Enhancing Effect of Trachelogenin from Trachelospermi caulis Extract on Intestinal Barrier Function

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Trachelospermi caulis is used widely as an herbal medicine in oriental countries to attenuate fever and pain. We wished to reveal the novel function of this herb and its active component on barrier function in intestinal epithelial cells. Monolayers of intestinal epithelial cells (Caco-2) were used to evaluate the transepithelial electrical resistance (TEER) and quantity of permeated ovalbumin (OVA) as indices of barrier function. T. caulis increased TEER values on cell monolayers and decreased OVA permeation across cell monolayers. To ascertain the active component of T. caulis, the extract was isolated to five fractions, and the effect of each of these fractions on intestinal barrier function examined. Chloroform and ethyl acetate fractions showed increased TEER values and decreased OVA flux. Chloroform and ethyl acetate fractions contained mainly trachelogenin and its glycoside, tracheloside. Trachelogenin increased TEER values and decreased OVA flux by enhancing the tight-junction protein occludin (but not tracheloside) in Caco-2 monolayers. These findings demonstrated that trachelogenin, an active component of T. caulis, might help to attenuate food allergy or inflammatory bowel disease through inhibition of allergen permeation or enhancement of the intestinal barrier.

Key words Trachelospermi caulis; trachelogenin; intestinal barrier; occludin; Caco-2 cell; ovalbumin

Trachelospermi caulis belongs to the Apocynaceae family. It is the dried leafy stem of Trachelospermum asiaticum var. intermedium NAKAI. T. caulis is used widely as a traditional herbal medicine in China, Japan, and Korea. It has been used to attenuate fever and pain because of its anti-pyretic and anti-nociceptive effects, respectively. Furthermore, it has been demonstrated that T. caulis can suppress inflammatory diseases such as rheumatoid arthritis.[1–4] T. caulis contains phenolic compounds, flavones, and lignans.[5–7] The major components of T. caulis have been identified to be trachelogenin and its glycoside form, tracheloside.[8] The physiological effects of trachelogenin and tracheloside, such as anti-inflammatory and anti-estrogenic effects, have rarely been reported.[9,10] Furthermore, the effects of T. caulis (or its components) on the intestinal epithelium are not known.

Intestinal epithelial cells absorb dietary nutrients and form a monolayer as a physical barrier. The intestinal barrier regulates absorption via the paracellular diffusion pathway. The barrier function defends against the permeation of xenobiotics and food allergens from eggs, milk, wheat, peanuts, soybeans, and rice.[11] Previously, we revealed that Scutellaria baicalensis enhanced intestinal barrier function through regulation of tight junctions (TJs), resulting in inhibition of allergen permeation.[12] The enhancing effect could suppress intestinal-related diseases such as inflammatory bowel disease (IBD) and food allergy.

In the present study, we examined nine natural-product extracts [including T. caulis extract (TCE)] from plants on intestinal barrier function using the human intestinal epithelial cell line Caco-2. We also investigated which components from TCE affect intestinal barrier function in monolayers of intestinal epithelial cells.

MATERIALS AND METHODS

Materials The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, non-essential amino acids (NEAA), phosphate-buffered saline (PBS), and Hank's balanced salt solution (HBSS) were purchased from WelGENE (Daegu, South Korea). Ovalbumin (OVA), bile salts (mixture of sodium cholate and sodium deoxycholate), horseradish peroxidase (HRP), Triton X-100, and Tween20 were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). 3-(4-Hydroxyphenyl)propionic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Trachelogenin (purity >95%) (PubChem CID: 452855) was obtained from Dr. Sang-Won Choi, Catholic University of Daegu. Tracheloside (purity >98%) (PubChem CID: 169511) was purchased from Chengdu Biopurify Phytochemicals Ltd. (Sichuan, China). Paraformaldehyde (16%) was from Electron Microscopy Sciences (Wilmington, DE, U.S.A.). Bicinchoninic acid (BCA) test kit and Protease inhibitor kit were purchased from Cell Signaling Technology (Beberly, MA, U.S.A.). Anti-occludin, β-actin, and fluorescein isothiocyanate (FITC)-labeled goat immunoglobulin G (IgG) antibodies were purchased from Santa.
Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and Invitrogen (Gaithersburg, MD, U.S.A.), respectively.

**Sample Preparation** The nine samples (Rosa multiflora, Ostericum koreanum, Hordeum vulgare, Broussonetia kazinoki, Trachelospermum asiaticum, Luffa cylindrica, Castanea crenata, Pinellia ternata, Quisqualis indica) used for the screening experiment were purchased from a Plant Extract Bank at the Korea Research Institute of Bioscience & Biotechnology (Daejeon, South Korea). T. caulis was purchased from Kyeong-dong Oriental Pharmacy (Seoul, Korea) and identified by Professor Y. Bu (Department of Herbal Pharmacology, Kyung Hee University, Seoul, South Korea). Dr. Bu analyzed components well-known from T. caulis (crude drug) using HPLC, especially arctiin. The specimen (KFRI-SL-2037) is stored by the Functional Materials Research Group of the Korea Food Research Institute. The extract was underwent reflux extraction twice in 95% ethanol for 5 min using a Soxwave100 apparatus. The ethanol extract was concentrated under vacuum in a rotary evaporator. The concentrated extract was lyophilized (−50°C) and kept at 4°C until needed. The dried ethanol extract was dissolved in saline before use.

**Sample Fractionation** We obtained 17.84 g of ethanolic extract from 200 g of T. caulis (crude drug). Its yield was 8.92%. And then 10 g of the extract was sequentially fractionated using other solvents such as hexane, chloroform, ethyl acetate, butanol, and water. Each solvent collected was concentrated under vacuum in a rotary evaporator, and then the concentrated fractions were lyophilized. Amounts dried from each hexane, chloroform, ethyl acetate, butanol, and water were 1.281, 1.645, 0.878, 2.248, and 3.948 g, respectively.

**HPLC Analysis** HPLC analysis was carried out with a Jasco PU-2080 plus liquid chromatography system (JASCO, Tokyo, Japan) equipped with a multi wavelength detector Jasco UV-2075 plus (JASCO). Samples were injected to 1 µg/mL by auto injector AS-2057 plus (JASCO), and ODS C18 column (250×4.6 mm) was used to separate compounds at 40°C in column adaptor CO-2060 plus (JASCO). Solvents were used mixture of acetonitrile (A) and water (B) in gradient mode (eluent A: 20 to 40% in 35 min), and the flow rate was 1 mL/ min. UV wavelength for detection was 280 nm.

**Cell Culture** Caco-2 cells were cultured at 37°C in humidified air containing 5% CO2. Cells were maintained in a 100-mm dish with DMEM containing 1000 mg/L of glucose and supplemented with 10% FBS, 1% NEAA, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Cells were seeded at 2×105 cells/mL on a 12-transwell or 24-well plate (Costar, Corning, NY, U.S.A.) and allowed to grow for 3 weeks; the cells were used if their TEER values were 300–500 (Millipore, Bedford, MA, U.S.A.). Monolayers of Caco-2 cells were used from 21 d after seeding. The integrity of Caco-2 cell monolayers was checked by measuring TEER using a Millicell-ERS device (Millipore, Bedford, MA, U.S.A.). Monolayers of Caco-2 cells were used if their TEER values were 300–500 Ω cm−2. Cell monolayers were washed twice with HBSS and then pre-incubated for 30 min at 37°C in a CO2 incubator to stabilize them. Cell monolayers were treated with each sample and bile salts for 60 min, and OVA added for 3 h at 37°C. Three hours after incubation with OVA, the TEER of cell monolayers was measured. The medium on the basolateral side of the monolayer (which contained the permeated OVA) was collected for the enzyme-linked immunosorbent assay (ELISA).

**ELISA** Monolayers of Caco-2 cells in 12-transwell plates were pre-incubated with bile salts and TCE for 1 h and then treated with 400 µg/mL OVA for 3 h in HBSS. After incubation, the level of permeated OVA on the basolateral side of the monolayer was determined using a commercial ELISA kit. Primary and secondary antibodies for ELISA were obtained from OVA-immunized rabbits. Secondary antibodies were conjugated with HRP according to a protocol described previously. The flash count between HRP and 3-(4-hydroxyphenyl)-propionic acid was detected by fluorometric means using a Fluoroskan Ascent Microplate Fluorescence Reader (Thermo Labsystems, Waltham, MA, U.S.A.) to set an excitation wavelength of 320 nm and emission wavelength of 405 nm.

**Induction of Anaphylactic Response by Oral Administration of OVA** Female BALB/c mice, weighing approximately 18–20 g, were purchased from OrientBio Inc. (Kyung-gi, Korea). BALB/c mice (6 weeks old) were housed in an air-conditioned room (23±2°C) with a 12-h light/dark cycle. Mice were allowed free access to food and water. All animal experiments were performed in accordance with the guidelines for animal care and use of the Korea Food Research Institute. Mice were divided into naïve (n=4), sham (n=4), TCE (n=4), and Dexa (n=4) groups. To induce an allergic response, mice were sensitized with 20 µg OVA adsorbed in 2 mg/mL Imject Alum (Pierce, Rockford, IL, U.S.A.) by intraperitoneal injection on day 0. From day 17, mice were orally challenged with 50 mg OVA in saline every 3 days, for a total of five times. To investigate the preventive effect of TCE, the TCE (25 mg/kg) was orally administered every day, from day 17 to 29. Diarrhea, anaphylaxis, and rectal temperature were measured as an index of food allergy symptoms. Diarrhea and anaphylaxis were observed by visually monitoring mice for 60 min after challenge. Rectal temperature was measured using a Thermalert TH5 monitoring thermometer (Physitemp, Clifton, NJ, U.S.A.).

**Immunohlottting and Fluorescence Microscopy** To detect occludin protein on cell monolayers, Caco-2 cells were incubated on cover glasses (18 mm; Marienfeld Superior, Marienfeld Germany) in six-well plates for 3 weeks. Cells were treated with 100 µmol/L of trachelogenin for 3 h, and cells washed thrice with PBS. Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and fixed cells treated with 0.2% Triton X-100 for 4 min at room temperature. After washing cells thrice with PBS-T (1% Tween-20 in PBS) for 5 min, blocking was carried out in 3% normal goat serum (Vector Laboratories, Burlingame, CA, U.S.A.) in PBS-T for 60 min. Cells were incubated with rabbit polyclonal anti-occludin antibodies (1:100) in blocking solution overnight at 4°C. After washing PBS-T, cells were incubated with FITC-labeled anti-rabbit goat IgG (1:200 dilution) in PBS-T for 60 min at room temperature in the dark. Finally, after washing with PBS-T, the cover glasses were mounted on glass plates with mounting medium containing 4’,6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories). Samples were observed using a fluorescence microscope (Axio Observer A1; Carl Zeiss, Jena, Germany).

**Western Blotting Analysis** For occludin protein detection, Caco-2 cells were cultured with trachelogenin for 6 h. And then the cells were washed with ice-cold PBS, scraped, and lysed in radio immunoprecipitation assay (RIPA) buffer.
containing 0.1% protease inhibitor cocktail. After centrifugation, total protein concentration from the cell lysate was determined by BCA assay according to the manufacturer’s instructions. Protein levels were quantified using an automated capillary-based size sorting system (WES; ProteinSimple, Santa Clara, CA, U.S.A.). All procedures were performed according to the manufacturer’s instructions. Four microliters of cell lysate and primary antibodies [occludin (1:100) and β-actin (1:500)] were used to detect occludin protein, and the data was analyzed with inbuilt Compass software (ProteinSimple).

Statistical Analyses Data are the mean±standard deviation (S.D.). Differences between experimental data were assessed by one-way ANOVA followed by the F-protected Fisher’s least significant difference test.

RESULTS

Effects of Natural-Product Extracts on Intestinal Barrier Function in Monolayers of Caco-2 Cells We examined the effects of extracts from nine plants (Rosa multiflora, Ostericum koreanum, Hordeum vulgare, Broussonetia kazinoki, Trachelospermum asiaticum, Luffa cylindrical, Castanea crenata, Pinellia ternata, and Quisqualis indica) on intestinal barrier function by measuring TEER in monolayers of Caco-2 cells. Of the 9 natural-product extracts, Trachelospermum asiaticum (T. caulis) increased TEER significantly in Caco-2 cell monolayers (Fig. 1). This result suggested that T. caulis enhances intestinal barrier function. In contrast, the other eight natural-product extracts did not change TEER in Caco-2 cell monolayers.

Effects of T. caulis Extract (TCE) on TEER and OVA Flux in Caco-2 Cell Monolayers We also investigated the effects of TCE on intestinal barrier function by measuring

![Fig. 1. Effects of Natural-Product Extracts on Intestinal Barrier Function in Caco-2 Cell Monolayers](image1)

Intestinal barrier function was measured in terms of TEER in Caco-2 cell monolayers for 3h. Nine natural-product extracts (Rosa multiflora, Ostericum koreanum, Hordeum vulgare, Broussonetia kazinoki, Trachelospermum asiaticum, Luffa cylindrical, Castanea crenata, Pinellia ternata, and Quisqualis indica) were screened using TEER values in Caco-2 cell monolayers. Each ethanolic extract treated at 400 µg/mL. Each value is the mean±S.D. (n=3). **p<0.01 vs. control. Data were analyzed using ANOVA followed by the F-protected Fisher’s least significant difference test.

![Fig. 2. Effects of TCE on TEER and OVA Flux in Caco-2 Cell Monolayers](image2)

Caco-2 cell monolayers were incubated with 100 µg/mL of TCE for 3h. TEER was measured at 3h (A). OVA flux on the basolateral side of the monolayer via paracellular diffusion for 3h was detected using ELISA (B). Each value is the mean±S.D. (n=3). *p<0.05 and **p<0.01 vs. control. Data were analyzed using ANOVA followed by the F-protected Fisher’s least significant difference test.
TEER in Caco-2 cell monolayers. TEER increased significantly in cells incubated with 100 µg/mL of TCE for 3 h (Fig. 2A). We also investigated the ability of TCE to inhibit allergen permeation in Caco-2 cell monolayers. We used OVA, the major allergen in eggs. OVA permeated the basolateral side of monolayers via paracellular diffusion after 3 h, which was detected using ELISA. OVA flux decreased significantly in cells incubated with 100 µg/mL of TCE (Fig. 2B). This result suggested that TCE may inhibit the allergic response by suppressing OVA permeation.

Next, we examined the effect of T. caulis in a mouse model of food allergy because the permeation of allergen triggers allergic responses in intestine. Food-allergy symptoms induced by OVA were evaluated and scored by the criteria for diarrhea, anaphylactic response, and rectal temperature after the fifth challenge. Severe symptoms of OVA-induced food allergy were observed in the sham group (diarrhea, 2.3 points; anaphylactic response, 2.5 points) (Table 1). In contrast, the TCE group showed suppressive tendency of oral OVA challenge-induced diarrhea and anaphylactic response (diarrhea, 1.5 points; anaphylactic response, 2.3 points). We also measured rectal temperature for 1 h after the fifth challenge with OVA. Rectal temperature in the sham group fell to 31.5°C (−5.2°C) compared with the naïve group. However, TCE treatment in our model of food allergy significantly ameliorated the decrease in rectal temperature induced by OVA to 35.5°C (−2.1°C). Furthermore, we investigated the IgE level in our food-allergy model. The IgE level was increased with food allergy in response to OVA, whereas TCE administration tended to suppress IgE (Table 1). These results demonstrated that TCE administration could help to attenuate food allergy through enhancement of intestinal barrier function.

Effects of TCE Fractions on TEER and OVA Flux in Caco-2 Cell Monolayers

We examined the effects of TCE fractions on intestinal barrier function by measuring TEER in Caco-2 cell monolayers. Five fractions (hexane, chloroform, ethyl acetate, butanol, and water) had significantly increased TEER values in Caco-2 monolayers. Fractions of hexane, chloroform, and ethyl acetate also showed strong increases in TEER (Fig. 3A). We also investigated the ability of TCE fractions to inhibit OVA permeation using ELISA. OVA flux was decreased significantly by chloroform and ethyl acetate fractions (Fig. 3B). To reveal active components of TCE, chloroform (C) and ethyl acetate (D) fractions were analyzed using HPLC. Each value is the mean ± S.D. (n=3). **p<0.01 and *p<0.05 vs. control. Data were analyzed using ANOVA followed by the F-protected Fisher’s least significant difference test.

Table 1. Effect of T. caulis in a Mouse Model of Food Allergy

<table>
<thead>
<tr>
<th></th>
<th>Naïve group</th>
<th>Sham group</th>
<th>TCE group</th>
<th>Dexamethasone (Dexa) group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrhea (score)</td>
<td>0</td>
<td>2.3±1.5</td>
<td>1.5±1.7</td>
<td>0</td>
</tr>
<tr>
<td>Anaphylactic response (score)</td>
<td>0</td>
<td>2.5±0.6</td>
<td>2.3±1.0</td>
<td>0</td>
</tr>
<tr>
<td>Rectal temperature (°C)</td>
<td>(1.7±0.8)</td>
<td>31.5±1.2</td>
<td>35.5±3.4*</td>
<td>38.4±0.3**</td>
</tr>
<tr>
<td>Δ°C</td>
<td>(−5.2±1.1)</td>
<td>(−2.1±3.2)</td>
<td>(0.04±0.4)</td>
<td></td>
</tr>
<tr>
<td>IgE level in serum (ng/mL)</td>
<td>5178.9±434.37</td>
<td>7269.95±2725.38</td>
<td>4302.06±2513.20</td>
<td>4340.1±704.49</td>
</tr>
</tbody>
</table>

*Dexamethasone (Dexa) was used as positive control. Each value the mean ± S.D. (n=4). **p<0.01, *p<0.05 vs. sham group. Data were analyzed using ANOVA followed by the F-protected Fisher’s least significant difference test.
This result demonstrated that the inhibitory effect of TCE on allergen permeation was caused by commonly occurring compounds in chloroform and ethyl acetate fractions.

Effects of Single Components (Trachelogenin and Tracheloside) on TEER and OVA Flux in Caco-2 Cell Monolayers

We analyzed the components in chloroform and ethyl acetate fractions by LC-MS (Agilent Technologies, Santa Clara, CA, U.S.A.) and HPLC (JASCO, Tokyo, Japan). The chloroform and ethyl acetate fractions mainly contained five components such as C1 (E1), C2 (E2), C3 (E3), tracheloside, and trachelogenin (Figs. 3C, D). Therefore, we investigated the effects of trachelogenin and tracheloside on TEER and OVA permeation across Caco-2 cell monolayers because the chloroform and ethyl acetate fractions mainly contained two compounds: trachelogenin (Fig. 4A) and tracheloside (Fig. 4B). Trachelogenin (but not tracheloside) significantly enhanced TEER and inhibited OVA permeation (Figs. 4C, D). Furthermore, we confirmed that trachelogenin increased TEER and reduced OVA permeation in a dose-dependent manner (Figs. 4E, F). We suggest that the trachelogenin in Trachelospermum caulis is an active component that enhances intestinal barrier function to suppress OVA permeation across Caco-2 cell monolayers.

Enhancing Effect of Trachelogenin on Expression of the TJ Protein Occludin within Caco-2 Cell Membranes

TJs comprise several transmembrane proteins such as occludin, claudins (claudin-1–24), junctional adhesion molecules (JAM-1–3), and zona occludens (ZO-1–3) for barrier function on intestinal epithelial cells. In particular, occludin has been reported to be a very important protein in the TJ protein network for regulation of the paracellular permeability of epithelial monolayers. Therefore, we investigated if trachelogenin regulates the expression or assembly of occludin protein within Caco-2 cell monolayers using fluorescence microscopy. Cell monolayers were treated with saline/trachelogenin for 3h and then stained. Occludin in cell monolayers treated with trachelogenin developed strong staining within the cell membrane compared with control (which had saline treatment only) (Figs. 5A–F). This result suggested that trachelogenin enhanced intestinal barrier function through up-regulation of occludin protein on Caco-2 cell monolayers. To assess the change in occludin staining quantitatively, the FITC-fluorescence intensity of occludin was measured: trachelogenin increased the fluorescence intensity of occludin by 1.74-fold compared with control (Fig. 5G).

Furthermore, we investigated occludin expression by trachelogenin using Western blotting analysis. As a result, the expression of occludin was increased by treatment of trachelogenin in a dose-dependent manner. Therefore, we could certainly confirm that trachelogenin reinforced intestinal barrier function by up-regulating occludin expression (Fig. 5H).

DISCUSSION

TJs are key determinants of intestinal barrier function in the complex protein networks connected to epithelial cells.
TJs are organized by specific interactions between various intracellular proteins and transmembrane proteins. Interactions have been identified with three transmembrane protein families: occludin, claudins, and junctional adhesion molecule. Regulation of TJ-related proteins can enhance intestinal barrier function and contribute to reduce the risks of inflammatory bowel disease (IBD) and food allergies. Recently, it was reported that food- and herbal plant-derived factors could regulate enhancement of intestinal barrier function through TJ-related proteins. For example, naringenin was reported to enhance zonula occludens-2, occludin, claudin-1, and claudin-4 in Caco-2 cells. Especially, the claudin-4 was increased both the expression and activation level via transcriptional factor Sp1 by naringenin. Furthermore, naringenin has been shown to protect against impairment of the intestinal barrier and inflammation in a dextran sulfate sodium-induced model of colitis. For example, naringenin was reported to enhance zonula occludens-2, occludin, claudin-1, and claudin-4 in Caco-2 cells. Especially, the claudin-4 was increased both the expression and activation level via transcriptional factor Sp1 by naringenin. Furthermore, naringenin has been shown to protect against impairment of the intestinal barrier and inflammation in a dextran sulfate sodium-induced model of colitis. These reports showed that food- or herb-derived components such as a naringenin could prevent intestinal inflammation (e.g., IBD) through enhancement of intestinal barrier function. Our results showed the enhancing effect of T. caulis on intestinal barrier function, and that T. caulis could contribute to attenuate food allergy. We suggest that T. caulis may be able to ameliorate intestine-related disorders such as Crohn’s disease and ulcerative colitis. In addition, trachelogenin, main component from T. caulis on intestinal barrier function, showed potential as the first lignan to regulate various disorders via enhancement of intestinal barrier function.

Regulation of the permeability of epithelial cells in respiratory, circulatory, and gastrointestinal (GI) systems is very important. For example, it has been reported that baikalin isolated from Scutellaria baicalensis reduces the permeability of the blood–brain barrier by increasing expression of claudin-5 and zonular occludens-1 TJ proteins in the endothelial cells of brain microvessels. That study suggested that baikalin could suppress the inflammatory reaction and tissue injury in the brain by reinforcing the blood–brain barrier. Our study showed that the trachelogenin from TCE could inhibit allergen permeation by enhancing the effect of occludin on intestinal barrier function, and that the effect of trachelogenin could be applied not only in intestinal epithelial cells (GI system) but also in other endothelial cells (respiratory and circulatory systems). We believe that TCE and its active compound trachelogenin could regulate allergens, xenobiotics or inflammatory inducers on endothelial cells in the GI, respiratory, circulatory, and cutaneous systems. In the future, TCE and trachelogenin will be studied for their enhancing effects on intestinal barrier function and applied to in vivo models of IBD and asthma to verify such enhancing effects.

Our results showed that trachelogenin as an active component in T. caulis enhanced intestinal barrier function. However, amount of trachelogenin in ethyl acetate fraction was relatively low level although ethyl acetate fraction showed enhancing effect on barrier function in Caco-2 cells. We suggest that peak E1 (C1) in ethyl acetate fraction of T. caulis could affect to increase intestinal barrier function. To identify peak E1, we analyzed components known from T. caulis, such as quercetin, chlorogenic acid, luteolin, and arctigenin. However, they were not matched with peak E1. In the further...
In the present study, we demonstrated that *T. caulis* suppresses OVA permeation by enhancement of barrier function in intestinal epithelial cells. We also revealed, for the first time, that the active component of *T. caulis* for inhibition of OVA permeation is trachelogenin: it can up-regulate expression of occludin in TJ protein networks. We suggest that the trachelogenin from *T. caulis* could help to prevent/treat allergic disorders and IBD.

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**Conflict of Interest** The authors declare no conflict of interest.

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