Development of cosmetics ingredient using Chamaecyparis obtuse leaves originated from Jeju island extract by subcritical water extraction

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Abstract

The purpose of this study is to develop harmless natural ingredient originated from Jeju Island for cosmetic products. This natural ingredient from the leaves of *Chamaecyparis obtusa* was extracted using subcritical water extraction (SWE).

Ground *Chamaecyparis obtusa* leaves (1.2g) were subjected to subcritical water extraction. The extraction efficiencies of different temperatures (120, 135, 150 ℃), and pressures (60, 80 bar) were investigated. The identification of components was carried out using HPLC analysis. And efficacy evaluation of each extract was measured by DPPH scavenging ability for antioxidant, NO inhibition for anti-inflammatory, and paper disc diffusion method for antibacterial.

Subcritical water extraction from *Chamaecyparis obtusa* leaves exhibited the highest extraction efficiency at temperature 150 ℃, pressure 80 bar, flow rate 1 mL/min, extract 15min. At these optimum extraction parameters, the maximum yield 29.1%. As a result of confirming the antioxidant effect under optimal conditions, it exhibited a DPPH radical scavenging effect of about 80% when treated with 400 μg/mL, and an inhibitory effect of about 52% when treated with a concentration of 20 μg/mL. In addition, as a result of confirming the size of the transparent area for antibacterial activity through the paper disc method, it was confirmed that the effect was shown on the skin-related strains *Staphylococcus epidermidis* and *Cutibacterium acnes*. And based on HPLC results of SWE extract was found quercitrin 5.65 mg/g, catechin 8.89 mg/g.

Better results have been obtained with proposed method-Subcritical water extraction in terms of its higher yields and rapidity of extract, efficiency and cleanliness.

Keywords: Subcritical water extraction; *Chamaecyparis obtusa;* anti-oxidant, anti-inflammatory, anti-bacterial, Jeju Island

Introduction.

Chamaecyparis obtusa (Siebold & Zucc.ENDL) is also known as the cypress or hibiscus. It is an evergreen coniferous tree of the family Cypressidae, which is native to Japan and grows mainly in Jeju Island and southern regions in Korea. It grows about 40 meters tall and 2 meters wide. *Chamaecyparis obtusa*, which was mainly planted and distributed in the southern region and Jeju Island in South Korea due to the forest greening policy in the 1970s, is a *Chamaecyparis obtusa* family, and it has become a relatively easy-to-access arboretum due to the creation of recreational forests.[1] However, as the planting area of *Chamaecyparis obtusa* trees increases, many by-products such as leaves, stems, and fruits from the process of forest clearing and logging are produced, and these by-products are not properly utilized and are discarded. [2] Activities for *Chamaecyparis obtusa* include antimicrobial effects on acne bacteria in *Chamaecyparis obtusa* extracts[3], brain nerve cell protection active ingredients in the extracts[4], antioxidants on atopic dermatitis, anti-inflammatory and immunomodulatory effects[5], and *Chamaecyparis obtusa* extracts on skin changes in atopy infants[6].

This study evaluated the availability of *Chamaecyparis obtusa* leaves extract as a functional cosmetic raw material through antioxidant efficacy evaluation and anti-inflammatory and antibacterial efficacy evaluation with the extract through subcritical extraction, and identified the difference from the extracts through other extraction methods.

A method of extracting functional components using subcritical water is known, but its utilization is not that large. Subcritical water extraction is a method of breaking down and extracting substances using ionized water under temperature and pressure conditions below the critical point of water (374℃, 22.1 Mpa). The advantages of SWE are that the solvent used is pure water, the extraction time required for extraction is about only 5 to 30 minutes, and it is efficient to extract a non-polar material such as a phenol compound. [7-10]

The purpose of this study is to develop cosmetics ingredient using subcritical water extraction (SWE) with *Chamaecyparis obtusa* leaves originated from Jeju-island and comparison of other methods of extraction. This SWE use only water and could extract active material without chemical solvent which is harmful to human’s skin. In this study the antioxidant, anti-

inflammatory and antibacterial effects of *Chamaecyparis obtusa* leaves extract of Jeju island native plant using SWE was confirmed.

Materials and Methods

Materials

The *Chamaecyparis obtusa* leaves were collected from Jeju in March 2020 in Aewoleup, Jeju Island (South Korea). The leaves were seperated from the branches and the air-dried leaves were stored at room temperature.

Sample preparation

To obtain the *Chamaecyparis obtusa* leaves extract, hydrothermal extraction was refluxed at 90℃ for 4 hours with distilled water of a certain ratio of the sample, low-temperature extraction was extracted at room temperature for 4 hours, and solvent extraction was performed with ethanol corresponding to a certain ratio of the sample.

Subcritical Water Extraction (SWE)

SWE was performed in a laboratory scale by SpeedExtractor E-916 (BUCHI, Switzerland). The cell was filled with ground *Chamaecyparis obtusa* leaves (1.2 g accurately weighed) and add sand (15.0 g) for extract. The pressure conditions were 60, 80 bar, the temperature was 120℃, 135℃, and 150℃ respectively and the extraction time (15min) was constant on each experiments. The extracts were obtained in six variables and used as samples.

HPLC analysis

In this study, HPLC (Waters 2695, USA) and PDA detector were used for HPLC analysis, HPLC column was used Kromasil 100-5-C-18, (5 μm, 4.6×250 mm, AkzoNovel), and mobile phase A was 0.1% formal acid in distilled water and mobile phase B was acetonitrile was used. As HPLC conditions for separating the surface components of the sample, the flow rate of the mobile phase was 1mL/min, the injection amount of the sample was 10 $\mu\ell$, the detection wavelength was 260 nm, and the column oven was 40℃,

and the analysis was conducted for 30 minutes. The ratio of the mobile phase was set to 95-70% A (0-30min) and 5-30% B (0-30min), and standard products of quercitrin (Sigma Aldrich, USA) and catechin (Sigma Aldrich, USA) were purchased to measure the content of the separated sample, dissolved in D.W, and diluted.

Determination of Antioxidant Activities by DPPH

An atomic group with hole electrons is called free radical, which is unstable and highly reactive because it has unpaired active electrons. This large reactivity of Free Radical damages not only skin but also body components and consequently accelerates aging. The DPPH(1,1-diphenyl-2-picrylhydrazyl) experiment method is a representative experiment confirming the erasure ability of the free radial, and has the advantage of being relatively simple.

Radical scavenging activity measurement using DPPH was performed by applying a method such as Blois[11]. In order to measure the electron donating ability of the sample to the DPPH free radical, the sample was reacted at room temperature by adding 180 μL of 0.2mM DPPH solution to 20 μL, and absorbance was measured at 515 nm using a microplate reader. The activity was used as a control when no sample was added and the sample was added as an experimental group, and the erase activity inhibition rate of DPPH was shown by the following equation.

Anti-inflammatory Measurement (Nitric Oxide Concentration)

RAW264.7 cells, a murine macrophage cell line, were sold from ATCC and incubated in a DMEM medium containing 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS at 37℃, and subcultured at 2-day intervals. In the cytotoxicity evaluation (MTT assay), cells were divided into 2.0×10^5 cells/well, cultured 18 hours before in a cell incubator under conditions of 37℃ and 5% CO_2, and then LPS and samples were treated by concentration and cultured. After incubation for 24 hours, the MTT solution was added at a concentration of 500 μg/mL to further react for 3 hours. DMSO was added to and dissolved in formazan precipitate formed by reacting with living cells, and absorbance was measured at 570 nm using a microplate reader. The NO production rate was measured by dividing RAW264.7 cells into 2×10^5 cells/well on a 24 well plate and

incubating 18 hours before under conditions of 37℃ and 5% CO_2. It was exchanged with a medium containing 1 μg/mL of LPS, and samples were treated respectively and cultured for 24 hours. Subsequently, the amount of NO produced was measured at 540 nm by mixing 100 μL of the cell culture supernatant (1% sulfanilamide, 0.1% naphthylethylene-diamine in 2.5% phosphorous acid) with 100 μL of Griess reagent, reacted at 96 well plate for 10 minutes. The amount of NO produced was measured in the form of NO_2- present in the cell culture and quantified through a standard test curve prepared using sodium nitrite ($NaNO_2$) as a standard material.

Anti-bacterial Measurement (Paper disc diffusion method)

Staphylococcus epidermidis (S. epidermidis, CCARM 3709) and Cutibacterium acnes (C. acnes, CCARM 0081) were used by the CCARM(Culture Collection of Antimicrobial Resistant Microbes). S. epidermidis was incubated every 24 hours at 37℃ using a culture medium as a TSB, and C. acnes was incubated every 48 hours using the culture medium as a GAM. In order to measure the antibacterial activity of the sample, the paper disc diffusion method was performed to confirm the growth inhibition for each strain. S. epidermidis (CCARM 3709) is placed in a TSB medium containing 0.8% agar by adjusting turbidity to 0.5 McFarland standard and poured onto a solid medium containing 1.5% agar. When the medium hardened, 8 mm paper disc containing the sample solution was raised, incubated at 37℃ for 24 hours, and the size of the formed growth inhibition ring was measured. C. acnes (CCARM0081) is poured onto a solid medium containing 1.5% agar in a GAM medium containing 0.8% agar by adjusting turbidity with 0.5 McFarland standard. When the medium hardened, the diameter of 8 mm paper disc containing the sample solution was raised, and the anaerobic culture was performed at 37℃ for 48 hours, and the size of the growth inhibition ring formed was measured. As a positive control, erythromycin was used.

Results

Extraction Yield

As for the extraction method, solvent extraction, hydrothermal extraction, low temperature extraction, and subcritical extraction were performed, and each extract was freeze-dried and used for the experiment. The extraction yield is shown in Table 1, and the yield of hydrothermal extraction was much higher than that of solvent extraction and low temperature extraction, which was similar to that of subcritical extraction. In the case of subcritical extraction, the yield also increased as the extraction temperature condition increased, and the highest yield result was shown at 150℃.

Method	**Condition**	**Yield (%, mg/g)**
Solvent extraction	70% ethanol; Room temperature; 4h	14.5
Hydrothermal extraction	Water; Boiling point(<100 ℃); 4h	31.5
low-temperature extraction	Water; Room temperature; 4h	11.9
SWE	120 ℃; 60 bar; 15min	20.1
	120 ℃; 80 bar; 15min	20.4
	135 ℃; 60 bar; 15min	25.0
	135 ℃; 80 bar; 15min	24.7
	150 ℃; 60 bar; 15min	28.2
	150 ℃; 80 bar; 15min	29.1

Table1. Comparison of different methods for the extraction of *Chamaecyparis obtusa* leaves extracts and Extraction yield from various extract process of SWE

HPLC analysis

Analysis of the index components of the *Chamaecyparis obtusa* leaves subcritical water extract using HPLC. Taxifolin, quercitrin, kaempferol, catechin, and epi-catechin, known to be contained in cypress leaves, were analyzed to confirm the indicator components of the cypress leaves subcritical water extract. As a result, quercitrin and catechin were analyzed in the subcritical water extract, and as a result of confirming the contents of the two compounds, the contents in the raw subcritical water were 27.89 mg/L and 52.44 mg/L, respectively.

Method	Yield (%, mg/g)	Content of Quercitrin (mg/g)	Content of Catechin (mg/g)
Solvent extraction	14.5%	11.90	12.83
Hydrothermal extraction	31.5%	7.33	10.12
low-temperature extraction	11.9%	5.86	13.26
SWE; 150 ℃; 80 bar	29.1%	5.56	8.89

Table2. Analysis of surface components of *Chamaecyparis obtusa* leaves subcritical water extracts with various extract methods and process.

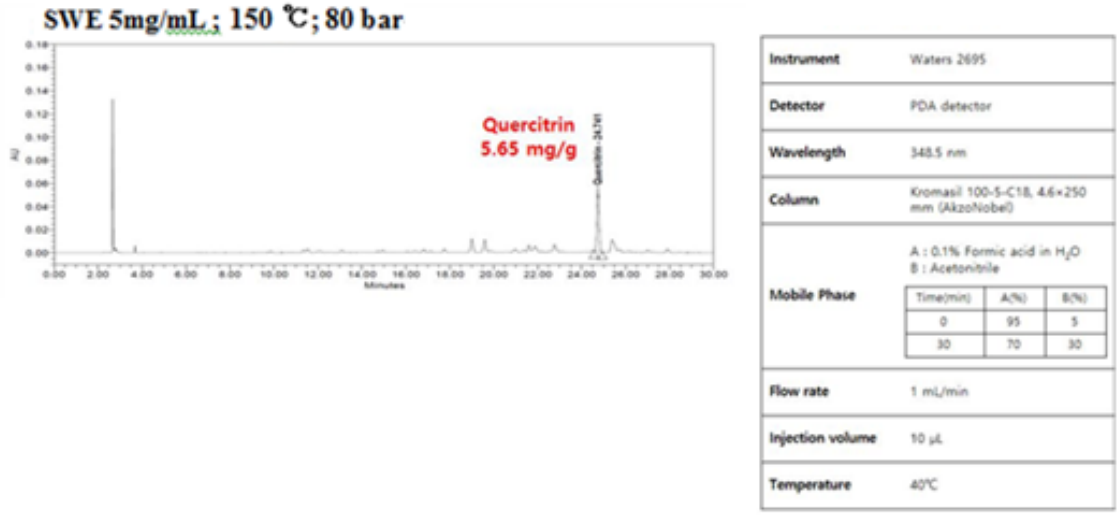

Figure1. Analysis of surface components of Quercitrin from *Chamaecyparis obtusa* leaves subcritical water extracts

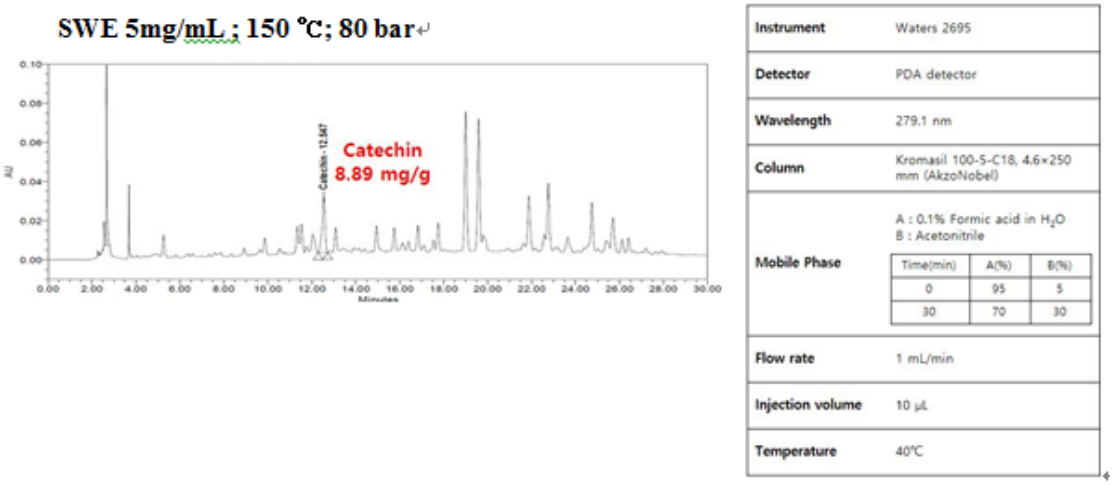

Figure2. Analysis of surface components of Catechin from *Chamaecyparis obtusa* leaves subcritical water extracts

Antioxidant activity

As a result of the DPPH radical erasure activity experiment of *Chamaecyparis obtusa* leaves subcritical water extract, it was confirmed that subcritical water extract had radical erasure activity similar to that of hot water and low temperature extract.

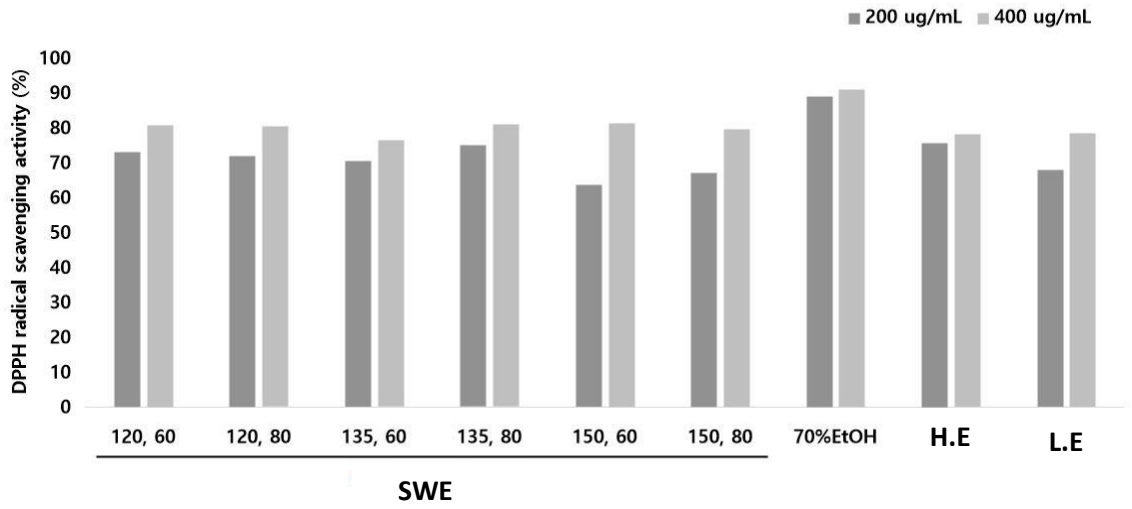

Figure3. DPPH radical scavenging test from *Chamaecyparis obtusa* leaves extracts with various extract methods and process.

Anti-inflammation

As a result of the NO production inhibition activity and cytotoxicity experiment of *Chamaecyparis obtusa* leaves subcritical water extract, 120℃, 60 bar, and 150℃, 80 bar extracts among subcritical water extract have excellent effects of inhibiting NO production without cytotoxicity at a concentration of 20 μg/mL.

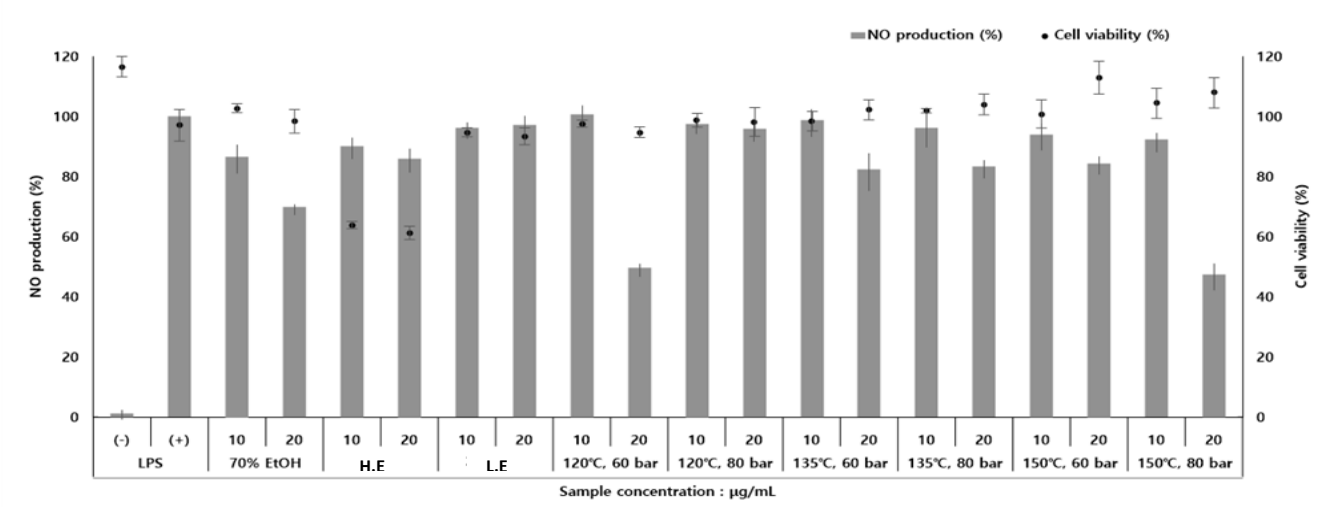

Figure4. NO production and cell viability in LPS-stimulated RAW 264.7 cells from *Chamaecyparis obtusa* leaves extracts with various extract methods and process.

Anti-bacterial

As a result of the antibacterial activity test of the *Chamaecyparis obtusa* leaves subcritical water extract, all subcritical water extract showed efficacy against *S.epidermidis.* However, it has been confirmed that *C.acnes* are effective only in extracts at 150℃.

Method and condition		Clear zone (mm)	
		S.epidermidis CCARM 3709	**C.acnes CCARM 0081**
70% EtOH Solvent extraction		10.0	11.0
Hydrothermal extraction		8.5	-
low-temperature extraction		-	-
SWE	120 ℃; 60 bar	8.5	-
	120 ℃; 80 bar	8.5	-
	135 ℃; 60 bar	8.5	-
	135 ℃; 80 bar	8.5	-
	150 ℃; 60 bar	9.0	8.5
	150 ℃; 80 bar	9.0	8.5

Figure5. Paper disc diffusion method from *Chamaecyparis obtusa* leaves extracts with various extract methods and process.

Discussion and Conclusion

In conclusion, this study demonstrated that the *Chamaecyparis obtusa* leaves SWE extract inhibitory effects of inflammatory mediator, antioxidant effects, and antibacterial effects. Moreover, the extraction process using subcritical extraction equipment is relatively shorter than the extraction time using general mass production equipment. It is considered to be an economical and efficient method for extraction. Based on the results of this study, it was possible to confirm the potential of *Chamaecyparis obtuse* leaves extract as a natural cosmetic ingredient by subcritical water extraction.

Acknowledgments

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