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Crocin Protects Cardiomyocytes against LPS-Induced Inflammation

Running Title: Attenuation of LPS-induced inflammation by crocin.

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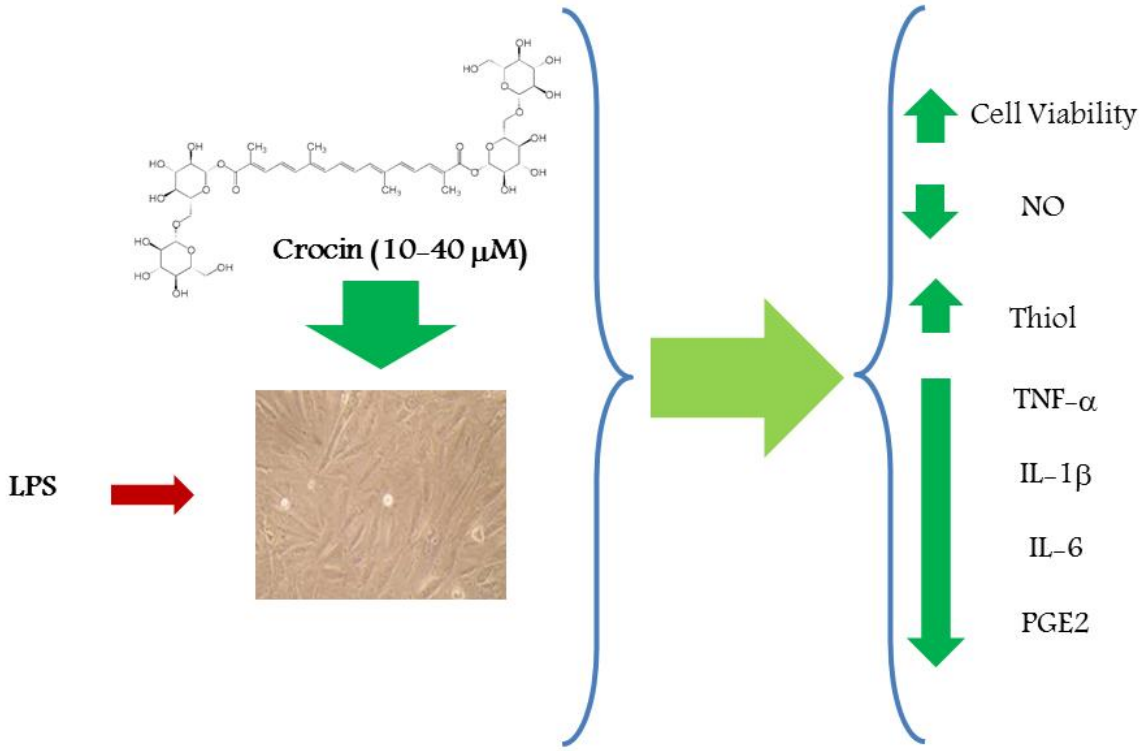
Graphical abstract



Abstract:

Background

Sepsis causes organ dysfunctions via elevation of oxidative stress and inflammation. Lipopolysaccharide (LPS) is the major surface molecule of most gram-negative bacteria and routinely used as a sepsis model in investigation studies. Crocin is an active compound of saffron which has different pharmacological properties such as anti-oxidant and anti-inflammatory. In this research, the protective effect of crocin was evaluated against LPS-induced toxicity in the embryonic cardiomyocyte cell line (H9c2).

Methods

The cells were pre-treated with different concentration of crocin (10, 20 and 40 µM) for 24 h, and then LPS was added (10 µg/ml) for another 24 h. Afterward, the percentage of cell viability

and the levels of inflammatory cytokines (TNF-α, PGE_2, IL-β, and IL-6), gene expression levels (TNF-α, COX-2, IL-β, IL-6, and iNOS), and the level of nitric oxide (NO) and thiol were measured.

Results

Our results showed that LPS reduced cell viability, increased the levels of cytokines, gene-expression, nitric oxide, and thiol. Crocin attenuated the LPS-induced toxicity in H9c2 cells via reducing the levels of inflammatory factors (TNF-α, PGE_2, IL-β, and IL-6, p <0.001), gene expression (TNF-α, COX-2, IL-β, IL-6, and iNOS, p <0.001), and NO (p <0.001), whereas increased the level of thiol content (p <0.001).

Conclusion

The observed results revealed that crocin has preventive effects on the LPS induced sepsis and its cardiac toxicity in-vitro model. Probably, these findings are related to anti-inflammatory and anti-oxidant properties of crocin. However, performing further animal studies are necessary to support the therapeutic effects of crocin in septic shock cardiac dysfunction.

Keywords: Sepsis; Lipopolysaccharide; Inflammation; H9c2; cardio-toxicity.

Introduction

Sepsis is a problem which leads to more than 30% mortality in the world [1]. There were several investigation notions that sepsis influences most of the organs especially on cardiac function disturbance which is common [2, 3]. Pro-inflammatory cytokines play a key role in the pathogenesis of sepsis. In this status, the immune system is stimulated and the inflammatory process is initiated [4]. Release of the inflammatory mediators leads to increase of vascular permeability, cardiac depression, and disruption of the coagulation system [4]. The myocardial function and structure are damaged by sepsis via elevation of inflammatory factors [5]. Some of novel inotropes and β-blockers may reduce the septic-induced myocardial dysfunction via reduction of heart rate and consumption of oxygen [5]. Indeed, the inflammation-interleukin (IL)-nitric oxide (NO)-reactive oxygen species (ROS) axis, could play a crucial role in the pathogenesis of cardiotoxicity [6, 7]. LPS is found in the outer membrane of gram-negative bacteria, it produces many types of mediators involved in septic shock. The toll-like receptor 4 (TLR-4) is widely expressed in the body including cardiomyocytes [8]. Alternatively, LPS binds to TLR-4 in cardiomyocytes and leads to secretion of TNF-α, IL-6, adhesion molecule-1 (ICAM-1) and inducible nitric oxide synthase (iNOS) via the activation of nuclear factor (NF)-κB [9, 10]. iNOS causes to production of nitric oxide which has an important role in septic shock [11], which eventually result in the depression of cardiac function [12]. Different investigations have shown that some of the natural products have a role in the reduction of LPS-induced toxicity [3, 7, 13, 14]. Crocin as a carotenoid is found in herbs such as *Crocus sativus* and *Gardenia jasminoides Ellis* [15]. This compound has different pharmacological properties, such as anti-hyperlipidemic, anti-atherosclerotic, anti-inflammatory, antitumor, scavenge of free radical, anti-atherosclerotic and antioxidant [16]. Also, it has beneficial effects on neuronal diseases such as Alzheimer's disease [17], memory loss [18], depression [19] and anxiety [20]. Other studies have shown cardioprotective effects of crocin involving, reduction of myocardial injury [21], improved isoproterenol-induced myocardial infarction [22] and decreasing of doxorubicin-induced myocardial injury [23]. Also, crocin reduced ischemia-reperfusion in isolated rat hearts via improvement of anti-oxidant capacity [24].

H9c2 cells are morphologically similar to immature embryonic cardiomyocytes. In fact, H9c2 cells have electrical and hormonal signaling pathways similar to adult cardiac cells; therefore these cells are considered an appropriate model for investigation of oxidative stress and

inflammation-induced cardiomyocyte damages [25, 26]. Given the anti-inflammatory and anti-oxidant nature of crocin, we investigated its possible protective effects against LPS-induced toxicity in H9c2 cardiomyocytes.

Materials and methods

Reagents and kits

DMEM culture media, penicillin plus streptomycin (pen/strep), amphotericin B, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), and LPS (*Escherichia coli* O55:B5 purified by phenol extraction, L2880 SIGMA) and other cell culture materials were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Proliferation assay kit (MTT), and ELISA kits (PGE_2, IL-6, IL-1β, and TNF–α) were provided from Roche Diagnostic (Mannheim, Germany) and eBioscience (San Diego, CA, USA), respectively. All other materials were of analytical and cell culture grade, which prepared from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Crocin was also purchased from Pure Crocin Co. (Mashhad, Iran).

Cell viability assay

The cell viability of H9c2 cells was evaluated in the presence of crocin (5-80 µM, [27, 28]) alone or in the presence of LPS stimulation (10 µg/ml, [26, 28]). In this assay, about 6000 cells were seeded in 96-wells plate. The cells were treated with different concentrations of crocin alone for 48 h, after this time, cell viability was evaluated by MTT assay. For the protective assay, the cells were pre-treated with crocin (5-80 µM) for 24 h, and then LPS was added, after 24 h, cell viability was determined via MTT assay. 10 µl of MTT solution (5 mg/ml) was added to each well, and then kept in the incubator for 3 h. After, this time, 100 µl of DMSO was added to each well for solving of formazan crystals. The absorbance was read by ELISA reader (Awareness Inc, USA) at 570 nm and 620 nm [3, 29, 30].

Experimental protocol

To evaluate the protective effects of crocin on LPS-induced inflammation and given its chemical structure, we used 24 h pre-incubation to monitor the changes in gene expression and in the level of pro-inflammatory and inflammatory factors, following 24 h LPS stimulation [28, 31, 32]. In

the present study, inflammation was induced using LPS (10 µg/ml), in H9c2 cells. Grouping was also performed as follows;

Group 1; H9c2 cells + vehicle (control group, this group received completed media culture and the same condition of others without LPS),

Group 2; H9c2 cells + vehicle incubation for 24 h then LPS (10 µg/ml) for 24 h

Group 3; H9c2 cells + highest concentration of crocin (40 µM) for 48 h

Groups 4-7; H9c2 cells + crocin (10, 20 and 40 µM) incubation for 24 h then added LPS (10 µg/ml) for another 24 h

Crocin solutions were prepared according to dissolving in DMSO and serially diluted with a complete medium in which the final concentration of DMSO was lower than 0.1 % v/v for all experiments. Crocin concentrations were selected according the preliminarily study (MTT assay) and previous reports on the protective effects of crocin (10-40 µM) [27, 32].

Assessment of Cytokines levels

Inflammatory cytokines consisting of TNF-α, IL-1β, and IL-6, as well as PGE_2, were assessed using sandwich ELISA method according to the manufacturer manual. In summary, the cells were cultured at a density of 10^6 cells per each 6-well plate and incubated with different concentrations of Crocin (10, 20 and 40 µM) according to the experimental design section. After that, the supernatant was collected and different cytokines were measured [3, 33].

Quantitative PCR for gene expression

To evaluate the effect of different concentrations of crocin on the gene expression, the levels of related mRNA of TNF-α, IL-1β, IL-6, and COX-2 as well as iNOS were also examined. GAPDH was considered a reference gene. The real-time PCR primers were obtained according to the previous studies designed with mRNA sequences [34]. The primers sequences were addressed in Table 1. The real-time PCR reactions were performed in glass capillaries (Qiagen, Germany) in a final volume of 10 µL containing 2 µL cDNA template, 0.4 µL of primer pairs (200 nM), and 5 µL of the SYBR green Master Mix. The Rotor-Gene 6000 software (Corbett Research, Australia) was used to analyze the standards and the unknown RNA copy numbers. The relative quantity of each mRNA was normalized to the relative quantity of GAPDH mRNA. The PCR conditions were as follows: 95 °C for 3 minutes, followed by 45 cycles of 95 °C for 20

seconds, annealing temperature (55-65 °C) for 5 seconds, and 72 °C for 10 seconds [34]. The values for gene expression levels were examined using the ΔCt method and fold-change values were reported as $2^{-(\Delta\Delta Ct)}$.

Determination of the level of nitric oxide and total GSH content

The amount of nitric oxide metabolites were measured as a concentration of produced nitrite using the method of Griess as described previously [33, 35-37]. The supernatants collected for cytokines assay were also used to examine the concentration of nitrite produced by the cells at 540 nm using Griess reagent (G4410 SIGMA) in a spectrophotometer. The concentration of nitrite was determined using sodium nitrite standard curve [36, 38]. Total intracellular amount of GSH was measured using commercial biochemistry kits (ZB-GSH-96A) ZellBio Company, Germany.

Statistical Analysis

Data were analyzed using GraphPad Prism ® 6 (GraphPad Software, San Diego, CA) software and presented as means±SEM. Comparisons between the groups were done using one-way analysis of variance (ANOVA) with Tukey-Kramer *post-hoc* multiple comparisons test. Statistically, *p* values (p) lower than 0.05, 0.01 and 0.001 were considered significant differences.

Results

Effects of crocin and LPS on cell viability

The cells were incubated with different concentrations of crocin (5-80 µM) for 48 h and cell viability was evaluated after this time. Crocin alone (5-80 µM) had no cytotoxicity effects on the cell viability (Figure 1a). As shown in figure1b, cell viability was decreased in the presence of LPS ($p<0.001$ *vs*. control). Pretreatment of the cells with crocin (5-80 µM) for 24 h decreased the LPS-induced toxicity compared to the LPS group.

Effects of crocin and LPS on the levels of TNF-α, PGE_2, IL-1β, and IL-6

As illustrated in Figures 2a-d, LPS significantly increased the levels of TNF-α (p<0.001), PGE2 (p<0.001), IL-1β (p<0.001) and IL-6 (p<0.001) in comparison to the control group. Our results revealed that crocin (10-40 μM) significantly reduced the levels of TNF-α (p <0.001, Fig.2a), PGE2 (p <0.001, Fig.2b), IL-1β (p <0.001, Fig.2c), and IL-6 (p<0.001, Fig.2d) compared to the LPS group in a dose-dependent manner.

Effect of Crocin on gene expression levels of TNF-α, COX-2, IL-1β, IL-6 and iNOS

We found that LPS (10 μg/ml) significantly increased the gene expression levels of TNF-α (p<0.001, Fig.3a), COX-2 (p<0.001, Fig.3b), IL-1β (p<0.001, Fig.3c), IL-6 (p<0.001, Fig.3d), and iNOS (p<0.001, Fig.3e) comparing to the control group. Interestingly, crocin (10-40 μM) significantly reduced the gene expression levels of TNF-α (p <0.001, Fig.3a), COX-2 (p <0.001, Fig.3b), IL-1β (p <0.001, Fig.3c) and IL-6 (p <0.001, Fig.3d) as well as iNOS (p <0.0001, Fig.3e) compared to the LPS group.

Effects of crocin and LPS on the levels of NO and thiol (GSH)

As shown in figure 4a, LPS significantly increased NO level in comparison to the control (p<0.001), while crocin (10-40 μM) notably reduced NO level following the LPS stimulation (p <0.001). On the other hand, LPS markedly reduced the level of thiol, which pretreatment with different concentrations of crocin (10-40 μM) significantly increased the thiol level in comparison to the LPS group (p <0.001, Fig.4b).

Discussion

To the best of our knowledge, this is the first study evaluating the protective effects of crocin in LPS-induced cardiomyocytes toxicity as a model of septic shock heart injury. Our findings indicated that LPS considerably reduced the cell viability of H9c2 cardiomyocytes, and increased the inflammatory cytokines TNF-α, PGE_2, IL-1β, and IL-6 and the expression of related genes. Intriguingly, we revealed that 24 h pre-incubation with crocin increased the cell viability,

inflammatory mediators, and NO production, and elevating the anti-oxidant defense system thiol group.

Evidence shows that LPS can interact to TLR-4 and provide an overexpression and over activation of transcriptional factors including NF-κB, Activator protein 1 (AP-1) and mitogen-activated protein kinase (MAPK). In this context, transcription and translation of pro-inflammatory cytokines are markedly elevated [39-44]. Therefore, in the present study, we measured the levels of pro-inflammatory cytokines in both protein and gene expression levels. We found that crocin notably reduced both protein and gene expression levels of inflammatory cytokines, in a concentration dependent manner. In agreement with our findings, it has been reported that crocin decreases the activation and expression of NF-κB and lead to a reduction in the levels of pro-inflammatory cytokines [31]. On the other hand, crocin has shown several anti-inflammatory properties through the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), which leads to the up-regulation and down-regulation of anti-inflammatory (IL-10) and inflammatory cytokines, respectively [31]. Interestingly, it has been also shown that the main part of protective effects of crocin is associated with the activation of Nrf2 transcription factor because the uses of Nrf2 siRNA and HO-1 antagonist (ZnPP) markedly abolished the protective effects of crocin [31]. In another study, in the presence of LPS stimulation, crocin also decreased the level of NF-κB activation and reduced both protein and gene expression levels of inflammatory cytokines [32]. Taken together, crocin likely decreases pro-inflammatory cytokines through the inhibition of inflammatory transcriptional factors including NF-κB and activation of Nrf2.

Under sepsis condition, myocardial dysfunction occurs as heart failure and adverse left ventricular remodeling [39]. Furthermore, it has been reported that LPS exposure has an important role in the pathogenesis of cardiomyopathy [40]. LPS acts via TLR-4 and NF-κB signaling pathways leading to the overexpression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α [41-44]. The studies have reported that the elevation of TNF-α level leads to negative inotropic effects [39]. In this regard, it has been shown that the short term administration of anti-TNF-α improves the cardiac function in sepsis patients [45]. In addition, elevation of the inflammatory cytokines can cause heart failure via increasing the occurrence of apoptosis in cardiomyocytes [46]. Cardiomyocytes have an important role in the function of the heart; however, their apoptosis leads to dysfunction of myocardial contractile and cardiac output

attenuation [47]. Several studies have reported that oxidative stress and especially ROS over-production occurs following the sepsis condition [48]. However, recent studies have shown cardio-protective effects of medicinal plants against toxic agents via reduction of inflammatory pathway and elevation of anti-oxidant capacity [49, 50].

In our experiment, crocin reduced the inflammatory cytokines in H9c2 cells in a concentration-dependent manner. There were different studies shown that exposure to the LPS increases the expression level of COX-2 producing and releasing the prostaglandins (e.g. PGE2) which are responsible for inflammatory symptoms [51]. In our study, crocin also reduced the expression levels of COX-2 and the levels of its inflammatory consequent product PGE_2. It has been shown that endotoxemia and septic shock increase the levels of inducible nitric oxide synthase (iNOS) and NO generation [52]. In this context, the endothelium-dependent vasodilatory response is attenuated due to NO increase and down-regulation of endothelial NOS (eNOS) [52]. Overexpression of iNOS leads to myocardial depression and cardiomyocyte apoptosis [53]. In addition, increasing of NO causes oxidative stress via production of peroxynitrites and other reactive nitrogen species (RNS) [54], which react with amino, thiol (SH), diazo, and tyrosyl moiety groups, as well as with heme or sulfur centers structures [55]. Therefore, the decreased level of thiol by LPS may be a result of NO over-production.

In the current study, crocin decreased the levels of iNOS, NO production while increasing the levels of thiol. According to these results, protective effects of crocin may be mediated via inhibition of inflammatory cytokines, downregulation of iNOS, COX-2 levels and reduction of oxidative stress which confirmed with recent studies [15, 56, 57]. *In vivo* studies have also revealed that treatment with crocin improves the myocardial function following the doxorubicin toxicity [23] and isoprenaline [22]. Crocin attenuated ischemia-reperfusion-induced oxidative stress in isolated rat hearts by regulation of iNOS, eNOS, and improvement of the anti-oxidant system [24, 58]. Furthermore, crocin alleviated the myocardial infarction by suppression of the inflammatory cytokines and the apoptosis levels as well as the regulation of iNOS expression [59, 60]. In another study, it has been also reported that treatment with crocin enhances myocardial function following streptozotocin (STZ)-induced diabetes via preventing the apoptosis [61].

A study demonstrated that crocin reduced LPS-induced inflammation in a murine macrophage cell line, RAW264.7, by inhibition of the nuclear translocation of NF-κB, and PGE_2 production

[57]. Also, crocin prevented NF-κB activation, reduction of NO, TNF-α, IL-1β, and ROS generation in rat brain microglial cells following the LPS stimulation [15]. Another study reported a protective effect of crocin against LPS-induced toxicity in retinal ganglion cells and BV2 microglial cells via modulation of inflammatory factors [62]. Moreover, crocin dose-dependently reduced oxidative stress in neurons via increasing the anti-oxidant enzymes, in both *in vitro* and *in vivo* studies [63, 64]. Therefore, the protective effects of crocin against LPS-induced inflammation can be related to its anti-oxidant and anti-inflammatory activities.

Conclusion

Results of the present study have showed that crocin an active ingredient of *Crocus sativus* had not toxicity effects on H9c2 cells and increased LPS-induced cell viability. The protective effect of crocin against LPS is related to the inhibiting of inflammatory factors synthesis and attenuating the oxidative stress. Therefore, crocin may have beneficial effects in the prevention or reduction of sepsis cardiomyopathy, although further in-vivo studies are still required to confirm its protection.

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Conflict of interest

The authors declare no conflict of interest**.**

Declaration of transparency and scientific rigor

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigor of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

Ethical Disclosure

This is an in-vitro (cell line) study. It is not applicable.

Declaration of Interest Statement

The authors (Vafa Baradaran Rahimi, Mohammad Taghi Khammar , Hassan Rakhshandeh, Alireza Samzadeh-Kermani, Azar Hosseini and Vahid Reza Askari) contributing to providing this article declare that agreed for submitting and publishing this article. Furthermore, the authors absolutely declare that there is no conflict of interest. The authors agreed that any subsequent publication of the Submission will credit the Journal as the site of first publication and provide a link to the Journal website.

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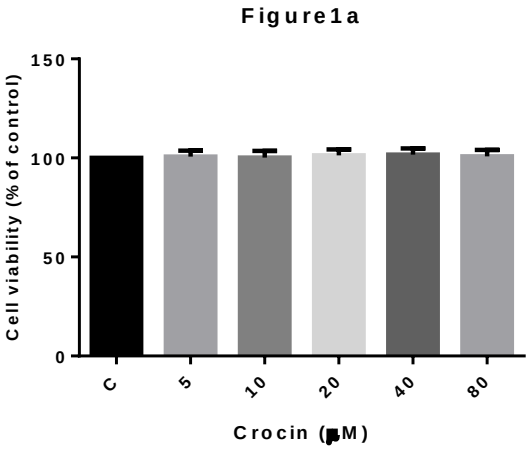
Legends



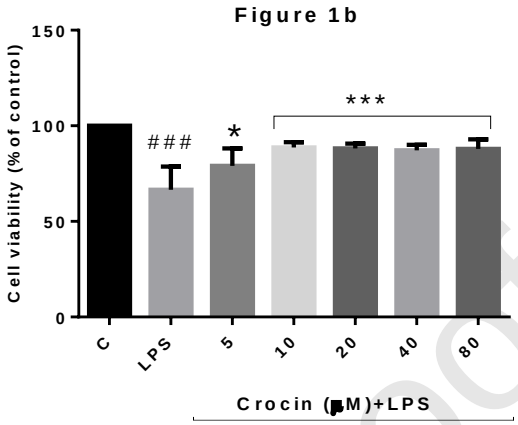
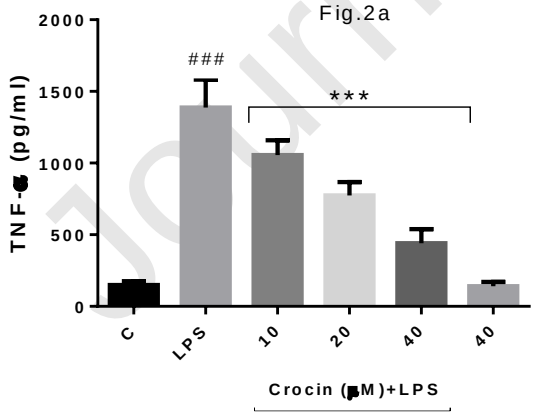
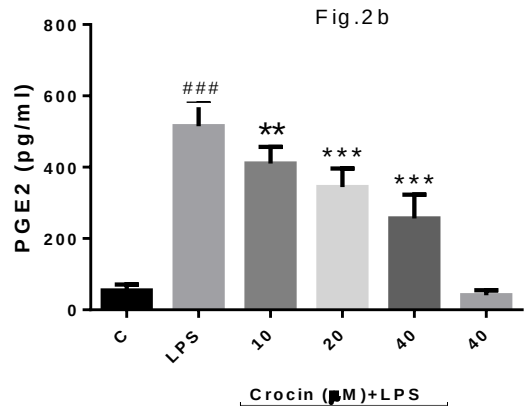
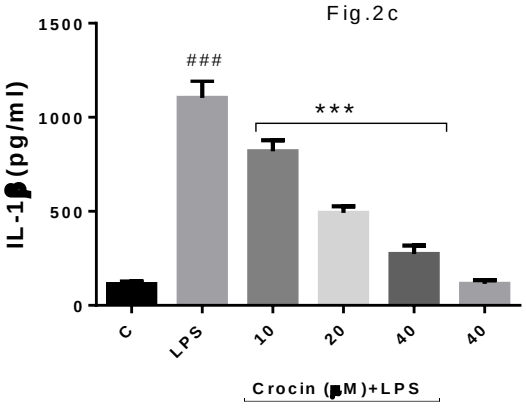


Figure1. Effect of crocin on cell viability without (Fig.1a) or with (Fig.1b) LPS; The cell viability was evaluated in the presence of crocin alone (Fig.1a), also cells were pretreated with different concentrations of crocin for 24 h and then exposed to LPS (10 µg/ml) for 24 h (Fig.1b). The cell viability was evaluated by MTT assay. Data are mean ± SEM (n = 3). ### $p < 0.001$ *vs.* control, * $p< 0.05$ and *** $p< 0.001$ *vs.* LPS.







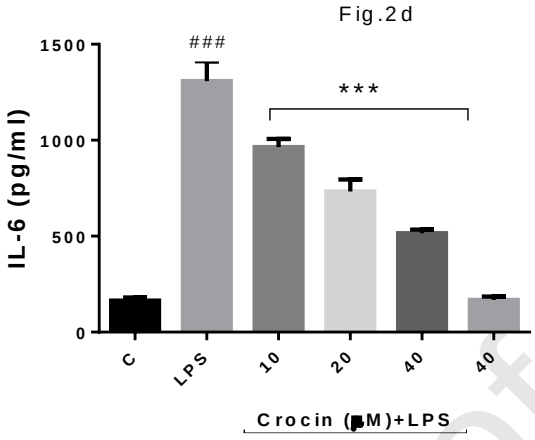
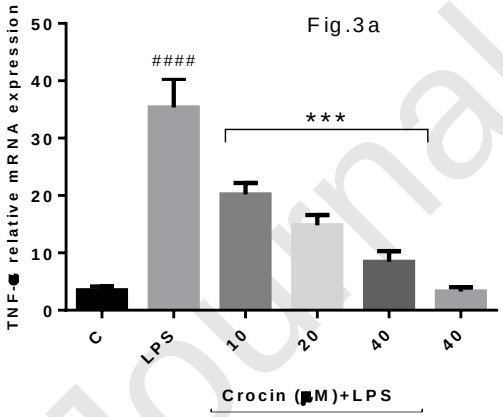
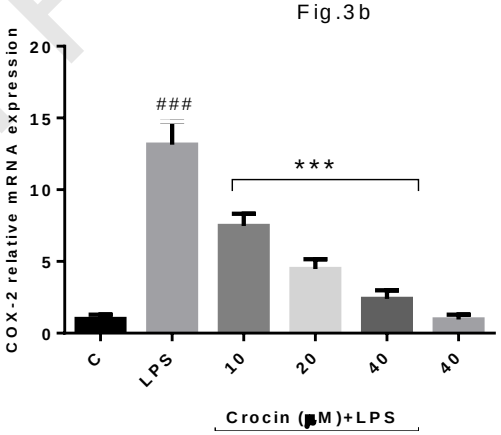
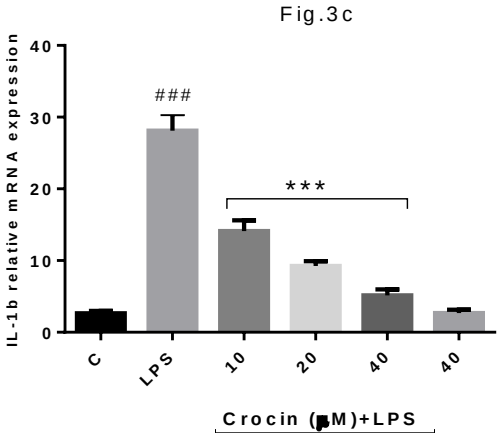
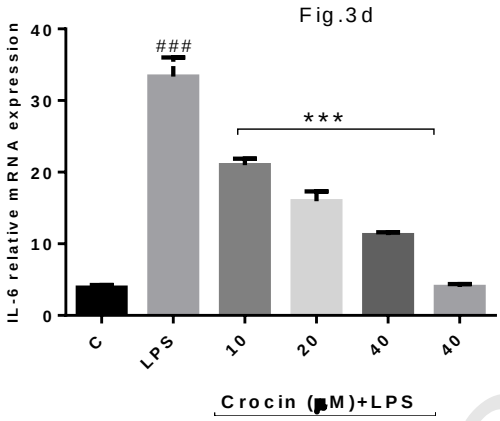


Figure2. Effect of crocin and LPS on the levels of TNF-α, PGE2, IL-1β, and IL-6; the cells were pretreated with crocin for 24 h and then incubated with LPS for 24 h. After 24 h, the levels of TNF-α, PGE2, IL-1β, and IL-6 were determined in presence of LPS. Data are mean ± SEM (n = 3). $^{###}p < 0.001$ *vs.* control, $^{**}p< 0.01$ and $^{***}p< 0.001$ *vs.* LPS.









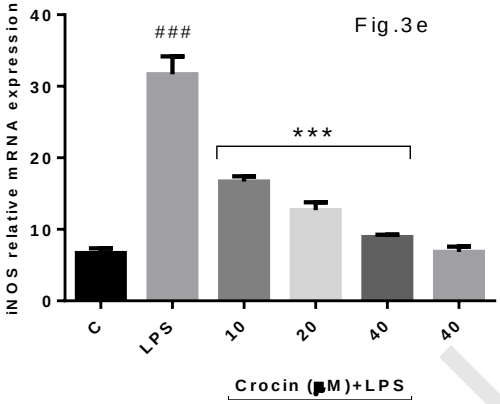


Figure3. Effects of crocin and LPS on the levels of gene expression of TNF-α, COX-2, IL-1β, IL-6, and iNOS; The cells were pretreated with crocin for 24 h then incubated with LPS for 24 h. After 24 h, the levels of gene expression of TNF-α, COX-2, IL-1β, IL-6, and iNOS were determined in presence of LPS. Data are mean ± SEM (n = 3). $^{###}p < 0.001$ *vs.* control and $^{***}p < 0.001$ *vs.* LPS.

Fig.4a

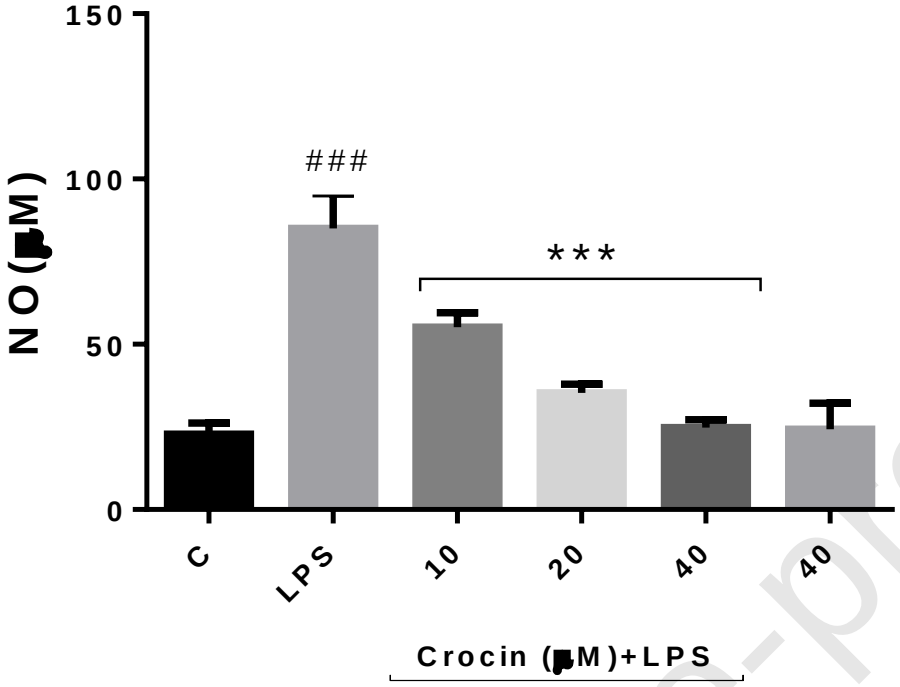


Fig.4b

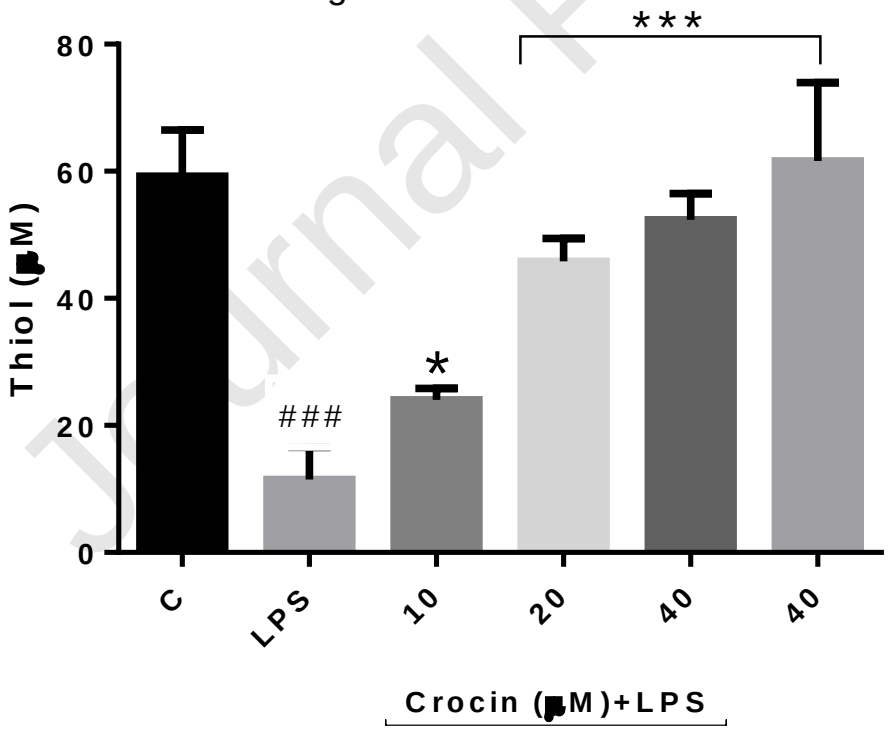


Figure4. Effect of crocin on NO and thiol in presence of LPS; The cells were pretreated with crocin for 24 h then incubated with LPS for 24 h. After 24 h, the levels of NO and thiol were measured in presence of LPS. Data are mean ± SEM (n = 3). $^{###}p < 0.001$ *vs.* control, $^{*}p< 0.05$ and $^{***}p< 0.001$ *vs.* LPS.

Table 1: The primers for Real-time PCR [34]

Gene name	5'-3' primer sequence		Accession Number
IL-1β	FW	CACCTCTCAAGCAGAGCACAG	M98820
	RW	GGGTTCCATGGTGAAGTCAAC	
IL-6	FW	TCCTACCCCAACTTCCAATGCTC	E02522
	RW	TTGGATGGTCTTGGTCCTTAGCC	
TNF-α	FW	AAATGGGCTCCCTCTCATCAGTTC	X66539
	RW	TCTGCTTGGTGGTTTGCTACGAC	
COX-2	FW	TGTATGCTACCATCTGGCTTCGG	S67722
	RW	GTTTGGAACAGTCGCTCGTCATC	
iNOS	FW	CATTGGAAGTGAAGCGTTTCG	L12562
	RW	CAGCTGGGCTGTACAAACCTT	
GAPDH	FW	GTATTGGGCGCCTGGTCACC*	AB017801
	RW	CGCTCCTGGAAGATGGTGATGG	

FW forward primer; RW reverse primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.