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Research article

Crocin attenuates the lipopolysaccharide-induced neuroinflammation via expression of AIM2 and NLRP1 inflammasome in an experimental model of Parkinson's disease

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ABSTRACT

The underlying mechanisms of inflammasome activation and the following dopaminergic neuron loss caused by chronic neuroinflammation remain entirely unclear. Therefore, this study aimed to investigate the impact of crocin on the inflammasome complex within an experimental model of Parkinson's disease (PD) using male Wistar rats. PD was induced by the stereotaxic injection of lipopolysaccharide (LPS), and crocin was intraperitoneally administrated one week before the lesion, and then treatment continued for 21 days. Open field (OF) and elevated plus maze tests were applied for behavioral assays. Furthermore, hematoxylin and eosin (H&E) and immunostaining were performed on whole brain tissue, while dissected substantia nigra (SN) was used for immunoblotting and real-time PCR to evaluate compartments involved in PD. The time spent in the center of test was diminished in the LPS group, while treatment with 30 mg/kg of crocin significantly increased it. H&E staining showed a significant increase in cell infiltration at the site of LPS injection, which was ameliorated upon crocin treatment. Notably, crocin-treated animals showed a reduced number of caspase-1 and IL-1β positive cells, whereas the number of positive cells was increased in the LPS group ($P < 0.05$). A significant decrease in tyrosine hydroxylase (TH) expression was also found in the LPS group, while crocin treatment significantly elevated its expression. IL-1β, IL-18, NLRP1, and AIM2 genes expression significantly increased in the LPS group. On the other hand, treatment with 30 mg/kg of crocin significantly downregulated the expression levels of these genes along with NLRP1 ($P < 0.05$). In summary, our findings suggest that crocin reduces neuroinflammation in PD by diminishing IL-1β and caspase-1 levels, potentially by inhibiting the expression of AIM2 and NLRP1 genes.

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1. Introduction

The second most prevalent progressive neurological disorder is Parkinson's disease (PD). The neuropathological hallmark of PD is marked by depletion of dopaminergic neurons(DA) in the pars compacta of the substantia nigra (SN) area, ultimately resulting in

Abbreviation

AIM2	Absent in melanoma 2
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
CARD domain	Caspase activation and recruitment domain
PD	Parkinson's disease
LPS	Lipopolysaccharides
NDD	Neurodegenerative diseases
NF-kB	Nuclear factor kappa light chain enhancer B
NLRC4	CARD domain-containing protein 4
NLRP1	Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) protein 1
NLRP2	NOD-like receptor protein 2
OFT	Open field test
TH	Tyrosine hydroxylase
SNc	Substantia nigra pars compacta
α-Syn	Alpha-synuclein

bradykinesia, stiffness, resting tremor, and cognitive impairment [1].

Recently, several theories have been proposed to explain the etiology of PD, such as chronic neuroinflammation, aging, oxidative stress, and mitochondrial damage [2,3]. However, the connection between activation of inflammasome and loss of dopaminergic neurons and chronic neuroinflammation is not entirely defined. Since compelling evidence indicates the correlation between neuroinflammation and dopaminergic degeneration, current investigations focus on the role of inflammasomes [4,5]. New findings have revealed that high levels of pro-inflammatory cytokines such as IL-1β and IL-18, can damage dopaminergic neurons in the SN. Furthermore, as the release of pro-inflammatory cytokines could trigger the cascades of neural death, the inflammasome complex carefully regulates it [4].

Nucleotide-binding oligomerization domain (NOD)-like receptor protein 1 (NLRP1), NLRP3, CARD domain-containing protein 4 (NLRC4; or IPAF), and absent in melanoma 2 (AIM2) are the most well-characterized inflammasomes. These inflammasomes mediate the breakdown of IL-1β and IL-18 [4,6]. Upon sensing the danger signal by the NOD-like receptor (NLR), autocatalytic cleavage of caspase-1 results in the release and processing of IL-1β and IL-18 [7]. The NLRP3 inflammasome can be activated by pathogenic misfolded α-synuclein aggregates, leading to PD neurotoxicity [8]. However, role of other inflammasome complexes, including NLRP1 and AIM2 in PD development is not well understood. To activate caspase-1, the cytoplasmic DNA receptor (AIM2) forms an inflammasome complex with its ligand and apoptosis-associated speck-like protein, which contains a C-terminal caspase recruitment domain (ASC) [9]. Evidence from animal and clinical studies plus epidemiological investigations in PD patients support the involvement of increased levels of pro-inflammatory mediators that contribute to neuronal cell death via inflammasome activation [10–12].

Despite numerous efforts, no effective treatment for PD has been found yet. Current dopaminergic treatments mainly focus on symptom alleviation and slowing down disease progression. Several studies have highlighted the beneficial impacts of crocin in neurodegenerative diseases due to its anti-inflammatory, antioxidant, and anti-apoptotic activities. Crocin is a cost-effective and safe natural product extracted from saffron, effectively crosses the blood-brain barrier (BBB), and has demonstrated promising neuroprotective properties [10,13]. Nam et al. revealed that crocin can inhibit NF-κB activation, and the production of nitric oxide and lipopolysaccharides (LPS)-induced tumor necrosis factor-α (TNF-α) and IL-1β in microglial cells [14]. In addition, Erdemli ME et al. confirm the ameliorating effects of crocin on the inflammation in periodontitis-induced kidney damage [15]. Another study demonstrated that administering crocin can prevent inflammatory processes and neuronal dysfunction induced by lipopolysaccharides (LPS) in the hippocampus [16]. Crocin also diminishes anxiety and depressive-like symptoms in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD [17]. However, the effects of crocin on inflammasome activation in neurodegeneration remain unclear.

Inflammasome products have recently been introduced as therapeutic targets for most neurodegenerative diseases (NDDs). Therefore, identifying inflammasome-driven neuroinflammation in PD could have significant implications for future research. Hence, this study was conducted to evaluate role of inflammasome pathways, including NLRP1, NLRP3, NLRC4, AIM2, and ASC, in LPS-induced PD. We provide evidence that increased gene expression levels of AIM 2 in our PD rodent model involve chronic neuroinflammation. Additionally, we revealed the effect of crocin treatment on the inflammasome and pro-inflammatory cytokines expression.

2. Materials and methods

2.1. Animals

Based on previous research and due to their higher susceptibility to LPS-induced neuroinflammation only adult male Wistar rats (200–250 g) were used in this study [18]. The rats were kept under 12 h light-dark cycles, 25 ± 2 °C with water and food accessible. According the National Institute of Health's Guide for the Care and Use of Laboratory Animals all experimental procedures were approved by the Animal Ethics Committee of the Babol University of Medical Sciences (Documentation No IR.MUBABOL.HRI.REC.1398.163).

2.2. Surgical procedure and intracerebral injection of LPS

After anesthesia with intraperitoneal (i.p) injection of Xylazin (10 mg/kg) and Ketamin 60 mg/kg, rats were secured onto a stereotaxic instrument (Stoleting, USA). The coordinates for the injection of LPS or saline into the left substantia nigra pars compacta (left SNc) were determined based on the Paxinos rat brain atlas and previously published protocols [19,20]. The stereotaxic apparatus with a Hamilton syringe enabled the delivery of LPS or saline into the desired location of the rat brain. The injection was performed at the following coordinates: AP = −5.5, ML = −1.8, DV = −8.3 from the skull. The injection speed was set to 0.5 μl/min and waited for 5 min after injection to minimize reflux along the injection track. Following recovery, the rats were placed back in a controlled environment.

2.3. Experimental design

The rats were randomly allocated into five groups (n = 10/group), including control, LPS, crocin-treated 15, 30 mg/kg groups, and sham. Rats in the control group received saline. The LPS-treated lesioned group received a single intranigral injection of 2 μl of LPS (1 μg/μl) in the SN area using a micro-dosing system (LPS group). The LPS concentration for unilaterally injection into the left SN was selected based on a previous study to induce a rat model of PD based [21]. The crocin-treated lesioned groups received crocin for one week before micro-injection and three weeks afterward. The protocol for crocin administration, involving pre- and post-injection, was based on the study by Azmand et al. that demonstrated the anti-inflammatory and antioxidant properties of crocin [22]. In the low-dose crocin-treated group, 15 mg/kg of crocin was administrated i.p. before and after LPS (1 μg/μl) micro-injection. In the high-dose treated group, rats received 30 mg/kg of crocin following the same order. Rats in the sham group received 30 mg/kg of crocin before and after micro-injection but received saline injection instead of LPS.

2.4. Behavioral assessment through the open field and elevated plus maze

Behavioral assessments were conducted three weeks after the treatment to determine motor and non-motor dysfunctions. Thirty minutes before each test, animals were transported to the testing site to adapt to the new surroundings. To assess the movement performance of the PD rats following the LPS injection, an OF test was performed on day 21. Every rat was positioned in the OF apparatus' central square (50*50*40 cm), as described by Dayane Pessoa et al. [23]. The rats were permitted to explore around the arena for 5 min while their motor coordinates were documented. During observation, the activity was monitored using data collection for velocity, distance moved, and cumulative duration in the central zone. The distance traveled by the rat in the open field is measured to assess its general activity level, while preference for the walls of the field indicated anxiety levels. The OF activity was recorded using a video camcorder (Sony Handycam) and later analyzed by Ethovision software.

Elevated plus maze was performed to monitor the number of entries into and the amount of time spent in the closed and open arms, as well as the latency to enter the open arms. The time spent in the open arms of the maze serves as an indicator of anxiety-like behavior, whereas a reduced time in the open arms suggests elevated levels of anxiety. The test was carried out as described previously by Walf and Frye [24]. In detail, the apparatus is constructed up of a central platform from with two open arms and two closed arms (approximate 50 × 10 × 40 cm) extended. Every animal was positioned in the center and allowed to explore the maze. The Ethovision software recorded the behavior for 10 min for later analysis.

2.5. Assigning of rats for pathological and molecular experiments

Upon completion of the treatment protocol, 10 rats in each group were sacrificed and randomly subdivided into three subgroups. For immunofluorescence staining four rats from each group were designated, while two rats were allocated for hematoxylin and eosin (H&E) staining. Additionally, four rats from each group were designated for real-time PCR and western blotting.

2.6. Rat brain histopathologic analysis

Rats were deeply anesthetized, and following sacrifice by rapid decapitation, their brain tissues were extracted, fixed in 10 % formalin, embedded in paraffin, and sliced into serial coronal sections (5 μm) using a microtome (Leica RM 2135, Germany). After deparaffinization with xylene, the sections were hydrated with different concentrations of alcohol, for 4 min stained by hematoxylin, followed by xylene clearing. The sections then underwent a washing process and were counterstained with eosin for 1.5 min. Following

another wash, they were covered and captured on camera for further examination. An expert in pathology evaluated the intensity of inflammatory cells while remained blinded to experimental groups.

2.7. IL-1β and caspase-1 immunofluorescence staining

Rats underwent deep anesthesia and were intracardially perfused with 0.1 M phosphate-buffered saline (PBS), then 4 % paraformaldehyde in 0.1 M PBS (H of 7.4). The rats' brains were carefully removed from the skull and then immersed in 4 % paraformaldehyde (PFA). Rat brain tissues were preserved for a night using 20 % sucrose/PBS solution, followed by embedding in an optimum cutting temperature compound (OCT compound), and stored at −80 °C. The coronal slices of the SN with a thickness of 7–10 μm were acquired and placed on superfrost plus slides (Thermo Scientific, USA). The SN sections underwent rehydration in 0.1 M PBS for 5 min, then a PBS solution containing 0.3 % Triton X100 and 4 % BSA was used for avoiding non-specific bindings. Subsequently, anti-Caspase-1 (1:100, Sc-392736) and anti- IL-1β (1:100, Sc-12742) antibodies (Santa Cruz Biotechnology, USA) were exposed to brain tissue sections for overnight at 4 °C. In the next step, secondary antibody (anti-mouse *m*-IgGκ- FITC, 1:100, Sc-516140) was applied, and incubation continued for 1 h at room temperature. The 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. Finally slides examined using a fluorescent microscope (NIKON E600, Japan). For quantifying number of positive cells, the acquired images underwent analysis through the Fiji software [25].

2.8. Expression of tyrosine hydroxylase via immunoblotting

The brain tissue was homogenized in RIPA buffer (Cytomatingene, Iran), and were clarified through centrifugation at 14,000×*g* at 4 °C for 15 min. The protein content in the resulting supernatant was assessed using Bradford's assay [26]. For immunoblotting, the protein contents of brain tissue were separated via employing 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [27]. Briefly, the protein lysates were diluted 1:2 in sample buffer (62.5 mM Tris–HCl, pH 6.8, 10 % glycerol, 5 % β-mercaptoethanol, 2 % SDS and 1 % bromophenol blue), heated at 95 °C for 5 min, and subjected to SDS-PAGE. The proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Subsequently, the PVDF membranes were incubated with 2.5 % bovine serum albumin (BSA) in PBS (pH 7.4) for overnight at 4 °C. Immunodetection was achieved by incubation of anti-tyrosine hydroxylase (TH) antibody (1:200, Rabbit Polyclonal IgG, Abcam, USA) and anti-β-Actin antibody (1:200, mouse monoclonal IgG_1 κ, Santa Cruz Biotechnology, Inc. USA) for 3 h at room temperature (RT). After that, PVDF membranes were washed 4 times with PBS, including 0.1 % tween 20, and then incubated with anti-rabbit IgG H&L (HRP) antibody (1:3000, goat IgG, Bio-Rad, USA) and goat anti-mouse IgG (HRP) (1:3000, goat IgG, cytomatingene, Iran) for 1 h at RT for detection of TH and β-Actin, respectively. Eventually, the signals were recorded through a G: box imaging system (Syngene, UK) through the chemiluminescent method according to the manufacturer's recommendations (ECL, Parstus Biotechnology, Iran).

2.9. Quantitative real-time PCR

The expression levels of AIM2, ASC, caspase-1, IL-1β, IL-18, NLRP1, NLRP3 and NLRC4 were assessed in the SN extracts via quantitative real-time polymerase chain reaction (qPCR). In detail, total RNA was isolated from the SN homogenates according to the manufacturer's protocol (Parstous Biotechnology, Iran). Afterward, Easy™ cDNA Reverse Transcription kit was used to synthesize first-strand cDNA (Pars Tous Biotechnology, Iran). The specific oligonucleotide primers were designed by Beacon Designer (PREMIER Biosoft, Palo Alto, CA, USA) [28]. The cDNA samples were amplified in duplicate by SYBR Green qPCR Master Mix 2X (Pishgam, Tehran, Iran) on an ABI StepOnePlus thermocycler (ABI, USA). Briefly, qPCR was performed with 10 μl of SYBR Master Mix, 0.4 μl of forward and reverse primers (10 pmol/μl), 7.8 μl of DEPC water, and 2 μl of cDNA. The temperature and cycling conditions were as

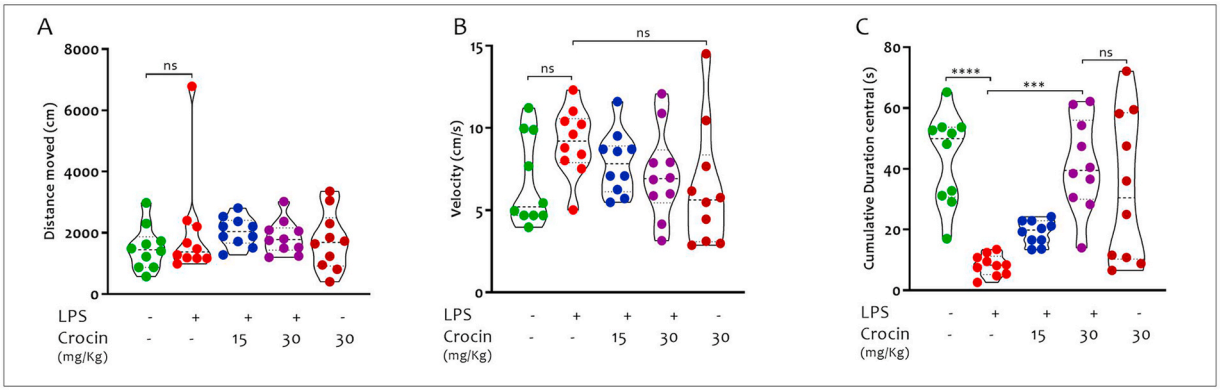

Fig. 1. Effects of i.p administration of crocin on (A) distanced moved, (B) velocity and (C) the cumulative duration central time. LPS-induced anxiety was significantly recovered after 3 weeks of treatment with 15 and 30 mg/kg of crocin (***$P < 0.001$; ****$P < 0.0001$; ns: non-significant, n = 10).

outlined below: initial denaturation at 95 °C for 5 min, then 40 cycles of 95 °C for 20 s, 58–64 °C for 30 s (annealing temperature), and extension at 72 °C for 30 s. Following the amplification reactions, melting curves was incorporated in each assay. to verify the presence of single PCR product [29]. The desired genes expression ratios were examined through the Pfaffl method [30].

2.10. Statistical method

The analysis of all data was conducted using the GraphPad Prism® 6.0 package for Windows (GraphPad Software Inc., La Jolla, CA, USA). The data were visually represented using violin plots. Comparisons between the different groups were assessed using the One-way ANOVA test, followed by the Tukey-Kramer test. Statistically significant changes were recognized at $P < 0.05$.

3. Results

3.1. Behavioral level results

3.1.1. Crocin attenuated LPS-induced anxiety-like behavior

Twenty-one days post LPS injection, the OF test were conducted to evaluate anxiety-like behavior. Evaluated parameters included average velocity, distance moved, and time spent in the center. No significant changes in distance moved and velocity were observed in different groups (Fig. 1A and B), while LPS administration reduced the cumulative duration in the center compared to the control group. Pre-treatment with 30 mg/kg ($p < 0.001$) crocin significantly increased the cumulative duration in the center compared to the LPS group. Moreover, there was no significant difference between the control and sham groups (Fig. 1C). This finding indicated that crocin exhibits protective effects against LPS-induced anxiety in the rats. The Elevated Plus Maze was utilized to monitor the animals for time spent and the number of entries in the open arms. The sham group demonstrated the longest duration spent. However, an evident improvement in behavior was not noticed among experimental groups. The analysis of the time spent in the open arm showed that the animals treated with 30 mg/kg crocin tended to spend less time in the open arm, but it was not statistically significant (Fig. 2A). In addition, there were no significant differences in the number of entries into open arm between different groups (Fig. 2B). Overall, the results of test highlighted that crocin can exert protective effects against LPS-induced anxiety in rats.

3.2. Brain tissue assay results

3.2.1. Crocin ameliorates LPS-induced inflammatory cell infiltration in the SN

Evaluation under light microscopy showed prominent differences in the presence of inflammatory cells in the SN and ventral tegmental area (VTA) regions between the experimental and control groups, where the red circle represents the SN and the green circle represents the VTA (Fig. 3A–D). The H&E-stained sections of the substantia nigra from the control group showed a normal appearance. The control groups showed no evidence of inflammatory cell infiltration, and neurons appeared normal, with preserved extracellular matrix (Fig. 3B). Conversely, in the LPS-treated group, a single injection of LPS induced tissue damage, accompanied by inflammatory cellular infiltration via microglial migration in the initial lesion. After a single injection of 2 μl of LPS, cell infiltration, accumulation of a large number of necrotic cells in the SN, and vacuolization were noticeably observed in LPS group (Fig. 3C). Both crocin-treated groups showed a decrease in inflammatory cell infiltration compared to the LPS group. Histopathological evaluations of brain tissue presented markedly lower necrosis, degenerative changes, and inflammatory cell infiltration in the high-dose (30 mg/kg) crocin-treated group (Fig. 3D and E).

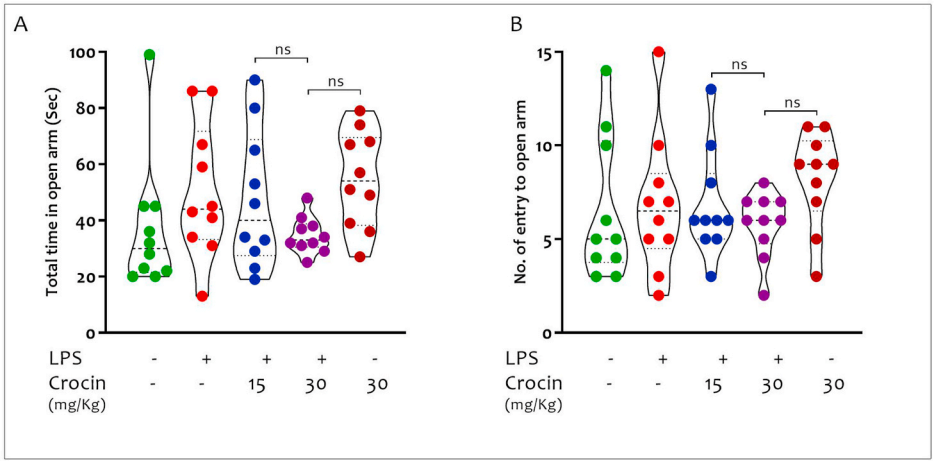

Fig. 2. Effects of crocin on anxiety-like behavior in the elevated plus maze. Treatment with 15 and 30 mg/kg of crocin had no significant effect on (A) time spent in the open arms and (B) the number of entries in the open arms 3 weeks after micro-injection of LPS ($P > 0.05$, n = 10).

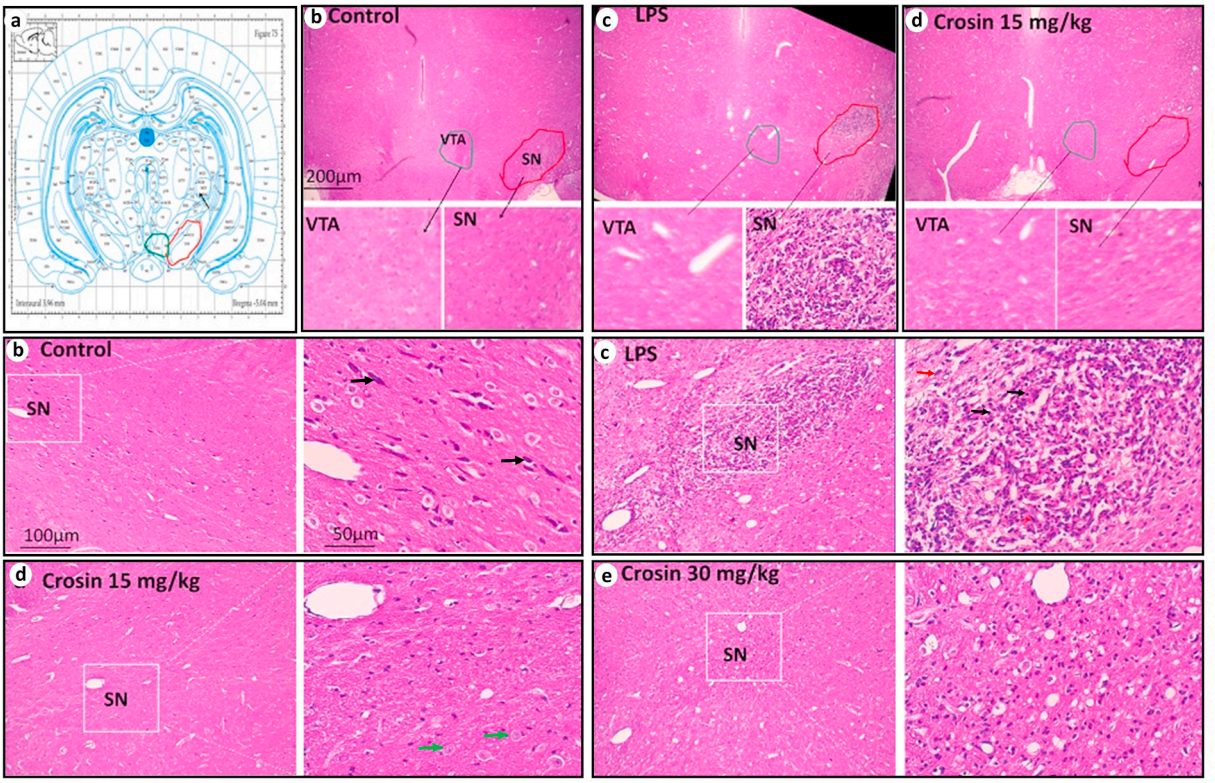

Fig. 3. H& E staining and the evaluation of cellular infiltration in different experimental groups the SN and VTA regions in. (a) Stereotaxic co-ordinates of the SN and VTA in histological analysis. The red circle shows the SN, and the green circle shows the VTA (b–d). The cross sections of the brain from the same stereotaxic coordinate in the control (b), LPS (c) and crocin 15 mg/kg (d) groups, the VTA and SN regions were marked with green and red circles, respectively, and magnified under the pictures. Cellular infiltration increased in the left SN of the LPS administrated group (c) compared to the control group (b), but the VTA region was unaffected. The presence of neurons is shown with black arrows in the control group (b). Severe cellular infiltration was observed in the LPS group. The necrotic cells are represented with black arrows and the presence of microglia cells in the SN region is shown with red arrows (c). Treatment with 15 mg/kg (d) and (e) 30 mg/kg crocin decreased the number of necrotic cells and increased the number of astrocytes represented with green arrows. The scale bar in the first-row pictures b-d is 200 μm, and in the second (b and c) and third rows is 100 μm. White squares show magnified regions (scale bar 50 μm) and are the same for third-row pictures. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Molecular detection results

3.3.1. Crocin reversed LPS-induced neuroinflammation in SN by reducing caspase 1 and IL-1β

To investigate the involvement of caspase-1 and IL-1β in the lesion area, serial brain specimens from the SN subregions were stained by specific antibodies, and quantification of positive cells was performed among all experimental groups. Three weeks after LPS injection, the results demonstrated a significantly higher number of positive cells for IL-1β and caspase-1 in the SN subregions of the PD animals compared to the control group. The immune-positive cells for IL-1β and caspase-1 were represented in Figs. 4 and 5, respectively. More positive cells for IL1-β and caspase-1 were found in the LPS group, while in the control and sham groups, positive cells for these proteins were reduced. IL-1β production was increased significantly in the LPS group ($P < 0.001$). Treatment with crocin led to a significant downward trend ($P < 0.001$) in IL-1β production in both crocin-treated groups (Fig. 6A). In addition, caspase-1 positive cells were increased following induction of disease compared to the control group. However, only the high-dose crocin-treated group (30 mg/kg) showed a significant decrease in caspase-1-expressing cells compared to the LPS group (Fig. 6B). Moreover, no significant difference was observed in the levels of caspase-1 and IL-1β between the sham and control groups.

3.3.2. Crocin reduced TH expression after LPS administration in the SN

TH is a key hallmark of pathological apoptosis of dopaminergic neurons, and is linked to neurodegenerative conditions. Western blotting was performed across all experimental groups to evaluate the TH expression in the SN. After protein extraction from brain tissues, the protein concentration was ranged from 0.50 to 0.75 μg/μl. The specific detection of TH (55 KDa) has been shown in Fig. 7A, which was normalized with β-Actin (Fig. 7B), (Fig. 1 supplementary). A comparison of protein expression revealed a decrease in TH expression in the LPS-treated group. Conversely, treatment with 30 mg/kg of crocin significantly increased TH expression compared to both the LPS-treated group and the 15 mg/kg crocin-treated group. Moreover, the expression of TH in the 15 mg/kg of crocin-treated

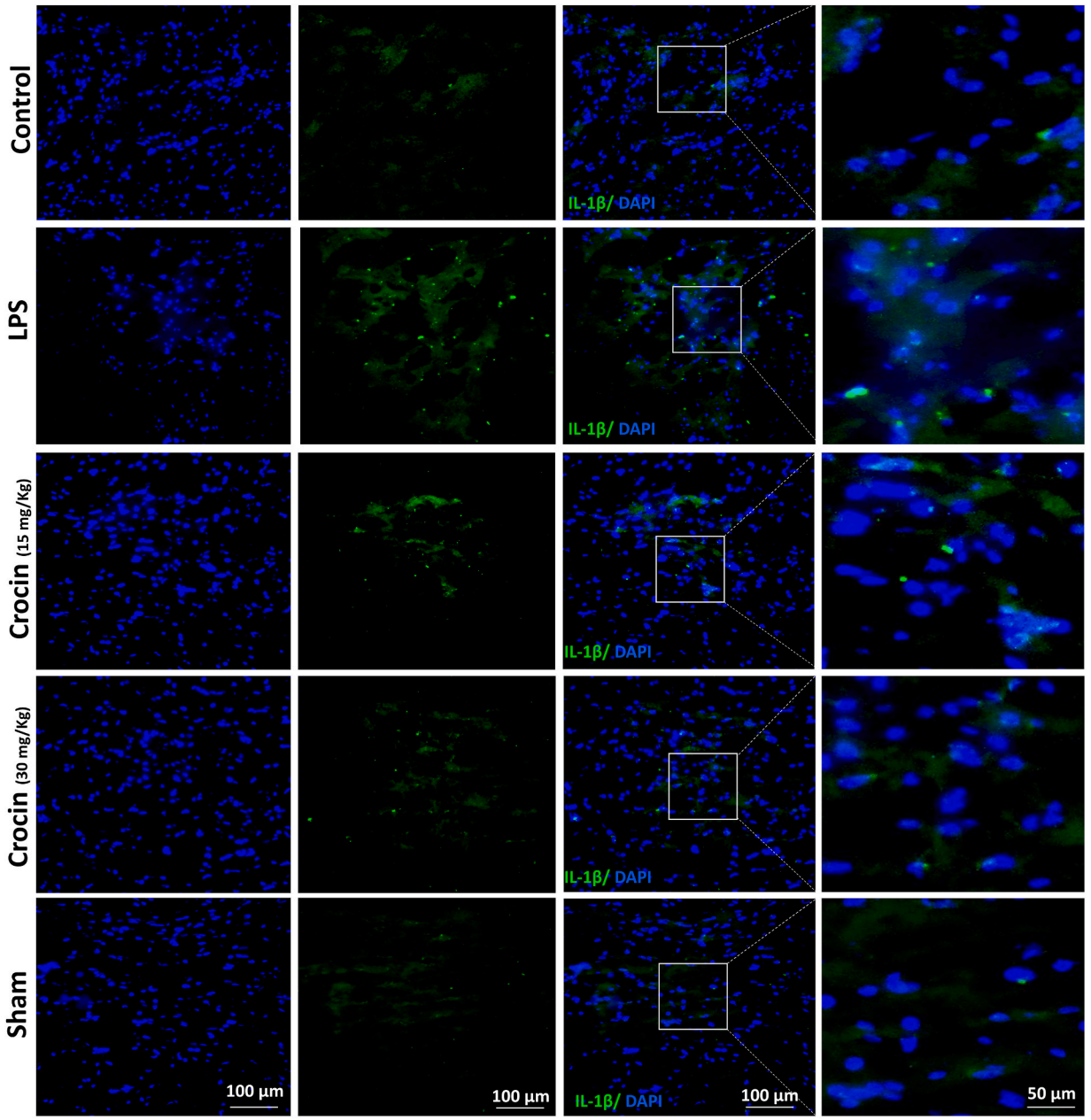

Fig. 4. The immunostaining of IL-1β, 21 days after LPS injection indicated that the number of IL-1β positive cells (IL-1β antibody (green) and nuclei are labeled with DAPI (blue)) was remarkably increased compared to the control group. The scale bars are 50 and 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

was significantly different from that of the LPS group (Fig. 7C). Therefore, western blotting results revealed that treatment with crocin can alleviate neural loss induced by LPS administration.

3.3.3. Crocin attenuates mRNA expression of IL-1β, Il-18, NLRP1 and AIM2 inflammasome components

The inflammasome complex's gene expression level and IL-1β production, such as NLRP1, NLRP3, AIM2, NLRC4, ASC, IL-18, and caspase-1 were evaluated by SYBR Green real-time PCR. The expression of two target inflammasome complexes consisting of IL-1β and IL-18 was explored (Fig. 8A and B). The analysis showed a significant increase in IL-1β ($P < 0.001$) and IL-18 ($p < 0.001$) expression in the PD group compared to the control. Notably, a notable reduction in IL-1β and IL-18 expression was detected in the crocin-treated group (30 mg/kg). No significant difference was found between the sham and control groups ($P > 0.05$). The analysis of caspase-1 and ASC expression in the tissue samples did not show any significant changes ($p > 0.05$) in the different groups compared to the control group (Fig. 8 C, D). Although crocin administration showed a tendency to reduce caspase-1 and ASC expression, the reduction was not

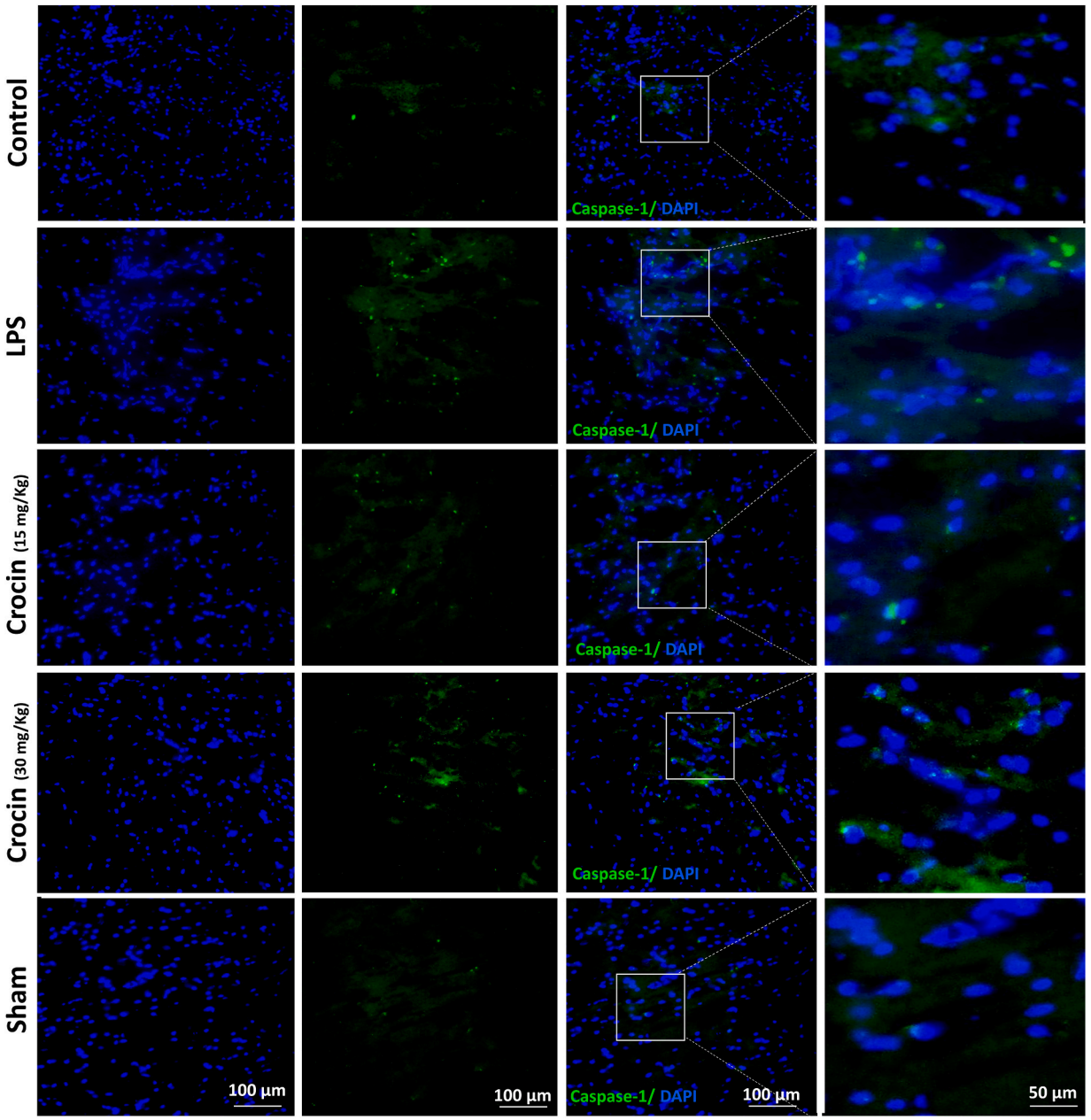

Fig. 5. The Immunostaining of caspase-1, 21 days after LPS injection indicated that the number of caspase-1 positive cells (caspase-1 antibody (green) and nuclei are labeled with DAPI (blue)) was remarkably increased compared to the control group. The scale bars are 50 and 100 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

significant ($P > 0.05$). NLRP expression analysis indicated a significant increase of the NLRP1 and AIM2-dependant inflammasome pathway following disease induction ($P < 0.05$). However, there was no significant increase observed for NLRP3 and NLRC4. Interestingly, NLRP1 and AIM2 expression was reduced in both crocin (15 mg/kg and 30 mg/kg) - treated groups (Fig. 8E–H).

4. Discussion

Neuroinflammation is a potential risk factor for the development and progression of PD [31–33]. Compelling evidence has confirmed the critical roles of IL-1β and IL-18 in the pathogenesis of PD [6,33–35]. Despite significant progress in managing Parkinson's disabilities, a definitive treatment remains elusive. Recent research suggests that herbal medicine, such as crocin, may reduce the risk of PD due to its anti-inflammatory effect [10,13,36]. In this study, we aimed to investigate the potential impact of crocin on inflammasome pathways in an LPS-induced model of PD.

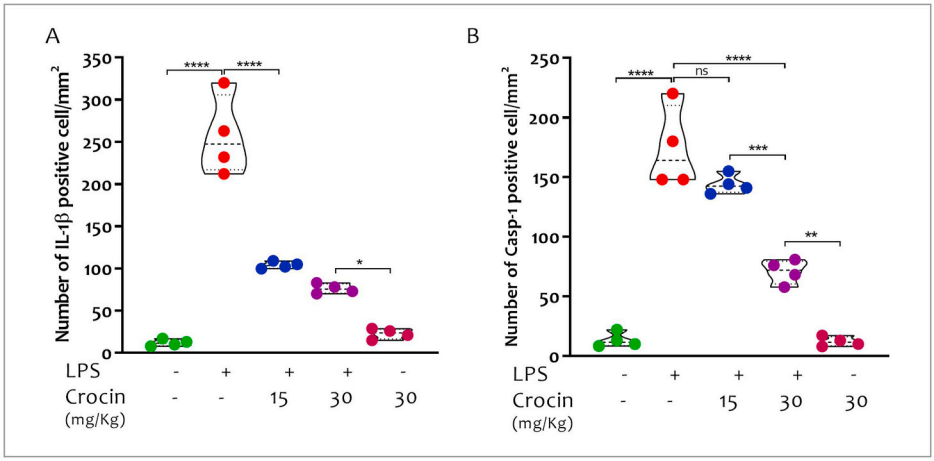

Fig. 6. Quantitative analysis of IL-1β and caspase-1 positive cells. (A) Number of IL-1β positive cells/mm2, (B) Number of caspase-1 positive cells/mm2; The LPS group showed higher IL-1β and caspase 1-expressing cells than the control group ($P < 0.0001$). The low-dose crocin-treated group showed a decrease in IL-1β -expressing cells compared to the LPS group ($p < 0.0001$), but the number of IL-1β -expressing cells in the low-dose crocin-treated group was not higher than that in the high-dose crocin-treated group. The sham group showed lower IL-1β-expressing cells than the high-dose crocin-treated group ($P < 0.05$). The high-dose crocin-treated group showed a decrease in caspase-1-expressing cells compared to the LPS ($p < 0.001$) and low-dose crocin-treated ($P < 0.01$) groups, but the number of these cells was higher when compared to the control ($p < 0.01$) and sham ($p < 0.01$) groups, ($n = 4$). (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$).

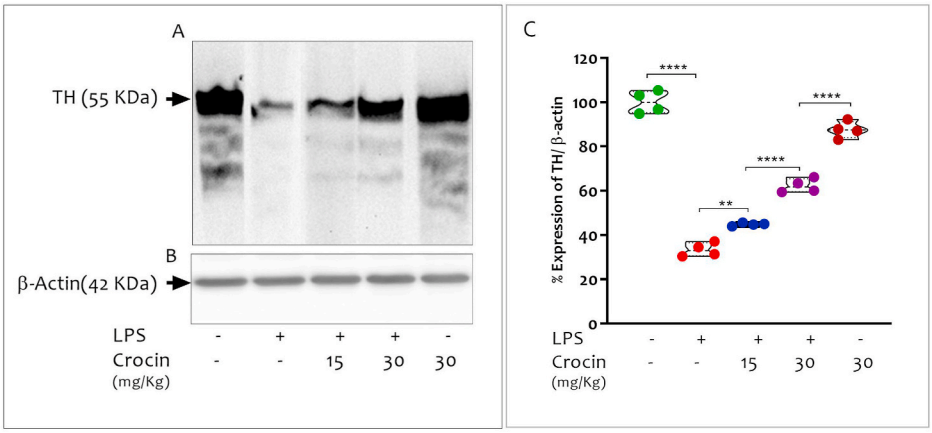

Fig. 7. Semi-quantitative analysis of TH expression between experimental groups. (A, B) The TH and β -actin expression in different groups was evaluated through western blotting. (C) TH expression was normalized to β-actin expression, and columns are plotted by percentage of protein expression in different groups. Treatment with 30 mg/kg of crocin significantly increased TH expression compared to the LPS group ($n = 4$). **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$, ns: non-significant.

Our findings proved that intranigral injection of LPS significantly reduced TH expression and boosted the gene expression of NLRP1 and AIM2 inflammasome, IL-18, and IL-1β. Furthermore, LPS increased the number of caspase-1 and IL-1β-expressing cells in the SN. However, crocin treatment significantly reduced the SN's LPS-induced neuroinflammation and DA neuronal loss. Animals treated with crocin also exhibited lower anxiety levels. H&E staining showed that crocin treatment decreased cell infiltration at the LPS injection site. These findings suggest that crocin may hold promise in developing new therapeutic strategies for PD using herbal medicine.

Our findings showed that LPS is a suitable model to induce chronic neuroinflammation, leading to dopaminergic neuronal death in PD [37]. LPS administration has been shown to induce neurodegeneration and neuroinflammation, resulting in selective loss of DA neurons that accompanied by up-regulation of pro-inflammatory cytokines [38]. In addition to motor symptoms, non-motor symptoms such as anxiety play a significant role in PD. Anxiety and depression often precede the onset of motor symptoms [39–41]. Our behavioral analysis indicated that anxiety levels in LPS-receiving animals were significantly higher than in the control group, which were significantly attenuated by crocin treatment [42]. Our study found that crocin treatment significantly attenuated anxiety-like behavior induced by LPS injection in rats, as indicated by reduced time spent in the center during the OF test. Our results are in agreement with the previous studies that demonstrated LPS injection is associated with anxiety-like behavior [41,43]. However, we did not observe significant changes in motor symptoms, possibly due to a single injection or a 3-week latency period after injury. It

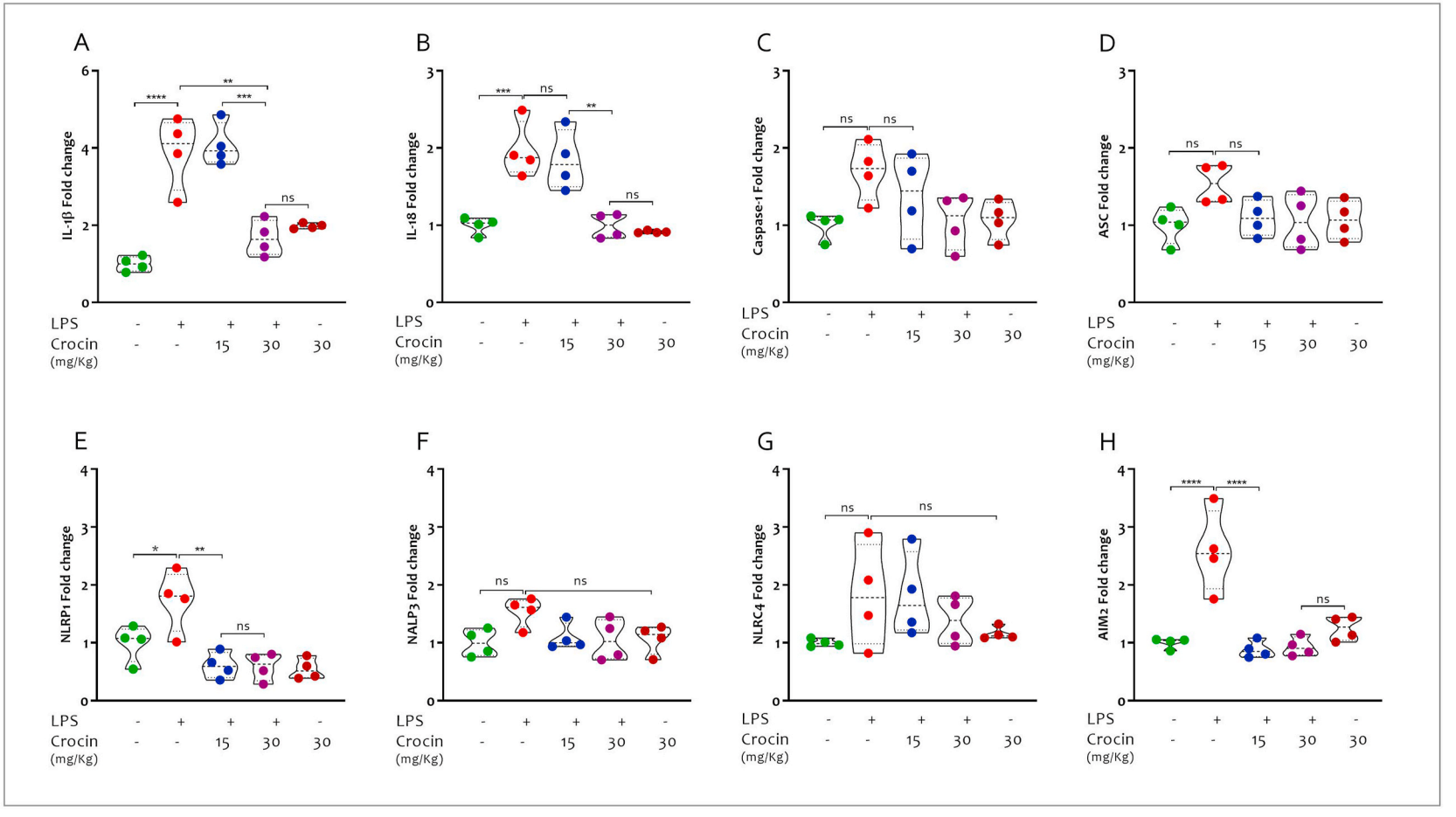

Fig. 8. Expression of genes involved in inflammasome pathway (n = 4). (A) Expression of IL-1β was significantly different between the control and LPS groups ($P < 0.01$). Treatment with 30 mg/kg crocin significantly decreased the expression of IL-1β ($P < 0.01$) compared to the LPS and 15 mg/kg crocin-treated groups. (B) The expression of IL-18 was similar to IL-1β in different groups. (C, D) Expression of caspase-1 and ASC did not show significant differences between experimental groups. (E–H) Comparison of expression levels of NLRP1, NALP3, NLRC4, and AIM2 genes among experimental groups. Treatment with 15 mg/kg crocin significantly reduced NLRP1 expression compared to the LPS group ($P < 0.01$). AIM2 expression was significantly higher in the LPS group than control ($P < 0.0001$). AIM2 expression was reduced following treatment with 15 or 30 mg/kg crocin ($P < 0.0001$). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ***: $P < 0.0001$, ns; non-significant).

should be noted that in some studies, the incubation period was more than one month. In addition, motor deficit phenotypes are generally considered for PD occurrence assessment in animal models. Indeed, through PD progression, neurons are constantly compensating for functions of the lost neurons, and in the early stages, motor dysfunction is not observed. When the striatal neuron destruction reaches 80 %, motor dysfunction appears. However, non-motor deficits manifest years before motor symptoms and have not received sufficient attention in research and clinics. Our study highlighted the potential of using anxiety symptoms alongside molecular tests for early detection of PD [44].

Non-motor symptoms in PD, including anxiety, are linked to abnormalities in anxiety-related structures, such as the locus coeruleus, hippocampus, and prefrontal cortex. The locus coeruleus, which is significantly reduced in the early stages of PD, is believed to play a role in anxiety responses [45]. Previous studies have suggested that the locus coeruleus is related to the early non-motor symptoms of PD, including anxiety [46–48] and depression [49]. This may be in part due to changes in the interaction between noradrenergic and dopaminergic areas in PD due to neuronal loss in both the SN and locus coeruleus, which could influence the function of the surviving neurons in both regions [50] and lead to pathological anxiety responses in the locus coeruleus. SN modulates of hippocampal activity is also implicated in anxiety [51]. In addition, LPS-induced anxiety-like behaviors have been associated with decreased brain-derived neurotrophic factor (BDNF) gene expression in the hippocampus [52]. In this study, we have observed that non-motor symptoms, such as anxiety, can be treated with crocin, while motor symptoms were not. However, crocin effects on neurotransmitter systems, such as the serotonergic and dopaminergic systems, may contribute to its improvement of non-motor symptoms. Additionally, crocin may have a specific effect on anxiety associated with PD through its anti-inflammatory and antioxidant mechanisms [52].

LPS activates inflammasome through the CD14/TLR-4 receptor complex, leading to the release of pro-inflammatory cytokines such as IL-1β and IL-18, resulting in chronic neuroinflammation [6,7,53]. Previous studies have reported an enhancement in IL-1β and IL-18 mRNA expression in PD, supporting their important role in PD development. Our findings indicate that LPS can initiate a destructive cycle of chronic neuroinflammation partially mediated by NLRP1 and AIM2 inflammasome activation. Clinical studies have reported the presence of AIM2 in CSF and blood samples of patients with PD [54]. NLRP3 inflammasome activation has also been implicated in the pathogenesis of neurological disorders, including PD [55],and selective NLRP3 inflammasome inhibitor, MCC950, has been proposed as a potential therapeutic agent for PD [56]. Research showed a considerable increase in NLRP3 expression levels in PD progression [57], while we observed an increase in NLRP3 expression in our study, but it was not statistically significant. In contrast, we noticed a higher increase in AIM2 and NLRP1 inflammasome than other inflammasome in the LPS-induced model of PD. However, some studies have reported NLRP3 activation. Therefore, further studies are needed to understand the role of inflammasome activation in PD pathogenesis completely. There could be some explanation for our obtained results. First, AIM2 inflammasome activation may be more specific to LPS, while NLRP3 inflammasome was activated by a wide range of stimuli [58]. Second, NLRP3 inflammasome activation may depend more on mitochondrial damage due to rotenone-induced model of Parkinson's disease [59]. In contrast, AIM inflammasome activation in LPS-induced Parkinson's disease warrants deeper mechanistic studies [60]. Owing to the critical role of inflammasomes in PD progression, our study showed an important role of NLRP1 and AIM2 inflammasomes in the immunopathology of PD. Therefore, targeting both AIM2 and NLRP1 alongside NLRP3 inflammasome, may provide a treatment approach for attenuating neuroinflammation in PD.

Our results revealed protective effects of crocin against PD caused by LPS by reducing IL-1β and IL-18 levels along with NLRP1 and AIM2 inflammasome. A considerable body of evidence confirmed the anti-inflammatory activity of crocin [14,61]. Ahmed. et al. demonstrated that crocin administration significantly reduces inflammatory markers [62]. Another study (Nam et al.) showed that crocin suppresses LPS-induced microglial activation and reduces TNFα and IL-1β production by activated microglia [14]. Although, Sangare. et al. reported no effect of crocin on NLRP1 or AIM2 inflammasome complex in urate-induced mouse peritonitis [63], our obtained results in rat model of PD suggest that crocin can decrease mRNA expression of IL-1β, IL-18, NLRP1, and AIM2, alleviates anxiety-like behavior, and subsequently inhibits the depletion of TH neurons in the SN post-injury. Our findings, in line with previous results, strongly recommend that the neuroprotective effects of crocin are likely due to its anti-inflammatory activity as well as its ability to restore the production of TH. LPS significantly decreased TH protein expression in the left SN [64]. This may explain the selective damage of dopaminergic neurons in the SN by LPS. Our data indicate that crocin mitigated the loss of TH neurons and enhanced the expression of TH proteins in the SN following insult.

However, our study has several limitations. First, we did not measure the expression of inflammasome genes at the protein level. Second, there was an inconsistency in the expression of caspase-1 in immunofluorescence staining and quantitative real-time PCR results. This inconsistency in the expression of caspase-1 could be due to the different sensitivity and specificity of these two assays or because the gene expression was performed specifically in the SN, while immunofluorescence was performed on the whole brain tissue. Third, we did not directly knock-out the AIM2 and NLRP1 genes to understand their exact role in PD. Therefore, it needs to be further explored. Fourth, we did not explore α-synuclein expression before and after crocin treatment, which could provide clues about inflammasome activation or protective mechanism of crocin.

5 Conclusion

Increased expression of IL-1β and caspase-1, along with elevated levels of TH, may contributed to immunopathology of PD, probably through to the activation of inflammasome complex via the NLRP1 and AIM2 pathway. Crocin may effectively treat PD by reducing inflammation and interfering with the inflammasome complex. These findings suggest that crocin-based interventions have the potential to mitigate neuroinflammation and impact the PD progression.

Ethics approval

Animal Ethics Committee of the Babol University of Medical Sciences approved this study (Documentation No IR.MUBABOL.HRI.REC.1398.163).

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CRediT authorship contribution statement

Solmaz Alizadehmoghaddam: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Fereshteh Pourabdolhossein:** Writing – review & editing, Methodology, Investigation, Data curation. **Hossein Najafzadehvarzi:** Writing – review & editing, Methodology, Investigation, Data curation. **Maryam Sarbishegi:** Writing – review & editing, Resources, Methodology. **Kiarash Saleki:** Writing – original draft, Methodology. **Hamid Reza Nouri:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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