

The Effect of Safranal on Th₁/Th₂ Cytokine Balance

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ABSTRACT

Background: Several biological and medical benefits of Saffron, *Crocus sativus* (Iridaceae), have been demonstrated. However, mechanisms of actions for purified constituents are greatly unknown. **Objective:** To examine the effects of Safranal, a main constituent of Saffron stigma, on cell viability and cytokine profile of peripheral blood mononuclear cells (PBMC) were examined. **Methods:** Effects of Safranal at 0.1, 0.5 and 1 mM concentrations were evaluated on cell viability and production of interleukin 4 (IL-4), IL-10 and interferon- γ (IFN- γ) from non-stimulated and phytohemagglutinin (PHA) stimulated PBMCs, compared to 0.1 mM dexamethasone and saline. **Results:** In stimulated cells, different concentrations of Safranal caused significant decrease of lymphocytes viability ($p < 0.001$ for all concentrations). All concentrations of Safranal inhibited IFN- γ and IL-10 secretion in stimulated cells ($p < 0.01$). In addition, high concentration of Safranal significantly decreased cell viability of non-stimulated PBMCs ($p < 0.001$). The effect of 1 mM Safranal on IL-4 secretion was less than dexamethasone ($p < 0.05$). Safranal showed a stimulatory effect on IFN- γ secretion in non-stimulated cells. The IFN- γ /IL-4 ratio at the presence of two higher Safranal concentrations both in non-stimulated and stimulated cells were significantly higher than those of control and PHA stimulated groups, respectively ($p < 0.05$). **Conclusion:** The IFN- γ /IL-4 ratio increases in the presence of Safranal which indicates an effect on Th1/Th2 balance. Therefore, Safranal may have therapeutic effects in inflammatory diseases associated with Th1/Th2 imbalance.

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Keywords: Cell Viability, Cytokine, Safranal, Th1, Th2

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INTRODUCTION

Crocus sativus L, is a small perennial plant from the iris family (Iridaceae) which is cultivated mainly in Iran with various medical and pharmacological properties. The main constituents of the stigma of this plant are crocin, Safranal, picrocrocin, ketoisophorone, isophorone and glycosidic terpenoids (1).

Safranal is a monoterpene aldehyde, formed by hydrolysis of picrocrocin during drying and storage which is responsible for the scent of Saffron (1). Safranal constitutes about 60% of the volatile fraction of the saffron and it may be responsible for some therapeutic effects of the plant which is used in traditional medicine for treatment of various disorders (2-4).

Several pharmacological and therapeutic effects for Safranal have been demonstrated previously including: anticonvulsant, anxiolytic, hypnotic, antioxidant, chemopreventive and anticancer effects (5-8). Antitussive activity of saffron's stigma and its components, Safranal and crocin, the relaxant effects of aqueous-ethanolic extracts of *C. sativus* and Safranal, their stimulatory effect on b-adrenoceptors and the inhibitory effect of the extract of the plant and Safranal on histamine (H1) receptors were also demonstrated previously (9-12).

T helper 2 (Th2) and other inflammatory cells are activated in asthma and other inflammatory diseases, and their mediators cause tissue inflammation, mucus secretion and airway hyperresponsiveness (13-15). T helper 1 proved to inhibit T helper 2 response and thus, as a goal of asthma therapy, the effort should focus on switching to T helper 1 response (16).

In previous studies the effect of the extract of *Crocus sativus* on Th1/Th2 balance of human PBMC was shown (17). The potential antiproliferative and cytotoxic effects of saffron in human lung cancer cell line was also demonstrated (18). In the present study, effect of Safranal, the constituent of *Crocus sativus*, on PBMC viability and Th1/Th2 balance was studied by measuring secreted cytokines including IL-4, IFN-g and IL-10.

MATERIALS AND METHODS

Isolation of Peripheral Blood Mononuclear Cells (PBMCs). Ten ml EDTA anticoagulated peripheral blood samples were collected from ten healthy male subjects (age 20-60 years). Whole blood samples were layered on to Ficoll-density gradient (Sigma, USA) and centrifuged for 15 min at 800×g in room temperature. PBMC layer was collected and cells were then washed three times by phosphate buffer saline (PBS) at 300×g for 10 minutes.

Treatment of PBMCs. The following groups of 5000 cells/well PBMCs were seeded and treated in 96 well microplates in RPMI-1640 (Sigma, USA) at 37°C and presence of %5 CO₂. Cells were treated with a range of Safranal concentrations (*Fluka, Italy, Catalogue No. C4915, purity 75%*), 0.1 mM dexamethasone (*Sigma Chemicals, LTD*) or saline as control at presence or absence of 5 µg/ml phytohemagglutinin (PHA) for 72 hours. Totally, 9 groups were examined:

- 1) PHA stimulated PBMCs: treated with 5 µg/ml PHA only
- 2) Non-stimulated PBMCs: treated with 0.1 mM dexamethasone (*Sigma Chemicals, LTD*)
- 3-5) Non-stimulated PBMCs: treated with 0.1, 0.5 and 1 mM safranal
- 6-8) PHA stimulated PBMCs treated with 0.1, 0.5 and 1 mM Safranal

9) Control group treated with RPMI with equal volumes of drug and/or PHA.

Cell Viability. After mentioned treatments, supernatants were collected by centrifuging samples at $1200\times g$, $4^{\circ}C$ for 5 min and immediately stored at $-20^{\circ}C$ until the time of cytokine analyses. One hundred micro liter of fresh 5 mg/ml MTT solution in RPMI was added to the remained cells in the wells and the plates were incubated for 4 hours. Resulting formazan crystals were solubilized in 100 μ l DMSO and the optical densities (OD) of the wells were measured at 570 nm with a reference of 620 nm. Percentage of viability for a sample was defined as following:

Percentage of viability = Mean of Sample OD * 100 / Mean of Control OD

Measurement of IL-4, IL-10 and IFN- γ in Cell Culture Supernatant. Concentrations of IL-4, IL-10 and IFN- γ were measured using enzyme linked immunosorbent assay (ELISA) (Bender MedSystems Inc, Austria) according to manufacturer's instruction.

Statistical Analysis. Data were expressed as mean \pm SEM. The ratio of IFN- γ to IL-4 was calculated in all experimental groups. Percent changes in the values of MTT, IFN- γ , IL-10 and IL-4 were calculated in proportion to control values in cultured PBMCs, treated with different concentrations of Safranal. Analysis of variance (ANOVA) with Tukey-Kramer multiple *post hoc* test was used for the comparison of results between control, PHA stimulated and Safranal treated groups. The effect of each concentration between PHA stimulated and non-stimulated lymphocytes was compared using unpaired t test. Comparisons of IFN- γ /IL-4 ratio between treated and control groups were performed using the nonparametric analysis of variance (Kruskal-Wallis) test. Statistical significance was considered at $p < 0.05$.

RESULTS

Effect of Dexamethasone and Safranal on Viability and Cytokine Secretion By lymphocytes. Viability (239.87 ± 17.35 vs. $100 \pm 0.00\%$), secretion of IFN- γ (82.58 ± 27.39 vs. 0.80 ± 0.27 pg/ml), IL-10 (13.033 ± 2.10 vs. 2.57 ± 0.38 pg/ml) and IL-4 (15.07 ± 1.65 vs. 7.13 ± 1.17 pg/ml) were significantly increased in PHA stimulated PBMC compared to control group ($p < 0.05$ to $p < 0.001$), (Figures. 1-4).

In dexamethasone treated group, viability of PBMC (92.03 ± 5.96 vs. $100 \pm 0.00\%$) and IL-10 secretion (3.52 ± 0.74 vs. 2.57 ± 0.38 pg/ml) were not significantly changed compared to the control group, but IL-4 was significantly decreased (2.60 ± 1.20 vs. 7.13 ± 1.17 pg/ml), and IFN- γ (7.25 ± 1.95 vs. 0.80 ± 0.27 pg/ml) was increased ($p < 0.01$ for both cases), (Figure 1-4).

In non-stimulated PBMC groups which were treated with 3 different concentrations of Safranal, significant inhibition of cell viability was observed only in treated group with high concentration of Safranal compared to control group (36.18 ± 2.46 vs. $100 \pm 0.00\%$, $p < 0.001$). There was not any other statistically significant difference in the measured parameters between treated groups with Safranal and control group (Figures 1-4).

In stimulated PBMC groups treated with 3 different concentrations of Safranal, viability of PBMCs ($p < 0.001$ for all concentrations), secretion of IFN- γ ($p < 0.01$ to $p < 0.001$) and IL-10 ($p < 0.01$ for all concentrations) were significantly inhibited. However, IL-4 secretion did not changed significantly in treated groups (Figures 1-4).

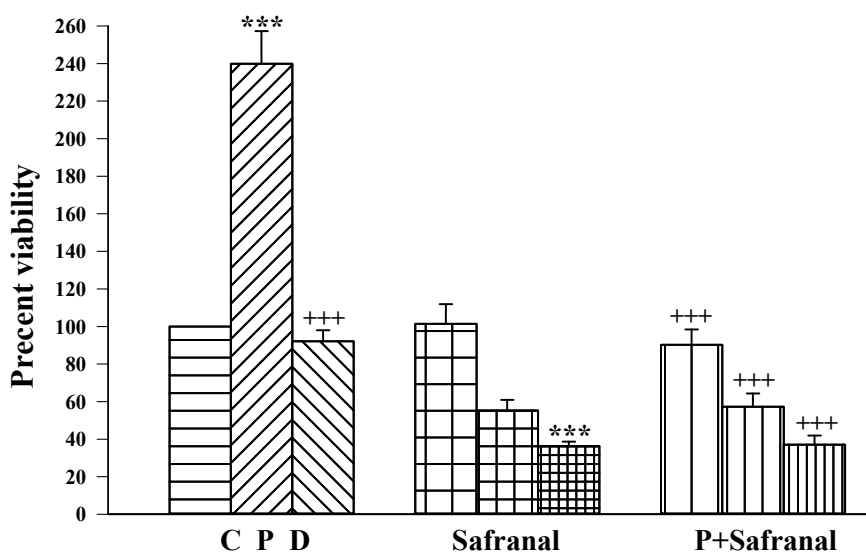


Figure 1. Viability of cultured PBMCs (mean \pm SEM, n=50) in control group (C), PHA stimulated group (P), dexamethasone treated group (D) and Safranal treated groups in the presence (P+Safranal) and absence (Safranal) of PHA (coarse, medium and fine filled bars are indicators for 0.1, 0.5 and 1 mM concentrations). ***, $p < 0.001$, compared to control non-stimulated group. +++, $p < 0.001$, compared to non-treated PHA stimulated cells. The statistical comparisons were made using analysis of variance (ANOVA) with Tukey–Kramer multiple *post hoc* test.

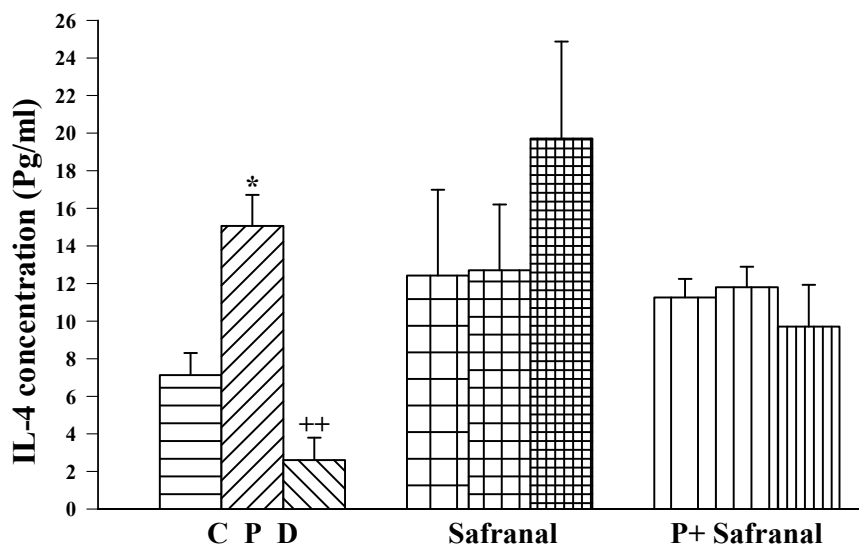


Figure 2. Concentration of IL-4 (mean \pm SEM, n=6) in supernatant of cultured PBMCs in control group (C), PHA stimulated group (P), dexamethasone treated group (D) and Safranal treated groups in the presence (P+Safranal) and absence (Safranal) of PHA (coarse, medium and fine filled bars are indicators for 0.1, 0.5 and 1 mM concentrations). *, $p < 0.05$, compared to non-stimulated control group. ++, $p < 0.01$, compared to non-treated PHA stimulated cells. The statistical comparisons were made using Analysis of variance (ANOVA) with Tukey–Kramer multiple *post hoc* test.

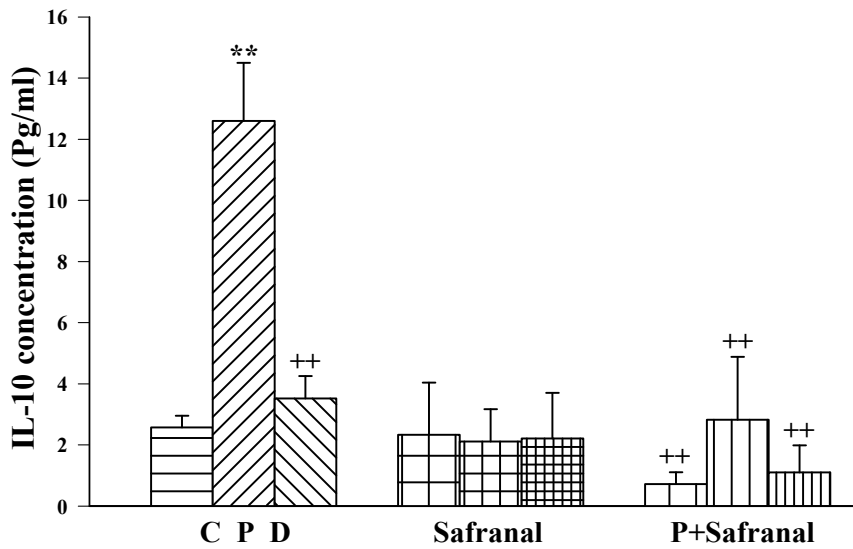


Figure 3. Concentration of IL-10 (mean \pm SEM, n=9) in supernatant of cultured PBMCs in control group (C), PHA stimulated group (P), dexamethasone treated group (D) and Safranal treated groups in the presence (P + Safranal) and absence (Safranal) of PHA (coarse, medium and fine filled bars are indicators for 0.1, 0.5 and 1 mM concentrations). **, $p < 0.01$, compared to non-stimulated control group. ++, $p < 0.01$, compared to non-treated PHA stimulated cells. The statistical comparisons were made using analysis of variance (ANOVA) with Tukey–Kramer multiple *post hoc* test.

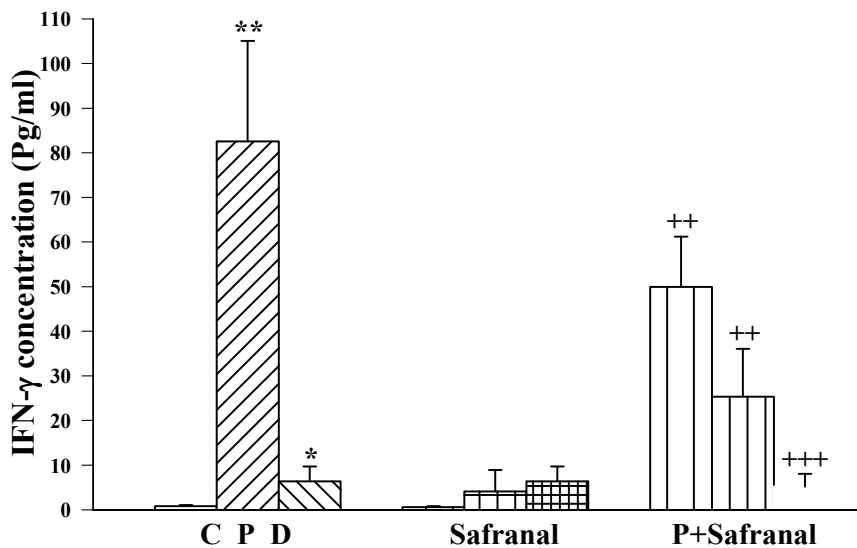


Figure 4. Concentration of IFN- γ (mean \pm SEM, n=9) in supernatant of cultured PBMCs in control group (C), PHA stimulated group (P), dexamethasone treated group (D) and Safranal treated groups in the presence (P + Safranal) and absence (Safranal) of PHA (coarse, medium and fine filled bars are indicators for 0.1, 0.5 and 1 mM concentrations). **, $p < 0.01$, ***, $p < 0.001$, compared to non-stimulated control group. ++, $p < 0.01$, ***, $p < 0.001$, compared to non-treated PHA stimulated cells. The statistical comparisons were made using analysis of variance (ANOVA) with Tukey–Kramer multiple *post hoc* test.

Effect of Safranal on IFN- γ /IL-4 ratio (Th1/Th2 balance). The IFN- γ /IL-4 ratio was significantly increased in treated groups with two higher concentrations of Safranal in both stimulated and non-stimulated conditions compared to control group ($p < 0.05$ to $p < 0.01$), (Figure 5).

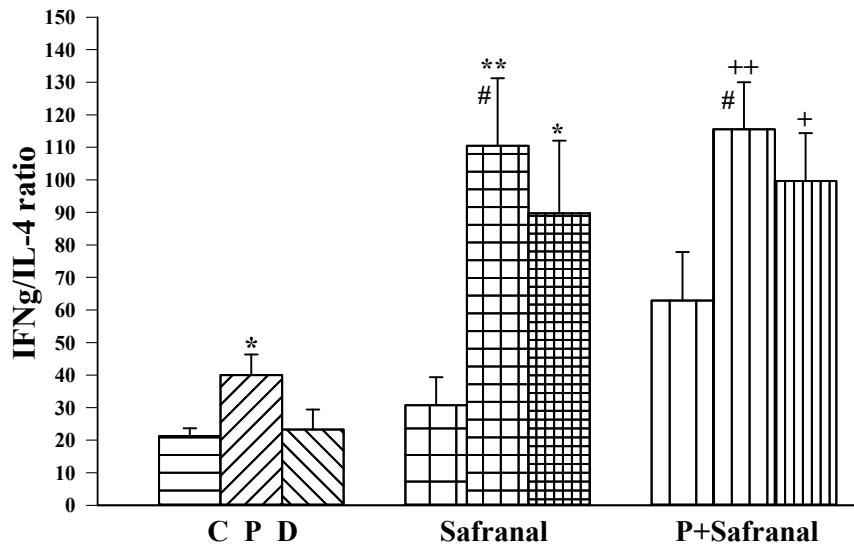


Figure 5. The ratio of IFN- γ /IL-4 (mean \pm SEM, $n=6$) in supernatant of cultured PBMCs in control group (C), PHA stimulated group (P), dexamethasone treated group (D) and Safranal treated groups in the presence (P + Safranal) and absence (Safranal) of PHA (coarse, medium and fine filled bars are indicators for 0.1, 0.5 and 1 mM concentrations). *, $p < 0.05$, **, $p < 0.01$, compared to non-stimulated control group. +, $p < 0.05$, ++, $p < 0.01$, compared to non-treated PHA stimulated cells. #, $p < 0.05$, compared to low Safranal concentration. The statistical comparisons were made using non parametric ANOVA (Kruskal-Wallis) test.

Comparison of the Effects of Safranal between Stimulated and non-Stimulated Lymphocytes. In non-stimulated cells, cell viability in treated groups with all concentrations of Safranal, IL-4 in treated with low concentration and IL-10 in high concentration (1 mM) were significantly lower than those of “stimulated” condition ($p < 0.001$ for cell viability and $p < 0.05$ for IL-4 and IL-10, Table 1).

Comparison of the Effects of Safranal with Dexamethasone. The effects of high concentration of Safranal (1 mM) on cell viability and IL-4 were higher than that of dexamethasone in “non-stimulated” cells ($p < 0.01$ for cell viability and $p < 0.5$ for IL-4, Table 2).

Comparison of the Effects of Three Concentrations of Safranal. The effect of lower concentration of Safranal (0.1 mM) on PBMC viability in “non-stimulated” and “stimulated” cells was significantly lower than medium and high concentrations (0.5 mM and 1.0 mM), ($p < 0.01$ to $p < 0.001$, Table 2).

Table 1. Percent change in the values of MTT, IFN- γ , IL-10 and IL-4 in proportion to control values in cultured PBMCs treated with three Safranal concentrations (S1, S2 and S3 are 0.1, 0.5 and 1 mM Safranal concentrations, respectively) and stimulated cells with PHA (5 μ g/ml), treated with three Safranal concentrations (PS1, PS2 and PS3).

Variable	S1	S2	S3	PS1	PS2	PS3
MTT	-30.08 \pm 8.83	-144.47 \pm 16.89	-235.91 \pm 18.35	-252.91 \pm 41.61 ***	-468.9 \pm 39.03 ***	-700.31 \pm 44.04 ***
IFN- γ	80.27 \pm 146.45	158.82 \pm 66.25	266.87 \pm 73.87	1857.92 \pm 927.09	427.12 \pm 688.69	19275.03 \pm 75.65
IL-4	108.00 \pm 82.03	122.77 \pm 77.84	168.99 \pm 64.84	-40.44 \pm 23.62	-36.17 \pm 25.82	-131.04 \pm 79.24 *
IL-10	-329.79 \pm 85.80	-267.88 \pm 93.15	-255.71 \pm 97.14	-664 \pm 365.17 *	-541.12 \pm 94.60	-264.82 \pm 93.40

Values are presented as mean \pm SEM. For MTT measurement, n=50, for IFN- γ and IL-10, n=9 and for IL-4, n=6. *, $p < 0.05$, ***, $p < 0.001$, compared to the same concentration in non-stimulated lymphocyte. The statistical comparisons were made using unpaired t test.

Table 2. The values of MTT, IFN- γ , IL-10 and IL-4 in cultured PBMCs treated with dexamethasone, three Safranal concentrations (S1, S2 and S3 are 0.1, 0.5 and 1 mM Safranal concentrations, respectively) and stimulated lymphocytes treated with three Safranal concentrations (PS1, PS2 and PS3).

Variable	D	S1	S2	S3	PS1	PS2	PS3
MTT	92.030 \pm 5.959	101.34 \pm 10.51 +++ †††	55.17 \pm 5.75	36.18 \pm 2.46 **	90.23 \pm 8.11 +++ ††	57.24 \pm 7.08	36.1 \pm 4.86
IFN- γ	7.25 \pm 1.947	0.59 \pm 0.24	4.10 \pm 1.35	6.36 \pm 3.348	11.25 \pm 4.23	10.71 \pm 2.96	2.91 \pm 1.15
IL-10	3.522 \pm 0.734	2.33 \pm 1.80	2.11 \pm 1.06	2.211 \pm 1.490	0.716 \pm 0.29	2.82 \pm 2.058	1.1 \pm 0.89
IL-4	2.6 \pm 1.200	12.42 \pm 4.57	12.7 \pm 3.50	19.72 \pm 5.16 *	11.25 \pm 0.99	11.8 \pm 1.08	9.7 \pm 2.230

Values are presented as mean \pm SEM. For MTT measurement, n=50, for IFN- γ and IL-10, n=9 and for IL-4, n=6. *, $p < 0.05$, **, $p < 0.01$, compared to dexamethasone. +++, $p < 0.001$, compared to low Safranal concentration. ††, $p < 0.01$, †††, $p < 0.001$, compared to medium Safranal concentration. The statistical comparisons were made using analysis of variance (ANOVA) with Tukey-Kramer multiple *post hoc* test.

DISCUSSION

The results of this study showed a significant inhibitory effect of high concentration of Safranal (1 mM) on the viability of non-stimulated cultured human PBMCs and all its concentrations on stimulated cells with PHA. The results showed no inhibitory effect of Safranal on IL-4 secretion in both stimulated and non-stimulated PBMCs. However, the ratio of IFN- γ to IL-4 was increased in the presence of two higher Safranal concentrations in both non-stimulated and stimulated cells. The effect of high concentration of Safranal (1 mM) on cell viability and IL-4 were higher than that of dexamethasone in non-stimulated cells. The effect of lower concentrations of Safranal (0.1 mM) on PBMC viability in non-stimulated and stimulated cells was significantly lower than its medium and high concentration.

Therefore, the results of the present study showed that Safranal, a constituent of *Crocus sativus* (saffron) increase IFN- γ /IL-4 ratio in both stimulated and not-stimulated human PBMCs. Safranal also showed potent inhibitory effect on cell viability in both stimulated and not-stimulated cells. Increased IFN- γ /IL-4 ratio indicates that Safranal can stimulate Th1 and/or suppress Th2 lymphocyte subtype. Anti-inflammatory effects of Safranal have also been shown in previous studies (19) which support the findings of the present study.

In many inflammatory diseases including asthma, Th1 and Th2 subtypes of lymphocytes have essential role in the pathological characteristics of the disease (12,13). Therefore, modulation of Th1 and Th2 subtypes has therapeutic effects in these inflammatory diseases. In asthma, one of the immunologic features is disturbance of Th1/Th2 balance. Th2 lymphocytes are activated in asthma, and many features of asthma such as airway inflammation, mucus secretion and airway hyperresponsiveness result from mediators secreted by these cells (14).

Th1 cells inhibit Th2 lymphocytes and thus, one goal of asthma therapy should be shifting to Th1 lymphocyte subtype (15). The inflammatory responses in asthma are regulated by the balance of Th1 and Th2 cells. Th2 cells promote the activity of macrophages and regulate the pro-inflammatory response, whereas Th1 cells inhibit the activity of Th2 and regulate the anti-inflammatory response (20). Th1 cells produce IL-2 and IFN- γ whereas Th2 cells produce IL-4 and IL-10 (21). In fact, the results of the present study show that Safranal enhances the IFN- γ /Th2 ratio, and may indicate stimulation of Th1 cells.

Relaxant effect of Safranal on tracheal smooth muscle, its stimulatory effect on β 2-adrenoceptors (9,10) and its inhibitory effect on histamine (H₁) receptors (12) as well as antitussive effect of Safranal on guinea pigs (17) have been demonstrated previously.

Our recent studies which indicate the anti-inflammatory property of the plant, showed that the extract of *Crocus sativus* and Safranal improve pathological changes as well as total and differential WBC counts in sensitized guinea pigs (22,23). In addition, the results of our other study which was similar to the findings of the present study showed improved Th1/Th2 balance in human PBMCs and in sensitized animals after treatment with extract of *Crocus sativus* (17,25).

The preventive effect of the plant on hematological parameters (26) and pulmonary inflammation (27) in experimental asthmatic animals as well as its cytotoxic effect on HepG-2 and Hep-2 cell lines (28) were also shown. The latter may be due to a decrease in the NO concentration (28). These findings may suggest that the observed effects for the plant is due to its constituent, Safranal. In addition, the effect of Safranal on

endothelin and Th1/Th2 balance in sensitized guinea pigs was demonstrated which support the findings of the present study (29,30).

The effects of Safranal including suppressing PBMCs and reducing IFN- γ /IL-4 ratio were comparable with those of dexamethasone. The effects of Safranal on cell viability were concentration dependent. However, the maximum effect of Safranal on IFN- γ /IL-4 ratio was observed in its medium concentration which may indicate non-selective effect of its high concentration.

Safranal at high concentration possibly leads to decrease in cell viability through mechanisms such as allergy or tolerance in non-stimulated cells, while in stimulated cells it is likely to have synergic effect with mitogens such as PHA. However, this suggestion should be evaluated in further studies.

According to the results of the present study, Safranal could have a therapeutic effect on obstructive pulmonary diseases mainly asthma due to both bronchodilation and changing Th1/Th2 balance.

While the major constituent of the plant is crocin and with regard to its anti-inflammatory and anti-oxidant properties (22), the effect of this constituent of *Crocus sativus* should be also examined on Th1/Th2 balance.

In conclusion, the results of this study show that Safranal, a constituent of *Crocus sativus* can increase IFN- γ /IL-4 ratio which indicate increasing Th1/Th2 balance. Therefore it may have therapeutic effect on inflammatory disorders such as asthma which are associated with Th1/Th2 imbalance.

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