The Prescription Theory of Sovereign, Minister, Assistant and Courier Principle: Anti-wrinkle formula Seungma-Galgeun-Tang.

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Summary

This study will provide the scientific evidences for the traditional Chinese prescription theory of Sovereign, Minister, Assistant and Courier Principle with the case of anti-wrinkle formula Seungma-Galgeun-Tang (SGT) water extract. This theory describe that four different roles for each composing herbs are arranged according to their own physiological contribution. Biological activity, diminution of toxicity and increase of bioactive components transportation through the barrier were evaluated for each herbs according to their roles in traditional prescription theory. From the results, the Sovereign herb and Minister showed most potent anti-wrinkle activities and Assistant herb diminished the cytotoxicity from other herb. The Courier herb enhanced the transportation of active component of Sovereign herb through the skin cell model. These results evidenced the reason why traditional herbal formula should be used as a combination instead of single use of most potent herb.

Introduction

The sovereign, minister, assistant and courier principle is a basic theory of traditional herbal formulation in China, Japan and Korea. This theory has four different roles are assigned for the composing herbs according to their own physiological contribution. The sovereign herbs represent major pharmacological efficacy. The minister herbs support the sovereign herbs in terms of bioactivity. The assistant herbs have a role of reducing side effects of the other herbs and the courier herbs help to

deliver other herbs to the right places such as target organ or tissues. In this study, we tried to evaluate this principle with experimental evidences on anti-wrinkle formula Seungma-Galgeun-Tang (SGT).

SGT has anti-allergic effect[1] and inhibited cytopathic effect of human respiratory syncytial virus in cell lines of human respiratory tract[2], Enterovirus 71 infection in human foreskin fibroblast cell line[3]. However, no previous study has been performed about anti-wrinkle efficacy of SGT.

Two major molecular markers for wrinkle formation, matrix metalloproteinase (MMPs) and Type-1 collagen were evaluated on dermal fibroblast cells exposed by UVB in order to clarify the anti-skin aging efficacy of sovereign and minister herbs, *Pueraria lobata* Ohwi and *Cimicifuga heracleifolia* Kom. The MTT assay was conducted on fibroblast cells to define the role of assistant herbs, *Paeonia lactiflora* Pallas. The human 3D tissues were used to measure how much of phytochemicals in sovereign herb were delivered to the dermis from the epidermis when composed with courier herb, *Glycyrrhiza uralensis* Fischer et DC.

Methods

Preparation of SGT water extract: The 125 g of SGT was composed of 25 g *Pueraria lobata* Ohwi (Leguminosae, root), 50 g *Cimicifuga heracleifolia* Kom (Ranunculaceae, rhizome), 25 g *Paeonia lactiflora* Pallas (Paeoniaceae, root) and 25 g *Glycyrrhiza uralensis* Fischer et DC. (Leguminosae, root and stolon), respectively. The four botanical sources were composed by sovereign, minister, assistant and courier Principle in sequence. SGT was extracted with distilled water at 100℃ for 4 h, using reflux extraction. The extract was filtered through filter paper and the filtrate was freeze-dried (yield 22 g) and kept at 4℃.

HPLC-ESI-MS analysis: SGT extract was dissolved in distilled water to prepare final concentration of 30 mg/mL and filtered using 0.45 μm syringe filter (Millipore, Bedford, MA, USA) before being subjected to HPLC system. The HPLC system consisted of UVD340U PDA detector, Dionex model P680 HPLC pump and ASI100 auto sampler operated by Dionex chromeleon software. The UV-VIS detection wavelength was set to 315 nm. The Waters μBondapak C18 (MA, USA) column (300 × 3.9 mm i.d.; 5 μm) was selected and it was placed in a column oven set at 25℃. The mobile phase comprised of following gradient method of acetonitrile in H_2O which were acidified with acetic acid (0.1%): 2% (0-120 min), 2-20% (120-160 min), 20-

100% (160-169 min) and 100-2% (169-170 min). The flow rate was 0.8 mL/min. The injection volume was 10 μL.

AccuTOF® single-reflectron time-of-flight mass spectrometer was equipped with an ESI source (Electrospray ionization, JEOL, USA) and was operated with Mass Center system version 1.3.7b (JEOL, USA). Operating parameters of the mass spectrometer were set as follows: In the positive ion mode, orifice 1 = 80 V, orifice 2 = 5 V and ring lens = 10 V, respectively. The ion guide potential and detector voltage were set to 2000 V and 2300 V, respectively. ESI parameters were set as follows: needle electrode = 2000 V, nitrogen gas was used as a nebulizer, desolvating and their flow rate were 1 and 3 L/min, respectively. The desolvating chamber temperature = 250℃ and orifice 1 temperature = 80℃, respectively. Mass scale calibration was accomplished with YOKUDELNA calibration kit (JEOL, Japan) for accurate mass measurements and calculations of the elemental composition.

Human skin fibroblast cell culture: Primary human foreskin dermal fibroblasts were established from biopsies of healthy donor of 22 years and in accordance with Institutional Review Board (IRB) approved by the Kyung Hee University Hospital (Seoul, Korea) (IRB approval no. 2012-01-006). The cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, streptomycin (100 mg/ml), 2 mM glutamine, and penicillin (100 U/ml), in a 37℃ humidified incubator containing 5% CO_2.

Collagen and MMP-1 protein expressions: To determine the anti-wrinkle effects of SGT water extract, we evaluated the expression of collagen and MMP-1 in human dermal fibroblasts using Western blot techniques. The cells were treated with SGT water extract for 48 h with a variety of concentration (0, 100, 200, and 400 ㎍/mL). The culture medium was collected after stimulation and used to examine MMP-1 concentration and type-1 procollagen. The collagen content was evaluated effective inhibition of MMP-1 used by colorimetric method and investigated using a procollagen type-1 C-peptide assay kit.

UVB irradiation: The cells were cultured in DMEM media for 24 hr. After overnight, the media was replaced by phosphate-buffered saline (pH 7.4). And then the cells were exposed to Ultra-violate irradiation (40 mJ/cm^2) light. After irradiation, the cells were cultured for 24hrs in serum-free DMEM media with or without various concentration of SGT water extracts (0, 100, 200, and 400 ㎍/mL)[4].

Human 3D skin layer system: To define the function of courier enhancing the power of delivery to dermis, 3D skin system was utilized. The major phytochemical of puerarin from sovereign was selected as an indicator and analyzed by HPLC system. All samples were dissolved in culture media for applying to the apical surface of EpiDerm™ tissue. The samples were sovereign as single, sovereign with minister, sovereign with assistant and sovereign with courier to compare which herb enhance the permeability of sovereign to dermis. The blended were followed by SGT composition ratio. The EpiDerm™ kit was obtained from MatTek Corporation (Ashland, MA). Fore exposure via the culture media, all samples were dissolved in media to total 1 mL volume. The composition of samples was listed in Table 1.

Table 1: Sample preparation in 3D skin layer study

No.	Composition
1	Sovereign 50 μg/mL
2	(Sovereign 50 μg + Minister 100 μg) /mL
3	(Sovereign 50 μg + Assistant 50 μg) /mL
4	(Sovereign 50 μg + Courier 50 μg) /mL

*The SGT composition ratio is Sovereign : Minister : Assistant : Courier = 1 : 2 : 1 : 1.

Statistics analysis: All values from at least three independent experiments were presented as mean ± S.E. Statistically significant differences between the groups were determined by statistical package for social sciences (SPSS) using one way analysis of variance (ANOVA). $P < 0.05$, $P < 0.01$ and $P < 0.001$.

Results

1. <u>HPLC-ESI-MS profile:</u> The optimal chromatographic profile of SGT was obtained by HPLC system and the six major peaks were identified by HPLC-ESI-MS study (Fig. 1). The retention time, observed mass, mass difference and proposed compounds of six peaks were listed in Table 2. The six major phytochemicals in SGT were identified as puerarin, 3'-methoxypuerarin and daidzin from sovereign as Puerariae Radix, isoferulic acid from minister as Cimicifugae Rhizoma, paeoniflorin from assistant as Paeoniae Radix and liquiritin from courier as Glycyrrhizae Radix. These six compounds were identified by comparing both UV and MS spectrum to their spectroscopic data in literatures[5, 6, 7]. The puerarin was observed as most abundant

phytochemical in SGT, so it was selected to calculate how much potential to permeate 3D skin layer in composed with sovereign and the other composed herbs.

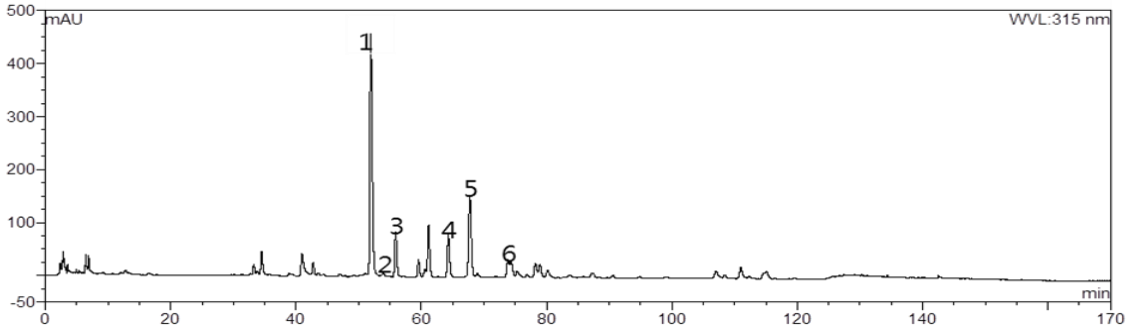

Figure 1: Typical chromatogram of SGT water extract.

Table 2: The observed and calculated mass numbers of SGT water extract.

Peak No.	Rt (min)	Theoretical mass $[M+H]^+$	Observed mass $[M+H]^+$	Mass difference (mmu)	Identification
1	51.93	417.11854	417.11338	-5.16	puerarin
2	53.96	481.17096	481.17640	5.44	paeoniflorin
3	55.91	447.12910	447.12316	-5.94	3'-methoxypuerarin
4	64.31	417.11854	417.11534	-3.20	daidzin
5	67.74	195.06573	195.05935	-6.38	isoferulic acid
6	73.83	419.13416	419.13338	-0.78	liquiritin

2. Reduced cytotoxicity as a role of an assistant herb, *Paeonia lactiflora.*:

As shown in Figure 2A, the extracts of SGT did not induce any cytotoxicity at concentrations up to 400 ㎍/mL but some of combination extracts showed cytotoxicity. Figure 2B showed that the cytotoxicity was only presented in the group where assistant herb was not included.

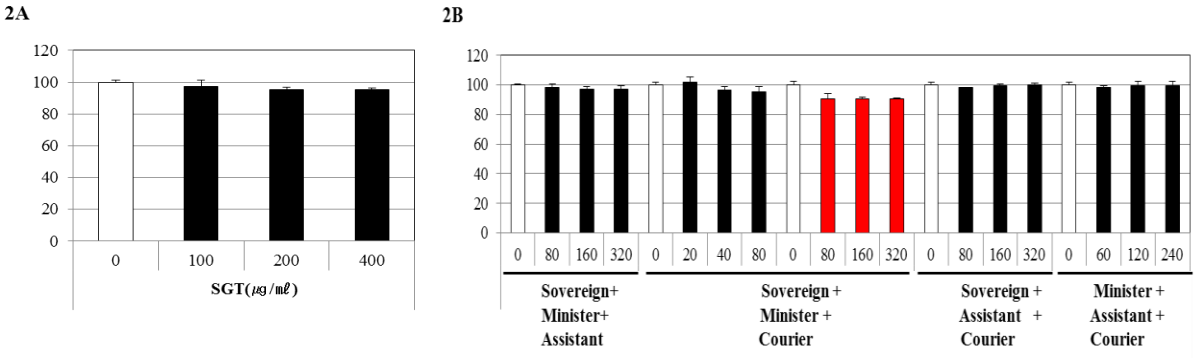

Figure 2: The effects on reducing the cell cytotoxicity for assistant herb *Paeonia lactiflora*.

3. <u>Collagen and MMP-1 protein expression in human dermal fibroblasts.</u>

The expression of Type-1 procollagen significantly increased by the treatment of SGT on UVB-irradiated human skin fibroblast cells in a dose-dependent manner via the inhibition of UVB-induced MMP-1 production. After UV-irradiation, the cells were incubated with 0, 100, 200, and 400 ㎍/mL of SGT for 1 day. As shown in Figure 3A, SGT dramatically inhibited UVB-induced MMP-1 expression; the MMP-1 expression level decreased to 30.7% at 200 ㎍/mL and 30.9% at 400 ㎍/mL compared with the only UVB-irradiation control.

It was investigated whether SGT inhibited MMP-1 production dose-dependently, compared with the non-treated control as the concentration increase. Figure 3B shows that SGT promoted effects the expression of collagen significantly increased in UVB-irradiated human skin fibroblast cells in a dose-dependent manner; the collagen expression level increased to 24.7% at 100 ㎍/ mL, 31.6% at 200 ㎍/ mL and 32.7% at 400 ㎍/ mL via inhibition of UVB-induced MMP-1.

As shown in Figure 4, combination extracts composed of sovereign, minister herbs and courier herb inhibited the production of MMP-1 and promoted type-1 procollagen synthesis in a dose-dependent manner.

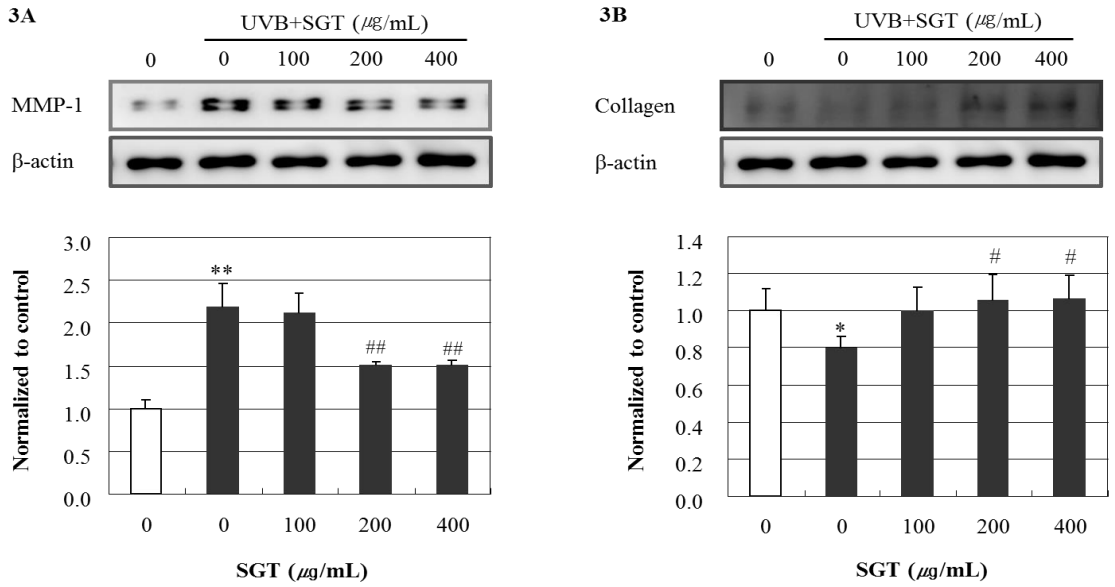

Figure 3: The effects of SGT water extract on UVB-irradiation induced production of MMP-1 and Collagen in human skin derma fibroblast cells. Each exterminations were conducted at least in triplicate: the data represent mean ± S.E. $^{*}p<0.05$ and $^{**}p<0.01$ vs. non UVB-irradiation, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. only UVB-irradiation.

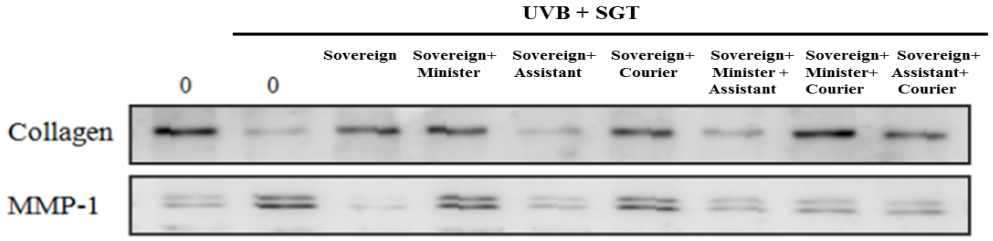

Figure 4: The effective on combination of SGT water extract on UVB-irradiation induced production of MMP-1 and Collagen

4. **<u>Effect on permeability of active component on human 3D skin tissues</u>**

The major active component of SGT was identified as puerarin from sovereign herb and the permeability through 3D skin system was enhanced by the combination with courier herb *Glycyrrhiza uralensis*. The puerarin delivery through dermis was impeded by the combination with minister or assistant herb.

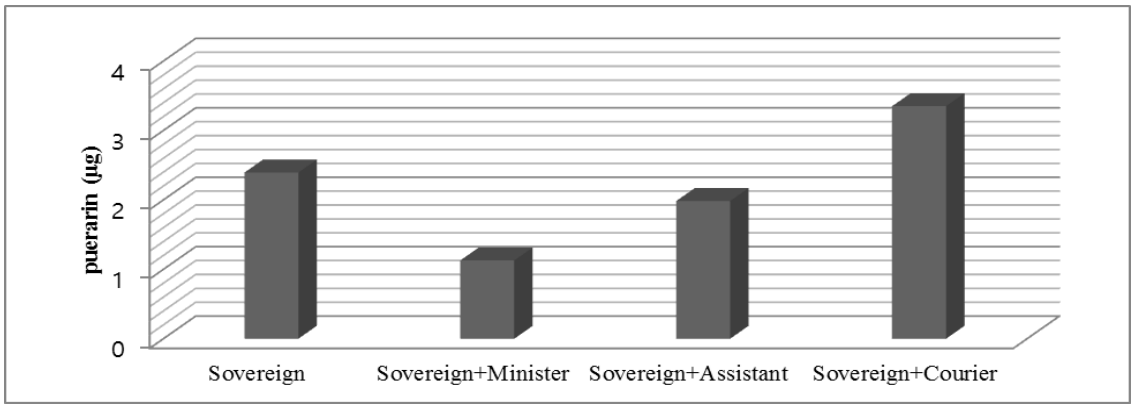

Figure 5: Determination of puerarin from the bottom media of EpiDerm^{TM} tissure after topical exposure. The skin tissue was exposed to Sovereign, Sovereign+Minister, Sovereign+Assistant and Sovereign+Courier samples for 24 h. The amount of puerarin which were permeated through the skin layer and conserved in bottom media was calculated by HPLC analysis.

Conclusion

We concluded that SGT formula protected human skin cells against UVB induced aging and had a potential to be developed as an active ingredient of oriental cosmetic products for skin aging. According to sovereign, minister, assistant and courier principle, we suggest that sovereign and minister herbs should exchange their role in the SGT formula when it is applied on human skin for anti-wrinkle purpose. *Pueraria lobata* corresponds to the sovereign herb because it provided anti-wrinkle effects, *Cimicifuga heracleifolia* corresponds to minister herb because it also showed anti-wrinkle effects but milder compared to *P. lobata*. The *Paeonia lactiflora* was assigned to the assistant herb because it reduced cytotoxicity of other herbs. The *Glycyrrhiza uralensis* was assigned to the courier herb because it enhanced the epidermis permeability of major phytochemicals in the sovereign herb. In this study, the traditional herbal formulation theory was evaluated by the experimental evidences and it can be applied to the development of diverse cosmetic products based on oriental herbal formulation principle.

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