Pharmaceutical Sciences, 2024, 30(4), 486-495
doi:10.34172/PS.2024.23
https://ps.tbzmed.ac.ir/

Research Article

Hypericum perforatum Alleviates Ovalbumin-Induced Asthma through Downregulating TH2 and Upregulating TH1 Related Parameters in BALB/C Mice

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Article Info

Article History:
Received: 25 Apr 2024
Accepted: 23 Jul 2024
ePublished: 13 Aug 2024

Keywords:
-Allergic Asthma
-*Hypericum perforatum*
-Ovalbumin
-TH1
-TH2

Abstract

Background: Allergic asthma is a chronic and inflammatory disease of the respiratory tract, which is characterized by inflammation, airway obstruction and swelling. Anti-inflammatory compounds can be used to improve the disease severity. *Hypericum perforatum* (HP)/ St. John's wort has been used in the treatment of many diseases due to its anti-inflammatory effects. Therefore, this study was performed to investigate the effects of HP on TH1 and TH2 parameters in an animal model of allergic asthma.

Methods: Thirty BALB/c mice were sensitized with subcutaneous injection and inhalation of ovalbumin and then administered different doses of HP post-sensitization. Histological analysis of the lungs was performed. The effects of HP on mRNA expression of T-bet and GATA3 (TH1 and TH2 transcription factors, respectively), IFN-γ, IL-4 and IL-10 in the spleen were determined by real-time PCR. The protein levels of IFN-γ, IL-4 and IL-10 cytokines were also determined by ELISA technique in the serum of mice.

Results: HP extract (HPE) reduced the clinical severity in asthma-induced mice, and a reduction in the inflammatory cell infiltration in the lung. Furthermore, treatment with HPE inhibited asthma-related mediators as well as increased the anti-inflammatory parameters compared to the control. The study also showed that treatment with HPE significantly increased IFN-γ, IL-10 and T-bet, while decreasing the level of IL-4 and GATA3 in mice treated with HPE.

Conclusion: The data indicated that HPE can attenuate allergic asthma autoimmune responses by inhibiting infiltration of immune cell in the lung and TH2-related parameters. It is reasonable to assume that HPE has significant effects in the treatment of allergic asthma.

Introduction

Asthma is a chronic and inflammatory disease of the respiratory tract that is associated with overreaction and reversible narrowing of the airways. Inflammation leads to airway obstruction, swelling and clinical manifestations such as wheezing, shortness of breath, cough, and chest tightness.[1] The prevalence of allergic asthma has been increasing in recent years. Asthma is a serious problem for global health and significantly affects people's lifestyles.[2,3] It is estimated that, allergic asthma affected about 262 million people in 2019, and caused 455,000 deaths.[4] Its prevalence varies according to geographical area and gender for example, it is more common in women than men.[5] Immune cells such as eosinophils, mast cells and lymphocytes play an important role in inflammatory responses.[6] In fact, these cells promote inflammation in the airways and cause bronchoconstriction and lung dysfunction by production of allergic inflammatory mediators.

In addition, CD4+T cells are involved in the disease pathogenesis.[7] These cells differentiate into different subtypes such as T helper1 (TH1), T helper2 (TH2), T helper17 (TH17), and regulatory T cells (T reg) based on the type of antigen and their milieu.[8] An imbalance between TH1 and TH2 responses with a tendency toward TH2 can be a major cause of inflammatory responses in allergic asthma. TH2 cells secrete cytokines, including interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 10 (IL-10) and interleukin 13 (IL-13) and promote allergic diseases.[9] IL-4

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(as the key cytokine of TH2) binds to the IL-4 receptor to activate STAT6, which ultimately leads to the induction of GATA3 expression and TH2 differentiation.[10] It has been documented that the level of this transcription factor increases in allergic asthma patients.[11] On the other hand, TH1 cells secrete the interferon gamma (IFN-γ), which activates macrophages and promotes cellular immunity. The studies have shown that the differentiation of naïve TCD4+ cells into TH1 depends on transcription factors such as T-bet, STAT1 and STAT4. In addition, IFN-γ and IL-12 activate STAT1 and STAT4 and subsequently lead to the expression of T-bet transcription factor and TH1 differentiation.[12]

It has been documented that TH2 and related cytokines have a pathologic role in allergic asthma, while TH1 and IFN-γ play fundamental part in amelioration of the disease.[13,14]

Contrary to intensive research, the treatment of asthma has yet remained as a challenge. Thus, there has been growing interest in using herbal remedies with anti-inflammatory or immune-modulatory properties for the treatment of asthma.[15] *H. perforatum* (HP) is a valuable perennial medicinal plant of the Hypericaceae family.[16] It is well now established that HP has various effects, including anti-inflammatory[17], antiviral[18], anti-depressant[19] and anti-bacterial.[20] This plant is one of the best-selling medicinal plants in the United States and other Western countries. Therefore, it has been highly regarded by researchers due to HP properties and its low side effects.[21]

According to the properties of HP, we hypothesize that HP may modulate immune responses in allergic asthma by downregulating TH2 and upregulating TH1 parameters.

Methods

Animals

In this study, 30 female BALB/c mice (aged 4-6 weeks) with a weight of 18-22 g, were purchased from the animal farm of Kerman University of Medical Sciences, Kerman, Iran. The mice were maintained in pathogen-free facilities at 21-23 °C with a 12-h light/dark cycle, and standard conditions in terms of adequate water and food for a week to adapt to environmental conditions.

The animals were randomly divided into five groups (n=6/group). The first group (control/non-asthmatic) received saline without induction of asthma. The second group (asthma) was asthma induced and received saline. Groups 3, 4 and 5 were asthma induced and received HP extract at doses 50, 150 and 300 mg/kg and were named HP-50, HP-150 and HP-300, respectively.

Preparation of hydroalcoholic extract of H. perforatum

HP was collected at a high altitude from Kerman (Iran). The samples were approved by a botanist in the herbarium department of Vali-e-Asr University of Rafsanjan, Iran. To prepare the hydro-alcoholic extract of the plant (HP), 100 g of the ground plant was dissolved in ethanol-water (70/30, v/v 1260 ml of 96% ethanol solvent and 540 ml of distilled water). Extraction was performed by soaking at 40 °C for 72 hours in the dark on a shaker. The resulting product was then filtered through filter paper. Finally, the solvent was removed from the extracts using a rotary evaporator at 40 °C until only a semisolid extract remained. The residue was stored in a refrigerator and the desired concentration of the extract was prepared in distilled water before administration to the animals.[22,23]

Asthma induction and treatment

Allergic asthma was induced in BALB/c mice in two steps: In the sensitization step, a suspension of ovalbumin (OVA, grade V, 98% pure; Sigma, St. Louis, MO, USA) and aluminum hydroxide, Al(OH3), was injected subcutaneously (s.c) on days 0, 7 and 14. This suspension was prepared by mixing ovalbumin (0.5 mg/ml) and aluminum hydroxide (20 mg/ml) in 9% NaCL. In the challenge step, the mice were exposed to 1% inhaled ovalbumin solution on days 18, 19, 20, and 21 using a whole-body exposure chamber (BTK-4267, Behboud Tahghigh Kerman, Iran). (Figure 1).

The construction method of the asthmatic mouse model was based on the study by Yu *et al.*,[24] with a simple but effective modification.

The disease was induced in all groups except the normal group or non-asthmatic control group. Animals in the asthmatic group received saline instead of ovalbumin solution. In the treatment groups, HP extract was administrated intraperitoneally (i.p) to mice in groups 3, 4 and 5 at 50, 150 and 300 mg/kg, respectively.

The drugs were given to the mice from the 17th day to the 22nd day, once a day 1 hour before each ovalbumin challenge. The doses were selected based on a research by Nosratabadi *et al.*[25] On the 23rd day, the mice were sacrificed according to animal ethics.

Histologic analysis

On day 23 after asthma induction, the mice were anesthetized with ketamine-xylazine mixture and their lungs were removed and immersed in 10% formalin. Paraffin-embedded sections (5-µm thick) were prepared from the lungs and stained with hematoxylin and eosin (H&E) to observe the infiltration of inflammatory cells and the airway morphology. Inflammation was determined using the following scales: 0=No inflammation; 1=Mild inflammation; 2=Moderate inflammation; 3=Severe inflammation.

Enzyme-linked immunosorbent assay (ELISA)

To determine the effect of HP on the cytokine level, animals were anesthetized with ketamine and xylazine 24 hours after the last inhalation. Then the mice were sacrificed and serum from peripheral blood was collected and stored at -80°C for quantitative determination of cytokines. Serum levels of IL-4, IL-10 and IFN-γ were determined using ELISA kits (Karmania pars gene, Iran) according to the manufacturer's protocol.

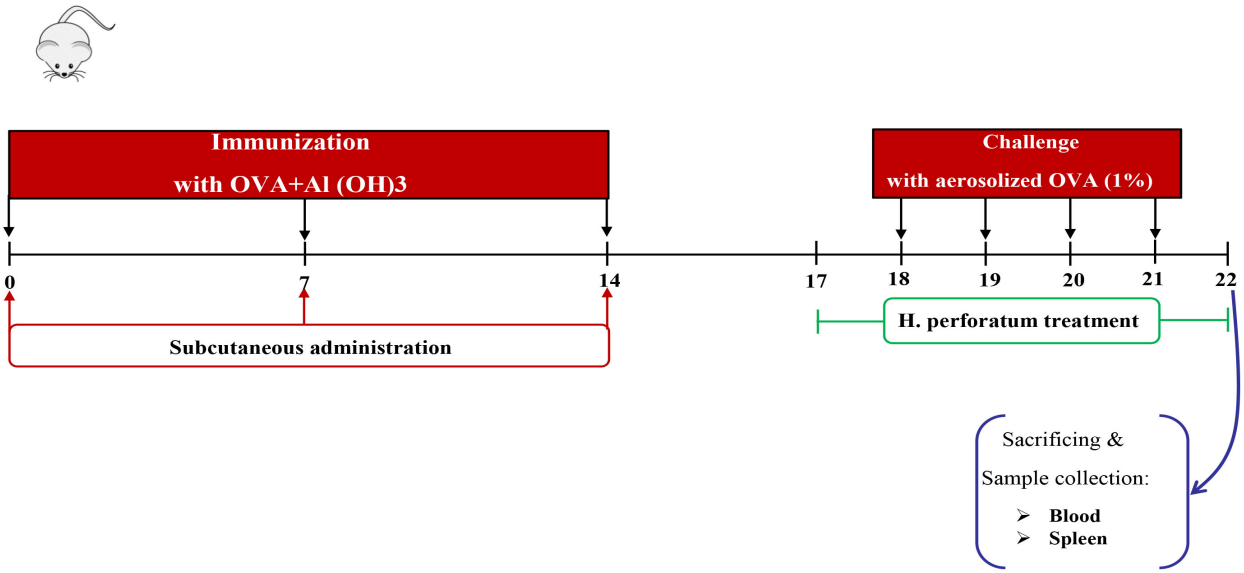

Figure 1. Schematic diagram of the experimental protocol of ovalbumin-induced asthma and HP treatment in mice.

Quantitative real-time PCR (qPCR)

The effect of HP on the expression of TH1 and TH2 transcription factors (T-bet and GATA3, respectively) and their key cytokines (IFN-γ, IL-4 and IL-10) were determined by SYBR green real-time-PCR method. The spleen cells were isolated from all mice and total RNA was extracted from the samples using TRIzol reagent (Karmania pars gene, Iran). Then cDNA was synthesized using Karmania pars gene kit (Iran) according to the manufacturer's instructions. To perform the real-time PCR reaction, a combination of template, primers, SYBR green master mix and sterile distilled water was distributed in the microtubes. The PCR vials were then placed in the ABI model of a real-time PCR device and the reaction was performed according to the following schedule: Initial denaturation was done at 95°C for 5 minutes, Denaturation; in this step, the reaction was heated to 95 °C for 20 seconds and repeated 35 times - Connection/ multiplication; reaction's temperature was lowered to 60°C for 30 seconds and repeated 35 times. Mouse beta-actin was used as an endogenous control for sample normalization. Results calculated the relative quantification delta-delta Ct based on the expression of target genes normalized to β actin. The results were reported as fold change compared to control. The sequence of primers used in this research is shown in Table 1.

Statistical analysis

SPSS software version 22 was used for statistical calculations. The distribution of data was examined using the Shapiro-Wilk statistical test. ANOVA statistical test was used to compare the variables. A significant level ($P<0.05$) was considered.

Table 1. The sequences of primers which are used in the study.

Genes		Sequences
Mouse GATA3	F	5′- CCTACCGGGTTCGGATGTAA-3′
	R	5′- CACACACTCCCTGCCTTCTGT-3′
Mouse T-bet	F	5′- ACCTGTTGTGGTCCAAGTTCAA-3′
	R	5′- GCCGTCCTTGCTTAGTGATGA -3′
Mouse IL-4	F	5′- TCACAGCAACGAAGAACACCAC-3′
	R	5′- TCTGCAGCTCCATGAGAACACTA-3′
Mouse IFN-γ	F	5′- ATTGCCAAGTTTGAGGTCAACAA-3′
	R	5′- ATCTCTTCCCCACCCCGAAT-3′
Mouse IL-10	F	5′- CAGGTGAAGACTTTCTTTC-3′
	R	5′- AACCCAAGTAACCCTTAA-3′
Mouse beta-actin	F	5′- CGATGCCCTGAGGCTCTTA-3′
	R	5′- TGGATGCCACAGGATTCCA-3′

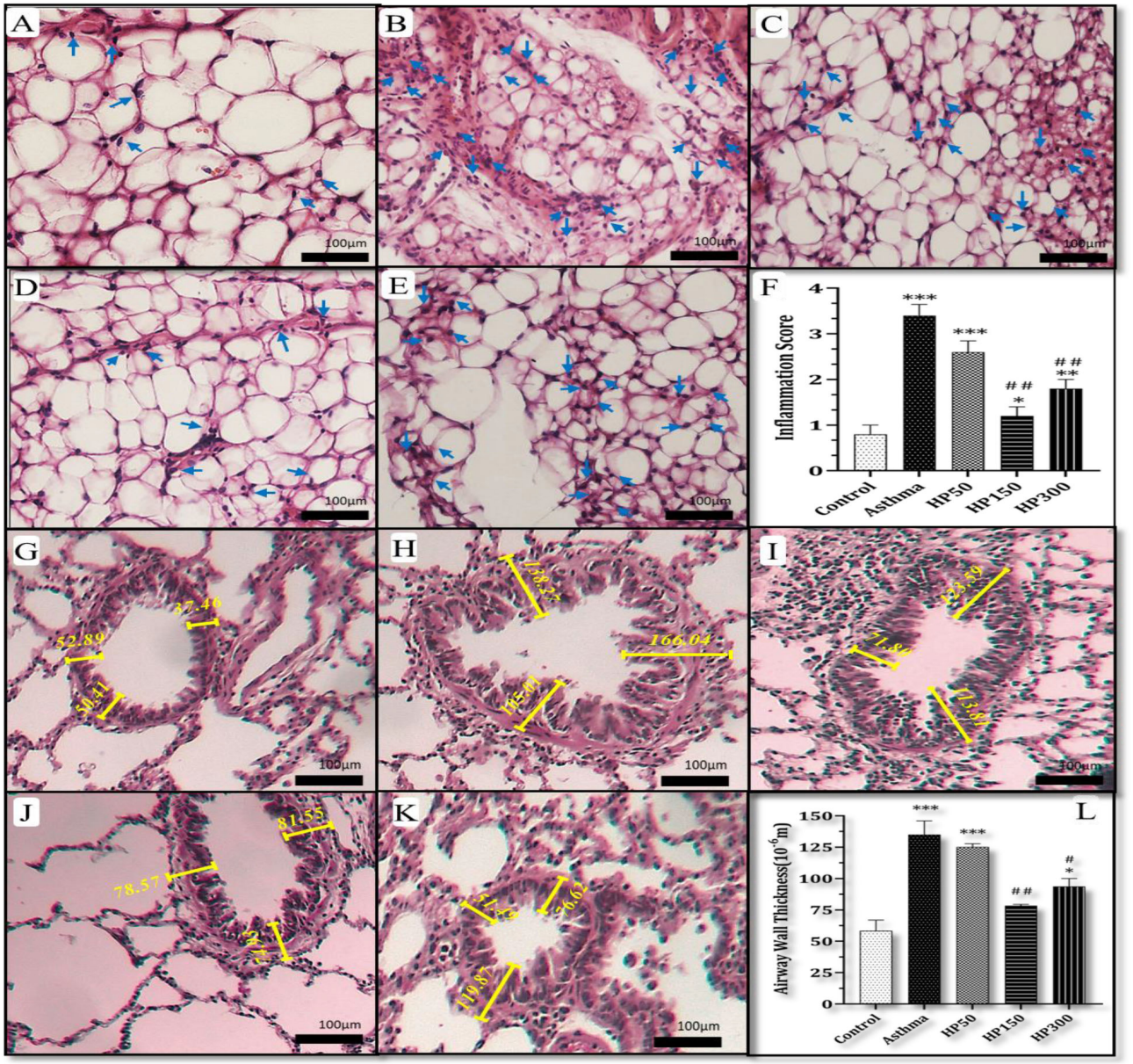

Figure 2. The inflammatory cell infiltration into the lungs after administration of HP. Lung tissue from each mouse (collected on Day 22 post-immunization) was fixed, embedded in paraffin, sections (5-µm) were prepared and the tissues were then stained with hematoxylin-eosin (H&E) and infiltration of inflammatory cells were evaluated. (A) Microscopic findings of lung tissue showed in the control group no cell infiltration was seen. (B) In the asthma group, an increase in bronchial wall thickness, and infiltration of leukocyte was observed. (C-E) Pathological changes in the treated groups, HP50, HP150 and HP300mg/kg, respectively. (F and L) Histologic features were also scored semi-quantitatively as described in the methods section. (G-K) Results for airway wall thickness in the lung tissue of mice (figures 2G-K, control, asthma, HP50, HP150 and HP300mg/kg, respectively). Values are shown as mean ± SEM. *P<0.05, ** P<0.01, *** P<0.001 vs. asthma group. # P<0.05, ## P<0.01, ### P<0.001 vs. control group.

Results

HP decreased infiltration of inflammatory cells into the lung

The results of lung histology showed that in the asthma group, a significant number of inflammatory cells was found, compared to the control group (Figure 2A and 2B). Increased bronchial wall thickness was also observed in this group (P<0.001). The results showed the effect of HP in a dose of 50 mg/kg on lung pathology was not significant compared to asthma group (Figure 2C), while infiltrating of inflammatory cells was significantly reduced in treatment with 150 and 300 mg/kg of HP (Figures 2D-2E, respectively). Similar results were observed for airway wall thickness in the lung tissue of mice (Figures 2G-(Figures 2G-K, control, asthma, HP50, HP150 and HP300 mg/kg, respectively). The results showed that HP in doses of 150 and 300 mg/kg can reduce the clinical severity of asthma due to the reduction of cellular infiltration into the lung. Histologic features were also scored semi-quantitatively as shown in the Figure 2F and 2L.

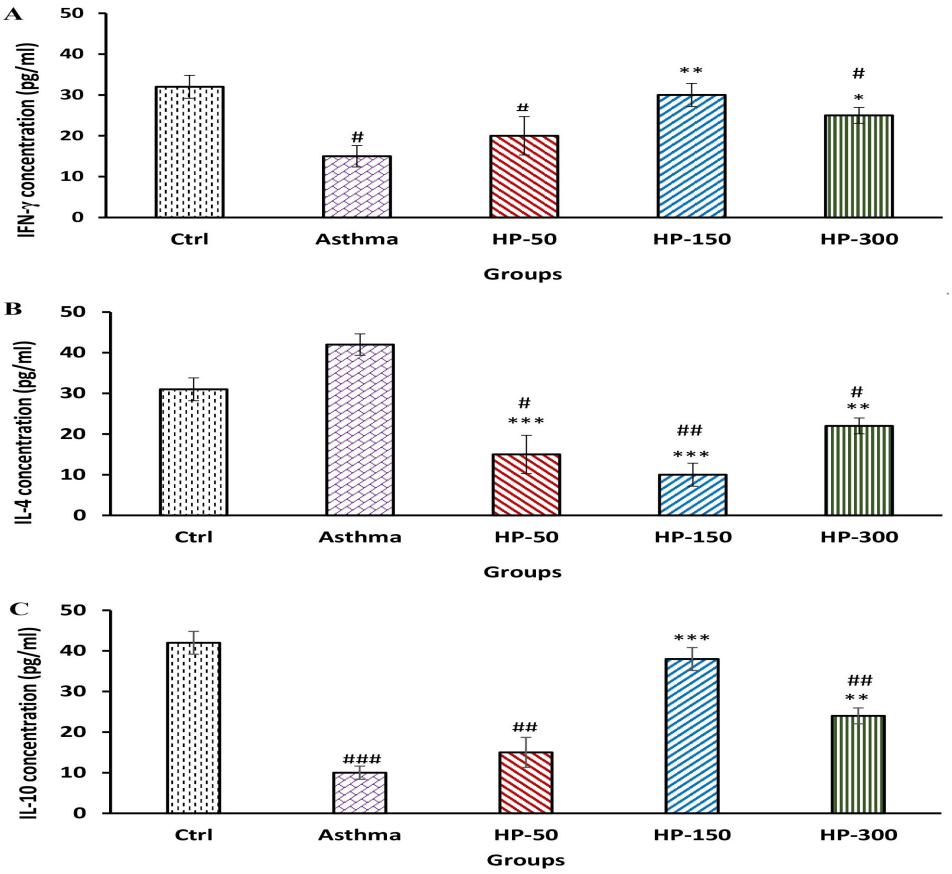

Figure 3. Effect of HP on serum levels of IFN-γ, IL-4 and IL-10 in the study groups. Serum from immunized mice from all groups were collected on day 22 post immunization. Cytokine levels of (A) IFN-γ, (B) IL-4 and (C) IL-10 were measured by ELISA. Results are shown as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs asthma group. # P<0.05, ## P<0.01, ### P<0.001 vs. control group. Statistical analysis was performed by one-way ANOVA followed by post hoc Tukey's test.

Effect of HP treatment on serum levels of IFN-γ, IL-4 and IL-10

To investigate the effect of HP on the level of IFN-γ, IL-4 and IL-10 in asthmatic mice, the serum from all animals was collected on day 22 post immunization and analyzed by ELISA technique. Results showed that the level of IFN-γ and IL-10 was decreased in the asthma group compared to control animals (P<0.05 for IFN-γ and P<0.001 for IL-10; Figures 3A and C), while the level of IL-4 was increased (P<0.05; Figure 3B). Treatment with HP significantly increased the level of IFN-γ and IL-10 in comparison with asthma mice (P<0.05; Figures 3A and C). There were also statistically significant differences in the levels of IFN-γ and IL-10 between the treatment groups and the control, except for the HP-150 group (P<0.05 for IFN-γ and P<0.01 for IL-10; Figures 3A and C). In contrast, the level of IL-4 was highly reduced in the serum of mice treated with HP compared to asthma mice (P<0.01; Figure 3B). These results indicate that HP induces the suppression of Th2 response through the inhibition of IL-4 production and promotion of Th1 responses via upregulation of IFN-γ.

Effect of HP on the polarization of TH1 and TH2 responses in asthma mice

To evaluate the therapeutic potential of HP on the polarization of TH1 and TH2, quantitative real-time PCR was performed. The asthma group showed reduced expression of T-bet and elevated expression of GATA3 in the spleen compared with the control mice (P<0.001 and P<0.01, respectively; Figures 4A and B). However, treatment with HP reversed the asthma-induced changes in the expression of T-bet and GATA3 (P<0.05). In addition, The mRNA expressions of IFN-γ and IL-10 were decreased (P<0.01 and P<0.001, respectively; Figures 4C and E) in the asthma group, while those of IL-4 were significantly increased compared with the normal control group (P<0.05; Figure 4D). Furthermore, treatment with HP led to elevated expression of both IFN-γ and IL-10 (Figures 4C and E), as well as decreased expression of IL-4 compared to the asthma group (P<0.05 for HP-150 and HP-300, Figure 4D). As a result, HP has a protective effect in the treatment of asthma disease by inhibiting Th2 cells and upregulating Th1 cells.

Discussion

In this research, the effect of *H. perforatum* extract on ovalbumin-induced asthmatic mouse model was investigated. To the best of our knowledge, this is the first report in which *H. perforatum* is used for asthma treatment. The results of our study found that in asthmatic animals, a significant increase in infiltration of inflammatory

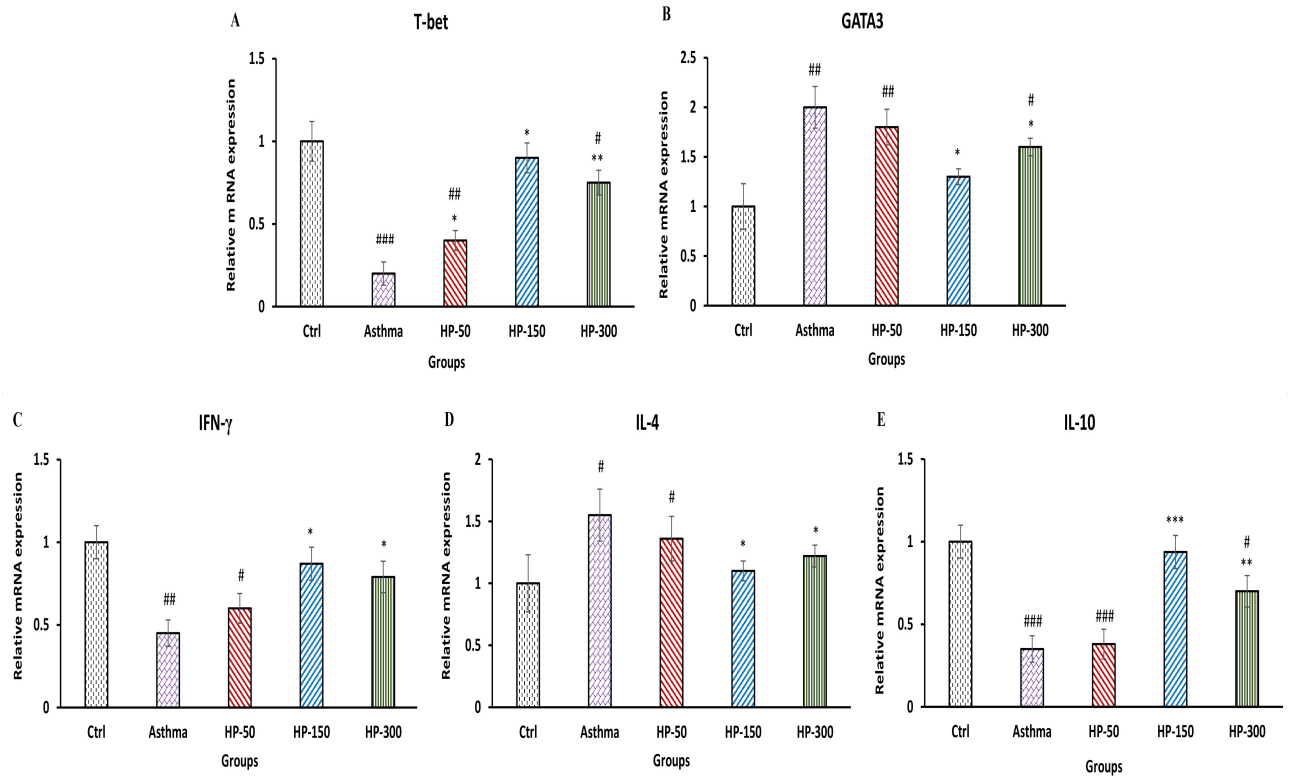

Figure 4. Expression of transcription factors (T-bet, GATA3) and related cytokines in the spleen of mice with asthma. Splenocytes were isolated from mice on day 22 post sensitization. Total RNA was extracted and reverse transcribed into cDNA. Equal quantities of cDNA from each sample analyzed by qPCR using β-actin as the internal control. mRNA expression levels of transcription factors were evaluated in the spleen of mice. * P<0.05, ** P<0.01, *** P<0.001 vs asthma group. # P<0.05, ## P<0.01, ### P<0.001 vs control group. Statistical analysis was performed by one-way ANOVA followed by post hoc Tukey's test.

cells into the lungs compared to the control group, while mice treated with HP significantly decreased clinical scores and reduced asthma severity. These changes were observed in connection with the decrease in infiltration of inflammatory cells into the lungs.

In accordance with our results a study on an animal model of lung injury reported that HP decreased PMNs infiltration, lipid peroxidation, and production of TNF-α and IL-1β.[26] Furthermore, bronchodilator properties of HP are well-known and have been studied in an animal model of bronchoconstriction.[27] This research reported that HP dose-dependently inhibited the carbachol-induced increase in the inspiratory pressure.

In addition, extensive studies and results have documented that the biological activity of HP is related to its active chemicals such as hyperforin and hypericin.[28,29] In line with these researches, Dell'Aica *et al.*,[30] in an animal model of bleomycin-induced pulmonary inflammation indicated that, hyperforin as an active ingredient of HP prevents neutrophil trafficking to the lungs and leads to a reduction in lung fibrosis.

In another study on animal model of edema, Zdunic *et al.*,[31] showed that, II8-biapigenin and quercetin, the two major oil extracts of HP, have anti-inflammatory and gastroprotective properties.

Today, researches have demonstrated that autoreactive T cells play a pivotal part in the pathogenesis of asthma and polarization of these cells toward TH1 or TH2 can significantly influence the disease.[32] A study by Gao *et al.*[33] showed an increased level of TH2 cytokines and a decreased level of TH1 cytokines in asthma-induced mice.TH2 cells can activate mast cells, eosinophils and stimulate B cells to switch to IgE production, while TH1 cells could inhibit TH2 cells by IFN-γ secretion and down-regulating mast cells and eosinophils.[34] The studies have documented that TH1 cells can prevent the development of allergic asthma by producing IFN-γ cytokine.[35,36] Another study has also shown that the expression of T-bet transcription factor and secretion of IFN-γ cytokine are decreased in patients with allergic asthma.[37] Our study showed that HP increases IFN-γ and T-bet expression in murine splenocytes. In line with our data, an animal study in influenza-infected mice showed that HP increased IFN-γ and IL-10 levels in the BALF and serum of infected animals.[38] Therefore, according to the results it seems that HP induces the production of TH1 cytokines via up-regulation of T-bet and subsequently differentiation of naïve CD4+T cells into TH1 subsets. A study by Ko *et al.*[39] reported a decrease in T-bet levels in the peripheral blood of asthmatic patients. In this study, T-bet levels were decreased both in people with asthma without steroids and in asthmatic patients who had inhaled corticosteroids. Therefore, stimulation

of immune responses toward TH1 cells, plays a role in balancing allergic responses induced by TH2 cells.[40] To date, no reports are available on the application of HP on differentiated T cells in asthma or other hypersensitive diseases. However, one study on atopic dermatitis patients showed the efficacy of HP in the treatment of mild to moderate atopic dermatitis.[41] The potential of HP in the treatment of asthma was also confirmed, in part, by the results of the evaluation of TH2 cells in the spleens of treated mice. Previous investigations demonstrated that elevated levels of TH2 cells are responsible for the pathogenesis of asthma, and a reduction in TH2 cells might be useful in controlling asthma disorders.[14] Therefore, the present study investigated the effect of HP on TH2 cells in asthma-induced animals. The data showed that administration of HP led to lower expression of GATA3, a TH2 transcription factor, in the spleen of treated animals in comparison to the asthma group. Our results also showed that this change was more significant in mice treated with an intermediate dose of HP. However, research in this field is still scarce, but the study by Menegazzi *et al.*,[26] demonstrated that HP suppressed activation of STAT3 and NF-kB, production of IL-1β and TNF-α and led to decreased inflammation in the lung of animal model of carrageenan-induced lung injury.

In addition, the possible mechanism involved in ovalbumin-induced asthma is the expression of IL-4, a key cytokine for Th2 differentiation. Our results indicated that HP improved the disease by significantly inhibiting the expression of IL-4. Previous studies have shown that inhibiting IL-4 leads to reduced TH2 differentiation and on the other hand results in the trafficking of TH1 cells into inflamed tissues.[42] Therefore, it seems that in addition to affecting TH2 cells, HP can improve the clinical symptoms of allergic asthma through an effect on TH1 cells.

In addition, our results revealed that the level of IL-10 was increased in the groups treated with HP. In parallel with our results, Xiuying indicated that HP can enhance IL-10 in bronchoalveolar lavage (BAL) fluid of mice infected with influenza virus.[38] Moreover, a study by Barathan *et al.*[43] on HepG2 cells showed that hypericin, an active component of HP, led to an increase in the level of IL-10 and IFN-γ in comparison to the control group. Additionally, the results revealed that although HP reduced the expression of GATA3 in asthmatic mice, the level of IL-10 increased in the serum and spleen of HP-treated mice. According to our results, it can be concluded that in addition to TH2 cells, other cells such as regulatory T cells (Treg) can produce IL-10 in large amounts. The results of a study by Nosratabadi *et al.*[44] also confirmed the conclusion. This research demonstrated hyperforin (a constituent of HP) enhances the level of IL-10 and TGFβ from regulatory T cells in the spleen of EAE mice treated with hyperforin. The other study also showed HP treatment led to an increase in the population of regulatory T cells in the spleen of EAE mice.[25]

So far, there is no study that shows the exact mechanism of HP in allergic asthma, but a study on the animal model of lung injury has shown that the HP leads to a decrease in the expression of NF-κB and STAT3.[26] In addition, the researchers have documented that cytokines start signaling through the activation of STAT molecules, which are transcription factors required for many biologic functions of T cells.[45,46]

On the other hand, many studies have shown that STAT3 leads to the inhibition of the expression of T-bet,[47,48] so it seems that HP by inhibiting STAT3 and increasing T-bet expression leads to the improvement of the disease symptoms, which this data is in line with our results that showed that HP leads to an increase in the expression T-bet and IFN-γ.

In this research, our team also investigated the effect of three concentrations of the extract on TH1 and TH2 transcription factors and related cytokines. The findings showed that although an intermediate dose of HP (150 mg/kg) was more effective in the treatment of asthmatic animals, the differences between three concentrations of HP were not statistically significant.

It can be pointed out that some herbal medicines in high doses due to increased cytotoxic effects or other side effects may have opposite effects. Therefore, the increased level of T-bet in this group increases the balance between TH1 and TH2 responses towards to TH1 cells and significantly reduces asthma symptoms.

Conclusion

It can be stated that HP as a valuable medicinal plant can ameliorate clinical and pathological manifestations of asthma, through decreasing the infiltration of allergic inflammatory cells in the lungs of asthmatic mice. Our finding also indicated that HP-treated mice showed high expression of TH1 transcription factor and its signature cytokine (IFN-γ), while TH2 transcription factor and its related parameters were decreased. Therefore, the results here demonstrated that HP could play crucial roles in the modulation of immune responses via the immune-deviation of immune responses from TH2 towards TH1 and, hence, have a promising therapeutic potency to be considered as a useful candidate for the treatment of asthma.

Although the present research showed that HP could alleviate allergic asthma mainly through an effect on TH1 and TH2 cells and their signature molecules, but due to limited sample size and lack of a placebo group, further study such as, long-term studies and potential clinical trials should be carried out to translate these findings into clinical practice.

Ethical Issues

Experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals with the approval of the Animal Ethic Committee of Rafsanjan University of Medical Sciences (IR.RUMS.REC.1398.127).

Acknowledgments

The authors wish to thank the authorities in the research unit of Rafsanjan University of Medical Sciences.

Author Contributions

Fahimeh Rostamabadi: Conceptualization, Methodology, Investigation, Formal Analysis, Writing - Review & Editing. Reza Nosratabadi: Formal Analysis, Writing - Review & Editing, Amir Rahnama: Formal Analysis, Writing - Original Draft. Vahid Mohammadi-Shahrokhi: Conceptualization, Methodology, Writing - Review & Editing.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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