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Effects of *Thymus quinquecostatus* Celakovski on Allergic Responses in OVA-Induced Allergic Rhinitis Mice

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Abstract: Allergic rhinitis (AR) is defined allergic disease that is mediated by Th2 cells. Its incidence rate is showing a growing tendency worldwide. Research on traditional medicine for AR is also being increasingly conducted. *Thymus quinquecostatus* Celakovski (TQ) has been used as an important medicinal and aromatic plant in the world. The purpose of this study was to assess whether TQ can alleviate AR. BALB/c mice were sensitized and challenged with ovalbumin (OVA) to provoke AR. Mice were treated with ethanol extract of TQ at 10 or 100 mg/kg after the intranasal OVA challenge. Their clinical symptoms such as nasal rubbing and sneezing were significantly reduced in the ethanol extract group (10 or 100 mg/kg) compared to the OVA group. Serum levels of Th1 (TNF-α) and Th2 (IL-4, IL-5, and IL-6) cytokines and IgE levels (both total and OVA-specific) were significantly reduced by administration of ethanol extract of TQ at 100 mg/kg. The thicknesses of the nasal septum and epithelium were significantly reduced by the administration of ethanol extract of TQ. These results suggest that TQ may inhibit early and late phases of AR reactions.

Keywords: allergic rhinitis; anti-inflammation; ovalbumin; *Thymus quinquecostatus* celakovski; Th1/2 cytokine

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1. Introduction

Allergy, which is a chronic inflammatory disease, is a type of hypersensitivity reaction caused by an abnormally occurring immunological mechanism [1]. It is interpreted as an immune response that causes stimulatory or harmful actions from a wide range of meanings such as altered states and adverse reactions due to repeated exposure to an external stimulus, that is, an antigen [2]. Causes of allergies include dust, pollen, grass, pet dander, mold, and so on. In addition, allergies are usually immunoglobulin E (IgE)-mediated hypersensitivity reactions that can cause abnormal hypersensitivity reactions to inflammation [3].

The prevalence rate of allergic diseases has been on the rise in recent years because of the development of industrialization. Notably, the incidence rate of allergic rhinitis (AR) has increased rapidly [4]. AR is a chronic inflammatory disease that occurs in the nasal cavity mediated by IgE. IgE antibodies can bind to antigens to form a complex that can

bind to the membrane surface of basophils and mast cells. After that, these cells produce histamine, prostaglandin, and leukotrienes. These chemical mediators cause various pathological outcomes such as smooth muscle contraction, vascular permeability, vasodilation and flushing, mucous secretion, and pruritus. Consequently, clinical symptoms of AR such as clear runny nose, paroxysmal sneezing, itching, rhinorrhea, and nasal congestion [5–7]. They begin at 1 to 2 min after antigen inflow. Symptoms usually disappear within one hour. If AR persists for a long time, various chronic inflammatory diseases such as sinusitis, asthma, nasal polyposis, and serous otitis media occur [8,9]. Current treatments for AR can only alleviate symptoms not eliminate the cause of disease, because it is almost caused by environmental factors. It is classified as seasonal and perennial factors. Seasonal factors of allergen originate from pollen of grasses, trees, and weeds. And, perennial factors of allergen are originated from dust mites, fungi, animal dandruff, and other occupational factors [10,11]. The symptomatic treatment uses medications such as corticosteroids, antihistamines, cromolyn, anti-cholines, and leukotriene receptor antagonists depending on symptoms. However, side effects are also occurring when drugs are used for a long time. Immunotherapy is considered if they do not respond to these medications [12]. It is effective in improving symptoms and quality of life. However, it has the disadvantages of requiring long-term treatment of 3 years or more and a high cost [13].

Experimental AR model in mice can be established by administering the unsensitized proteins to the nasal cavity and respiratory tract in mice because AR is not spontaneously caused in mice [14]. OVA, house dust mite, and fungus are the widely used allergens to induce the experimental AR model in mice. Among them, OVA is the universally used reagent for experimental allergen because of its low cost, high purity, and advantage to the study mechanism by using the genetic manipulation mice for OVA [15]. It is the major constituent protein in egg white, which represents 60~65% of the total protein [16]. So, it can work as an allergen to the mice, which are bred on the OVA-free feed. LPS-eliminated pure OVA is ordinarily co-sensitized with an adjuvant such as aluminum hydroxide (alum), heat-killed *Bordetella pertussis*, and complete Freund's adjuvant (CFA) in order to enhance the inflammatory reaction. Alum and *Bordetella pertussis* boost Th2 responses, and CFA boosts Th1 responses. [17–19]. A two-step procedure to administrate allergen is commonly accepted and used: sensitization phase (general sensitization) and challenge phase (local sensitization). The sensitization phase is generally accomplished by administrating allergen through intranasal (IN), intraperitoneal (IP), or subcutaneous (SC) [20]. IP injection of antigen is universally adopted in asthma and AR models because it can produce IgE more than IN [21]. Antigen-presenting cells (APCs) migrate to the local lymph node after being sensitized to allergen. APCs present allergen to T cells followed by T cells differentiating to Th2 cells. Th2 cells produce cytokines such as IL-4, IL-5, and IL-13, and present allergen to B cells. Accordingly, B cells produce antigen-specific IgE. The purpose of the sensitization phase establish the IgE-mediated sensitive immune response system by generating memory lymphocytes. The challenge phase is accomplished by administrating the same allergen as the sensitization phase through IN, transtracheal, or inhalation injection. Accordingly, Th2-dominant atopic reactions can be developed in specific sites such as nasal or skin. IN instillation of antigen is generally adopted method for airway allergy models such as AR and asthma [14,22–24]. Therefore, we adopted a two-step procedure to induce AR in mice by using OVA and alum.

Thymus quinquecostatus Celakovski (TQ) is a perennial woody plant belonging to the family Labiatae, named 'Thyme' in Europe. It has been used as an important herbal medicine in East Asia, including Korea. In particular, the essential oil of thyme has been widely used for treating digestive disorders, excitatory neurological diseases, high blood pressure, and respiratory inflammatory diseases [25]. Thyme's essential oil contains about 25~42% of a variety of phenolic compounds such as thymol, so it is very effective in inhibiting lipid oxidation [26,27]. Since thyme's essential oil contains a large number of active substances, studies on its anti-inflammatory [28], cell regeneration [29], antibacterial [30], antifungal [31], and antioxidant effects [10] have been conducted. Until now, studies on TQ

have been mainly conducted on the analgesic, anti-inflammatory, and sedative properties of the essential oil extract. Therefore, research on the anti-allergy effects of TQ extracts is insufficient.

Accordingly, this study aimed to investigate the anti-allergic effect of TQ extract using an animal model of ovalbumin (OVA)-induced AR to confirm the regulatory effect of TQ on allergic reactions.

2. Materials and Methods

2.1. Reagents

Chicken egg albumin (ovalbumin, OVA) and aluminum hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse IL-4 and IL-5 ELISA kits were purchased from Invitrogen (Carlsbad, CA, USA). TMB (3,3′,5,5′-Tetramethylbenzidine) single-solution substrate reagent was purchased from Thermo Fisher Scientific (Frederick, MD, USA). Anti-ovalbumin IgE (mouse) ELISA kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). The mouse IgE ELISA set, mouse TNF-α kit, and mouse IL-6 ELISA kit were obtained from BD Bioscience (San Diego, CA, USA).

2.2. Preparation of TQ

The ethanol extract of TQ was obtained by a reflux decoction method. Briefly, TQ (200 g) was sonicated with 2000 mL 50% ethanol (% v/v) for 2 h. The solvent was filtered with Whatman No.2 filter paper and evaporated by a rotary evaporator. Finally, it was lyophilized (Batch method) and stored at 4 °C. The yield of dried extract was 6.5%. The ethanol extract of TQ was diluted in purified water and filtered with a 0.45 μm syringe filter (HYUNDAI Micro, Seoul, Republic of Korea) before the experiment.

2.3. Animals

Female BALB/c, 6-week-old mice (19–21 g, n = 24) were purchased from SAMTACO (Osan, Republic of Korea). These mice were acclimatized in a specific pathogen-free (SPF) environment at 22 ± 2 °C with a 12-h light/dark cycle for one week. Animal care and all experimental procedures were approved by the Committee on the Care of Laboratory Animal Resources, Jeonbuk National University, Korea (approval number: JBNU 2022-05).

2.4. Experimental Group Design

A total of 24 mice were randomly divided into four groups (6 mice per group): Blank group (normal control + saline), AR group (OVA-induced AR + saline), TQ 50% EtOH 10 group (OVA-induced AR + 10 mg/kg TQ 50% EtOH extract treatment), and TQ 50% EtOH 100 group (OVA-induced AR + 100 mg/kg TQ 50% EtOH extract treatment).

2.5. The OVA-Induced AR Model

After stabilizing mice for one week, mice were sensitized by intraperitoneal injection of OVA (250 μg) in 200 μL of phosphate buffer solution (PBS) containing 10 mg of aluminum hydroxide on days 0, 7, and 14 as shown in Figure 1a. One week later from the last injection of OVA, mice were challenged for 10 days (from day 21 to day 30) with nasal instillation of OVA at 50 μg in 20 μL of PBS into bilateral nasal cavities, and the absorption of the drug was waited for 2 min. TQ extract was given orally to mice after the nasal cavities challenge on days 21–30. The control group was treated with PBS. Thereafter, nasal rubbing time and number of sneezing known to be behavioral symptoms of allergic rhinitis were measured for 10 min. Behavioral changes were observed until the end of the experiment. At the end of each experimental day, a blood sample was drawn from the orbital vein under diethyl ether anesthesia. Nasal mucosa and tissues were collected.

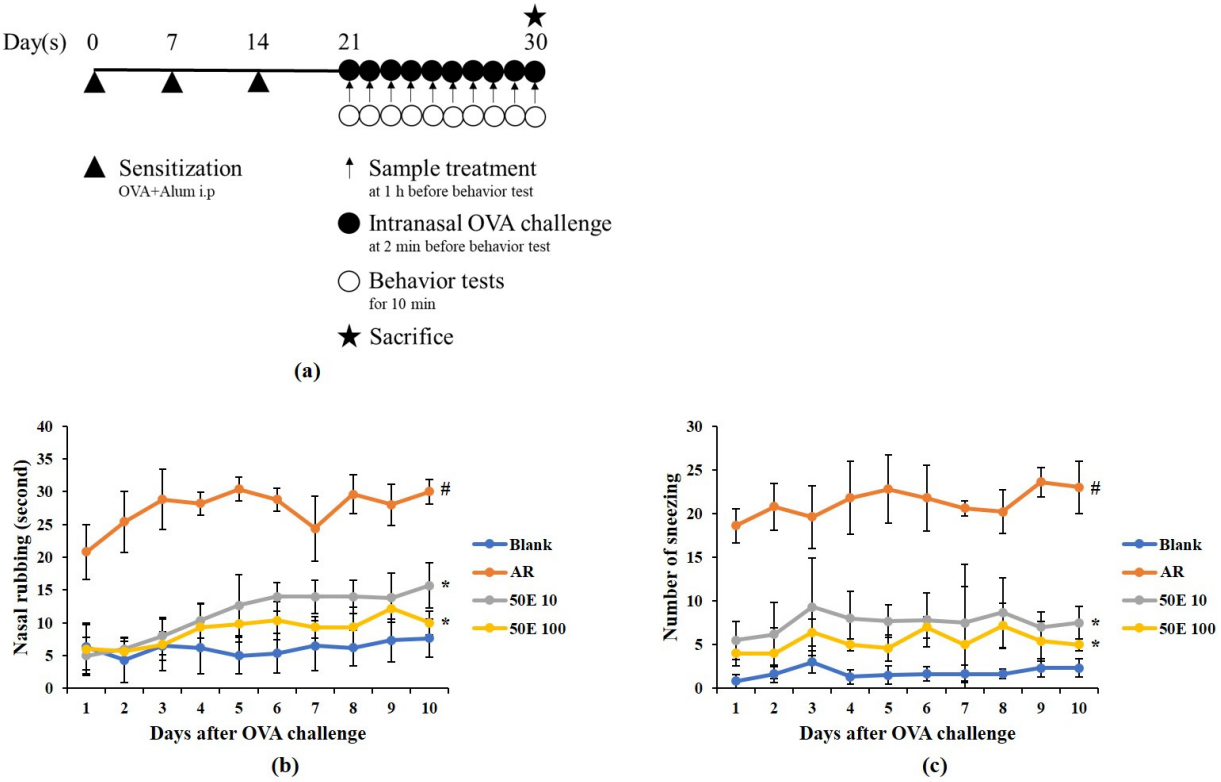

Figure 1. Effect of TQ extract on the nasal symptom score of ovalbumin (OVA)-induced AR mouse. (**a**) Schematic diagram of the experiment. The number of mice rubbing motions (**b**) and nose sneezing (**c**) were measured for a 10-minute period. Normal group: Blank; OVA-induced AR group: AR; 10 mg/kg TQ 50% EtOH extract treated AR group: 50E 10; 100 mg/kg TQ 50% EtOH extract treated AR group: 50E 100. Values are presented as mean ± SEM (n = 6 in each group). # $p < 0.05$ compared with Blank group; * $p < 0.05$ compared with AR group.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA) Measurement in Serum

Serum levels of IL-4 and IL-5 (Invitrogen, Carlsbad, CA, USA), OVA-specific IgE (Cayman Chemical, Ann Arbor, MI, USA), total IgE, TNF-α, and IL-6 (BD Bioscience, San Diego, CA, USA) were measured using ELISA kits according to the manufacturer's instructions.

2.7. Histopathological Evaluation of Nasal Mucosa Tissue

The nose of each mouse was removed and then fixed in 10% paraformaldehyde for two days, followed by decalcification in an ethylenediamine triacetic acid decalcifying solution for 5 days. Specimens were then embedded in paraffin wax. Nasal tissues were coronally sectioned into 5 µm slices and stained with hematoxylin and eosin for general morphology. After that, infiltration of inflammatory cells and epithelial damage in the nasal tissue were evaluated in randomly selected high-power fields at 4× and 200× magnifications.

2.8. Liquid Chromatography-Mass Spectrometry Analysis (LC/MS)

The standard compounds of caffeic acid (CA) and rosmarinic acid (RA) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Sigma-Aldrich Co. LLC (St. Louis, MO, USA), respectively. Samples and standards were dissolved in methanol. Ultra-performance liquid chromatography (UPLC) was performed on an ACQUITY UPLC system (Waters, MA, USA) with an ACQUITY binary solvent manager pump (ACQ-BSM), an ACQUITY PDA detector (ACQ-PDA), and a Waters micromass ZQ spectrometer (Waters) coupled with an electrospray ionization (ESI) interface and an ion trap mass analyzer. Samples and standards were analyzed under the following conditions: column, ACQUITY UPLC BEH Shield RP18 (2.1 × 100 mm, 1.7 µm; USA); mobile phase, distilled

water (solvent system A) and acetonitrile (CH_3CN, solvent system B) in a gradient mode (B 3% from 0 to 10 min, B 30% from 10 to 15 min, B 100% from 15 min to 18 min, B 3% from 18 to 20 min); sample injection volume, 5 μL; flow rate, 0.3 mL/min; column temperature, 40 °C, UV wavelength, 325 nm. Conditions for MS analysis in the negative ion mode were as follows: capillary voltages, 3.30 kV; cone voltage, 50 V; extractor voltage, 3 V; RF lens voltage, 0.2 V; source temperature 120 °C, desolvation temperature 300 °C, gas flow desolvation 600 L/h, gas flow cone 30 L/h.

2.9. Statistical Analysis

The results are presented as mean ± SEM of at least three independent experiments. The data were analyzed using SPSS Statistics 20.0. A Tukey test was used to determine statistically significant differences. $p < 0.05$ was considered significant.

3. Results

3.1. Mitigating Effect on Nasal Rubbing Time and Number of Sneezing

Itching, nasal congestion, rhinorrhea, runny nose, and sneezing are appeared during AR [11]. As shown in Figure 1b,c, the time of nasal rubbing and sneezing was significantly increased in the AR group compared to the Blank group. However, both symptoms including nasal rubbing and sneezing were significantly decreased in both groups treated with 50% EtOH extract of TQ at 10 and 100 mg/kg compared to the AR group.

3.2. Effect of TQ Extracts on Production of Th1-and Th2-Related Cytokines in Serum

As shown in Figure 2, both Th1 and Th2-related cytokines in serum were significantly increased in the AR group compared to the Blank group. However, both Th-1 (TNF-α) and Th-2 (IL-4, IL-5, and IL-6) related cytokines were significantly decreased in groups treated with 50% EtOH extract of TQ at 10 and 100 mg/kg compared to the AR group.

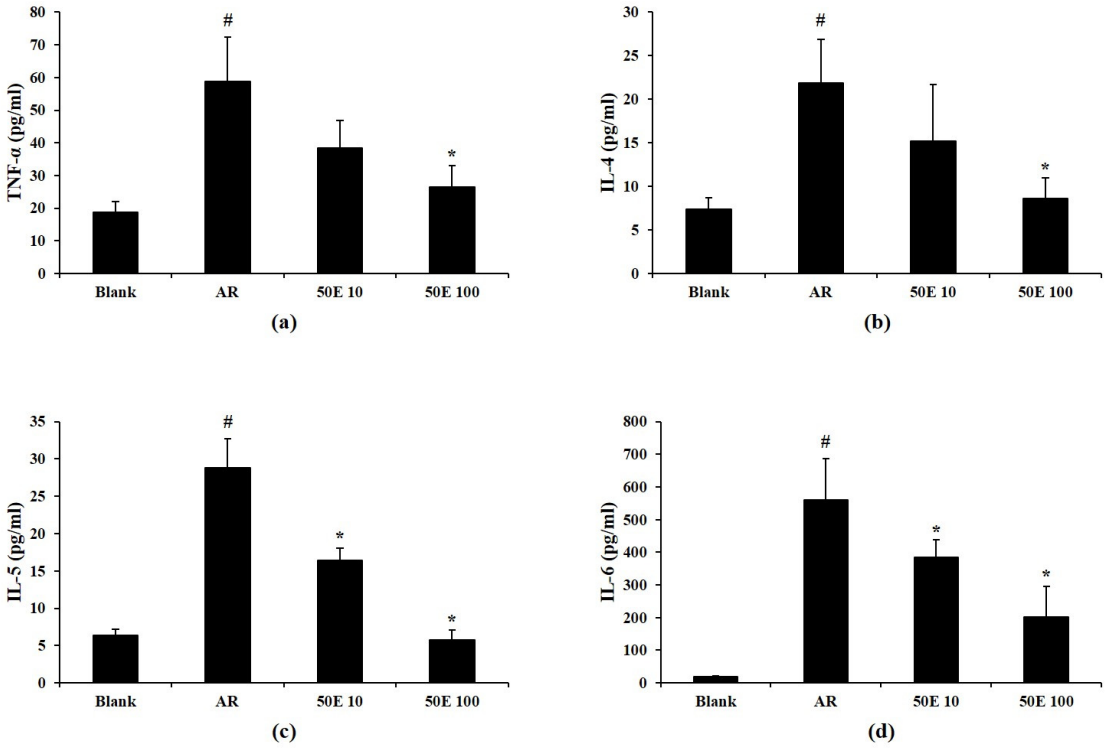

Figure 2. The effect of TQ extract on the production of Th1-and Th-2 related cytokines in serum. TNF-α (**a**), IL-4 (**b**), IL-5 (**c**), and IL-6 (**d**) levels in the serum are shown. Values are presented as mean ± SEM ($n = 6$ in each group). # $p < 0.05$ compared with the Blank group; * $p < 0.05$ compared with the AR group.

3.3. Effect of TQ Extracts on OVA-Specific IgE and Total IgE

Serum levels of total IgE and OVA-specific IgE were significantly increased in the AR group compared to the Blank group. Both total IgE and OVA-specific IgE levels in the serum after treatment with 50% EtOH extract of TQ at 10 mg/kg were decreased, although such decreases were not statistically significant. Both total IgE and OVA-specific IgE levels in the serum after treatment with 50% EtOH extract of TQ at 100 mg/kg were significantly decreased compared to the AR group (Figure 3).

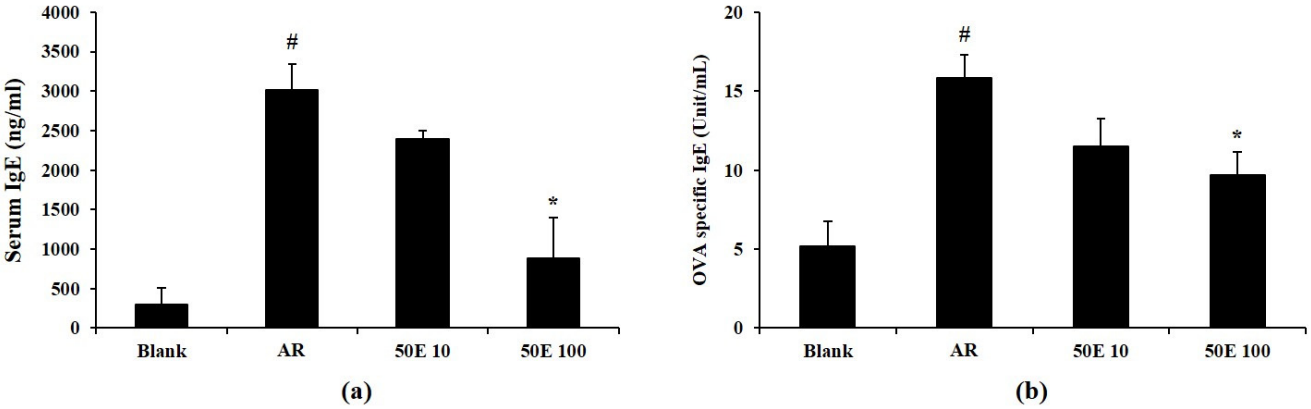

Figure 3. The effect of TQ extract on IgE and OVA-specific IgE levels in the serum of an allergic rhinitis mouse model. Total serum IgE levels (**a**) and OVA-specific IgE levels (**b**) in serum were measured by ELISA. Values are presented as mean ± SEM (n = 6 in each group). # $p < 0.05$ compared with the Blank group; * $p < 0.05$ compared with the AR group.

3.4. Effects of TQ Extract on Histological Transformations in Nasal Tissues

We evaluated whether TQ extract enhances histopathological transformations in the nasal mucosa during AR using H&E staining. The thickness of the nasal septum and the infiltration of the inflammatory cells were increased in the nasal mucosa of the AR group compared to the control group. However, these were decreased in the group treated with 50% EtOH extract of TQ at 10 and 100 mg/kg compared to the AR group (Figure 4a,b). Also, the thickness of epithelial cells in the nasal mucosa was reduced in the group treated with 50% EtOH extract of TQ at 10 or 100 mg/compared to the AR group (Figure 4a,c).

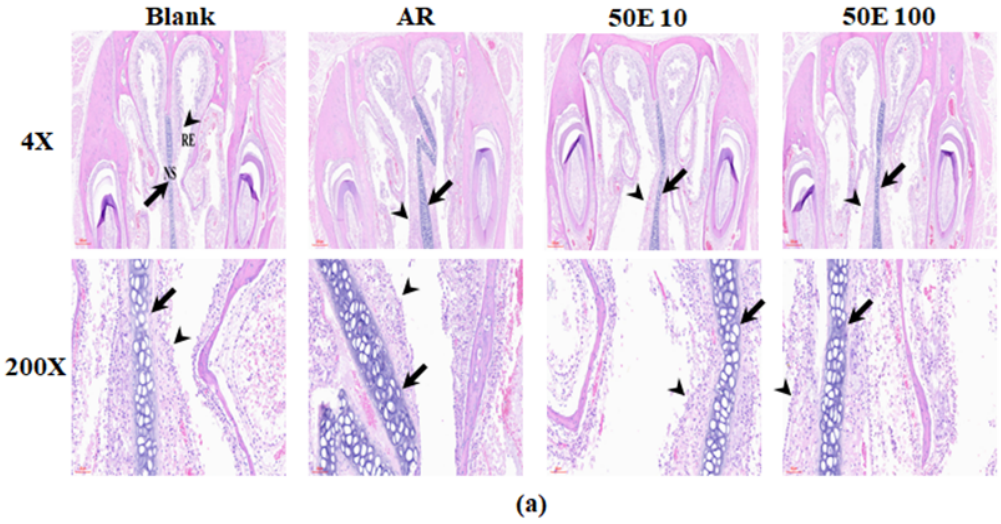

Figure 4. *Cont.*

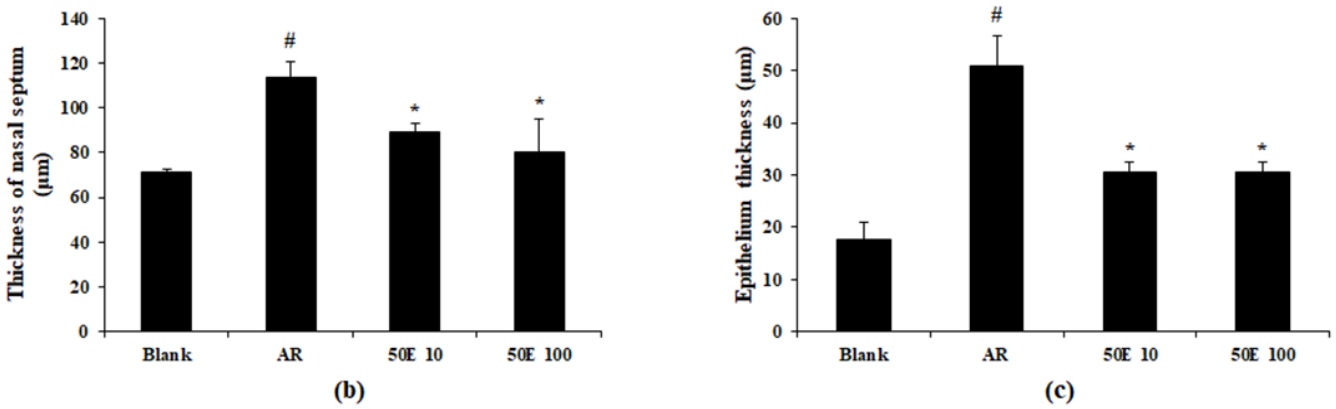

Figure 4. The effects of TQ extract on histological transformations in OVA-induced AR nasal tissues. Nasal tissues were stained with hematoxylin and eosin. NS, nasal septum (arrow); RE, respiratory epithelium (arrowhead). Scale bar = 300 μm (upper row) and 60 μm (lower row) (**a**). Thickness of nasal septum in nasal mucosa (**b**) and epithelium thickness of nasal mucosa (**c**). Values are presented as mean ± SEM (n = 3 in each group). # $p < 0.05$ compared with the control group; * $p < 0.05$ compared with the AR group.

3.5. UPLC Analysis of the TQ Extract

CA and RA known to be main compositions of TQ were analyzed by UPLC-MS. The retention time of CA and RA were respectively observed at 6.100 and 9.650 min in both the internal standard component and the TQ extract (Figure 5a–c). Furthermore, quantitative analysis indicated that CA and RA levels contained 0.26 and 8.51 mg/g in TQ, respectively (Figure 5d,e).

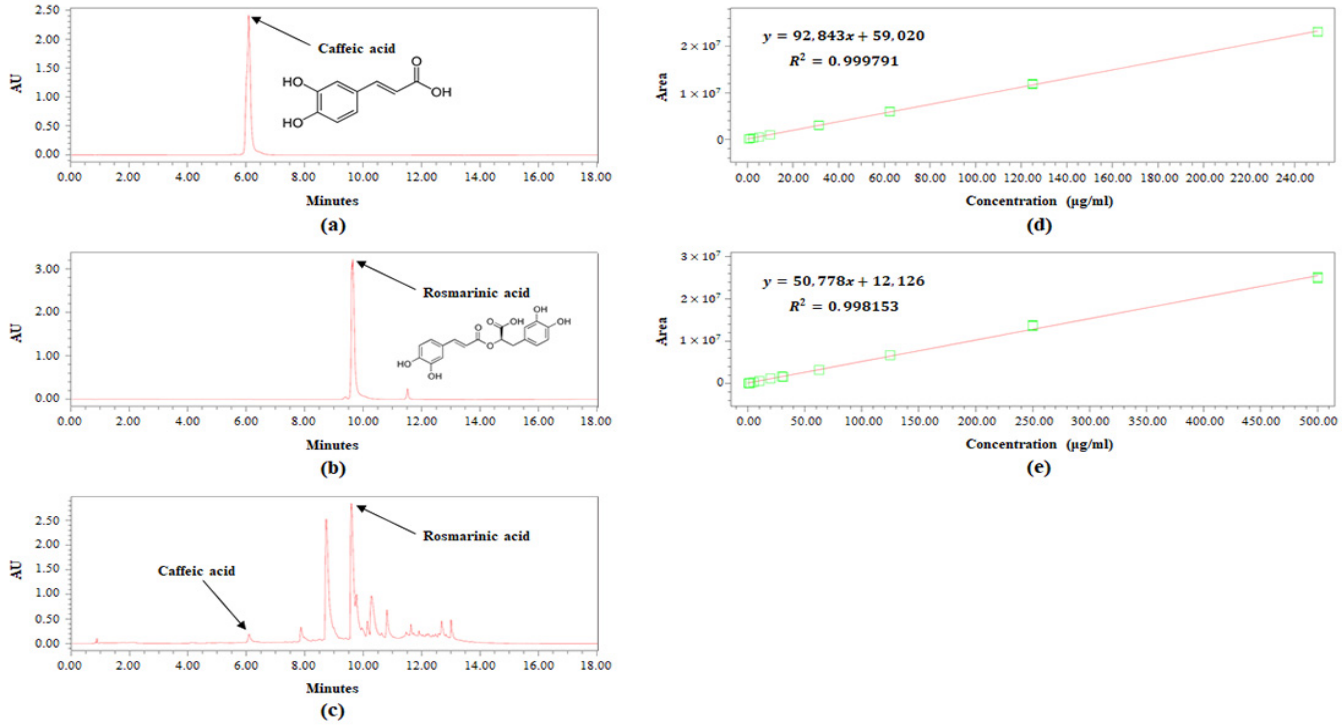

Figure 5. Analysis of caffeic acid and rosmarinic acid in TQ. UV chromatograms of the internal standard of caffeic acid (**a**), rosmarinic acid (**b**), and TQ ethanol extract (**c**) are shown. The calibration curve of caffeic acid (**d**) and rosmarinic acid (**e**) are shown.

4. Discussion

We found that TQ alleviated inflammation in mice with OVA-induced AR. Particularly, TQ ethanol extract alleviated AR-related symptoms such as nasal rubbing and sneezing and histological transformations such as thickness of nasal mucosa and epithelial cells. It also reduced serum levels of Th1/2-type inflammatory cytokines and OVA-specific and total IgE.

AR is characterized as a chronic inflammatory condition of the nasal mucosa, resulting from an overproduction of Th2 cytokines, including IL-4, IL-5, and IL-13, in comparison to Th1 cytokines. Eosinophils are known to cause clinical features of AR including tissue damage. IL-4 is generally produced in Th2 cells. It plays a crucial role in B cell activation and IgE antibody generation by promoting B cell differentiation and proliferation [32]. IL-5 is released by Th2 cells and mast cells [33]. It promotes the growth and differentiation of eosinophils and activates mature eosinophils [34]. IgE is mainly regulated by IL-4, IL-13, and IFN-γ. It binds strongly to the cell surface of mast cells or basophils to secrete toxic amine compounds such as histamine and serotonin, causing severe hypersensitivity symptoms. Thus, it is an important factor in allergic reactions [35]. In this study, serum levels of TNF-α, IL-4, IL-5, IL-6, and IgE were significantly reduced in the group treated with 50% EtOH extract of TQ compared to the AR group (Figures 2 and 3). Also, we considered that a significant decrease in IgE levels in only a high dose of TQ 50% EtOH extract may be due to the regulation of Th2 cytokines. Only a high dose of TQ 50% EtOH extract significantly inhibited both Th2 cytokines such as IL-4 and IL-5. This result is similar to previous reports that the production of IgE is regulated by Th2 cytokines [36,37]. These results indicate that the TQ ethanol extract can inhibit the Th1/2 cytokines and IgE, thereby suppressing inflammation and allergic reactions in rhinitis.

Pathological changes such as severe submucosal edema, damage to the nasal mucosal epithelium, loss of ciliated cells, thickening of olfactory groove, and increased number of mast cells, eosinophils, and mucus-secreting goblet cells will appear in nasal mucosal tissues during AR [38]. In Figure 4, the epithelium thickness of nasal mucosa was increased due to damage caused by infiltration of eosinophilic leukocytes in the nasal mucosa of the AR group. However, the group treated with TQ 50% EtOH extract showed a significant reduction of the thickness of nasal septum and epithelium in nasal mucosa compared to the AR group (Figure 4). This result means that TQ can alleviate AR by reducing pathological changes.

Caffeic acid, a water-soluble natural polyphenolic compound, and rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, are extensively existed in herbal medicines [39,40]. They are commonly found in plants of the Labiatae family [41–43]. Therefore, CA and RA have been used as standard components of *Thymus* species [44,45]. With reference to previous reports, we analyzed both components in the ethanol extract of TQ. We found that CA and RA were present in the ethanol extract of TQ at 0.26 and 8.51 mg/g, respectively (Figure 5). Indeed, some previous studies have reported that CA has an anti-allergic effect against asthma and dermatitis [46–48]. RA can also alleviate AR in clinical trials and animal experiments [49–52]. Therefore, we suggest that the therapeutic effect of TQ ethanol extract upon AR is attributed to CA and RA.

The treatment goal of current medicines for AR is to rest on symptoms. Anti-histamines, anti-leukotrienes, nasal or oral glucocorticoids, and decongestants are the commonly used therapeutic options to relieve of symptoms during AR [53]. However, they have only partially inhibiting effects as well as often side effects such as headache, nasal and throat irritation, nosebleeds, and dryness of eyes, mouth, and nasal [54]. Therefore, safer and more effective agents against AR need to be developed and should be supported by the patients or healthcare providers. According to this, natural products such as herbal or folklore medicines have advantages. Herbal medicines are commonly considered less toxic and having fewer side effects compared to synthetic agents [55]. TQ is one of the herbal medicines and has been used in pharmaceutical industries in Asia including China, Korea, Japan, Taiwan, and eastern Russia [54,56]. Additionally, since it has been used as food to

enhance the flavor of tea or spice, it is one of the reasons to support the safety of TQ [57]. However, an overdose of TQ can cause side effects. Lu et al. [58] assessed the toxicological safety test including acute toxicity, genotoxicity, and subacute toxicity of TQ. TQ extract did not significantly induce acute toxicity, genotoxicity, and teratogenic on spermatozoa. However, a high dose of TQ extract can cause toxicity to the micronucleus of bone marrow cells, liver and kidney, hypoglycemia, and abnormal hematopoietic system. Taken together, we suggest that appropriate consumption of TQ such as complex prescription, over-the-counter (OTC) medicines, and health-functional foods may support remedying AR without potential complications.

We revealed the anti-allergic effect of TQ ethanol extract against AR, but some limitations still remain in this study. First, a detailed anti-allergic mechanism of TQ against AR should be conducted. We revealed that TQ ethanol extract inhibits Th2-related cytokines, OVA-specific and total IgE levels, and allergic symptoms during AR. So, we can suppose that the anti-allergic rhinitis effect of TQ ethanol extract through the regulation of Th2-related cytokines. However, our results could not explain how TQ ethanol extract regulates the Th2-related cytokines, what is the detailed molecular cascade stream, and the direct relationship between cellular molecules and other cellular molecules or cells. Also, the main components with anti-allergic effects in the TQ should be revealed. We identified that CA and RA are included in the TQ ethanol extract. Therefore, we suggested that the anti-allergic effect in the TQ ethanol extract against AR is attributed to CA and RA before because these have anti-allergic effects. However, we only suggested two components with anti-allergic effects among the numerous components within TQ. Besides, our results could not explain why CA and RA are the main components with the anti-allergic effects in the TQ ethanol extracts against AR because standard components are not the main components with effect upon all diseases.

5. Conclusions

Through this study, it was confirmed that the TQ ethanol extract had a significant effect on the hematological, behavioral, and histological aspects of an OVA-induced AR animal model. Therefore, TQ could be considered as a potential candidate for a remedy for AR. If various mechanisms of TQ are identified through continuous research in the future, prevention and treatment of AR will be more effective.

Author Contributions: Conceptualization, S.-H.K., H.-S.K. and J.-S.J.; acquisition, N.-Y.L. and H.-J.O.; validation, D.-K.K.; formal analysis, S.-H.K. and D.-K.K.; resources, N.-Y.L. and H.-J.O.; writing—original draft preparation, S.-H.K.; writing—review and editing, D.-G.K., H.-S.K., S.-I.Y. and J.-S.J.; visualization, S.-H.K. and D.-G.K.; supervision, J.-S.J. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Experimental animal procedures were conducted under the guidelines and protocol approved by the IACUC of Jeonbuk National University (approved number: JBNU 2022-05).

Informed Consent Statement: Not applicable.

Data Availability Statement: Datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request. The data are not publicly available due to privacy.

Conflicts of Interest: The authors declare no conflict of interest.

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