

ANTI-OXIDANT, ANTI-BACTERIAL, AND ANTI-INFLAMMATORY ACTIVITY OF *Scutellaria baicalensis* ROOT SUPERCRITICAL FLUID EXTRACTS

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ABSTRACT

Scutellaria baicalensis (*S. baicalensis*) root extract has been used for a long time in Oriental medicine and the cosmetics industry due to its natural anti-oxidant properties in disease treatment. However, comprehensive studies comparing the physiological activities of different *S. baicalensis* root extracts are lacking. Specifically, there is a need to compare *S. baicalensis* root hydrothermal extract (SBHE), *S. baicalensis* root 75% ethyl alcohol extract (SBAE), and *S. baicalensis* root supercritical fluid extracts (SBSEs) simultaneously. In this study, we extracted *S. baicalensis* roots using three different methods: hydrothermal, 75% ethyl alcohol, and supercritical fluid extraction. We then evaluated their radical scavenging activities, SOD-like activity, anti-bacterial activities, and the production of inflammatory factors. Among the extracts tested, the *S. baicalensis* root supercritical fluid 35°C extract (SBSE35) exhibited superior overall physiological activities. Notably, only the SBSEs demonstrated anti-bacterial activity against *Staphylococcus aureus*, whereas SBHE and SBAE did not. SBHE and SBAE increased interleukin (IL)-6 levels ($p \leq 0.05$). Additionally, SBHE and SBAE showed a slight decrease in IL-8 at low concentrations, but an increase in IL-8 production in a concentration-dependent manner. In contrast, SBSE35 significantly reduced both IL-6 and IL-8 levels ($p \leq 0.05$). These findings suggest that SBSE35 has greater potential for improving human health in the cosmetics and pharmaceutical fields.

Keywords: *Scutellaria baicalensis* root; supercritical fluid extraction; anti-oxidant; anti-bacterial; anti-inflammation

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INTRODUCTION

Throughout the world, natural products are extensively employed in the fields of food, cosmetics, and pharmaceuticals for disease prevention and treatment due to their inherent properties as natural antioxidants, particularly phenolic compounds (Chaughule *et al.*, 2023; Andrade *et al.*, 2023; Jing *et al.* 2023; Rahaman *et al.*, 2023). Another reason behind the preference for natural products is the belief that they are natural itself and safe for the human body, in contrast to the hazards associated with chemical synthetic substances (Rahaman *et al.*, 2023; Banfonan *et al.*, 2023). Moreover, the utilization of discarded by-products (such as fruit seeds and stalks) from plant consumption contributes to environmental sustainability and economic benefits through upcycling practices (Rashid *et al.*, 2023).

Scutellaria baicalensis (*S. baicalensis*) root has been widely utilized from the Orient since ancient times (Zhao *et al.*, 2016). It is renowned for its potent anti-convulsant, anti-cancer, anti-oxidant, anti-bacterial, and anti-inflammatory effects (Zhao *et al.*, 2016; Liu *et al.*, 2023; Chmiel *et al.*, 2023). The main active constituents

of *S. baicalensis* root are flavonoids such as glycoside (baicalin, wogonoside) and aglycone (baicalein, wogonin) which have been reported to exhibit various physiological activities such as anti-inflammatory diseases (Tong *et al.*, 2012; Wen *et al.* 2023). Moreover, more than 40 flavonoids have been identified in *S. baicalensis* root (Li *et al.*, 2011).

Since extracting the maximum yield of active compounds contained in plants determines the efficacy of the extract, leading to numerous studies aiming to enhance active compounds extraction efficiency (Oracz *et al.*, 2023). In recent time, researches on separating phytochemicals using supercritical carbon dioxide (SC-CO₂) extraction are ongoing (Oman *et al.* 2013). Previous studies have applied SC-CO₂ extraction to isolate compounds such as baicalin, wogonoside, and wogonin (Gao *et al.*, 2016; Vinitha *et al.*, 2022).

Supercritical fluids exhibit exceptional solubility due to the properties of gases (low viscosity, high diffusivity, and low surface tension), as well as the properties of liquids (high density) (Temelli, 2009). These distinctive characteristics facilitate rapid penetration into the raw material and form clustering

around the active ingredients, subsequently enabling their dissolution and extraction (Uwineza *et al.*, 2020). Carbon dioxide is commonly employed as a supercritical fluid than alternative solvents due to its relatively low critical temperature and pressure (31.1°C and 73.8 bar) (Esfandiari, 2015). These critical parameters enable carbon dioxide to more readily attain a supercritical fluid state compared to other solvents (Esfandiari, 2015). These properties of carbon dioxide necessitate low energy consumption for maintaining a supercritical fluid state, making it an economical choice. Furthermore, it allows extraction without compromising the heat-sensitive active ingredients that may be destroyed during hydrothermal extraction (Cossuta *et al.*, 2008).

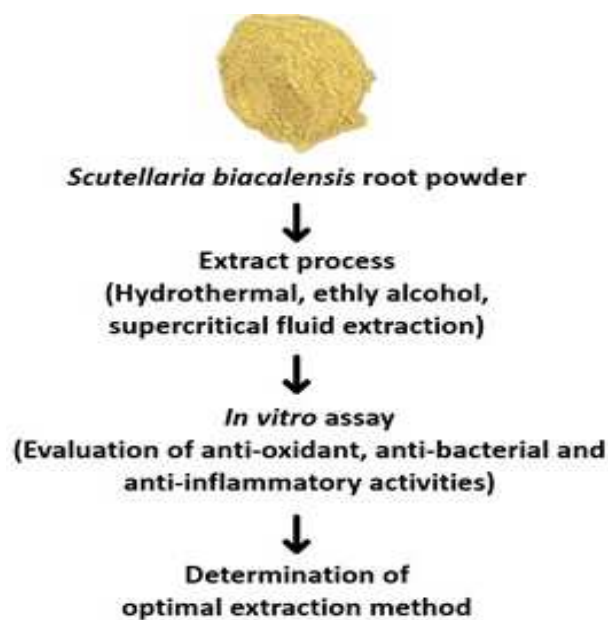


Figure 1. The research flowchart of this study.

Based on the results in the existing literature, we aimed to confirm that active compounds can be isolated from *S. baicalensis* roots using water, organic solvent, and SC-CO₂ by evaluating the efficacy (Shen *et al.*, 2019; Yoon *et al.*, 2009). However, organic solvents are very hazardous to the human health, and there is a risk of organic solvents remaining in the extract. (Joshi *et al.*, 2019). Considering these concerns, ethyl alcohol, which is commonly used in the cosmetic industry and comparatively safer than other organic solvents, was selected as the organic solvent in this study. Therefore, the extraction of *S. baicalensis* root was conducted using biocompatible solvents (water and SC-CO₂) and the organic solvent (ethyl alcohol). No prior studies have been reported that simultaneously compare *S. baicalensis* root extracts obtained through hydrothermal extraction, ethyl alcohol extraction, and supercritical fluid extraction.

Inflammation is one of the immunological

responses triggered by external factors, including microbiological antigens, physical stimuli, or chemical stimuli (Suzuki *et al.*, 2020; Gopinath *et al.*, 2023). Especially, excessive inflammatory response promotes skin aging and induces pruritus and pain, and subsequently diminishing the overall the quality of life (Kanaki *et al.* 2016; Borg *et al.* 2013). There are representative skin inflammation diseases, as exemplified by psoriasis, atopic dermatitis, and allergic contact dermatitis (Albanesi *et al.*, 2005). Given the exposure to external harmful environments (e.g., fine dust, exhaust gas) and the frequent use of masks in the 21st century, effectively managing inflammation diseases with a disrupted skin barrier has become a challenging task (Alves *et al.*, 2023; Lugović-Mihić *et al.*, 2023). Therefore, constant management of skin lesions becomes necessary. This can be achieved through the continuous use of effective skincare products or medications.

Our objective was to determine the optimal extraction method by identifying the most effective extract from an efficacy perspective. To achieve this, we adopted three different extraction methods (hydrothermal, ethyl alcohol, and supercritical fluid extraction) and conducted *in vitro* experiments to evaluate their biological activities, including anti-oxidant, anti-bacterial, and anti-inflammatory properties. The ultimate goal of our study was to develop a valuable material that can contribute to achieving healthy and well-balanced skin for individuals seeking such benefits.

MATERIALS AND METHODS

Extraction Method

Hydrothermal extraction: Hydrothermal extraction method is inexpensive, easy to handle, non-toxic, and abundant availability (Yulianto *et al.*, 2019). Plus, water can dissolve water soluble compounds and polar substances (Chuo *et al.*, 2022). *S. baicalensis* root used in this study was obtained from a Korean company (Kunhwa Pharm, Gwangju, Korea). Initially, we pulverized *S. baicalensis* root finely with a blender. In the next step, 20 g of the pulverized root was mixed with 200 g of triple distilled water and extract it with hot water (70°C) for 2 h, using a thermostat. The ratio of raw material to solvent was set at 1:10, taking into account the amount of solvent necessary to sufficiently immerse the raw material. To ensure effective extraction of active compounds while minimizing their degradation due to heat, the extraction temperature was set at 70°C (Antony *et al.*, 2022). The resulting mixture was then filtered with a sieve net, followed by centrifuge (Hanil Science, Gimpo, Korea), and further filtration through 5 µm filter (Advantec, Tokyo, Japan) to remove the solid residue. As a final step, the frozen aqueous extract was sublimed in a freeze dryer and obtained *S. baicalensis* root hydrothermal extract (SBHE).

Ethyl alcohol extraction: Ethyl alcohol exhibits good miscibility with water and can efficiently extract the target substance by adjusting the water-ethyl alcohol ratio (Hashim *et al.*, 2021; Lyu *et al.*, 2022). Additionally, ethyl alcohol has the capability to dissolve both polar and non-polar substances (Le *et al.*, 2021). Based on previous studies demonstrating excellent results with plant extractions using 70% ethyl alcohol, this experiment utilized 75% ethyl alcohol. This adjustment was made to account for the evaporation of ethyl alcohol during the extraction process (Oracz *et al.*, 2023; Shen *et al.*, 2019; Stoica *et al.*, 2013; Zhang *et al.*, 2007). *S. baicalensis* root was finely pulverized using a blender with a blender. Subsequently, 20 g of the crushed *S. baicalensis* root was added to an extraction container containing 200 g of 75% ethyl alcohol, and the mixture was thoroughly combined. It was immersed and allowed to extract at room temperature for 2 h, while being shielded from ultraviolet rays. The resulting mixture was filtered using a sieve net, followed by centrifugation, and further filtration through a 5 µm filter to eliminate solid residues. The 75% ethyl alcohol solution containing the extracts was subjected to vaporization using a rotary vacuum evaporator (EYELA, Tokyo, Japan) at 50°C. As the final step, the remaining solvent was frozen in a deep freezer and subsequently sublimed in a freeze dryer. This process yielded the *S. baicalensis* root 75% ethyl alcohol extract (SBAE).

Supercritical carbon dioxide extraction: We conducted the extraction of *S. baicalensis* root using two different temperatures. A total of 100 g of pulverized *S. baicalensis* root was placed into the extraction container, and the SC-CO₂ extraction process was performed using the SC-CO₂ extraction system (Ari instrument, Namyangju, Korea) for 2 h for each extract. The extraction conditions were as follows. The extractor temperature was set to 35°C and 60°C respectively, with a pressure of 350 bar. Under fixed pressure conditions, increasing the temperature of the supercritical fluid decreases the solvent density, thereby reducing its solvating power, while simultaneously increasing the vapor pressure, which facilitates compound extraction. To determine whether the changes in the solvent's solvating power or the compounds' vapor pressure predominantly influence extraction, the temperature was set between 35°C and 60°C. Since temperatures above 60°C can lead to the degradation of thermolabile compounds, the extraction was conducted at 35°C, near the critical point of carbon dioxide, and 60°C to prevent compound degradation due to excessive heat. (Reverchon *et al.*, 2006). The separator was maintained at a temperature of 25°C and a pressure of 30 bar. Ethyl alcohol was used as a co-solvent. The ethyl alcohol with the extracts was vaporized using a rotary vacuum evaporator at 50°C, resulting in the production of *S. baicalensis* root supercritical fluid extracts (SBSEs).

These SBSEs include the *S. baicalensis* root supercritical fluid 35°C extract (SBSE35) and the *S. baicalensis* root supercritical fluid 60°C extract (SBSE60). The process diagram for supercritical fluid extraction is depicted in Figure 2.

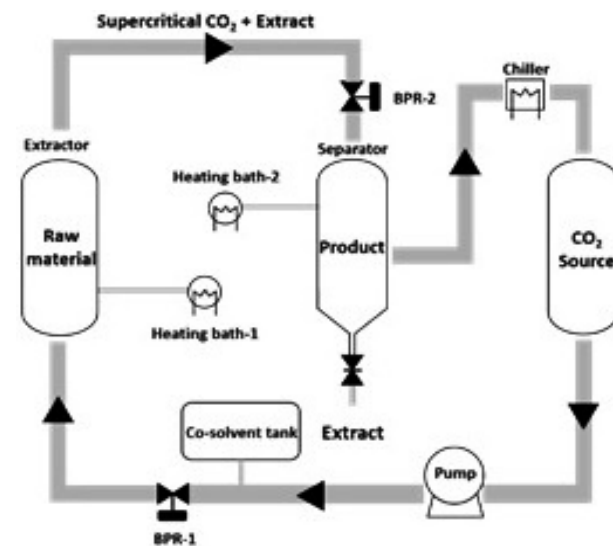


Figure 2. Summarized schematic diagram of supercritical fluid extraction for extracting natural products.

Sample preparation: To dissolve the extracts obtained from various solvents, which have differing solubilities, dimethyl sulfoxide (DMSO) was used as a solubilizing agent to aid in their dissolution. (Di, L. *et al.*, 2006). These extracts were dissolved in 99.50% DMSO beforehand and stored in a deep freezer at -80°C. The stock solutions, prepared at a concentration of 40 mg/mL were thawed at room temperature immediately before the commencement of experiment. For the assays, the samples were diluted to specific concentrations using either DMSO or a medium (for cell-based assays). It has been reported that DMSO concentrations below 2% have only a negligible effect on cell viability and other biological mechanisms. (Hoyberghs *et al.*, 2021; Sumida *et al.* 2011). Nevertheless, to account for any potential effects caused by DMSO, an equal amount of DMSO was added to the control group in all experiments.

Anti-oxidant Assays

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay: We prepared 0.2 mM DPPH solution by dissolving DPPH powder (Sigma Aldrich, St. Louis, USA) in 99.90% ethyl alcohol. 10 µL of four *S. baicalensis* root extracts (SBREs) was placed in a transparent 96-well microplate (max volume of 300 µL) in triplicate. Subsequently, 190 µL of DPPH solution was added to the wells, and allowed to react in the absence of light for a half hour (25°C). The 96-well microplate containing the reaction solution was

inserted into a microplate reader (BioTek, Winooski, USA) and shaken for 1 min. Then, we measured the absorbance of reacted solution (DPPH solution + SBREs) at 520 nm ($n = 3$). L-ascorbic acid (Daejung, Siheung, Korea) was employed as a positive control (PC). The values of the 50% inhibitory concentration (IC₅₀) values were calculated using the log-linear interpolation method.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay: Each 14.80 mM ABTS and 5.20 mM potassium persulfate in phosphate buffered saline (PBS) were prepared. Next, we mixed them and allow them to react to form ABTS cation radical for 20 h in a dark refrigerator (4°C). The produced ABTS⁺ radical solution was diluted 1/45 in PBS, and the absorbance of it at the wavelength of 734 nm (Ab734) was adjusted to 0.70–0.75 and used in the experiment. 10 µL of SBREs was mixed with 190 µL of ABTS⁺ radical solution (Ab734 = 0.70–0.75) and left to react in a 96-well microplate in the absence of light for 20 min (25°C). The 96-well microplate containing the reaction solution was inserted into a microplate reader and shaken for 1 min. Then, we measured the absorbance of reacted solution (ABTS working solution + SBREs) at 734 nm ($n = 3$). L-ascorbic acid was used as a PC.

Superoxide dismutase (SOD) assay: SOD assay was conducted by modifying the method of the OxiTec™ SOD Assay Kit (Biomax CO., Guri, Korea). 170 µL of water-soluble tetrazolium salt (WST)-1 solution was combined with 10 µL of sample on a transparent 96-well microplate. Subsequently, 20 µL of enzyme working solution was dispensed, and then the mixture was allowed to react for 40 min in an incubator at 37°C. We inserted the 96-well microplate containing this reaction solution into a microplate reader and mixed it for 1 min. We gauged the absorbance of reacted solution at 450 nm ($n = 3$). L-ascorbic acid was used as a PC.

Total polyphenol content assay: 10 µL/well of each SBRE was placed, followed by the addition of 40 µL of 1 N Folin-ciocalteu's reagent diluted with triple distilled water (Sigma Aldrich, St. Louis, USA). They were allowed to react for 3 min. After that, we dispensed 150 µL of 20% Na₂CO₃ to each well. Immediately after shaking for 1 min with a microplate reader, we gauged the absorbance of reacted solution at 760 nm. A linear standard curve was prepared using gallic acid ($R^2 = 0.9962$). The total polyphenol content in four extracts was expressed as gallic acid equivalents (mg) per weight (g) of the extract (mg GAE/g extract).

Anti-bacterial Assays

Paper disc diffusion assay: *Staphylococcus aureus* (*S. aureus*, ATCC 6538) is a common microorganism that can cause food poisoning, skin infections, and atopic dermatitis (Liu, A. *et al.*, 2024; Braun *et al.*, 2024) In

other words, this bacterium contributes to the induction of inflammation in the human body. To evaluate the anti-bacterial property, the disc diffusion method was performed. Using sterile Mueller-Hinton (MH) medium (KisanBio, Seoul, Korea), *S. aureus* strain was subcultured once after culturing for 24 h in a shaking incubator (500 rpm) at 37°C. The experiment was performed once the bacteria reached the mid-exponential phase, indicated by an optical density at 600 nm of 0.5 (OD₆₀₀ = 0.5). After inoculating 150 µL of the bacterial suspension on a semi-solid agar plate medium, it was uniformly spread on all sides with a spreader. After sufficient drying, an 8 mm paper disc (Advantec, Tokyo, Japan) was put on the plate and it was impregnated with 50 µL of the sample (10–20 mg/mL). In the case of *S. aureus* strain experiment, 40 mg/mL of methylparaben was adopted as a PC (Lee *et al.*, 2018). Methylparaben is widely used as a preservative in cosmetics and pharmaceuticals. After incubation (37°C) for 18 h, the petri dishes with paper disc impregnated were observed for measuring the diameter of the inhibition zones.

Minimal inhibitory concentration (MIC) assay: In order to find out the minimum growth inhibitory concentration, MIC assay was carried out by applying the broth dilution method in a transparent and aerated 96-well microplate. *S. aureus* strain was cultured in a shaking incubator for 24 h and subsequently subcultured once. It was confirmed that the exponential phase was entered (OD₆₀₀ = 0.5). The bacterial suspension (OD₆₀₀ = 0.5) was inoculated into sterile MH medium to achieve a bacterial suspension concentration of 1.00%. 100 µL of this suspension was mixed with 100 µL of SBREs (6.25–800 µg/mL) and a PC (625–5000 µg/mL), which were diluted using a two-fold serial dilution method. After culturing in an incubator at 37°C for 12 h, the results of the MIC assay for *S. aureus* strain were visually confirmed.

Cell-based Assays and Anti-inflammatory Assays

Cell culture: RAW 264.7 cells (mouse macrophage) were received from Korean cell line bank (Seoul national university, Seoul, Korea). RAW 264.7 cells are suitable for immunological research as they are macrophages and play a crucial role in immune responses (Shi *et al.*, 2017). HaCaT cells (human keratinocyte cell line) were obtained from a cosmetic R&D center (Amore Pacific, Yongin, Korea). HaCaT cells were selected for this experiment to examine changes in the production of factors related to inflammatory responses when treated with SBREs from the perspective of skin cells. RAW 264.7 cells were incubated in Dulbecco's modified eagle high glucose medium (DMEM; Welgene, Gyeongsan, Korea), supplemented with 10% fetal bovine serum (FBS; Gibco, New York, USA) and 1% penicillin/streptomycin (PS; Lonza, Basel, Switzerland) in a humidified atmosphere

with 37°C and 5% CO₂. HaCaT cells were incubated in DMEM, supplemented with 10% FBS (Samchun, Seoul, Korea) and 1% PS in the same incubation conditions adopted for RAW 264.7 cells. After we observed cell density (70-80%) and normal cell morphology using by TS100 optical microscope (Nikon, Tokyo, Japan) with a 4× magnification lens, subculture was carried out.

Cell cytotoxicity: We investigated changes in cell viability after treating cells with our samples (25-200 µg/mL for RAW 264.7 cells and 2.5-80 µg/mL for HaCaT cells). Each RAW 264.7 cells and HaCaT cells were seeded onto an aerated 96-well microplate for 24 h, respectively (1.0×10^4 cells/well). We utilized the Quanti-Max™ WST-8 assay kit (Biomax Co., Guri, Korea), which measures cell viability by the conversion of WST-8 to WST-8 formazan dye (orange color) in the presence of active cells, as electrons are transferred from the living cells (Teo *et al.*, 2014). After 24 h in the incubator, the medium was suctioned. Cells attached to the surface of the plate were treated with SBREs for 48 h. Subsequently, after the medium was aspirated, 100 µL of 10% WST-8 solution was treated for 2 h. Throughout the experiment, except for the time of treating cells with substances, they were maintained at 37°C and 5% CO₂. We monitored the absorbance of WST-8 formazan at 450 nm at ambient temperature. The concentration at which the cell viability of the experimental groups treated with the extracts started to decrease statistically significantly was regarded as the toxic concentration of SBREs on cells.

NO assay: The subsequent experiments were conducted at concentrations below the cytotoxic levels of SBREs to RAW 264.7 cells, as determined by the cytotoxicity assay results. To ensure accurate comparisons, the experiments were performed at the same concentrations, specifically 12.5-50 µg/mL, which are below the toxic levels for RAW 264.7 cells. 1×10^4 RAW 264.7 cells per well were seeded on a transparent 96-well microplate for 24 h. After that, our samples (12.5-50 µg/mL) were treated for 48 h with lipopolysaccharide (LPS; Sigma Aldrich, St. Louis, USA) of 100 ng/mL, and the supernatants were collected. We used Griess Reagent System (Promega, Madison, USA) and followed the manufacturer's protocol instructions. 50 µL of the supernatant and standard solution were placed in wells, and 50 µL of sulfanilamide solution was rapidly added with a multi-channel pipette (Eppendorf, Hamburg, Germany). After 10 min, 50 µL of N-1-naphthylethylenediamine dihydrochloride (NED) solution was quickly added with a multi-channel pipette and allowed to react for 10 min. The light was blocked during the reaction. We measured the absorbance of reacted solution at 540 nm ($\lambda_{\text{max}} = 520-550\text{nm}$) to determine the NO production.

Enzyme-linked immunosorbent assay (ELISA; IL-6

and IL-8): Based on the cytotoxicity assay results, the experiments were conducted at concentrations of SBREs below the toxic levels for HaCaT cells. To accurately compare the efficacy of SBREs, all experiments were performed at the same concentrations, specifically 0.625-2.5 µg/mL. 4×10^4 cells per well were seeded on a transparent 24 well cell culture plate for 24 h. After ensuring that HaCaT cells are attached to the surface, we removed the medium. Then 5 nanograms of tumor necrosis factor-alpha (TNF- α ; R&D systems, Minneapolis, USA) per mL were simultaneously treated with the SBREs (0.625-2.5 µg/mL) for 48 h. After the incubation period, the supernatants were taken and the cells were spun down by a centrifugal separator, and then the supernatants were used for the experiment. The ELISA for interleukin (IL)-6 and IL-8 kits were employed according to the manufacturer's protocol, and the principle of a sandwich-type ELISA was used.

Statistical Analysis: All data were represented as mean and standard deviation (SD). All experiments were conducted in triplicate except for paper disc diffusion assay ($n = 4$). Statistical analysis was carried out to analyze the differences in values between the control group and experimental group using the student t-test. Additionally, the differences in values between the groups were evaluated using one-way analysis of variance (ANOVA). In cases where a significant difference was observed following ANOVA analysis ($p \leq 0.05$), Tukey's post-hoc test was applied. P-value less than 0.05 ($p \leq 0.05$) was considered statistically significant, and the analysis was performed using SPSS 29 version (IBM, New York, USA).

RESULTS

Evaluation of Anti-oxidant Efficacy

DPPH assay: Four different extracts scavenged DPPH free radical, which is very stable, in a concentration-dependent manner (Figure 3A,B,C,D). IC₅₀ of SBHE, SBAE, SBSE35, and SBSE60 were determined as 67.51 µg/mL, 92.28 µg/mL, 42.72 µg/mL, and 111.05 µg/mL, respectively. Among them, SBSE35 showed the best DPPH free radical scavenging ability. SBSE35 exhibited 86.16% scavenging ability at 100 µg/mL (Figure 3C), and SBHE and SBSE35 at 200 µg/mL showed over 90% radical scavenging ability, effectively removing almost all radicals (Figure 3D). On the other hand, SBAE and SBSE60 showed only 80.18% and 71.62% radical scavenging, respectively, at the highest concentration of 200 µg/mL (Figure 3D). ANOVA demonstrated significant differences in the scavenging power of the SBREs ($p \leq 0.001$).

ABTS assay: All extracts scavenged ABTS cation radical, which is lipophilic and hydrophilic, as the

concentration of the extracts was elevated (Figure 3E,F,G,H). The IC₅₀ values of SBHE, SBAE, SBSE35, and SBSE60 were determined as 16.09 µg/mL, 16.58 µg/mL, 5.08 µg/mL, and 7.30 µg/mL, respectively. At the highest concentration of 20 µg/mL, SBSE35 and SBSE60 scavenged more than 90% of radicals (very transparent ABTS working solution) (Figure 3H). However, SBHE and SBAE did not scavenge less than 60% at the same concentration (Figure 3H). ANOVA showed highly significant differences in the scavenging activity of the SBREs ($p \leq 0.001$). SBSE35 exhibited the most excellent scavenging activity against ABTS⁺ radical, as confirmed at the four different concentrations tested. At concentrations of 2.5 µg/mL, 5 µg/mL, and 20 µg/mL, no significant difference was observed between SBHE and SBAE (Figure 3E,F,H).

SOD assay: SBREs demonstrated SOD-like activity surpassing 70% at the highest concentration of 400 µg/mL (Figure 3I,J,K,L). The SOD-like activity increased with the escalating concentrations of SBHE, SBAE, SBSE35, and SBSE60. Among them, SBHE was the lowest the IC₅₀ value (46.57 µg/mL), while SBAE, SBSE35, and SBSE60 had IC₅₀ values of 109.14 µg/mL, 61.78 µg/mL, and 143.18 µg/mL, respectively. At a concentration of 50 µg/mL, SBHE, SBAE, SBSE35, and SBSE60 did not show significant differences in SOD-like

activity ($p > 0.05$). However, at concentrations above 100 µg/mL, a statistical significance began to emerge between SBSE35 and SBSE60 groups (Figure 3J), and at concentrations 400 µg/mL, SBAE and SBSE35 groups also exhibited statistical significance (Figure 3L). Notably, although the IC₅₀ value of SBHE was lower than that of SBSE35, post-hoc tests revealed no significant differences within the range of 50–400 µg/mL. SBSE35 displayed superior SOD-like activity compared to SBAE and SBSE60 at a concentration of 400 µg/mL (Figure 3I,J,K,L) ($p \leq 0.05$). ANOVA indicated statistically significant differences in the SOD-like activity among SBREs ($p \leq 0.05$).

Total polyphenol assay: The total polyphenol content of SBREs were shown in Table 1. Total polyphenol content differed among extracts obtained by different solvents and temperatures. Among the four extracts, SBSE35 had the highest polyphenol content. SBSE35 contained approximately 2.40, 3.02, and 1.65 times more polyphenols compared to SBHE, SBAE, and SBSE60. The amount of polyphenol in SBREs followed this order: SBSE35 > SBSE60 > SBHE > SBAE. ANOVA revealed significant differences in the amount of polyphenols in SBREs expressed mg GAE/g ($p \leq 0.001$). Tukey's post-hoc test confirmed significant differences in total polyphenol content among all groups ($p \leq 0.05$).

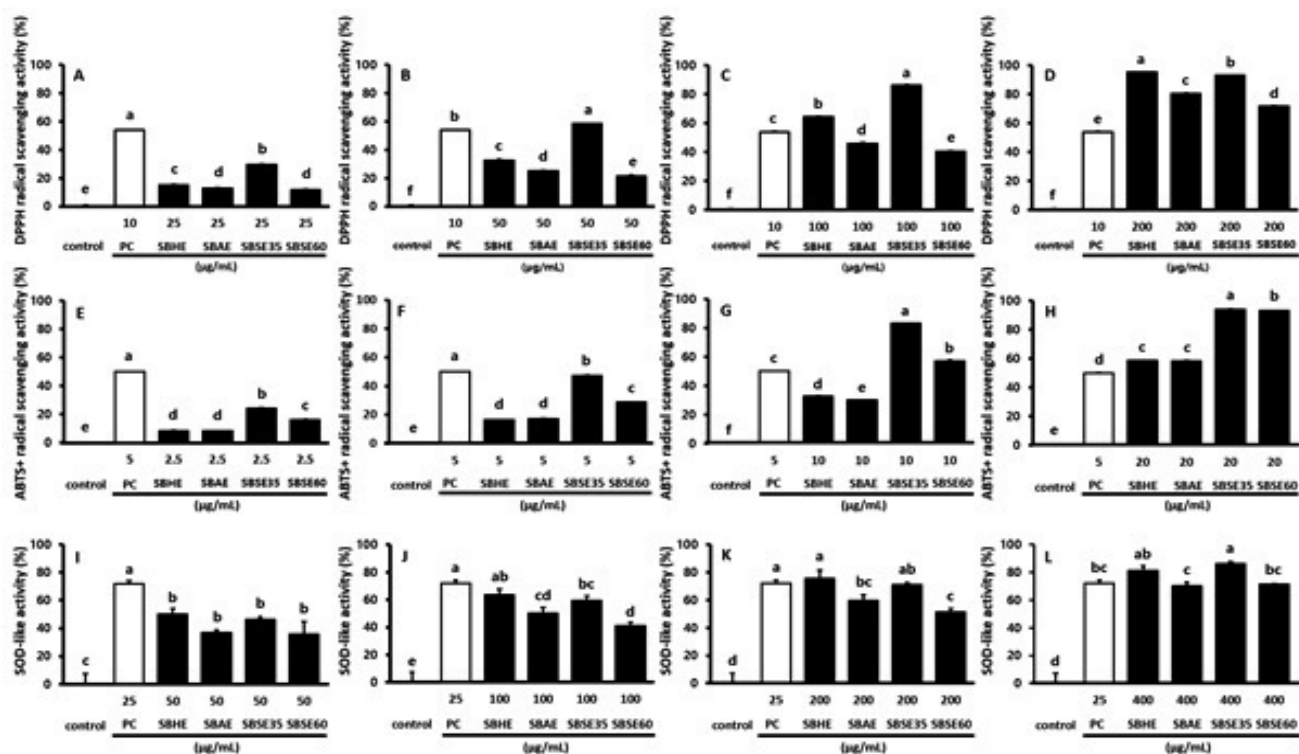


Figure 3. Evaluation of Anti-oxidant Efficacy: (A–D) DPPH free radical scavenging effect of *S. baicalensis* root extracts; (E–H) ABTS⁺ radical reduction effect of *S. baicalensis* root extracts; (I–L) SOD-like activity of *S. baicalensis* root extracts; Data offered are the means and SD. Same letters represent non-significant differences according to the post-hoc Tukey's HSD test ($p > 0.05$).

Evaluation of Anti-bacterial Efficacy

Paper disc diffusion assay: As a result of the paper disc diffusion assay on *S. aureus*, only SBSEs showed a detectable anti-bacterial activity, as evidenced by the formation of inhibition zones. It was observed that the size of the inhibition zone is expanded as the concentration in-creased from 10 mg/mL to 20 mg/mL. For comparison and ensuring the reliability of the experiment, methylparaben, a preservative commonly used in cosmetics and drugs, was used as a PC. The inhibition zone diameter of methylparaben (40 mg/mL) was measured as 10.38 ± 0.48 mm, while the inhibition zones of SBSE35 and SBSE60 (20 mg/mL) were 13.38 ± 0.48 mm and 11.88 ± 0.75 mm, respectively. SBSE35 (20 mg/mL) shows a significant difference in the size of inhibition zone compared to SBSE60 (20 mg/mL) ($p \leq 0.05$). Further details of the experimental results are as follows (Table 2).

MIC assay: The following table shows the experimental results of minimal growth inhibitory concentration of *S. aureus* (Table 3). Consistent with the findings from the paper disc diffusion assay, we could not verify the MIC values of SBHE and SBAE at 6.25-800 $\mu\text{g/mL}$ concentrations. However, we confirmed the MIC values of SBSE35 at 200 $\mu\text{g/mL}$. It was visually observed that SBSE60 inhibited the growth of bacteria in a concentration-dependent manner, but did not completely inhibit it at the highest tested concentration of 800 μg of SBSE60 per mL. For this reason, SBSE60 was marked as “> 800 $\mu\text{g/mL}$ ” in Table 3.

Table 3. MIC values on *S. aureus* treated with four *S. baicalensis* root extracts.

Strain	Sample	Concentration ($\mu\text{g/mL}$)
<i>S. aureus</i>	PC	2500
	SBHE	> 800
	SBAE	> 800
	SBSE35	200
	SBSE60	> 800

Cytotoxicity and Evaluation of Anti-inflammatory Efficacy

Cell cytotoxicity in RAW 264.7 cells: The toxic concentrations of the extracts in RAW 264.7 cells were shown graphically (Figure 4A,B,C,D). SBHE and SBAE exhibited a statistically significant decrease in cell viability above the concentration of 200 $\mu\text{g/mL}$ (Figure 4A,B). Similarly, in the case of SBSEs, the cell viability showed a statistically significant decrease compared to the control group at concentrations above 100 $\mu\text{g/mL}$ (Figure 4C,D). Therefore, to ensure a fair comparison of the efficacy of SBREs under identical conditions, NO assay was conducted at the same concentration (12.5–50 $\mu\text{g/mL}$), which are below the concentrations that showed

Table 1. Total polyphenolic content contained in four extracts.

Sample	Extraction method	Total polyphenol (mg GAE/g)
SBHE	Hydrothermal extraction	134.17 ± 1.69
SBAE	Ethyl alcohol extraction	106.47 ± 4.27
SBSE35	Supercritical fluid extraction	321.36 ± 16.20
SBSE60	Supercritical fluid extraction	194.25 ± 10.83

Table 2. The diameter values of the inhibition zone of paper disc impregnated with four *S. baicalensis* root extracts on *S. aureus*.

Sample	Concentration (mg/mL)	Inhibition zone (mm)
DW	-	-
DMSO	-	-
PC	40	10.38 ± 0.48
SBHE	10	-
	20	-
SBAE	10	-
	20	-
SBSE35	10	11.00 ± 0.48
	20	13.38 ± 0.48
SBSE60	10	10.63 ± 0.48
	20	11.88 ± 0.75

cytotoxicity.

Cell cytotoxicity in HaCaT cells: The toxic concentrations of the extracts in HaCaT cells were depicted graphically (Figure 4E,F,G,H). The concentrations at which the cell viability began to decrease statistically significantly, in comparison to the control group, varied among the extracts. For SBHE, cell viability exhibited a significant decrease at a concentration of 80 $\mu\text{g/mL}$ ($p \leq 0.05$), while for SBAE, the decrease was observed at a concentration of 40 $\mu\text{g/mL}$ ($p \leq 0.05$) (Figure 4E,F). On the other hand, SBSE35 and SBSE60 demonstrated cytotoxic effect on HaCaT cells, beginning at concentrations of 5 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$ ($p \leq 0.05$), respectively (Figure 4G,H).

Therefore, in order to compare the efficacy of the extracts under identical conditions, ELISA experiments for both

IL-6 and IL-8 were conducted at the same concentration (0.625-2.5 µg/mL), which exhibited no cytotoxicity.

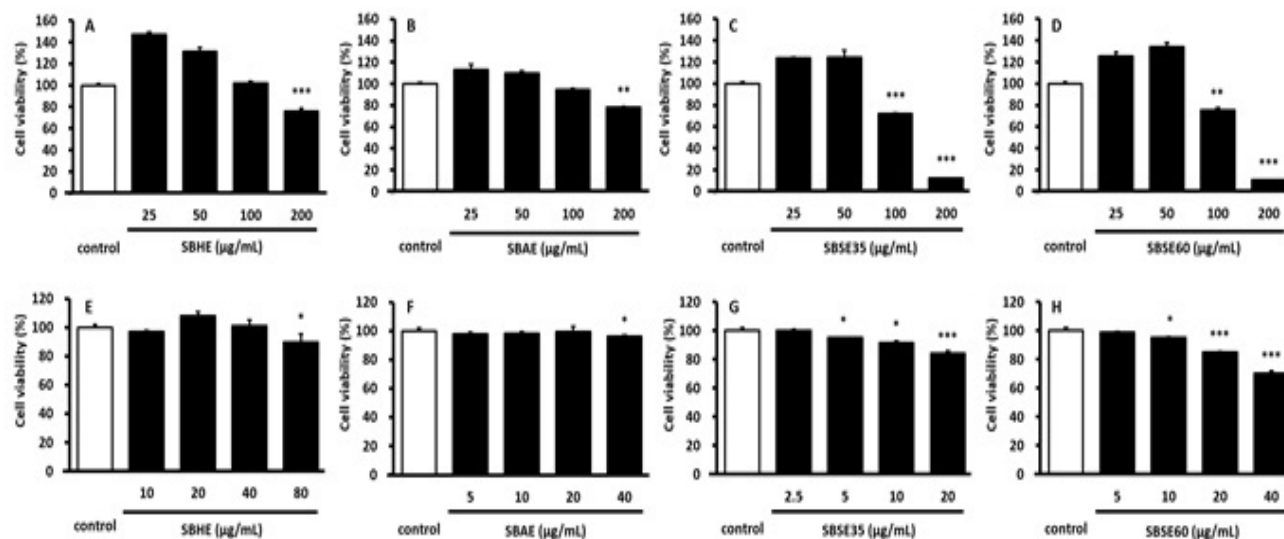


Figure 4. Cell cytotoxicity in RAW 264.7 cells and HaCaT cells treated with four extracts for 48 h: (A) Toxic concentration in cells treated with SBHE (25–200 µg/mL); (B) Toxic concentration in cells treated with SBAE (25–200 µg/mL); (C) Toxic concentration in cells treated with SBSE35 (25–200 µg/mL); (D) Toxic concentration in treated with SBSE60 (25–200 µg/mL); (E) Toxic concentration in cells treated with SBHE (10–80 µg/mL); (F) Toxic concentration in cells treated with SBAE (5–40 µg/mL); (G) Toxic concentration in cells treated with SBSE35 (2.5–20 µg/mL); (H) Toxic concentration in cells treated with SBSE60 (5–40 µg/mL). All data offered are the means and SD.; * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. control.

NO production: All extracts were evaluated for their ability to inhibit NO production (Figure 5). Among the extracts, SBSEs were particularly effective in inhibiting NO production. At 12.5 µg/mL concentration, SBSE35 and SBSE60 reduced NO levels by 60.37% and 58.88%, respectively, compared to the only LPS treatment group. Furthermore, at the highest concentration of 50 µg/mL, SBSE35 and SBSE60 demonstrated substantial reductions in NO production, achieving levels of 90.93% and 78.26%, respectively, versus the only LPS treatment

group after 48 h (Figure 5C). On the other hand, SBHE and SBAE only reduced NO production in RAW 264.7 cells by 55.15% and 52.92%, respectively, at 50 µg/mL after 48 h (Figure 5C). ANOVA revealed significant differences among groups treated with SBREs ($p \leq 0.001$). These results indicate that SBSEs exert a potent inhibitory effect on NO production compare to SBHE and SBAE ($p \leq 0.01$). Notably, SBSE35 exhibited the highest level of inhibition in NO production, highlighting its exceptional ability to suppress NO production.

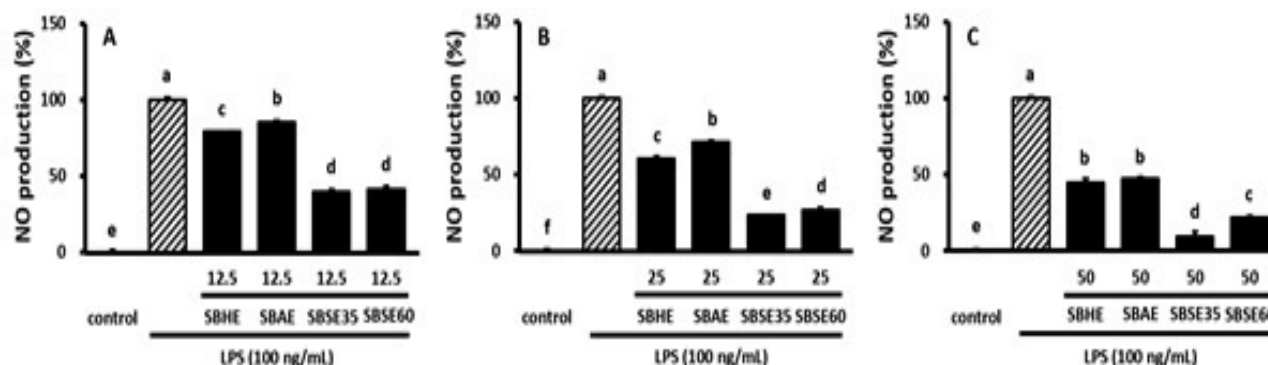


Figure 5. Change of NO production in RAW 264.7 cells treated with LPS and LPS + four extracts: (A) The reduction of NO in cells treated with SBHE, SBAE, SBSE35, and SBSE60 (12.5 µg/mL); (B) The reduction of NO in treated with SBHE, SBAE, SBSE35, and SBSE60 (25 µg/mL); (C) The reduction of NO in cells treated with SBHE, SBAE, SBSE35, and SBSE60 (50 µg/mL). All data offered are the means and SD. Same letters represent non-significant differences according to the post-hoc Tukey's HSD test ($p > 0.05$).

IL-6 production: IL-6 is a cytokine involved in the pathogenesis of inflammatory and autoimmune diseases (Nishi *et al.*, 2023; Kimura *et al.*, 2010). Hence, reducing overproduction of IL-6 is crucial for managing skin barrier disruption caused by inflammation. To investigate the effect of SBREs on the reduction of the inflammatory cytokine levels, we induced the inflammation reaction in HaCaT cells by TNF- α . SBHE and SBAE did not exhibit any reduction in IL-6 production but rather increased the levels of IL-6 in HaCaT cells (Figure 6A,B,C) ($p \leq 0.05$). However, when HaCaT cells were treated with SBSEs, it was confirmed that IL-6 was statistically significantly decreased ($p \leq 0.05$) (Figure 6A,B,C). Treatment of TNF- α with SBSE35 and SBSE60 in HaCaT cells for 48 h led to a decline in IL-6 levels as the concentration of extracts increased. At the concentration of 2.5 $\mu\text{g/mL}$ of SBSE35 and SBSE60, IL-6 production was reduced by 45.24% and 28.76%, respectively, compared to the control induced by TNF- α . ANOVA showed significant differences in inhibitory effect of SBREs on IL-6 production ($p \leq 0.001$).

IL-8 production: It has been reported that a lot of IL-8 chemokine is produced in patients with various

inflammatory diseases (Liao *et al.*, 2023). Thus, it is important to inhibit the levels of IL-8 in order to alleviate inflammatory conditions. In our study, HaCaT cells were stimulated with TNF- α , resulting in an increase in IL-8 production. To investigate the effect of SBREs on regulating IL-8 secretion in HaCaT cells, we performed an ELISA for IL-8. We confirmed the group treated with TNF- α statistically significantly increased IL-8 production compared to the untreated group. As a result of IL-8 ELISA, SBHE and SBAE incurred the rise of IL-8 production. In other words, SBHE and SBAE showed a slight decrease in IL-8 at 0.625 $\mu\text{g/mL}$ and 1.25 $\mu\text{g/mL}$ ($p \leq 0.05$) (Figure 6D,E), but an increase in IL-8 production in a concentration-dependent manner ($p > 0.05$) (Figure 6D,E,F). Conversely, SBSEs showed the efficacy of reducing the levels of IL-8 as the concentration of extracts was elevated (Figure 6D,E,F). At the concentration of 2.5 $\mu\text{g/mL}$ of SBSE35 and SBSE60, IL-8 production was reduced by 38.36% and 10.00%, respectively, compared to the control induced by TNF- α . ANOVA revealed significant differences in suppressive effect of SBREs on IL-8 production ($p \leq 0.001$).

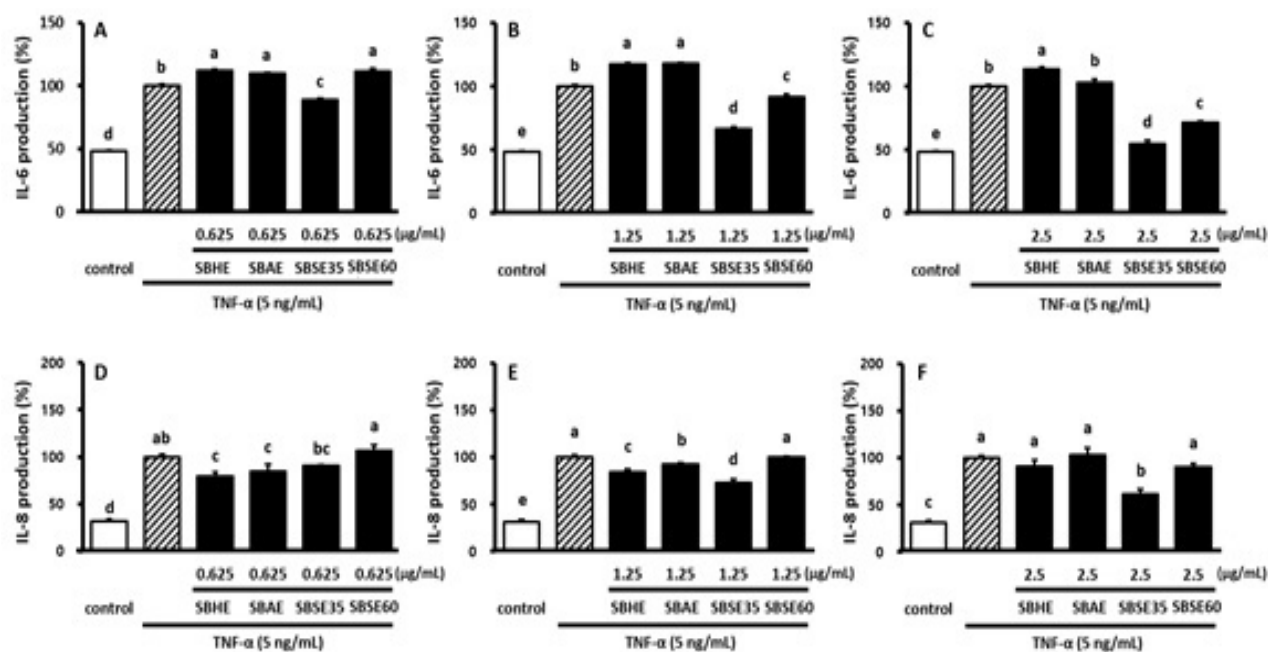


Figure 6. Change of IL-6 and IL-8 production in HaCaT cells treated with TNF- α and TNF- α + four extracts: (A) Change of IL-6 production in cells derived from human keratinocytes treated with SBHE, SBAE, SBSE35, and SBSE60 (0.625 $\mu\text{g/mL}$); (B) Change of IL-6 production in cells treated with SBHE, SBAE, SBSE35, and SBSE60 (1.25 $\mu\text{g/mL}$); (C) Change of IL-6 production in cells treated with SBHE, SBAE, SBSE35, and SBSE60 (2.5 $\mu\text{g/mL}$). (D) Change of IL-8 levels in cells treated with SBHE, SBAE, SBSE35, and SBSE60 (0.625 $\mu\text{g/mL}$); (E) Change of IL-8 levels in cells treated with SBHE, SBAE, SBSE35, and SBSE60 (1.25 $\mu\text{g/mL}$); (F) Change of IL-8 levels in cells treated with SBHE, SBAE, SBSE35, and SBSE60 (2.5 $\mu\text{g/mL}$). All data offered are the means and SD. All data offered are the means and SD. Same letters represent non-significant differences according to the post-hoc Tukey's HSD test ($p > 0.05$).

DISCUSSION

All extracts exhibited significant DPPH radical (relatively hydrophobic) and ABTS⁺ radical (hydrophilic and lipophilic) scavenging activity. In the DPPH experiment, the DPPH radical scavenging abilities of SBHE and SBSE35 at a concentration of 200 µg/mL were found to be 95.37% and 92.98%, respectively ($p \leq 0.05$). However, at concentrations of 25-100 µg/mL, SBSE35 exhibited better DPPH radical scavenging activity than SBHE ($p \leq 0.05$). Based on the IC₅₀ values, it can be concluded that among SBREs, SBSE35 (IC₅₀ = 42.72 µg/mL) demonstrated the best DPPH radical scavenging activity. Since the scavenging abilities of the extracts for DPPH radicals and ABTS⁺ radicals were different, the concentration used in the ABTS assay was one-tenth that of the DPPH assay. In all tested concentrations in the ABTS assay, SBSE35 showed the highest ABTS⁺ radical scavenging ability. Therefore, based on the results of these two radical scavenging experiments, it can be confirmed that SBSE35 possesses the strongest anti-oxidant power. In the SOD assay, no significant differences were observed among the four extracts at a concentration of 50 µg/mL, but as the concentration increased, significant differences became apparent. However, it was concluded that even at a concentration of 400 µg/mL, SBHE and SBSE35 did not exhibit a significant difference, indicating similar SOD-like activity. As the result of the SOD assay did not show a trend consistent with the results of radical scavenging activities, it can be inferred that the compounds responsible for SOD-like activity are distinct from the compounds involved in radical scavenging.

We confirmed the anti-bacterial effect of only SBSEs against *S. aureus*. *S. aureus*, which is a Gram-positive, is a bacterium widely distributed in nature, especially on the skin and nasal cavity (Moreno-Grúa *et al.*, 2020). It is associated with various infections such as otitis media, pneumonia, food poisoning, and atopic dermatitis (Zhou *et al.*, 2023; Andersen *et al.*, 2015; Alminderej *et al.*, 2021; Shimamori *et al.*, 2020; Demessant-Flavigny *et al.*, 2023). However, it is important to note that the human body harbors both beneficial and pathogenic microbes, and maintaining a balanced microbial ecosystem is essential (Panthee *et al.*, 2022). Therefore, excessive anti-bacterial activity can have unintended consequences and side effects. Given that SBSE35 (MIC = 200 µg/mL) has moderate anti-bacteria effect, it is believed that it can contribute to preventing bacterial colonizations, infections, and the release of superantigens by *S. aureus*, avoiding adverse effects when used as an additive in cosmetics and topical drug.

We demonstrated that only SBSEs could lower the levels of IL-6 and IL-8. The classical mechanism of polyphenols assumes that the anti-oxidant capacity

required transfer of electron from the polyphenol structure to the radical (Lamien-Meda *et al.*, 2008; Gebicki *et al.*, 2021). Polyphenols exhibit anti-inflammatory activity as well as anti-oxidant activity, generally (Afonso *et al.*, 2020). Although we confirmed the presence of polyphenols in all four extracts by applying the Folin-ciocalteu method (Table 1), SBHE and SBAE did not decrease IL-6 production, but rather increase the IL-6 cytokine levels ($p \leq 0.05$) and also exhibited in a concentration-dependent increase in IL-8 production. In contrast, SBSE35 reduced the production of IL-6 and IL-8 ($p \leq 0.05$). SBSE60 decreased IL-6 production ($p \leq 0.05$). IL-6 plays a crucial role in regulating Th17/Regulatory T cell (Treg) (Kimura *et al.*, 2010). Balanced Th17 cell and Treg are crucial for immune homeostasis, and if the balance is disrupted, inflammatory diseases are triggered (Kiselova *et al.*, 2006; Cardoneanu *et al.*, 2022). Also, it is reported that IL-8 correlates with angiogenesis, neutrophil activation, tumorigenicity, metastasis of tumor, and dry skin (Ghazy *et al.*, 2023; Waugh *et al.*, 2008; Murata *et al.*, 2021). In addition, excessive production of NO is known to act as a vasodilator and promote an inflammatory response (Fan *et al.*, 2023). In our study, the supernatant of RAW 264.7 cells stimulated with LPS and treated with 50 µg/mL of SBSE35 reduced NO production by 90.93% compared to control stimulated with LPS. It is deduced that the inflammatory response can be alleviated due to the decrease in the substances that induce the vasodilation, thereby suppressing the migration of cells and substances involved in various immune responses at the site of skin lesions. It is imperative to note that SBHE and SBAE pose a safety concern as they elevate inflammatory cytokine and chemokine. Consequently, SBSE35, which demonstrates effective regulation of NO, IL-6, and IL-8, holds significant potential for application in various industries aimed at improving human health, avoiding adverse effects.

Based on the assessment of anti-oxidant, anti-bacterial, NO assay and ELISA (IL-6 and IL-8), SBSE35 exhibited superior effects in terms of anti-oxidative stress, anti-bacterial properties, and anti-inflammatory activity compared to SBHE and SBAE. This superiority can be attributed to the extraction of specific components with exceptional biological activities, facilitated by the unique properties of supercritical fluid. In other words, supercritical fluids with gas-like and liquid-like characteristics can continuously change their density, enabling efficient extraction of bioactive compounds (Uwineza *et al.*, 2020; Cooper *et al.*, 2009; Okumura *et al.*, 2001). In particular, when comparing the efficacy of SBSE35, and SBSE60, SBSE60 demonstrated lower physiological activities than SBSE35. This disparity could be attributed to the degradation of bioactive ingredients, which exhibit excellent effects, caused by high temperatures (60°C) during supercritical fluid

extraction, similar to the destruction of heat-sensitive components by hydrothermal extraction (Cossuta *et al.*, 2008). Moreover, the supercritical carbon dioxide fluid at 60°C exhibits a lower solvent density compared to that at 35°C, leading to a decrease in solvating power, while the vapor pressure of the compounds increases. However, based on the results of *in vitro* assays, this paper suggests that the strong solvating power due to higher density is deemed more crucial for the extraction of bioflavonoids than the increase in vapor pressure of the compounds. (Reverchon *et al.* 2006). Therefore, it is believed that the extraction of more active ingredients, without degradation, can be achieved by conducting the extraction process near the critical temperature of carbon dioxide (relatively low temperature) (Quitério *et al.*, 2022).

To clearly interpret the experimental results of this study, future research should incorporate quantitative analysis of the bioactive constituents contained in the extracts. Furthermore, conducting anti-oxidant, anti-bacterial, and anti-inflammatory experiments using the active ingredients would enable the determination of which specific components are responsible for the anti-oxidant, anti-bacterial, and anti-inflammatory effects exhibited by the extracts.

Conclusion: In the current work, we tried to find the optimal extraction method. To investigate, four types of extracts were obtained by three different extraction methods. We demonstrated SBSE35 had a powerful radical scavenging activity and significant SOD-like activity. Moreover, only SBSE35 had moderate anti-bacterial activity, while SBHE and SBAE had no anti-bacterial effect against *S. aureus*. Furthermore, we demonstrated that SBSE35 remarkably suppresses inflammation *in vitro* by reducing NO, IL-6, and IL-8. Based on these results, we concluded that SBSE35 holds great abilities as a valuable natural resource and a highly promising agent in the fields of cosmetics and pharmaceuticals, particularly for its anti-inflammatory properties.

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