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Original Research

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Extracts of *Grifola frondosa* inhibit the MAPK signaling pathways involved in keratinocyte inflammation and ameliorate atopic dermatitis

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Conflict of Interest
The authors declare no potential conflicts of interests.

ABSTRACT

BACKGROUND/OBJECTIVES: *Grifola frondosa*, commonly referred to as the maitake mushroom, has been studied extensively to explore its potential health benefits. However, its anti-inflammatory effects in skin disorders have not been sufficiently elucidated. This study aimed to elucidate the anti-inflammatory role of the ethanol extract of *G. frondosa* in atopic dermatitis (AD) using in vivo and in vitro models.
MATERIALS/METHODS: We investigated its impact on skin and spleen inflammatory responses in *Dermatophagoides farinae* extract (DFE)/1-chloro-2,4 dinitrochlorobenzene (DNCB)-induced AD-like skin lesions in a mouse model. Additionally, we determined the immunosuppressive response and mechanism of *G. frondosa* by inducing atopic-like immune reactions in keratinocytes through tumor necrosis factor (TNF)-α/interferon (IFN)-γ stimulation.
RESULTS: Our study revealed that *G. frondosa* ameliorates clinical symptoms in an AD-like mouse model. These effects contributed to the suppression of Th1, Th2, Th17, and Th22 immune responses in the skin and spleen, leading to protection against cutaneous inflammation. Furthermore, *G. frondosa* inhibited the production of antibodies immunoglobulin (Ig)E and IgG2a in the serum of AD mice. Importantly, the inhibitory effect of *G. frondosa* on inflammatory cytokines in TNF-α/IFN-γ-stimulated AD-like keratinocytes was associated with the suppression of MAPK (Mitogen Activated Protein Kinase) pathway activation.
CONCLUSIONS: Collectively, these findings highlight the potential of *G. frondosa* as a novel therapeutic agent for AD treatment and prevention.

Keywords: *Grifola frondosa*; atopic dermatitis; inflammation; cytokines; keratinocytes; mitogen-activated protein kinase

INTRODUCTION

Atopic dermatitis (AD) stands as the most prevalent long-term skin inflammation disorder globally. Its defining features include the accumulation of immune cells, heightened skin sensitivity, thickening of the skin layers, excessive skin cell growth, and typical eczema-like rashes. Additionally, intense itching is a significant symptom of this condition [1]. Keratinocytes react to both external and internal threats by producing pro-inflammatory molecules that are part of both the innate and adaptive immune responses. These molecules

Author Contributions

Conceptualization: Choi JK; Data curation: Choi EJ; Formal analysis: Choi EJ; Funding acquisition: Choi JK; Investigation: Choi EJ; Methodology: Choi EJ; Project administration: Choi JK; Resources: Choi EJ; Software: Choi EJ; Supervision: Choi JK; Validation: Choi JK, Choi EJ; Visualization: Choi EJ; Writing - original draft: Choi JK; Writing - review & editing: Choi JK.

are crucial in attracting certain cells, which is a fundamental process in the development and progression of AD. This mechanism is essential for the initiation of AD [1]. Furthermore, the production of immunoglobulin(Ig)E antibodies in AD induced by environmental allergens or autoantigens is correlated with skin inflammation and disease severity [2].

The dominant Th2 immune response that governs AD includes the production of cytokines such as interleukin (IL)-4, IL-13, and IL-31, which induce skin inflammation and further amplify IgE [2]. Moreover, augmentation of the adaptive immune responses of Th1, Th17, and Th22 cells contributes to a more severe trajectory of atopic onset. Therefore, strategies aimed at modulating responsive T cells are appealing options for the treatment of AD [2].

At present, treatments that block IL-4 and IL-13, including the IL-4Rα blocker dupilumab and the anti-IL-13 drug tralokinumab, have shown positive results in individuals with AD [3]. However, the efficacy of these therapeutic approaches in fully mitigating all symptoms of the condition and whether they will sustain long-term benefits remain unclear [1]. Furthermore, owing to the limited response and potential side effects, achieving a complete cure for AD through these treatments remains a formidable challenge.

Natural products have served as abundant sources of anti-inflammatory agents and have been employed for millennia in the treatment and prevention of various diseases [4]. These natural products exert their anti-inflammatory effects by reducing ILs produced by neutrophils, lymphocytes, monocytes, and inflammatory cells in AD [5]. This effect blocks the inflammatory response primarily through inhibition of the MAPK and NF-kB signaling pathways [5].

Wild mushrooms have been used for a long time in worldwide as folk medicine and functional foods [6, 7]. *Grifola frondosa*, a large edible mushroom of the Meripilaceae family, has garnered recent attention owing to its long-term consumption, which positively influences immunity and metabolism [8], and exhibits anti-tumor activity [9], anti-obesity effects [10], anti-aging and neuroprotective effects [11], and inhibits of skin fibroblast inflammation and cytotoxicity [12]. It was therefore of interest to investigate whether *G. frondosa* can suppress the production of inflammatory cytokines in AD, thereby potentially controlling the condition.

In this study, we evaluated the anti-inflammatory properties of an ethanolic extract from *G. frondosa* on AD-like skin lesions, using both *in vivo* and *in vitro* models. *G. frondosa* inhibited proinflammatory cytokines both *in vivo* and *in vitro* in an AD-like skin lesion model, exerting regulatory effects via mitogen-activate protein kinase (MAPK) signaling. Thus, the data presented here demonstrate the potential of *G. frondosa* as a natural candidate substance for future AD prevention and effective therapeutic strategies.

MATERIALS AND METHODS

Preparation of plant extracts

Specimens of *G. frondosa* were sourced from the National Institute of Horticultural and Herbal Sciences in Korea. After lyophilization, the specimens underwent three separate 2-h extractions using a 70% ethanol solution. The extracted solution was then filtered through a 0.25 μm filter and freeze-dried for a period of 5 days.

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Analysis of protein content and amino acids

The protein content of the extract was measured using the Bradford technique, with BSA (ranging from 0.1 to 1.0 mg/mL) forming the baseline calibration curve. To assess amino acids, the extract was broken down with 6N hydrochloric acid under vacuum conditions at 110°C over 24 h. The content of amino acids in the extract was evaluated using an L-8900 amino acid analyzer from Hitachi, Tokyo, Japan. This involved a post column reaction with ninhydrin. The settings for the amino acid analysis were an ion exchange column measuring 4.6 × 60 mm; an injection volume of 20 µL; and a UV detector set at VIS1:570 nm and VIS2:440 nm. A standard amino acid solution from Wako Osaka, Japan, served to identify and calculate the amino acid levels.

FT-IR spectroscopy

The FT-IR spectral details were collected with an Agilent Cary 630 spectrophotometer from Agilent Technologies, USA. This instrument is equipped with a single-bounce diamond ATR module designed for KBr and is complemented by MicroLab and Resolutions Pro Software. Spectra were recorded in the range of 350 to 6,300 cm-1 with a 2 cm-1 resolution, using the transmission method.

Animals

Female BALB/c mice that were 8-wk-old were purchased from Samtako in Osan, Republic of Korea, and maintained in an environment free from specific pathogens. All experimental procedures received the green light from Konkuk University's Institutional Animal Care and Use Committee under the approval number KU14012.

Induction of AD-like lesions in mice

Mice were subjected to AD by recurrently exposing their ears to *Dermatophagoides farina* extract (DFE; house dust mite extract) and 2,4-dinitrochlorobenzene (DNCB) in accordance with the method outlined by Choi et al. (2017). For AD induction, the mice were categorized into four distinct groups: control, AD, AD with *G. frondosa*, and AD supplemented with ceramide (Cera). Cera served as a benchmark treatment for positive results. The onset of AD was triggered by stripping both ear surfaces five times using surgical tape (Nichiban, Tokyo, Japan). Post this, 20 µL of 1% DNCB was applied to each ear and, after 4 days, it was followed by a 20 µL dose of DFE (10 mg/mL). Over the next 4 weeks, the DNCB and DFE treatments were alternated weekly. During this AD induction phase, an ethanolic extract of *G. frondosa* (20 µL, 1 mg/mL) was topically applied to each ear. From day 7 to day 28, *G. frondosa* and Sera were applied every other day, for a total of 12 times.

One day after DNCB or DFE treatment, ear thickness was assessed using a dial thickness gauge (Kori Seiki MFG Co., Tokyo, Japan). On the 14th and 28th days, blood was drawn from the mice through orbital puncture, processed to obtain serum samples, and then preserved at −70°C. After blood extraction, ear tissues and splenocytes were harvested for further histological evaluation, as well as mRNA and protein analyses.

Histology

The removed ears were maintained in 4% paraformaldehyde for a duration of 16 h, after which they were encased in paraffin. Thin slices, measuring 5 µm each, were cut and stained with hematoxylin and eosin. The thickness of both the epidermis and dermis was measured under a microscope. For evaluating the invasion of mast cells, the tissue slices were stained with toluidine blue. Following this, mast cells were tallied in five randomly selected viewing areas.

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Cell culture of stimulation of keratinocytes

HaCaT cells were cultured in Dulbecco's modified Eagle's medium enriched with 10% fetal bovine serum and an antibiotic mix of 10 μg/100 mL of penicillin/streptomycin. They were maintained in an environment with 5% CO2 at a temperature of 37°C. Before exposure to TNF-α (10 ng/mL) and IFN-γ (10 ng/mL) for a duration of 30 min or 6 h, the HaCaT cells were first pre-treated with *G. frondosa* and cyclosporin A for an hour. Subsequently, the mRNA expression of various cytokines and chemokines were examined.

Cell viability

Cell viability was assessed using the 3-(4,5-dimethylthiazolyl-2)2,5-diphenyl tetrazolium bromide assay (MTT) assay. HaCaT cells (5×10^4 per well) were treated with various concentrations of *G. frondosa* After 24 h, MTT (5 mg/mL) was added to each well and further incubated for 4 h. The formed formazan crystals were dissolved by adding 100 μL of isopropanol to each well. The absorbance values of each sample were calculated in relation to the average absorbance of the untreated control group and presented as a percentage.

RNA isolation and polymerase chain reaction (PCR)

RNA was extracted from ear tissue, and splenocytes of every treatment group utilizing TRIzol, adhering to the guidelines provided by the manufacturer. Splenocytes were derived by mincing and pulverizing the spleen tissue before RNA extraction. Superscript II reverse transcriptase (Invitrogen, Waltham, MA, USA) was employed to produce the first-strand cDNA. Quantitative real-time PCR was carried out with a Thermal Cycler Dice TP850 (Takara Bio Inc., Shiga, Japan) in line with the provided instructions. PCR conditions have been previously described [13]. Briefly, a 25-μL reaction mixture was prepared for every sample by blending 2 μL of cDNA (100 ng), 1 μl of both sense and antisense primers (0.4 μM each), 12.5 μL of SYBR Premix Ex Taq (Takarabio Inc., Shiga, Japan), and 9.5 μL of distilled water. The PCR settings were an initial step of 10 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C, and concluding with 15 s at 95°C, 30 s at 60°C, and another 15 s at 95°C. The mRNA quantities of the investigated genes were balanced against GAPDH, calculated as: relative mRNA expression = $2^{-(\Delta Ct \text{ of target gene} - \Delta Ct \text{ of GAPDH})}$, with Ct denoting the threshold cycle value. The ex-pression rate of the evaluated gene in each specimen was standardized against GAPDH and is shown as relative mRNA quantities.

Western blotting

Western blotting was conducted on whole cell lysates as previously described [13]. Briefly, cells (2×10^6 cells/6-well plate) were treated for 20 min to study the activation of p38, ERK, and JNK. Following treatment, cells were rinsed twice using cold PBS, and lysates were obtained in 200μl of lysis buffer. After centrifuging these lysates at 4°C for 20 min, the clear supernatants were gathered. Proteins were then run on an 8–12% SDS-PAGE and subsequently moved onto a nitrocellulose membrane. To confirm consistent sample loading, the membrane was marked using reversible Ponceau S staining. The ECL-PLUS system (Amersham, Arlingon Heights, IL, USA) was employed for immunodetection. The antibodies chosen for the western blot included: phospho-p38 MAPK, p38 MAPK, phospho-p44/42 MAPK (Erk1/2), p44/42 MAPK, phospho-SAPK/JNK, and SAPK/JNK, all sourced from Cell Signaling Technology.

Enzyme-linked immunosorbent assay

Serum levels of immunoglobulin IgE and IgG2a were measured on day 28 after the first induction using an IgE and IgG2a enzyme-linked immunoassay kit (BD Biosciences, Franklin Lakes, NJ, USA), following the manufacturer's instructions.

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Statistical analysis

Data analysis was carried out using GraphPad Prism 9. A one-way ANOVA was performed, followed by Dunnett's multiple comparison test. All data are displayed as the average ± SD of relative fold variations.

RESULTS

Characterization of *G. frondosa*

We examined the chemical composition of *G. frondosa* after ethanol extraction. *G. frondosa* has considerable nutritional value because it is rich in proteins, carbohydrates, and selected phytochemicals (**Supplementary Table 1**). The ethanolic extract of *G. frondosa* contained 195.46 ± 0.83 μg/g of carbohydrates. The monosaccharide constituents of the *G. frondosa* extract were primarily glucose (226.00 ± 3.22 μg/mL) and mannose (5.68 ± 0.37 μg/mL) (**Supplementary Table 1**). The ethanolic extract of *G. frondosa* also contained 208.50 ± 1.25 μg/g of protein, predominantly composed of glutamic acid (5.0980 g/100 g), as shown in **Supplementary Table 1**. Polyphenols constituted the primary constituents of the *G. frondosa* ethanolic extract (10.72 ± 0.028 mg/g), followed by flavonoids (728.08 ± 13.21 μg/g), while the β-carotene and lycopene contents were 22.80 ± 0.75 μg/g and 2.27 ± 0.11 μg/g, respectively.

The ethanolic extract of *G. frondosa* displays its FTIR spectrum in **Fig. 1**. At 3375 cm-1, the band signifies hydroxyl stretching attributed to hydrogen bonds and N-H vibrations. The band at 2931 cm-1 denotes C–H stretching. The 1622 cm-1 band reflects the asymmetric vibrations related to carboxyl groups. The stretch at 1,397 cm-1 is associated with the OH groups in phenolic components. Finally, the peaks at 1,079, 1,044, and 992 cm-1 are related to the C-O-C bonds from sugars present in the extract [14].

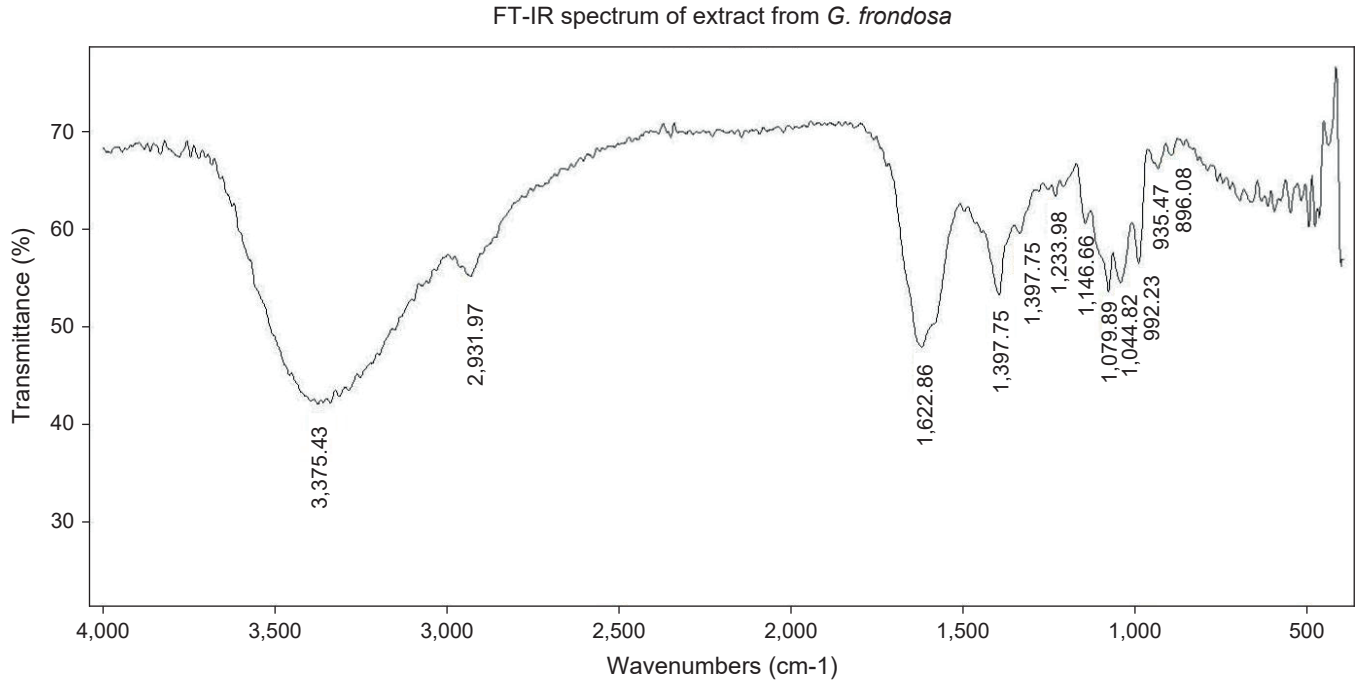

Fig. 1. FT-IR spectrum of the crude ethanolic extract of *G. frondosa*.

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G.frondosa suppressed symptoms of AD-like skin lesions *in vivo*

In this study, we investigated the potential *in vivo* inhibitory function of *G. frondosa* using an AD-like skin lesion model. AD was induced in the ears of BALB/c mice using DFE and DNCB for 28 days. Starting from day 7, as indicated in the schematic representation of disease progression, *G. frondosa* and Cera were used as treatment groups (**Fig. 2A**). DNCB/DFE-induced AD mouse model, Cera, which demonstrated effects in reducing clinical symptoms, regulating inflammation, and inhibiting skin moisture loss, was used as a control [15]. To assess changes in symptoms, ear thickness was measured for 28 days, and photographs of the mouse ears were taken on day 28. The ear thickness of the DFE/DNCB-induced AD and Cera groups was significantly increased (**Fig. 2B**), and images of the mouse ears exhibited pronounced inflammation with erythema and scaling (**Fig. 2C**). In contrast, the *G. frondosa* group exhibited significantly lower ear thick-ness compared to both the AD and control mice groups and displayed image results similar to those of the control mice. Upon examining the ear tissue histopathologically, it was observed that the thickness of both the epidermis and dermis was notably greater in mice with AD, accompanied by an elevation in mast cell infiltration. However, in mice treated with *G. frondosa*, the thickness of the epidermis and dermis decreased and mast cell infiltration was suppressed (**Fig. 2D and E**).

G. frondosa suppressed AD-like skin lesions by inhibiting the production of inflammatory cytokines associated with Th1, Th2, Th17, and Th22 responses

AD is marked by varying inflammatory stages that transition from initial, acute phases dominated by Th2 and Th22 cells to a prolonged chronic phase where Th1, Th2, and Th17 cells are prominent [16]. In particular, the Th1-driven IFN-γ promotes the formation of IgG2a antibodies, while the Th2-related cytokine IL-4 encourages the production of IgE. Consequently, each antibody type serves as a marker for different T cell reactions [17]. Numerous studies have interpreted the DFE/DNCB-induced AD skin lesion model as a chronic model, owing to the elevated production of IgE and IgG2a [13,18]. Therefore, we analyzed whether *G. frondosa* could inhibit the increased production of IgE and IgG2a in mouse serum induced by cytokines produced by T cells. As expected, elevated levels of IgE and IgG2a were observed in mouse serum 28 days after AD induction but was significantly suppressed in the serum of mice treated with *G. frondosa* (**Fig. 3A**). Subsequently, we isolated cells from mouse skin tissue and spleen to investigate whether the reduction in the clinical symptoms of AD induced by *G. frondosa* was associated with the inhibition of cytokine production in T cells. In AD mice, increased levels of IFN-γ (Th1), IL-4, IL-13, IL-31 (Th2), IL-17 (Th17), and IL-22 (Th22) have been observed in both the skin and spleen. In contrast, AD mice treated with *G. frondosa* exhibited a significantly lower mRNA expression of Th1, Th2, Th17, and Th22-related cytokines in these tissues and spleen cells (**Fig. 3B and C**). These results indicated that *G. frondosa* regulates the production of diverse cytokines derived from T cells within the skin and lymphoid tissues, even during the chronic phase of atopy, suggesting its potential to attenuate AD inflammation.

G. frondosa reduced the production of pro-inflammatory cytokines and chemokines in HaCaT cells

Human keratinocyte HaCaT cells stimulated with TNF-α and IFN-γ were utilized as an *in vitro* AD mimic model, featuring the expression of various inflammatory cytokines and Th2 chemokines such as CCL17 [13]. We first performed an MTT assay to assess the viability of HaCaT cells treated with different concentrations of *G. frondosa* to determine the effective concentration within the cells. *G. frondosa* treatment at various concentrations (0.25, 0.5, and 1 mg/mL) for 24 h did not affect the survival rate of HaCaT cells (**Fig. 4A**). To investigate the anti-inflammatory effects of *G.*

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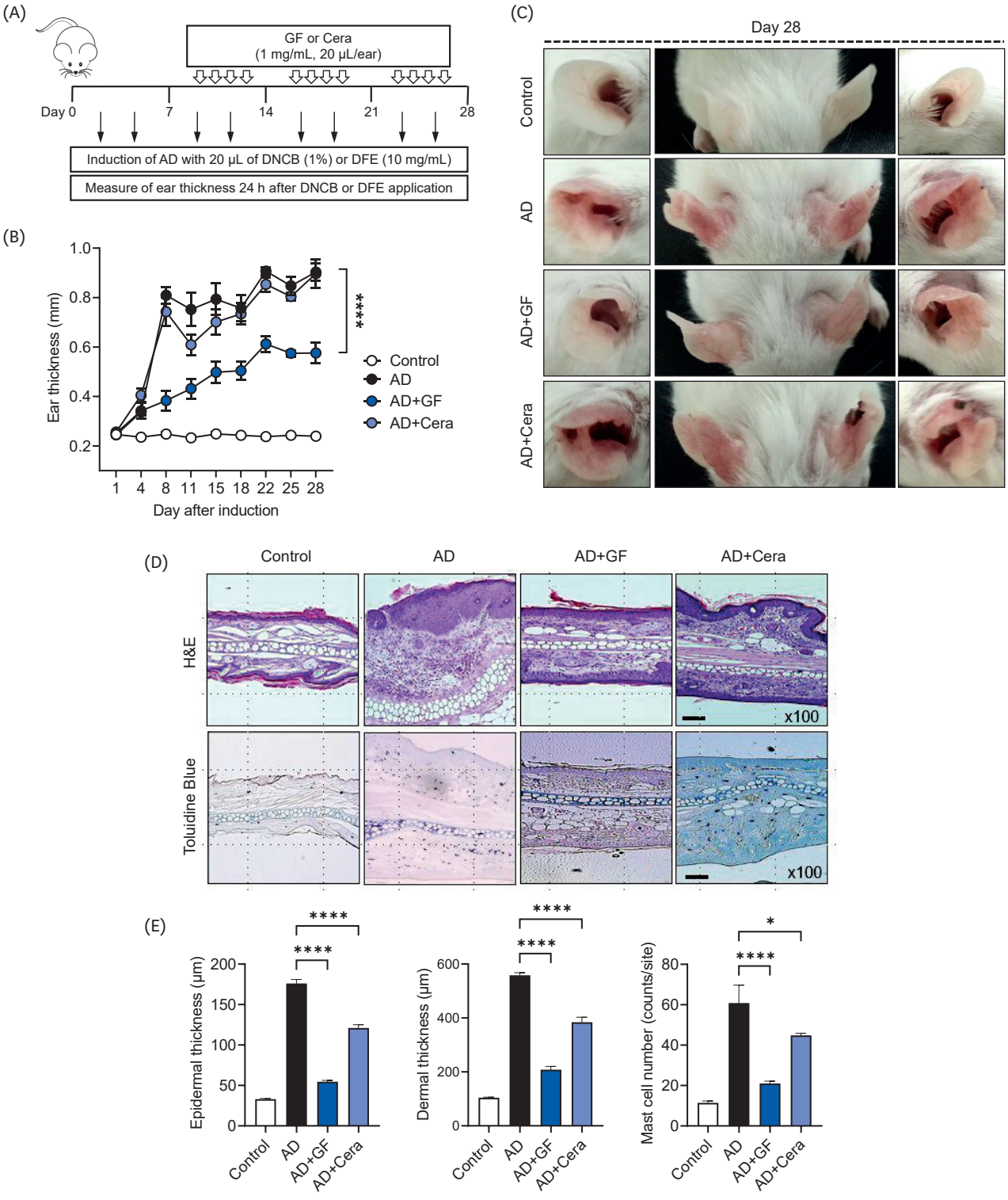

Fig. 2. *G. frondosa* treatment alleviates clinical symptoms in the AD-like skin lesion mouse model. (A) Study design for inducing AD-like skin lesions. Mice were grouped into four sets with 5 mice each. (B) 24 h post DNCB or DFE treatment, the ear thickness was gauged using a dial thickness instrument. (C) A photo representation of mouse ears from each typical group on the 28th day. (D) Microscope images of ear tissues stained with H&E or toluidine blue (at a 100× zoom, scale marker is 20 μm). (E) The thickness of both the epidermis and dermis was gauged from the H&E-stained tissue images. The count of invading mast cells was ascertained through toluidine blue staining. Data are presented as the mean ± SEM.
Cera, ceramide; AD, atopic dermatitis; DNCB, 2,4-dinitrochlorobenzene; DFE, *Dermatophagoides farina* extract; H&E, hematoxylin and eosin.
$^{*}P < 0.05$; $^{****}P < 0.0001$ indicates significant reduction compared to AD.

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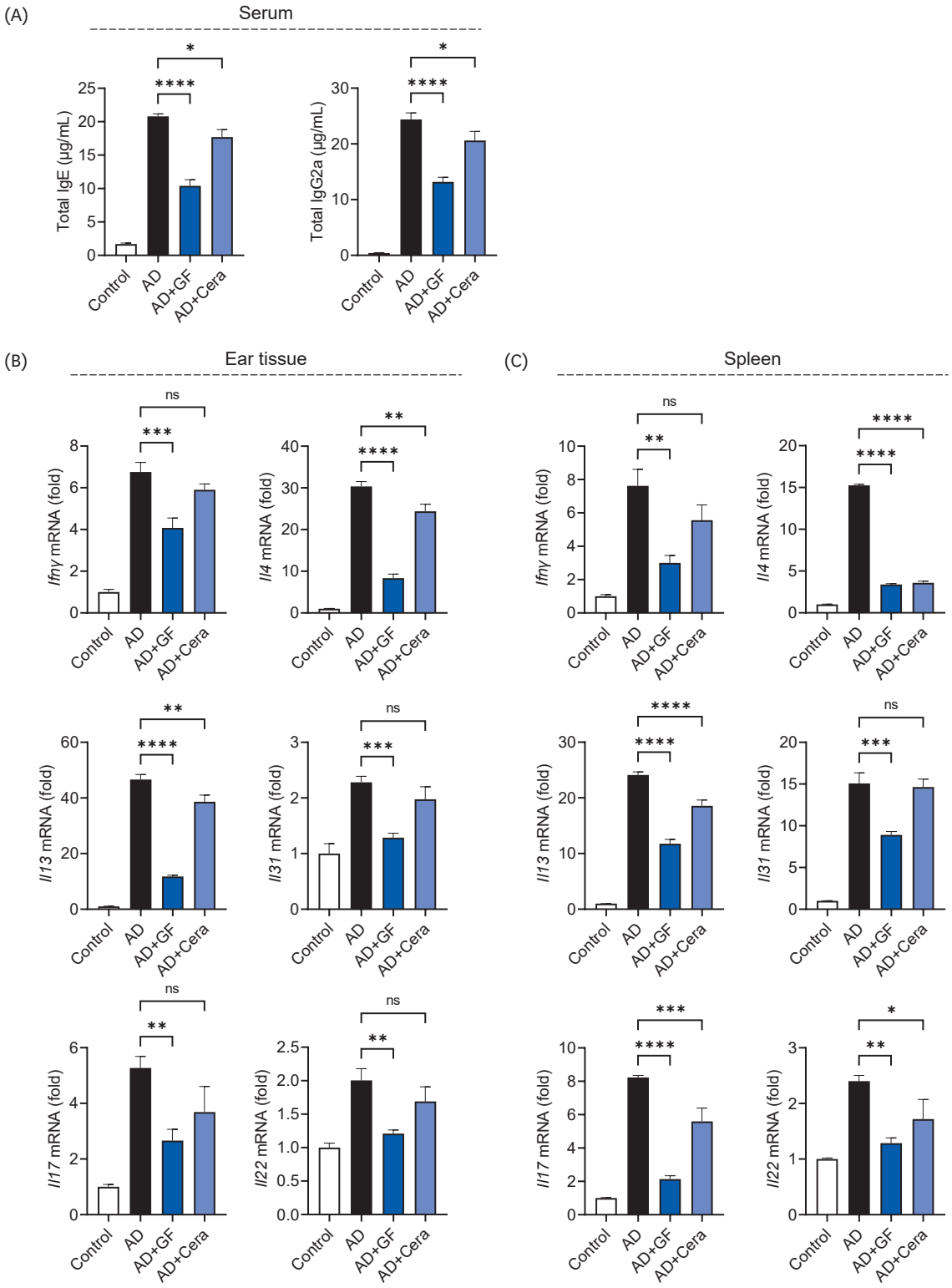

Fig. 3. *G. frondosa* treatment reduces immunoglobulin levels in the blood and suppresses cytokines related to Th1, Th2, Th17, and Th22. *G. frondosa* treatment reduces immunoglobulin levels in the blood and suppresses cytokines related to Th1, Th2, Th17, and Th22. (A) Total serum IgE and IgG2a levels were measured by enzyme-linked immunosorbent assay. (B) Cytokine expression in the ears and spleen of mice exhibiting AD-like skin lesions. On the 28th day, the ears and spleen were removed and total RNA was extracted. The quantitative real-time polymerase chain reaction was conducted following the procedures outlined in the Methods section. Data are presented as the mean ± SEM.
Ig, immunoglobulin; AD, atopic dermatitis; Cera, ceramide; ns, not significant.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ indicates significant reduction compared to AD.

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frondosa in *in vitro* AD-like model, we cultured HaCaT cells in a medium containing *G. frondosa* or cyclosporine A (CsA) after stimulation with TNF-α/IFN-γ. In AD patients, CsA, which effectively regulates pro-inflammatory cytokines, was used as a drug control [19]. After 6 h of stimulation, mRNA expression was measured using RT-PCR and qPCR. The results revealed that at concentrations of 0.5 and 1 mg/mL of *G. frondosa*, the expression of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, along with the CCL17 chemokine, was significantly suppressed in PCR analyses compared to the group stimulated only with TNF-α/IFN-γ (**Fig. 4B and C**). In addition, a more pronounced effect was observed compared to that of CsA (**Fig. 4B and C**).

G. frondosa controlled inflammatory cytokines and chemokines by inhibiting the activation of MAPK signaling

Stimulation with TNF-α and IFN-γ-stimulated HaCaT cells activate the p38, ERK, and JNK signaling pathways in the MAPK signaling cascade when producing pro-inflammatory cytokines and chemokines [20]. Given this, we investigated whether *G. frondosa* inhibits MAPK activation and suppresses pro-inflammatory cytokines and CCL17 in HaCaT cells stimulated with TNF-α and IFN-γ for 30 min in media containing *G. frondosa*. Using western blotting, we observed significant inhibition of p38 and ERK activation in cells treated with *G. frondosa* (**Fig. 5**). However, *G. frondosa* did not inhibit the JNK expression. These results suggest

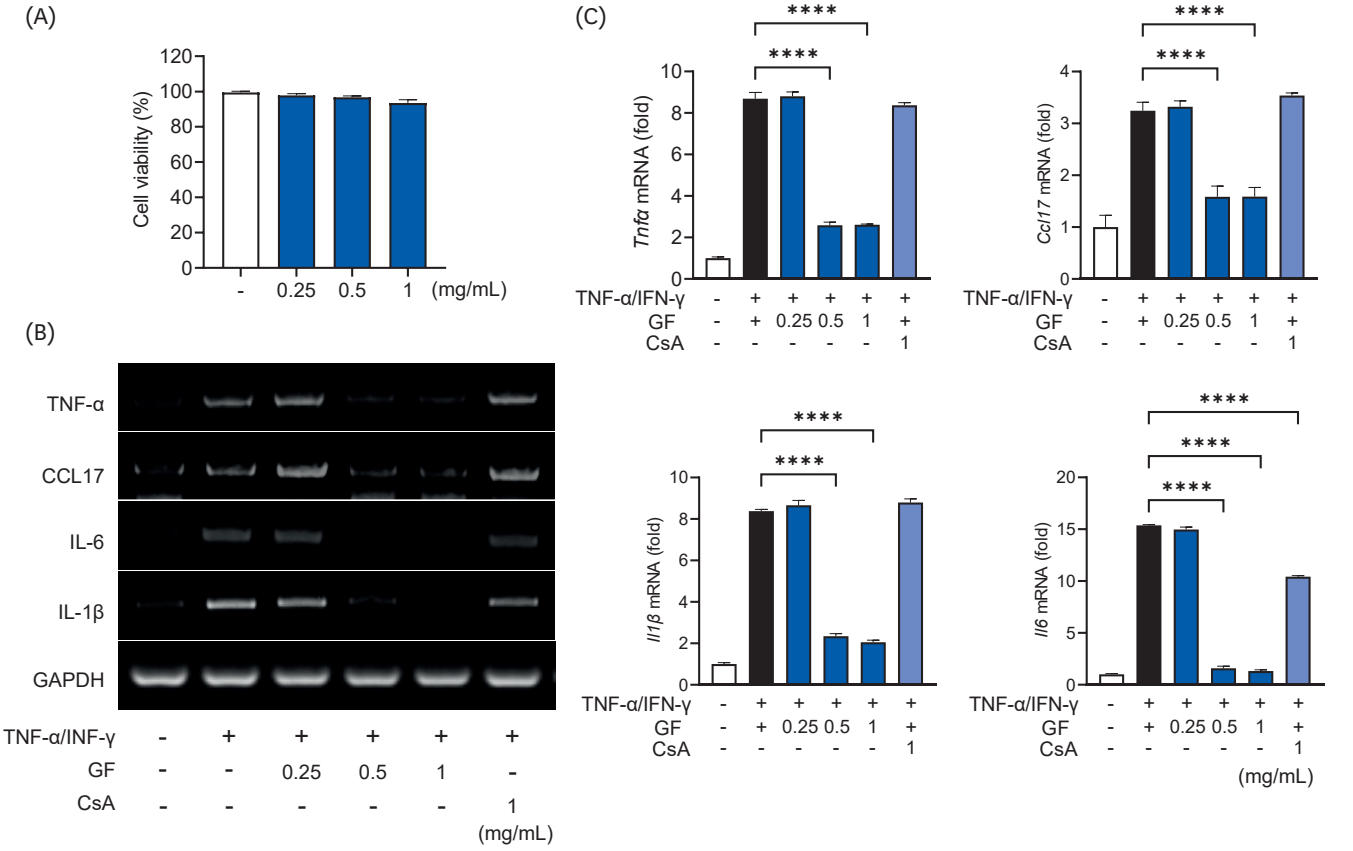

Fig. 4. *G. frondosa* suppresses chemokines and cytokines in keratinocytes. (A) The cytotoxic effects of *G. frondosa* on HaCaT cells were examined. The MTT assay was used to cell viability over a 24-h period. (B and C) HaCaT cells with or without *G. frondosa* or CsA pre-treatment for 1 h were stimulated with TNF-α (10 ng/mL) and IFN-γ (10 ng/mL) for 6 h to analyze gene expression. Pro-inflammatory cytokine gene expressions (TNF-α, IL-1β, and IL-6) and CCL17 were assessed by quantitative real-time polymerase chain reaction analysis (C). Data are presented as the mean ± SEM.
TNF, tumor necrosis factor; IFN, interferon; CsA, Cyclosporine A; IL, interleukin.
$^{****}P < 0.0001$ indicates significant reduction compared to atopic dermatitis.

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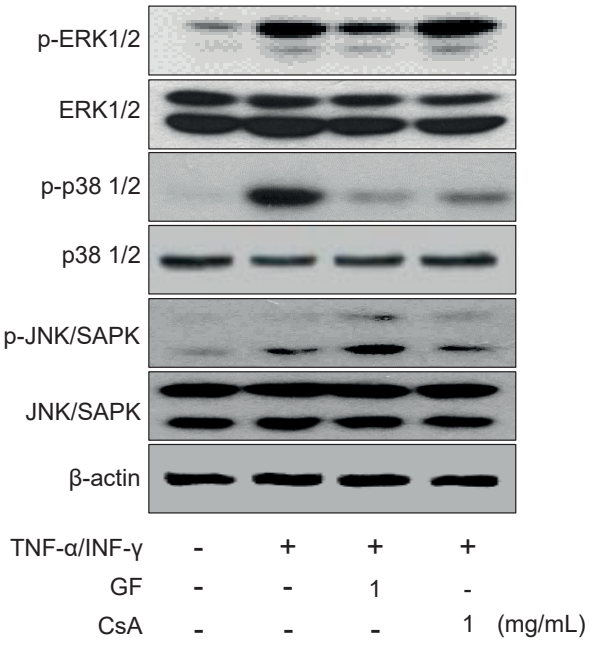

Fig. 5. G. *frondosa* downregulates the phosphorylation of MAPK signaling molecules such as p38 and ERK in keratinocytes. Inhibition of the p38, ERK, and JNK MAPKs pathways. Western blotting analysis of whole-cell extracts of HaCaT cells. Before being stimulated with TNF-α (10 ng/mL) and IFN-γ (10 ng/mL) for 30 min, cells were pre-treated with either G. *frondosa* (1 mg/mL) or CsA (1 mg/mL) for an hour.
TNF, tumor necrosis factor; IFN, interferon; CsA, Cyclosporine A.

that *G. frondosa* inhibits pro-inflammatory cytokines and chemokines by interfering with or suppressing p38 and ERK MAPK signaling during inflammation activation in keratinocytes.

DISCUSSION

In this study, we investigated the inhibition of inflammation in AD and the underlying mechanisms of *G. frondosa*, which has a wide range of therapeutic activities such as immunomodulatory, antioxidative, and anti-inflammatory properties [12,21,22]. Many studies reported that *G. frondosa* harbors essential bioactive constituents, including β-glucans, with a relatively high content [23-25]. In addition, the application of β-glucans has been shown to result in significant improvements in the clinical symptoms of AD, particularly in the notable alleviation of pruritus in the treated half of the patients [26]. Our analysis of the ethanol extract of *G. frondosa* revealed a carbohydrate content of 195.46 ± 0.83 μg/g, and this extract exhibited higher levels of flavonoids, lycopene, and β-carotene compared to ethanol extracts from dried mushrooms. These components exert various therapeutic effects. Hence, we hypothesized that the constituents and contents of this *G. frondosa* ethanol extract would have a positive impact on the inhibition of inflammation in AD.

AD is a chronic inflammatory skin disorder characterized by dryness, itching, and rash [2]. The pathogenesis of AD is intricately linked to complex immune system dysfunction, in which T cells play a central role [2]. T cells are vital constituents of the immune system and are categorized into diverse sub-types, with particular emphasis on Th2 cells in the context of AD [27]. Overactivation of Th2 cells triggers an inflammatory cascade, impairing skin barrier function and enhancing sensitivity to allergens [2]. Although Th2 dominance is a

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hallmark of the acute stages of AD, the transition from Th2 to Th1 is recognized as driving the chronic stage [27]. Progression from acute to chronic AD involves heightened Th2, Th1, Th17, and Th22 adaptive immune responses, accompanied by increased cytokine production [2]. Currently, therapies such as Th2 cytokine inhibitors, steroids, and immunosuppressants have demonstrated efficacy in AD and have become standard treatments [2]. However, owing to potential long-term side effects and limited effectiveness, the ongoing development of AD treatments is essential. Natural products, often associated with fewer side effects than conventional medications, hold promise as effective treatments when properly dosed [28]. In particular, *G. frondosa* exhibits anticancer, antimicrobial, antioxidant, and anti-inflammatory actions [25]. In this study, we demonstrate the immunosuppressive activity of *G. frondosa* during the onset of AD, highlighting its potential as a natural remedy for dermatological conditions. The ap-plication of *G. frondosa* during AD induction in mice impeded inflammatory cell infiltration, including edema, erythema, and mast cell activation, thereby providing protection against AD. Moreover, *G. frondosa* suppresses the expression of cytokines produced by Th1 (IFN-γ), Th2 (IL-4, IL-13, and IL-31), Th17 (IL-17A), and Th22 (IL-22) cells in skin and lymphoid tissues. Notably, IFN-γ and IL-4 stimulate B-cell differentiation into plasma cells, enhancing IgE and IgG2a production [29], which can amplify chronic AD inflammatory responses [2]. However, *G. frondosa* potentially contributes to the effective control of chronic AD by inhibiting all these processes.

Keratinocytes play an initiating role in triggering inflammatory cascades in AD, given that they are a major source of chemokines and inflammatory cytokines that recruit inflammatory cells, such as dendritic cells, adipocytes, eosinophils, and T cells, to the AD skin [30-32]. Among them, a chemokine known as CCL17 is not expressed in normal skin but is found in both acute and chronic AD lesions [31]. Elevated levels of CCL17 drive Th2 polarization, which is crucial in AD pathogenesis, and exacerbates AD by inducing pruritus [33]. While several mechanisms underpin CCL17 contribution to AD pathogenesis, numerous studies have reported that in HaCaT cells, TNF-α and IFN-γ, upon binding to their specific receptors, induce the activation of p38, ERK, or JNK through phosphorylation [20,34]. This triggers CCL17 synthesis, accompanied by inflammatory cytokines such as TNF-α, IL-1β, and IL-6, and is employed as an *in vitro* model mimicking AD [34]. In line with this, our results showed a significant increase in CCL17 and inflammatory cytokines in TNF-α and IFN-γ stimulated HaCaT cells, with *G. frondosa* demonstrably inhibiting these elevated cytokines. *G. frondosa* also distinctly suppressed the activation of MAPK pathways, notably the p38 and ERK pathways. This is consistent with recent reports showing that some constituents of *G. frondosa* inhibit p38, ERK, and JNK, demonstrating their anti-inflammatory and immunomodulatory effects [22]. In contrast, our *in vitro* results indicated that cyclosporine A, which is used for the treatment of immune disorders, such as AD, did not inhibit the activity of cytokines, including CCL17 and MAPK. These results suggest the potential of *G. frondosa* to be more effective in sup-pressing AD, emphasizing the value of natural product-based therapies.

In summary, *G. frondosa* is capable of suppressing AD, not merely as a single compound, but also as its own extract. Natural products typically contain various beneficial ingredients and are more cost-effective than most pharmaceuticals. Thus, when judiciously utilized at appropriate doses, these natural products present potential alternative therapeutic strategies for the management of AD. Our evidence suggests that *G. frondosa*, by inhibiting cytokine production across various T cell types, offers an appealing option for therapeutic strategies in both acute and chronic AD, as well as in other skin disorders.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1

Content of carbohydrates, proteins and selected phytochemicals of aqueous extract from *G. frondosa*

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