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Chinese Propolis Attenuates *In-Vivo* and *In-Vitro* Asthmatic Reactions

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Abstract

This study was designed to evaluate the inhibitory effects of Chinese Propolis (prepared as an ethanolic extract) on asthmatic reactions in-vivo and in-vitro. Ethanolic extract of propolis (EEP) significantly inhibited OVA-induced contractions of passively sensitized guinea pig tracheal zigzag preparations producing significant increase and decrease in EC50 and Emax, respectively. EEP appeared to exhibit significant inhibitory effects on allergic and inflammatory reaction associated murine model of asthma. EEP significantly reduced aggregation of inflammatory cells in bronchoalveolar lavage (BAL) fluid and in lung tissues with marked dilated bronchia. Also, EEP markedly reduced serum IgE and lung mRNA levels of inducible nitric oxide synthase (iNOS), transforming growth factor-β1 (TGF-β1) and tumour necrosis factor-α (TNF-α) in mice. These results suggest that EEP is a potent inhibitor of the inflammatory changes associating asthma and it could be used as an adjuvant therapy for patients with allergic airway inflammation.

Keywords: Propolis; Asthma; Airway Inflammation; IGE

Introduction

Asthma is an inflammatory disease of the airways whose common characteristics include reversible airway obstruction, airway hyperresponsiveness, airway and lung inflammation and increased mucus production [1]. It is one of the most common chronic inflammatory diseases, affecting approximately 4% to 10% of the population [2]. Although asthma is generally well controlled with existing therapies (such as inhaled corticosteroids and β2-agonists), there are several reasons why a research for novel treatment modalities for asthma is still ongoing: (a) the disease is still poorly controlled in patients with severe, corticosteroid-insensitive asthma; (b) there is a constant need for improvement of existing therapies in terms of more favorable side effect profile or oral formulation; and (c) the current asthma therapies are not cures and symptoms return soon after the treatment is stopped, even after long-term therapy [3]. Airflow obstruction is also a hallmark in asthma that is caused by constriction of bronchial smooth muscles and infiltration of leukocytes that fill the airways and induce epithelial damage and desquamation into the lumen of the airways [4].

Propolis (bee glue) is a resinous hive product collected by honey bees from many plant sources. Propolis contains a variety of different chemical compounds, including phenolic acids or their esters, flavonoids, terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes and β-steroids. Propolis cannot be used in its crude form, so it must be purified by extraction to remove the inert material and preserve the polyphenolic fraction.

Furthermore, propolis ethanol extract (EEP), alone or incorporated in another dosage form, is commonly utilized as therapeutics [5]. EEP has shown several biological and pharmacological properties, such as immunomodulatory [6], anti-cancer [7], anti-inflammatory and anti-oxidant effects [5].

Therefore, we hypothesized that the efficacy of propolis in asthma results from its possible immunomodulatory/anti-inflammatory activities. In order to test this hypothesis, we have investigated the influence of EEP on antigen-induced contractions of passively sensitized guinea pig isolated tracheal zigzag preparations and ovalbumin (OVA)-induced airway hyperresponsiveness and airway inflammation in a mouse model of asthma.

Material and Methods

Animals

All protocols described in this study followed the protocols approved by the University of Mansoura, Department of Pharmacology Committee for Animal Experimentation. All animals used in this study were maintained under standard conditions of temperature, about 25°C, with regular 12 h light/12 h dark cycle and allowed free access to food and water.

Mice: Adult male albino mice weighing 20-25 g were used in this study. They were purchased from "Urology and Nephrology Center", Mansoura University, Egypt.

Guinea pigs: Adult male guinea pigs weighing 300-500 g were used in this study. They were purchased from a local breeder.

Drugs: Chinese propolis crude powder (Dalion Garo International Trade Co. Ltd., Dalion, Liaoning, China). acetylcholine (ACh) hydrochloride, alum, OVA grade V, urethane, Quercetin, apigenin, kaempferol, chrysin, p-coumaric acid, caffiec acid phenethyl ester and artepillin C (Sigma Chemicals Co., St. Louis, MO., USA).

Preparation of EEP

The extract was prepared as previously described by Bankova et al. [8]. Briefly, propolis crude powder (10 g) was mixed with 100 mL of 96% ethanolic solution for extraction. The mixture was left for 4 days with a one-hour shaking period every day. The mixture was filtered and the extract was evaporated under vacuum till the volume reached 15 mL. Before use, this stock was diluted with distilled water to give the desired concentration and the obtained milky solution was used for *in-*

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vitro and *in-vivo* experiment. The final concentration of ethanol in this solution did not exceed 5%.

High-performance liquid chromatographic assay

The ethanolic extracts of propolis were analysed using Shimadzu™ LC-20A Series Chromatoghraph equipped with a Rheodyne injector valve with a 20 µl loop and a SPD-20A UV detector. Separation as achieved on a Shim-pack VP-ODS column (5 µm) (150X 4.6 mm) combined with a gaured column (Nishunokyo-Kuwabaracho, Nakagypku, Kyoto 604-8511, Japan). LC Workstation (Nishunokyo-Kuwabaracho, Nakagypku, Kyoto 604-8511, Japan) was applied for data collecting and processing. The solvent used is formic acid (95:5, v/v) (solvent A) and methanol (solvent B). The elution was carried out with a linear gradient and a flow rate of 1 ml/min. Spectrophotometric detection was conducted at 254 nm. Quercetin, apigenin, kaempferol, chrysin, p-coumaric acid, caffiec acid phenethyl ester, galangin and artepillin C were used as authentic standards, and the major peaks eluted by HPLC were characterized by the analysis of ultraviolet absorbance at 200 to 400 nm.

Experimental protocol

Antigen-induced contractions of passively sensitized guinea pig isolated tracheal zigzag preparations:

a. Active sensitization procedure: Guinea pigs were actively sensitized with OVA (10 µg/mL) plus alum (100 mg/mL) in 0.9% sodium chloride. The suspension was stirred for 2 h before the guinea pigs received 1 mL, I.P. injection (regardless of the weight). The guinea pigs were used 14–21 days after sensitization. This method of sensitization in guinea pigs showed a rise in the serum titre of both immunoglobulin (Ig) E and IgG [9].

b. Passive sensitization procedure:

• **Blood collection and serum preparation:** Blood was collected from the hearts of actively sensitized guinea pigs after being anaesthetized with ether. Blood was left to clot for 20 min before being centrifuged at 5000 rpm for 20 min at 21°C and serum was seprated. Before use, serum was diluted in a ratio of 1:5 with modified Krebs-Henseleit Solution (KHS) of the following composition in mM: NaCl 118, KCl 5.4, NaH_2PO_4 1, $CaCl_2.2H_2O$ 1.9, $NaHCO_3$ 25 and glucose 11.1 [10]. This dilution was made to avoid excessive formation of bubbles during subsequent incubation and aeration.

• **Test for effective active sensitization:** The titres of antibodies in the serum (from actively sensitized guinea pigs) were not measured directly. However, the tracheae from the actively sensitized guinea pigs, from which the serum was taken, were isolated, immediately after blood collection, and cleared from extraneous connective tissue and blood vessels. The tracheal zigzags were then prepared according to the method described by Emmerson and Mackay, [11] and suspended in 20 mL organ bath. The tracheal tension was set and kept at 1g tension throughout a stabilization period of 60 min during which the modified KHS, aerated with a mixture of 95% O_2 and 5% CO_2, was refreshed every 10 min. The tracheal zigzag preparations were tested for OVA sensitivity by constructing a cumulative concentration–response curve for OVA (3-300 ng/mL) [12]. If OVA caused *in-vitro* contractions of tracheal zigzag preparations, this is a strong indication that the animals had been successfully actively sensitized to OVA and that the antibody titre of the serum was raised. Serum was not used for subsequent incubation if there was no *in-vitro* contraction to OVA in the donor trachea [13].

• **Tissue preparation and incubation:** Non-sensitized guinea pigs were anaesthetized with ether, the thorax was opened, the trachea removed and placed in modified KHS and prepared as previously described. The resulting tracheal zigzag preparation was incubated in 2 mL of previously collected serum (from actively sensitized guinea pigs) diluted with 10 mL of modified KHS gassed with a mixture of 95% O_2 and 5% CO_2 for 4 h at 37°C.

• **Drug addition:** After an incubation period of 4 h, the tracheal zigzag was removed from the diluted serum and suspended in 20 mL organ bath. The tracheal tension was set and kept at 1g tension throughout a stabilization period of 60 min during which the modified KHS was refreshed every 10 min. Isometric tension was measured by means of an isometric transducer (Harvard Apparatus) connected to a 2–channel oscillograph (Harvard Apparatus LTD, South Natick, MA, USA). The trachea was initially contracted with 0.1 mM ACh to check its functional integrity. The preparation was then washed several times and left until the tension returned to baseline. Fifteen tracheal zigzag preparations were prepared as described above and divided into the following groups (each group consists of 5 preparations): a) Control group: Tracheal zigzags were incubated with drug-free modified KHS. b) EEP group: Tracheal zigzags were incubated with 100 µg/mL of EEP [14]. c) Ethanol group: Tracheal zigzags were incubated with ethanol at final bath concentration of 0.015% (representing the concentration of ethanol added in the organ bath as a part of the EEP)

After incubation with drugs for 30 min, a cumulative concentration–response curve for OVA, 3-300 ng/mL, was constructed for each preparation. Smooth muscle contraction was calculated as % of maximal contraction induced by 0.1 mM ACh.

OVA-induced asthma in mice:

a. Sensitization and airway challenge: Mice were grouped into 3 groups; control, OVA and OVA-EEP treated groups, where each group is comprised of 10 mice. Mice were sensitized by subcutaneous injections with 25 µg of OVA adsorbed on 1 mg of alum in 200 µL of normal saline per mouse on days 0, 7, 14 and 21 (only drug free normal saline in control group). Intranasal challenges with OVA (20 ng/50 µL saline) were carried out on days 31, 33, 35 and 37 [only drug free normal saline in control-group] [15]. Mice in the EEP-treated group were treated with oral administration of 400 mg/kg/day of EEP, starting from day 30 to day 38 (1 hour before challenge on days of challenges) while control group received 12 mL/kg/day of 5% ethanolic solution (EEP vehicle).

b. Inflammatory cell counts in bronchoalveolar lavage (BAL) fluid: Twenty-four hours after the last OVA or saline challenge, mice were anaesthetized with urethane (2.5 g/kg, I.P.). Tracheae were exposed and cannulated with polyethylene cannula for BAL that was performed by instillation of 0.5 mL of phosphate-buffered saline (PBS). The thorax was gently massaged then the BAL fluid was withdrawn. The process was repeated 4 times. About 1 mL of the instilled fluid was retrieved from each mouse. The retrieved BAL fluid was centrifuged at 500 g for 10 min at 4°C. The cell free supernatant was removed and the cell pellet was resuspended in 200 µL PBS and used for total and differential leukocyte counts. Total leukocytes in BAL fluid were counted using a hemocytometer. Different cell types were identified by differential staining microscopy with Diff-Quick. The cell counts of lymphocytes, eosinophils and monocytes in BAL fluid were obtained.

Lung tissue histopathology

Lungs were harvested after performing BAL, the right lung was fixed with 10% buffered formalin, cut into sections, stained with hematoxylin

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and eosin and examined under microscope to evaluate the severity of inflammation. Inflammatory infiltrates were further characterized according to cell type on a morphologic basis using fluorescence microscopy (Leica DM 5000 B). Inflammatory changes were expressed as scores of different cell types, namely eosinophils and plasma cells, this semiquantitative scale was from 0 to 3 (0 = no, 1=mild, 2=moderate, 3=marked) for each cell type. The total inflammation score for each animal was calculated as the mean of the scores for 6 lungs [16].

Measurement of IgE in serum

Levels of IgE in sera were determined by enzyme immunoassays kits (Costar, Cambridge, MA) according to the manufacturer's protocol.

Isolation, purification and reverse transcription (RT) of RNA

The lungs were isolated and flash-frozen in liquid nitrogen and stored at -80°C. Lung samples (about 20-30 mg of tissue for each sample) were mechanically homogenized using a variable speed homogenizer (model 125, OMNI international). Total RNA was isolated from the homogenized lungs and purified using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. To remove the contaminating genomic DNA (gDNA), RNA samples (1 µg each) was added to 2 µL genomic DNA (gDNA) wipeout buffer at 42°C for 2 minutes. RNA samples were reverse transcribed at 42°C for 25 minutes in 20 µL containing 1 µL quantiscript reverse transcriptase, 4 µL quantiscript RT buffer and 1 µL RT primer mix. The reaction was terminated by heating at 95°C for 3 minutes. Real-time polymerase chain reaction (RT-PCR) was carried out in a 23 µL final volume in duplicates using SYBR Green I as a fluorescent detection dye. The reactions contained 2 µL cDNA, 12.5 µL Rotor-Gene SYBR Green PCR Master Mix, 8.5 µL RNase-free water and forward and reverse primers in final concentration 1 µM each. Primer sequences are described in table 1. PCR amplification was performed in the thermocycler RotorGene Q (Qiagen, Hilden, Germany). After an initial activation step at 95°C for 5 minutes (hot start DNA polymerase activation), 40 cycles with the following thermocycling conditions were carried out: denaturation at 95°C for 5 seconds, combined annealing/ extension at 60°C for 10 seconds at which the fluorescence was acquired. Amplification specificity was checked by generation of a melting curve by heating the PCR product slowly at a rate of 1°C sec-1 from 60°C to 95°C which causes melting of the double-stranded DNA and a corresponding decrease in SYBR Green fluorescence. The relative quantification values for calibrator-normalized target gene expression were normalized to β-actin. PCR efficiency of both the target (iNOS, TGF-β1 and TNF-α) and reference (β-actin) genes was calculated from the derived slopes of the standard curves.

Primer		Sequence	Melting temperature (T_m)	Product size (bp)
Reference gene				
β-actin	Forward	TGGGTATGGAATCCTGTGG	55.78	96
	Reverse	GCACTGTGTTGGCATAGAGG	56.18	
Target genes:				
iNOS	Forward	GGAGCCTTTAGACCTCAACAGA	58.03	104
	Reverse	GGCTGGACTTTTCACTCTGC	57.17	
TNF-α	Forward	CCACCACGCTCTTCTGTC	57.79	114
	Reverse	ATCTGAGTGTGAGGGTCTGG	57.62	
TGF-β1	Forward	GCTAATGGTGGACCGCAAC	58.57	100
	Reverse	CACTGCTTCCCGAATGTCTG	58.72	

Table 1: Primers used in real-time polymerase chain reaction.

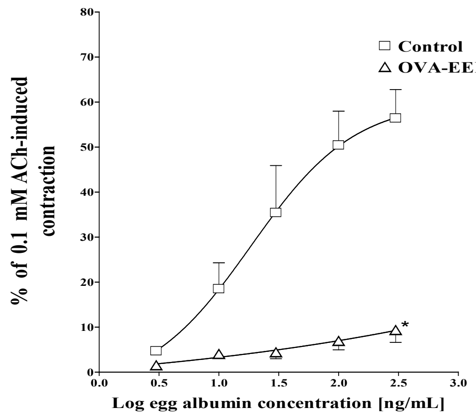

Figure 1: Effect of EEP on antigen-induced contractions of passively sensitized guinea pig tracheal zigzag preparations
EEP: Ethanolic Extract of Propolis.
Each point represents the mean ± S.E. of 5 preparations.
*Significantly different when compared with the control group using Student t test at P<0.05.

Statistical analysis

Statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test or Student t test. Statistical significance was considered when P<0.05. The results are presented as mean ± standard error of mean (S.E.M). The best curve fitting for concentration–response curve for each experimental condition was plotted and from it the values of maximal-agonist induced response (E_{max}) and the concentration of the agonist (expressed as negative log molar) producing 50% of E_{max} were deduced. The highest response obtained was considered as the maximum response (E_{max}). Non-linear regression analysis was carried out using Graphpad Prism software (Graphpad Software Inc., San Diego, CA, USA). Statistical analysis was carried out using Instat-3 computer program (GraphPad Software Inc., V3.05, San Diego, CA, USA).

Results

Chemical composition of EEP

The chemical constitution of propolis was evaluated by HPLC analysis, and the chromatogram showed the characteristic composition of propolis sample. The sample used in this experiment presented the following phenolic compound composition in mg%: Quercetin (89), apigenin (50), kaempferol (102), chrysin (1700), p-coumaric acid (90), caffiec acid phenethyl ester (790), galangin (778) and artepillin C (200).

Effect of EEP on antigen-induced contractions of passively sensitized guinea pig isolated tracheal zigzag preparations

OVA induced concentration-dependant contractions of passively sensitized guinea pig tracheal zigzag preparations. Incubation of the passively sensitized tracheal zigzag with EEP (100 µg/ml) resulted in a significant decrease in OVA-induced contractions (Figure 1) compared to control group. EC_{50} value was significantly decreased and E_{max} significantly increased by incubation with EEP when compared with the control group (Table 2). Ethanol at final bath concentration of 0.015% did not produce any significant effect, on the contractility of the isolated passively sensitized guinea pig trachea, when compared with the control group.

Treatment	EC_{50} (mg/mL)	E_{max}
Control	0.048 ± 0.005	55.7 ± 6.1
EEP (100 µg/mL)	0.246 ± 0.073π	9.4 ± 2.7π

EEP: Ethanolic Extract of Propolis; E_{max}: (% of contractions induced by 0.1 mM ACh)
Values represent mean ± S.E. of 5 preparations.
πSignificantly different when compared with the control group respectively using Student t test (P<0.05).

Table 2: Effect of EEP on EC50 and Emax of antigen-induced contractions of passively sensitized guinea pig isolated tracheal zigzag preparations.

	Total leukocyte count (x 10^4)	Lymphocyte count (x 10^4)	Eosinophil count (x 10^2)	Monocyte count (x 10^3)
Control	14 ± 0.82	10.95 ± 0.43	0 ± 0	0 ± 0
OVA	24.4 ± 2.14 π	18.53 ± 1.91π	71.2 ± 5.54π	12.28 ± 0.75π
OVA-EEP	14 ± 0.82*	11.01 ± 0.55*	0 ± 0*	0 ± 0*

EEP: Ethanolic Extract of Propolis; BAL: Bronchoalveolar Lavage
Values represent mean ± S.E.M
π*Significantly different compared to control and OVA group respectively using one way ANOVA followed by Tukey-Kramer multiple comparisons test (P<0.05).

Table 3: Effects of EEP on total and differential leukocyte counts in BAL fluid of mice in a murine model of asthma.

	Score of Eosinophils	Score of Plasma Cells
Control	0 ± 0	0.8 ± 0.2
OVA	2.8 ± 0.2π	2.33 ± 0.21π
OVA-EEP	0.5 ± 0.22*	1 ± 0*

EEP: Ethanolic Extract of Propolis
Values represent mean ± S.E.M
π*Significantly different compared to control and OVA group respectively using one way ANOVA followed by Tukey-Kramer multiple comparisons test (P<0.05).

Table 4: Effect of EEP on histopathologic scores of eosinophils and plasma cells in lung tissues of mice in a murine model of asthma.

Effect of EEP on total and differential leukocyte counts in BAL fluid

Total leukocyte count in BAL fluid of OVA challenged group was significantly increased when compared with control group (Table 3). Diff-Quick staining of the cells revealed that most of the increased cells in OVA group were eosinophils, monocytes and lymphocytes. EEP could significantly decrease the total number of leukocytes and the three measured cell types.

Effect of EEP on lung tissue histopathology

Light microscopic examination of sections of lungs of mice in the control group revealed marked infiltration of eosinophils and accumulation of plasma cells. It was found that the OVA group showed significant increase in the mean scores of eosinophils and plasma cells when compared with the control group. EEP produced significant decrease in the scores of eosinophils and plasma cells compared with OVA group. Also dilated bronchioles were observed in the lungs of mice in the EEP-OVA group. The results of this experiment are demonstrated in table 4 and figure 2.

Effect of EEP on serum IgE levels

As shown in figure 3, control treated mice exhibit minor detectable OVA-specific IgE in sera. The levels of sera IgE were found to be significantly increased in OVA group compared with control group. However, administration of EEP significantly decreased the levels of serum IgE compared with OVA group but there is still significant higher level of serum IgE than control.

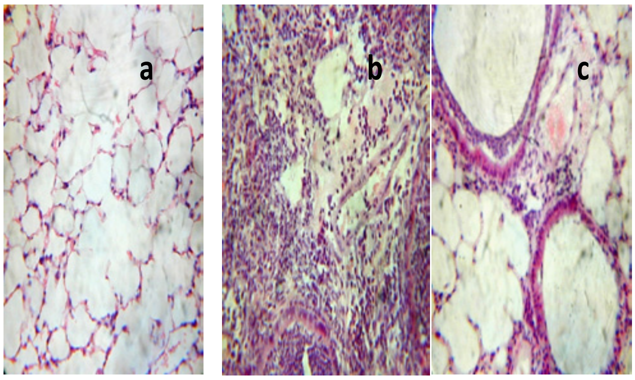

Figure 2: Histology of lung tissue of mice in a murine model of asthma; a. Control group showing no inflammation, b. OVA group showing marked inflammation, c. EEP-OVA group showing mild inflammation and dilated bronchi (H&E stain, 40x).
OVA: Ovalbumin; EEP: Ethanolic Extract of Propolis; H&E: Heamatoxylin and Eosin.

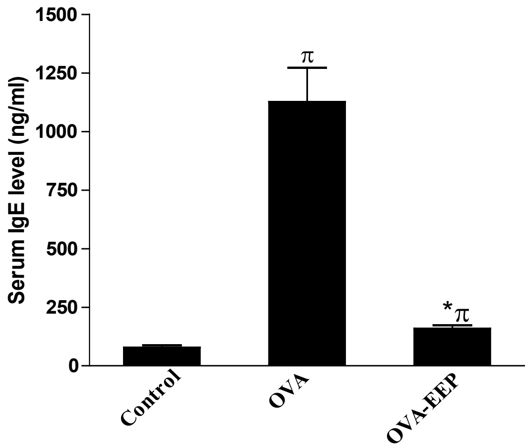

Figure 3: Effects of EEP on serum IgE of asthmatic mice.
Values represent mean ± S.E.
EEP: Ethanolic Extract of Propolis; IgE: Immunoglobulin E
π *Significantly different compared to control and OVA group respectively using one way ANOVA followed by Tukey-Kramer multiple comparisons test (P<0.05).

Effect of EEP on lung iNOS, TGF-β1 and TNF-α mRNA expression levels

Gene expression levels of iNOS, TGF-β1 and TNF-α in lung tissue were significantly higher in OVA challenged mice compared with control group. Significant decrease of iNOS, TGF-β1 and TNF-α mRNA expression was observed with OVA-EEP group compared to OVA group. These results are demonstrated in figure 4.

Discussion and Conclusions

Asthma is a chronic inflammatory condition of the lung airways with significant clinical impact and growing incidence. It is characterized by a complex interplay of different environmental and genetic factors that affect airway structure and function leading to airway obstruction, airway inflammation and airway hyper responsiveness (AHR) [4,17]. This study was designed to evaluate the ability of EEP to inhibit different pathologic changes associating asthma. EEP could induce relaxation of OVA-sensitized guinea pig tracheal zigzag constricted by

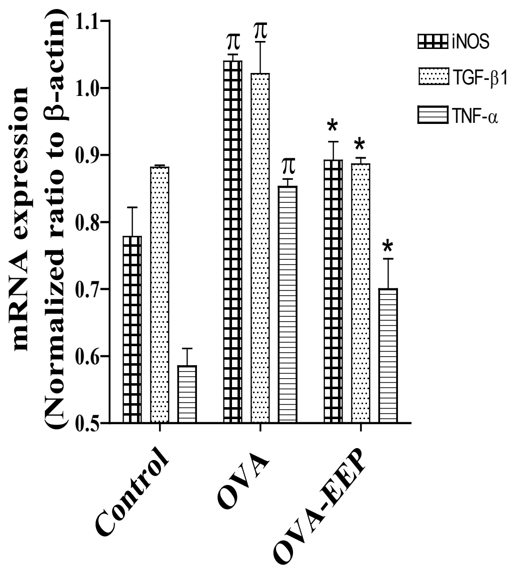

Figure 4: Effect of EEP on iNOS, TGF-β1 and TNF-α mRNA expression levels. EEP: Ethanolic Extract of Propolis; iNOS: Inducible Nitric Oxide Synthase; TGF-β1: Transforming Growth Factor-β1; TNF-α: Tumor Necrosis Factor-α. Values represent mean ± S.E.M
π *Significantly different compared to control and OVA group respectively using one way ANOVA followed by Tukey-Kramer multiple comparisons test ($P<0.05$).

OVA. EEP was also able to effectively suppress allergic inflammation in rodent model of allergic airway inflammation through decreasing total leukocyte, eosinophil, monocyte and lymphocyte counts in BAL fluid, eosinophil and plasma cell scores with dilated bronchi in lung tissue, IgE serum level and the magnitude of gene expression of iNOS, TGF-β1 and TNF-α in lung that was increased during OVA sensitization and challenge. The results of this study revealed that EEP may be used as adjuvant therapy for patients with allergic airway inflammation.

Mast cells are widely distributed throughout vascularized tissues and certain epithelia and play a major role in allergic asthma [18]. Immunologic mast cell stimulation is initiated upon the interaction of a multivalent antigen (allergen) with its specific antibody attached to the cell membrane via its high affinity receptor, Fc”RI [19,20]. In passive sensitization, the isolated tissue from a non-sensitized subject is incubated with serum containing elevated levels of antibodies to a specific allergen. This is sufficient to cause the passive transfer of specific Ig from the sensitizing serum, which binds with high affinity to solubilized Fc receptors on the surface of mast cells in non-sensitized tissues [13]. The degranulation of mast cells leads to the release of inflammatory mediators, such as eicosanoids, proteoglycans, proteases and several proinflammatory and chemotactic cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-4, IL-13, and transforming growth factor (TGF)-β1 [21].

According to our results, EEP inhibited OVA-induced contractions of passively sensitized tracheal smooth muscles. The ability of EEP to inhibit OVA-induced contractions of tracheal zigzag preparations could be attributed, at least in part, to its direct broncho relaxant effect proved by Paulino et al. [22], who showed that EEP has a direct relaxant effect on guinea pig trachea *in-vitro*. This effect involves the release of nitric oxide, the activation of soluble guanylate cyclase and activation of Ca^{2+}- and ATP-sensitive K^+ channels [22] as well as the control of calcium mobilization [14].

Mice sensitized and challenged with OVA showed a significantly increased influx of total leukocytes, eosinophils and lymphocytes, and the secretion of inflammatory mediators. Histological examination of lung tissues paralleled the results of analysis of the cell count in the BAL fluid which showed marked influxes of eosinophils and plasma cells in OVA group. EEP supplementation can reverse established airway inflammation where EEP treatment reduces total infiltrating leukocytes, eosinophils, monocytes and lymphocytes in BAL fluid, also the influx of eosinophils and plasma cells in lung tissue were decreased with marked dilated bronchi.

Those findings agree with the findings of Jung et al. [23] where caffeic acid phenethyl ester, a biologically active ingredient of propolis, showed inhibitory effects on the inflammatory cells in BAL fluid in a murine model of asthma. It could significantly decrease the total leukocyte, eosinophil, lymphocyte and macrophage counts. Mice treated with caffeic acid phenethyl ester also showed marked reduction in the infiltration of inflammatory cells within the peribronchiolar and perivascular regions [23].

Anti-OVA IgE antibody levels were reduced in serum of OVA sensitized and challenged mice following oral administration of EEP. It has been reported that Th2 cells encourage production of IgE antibody [24]. These results indicate that EEP has a function that modulates the proliferation of lung Th cells from Th2-cell-dominant to Th1-cell-dominant in our airway inflammation model of mice. These results suggest that EEP has an effect on allergic asthma that developed in an IgE-dependent manner.

The role of iNOS expression leading to the subsequent generation of NO during allergic lung responses is unclear. iNOS may be involved in the complex balance between Th1 and Th2 cells in immune and inflammatory states, which ultimately favours a Th2 cell outcome [25]. Deficient iNOS also suppressed ozone-induced airway tissue injury and LPS-induced acute airway inflammation in mice [26,27]. Therefore, potent iNOS inhibitors have long been considered to be an effective treatment of airway inflammation. In this study, the administration of EEP significantly attenuated OVA-induced asthmatic inflammation accompanied by the suppression of iNOS expression. Administration of EEP significantly inhibited leukocyte recruitment into the lung and regulated the Th1/Th2 balance to suppress OVA-induced airway inflammation. These results suggest that inhibiting iNOS expression by EEP attenuates airway inflammation through the infiltration of inflammatory cells into the lung. Nevertheless, EEP may inhibit airway hyper responsiveness (AHR) of OVA-induced asthma, regardless of suppression of iNOS, because AHR is not linked with NO in asthma [28]. Therefore, we consider that the suppression of iNOS expression by EEP is one of many inflammatory effects in asthma. Thus, the mRNA levels of TGF-β1 and TNF-α were also measured. Both TGF-β1 [29] and TNF-α [30] are found to be a proinflammatory and powerful chemotactic agents for neutrophils, eosinophils, macrophages and mast cells. Furthermore, there is increased expression of TNF-α [31] and TGF-β1 [32] in the airways of asthmatic patients. Also, there is increased expression of both TGF-β1 and TNF-α in LPS-induced acute airway inflammation in rat [33]. In our study, the upregulated TNF-α and TGF-β1 mRNA levels in the lungs of asthmatic mice were significantly decreased by EEP. This provide anther pathway by which EEP can inhibit OVA-induced airway inflammation in a murine model of asthma. These findings suggest that EEP may be useful as an adjuvant therapy for asthmatic patients in the future.

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