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ARTICLE

Comparative study on the impacts of visnagin and its methoxy derivative khellin on human lymphocyte proliferation and Th_1/Th_2 balance

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Abstract

Background Visnagin is a phenolic and natural compound in turmeric and fenugreek, and its anti-inflammatory effect has been indicated. Therefore, this study aimed to investigate and compare the anti-inflammatory properties of visnagin and its methoxy derivative khellin on human lymphocytes.

Methods Human lymphocytes were treated with khellin, visnagin (10, 30, and 100 μM), and dexamethasone (0.1 mM) in the presence of phytohemagglutinin (PHA). The levels of cell proliferation, nitric oxide (NO), glutathione (GSH), malondialdehyde (MDA), and MDA/GSH ratio were measured using biochemistry methods. Furthermore, the mRNA levels of interferon-γ (IFN-γ), interleukin (IL)-4, and IL-10 were assessed using real-time PCR, while IFN-γ/IL-4(Th_1/Th_2), IFN-γ/IL-10(Th_1/T_{reg}), and IL-4/IL-10(Th_2/T_{reg}) ratios were made by dividing their exact values.

Results In the PHA-stimulated group, GSH and IFN-γ/IL-4 levels were markedly diminished, but other variables were significantly elevated compared to the control group. Khellin and visnagin significantly declined the levels of cell proliferation, MDA, MDA/GSH ratio, and NO production. Khellin and visnagin concentration-dependently diminished IFN-γ and IL-4 levels and increased IL-10 levels compared to the PHA-stimulated group. Two higher concentrations of khellin and visnagin (30 and 100 μM) considerably diminished the IFN-γ, IFN-γ/IL-10, and IL-4/IL-10 values compared to the PHA-stimulated group. However, 100 μM of khellin and visnagin significantly increased GSH level compared to the PHA-stimulated group.

Conclusions In PHA-stimulated lymphocytes, representing Th_2 dominant allergic diseases, khellin and visnagin provides more specific anti-oxidant, anti-inflammatory, and immunomodulatory functions than dexamethasone. In addition, the effects of khellin were more prominent than visnagin.

Keywords Khellin · Visnagin · Immunomodulatory · Inflammation · Oxidative stress · T helper cells

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Abbreviations

AP-1	Activator protein 1
CMI	Cell-mediated immunity
CD	Cluster of differentiation
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
GSH	Glutathione
iNOS	Inducible nitric oxide synthase
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MDA	Malondialdehyde
NO	Nitric oxide
NF-κB	Nuclear factor-kappa light chain enhancer of activated B cells
TNF	Tumor necrosis factor

Introduction

T lymphocytes are one of the adaptive immune system's primary arms and act as the central mediator of cellular immunity (CMI) [1–3]. T helper (Th) cells are a subset of the cluster of differentiation (CD)4^+ T lymphocytes that play a vital role in prompting distinct adaptive immunity. This subpopulation consists of influential subgroups, such as Th1, Th2, and Th17, and exerts various biological defensive and inflammatory functions [4–14]. In this regard, Th1 lymphocytes play a crucial role in regulating cellular immunity against tumors and intracellular pathogens by releasing interferon (IFN)-γ and interleukin (IL)-2 [15, 16]. On the contrary, Th2 cells stimulate the humoral immune response to combat extracellular infections, such as fungi and parasites, and comprise allergic reactions, including asthma and atopic dermatitis, mainly by producing IL-4, IL-5, and IL-13 [17, 18]. Numerous studies have described the essential function of Th2 cells in initiating, maintaining, and promoting allergic inflammatory responses and cancer [1, 17–20]. It has been revealed that changes in the ratio pattern of IFN-γ/IL-4 cytokines (Th1/Th2 balance) to IL-4 (main cytokine secreting from Th2 cells) lead to allergic as well as atopic diseases including asthma, anaphylactic shock, atopic dermatitis rhinitis, and cancer. Th1 susceptibility may also cause autoimmune diseases, the most important of which are rheumatoid arthritis (RA) and multiple sclerosis (MS) [5–14, 21]. Experimentally, there is ample evidence indicating that Th1 and Th2 lymphocytes antagonize each other's responses reciprocally [1, 6–14, 17, 18].

Pyrones are the major constituents of *Ammi visnaga,* and their immunostimulatory effects have been demonstrated previously [22]. Two of the main pyrones of *A. visnaga* are khellin and visnagin [23–27]. Therapeutic applications of khellin and visnagin were indicated for photochemotherapy of vitiligo and psoriasis using khellin [28], the photodynamic properties of visnagin and khellin in their optical reaction with DNA [29], and the use of khellin as an antispasmodic agent and for the treatment of kidney stones [30]. Furthermore, both khellin and visnagin showed vasodilator activities owing to their calcium channel blocking actions and also anti-inflammatory effects by inhibiting activator protein 1 (AP-1) and nuclear factor-kappa light chain enhancer of activated B cells (NF-κB) signaling [31, 32]. Contextually, it has been demonstrated that visnagin isolated from *A. visnaga* induces anti-inflammatory reactions and reduces mRNA expression of tumor necrosis factor-α (TNF-α), IFN-γ, and IL-1β [33]. Furthermore, it has been reported that visnagin reduced the expression of lipopolysaccharide (LPS)-induced inflammatory genes, including TNF-α, IL-1β, IFN-γ, and inducible nitric oxide synthase (iNOS), as well as IL-6 by inhibiting the AP-1 and NF-κB pathways, and led to release of anti-inflammatory cytokine IL-10 [33]. In addition, khellin restrained the mutagenicity of benzo [a] pyrene, 2-aminoflurane, and 2-aminoanthracene promoters in *Salmonella typhimurium* T98 [34].

Due to the toxicity and adverse effects of chemical compounds, herbal remedies can be a valuable choice in permanently altering the response of T helper cells with at least toxicity. Therefore, this comparative study aimed to investigate the immunomodulatory properties of khellin and visnagin on phytohemagglutinin (PHA)-stimulated human lymphocytes.

Materials and methods

Chemicals and reagents

Roswell park memorial institute (RPMI)-1640 growth medium, PHA, fetal bovine serum (FBS), penicillin–streptomycin (pen/strep), dexamethasone, Ficoll, dimethylsulfoxide (DMSO), khellin and visnagin, and other materials were cellular and molecular grade (purity of > 97%) and obtained from Sigma Aldrich Chemical Company (St. Louis, MO, USA). Red blood cell (RBC) lysis buffer was provided by Biolegend company (San Diego, CA, USA). The cell proliferation assessment kit (WST-1) was from Roche Diagnostic (Mannheim, Germany).

Subjects and enrolling criteria

In the current study, inclusion criteria were healthy men with body mass index (BMI) lower than 25 and lack of history of inflammatory, autoimmune and infectious ailments, respiratory disorders, history of smoking, chest X-ray adducts, hypertension, and diabetes, as well as people being treated with glucocorticoids (GC), other immunosuppressants, anti-histamines, and herbal medicines. Healthy male volunteers' characteristics were 23.0 ± 5.4 years (age ± SD), 178.5 ± 13.4 cm (height ± SD), and 75.6 ± 11.4 kg (weight ± SD). They fasted for about 10–12 h before blood sampling. This study was permitted by the ethics committee of Mashhad University of Medical Sciences (IR.MUMS.fm.REC.1395.593), and all individuals agreed with informed written consent.

Isolation of human lymphocytes and grouping

First, 10 mL of blood was collected from each volunteer in an anti-coagulant tube (heparinized). Then, collected blood cells were layered employing Ficoll (Sigma, St. Louis, MO, USA) and centrifuged at 400 g at controlled

room temperature (20–25 °C) for 20 min. Eventually, lymphocytes were collected according to previous studies [1, 7, 9, 10, 35–37]. Experimental groups were justified as follows. Group 1: lymphocytes + culture medium + vehicle; Group 2: lymphocytes + culture medium + PHA (5 µg/ml at final concentration, [1, 38]) + vehicle; Groups 3, 4, and 5: lymphocytes + culture medium + PHA + khellin (10, 30 and 100 µM); Groups 6, 7, and 8: lymphocytes + culture medium + PHA + visnagin (10, 30 and 100 µM); and Group 9: lymphocytes + culture medium + PHA + dexamethasone (0.1 mM). The levels of DMSO, as a vehicle, were less than 0.1%. Moreover, the vehicle group contained the concentration specified for DMSO.

Cell proliferation assay

Cell proliferation was assessed based on the manufacturer's guidelines utilizing a WST-1 colorimetric test kit. Briefly, lymphocytes were inserted into RPMI-1640 enriched and cultured in 10^5 cells/100 µl/well in 96-well plates. Each well was treated based on the experimental group in a final volume of 200 µl/well, and incubated for 48 h. By ending the incubation time, the lymphocytes were co-incubated with WST-1 reagent for 4 h at 10% of the final volume of each well. Ultimately, the optical density was measured at 450 nm against 630 nm [1, 7, 9, 10, 35–37, 39].

Biochemical assessments

Lymphocytes were cultured in 5×10^6 cells/well in 6-well plates and treated based on the experimental group in a final volume of 1500 µl/well for 48 h. Later, the supernatant and cells were collected separately for biochemical and real-time PCR assays, respectively. The nitrite concentration was determined utilizing a Griess reagent (sulfanilamide and *N*-(1-Naphthyl)-ethylenediamine in 2N hydrochloric acid) by a spectrophotometer [1, 7, 9, 10, 35–37]. In addition, oxidative stress was evaluated based on the levels of malondialdehyde (MDA, an index of lipid peroxidation) and glutathione (GSH, an index of anti-oxidant) using commercial biochemistry kits (ZellBio Company, Germany) based on the manufacturer's guidelines [1, 7, 9, 10, 35–37], in the supernatant.

RNA extraction, synthesis of cDNA, and gene expression study

Based on the manufacturer's guidelines, total RNA was isolated from spleen cells using a tripure isolation reagent (Roche Applied Science, Germany) according to the previous reports [1, 7, 9, 10, 35–37]. The mRNA extracted by the reverse transcriptase enzyme was converted to cDNA. Afterward, this construct was used as a template in PCR. The RNA volume for all reactions was a constant volume of 1 µl. The reverse transcription was performed through Revert Aid™ H Minus M-MulV First-Strand (Fermentase, Germany) based on the manufacturer's guidelines. Real-time PCR primers and quantitative real-time PCR (qPCR) were consistent with our previous studies [1, 7, 9, 10, 35–37].

Data analysis

All collected data were analyzed using Graph Pad Prism® 8 (Graph Pad Software, San Diego, CA, USA) software. First, all data were evaluated for normal distribution using Kolmogorov–Smirnov's test. All data followed normal distributions. The results are presented as mean ± standard deviation (SD). Furthermore, Levene's test was used to assess the equality of variances for the groups. If the data had equal variance, they were analyzed using a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post *hoc* test [5, 40, 41]. On the other hand, the data with no equal variance were analyzed using the Brown–Forsythe ANOVA test, followed by Games–Howell's multiple comparisons tests [5, 40, 41]. Significance levels were considered when p-values < 0.05. For one-way ANOVA, F values are reported as $F_{\text{df of treatment, residual}}$, and For Brown–Forsythe ANOVA, F^*values are reported as $F^*_{\text{df of treatment, residual}}$. In addition, raw data and the following analysis were reported as supplements 1 and 2, respectively.

Results

Effects of visnagin and khellin on cell proliferation

In non-stimulated lymphocytes, no significant changes were observed in cell proliferation for all control and treatment groups ($F_{8, 99} = 0.2977$, $p = 0.9652$, one-way ANOVA, Fig. 1a). However, in the case of PHA stimulation of lymphocytes (Fig. 1b), all three concentrations of khellin and visnagin significantly and concentration-dependently diminished cell proliferation ($F^*_{9, 88.11} = 109.6$, $p < 0.0001$, Brown–Forsythe ANOVA, Fig. 1b) ($p < 0.001$ for all concentrations of khellin, and two higher concentrations of visnagin, Games–Howell's multiple comparisons tests).

Effects of visnagin and khellin on NO levels

In the case of PHA stimulation of lymphocytes, NO production levels were significantly increased compared to the non-stimulation state ($F_{9, 110} = 43.20$, $p < 0.0001$, one-way ANOVA, $p < 0.001$, Tukey–Kramer post *hoc* test, Fig. 2). On the other hand, khellin (10, 30, and 100 µM) and visnagin (30 and 100 µM) dramatically reduced NO production in a concentration-dependent manner compared to the

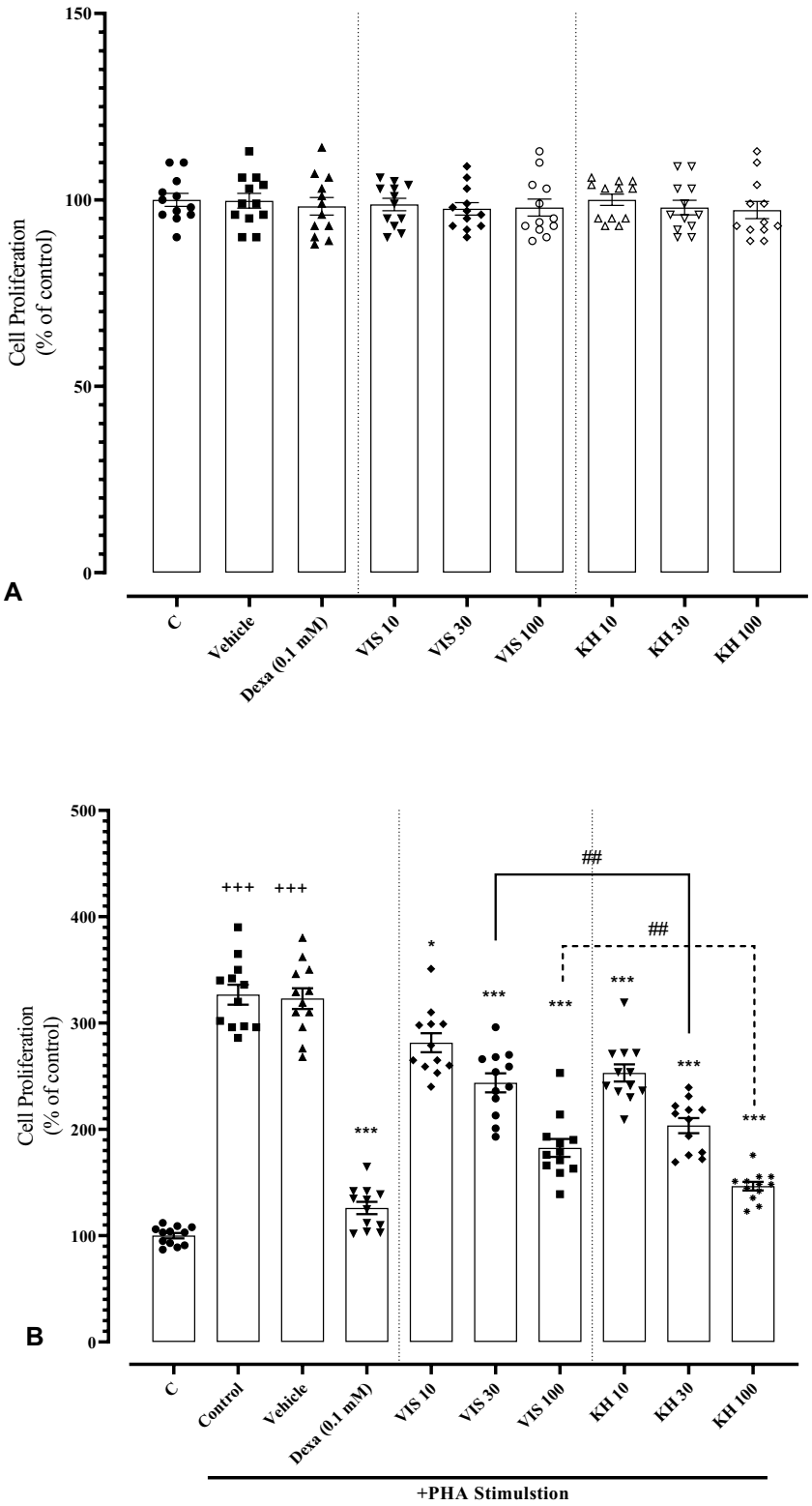


Fig. 1 The effects of khellin and visnagin on cell proliferation in the absence (**A**) and presence (**B**) of phytohemagglutinin stimulation. Data were presented as mean ± SD values (for each group, $n = 12$). Data had equal variances in the absence of PHA stimulation (**A**). Therefore, statistical analyses employed one-way ANOVA followed by Tukey–Kramer's post *hoc* test. In contrast, in the presence of PHA (**B**), data had no equal variances and did not pass Levene's test. Thus, statistical analyses employed the Brown–Forsythe ANOVA test, followed by Games–Howell's multiple comparisons tests. $^{+}$ Compared to the non-stimulated control group; $^{+++}p < 0.001$. $^{\#}$ Compared respected concentrations of khellin with visnagin; $^{\#\#}p < 0.01$. *Compared to the PHA-stimulated control group; $^{***}p < 0.001$. Non-stimulated control group (C), phytohemagglutinin (PHA), PHA-stimulated control group (Control), dimethylsulfoxide (DMSO), dexamethasone-treated group (Dexa), visnagin-treated groups (VIS), khellin-treated groups (KH)

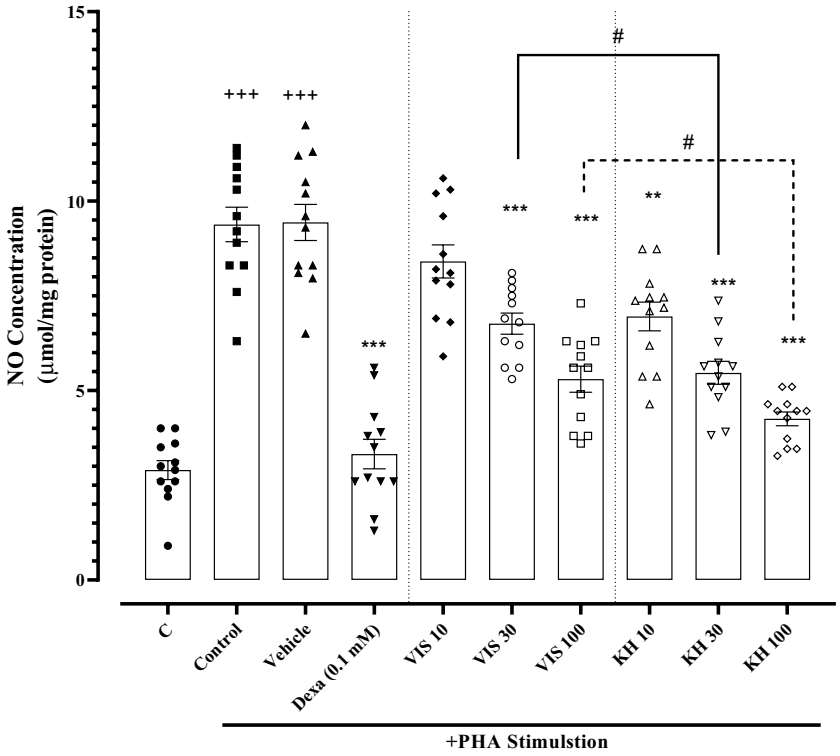


Fig. 2 The effects of khellin and visnagin on nitric oxide concentration in the presence of phytohemagglutinin stimulation. Data were presented as mean ± SD values (for each group, $n = 12$). Data had equal variances. Statistical analyses employed one-way ANOVA followed by Tukey–Kramer's post *hoc* test. $^{+}$Compared to the non-stimulated control group; $^{+++}p < 0.001$. $^{\#}$Compared respected concentrations of khellin with visnagin; $^{\#}p < 0.05$. *Compared to the PHA-stimulated control group; $^{**}p < 0.01$, $^{***}p < 0.001$. Nitric oxide (NO), non-stimulated control group (C), phytohemagglutinin (PHA), PHA-stimulated control group (Control), dimethylsulfoxide (DMSO), dexamethasone-treated group (Dexa), visnagin-treated groups (VIS), khellin-treated groups (KH)

PHA-stimulated control group ($F_{9, 110} = 43.20$, $p < 0.0001$, one-way ANOVA, $p < 0.01$ for 10 µM, $p < 0.001$ for other cases, Tukey–Kramer post *hoc* test, Fig. 2).

Effects of visnagin and khellin on levels of MDA and GSH and MDA/GSH ratio

In the presence of PHA, levels of MDA ($F_{9, 110} = 37.79$, $p < 0.0001$, one-way ANOVA, Fig. 3a) and MDA/GSH ratio ($F^*_{9, 29.21} = 9.415$, $p < 0.0001$, Brown–Forsythe ANOVA, Fig. 3c) were significantly elevated in comparison to the non-stimulation state ($p < 0.001$ for both parameters, Tukey–Kramer post *hoc* test, and Games–Howell's multiple comparisons tests, respectively, Fig. 3a and c). However, the GSH level ($F_{9, 110} = 12.99$, $p < 0.0001$, one-way ANOVA, Fig. 3b) was diminished considerably in contrast to the non-stimulation state ($p < 0.001$, Tukey–Kramer post *hoc* test, Fig. 3b). Our results also demonstrated that khellin (10, 30, and 100 µM) and visnagin (30 and 100 µM) notably reduced the levels of MDA and MDA/GSH ratio in a concentration-dependent manner ($p < 0.001$ for all cases, Tukey–Kramer post *hoc* test, Fig. 3a and b). Besides, khellin at two higher concentrations significantly reduced the MDA concentration ($p < 0.05$ for 30 µM, and $p < 0.01$ for 100 µM, Tukey–Kramer post *hoc* test, Fig. 3a) than visnagin. Moreover, khellin (30 µM; $p < 0.05$, and 100 µM; $p < 0.001$, Games–Howell's multiple comparisons tests, Fig. 3c) and visnagin (100 µM; $p < 0.001$, Games–Howell's multiple comparisons tests, Fig. 3c) significantly attenuated the MDA/GSH ratio compared to the PHA-stimulated control group. Nevertheless, khellin (100 µM; $p < 0.001$, Tukey–Kramer post *hoc* test, Fig. 3b) and visnagin (100 µM; $p < 0.001$, Tukey–Kramer post *hoc* test, Fig. 3b) remarkably increased GSH concentration compared to the PHA-stimulated control group.

Effects of visnagin and khellin on IFN-γ, IL-4, and IL-10 levels

In the gene expression study, the mRNA levels of IL-10 ($F^*_{9, 89.33} = 43.22$, $p < 0.0001$, Brown–Forsythe ANOVA, Fig. 4a), IL-4 ($F^*_{9, 90.55} = 38.55$, $p < 0.0001$, Brown–Forsythe ANOVA, Fig. 4b), and IFN-γ ($F^*_{9, 84.52} = 17.75$, $p < 0.0001$, Brown–Forsythe ANOVA, Fig. 4c) were significantly elevated in the PHA-stimulated control group in comparison to the non-stimulated conditions ($p < 0.001$ for all cases, Games–Howell's multiple comparisons tests, Fig. 4a, b, and c). In the stimulated state, the highest concentration (100 µM) of both khellin ($p < 0.001$, Games–Howell's multiple comparisons tests, Fig. 4a) and visnagin ($p < 0.001$, Games–Howell's multiple comparisons tests, Fig. 4a) significantly elevated the mRNA levels of IL-10, but dexamethasone (0.1 mM, $p < 0.001$, Games–Howell's multiple comparisons tests, Fig. 4a) considerably diminished IL-10 mRNA levels, compared to the PHA-stimulated control group. IL-4 mRNA expression levels were significantly decreased in dexamethasone (0.1 mM; $p < 0.001$,

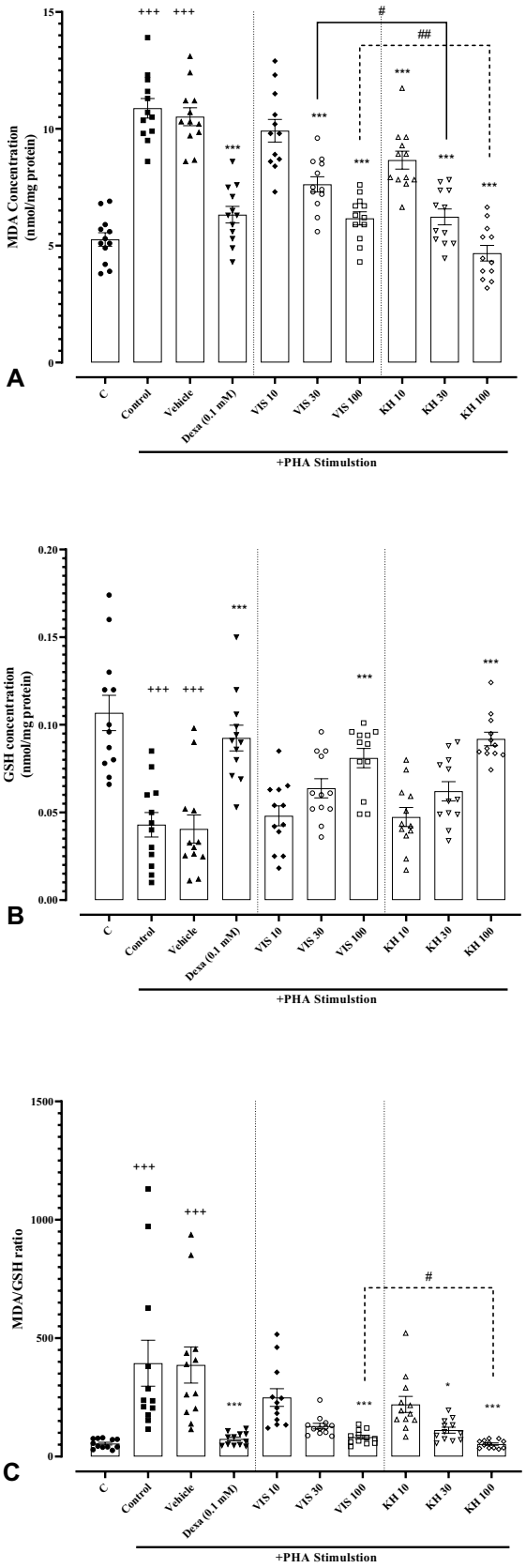


Fig. 3 The effects of khellin and visnagin on malondialdehyde (**A**) and glutathione (**B**) concentrations and their ratio (**C**) in the presence of phytohemagglutinin stimulation. Data were presented as mean ± SD values (for each group, $n = 12$). Data had equal variances in malondialdehyde (**A**) and glutathione (**B**) concentrations. Therefore, statistical analyses employed one-way ANOVA followed by Tukey–Kramer's post *hoc* test. In contrast, data for MDA/GSH ratio (C) had no equal variances and did not pass Levene's test. Thus, statistical analyses employed the Brown–Forsythe ANOVA test, followed by Games–Howell's multiple comparisons tests. $^{+}$Compared to the non-stimulated control group; $^{+++}$ $p < 0.001$. $^{\#}$Compared respected concentrations of khellin with visnagin; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$. *Compared to the PHA-stimulated control group; $^{*}p < 0.05$, $^{***}p < 0.001$. Malondialdehyde (MDA), glutathione (GSH), non-stimulated control group (C), phytohemagglutinin (PHA), PHA-stimulated control group (Control), dimethylsulfoxide (DMSO), dexamethasone-treated group (Dexa), visnagin-treated groups (VIS), khellin-treated groups (KH)

Games–Howell's multiple comparisons tests, Fig. 4b), visnagin (100 µM; $p < 0.01$, Games–Howell's multiple comparisons tests, Fig. 4b), and khellin-treated groups (10 µM; $p < 0.01$, 30 and 100; $p < 0.001$, Games–Howell's multiple comparisons tests, Fig. 4b). The suppressive effect of khellin on IL-4 was more pronounced than the equal concentrations of visnagin ($p < 0.01$ for 10 µM and 30 µM, and $p = 0.07$ for 100 µM, Games–Howell's multiple comparisons tests, Fig. 4b). IFN-γ mRNA expression was significantly diminished in the dexamethasone-treated group (0.1 mM; $p < 0.01$, Games–Howell's multiple comparisons tests, Fig. 4c) and two high concentrations of khellin ($p < 0.05$ for 30 µM and $p < 0.001$ for 100 µM, Games–Howell's multiple comparisons tests, Fig. 4c) compared to the PHA-stimulated group. The highest concentration of khellin had a more reducing effect on IFN-γ mRNA expression than visnagin at the same concentration (100 µM; $p < 0.01$, Games–Howell's multiple comparisons tests, Fig. 4c).

Effects of visnagin and khellin on IFN-γ/IL4 (Th1/Th2), IFN-γ/IL10 (Th1/Treg), and IL4/IL10 (Th2/Treg) ratios

The IFN-γ/IL-4 ($F_{9,\ 110} = 2.553$, $p = 0.0106$, one-way ANOVA, Fig. 5a) gene expression ratio was significantly diminished in PHA-stimulated lymphocytes in comparison to the non-stimulated group ($p < 0.01$, Tukey–Kramer post *hoc* test, Fig. 5a). On the other hand, the IFN-γ/IL-10 ($F^{*}_{9,\ 46.40} = 8.316$, $p < 0.0001$, Brown–Forsythe ANOVA, Fig. 5b) and IL-4/IL-10 ($F^{*}_{9,\ 52.20} = 17.86$, $p < 0.0001$, Brown–Forsythe ANOVA, Fig. 5c) gene expression ratios were significantly elevated in the PHA-stimulated cells ($p < 0.05$ and $p < 0.001$, respectively, Games–Howell's multiple comparisons tests, Fig. 5b and c). In the case of stimulation of lymphocytes with PHA, khellin (30 µM; $p < 0.05$, and 100 µM; $p < 0.01$, Games–Howell's multiple comparisons tests, Fig. 5b) and visnagin (100 µM; $p < 0.05$,

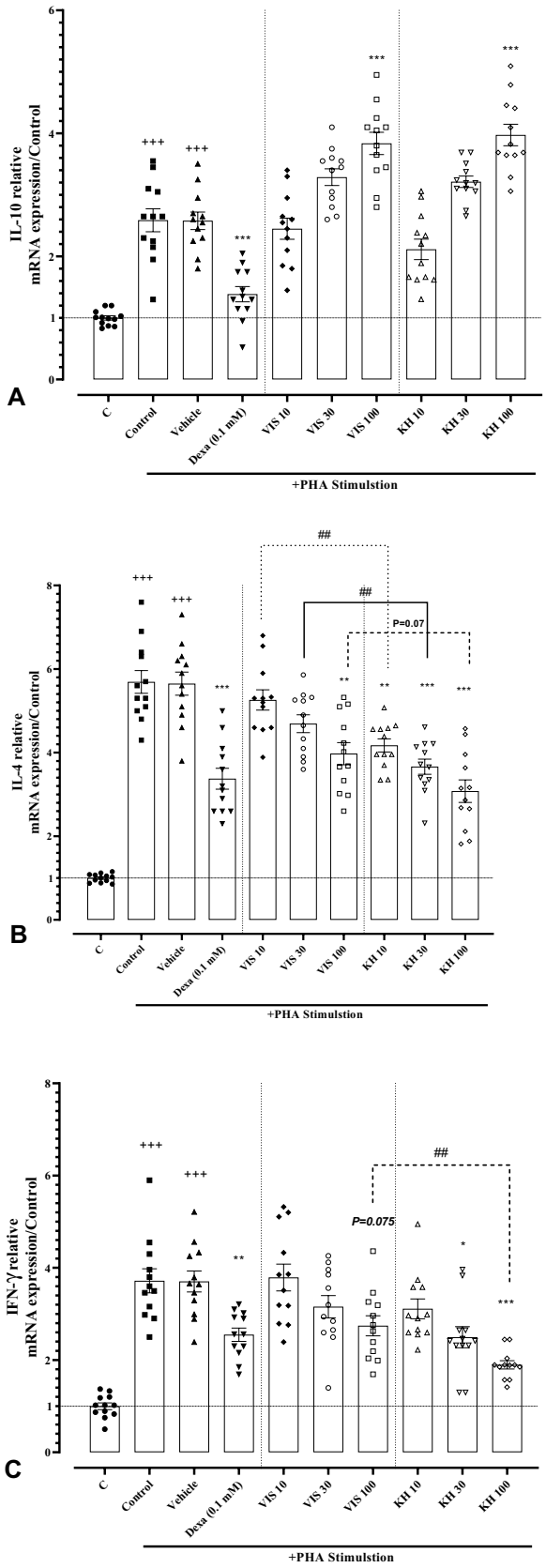


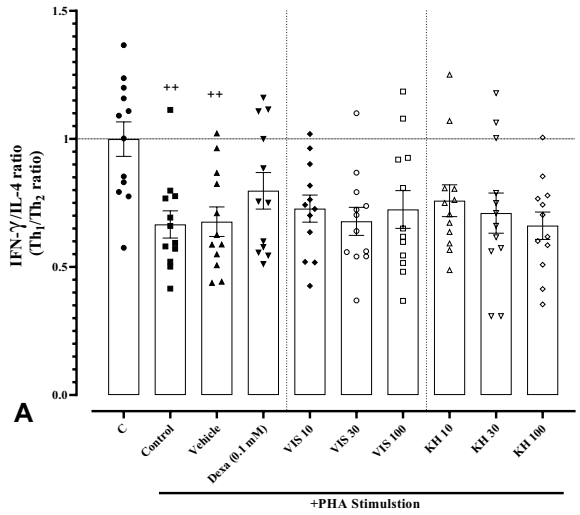
Fig. 4 The effects of khellin and visnagin on IL-10 (**A**), IL-4 (**B**), and IFN-γ (**C**) relative mRNA gene expression levels in the presence of phytohemagglutinin stimulation. Data were presented as mean ± SD values (for each group, $n = 12$). Data had no equal variances and did not pass Levene's test. Thus, statistical analyses employed the Brown–Forsythe ANOVA test, followed by Games–Howell's multiple comparisons tests. + Compared to the non-stimulated control group; + + + $p < 0.001$. # Compared respected concentrations of khellin with visnagin; ## $p < 0.01$. *Compared to the PHA-stimulated control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Interleukin-4 (IL-4), interleukin-10 (IL-10), and interferon-gamma (IFN-γ), non-stimulated control group (C), phytohemagglutinin (PHA), PHA-stimulated control group (Control), dimethylsulfoxide (DMSO), dexamethasone-treated group (Dexa), visnagin-treated groups (VIS), khellin-treated groups (KH)

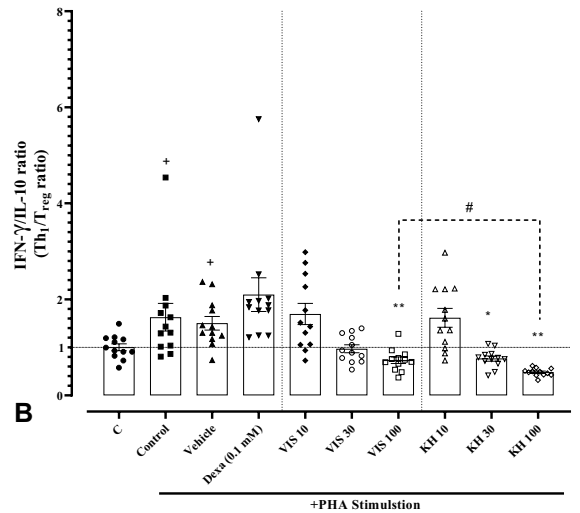
Games–Howell's multiple comparisons tests, Fig. 5b) significantly diminished the IFN-γ/IL-10 ratio compared to the control group. However, the impacts of the highest concentration of khellin and visnagin on the IFN-γ/IL-10 ratio were significantly different, with the more significant implications of khellin (100 µM, $p < 0.05$, Games–Howell's multiple comparisons tests, Fig. 5b). Furthermore, khellin (30 and 100 µM, $p < 0.001$ for both cases, Games–Howell's multiple comparisons tests, Fig. 5c) and visnagin (30 µM; $p < 0.01$, 100 µM; $p < 0.01$, Games–Howell's multiple comparisons tests, Fig. 5c) significantly diminished the IL-4/IL-10 ratio in comparison to the PHA-stimulated group. The attenuation effects of two higher concentrations of khellin ($p < 0.05$ for 30 µM and $p = 0.08$ for 100 µM, Games–Howell's multiple comparisons tests, Fig. 5c) on the IL-4/IL-10 ratio were more significant than the same concentrations of visnagin.

Discussion

The present study is the first on the immunomodulatory effects of khellin and visnagin on human lymphocytes in non-stimulated and PHA-stimulated states. In the current experiment, we examined and compared the properties of three equal concentrations of khellin and visnagin on cell proliferation, oxidative stress, NO production, IFN-γ, IL-4, and IL-10 mRNA levels, and IFN-γ/IL4 (Th_1/Th_2), IFN-γ/IL10 (Th_1/T_{reg}), and IL4/IL10 (Th_2/T_{reg}) ratios.

In numerous studies, PHA, a known T-cell mitogen activator, induces T-cell subtypes proliferation [1, 20] and activation, which provides Th_2 dominant models like cancers and inflammatory allergic diseases [1, 20, 38, 42]. Nevertheless, several studies have shown that PHA at a concentration of 5 µg/ml provides lymphocyte stimulation and leads to an imbalance between the three subtypes of T cells ($Th_1/Th_2/T_{reg}$) toward Th_2 cells and perturbation of its cytokines releases and productions [1, 43–45]. The more significant effect of PHA-induced cellular stimulation on Th_2 (IL-4) than on Th_1 (IFN-γ) leads to a substantial reduction in $Th_1/$





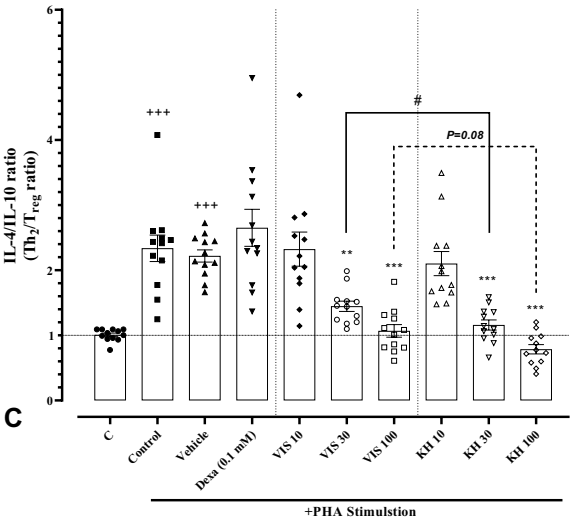


Fig. 5 The effects of khellin and visnagin on IFN-γ/IL-4 **A** Indicating Th1/Th2 balance, IFN-γ/IL-10 **B** Indicating Th1/Treg balance, and IL-4/IL-10 **C** Indicating Th2/Treg balance levels in the presence of phytohemagglutinin stimulation. Data had equal variances in IFN-γ/IL-4 (**A**). Therefore, statistical analyses employed one-way ANOVA followed by Tukey–Kramer's post *hoc* test. In contrast, data for IFN-γ/IL-10 (**B**) and IL-4/IL-10 (**C**) levels had no equal variances and did not pass Levene's test. Thus, statistical analyses employed the Brown–Forsythe ANOVA test, followed by Games–Howell's multiple comparisons tests. $^{+}$Compared to the non-stimulated control group; $^{+}$ $p<0.05$, $^{++}$ $p<0.01$, $^{+++}p<0.001$. $^{\#}$Compared respected concentrations of khellin with visnagin; $^{\#}p<0.05$. *Compared to the PHA-stimulated control group; $*p<0.05$, $**p<0.01$, $***p<0.001$. T helper-1 (Th1), T helper-2 (Th2), regulatory T cells (Treg), interleukin-4 (IL-4), interleukin-10 (IL-10), and interferon-gamma (IFN-γ), non-stimulated control group (C), phytohemagglutinin (PHA), PHA-stimulated control group (Control), dimethylsulfoxide (DMSO), dexamethasone-treated group (Dexa), visnagin-treated groups (VIS), khellin-treated groups (KH)

Th_2 (IFN-γ/IL-4 ratio) and T_{reg}/Th_2 balance (IL-10/IL-4) is approved. In this way, the results of PHA stimulation in the present study also support this finding [35, 43, 45, 46]. Our research findings revealed an increase in the rate of cell proliferation and gene expression of cytokines such as IL-4, IL-10, and IFN-γ, as well as nitric oxide production following incubation with PHA. Comparing the stimulation state with the non-stimulatory state indicates the development of an inflammatory condition by reducing the IFN-γ/IL-10 (Th_1/T_{reg}) and IL-4/IL-10 (Th_2/T_{reg}) ratios relative to Th_2 by further increasing the amount of IL-4.

In this study, three equal concentrations (10, 30, and 100 μM) of khellin and visnagin showed no significant anti-proliferative activities in non-stimulated lymphocytes through WST-1 colorimetric assay, which indicates no toxicity of these substances on normal cells. These findings are supported by Laura Risaliti and coworkers' study that demonstrated khellin loaded in ASC10 ascosomes and formulated in hydrogel hydroxyethyl cellulose has no toxic effects according to the liver and dermal histological and pathological evaluations [47].

Under PHA stimulation, the number of cells was increased. After that, treatment with khellin and visnagin concentration-dependently inhibited cell growth. Several studies have shown the therapeutic effects of khellin and visnagin. For example, the Qingling Qi et al. study confirmed that visnagin is a powerful anti-oxidant with specific anti-cancer activity. In fact, visnagin eventually causes apoptosis and migration prevention of HeLa cancer cells by modulating anti-oxidant systems and inhibiting the PI_3K/AKT/mTOR and MAPK signaling pathways, which are distinctive properties of a perfect anti-cancer medicines[48].

Nitric oxide and inducible nitric oxide synthase (iNOS) can play an essential role in the pathogenesis of inflammation, multiple sclerosis, cancer, and allergic diseases such as asthma and atopic dermatitis [46, 49–52]. It has also

been shown that a decrease in NO levels leads to reducing and treating an unbalanced Th_1/Th_2 ratio to a dominant Th_2 disease like asthma and cancers, especially leukemia [46, 49–52]. A significant increase in NO levels following stimulation of lymphocytes with PHA compared with unstimulated cells supported by previous studies. In this regard, it has been plentifully reported that PHA or LPS/IFN-γ (RAW264.7) stimulates lymphocytes and macrophages and increases NO levels [1, 43, 46, 50, 53]. In our current study, dexamethasone, a steroidal anti-inflammatory drug at a concentration of 0.1 mM as a positive control, significantly reduces the NO level of PHA-stimulated lymphocytes. However, all three concentrations of khellin and two higher concentrations of visnagin significantly decreased nitric oxide levels in PHA-stimulated lymphocytes.

To evaluate the immune response, T helper cell subsets, including Th_1, Th_2, and T_{reg} cells, and associated cytokine gene expression comprising IFN-γ, IL-4, and IL-10 were assessed. Th_2 cells have a protective role against worms and cause acute and chronic allergic reactions [1, 20]. On the other hand, overexpression of Th_2 cells reduces the population of Th_1 cells and prevents the activation of Th_1 cells. This condition shifts the Th_1/Th_2 balance toward Th_2 activity, compromises the body's immune system against cellular defenses and infections, and promotes cancer [1, 19, 46, 50, 51, 54]. The subset of regulatory T cells with the secretion of cytokines IL-10 and TGF-β, depending on the regulation of the Th_1/Th_2 ratio, have antagonistic effects on Th_1 and Th_2 cells [6–14, 55–57]. Therefore, manipulating Th responses toward a suitable immune response may be the best way to transcend disease immunopathology. For example, T_{reg} cells can reduce allergic reactions by secreting TGF-β and IL-10, thereby diminishing the Th_2 response or increasing Th_1 activity, IL-4 production, and IgE inhibition. On the other hand, the overactive Th_1 lymphocytes, regulatory T cells, and Th_2 subpopulations, by secreting IL-10, TGF-β, IL-4, and IL-13, can suppress the Th_1 response, respectively. Herbal agents, exclusively pure compounds, are valuable for modulating immunity in the selective rearrangement of diseases associated with an unbalanced immune response from allergy to autoimmunity and from cancer to infection [6–14, 52, 54, 56, 57].

The findings of this study confirmed that dexamethasone at 0.1 mM concentration did not significantly affect the expression of lymphocyte cytokine genes, and also Th_1/Th_2 (IFN-γ/IL-4) and T_{reg}/Th_2 (IL-10/IL-4) balance in the non-stimulation state. However, in the stimulation state by PHA, dexamethasone (0.1 mM) significantly diminished cell proliferation, the gene expression level of IFN-γ, IL-4, and IL-10, and altered Th_1/T_{reg} (IFN-γ/IL-10) and Th_2/T_{reg} (IL-4/IL-10) balance toward Th_1 and T_{reg}. Therefore, non-specific inhibitory impacts of dexamethasone were seen on gene levels (IL-4, IL-10, and IFN-γ), and its effect on Th_2 (IL-4) was more potent than on Th_1 (IFN-γ) cells. Various studies support the non-specific activities of dexamethasone perceived in this study by proving the same non-specific effect on imbalanced Th_1/Th_2 (IFN-γ/IL-4) and T_{reg}/Th_2 (IL-10/IL-4) toward Th_1 and T_{reg}, respectively [1, 6–14, 20]. Besides, Ramirez et al. have revealed that pretreatment of T cells with dexamethasone diminished IFN-γ secretion [58]. A similar study also supports its inhibitory activity on IL-4, IL-10, and IFN-γ secretion in non-stimulated and mitogen-activated splenocytes [59]. The inhibitory effect of dexamethasone on IL-4 and IFN-γ production in conditions of stimulation with PHA and alternation of the Th_1/Th_2 balance toward Th_1 and its inhibitory effect on proliferation was also confirmed previously [43, 45].

In the PHA-stimulated lymphocytes, the IL-10 gene expression level was elevated for all three tested khellin concentrations in a concentration-dependent manner, with a significant increase in the highest concentration. These results demonstrated that khellin, at all three concentrations, could significantly diminish the IL-4 gene expression level, at two higher concentrations, could substantially diminish the gene expression of IFN-γ, and at the highest concentration, could dramatically elevate the IL-10 gene expression levels. IFN-γ/IL-10 and IL-4/IL-10 significantly declined due to two higher concentrations of khellin. In stimulated lymphocytes, visnagin declined IFN-γ and IL-4 levels in a concentration-dependent manner, with a significant decrease at the highest concentration for reducing the IL-4 levels. In addition, visnagin significantly increased IL-10 at the highest concentration. Visnagin at two higher concentrations also diminished IFN-γ/IL-10 and IL-4/IL-10. Notably, the frictional difference of the highest concentration of khellin (100 μM) compared to visnagin was significant. IFN-γ/IL4, IFN-γ/IL10, and IL4/IL10 ratios were improved as a result of treatment with two high concentrations of khellin and visnagin, which were not affected by dexamethasone. The effects of two higher concentrations of khellin were significantly higher than the same visnagin-related concentrations. The findings of this study confirmed that treatment of the Th_2 predominant imbalance state with khellin and visnagin could adjust the Th_1/T_{reg} (IFN-γ/IL-10) and Th_2/T_{reg} (IL-4/IL-10) balance toward Th_1 and Th_2 polarization with relatively selective activity on IFN-γ and IL-4 regarding gene expression data.

Furthermore, several studies have shown that the visnagin concentrated of *A. visnaga* extract provided anti-inflammatory effects by reducing mRNA expression and releasing TNF-α, IL-1β, and IFNγ. In addition, visnagin decreased IL-6 and MCP-1 mRNA levels with LPS-induced inflammation. These findings indicate that the probable cause of the anti-inflammatory effect of visnagin is related to the inhibition of transcription factors such as AP-1 and NF-κB [33]. Moreover, the neuroprotective effect of visnagin in terms of

disease suppression caused by kainic acid in the brain has been revealed. It has been found that this neuroprotective effect of visnagin was associated with its anti-inflammatory effects [60]. In one study, khellin inhibited the mutagenicity of promutagens benzo[a]pyrene, 2-aminofluorene, and 2-aminoanthracene in Salmonella typhimurium T98, while visnagin represented higher toxicity [34].

However, as a study limitation, we measured the gene expression level, which suggested verifying cytokine gene expression at the protein levels and mRNA levels in future studies. Furthermore, as another limitation of the current research, all the changes in NO production, redox potential, cytokine production, and gene expression following visnagin and khellin treatment were consequences of changes in cell proliferation/activation (Fig. 1b). Therefore, the alternative explanation to these observed changes is that visnagin and khellin treatment could inhibit T-cell activation, reducing both proliferation and expression of Th_1/Th_2 cytokines. Noteworthy, previous studies showed that visnagin eventually causes apoptosis of HeLa cancer cells by modulating anti-oxidant systems and inhibiting the PI_3K/AKT/mTOR and MAPK signaling pathways, which are distinctive properties of a perfect anti-cancer drug[48]. Besides, it has been demonstrated that visnagin attenuates LPS-induced inflammation and IL-6 and MCP-1 mRNA levels. These findings indicate that the probable cause of the anti-inflammatory effect of visnagin could be described through the inhibition of transcription factors such as AP-1 and NF-κB [33]. Therefore, further experiments can be suggested, including apoptosis assay using Annexin-PI staining and evaluating the same concentrations and parameters in unstimulated lymphocytes.

In conclusion, the whole findings of this study together could prove that khellin at two higher concentrations (30 and 100 μM) and visnagin at the highest concentration (100 μM), without any cytotoxicity, suppress Th_1 and Th_2 immune cell responses. Consequently, these natural compounds have therapeutic values in immunopathologic disorders in which Th_2 or Th_1 is predominant such as atopic dermatitis, cancers, and autoimmune diseases. Moreover, the results of our study presented the selective suppression of proinflammatory cytokines (IFN-γ) and relatively particular anti-inflammatory cytokines (IL-10) by khellin and visnagin.

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Data availability Data are available by the corresponding author upon request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This study was permitted by the ethics committee of Mashhad University of Medical Sciences (IR.MUMS.fm.REC.1395.593), and all individuals agreed with informed consent.

References

1. Askari VR, Rezaee SA, Abnous K, Iranshahi M, Boskabady MH. The influence of hydro-ethanolic extract of *Portulaca oleracea* L. on Th(1)/Th(2) balance in isolated human lymphocytes. J Ethnopharmacol. 2016;194:1112–21.
2. Gleeson M. Immune function in sport and exercise. J Appl Physiol. 1985;2007(103):693–9.
3. Knight JA. Review: free radicals, antioxidants, and the immune system. Ann Clin Lab Sci. 2000;30:145–58.
4. Annunziato F, Cosmi L, Romagnani S. Human and murine Th17. Curr Opin HIV AIDS. 2010;5:114–9.
5. Ivanova EA, Orekhov AN. T helper lymphocyte subsets and plasticity in autoimmunity and cancer: an overview. Biomed Res Int. 2015;2015: 327470.
6. Askari VR, Fereydouni N, Baradaran Rahimi V, Askari N, Sahebkar AH, Rahmanian-Devin P, et al. beta-Amyrin, the cannabinoid receptors agonist, abrogates mice brain microglial cells inflammation induced by lipopolysaccharide/interferon-gamma and regulates M(1)/M(2) balances. Biomed Pharmacother. 2018;101:438–46.
7. Askari VR, Rahimi VB, Rezaee SA, Boskabady MH. Auraptene regulates Th1/Th2/TReg balances, NF-κB nuclear localization and nitric oxide production in normal and Th2 provoked situations in human isolated lymphocytes. Phytomedicine. 2018;43:1–10.
8. Askari VR, Rahimi VB, Tabatabaee SA, Shafiee-Nick R. Combination of Imipramine, a sphingomyelinase inhibitor, and β-caryophyllene improve their therapeutic effects on experimental autoimmune encephalomyelitis (EAE). Int Immunopharmacol. 2019;77: 105923.
9. Askari VR, Rahimi VB, Zargarani R, Ghodsi R, Boskabady M, Boskabady MH. Anti-oxidant and anti-inflammatory effects of auraptene on phytohemagglutinin (PHA)-induced inflammation in human lymphocytes. Pharmacol Rep. 2021;73:154–62.
10. Askari VR, Rezaee SA, Abnous K, Iranshahi M, Boskabady MH. The influence of hydro-ethanolic extract of *Portulaca oleracea* L. on Th1/Th2 balance in isolated human lymphocytes. J Ethnopharmacol. 2016;194:1112–21.
11. Askari VR, Shafiee-Nick R. Promising neuroprotective effects of beta-caryophyllene against LPS-induced oligodendrocyte toxicity: a mechanistic study. Biochem Pharmacol. 2019;159:154–71.
12. Askari VR, Shafiee-Nick R. The protective effects of beta-caryophyllene on LPS-induced primary microglia M(1)/M(2) imbalance: a mechanistic evaluation. Life Sci. 2019;219:40–73.
13. Baradaran Rahim V, Khammar MT, Rakhshandeh H, Samzadeh-Kermani A, Hosseini A, Askari VR. Crocin protects cardiomyocytes against LPS-Induced inflammation. Pharmacol Rep. 2019;71:1228–34.
14. Gholoobi A, Askari VR, Naghedinia H, Ahmadi M, Vakili V, Baradaran RV. Colchicine effectively attenuates inflammatory biomarker high-sensitivity C-reactive protein (hs-CRP) in patients

with non-ST-segment elevation myocardial infarction: a randomised, double-blind, placebo-controlled clinical trial. Inflammopharmacology. 2021;29:1379–87.
15. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature. 1996;383:787–93.
16. Oestreich KJ, Weinmann AS. Transcriptional mechanisms that regulate T helper 1 cell differentiation. Curr Opin Immunol. 2012;24:191–5.
17. Braun CM, Huang SK, Bashian GG, Kagey-Sobotka A, Lichtenstein LM, Essayan DM. Corticosteroid modulation of human, antigen-specific Th1 and Th2 responses. J Allergy Clin Immunol. 1997;100:400–7.
18. Cosmi L, Liotta F, Maggi E, Romagnani S, Annunziato F. Th17 cells: new players in asthma pathogenesis. Allergy. 2011;66:989–98.
19. Cohn L, Homer RJ, Niu N, Bottomly K. T helper 1 cells and interferon gamma regulate allergic airway inflammation and mucus production. J Exp Med. 1999;190:1309–18.
20. Kianmehr M, Rezaei A, Hosseini M, Khazdair MR, Rezaee R, Askari VR, et al. Immunomodulatory effect of characterized extract of Zataria multiflora on Th(1), Th(2) and Th(17) in normal and Th(2) polarization state. Food Chem Toxicol. 2017;99:119–27.
21. Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F, et al. Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother. 2007;56:641–8.
22. Daoudi A, Aarab L, Abdel-Sattar E. Screening of immunomodulatory activity of total and protein extracts of some Moroccan medicinal plants. Toxicol Ind Health. 2013;29:245–53.
23. Franchi G, Ferri S, Bovalini L, Martelli P. *Ammi visnaga* (L.) Lam.: occurrence of khellin and visnagin in primary rib channels and endosperm, and emptiness of vittae, revealed by UV microscopy. Int J Crude Drug Res. 1987;25:137–44.
24. Mk M. Determination of khellin and visnagin in ammi visnaga fruits and in renal teas by high performance liquid chromatography. Egypt J Pharm Sci. 1992;33:897–904.
25. Gunaydin K, Beyazit N. The chemical investigations on the ripe fruits of *Ammi visnaga* (Lam.) Lamarck growing in Turkey. Nat Prod Res. 2004;18:169–75.
26. Zrira S, Elamrani A, Pellerin P, Bessière J-M, Menut C, Benjilali B. Isolation of Moroccan *Ammi visnaga* oil: comparison between hydrodistillation, steam distillation and supercritical fluid extraction. J Essent Oil-Bear Plants. 2008;11:30–5.
27. Hashim S, Jan A, Marwat KB, Khan MA. Phytochemistry and medicinal properties of *Ammi visnaga* (Apiacae). Pak J Bot. 2014;46:861–7.
28. Vedaldi D, Caffieri S, Dall'Acqua F, Andreassi L, Bovalini L, Martelli P. Khellin, a naturally occurring furochromone, used for the photochemotherapy of skin diseases: mechanism of action. Farmaco Sci. 1988;43:333–46.
29. Trabalzini L, Martelli P, Bovalini L, Dall'Acqua F, Sage E. Photosensitization of DNA of defined sequence by furochromones, khellin and visnagin. J Photochem Photobiol B. 1990;7:317–36.
30. Vanachayangkul P, Byer K, Khan S, Butterweck V. An aqueous extract of *Ammi visnaga* fruits and its constituents khellin and visnagin prevent cell damage caused by oxalate in renal epithelial cells. Phytomedicine. 2010;17:653–8.
31. Duarte J, Perez-Vizcaino F, Torres AI, Zarzuelo A, Jimenez J, Tamargo J. Vasodilator effects of visnagin in isolated rat vascular smooth muscle. Eur J Pharmacol. 1995;286:115–22.
32. Rauwald HW, Brehm O, Odenthal KP. The involvement of a Ca2+ channel blocking mode of action in the pharmacology of *Ammi visnaga* fruits. Planta Med. 1994;60:101–5.
33. Lee JK, Jung JS, Park SH, Park SH, Sim YB, Kim SM, et al. Anti-inflammatory effect of visnagin in lipopolysaccharide-stimulated BV-2 microglial cells. Arch Pharm Res. 2010;33:1843–50.
34. Schimmer O, Rauch P. Inhibition of metabolic activation of the promutagens, benzo [a] pyrene, 2-aminofluorene and 2-aminoanthracene by furanochromones hi Salmonella typhimurium. Mutagenesis. 1998;13:385–9.
35. Boskabady MH, SeyedhosseiniTamijani SM, Rafatpanah H, Rezaei A, Alavinejad A. The effect of Crocus sativus extract on human lymphocytes' cytokines and T helper 2/T helper 1 balance. J Med Food. 2011;14:1538–45.
36. Guo CH, Han LX, Wan MR, Deng GJ, Gan JH. Immunomodulatory effect of bone marrow mesenchymal stem cells on T lymphocytes in patients with decompensated liver cirrhosis. Genet Mol Res. 2015;14:7039–46.
37. Askari VR, Baradaran Rahimi V, Assaran A, Iranshahi M, Boskabady MH. Evaluation of the anti-oxidant and anti-inflammatory effects of the methanolic extract of Ferula szowitsiana root on PHA-induced inflammation in human lymphocytes. Drug Chem Toxicol. 2020;43:353–60.
38. Movafagh A, Heydary H, Mortazavi-Tabatabaei SA, Azargashb E. The significance application of Indigenous phytohemagglutinin (PHA) mitogen on metaphase and cell culture procedure. Iran J Pharm Res. 2011;10:895–903.
39. Rahimi VB, Askari VR, Emami SA, Tayarani-Najaran Z. Anti-melanogenic activity of *Viola odorata* different extracts on B16F10 murine melanoma cells. Iran J Basic Med Sci. 2017;20:242–9.
40. Baradaran Rahimi V, Mousavi SH, Haghighi S, Soheili-Far S, Askari VR. Cytotoxicity and apoptogenic properties of the standardized extract of *Portulaca oleracea* on glioblastoma multiforme cancer cell line (U-87): a mechanistic study. Excli J. 2019;18:165–86.
41. Cichoń M. Reporting statistical methods and outcome of statistical analyses in research articles. Pharmacol Rep. 2020;72:481–5.
42. Sofuni T, Yoshida MC. Combined use of several mitogens for mitotic stimulation to human lymphocytes. J Radiat Res. 1992;33:222–30.
43. Boskabady MH, Mehrjardi SS, Rezaee A, Rafatpanah H, Jalali S. The impact of *Zataria multiflora* Boiss extract on in vitro and in vivo Th1/Th2 cytokine (IFN-gamma/IL4) balance. J Ethnopharmacol. 2013;150:1024–31.
44. Feyzi R, Boskabady MH, SeyedhosseiniTamijani SM, Rafatpanah H, Rezaei SA. The effect of Safranal on Th1/Th2 cytokine balance. Iran J Immunol. 2016;13:263–73.
45. Gholamnezhad Z, Rafatpanah H, Sadeghnia HR, Boskabady MH. Immunomodulatory and cytotoxic effects of *Nigella sativa* and thymoquinone on rat splenocytes. Food Chem Toxicol. 2015;86:72–80.
46. Zamani Taghizadeh Rabe S, Iranshahi M, Mahmoudi M. In vitro anti-inflammatory and immunomodulatory properties of umbelliprenin and methyl galbanate. J Immunotoxicol. 2016;13:209–16.
47. Risaliti L, Yu X, Vanti G, Bergonzi MC, Wang M, Bilia AR. Hydroxyethyl cellulose hydrogel for skin delivery of khellin loaded in ascosomes: characterization, in vitro/in vivo performance and acute toxicity. Int J Biol Macromol. 2021;179:217–29.
48. Qi Q, Wang Q, Wang Z, Gao W, Gong X, Wang L. Visnagin inhibits cervical cancer cells proliferation through the induction of apoptosis and modulation of PI3K/AKT/mTOR and MAPK signaling pathway. Arab J Chem. 2022;15: 103684.
49. Hong CH, Hur SK, Oh OJ, Kim SS, Nam KA, Lee SK. Evaluation of natural products on inhibition of inducible cyclooxygenase

(COX-2) and nitric oxide synthase (iNOS) in cultured mouse macrophage cells. J Ethnopharmacol. 2002;83:153–9.
50. Kohno S, Murata T, Sugiura A, Ito C, Iranshahi M, Hikita K, et al. Methyl galbanate, a novel inhibitor of nitric oxide production in mouse macrophage RAW264.7 cells. J Nat Med. 2011;65:353–9.
51. Phan-Lai V, Dang Y, Gad E, Childs J, Disis ML. The antitumor efficacy of IL2/IL21-cultured polyfunctional Neu-Specific T cells is TNFα/IL17 dependent. Clin Cancer Res. 2016;22:2207–16.
52. Zou Q, Jin J, Xiao Y, Zhou X, Hu H, Cheng X, et al. T Cell intrinsic USP15 deficiency promotes excessive IFN-gamma production and an immunosuppressive tumor microenvironment in MCA-induced fibrosarcoma. Cell Rep. 2015;13:2470–9.
53. Lyu SY, Park WB. Production of cytokine and NO by RAW 264.7 macrophages and PBMC in vitro incubation with flavonoids. Arch Pharm Res. 2005;28:573–81.
54. Zhao R, Gao X, Cai Y, Shao X, Jia G, Huang Y, et al. Antitumor activity of *Portulaca oleracea* L. polysaccharides against cervical carcinoma in vitro and in vivo. Carbohydr Polym. 2013;96:376–83.
55. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol. 2003;33:1205–14.
56. Shalev I, Schmelzle M, Robson SC, Levy G. Making sense of regulatory T cell suppressive function. Semin Immunol. 2011;23:282–92.
57. Wolff MJ, Leung JM, Davenport M, Poles MA, Cho I, Loke P. TH17, TH22 and treg cells are enriched in the healthy human cecum. PLoS ONE. 2012;7: e41373.
58. Ramirez F, Fowell DJ, Puklavec M, Simmonds S, Mason D. Glucocorticoids promote a TH2 cytokine response by CD4+ T cells in vitro. J Immunol. 1996;156:2406–12.
59. Moynihan JA, Callahan TA, Kelley SP, Campbell LM. Adrenal hormone modulation of type 1 and type 2 cytokine production by spleen cells: dexamethasone and dehydroepiandrosterone suppress interleukin-2, interleukin-4, and interferon-gamma production in vitro. Cell Immunol. 1998;184:58–64.
60. Kwon MS, Lee JK, Park SH, Sim YB, Jung JS, Won MH, et al. Neuroprotective effect of Visnagin on Kainic acid-induced neuronal cell death in the mice hippocampus. Korean J Physiol Pharmacol. 2010;14:257–63.