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RESEARCH ARTICLE

Enhancement of Anti-skin inflammatory Activities of *Scutellaria baicalensis* using an Alkaline Reduced Water Extraction Process

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Abstract WE (water extraction at 100°C for 24 h), EE (70% ethanol extraction at 80°C for 24 h), and AWE (alkaline reduced water extraction at 100°C for 24 h) were performed. Nitric oxide production from macrophages of AWE extracts at 1.0 mg/mL was decreased to 3.1 μmol. Secretion of PGE_2 from human fibroblasts was also reduced to 496.47 pg/mL at 1.0 mg/mL of AWE extracts with UV irradiation. Anti-skin inflammatory activities were enhanced by extraction of 8.894 mg/g more baicalein, the major bioactive component in *Scutellaria baicalensis,* using alkaline reduced water (AWE), compared to 1.157 mg/g for WE and 2.215 mg/g for EE. Improvement was caused by better and more elution of both hydrophobic and hydrophilic substances using alkaline reduced water and also by a synergistic effect between the antioxidant and anti-inflammatory activities of AWE extracts.

Keywords: *Scutellaria baicalensis*, baicalein, alkaline reduced water, anti-skin inflammatory activity, antioxidative activity

Introduction

Scutellaria baicalensis is the root of a perennial herbaceous plant from the Labiatae family that originates from China. This root has been introduced to Korea as a medicinal plant, and is now cultivated nationwide (1). The main components of *S. baicalensis*, (baicalin, baicalein, and wogonin) are widely used in traditional herbal medicine for anti-skin inflammatory activity. This root is known to have anti-inflammatory, anti-pyretic, anti-bacterial and diuretic effects (2-6). The main ingredients of *S. baicalensis*, including baicalin and baicalein, are fat-soluble substances. Thus, ethanol and other organic solvents must be used for extraction. However, organic substances, such as ethanol, are not desired in cosmetic development. This has lead to recent interest in water, and research on functional water with different manufacturing and treatment methods has increased (7).

As a solution for effective *S. baicalensis* extraction, alkaline reduced water, which differs from normal water, is a functional type of water that is known as 'Water of the Wonder' (8). Alkaline reduced water was first made in Japan for drinking. Functional water includes electrolyzed alkaline reduced water, acidic water, and ionic water. The uses of functional water vary based on characteristics, and functional water is also used for sterilization and disinfection. Alkaline reduced water can be manufactured using different methods, the most common being electrolysis. It was reported that the minerals generated at the negative electrolytic pole cause the structure of electrolyzed alkaline reduced water to become hexagonal, which provides greater strength (8). This structure allows water to easily deliver hydrogen, the active component, by penetrating cells or other micro-substances, and the hexagonal structure facilitates absorption and excretion of substances. Additionally, alkaline reduced water has a higher oxygen content than other functional waters and contains a small amount of the essential minerals necessary for living organisms and stimulation of metabolism (9).

The effects of alkaline reduced water include elimination

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of free radicals due to the strong reducing power of the hydrogen that is generated in the water using an electric device, an improvement in hyperlipidemia, and an atopic dermatitis effect (7,8,10). This water is also used for hydrotherapy to eliminate the free radicals that accelerate oxidation. Thus, hydrotherapy is an auxiliary method for treating diseases. Alkaline reduced water is frequently used to treat chronic disease (11).

The distinguishing characteristics of alkaline reduced water include size of water clusters (or water molecule groups) of 54-60 Hz (the unit of water clusters). This is tiny size of water clusters were absorbed in the body. In general, one cluster has a group of 10-16 water molecules. This cluster size of alkaline water is smaller than the 280 Hz size of acidic water, and also smaller than the sizes of general distilled water (100 Hz), rain water (118 Hz), and even mineral water (94 Hz) (12). Therefore, the small size of alkaline water facilitates absorption, extraction, and dissolution through rigid plant cell walls of *S. baicalensis,* or other natural substances (13). Additionally, permeation is also easier for alkaline reduced water than for regular distilled water because the smaller cluster size has less surface tension and can penetrate dehydrated body tissues more effectively. Therefore, alkaline reduced water can increase both the efficiency of extraction and the capacity to break down lipids and fatty acids, such as cholesterol (14,15). A previous study on alkaline reduced water demonstrated an anti-skin inflammatory effect, including atopic dermatitis effects (8). Therefore, extraction of both water-soluble and fat-soluble substances may be possible (16). Therefore, in this work, alkaline reduced water was used as an extraction solvent to improve extraction of biologically active substances from *S. baicalensis*. An increase in anti-skin inflammatory activities was obtained.

Materials and Methods

Cell cultures and sample preparation The cell lines used were mouse macrophage RAW 264.7 cells (KCLB 40071; Korean Cell Line Bank, Seoul, Korea) and human fibroblast CCD-986sk cells (KCLB 21947; Korean Cell Line Bank). Media used for culturing cells were RPMI 1640 (Gibco; Bend, OR, USA), gentamycin sulfate (G1914; Sigma, St. Louis, MO, USA), and HEPES buffer (90909C; Sigma) enriched with 10% fetal bovine serum (FBS; Gibco). *S. baicalensis* was purchased at Daegwang Pharmacy (Chuncheon, Korea) and was ground to a size of 2-3 mm using a grinder (HMF-1000A; Hanil Electric, Seoul, Korea) before use. Alkaline water for extraction was generated at the negative terminal during electrolysis (AK-8000; Nexus Co., Incheon, Korea). The amount of alkaline reduced water used for the process was 10x the weight of ground *S. baicalensis*. Hot water extraction was performed at 100°C for 24 h using alkaline reduced water or distilled water (W0003; Samchun Pure Chemical Co., Pyeongtaek, Korea), and at 80°C for 24 h using ethanol (02860; Sigma). Obtained extracts were filtered through 20-25 μm filter paper (Whatman, Fairfield, CT, USA), then condensed using a rotary vacuum evaporator (Rotary Vacuum Evaporator N-N series; Eyela, Tokyo, Japan). Extracts were freeze dried using a lyophilizer (TFD series; IlShinBioBase, Dongduchun, Korea) before use (17).

Measurement of cell toxicity Cell cytotoxicity was measured using the MTT method (18). Human fibroblast CCD-986sk cells (KCLB 21947; Korean Cell Line Bank) that were cultured at 1.0×10^6 cells/well in 96-well plate (353274 384; BD FALCON, Colorado Springs, CO, USA), and cultured for 24 h. Then, extracts of *S. baicalensis* from alkaline reduced water extraction or 70% ethanol extraction were injected into each well at different concentrations (0.2-1.0 mg/mL), and cells were cultured in a CO_2 incubator (CB150; Binder, Tuttlingen, Germany) for 24 h. MTT solution (88417; Sigma) at a concentration of 5 μg/mL was added to each well, and the supernatant was reduced after 4 h. Then, 10 μL of acid-isopropanol (0.04 N HCl in isopropanol, W292907; Sigma) was added to each well, and the absorbance was measured at 565 nm using a microplate reader (M1000 PRO; Infinite, Mannedorf, Switzerland).

Measurement of antioxidant activities The DPPH radical scavenging activity was measured using a modified Dietz assay (19). First, 80 μL of each sample (100°C *S. baicalensis* hot water, 80°C *S. baicalensis* ethanol, and 100°C *S. baicalensis* alkaline reduced water extracts), ascorbic acid (1043003, 465119; Sigma), and baicalein (465119; Sigma), which is an indicator of *S. baicalensis* activity, were mixed with 200 μL of 0.1 mM DPPH (dissolved in ethanol, D9132; Sigma) and the extraction solvent (0.5 mg/mL) in a 96-well plate (353274 384; BD FALCON). The mixture was left in a dark room for 20 min at 25°C. The absorbance of the mixture was measured at a wavelength of 525 nm using a microplate reader (EMax Endpoint ELISA Microplate Reader, Sunnyvale, CA, USA). The result was calculated using the DPPH radical scavenging activity (%) equation as:

$$\text{DDPH radical scavenging activity (\%)} = \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \times 100$$

Measurement of nitric oxide (NO) production Measurement was performed using a modified Green assay with mouse macrophage RAW264.7 cells (20). First, 1.0×

10^6 cells/mL were placed into a 96-well plate (353274 384; BD FALCON) with RPMI 1640 (31800022; Gibco) containing 100 U/mL of penicillin (A1593; Sigma), 100 μg/mL of streptomycin (85886; Sigma), and 10% (v/v) FBS (10437028; Gibco). Macrophages were then cultured for 2 h. *S. baicalensis* alkaline reduced water, *S. baicalensis* ethanol, and *S. baicalensis* hot water extracts at different concentrations (0.2-1.0 mg/mL) were inoculated into the cultured cells, which were then cultured for an additional 1 h. Afterwards, LPS (1 μg/mL, L2630; Sigma) was added to this mixture, followed by culturing at 37°C and 5% CO_2 in an incubator (CB150; Binder) for 24 h. When culturing the cells, the amount of NO generated was measured and used as a control. Resveratrol (R5010; Sigma) was used as a positive control, and baicalein (465119; Sigma) was used as a standard. Then, 50 μL of the cell culture supernatant was obtained using centrifugal separator (Combi 514R; Hanil Science Medical., Daejeon, Korea) and allowed to react with 50 μL of Griess reagent (G4410; Sigma) for 5 min before the absorbance of the mixture was measured at 540 nm using a microplate reader (EMax). $NaNO_2$ (35273; Sigma) was used as a standard.

Measurement of prostaglandin E_2 (PGE_2) production Production of PGE_2 was measured using a Correlate-EIA™ Prostaglandin E_2 kit Assay (R&D Systems Inc., Minneapolis, MN, USA). Approximately 5.0×10^5 cells/mL of mouse macrophage RAW264.7 cells (KCLB 40071; Korean Cell Line Bank) was incubated in a 96-well plate (353274 384; BD FALCON) for 24 h, then the cells were then treated with different concentrations (0.2-1.0 mg/mL). Afterwards, the mixtures were allowed to react for 4 h, then 1 μg/mL of LPS (L2630; Sigma) was added before the macrophages were incubated at 37°C in CO_2 in an incubator (CB150; Binder) for 18 h. The medium from this incubation was used as a sample for an EIA kit (KGE004B; R&D Systems Inc.) to measure the amount of PGE_2.

HPLC analysis of extracts using different extraction solvents HPLC was used to compare the peak profiles of alkaline-reduced water, distilled water, and 70% ethanol extracts. Extracts and standards of *S. baicalensis* and baicalein were dissolved in methanol (34860; Sigma) for HPLC analysis at 100 ppm. The solutions were then filtered through a 0.2 μm syringe filter (ALG422A; Gilson Scientific Ltd., Luton, UK). HPLC was performed using a Bio-Tek 500 series (BioTek; Winooski, VT, USA), a Bio-Tek 522 controller pump, a Bio-Tek HPLC 535 UV Detector (277 nm), and a Prevail C18 column (Prevail C18 5 μm 4.6×250 mm, A-99301; Alltech, Nicholasville, KY, USA). Acetonitrile (34967; Sigma) (A) and triple distilled water (W0006; Samchun Pure Chemical Co.) (B) containing 1% acetic acid (320099; Sigma) were used as the mobile phase at 1 mg/mL. Proportions of components during the gradient elution were 30% A and 70% B for 15 min, 65% A and 35% B for 25 min, and 30% A and 70% B for 45 min (16).

Statistical analysis Statistical analysis was performed on data from 3 replicates. The mean value of test results was obtained using the SAS (Statistical Analysis System) program (SAS Institute, Cary, NC, USA) and statistical analysis was used to determine significant differences ($p<0.05$).

Results and Discussion

Comparison of extraction yields using different solvents Extraction yields for distilled water, 70% ethanol, and alkaline reduced water are shown in Table 1. The highest yield of 21.8% (w/w) was obtained using electrolyzed alkaline reduced water. A yield of 20.3% was estimated for 70% ethanol, followed by 19.2% for hot distilled water extraction. This result was similar to results for fat-soluble substance extraction where increases from a minimum of 2% to a maximum of 5% with a non-polar solvent were reported (21), probably caused by the small cluster size of alkaline reduced water of 54-60 Hz that allowed the water to easily penetrate the cell wall (13, 21).

Use of natural substances extracted using alkaline reduced water can replace substances extracted using other organic solvents, which are not desirable for cosmetic purposes. The major biologically active components in *S. baicalensis*, hydrophobic baicalin and baicalein, can be extracted more easily using alkaline water rather than using ethanol.

Effect of extraction solvents on cytotoxicity The cell toxicity of each extract against human fibroblasts is shown in Fig. 1. The ethanol extract showed the highest cell toxicity of 15.6%. In general, the lowest cytotoxicity was observed for extracts from alkaline water. Ethanol extracts showed the highest cytotoxicity values, even when the

Table 1. Comparison of the extraction yields of *S. baicalensis* using different extraction processes

Extraction process	Extraction yields (%)*
WE[1)]	19.2±0.48[A4)]
EE[2)]	20.3±0.17[B]
AWE[3)]	21.8±0.56[C]

[1)]WE, *S. baicalensis* from water extraction at 100°C
[2)]EE, *S. baicalensis* from 70% ethanol extraction at 80°C
[3)]AWE, *S. baicalensis* from alkaline reduced water extraction at 100°C
[4)]Mean values±SD from triplicate independent experiments are shown; means with different letters (A-C) within the same concentration are significantly different at $p<0.05$.

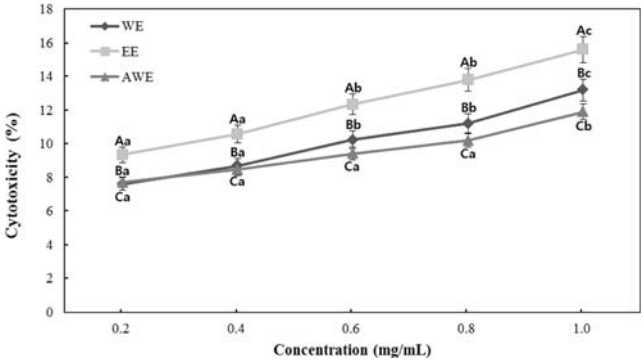

Fig. 1. Cytotoxicity of *Scutellaria baicalensis* extracts against human skin fibroblast CCD-986 cells under different extraction conditions. WE, *S. baicalensis* from water extraction at 100°C; EE, *S. baicalensis* from 70% ethanol extraction at 80°C; AWE, *S. baicalensis* from alkaline reduced water extraction at 100°C; mean values±SD from triplicate independent experiments are shown; means with different letters (A-C) within the same concentration are significantly different at $p<0.05$, and means with different letters (a-c) within the same sample are significantly different at $p<0.05$.

extraction yield from ethanol was similar to the yield from alkaline water (the highest extraction yield). Thus, alkaline water eluted both hydrophobic and hydrophilic components, but lesser amounts of hydrophobic substances since more toxic hydrophobic substances were extracted using 70% ethanol. Furthermore, there were no significant differences ($p<0.05$) between hot water and alkaline reduced water extracts, supporting the hypothesis that harmful substances were only extracted using 70% ethanol. Moreover, pure water can not be used to elute hydrophobic baicalin, which also results in a decreased biological activity of extracts using distilled water. For the anti-inflammatory activity, the low cell toxicity effect of *S. baicalensis* is an important characteristic when comparing this material to other natural substances (22,23).

Measurement of antioxidant activities To verify the increased anti-inflammatory activities of *S. baicalensis* alkaline reduced water extracts, antioxidant activities were determined (Table 2). Higher antioxidant activities, based on DPPH free radical scavenging, impacted the anti-inflammatory effect (24). Ascorbic acid, a positive control, had the maximum DPPH free radical scavenging activity of 80%, followed by 66% for baicalein (a main component of *S. baicalensis*), 61% for alkaline reduced water extracts, 57% for 70% ethanol extracts, and 50% for hot water extracts. Baicalein is known to be a highly antioxidant fat-soluble substance, and the high DPPH radical scavenging ability of the alkaline reduced water extracts, as well as the high level of baicalein extraction, a fat-soluble extract, is believed to correspond to a high antioxidative activity. In agreement with this study, the *S. baicalensis* methanol extract reported by Kim (25) showed a higher value than

Table 2. Estimation of free radical scavenging activities of *S. baicalensis* using different extraction processes

Extraction process	The DPPH radical scavenging activity (%)*
WE[1)]	50.28±0.31[A6)]
EE[2)]	57.39±0.28[B]
AWE[3)]	61.06±0.38[C]
Baicalein[4)]	66.07±0.29[D]
Ascorbic acid[5)]	79.84±0.19[E]

[1)]WE, *S. baicalensis* from water extraction at 100°C
[2)]EE, *S. baicalensis* from 70% ethanol extraction at 80°C
[3)]AWE, *S. baicalensis* from alkaline reduced water extraction at 100°C
[4)]Baicalein, standard
[5)]Ascorbic acid, positive control
[6)]Mean values±SD from triplicate independent experiments are shown; means with different letters (A-E) within the same concentration are significantly different at $p<0.05$.

the 12.5% DPPH radical scavenging activity measured at the 500 μg/mL concentration in this study (25). Thus, baicalein, an indicator of *S. baicalensis* and a fat-soluble substance, does not have a high antioxidative activity, even when extracted using an organic solvent. However, substances obtained from AWE of *S. baicalensis* demonstrated higher antioxidative activities than substances extracted using hot water ethanol. This increased antioxidative activity is attributed to a strong synergistic effect between the antioxidative activity of baicalein in *S. baicalensis* and the strong antioxidative activity of alkaline reduced water.

Measurement of NO production in macrophages The anti-inflammatory activity was verified by measuring NO production in mouse microphage RAW264.7 cells. The NO production ability was verified by studying *S. baicalensis* extracts obtained using different solvents and by investigating the effects of LPS treatment. Resveratrol, a positive control, and baicalein, a standard, were used to compare experimental groups.

The results of comparative NO production are shown in Fig. 2. Differences were verified by comparison of results from the resveratrol-administered group (control) with sample groups. The LPS-treated *S. baicalensis* ethanol extract group exhibited 7.12 μmol nitric oxide production when using a 1.0 mg/mL extract concentration as a standard. The LPS-treated *S. baicalensis* alkaline reduced water extract exhibited a 6.71 μmol nitric oxide production. The experimental group that was not treated with LPS exhibited in a high 3.92 μmol NO production with the *S. baicalensis* hot water extract. NO production values for the ethanol and alkaline reduced water extracts were 3.61 and 3.25 μmol, respectively. Comparing nitric oxide production between the LPS-treated group and the non-LPS-treated group indicated that the *S. baicalensis* alkaline reduced water extract reduced the 6.71 μmol nitric oxide production to 3.25 μmol, verifying a decrease in nitric oxide generation.

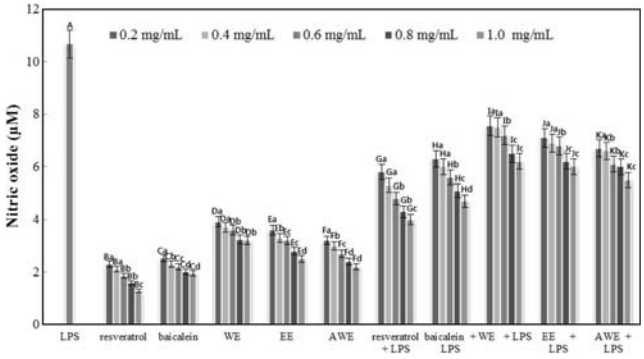

Fig. 2. Comparison of NO Production in *S. baicalensis* extracts under different extraction conditions. WE, *S. baicalensis* from water extraction at 100°C; EE, *S. baicalensis* from 70% ethanol extraction at 80°C; AWE, *S. baicalensis* from alkaline reduced water extraction at 100°C; resveratrol, positive control; baicalein, standard;mean values±SD from triplicate independent experiments are shown; means with different letters (A-C) within the same concentration are significantly different at $p<0.05$, and means with different letters (a-c) within the same sample are significantly different at $p<0.05$.

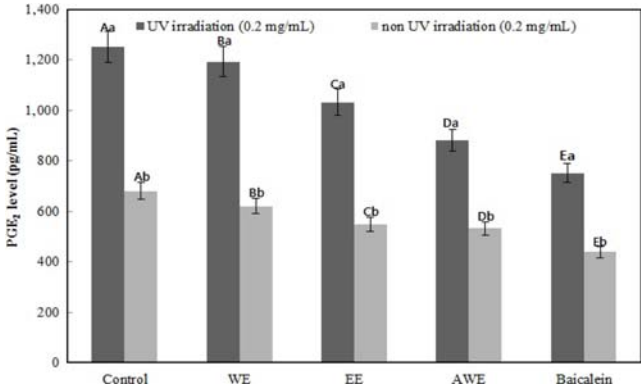

Fig. 3. Effect of *S. baicalensis* extracts on production of PGE_2 in macrophages. Control, no treatment; WE, *S. baicalensis* from water extraction at 100°C; EE, *S. baicalensis* from 70% ethanol extraction at 80°C; AWE, *S. baicalensis* from alkaline reduced water extraction at 100°C; baicalein, standard; mean values±SD from triplicate independent experiments are shown; means with different letters (A-C) within the same concentration are significantly different at $p<0.05$, and means with different letters (a-c) within the same sample are significantly different at $p<0.05$.

In addition, the group treated with LPS-only was compared with the group treated with LPS and the *S. baicalensis* extracted using different solvents. All experiments reduced the NO production by more than 2.4 μmol. Thus, the antioxidative activity of *S. baicalensis* extracted using alkaline reduced water lowered NO production to 3.25 μmol, which was lower than values for the hot water and ethanol extracts. Therefore, alkaline reduced water extracts exhibited increased antioxidative effects (26-28). The strong antioxidative activities of baicalin and baicalein in *S. baicalensis* prevented the oxidative damage caused by free radicals and the skin barrier effect, and induced the skin immune reaction that substantially reduces NO production (2-6,29,30).

Measurement of prostaglandin E_2 (PGE_2) production

PGE_2 production was measured to determine the anti-inflammatory activity of *S. baicalensis* extracted using alkaline reduced water. PGE_2 production showed the ability of each sample to inhibit PGE_2 production. Measured PGE_2 production at a low extract concentration of 0.2 mg/mL is shown in Fig. 3. Measurements of PGE_2 production at each concentration (based on Fig. 3) are shown in Table 3.

Activation of anti-inflammatory activity by a low alkaline reduced water extract concentration (0.2 mg/mL) resulted in PGE_2 production of 881.25 pg/mL with UV treatment, and 532.19 pg/mL without UV treatment. Thus, reductions in PGE_2 production of 370 and 150 pg/mL with and without the UV treatment, respectively, compared with controls, demonstrated the anti-inflammatory effect. In addition, the hot water extract reduced PGE_2 production by 58 and 60 pg/mL with and without UV treatment, respectively. The

Table 3. Effects of *S. baicalensis* extracts on PGE_2 production in human fibroblasts with and without UV exposure

Extraction process	PGE_2 level					
	UV irradiated	non-UV irradiated	UV irradiated	non-UV irradiated	UV irradiated	non-UV irradiated
	0.2 mg/mL	0.2 mg/mL	0.6 mg/mL	0.6 mg/mL	1.0 mg/mL	1.0 mg/mL
Control[1)]	1250.21±0.18$^{Aa6)}$	682.17±0.18^{Ab}				
WE[2)]	1192.19±0.18^{Ba}	621.41±0.25^{Bb}	1154.91±0.31^{Bc}	601.20±0.28^{Bd}	1115.42±0.32^{Be}	592.00±0.21^{Bf}
EE[3)]	1032.80±0.41^{Ca}	548.42±0.19^{Cb}	981.70±0.42^{Cc}	542.42±0.33^{Cd}	942.47±0.24^{Ce}	532.41±0.15^{Cf}
AWE[4)]	881.25±0.26^{Da}	532.19±0.43^{Db}	812.30±0.18^{Dc}	508.53±0.17^{Dd}	753.74±0.11^{De}	498.00±0.26^{Df}
Baicalein[5)]	752.41±0.31^{Ea}	439.81±0.21^{Eb}				

[1)]Control, non treatment
[2)]WE, *S. baicalensis* from water extraction at 100°C
[3)]EE, *S. baicalensis* from 70% ethanol extraction at 80°C
[4)]AWE, *S. baicalensis* from alkaline reduced water extraction at 100°C
[5)]Baicalein, standard
[6)]Mean values±SD from triplicate independent experiments are shown; means with different letters (A-E) within the same concentration are significantly different at $p<0.05$, and means with different letters (a-f) within the same sample are significantly different at $p<0.05$.

ethanol extract reduced the amount of PGE_2 production by 217 and 133 pg/mL with and without the UV treatment, respectively. Thus, the anti-inflammatory activity of alkaline reduced water extracts was higher than that of other extracts. However, the positive control baicalein reduced PGE_2 production of the control group by 500 and 243 pg/mL with and without UV treatment, respectively, the largest reductions in PGE_2 production, indicating the better anti-inflammatory activity of the artificial substance baicalein. The *S. baicalensis* alkaline reduced water extract, a natural substance, showed less anti-inflammatory activity than baicalein. However, the alkaline reduced water extract showed a higher anti-inflammatory effect than the other types of extracts.

As the sample concentration increased linearly, the PGE_2 production decreased (Table 3), demonstrating an increase in the anti-inflammatory activity. Without UV treatment and at the highest extract concentration of 1.0 mg/mL, samples showed reductions in PGE_2 production of; hot water extract, 90.17 pg/mL; ethanol extract, 149.76 pg/mL; alkaline reduced water extract, 184.17 pg/mL; and baicalein 269.56 pg/mL. The highest anti-inflammatory activity was observed for baicalein, followed by the alkaline reduced water, ethanol, and hot water extracts. Furthermore, when PGE_2 production was increased using UV treatment, the hot water extract reduced PGE_2 production by 134.79 pg/mL, the ethanol extract reduced PGE_2 production by 307.74 pg/mL, and the alkaline reduced water extract reduced PGE_2 production by 496.47 pg/mL. More obvious sample differences in the anti-inflammatory effect with UV treatment were evident than for samples not treated with UV. In addition, when PGE_2 production was increased using UV stimulation, reductions in PGE_2 production for extracts were 134.79 pg/mL for the hot water, 307.74 pg/mL for the ethanol, and 496.47 pg/mL for the alkaline reduced water extracts, indicating the increased anti-inflammatory effect of UV treatment compared with decreases without UV treatment.

These results might be attributable to a UV-induced peptide that inhibits secretion of cytokines (IL-1 and TNF) and future PLA_2 activation, or to final treatment with arachidonic acid, a direct inflammatory substance that is secreted from phospholipids and is an inflammatory mediator that is generated during inflammation to accelerate skin damage (worsening of inflammatory diseases and atopic dermatitis by oxidative stress and oxidative substances). However, the anti-inflammatory effect was assumed to be increased because oxidative substances were removed by strong antioxidants, such as baicalin and baicalein in *S. baicalensis* (31). A more detailed study of the anti-skin inflammatory mechanism of extracts should be performed with consideration of RNA and protein expression levels related to anti-inflammation genes.

Comparison of extract HPLC profiles Comparison of HPLC chromatograms used to measure changes in the content of useful active ingredients obtained from different extraction methods are shown in Fig. 4. Chromatograms for baicalein (the main ingredient of *S. baicalensis*), the hot water, the 70% ethanol, and the alkaline reduced water extracts are shown in Fig. 4A-4D. In Fig. 4, *S. baicalensis* extraction were peaked at around the 11.2 min retention time, and sample of *S. baicalensis* values of 1.157, 2.215, and 8.894 mg/g were measured for B, C and D, respectively.

Baicalein in *S. baicalensis* is a fat-soluble substance, the content of which was expected to be highest in the 70% ethanol extract. However, the content was 8× higher in the alkaline reduced water extract, presumably due to the small size of the alkaline reduced water cluster (approximately 60 Hz). This size enabled fast absorption, extraction, and solubility through the cell walls of *S. baicalensis*, thus increasing the baicalein content in alkaline reduced water

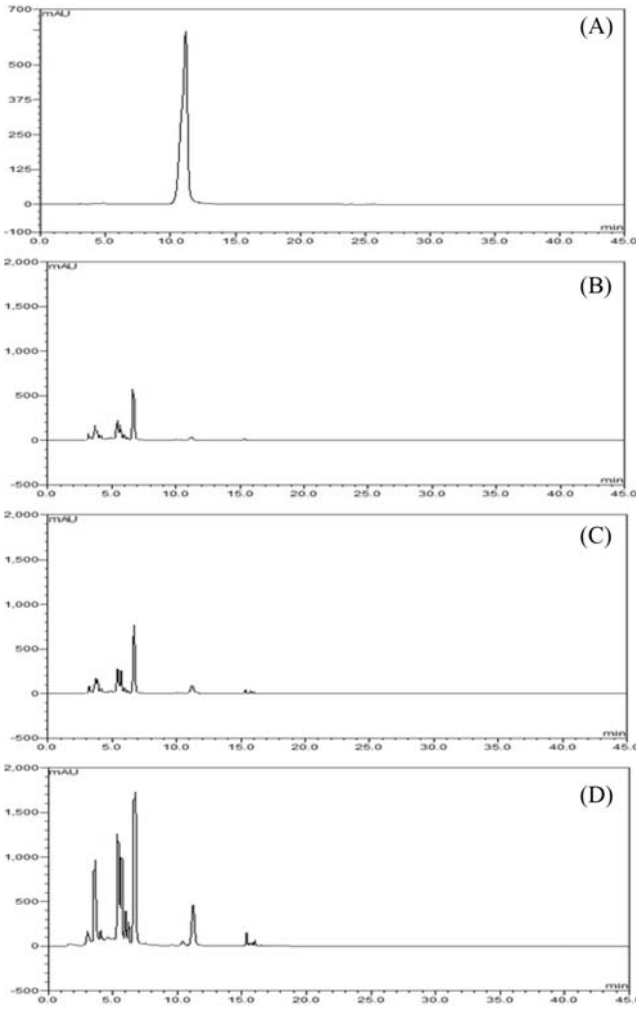

Fig. 4. Comparison of HPLC peaks of *S. baicalensis* extracts using different extraction Solvents. (A) Baicalein 100 ppm-standard; (B) 100°C water extracts; (C) 80°C 70% ethanol extracts; (D) 100°C alkaline reduced water extracts

extracts (12). Additionally, elution of baicalein indicated that alkaline reduced water was able to extract hydrophobic substances.

Quantitative analysis using *S. baicalensis* 70% methanol extracts with ultrasonic treatment was conducted to determine retention times. The time was 7.5 min for baicalin and 15.6 min for baicalein. *S. baicalensis* hot water extracts showed retention times of 7.65 min for baicalin and 11.65 min for baicalein, indicating the lower molecular weight of baicalin relative to baicalein (32,33).

Baicalein, which is hydrolyzed to glucuronic acid and baicalein, had a high peak that appeared prior to the 11.2 minute peak of baicalein. This peak was due to low Mw flavonoids and polyphenols, including baicalin and glucuronic acid. The extract amount presumably increased due to AWE (34).

The amount of baicalein, a main bioactive substance in alkaline reduced water extracts, other minor bioactive substances, such as the low M_W substances baicalin, glucuronic acid, and other molecules, They were higher than amounts from conventional WE and EE. Alkaline reduced water can be used to extract both water-soluble and fat-soluble substances. Complex substances in extracts demonstrated the synergistic effect between the activities of fat-soluble substances and water-soluble substances, which increased the skin anti-inflammatory activities of alkaline reduced water extracts, compared to hot water and 70% ethanol extracts.

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Disclosure The authors declare no conflict of interest.

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