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RESEARCH

Dissecting the antidepressant effect of troxerutin: modulation of neuroinflammatory and oxidative stress biomarkers in lipopolysaccharide-treated mice

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Abstract

The role of neuroinflammation in the pathogenesis of depression has prompted the search for new antidepressants. Troxerutin, a bioflavonoid with anti-inflammatory and antioxidant properties, has shown promise, but its impact on neurobehavioral functions remains poorly understood. This study aimed to investigate the antidepressant potential of troxerutin and its effect on the neuroinflammatory response. Here, we exposed male Swiss mice ($n = 5$/group) to various treatments, including naive and negative controls receiving distilled water, troxerutin-treated groups administered at different doses (10, 20, 40 mg/kg, i.p.), and an imipramine-treated group (25 mg/kg, i.p.). After seven days of treatment, with the exception of the naive group, mice were administered a single dose of lipopolysaccharide (LPS, 0.83 mg/kg). Behavioral evaluations, consisting of the novelty-suppressed feeding (NSF) test, forced swim test (FST), and open field test (OFT), were conducted. Additionally, brain samples were collected for biochemical and immunohistochemical analyses. Troxerutin significantly reduced immobility time in the FST and mitigated behavioral deficits in the NSF test. Additionally, troxerutin increased glutathione (GSH) and superoxide dismutase (SOD) levels while reducing nitrite, malondialdehyde (MDA), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interferon-gamma (IFN-γ) levels compared to the negative control. Immunohistochemistry analysis revealed decreased expression of inducible nitric oxide synthase (iNOS) and nuclear factor-kappa B (NF-κB) in troxerutin-treated mice. Overall, these findings suggest that troxerutin exerts significant antidepressive-like effects, likely mediated by its anti-inflammatory and antioxidant mechanisms. The reduction in neuroinflammatory and oxidative stress biomarkers, along with the improvement in behavioral outcomes, underscores troxerutin's potential as a therapeutic agent for depression.

Keywords Depression · Oxidative stress · Neuroinflammation · Neurobehavior · Troxerutin

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Abbreviations

Akt/GSK3	Protein kinase B/glycogen synthase kinase 3
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CREB	CAMP response element–binding protein
ELISA	Enzyme-linked immunosorbent assay
EPK	Eukaryotic protein kinase
FST	Forced swimming test
GSH	Glutathione
HO-1	Heme oxygenase-1
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MDA	Malondialdehyde
NF-κB	Nuclear factor-kappa B

NO	Nitric oxide
NQO1	NAD(P)H quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2–related factor 2
NSF	Novelty-suppressed feeding
OFT	Open field test
PMF	Post-mitochondrial fraction
ROS	Reactive oxygen species
SIRT1/SIRT3	Sirtuin 1/3
SOD	Superoxide dismutase
TCA	Tricyclic antidepressant
TNF-α	Tumor necrosis factor-alpha
TRD	Treatment-resistant depression
Tx	Treatment

Introduction

Depression is a common neurobehavioral disorder, significantly impacts daily life through persistently low mood, diminished interest in activities, anxiety, hypersomnia, loss of appetite, inattention, and reduced energy levels (Brent and Maalouf 2015; Benasi et al. 2021). With a lifetime prevalence of up to 20.6% (Hasin et al. 2018), its onset can span from childhood to late life, leading to severe impairment without effective therapeutic intervention (Forbes and Dahl 2005). The disorder arises from a complex interplay of genetic, biological, psychological, and environmental factors (Malhi and Mann 2018).

Several hypotheses have been proposed to understand depression, including the monoamine hypothesis, which emphasizes neurotransmitter imbalances, and the neurotrophic hypothesis, which focuses on neuronal growth factors (Jeon and Kim 2016). However, the pathophysiology of depression also involves elevated levels of pro-inflammatory cytokines (Malhi and Mann 2018), suggesting a link between inflammation and depressive symptoms. This inflammatory response may precede and exacerbate neuronal damage, leading to depressive episodes. The macrophage hypothesis suggests that the inflammatory process triggers depressive disorders through pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α), released by macrophages in response to inflammation (Anisman and Merali 2003). These cytokines impact the central nervous system, and understanding their mechanisms is crucial for developing novel antidepressants, particularly for treatment-resistant depression (TRD), where excessive interleukin levels are often observed (Osimo et al. 2020).

The administration of lipopolysaccharide (LPS), a bacterial endotoxin, induces symptoms resembling depression (Lasselin et al. 2020). Mechanistic evaluation shows that the immune chemotactic response to LPS prompts the release of pro-inflammatory cytokines, thus mediating LPS-induced depression (Sangaran et al. 2021). LPS also initiates the release of nitric oxide (NO), free radicals (ROS), and an array of inflammatory mediators that activate eukaryotic protein kinase (EPK) nuclear factor signal transduction pathways. This activation downregulates the transcription factor, cyclic-AMP response element–binding protein (CREB) (Noworyta-Sokołowska et al. 2013). Consequently, the diminished neurotrophic transcription, marked by increasing neuronal atrophy, is likely associated with pro-inflammatory cytokines serving as underlying triggers (Ge et al. 2015; Motaghinejad et al. 2016). Therefore, a controlled inflammatory response could potentially alleviate neurobehavioral impairment and preserve cognitive function in depression.

Previous preclinical studies have demonstrated that specific categories of plant-derived flavonoids can permeate the blood–brain barrier, influencing the CNS by orchestrating neuroprotective responses to safeguard neuronal survival against injuries induced by inflammation and oxidative stress (Panche et al. 2016; Karthika et al. 2022; Isibor et al. 2022; Oredeko et al. 2023). These flavonoids exert neuroprotective effects by inhibiting oxido-inflammatory pathways in neuronal cells such as microglia and astrocytes, or by enhancing synaptic plasticity, thereby fostering memory and cognitive functions (Jang and Johnson 2010; Hritcu et al. 2017; Omeiza et al. 2023) and one of the plant-derived flavonoids that has shown promising neuroprotective effects is troxerutin.

Troxerutin is a well-studied flavonoid that has been shown to possess anti-inflammatory, antioxidant, and neuroprotective properties, making it a potential therapeutic agent for various neurological disorders. It is derived from the Sophora japonica tree, and has been hypothesized to have potential neuromodulatory activities due to its ability to freely traverse into the brain (Jamali-Raeufy et al. 2019; Masood et al. 2020; Malik et al. 2023). The European Pharmacopoeia reports that troxerutin contains mainly tris(hydroxyethyl)rutin, while other trace components (mono, tetra, and bis-hydroxyethyl derivatives) are less than 20%. Its structural relationship to the naturally occurring bioflavonoid rutin, which exerts antidepressant-like effects via the glutamatergic pathway rather than the monoaminergic system, further suggests its antidepressant potential (Bianchi et al. 2018; Anjomshoa et al. 2020). Troxerutin is chemically denoted as vitamin P4 and has drawn attention for its diverse pharmacological profile, encompassing antioxidant, anticancer, and anti-inflammatory properties (Zhang and Stackman 2015).

The interplay of various mechanisms, including neuroinflammatory maladaptation, emerges as a plausible pathology preceding monoamine depletion, providing additional insight into why targeting oxido-inflammatory attenuation could serve as a promising primary strategy for reversing neurobehavioral deficits in depressive disorders (Caspani

et al. 2019; Omeiza et al. 2023). Furthermore, oxidative stress plays a significant role in neurodegeneration associated with depression, as highlighted by recent reviews discussing the neuroprotective potential of compounds like lithium (Ghanaatfar et al. 2023) and the importance of evaluating cognitive performance in animal models to link these findings to human cognition (Ghafarimoghadam et al. 2022).

This study assesses the effect of troxerutin on neurobehavioral activity in a neuroimmune model of depression in mice, aiming to elucidate its therapeutic potential through modulation of oxidative stress and neuroinflammation pathways.

Material and methods

Animals

The study utilized male Swiss mice with an average weight range of 20–25 g. The animals were housed in standard conditions, including ventilated propylene cages (42 cm in length, 30 cm in breadth, and 27 cm in height) with a 12-h light/dark cycle and a room temperature of 25 ± 1 °C. Animals were provided continuous free access to food and water and allowed to acclimatize for 2 weeks before exposing them to experimental protocols. The animal's handling was in accordance with the National Research Council's Guide for care and use of laboratory animals in biomedical research, and ethical approval was obtained from the University of Ibadan's local ethical committee on preclinical biomedicine with certification number UI/ACUREC/22–034. All investigational procedures were designed and done in compliance with the guidelines of Animal in Research: Reporting In-Vivo Experiments (ARRIVE).

Drugs and chemicals

Troxerutin was obtained from Xi'an Biological Technology in China, lipopolysaccharide from Sigma-Aldrich in the USA, imipramine from Shanghai Zhongxi Pharmaceutical Co., Ltd. in China, ELISA kits from Biobase Inc. in China, trichloroacetic acid and thiobarbituric acid from Sigma-Aldrich in the USA, immunohistochemical mouse IgG antibody from Elabscience in the USA, and chromogen diaminobenzidine tetrahydrochloride from Sigma-Aldrich in the USA.

Lipopolysaccharide-induced depression model

Lipopolysaccharide, an endotoxin of bacterial origin, at non-lethal dose induces depressive symptoms in mice and this experimental model represents the neuroinflammatory pathogenesis of depression. In this study, we administered LPS, 0.83 mg/kg, i.p, as described in a previous study (Sulakhiya et al. 2014). Animals were assigned into 6 treatment groups of 5 mice each, Group 1 received the vehicle, distilled water only, Group 2 received distilled water and LPS, Group 3 received troxerutin (10 mg/kg, i.p) and LPS, Group 4 received troxerutin (20 mg/kg, i.p) and LPS, Group 5 received troxerutin (40 mg/kg, i.p) and LPS, and Group 6 received imipramine (25 mg/kg, i.p) and LPS. We administered the respective treatments for 6 consecutive days without administration of LPS. On the 7th day, LPS was dissolved in distilled water and was administrated 1 h after treatment with distilled water, troxerutin, or imipramine as indicated and animals were fasted (acute food deprivation) on the 7th day till the following morning. The neurobehavioral tests, novelty-suppressed feeding (NSF) and forced swim test (FST), were then assessed 24 h post-LPS exposure (Fig. 1). To rule out non-specific effects of interventions, the test animals were also subjected to a locomotor activity test. A separate set of animals exposed to troxerutin and/or LPS, not participating in behavioral tests, were sacrificed for biochemical and immunohistochemistry assays.

Novelty-suppressed feeding

Hyponeophagia is a common depressive behavior and the assessment was here done using the method of Samuels and Hen (2011). In the NSF, after the 24-h acute food deprivation and LPS challenge, animals were singly introduced at corner end in open field which has a normal chow diet pellet dropped in the center; the arena has a dimension of 40 cm × 40 cm. The lag time taken for each mouse to have a first bite of the chow within a 5-min test period was recorded as latency to feed. Animals were returned individually into their respective home cages which contained pre-weighed chow diet pellet feed and the amount of feed consumed by the animal in 5 min was determined and recorded.

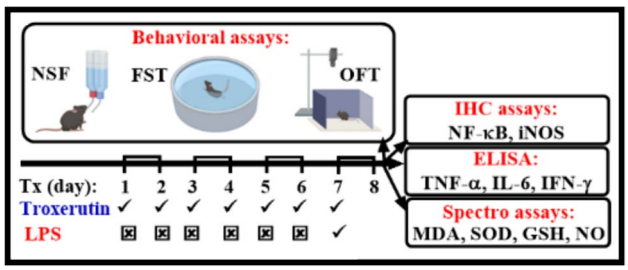


Fig. 1 The methodology employed to explore the effect of troxerutin against LPS-induced depressive-like symptoms in mice. NSF novelty-suppressed feeding, FST force swimming test, OFT open field test, Tx treatment, LPS lipopolysaccharide, MDA malondialdehyde, SOD superoxide dismutase, iNOS inducible nitric oxide synthase, TNF-α tumor necrosis factor-alpha, IL-6 interleukin-6, IFN-γ interferon-gamma

Forced swimming test

When subjected to inescapable stress, rodents tend to exhibit characteristic despair behavior which has been modeled by Porsolt et al. (1977) to investigate predictive validity of antidepressants. We utilized the FST inescapable stress model to evaluate antidepressant activity of troxerutin in this study. Animals were allowed to swim in water at room temperature inside a transparent plexigas cylinder having a dimension of 40 by 18 cm with the water level filled up to about 15-cm depth. Each animal was allowed to swim for 6 min and the measure of behavioral despair was recorded with a stopwatch by an independent observer as duration of immobility within the last 4 min of swimming. Criteria for immobility were absence of movement of any form except those necessary to keep afloat.

Locomotor activity study

Enhanced locomotor activity may precipitate false positive outcomes in the neurobehavioral study. Locomotor activity of treated mice in OFT test was done using a model described by Akanmu et al. (2011) to determine specificity of antidepressant-like activity of troxerutin. The open field consists of an activity cage having three opaque sides and one other side made of transparent Plexiglas to form a box shape with dimensions of 28 cm by 28 cm by 28 cm. the floor of the box was painted into grid lines with a black paint to form 16 squares with each having a dimension of 7 cm by 7 cm. In the experiment, we introduced animals individually to the center of activity box 30 min after treatment and were allowed to explore freely for 5 min during which number of squares completely crossed with all four paws were counted and recorded by an independent observer.

Sample preparation for biochemical assays

Animals from troxerutin and/or LPS groups were sacrificed by cervical dislocation; their craniums were opened to harvest the brains which was rinsed with ice-cold 1.15% KCl, and parched with tissue paper. The brains were weighed individually and recorded, after which they were individually placed in specimen bottle containing ice-cold phosphate buffer solution adjusted to a 7.4 pH. The brains were minced and then homogenized completely. Homogenates were afterwards centrifuged in a 4 °C cold centrifuge for 10 min at rpm of 10,000 g. The resulting supernatant from the centrifuged homogenate was decanted as the mitochondrial fraction (PMF) into Eppendorf tubes and this supernatant was used in the respective spectrophotometric biochemical analysis of the levels of glutathione (GSH) according to protocol of Moron et al. (1979), malondialdehyde (MDA) according to protocol of Ohkawa et al. (1979), superoxide dismutase (SOD) according to protocol of Misra and Fridovich (1972), and nitrite according to protocol of Green et al. (1982), while the concentrations of TNF-α, IL-6, and IFN-γ in the PMF were measured using commercially available ELISA kit. The ELISA procedure was done in a 96-well as described by the manufacturer and endpoints were read with microplate reader Spectramax M-5 (Molecular Devices, Sunnyvale, CA) equipped with Softmax Pro v 5.4 (SMP 5.4).

Immunohistochemistry analysis

Immediately after the behavioral studies, mice were deeply anesthetized and sacrificed. Each animal was administered 50 mg/kg pentobarbital sodium intraperitoneally and was subsequently administered 4% paraformaldehyde in 0.01 M phosphate buffer via transcardiac perfusion in order to achieve in situ tissue fixation. The brains were excised and postfixed in buffered formalin until sectioning for study. Immunohistochemistry staining was carried out by rapidly removing the hippocampus and prefrontal cortex, and fixing the brain regions individually for 48 h in 4% paraformaldehyde solution after which serial coronal sections were obtained by cutting each individual brain serially into multiple sections of 2 to 5 µm thickness. Tissue blocks were prepared as free-floating slices by processes including paraffin embedding, deparaffinising, rehydrating, and pretreatment with hydrogen peroxidase. The prepared free-floating sections were immunostained according to the kit manufacturer's guideline. For each test animal, three independent sections were prepared. Using a binocular microscope (Nikon, Tokyo, Japan) with a mounted digital camera for capturing the positively stained expressions on micrographs, the NF-κB-positive nuclei or iNOS-positive nuclei were captured at × 400 magnifications and quantified using Image J analyzer software (NIH Image, MD, US). The field was viewed at an area of 500 by 500 mm^2 in each section and mean value for the three sections was calculated as expression activity.

Data analysis

The data were presented as means ± standard error of the mean (SEM). The normality of the data was assessed using the Shapiro–Wilk test, and the homogeneity of variance was evaluated using Levene's test. Since the data met the assumptions of normality and homoscedasticity, a one-way analysis of variance (ANOVA) was used to compare mean values across the groups, followed by a Tukey post hoc test to determine significant differences between groups. The analysis was performed using GraphPad Prism Biostatistics software version 8. The significance level was set at $P < 0.0001$, with a 95% confidence interval.

Results

Troxerutin (TXN) reversed neurobehavioral deficit in novelty-suppressed feeding

Exposure to LPS markedly prolonged ($P < 0.05$) the latency [F (5, 24) = 51.71, $P < 0.0001$] to feed in mice treated with the vehicle, as compared to the LPS-free group. However, this latency was significantly attenuated with the administration of troxerutin (10, 20, 40 mg/kg, i.p) and imipramine (25 mg/kg, i.p). In the case of observed feed consumption [F (5, 24) = 3.800, $P = 0.0112$], vehicle-treated mice exposed to LPS exhibited a noteworthy reduction compared to the LPS-free group. Nevertheless, this consumption was significantly increased upon treatment with troxerutin (10, 20, 40 mg/kg, i.p) and imipramine (25 mg/kg, i.p). Detailed results are presented in Table 1.

Table 1 Effect of troxerutin on feed latency and consumption in mice treated with LPS

Treatment	Latency to feed (s)	Feed consumption (g)
VEH (10 mL/kg)	99.4 ± 5.99	1.24 ± 0.1691
LPS + VEH	216 ± 4.94*	0.32 ± 0.037*
LPS + TXN (10 mg/kg)	101.4 ± 6.99#	1.14 ± 0.1833#
LPS + TXN (20 mg/kg)	100 ± 3.06#	1.2 ± 0.1871#
LPS + TXN (40 mg/kg)	99.6 ± 7.03#	1.14 ± 0.2337#
LPS + IMI (25 mg/kg)	106.4 ± 9.32#	1.28 ± 0.2354#

*$P < 0.0001$ compared to vehicle-treated LPS-free group. #$P < 0.0001$ compared to vehicle-treated LPS-exposed group. Values are expressed as mean ± S.E.M ($n = 5$). *VEH* vehicle, *LPS* lipopolysaccharide, *TXN* troxerutin, *IMI* imipramine

Troxerutin reduced immobility and restored locomotor activity in mice treated with LPS

In the forced swimming paradigm, the administration of LPS significantly increased the duration of immobility [F (5, 24) = 40.43, $P < 0.0001$] in mice compared to the vehicle control group. However, both troxerutin-treated and imipramine-treated groups exhibited a decrease in immobility time, as depicted in Fig. 2A. Furthermore, in the open field test, the number of line crossings [F (5, 24) = 15.82, $P < 0.0001$], metric of locomotor activity, decreased in the LPS-treated group compared to the vehicle control group. This LPS-induced effect was reversed by troxerutin and imipramine, as illustrated in Fig. 2B. The observed reduction in immobility time and normalization of locomotor activity suggests troxerutin's antidepressant-like activity, akin to the standard antidepressant drug imipramine.

Troxerutin mitigates oxidative biomarkers in the brains of mice treated with LPS

Troxerutin administration in mice resulted in the enhancement of endogenous antioxidants in the brain, as depicted in Fig. 2. Mice subjected to lipopolysaccharide (LPS) without any intervention exhibited a depletion in GSH levels [F (5, 24) = 21.45, $P < 0.0001$] and SOD activity [F (5, 24) = 13.86, $P < 0.0001$] compared to vehicle control mice. However, this LPS-induced effect was effectively prevented in mice treated with troxerutin at doses of 10, 20, and 40 mg/kg, as well as imipramine at 25 mg/kg (Fig. 3A and B). In addition, the oxidative stress markers, MDA levels [F (5, 24) = 71.52, $P < 0.0001$] and nitrite production [F (5, 24) = 10.01, $P < 0.0001$], showed a marked increase in LPS-exposed mice. Pretreatment with troxerutin and imipramine

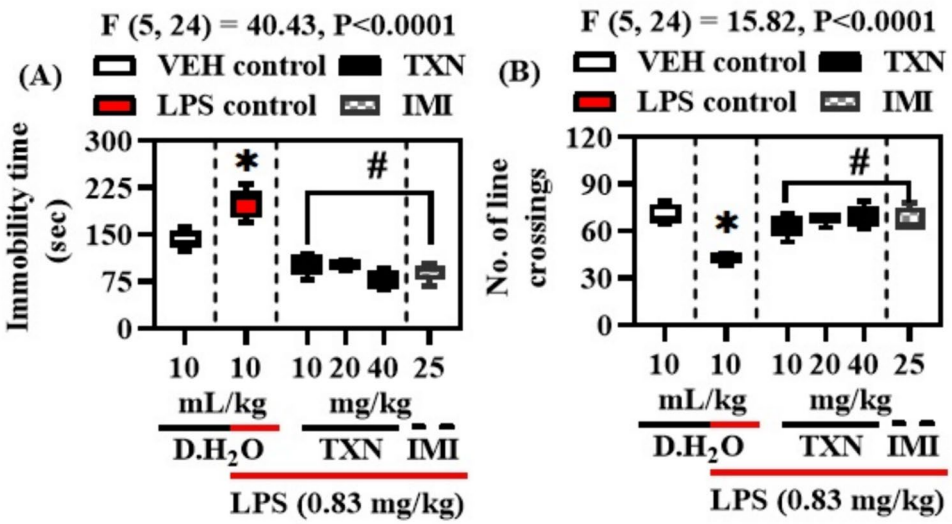


Fig. 2 Effect of troxerutin (10–40 mg/kg, i.p) on immobility time (**A**), and number of line crossings (**B**) in post-LPS exposure. Values are expressed as mean ± S.E.M ($n = 5$). *$P < 0.0001$ compared to vehicle-treated LPS-free group. #$P < 0.0001$ compared to vehicle-treated LPS-exposed group. VEH vehicle, LPS lipopolysaccharide, TXN troxerutin, IMI imipramine

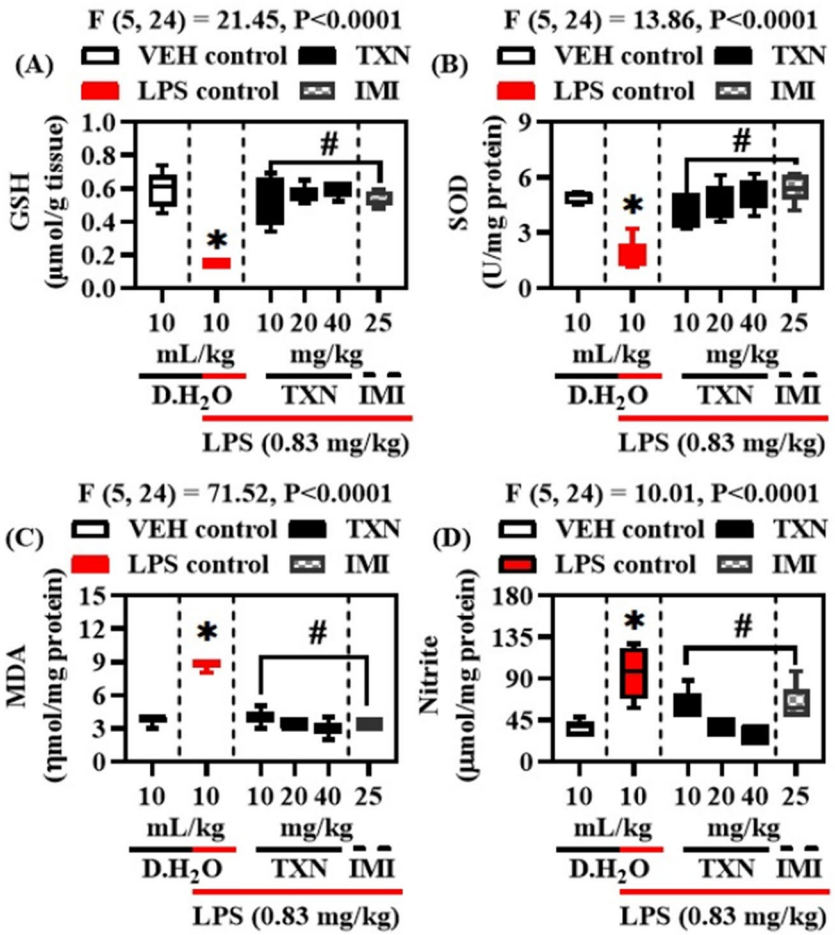


Fig. 3 Effect of troxerutin (10–40 mg/kg, i.p) on the levels or activity of **A** GSH, **B** SOD, **C** MDA, and **D** nitrite in LPS-treated mice brains. Values are expressed as mean ± S.E.M ($n = 5$). *$P < 0.0001$ compared to vehicle-treated LPS-free group. #$P < 0.0001$ compared to vehicle-treated LPS-exposed group. VEH vehicle, LPS lipopolysaccharide, TXN troxerutin, IMI imipramine, GSH reduced glutathione, SOD superoxide dismutase, MDA malondialdehyde

significantly reduced the levels of MDA and nitrite in the mice brain, as depicted in Fig. 3C and D, respectively.

Troxerutin reduced the levels of pro-inflammatory cytokines in the brains of mice exposed to LPS

Figure 4 illustrates how troxerutin, a promising therapeutic agent, alleviates neuroinflammation induced by LPS. Intoxication of mice with LPS resulted in elevated levels of TNF-α [F (5, 24) = 9.741, $P < 0.0001$], IL-6 [F (5, 24) = 17.84, $P < 0.0001$], and IFN-γ [F (5, 24) = 52.52, $P < 0.0001$], compared to the LPS-free group. Notably, these effects were counteracted in the brains of mice pretreated with troxerutin (10, 20, 40 mg/kg) or imipramine (25 mg/kg), as evidenced in Fig. 4A–C, when compared to vehicle-treated controls exposed to LPS.

Troxerutin reduced the NF-κB expression in prefrontal cortex and hippocampusin the brains of mice exposed to LPS

The examination of prefrontal cortex and hippocampus from the brain slice revealed a substantial presence of NF-κB positive cells expression in the LPS-exposed group. In contrast, the groups treated with troxerutin displayed a scarcity of positively stained cells, as shown in Fig. 5A and C. The quantification of NF-κB positive cell expression, depicted in Fig. 5B and D, respectively, indicated a significant decrease in NF-κB expression in both the prefrontal cortex [F (5, 24) = 52.42, $P < 0.0001$] and the hippocampus [F (5, 24) = 28.81, $P < 0.0001$] following treatment with troxerutin or imipramine compared to the LPS-exposed vehicle group.

Troxerutin decreased the iNOS expression in prefrontal cortex and *hippocampus* in the brains of mice exposed to LPS

Examination of the prefrontal cortex and hippocampus in brain slices revealed a notable presence of cells expressing iNOS positivity in the LPS-exposed group. Conversely, groups treated with troxerutin exhibited a scarcity of positively stained cells, as depicted in Fig. 5A and C. The quantification of iNOS-positive cell expression, illustrated in Fig. 6B and D, respectively, indicated a significant reduction in iNOS expression in both the prefrontal cortex [F (5, 24) = 30.05, $P < 0.0001$] and the hippocampus [F (5,

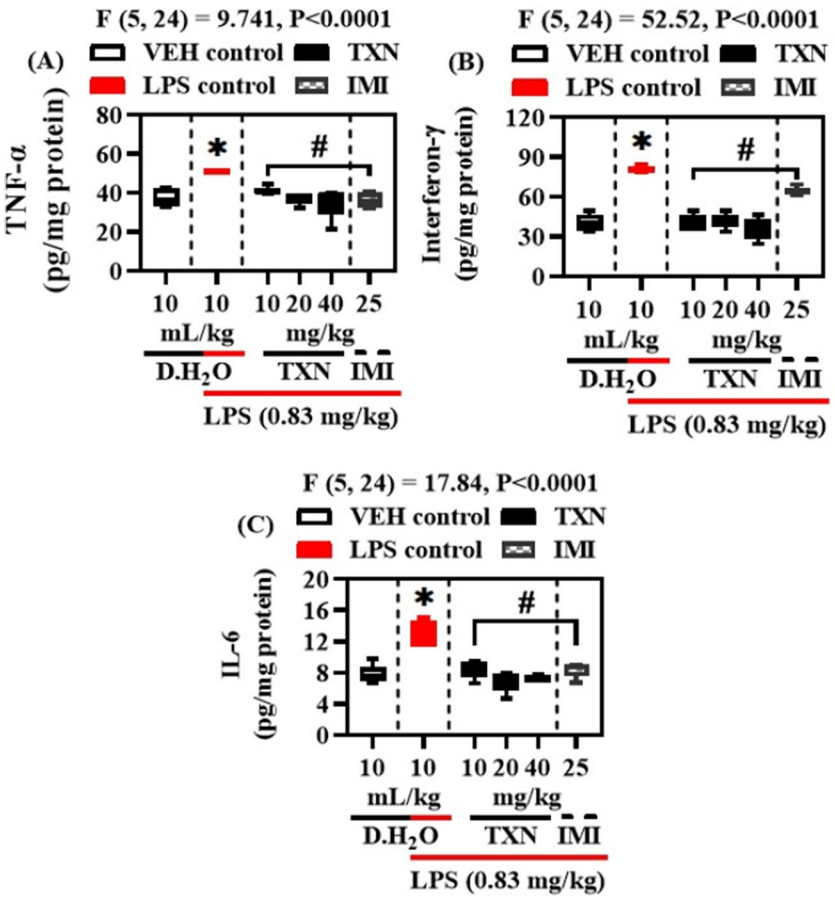


Fig. 4 Effect of troxerutin (10–40 mg/kg, i.p) on the levels of **A** TNF-α, **B** Interferon-γ, and **C** IL-6, in LPS-treated mice brains. Values are expressed as mean ± S.E.M ($n = 5$). *$P < 0.0001$ compared to vehicle-treated LPS-free group. #$P < 0.0001$ compared to vehicle-treated LPS-exposed group. VEH vehicle, LPS lipopolysaccharide, TXN troxerutin, IMI imipramine, TNF-α tumor necrosis factor-alpha, Interferon-γ interferon-gamma, IL-6 interleukin-6

24) = 24.33, $P < 0.0001$] following treatment with troxerutin or imipramine compared to the LPS-exposed vehicle group.

Discussion

Lipopolysaccharide (LPS), a constituent found in the outer membrane of Gram-negative bacteria, is renowned for its potent inflammatory properties (Matsuura 2013). A wealth of existing data consistently reveals that the administration of LPS in experimental models triggers inflammation, leading to heightened neuroinflammation, even with a single dose (Sulakhiya et al. 2014). This augmented inflammatory response results in an excess of pro-inflammatory cytokines and oxidative stress, ultimately manifesting as neurobehavioral impairment resembling depressive disorder (Maria Michel et al. 2012; Jazvinšćak Jembrek et al. 2023). Our recent findings underscore the substantial effect of a single LPS dose on impairing the neurobehavioral activities of animals treated with the vehicle alone. However, troxerutin, mimicking the effectiveness of the reference drug imipramine [tricyclic antidepressant (TCA)], adeptly prevents LPS-induced behavioral impairment in forced swimming (FST), open field (OFT), and novelty-suppressed feeding (NSF) paradigms by inhibiting oxido-inflammatory signaling and mitigating cellular damage.

The commonly adopted neurobehavioral paradigm for assessing antidepressant activity in mice is the FST (Yankelevitch-Yahav et al. 2015). This test is predictive of behavioral despair, and various studies have demonstrated that following LPS exposure, agents with antidepressant properties reduce the duration of immobility in animals subjected to FST, as seen with the selective serotonin reuptake inhibitor fluoxetine (Ohgi et al. 2013). In our study, firstly, LPS induces a prolonged duration of immobility in animals without any intervention when subjected to FST. Furthermore, we expanded our investigation by incorporating the OFT to assess the impact of LPS on locomotor activity. The OFT is a valuable tool for evaluating general activity levels (Omeiza et al. 2022) and exploring whether the observed immobility in the FST is linked to a broader decrease in motor function. Our findings revealed that LPS exposure induced hypoactivity in animals, as evidenced by a significant reduction in the number of line crossings in the open field. This decrease in locomotor activity is indicative of a broader behavioral alteration beyond the specific context of the FST. Importantly, the reduction in line crossings in the OFT aligns with the prolonged immobility observed in the FST after LPS

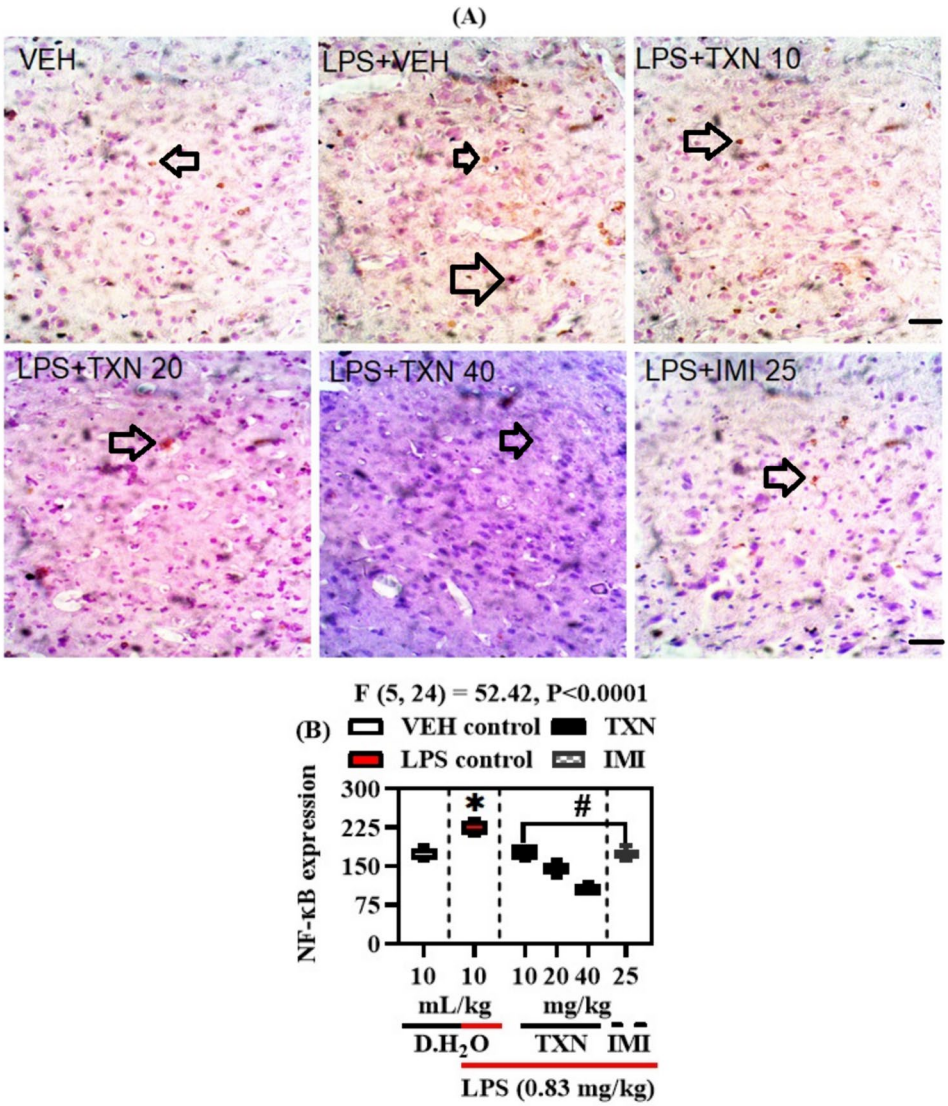


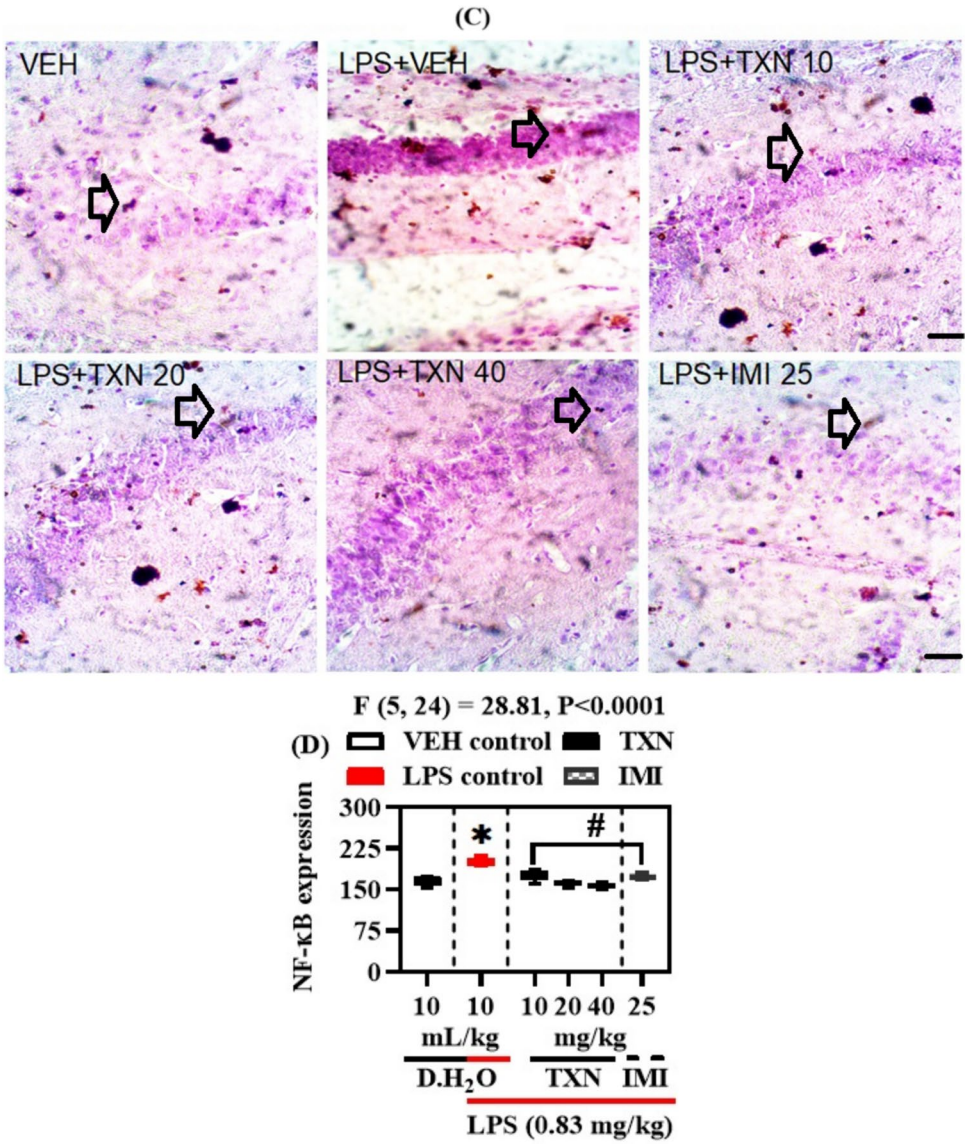
Fig. 5 Photomicrographs vividly depict the impact of troxerutin on LPS-induced immunohistochemical alterations and NF-κB immunopositive cell expressions in the prefrontal cortex (**A**) and hippocampus (**C**) of mice brains. Higher intensity reflects elevated NF-κB immunopositive cell expression, while lower intensity signifies reduced expression. Troxerutin effectively diminishes LPS-induced NF-κB expression in the prefrontal cortex (**B**) and cornu-ammonis region of the hippocampus (**D**) in mice brains. Magnification = × 400. Black arrow in panels **A** and **C** indicates immunopositive cell expression. Data is presented as mean ± S.E.M ($n = 3$). *$P < 0.0001$ compared to the vehicle-treated LPS-free group. #$P < 0.0001$ compared to the vehicle-treated LPS-exposed group. Abbreviations: VEH vehicle, LPS lipopolysaccharide, TXN troxerutin, IMI imipramine, NF-κB nuclear factor-kappa B

exposure. The correlation between LPS-induced hypoactivity in the OFT and increased immobility in the FST suggests a potential link between reduced locomotor activity and depressive-like behavior in our animals. It implies that the behavioral despair observed in the FST might be associated with a more generalized decrease in overall activity, reflecting aspects of depression-like symptoms (Stepanichev et al. 2018).

Furthermore, to deepen our understanding of LPS-induced depression, we incorporated the NSF paradigm into our study. The NSF test is recognized as a model for assessing depressive-like behavior, and it specifically evaluates an animal's response to an aversive stimulus, such as the introduction of a novel food source in an unfamiliar environment (Bodnoff et al. 1988; Sellmeijer 2016). In our investigation, we sought to explore whether LPS-induced depression exhibited a sickness behavior endophenotype, mirroring human depressive symptoms such as anorexia and disinterestedness. Our results revealed that animals exposed to LPS displayed a significant increase in the latency to approach and consume food in the NSF test compared to the control animals. This delayed feeding response is indicative of depressive-like behavior, suggesting that LPS exposure not only impacts locomotor activity but also influences the animal's approach to food in a novel environment. The observed sickness behavior phenotype aligns with clinical manifestations of depression in humans, where symptoms often extend beyond emotional aspects to include disruptions in appetite and interest (Sharpley and Bitsika 2013).

The behavioral changes observed in response to LPS insult, as seen in the FST, OFT, and NSF results, are likely the result of a complex interplay of neurobiological mechanisms. To investigate this further, we examined the underlying mechanisms associated with LPS-induced neurobehavioral impairment. LPS, known for inducing an immune response and subsequent neuroinflammation, triggers a

Fig. 5 (continued)



cascade of events affecting various aspects of brain function. Our data reveal that the release of pro-inflammatory cytokines, including TNF-α, IFN-γ, and IL-6, and the overexpression of iNOS and NF-κB in brain tissues during the immune response, may directly impact neural circuits governing behaviors. This is consistent with previous studies that reported depression to have a significant inflammatory component (Batista et al. 2019; Jeon and Kim 2016; Montoya et al. 2021; Wang et al. 2019; Omeiza et al. 2023). These inflammatory cytokines and chemokines affect neurotransmitter systems and neuronal activity, contributing to depressive-like behaviors. Notably, the inflammatory response involves the activation of macrophages, which release pro-inflammatory cytokines like IL-6, creating an inflammatory environment in regions like the prefrontal cortex and hippocampus (Prestwood et al. 2021; Eneni et al. 2023). TNF-α and IFN-γ also play key roles in this process, with TNF-α contributing significantly and IFN-γ intensifying the pro-inflammatory state (Dantzer et al. 2008; Wei et al. 2020). iNOS and NF-κB are also central to this response, leading to neuroinflammation, neuronal cell death, and reduced serotonin levels (Kaster et al. 2012; Venkataramana et al. 2015; Carabelli et al. 2020; Jamali-Raeufy et al. 2021; Sangaran et al. 2021; Stone and Williams 2023).

In addition to inducing neuroinflammation, previous data indicated that a single dose of systemic LPS could prompt oxidative stress through various mechanisms: activation of NADPH oxidase, causing superoxide radical production; promotion of mitochondrial dysfunction, leading to ROS generation; induction of iNOS, increasing NO and forming damaging peroxynitrite; activation of inflammatory pathways like NF-κB, boosting ROS production; triggering metal ion release, contributing to hydroxyl radical formation via Fenton reactions (Kaur et al. 2006a,

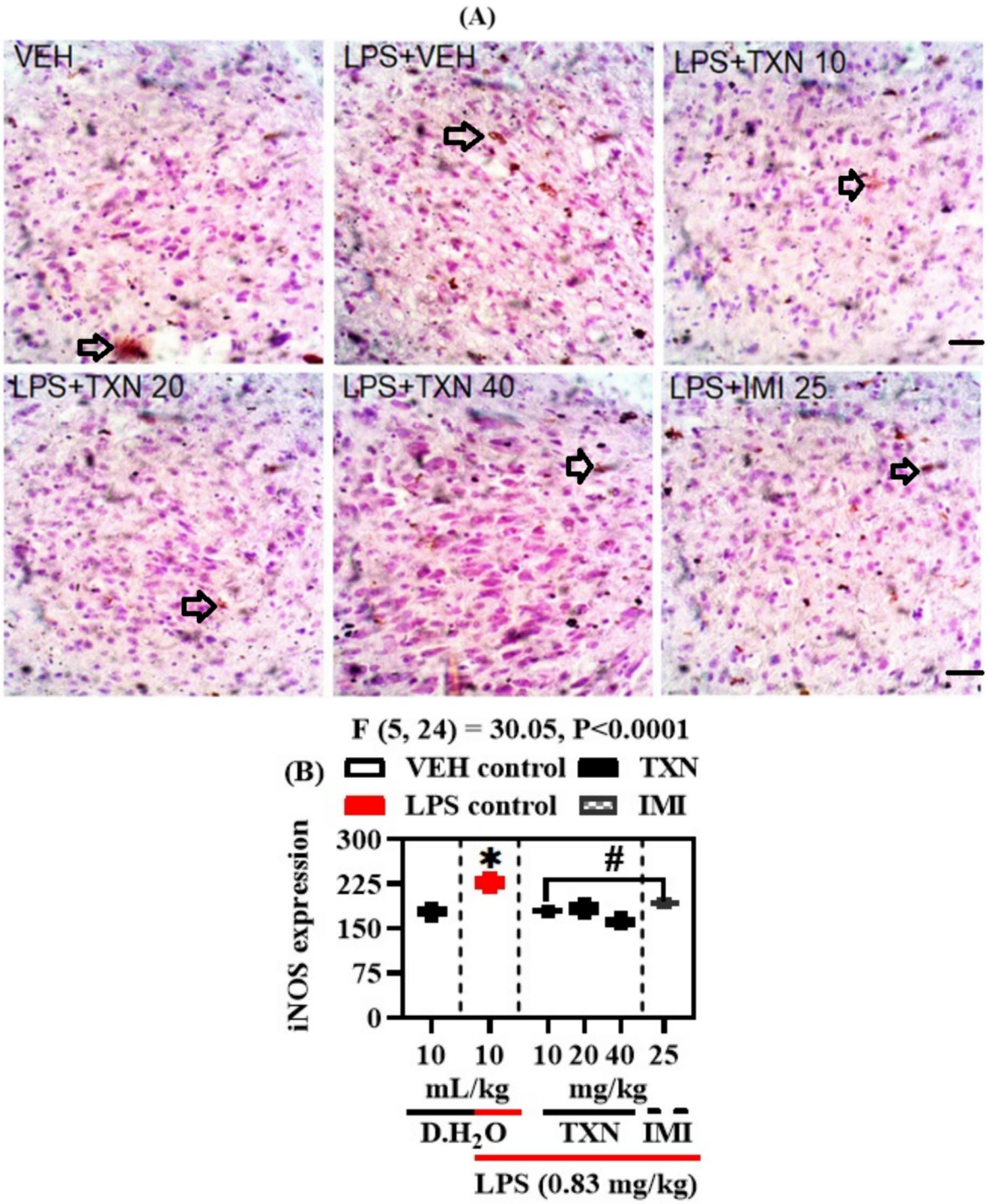


Fig. 6 Photomicrographs vividly depict the impact of troxerutin on LPS-induced immunohistochemical alterations and iNOS immunopositive cell expressions in the prefrontal cortex (**A**) and hippocampus (**C**) of mice brains. Higher intensity reflects elevated iNOS immunopositive cell expression, while lower intensity signifies reduced expression. Troxerutin effectively diminishes LPS-induced iNOS expression in the prefrontal cortex (**B**) and cornu-ammonis region of the hippocampus (**D**) in mice brains. Magnification = × 400. Black arrow in panels **A** and **C** indicates immunopositive cell expression. Data is presented as mean ± S.E.M ($n = 3$). *$P < 0.0001$ compared to the vehicle-treated LPS-free group. #$P < 0.0001$ compared to the vehicle-treated LPS-exposed group. Abbreviations: VEH vehicle, LPS lipopolysaccharide, TXN troxerutin, IMI imipramine, iNOS inducible nitric oxide synthase

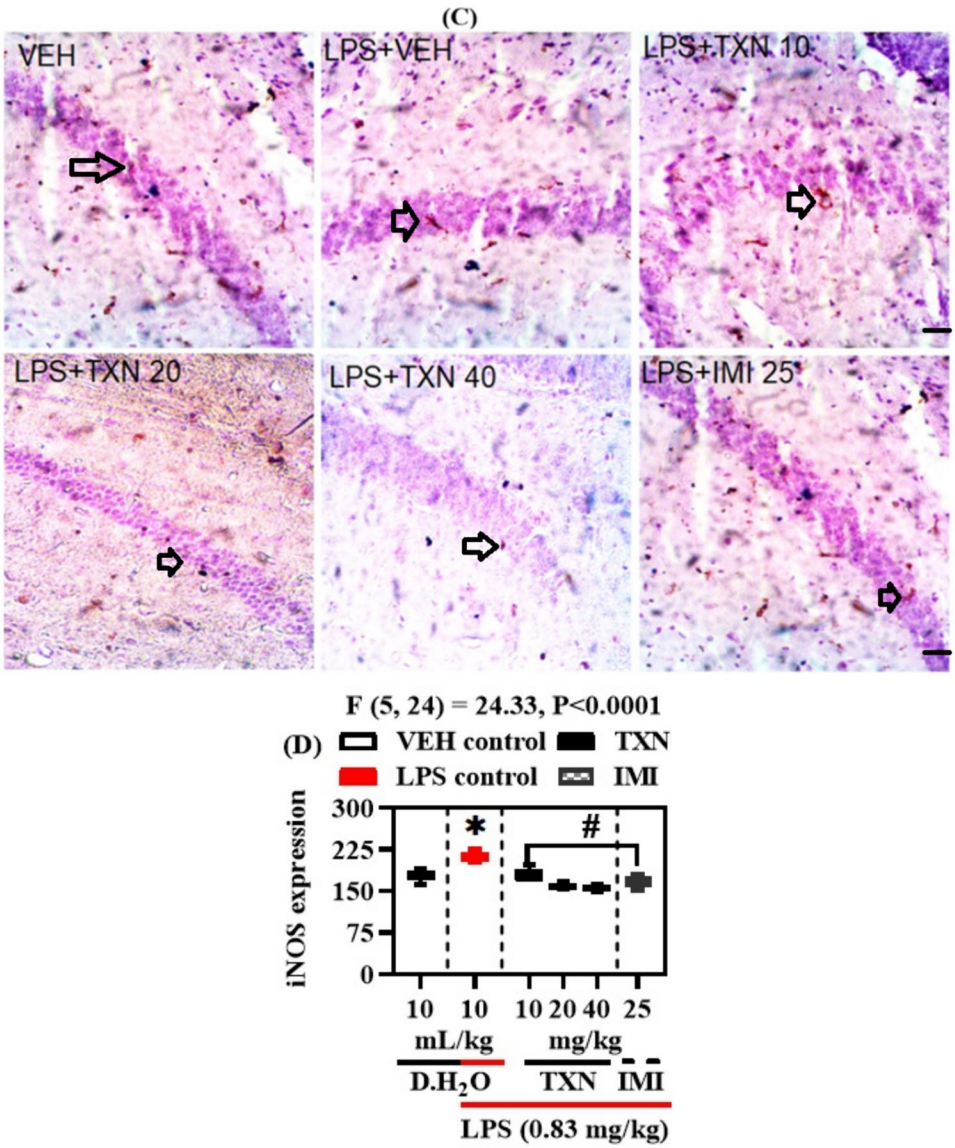


Fig. 6 (continued)

2006b; Salzano et al. 2014; Ingram et al. 2019; Mokhtari-Zaer et al. 2020; Wu et al. 2020). Additionally, LPS may impair antioxidant enzyme activity, compromising cellular defense against ROS (Ilçe et al. 2019). These processes collectively result in oxidative stress, potentially damaging cellular components, particularly in the brain, which is vulnerable to oxidative attack (Abdulrahim et al. 2021; Adebayo et al. 2023; Ben-Azu et al. 2024). In our current study, mice subjected to LPS without intervention exhibited depleted GSH and SOD activity, and elevated MDA levels, and nitrite, indicating heightened oxidative stress. Therefore, the outcomes of these LPS-mediated processes not only result in neuronal cell death as a result of neuroinflammation and oxidative stress but also lower monoamine biosynthesis, a consistent finding in depressed subjects across various studies (Dantzer et al. 2008; Sun et al. 2022).

Interestingly, certain therapeutic agents and supplements have shown promise in reversing crucial biomarkers associated with depressive behaviors in various paradigms (Han et al. 2020; Omeiza et al. 2023). Previous studies have documented the reversal of behavioral despair in the FST and hypoactivity in the OFT, along with the suppression of appetite in the NSF paradigms (Li et al. 2018). These behavioral changes are indicative of potential antidepressant efficacy, particularly when targeting the monoaminergic pathway (Gerhard et al. 2016). It is worth noting that specific antidepressants, particularly those affecting serotonin, may also possess anti-inflammatory properties (Roumestan et al. 2007). In this study, troxerutin, a flavonoid and dietary

supplement, effectively counteracts the behavioral disruptions induced by LPS, highlighting its anti-inflammatory and antioxidant attributes in the FST, OFT, and NSF paradigms. This robustly supports troxerutin's antidepressant properties, evident in its similarity to the effects observed with the reference drug imipramine. These findings align with previous reports involving substances or supplements such as arctigenin, paroxetine, melatonin, *Carpolobia lutea*, and minocycline, which demonstrated the reversal of depressive-like behaviors. These interventions achieved antidepressant effects by diminishing NF-κB expression, suppressing iNOS expression, reducing IL-1β and IL-6 levels, and inhibiting IDO through the modulation of pro-inflammatory cytokines (TNF-α and IFN-γ), respectively (Henry et al. 2008; Strawbridge et al. 2015; Omeiza et al. 2021; Omeiza et al. 2023).

Moreover, troxerutin has been shown to inhibit NF-κB, leading to reduced inflammation and oxidative stress in mice exposed to LPS. Its neuroprotective effects have been demonstrated through its ability to mitigate LPS-induced neuroinflammation and oxidative damage, mediated by the SIRT1/SIRT3 signaling pathway (Jamali-Raeufy et al. 2019). Troxerutin's activation of the Nrf2 pathway upregulates antioxidant enzymes, such as HO-1 and NQO1, and enhances the activity of sirtuin proteins, specifically SIRT1 and SIRT3 (Jamali-Raeufy et al. 2019; Thomas et al. 2019; Wang et al. 2021; Yu et al. 2022). Consistent with these findings, our data indicate that troxerutin, a derivative of rutin, effectively scavenges ROS and enhances the activity of endogenous antioxidant enzymes, including SOD and GSH. This, coupled with its anti-inflammatory effects, ultimately prevents LPS-induced depressive-like phenotypes.

This study has several limitations, most notably the lack of exploration into the effects of troxerutin on other rodent models of depression, such as the unpredictable chronic mild stress model, which would help validate the data collected from the LPS mouse model of depression. Additionally, there is limited data on the impact of troxerutin on other key pathways involved in depression pathophysiology, including CREB/BDNF, Akt/GSK3, and monoaminergic (e.g., serotonergic and noradrenergic) signaling pathways. Addressing these areas would provide a more comprehensive understanding of its mechanism of action. Despite these limitations, our findings highlight the significant antidepressive-like activity of troxerutin in mitigating LPS-induced neurobehavioral impairments, similar to the reference drug imipramine. Troxerutin effectively counteracts depressive-like behaviors in the FST, OFT, and NSF paradigms. These effects are linked to troxerutin's anti-inflammatory and antioxidant properties, evidenced by its ability to inhibit NF-κB and iNOS expressions, reduce pro-inflammatory cytokines levels, and modulate oxidative stress signaling, thereby indirectly eliciting antidepressant-like effects. Given this background, troxerutin holds promise as a potential molecule or adjunct for the treatment of depression. Further studies should aim to address the current study's limitations and provide a thorough understanding of troxerutin's antidepressive-like mechanisms at both the molecular and connectivity levels.

Author contributions AS: conceptualization, experimental design, data curation, investigation, methodology, data analysis, and original draft writing; NAO: data curation, data analysis, original draft writing, review, and editing; AB: experimental design, data analysis, and co-supervision; HAA: softwares source, data analysis, review and editing of writing; AOA: conceptualization, experimental design, supervision, and review and editing of writing. All authors read the reviewed manuscript and declare that all data were generated in-house, with no paper mill used.

Data Availability The authors declare that the databases that support the results will be available when requested from the corresponding author.

Declarations

Ethical approval The study received approval from the local ethical committee on preclinical biomedicine at the University of Ibadan, with certification number UI/ACUREC/22–034. All investigative procedures were conducted in accordance with the Animal in Research: Reporting In-Vivo Experiments (ARRIVE) guidelines.

Competing interests The authors declare no competing interests.

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