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Original Articles

十全大補湯 藥鍼液의 사람 피부아세포의 콜라게나제 활성 및 프로콜라겐 합성과 티로시나제 활성에 미치는 영향

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Effects of Sipgeondaebo-tang Pharmacopuncture Extracts on the Collagenase Activity and Procollagen Synthesis in HS68 Human Fibroblasts and Tyrosinase Activity

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ABSTRACT

Objectives : This study was designed to investigate the collagen metabolism and tyrosinase activity of Sipgeondaebo-tang Pharmacopuncture extracts (SP).

Methods : The effect of SP on type I procollagen production and collagenase activity in human normal fibroblasts HS68 after UVB (312 nm) irradiation was measured by ELISA method. The tyrosinase activity after treatment of SP was measured as well.

Results : Type I procollagen production was recovered by SP in UVB damaged HS68 cells. The increased collagenase activity after UVB damage was significantly recovered by SP. The tyrosinase activity was significantly reduced as well. However, the L-DOPA oxidation was not changed.

Conclusion : SP showed the anti-wrinkle effects and whitening effects in vitro. These results suggest that SP may be a potential pharmacopuncture as an anti-aging pharmacopuncture treatments.

I. Introduction

Sipgeondaebo-tang pharmacopuncture extracts (Shiquandabu-tang in chinese, SP) are composed of ten herbal medicines. Its effects are to warm and tonify the qi and blood for qi and blood deficiency[1,2].

Every man and woman has the desire to look and feel at least ten years younger than their chronological age. Therefore, in these days, aging seems to be treated as not a nature to accept but a disease or a disorder to overcome. Theories of aging fall into two categories, (1) programmatic theory and (2) stochastic theory. The programmed theory proposes a clock in our bodies that controls not only our process of development but also triggers our self-destruction. The stochastic theory proposes that the cross-linking of proteins and other cellular macromolecules leads to age-dependent diseases and disorders. Processes that are associated with cellular damage and aging are the production of free radicals (a process much enhanced after ultraviolet irra-

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diation) and an increasing number of errors during DNA replication. Cellular manifestations of intrinsic aging include decreased life span of cells, decreased responsiveness of cells to growth signals, which may reflect loss of cellular receptors to growth factors, and increased responsiveness to growth inhibitors. All these findings are more pronounced in cells derived from photo-damaged skin[3]. Aging process in skin (extrinsic aging) is generally referred to as photo-aging due to chronic exposure to short wavelength UV light (UVB) and is characterized by severe wrinkling and pigmentary changes, such as solar lentigo and mottled pigmentation on exposed areas such as the face, neck, and forearm[4]. It has been shown that UV irradiation leads to the formation of reactive oxygen species (ROS) that activate the mitogen-activated protein (MAP) kinase pathway, which subsequently induces the expression and activation of matrix metalloproteinases (MMPs) in human skin in vivo[5,6]. MMPs including collagenase are considered key factors in the photo-aging process. Melanogenesis was induced after UV irradation as well. The key regulator in melanogenesis is well known as a type of enzyme, tyrosinase. Tyrosinase is a copper-containing enzyme present in animal tissues that catalyzes the production of melanin[7].

In the present study, the effect of SP on type I procollagen production and collagenase activity in human normal fibroblasts HS68 after UVB (312 nm) irradiation were investigated. The tyrosinase activity after treatment of SP was measured as well.

II. Materials and methods

1. Sample preparation

The Ginseng Radix, Atractylodis Rhizoma Alba, Hoelen, Glycyrrhizae Radix, Angelicae Gigantis Radix, Paeonia Radix, Rehmanniae Radix Preparata, Astragali Radix, and Cinnamomi Cortex were purchased from Omniherb (Korea). SP was prepared as follow. The Ginseng Radix (12 g), Atractylodis Rhizoma Alba (12 g), Hoelen (12 g), Glycyrrhizae Radix (12 g), Angelicae Gigantis Radix (12 g), Paeonia Radix (12 g), Rehmanniae Radix Preparata (12 g), Astragali Radix (10 g) and Cinnamomi Cortex (10 g) in 2,000 ml distilled water was heated in a heating extractor for 3 hours. The extract was filtered and concentrated by using the rotary evaporator. The extracts were lyophilized by using freeze dryer (19.5 g). The extract was dissolved in water and filtered three times through microfilter paper and syringe filter (Whatman #2, 0.45 μm to 0.2 μm). Filtered material was placed in the disinfected vial and was sealed for further study.

2. Reagents

All reagents were purchased from Sigma-Aldrich except as mentioned below (St. Louis, MO, USA).

3. Cell culture

HS68 human fibroblasts (Health Protection Agency Culture Collections, UK) were cultured in Dulbecco's Modified Eagle's medium (Gibco, USA) containing 10% fetal bovine serum, 1% antibiotics at 37° C in a humidified atmosphere of 5% CO_2. When cells reached above confluency, subculture was conducted at a split ratio of 1:3.

4. UVB irradiation

A UVB lamp (Vilber Lourmat, France) was used as a UVB source. In brief, HS68 cells were rinsed twice with phosphate-buffered saline (PBS), and all irradiations were performed under a thin layer of PBS (200 μl/well). Immediately after irradiation, fresh serum-free medium was added to the cells.

After 24 hours incubation period, responses were measured. Mock-irradiated blanks followed the same schedule of medium changes without UVB irradiation.

5. Cell viability

General viability of cultured cells was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. The human fibroblast cells (HS68) were seeded in 24-well plates at a density of 2×10^5/ml per well and cultured at 37° C in 5% CO_2. Cells were pretreated with the sample at a concentration of 100, 10, 1 μg/ml for 24 hours prior to UVB irradiation. After UVB irradiation, cells were retreated with the sample and incubated for additional 24 hours, before being treated with 0.05 mg/ml (final concentration) of MTT. The blank and control group was cultivated without sample treatment. The cells were then incubated at 37° C for additional 4 hours. The medium containing MTT was discarded, and MTT formazan that had been produced was extracted with 200 μl of DMSO. The absorbance was read at 595 nm with a reference wavelength of 690 nm. The cell viability was calculated as follows:

Cell viability (%)
=[(OD595 of sample)/(OD595 of control)]$\times$100

6. Assays of collagen type I synthesis and collagenase inhibition

HS68 human fibroblasts were inoculated into 24-well plate (2×10^5 cells/well) and cultured at 37° C in 5% CO_2. Cells were pretreated with the sample at a concentration of 10, 30, and 100 μg/ml for 24 hours prior to UVB irradiation. After UVB irradiation, cells were retreated with the sample and incubated for additional 24 hours. The blank and control group was cultivated without sample treatment. After culturing, the supernatant was collected from each well, and the amount of pro-collagen type I was measured with a procollagen type I C-peptide assay kit (Takara Bio, Japan). The activity of collagenase was measured with a matrix metalloproteinase-1 (MMP-1) human biotrak ELISA system (Amersham life science, USA).

7. Tyrosinase inhibition assay

Tyrosinase activity was determined essentially as previously described[8]. The reation mixtures were prepared by adding 40 U of mushroom tyrosinase to 20 μl of SP dissolved in distilled water, and then adding 40 μl of 1.5 mM L-tyrosine and 220 μl of 0.1 M sodium phosphate buffer (pH 6.5). The resul ting mixture (300 μl) was incubated for 10 min at 37° C and then absorbance at 490 nm was measured. The same mixture, but without SP extract, was used as a control.

8. Inhibition of L-DOPA oxidation

The inhibitory effect of SP on L-DOPA oxidation was determined according to the method of Joshi with a slight modification[9]. 50 μl of SP dissolved in 0.1 M sodium phosphate buffer was added to 40 U of mushroom tyrosinase in 900 μl of 0.1 M sodium phosphate buffer (pH 6.5). After 6 min of incubation at 37° C, 3 mM of L-DOPA was added. Then the mixture was incubated at 37° C for 15 min. Activities were quantified by measuring absorbance at 475 nm. The same mixture, but without SP extract, was used as a control.

9. Statistical analysis

The results were expressed as means ± standard error of the mean (SEM). Significances of changes were determined using the one-way ANOVA with a Dunnett's post hoc test. Values of $p < 0.05$ were considered significant.

III. Results

1. Cytotoxicity on HS68 human fibroblasts

In order to evaluate the cytotoxicity of SP, samples were prepared at various concentrations and used to treat human fibroblasts (HS68). The results of this evaluation are shown in Figure 1 at concentrations of 10, 30, 100 μg/ml. The cell viability was recalculated into 100% of control group. The cell viabilities of SP 10 μg/ml treated, SP 30 μg/ml treated, SP 100 μg/ml treated are 105.2 ± 2.0%, 104.5 ± 0.7%, and 106.2 ± 0.5%, respectively. SP showed no cytotoxicity up to the effective concentration for anti-wrinkle activity (less than 100 μg/ml).

2. Assay of collagen type I synthesis

To evaluate the amount of collagen type I synthesis that occurred upon exposure to the sample, collagen type I was quantitatively detected by using the previously described procollagen type I C-peptide assay kit. Collagens are synthesized as precursor molecules, called procollagens. These molecules contain additional peptide sequences, usually referred to as 'propeptides', at both the amino-terminal end and the carboxy-terminal end. These propeptides are cleaved from the collagen triple-helix molecule during its secretion, after which the triple-helix collagens are polymerized into extracellular collagen fibrils. Thus, the amount of free propeptide stoichiometrically reflects the amount of collagen molecules synthesized[10]. The amounts of type I collagen synthesis of SP were shown in Figure 2. SP did not increase the expression of type I collagen at all concentrations of 10, 30, and 100 μg/ml (11.5 ± 2.3 ng/ml, 12.1 ± 2.4 ng/ml, and 13.9 ± 2.3 ng/ml) compared with control group (15.3 ± 1.6 ng/ml, Fig. 2).

3. Assay of collagenase activity

To evaluate the collagenase activity, matrix metalloproteinase-1 (MMP-1) activity was quantitatively measured by using the previously described matrix metalloproteinase-1 assay kit. The activities of MMP-1 of SP treatment were recalculated into 100% of control group (Figure 3). SP significantly reduced the MMP-1 activity at concentrations of 10 μg/ml (59.1 ± 1.9%, $p < 0.05$). Both 30 μg/ml, and 100 μg/ml showed the tendency of reducing patterns. However, there were no significances (62.8 ± 9.1%, and 55.9 ± 16.2% respectively, Fig. 3).

4. Tyrosinase activity assay

The activities of SP on tyrosinase activity were recalculated into 100% of control group (Fig. 4). SP significantly reduced the tyrosinase activity at concentrations of 1 and 10 mg/ml (74.4 ± 4.6% and 54.0 ± 14.4%, $p < 0.05$) in a dose dependent manner. The tyrosinase activity of SP 0.1 mg/ml treated group did not show any significance (94.4 ± 0.3%).

5. L-DOPA oxidation

The activities of SP on L-DOPA oxidation were recalculated into 100% of control group (Figure 5). SP significantly reduced the L-DOPA oxidation activity at concentrations of 10 mg/ml (47.9 ± 16.5%, $p < 0.05$). SP 0.1 and 1 mg/ml treated

groups did not show any activity (111.3 ± 2.9% and 100.9 ± 0.5% respectively).

IV. Discussion and conclusion

Sipgeondaebo-tang pharmacopuncture is composed of ten herbal medicines; Ginseng Radix, Atractylodis Rhizoma Alba, Hoelen, Glycyrrhizae Radix, Angelicae Gigantis Radix, Paeonia Radix, Rehmanniae Radix Preparata, Astragali Radix, and Cinnamomi Cortex[1]. Its effects are to warm and tonify the qi and blood for qi and blood deficiency in consumptive disorders with coughing, reduced appetite, spermatorrhea, and weakness of the lower extremities. The qi and blood deficiency is commonly showed in aging-process. This decoction is a very commonly used formula for qi and blood deficiency with a predominance of deficient qi tending toward cold[1,2].

The skin aging is one of the most obvious evidence of aging. The skin is increasingly exposed to ambient UV-irradiation thus increasing risks for photo-oxidative damage with long-term detrimental effects like photo-aging, characterized by wrinkles, loss of skin tone and resilience. Photo-aged skin displays alterations in the cellular component and extracellular matrix with accumulation of disorganized elastin and its microfibrillar component fibrilin in the deep dermis and a severe loss of interstitial collagens, the major structural proteins of the dermal connective tissue. MMPs are known to be upexpressed in human fibroblasts within hours after exposure to UV irradiation and are, therefore, considered key factors in the photo-aging process. Therefore, agents with the ability to elevate ECM protein levels or inhibit the major collagen-degrading enzymes like MMPs would prove to be useful in the development of effective anti-aging agents. Collagen is a group of naturally occurring proteins. In nature, it is found exclusively in animals, especially in the flesh and connective tissues of mammals[11]. It is the main component of connective tissue, and is the most abundant protein in mammals, making up about 25% to 35% of the whole-body protein content[12]. Collagen, in the form of elongated fibrils, is mostly found in fibrous tissues such as tendon, ligament and skin, and is also abundant in cornea, cartilage, bone, blood vessels, the gut, and intervertebral disc. In muscle tissue it serves as a major component of endomysium. Collagen constitutes 1% to 2% of muscle tissue, and accounts for 6% of the weight of strong, tendinous muscles[13]. Collagen occurs in many places throughout the body. So far, only 29 types of collagen have been identified and described. Over 90% of the collagen in the body, however, is of type I, II, III, and IV. Among them, collagen type I is placed at skin, tendon, vascular, ligature, organs, and bone (main component of bone). Collagen-related diseases most commonly arise from genetic defects or nutritional deficiencies that affect the biosynthesis, assembly, postranslational modification, secretion, or other processes involved in normal collagen production .

In this study, we evaluate the cytotoxicity of SP on human fibroblasts (HS68) at various concentrations. There was no cytotoxicity in all SP-treated concentrations. However, the amount of collagen type I was not increased at all concentration of SP treatments.

To evaluate the collagenase activity, matrix metalloproteinase-1 (MMP-1) activity was quantitatively measured. SP significantly reduced the MMP-1 activity at a concentration of 10 μg/ml. However, 30 μg/ml and 100 μg/ml treatments showed the almost same effects as a 10 μg/ml treatment. The reason of the insignificance is thought to due to the variances of each trial.

Tyrosinase is one of the important enzymes that has a key role in pigmentation process[14]. L-DOPA oxidation was also undertaken by tyrosinase. The effects of SP on tyrosinase activity and L-DOPA oxidation were significantly effective at both 1 and

10 mg/ml treatments, and 10 mg/ml treatment, respectively.

In conclusion, SP showed the anti-wrinkle and whitening effects. These results suggest that SP may be a potential pharmacopuncture as an anti-aging pharmacopuncture treatments. We think further studies will be needed to unravel exactly under the effects in vivo and clinical experiments and the molecular mechanisms of the effects.

V. Acknowledgement

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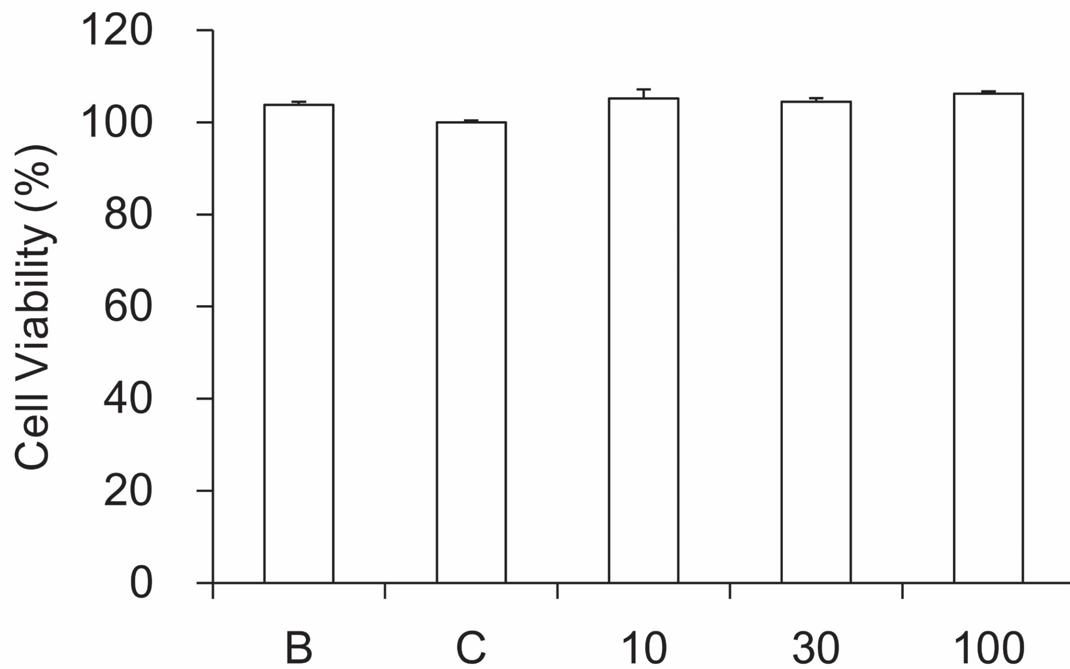

Figure 1. Cell viability of SP on HS68 human fibroblasts. B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: Sipgeondaebo-tang pharmacopuncture extracts (SP 10, 30, and 100 μg/ml) treated group. Data are expressed as the mean ± SEM of three experiments.

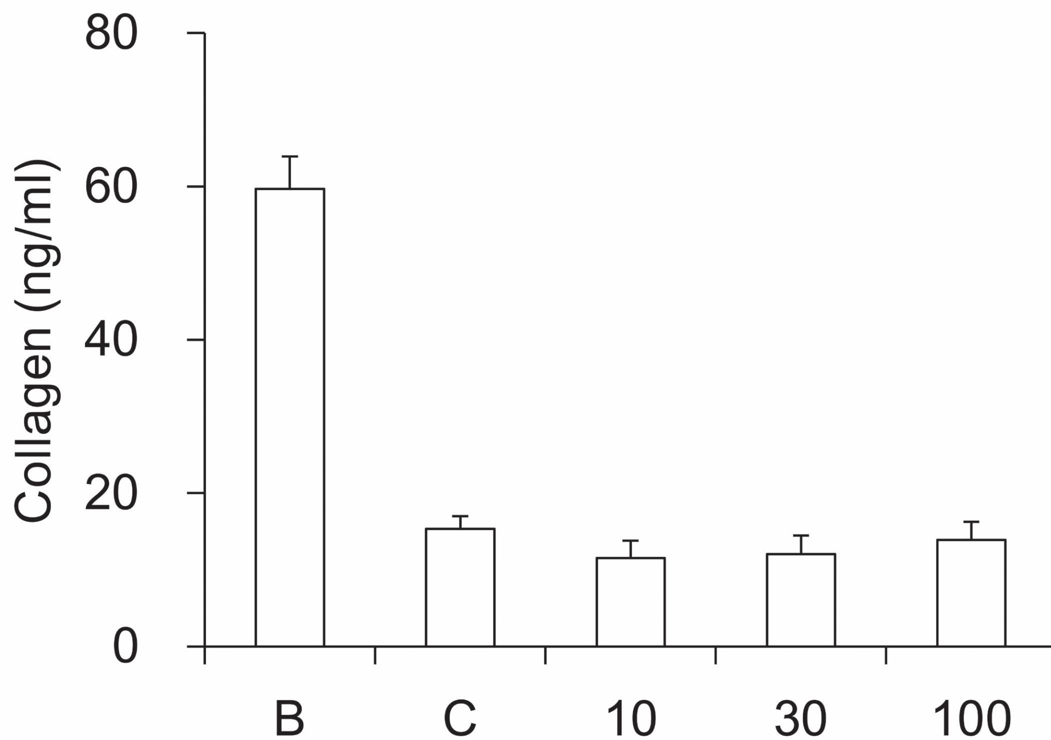

Figure 2. Effect of SP on collagen type I synthesis in human fibroblast cells. B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: Sipgeondaebo-tang pharmacopuncture extracts (SP 10, 30, and 100 μg/ml) treated group. Data are expressed as the mean ± SEM of three experiments.

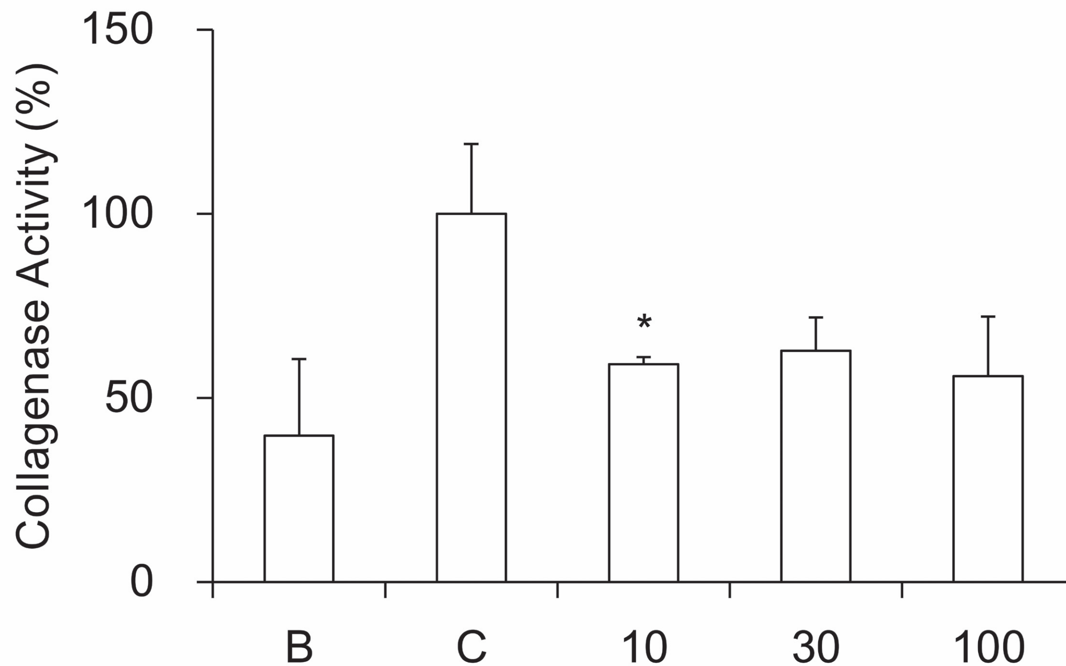

Figure 3. Effect of SP on collagenase activity in human fibroblast cells. B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: Sipgeondaebo-tang pharmacopuncture extracts (SP 10, 30, and 100 μg/ml) treated group. Data are expressed as the mean ± SEM of three experiments. *: significantly different from the control, $p < 0.05$.

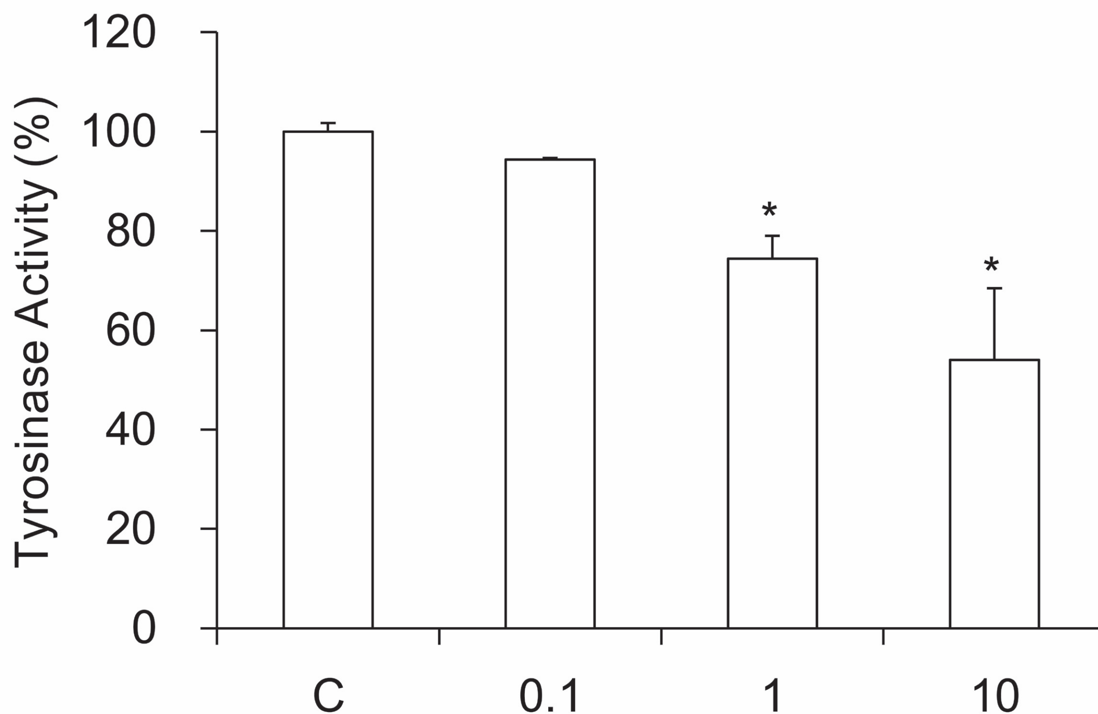

Figure 4. Effect of SP on tyrosinase activity. C: control, distilled water treated group. 0.1, 1, and 10: Sipgeondaebo-tang pharmacopuncture extracts (SP 0.1, 1, and 10 mg/ml) treated group. Data are expressed as the mean ± SEM of three experiments. *: significantly different from the control, $p < 0.05$.

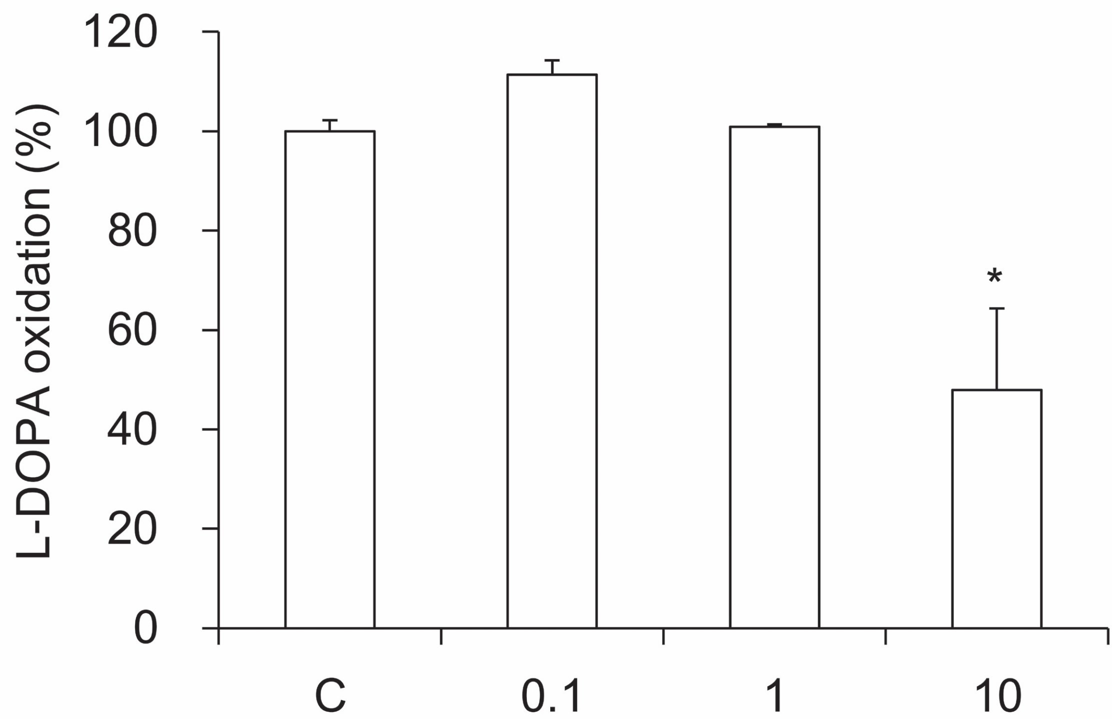

Figure 5. Effect of SP on L-DOPA oxidation. C: control, distilled water treated group. 0.1, 1, and 10: Sipgeondaebo-tang pharmacopuncture extracts (SP 0.1, 1, and 10 mg/ml) treated group. Data are expressed as the mean ± SEM of three experiments.