

ORIGINAL ARTICLE

The *In Vitro* Impact of Glycyrrhizic Acid on CD4+ T Lymphocytes through OX40 Receptor in the Patients with Allergic Rhinitis

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Abstract— Glycyrrhizic acid (GA), the major bioactive component of glycyrrhiza, possesses anti-inflammatory, anti-allergic, and immunomodulatory activities. This study aimed to investigate the *in vitro* anti-allergic effect of GA through the OX40 receptor in patients with allergic rhinitis. Purified naive CD4+ T cells of patients with allergic rhinitis ($n = 12$) were activated with anti-CD3/anti-CD28 with and without anti-OX40 agonist mAbs and then treated with 50, 100, and 200 μM GA and 0.1 μM dexamethasone. Cells were incubated (72 h) to measure cell proliferation. Expression of OX40 in anti-OX40 mAb stimulated CD4+ T cells was evaluated by flow cytometry. mRNA expression of the OX40 receptor and T-bet, GATA-3, and forkhead box P3 (FoxP3) transcriptional factors were measured by a quantitative polymerase chain reaction. The levels of interleukin (IL)-4, IL-10, and interferon- γ (IFN- γ) were also measured. GA inhibited significantly the augmented T cell proliferation induced with anti-OX40 mAb. Protein and gene expression of OX40 was also decreased significantly. Dexamethasone and GA inhibited T-bet and GATA-3 genes expression, but this inhibition was only significant for GATA-3. In contrast, enhanced gene expression of FoxP3 was seen using 200 μM GA and dexamethasone. The levels of IL-4, IL-10, and IFN- γ decreased after treatment with both dexamethasone and GA, but the ratio of IFN- γ /IL-4 (Th1/Th2 balance) increased significantly due to 200 μM GA treatment. This study suggests that GA may have a therapeutic effect on allergic rhinitis, partly by modulation of the Th1/Th2 balance through suppression of OX40 and increasing the activity of regulatory T cells.

KEY WORDS: glycyrrhizic acid; OX40 receptor; allergic rhinitis; CD4+ T cells.

INTRODUCTION

Allergic rhinitis is an antigen-mediated inflammation in the nasal mucosa, which may also develop into paranasal

sinuses. It is one of the most common diseases in children as well as adults, and affects 10–40% of young people around the world. This disease has a considerable effect on the quality of life in terms of sleep, performance of daily tasks, social activities, as well as the physical and emotional health of patients [1, 2]. The most important common signs and symptoms of this disease include nasal congestion, runny nose accompanied by sneezing, and itching of the nose, eyes, and the pharynx [3].

The most important allergens that cause allergic responses in people with allergic rhinitis include plant pollens,

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fungi, and household allergens, such as mites present in dust and pets. Most people with asthma have rhinitis and allergic rhinitis (seasonal or annual), which increases significantly the probability of developing asthma. In this regard, over 40% of people with allergic rhinitis have asthma or they will be affected [4, 5].

Generally, the process of sensitization in people with rhinitis involves the uptake of the allergen by the antigen-presenting cells in nasal mucosa, which causes the activation of allergen-specific T cells. Simultaneously, the activity of epithelial cells through non-antigenic pathways (such as proteases) leads to the release of epithelial cytokines, such as thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, and IL-33, and eventually directs cellular response toward T-helper (Th)2 cells. This altered direction of immune responses causes dendritic cells and type 2 innate lymphoid cells (ILC-2) along with basophils, to generate cytokines such as IL-4 and IL-13. This results in the production of Th2 cells and the conversion of B cells to allergen-specific IgE-producing plasma cells [6].

Th2 cells play an important role in the development of the allergic diseases and produce large amounts of IL-4, IL-5, and IL-13 cytokines, causing the production of allergen-specific IgE and release of mast cell mediators, where the GATA-3 transcription factor is involved in Th2 lymphocyte differentiation [7]. Th1 cells inhibit the Th2 response by secreting interferon-gamma (IFN- γ), where the T-bet transcription factor is involved in their differentiation. The estimation of IgE production and the balance of Th1/Th2 cytokines are important tools in assessing allergic diseases [8]. Among the CD4⁺ T lymphocytes, regulatory T cells (Tregs) are mostly known for the production of IL-10 and tumor growth factor (TGF)- β cytokines as well as forkhead box P3 (FoxP3) transcription factor. These cells regulate and inhibit the function of Th1, Th2 cells, mast cells, basophils, and eosinophils [9].

OX40 is a type-1 transmembrane glycoprotein that belongs to the tumor necrosis factor (TNF) receptor family. Once T lymphocytes are activated through T cell receptor (TCR), this co-stimulatory receptor is expressed on these cells, which is required for their proliferation and survival. It seems that the OX40 receptor permanently exists on regulatory T lymphocytes. OX40 ligand (OX40L) can be expressed on dendritic cells, macrophages, activated B lymphocytes, and other cells, including mast cells and endothelial cells [10, 11]. Researchers have also found that functional OX40L can also be induced on the activated CD4⁺ T and CD8⁺ T cells, whose expression increases due to TGF β -1 [12]. The interaction between OX40 and its ligand can participate in the immune response by regulation of the Th1/Th2

balance [13]. On the other hand, studies show that OX40 signaling amplification may inhibit the production of IL-10 and the inhibitory function of Treg lymphocytes [14]. In general, the interaction between OX40 and its ligand can be effective in the cytokines production and thus the differentiation of T cells [10, 13].

Previous studies have shown that the inhibition of OX40 in the animal model of lung inflammation has resulted in a diminished number of eosinophils and neutrophils in the bronchial alveolar lavage fluid (BALF) and the lungs of mice sensitized with house dust mite (HDM). In addition, the inhibition of OX40 has caused the inhibited production of Th2 cytokines, such as IL-5 and IL-13 from the cells of allergic people sensitized with mites under *in vitro* conditions. The use of antibodies against OX40 has led to diminished IL-4 level and enhanced IFN- γ in HDM-sensitized mice [15]. OX40L-treated and OX40⁺ T cell-treated ovalbumin (OVA)-induced mouse asthma model exhibited increased asthma through OX40/OX40L signaling [16]. Thus, OX40 may be a suitable therapeutic target for repression of Th2 cell-mediated allergic rhinitis.

Licorice is a plant with an ancient origin, whose most important bioactive compound, glycyrrhizic acid (GA), is widely used in food industries and traditional herbs [17–19]. Clinical and experimental studies suggest that licorice extracts and its principle component, GA, enjoys different pharmacological properties that cause diminished oxidative stress, viral infections, fibrosis, tumor growth, and mutagenesis [20–25]. Furthermore, certain other characteristics, such as anti-inflammatory, anti-allergic, and immunomodulatory properties, have also been mentioned with regard to glycyrrhizic acid [26–31]. It seems that the GA present in the extract of licorice root through the OX40/OX40L axis, and affecting proliferation and function of CD4⁺ T lymphocytes, which have a role in treating allergic asthma [32]. This study aimed to investigate the anti-allergic effect of GA on proliferation and production of cytokines of the peripheral blood naive CD4⁺ T cells through its possible effect on the OX40 receptor in patients with allergic rhinitis in *in vitro* conditions.

METHODS AND PATIENTS

Selection of Patients

Twelve patients with allergic rhinitis (consisting of seven male and five female subjects; 27–50 years old, with a mean age of 36.3 ± 11.58 years) were included in the study. The inclusion criteria included diagnosing allergic rhinitis according to clinical examinations and disease history,

specific signs and symptoms of allergic rhinitis, positive prick test, and the absence of non-allergic diseases of nasopharynx and other organs.

The exclusion criteria included chronic rhinosinusitis, nasal polyps, and non-allergic rhinitis (infective), smoking, presence of other allergic diseases, existence of systemic diseases affecting the level of cytokines, consumption of steroids up to 6 weeks before the blood test, and antihistamine consumption up to 1 week before sampling. The study protocol was confirmed by the Ethics Committee of Isfahan University of Medical Sciences. Written informed consent was taken from each patient before taking a sample.

Methods

GA Preparation

First, GA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 40% ethanol to prepare the stock solution of 5 mM. The stock solution was thereafter filtered and then aliquoted, and finally preserved at -20°C .

Isolation of Peripheral Blood Naive CD4⁺ T Cells

Twenty milliliters of the peripheral blood containing EDTA was taken from the patients. First, the peripheral blood mononuclear cells (PBMCs) were separated by using Ficoll density gradient centrifugation (Sigma, USA). The viability of the cells was investigated by using the Trypan blue dye exclusion method, in which a viability of over 95% was considered acceptable for further investigation. The naive CD4⁺ T cells were separated using magnetic beads and MACS® columns (Miltenyi Biotec GmbH, Germany) based on negative selection, according to the instructions of the kit. The cells were thereafter stained with fluorescein isothiocyanate (FITC)-labeled anti-human CD4 and phycoerythrin (PE)-labeled anti-human CD45RA antibodies (Biolegend, San Diego, CA, USA) and then investigated by a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA); finally, their purity was determined as over 90%.

Cell Proliferation Assay

The isolated naive CD4⁺ T cells were cultured in the RPMI-1640 medium (Bioidea, Tehran, Iran), which contained 10% fetal bovine serum and 1% penicillin-streptomycin (Bioidea, Iran) under standard culture conditions (37°C ; humid air (95%) and CO_2 (5%)). To perform the cell proliferation assay, TetraZ™ Cell Counting Kit (Biolegend, San Diego, CA, USA) was used. The T cells (2×10^5 cells per well) were cultured in triplicate in flat-bottomed 96-well

plates that had been stimulated with the anti-CD3 monoclonal antibody (mAb) (Mabtech, Sweden) at $1\text{ }\mu\text{g/ml}$ as pro-coat overnight and soluble anti-CD28 mAb (Mabtech) at $0.1\text{ }\mu\text{g/ml}$ in the absence or presence of the anti-OX40 agonist mAb at $2.5\text{ }\mu\text{g/ml}$ (Creative Biolabs, Cat. No: MOM-18455, USA). The cells were then treated with GA concentrations of 50, 100, and $200\text{ }\mu\text{M}$ and $0.1\text{ }\mu\text{M}$ of dexamethasone (Abidi, Tehran, Iran) according to the following plan (Experimental Groups, Table 1). After 72 h of incubation, $10\text{ }\mu\text{l}$ of TetraZ™ solution was added to each well and the plate was incubated at 37°C for 4 h. Finally, the absorbance was measured at the wavelength of 450 nm by using a plate reader (Stat Fax 2100, USA).

Flow Cytometry Analysis

The isolated naive CD4⁺ T cells were cultured in (1×10^6), according to experimental group 2 for 72 h. The supernatant was collected by centrifuging the samples at $1200g$ at 4°C for 5 min. The centrifuged supernatant was then immediately preserved at -20°C to be used for investigating cytokines. The cells were washed with phosphate-buffered saline (PBS) for the flow cytometry; they were then stained with the PE-anti-human OX40 antibody, and PE Mouse IgG1, k Isotype Control (Biolegend, San Diego, CA, USA). The cells were then incubated for 20 min at 4°C ; after this, they were centrifuged at 4°C for 5 min at 1500 RPM. Once again, the cells were then washed twice with PBS. After staining, the cells were resuspended in PBS and the analysis of the cells was performed by flow cytometer. The data were also analyzed by the FlowJo 7.6 software (FlowJo, USA).

RNA Purification and Quantitative Real-Time Polymerase Chain Reaction

The total RNA was extracted by using the YTA total RNA Purification Mini Kit (Yektatajhez, Tehran, Iran), according to the kit instructions. Thereafter, cDNA synthesis was conducted by using the PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan) according to the kit protocol. Quantitative real-time PCR (qRT-PCR) was performed by the SYBR® Premix Ex kit (Takara Bio Inc., Japan) on the cDNA samples by an Applied Biosystems StepOne™ machine (ABI StepOne, CA, USA). The extent of the of *GATA-3*, *T-bet*, *OX40*, and *FoxP3* genes expression was measured in the cells undergoing the treatment under the aforementioned conditions (experimental group 2). The primers were designed by the AlleleID 7.0 software (Premier Biosoft, USA). Primers are listed below (Table. 2).

Table 1. Experimental Groups in the Study

Experimental groups	
Group 1 (without OX40 mAb)	Unstimulated naive CD4+ T cells
	Naive CD4+ T cells cultured with anti-CD3 and anti-CD28 mAbs
	Naive CD4+ T cells cultured with anti-CD3, anti-CD28 mAbs, and ethanol (as vehicle)
	Naive CD4+ T cells cultured with anti-CD3, anti-CD28 mAbs, and GA (50, 100, and 200 μ M)
	Naive CD4+ T cells cultured with anti-CD3, anti-CD28 mAbs, and 0.1 μ M Dex
Group 2 (with OX40 mAb)	Unstimulated naive CD4+ T cells
	Naive CD4+ T cells cultured with anti-CD3 and anti-CD28 mAbs
	Naive CD4+ T cells cultured with anti-CD3, anti-CD28 mAbs and mouse isotype control IgG
	Naive CD4+ T cells cultured with anti-CD3, anti-CD28 and anti-OX40 mAbs
	Naive CD4+ T cells cultured with anti-CD3, anti-CD28 and anti-OX40 mAbs and Ethanol (as vehicle)
	Naive CD4+ T cells cultured with anti-CD3, anti-CD28 and anti-OX40 mAbs and GA (50, 100, and 200 μ M)
	Naive CD4+ T cells cultured with anti-CD3, anti-CD28 and anti-OX40 mAbs and 0.1 μ M Dex

GA glycyrrhizic acid; Dex dexamethasone

To normalize the results of the extent of the target gene expression, the *GAPDH* gene expression level and its related primers were used (internal control). In addition, the $2^{-\Delta\Delta C_t}$ method was utilized for the calculation of the relative mRNA levels.

Measuring the Levels of Cytokines in the Cellular Cultivation Supernatant

The concentrations of IL-4, IL-10, and IFN- γ were measured by the enzyme-linked immunosorbent assay (ELISA) according to the kit protocol (Biolegend, San Diego, CA, USA). The minimal detection levels of cytokines were 4 pg/ml for IFN- γ , 2.4 pg/ml for IL-4, and 2 pg/ml for IL-10. In addition, the ratio of IFN- γ /IL-4 was calculated as the Th1/Th2 index.

Statistical Analysis

Data analysis was conducted using IBM SPSS 21 Statistics (IBM, USA). The values were shown as mean $\pm$ SD. The difference between the groups was examined through the one-way analysis of variance (ANOVA) test along with the

Tukey HSD *post hoc*; $p < 0.05$ was considered as the significance level.

RESULTS

The Effect of GA on the Proliferation of the Peripheral Blood Naive CD4+ T Lymphocytes

To evaluate the extent cells proliferation, after cells cultivation, as two experimental groups, using the TetraZTM Cell Counting Kit, the effect of different GA concentrations (50, 100, and 200 μ M) was examined in the presence and the absence of the agonist anti-OX40 mAb (the OD was read at 450 nm). Cells cultured with anti-OX40 antibody resulted in the increased proliferation of T cells compared to the group of cells, which were stimulated with only anti-CD3 and anti-CD28 mAbs (the mean $\pm$ standard deviation (SD) of OD values were 1.35 ± 0.21 vs. 1.09 ± 0.23 respectively) (Fig. 1b). Our results indicated that GA, in a dose-dependent way, could inhibit the augmented proliferation of the CD4+ T cells induced with anti-OX40 mAb (Fig. 1). Therefore, the rest of the assays were performed in experimental group 2. Dexamethasone, which was employed as the positive control,

Table 2. Sequences of Primers Used in qRT-PCR Analysis

Gene	Forward primer	Reverse primer
<i>GAPDH</i>	5'-GAAATCCCATCACCATCTTCCA-3'	5'-CAAATGAGCCCCAGCCTTC-3'
<i>OX40</i>	5'-CGTGGTGTAACCTCAGAAAGT-3'	5'-CAGTCAACTCCAGGCTTGTA-3'
<i>T-bet</i>	5'-ACCAAGAATGCCGAGATTACT-3'	5'-GGTAGGAGAGGAGAGTAGTGA-3'
<i>GATA-3</i>	5'-GTC CTC CCT GAG CCA CAT CT-3'	5'-GTG GTC CAA AGG ACA GGC TG-3'
<i>FOXP3</i>	5'-GCTGGTGCTGGAGAAGGAG-3'	5'-TCGGATGATGCCACAGATGAA-3'

qRT-PCR quantitative real-time polymerase chain reaction, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *OX40* a member of the tumor necrosis factor receptor superfamily (TNFRSF), *T-bet* a T-box transcription factor, *GATA-3* GATA-binding protein 3, *FoxP3* Forkhead box P3

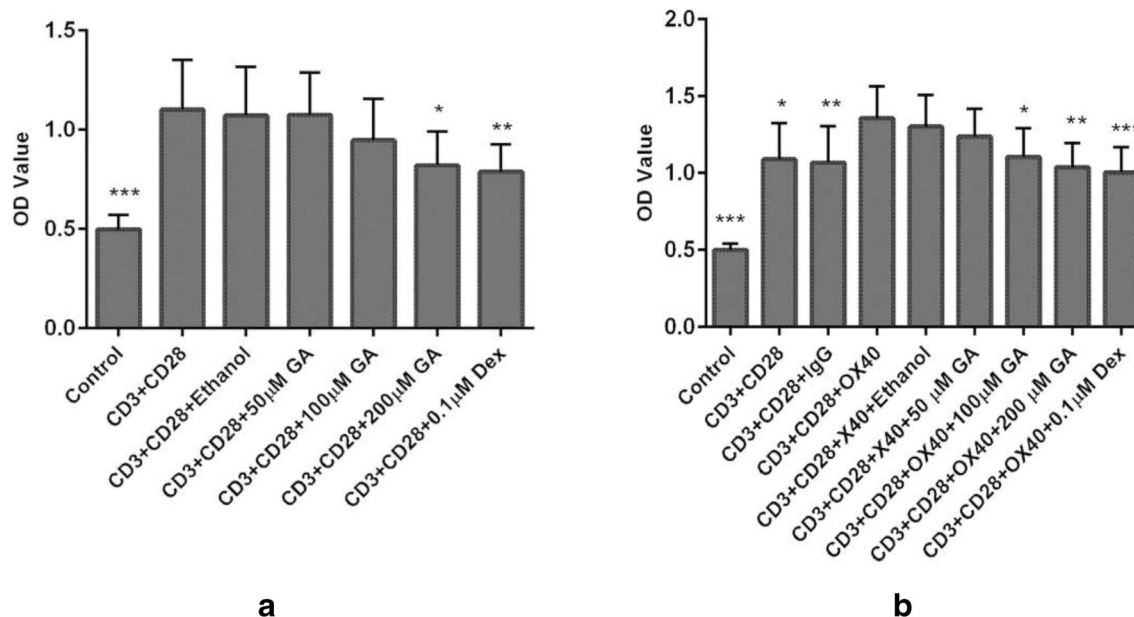


Fig. 1. The effect of glycyrrhizic acid (GA) on proliferation of CD4+ T cells. The proliferation of CD4+ T cells was evaluated after 72 h of culture with anti-CD3, anti-CD28 mAbs, and different concentrations of GA in the absence (a) or presence (b) of anti-OX40 mAb. Control, unstimulated CD4+ T cells; CD3+CD28, CD4+ T cells cultured with anti-CD3 and anti-CD28 mAbs; CD3+CD28+IgG, CD4+ T cells cultured with anti-CD3, anti-CD28 mAbs and isotype control IgG; CD3+CD28+OX40, CD4+ T cells cultured with anti-CD3, anti-CD28, and anti-OX40 mAbs; CD3+CD28+OX40 + ethanol, CD4+ T cells cultured with anti-CD3, anti-CD28, and anti-OX40 mAbs and ethanol; CD3+CD28+OX40+GA, CD4+ T cells treated with anti-CD3, anti-CD28 and anti-OX40 mAbs and GA (50, 100, or 200 μM); CD3+CD28+OX40+Dex 0.1, CD4+ T cells treated with anti-CD3, anti-CD28, and anti-OX40 mAbs and 0.1 μM dexamethasone. Data indicated as mean ± standard deviation (SD) ($n = 12$ per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ showed significant differences with CD3+CD28 (a) and CD3+CD28+OX40 (b).

also led to a significant reduction in T cells proliferation ($p < 0.001$).

The Effect of GA on Levels Expression of OX40 Gene and Protein

To measure the extent of the expression of OX40 protein across CD4+ T lymphocytes, the cells were stained by PE-anti-human OX40 and PE Mouse IgG1, k Isotype control, and then investigated through the flow cytometry method. The extent of OX40 gene expression was examined in the cells that treated under the aforementioned conditions by qRT-PCR.

GA caused the diminished expression of OX40 both at the protein and the gene levels in T cells. This reduction was significant at high concentrations of GA (100 and 200 μM) ($p < 0.05$; $p < 0.001$). Note that dexamethasone also resulted in a significant reduction of the OX40 expression in both cases ($p < 0.001$) (Fig. 2a–c).

The Effect of GA on the Extent of *FoxP3*, *GATA-3*, and *T-bet* Genes Expression

To investigate the effect of GA on Treg, Th2, and Th1, the extent of transcription factors of FoxP3, GATA-3, and T-bet associated with these cells, was examined in the experimental groups by using qRT-PCR. Data represented compared to the cells were treated with the anti-OX40 mAb.

With the enhanced signaling of OX40 in the cells, which treated with anti-CD3, anti-CD28, and anti-OX40 mAbs, the extent of *T-bet* gene expression of diminished compared to the group of cells, which were cultured only with anti-CD3 and anti-CD28 mAbs ($p < 0.01$).

The extent of the *T-bet* gene expression diminished across the different GA concentrations, which was not significant, even at the concentration of 200 μM of GA ($p = 0.178$). Furthermore, the extent of this gene the expression declined in the cells treated with dexamethasone, where the level of this reduction was higher than the level of the cells treated with GA ($p = 0.161$) (Fig. 3a).

It should be noted that the extent of *GATA-3* gene expression grew by elevation of OX40 signaling in the T

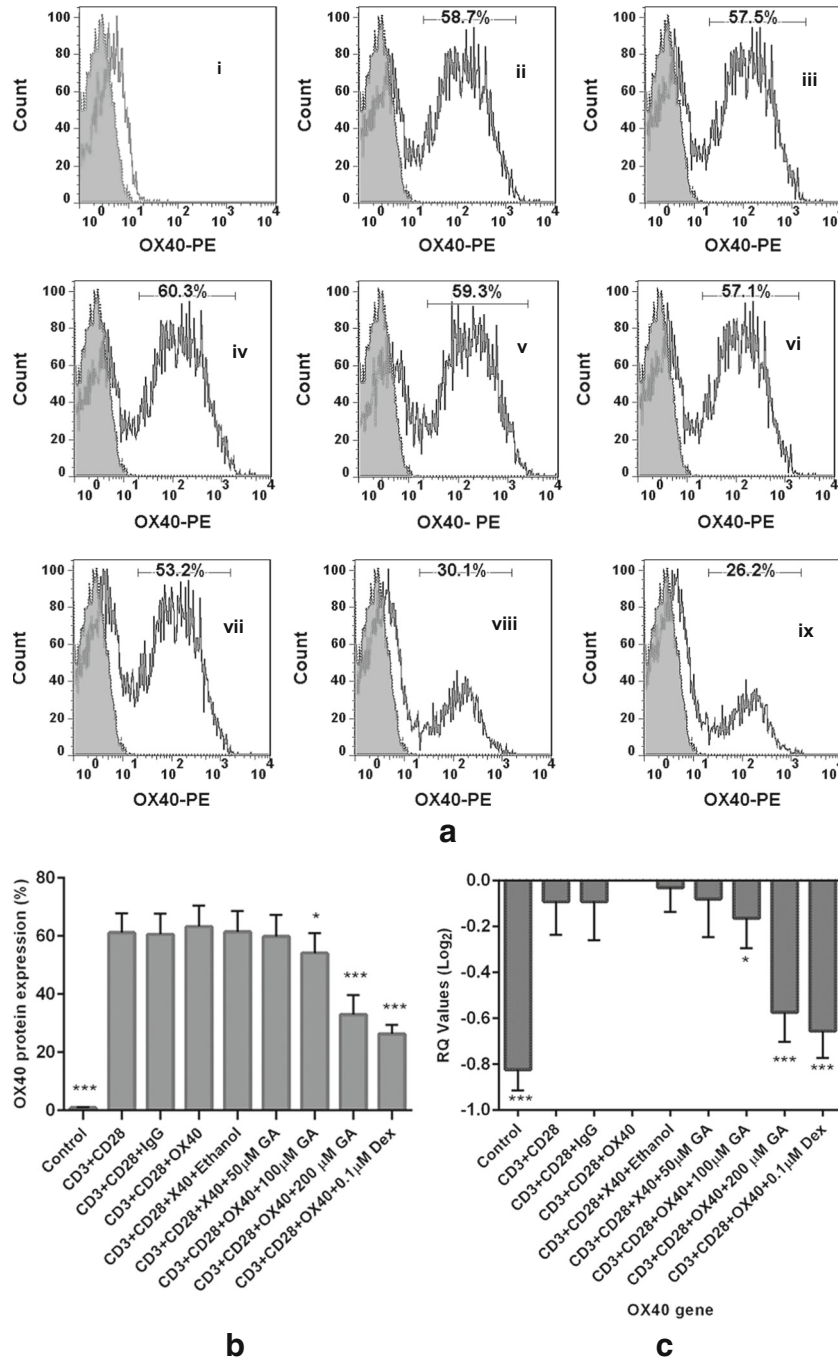


Fig. 2. The effect of glycyrrhizic acid (GA) on the expression level of the OX40 protein and gene. **a** Representative flow cytometric analysis of CD4⁺ T cells labeled with PE-anti-human OX40 (solid line histogram) and PE mouse IgG1, k isotype control (filled histogram). Control (i), CD3+CD28 (ii), CD3+CD28+IgG (iii), CD3+CD28+OX40 (iv), CD3+CD28+OX40+ ethanol (v), CD3+CD28+OX40+ GA (50, 100, or 200 μ M) (vi–viii), and CD3+CD28+OX40+ Dex 0.1 μ M (ix). **b** OX40 protein expression (%) and **c** OX40 gene expression. Mean log 2 relative expression (log 2 RQ) values for quantitative real-time PCR (qRT-PCR) represented. Data presented as mean $\pm$ standard deviation (SD) (n = 12 per group). * p < 0.05, ** p < 0.01, *** p < 0.001 showed significant differences with CD3+CD28+ OX40.

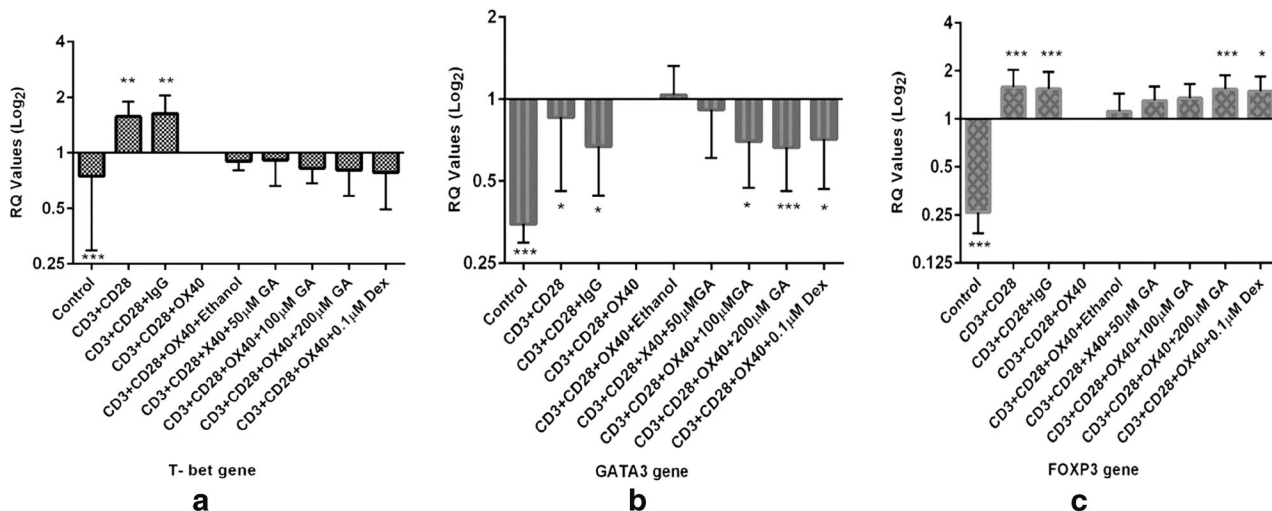


Fig. 3. The effect of glycyrrhizic acid (GA) on the expression level of **a** T-bet, **b** GATA-3, and **c** FoxP3 genes. Mean log 2 relative expression (log 2 RQ) values for quantitative real-time PCR (qRT-PCR) represented. Data represented as mean $\pm$ standard deviation (SD) ($n = 12$ per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ showed significant differences with CD3+CD28+OX40.

lymphocytes compared to the cells, which were not treated with anti-OX40 mAb ($p < 0.05$). The extent of *GATA-3* gene expression in the cells treated with different GA concentrations declined at the concentrations of 100 and 200 μ M of GA ($p < 0.05$, $p < 0.001$). Note that the extent of this gene expression in the lymphocytes, which adjoined dexamethasone, decreased significantly. However, this reduction was lower than 200 μ M of GA and was comparable to 100 μ M of GA ($p < 0.05$) (Fig. 3b).

The extent of *FoxP3* gene expression also decreased along with the enhanced OX40 signaling in anti-OX40 mAb stimulated CD4⁺ T cells. However, the extent of the expression of this gene increased in the cells treated with GA, and at the concentration of 200 μ M of GA, this growth was significant ($p < 0.001$). Note that the extent of *FoxP3* gene expression in the cells treated with dexamethasone also increased significantly ($p < 0.05$) (Fig. 3c).

The Effect of GA on the Cytokines Levels

To investigate the effect of GA on the function of T lymphocytes and the levels of produced cytokines, the levels of IL-4, IL-10, and IFN- γ were measured by ELISA method. The IFN- γ level diminished along with the reduced expression of the *T-bet* gene in the cells treated with the anti-OX40 mAb compared to the cells, which were cultured only with anti-CD3 and anti-CD28 mAbs (746.68 ± 80.1 vs. 857.73 ± 67.23 pg/ml respectively, $p = 0.017$).

The IFN- γ level dropped in the cells treated with different concentrations of GA, though it was not significant, even

at the concentration of 200 μ M of GA (694.96 ± 86.82 vs. 746.68 ± 80.1 pg/ml, $p = 0.775$). Dexamethasone also lowered the IFN- γ level, with this reduction, as with the extent of the *T-bet* gene was higher than the extent of the cells treated with GA (690.97 ± 88.48 vs. 746.68 ± 80.1 pg/ml, $p = 0.697$) (Fig. 4a).

As expected, the IL-4 level grew in the cells cultured with the anti-OX40 mAb compared to the cells lacking this antibody (9.27 ± 3.24 vs. 5.1 ± 2.24 pg/ml, $p < 0.01$). The IL-4 level decreased in the cells were treated with GA. This reduction was significant at the concentrations of 100 and 200 μ M of GA and was higher at the latter concentration (6.05 ± 2.43 vs. 9.27 ± 3.24 and 2.83 ± 1.35 vs. 9.27 ± 3.24 pg/ml, respectively) ($p = 0.031$ – $p < 0.001$). Dexamethasone also significantly diminished the IL-4 level, while this reduction was comparable to the concentration of 100 μ M of GA and lower than the concentration of 200 μ M of GA (5.95 ± 1.78 vs. 9.27 ± 3.24 pg/ml, $p = 0.022$) (Fig. 4b).

The cells were stimulated both with anti-CD3 and anti-CD28 along within anti-OX40 mAbs, which had lower IL-10 compared to the cells treated in the absence of OX40 mAb (167.2 ± 50.32 vs. 249.27 ± 62.84 pg/ml, $p < 0.01$). The level of this cytokine increased in the cells that were affected by the different concentrations of GA (though this growth was not significant ($p > 0.05$)). Dexamethasone also enhanced IL-10 production, which was not significant (217.93 ± 57.9 vs. 167.2 ± 50.32 pg/ml, $p > 0.05$) (Fig. 4c).

The IFN- γ /IL-4 ratio increased in cells treated at different concentrations of GA, but this growth was significant only

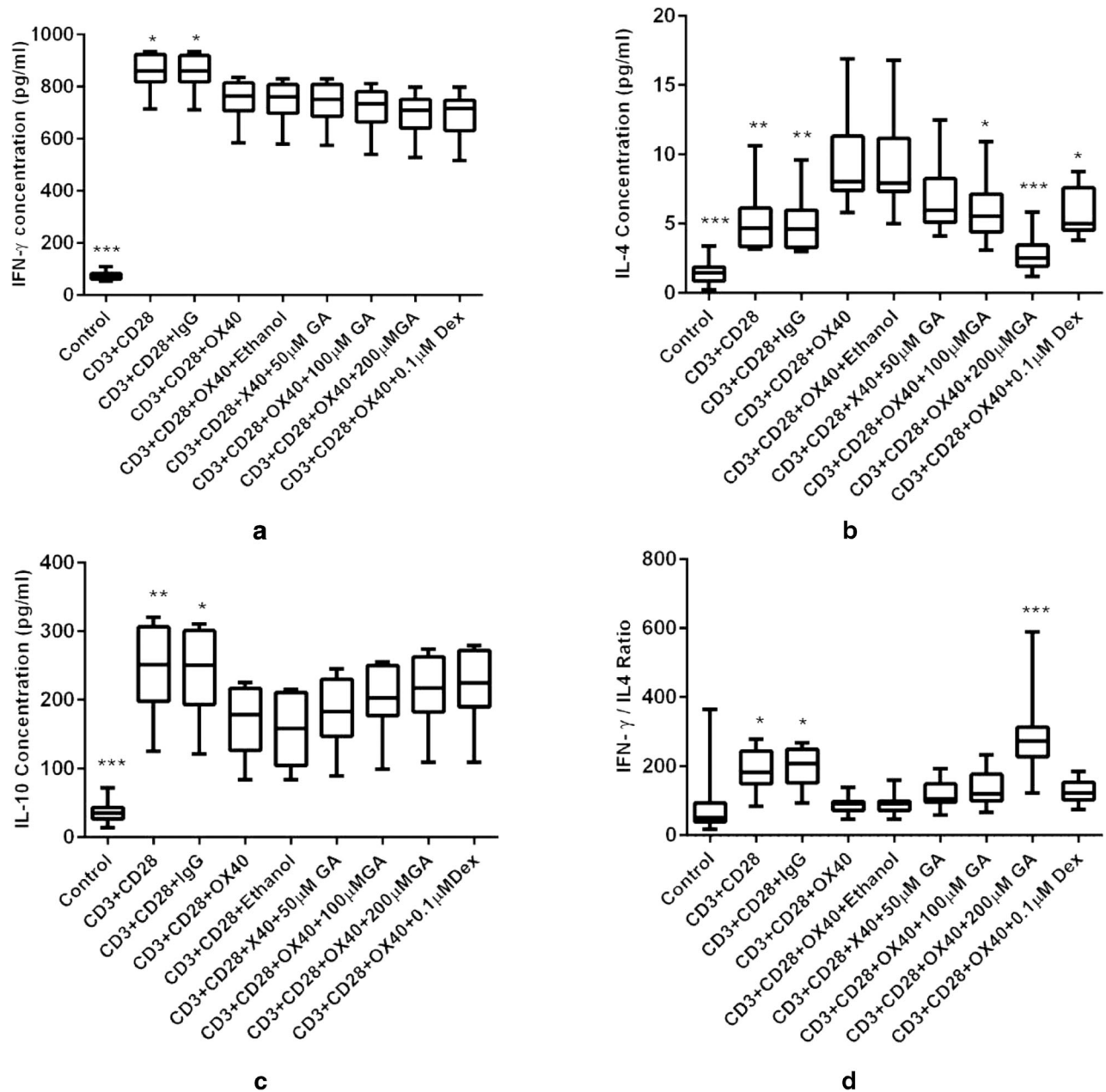


Fig. 4. The effect of GA on the levels of **a** IFN- γ , **b** IL-4, and **c** IL-10 cytokines. **d** IFN- γ /IL-4 ratio is also represented as the Th1/Th2 index. Data presented as mean $\pm$ standard deviation (SD) ($n = 12$ per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ showed significant differences with CD3+CD28+ OX40. GA = Glycyrrhizic acid; IFN- γ =interferon- gamma; IL-4 = interleukin 4; IL-10 = interleukin 10.

at the concentration of 200 μ M of GA compared to the cells cultured with anti-CD3, anti-CD28, and anti-OX40 mAbs (312.39 ± 159.2 vs. 87.25 ± 24.14 , respectively, $p < 0.001$). The dexamethasone also elevated this ratio, but the lift was not significant (181.06 ± 85.28 vs. 87.25 ± 24.14 , $p = 0.062$) (Fig. 4d).

DISCUSSION

This study aimed to investigate the anti-allergic effect of GA on proliferation and production of cytokines of the peripheral blood naive CD4⁺ T cells through its possible effect on the OX40 receptor in patients with allergic rhinitis in

in vitro conditions. Allergic rhinitis is a common disease with growing incidence and significantly affects the life of the patients. The chronic nature of the disease, long-term therapeutic measures are required [33]. Accordingly, we selected a natural GA compound, which is an anti-inflammatory compound in licorice, and is well-known in traditional Persian medicine (TPM) [34]. Licorice has also been used in traditional Chinese medicine (TCM) as a safe compound that has both pharmacological and anti-allergic properties [35–37].

This study showed that the cells cultured with an anti-OX40 mAb resulted in the increased proliferation of T cells. GA, especially at the concentrations of 100 and 200 μ M, could inhibit the augmented proliferation of CD4+ T cells induced with anti-OX40 mAb. As the increased signaling of OX40 results in the greater proliferation and survival of T cells, the effect of GA through the reducing expression of this molecule on T cell proliferation seems to be logical [10]. The results of this study were in line with another study in the mouse model of asthma [32]. Furthermore, we also employed anti-CD28 mAb to stimulate the cells as studies have shown that CD28 brings about the preservation of OX40 across the T cells, and enhances survival and proliferation that results from OX40 signaling [10]. In the present study, dexamethasone, which is used as a positive control in the treatment of allergic diseases, also led to the diminished proliferation of T cells.

Investigating the effect of GA on the extent of OX40 receptor expression showed that GA resulted in the diminished expression of this molecule both at the protein and the gene levels, with the maximum effect of being observed at the concentration of 200 μ M of GA. These results were consistent with the mentioned study, which was performed in the animal model. Receiving GA by the mouse model of asthma resulted in a diminished expression of this molecule and its ligand [32]. The results of this study also showed that dexamethasone led to the diminished expression of this receptor. Furthermore, there is no report which focuses on the effect of dexamethasone on this co-stimulatory molecule.

It seems that a part of the anti-allergic properties of GA is related to improving the Th1/Th2 balance through the modulation of the GATA-3 and the T-bet transcription factors as well as their associated cytokines, *i.e.*, the ratio of IFN- γ /IL-4; another part is also related to increased levels of IL-10 and *FoxP3* gene expression.

T cells, especially Th1, Th2, and Treg cells, are the key cells in the allergic responses. Th1 and Th2 subgroups cross-regulate each other. Therefore, the balance between the IFN- γ /IL-4 cytokines specify whether the immune response is suitable or is acting pathologically. In the allergic diseases, the balance shifts toward Th2, thereby therapeutic measures

are useful in resolving the imbalance [38, 39]. Treg cells also produce IL-10 and are eligible for the FoxP3 transcription factor, which interactively inhibit the *T-bet* and *GATA-3* genes expressions. When *FoxP3* gene expression increases, it modulates the immune response [40]. As observed in the previous studies, it has been found that the OX40 molecule is involved in the differentiation of the Th1 and Th2 cells as well as the production of cytokines by them; it is expected that GA will be effective on the Th1/Th2 balance by affecting the extent of the expression of this molecule [13].

This study examined the extent of transcription factors expression of T-bet, GATA-3, and FoxP3 as well as the levels of IFN- γ , IL-4, and IL-10 cytokines and the results showed that although GA, especially at high concentration (200 μ M), decreased the extent expression of the *T-bet* and *GATA-3*, this reduction was significant only for the latter, causing the Th1/Th2 ratio to shift toward the elevation of Th1 and the reduction of Th2, in which this ratio, regarding GA, was higher compared to dexamethasone. It seems that the reduced expression of the OX40 receptor and its signaling leads to the altered function of T cells. GA causes the reduction of *GATA-3* and its inhibitory effect on *T-bet* by affecting the extent of the expression of this co-stimulatory molecule, especially in Th2 in the allergic rhinitis [39].

The results of this study showed that GA, especially at the concentration of 200 μ M, decreased the production of IFN- γ and IL-4. This finding is consistent with their relevant transcription factors, causing improvement of Th1/Th2 imbalance.

Several reports have shown the levels of IL-13, IL-5, and IL-4 cytokines decreased in BALF, while the IFN- γ level increased by GA. This can be associated with certain biological roles, such as inhibiting the stimulation of proliferation of B and T cells as well as the differentiation of CD4+ T cells to Th2 cells [32, 41, 42].

One study showed that in the mouse model of asthma, GA had a therapeutic effect; a part of this effect was related to modulation of Th1/Th2 balance through the inhibition of OX40-OX40L signaling as well as the P38 MAPK activity [32]. In another study, 18 β -glycyrrhetic acid caused a significant reduction in the *GATA-3* expression in the lung tissue of the affected mice, while the extent of the *T-bet* expression decreased only to a small extent [43].

Similarly, other studies showed the GA modulatory effects on the T cells, but in most of these studies, IFN- γ increased. This finding is inconsistent with the finding of this study. This discrepancy could be due the general difference in the human and animal model or due to the varied behavior of cells between *in vivo* and *in vitro* as well as the role of cytokines

and other cells present in *in vivo* on the differentiation and function of the lymphocytes. The use of anti-asthma herbal medicine intervention (ASHMI) (in which licorice and NOT GA as a pure form is one of its main constituents) showed that the consumption of the herb in patients with asthma had useful effects on regulation of Th1/Th2 balance, causing a reduction of Th2 cytokines and IgE, while enhancing the serum level of IFN- γ [44, 45]. However, this compound caused the inhibition of IL-5 production, it had no effect on IFN- γ levels in *in vitro* [46]. This suggests different findings under different *in vivo* and *in vitro* conditions. Furthermore, these studies also suggest the necessity of investigating the effect of different pure compounds under different conditions.

In the present study, the level of expression of *FoxP3* increased in the groups treated by GA (200 μ M) in comparison to the other groups. Dexamethasone also resulted in an elevated extent of the *FoxP3* expression. Although there is controversy concerning the regulatory effect of OX40 on Treg cells, most studies show that with increased OX40 signaling, the functions of the Treg cells decline; others consider it to be context-dependent [10]. Therefore, neutralizing this effect could reverse the functions of the mentioned cells. In this study, by adding the OX40 agonist mAb in addition to anti-CD3 and anti-CD28 mAbs to the cells, the extent of *FoxP3* gene expression decreased ($p < 0.001$). However, by adding GA to the cells and reducing the extent of the expression of the OX40 receptor, the extent of *FoxP3* expression increased. This growth in the expression may result in the diminished expression of *GATA-3* and *T-bet* genes, and their associated cytokines. As the extent of the expression of *FoxP3* gene generally drops in people with allergic rhinitis, this enhanced expression by GA can be promising for strengthening the activity of the Treg cells [8].

The results of this study are consistent with the relevant study showing that GA caused enhanced *FoxP3* expression [47]. Furthermore, in another study using 18 β -glycyrrhetic acid, this growth was also observed [43].

The results of this study also showed that IL-10 level decreased in the culture medium of eligible cells of the anti-OX40 agonist mAb compared to the group lacking this antibody ($p < 0.001$). In addition to the inhibitory activity of this antibody on Treg cells, it suggests that OX40 agonist antibody may also prevent the induction of iTreg cells and IL-10 secretion [14]. We showed that the level of IL-10 under the effect of GA increased, but this growth was not significant. It could be due to diminished number of Treg, especially iTreg cells in the allergic subjects [48]. Furthermore, it should be noted that IL-10 is also produced by other CD4+ T cells, especially Th2 and it seems that IL-4 and *GATA-3* are required for IL-10 production by Th2 cells [49], where, with reduced OX40 expression

and the diminished expression levels of the aforementioned molecules, the IL-10 level also falls.

It should be noted that this study had some limitations: The extent of T-bet, *GATA-3*, and *FoxP3* transcription factors expression was not investigated by flow cytometry method. In addition, the effect of GA on upstream and downstream molecules involved in OX40 signaling pathway was not studied. Also, the measurement of some other important immunological parameters in allergic rhinitis such as Th17 and Th9 cells was not a part of our protocol that should be investigated in future studies. However, the strong point of our study was to investigate the anti-allergic effects of GA through OX40 receptor in human allergic rhinitis.

In conclusion, it can be stated that GA can apply parts of its anti-allergic effects by affecting the OX40 receptor and improving the Th1/Th2 imbalance, and enhancing the regulatory activity of Treg cells. It can be propounded as a medication or therapeutic supplement for treating people with allergic rhinitis.

Funding This work was supported by grant 394902 from Isfahan University of Medical Sciences.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no competing interests.

Ethical Approval. All procedures performed in studies involving human participants were in accordance with the Ethics Committee of Isfahan University of Medical Sciences (Code of Ethics: IR.MUI.REC.1394.3.902) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent. Informed consent was obtained from all individual participants included in the study.

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