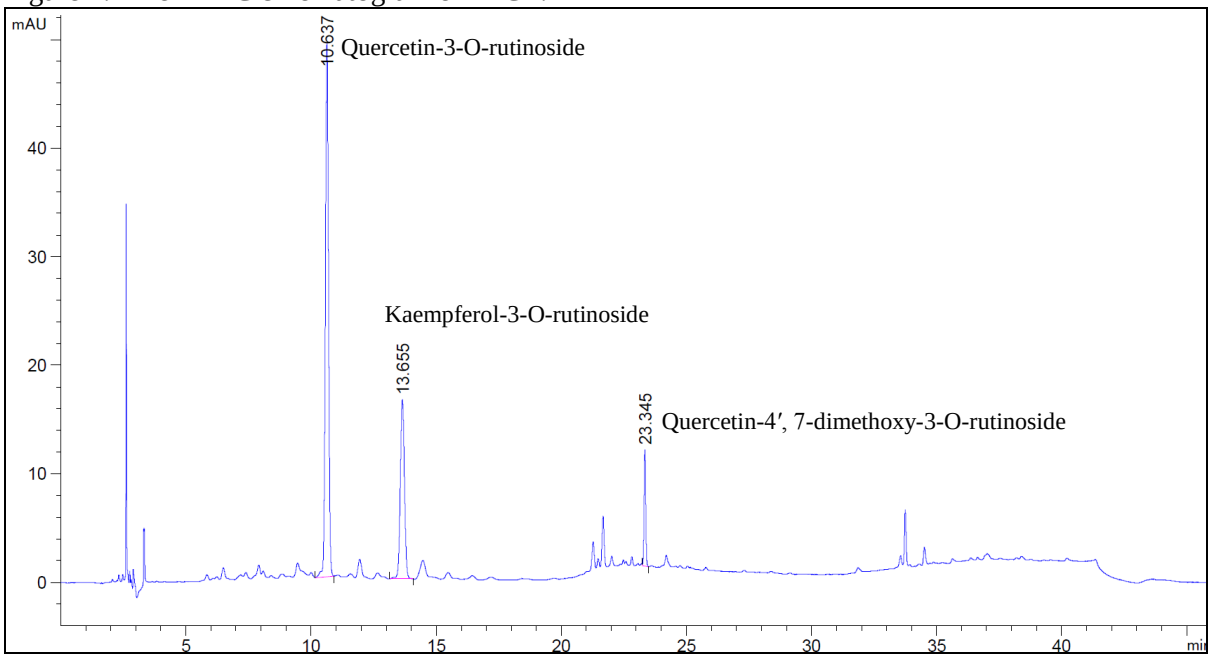
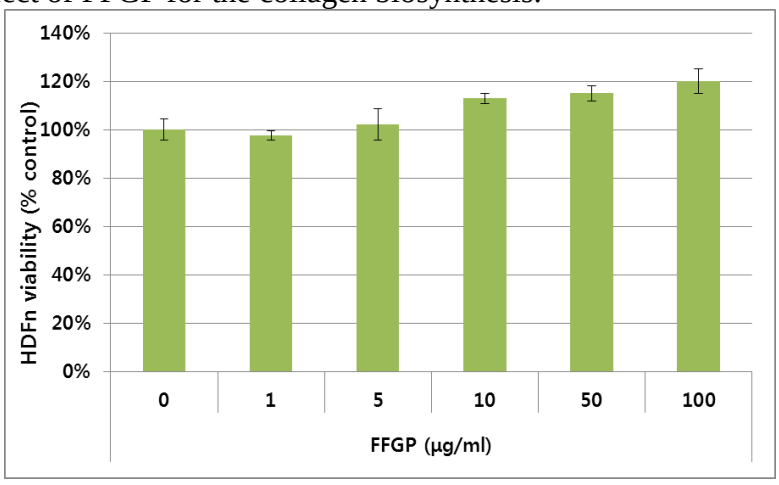


Table 2. Flavonoids contents in FFGP.

Flavonoid	Contents (mg/100mg)
Quercetin-3-O-rutinoside **(1)**	13.4
Kaempferol-3-O-rutinoside **(2)**	5.9
Quercetin-4′, 7-dimethoxy-3-O-rutinoside **(3)**	1.5

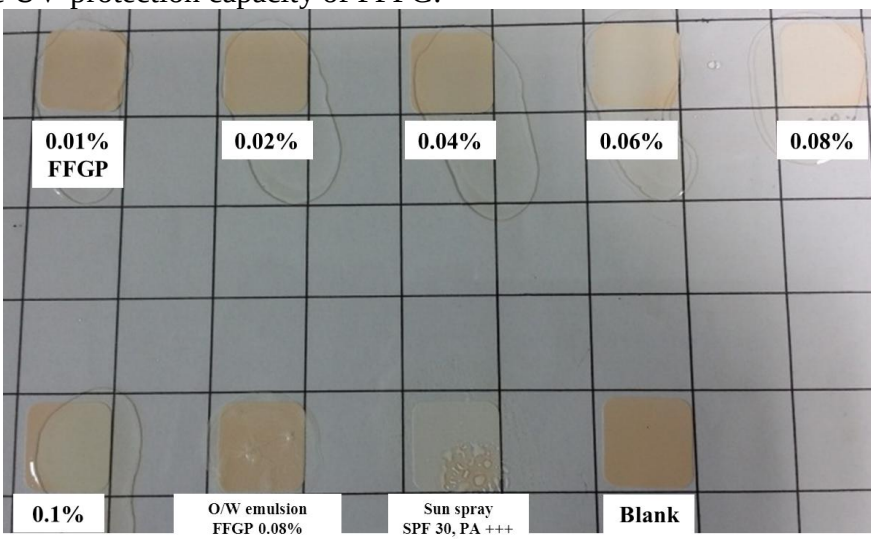
In many studies, flavonoids from natural plant sources are effective on the improvement of wrinkle [4] [5]. Since collagen gives a resiliency to skin, measuring collagen production activity could be the most direct method to estimate the wrinkle improvement capacity of the candidates. A procollagen, a precursor type of collagen, contains a propeptide, which is the peptide-base sequence onto the amino-terminal and the carboxy-terminal [6]. Propeptide has the function to help the propeptide molecules fold together in cell and it is cut off from the collagen molecule and separated when the polymerization of collagen occurs. Therefore, measuring the amount of separated propeptide, it is possible to catch on to the degree of the collagen biosynthesis. Fig. 3 shows the effect of FFGP for the procollagen synthesis according to its concentration in comparison to control. FFGP showed collagen biosynthesis stimulating activity in a dose-dependently manner (10~100 μg/ml).

Figure 3. The effect of FFGP for the collagen biosynthesis.



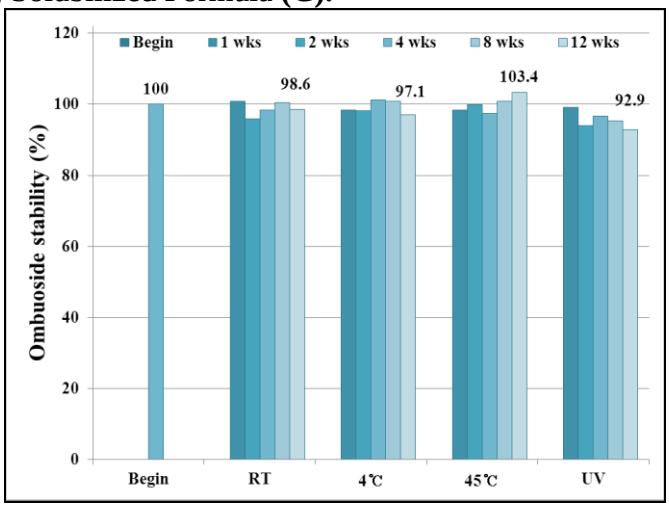
The sunlight ultraviolet radiation in the range between 100 and 400 nm is subdivided into UVC (100-280 nm), UVB (280-315 nm), and UVA (315-400 nm) when considering the effect of ultraviolet radiation on human health and the environment [7]. Because UV light is the major cause of typical collagen degradation, UV-protection capacity of FFGP could plays quite significantly on its collagen synthesis stimulating activity. When the samples were treated more than 0.06% as FFGP powder, surprisingly outstanding UV-protection capacity was shown (Fig. 4). However, even though 0.08% of FFGP was treated UV-protection capacity of FFGP was not remarkable when it was applied as O/W emulsion. For utilization of FFGP as UV protection product, an in-depth study about optimum formulation which can boost UV-protection capacity of FFGP should be carried out.

Figure 4. The UV-protection capacity of FFPG.

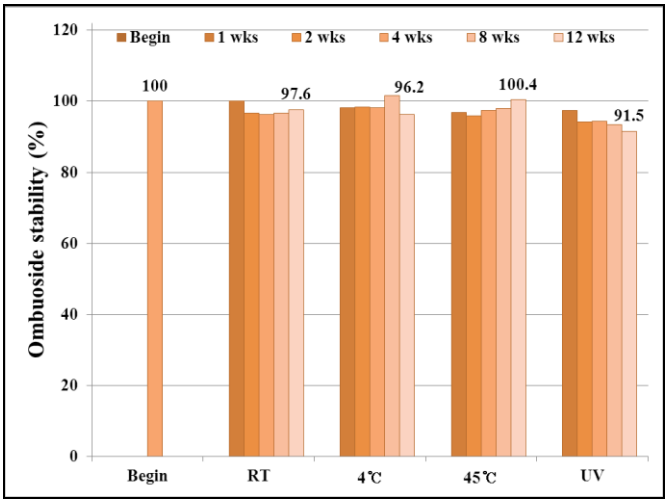


We finally selected ombuoside as an index compound of FFGP among identified flavonoids and estimated its stability in cosmetic formulation. Securing a storage stability of an index compound is considerably important because the index compound could represent a total quality of the cosmetic products. In all types of cosmetic formulae, ombuoside showed excellent thermal stability at every conditions of storage for 12 weeks maintaining its contents above 90% compared to beginning (Fig. 5). These means that the collagen biosynthesis stimulating effect of FFGP could be maintained in cosmetic formulae and the verifications about this hypothesis through a clinical test should be accomplished soon.

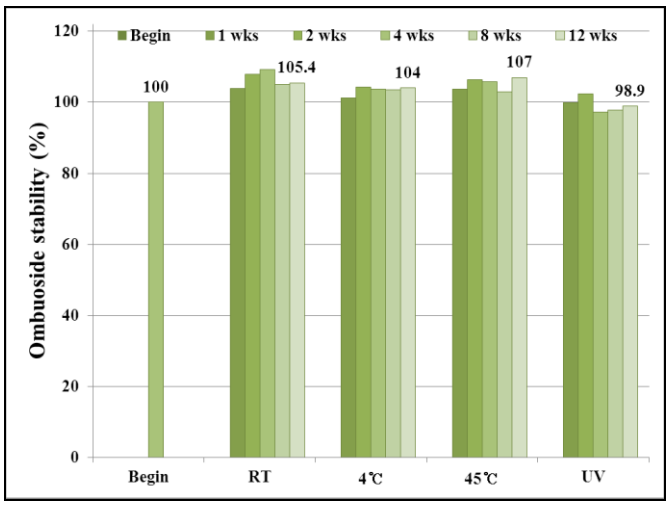
Figure 5. The Stability of ombuoside in High viscosity O/W emulsion **(A)**, Low viscosity O/W emulsion **(B)**, Solubilized Formula **(C).**



(A)



(B)



(C)

CONCLUSION

We have shown that the flavonoids-rich fraction from *G. Pentaphyllum* stimulates the collagen biosynthesis in human dermal fibroblast and its main flavonoids are being maintained in several thermal storage conditions. The UV-protection capacity of FFGP suggested that the possibilities in the invention of another natural born sun protection product. For the next research, the tracking of effective single compound, the development of optimal cosmetic formulations for the UV protection capacity of FFGP and clinical test about the improvement wrinkles would be conducted.

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