

Propolis extracts exhibit an immunoregulatory activity in an OVA-sensitized airway inflammatory animal model

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Abstract

Propolis, which has been used widely in folk medicine, has been shown to exhibit various biological activities but its immunoregulatory and anti-inflammatory activities in intact animals have not been well studied. We investigated these activities of propolis using an ovalbumin-induced asthma animal model. Mice were immunized and sensitized by exposure to ovalbumin (OVA) antigen and administered with low- (65 mg/kg body weight) and high-dose (325 mg/kg body weight) propolis water extracts by tube feeding. The serum OVA-specific IgE titer and cytokine profiles in cultured splenocytes and bronchoalveolar lavage fluids (BALF) were analyzed. The number of eosinophils in BALF was counted. Here we demonstrate that propolis extracts can suppress the serum levels of OVA-specific IgE and IgG₁, and airway hyperresponsiveness (AHR) in OVA-sensitized mice. There are no significant differences in the concentration of eotaxin or the number of eosinophils in BALF among the four groups. However, the higher dose of propolis extracts decreases the level of IL-5 in BALF. The splenocytes from mice administered with propolis extracts (low- and high-dose groups) exhibit a strong inhibition of IL-10 secretion and up-regulation of IFN- γ secretion in splenocytes stimulated with concanavalin A (ConA). In addition, cytokine (IFN- γ , IL-6, and IL-10) secretion in OVA-stimulated splenocytes from the propolis groups was significantly lower than that in the control group. These results suggest that propolis extracts may be a potential novel therapeutic agent for asthma.

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1. Introduction

Asthma is a chronic inflammatory disorder of the pulmonary airways caused by altered airway hyper-

sponsiveness to inhaled allergens. It is characterized by reversible airflow obstruction and airway inflammation, persistent airway hyperactivity and airway remodeling [1]. Ovalbumin-induced airway hypersensitivity in mouse models is frequently used as a tool to study bronchial asthma. Overwhelming evidence suggests that Th2 (type II T helper) cells and IL-4 and IL-5 produced by these cells are main mediators for mediating the allergen-

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induced hypersensitivity in lung through eosinophils, CD8⁺ T cells, mast cells and B cells [2–4] and that IgE [5] may not be involved in mediating this hypersensitivities. Successful specific immunotherapy has been associated with changes from a prevalent Th2 to a prevalent Th1 response of allergen-reactive Th cells [6].

The use of propolis, the resinous hive and glue-like substances produced by bees, as a folk medicine dates back at least to 300 BC. Propolis is produced by bees from collecting parts of plants, buds and exudates [7]. The chemical substances found in propolis include waxes, resins, balsams, aromatic and ethereal oils, pollen and other organic components [8]. Propolis and its phenolic constituents and their derivatives have been shown to exhibit several biological activities, including anti-proliferative activity toward human tumor cells, anti-inflammatory, antibacterial, antiviral, immunomodulatory, antioxidant [9], microbicidal [10] and radioprotection [11] activities. Since immunomodulatory and anti-inflammatory agents have been used to treat patients with asthma, we hypothesize that propolis extracts may be a novel agent for treating asthma. In this study, we demonstrate that propolis extracts treatment attenuates airway inflammation in OVA-sensitized mice probably via its ability to modulate cytokine production.

2. Materials and methods

2.1. Mice

Inbred BALB/c female mice (6–8 weeks old and weighed 15–20 g) were obtained from the Animal Center of the College of Medicine of National Taiwan University and maintained in the Department of Nutrition and Food Sciences, Fu Jen Catholic University. Mice were maintained on standard laboratory chow ad libitum. Room temperature was maintained at 19–24°C and relative humidity at 50–70%. Animal experiments were performed according to the Guidelines for the Care and the Use of Laboratory Animals, Fu-Jen University.

2.2. Establishment of the experimental animal model of asthma

The immunization protocol was similar and modified to that described previously [12,13]. Briefly, the BALB/c mice were immunized with an intraperitoneal injection of 20 and 50 µg/ml OVA (ovalbumin, Sigma) in phosphate-buffered saline (PBS) combined with the adjuvant aluminium hydroxide (Pierce) at 10 and 12 weeks old, respectively. Negative control mice were given intraperitoneal injection of 1× PBS on each immunization. All mice were exposed to 6–8 ml of 2% OVA aerosols over a 20 min period by placing them in a chamber using an ultrasonic nebulizer (DeVilbiss Pulmo-Aide,

USA) at 14 and 20 weeks old. On weeks 13, 16, 18 and 21, mice were bled from the retro-orbital venous plexus. Collected blood samples were tested for OVA-specific IgE, IgG₁ and IgG_{2a}.

2.3. Propolis extracts treatment of mice with asthma

Propolis water extracts (powder) provided by the Uni-President Enterprises Corp. (Taiwan) was dissolved in H₂O. BALB/c mice were divided into four groups (6 mice in a group), including a positive control group, a low-dose (65 mg/kg body weight) or a high-dose (325 mg/kg body weight) propolis groups, and a negative control group which is referred as the unimmunized group. Propolis groups (low or high doses) were administered with propolis extracts daily by tube feeding that started at 12 weeks of age and continued up to the day of sacrifice.

2.4. Quantification of OVA-specific IgE, IgG1 and IgG2a

OVA-specific IgE, IgG₁ and IgG_{2a} in serum were measured by a capture ELISA assay (BETHYL, Montgomery, TX, USA) as described previously [14] with slight modification. ELISA Plates (Costar, Cambridge, MA) were coated with 200 µg/ml of OVA in 0.1 M NaHCO₃ (pH 9.6). Following incubation at 4°C overnight, the plates were washed and further blocked with 1% bovine serum albumin in 1× PBS (blocking buffer) for 2 h at room temperature. Collected serum samples or pooled high-OVA-specificity antibodies were serially diluted in blocking buffer, applied to the blocked wells and incubated at 4°C overnight. Then, the plates were washed and incubated with horseradish peroxidase-conjugated anti-mice IgE, IgG₁ or IgG_{2a} antibodies and incubated at 4°C overnight. After washing, wells were developed using tetramethylbenzidine and the absorbance at 450 nm was measured using a microplate autoreader (MRX II microplate reader, Dynex, USA). An internal standard was included in the sera of OVA-immunized BALB/c mice for each assay. The data were presented as ELISA unit (EU) by calculate the ratio of an OD450 value of optima diluted sera sample to the value of OD450 which were high-titers of OVA-specificity-antibodies pooled from previous collected sera of OVA-sensitized asthma mice.

2.5. Airway hyperresponsiveness test

Airway hyperresponsiveness (AHR) was tested with the Buxco system (Biosystem XA; Buxco Electronics Inc. Sharon, CT, USA) according to protocol provided by the manufacturer. The enhanced pause (P_{enh}) value for each minute was recorded when the third recorded value was noted. The value of P_{enh} is calculated by collecting data information derived from transducer (differential pressure transducer; Buxco) and preamplifier (MAX II, Buxco). $P_{\text{enh}} = \text{Pause} \times \text{PIF} / \text{PEF}$; $\text{Pause} = (T_e - T_r) / T_r$, (PIF, peak inspiratory flow; PEF, peak expiratory flow; T_e , expiratory time; T_r , relaxation time). A day before sacrifice, mice were tested for AHR by giving

aerosolized normal saline, 12.5, 25 and 50 mg/ml methacholine in normal saline for 3 min. The highest P_{enh} value obtained during each methacholine challenge was expressed as a relative increase ratio in response to PBS challenge.

2.6. Bronchoalveolar lavage fluids (BALF) analysis and histopathological studies

At 21 weeks old, all groups of mice were sacrificed. The lung was immediately lavaged via the trachea cannula with 1 ml of Hank's balanced salt solution (HBSS, Atlanta Biologicals, Norcross, GA), which is free of calcium and magnesium ions, containing 2% fetal bovine serum (FBS). The BALF was centrifuged at 1500 rpm for 5 min at 4°C. Collected supernatants of BALF were stored at -20°C for determining the levels of eotaxin and IL-5 by using ELISA assays. After washing, the cells were re-suspended in 1 mL 1× HBSS and the total cell numbers were determined using a hemocytometer. BALF cells were resuspended in culture medium (100 µl), centrifuged (Cytospin 4 cytocentrifuge, Thermo Shandon, USA) at 500 rpm for 5 min, and stained with Liu's stain solution (Liu A & Liu B, Delta, Japan). Cell types were identified using morphological criteria. 200–300 leukocytes were counted on each slide. The numbers of eosinophils, lymphocytes, and monocytes in BALF were counted.

To evaluate the effects of propolis extracts on the lung inflammation, the lungs were immediately injected with 0.7 ml of 4% formaline before removal. The lung tissue was subsequently embedded in paraffin and cut into 5 µm-thick sections. These frozen sections were stained with hematoxylin–eosin and examined for histological changes using a light-microscope (Olympus, BX41TF, Japan).

2.7. Preparation of splenocytes

Animals were sacrificed by cervical spine dislocation. The spleen was removed and crushed into a single cell suspension, and red blood cells lysed with Tris-buffered ammonium chloride before washing (three times) with Hank's Balanced Salts solution. 5 µg/ml concanavalin A (ConA) or 50 µg/ml OVA were used to stimulate the 48-h cultured splenocytes.

2.8. Cytokine levels in the cultured supernatants of splenocytes and in BALF

To analyze the Th1 and Th2 responses of mice administered with or without propolis extracts, splenocytes (1×10^7 cells/ml) were treated with either 5 µg/ml ConA or 50 µg/ml OVA for 48 h and cytokines such as IL-2, IL-4, IL-5, IL-6, IL-10, TNF-α and IFN-γ in the supernatants were assayed. The eotaxin and IL-5 levels in BALF were assayed. Above assay were done by ELISA kit (R&D) according to the manufacturer's instructions. Briefly, supernatants or the BALF was added to wells precoated over night at 4°C with specific monoclonal antibodies. After 2-h incubation, the plates were washed and biotin-conjugated antibodies were added. After further incubation at room temperature for 2 h, HRP–avidin

was added to each well. The substrate tetramethylbenzidine was then added and the OD (at 450 nm) values were converted to the concentrations of cytokines in the assays. The sensitivities of detection were as follows: ~15 pg/ml for IL-4, IL-5, IL-6 and eotaxin, ~30 pg/ml for IL-10, TNF-α, IFN-γ and eotaxin, and ~5 pg/ml for IL-2.

2.9. Statistics

Results are expressed as mean ± SD. Statistical Analysis System (SAS) including unpaired Student's *t*-test and one-way analysis of variance (one-way ANOVA) were used to analyze the results. If there was a difference among the results, we then used Duncan's multiple range method to compare the groups and $p < 0.05$ represent a significant difference in comparison with the positive control group.

3. Results

3.1. Effects of propolis extract administration on the titers of OVA-specific IgE, IgG₁ and IgG_{2a} in the sera of OVA-sensitized mice

Propolis extract administration had no influence on the growth of OVA-sensitized mice as determined by weekly measurement of their body weights. On weeks 13, 16, 18 and 21, sera of mice were examined for OVA-specific IgE, IgG₁ and IgG_{2a} titers. As shown in Fig. 1, the titer of OVA-specific IgE increased with time in OVA-sensitized mice fed without propolis extract (the positive control group). Mice fed with a low dose of propolis extracts for 18 and 21 weeks exhibited a significant decrease of the OVA-specific IgE titer as compared with the positive control group ($p < 0.05$). Mice that were not sensitized with OVA and not fed propolis extracts (the negative control group) did not exhibit detectable OVA-specific IgE in sera. Thus, propolis extracts had an inhibitory effect on OVA-specific IgE production. To analyze Th1 and Th2 responses in mice, the titers of OVA-specific IgG₁ and IgG_{2a} were determined. As shown in Fig. 2, mice fed with a low dose of propolis for 18 and 21 weeks had a lower level of the OVA-

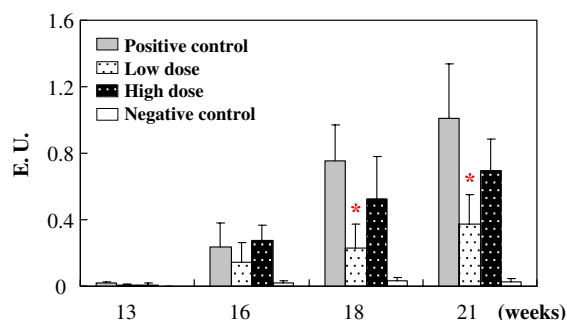


Fig. 1. Effects of propolis extracts on the serum levels OVA-specific IgE in mice sensitized with OVA.

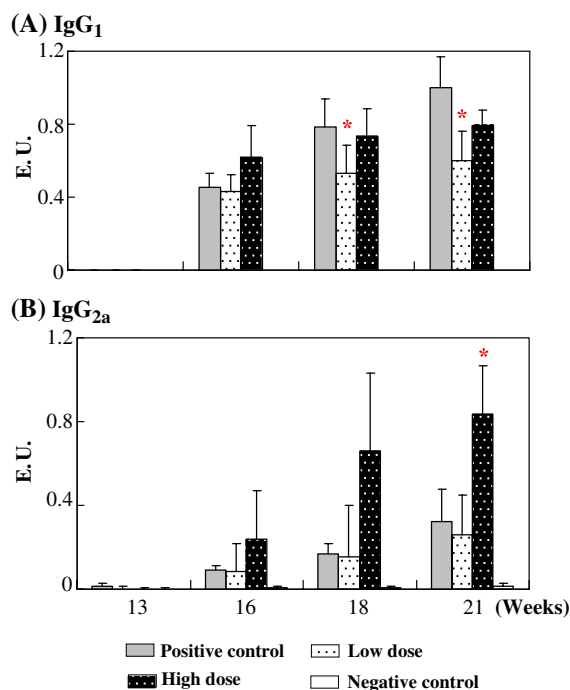


Fig. 2. Effects of propolis extracts on the serum levels of OVA-specific IgG₁ and IgG_{2a} in mice sensitized with OVA.

specific IgG₁ titer which represents a Th2 response. The OVA-specific IgG_{2a} titer of the Th1 response in mice fed with a high dose of propolis extracts was significantly higher than that of mice in the positive control group. This suggests that propolis extracts may contain complex active components, including Th2 response inhibitors and Th1 response stimulators (which are active in the high dose of propolis extracts). ELISA assays revealed that the amounts of OVA-specific IgE and IgG₁ were lower in the propolis groups than those in the positive control group, indicating that propolis extracts attenuated the Th2 response dominant in OVA-sensitized mice. So it can be concluded that propolis extracts administration shifts the Th2 to Th1 response in the asthma animal model. These results also suggest that propolis extracts may have a therapeutic effect on asthma.

3.2. Effect of propolis extracts on airway hyperresponsiveness (AHR) in an asthma animal model

Experimental evidence indicates that the maximum degree of airway narrowing in response to methacholine is greater in asthmatics than in normal subjects [15]. As shown in Fig. 3, increasing concentrations of methacholine correspondingly augment the AHR in asthma mice. Propolis extracts appeared to inhibit methacholine-induced AHR in the OVA-induced-asthma mice. Mice fed with a low dose of propolis extracts had a lower airway resistance compared to the positive control group after they had been given 25 mg/ml methacholine.

3.3. Cytokine release in ConA-stimulated splenocytes

Many studies have indicated that asthma attack is the result of imbalanced immune responses related to Th1/Th2 cytokines production. In the lung tissue, Th2 cells can respond to inhaled allergens by secreting IL-4, IL-5, and other mediators, representing chronic airway inflammation [16]. Thus, the Th1 and Th2 immune responses in OVA-sensitized mice can be determined by identification of cytokines released from splenocytes. To determine the effect of propolis extracts on the Th2 response, we analyzed the cytokines released from splenocytes of OVA-sensitized mice. The identification of IL-2 and IFN- γ in the supernatants of splenocytes was used as an indicator of the Th1 response while the presence of IL-4, IL-5 and IL-10 in the supernatants indicates the Th2 response. As shown in Table 1, the OVA-sensitized mice fed with propolis extracts had a significantly higher IFN- γ level than that in the positive control mice. No difference was seen in the levels of IL-2, IL-4 and IL-5. In addition, the levels of Th2 cytokines such as IL-10 were lower in propolis extracts-administered mice than those in the positive control mice (Table 1). These results suggest that propolis extracts have an inhibitory effect on the Th2 cytokine response. It is important to note that the negative control group had higher IFN- γ and lower Th2 cytokine release than the positive control group. This was due to that the negative control group had no allergen stimulation or asthma.

3.4. Cytokine release in OVA-stimulated cultured splenocytes

In order to analyze the OVA-specific cytokine release in mice, splenocytes (1×10^7 cells/ml) were treated with 50 μ g/ml OVA for 48 h. The supernatant was collected for assaying pro-inflammatory cytokines such as IL-6 and TNF- α , and other cytokines like IFN- γ , IL-4 and IL-10. As shown in Table 2, the levels of IL-6 and IL-10 in the propolis groups (including low- and high-dose groups) were lower than those in the positive control group but no differences were

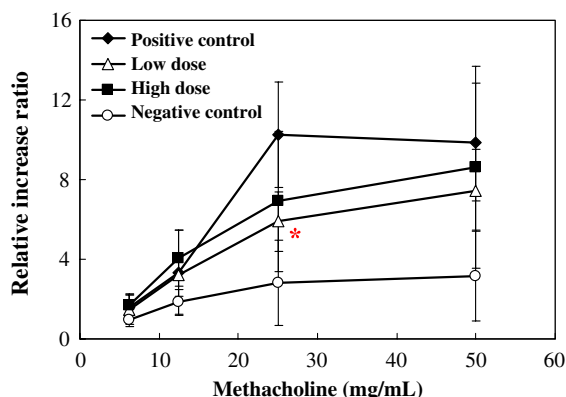


Fig. 3. Effects of propolis extracts on airway hyperresponsiveness (AHR) in mice sensitized with OVA.

Table 1

The effect of propolis on Th1 and Th2 cytokine production by splenocytes of mice stimulated with ConA

Cytokine	Positive control	Low dose	High dose	Negative control
	pg/ml ¹			
IL-2	3290.0±414.4	3070.5±691.5	3174.0±971.3	3483.5±776.1
IFN-γ	9279.0±1326.7	12407.7±2536.0*	12625.4±2204.0*	13658.8±1350.5*
IL-4	321.6±174.3	279.4±161.5	242.5±94.0	181.6±87.9
IL-5	908.6±326.8	707.5±218.2	710.7±145.2	440.7±192.0*
IL-10	589.6±68.4	362.2±125.6*	345.7±113.0*	209.7±86.0*

Splenocytes were stimulated with 5 µg/ml Con A and culture supernatants were collected, and the levels of cytokine IL-2, IFN-γ, IL-4, IL-5, and IL-10 were determined using ELISA assays.

¹The values were presented as mean±SD, *n*=6.

*Data were significantly different from those of the positive control group based on between-group comparisons by ANOVA with Dunnet post-test (*p*<0.05).

seen in the levels of TNF-α between these groups (Table 2). Unlike the cytokine secretion profile seen in ConA-stimulated splenocytes, the OVA-specific IFN-γ secretion was significantly inhibited by propolis extracts administration compared to that of the positive control group. The negative control group has not been immunized with OVA and secretes baseline levels of IL-4, IL-10, and IFN-γ. (Table 2).

3.5. Bronchoalveolar lavage fluids (BALF) analysis and histopathological studies

Our asthma animal model exhibited eosinophilia in BALF while the negative control group showed predominance of lymphocytes and a few polymorphonuclear cells. There is no difference in the eosinophil number among the studied groups (data not shown). In our asthma animal model, the levels of RANTES, MCP-1 and IL-10 were too low to be detected in BALF. As shown in Fig. 4, the level of eotaxin in the BALF (15–20 pg/ml) was not much different from the other studied groups. However, with high-dose propolis extract administration, the IL-5 level in BALF was significantly decreased and almost as low as that in the negative control group (*p*<0.05). IL-5 secreted by both mature and immature eosinophils from the bone

marrow is known to regulate the expression of the transmembrane isoform of its own receptor and is essential for the terminal differentiation of committed eosinophil precursors [17]. This suggests that the higher dose of propolis extracts decreases secretion of IL-5. Histological analysis of the lung sections with H & E staining revealed that the propolis groups had a less inflammatory cell infiltration in the peribronchial and peribronchiolar regions compared to the control group (Fig. 5). The negative control group did not exhibit inflammation.

4. Discussion

Natural products such as propolis have been used for thousands of years in folk medicine due to its wide range of beneficial effects against diseases. Considering that only a few studies have been reported on the in vivo effect of propolis on airway inflammation, our aim in this study was to evaluate the potential therapeutic effects on asthma and to determine mechanisms for affecting asthma.

Th2 type cells, which can produce several cytokines after activation, are activated in a greater proportion in

Table 2

The effect of propolis on cytokine production in splenocytes of mice stimulated with OVA antigen

Cytokine	Positive control	Low dose	High dose	Negative control
	pg/ml ¹			
IL-6	521.5±76.9	262.7±101.0*	314.2±128.8*	47.0±20.3*
TNF-α	231.5±52.6	180.0±59.0	181.0±38.9	119.2±57.8*
IFN-γ	423.3±155.9	66.8±23.5*	91.3±60.3*	61.3±28.9*
IL-4	19.5±9.9	18.2±10.7	16.5±10.2	14.9±12.8
IL-10	2485.2±564.2	1299.2±352.9*	1269.8±495.4*	219.0±38.1*

Splenocytes were stimulated with 50 µg/ml OVA and culture supernatants were collected, and the levels of cytokine IL-2, IFN-γ, IL-4, IL-5, and IL-10 were determined using ELISA assays.

¹The values were presented as mean±SD, *n*=6.

*Data were significantly different from those of the positive control group based on between-group comparisons by ANOVA with Dunnet post-test (*p*<0.05).

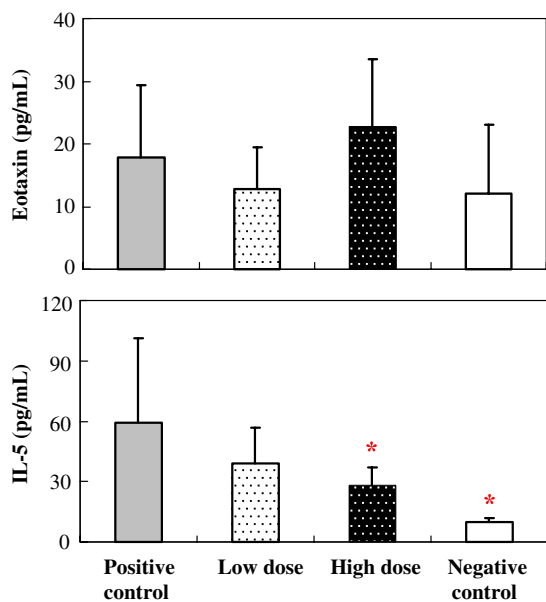


Fig. 4. Effects of propolis extracts on levels of eotaxin and IL-5 in BALF of OVA-sensitized mice.

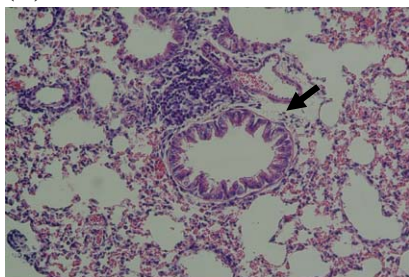
asthmatic individuals [18]. The groups of mice administrated with propolis extracts had significantly higher levels of IFN- γ in ConA-stimulated splenocytes in comparison with the control group (Table 1). Our results imply that propolis extracts may not only directly enhance the Th1 response in CD4⁺ T cells but also have

an inhibitory effect on Th2 cytokine release in splenocytes such as decreased secretion of IL-10 (Table 1) and decreased level of IL-5 in BALF (Fig. 4). In addition to its effects on Th1 and Th2 cell cytokine release, IL-10 stimulates B cell isotype switch and production of IgG [19]. This may explain why the amounts of OVA-specific IgG₁ in the propolis groups were lower than that in the control group (Fig. 2).

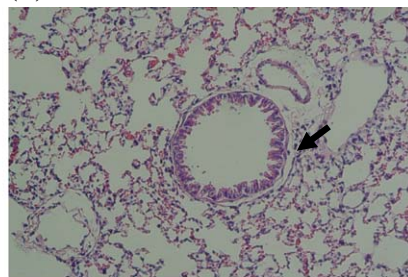
In these above laboratory results, we demonstrate that the amount of OVA-specific IL-6 released from splenocytes of asthma mice was lower than that of the positive control group. However, no difference is found in the amounts of released OVA-specific TNF- α between asthma and the positive control mice (Table 2). These findings are consistent with a clinical pharmacological study that demonstrated decreased levels of IL-6 in sera of asthma patients treat with propolis as an adjuvant [20]. IL-6 can stimulate B lymphocytes to synthesize antibodies and can stimulate production of acute-phase proteins in human liver for defending against infection [21]. We hypothesize that mice administrated with propolis extracts can block secretion of pro-inflammatory cytokines like IL-6 in splenocytes, diminish the OVA-stimulated B cell activation and decrease the levels of OVA-specific IgE and IgG₁ in sera (Figs. 1 and 2).

Caffeic acid phenethyl ester (CAPE), an anti-inflammatory component of propolis, is known to be an inhibitor of nuclear factor-kappa B and significantly

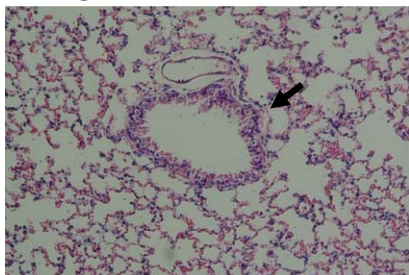
(A) Positive control



(B) Low dose



(C) High dose



(D) Negative control

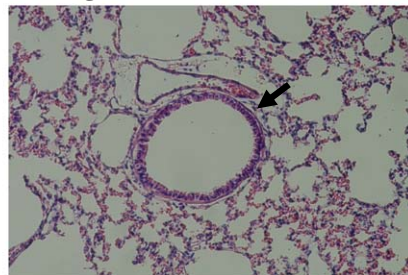


Fig. 5. Histological analysis of the lungs in OVA-sensitized mice treated with propolis extracts.

reduces the levels of pro-inflammation cytokines (TNF- α and IL-1 β) in rats [22]. In our studies, these cytokines are also attenuated in mice administrated with propolis extracts. Since water extracts of propolis are used in this study, the activities of propolis extracts may not be contributed by CAPE, which is mainly present in the oil extracts of propolis.

Clinical investigations have revealed a correlation between the presence of inflammatory cells and histological changes in pulmonary tissues, and the severity of airway hyperactivity [23,24]. The levels of pro-inflammatory cytokines can be an indicator for the severity of airway inflammation [25]. Recent studies suggest that chemoattractant cytokines may be important in eosinophil influx as evidenced by increased levels of MCP-1, MIP-1 α , RANTES, and eotaxin in BALF of asthmatics [26]. We demonstrate that with high dose of propolis extracts administration, the IL-5 level in BALF is significantly decreased compared to that in the positive control group and is almost as the low as that found in the negative control group ($p < 0.05$). Since IL-5 is known to be a selective activator of eosinophils function [27] and since IL-5 deficiency abolishes airway hyperreactivity in a mouse asthma model [28], this result suggests that the higher dose of propolis extracts attenuate the local inflammation by decreasing the level of IL-5.

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