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Antimicrobial, Antioxidative, Elastase and Tyrosinase Inhibitory Effect of Supercritical and Hydrothermal *Asparagopsis Armata* Extract

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Abstract

In this paper, we present to evaluate physiological activity of Asparagopsis armata extraction. After extraction with Asparagopsis armata using hydrothermal and supercritical carbon dioxide, various physiological activities were examined. The total concentration of polyphenol compounds was determined to be 18.85 mg/g of hydrothermal Asparagopsis armata extraction and 14.74 mg/g of supercritical Asparagopsis armata extraction. In DPPH radical scavenging assay, ascorbic acid was used as positive antioxidant control. In ABTS radical scavenging assay, ascorbic acid was used as positive antioxidant control. The percentage of inhibition and IC50 were measured. The IC50 of Asparagopsis armata extraction is 261.44ppm and the IC50 of supercritical Asparagopsis armata extraction is 153.98 ppm. The elastase inhibitory assay showed concentration dependence and the IC50 of hydrothermal Asparagopsis armata extraction is 3387 ppm and the IC50 of supercritical Asparagopsis armata extraction is higher than 2500 ppm. In mushroom tyrosinase inhibition experiments, tyrosinase inhibition's IC50 of supercritical Asparagopsis armata extraction was 248.06. In the SOD-like experiments, the concentration-dependent results were showed and IC50 of hydrothermal Asparagopsis armata extraction is 845.29 ppm. In the antimicrobial experiments, maximum clear zones of supercritical Asparagopsis armata extraction represented 23.00 mm in Propionibacterium acnes. In the other hand, in experiments with the same conditions, hydrothermal Asparagopsis armata extraction had no effect in all strains.

Keywords: *Asparagopsis Armata, Seaweed, Supercritical, Antimicrobial, , Cosmetics*

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1. Introduction

Recently, as interest in Lifestyle of Health and Sustainability (LOHAS) has increased, the number of wellness people based on naturalism has increased, and various industrial fields have been studying and developing various natural materials[1-3]. According to the report of the Agricultural Technology Commercialization Foundation, the global natural cosmetics market is expected to increase by 7.2% compared to the previous year to about 11.73 trillion won in 2012, and to about 14.3 trillion won in 2015. As of 2012, the domestic natural cosmetic market size was about 2,337 trillion won, and the average annual growth rate was 9.0%, which is expected to reach 3,271 billion won in 2015. Natural cosmetics, which are showing such steady growth trend, have a high share in areas related to wrinkle improvement, whitening, and acne worldwide[4].

Hydrothermal extraction and solvent extraction are mainly used to obtain high purity functional extracts required in the cosmetics and food industry, and solvent extraction methods are used often to obtain various active ingredients. However, there are problems with solvents such as environmental pollution caused by large amounts of waste solvents and residual solvents in extracts. Interest in supercritical fluid extraction process using supercritical carbon dioxide is increasing as an environment-friendly alternative technology for compensate and improve the shortcomings of existing natural effective extraction process. Extracting supercritical fluids uses carbon dioxide that is harmless to the human body. In addition, when extracting active ingredients with a range of molecular weights from natural materials, there are several application ranges depending on the extraction method. For supercritical carbon dioxide, it is possible to extract the active ingredient for almost any range of molecular weight. Currently, studies are actively conducted on various natural extracts using supercritical carbon dioxide

Seaweed has been widely used for food in Asia since ancient times, has low calories, is rich in vitamins, minerals, and dietary fiber, and contains non-digestible viscous polysaccharides that are not found in land plants. Among seaweeds, seaweed, seaweed, and kelp are the most common. Seaweeds contain about 32 to 75% of dietary fiber, and 51 to 85% of them are known to be water-soluble dietary fiber [5]. Most of the dietary fiber of seaweed is a substance that is not digested and absorbed, but it is reported that laminine, a substance contained in brown algae, has an effect of lowering blood pressure [6]. In addition, research has been reported that green algae and red algae contain betaine, which reduces blood cholesterol [7]. In this context, *Asparagopsis armata* , a red macroalgae, is already known to produce bioactive compounds with antitumor and antimicrobial potential [8-10].

Therefore, this study intends to evaluate the antibacterial and antioxidant properties of *Asparagopsis armata* as a cosmetic material and to provide basic data for the development of cosmetic materials based on this.

2. Materials and Experiments

2.1 Instruments and Reagents

The equipment and reagents used for each experiment are as follows. The solutions used for the polyphenol, DPPH test used in the antioxidant and antibacterial tests were obtained from Sigma Aldrich (USA). The equipment used in the experiment is as follows. Supercritical fluid extraction (ARI instrument, Namyangju, Korea), Absorption spectrophotometer (SYNERGY HTX multi-mode reader, Bio Tek, Seoul, Korea), Centrifugal separator (Supra-25K, Hanil Scientific Inc., Gimpo, Korea). Thermostat (Changshin Science, Seoul, Korea), High pressure processor (Microfluidizer, Picomax, Seoul, Korea), Particle size analyzer (Nanoctrac Flex, DREAM Co., Suwon, Korea), Particlemetrix (Stabino® Paticle Charge Mapping, DREAM Co., Suwon, Korea), Franz Diffusion Cells and Systems (PermeGear, USA).

2.2 Sample Extraction

In the hydrothermal extraction method, purified water was added to the *Asparagopsis armata* powder and extracted for 4 hours in a thermostat at 80℃ and filtered and freeze dried. In the supercritical extraction method, the pressure of the extractor was set to 350 bar and the temperature to 60℃. The pressure of the separator was set to 40 bar, the temperature was set to 25℃, and the flow rate of CO_2 was extracted at 60 mL/min for 150 minutes.

2.3 Total Polyphenol Content Measurement

Quantification of polyphenol was measured by Folin-danis' method [11]. To 100 μL of the Folin-Ciocalteu reagent, add 100 μL of the diluted sample solution and reacted at room temperature for 3 minutes. 100 μL of Na_2CO_3 solution was added and the absorbance was measured at 760 nm with an ELISA reader. The average value of polyphenol contents by concentration was calculated. The calibration curves were quantitatively analyzed using garlic acid as a standard.

2.4 Antioxidant Activity Measurement

1) Measurement of DPPH Radical Scavenging Ability

The effect about DPPH radical scavenging was measured by Blois method [12]. To 100 μL of the extract solution, 120 μL of 0.45 mM 2,2-diphenyl-1-picrylhydrazyl solution was added and reacted in the dark room for 30 minutes. Absorbance was measured at 530 nm with an ELISA reader.

DPPH radical scavenging activity (%) = [(Absorbance of DPPH solution - absorbance of samples) / absorbance of DPPH solution] × 100

2) Measurement of ABTS Radical Scavenging Ability

The effect about ABTS radical scavenging was measured by Van den Berg method [13]. The ABTS radical cation (ABTS+) was produced by reacting 7.2 mM stock solution ABTS with 2.64 mM potassium persulphate (final concentration) and allowed the mixture to stand in the dark for at least 24 hours at room temperature before use. The ABTS+ solution was diluted to an absorbance 0.7 ± 0.05 at 745nm. Absorbance was measured 20 minutes after the initial mixing of different concentrations of the extracts. The ABTS+ decolorisation capacity of the extracts were compared with the standard ascorbic acid.

ABTS radical scavenging activity (%) = [(Absorbance of ABTS solution - absorbance of samples) / absorbance of ABTS solution] × 100

2.5 Measurement of Superoxide Dismutase (SOD)

SOD-like activity was performed by modifying Marklund's method [14]. The experiment was carried out using SOD Assay Kit (BCBV5418). 20 μL of buffer solution and 20 μL of enzyme working solution were added to 20 μL of each sample solution, and incubation was carried out at 37℃ for 20 minutes. The absorbance at 420 nm was measured by an ELISA reader.

SOD similar activity (%) = [1 - (Absorbance in the sample addition group / absorbance in the no additives)] × 100

2.6 Measurement of Elastase Inhibitory Activity

Elastase inhibitory activity was measured by Cannell's method [15]. The experiment was carried out using EnzCheck® elastase Assay Kit (E-12056). 1x Reaction buffer was used to dilute the *Asparagopsis armata* extract sample and incubated in a 96-well black plate using 100 mg/L DQ elastin solution and 0.2 U/mL elastase for 30 minutes at room temperature. Absorbance was measured at 485 nm excitation and 528 nm emission fluorescence with an ELISA reader.

Inhibition rate of elastase (%) = [1-(Absorbance in the sample addition group / absorbance in the no additives)] x 100

2.7 Measurement of Tyrosinase Inhibitory Activity

Tyrosinase activity was modified by the method of Kubo [16]. 0.1 M sodium phosphate buffer (pH 6.5) and 50 µl with purified water to 40 µl, 2000U mushroom tyrosinase (Sigma, T3824) 5 µl pre-manufacturing a total of 95 µl to minutes after the extract 5 µl was placed. 0.03% L-tyrosine 50 µl was added and incubated at 37℃ for 10 minutes. Kojic acid was used as positive control . Absorbance was measured at 475 nm with an ELISA reader.

Inhibition rate of tyrosinase (%) = $[100 - (\frac{b-b'}{a-a'}) \times 100]$

a: Absorbance after reaction of blank

b: Absorbance after sample liquid reaction

a′,b′: Absorbance measured by replacing with buffer solution

2.8 Antimicrobial Experiment

The disc diffusion test was performed to determine the antimicrobial activity of *Asparagopsis armata* [17]. *Staphylococcus aureus, Escherichia coli, Bacillus subtilis*, and *Propionibacterium acnes* were purchased from KCM and KCTC. The strains *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* were cultured in Muller-Hinton medium at 37℃ for 24 hours, re-cultured once, and then absorbed at 600 nm using a spectrophotometer. *Propionibacterium acnes* was incubated in a sealed container for 72 hours, re-incubated once, and then absorbed at 600 nm using a spectrophotometer. The culture conditions are shown in Table 1.

Table 1. List of strains and cultivation condition used for antimicrobial experiments

Strains	Media	Temperature (℃)	Time (h)
Staphylococcus aureus (ATCC6538)	MH	37	24
Escherichia coli (ATCC23726)	MH	37	24
Bacillus subtilis (ATCC19659)	MH	37	24
Propionibacterium acnes (ATCC6919)	RC	37	48

Media was used by MH (Muller-Hinton medium) and RC (Reinforced Clostridial medium)

2.9 Statistical Processing

All experiments were repeated 3 times. All values were expressed as mean and standard deviation and the difference between the values was analyzed by t-test, one-way analysis of variance (ANOVA) with Post hoc(LDS) respectively.

3. Results

3.1 Yield

Asparagopsis armata was extracted with hydrothermal and supercritical. Each yield was 24 % in HAE (Hydrothermal *Asparagopsis armata* extraction) and 0.24 % in SAE (Supercritical fluid *Asparagopsis armata* extraction).

3.2 Total Polyphenol Content

To measure the total polyphenol content, the results of the comparison of the extraction process of *Asparagopsis armata* extract are shown in Table 6. In 1.5 mg/ml, 22.22±1.07 mg/g of polyphenol was extracted from hydrothermal extraction and 18.08±1.08 mg/g of polyphenol was detected in supercritical extraction (Table 4).

Table 4. Total polyphenols of extracts from *Asparagopsis armata*

Samples	Method	Total polyphenols (mg/g)
HAE[a]	Hydrothermal extract	18.85 ± 2.56
SAE[b]	Supercritical fluid extract	14.74 ± 4.31

Values represent the mean ± SD of three independent experiments.
[a] HAE : Hydrothermal *Asparagopsis armata* extraction
[b] SAE : Supercritical fluid *Asparagopsis armata* extraction

3.3 Antioxidant Efficacy of *Asparagopsis Armata*

1) DPPH Radical Scavenging Ability

DPPH radical is a method of measuring the activity of a hydrogen donor. When they get electron from phenolic compounds or aromatic amines, the color is turned purple to yellow by proton-radical scavengers [18]. Figure 1 represented the antioxidant activity of the extracts was shown between 0.31 ~ 5 mg/ml. In 5 mg/ml, the radical scavenging activity was 41 % in hydrothermal extraction and 31% in supercritical extraction.

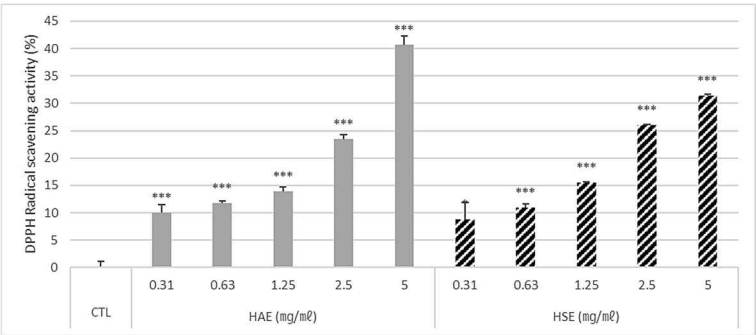

Figure 1. Scavenging effect of *Asparagopsis armata* on DPPH assays

Values represent the mean ± SD of three independent experiments. Positive control : Ascorbic acid 50 μg/ml to 96%. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
[a] HAE : Hydrothermal *Asparagopsis armata* extraction
[b] SAE : Supercritical fluid *Asparagopsis armata* extraction

2) Measurement of ABTS Radical Scavenging Ability

ABTS radical cation is a deep blue-green radical that reacts with antioxidants and is characterized by discoloration by light green. The antioxidant effect of ABTS free radicals produced by the reaction of potassium persulfate is measured by the degree of discoloration of the radical-specific cyan color to light green [19]. As a result, it was found that the concentration dependent of the extracts at 62.5 ~ 500 μg/ml. The highest radical scavenging activity is shown the SAE at 500 μg/ml shown in Figure 2.

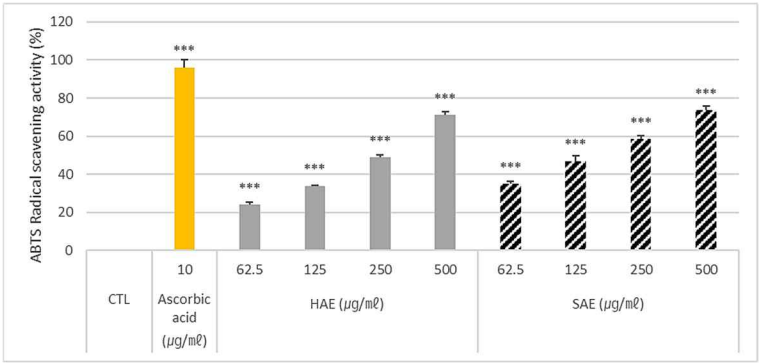

Figure 2. Scavenging effect of *Asparagopsis armata* on ABTS assays

Values represent the mean ± SD of three independent experiments. *p＜0.05, **p＜0.01, ***p＜0.001.

[a] HAE : Hydrothermal *Asparagopsis armata* extraction

[b] SAE : Supercritical fluid *Asparagopsis armata* extraction

3.4 Superoxide Dismutase (SOD)

SOD-like activity assay is an antioxidant activity assay using color development by automatic oxidation [20]. The substances that inhibit superoxide in the samples used in the experiment can inhibit the oxidation by oxidation in the presence of SOD or SOD-like active substances. In the SOD-like experiments, the concentration-dependent results were represented and IC50 of HAE is 845.29 ppm and IC50 of SAE is 1800.86 ppm as shown in Figure.

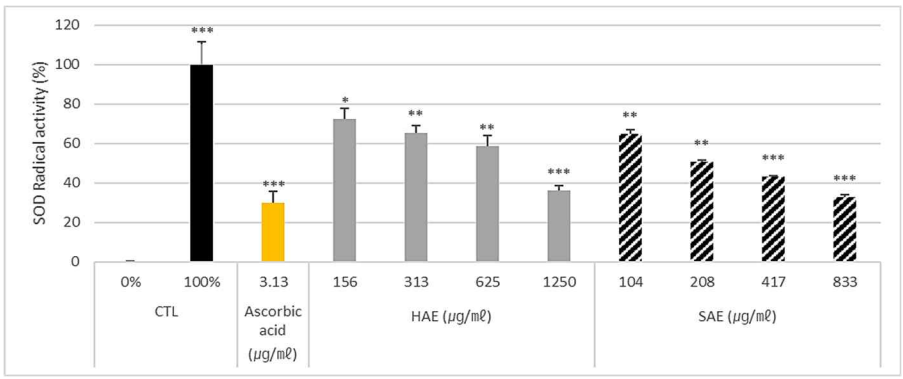

Figure 3. Scavenging effect of *Asparagopsis armata* on SOD assays

Values represent the mean ± SD of three independent experiments. *p＜0.05, **p＜0.01, ***p＜0.001.

[a] HAE : Hydrothermal *Asparagopsis armata* extraction

[b] SAE : Supercritical fluid *Asparagopsis armata* extraction

3.5 Elastase Inhibitory Activity

The elastase which is presented in the dermis of the skin is an enzyme capable of degrading various proteins including fibronectin, collagen and elastin which maintains elasticity of the skin in the dermis [21]. The elastase inhibitory effect which is effective for improving the wrinkles of the skin was measured according to the concentration dependent as shown in Figure 4.

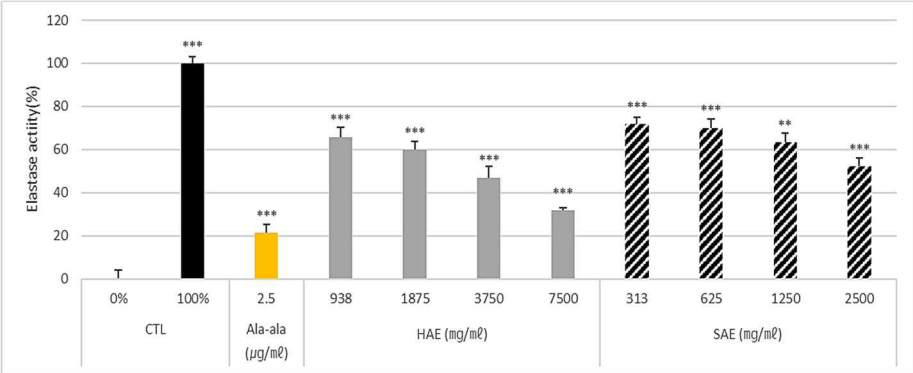

Figure 4. Scavenging effect of *Asparagopsis armata* on elastase assays

Values represent the mean ± SD of three independent experiments. *p＜0.05, **p＜0.01, ***p＜0.001.

[a] HAE : Hydrothermal *Asparagopsis armata* extraction

[b] SAE : Supercritical fluid *Asparagopsis armata* extraction

3.6 Tyrosinase Inhibitory Activity

Melanin is a pigment produced by melanomas, one of the cell organelles. Melanin is produced by the action of various enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP1) and tyrosinase-related protein 2 (TRP2) in melanomas [22]. Among them, tyrosinase is a major regulatory enzyme that plays a role in the oxidation of tyrosine to DOPA quinone after being hydrolyzed with DOPA and is related to melanin. The lowest tyrosinase activity of 39% was observed at the concentration at 333 mg/ml of SAE and HAE had no effect as shown in Figure 5.

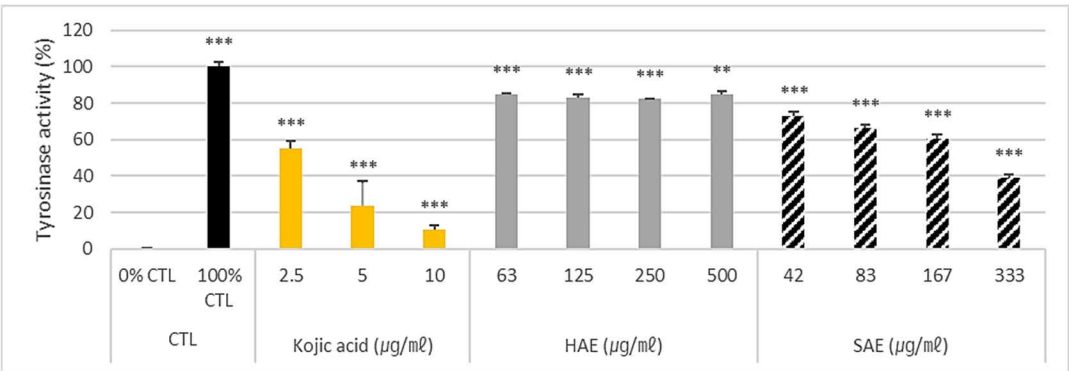

Figure 5. Scavenging effect of *Asparagopsis armata* on tyrosinase assays

Values represent the mean ± SD of three independent experiments. *p＜0.05, **p＜0.01, ***p＜0.001.

[a] HAE : Hydrothermal *Asparagopsis armata* extraction

[b] SAE : Supercritical fluid *Asparagopsis armata* extraction

3.7 Antimicrobial Experiment

The antimicrobial test was conducted three times using the paper disc method. The results of the clear zone measurement are shown in Table 5. As a result of the antimicrobial test, the supercritical fluid *Asparagopsis armata* extraction had antimicrobial effect in the four strains. In the case of *Propionibacterium acnes* strain, the largest clear zone of 23.00±1.00 mm was found at the concentration of 20 mg/ml. In the other hand, in experiments with the same conditions, hydrothermal *Asparagopsis armata* extraction had no effect in *all* strains.

Table 6. The effect of Supercritical fluid *Asparagopsis armata* extraction amount on area of clear zone

Strain	Clear zone (mm)			
	10 mg/mL	5 mg/mL	2.5 mg/mL	1.25 mg/mL
Staphylococcus aureus	11.07 ± 1.85 [a]	-	-	-
Escherichia coli	10.97 ± 0.15 [a]	-	-	-
Bacillus subtilis	12.80 ± 0.20 [a]	11.07 ± 0.06 [a]	-	-
Propionibacterium acnes	23.00 ± 1.00 [a]	17.33 ± 0.58 [a]	-	-

Values represent the mean ± SD of three independent experiments. Positive control: *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* are used methyl paraben, *Propionibacterium acnes* is used salicylic acid.
[a] Growth inhibition line

4. Conclusion

The purpose of this study was to investigate the efficacy of *Asparagopsis armata* as a cosmetic material and to provide basic data for the development of cosmetic materials based on this. The effect of antioxidant, antimicrobial, wrinkle and whitening on *Asparagopsis armata* was investigated through this study. The antioxidant activity was slightly different in each experiment according to the extraction method. However, in the elastase assay and tyrosinase inhibition assay, supercritical fluid *Asparagopsis armata* extraction showed a superior effect than the hydrothermal *Asparagopsis armata* extraction. And, in the antimicrobial experiments, maximum clear zones of SSE represented 23.00 mm in *Propionibacterium acnes*, 12.90 mm in *Bacillus subtilis*, 11.00 mm in *Escherichia coli*, 9.20 mm in *Staphylococcus aureus*. And maximum clear zones of SSE represented 23.00 mm in *Propionibacterium acnes*, 12.80 mm in *Bacillus subtilis*, 10.97 mm in *Escherichia coli*, 11.07 mm in *Staphylococcus aureus*. And had no effect about the rest of the strains.

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