

The Protective Effect of α -Hederin, the Active Constituent of *Nigella sativa*, on Lung Inflammation and Blood Cytokines in Ovalbumin Sensitized Guinea Pigs

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In the present study, the preventive effect of two different concentrations of α -hederin, the active constituent of *Nigella sativa*, on lung inflammation and blood cytokines in ovalbumin sensitized guinea pigs was examined. Forty eight male adult guinea pigs were divided into control (C), sensitized (S) and sensitized pretreated groups; with thymoquinone (S+TQ), low dose (S+LAH) and high dose of α -hederin (S+HAH) and inhaled fluticasone propionate (S+FP). The lung histopathology and blood levels of IL-4, IFN- γ and IL-17 were assessed. Compared to sensitized animals, all pathological changes improved significantly in pretreated groups ($p < 0.001$ to $p < 0.05$). These improvements in α -hederin pretreated groups were similar to S+TQ and S+FP groups except cellular infiltration in S+LAH and S+HAH groups which was lower than S+TQ group ($p < 0.05$). The blood IL-4 and IL-17 levels in S+HAH groups showed a significant decrease compared to S group ($p < 0.05$) which were similar to S+TQ and S+FP groups. The level of IFN- γ in S+LAH and S+HAH groups increased significantly compared to S group ($p < 0.05$) which was higher than S+FP group ($p < 0.05$). Blood IL-4 in S+HAH group was significantly lower than S+LAH group ($p < 0.05$). In conclusion, α -hederin could attenuate the lung inflammation and improve the changes of cytokines like thymoquinone and fluticasone in used dosages. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: asthma; α -hederin; lung inflammation; IL-4; IL-17; IFN- γ .

INTRODUCTION

Asthma is a heterogeneous chronic airway disorder characterized by bronchoconstriction, airway hyper-responsiveness, increased mucus secretion, inflammation and remodeling (Olin and Wechsler, 2014) and marked by recurrent episodes of wheezing, coughing and shortness of breath (Miller, 2001). Airway remodeling in asthma increased thickness of the basement membrane, epithelial injury, goblet cell metaplasia, airway smooth muscle hyperplasia and increased airway lymphangiogenesis and angiogenesis (Olin and Wechsler, 2014). There is an imbalanced Th1/Th2 ratio of immune activity in asthmatic patients. CD4⁺Th2 cells produce a series of cytokines, including IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. CD4⁺Th1 cells are responsible for production of IFN- γ and IL-2 (Miller, 2001). Th17 cells that express IL-17 are CD1⁺ T cells and play a role in a subset of patients with asthma disease (Olin and Wechsler, 2014).

Both systemic and local administration of the essential oil of *Nigella sativa* and one of its constituents, thymoquinone, showed an antiinflammatory and anti-

asthmatic activity on laboratory animals and humans (Randhawa and Al-Ghamdi, 2002; Boskabady *et al.*, 2004; Boskabady *et al.*, 2007; Hosseinzadeh *et al.*, 2008; Ahmad *et al.*, 2010; Boskabady *et al.*, 2010; Aziz *et al.*, 2011; Boskabady *et al.*, 2011a, 2011b; Al-Jawad *et al.*, 2012; Keyhanmanesh *et al.*, 2014). However the exact mechanism of this plant is not identified yet. Another bioactive constituent of *N. sativa* is α -hederin (Rooney and Ryan, 2005). Also, α -hederin is one of the principal bioactive constituents of *Hedera helix*. *H. helix*, a member of the Araliaceae family, is a well-known plant as ivy plant or English ivy (Hocaoglu *et al.*, 2012).

In the present study, the preventive effect of two different concentrations of α -hederin on blood cytokines levels and lung histopathology in ovalbumin sensitized guinea pigs was examined.

MATERIALS AND METHODS

Forty eight male adult Dunkin–Hartley guinea pigs (400–700 g) were selected for the study. They were accommodated in individual cages kept at $22 \pm 2^\circ\text{C}$ on a 12 h light/dark cycle. After 10 days, animals randomly divided to six groups; control group (C), sensitized group (S), sensitized groups pretreated with thymoquinone (Sigma Chemical Ltd., UK); 3 mg/kg (Aziz *et al.*, 2011)

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(S+TQ), low dose of α -hederin (Extrasynthese Co., France); 0.3 mg/kg (S+LAH) and high dose of α -hederin; 3 mg/kg (S+HAH). These were injected intraperitoneally on 10th day of induction protocol. The last sensitized group received inhaled fluticasone propionate 250 μ g twice a day (GlaxoSmithKline) during the sensitization period (Keyhanmanesh *et al.*, 2010a) (group S+FP).

This study was approved by the ethical committee of the Tabriz University of Medical Sciences.

Animal sensitization. Sensitization of the animals to ovalbumin was done by the use of the method explained before (Keyhanmanesh *et al.*, 2010b). Briefly, guinea pigs were sensitized to ovalbumin (Sigma Chemical Ltd., UK) by injecting 100 mg, i.p. and 100 mg, s.c. on 1st day and a further 10 mg, i.p. on 8th day. From 14th day, sensitized animals were exposed to an aerosol of 4% ovalbumin for 18 ± 1 days for 4 min daily. The aerosol was administered in a closed chamber of dimensions $30 \times 20 \times 20$ cm using a nebulizer (Omron Healthcare, Inc., USA). Control animals were treated similarly, but saline was used instead of the ovalbumin solution.

Lung histopathological evaluation. Guinea pigs were sacrificed by a cervical dislocation, and their lungs and trachea were removed and placed into 10% buffered formalin (37%, Merck, Germany). Seven days later, the tissues were dried by passage through 70% to 100% ethanol. After that the tissues were cleared by xylene. Then the tissues were embedded in paraffin. Finally the specimens were cut into 4- μ m slices using an Autotechnicon apparatus and stained with hematoxylin and eosin (H&E stain). The tissues were then evaluated under a light microscope. The pathologic changes in the lung of sensitized and pretreated groups included the vascular and airway membrane hyperplasia, the presence of mucosal plug and atelectasis, respiratory epithelial denudation and cellular infiltration. The pathological changes were scored according to as follows: (1) no pathologic changes, 0; (2) patchy changes, 1; (3) local changes, 2; (4) scattered changes, 3; (5) severe changes (in the most parts of the lung), 4.

Measurement of blood IL-4, IFN- γ and IL-17 levels. Five millilitres of peripheral blood was obtained immediately after sacrificing the animals and placed at room temperature for 1 h. The samples were then centrifuged at 3500 rpm at 4 °C for 10 min. The supernatant was collected and immediately stored at -70 °C until analysis. Finally, blood IL-4, IFN- γ and IL-17 levels were measured using the sandwich enzyme-linked immunosorbent assay (ELISA) method with commercial kits (Boskabady *et al.*, 2011a).

Statistical Analysis. All the results were considered as mean $\pm$ SEM. The data of five sensitized groups were compared with controls using one-way analysis of variance (ANOVA) with Tukey–Kramer post-test. Furthermore, the data of four pretreated groups were compared with sensitized guinea pigs using one-way analysis of variance (ANOVA) with Tukey–Kramer post-test. The

data of α -hederin pretreated groups were compared with S+TQ and S+FP groups using the unpaired Student's *t*-test. In addition, the data between α -hederin pretreated groups were compared using the unpaired Student's *t*-test.

RESULTS

Lung histopathology

With regard to this scoring, all pathological changes in the S group, including the vascular membrane hyperplasia, airway membrane hyperplasia, the presence of mucosal plug, respiratory epithelial denudation, cellular infiltration and atelectasis were significantly higher than those in the group C ($p < 0.001$ for all cases, Figs. 1 and 2).

In comparison to sensitized animals, all pathological changes improved significantly in pretreated groups ($p < 0.001$ to $p < 0.05$). These improvements in α -hederin pretreated groups were similar to thymoquinone and fluticasone pretreated groups except cellular infiltration in S+LAH and S+HAH groups which was lower than S+TQ group ($p < 0.05$, Table 1).

Levels of blood cytokines

The mean value of blood IL-4 levels in group S (33.66 ± 0.99) was significantly higher than that in group C (29.16 ± 1.06 , $p < 0.05$). The blood IL-4 level in S+TQ (30.25 ± 1.05), S+HAH (30.08 ± 1.14) and S+FP (31.08 ± 0.84) groups showed a significant decrease compared to that of S group ($p < 0.05$ for all). But the mean value of blood IL-4 level in S+LAH group (33.33 ± 0.85) did not show a significant difference compared to that of the S group. The mean value of IL-4 in S+LAH and S+FP groups showed significant difference compared to S+TQ group ($p < 0.05$). The significant difference was seen among S+LAH and S+HAH groups ($p < 0.05$).

The mean value of the blood IFN- γ level of group S (32.41 ± 0.47) was significantly higher than that of group C (29.85 ± 1.12 , $p < 0.05$). Pretreated groups, except S+FP, showed a significant increase in blood IFN- γ (35.66 ± 1.05 for S+TQ, 35.25 ± 1.11 for S+LAH and 35.33 ± 0.97 for S+HAH) compared to sensitized animals ($p < 0.05$) although the decrease in S+FP group (31.50 ± 0.76) was not statistically significant. In addition, the mean value of IFN- γ in S+TQ, S+LAH and S+HAH groups was significantly higher than that in group C ($p < 0.01$). However blood IFN- γ level did not show any significant difference between thymoquinone and α -hederin pretreated groups, these levels were significantly higher than that of S+FP group ($p < 0.01$ to $p < 0.05$).

The mean value of the blood IL-17 levels in group S (4.85 ± 0.21) was significantly higher than that in group C (4.06 ± 0.15 , $p < 0.05$). The blood IL-17 level in S+TQ (4.25 ± 0.07), S+HAH (4.10 ± 0.17) and S+FP (4.18 ± 0.12) groups showed a significant decrease compared to that of the S group ($p < 0.05$ for all). But the mean value of the blood IL-17 level in S+LAH group (4.41 ± 0.08) did not show a significant difference compared to that of the S group. Also, pretreated groups did not show a significant difference compared to that

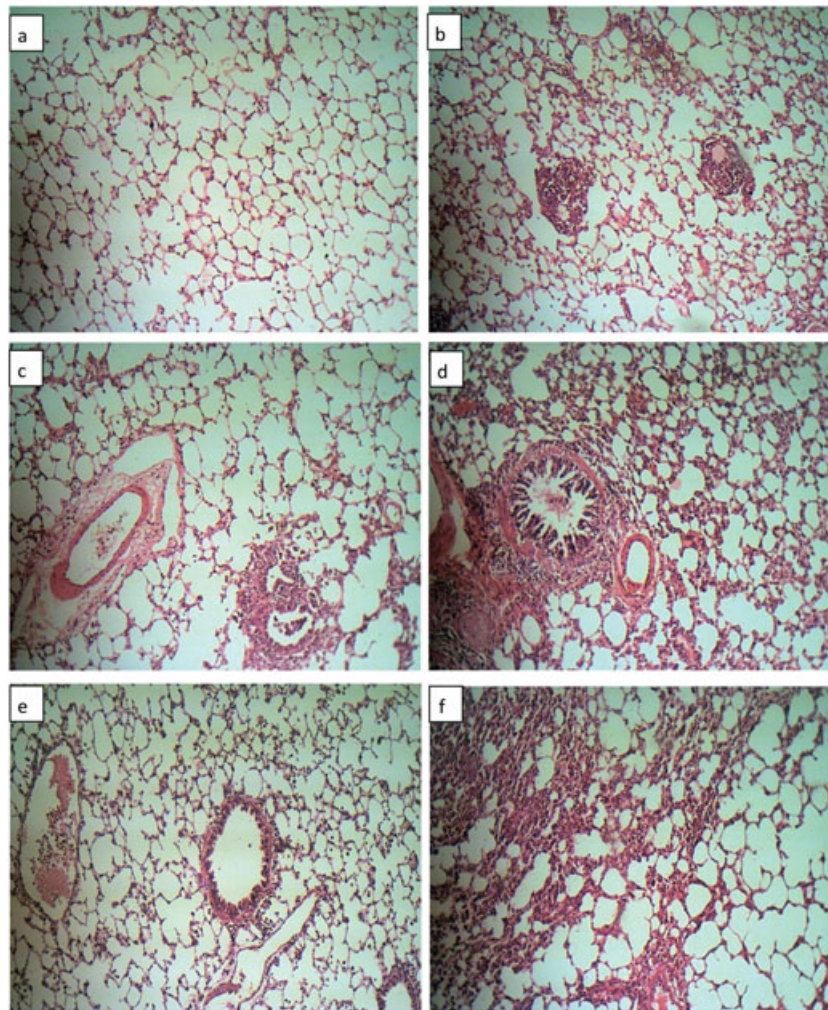


Figure 1. Photographs of a lung specimen in different groups (magnification for each group; 10×20). Normal lung tissue in control group (a), cell infiltration in sensitized group (b), remodelling and oedema in S+TQ group (c), cell infiltration in S+LAH (d) and in S+HAH (e) and atelectasis in S+FP group (f). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Table 1. Pathological finding scores in the control (C), sensitized (S), S pretreated with thymoquinone (S+TQ), S pretreated with low dose of α -hederin (S+LAH) and high dose of α -hederin (S+HAH) and pretreated with inhaled fluticasone propionate (S+FP) guinea pigs

Pathological findings	Scores in groups					
	C	S	S+TQ	S+LAH	S+HAH	S+FP
Vascular membrane hyperplasia	0.38 ± 0.18	3.00 ± 0.33	1.38 ± 0.32	1.25 ± 0.37	1.13 ± 0.40	1.50 ± 0.33
Airway membrane hyperplasia	0.25 ± 0.16	2.75 ± 0.31	1.00 ± 0.26	1.13 ± 0.29	1.00 ± 0.38	1.00 ± 0.27
Mucosal plug	0.38 ± 0.18	2.50 ± 0.33	0.63 ± 0.18	0.63 ± 0.26	0.88 ± 0.29	1.25 ± 0.37
Respiratory epithelial denudation	0.25 ± 0.16	3.00 ± 0.27	1.25 ± 0.25	1.13 ± 0.23	0.88 ± 0.29	1.5 ± 0.33
Cellular infiltration	0.25 ± 0.16	2.13 ± 0.40	1.63 ± 0.26	0.88 ± 0.29	$0.63 \pm 0.32^{\#}$	$0.88 \pm 0.23^{\#}$
Atelectasis	0.13 ± 0.12	2.38 ± 0.37	1.13 ± 0.23	1.00 ± 0.27	0.63 ± 0.26	0.88 ± 0.29

Values are presented as mean $\pm$ SEM ($n = 8$ for each group).

Statistical differences between pretreated groups vs. S+TQ group:

$^{\#}$; $p < 0.05$.

of the C group. Blood IL-17 levels did not show any significant differences among pretreated groups (Fig. 3).

DISCUSSION

In the present study, the effect of α -hederin on blood IL-4, IFN- γ and IL-17 levels and pathological changes in lung tissue of ovalbumin sensitized guinea pigs were examined

in comparison to thymoquinone and fluticasone as positive controls. The results indicated that the induction of asthma caused a significant increase in the level of blood cytokines compared to control group. Moreover, pathological changes including increased thickness of blood vessels and airways' wall, the mucus plaque, denudation of respiratory epithelium, inflammatory infiltration and atelectasis confirmed asthma in this group. All these changes conformed to previous studies (Keyhanmanesh *et al.*, 2010a; Boskabady *et al.*, 2011b).

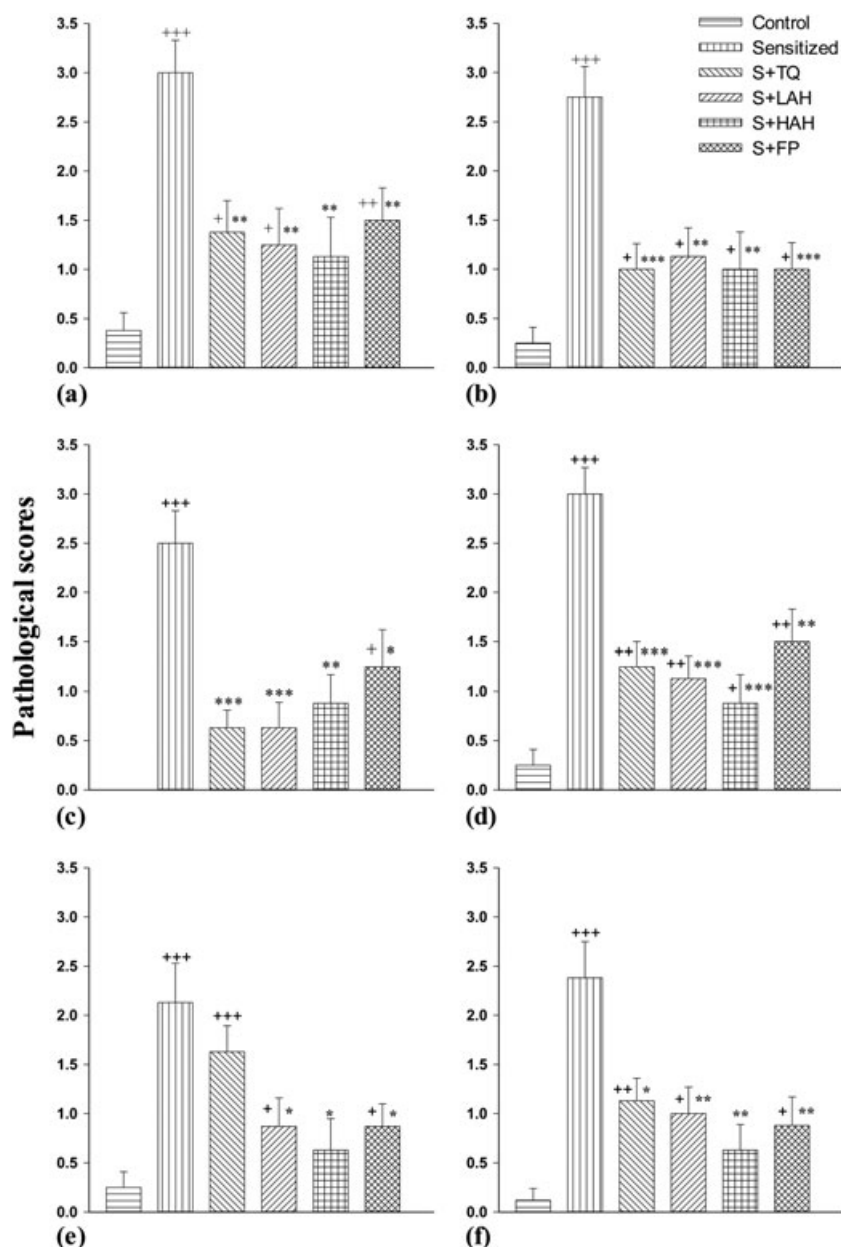


Figure 2. The vascular (a) and airway membrane hyperplasia (b), mucosal plug (c), local epithelial denudation(d), cellular infiltration (e) and atelectasis (f) of lungs in control (C), sensitized (S), S treated with thymoquinone (S+TQ), S treated with low dose of α -hederin (S+LAH) and high dose of α -hederin (S+HAH) and S pretreated with inhaled fluticasone propionate (S+FP) guinea pigs (for each group, $n = 8$). Statistical differences between different groups vs. control: +; $p < 0.05$, ++; $p < 0.01$, +++; $p < 0.001$. Statistical differences between pretreated groups vs. sensitized group: *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.

Airway inflammation is a key factor in pathogenesis of asthma as recent asthma management strategies have been emphasized on suppression of airway inflammation (Hocaoglu *et al.*, 2012). Preventive treatments for asthma should be able to diminish the airway inflammation which is the main feature of this disease. In this study, most of the above-mentioned changes have improved by administration of α -hederin, thymoquinone and fluticasone propionate. Antiinflammatory effect of thymoquinone and fluticasone propionate in previous studies has been shown on different models of asthma (El Gazzar *et al.*, 2006a, 2006b; El Mezayen *et al.*, 2006; Keyhanmanesh *et al.*, 2010b, Ammar *et al.*, 2011; Woo *et al.*, 2012).

In this study, the induction of asthma increased blood IL-4 and IFN- γ levels. Administration of α -hederin could decrease blood IL-4 level and increase serum IFN- γ level compared to sensitized group. The reduction in IL-4 level in S+LAH group was lower than

thymoquinone in used dose and similar to the effect of fluticasone propionate although IFN- γ level rise was similar to the effect of thymoquinone and higher than fluticasone in used dosages. α -Hederin is a triterpenoid saponin, which widely exist in nature and are the major active component of some traditional medicinal herbs such as *N. sativa* and *H. helix* (Jeong and Park, 1998). The seeds of *N. sativa* are characterized by a very low degree of toxicity. Administration of either the seed extract or its oil has not been shown to induce significant adverse effects on liver or kidney functions (Ali and Blunden, 2003). A literature search has revealed that many studies have been carried out related to the anticancer activities of *N. sativa* and some of its active compounds, such as thymoquinone and α -Hederin. Acute and chronic toxicity studies have recently confirmed the safety of *N. sativa* oil, particularly when given orally (Randhawa and Al-Ghamdi, 2002).

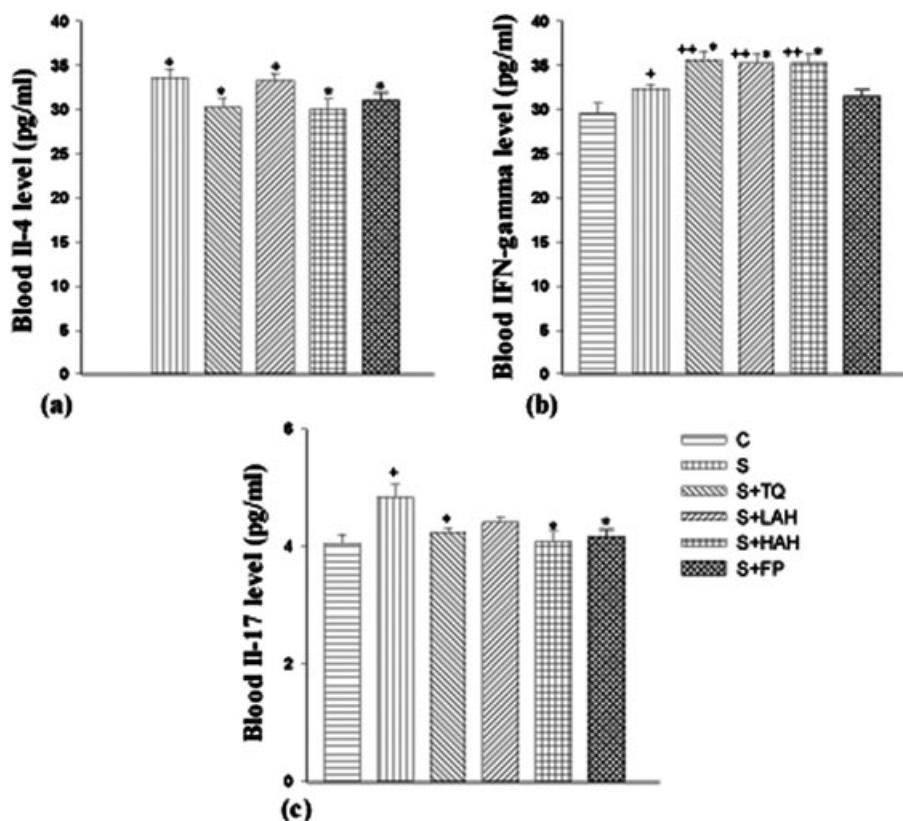


Figure 3. The blood IL-4 (a), IFN- γ (b) and IL-17 (c) levels (pg/ml) in control, sensitized (S), S pretreated with thymoquinone (S + TQ), S pretreated with low dose of α -hederin (S + LAH) and high dose of α -hederin (S + HAH) and S pretreated with inhaled fluticasone propionate (S + FP) guinea pigs (for each group, $n = 8$). Statistical differences between different groups vs. control: +; $p < 0.05$, ++; $p < 0.01$. Statistical differences between pretreated groups vs. sensitized group: *; $p < 0.05$.

Gepdiremen and his colleagues indicated the acute antiinflammatory activity of saponins isolated from ivy such as α -hederin in carrageenan-induced rat paw edema (Gepdiremen *et al.*, 2005). In clinical studies, dry extract of ivy leaves treated acute and chronic inflammatory bronchial diseases and improved symptoms like coughing and viscous mucus as well as lung function parameters (Gulyas *et al.*, 1997; Büechli *et al.*, 2005; Fazio *et al.*, 2009; Holzinger and Chenot, 2010; Cwientzek *et al.*, 2011; Stauss-Grabo *et al.*, 2011).

Asthmatic airway inflammation is regulated by the balance of Th1/Th2 cells. Th2 cells release IL-4, which releases IgE from B cells and stimulates the pro-inflammatory responses mediated by inflammatory cells such as eosinophils. However, Th1 cells release IFN- γ and regulate the antiinflammatory response (Romagnani, 1999; Cohn *et al.*, 2004; Schmidt-Weber and Blaser, 2005). An inappropriate change in Th1/Th2 balance can cause hyper or hypo-reactivity result in immune-pathological diseases such as allergy, autoimmunity or cancer (Jalali *et al.*, 2013). Administration of IFN- γ during allergic sensitization prohibited airway inflammation. So, it is possible that IFN- γ induced by thymoquinone will play a protective role in ameliorating the inflammatory response observed in ovalbumin sensitized and challenged mice (El Gazzar *et al.*, 2006a). Therefore, the results of the present study suggest that α -hederin and thymoquinone, the constituents of *N. sativa*, have inhibitory effect on IL-4 but enhance the production of IFN- γ , indicating the inhibitory effect on Th2 and stimulatory effect on Th1 helper cells. Moreover, these results indicate that α -hederin causes inhibitory effect on Th2 similar to fluticasone but has a stimulatory effect on Th1 helper cells which is

different with the effect of fluticasone. Therefore, the results of the present study showed that in inflammatory disorders associated with decreasing the balance of Th1/Th2, α -hederin may show even more therapeutic values compared to fluticasone. Undoubtedly, there is a need for additional studies to substantiate the effects.

The results showed that asthma induction increased the levels of IL-17 and administration of high dose of α -hederin could reduce IL-17 level compared to sensitized group. This reduction was similar to the effect of thymoquinone and fluticasone in used dosages. Recent studies have shown a link between Th17 immune response and allergic airway inflammation (Neveu *et al.*, 2010). Hellings and his colleagues demonstrated that the expression of IL-17 in airways increased during allergen inhalation, and this formed a relation between allergen-induced T cells and neutrophil activity because neutrophils might have important roles in airway remodeling in severe chronic asthma (Hellings *et al.*, 2003). Another study in 2006 showed that IL-17 was a negative regulator in allergic asthma and IL-23 induced IL-17 secretion in lung. This study also indicated that IL-17 was necessary for developing allergic asthma during sensitization by antigen, conversely; exogenous IL-17 decreased pulmonary eosinophil count and increased bronchial responsiveness. This is a new regulatory role for IL-17 (Schnyder-Candrian *et al.*, 2006).

The exact mechanism of α -hederin effect is not clear. In addition to regulation of the Th1/Th2 balance, α -hederin could inhibit the internalization of β 2-adrenergic receptors in stimulated airway smooth muscle cells (Sieben *et al.*, 2009). The previous study reported an elevated β 2-adrenergic responsiveness in α -hederin pre-treated

human airway smooth muscle cells after stimulation with terbutaline and forskolin (Sieben *et al.*, 2009). Another study suggested that α -hederin elevated β 2-adrenergic responsiveness by increasing cAMP levels (Wolf *et al.*, 2011).

Another involved mechanism is the antioxidant effect of α -hederin. Many inflammatory cells that produce active oxygen species such as superoxide and hydrogen peroxide are involved in airway inflammation of asthma. These active species of oxygen directly caused bronchoconstriction, histamine release from mast cells and mucus secretion from epithelial airways cells (Cluzel *et al.*, 1987). In addition, oxidative stress played an important role in airway inflammation of asthma (Talati *et al.*, 2006). Various components of antioxidant defense (enzymatic and non-enzymatic) reduced in asthmatic patients (Sackesen *et al.*, 2008). α -Hederin showed anti-cancer effects and could modulate the immune system (Jeong and Park, 1998). Other study showed that α -hederin and thymoquinone affected cancer cells as prodrugs, and these compounds had no risk of toxicity and apoptosis (Rooney and Ryan, 2005).

Airway remodeling is a potentially important consequence of asthma which can be defined as the presence of persistent changes of airway structure consisting of goblet cell hyperplasia, basement membrane thickening, sub-epithelial fibrosis, angiogenesis and airway smooth muscle hypertrophy and hyperplasia (Hocaoglu *et al.*, 2012). Although current treatments for asthma therapies are effective in reducing inflammation, airway remodeling is poorly decreased by them. Thus, new therapeutic modalities, which can directly target the remodeling processes and reverse permanent changes in lung, are essential (Hocaoglu *et al.*, 2012).

In this study, administration of α -hederin with two different doses decreased pathological changes of lung tissue due to asthma induction, such as reduced thickness of vessels and airways wall, mucus plaque, respiratory epithelium denudation, inflammatory infiltration and atelectasis. The other study indicated that one-week *H. helix* gavage significantly reduced goblet cell count and thickness of basement membrane in ovalbumin-sensitized mice (Hocaoglu *et al.*, 2012). Administration of corticosteroids has also been shown to inhibit the structural changes associated with airway fibrosis in

animal models (Hocaoglu *et al.*, 2012). Hocaoglu and colleagues evaluated the effects of *H. helix* on histopathologic changes of lungs in an allergic asthma animal model. Results showed that although *H. helix* administration reduced only goblet cell counts and the thicknesses of basement membrane in the asthmatic airways, but dexamethasone ameliorated all histopathologic parameters except thickness of basement membrane better than *H. helix* (Hocaoglu *et al.*, 2012). Moreover, Fazio reported that 7-day administration of syrup containing dried ivy leaf extract could treat patients with acute or chronic bronchitis and improve most of their symptoms (Fazio *et al.*, 2009). In our current study, the protective effect of single i.p. administration of α -hederin demonstrated on tracheal responsiveness and lung inflammation in ovalbumin sensitized guinea pigs (Saadat *et al.*, 2015).

These improvements of pathological changes in both groups (S+LAH and S+HAH) were almost identical and similar to thymoquinone and fluticasone pretreated groups except cellular infiltration which was lower than S+TQ group. As there are not any differences between high and low doses of α -hederin, perhaps the effective dose of α -hederin for eliminating asthmatic symptoms is 0.3 mg/kg.

In conclusion, α -hederin could attenuate the lung inflammation and improve the cytokines' changes like thymoquinone and fluticasone. These results indicate the effect of α -hederin on pathological and cytokine changes in sensitized guinea pigs that could indicate the prophylactic effects of this substance on asthma.

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Conflict of Interest

Non declared.

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