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Cudrania tricuspidata leaf extracts and its components, chlorogenic acid, kaempferol, and quercetin, increase claudin 1 expression in human keratinocytes, enhancing intercellular tight junction capacity

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Abstract

Dysfunction of tight junctions and their components can cause diverse skin diseases. Here, we investigated the expression of claudin 1, a major tight junction protein, and changes of tight junction capacity upon treatment of the extracts of *Cudrania tricuspidata* (*C. tricuspidata*) and its components, chlorogenic acid, kaempferol, and quercetin. The effects of ethanol extracts of *C. tricuspidata* (EECT) and water extracts of *C. tricuspidata* (WECT) on the viability of human keratinocyte HaCaT cells were assessed by cell proliferation assay. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted to measure the expression of claudin 1 mRNA. The protein expression of claudin 1 was analyzed by western blot and its tight junctional distribution was observed with immunofluorescence microscopy analysis. The tight junction capacity was analyzed by dispase assay. Upon treatment of WECT to HaCaT cells, the mRNA and protein expressions of claudin 1 were increased. In addition, chlorogenic acid, kaempferol, and quercetin increased claudin 1 protein expression levels in a dose-dependent manner. WECT and these three compounds enhanced the tight junction capacity of HaCaT cells in dispase assay. WECT, and its components, such as chlorogenic acid, kaempferol, and quercetin, upregulates both mRNA and protein expressions of claudin 1, which leads to the enhancement of tight junction capacity. Thus, WECT could be a therapeutic approach for treating tight junction-disrupted conditions such as atopic dermatitis and psoriasis.

Keywords: *Cudrania tricuspidata*, Claudin 1, Tight junction, Chlorogenic acid, Kaempferol, Quercetin, Dispase assay

Introduction

Cudrania tricuspidata is a thorny, deciduous tree that is found throughout Japan, China, and Korea. The root bark and cortex of *C. tricuspidata* are used as crude Chinese

drugs and ubiquitous traditional herbal remedies in East Asia [1]. Several studies found that root bark extracts induce apoptosis in human leukemia cells (HL-60 cells) and function as a hepatoprotective compound [2, 3]. In addition, *C. tricuspidata* fruit was found to have anti-bacterial effects, and can suppress the development of atopic dermatitis [4, 5]. Many studies have investigated the physiological and biochemical effects of *C. tricuspidata* in relation to its anti-inflammatory and anti-cancer activities [6–9]; however, the effects of *C. tricuspidata* on tight junction function are largely unknown.

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Tight junctions regulate the passage of water, ions, and molecules through the paracellular pathway. The permeability properties of tight junctions are variable among the many types of epithelia, and depend on specific requirements for transepithelial or transendothelial solute transport [10]. Several studies have identified more than 40 different proteins located within the tight junctions of epithelial, endothelial, and myelinated cells [11–15]. Among these proteins, members of the claudin family are directly involved in tight junction function. In particular, claudin 1 maintains the integrity of the paracellular barrier and regulates water homeostasis. Loss of claudin 1 in epithelial cells leads to a high susceptibility to external pathogens, and malfunctions of the skin barrier [16–18]. Several studies have shown that skin diseases, such as atopic dermatitis, skin tumors, and dehydration, are caused by a loss of claudin 1 in tight junctions [19–21]. Interestingly, a recent study found that the symptoms of dinitrochlorobenzene (DNCB)-induced atopic dermatitis mice could be improved by increasing claudin 1 expression [22]. Thus, studying of the regulation of claudin 1 could be a considerably important factor for a better understanding on how to treat tight junction-mediated skin diseases.

In the present study, we investigated the effect of *C. tricuspidata* leaf extracts on tight junctions. We found that *C. tricuspidata* leaf extracts caused an increase in claudin 1 expression in HaCaT cells. In addition, the representative components of *C. tricuspidata* leaf extracts; chlorogenic acid, kaempferol, and quercetin also showed the similar activity on claudin 1 expression. Moreover, this increase in claudin 1 resulted in an enhancement of tight junction formation, where claudin 1 was predominantly located in the cellular membrane. We propose *C. tricuspidata* leaf extracts as a therapeutic treatment for tight junction-mediated skin diseases, due to their ability to increase claudin 1 expression.

Materials and methods

Cudrania tricuspidata leaf extracts preparation and yield

Cudrania tricuspidata leaves were gathered from farms in Gongju, Korea, in August 2017. Plant was botanically authenticated by Professor Dr. Myoung Soo Nam and deposited in the Division of Animal Resource Science, Chungnam National University, Daejeon, South Korea.

The leaves were washed with water and dried at room temperature. The dried leaves were ground into a fine powder, of which 10 g was mixed with 30 ml distilled water or 90 ml ethanol for 30 min. Water extracts were centrifuged at 4000×g for 15 min, followed by freeze-drying of the supernatant. Ethanol extracts were concentrated in vacuum evaporator at 55 °C until the ethanol was completely evaporated, followed by freeze-drying. The yield of each extract was measured using the

following equation: water extracts of *C. tricuspidata* leaf (WECT), 13.2 ± 0.125%; ethanol extracts of *C. tricuspidata* leaf (EECT), 11.2 ± 0.025%.

$$\text{Yield (\%)} = \frac{\text{Freeze-dried powder (g)}}{\text{Ground fine powder (10 g)}} \times 100$$

Cell culture and chemicals

Human keratinocytes (HaCaT cells) were purchased from AddexBio (cat# T0020001, CA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, NY, USA), at 37 °C, in 5% CO₂. The main phenolic components of WECT; chlorogenic acid, kaempferol, and quercetin were purchased from Sigma-Aldrich (MO, USA). Chlorogenic acid, kaempferol, and quercetin were diluted in DMSO and treated to HaCaT cells with DMSO control.

MTS assay

HaCaT cells were cultured in 96-well plates and treated with WECT or EECT. After 24 h, 20 µl MTS solution (Promega, WI, USA) was added and cells were incubated at 37 °C for 1 h. Using a microplate reader (Molecular Devices EMax Plus, CA, USA), absorbance was measured at 490 nm. Cell viability was measured using the following equation:

$$\text{Cell viability (\%)} = \left(\frac{A_{\text{Experimental group}} - A_{\text{Sample blank}}}{A_{\text{control group}}} \right) \times 100$$

RNA preparation and quantitative RT-PCR analysis

Total RNA was extracted from HaCaT cells, using a Hybrid-R™ Kit (GeneAll, Korea) according to the manufacturer's instructions. cDNA was synthesized using M-MLV reverse transcriptase (Promega, WI, USA) with random hexamers. To investigate changes in tight junction-related genes, primers were designed (Table 1) and quantitative real-time PCR (qRT-PCR) was performed. Briefly, synthesized cDNA, 2X Prime Q-master mix (Genet Bio, Korea), and 10 pmol/µl forward and reverse primers were mixed and applied to qRT-PCR using AriaMx (Agilent, USA). Temperature and cycle settings are as follow: 95 °C, 20 s; 58 °C, 20 s; 72 °C, 20 s; 40 cycles. β-Actin was used as internal standard. The specificity of each PCR product was confirmed by melting curve analysis.

Immunoblot and immunofluorescence microscopy analysis

Cells were washed with phosphate-buffered saline (PBS) and lysed with Laemmli Sample Buffer (Bio-Rad, CA, USA). Total cell lysates were separated using SDS/PAGE, transferred to a nitrocellulose membrane, and

Table 1 Primers used for quantitative real-time PCR analysis of tight junction-related genes

Gene		Primer sequence (5' to 3')	Product size (bp)	Annealing temp. (°C)
ZO-1	F	AGA GCA CAG CAA TGG AGG AA	133	58
	R	GAC GTT TCC CCA CTC TGA AA		
Occludin	F	TTT GTG GGA CAA GGA ACA CA	137	58
	R	ATG CCA TGG GAC TGT CAA CT		
Claudin 1	F	GCA GAT CCA GTG CAA AGT CT	136	58
	R	CAT ACA CTT CAT GCC AAC GG		
Claudin 4	F	CGC ATC AGG ACT GGC TTT AT	131	58
	R	AGT TGA GGA CCT GGA AGG CT		
Claudin 6	F	GGC CCT CTG AGT ACC CTA CC	136	58
	R	GCA GGA GGC AGA AAC AAA AG		
Claudin 8	F	GGC TGT TTC TTG GTG GTG TT	137	58
	R	CAC GCA ATT CAT CCA CAG TC		
β -Actin	F	TCA CCC ACA CTG TGC CCA TCT ACG A	295	58
	R	CAG GGG AAC CGC TCA TTG CCA ATG G		

blocked with 5% non-fat dry milk (Rockland, PA, USA). For immunofluorescence microscopy analysis, cells were cultured in 4-well chamber slides (Lab-Tek, NY, USA), fixed in 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100. Then, cells were blocked with a 1% BSA and 5% goat serum solution. Antibodies for immunoblot and immunofluorescence analysis were as follows: anti-claudin 1 (Cell Signaling Technologies, MA, USA) and anti-tubulin alpha (Sigma-Aldrich, MO, USA).

Dispase assay

Confluent HaCaT cells were seeded in triplicate onto 12-well plates and treated with WECT, chlorogenic acid, kaempferol, and quercetin for 24 h. The HaCaT cell monolayer was washed with PBS and treated with 1 ml dispase solution (2.4 units/ml; Roche Diagnostics, Mannheim, Germany) for 1 h. Cells were detached from the plate, physical stress was applied to split the cell layer into pieces, and fragments were manually counted.

Statistical analysis

Unpaired two-tailed Student's *t*-test was used for statistical analysis of data. *p* value lower than 0.01 was considered statistically significant.

Results

Effect of *C. tricuspidata* leaf extracts on cell viability

Although several studies have shown that *C. tricuspidata* leaf extracts have biochemical and physiological activities, their effects on skin, such as in the maintenance of water homeostasis and protection against

infection, are still unknown. Thus, we investigated the effects of *C. tricuspidata* leaf extracts on tight junctions, the most important component of the skin barrier. We evaluated cell viability using human epidermal keratinocytes (HaCaT cells). HaCaT cells were treated with WECT or EECT for 24 h, followed by an MTS assay. We found that WECT did not decrease cell viability to below 80%, whereas cell viability was decreased to 51.6% at a 2.0 mg/ml concentration (Fig. 1). However, EECT strongly decreased cell viability at a 0.125 mg/ml concentration, and at a concentration range of 0.25 to 2.0 mg/ml of EECT, cell viability was decreased to below 20%.

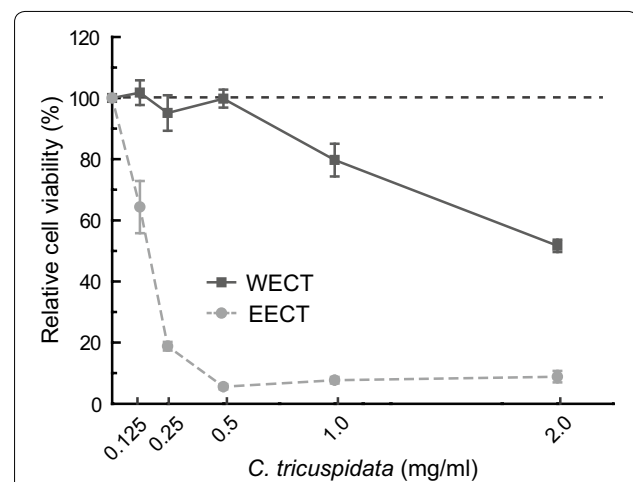


Fig. 1 Effect of *C. tricuspidata* leaf extracts on cell viability. HaCaT cells were treated with indicated concentrations of extracts for 24 h, followed by an MTS assay

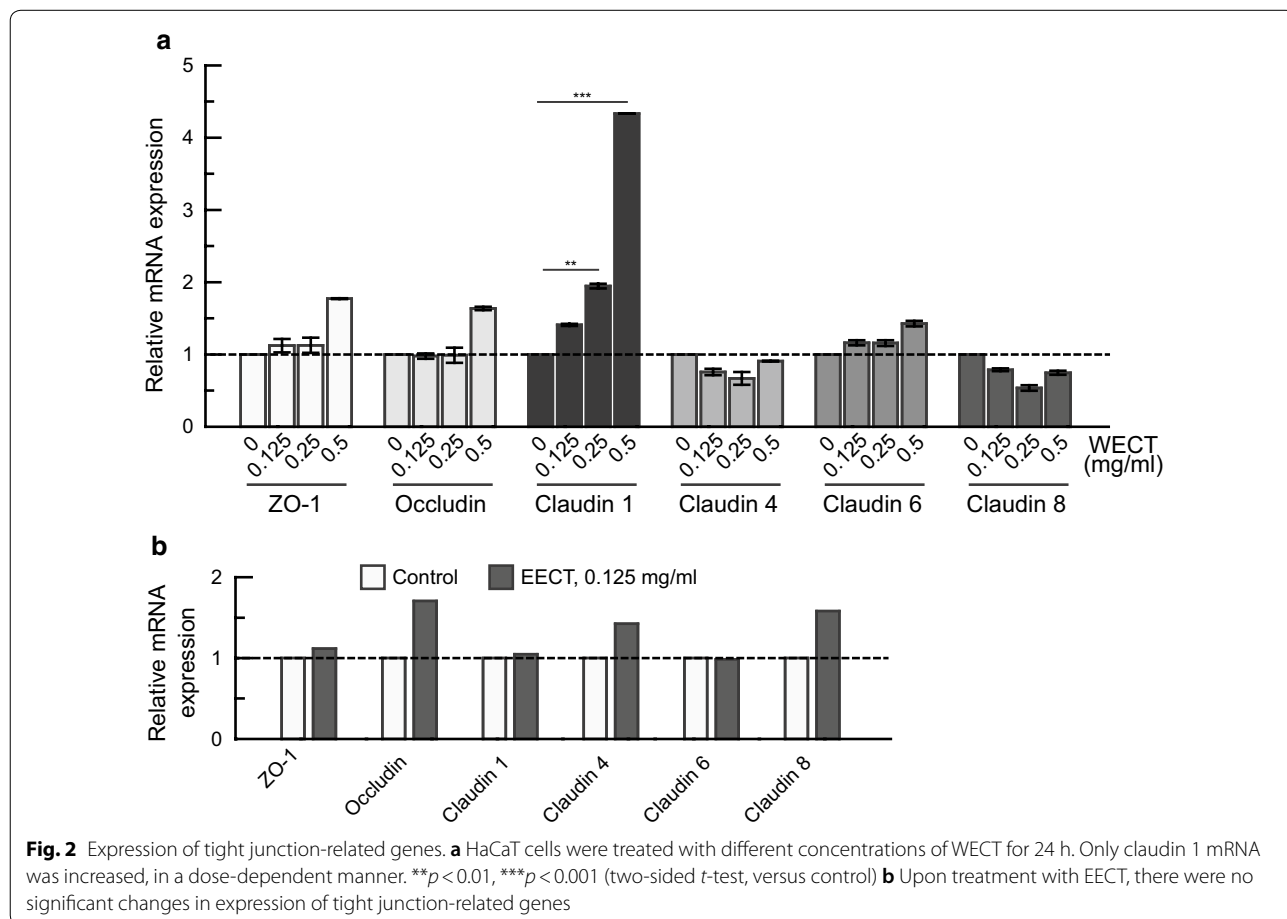
Claudin 1 mRNA expression is increased by WECT in a dose-dependent manner

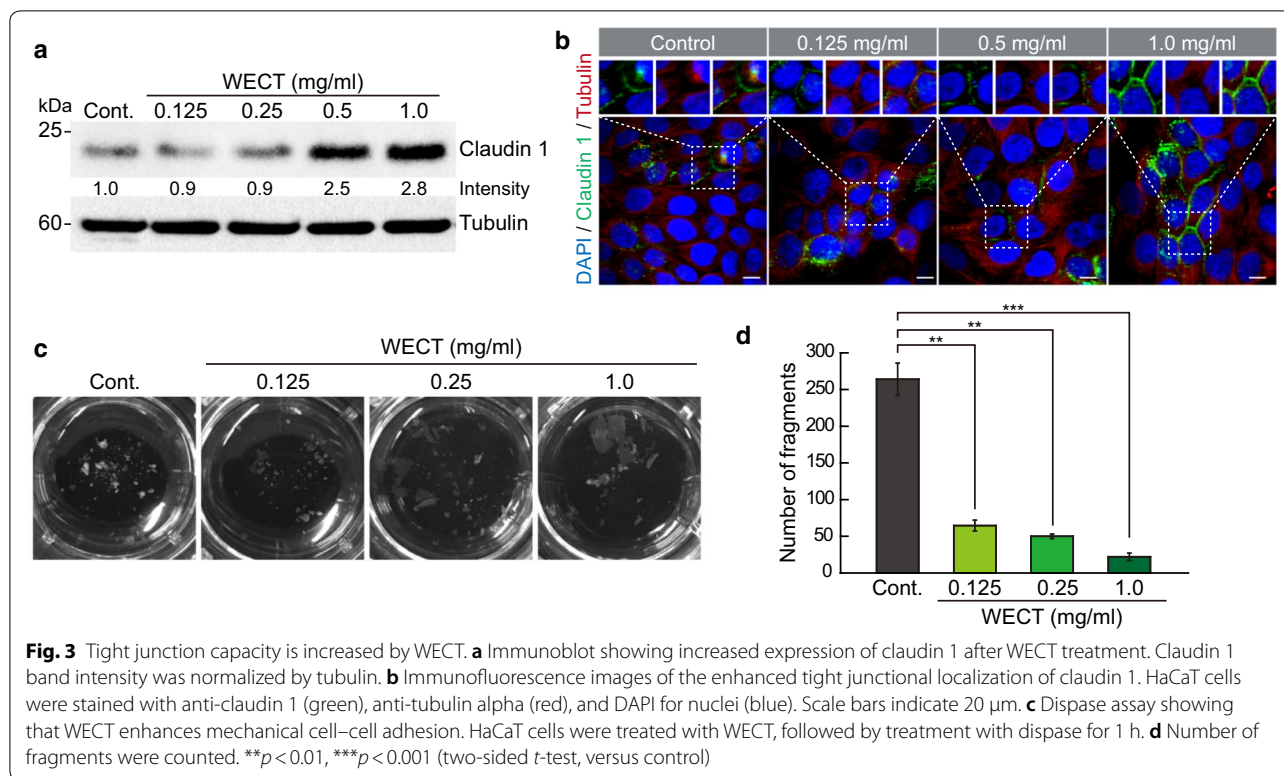
Tight junctions are composed of several transmembrane proteins, including claudin 1 and occludin. Here, we performed qRT-PCR to examine the regulatory effect of *C. tricuspidata* leaf extracts on the expression level of tight junction-related genes. As shown in Fig. 1, cell viability was not affected by treatment with 0.125, 0.25, or 0.5 mg/ml WECT. When we treated HaCaT cells with the same concentrations of WECT for 24 h, only claudin 1 mRNA expression showed a dose-dependent increase (Fig. 2a). When HaCaT cells were treated with 0.125 mg/ml EECT, no significant changes in mRNA expression of tight junction-related genes were observed (Fig. 2b). Thus, we found that WECT upregulates the expression of claudin 1 in a dose-dependent manner.

WECT increases tight-junctional claudin 1 expression and tight junction capacity

We found that only WECT caused an increase in claudin 1 mRNA expression in HaCaT cells (Fig. 2a, b). Claudin 1, the most important component of tight junctions,

was significantly increased, meaning that treatment with WECT could enhance cell–cell adhesion in HaCaT cells. To investigate not only mRNA levels but also claudin 1 protein levels, we treated HaCaT cells with different concentrations of WECT, and assessed protein expression by western blot analysis. We found that claudin 1 protein was also increased in a dose-dependent manner, consistent with the increase in mRNA expression (Fig. 3a). Claudin 1 protein expression was increased 2.5 and 2.8 times following treatment with 0.5 and 1.0 mg/ml WECT, respectively, compared with control. Next, we investigated the subcellular localization of this increase in claudin 1 expression upon treatment with WECT. Using immunofluorescence microscopy analysis, we found that the increased claudin 1 was predominantly localized to tight junctions (Fig. 3b). This increase in claudin 1 means that the anchoring capacity of the tight junctions, which maintains cell–cell adhesion, is enhanced. To assess tight junction ability after treatment with WECT, we performed a dispase assay. WECT was added to HaCaT cells for 24 h, then the dispase solution was added. After 1 h, cells were detached from the bottom of the plate and





physical stress was applied to break the cell layer into pieces. Claudin 1, located at the cellular membrane as shown in Fig. 3b, was found to considerably enhance the tight junction capacity of HaCaT cells (Fig. 3c, d).

The enhancement of tight junction capacity by the three major components of WECT

Since it has been investigated that the components of *C. tricuspidata* water extract includes several bioactive phenolic compounds, we assessed whether those phenolic components are responsible for the increase of claudin 1 expression [23–25]. Among them, we treated chlorogenic acid, kaempferol, and quercetin to HaCaT cells, because they are the most abundance components and previously shown to have several bioactive effects [25]. The increase in claudin 1 protein expression was also shown in all of three components treated HaCaT cells (Fig. 4b). Furthermore, the increased claudin 1 was shown to be more localized in tight junctions, which resulted in the enhancement of tight junction ability (Fig. 4c, d).

Taken together, these data indicate that claudin 1 is increased at both the mRNA and protein level by WECT and its components, and that the increased claudin 1 is localized to the cellular membrane and enhances tight junction function in HaCaT cells. From our data, we demonstrate the potential possibility of WECT as an

herbal therapeutic treatment for tight junction-related skin diseases such as atopic dermatitis, psoriasis, and erythroderma.

Discussion

Tight junctions mediate the selective movement of solutes across the epithelium, and form an intercellular barrier between adjacent epithelial cells. Claudin 1-deficient mice die within one day of birth from severe dehydration [26]. In addition, alterations in tight junctions and their related proteins in the skin lead to psoriasis, ichthyosis, and erythroderma. The increase in cell-to-cell binding capacity by modulating tight junction-related proteins, could be a double-edged sword. However, regarding the advantage of increasing tight junction capacity upon skin damage, we focused on the effects of *C. tricuspidata* leaf extracts on tight junctions and claudin 1 expression, as a potential therapeutic treatment of the disease showing decreased level of tight junction proteins. We found that extracts of *C. tricuspidata* the leaf showed high free radical scavenging activity, similar to resveratrol (data not shown). Free radicals are highly reactive oxygen species (ROS) associated with cell damage and senescence. Accordingly, the elimination of ROS by *C. tricuspidata* leaf extracts could prevent cellular damage and senescence of skin. Our data demonstrated high antioxidant effects of the *C. tricuspidata* leaf, indicating that the leaf

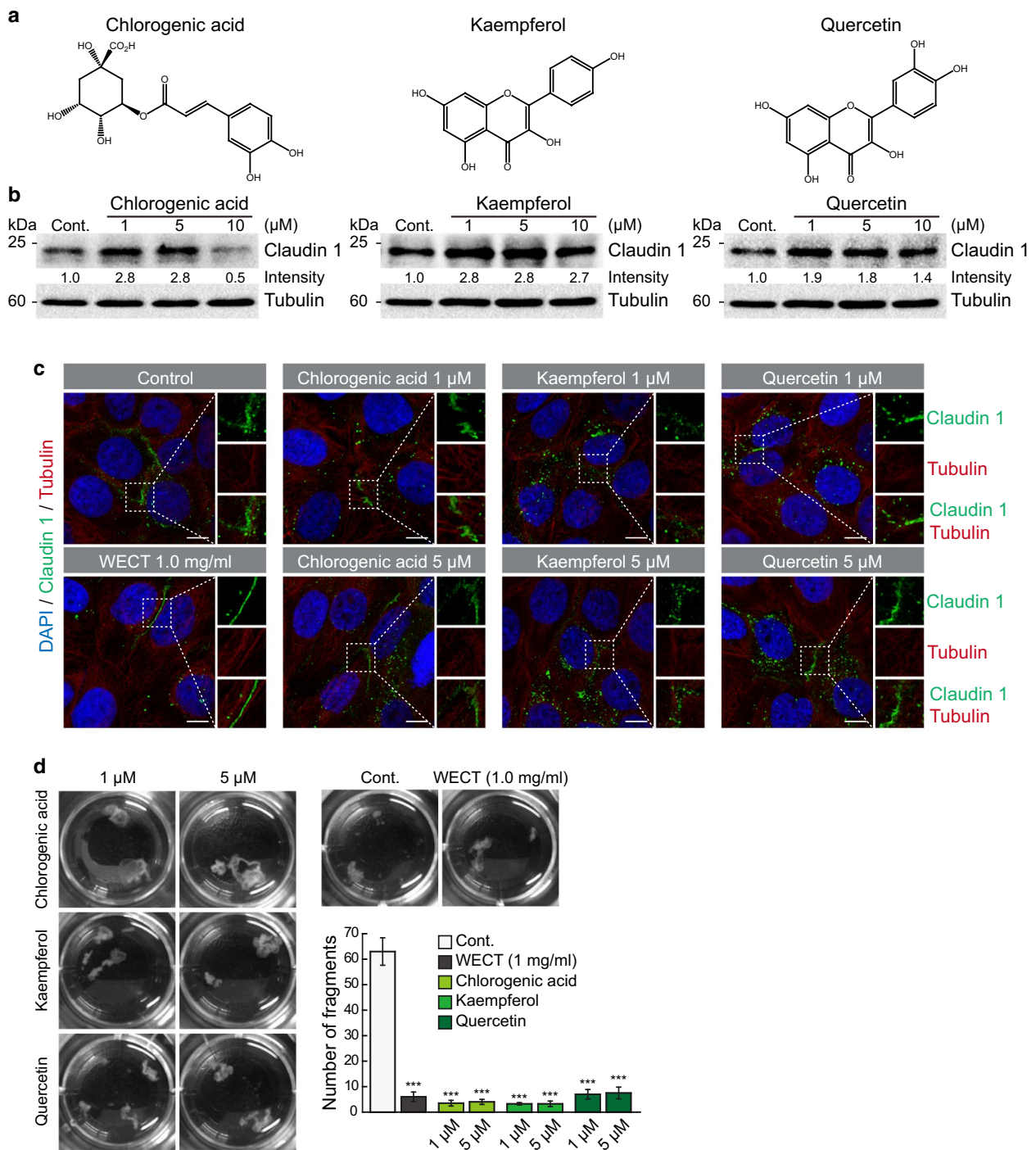


Fig. 4 The effect of three major components of WECT on tight junction. **a** Chemical structure of chlorogenic acid, kaempferol, and quercetin. **b** Immunoblot analysis showing increased claudin 1 protein by chlorogenic acid, kaempferol, and quercetin. Band intensity of claudin 1 was normalized by tubulin. **c** Increased claudin 1 distribution throughout the tight junction. WECT were treated for positive control. Cells were stained with anti-claudin 1 (green), anti-tubulin alpha (red), and DAPI for nuclei (blue). Scale bars indicate 10 μ m. **d** Disperse assay showing that enhanced tight junction capacity by the components of WECT. Number of fragments were counted. *** $p < 0.001$ (two-sided t -test, versus control)

extracts can regulate the progression of ROS-mediated senescence or cellular damage. To investigate the biochemical and physiological effects of *C. tricuspidata*, we first examined the cytotoxicity of the extracts in HaCaT cells, in order to optimize the treatment concentration for further studies. WECT showed low cytotoxicity up to 0.5 mg/ml, whereas EECT showed considerably higher cytotoxicity, even at the lowest concentration (Fig. 1). Although we equally adjusted the total volume of ethanol for each sample, it still significantly decreased cell viability. Thus, we speculated that the cytotoxic components of *C. tricuspidata* leaf, which have different solubilities in water and ethanol, might also have different effects on cell viability, causing the discrepancy between the two extracts. Based on our MTS data, we set the optimal concentrations to 0 to 0.5 mg/ml WECT, and 0 to 0.125 mg/ml EECT.

The formation of tight junctions in the uppermost portion of the cellular membrane is established by the polarized insertion of diverse proteins, which are related to tight junctions. Thus, we proposed that if *C. tricuspidata* leaf extracts could upregulate the expression of those types of proteins, it could be a useful natural compound for treating tight junction-mediated diseases such as atopic dermatitis. Accordingly, we investigated changes in tight junction-related genes after treatment with *C. tricuspidata* leaf extracts. To our surprise, claudin 1 expression was gradually increased by WECT, whereas other genes were not affected (Fig. 2a). EECT was shown to have high cytotoxicity even at the lowest concentration (Fig. 1). Nevertheless, we evaluated its potential regulatory effects on tight junction-related genes, due to the fact that it could contain the same component as WECT that is responsible for the claudin 1 increase. However, the expression of tight junction-related genes was not significantly affected by treatment with EECT (Fig. 2b). In our previous study, we investigated the effect of the fruit extract of *C. tricuspidata* on tight junction-related genes. Therefore, effective ingredients and mechanism of action on the tight junction of the extract of *C. tricuspidata* by different parts are thought to be different. When EECT was treated for 48 h, claudin 6 mRNA expression level was increased [27]. If the composition of the *C. tricuspidata* extract of the two sites is similar, this discrepancy may be caused by difference in treat time and if we treated EECT for longer time, we could see the same effect of EECT on claudin 6 expression level. Taken together, we speculate that the main components of *C. tricuspidata* leaf, which cause the increase in claudin 1 expression in HaCaT cells, are present only in WECT.

Given our observation that claudin 1 mRNA expression was increased in a dose-dependent manner, we speculated that *C. tricuspidata* leaf extracts would also

upregulate its protein expression. Thus, we performed western blot analysis to investigate whether the level of claudin 1 protein also increased. As expected, claudin 1 expression was upregulated in HaCaT cells after WECT treatment, and expression levels were consistent with the qRT-PCR results (Fig. 3a). However, several studies have reported that claudin 1 is localized to the cytoplasm; therefore, we sought to determine the site of the increased claudin 1 expression after WECT treatment. Using immunofluorescence microscopy analysis, we found that the increased claudin 1 was predominantly located in the cellular membrane, indicating that claudin 1-involved tight junction formation is strengthened by WECT (Fig. 3b–d). Natural material extract like WECT consists of many compounds. Thus, it was needed to identify the components of WECT that is responsible for the increase in claudin 1 expression. The major components of WECT and EECT were previously well studied and their functions were involved in anti-inflammation, cancer treatment, and several bioactivities [23, 28, 29]. Even though, the three active compounds are contained differently in WECT or EECT [23–25], the purpose of our study is to suggest the activity of the extracts on tight junction which could be applied to functional food and treatment of tight junction-mediated diseases. Thus, we chose only WECT, due to its low cytotoxicity. As shown in Fig. 4b and c, all of those well-known components of WECT, chlorogenic acid, kaempferol, and quercetin increased claudin 1 expression, even when treated at the lowest concentration. Same with the effect of WECT on tight junction capacity, the major three components also enhanced tight junction ability (Fig. 4d). However, it has been demonstrated that component of *C. tricuspidata*, oxyresveratrol, has tyrosinase inhibition activity [30] which is required for tight junction [31, 32] and that oxyresveratrol improve tight junction integrity via MAPK pathways [33]. In this study, we addressed other three components of *C. tricuspidata* that also increase tight junction integrity. We speculate that the underlying mechanism of the enhancement of tight junction by the three components would be similar with oxyresveratrol due to their structures, phenolic compounds; nonetheless, it will be necessary to further investigate the mechanism of the three compounds on tight junction enhancement. Several papers showed that the three compounds could induce the generation of ROS that could damage cells and alter gene expression [34–36]. We confirmed that the three active compounds did not show significant cytotoxicity on HaCaT cells (data not shown); however, it still needs to be investigated the gene expression profile by the treatment of the compounds. We, currently, investigate the effect of the three compounds on cell signal pathway whose action might be result in the

increase of claudin 1 and tight junction capacity. All together, we found that WECT could increase claudin 1 expression by which its representative components have synergetic effect on increase of claudin 1 expression.

Loss of claudin 1 in tight junctions can cause dehydration of the skin, leading to several skin diseases. Previous reports shown that by simply increase claudin 1 expression, it could alleviate the symptoms of those skin diseases. In the present study, we identified a novel regulatory effect of *C. tricuspidata* leaf extracts on claudin 1 expression and tight junction capacity. Our data suggest that WECT and its components could be used as a valuable treatment for preventing or improving tight junction-mediated skin diseases like dehydration and atopic dermatitis of the skin and as an additive for functional food or cosmetic for skin care.

Authors' contributions

Conceived and designed the experiments: EMK, KSP, MSN, KKK. Performed the experiments: JK, NC, EMK, KSP, YWK. Analyzed the data: JK, NC. Contributed plants materials: JHN. Wrote the paper: JK, NC, KKK. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed in this study are available from the corresponding authors on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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