

A Quantitative Analysis of the Active Ingredients of Broad-leaved Bamboo and Study on Its Skin Condition Improvement Effect

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ABSTRACT: Je-ju-jo-rit-dae is a medicinal plant widely used in oriental medicine. However, its high reproductive rate has become a problem for the environmental ecosystem in Jeju. The extracts of the je-ju-jo-rit-dae are known for their antioxidant and anti-cancer effects. Still, no research has been conducted on significant impact and efficacy as an external skin agent, which has yet to be used commercially. This study performed a highly anti-oxidizing analysis by extracting the handle with methanol as a non-skin material at Je-ju-jo-rit-dae and identified its anti-inflammatory function and collagen synthesis ability. The HPLC analysis of these functional divisions shows Asiatic acid and Asiaticoside among the high levels of flavonoids and triterpenoid. The efficiency of Je-ju-jo-rit-dae found in the study is likely a good material for high-functioning skin external agents.

KEYWORDS: Nutrition and Natural Products; Immunology; Anti-inflammation; Collagen-neogenesis; *Sasa-Borealis*.

■ Introduction

Sasa borealis, a bamboo genus plant belonging to the Gramineae, is also called *Phyllostachys nigra* var. hennis Stapf, *Phyllostachys bambusoides* S. et Z, or *Sasa borealis* in oriental medicine. Among these, *Sasa borealis* is classified into the species of *Sasa borealis* Hackel Makino, *Sasa kurilensis* Makino et Shibata, and *Sasa quelpaertensis* Nakai. The leaves used as medicine are narrow needle-shaped, 7-15 cm long, 1-2 cm wide, sharp at one end, and a leafstalk attached to the other. Overall, it is green, and the back is light green and observed from the base.¹ *Sasa borealis* in the plant ecosystem on Jeju Island is not welcomed. In the plains with low altitudes, it is not a significant threat because the growth rate of the *Sasa borealis* can be relatively controlled. On the other hand, at the high altitude of Hallasan Mountain, where there are many natural protected species and rare plants, the strong vitality of *Sasa borealis* gradually dominates the growing area of the native species growing in the region is a problem because their numbers are increasing. However, the range of use of the naturally existing *Sasa borealis* has not been expanded to a particular industrial area, and thus the number of populations has not been controlled.^{2,3}

Human skin is a multi-layered tissue composed of stratum corneum, epidermal, dermal, and basal layers, and the fat and muscle layers below it. It is a vital organ that protects the human body from external stimuli and mediates sensations such as touch and pain.⁴ Skin naturally loses its function and structure due to aging as well as stress and toxic environmental stimulation, and in this process, inflammation occurs, and collagen loss occurs.⁵ It has been shown that *Sasa borealis* contains polyphenols, including flavonoids, isoorientine, triclin, luteolin, etc. Whether *Sasa borealis* can induce skin inflammation relief and collagen resynthesis has not been studied.^{1,6} This study aimed to increase the need for ecological control of Je-ju-jo-rit-dae by applying external skin preparations for Je-ju-jo-rit-dae and to

discover new active ingredients that exist in Je-ju-jo-rit-dae by sorting the methanol extract of Je-ju-jo-rit-dae and confirming the stimulating activity of anti-inflammatory and collagen synthesis.

■ Methods

1. Reagents and research substances:

1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), gallic acid, Folin & Ciocalteu's phenol reagent, sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3 \cdot 6H_2O$), quercetin, quercetin-3-O-rutinoside, myricetin, luteolin, kaempferol, madecassic acid, madecassoside, Asiatic acid, Asiaticoside used in this experiment were purchased and used from Sigma-Aldrich (St. Louis, MO, USA).

2. Method and preparation for extracting *Sasa borealis*:

Sasa borealis used in this experiment was collected from September to October 2019 in the area of Hallasan in Jeju Island, was washed with purified water, dried at room temperature, cut into 1~2 cm in size, and dried at 40°C for two days. The completely dried *Sasa borealis* was weighed in units of 500g with a precision balance, mixed with 3 liters of methanol, and extracted at room temperature. After extraction and filtering with Whatman Filter paper, the filtrate was concentrated until completely evaporated with a rotary vacuum evaporator (N-1200, EYELA, Tokyo, Japan) at 40°C. The remaining solid extract was dissolved in distilled water and methanol. The same amount of n-hexane, chloroform, ethyl acetate, and n-butanol were sequentially solvent fractionated to obtain a fraction (Figure 1).

3. The fixed amount of flavonoids:

The content of total phenolic compounds was measured using the Folin-Denis method.⁷ After each extract was prepared at a concentration of 1 mg/ml, 72 µl of distilled water was added to 24 µl of the sample solution, and 24 µl of Folin-ci-

calteu reagent was added and mixed, followed by incubation for 5 minutes. After adding 24 μ l of Na_2CO_3 and 56 μ l of distilled water, the mixture was left in the dark for 1 hour and 30 minutes, and the absorbance was measured at 725 nm (Perkin Elmer, Victor2 1420 Multilabel counter). Then, the amount was converted from a standard curve using Gallic acid.

The total flavonoid content was measured using the Zhishen method.⁸ After adding 50 μ l of distilled water and 7.5 μ l of a 5% NaNO_2 solution to 75 μ l of the sample solution, the mixture was allowed to stand for 5 minutes. Then 15 μ l of a 10% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ aqueous solution was added and reacted for 6 minutes. After 6 minutes, 50 μ l of 1N- NaOH aqueous solution was added and reacted at room temperature for 10 minutes, and the absorbance was measured at 450 nm. Then, the Quercetin was compared with the standard solution, and the content was calculated.

Confirmation of antioxidant activity: DPPH and ABTS assay:

The method of Wayner nine was modified to measure the antioxidant efficacy. In the case of the hydrogen donating activities for DPPH, 100 μ l of the sample solution and 100 μ l of the DPPH solution were mixed in a 96-well plate, left to stand at room temperature for 30 minutes, and then measured with a spectrophotometer (Perkin Elmer, Victor2 1420 Multilabel counter) at 540 nm. After comparison with the control group to which the sample was not added, the free radical scavenging effect was calculated as follows and expressed as a percentage (%).

$$\text{Inhibition(\%)} = \left(1 - \frac{\text{absorbance of sample solution} - \text{absorbance of blank test solution}}{\text{absorbance of no additives}}\right) \times 100$$

In the case of ABTS radical scavenging effect, 2.6 mM $\text{K}_2\text{S}_2\text{O}_8$ was mixed with 7.4 mM ABTS solution and reacted in the dark for about 12 hours, and then an ABTS solution was adjusted to have an absorbance value of 1.0 ± 0.1 at 734 nm was used. 285 μ l of ABTS solution was mixed with 15 μ l of samples for each concentration and reacted in the dark for 30 minutes to measure absorbance at 734 nm. The resulting value was expressed as a percentage (%) of the radical scavenging effect after comparing the extract-added and non-added groups.

5. Confirmation of in vitro anti-inflammatory effects:

To determine the anti-inflammatory effect of the extract, the extracts were distributed and used in RAW264.7 cells (ATCC, American Type Culture Collection, USA). For culture, an RPMI medium containing 10% FBS (fetal bovine serum, Gibco, USA) and 1% antibiotics was used. Then, it was cultured under conditions of 37°C and 5% CO_2 . In the case of LPS (Lipopolysaccharide derived from *E. coli*, Sigma-Aldrich), it was diluted to 200 ng/ml in the medium on the second day of cell stabilization and treated. For 6 hours after treatment, the activation of cells was observed and calculated based on the degree of adhesion of the cells and the formation of dendritic structures.

6. Immunofluorescence measurement of collagen formation:

To confirm the collagen formation effect of the extract, human fibroblasts were received from ATCC and used.

Twenty-four hours after the sample treatment, each cell was fixed with 4% formalin for 5 minutes at room temperature, washed several times with PBS (Phosphate buffered saline), and then treated with 0.05% Triton-X100 to permeabilize for 5 minutes at room temperature. The treated cells were blocked in 1% albumin solution for 1 hour, and the primary antibody was treated with 1:200 (Collagen I/IV antibody, 9007-34-5, Sigma-Aldrich) at 4° C for 12 hours. After treatment, it was washed several times with PBS, and Alexafluor-488 conjugated rabbit antibody (cellsignaling, USA) as a secondary antibody was treated at room temperature at 1:250 for 3 hours. Then the nuclei were stained with DAPI (1mg/ml) at room temperature for 5 minutes. The treated sample was observed with confocal laser microscopy (LSM800, Zeiss, Germany), and quantitative analysis was performed with ZEN blue software.

7. HPLC analysis:

For analysis, the extracts were centrifuged, and the supernatant was filtered through a 0.45 μ m filter and used as a sample solution. Then, 15 μ l was injected into the HPLC for analysis. Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) was used as the HPLC analyzer, and the column was Phenomenex Gemini NX C18 (4.6 $\times$ 150 mm, three μ m), and the temperature was 30°C. Agilent DAD was used as the detector, and the wavelength was set to 254 nm. The flow rate was 0.6 ml/min, and 15 μ l was set as an injection volume. The moving bed was 0.5% aqueous formic acid solution (A) and acetonitrile (B), 5% B-3 min, 10% B-8 min, 10% B-12 min, 15% B 15 min, 15% B-20 min, 40% B-27 min, 40% B-32 min, 65% B-39 min, 65% B-43 min, 30% B-48 min, 10%-B 50 min were analyzed under gradient conditions. As external standards, quercein-3-O-rutinoside, kaempferol-3-O-glucoside, myricetin, luteolin, kaempferol, Madecassoside, Madecassic acid, Asiatic acid, and Asiaticoside were used. The qualitative and quantification of each standard were compared with the original standard's retention time. The result of the content of each fraction and the fraction using silica column chromatography was obtained from the calibration curve.

8. Statistical analysis:

All experiments were repeated at least three times and expressed as mean and standard deviation (S.D). Statistical analysis was done using ANOVA (one-way analysis of variance) and Student's t-test, which was determined to be significant when $p < 0.05$.

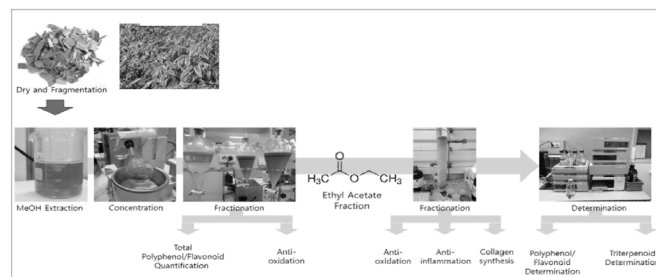


Figure 1: Design and progress of the experiment.

■ Results

1. Extraction and extraction yield of Broad-leaved Bamboo:

500 g of dried leaves of *Sasa borealis* were pulverized, immersed in 3 liters (L) of methanol solvent for 24 hours at room temperature, filtered, and reduced pressure to obtain an extracted solid in an average yield of 21.30% (S.D = 7.24, N = 5). After stirring and dissolving the extract in a mixture of distilled water and methanol (9:1 v/v), the fractions are kept for 3 hours in 4 solvents with different polarities: n-hexane (Hx), CHCl₃ (Cf), Ethyl acetate (EA) and n-BuOH (Bu), and then extracted, filtered, and reduced pressure to obtain a solid as shown in Table 1. Among the fractions, the yields of EA and Bu fractions were the highest.

Table 1: Total extract and TPC, TFC result.

Sample	Yield (%)	Total Polyphenolic compounds (mg Gallic acid equivalent/g)	Total Flavonoid compounds (mg Quercetin equivalent/g)
Methanol	21.30	78.54±5.0	248.39±3.5
n-hexane	0.54	15.50±0.4	247.23±3.9
Chloroform	0.02	28.01±1.0	219.64±0.2
Ethyl acetate	2.95	374.30±7.1	213.20±2.4
n-butanol	4.37	128.58±2.1	294.34±7.8

2 Measurement of flavonoid content in total extract of *Sasa borealis*:

According to a study by Nam *et al.*, it was found that phenolic compounds were present in the extract of *Sasa borealis*, but an accurate analysis of the content was not performed.¹⁰ Therefore, the amount of total phenolic compounds (TPC) and the number of total flavonoids (TFC) were compared and analyzed in the extracts and extracted fractions used in this study. In the case of TFC, it was generally about 220 mg/g (SD = 45.35) as a result of quantification by the Folin-Ciocalteu method using Quercetin as a reference substance. In contrast, in the case of TPC, it showed the highest impact, 374.30 mg/g (SD = 7.1) in the EA fraction (SD = 7.1).

3 Measurement of Antioxidant Activity of Total Extract of *Sasa borealis*:

DPPH and ABTS assays were performed to evaluate antioxidant activity. DPPH turns purple depending on the presence of antioxidant activity and is chemically stabilized, water-soluble, and is mainly used to detect antioxidants and aromatic amine substances. ABTS radical scavenging effect method developed based on the fact that antioxidants inhibit the absorbance of ABTS cationic radicals in plasma can be compared with Trolox, a positive control. It can be expressed by measuring the level at which the turquoise color gradually disappears since it has the opposite color development tendency as DPPH.

In the case of the antioxidant activity of the extract of *Sasa borealis*, as a result of comparing vitamin C (Ascorbic acid; AA) as positive control by the DPPH method, it was confirmed that the total extract (MeOH), EA, and Bu fractions had relatively high antioxidant activity (Figure 2A). Similarly, compared with the positive control Trolox in the ABTS method, overall higher antioxidant activity was detected compared to the DPPH method, and relatively high antioxidant activity was also confirmed in EA and Bu fractions (Figure 2B).

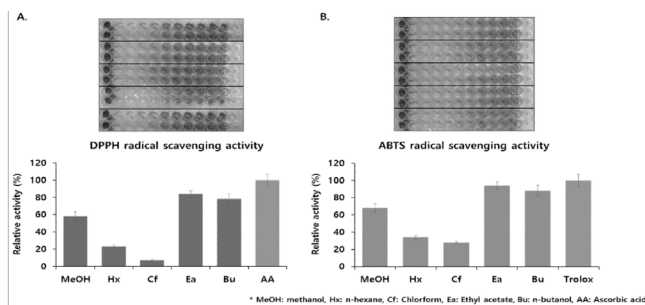


Figure 2: Antioxidant Activity of total extracts and fractions (A) DPPH assay result (B) ABTS assay result

4. Fraction of EA fraction and measurement of antioxidant Activity of Fraction:

Five fractions were obtained using Silica gel column chromatography (CHCl₃: Methanol = 100:0 to 0:100 gradient) for further separation of the EA fraction, showing consistent antioxidant activity among the fractions of the extract of *Sasa borealis* (Figure 3A).

The antioxidant activity of each fraction was high in F3 and F4 in the DPPH method (Figure 3B) and F3, F4, and F5 in the ABTS method (Figure 3C).

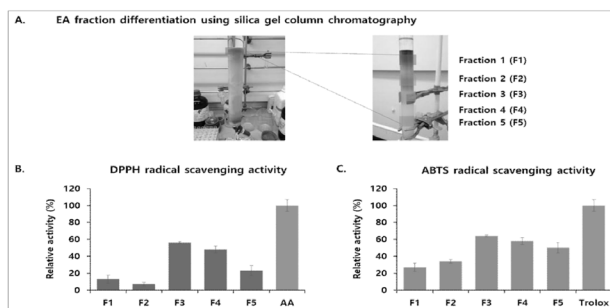


Figure 3: Ethyl acetate Fraction (A) Result of Silica gel chromatography (B) DPPH assay result for each fraction (C) ABTS assay result for each fraction

5. Confirmation of the differentiation inhibitory ability of fractional extract against LPS-stimulated macrophages:

Activating macrophage cell line RAW264.7 cells by treatment with Lipopolysaccharide (LPS), a cell membrane component of the infectious agent, is a frequently used method for measuring anti-inflammatory activity.¹² To identify the active ingredient in the EA fraction, it was determined whether the activation of macrophages occurred in cells treated with or without LPS after treating the RAW264.7 cell line with LPS. As shown in Figure 4A, in the group treated with the same amount of DMSO as the vehicle without any fraction treatment, the cells reacted to LPS and dendritic differentiation. Still, in the group treated with the fraction at a low concentration (5% v/v), the number of activated cells drops sharply. In particular, when fractions were treated at a high concentration (20% v/v), macrophage activity decreased to a level similar to that of the negative control (Figure 4B).

6 Confirmation of the collagen new synthesis promoting effect of the fractional extract on fibroblasts:

Polyphenols and flavonoids may cause the anti-inflammatory effect of the fraction. Still, it is well known that Triterpenoid, a representative, effective polysaccharide present in plants, also has excellent anti-inflammatory effects and the effect of

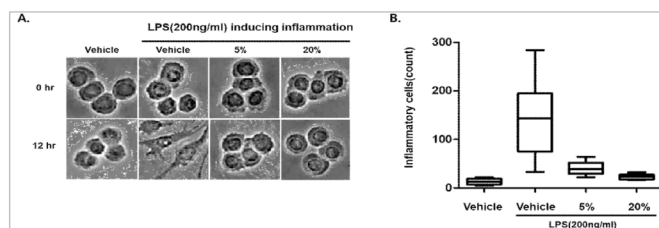


Figure 4: Determination of the anti-inflammatory activity of ethyl acetate activity (A) Optical microscope showing the degree of macrophage activation (B) Quantitative analysis result of activation energy promoting collagen synthesis.^{13,14} To confirm this, the fractions were treated by concentration in human fibroblast cells and then quantified by immunofluorescence compared to the positive control vitamin C (AA) (Figure 5A). As a result, it was confirmed that in cells treated with AA around the cell's nucleus labeled (blue fluorescence), high levels of collagen were expressed (green fluorescence) in the entire cytoplasm and even in cells treated with a low concentration (5% v/v) fraction, a slightly increased level of collagen was observed compared to the negative control group. Surprisingly, it was found that higher levels of collagen were synthesized in cells treated with the fraction at a high concentration (20% v/v) than in the positive control group, indicating that the fraction had a higher content of components that induce collagen synthesis (Figure 5B).

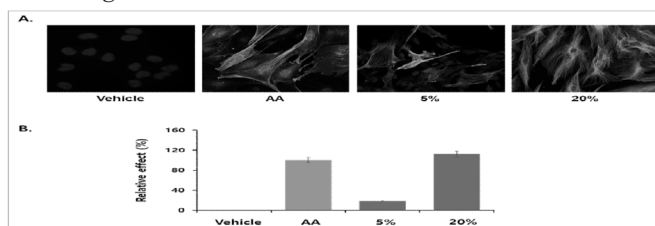


Figure 5: Measurement of ethyl acetate fraction's ability to induce collagen synthesis (A) Immunofluorescence Image of collagen1. Blue: Nucleus, Green: Collagen (B) Quantitative analysis result for expression level

7. Isolation of active ingredients from fractional extract: Triterpenoids and Flavonoids:

To separate the previously identified polyphenols, flavonoids, and components having anti-inflammatory and collagen synthesis promoting effects, the corresponding fractions were analyzed using HPLC. The chromatogram of the standard components detected in the standard solution and sample extracts of each sample is shown in Figure 6. The calibration level of each element in the standard solution was checked to establish the analysis method of each standard component. During the precision (%), the yield for QC, RE, and CV of LLOQ were less than 20%, and LQC, MQC, and HQC were less than 10%.

As a result of the analysis, the contents of Kaempferol-3-glucoside and quercetin-3-rutinoside as flavonoids were high, 44 ug/ml and 27 ug/ml, respectively (Figure 6A), and as a Triterpenoid, the contents of Asiatic acid and Asiaticoside among the reference substances were high, 637 ng/ml and 213 ng/ml respectively (Figure 6B).

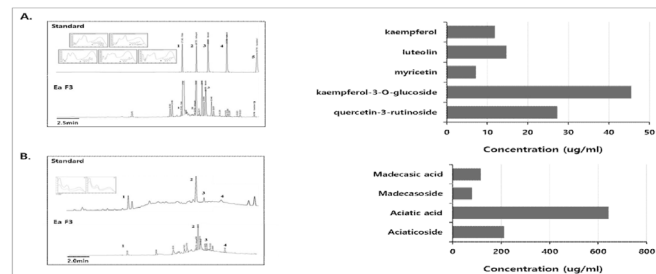


Figure 6: HPLC analysis of Polyphenol, Flavonoid, and Triterpenoid in Ethyl Acetate Fraction (A) HPLC analysis results for flavonoids and polyphenols (B) HPLC analysis results for representative triterpenoids

Discussion

Broad-leaved Bamboo has been recognized for its efficacy in the past. It has been used as a medicinal material in oriental medicine but has not received attention for its pharmacological use due to its wide growing area and high fertility.¹⁵ In particular, the extract of *Sasa borealis* has been studied not only for metabolic diseases such as diabetes and high blood pressure but also for its anticancer effect.^{1,10,16}

This study aimed to expand the scope of industrial use of Broad-leaved Bamboo, confirm whether Broad-leaved Bamboo is effective in human skin health, and isolate and identify active ingredients that cause the efficacy.

First, this study focused on non-aqueous active ingredients by extracting them with methanol. Previous studies have found that antioxidants of polyphenols can be identified by hot water extraction. In particular, to be used in skin health products that mainly use the emulsion properties of water, oils, and fats, it is also essential to confirm a non-aqueous active ingredient. As a result of the experiment, TPC and TFC, like hot water extraction, were also found in fractions of different solvents for the total methanol extract. It was found that extraction of phenolic compounds higher than hot water extraction was possible in the entire content portion.¹

Ethyl acetate is a solvent mainly used to remove caffeine from tea and coffee, and the content of the active ingredient in the extract of *Sasa borealis* was the highest.¹⁷ The fraction by Silica gel chromatography showed high antioxidant activity and anti-inflammatory and collagen synthesis promoting effects, confirming the possibility as an active ingredient for external skin preparations.

Polyphenols and flavonoid compounds, known mainly as active ingredients of *Sasa borealis*, have high antioxidant activity but relatively low anti-inflammatory effects. In particular, little is known about the impact of promoting collagen synthesis on fibroblasts.¹⁸ This study confirmed high levels of anti-inflammatory and collagen synthesis in certain fractions of Ethyl acetate from *Sasa borealis*. It established the hypothesis that these effects were not simply due to the high polyphenol content. Triterpenoid is a compound commonly known to promote collagen synthesis among many plant-derived active ingredients. In particular, Madecassic acid, Madecassoside, Asiatic acid, and Asiaticoside have excellent efficacy enough to be used in pharmaceuticals.¹⁹ Therefore, it has the possibility that the high collagen synthesis induction ability of *Sasa borealis* may be caused by these Triterpenoids. As a result of

HPLC analysis of the EA fraction, it was confirmed that the content of Madecassic acid or Madecassoside is low in the extract of *Sasa borealis*. Still, the content of Asiaticoside or Asiatic acid is high.

This study expanded the industrial applicability of Broad-leaved Bamboo by studying the content of previously unknown active ingredients and new functions of anti-inflammatory and collagen synthesis-inducing ability through fractional extraction from the native Broad-leaved Bamboo of Hallasan Mountain. However, in this study, since only *Sasa borealis* harvested from Hallasan Mountain around September 2019 were used, additional studies are needed to determine whether *Sasa borealis* harvested in different regions of Jeju or at other times have the same content or distribution of active ingredients.

In summary, this study aimed to expand the scope of industrial use of *Sasa borealis* through the development of natural vegetable raw materials that have anti-inflammatory and collagen synthesis induction ability necessary for skin inflammation or wrinkle improvement, confirmed the antioxidant efficacy of *Sasa borealis* through methanol extraction and fraction extraction, the anti-inflammatory efficacy and collagen synthesis induction ability in the Ethyl acetate fraction with high antioxidant activity to identify new functions of *Sasa borealis*, and not only investigated the new function of *Sasa borealis* but also reported the previously unknown contents of Asiatic acid and Asiaticoside as well as the high content of formerly known phenols in the fraction. As a result, the methanol extract of *Sasa borealis* appears to have high antioxidant, anti-inflammatory, and collagen synthesis ability due to its active ingredients. Thus it is highly likely to be used as an essential material for functional skin external preparations.

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