Inhibitory effect of *Salvia plebeia* leaf extract on ultraviolet-induced photoaging-associated ion channels and enzymes

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Abstract. In traditional Korean/Asian medicine, *Salvia plebeia* R.Br. (*S. plebeia*) leaves are used to treat inflammatory diseases, including dermatitis, cough, asthma and toothache. Recently, *S. plebeia* leaves have been applied in skin care, as they promote skin lightening and elasticity. Therefore, the present study investigated the anti-aging effects of *S. plebeia* leaf methanolic extract and its fractions (dichloromethane, ethylacetate and n-butanol). The results of a whole-cell patch clamp analysis indicated that the methanolic extract mediated ultraviolet (UV)-induced photoaging-associated ion channels, transient receptor potential vanilloid 1 (TRPV1) and calcium release-activated calcium channel protein 1 (ORAI1) channel activity in HEK293T cells overexpressing TRPV1 or ORAI1 and STIM1. Electrophysiological analysis revealed that the butanol fraction inhibited capsaicin-induced TRPV1 (84±8% at -60 mV/86±1% at 100 mV at 100 μg/ml) and ORAI1 (87±2% at -120 mV at 100 μg/ml) currents. Furthermore, the dichloromethane and hexane fractions inhibited tyrosinase activity by 32.4±0.69 and 22.6±0.96% at 330 μg/ml, respectively. Furthermore, the ethylacetate and butanol fractions inhibited elastase activity by 65.2±1.30 and 31.7±1.23% at 330 μg/ml, respectively. Tyrosinase and elastase, which are UV-induced photoaging-associated enzymes, regulate skin pigmentation and wrinkle formation, respectively. The results of the present study indicated that *S. plebeia* leaves may be a novel treatment for UV-induced photoaging.

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Abbreviations: TRPV1, transient receptor potential vanilloid 1; ORAI1, calcium release-activated calcium channel protein 1; AP-1, activate the nuclear activator protein; MMPs, metalloproteinases; MMP-1, collagenase; MMP-9, gelatinase B; ET, endothelin; STIM, stromal interaction molecule; ER, endoplasmic reticulum; PKC, calcium-dependent protein kinase C

Key words: Salvia plebeia, transient receptor potential vanilloid 1, calcium release-activated calcium channel protein 1, tyrosinase activity, elastase activity, ultraviolet-induced photoaging, calcium channel

Introduction

The elderly population is rapidly growing, which has led to an increase in dermatological problems, including eczema, wrinkling and pigmentation. Skin aging is associated with several factors, including genetics, environment, hormonal changes and metabolic processes. Although all of these factors can contribute to skin aging, the environment, particularly solar ultraviolet (UV) radiation is a prominent mediator of skin aging (1-3). UV radiation exposure activates fibroblast and keratinocyte growth factor receptors, which activate the nuclear activator protein (AP)-1. This produces matrix metalloproteinases (MMPs), including collagenase (MMP-1), gelatinase B (MMP-9) and stromelysin-1 (MMP-3), which degrade collagen and elastic fibers, thereby inducing skin wrinkling and laxity (4,5). Neutrophils also mediate sun-induced skin aging. Following UV radiation, neutrophils infiltrate the skin and release dermal elastase, a key elastolytic enzyme (6-8). Following skin exposure to UV radiation, the keratinocytes produce endothelins (ETs), which induce melanocyte proliferation and tyrosinase production.

Tyrosinase is a key melanogenic enzyme that controls skin pigmentation (9,10). Moreover, photoaged skin is functionally degenerated. The differentiation of keratinocytes, a component of the epidermis, is critical for the skin barrier function. As the skin ages, inhibition of keratinocyte differentiation disturbs the skin barrier, leading to allergen invasion, immunological reactions and inflammation. Therefore, skin barrier alterations induce inflammatory skin disease (11).

Intracellular calcium signaling mediates diverse skin processes, including barrier formation, cell differentiation, melanogenesis and tumor progression. These processes are regulated by several ion channels, including the transient receptor potential vanilloid 1 (TRPV1), which is associated with skin aging. TRPV1 is a nonselective cation channel with high calcium permeability, which responds to temperature, pH and vanilloids including capsaicin (12-14). TRPV1 in the skin mediates heat shock and UV radiation-induced MMP-1 expression (15). Furthermore, UV radiation, growth factors and cytokines induce MMP-mediated deterioration of dermal collagen, leading to skin wrinkling via inflammation, skin aging and tumor invasion (5,16,17). Calcium also mediates epidermal melanin production in melanocytes. Of note, TRPV1 expression is functionally associated with calcium influx (18). In addition, the stimulation of TRPV1 by capsaicin or heat delays skin barrier recovery (19). Furthermore, capsaicin-mediated TRPV1 activation in keratinocytes produces inflammatory cytokines, suggesting a role in inflammatory skin disease (20). Calcium release-activated calcium modulator 1 (ORAI1) channels also perform important functions in the skin. Stromal interaction molecule (STIM)-gated ORAI1 channels are highly selective calcium channels that are activated by endoplasmic reticulum (ER) calcium depletion (21,22). ORAI1 is essential for immune-cell function; however, a recent study indicated that ORAI1-mediated calcium uptake regulates keratinocyte proliferation and differentiation (23). ORAI1 calcium influx also regulates neutrophil activation, which triggers elastase release (24,25) and endothelin-1 (ET-1)-mediated melanogenesis (9). Therefore, modulators of TRPV1 and ORAI1 channels may represent novel therapeutic agents for UV-induced photoaging.

Salvia plebeia R.Br. (Lamiaceae) (*S. plebeia*) is an annual or biennial plant that grows in numerous countries, including China, Korea and India. It is used as a traditional medicine to treat inflammatory diseases, including hepatitis, cough and hemorrhoids (26). Pharmacological investigations have revealed that of the leaf extract of *S. plebeia* has anti-oxidative (27), anti-tyrosinase (28), anti-cancer (29) and hepatoprotective effects (26). The active components of *S. plebeia* comprise flavonoids (30,31), diterpenoids (32), lignin (33), aliphatic compounds (34) and sesquiterpenoids (35). Although *S. plebeia* leaves are used to prevent and treat skin aging-associated conditions, including inflammation, pigmentation and wrinkle formation, the exact underlying physicochemical and molecular mechanisms of its biological activity have remained elusive.

Therefore, the present study investigated whether *S. plebeia* leaves regulate the calcium concentration, a critical mediator of skin aging signaling pathways, via TRPV1 and ORAI1 channels. Its effects on tyrosinase and elastase, which are the major downstream enzymes in UV-induced photoaging, were also evaluated.

Materials and methods

Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except 3,5-bis(trifluoromethyl)pyrazole (BTP2), which was obtained from Tocris (Bristol, UK). Stock solutions of capsaicin (10 mM), inositol triphosphate ($InsP_3$; 20 mM), BTP2 (10 mM), and 4-(3-chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide (BCTC; 10 mM) were prepared in dimethyl sulfoxide (DMSO). All stock solutions were stored at -20˚C.

Extraction. The dried and pulverized leaves of *S. plebeia* (200 g) were purchased from Omniherb drug store (Seoul, Korea) and extracted twice with 70% methanol (1 l) under reflux to obtain the methanolic extract. After evaporation of the solvent, the solid extracted material was obtained (yield, 43.0 g), which was then successively partitioned with n-hexane (200 ml), dichloromethane (200 ml), ethylacetate (100 ml) and n-butanol (100 ml) to obtain the corresponding fractions with yields of 1.8, 1.5, 2.4 and 5.0 g, respectively. Dried extracts were diluted with DMSO to prepare stock solutions (30 and 100 mg/ml) of each extract.

Gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS (Agilent GC/Pegasus 4D, Agilent Technologies, Santa Clara, CA, USA) analyses of the dichloromethane and ethylacetate fractions were performed under the following conditions: Injector split ratio, 10:1; injection volume, 1.0 μl dichloromethane solution; injector temperature, 250˚C; column, Agilent J&W DM-5MS (30 m x 0.25 mm ID x 0.25 μm; Agilent Technologies); carrier gas, helium; flow rate, 1.0 ml/min; oven temperature programming, 50˚C (3 min)→10˚C/min→320˚C (18 min). For the MS analysis, the following conditions were used: Ion source, electron impact; 230˚C; analyzer, quadrupole; 150˚C; mass range, 35-800 *m/z* (mass-to-charge ratio).

Cell culture. HEK293T cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified incubator containing 20% O_2 and 10% CO_2 (as 3.7 g/l $NaHCO_3$ requires 10%). All media were supplemented with 10% fetal bovine serum (WelGENE, Daegu, South Korea), 100 U/ml penicillin and 100 g/ml streptomycin (Thermo Fisher Scientific, Inc.). The cells were subcultured every 2-3 days.

Transfection of TRPV1, ORAI1 and STIM1. For the patch clamp studies, the cells were transferred in 25-cm^2 culture flasks (Thermo Fisher Scientific, Inc.) 1 day prior to transfection. HEK293T cells were transiently transfected with a mammalian expression vector carrying human (h)TRPV1 or hORAI1 and hSTIM1 using the Turbofect transfection reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. In all transfection studies, the cells were co-transfected with a plasmid vector coding the enhanced green fluorescent protein (pEGFP-N1) to sort out the transfected cells. To record TRPV1 currents (I_{TRPV1}),

hTRPV1 and pEGFP-N1 were co-transfected at a ratio of 9:1. To record ORAI1 currents (I_{ORAI1}), hORAI1, hSTIM1 and pEGFP-N1 were triple-transfected at a ratio of 4.5:4.5:1, respectively. Human TRPV1 (hTRPV1) plasmid (pcDNA5/FRT) was generously donated by Dr Sung Joon Kim (Seoul National University, Seoul, Korea). Human ORAI1 (hOrai1) and human STIM1 (hSTIM1) were purchased from Origene Technologies (Rockville, MD, USA). hSTIM1 and hORAI1 cDNA were subcloned into pcDNA3.1 (Thermo Fisher Scientific, Inc.). Experiments were performed within 24-36 h of transfection.

Electrophysiology. Conventional whole-cell patch clamp methods were used to measure the hTRPV1 and hORAI1 currents at room temperature. Recordings were acquired using an Axopatch 700B amplifier interfaced with a Digidata 1440A (Molecular Devices, Sunnyvale, CA, USA). The recorded data were digitized at 10 kHz and low-pass filtered at 5 kHz using the pCLAMP 10.4 software (Molecular Devices). Patch pipettes were produced from thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL, USA) using a horizontal Flaming Brown micropipette puller (model P-97). Pipette tips were fire-polished to a resistance of 2-3 MOhm to facilitate gigaseal formation (Narishige, East Meadow, NY, USA). The transfected cells were transferred to a perfusion chamber (Warner Instruments, Hamden, CT, USA) mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan). Bath solutions were perfused at 3 ml/min. To measure the hTRPV currents, voltage ramp protocols ranging from -100-100 mV over 100 msec were applied every 20 sec at a -10-mV holding potential. For hORAI1, ramp-like pulses from -130-70 mV over 100 msec were applied every 30 sec at a -10-mV holding potential to obtain the current-voltage association. The junction potentials were canceled prior to patch formation and pipette capacitances were compensated for electronically after gigaseal formation. All the voltage and current trace data were analyzed using the Clampfit software 10.4, Prism 6.0 (GraphPad, Inc., La Jolla, CA, USA) and Origin 8.0 (MicroCal, Northampton, MA, USA).

For the hTRPV1 whole-cell patch clamping, the external solution contained 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 5 mM D-glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4). The internal solution contained 140 mM CsCl, 10 mM NaCl, 5 mM EGTA, 3 mM adenosine triphosphate magnesium salt and 10 mM HEPES (pH 7.2). The capsaicin-evoked activity was observed by applying capsaicin (1 μM) in the external solution after confirming the basal current. For the hORAI1 whole-cell patch clamp recording, the external solution contained 3.6 mM KCl, 10 mM $CaCl_2$, 1 mm $MgCl_2$, 5 mM D-glucose, and 10 mM HEPES (pH 7.4). The internal solution contained 130 mM Cs-glutamate, 20 mM 1,2-bis (o-aminophenoxy) ethane-*N*,*N*,*N*',*N*'-tetracetic acid, 1 mM $MgCl_2$, 3 mM MgATP, 0.002 mM sodium pyruvate and 20 mM HEPES (7.2). To activate the hORAI1 currents, 20 μM $InsP_3$ was added to the internal solution.

Tyrosinase assay. The tyrosinase inhibitory activity of *S. plebeia* was measured using a modified method (36). In brief, a mixture of sodium phosphate buffer (pH 6.8; 0.066 M), L-3,4-dihydroxyphenylalanine (1.5 mM) and DMSO (10 mg/ml) was added to the enzyme solution (500 U/ml), and the mixture was incubated at 30˚C for 10 min. After enzyme inactivation using an ice bath, the optical density was measured using spectrophotometer (Ultraspec 2000; Amersham Pharmacia Biotech, NJ, USA) at a wavelength of 475 nm. Kojic acid (200 μg/ml) was used as a positive control.

Elastase assay. Inhibition of porcine pancreatic elastase was estimated using a modified method (37). In brief, a mixture of Tris-HCl buffer (pH 8.0; 0.1 M), *N*-succinyl-Ala-Ala-Ala-p-nitroanilide (1 mM) and sample solution (plant extract or fractions, 10 mg/ml in DMSO) was added to the enzyme solution (elastase in Tris-HCl buffer, 0.1 U/ml), followed by incubation at 25˚C for 20 min. After enzyme inactivation using an ice bath, the optical density was measured at a wavelength of 410 nm using an Ultraspec 200 spectrophotometer. Ursolic acid (200 μg/ml) was used as a positive control.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. The N values indicate the number of separate cells used in the experiment. Comparison tests were performed using one-way analysis of variance with Bonferroni's *post-hoc* comparison. $P<0.05$ was considered to indicate a statistically significant difference. Prism 6.0 software (GraphPad, Inc.) and Origin 8.0 (MicroCal) were used for statistical analyses.

Results

S. plebeia extracts inhibit the basal I_{TRPV1} and I_{ORAI1}. To evaluate whether TRPV1 and ORAI1 are affected by the *S. plebeia* leaf crude extract and fractions, whole-cell currents in HEK293T cells overexpressing TRPV1 or ORAI1 and STIM1 were measured. To evaluate the inhibitory effects of this herbal extract on the basal I_{TRPV1}, 1 mM capsaicin was used to activate I_{TRPV1}. After confirming the I_{TRPV1} steady state, 10, 30 or 100 μg/ml of the extract or fractions were added to the external solution. A total of 1 μM of the selective TRPV1 inhibitor BCTC was used as a positive control. Fig. 1A shows a typical data recording for the capsaicin-induced I_{TRPV1} and its inhibition by the butanolic fraction. The associated I-V curves at (a) the peak I_{TRPV1} and at (b) 10, (c) 30, and (d) 100 μg/ml butanolic fraction were achieved using a ramp-like pulse protocol from -100-100 mV (Fig. 1B). Fig. 1B further shows that the application of capsaicin produced a strong outward rectifying current potential reversal (0 mV). To analyze the fraction-induced inhibition, the normalized amplitudes of the fraction-treated currents (I/I_{con} x100%) were obtained at 100- and -60-mV clamp voltages. As shown in Fig. 1C, the level of I_{TRPV1} inhibition by the butanolic fraction at -60 mV was 5±11, 39±10 and 84±8% at 10, 30 and 100 μg/ml, respectively, and at 100 mV, it was 4±9, 37±4 and 86±1% at 10, 30 and 100 μg/ml, respectively.

To evaluate the inhibition of I_{ORAI1}, 20 μM $InsP_3$ was added to the internal solution in order to deplete ER calcium stores. When the ER calcium concentration drops, STIM1 binds to and activates ORAI1, and thereby allows for calcium entry. After obtaining the whole-cell configuration, an inwardly rectifying current with reversal potential at 50 mV was obtained

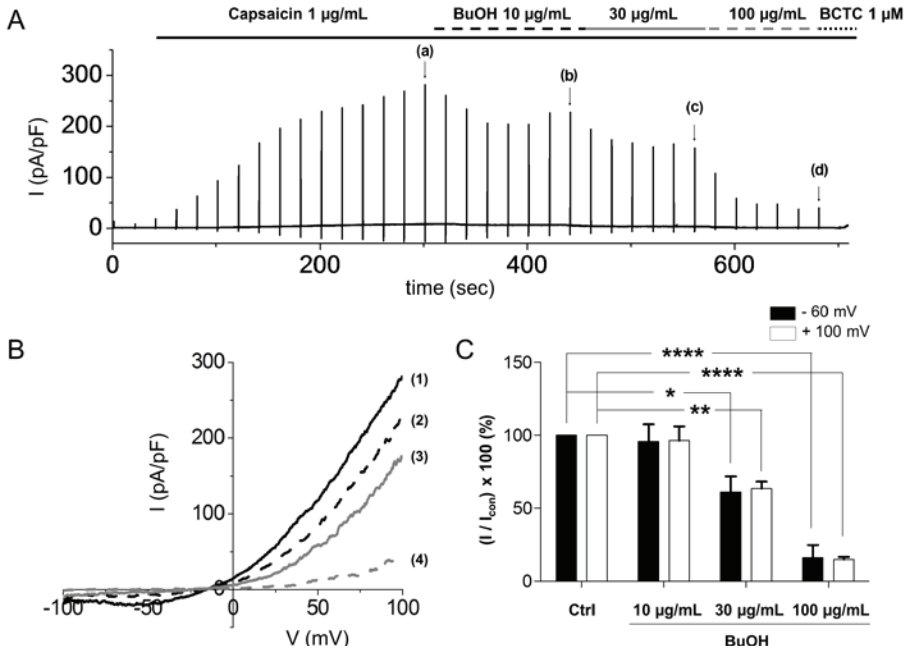

Figure 1. Effects of *Salvia plebeia* butanolic fraction on I_{TRPV1}. (A) Representative tracing of I_{TRPV1} inhibition by *S. plebeia* butanol fraction. After confirming I_{TRPV1} activation using 1 μM capsaicin, 10, 30 or 100 μg/ml butanolic fraction was applied to hTRPV1-overexpressing HEK293T cells. (B) Corresponding I-V association curve of control (a) peak current and (b) 10, (c) 30 and (d) 100 μg/ml butanolic fraction. (C) Summary of butanolic fraction-induced I_{TRPV1} inhibition rates at -60 and 100 mV. *P<0.05, **P<0.01 and ****P<0.0001 vs. control for each. I, current; V, voltage; Ctrl/Con, control; hTRPV1, human transient receptor potential vanilloid 1; BCTC, 4-(3-chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide; BuOH, butanolic fraction of *Salvia plebeia*.

in the ORAI1/STIM1 co-transfected cells. After confirming the steady state of the I_{ORAI1}, 10, 30 or 100 μg/ml extract or fraction of *S. plebeia* leaf was added to the bath solution to analyze the *S. plebeia*-mediated inhibition of ORAI1. BTP2 (10 μM), which is a selective ORAI1 inhibitor, was added to the external solution to confirm the basal current. As presented in Fig. 2A, the serial application of 10, 30 and 100 μg/ml of the butanolic fraction inhibited I_{ORAI1}. Fig. 2B shows the I-V association curve between the (a) control and butanolic fraction treatment at (b) 10, (c) 30 and (d) 100 μg/ml. At -120 mV, the butanolic fraction inhibited I_{ORAI1} by 31±5, 62±5 and 87±2% at 10, 30 and 100 μg/ml, respectively (Fig. 2C; n=6). The hexane fraction had a greater inhibitory effect on I_{ORAI1} than the butanolic fraction (Fig. 3); at -120 mV, the hexane fraction inhibited I_{ORAI1} by 56±2, 82±1 and 92±2% at 10, 30 and 100 μg/ml, respectively (Fig. 3C; n=7). The TRPV1 and ORAI1 inhibition rates are presented in Table I. The butanolic fraction had the strongest effect on I_{TRPV1} and I_{ORAI1}.

S. plebeia extracts inhibit t*yrosinase*. To verify the skin-lightening effects of the *S. plebeia* leaves tyrosinase assays were performed using the extract and fractions. Tyrosinase is a key enzyme that initiates melanogenesis. Decreased tyrosinase activity corresponds with reduced melanin production. kojic acid, a tyrosinase inhibitor, was used as a positive control (80.7±0.69%). As shown in Fig. 4, the dichloromethane (32.4±0.69%) and hexane (22.6±0.96%) fractions inhibited tyrosinase activity.

S. plebeia extracts inhibit elastase. Elastase regulates wrinkle formation by deteriorating and remodeling the extracellular matrix. Therefore, elastase assays can be used to evaluate the effect of *S. plebeia* on wrinkle formation. Ursolic acid, an elastase inhibitor, was used as a positive control (83.9±0.79%). As presented in Fig. 5, the ethylacetate and butanolic fractions inhibited elastase by 65.2±1.30 and 31.7±1.23%, respectively.

GC-MS

Molecular composition of S. plebeia extracts. As outlined in Fig. 6, the total ion GC-MS chromatogram of the most active dichloromethane fraction revealed phytol as a main compound (5.37%) in addition to minor sterol components, including 23-ethylcholest-5-en-3-ol (0.60%), stigmasterol (0.39%) and ergost-5-en-3-ol (0.24%). The ethyl acetate fraction contained hispidulin (4',5,7-trihydroxy-6-methoxyflavone) as a major constituent (30.4%) in addition to minor amounts of phenolic compounds (including 4-vinylguaiacol and 3-allyl-2-methoxyphenol).

Discussion

The use of natural products in the prevention and treatment of skin aging, particularly pigmentation and wrinkling, has aroused great interest. Solar UV radiation damage is of considerable importance in skin aging (1-3). Recently, *S. plebeia*, which is traditionally used to treat inflammatory diseases (26), has gained attention owing to its skin-whitening and elasticity effects. However, its anti-skin aging effect and the underlying biological mechanisms have remained elusive. Therefore, the present study examined the effects of *S. plebeia* leaves on the photoaging-associated ion channels TRPV1 and ORAI1, as

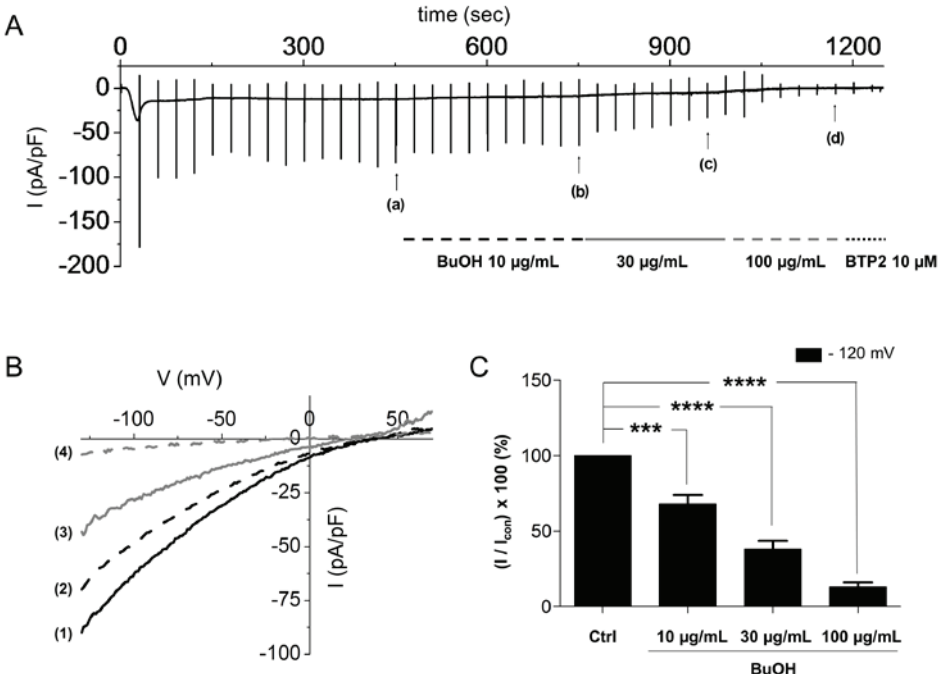

Figure 2. Effects of *Salvia plebeia* butanolic fraction on I_{ORAI1}. (A) Representative tracing of I_{ORAI1} inhibition by *S. plebeia* butanolic fraction. After confirming I_{ORAI1} activation using 20 μM inositol triphosphate, 10, 30 or 100 μg/ml butanolic fraction was applied to hORAI1- and stromal interaction molecule-overexpressing HEK293T cells. (B) Corresponding I-V association curve of (a) control current and (b) 10, (c) 30 and (d) 100 μg/ml butanolic fraction. (C) Summary of butanol fraction-induced I_{ORAI1} inhibition rates at -120 mV. ***P<0.001 and ****P<0.0001 vs. control for both. I, current; V, voltage; Ctrl/Con, control; hTRPV1, human transient receptor potential vanilloid 1; BTP2, 3,5-bis(trifluoromethyl)pyrazole; hORAI1, human calcium release-activated calcium modulator 1; BuOH, butanolic fraction of *Salvia plebeia*.

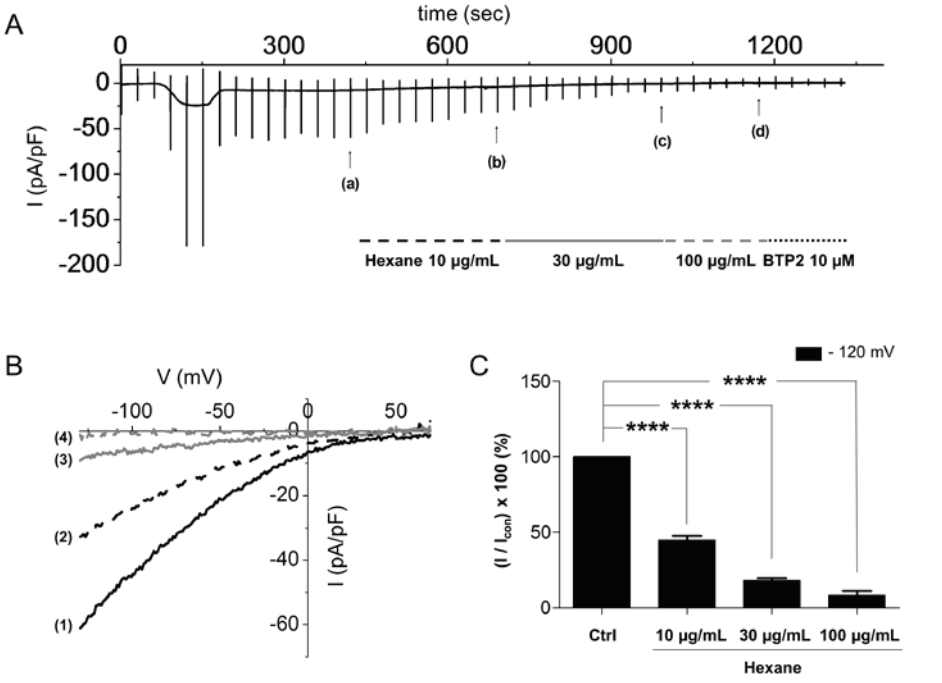

Figure 3. Effects of the *Salvia plebeia* hexane fraction on I_{ORAI1}. Representative tracing showing effect of hexane fraction on (A) I_{ORAI1} and (B) the corresponding I-V association curve at different times after treatment. (C) Summary of percentage change in I_{ORAI1} caused by hexane fraction at -120 mV. ****P<0.0001 vs. control. I, current; V, voltage; Ctrl/Con, control; BTP2, 3,5-bis(trifluoromethyl)pyrazole; hORAI1, human calcium release-activated calcium modulator 1; hexane, hexane fraction of *Salvia plebeia*.

well as on the photoaging-associated enzymes tyrosinase and elastase.

In keratinocytes, TRPV1 mediates UV-induced MMP-1, which destroys collagen. Following exposure to UV

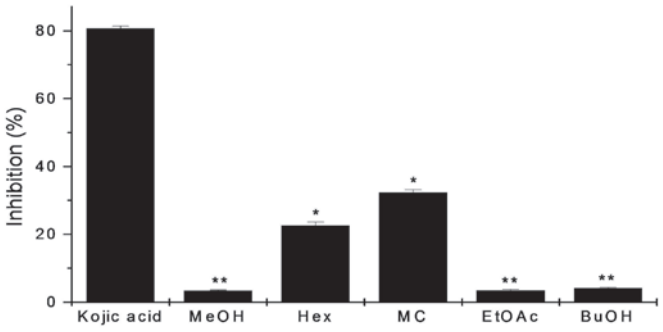

Figure 4. Inhibitory activity of *Salvia plebeia* extract and fractions on tyrosinase activity. MeOH, methanolic extract; MC, dichloromethane fraction; EtOAc, ethyl acetate fraction; BuOH, n-butanol fraction; Hex, hexanol fraction. Kojic acid was used as a positive control. *P<0.05 and **P<0.01 compared to kojic acid.

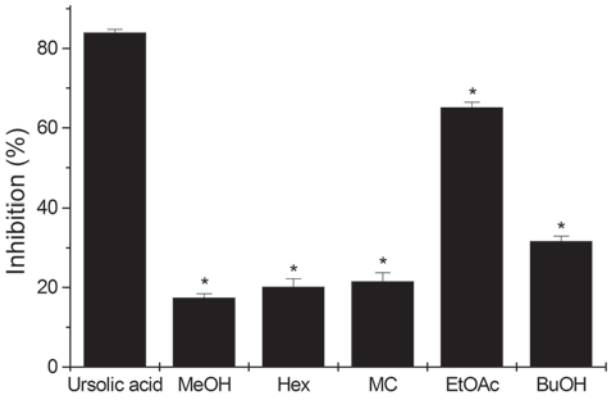

Figure 5. Inhibitory activity of *Salia plebeia* extract and fractions on elastase activity. MeOH, methanolic extract; MC, dichloromethane fraction; EtOAc, ethyl acetate fraction; BuOH, n-butanol fraction; Hex, hexanol fraction. Ursolic acid was used as a positive control. *P<0.05 compared to ursolic acid.

radiation, TRPV1 induces calcium influx, thereby activating calcium-dependent protein kinase C and promoting MMP-1 expression (15,38). A previous study suggested that TRPV1 is associated with melanogenesis due to changes in TRPV1 expression and calcium uptake (18). ORAI1, which regulates calcium uptake, regulates melanogenesis and neutrophil activation (9,24,25). Following UV radiation, the ORAI1-dependent calcium influx mediates ET-1-induced melanogenesis and the release of collagen and elastic fiber-degrading enzymes in neutrophils (9). In the present study, electrophysiological analysis indicated that the butanolic fraction (100 μg/ml) inhibited TRPV1 activity by 84±8 and 86±1% at -60 and 100 mV, respectively. Furthermore, -120 mV, the hexane and butanolic fractions (100 μg/ml each) strongly inhibited ORAI1 activity by 92±2 and 87±2%, respectively. Therefore, *S. plebeia* leaves inhibited the upstream signaling pathways regulating UV-induced skin wrinkle formation and pigmentation. Studies indicated that TRPV1 activation is involved in delayed skin barrier recovery and cutaneous inflammation (19,20). Therefore, *S. plebeia* may also prevent skin inflammation. In the present study, to determine whether the leaf extract and fractions of *S. plebeia* were able to inhibit tyrosinase and elastase, which are the mediators of UV photoaging, tyrosinase and elastase assays were performed. Tyrosinase regulates the synthesis of melanin, thereby determining skin pigmentation (10). Although the inhibitory efficacy was not strong, the dichloromethane and hexane fractions inhibited tyrosinase activity by 32.4±0.69 and 22.6±0.96%, respectively. Elastase activity has a pivotal role in UV-induced wrinkling (4,5) and the effect of *S. plebeia* on elastase was therefore assessed in the present study. The ethylacetate and butanolic fractions inhibited elastase by 65.2±1.30 and 31.7±1.23%, respectively; however, these fractions were less effective than ursolic acid, but had a relatively high efficacy. In addition, the GC-MS results revealed that the dichloromethane and ethyl acetate fractions contained phytol and hispidulin, respectively, as a main component. Phytol, a diterpene compound, has been reported to have a skin-whitening effect, which was determined by measuring tyrosinase promoter activity (39). To the best of our knowledge, the elastase inhibitory activity of hispidulin has not yet been reported. Furthermore, it has not been reported that phytol and hispidulin regulate the activity of TRPV1 and ORAI1 channels. Therefore, *S. plebeia* leaves may contain chemical constituents that directly inhibit enzyme activity associated with UV-induced photoaging.

In addition to its role in skin aging, TRPV1 is well known as a nociceptor activated by pain-producing stimuli, such as vanilloid compounds, moderate heat (43˚C) and low pH (<5.9) (12,40). TRPV1 is enriched in small-diameter, Aδ and C fiber sensory neurons and has a significant role in pain sensation and neurogenic inflammation (40-42). Activation of TRPV1 in sensory fibers results in the release of neuropeptides, such as substance P and calcitonin gene-related peptide (43). The released peptides induces the release of pro-inflammatory and vasoactive substances causing neurogenic inflammation, such as increased blood flow and edema in cells including keratinocytes, mast cells and fibroblast (43). Analysis of TRPV1 knockout mice demonstrated that TRPV1 has a functional role in pain transduction and neurogenic inflammation, as indicated by an increased threshold to noxious heat and decreased tissue swelling in inflammation (13,44,45). Therefore, studies have been performed to identify agents with the ability to inhibit TRPV1 channel activity for the development of novel analgesic therapeutics. ORAI1 is also a representative ion channel-regulating immune-cell activator. ORAI1 is distributed in T cells, B cells and mast cells, and ORAI1-mediated calcium signaling is essential to the immune response (46-49). Based on previously reported findings, T- and B-cell mediated immunity is severely impaired in human as well as mice in the absence of ORAI1 function (46,47,50). Therefore, much work has focused on the inhibition of ORAI1 channel activity to suppress undesirable immune responses. Also, *S. plebeia* has been traditionally used to treat inflammatory diseases such as hepatitis, cough, and hemorrhoids (26) and recently, pharmacological studies have demonstrated that the leaf extract has anti-inflammatory and anti-nociceptive activities (51,52). In addition, it was reported that its extract has an inhibitory effect on inflammatory arthritis in human rheumatoid synovial fibroblasts and a murine model (53), and in a mouse model of atopic dermatitis, its oral administration lessened atopic dermatitis symptoms and suppressed the expression of cytokines and chemokines (54). Thus, given these additional effects of

Table I. Effects of *Salvia plebeia* extract and organic fractions on I_{TRPV1} and I_{ORAI1} currents [$(I/I_{con})x100(\%)$].

	Clamp voltage (mV)								
	TRPV1						Orai1		
	-60			100			-12		
Group	*Salvia plebia* extract concentration (μg/ml)								
	10	30	100	10	30	100	10	30	100
Control	100	100	100	100	100	100	100	100	100
MeOH extract (n≤7)	203±49	268±80	278±66	138±8	162±11	164±15	90±1	73±2	33±6
n-BuOH fraction (n≤6)	95±11	61±10	16±8	96±9	63±4	14±1	69±5	38±5	13±2
CH_2Cl_2 fraction (n≤8)	157±16	261±49	330±84	119±3	140±12	139±21	84±2	51±6	26±5
EtOAc fraction (n≤6)	155±15	250±40	434±112	128±7	166±19	205±28	88±4	77±5	64±4
Hexane fraction (n≤7)	158±18	206±31	273±50	131±10	152±8	154±11	44±2	18±1	8±2

Results are normalized amplitudes of fraction-treated currents ($I/I_{con}x100\%$) measured at -60 and 100 mV clamp voltage for TRPV1 current and -120 mV clamp voltage for ORAI1 current, and are presented as the mean ± standard error of the mean. TRPV1, transient receptor potential vanilloid 1; ORAI1, calcium release-activated calcium modulator 1; I, current.

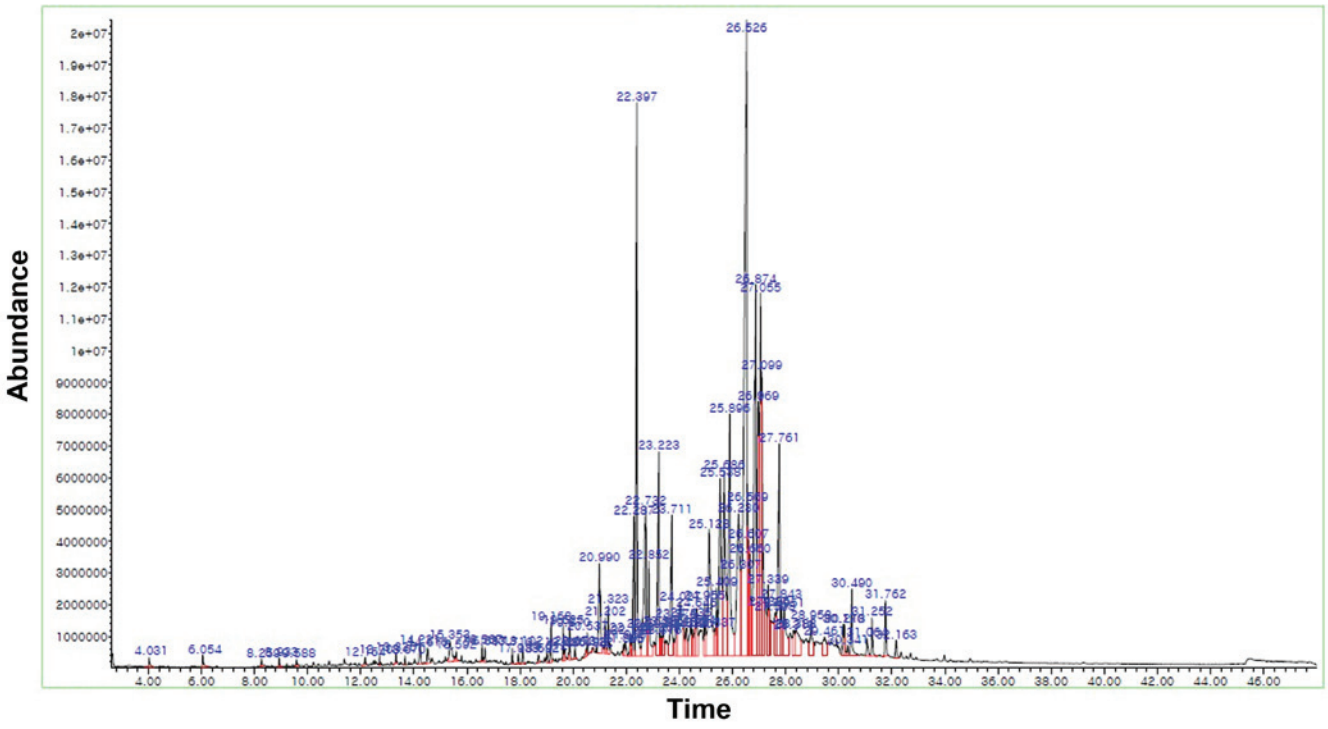

Figure 6. Total ion chromatogram of the dichloromethane fraction of *Salia plebeia* extract. Peaks having more than 95% quality are presented as follows: Rt (min)=22.40: phytol; 22.73: linolenic acid; 30.22: 0-tocopherol; 31.07: ergost-5-en-3-ol; 31.26: stigmasterol, 31.76; and 23-ethylcholest-5-en-3-ol.

S. plebeia, it may be applied not only as an anti-skin aging agent but also as an anti-inflammatory or analgesic agent.

In conclusion, the results of the present study suggested that *S. plebeia* leaves may have therapeutic effects on UV-induced photoaging mediated by inhibiting upstream signaling pathway-associated ion channels and downstream enzyme activity. These findings may explain the mechanism of the anti-skin aging effects of this herbal drug and suggest a novel application of this extract. Furthermore, the present study was the first to provide an electrophysiological assessment of the effects of *S. plebeia* leaves on TRPV1 and ORAI1 channels.

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