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Modulatory effects of the fruits of *Tribulus terrestris* L. on the function of atopic dermatitis-related calcium channels, Orai1 and TRPV3

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ABSTRACT

Objective: To examine the effects of *Tribulus terrestris* L. (*T. terrestris*) extract on the modulation of calcium channels to evaluate its use in topical agents for treatment of atopic dermatitis.

Methods: The 70% methanol extract of *T. terrestris* was prepared. Human HEK293T cells with over-expressed calcium release-activated calcium channel protein 1 (Orai1), transient receptor potential vanilloid 1, or transient receptor potential vanilloid 3 (TRPV3) were treated with *T. terrestris* extract. Modulation of ion channels was measured using a conventional whole-cell patch-clamp technique.

Results: *T. terrestris* extract (100 μg/mL) significantly inhibited Orai1 activity in Orai1-stromal interaction molecule 1 co-overexpressed HEK293T cells. In addition, *T. terrestris* extract significantly increased the TRPV3 activity compared with 2-Aminoethyl diphenylborinate (100 μmol/L), which induces the full activation of TRPV3.

Conclusions: Our results suggest that *T. terrestris* extract may have a therapeutic potential for recovery of abnormal skin barrier pathologies in atopic dermatitis through modulating the activities of calcium ion channels, Orai1 and TRPV3. This is the first study to report the modulatory effect of a medicinal plant on the function of ion channels in skin barrier.

1. Introduction

Atopic dermatitis (AD) is an allergic disease characterized by dry skin, intense itching, and recurrent skin inflammation [1]. The primary drivers of AD are still controversial; however, it seems clear that there is close interaction between impaired skin barrier functionality and an inappropriate immune response that induces cutaneous inflammation [2,3]. They are mutually reinforcing processes; disruption of the cutaneous barrier causes activation of keratinocytes, which release pro-inflammatory cytokines that attract T cells, particularly $CD4^+$ cells such as T-helper-2 (Th2) cells, and Th17 cells, ultimately leading to cutaneous inflammation [4,5]. Therefore, the therapeutic strategy is focused on a combination of skin barrier maintenance with emollients, and immune suppression therapy with topical corticosteroids or calcineurin inhibitors [1]. Although these therapeutic agents have greatly improved patient outcomes, the current treatment for allergic diseases including allergic rhinitis and asthma is still not ideal, and novel therapeutic strategies are required in the search for better drugs with safety and efficacy.

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Recently, three ion channels, calcium release-activated calcium channel protein 1 (Orai1), transient receptor potential vanilloid 1 (TRPV1), and transient receptor potential vanilloid 3 (TRPV3), have shed new light on potential targets in the treatment of inflammatory skin diseases. These ion channels have been shown to directly modulate epidermal proliferation, differentiation, barrier homeostasis, and inflammation [6–8]. Ca^{2+} influx through these channels eventually generates intracellular Ca^{2+} signaling that results in different outcomes dependent on the individual Ca^{2+} channel type; for example, lymphocyte activation through Orai1 [9], epidermal barrier formation and keratinocyte differentiation through TRPV3 [10,11], and itch generation through TRPV1 [12]. Therefore, a specific agonist/antagonist for each calcium channel is required for maintenance of skin barrier homeostasis and for treatment of dermatological diseases including AD.

Recently, increasing interest in various medicinal plants and their bioactive compounds has led to increased attention to their safety and efficacy in the treatment of allergic diseases. The fruits of *Tribulus terrestris* L. (*T. terrestris*) belonging to Leguminose are used traditionally in treatment of problems with the eye, cutaneous pruritus, edema, inflammation, and tracheitis, as well as to promote blood circulation and eliminate stasis [13,14]. *T. terrestris* fruit is also used as a tonic, aphrodisiac, analgesic, astringent, stomachic, antihypertensive, diuretic, lithontriptic, and urinary anti-infective agent [15–21]. *T. terrestris* fruit extract is used mainly for kidney disorders, as the fruit can remove gravel from the urine and treat kidney stones in the bladder [18]. In modern pharmacological studies, this plant has been reported to have a variety of protective effects on tacrine-induced liver toxicity [10], cisplatin-induced renal damage [19], cadmium intoxication-induced liver and kidney damage [20], oxalate-induced oxidative stress [21], and streptozotocin-induced diabetes [22]. This plant contains antifungal saponins [23], anthelmintic saponin [24], cytoprotective lignanamides [25], and anti-inflammatory N-trans-ρ caffeoyl tyramine [26].

Therefore, as a part of our ongoing research to find ion channel-modulating agents from natural sources [27,28], we examined the effect of *T. terrestris* fruit extract on the activities of ion channels Orai-1, TRPV1, and TRPV3, which are known to contribute to skin homeostasis [7]. To the best of our knowledge, this is the first electrophysiological study on whether a medicinal plant extract can modulate ion channel activity.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma–Aldrich (St. Louis, MO), unless otherwise stated. 3,5-bis(trifluoromethyl) pyrazole (BTP2) and 2-aminoethoxydiphenyl borate (2-APB, > 98% purity) were purchased from Tocris (Bristol, UK). Inositol 1,4,5,-triphosphate (InsP3) was purchased from Merck Millipore (Billerica, MA, USA). Stock solutions of capsaicin (10 mmol/L), 2-APB (50 mmol/L), BTP2 (10 mmol/L), allyl isothiocyanate (3 mmol/L), and 4-(3-chloro-2-pyridinyl)-N-(4-[1,1-dimethylethyl]phenyl)-1-piperazinecarboxamide (BCTC, 10 mmol/L) were prepared in dimethyl sulfoxide. A stock solution of InsP3 (20 mmol/L) was prepared in distilled H_2O. All stock solutions were stored at −20 °C.

2.2. Preparation of T. terrestris fruit extract

Dried fruits of *T. terrestris* were purchased from the Medicinal Materials Company (Kwangmyungdang Medicinal Herbs, Ulsan, Republic of Korea). The fruits (200 g) were extracted with methanol for 3 h and filtered through Whatman No.1 paper. The resulting product was freeze-dried (yield = 26%, *T. terrestris* fruit extract).

2.3. Cell culture

HEK-293T cells were purchased from American Type Cell Culture (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA) in a humidified incubator at 37 °C with 10% CO_2/20% O_2. All media contained 10% fetal bovine serum (WelGENE, Daegu, South Korea), 100 IU/mL penicillin, and 100 g/mL streptomycin (Life Technologies).

2.4. DNA constructs

Human TRPV1 (hTRPV1) plasmid (pcDNA5/FRT) was kindly provided by Dr. Sung Joon Kim (Seoul National University). Human Orai1 (hOrai1) and human stromal interaction molecule 1 (hSTIM1) were purchased from Origene Technologies (Rockville, MD, USA). hSTIM1 and hOrai1 cDNA was subcloned into pcDNA3.1 (Life Technologies). Human TRPV3 (pReceiver-M02) was purchased from Genecopoeia (Rockville, MD, USA). All constructs were confirmed by sequencing before transfection.

2.5. Transfection

For the whole-cell patch-clamp studies, hTRPV1 and hTRPV3 were transiently transfected into HEK293T cells using the Lipofectamine Plus reagent (Life Technologies) as described [27]. The *hOrai1* gene was co-transfected with STIM1 at a ratio of 1:1. Measurements were performed 24 h following transfection.

2.6. Electrophysiology

Electrophysiological recordings were performed using conventional whole-cell patch-clamp recordings. Experimental data were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and digitalized using Digidata 1440A (Molecular Devices) at 10 kHz. To reduce electrical noise, the data were further filtered by a low-pass filter at 5 kHz using pCLAMP 10.4 software (Molecular Devices). Micro electrodes (patch pipette) were fabricated with thin-wall borosilicate glass capillaries (World Precision Instruments, Sarasota, FL, USA) in four stages using a programmable horizontal puller (Model P-97, Shutter Instruments, Novato, CA, USA). Pulled patch pipettes were fire-polished to exhibit a resistance of 2.5–3.0 MΩ when immersed in extracellular solution. The transfected cells were transferred to a perfusion chamber (Warner Instruments, Hamden, CT, USA) mounted on the stage of an inverted

microscope (Nikon, Tokyo). Extracellular solutions were perfused to the bath chamber at 3 mL/min by gravity feed. Liquid junction potentials were nullified to zero before gigaseal formation. Pipette capacitances were compensated electronically after gigaseal formation. After confirming the whole-cell configuration, cell membrane capacitance was measured and then nullified using an Axopatch 200B amplifier. For measurement of TRPV1 and TRPV3 currents, voltage clamp protocols were applied every 20 s from −100 mV to 100 mV over 100 ms. Holding potential was applied for each current measurement to −10 mV for TRPV1 and 0 mV for TRPV3. For the hORAI1 current, ramp-like pulses from −130 to 70 mV over 100 ms were applied every 30 s at a holding potential of −10 mV to obtain the current–voltage (I–V) relationship. All voltage and current trace data were saved on a desktop computer and analyzed using Clampfit software 10.4, Prism 6.0 (GraphPad, La Jolla, CA, USA), and Origin 8.0 (Microcal, Northampton, MA, USA). All experiments were performed at room temperature.

2.7. Experimental solution for whole-cell patch clamp

Individual solutions were used for measurement of TRPV1, TRPV3, and Orai1 currents: (i) pipette solutions [140 mmol/L CsCl, 10 mmol/L NaCl, 5 mmol/L ethylene glycol tetraacetic acid (EGTA), 3 mmol/L adenosine 5′-triphosphate magnesium salt (MgATP), and 10 mmol/L 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) adjusted to pH 7.2] for current of TRPV1 (I_{TRPV1}) measurements; (ii) pipette solutions (140 mmol/L CsCl, 10 mmol/L EGTA, 4.85 mmol/L $CaCl_2$, 3 mmol/L MgATP, and 10 mmol/L HEPES adjusted to pH 7.2) for current of TRPV3 (I_{TRPV3}) measurements; (iii) pipette solutions (130 mmol/L Cs-glutamate, 20 mmol/L 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 1 mmol/L $MgCl_2$, 3 mmol/L MgATP, 0.002 mmol/L sodium pyruvate, and 20 mmol/L HEPES adjusted to pH 7.2) for current of Orai1 (I_{Orai1}) measurements; (iv) bath solutions for I_{TRPV1} measurements (140 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 5 mmol/L D-glucose, and 10 mmol/L HEPES adjusted to pH 7.4 with NaOH). Capsaicin-evoked activity was observed by capsaicin (1 μmol/L) in the external solution after adding the basal current; (v) bath solutions (139 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, 3 mmol/L $BaCl_2$, 2 mmol/L $MgCl_2$, 1 mmol/L EGTA, and 10 mmol/L glucose adjusted to pH 7.4 with NaOH) for I_{TRPV3} measurement; (vi) bath solutions (135 mmol/L NaCl, 3.6 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L $CaCl_2$, 5 mmol/L D-glucose, and 10 mmol/L HEPES adjusted to pH 7.4) for I_{Orai1} measurements. For activation of TPRV1 and TRPV3 currents, 1 μmol/L capsaicin (for hTRPV1) and 100 μmol/L 2-APB (for hTRPV3) was applied to each ion channel-transfected HEK293T cell. For activation of the hOrai-1 currents, 20 μmol/L InsP3 was added to the internal solution immediately before experimentation. The InsP3-containing pipette solution was kept on an ice block.

2.8. Statistical analysis

Data were analyzed by One-way ANOVA with Bonferroni's *post hoc* comparison. Results were expressed as mean ± SEM. *P* values less than 0.05 were considered significant.

3. Results

3.1. The effect of T. terrestris fruit extract on activation of I_{TRPV3}

In this study, to examine whether *T. terrestris* fruit extract can modulate the activity of TRPV3 ion channel, whole-cell patch clamp assay was performed in hTRPV3-overexpressed HEK293T cells (Figure 1). After confirming no basal current (Figure 1A, black arrow), *T. terrestris* fruit extract was added at 100 μg/mL to the bath solution, or 2-APB, TRPV3 agonist as a reference at the end of each experiment to confirm the full activation of I_{TPRV3}. A representative chart tract recording of I_{TRPV3} and related I–V relationship curve revealing the point peak of I_{TRPV3} generation and 2-APB induced peak current is shown in Figure 1A,B. According to the result, treatment of *T. terrestris* fruit extract at 100 μg/mL significantly ($P < 0.01$) increased the I_{TRPV3} activation with the level of (73.54 ± 8.92)% (−100 mV) compared to maximum current which was induced by 2-APB (I_{2APB}). Treatment of 2-APB also significantly increased its activity compared to control (Figure 1C). These results indicate that *T. terrestris* fruit extract can improve an impairment of skin barrier through activation of the TRPV3 ion channel.

3.2. The effect of T. terrestris fruit extract on I_{TRPV1}

In this study, we examined whether *T. terrestris* fruit extract can inhibit the activation of TRPV1 ion channels in hTRPV1-overexpressed HEK293T cells by whole-cell patch clamp assay. A typical chart trace record and its I–V curve are shown in Figure 2A,B. For activation of I_{TRPV1}, hTRPV1-overexpressed HEK293T cells were treated with 1 μmol/L of capsaicin, a specific agonist for TRPV1. After confirming the steady state current of TRPV1 (Figure 2A,B), *T. terrestris* fruit extract at 100 μg/mL was added to 1 μmol/L capsaicin solution, and then added to the bath chamber. However, treatment with *T. terrestris* fruit extract resulted in slight activation of the outward current of TRPV1. We also evaluated the effects of *T. terrestris* fruit extract on the inhibition rates of I_{TRPV1} at −60 mV. As a result, the inward part of I_{TRPV1} was not significantly inhibited by *T. terrestris* fruit extract (Figure 2C). From these results, *T. terrestris* fruit extract cannot inhibit the activation of TRPV1 ion channels.

3.3. The effect of T. terrestris fruit extract on the inhibition of I_{Orai1}

We next assessed the inhibitory effects of *T. terrestris* fruit extract on the activation of I_{Orai1} in hOrai1-STIM1-overexpressed HEK293T cells. As mentioned above, endoplasmic reticulum Ca^{2+} store depletion leads to activation of I_{Orai1} [9]. Therefore, endoplasmic reticulum Ca^{2+} depletion was induced by addition of 20 mmol/L 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, a strong Ca^{2+} chelator, and 20 μmol/L inositol trisphosphate. Under this condition, the inward rectifying current, I_{Orai1}, was slowly generated in the cells (Figure 3A,B). After confirming the steady state of I_{Orai1}, bath solutions were treated with *T. terrestris* fruit extract to determine the inhibitory effect. Treatment of 100 μg/mL *T. terrestris* fruit extract resulted in significantly inhibited activation of I_{Orai1} with levels of (37.90 ± 10.77)% compared to control (Figure 3C). At the end of the experiment, BTP2 was also added to confirm the

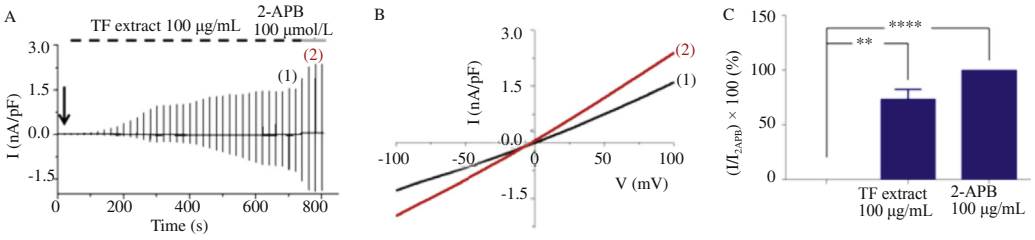

Figure 1. Effect of *T. terrestris* fruit extract on activation of I_{TRPV3} in HEK293T cells.
A: Representative chart recording of I_{TRPV3} activation by *T. terrestris* fruit extract. After confirming a steady state of I_{TRPV3} (1), 100 μmol/L 2-APB was applied to confirm its maximal current activation (2); B: Related I−V relationship curve at steady state current for 100 μg/mL *T. terrestris* fruit extract treatment (1) and 2-APB (2); C: Summarized column graph of I_{TRPV3} activation by *T. terrestris* fruit and 2-APB at −100 mV. The normalized amplitudes of remained currents (I/I_{2APB}) were measured at −100 mV clamp voltage. TF: *T. terrestris* fruit extract. Values are represented as mean ± SEM (n = 5). **: $P < 0.01$; ****: $P < 0.000\ 1$ compared with the control.

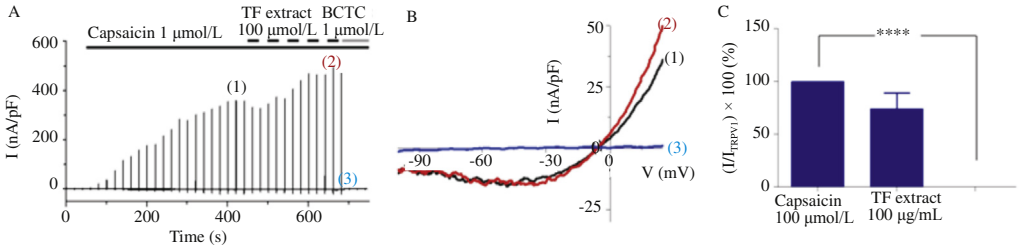

Figure 2. The inhibitory effect of *T. terrestris* fruit extract on allyl isothiocyanate-induced activation of I_{TRPV1} in HEK293T cells.
A: Representative chart trace of I_{TRPV1} modulation by *T. terrestris* fruit extract. After confirming steady state of I_{TRPV1} activation by 1 μmol/L capsaicin (1), 100 μg/mL *T. terrestris* fruit extract was added to the bath solution (2). At the end of the experiment, BCTC was also added to the bath solution to determine the basal current (3); B: Related I–V relationship curve at steady state current for TRPV1 (1), 100 μg/mL *T. terrestris* fruit extract (2) and 1 μmol/L BCTC (3) treatment; C: Summarized effects of *T. terrestris* fruit extract on I_{TRPV1} at −60 mV. The normalized amplitudes of remained currents (I/I_{TRPV1}) were measured at −60 mV clamp voltage. TF: *T. terrestris* fruit extract. Values are represented as mean ± SEM (n = 5). ****: $P < 0.000\ 1$ compared with the control.

basal current. From these results, *T. terrestris* fruit extract can inhibit the activation of Orai1 ion channels.

4. Discussion

The prevalence of AD ranges from 10% to 20% in developed countries, and in the case of many developing countries, it is currently lower, but successively increases every year [9,29]. AD is a common chronic inflammatory skin disease involved the multiple factors including genetic factors, immune system dysregulation, and skin barrier dysfunction. Although several conventional medications include topical agents and oral antiallergic drugs have been applied for the treatment of AD, current therapies are still rather limited and novel therapeutic strategies are required. Recently, some traditional medicines are useful for treating inflammatory skin diseases, and there has been increased interest in using them to develop new therapeutic agents for AD. *T. terrestris* has traditionally been used to treat cutaneous pruritus, edema, inflammation, and tracheitis [13,14]. Although some efficacies of *T. terrestris* in treating liver toxicity, and renal damage based on the herbological properties have been reported [19–21], little is known about its effects on inflammatory skin diseases including AD. In this study, we investigated the modulatory effects of *T. terrestris* fruit extract on activities of the ion channels, Orai-1, TRPV1, and TRPV3, which are known to contribute to skin homeostasis [7].

Traditionally, AD was thought to be a disorder with skin barrier dysfunction which purely involves keratinocytes. In the past 20 years, many researchers have proposed a more complex view of AD that arises from a complex interplay between immunological

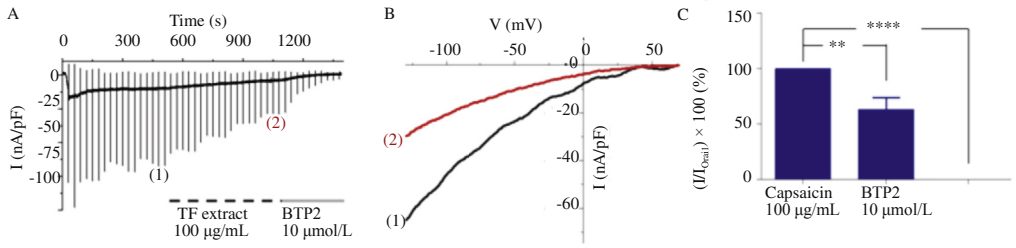

Figure 3. Inhibitory effect of *T. terrestris* fruit extract on inwardly-rectifying I_{Orai1} in HEK293T cells.
A: Typical chart trace recordings of I_{Orai1} (1) and their inhibition by 100 μg/mL *T. terrestris* fruit extract (2); B: Related I–V relationships of the control (1), and the cell treated with *T. terrestris* fruit extract at a concentration of 100 μg/mL (2); C: Summary of inhibition rate of I_{Orai1} by *T. terrestris* fruit extract and BTP2 at −120 mV. The normalized amplitudes of remained currents (I/I_{Orai1}) were measured at −120 mV clamp voltage. TF: *T. terrestris* fruit extract. Values are represented as mean ± SEM (n = 5). **: $P < 0.01$, ****: $P < 0.000\ 1$ compared with the control.

cells and keratinocytes [1]. Therefore, many of the current researches focus on the direct or indirect interplay between keratinocyte-derived proinflammatory mediators, such as thymic stromal lymphopoietin, and Th2 or Th17 T-cell derived cytokines [interleukin 4 (IL-4), IL-5, IL-13, and IL-17], which are closely related to epidermal barrier dysfunction and chronic inflammation [1,2]. Also, the studies on the underlying mechanism of T-cell activation and keratinocyte differentiation revealed that complex signaling pathways in AD pathogenesis are usually involved in increase of the intracellular calcium concentration as a key step [7].

Three Ca^{2+} ion channels, Orai-1, TRPV1, and TRPV3 are involved in T-cell activation [9], histamine-dependent itch development [12], and keratinocyte differentiation [11,30]. Orai-1 is a pore-forming subunit for Ca^{2+} release-activated Ca^{2+} channels, which is crucial for immune cell activation [9]. Mutation of the Orai-1 channel causes a severe immunodeficiency-like disease and autoimmunity [30]. TRPV1 channels are usually expressed in dorsal root ganglion neurons, which are activated at temperatures exceeding 43 °C, and also mediate nociception [31]. Moreover, it was recently revealed that IL-31, a Th2 cell-derived cytokine, directly binds to the IL-31 receptor expressed in dorsal root ganglion neurons. It promotes an itch sensation via TRPV1 activation, which is a form of direct neuro-immune crosstalk between T cells and sensory nerves [9]. TRPV3 was also originally reported to be a thermosensitive non-selective cation channel with high Ca^{2+} permeability that is activated at 33–40 °C, which mediates the sensing of warmth [32]. Recently, it was reported that the TRPV3 channel also participates in non-sensory functions, such as skin-barrier formation or wound healing [11,30]. Therefore, we investigated whether *T. terrestris* fruit extract can modulate Ca^{2+} ion channels such as Orai-1, TRPV1, and TRPV3 which are involved in AD pathogenesis. In our study, *T. terrestris* fruit extract (100 μg/mL) significantly inhibited the activation of Orai1 ion channel on I_{Orai1} in STIM1 and Orai-1 co-transfected HEK293T cells, and strongly increased the activation of I_{TRPV3} in TRPV3-overexpressed cells. However, *T. terrestris* fruit extract did not inhibit capsaicin-induced I_{TRPV1} in TRPV1-overexpressed HEK293T cells. These results indicate that *T. terrestris* fruit extract can modulate the activation of ion channels of Orai-1 and TRPV3 in immune cells such as T cells, and keratinocytes. Taken together, our finding implies that the topical application of *T. terrestris* fruit extract may be useful for the treatment of inflammation and for enhancing skin-barrier formation in AD.

In conclusion, our results suggest that *T. terrestris* fruit extract may be of therapeutic value for managing AD-associated pathogenesis with abnormal skin barrier function via modulation of the ion channels of Orai-1 and TRPV3. To our knowledge, this is the first electrophysiological study to evaluate the role of an herbal medicine in the modulation of ion channel activity. The findings will be helpful in the search for new therapeutic agents for the treatment of AD.

Conflict of interest statement

We declare that we have no conflict of interest.

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