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Effects of Sibseonsan as an Anti-Inflammatory, Anti-Wrinkle, and Skin Whitening Treatment

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ABSTRACT

Background: The purpose of this study was to investigate whether Sibseonsan (SSS) is an effective anti-inflammatory, anti-wrinkling, and whitening agent.
Methods: To determine whether SSS had an anti-inflammatory effect, a murine macrophage cell line was used (RAW 264.7) and production of DPPH, NO, TNF-α, and PGE_2 were measured. To ascertain potential anti-wrinkle effects of SSS in these cells, collagenase and elastase production were measured. To verify whether SSS had a whitening effect, tyrosinase activity and DOPA staining were performed using a melanoma cell line (B16/F10).
Results: There was no significant reduction in survival of SSS-treated RAW 264.7 cells, up to 400 μg/mL. Free radical scavenging (23.96 ± 1.85%) was observed in RAW 264.7 cells treated with SSS at a concentration of 400 μg/mL. The SSS treatment group (400 μg/mL) significantly inhibited NO production compared with the LPS stimulated treatment group. The SSS treatment of macrophage cells appeared to reduce production of TNF-α in a concentration dependent manner. There was a significant reduction in the concentration of PGE_2 by about 25% in the SSS treatment (400 μg/mL) group ($p = 0.05$). Compared with the control, the production of collagenase and elastase in B16/F10 cells treated with SSS (400 μg/mL) was greater by 26.37% and 45.71%, respectively. The SSS treatment (400 μg/mL) group showed a significant reduction by about 17% in tyrosinase production in B16/F10 cells. The SSS treatment group showed little change in DOPA staining.
Conclusion: SSS extract may be useful for the treatment and prevention of inflammatory diseases and may have anti-wrinkle and whitening effects. These results may support the use of SSS in clinical practice.

Introduction

Skin and mucous membranes protect individuals from harmful external factors. The disruption of the skin's defense system may lead to skin disease. That is, the skin and mucous membranes are the external barrier which respond to various external changes [1].

In the human body, free radicals are the by-products of various oxidation reactions during normal cell metabolism and play a protective role. However, free radicals can attack tissues during the body's response to disease, such as inflammatory diseases, cancer, hepatitis, arteriosclerosis, and gastritis. Free radicals also play an important role in aging [2]. Inflammatory reactions occur in many diseases and in many locations such as in the skin, in the brain causing neurological diseases and in the joints causing arthritis [3]. In recent years, acute and chronic inflammatory diseases have increased due to an aging population and changes in diet. Therefore, there is a growing interest in anti-inflammatory substances [4].

The explanation for the inflammatory response in Korean medicine, is mainly described in skin disorders. Representative inflammatory reactions, such as fever, edema, redness, and pain, are explained step by step [5].

The appearance of the skin is thought to be a key element of beauty. Recently, anti-wrinkle studies [6], prevention of melanin production [7], antioxidant studies [8], and skin whitening assessments [9] have been published. Research on the "whitening effect" has been conducted using tyrosinase activity inhibition [10], DOPA oxidation inhibition [11], stratum corneum removal [12], and UV protection [13]. The "whitening effect" is thought to result from the inhibition of melanin formation [14].

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Wrinkles that occur naturally with age have been studied in association with ultraviolet rays. Ultraviolet rays breaks down collagen in the skin and causes elastin degeneration, leading to wrinkles [15]. Thus, research has centered on the development of substances that inhibit the action of collagenase and elastase to reduce wrinkles.

In Korean medicine, the condition of the skin is determined by both external and internal factors. This is a concept that includes both disease and the environment. In Korean medicine the skin's glow is related to health. This is not only a measure of health but of aging [16]. Thus, skin is an important measure associated with aging.

Sibseonsan (SSS) is recommended for treating wounds and reducing pain in the Dongui Bogam. It promotes the production of new tissue and reduces inflammation. Therefore, it is recommended for use on injuries or for skin disease [17].

In this study antioxidant, anti-inflammation, skin wrinkle inhibition, and enzyme and melanocyte effects on skin whitening and cytotoxicity effects of SSS was determined in vitro using murine macrophage and melanocyte cell lines.

Materials and Methods

SSS extract

Five packs of herbs (OmniHub, Gyeongbuk, Korea) were extracted with 2 L of water at 100°C (approximately 1:10 weight/volume) for 4 hours and filtered using Whatman filter paper No. 4 (Table 1). The filtered extract was concentrated under reduced pressure using a rotary evaporator. The concentrated extract was lyophilized and used as a powder. The yield was 17.79% (Table 1)

Cell culture

Murine macrophage cell line RAW 264.7 and murine melanoma cell line (B16/F10 cells) were used (Korea Cell Line Bank, Korea). Dulbecco's modified eagle's medium (DMEM; GenDEPOT, USA) containing 10% fetal bovine serum (FBS; GenDEPOT, USA) and penicillin/streptomycin (GenDEPOT, USA) was used. The culture was incubated at 37°C in 5% CO_2 conditions.

Table 1. Compositions and Extracting Yield of Sibseonsan.

	Formula	Weight ratio (g)	Yield (%)
Sibseonsan	*Ginseng Radix*	4	17.79
	Astragali Radix	4	
	Angelicae Gigantis Radix	4	
	Magnoliae Cortex	4	
	Platycodi Radix	4	
	Cinnamomi Cortex	4	
	Cnidii Rhiaoma	4	
	Saposhnikoviae Radix	4	
	Angelicae Dahuricae Radix	4	
	Glycyrrhizae Radix	4	

Cell viability

To evaluate the cytotoxicity and viability of RAW 264.7 cells, a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed. RAW 264.7 cells were incubated in 96-well plates seeded with 5×10^4 cells/well. Cells were incubated for 24 hours in a 5% CO_2 incubator at 37°C. After 24 hours, 100 μL of SSS (50, 100, 200, 400, 500 and 1,000 μg/mL) was used to treat the cells for a further 24 hours, after which 20 μL of MTT solution (4 mg/mL) was used to treat the cells for 4 hours. The supernatant was removed and the cells were lysed using 100 μL of dimethyl sulfoxide (DMSO). The formazan produced by MTT reduction was measured at 570 nm using a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, California, USA).

DPPH

Free radical scavenging activity (the DPPH method) was used to determine homeostasis. 5×10^4 M 1,1-diphenyl-2-picryl-hyrazyl (DPPH) solution in 50 μL of SSS was diluted to 0.01% with anhydrous ethanol (absorbance at 518 nm, 0.94–0.98). After the addition of 450 μL, absorbance was measured at 518 nm for 10 minutes. Ascorbic acid (which is known for its excellent free radical scavenging effect) was used as a positive control. Antioxidant activity (free radical scavenging activity) was used in the following equation, and were conducted in triplicate.

$$Radical\ Scavensing\ Activity\ (\%) = 100 - \{\frac{(Abs\ sample - Abs\ blank) \times 100}{\text{Abs control}}\}$$

Nitric oxide

Nitric oxide production was determined using the Griess reaction method. RAW 264.7 cells which were incubated with DMEM supplemented with 10% FCS and seeded in 24-well plates at a concentration of 5×10^4 cells/mL. They were incubated for 24 hours at 37°C in a 5%, CO_2 incubator. After removing the medium it was replaced with FBS-free DMEM for 12 hours, the test substance diluted in phenol red free DMEM was added to concentrations of 100 μg/mL and 10 μg/mL, and treated for 15 minutes. Lipopolysaccharide was added at a concentration of 0.5 μg/mL and incubated for 16 hours, 100 μL of the supernatant was transferred to a 96-well plate, and 100 μL of GRIESS reagent was added, and left to react at room temperature for 15 minutes. The absorbance of each sample was determined at 543 nm using a spectrophotometer (SpectraMax 190).

TNF-α

RAW 264.7 cells incubated with DMEM supplemented with 10% FCS were seeded in 24-well plates at a concentration of 5×10^4 cells/mL, for 24 hours at 37°C in a 5% CO_2 incubator. After removing the medium and replacing it with FBS-free DMEM media for 12 hours, the test substance was diluted in DMEM without phenol red and added at a concentration of 10 μg/mL, and left for 15 minutes. Lipopolysaccharide was added at a concentration of 0.5 μg/mL, followed by incubation for 16 hours at 37°C in 5% CO_2, and the supernatant was collected and TNF-α was quantified using an ELISA kit.

Prostaglandin E_2

RAW 264.7 cells were seeded in a 96-well plate at a concentration

of 1×10^4 cells/well. They were incubated for 4 hours in a 5% CO_2 incubator at 37°C, then the cell culture was removed. After washing with PBS, 200 μL of DMEM medium (to which 3% FBS was added) was added per well, followed by incubation for 18 hours with arachidonic acid at 37°C in 5% CO_2. Lipopolysaccharide and SSS, which activate macrophages, were added at a concentration of 10 μg/mL and incubated for 18 hours in a 5% CO_2 incubator at 37°C. The supernatant was taken and prostaglandin E_2 was quantified using an ELISA kit.

Collagenase

SSS was dissolved in DMSO and diluted with an experimental concentration using collagenase assay buffer (150 mM Tris, 10 mM NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.01% Brij). A combination of 5 μL of test solution, 500 μM substrate buffer (DABYCL-γ-Abu-Pro-Gln-Gly-Leu-Glu (EDANS)-Ala-Lys-NH2), and 5 μL of enzyme (125 ppm) was left to react at 37°C for 1 hour. A total of 10 μL of 0.5 M EDTA was added to this to terminate the reaction, and the results were measured on 520 nm. The inhibition rate was calculated using the equation below.

$$\text{Inhibition rate}(\%) = \{\frac{(\text{eubstrate} + \text{enzyme}) - (\text{substrate} + \text{enzyme} + \text{SSS extract})}{(\text{substrate} + \text{enzyme}) - (\text{substrate})}\} \times 100$$

Elastase

SSS was diluted to the concentration to be tested with anhydrous ethanol. To 50 μL of the prepared test solution, 330 μL of 0.4 M HEPES buffer (pH 6.8) and 100 μL of Suc-(Ala) 3-PNA were mixed and pre-incubated at 25°C for 3 minutes 40 seconds. A total of 20 μL (0.5 U) of elastase was added and was measured for 1 minute at an absorbance at 410 nm on a spectrophotometer (SpectraMax 190). The "blank" HEPES buffer used did not contain extracts, and the enzyme inhibition rate was calculated using the following equation.

$$Inhibition(\%) = (1 - \frac{\Delta A/\min test}{\Delta A/\min blank}) \times 100$$

Tyrosinase

SSS was dissolved in 5% DMSO and diluted with 100 mM sodium phosphate buffer. A total of 100 μL of each test substance and 100 μL of the substrate (4 mM L-Dopa solution) were mixed. Then, 2 μL of mushroom tyrosinase (2 mg/mL) solution was added and mixed. Absorbance was measured at 472 nm for 2 minutes using a spectrophotometer (SpectraMax 190) to obtain a reaction gradient. To correct the inhibitory effect of the DMSO used as a solvent, a DMSO solution corresponding to the amount of the sample to be measured, was added as a control.

DOPA staining

B16/F10 melanoma cells were incubated on a chamber slide (8 chambers; Nunc) and treated with a sample for 12 hours and then fixed with 5% formalin solution. They were stained with 0.1% DOPA solution, dehydrated and sealed, and observed with an optical microscope.

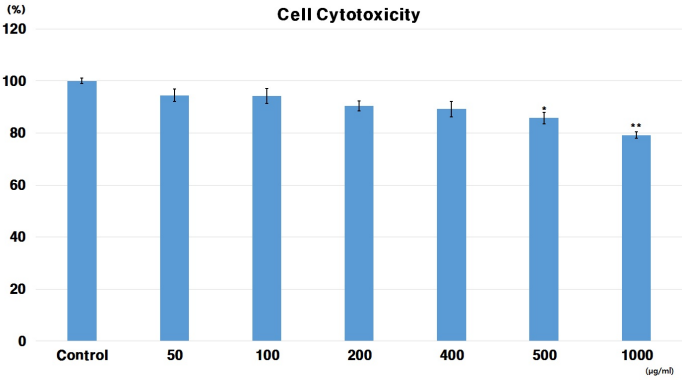

Fig. 1. Cell cytotoxicity of Sibseonsan treated RAW 264.7 cells. The cell cytotoxicity of the RAW 264.7 cells was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay.
Results were expressed as % of the control. *$p < 0.05$, **$p < 0.01$.

Statistics

Results were analyzed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± SD. To determine the effect of SSS a student's *t*-test was used. The control groups were compared with the groups treated with SSS concentrations of 50, 100, 200, and 400 μg/mL, respectively.

Results

Cell viability

The cell viabilities of RAW 264.7 cells treated with SSS at concentrations of 50, 100, 200, 400, 500 and 1,000 μg/mL were 100 ± 1.07%, 94.49 ± 2.44%, 94.24 ± 2.84%, 90.42 ± 1.95%, 89.22 ± 2.93%, 85.79 ± 2.21% and 79.21 ± 1.27%, respectively. There was no significant decrease in the cell viability at 50 to 500 μg/mL of SSS (Fig. 1). However, concentrations of SSS up to 400 μg/mL were used in this study.

DPPH

Free radical scavenging was measured using the DPPH method. The ascorbic acid (used as a positive control) showed 93.48 ± 0.03% free radical scavenging. Treatment with SSS at concentrations of 50, 100, 200 and 400 μg/mL showed free radical scavenging at 12.52 ± 0.51%, 14.87 ± 1.28%, 16.79 ± 1.17% and 23.96 ± 1.85%, respectively (Fig. 2).

Nitric oxide

As a result of measuring the inhibitory effect of SSS on NO production in LPS-stimulated RAW 264.7 cells, the NO production rate of the LPS treated group was 43.27 uM. The NO production was adjusted to 100% ± 1.94%, 96.79 ± 2.26%, 91.43 ± 2.19%, 85.43 ± 1.57%, 50, 100, 200, and 400 μg/mL, respectively, and 74.97 ± 2.11% in the control group (Fig. 2). NO was significantly decreased at a concentration of 400 μg/mL ($p < 0.05$; Fig. 3).

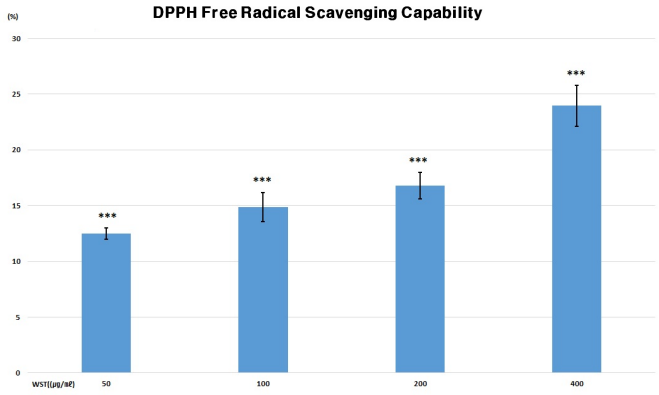

Fig. 2. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging in Sibseomsan extract treated RAW 264.7 cells.
Results were expressed as % of the control. ***$p < 0.001$.

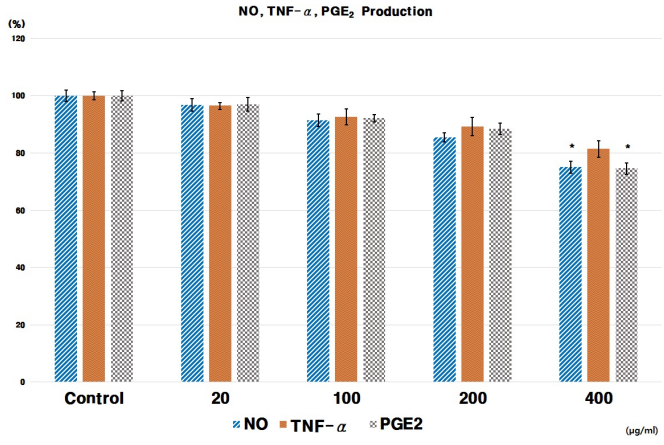

Fig. 3. The production of NO, TNF-α and PGE_2 tyrosinase in Sibseonsan extract treated RAW 264.7 cells.
Results were expressed as % of the control. *$p < 0.05$.
NO, Nitric Oxide; PGE_2, Prostaglandin E_2; TNF- α, Tumor necrosis factor-α.

Table 2. Effect of Sibseonsan Extract on Collagenase Production.

Samples	Absorbance (nm)	Reduction rate (%)
Substrate only	12,578482	
Substrate + enzyme	134,9371,174	26.37
SSS extract	99,3541.279	

Result were expressed as % of the control. *$p < 0.05$.

Table 3. Effect of Sibseonsan Extract on Elastase Production.

Samples	Absorbance	Reduction rate (%)
Control (Substrate + enzyme)	0.2740.04	45.71
SSS extract	0.1480.03	

Result were expressed as % of the control. *$p < 0.05$.

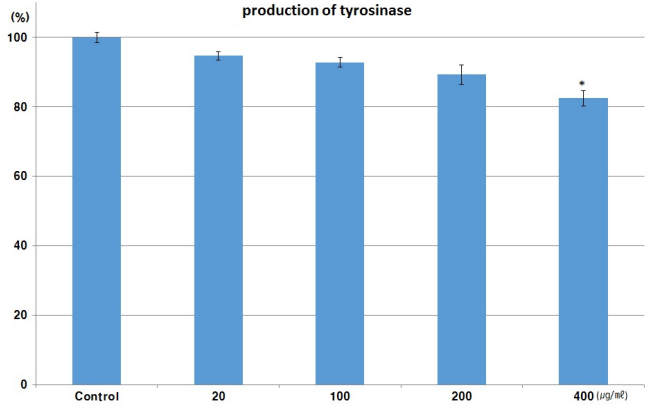

Fig. 4. The production of tyrosinase in Sibseonsan extract treated B16/F10 cells.
Results were expressed as % of the control. *$p < 0.05$.

TNF-α

The means of TNF-α concentration treated with LPS alone was 3.888 ng/mL. The TNF-α production was adjusted to 100 ± 1.48%, 96.49 ± 1.18%, 92.55 ± 2.79%, 89.26 ± 3.19%, 50, 100, 200, and 400 μg/mL, respectively and 81.45 ± 2.92% in the control group. TNF-α concentration was significantly decreased between 50 to 400 μg/mL in a concentration dependent manner (Fig. 3).

Prostaglandin E_2

The mean concentration of PGE_2 treated with LPS was 1.027 ng/mL. The PGE_2 production was 100 ± 1.85%, 96.95 ± 2.38%, 92.16 ± 1.27%, 88.46 ± 2.08%, and 74.59 ± 1.97% in the control group, 50, 100, 200, and 400 μg/mL, respectively. SSS extract showed a significant decrease in the amount of PGE_2 produced at 400 μg/mL compared with the control (Fig. 3).

Collagenase

Collagenase production was 26.37% (expressed as % of the control) using SSS at 400 μg/mL (Table 2).

Elastase

Elastase production was expressed as 45.71% compared with the control at 400 μg/mL concentrations (Table 3).

Tyrosinase

Tyrosinase production was expressed as 100 ± 1.51%, 94.62 ± 1.25%, 92.79 ± 1.46%, and 89.25 ± 2.77%, at 50, 100, 200, and 400 μg/mL concentrations, respectively, and 82.46% ± 2.18% for the control (Fig. 4).

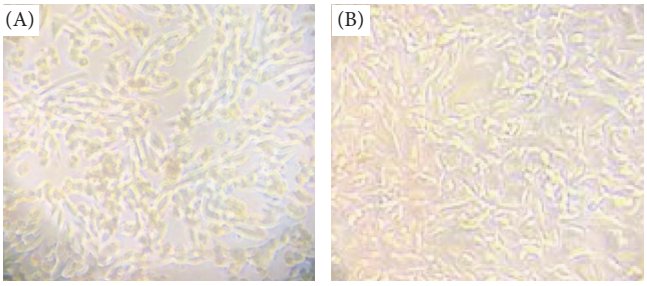

Fig. 5. The effect of Sibseonsan extract treatment on the morphology of the B16/F10 cells. Morphological change was observed up to a concentration of 400 μg/mL. (A) Non-treated group (control), (B) Galgeungyulpitang extract treated concentration of 400 μL.

DOPA staining

Projections on the cells decreased when treated with SSS at a concentration of 400 μg/mL compared with the control group, and DOPA staining decreased. It was observed that the intensity reduced (Fig. 5).

Discussion

The skin protects the individual from harmful biological, physical, and chemical factors that originate from outside the body [1].

Free radicals are unstable atoms that stabilize after reaction with other substances i.e. have a strong reactivity. These free radicals are by-products of various oxidation reactions during the metabolism of normal cells in the human body. They are produced by macrophages and play a productive role in controlling a response to infection. However, they attack tissues and are present in various diseases, such as inflammatory diseases, cancer, and liver disease. They are also known to be involved in the process of aging and are associated with age-related disease [3].

In Korean medicine, the causes of inflammation are external and internal factors. Internal factors include immunodeficiency, and external factors include bacteria and viruses [5]. In this study, SSS extract was investigated to determine whether it reduced free radicals. The DPPH method was used to test active oxygen removal. As a result, SSS extract showed 23.96 ± 1.85% active oxygen removal at 400 μg/mL (Fig. 2). Therefore, SSS may be used as an antioxidant for treating and suppressing inflammation in the future.

Inflammation is the defense mechanism of a body against invasion, which results in a change in the matrix of living tissue. Inflammation occurs with redness, fever, pain, and edema. In particular, the inflammatory response shows almost the same symptoms regardless of the cause and or the tissue. This change results in tissue damage. The damage produces a common substance in vivo. These chemical mediators include free radicals, nitric oxide (NO), prostaglandin (PG), and several cytokines that cause inflammation [2].

NO is produced by nitric oxide synthase (NOS) enzymes. Inflammatory processes in the body produce large amounts of NO, which plays an important role during various acute or chronic inflammatory diseases. There are 3 types of NOS: Type I, Type II, and Type III. Type I or III play a role in maintaining the homeostasis of individuals. Type II is inducible NOS (iNOS), which is induced by bacterial LPS, cytokines or calcium ionophores, produces excess NO, associated with various inflammatory diseases. The excess NO produced damages genes and proteins. It also reacts with superoxide anion (O2-) to produce peroxynitrite (ONOO-), which is highly toxic. It is transformed into a more potent toxin, associated with cancer. Therefore, it is important to reduce the incidence of NO for the inhibition and treatment of various inflammatory events [3].

This study investigated the effect of SSS extract on NO production. SSS treatment of RAW 264.7 cells at concentrations of 200 μg/mL and 400 μg/mL resulted in an adjusted NO production of 85.43 ± 1.57%, and 84.97 ± 2.11%, respectively. Therefore, SSS was determined to have a concentration-dependent inhibitory effect on NO production in macrophages (Fig. 3).

Inflammatory Stage 1 increases the permeability of blood vessels and in Stage 2, blood cells are mainly active. In Stage 3, tissues are regenerated. Several chemicals are involved in this process. The harmful irritation of inflammation acts locally causing direct damage. Most chemicals, however, are indirectly delivered to local blood vessels or cells. The major chemical transporters of the inflammatory response belong to amines (histamine, serotonin) and kinin (bradykinin), which are involved in immediate vascular permeability, and cytokine, prostaglandin, and leukotriene, which are mainly responsible for the delayed response. Among the various cytokines involved in immunity and inflammation, TNF-α is a major inflammatory cytokine produced by macrophages [18]. TNF-α is a glycoprotein with a molecular weight of less than 30κD, it binds to specific TNF-α-receptors and can regulate inflammatory and immune responses strongly.

Changes in the concentration of TNF-α were used to evaluate the effect of SSS extract on inflammatory mediators. The resulting SSS extract inhibited the expression of TNF-α in a concentration-dependent manner (Fig. 3). In addition, TNF-α showed inhibitory effects of SSS at 200 μg/mL and 400 μg/mL, 89.26 ± 3.19%, and 81.45 ± 2.92%, respectively.

Prostaglandin is derived from arachidonic acid. It is an intercellular and intracellular messenger that is involved in inflammation during the immune response, smooth muscle tone, vascular permeability, and cellular proliferation. Prostaglandin is made from arachidonic acid, the lipid component of cell membranes produced by phospholipase A2. That is, arachidonic acid synthesizes PG under the action of the cyclooxygenase (COX) enzyme. PGE_2 and PGI_2 increase vascular permeability. Cyclooxygenase has 2 isoforms, Type I and Type II. Cyclooxygenase-1, a Type I enzyme, is a housekeeping enzyme that maintains anti-tumor properties. Cyclooxygenase-2, on the other hand, plays an important role in various inflammatory diseases [19].

SSS extract was used to evaluate the inhibitory activity of Prostaglandin E_2 activity of macrophages after treatment. SSS extract inhibited Prostaglandin E_2 activity by 84.59 ± 1.97% at a concentration of 400 μg/mL (Fig. 3).

Wrinkles are caused by a decrease in the amount of collagen and elastic fibers that support the skin in its dermal layer and is a phenomenon of aging. Ultraviolet rays damage matrix proteins such as collagen and elastin in the skin dermis. It is claimed that ultraviolet rays reduce the amount of collagen in the skin and cause elastin degeneration. Ultraviolet rays reduce the synthesis of elastin and increase the expression of collagen, degrading enzymes, leading to wrinkles. Thus, the study of wrinkles relates to substances that inhibit the action of collagenase and elastase [15].

In this study, SSS extract was tested to determine whether it is effective in preventing skin wrinkles and maintaining elasticity by studying the action of collagenase and elastase. As a result, SSS extract was evaluated to have 26.37% enzyme inhibitory activity at a concentration of 400 μg/mL for collagenase (Table 2). For

elastase, SSS extract was evaluated to have an enzymatic activity inhibition of 45.71% at a concentration of 400 μg/mL (Table 3). Therefore, since SSS extract inhibits the degradation of collagen and elastin, it may be effective in preventing skin regeneration and aging.

Human skin color is determined by pigments such as melanin, carotene, and hemoglobin, present in the skin. The most influential pigment is melanin, which is produced by the enzymatic and non-enzymatic oxidation of tyrosine in melanocytes in the basal layer of the skin epidermis. It spreads to the keratinocytes that compose the epidermis, resulting in skin color. Tyrosinase plays the most important role in this process. In this study, the inhibitory effect of SSS extract on tyrosinase activity was investigated and showed that 82.46 ± 2.18% of tyrosinase activity was inhibited by SSS at a concentration of 400 μg/mL.

Melanocytes are not stained by the normal staining method, but when they are treated with DOPA, they are oxidized by the tyrosinase enzyme of melanocytes to form dark-brown deposits. Melanocytes were treated with SSS at each concentration and then incubated for 3 days. DOPA staining after incubation of the cells was carried out to observe the morphological activity of intracellular tyrosinase.

SSS not only has an anti-inflammatory effect and antioxidant activity in murine cells, but may also play an important role in anti-aging, in addition to producing a whitening effect. In the future, SSS may be used as an additive for cosmetics or foods. Future experiments may evaluate the safe and effective dose and concentration of SSS for therapeutic benefits.

Conclusion

As a result of evaluating the effect of SSS extract, treatment of murine cells in vitro, anti-inflammation, antioxidant, anti-skin wrinkle, and whitening effects were observed.

1. Cell viability was not significantly decreased at 400 μg/mL of SSS extract.
2. Radical scavenging activity of 23.96 ± 1.85% was observed at 400 μg/mL of SSS extract.
3. NO production, TNF-α secretion and PGE_2 production were 74.97 ± 2.11%, 81.45 ± 2.92% and 74.59 ± 1.97% each at 400 μg/mL of SSS extract.
4. Collagenase, elastase. and tyrosinase production amounts were 26.37%, 45.71% and 82.46 ± 2.18%, respectively, at 400 μg/mL of SSS extract.
5. SSS reduced the projections of melanocytes and inhibited melanogenesis.

SSS extract had anti-inflammatory and antioxidant effects and inhibited collagenase and elastase activity. Therefore, SSS extract may be useful for the treatment and prevention of inflammatory diseases. It is also thought to have an anti-wrinkle and whitening effect.

Conflicts of Interest

The author has no conflicts of interest to declare.

Acknowledgments

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