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Original research

Crocin exhibits neuroprotective potential against lipopolysaccharide-induced inflammatory responses

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Abstract:

Lipopolysaccharide (LPS) can induce neuroinflammation associated with Alzheimer's disease (AD). Crocin has neuroprotective properties due to its strong antioxidant, anti-inflammatory, and anti-apoptotic activities. The goal of this study is to find out if crocin can reverse the pathophysiological features of AD and the hippocampal neuroinflammation brought on by LPS. The rats were randomly divided into five equal groups. Group (I) served as a control, Group (II) received LPS (1 mg/kg/day) for one week, Following LPS exposure, Group III and IV received 50 mg/kg/day of captopril and 50 mg/kg/day of crocin, respectively, while Group V received a 30-day combination of both therapies. Nitric oxide (NO) and acetylcholine (Ach) levels were assessed in hippocampal samples, and Aurora kinase was found by immunohistochemical analysis. In the hippocampal homogenate, the LPS group exhibited a highly significant decrease in Ach level and a highly significant increase in NO concentration. Aurora expression in the hippocampus tissues increased concurrently with these results.. On the other hand, rats in the LPS+CAP group showed minor improvements in immunohistochemistry aurora expression along with a significantly higher Ach level and a significantly lower NO concentration.\. Rats in the LPS+CR and LPS+CAP+CR groups, however, exhibited a mild immunoreactivity to aurora expression along with a highly significant increase in Ach level and a decrease in NO concentration. Crocin co-treatment attenuates the LPS-induced neuroinflammation, which may be explained by increased NO, decreased Ach, and excessive aurora expression.

Keywords: Crocin, Captopril, \L\ipopolysaccharide, Neuroinflammation, Alzheimer's disease.

1- Introduction

Lipopolysaccharide (LPS) is a component of gram-negative bacteria's outer membrane. It is a strong endotoxin that is very resistant to being broken down via mammalian enzymes. Toll-like receptor (TLR)-4 is one of the receptors that is found on the membrane of the microglia.

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LPS enters the brain and binds to TLR-4 on microglia**.** TLR-4, in turn, stimulates microglia by beginning signal transduction cascades that result in rapid transcription and release of proinflammatory cytokines **(Sun *et al*., 2015).** These cytokines can also start a chain of harmful events in the peripheral and central nervous systems, which in turn causes memory loss and cognitive impairment (**Bahaidrah *et al.*, 2022).**

Acetylcholine (Ach) is a neurotransmitter critical for both the central and peripheral nervous systems, which has roles in synaptic transmission, learning, and memory. Ach also plays a vital role in modulating immune responses through the cholinergic anti-inflammatory pathway. The excessive inflammation can affect cholinergic signaling by disrupting Ach synthesis, release, or receptor expression. Impairment of forebrain cholinergic neurons, regulating innate immune responses and inflammation, leads to cholinergic dysfunction linked to Alzheimer's disease (AD) **(Zhi *et al*., 2022)**.

Nitric oxide (NO) is a multifunctional signaling molecule that plays important roles in many physiological and pathological processes. LPS-induced activation of immune cells results in elevated NO production by activation of inducible nitric oxide synthase (iNOS) as part of the inflammatory response. Excessive NO production, particularly during neuroinflammation, leads to the progression of neurodegenerative diseases such as Alzheimer's disease in the central nervous system **(Lin *et al.*, 2021).**

Many studies suggested that it may take many years before Alzheimer's disease (AD) is clinically diagnosed. Recently, the prospective use of different drugs like captopril has been explored for treating or preventing AD in patients due to its ability to modulate pathways implicated in renin angiotensin system (RAS) modulation and anti-inflammatory effect **(McGeer *et al*., 2016).**

The natural products, such as plants and their bioactive compounds, have been a cornerstone of medicine and therapeutic discovery for centuries. Their bioactive compounds possess varied chemical structures and biological activities, making them invaluable in understanding disease mechanisms and developing new treatments (**Kar *et al.*, 2019).** Saffron (*Crocus sativus Linne),* one of the best natural products, is still used as a food coloring, flavoring agent, and herbal medicine in many parts of the world today. Crocin (CR) is one of the main pharmacologically active components in saffron. It is also a naturally occurring carotenoid molecule that has been shown to have therapeutic potential in the treatment of neurological disorders. It is acting as an anti-inflammatory, anti-cholinergic, anti-oxidative stress, and anti-apoptotic agent **(Cho *et al*., 2018).**

Crocin has a neuroprotective effect, which may aid in preserving the integrity of cholinergic neurons by alleviating the destruction of cholinergic pathways. It supports and elevates Ach function through reducing oxidative stress and inflammation that is particularly vulnerable in Alzheimer's disease. Furthermore, CR can indirectly help in cholinergic transmission by altering other neurochemical pathways. This could improve cognitive function, especially when considering age-related cognitive decline and neurodegenerative diseases **(Noori *et al.*, 2022)**. Also, CR has beneficial properties that enable it to decrease the overexpression of iNOS, which reduces excessive NO production via inflammatory responses. This action helps to scavenge reactive nitrogen

species derived from NO, such as peroxynitrite ($ONOO^-$), which are highly reactive and damaging to cellular components **(Ahmed *et al.*, 2020).** All things considered, our research indicates that CR may be able to stop or lessen the neuronal damage and cognitive deterioration brought on by LPS. Therefore, it may be developed as a prospective therapeutic drug for the treatment of LPS-induced Alzheimer's disease.

2-Materials and methods

2.1-Chemicals

Lipopolysaccharide (LPS), captopril (CAP) and crocin (CR) were obtained from Sigma Aldrish Co., USA, with CAS numbers 17304, 441600 and 2630, respectively. Commercial Eliza kit of acetylecholine (Ach) was purchased from Bioassay Technology Laboratory, Egypt, with Cat. number E1290 Hu. Griess reagent was purchased from Srichem Co., India. The other chemicals were all of the best caliber.

2.2-Animals

Forty male albino rats, weighing 140 ± 20 g, were obtained from the Animal House, Faculty of Science, South Valley University. Animals were housed in a well-ventilated and clean cages maintained under a 12 h:12 h schedule of light: dark cycle at 25 ± 2ºC with a relative humidity of 15 ± 5% at the Animal House, Faculty of Science, Aswan University. All experimental protocols that were held on animals were done according to the guidelines of the Animal House of Aswan University, where standard commercial pellets were used for feeding and water ad libtum. Rats were kept for acclimation for around two weeks prior to the start of the investigation according to Ethical approval code is **ASWU/05/SC/ZO/24-01/0.**

2.3-Experimental Design:

Five groups of eight rats each were created from the included animals. The control group received no material treatment. The LPS group was injected intraperitone ally (ip) with lipopolysaccharide (LPS) (1 mg/kg b.w.) for 7 days (Abareshi *et al*., 2016). Following a 7-day LPS injection, the LPS+CAP, LPS+CR, and LPS+CAP+CR groups received oral treatment for 30 days with captopril (CAP) at a dose of 50 mg/kg b.w. **(Abareshi *et al.*, 2016**), crocin (CR) (50 mg/kg b.w., (**Yuan *et al.*, 2020**), and a combination of captopril and crocin, respectively.

Note: Lipopolysaccharide was freshly dissolved in normal saline, and both crocin and captopril were freshly dissolved in distilled water.

2.4-Collection of brain tissue:

Animals were sacrificed by decapitation 24 h after the last dose of administration. Brains were immediately dissected on an ice-cold plate, and the hippocampus was isolated and divided into two parts, one of them stored at -80°C for biochemical assays, and the other part was used for immunohistochemical studies.

2.5-Biochemical assessments:

Estimation of acetylecholine (Ach):

The demonstration of Ach in the hippocampus homogenates was carried out according to the BT lab ELISA kit method (Bioassay Technology Laboratory, Egypt).

Estimation of nitric oxide (NO):

Determination of NO levels was carried out in the hippocampus homogenates according to the method of **Montgomery and Dymock (1961).**

2.6-Immunohistochemical investigations:

The demonstration of Aurora expression in the hippocampus was carried out using the AB clonal kit method (ABclonal Technology Company, USA) catalog number #534056).. Formalin-fixed, paraffin-embedded hippocampal tissue slices were sectioned, 4-5 µm thick. The sections were deparaffinized and rehydrated. After applying the antigen retrieval solution, the endogenous peroxidase was inactivated for 15 minutes using 3% hydrogen peroxide, and then the solution was blocked for an hour. The rabbit polyclonal primary antibody (2.5% normal goat serum diluted in PBS/TBS supplemented with 0.3% Triton™ X-100, pH = 7.2) with catalog number (Aurora 1:150, Sigma, a5102) was applied to each section and incubated overnight in a humidified chamber at 4ºC. Then, after applying the peroxidase-labeled secondary antibody [HRP goat-anti-rabbit IgG (H+L)] with catalog number Dako, EnVision FLEX, High 800021 at a 1:200 dilution, the samples were incubated for 30 minutes. Freshly prepared DAB substrate and chromogen (Dako, EnVision FLEX, High pH (Link) kit # K800021) were used at room temperature for 2 to 5 minutes. Hematoxylin was used as a counterstain; sections were dried and then mounted.

2.7-Morphometric study and image analysis:

Histomorphometric analysis was used to assess the quantification of structural changes determined by histological analysis of hippocampal tissue. After the hippocampus of the brain was histologically processed, digital images were taken under the objective lens magnification of 40x using a digital camera connected to a light microscope. Morphometric analysis was done with a computerized image analysis software system, Image J, version 6. Spatial calibration with an object micrometer was performed before each analysis. Five pictures were selected from each animal in each group to measure % aurora intensity/surface area.

2.8-Statistical analysis:

All quantitative data were expressed as means ± standard error. One-way ANOVA was used to analyze mean differences, followed by Prism 6.0 (Graph and Software, Inc., San Diego, USA) and Microsoft Excel. The information gathered can be compared, statistically examined, and statistical significance was determined at $p < 0.05$.

3-Results and discussion

3.1-Nitric oxide (NO) concentration in various study groups:

Administration of LPS showed a highly significant increase (4.353 ± 0.128 nmole/mg tissue, with $P < 0.001$, for all) in NO levels compared with the control group (1.204 ± 0.021 nmole/mg tissue). However, the concentration of NO in the LPS+CAP group exhibited a significant decrease (3.377 ± 0.107 nmole/mg tissue, with $P < 0.01$ for all) compared with the LPS group. Additionally, the concentration of NO exhibited a highly significant decrease (2.51 ± 0.035 nmole/mg tissue and 1.68 ± 0.024 nmole/mg tissue, respectively, $P < 0.001$, for all) in the LPS+CR group and the LPS+CAP+CR group compared to those in the LPS group **(Fig. 1)**.

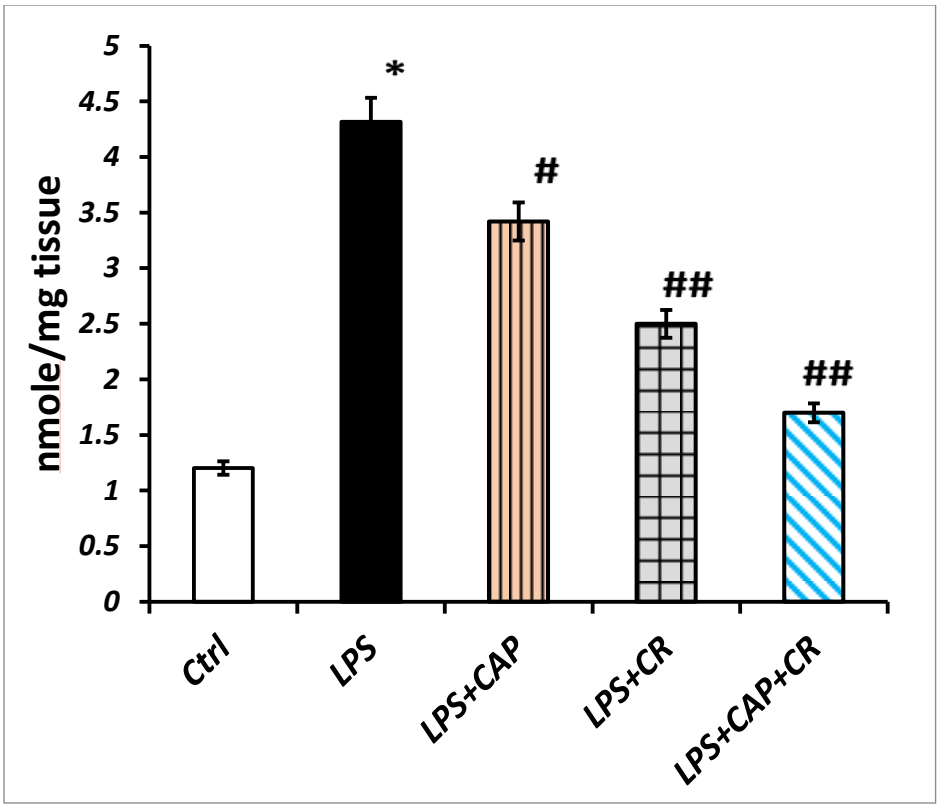

Fig (1): Concentration of NO in the hippocampus of adult male rats treated with lipopolysaccharide (LPS) (1 mg/kg b. wt.), LPS and captopril (CAP) (50 mg/kg b. wt.), LPS and crocin (CR) (50 mg/kg b. wt.) or LPS, CAP and CR. Values are means ± S. E. of 6 animals in each group. *Highly significant compared with the control group (P <0.001). # Significant compared with the LPS group (P <0.01). ## Highly significant compared with LPS group (P < 0.001).

3.2-Acetylecholine (Ach) concentrations in various study groups:

Values of the Ach concentrations in the LPS group showed a highly significant reduction (0.534 ± 0.008 µg/ml, P < 0.001) compared with the control group (1.87 ± 0.011 µg/ml). Meanwhile, the values in the LPS+CAP group were detected to be significantly elevated (0.987 ± 0.020 µg/ml, P < 0.01) compared with the LPS group. In addition, there was a highly significant elevation in the LPS+CR and LPS+CAP+CR groups (1.43 ± 0.013 µg/ml and 1.81 ± 0.012 µg/ml, respectively, with P < 0.001 for all) compared to those in the LPS group **(Fig. 2)**.

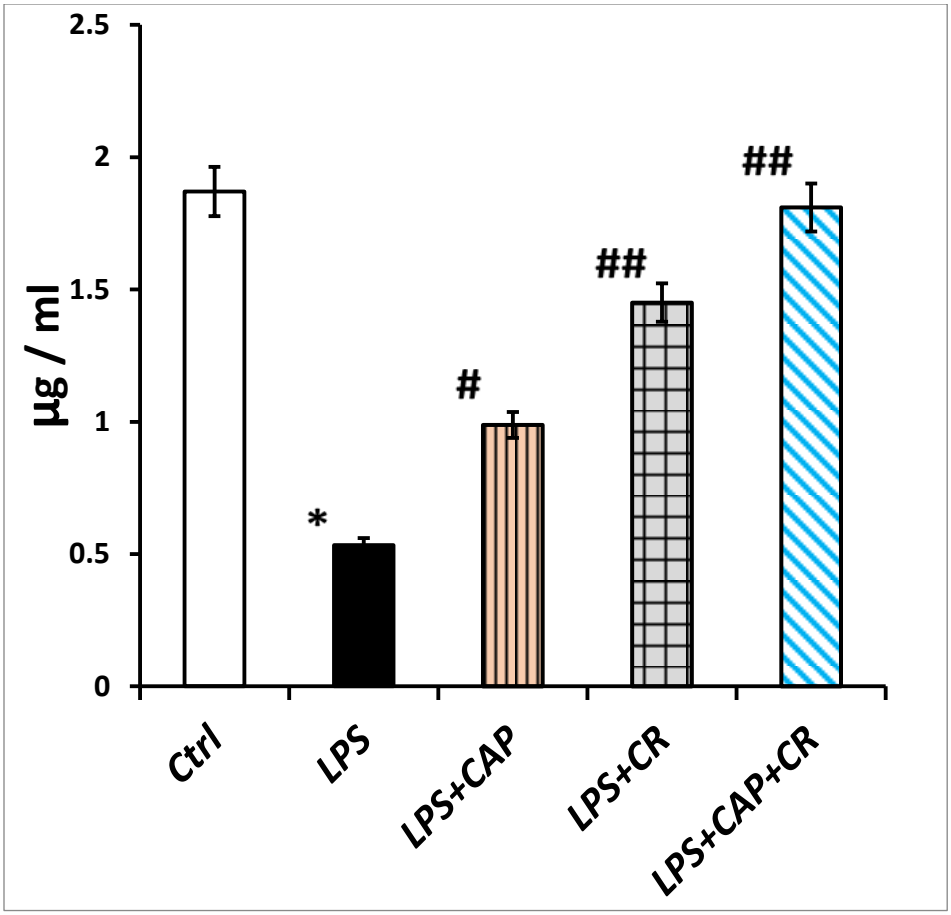

Fig (2): Concentration of Ach in the hippocampus of adult male rats treated with lipopolysaccharide (LPS) (1 mg/kg b. wt.), LPS and captopril (CAP) (50 mg/kg b. wt.), LPS and crocin (CR) (50 mg/kg b. wt.) or LPS, CAP and CR. Values are means ± S. E. of 6 animals in each group. *Highly significant compared with the control group (P <0.001). # Significant compared with the LPS group (P <0.01). ## Highly significant compared with LPS group (P < 0.001).

Table 1. NO and Ach in various studied groups.

Parameters / groups	NO	Ach
Ctrl	1.204 ± 0.021	1.87 ± 0.011
LPS	4.353 ± 0.128*	0.534 ± 0.008*
LPS+CAP	3.377 ± 0.107#	0.987 ± 0.020#
LPS+CR	2.51 ± 0.035##	1.43 ± 0.013##
LPS+CAP+CR	1.68 ± 0.024##	1.81 ± 0.012##

Values are means ± S.E. of 6 animals in each group.* Highly significant compared with control group (P <0.001). # Significant compared with LPS group (P <0.01). ## Highly significant compared with LPS group (P <0.001).

3.3-Immunohistochemical and morphometrical findings:

Immunohistochemical staining with the aurora antibody exhibited faint brown staining in the control group, marked by minimal immunoreactivity within the stratum pyramidale, stratum oriens, and stratum radiatum layers **(Fig. 3a).** On the other hand, the LPS-treated group showed visible brown staining and high immunoreactivity, indicating increased aurora expression in the same layers. **(Fig.3b). The LPS+CAP-treated group exhibited light brown staining, which indicates moderate immunoreactivity and reduced aurora expression. (Fig. 3c).** The group treated with LPS+CR showed a further decrease in aurora expression, as seen by weak antibody and extremely mild brown staining. **(Fig. 3d).** Nearly no aurora expression was observed in the LPS+CAP+CR-treated group, which showed faint brown staining, indicating negative immunoreactivity in the analyzed layers **(Fig. 3e).**

The LPS group's CA1 area had a highly significant increase (6.04 ± 0.222, P < 0.001) in comparison to the control group (1.283 ± 0.154), according to morphometric analysis for aurora intensity in the hippocampus sections. In contrast, the LPS+CAP group, the LPS+CR group, and the LPS+CAP+CR group exhibited a notably highly significant reduction (2.45 ± 0.143, 1.86 ± 0.087, and 1.557 ± 0.065, respectively, P < 0.001 for all) in the aurora intensity color relative to the LPS group **(Fig. 3f, Table 1).**

Table (2). Morphometrical results of the intensity color of immunohistochemical staining for Aurora in the CA1 region of the hippocampal tissues of the rats in various studied groups.

Groups	Intensity of Aurora
Ctrl	**1.283 ± 0.154**
LPS	**6.04 ± 0.222***
LPS+CAP	**2.45 ± 0.143##**
LPS+CR	**1.86 ± 0.087##**
LPS+CAP+CR	**1.557 ± 0.065##**

Values are means ± SE, of animals in each group. * Highly significant compared with the control-group (P < 0.001). ## Highly significant compared with the LPS-group (P < 0.001).

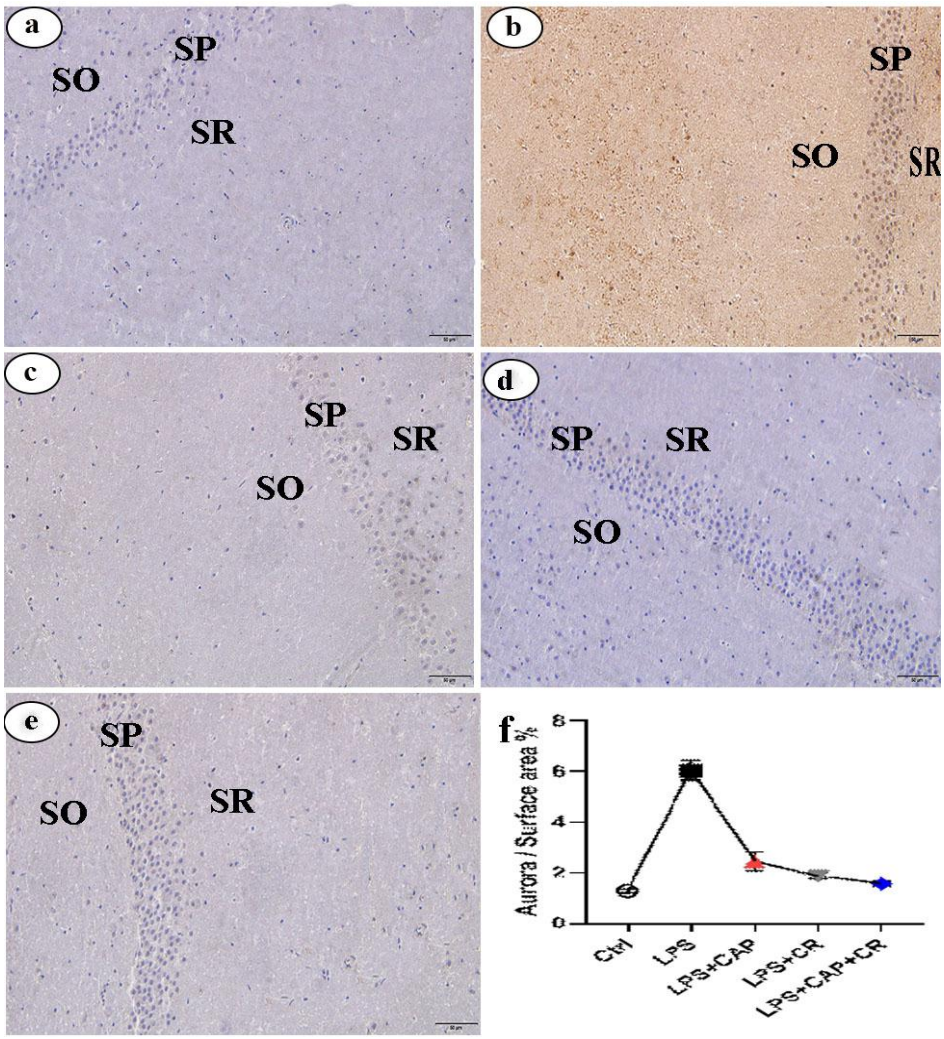

Fig (3): Photomicrographs of hippocampus sections of immunohistochemistry staining of (Aurora) (bar ¼ 50 mm). (a) Hippocampus section of control, (b) Section of LPS-induction group, (c) Section of LPS+CAP treated group, (d) Hippocampus section of LPS+CR treated group. (E) Section of LPS+CAP+ CR treated group, (C, D, E) showing the effects of CAP, CR or CAP and CR in combination after induced by LPS on hippocampus and (F) Intensity of CA1 region.

It has been demonstrated that LPS injection enhanced the level of pro-inflammatory cytokine expression in the hippocampus. Pro-inflammatory cytokines are raised in an excessive inflammatory response, which is characterized by increases in mitochondrial dysfunction, reactive oxygen species (ROS), and nitric oxide (NO) **(Daulatzai, 2016).** The present data showed a highly significant increase in the NO concentration after LPS exposure for one week. Our results are in agreement with the findings of **Lowes *et al.* (2013)** and **Lin *et al.* (2021),** who postulated that after LPS exposure, the inflammatory response occurs and some cytokines are released. This leads to abnormal nitric oxide synthase (NOS) activation, resulting in an increase in NO levels. Our results could be attributed to the explanation of **Wu *et al.* (2022),** who demonstrated the ability of LPS to stimulate the immune system, specifically macrophages and other

immune cells, through activation of transcription factors such as nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs). These transcription factors translocate to the nucleus and endorse the expression of numerous pro-inflammatory genes, such as iNOS which is responsible for producing large amounts of nitric oxide from L-arginine.

LPS-induced cholinergic dysfunction is a crucial mechanism in neuroinflammatory and neurodegenerative diseases. In the current study, LPS injection resulted in a highly significant decrease in Ach levels in the hippocampal homogenate of the brain. Our findings are consistent with **Park *et al.* (2020),** who discovered that there were decreases in Ach levels and increases in acetylcholine esterase (AchE) activity following LPS exposure. Additionally, **Tyagi *et al.* (2018)** found that LPS enhances the generation of reactive oxygen species (ROS) in the brain, causing oxidative damage to neuronal structures such as cholinergic neurons.

According to Liu *et al.* (2020), who showed that LPS stimulates microglia, causing the release of pro-inflammatory cytokines, the results of the current study's decrease in Ach levels could be interpreted similarly. Inflammatory cytokines limit the action of choline acetyltransferase (ChAT), which produces Ach. Additional explanation by **Xia *et al.* (2022),** who confirmed that LPS diminishes Ach levels and signaling in the brain, predominantly through pro-inflammatory effects and interactions with the cholinergic system.

LPS injection causes a variety of immunohistochemistry alterations in the brain, making it an ideal model for researching neuroinflammation. Aurora kinases regulate cell division and have been connected to a wide range of cellular processes **(Beheshti *et al*., 2019).** The present findings showed that LPS administration increased aurora expression in the hippocampal tissues. In the same line, **Bahaidrahet *et al*. (2022)** revealed that LPS-induced neuroinflammation leads to alterations in Aurora kinase expression in the brain. This result is in harmony with **Zhang *et al*. (2018),** who showed that LPS-induced damage and the cell cycle-related proteins, including Aurora kinases, are upregulated. Our results are attributed to the explanation of **Muhammad *et al*. (2019),** who revealed that LPS exposure upregulates aurora kinase through stimulation of the production of pro-inflammatory cytokines. These cytokines can activate signaling cascades related to aurora, increase oxidative stress and DNA damage, and alter synaptic function and plasticity.

As expected, there was a significant reduction in NO concentration in the LPS+CAP group in the homogenate of the brain hippocampus. Supporting evidence was reported by **Abdel-Zaher *et al*. (2017),** who confirmed that captopril decreased NO metabolites as a result of decreased iNOS. Also, our findings are attributed to the illumination of **Abareshi *et al.* (2017),** who revealed that captopril diminishes NO concentration by overwhelming inflammatory signaling pathways. It can reduce iNOS expression, modulate the cholinergic system via endothelial NOS, and reduce angiotensin II-mediated signaling in the renin angiotensin system (RAS).

As expected once more, the present data for the LPS+CAP group revealed a notable rise in the Ach levels in the hippocampal homogenate

These results were consistent with those of **Baroni *et al.* (2011)** and **Akbari et al. (2019),** who demonstrated that captopril protects cholinergic neurons indirectly, allowing for the synthesis and release of Ach.

In the same vein, **Abareshi *et al.* (2019) s**hown that captopril's anti-inflammatory and antioxidant activities provide noteworthy therapeutic benefits to raise Ach levels and shield cholinergic neurons from oxidative damage.

As expected, the LPS+CAP group showed a moderate expression of aurora color intensity. Similar findings were made by **Godsel *et al.* (2013), Wysocki *et al.* (2016), and Asraf *et al.* (2018),** who found that captopril inhibited aurora kinases, which in turn affected apoptosis and cellular proliferation.

Remarkably, the current data exhibited a highly significant reduction in NO concentration in the LPS+CR and LPS+CAP+CR groups. Similarly, **Nam *et al*. (2010) and Ghotbeddin *et al*. (2021)** authenticated the ability of crocin to block the production of iNOS and returned the elevated NO levels caused by LPS administration to control levels. The probable elucidation by **Dai *et al.* (2021)** showed that the effects of crocin make it a valuable product to scavenge NO, suppress NF-κB, and inflammatory cytokines, and reduce iNOS expression. Also, it acts as an antioxidant and moderates pathways related to angiotensin II and neuroinflammation. Additional explanation is provided by **Yang *et al.* (2023),** who illuminated that crocin improves mitochondrial health by reducing oxidative stress and preventing mitochondrial dysfunction, which is related to excessive NO release.

Interestingly, the LPS+CR and LPS+CAP+CR groups revealed a highly significant elevation in Ach levels**.** In parallel with our data, **Geromichalos *et al*. (2012)** and **Andrade *et al*. (2019)** showed that crocin has the ability to restore Ach levels by increasing the CHAT and decreasing AchE activities. The elevation of Ach levels in crocin groups in the present study may be due to its ability to cross the blood-brain barrier. It has potential neuroprotective effects through its antioxidant and anti-inflammatory properties. It also regulates choline uptake, a precursor for Ach production, by neurons as documented by **Saeedi et al. (2021)**.

The current study's findings showed that when exposed to aurora antibody, the hippocampus tissues of the LPS+CR and LPS+CAP+CR groups showed a poor expression. Our findings support those of **Dastan *et al*. (2024),** who demonstrated that crocin inhibits aurora expression via controlling pathways related to cell survival and proliferation. According to **Yang *et al*. (2023),** the anti-inflammatory, anti-oxidative stress, and anti-cholinergic qualities of crocin can be credited by the present investigation with modulating aurora expression in crocin groups.

Conclusions

LPS has a significant impact on brain biochemistry and cellular transmission, making it a popular model for investigating neuroinflammation. LPS reduces Ach levels, disrupting cholinergic signaling and cognitive function. It increases NO levels through the activation of iNOS, contributing to oxidative stress and neurotoxicity. Furthermore, LPS increases aurora kinase expression in the hippocampus, possibly as part of a stress response that disrupts normal cell cycle regulation and causes neuronal injury. Together,

these impacts aggravate neuroinflammatory processes and may hasten neurodegenerative alterations. On the other hand, crocin, a bioactive substance obtained from saffron, exhibits neuroprotective abilities. Crocin promotes cholinergic transmission and cognitive function by raising Ach levels. It lowers NO generation and shields neurons from nitrosative damage by reducing oxidative stress and blocking inflammatory signaling pathways. Additionally, crocin may control the expression of aurora kinase, preserving healthy cell cycle function and lessening cellular disturbances in the brain caused by inflammation. These various effects demonstrate crocin's potential as a treatment for neuroinflammation and associated neurological conditions. So according to the current study, people should consume foods high in crocin, such saffron, or take crocin supplements under a doctor's supervise.

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