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Antioxidant, Collagen Synthesis Activity *in Vitro* and Clinical Test on Anti-Wrinkle Activity of Formulated Cream Containing *Veronica officinalis* Extract

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Abstract

In this study, our objective was to evaluate the antioxidant, cytotoxicity and collagen synthesis activity *in vitro* and also to test the anti-wrinkle effect of formulated cream containing *Veronica officinalis* extract *in vivo.* Antioxidant evaluation was based on the scavenging activity of free radicles (DPPH) and procollagen type 1 protein (P1P) synthesis test was performed in fibroblast cell. Clinical anti-wrinkle activity was performed on female subjects in placebo-controlled trail. Verbascoside (an isolated compound) showed higher (IC_{50} value of 36.24 ± 1.81 µg/ml) free radicle inhibition activity but weaker collagen synthesis activity. The ethanolic extract showed good inhibition to DPPH free radicals and also showed a significant effect in collagen synthesis activity without cytotoxicity. In the *in vivo* study, treatment with the formulated cream (Scoti-Speedwell) for 56 days significantly reduced the percentage of wrinkle area and length with 18.0% and 16.05%, respectively. Overall, *Veronica officinalis* extract containing product (Scoti-Speedwell™) can be regarded as a potent anti-wrinkle agent in human skin.

Keywords

Antioxidant, Antiwrinkle, Collagen Synthesis, *Veronica officinalis*, Scoti-Speedwell™

1. Introduction

Skin aging (whether extrinsic or intrinsic type) causes wrinkling, sagging, laxity, dyspigmentation, and telan-

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giectasia [1]. As the skin ages, the collagen (a major component of skin) and elastin in the dermis lose elasticity resulting in wrinkles. To prevent the skin from aging or wrinkles, natural phytochemical source is desirable. Plant extracts rich in phytochemicals like flavonoids, phenolic acids, tocopherols, alkaloids, monoterpenes, having antioxidant activity are being widely used for the development of anti-wrinkle topical cosmetic products [2].

Veronica officinalis belongs to the family Scrophulariaceae and is commonly called as Speedwell. The plant is herbaceous and perennial in nature and distributed mostly in Europe and western Asia. In some part of the Europe (France), the plant is used as tea substitute called "Europe tea" and is considered as a medicinal herb [3]. According to Romanian folk medicine, *Veronica officinalis* was used for kidney diseases, cough and catarrh and wound healing purposes [4]. Recently, the literature showed that the plant was rich in phytochemicals and had moderate nitric oxide scavenging activity and strong anti-inflammatory (TNF-*α*) activity [5]. The plant is also reported to have higher antioxidant activity [6]. In this study, our main objective was to evaluate the efficacy of plant extract and isolated compound on antioxidant, cytotoxicity and collagen synthesis activity *in vitro* and also to test the anti-wrinkle effect of formulated cream containing *Veronica officinalis* extract *in vivo.*

2. Materials and Methods

2.1. Plant Materials, Extracts Process

Dried sample of *Veronica officinalis* (whole plant) were supplied from Royal Botanical Garden Scotland, in November 2012. The plant material was air-dried at room temperature, milled, extracted with ethanol (70%) overnight, and filtered, and the process was repeated three times. The resulting ethanol extract was concentrated at reduced pressure in a rotatory evaporator at 40˚C. A portion of the ethanol extract (410 g) was dissolved in distilled water, and placed in a separator funnel, and washed with n-hexane (200 mL, 15 times). The n-hexane phases were then combined and concentrated under reduced pressure. An identical process was repeated with chloroform, ethyl acetate, and butanol leaving a residual mixture of ethanol-water.

2.2. Isolation of Verbascoside Compound from *Veronica officinalis* (Speedwell)

Ethyl acetate extract (56.1 g) was fractionated by open CC using silica gel. Elution was carried out with addition of methanol to hexane-ethyl acetate mixtures in different ratios of increasing polarity until 100% methanol was reached. All fractions were analyzed by TLC. Verbascoside was obtained as the major compound by successive washes with CH_2Cl_2-MeOH (7:3) (5.02 g, 8.94% of the ethyl acetate extract). This compound was purified further by silica gel TLC using CH_2Cl_2-MeOH. The chemical structures of verbascoside was determined by comparison of spectroscopic and chromatographic data with those of authentic samples and were reported previously [7] [8].

2.3. Topical Formulation

Two percent of *Veronica officinalis* extract was mixed with a formulation containing water, Carbomer, Glycerine, Disodium EDTA (ethylene diamine tetra-acetic acid), Methylparaben, Trithanolamine, Tocopheryl acetate, Polysorbate 60, Stearyl alcohol, PEG-100 (polyethylene glycol-100) stearate, Sorbitan stearate, caprylic/capric triglyceride, Dimethicone, Mineral oil, Propylparaben, Butylene Glycol, Beeswax, and Fragrance. The placebo (control) was identical in composition, except plant extract.

2.4. Cell Culture and Cytotoxicity Determination

Fibroblast cell was purchased from the Korea cell line bank, Seoul, Korea were cultured in 96-well plates containing Dulbecco's Modified Eagle Medium (DMEM, 200 µl/well) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin sulfate (100 µg/ml) in a humidified atmosphere of 5% CO_2. The cell viability assay of the Speedwell extract was performed with MTT (3-(4,5-dimethylthiaol-2-yl)-2-5-diphenyltetrazolium bromide) reagent following the protocol of Mosmann *et al.* [9].

2.5. Collagen Synthesis Activity in Fibroblasts

Procollagen type 1 protein synthesis test was performed according to the protocol of Tanayama *et al.* [10] with

slight modification. 5×10^4 cell/well were seeded in a 24 well plate with DMEM (containing 10% FBS and 100 unit/ml penicillin-streptomycin). The plates were incubated overnight at 37˚C in a humidified incubator, 5% CO_2. After incubation, the test extract or compound was added to the plate in serum free media. After incubation the plate for 24 hour at 37˚C in a humidified incubator, 5% CO_2, the culture supernatant was collected after centrifuging at 13,000 rpm in 4˚C for 20 min. The resultant supernatant was measured in duplicate according to the supplier's instructions (Takara, MK101). Negative control was performed with buffer and substrate but without enzyme. All assays were performed independently in duplicate.

2.6. *In Vitro* Antioxidant Activity

2.6.1. DPPH Free Radical Scavenging Assay

The antioxidant activity of extract was determined according to the method described by Bracca *et al.* [11], with slight modification. Briefly, a dilution series of ethanolic extract and isolated compound verbascoside was prepared in a 96 well plate. The reaction mixture consisted of 0.1 ml extract with 0.2 ml DPPH solution (0.15 mM in 80% methanol solution). The mixture was shaken vigorously and left to stand for 30 min at room temperature in the dark. Ascorbic acid was taken as positive control. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm and the percent inhibition activity was calculated.

2.6.2. *In Vivo* Human Clinical Study (Wrinkle Area, Length Differences and Visual Score)

In vivo study was conducted in Guangzhou City, Land Proof Test Technology Co. Ltd., China. The study was a randomized, open, single-blinded, placebo-controlled, observer-blinded study which was approved by Guang-Dong light industry association institutional review committee for human testing. Twenty-one female subjects aged 45 - 65 years (without the history of serious diseases or allergic to cosmetics or pregnant women) participated in the study. The subjects' crow's feet area on both sides (right & left) were selected in which the wrinkles must not cross each other and the length of the main wrinkle must be at least 2 cm long. All subjects gave written informed consent prior to the study and evaluated for tolerance. Subjects were treated with Scoti-Speed well cream which contain 2% speedwell extract on the one side of the face (crow's feet) and with placebo (ingredients without plant extract) on the other side twice a day for 58 days. Clinical evaluations and measurements were performed on D0 (before treatment), D28 and D56. The anti-wrinkle effect of cream and placebo on wrinkled skin was evaluated by using Cutometer MPA 580 (CK Germany), Visioline VL650 (CK Germany), and SILFLO (cuDern USA).

2.7. Statistical Analysis

Statistical analyses were carried out using SPSS software (version 11.5; SPSS Inc., Chicago, IL, USA). The differences among samples were statistically evaluated via one-way analysis of variance (ANOVA) followed by Dunnett's posthoc test or Wilcoxon's test when appropriate. The level of significance was set at $p < 0.05$. Data are expressed as means ± standard errors.

3. Result

3.1. *In Vitro* Antioxidant Activity

The antioxidant efficacy of ethanolic extract of speedwell and isolated single compound verbascoside (**Figure 1**) is given in **Figure 2**. The isolated compound verbascoside showed a higher free radicle (DPPH) scavenging activity with the IC_{50} value of 36.24 ± 1.81 µg/ml. Similarly, the ethanolic extract of speedwell also showed good inhibition to DPPH radicles in dose dependent manner whose IC_{50} value was 103.50 ± 2.43 µg/ml.

3.2. Cell Viability and Collagen Synthesis Activity in Fibroblasts

The single compound verbascoside isolated from speedwell did not show significant result on Collagen synthesis activity in Fibroblasts (data not shown). However, the significantly increased in procollagen type 1 protein synthesis was observed due to the ethanolic extract of speedwell in fibroblast cell without cytotoxic effect (**Figure 3**). At a concentration of 2%, extract showed 44.6 % increase in collagen synthesis compare to the control (without extract). This activity of extract on PIP could be due to the interactions and synergistic effect of phytochemicals other than verbascoside present in the sample [12]-[14].

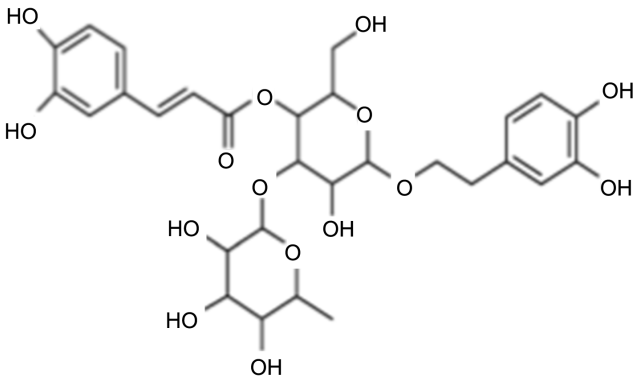

Figure 1. Verbascoside compound isolated from *Veronica officinalis*.

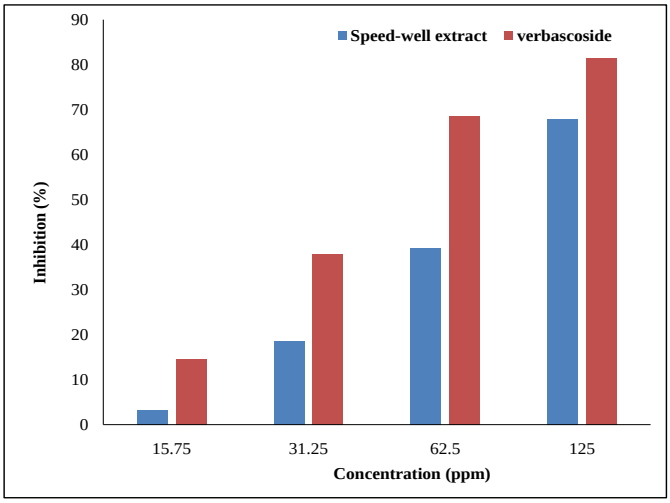

Figure 2. Antioxidant (DPPH free radical scavenging) activity in dose dependent manner exhibited by ethanolic extract of Speed-Well and the isolated compound Verbascoside.

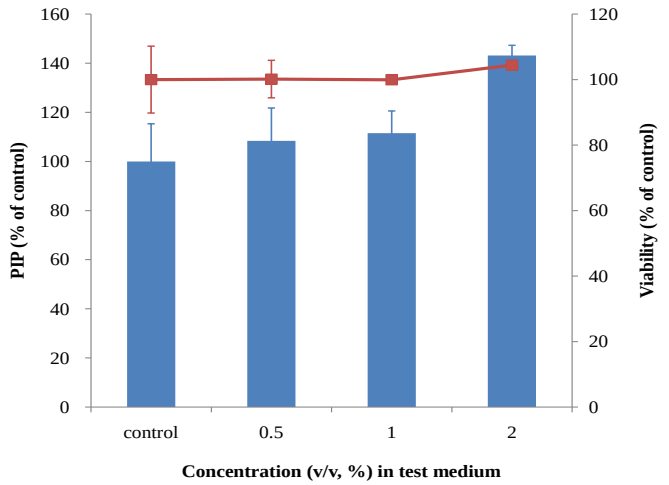

Figure 3. Pro Collagen type 1 protein synthesis test and cell viability test (MTT assay) in fibroblast cell shown by ethanolic extract of Speedwell.

3.3. Human Clinical Study

We examined the effect of topical formulated Scoti-speedwell cream on the wrinkles of crow's feet site of the eyes (**Figure 4**). The wrinkle area and length difference at the base line were analyzed to identify the differences at sample treated sites. Treatment with formulated cream for 28 days did not show any significant difference with the placebo. However, treatment for 56 days reduced significantly the percentage of wrinkle area (**Figure 5**) and wrinkle length (**Figure 6**) by 18.0% and 16.05% respectively compared with the placebo. The dermatological scores of the sides treated by the extract containing cream decreased significantly on 56 days with 66% lower than that of placebo treatment (**Figure 7**).

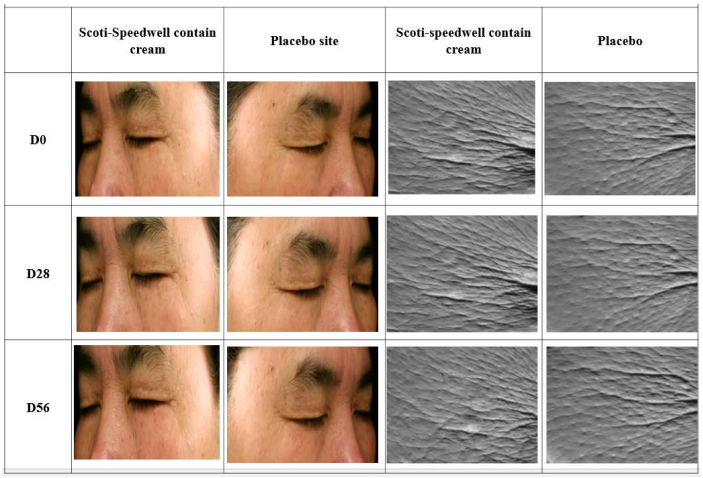

Figure 4. Photograph showing the images of wrinkles used for assessment of wrinkle area and length in the crow's feet region of the subject's eyes treated with 2% topically formulated Scoti-Speedwell extract and placebo treated for 56 days. Clinical evaluations and measurements were performed on D0 (before treatment), D28 and D56.

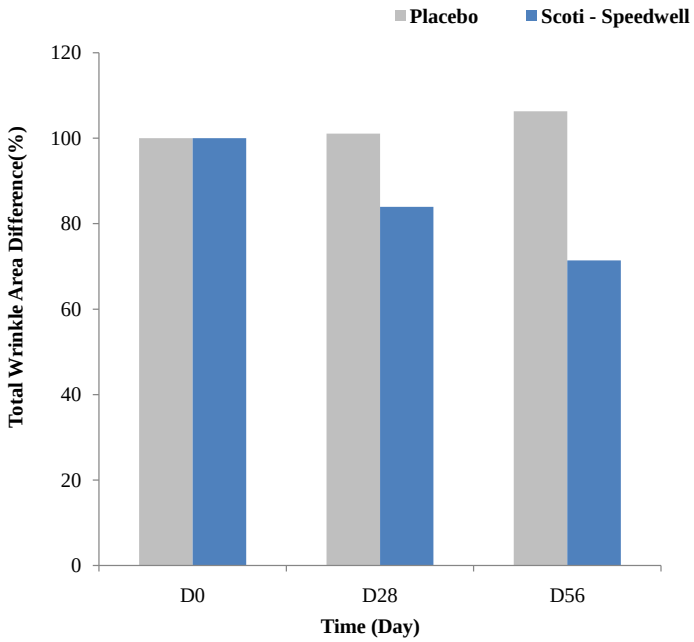

Figure 5. Differences in the wrinkle area after the treatment of topical formulated Scoti-speedwell cream or the placebo randomly on crow's feet region of eyes.

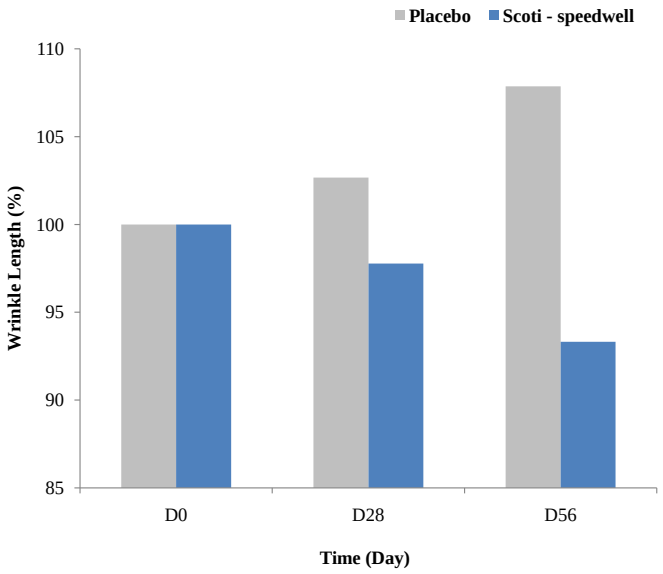

Figure 6. Differences in the wrinkle length after the treatment of topical formulated Scoti-speedwell cream or the placebo randomly on crow's feet region of eyes.

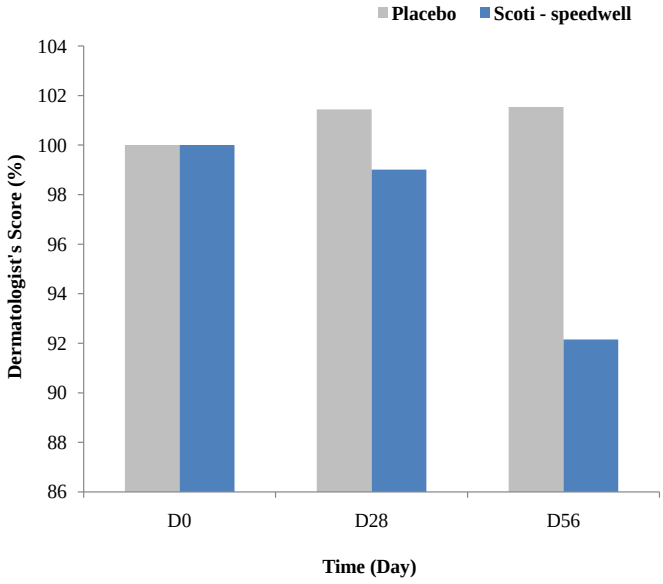

Figure 7. Differences in the visual score after the treatment of topical formulated Scoti-speedwell cream or the placebo randomly on crow's feet region of eyes.

4. Discussion

The speedwell extract showed significant anti-wrinkle activity *in vivo*. This could be due to the presence of higher radical scavenging activity of speed well extract which quenched the free radicals from the skin and thereby protected the collagen from degradation. And also, the phytochemicals present in the extract may have possible interactions with the special enzymes, mediators in the signal transduction pathway and thereby initiate the anti-wrinkle effects on skin [3]. Overall, *Veronica officinalis* extract containing product (Scoti-speedwell™) can be regarded as a potent anti-wrinkle agent in human skin.

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Conflict of Interest

There are no conflicts of interest.

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