

Comparative Metabolomic Profiling of Ocimum Sanctum Leaves Identifies Luteolin As A Neuroprotective Agent Against Neuroinflammation Via NLRP3 Inflammasome Modulation

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Abstract

Neuroinflammation is a critical pathological process associated with neuronal dysfunction and progressive neurodegeneration, with NLRP3 inflammasome activation representing an important molecular driver. The present study employed a metabolomics-guided approach to identify bioactive constituents of *Ocimum sanctum* leaves and evaluate their neuroprotective potential against NLRP3-mediated neuroinflammation. LC-MS/MS-based comparative metabolomic profiling identified multiple flavonoids and phenolic metabolites, among which luteolin exhibited prominent differential enrichment and was confirmed by accurate mass, retention time, and characteristic fragmentation pattern. Targeted quantification demonstrated substantial luteolin abundance in the hydroalcoholic extract. In an LPS-induced BV2 microglial model, luteolin reduced inflammatory activation and suppressed TNF- α , IL-6, IL-1 β , and IL-18 production without significant cytotoxicity. Mechanistic evaluation demonstrated attenuation of NF- κ B signaling, NLRP3 and ASC expression, and pro-caspase-1 processing, indicating suppression of inflammasome assembly and caspase-1 activation. Luteolin additionally reduced intracellular and mitochondrial ROS, lipid peroxidation, and mitochondrial dysfunction while restoring GSH, SOD, catalase, and mitochondrial membrane potential. In vivo validation further demonstrated reduced microglial activation, inflammatory cytokine production, NLRP3-associated signaling, and neuronal damage, accompanied by improved neurofunctional outcomes. Correlation analysis indicated an association between luteolin enrichment, reduced NLRP3 activation, and improved neuroprotective responses. Collectively, the findings identify luteolin as a prominent neuroprotective constituent of *O. sanctum* and suggest that coordinated regulation of oxidative stress, NF- κ B signaling, and NLRP3 inflammasome activation contributes to its anti-neuroinflammatory activity.

Keywords: *Ocimum sanctum*; Luteolin; Neuroinflammation; NLRP3 Inflammasome; Microglia; Metabolomics.

1. Introduction

Neuroinflammation is increasingly recognized as a fundamental pathological process underlying the initiation and progression of several neurological and neurodegenerative disorders. Although transient inflammatory responses within the central nervous system (CNS) are essential for tissue repair, immune surveillance, and restoration of cellular homeostasis, persistent or dysregulated neuroinflammation can promote neuronal dysfunction, synaptic impairment, oxidative damage, and progressive neuronal loss (Heneka et al., 2025; Zhang et al., 2023). Microglia, the resident immune cells of the CNS, play a central role in this process by sensing endogenous danger signals, environmental stimuli, damaged cellular components, and inflammatory mediators. Upon persistent activation, microglia undergo profound phenotypic and functional changes and release pro-inflammatory cytokines, reactive oxygen species (ROS), chemokines, and other mediators that can amplify neuronal injury. Increasing evidence indicates that inflammasome signaling, particularly the NOD-like receptor protein 3 (NLRP3) inflammasome, represents a critical molecular interface linking cellular stress, innate immune activation, and neurodegenerative pathology (DiSabato et al., 2016).

The NLRP3 inflammasome is a multiprotein intracellular signaling complex composed primarily of the NLRP3 sensor, apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1. Its activation generally involves two interconnected processes. The initial priming phase is frequently mediated by pattern-recognition receptors such as Toll-like receptor 4 (TLR4), which activates downstream MyD88-dependent nuclear factor- κ B (NF- κ B) signaling. NF- κ B subsequently increases the transcription of NLRP3 and pro-inflammatory cytokine precursors, particularly pro-IL-1 β and pro-IL-18 (W. Xu et al., 2025). The second activation phase is triggered by cellular danger signals, including mitochondrial dysfunction, excessive ROS generation, potassium efflux, calcium imbalance, lysosomal disruption, and accumulation of damage-associated molecular patterns. These signals promote NLRP3 oligomerization and recruitment of ASC, resulting in the conversion of pro-caspase-1 into active caspase-1. Activated caspase-1 subsequently cleaves pro-IL-1 β and pro-IL-18 into their biologically active forms, thereby promoting a potent inflammatory response. Persistent activation of this pathway can additionally contribute to gasdermin-D-dependent pyroptotic signaling and further propagation of neuroinflammatory injury (Y. He et al., 2016; Zahid et al., 2019).

The pathological importance of NLRP3 signaling extends beyond cytokine production because inflammasome activation is closely interconnected with oxidative stress, mitochondrial dysfunction, apoptosis, and neuronal homeostasis. Excessive mitochondrial ROS can act as an important secondary signal for NLRP3 activation, while inflammasome-derived inflammatory mediators can further impair mitochondrial function, creating a self-amplifying inflammatory cycle. IL-1 β and IL-18 released from activated microglia can influence neuronal excitability, synaptic function, oxidative metabolism, and cell survival. Consequently, sustained NLRP3 activation may contribute to the progression of neuronal damage by integrating inflammatory and metabolic disturbances. Modulation of the NLRP3 inflammasome therefore represents an attractive therapeutic strategy for controlling neuroinflammation and limiting inflammation-associated neuronal injury (Y. He et al., 2016; Paik et al., 2025).

Despite considerable progress in understanding inflammatory signaling, currently available pharmacological approaches for neuroinflammatory disorders remain associated with important limitations. Conventional anti-inflammatory agents may provide symptomatic or partial suppression of inflammatory responses but frequently lack specificity toward the intracellular signaling networks responsible for inflammasome activation. Moreover, long-term modulation of immune pathways can potentially interfere with physiological immune functions. These limitations have stimulated increasing interest in naturally occurring bioactive compounds capable of simultaneously regulating oxidative stress, inflammatory signaling, mitochondrial dysfunction, and neuronal survival (Paik et al., 2025). Medicinal plants represent a particularly valuable source of structurally diverse phytochemicals with multitarget pharmacological activities. However, conventional phytochemical screening approaches may overlook biologically important metabolites present at relatively low concentrations or fail to establish relationships between metabolite abundance and biological activity (Shao et al., 2015; Xia et al., 2023).

Metabolomics provides a powerful strategy for overcoming these limitations by enabling comprehensive characterization of small-molecule metabolites within biological or botanical systems. Mass spectrometry-based metabolomics, particularly liquid chromatography coupled with high-resolution tandem mass spectrometry (LC-MS/MS), can simultaneously detect numerous metabolites and generate characteristic chemical fingerprints of medicinal plants. Comparative metabolomic profiling can further identify metabolites that are differentially enriched between plant preparations, geographical samples, extraction conditions, or biologically active fractions. Integration of metabolomic data with pharmacological evaluation provides a rational approach for identifying candidate compounds responsible for specific biological effects. Such metabolomics-guided investigation is particularly relevant for medicinal plants with complex chemical compositions, where multiple flavonoids, phenolic compounds, terpenoids, and other secondary metabolites may contribute to therapeutic activity (Wu et al., 2022; Yang et al., 2019).

Ocimum sanctum L., commonly known as Tulsi or holy basil, is a medicinal plant extensively investigated for its antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, and neuroprotective properties. Its leaves contain a chemically diverse spectrum of secondary metabolites, including flavonoids, phenolic compounds, terpenoids, and other specialized metabolites. Several constituents of *O. sanctum* have demonstrated the ability to regulate oxidative stress and inflammatory pathways, supporting the potential of this plant as a source of neuroprotective molecules. Nevertheless, the precise metabolite responsible for modulation of inflammasome-mediated neuroinflammation remains insufficiently characterized. Comprehensive metabolomic profiling combined with mechanistic validation may therefore provide a more systematic approach for identifying the principal bioactive constituents of *O. sanctum* leaves (Hasan et al., 2023; Khan et al., 2025).

Among naturally occurring flavonoids, luteolin has attracted considerable attention because of its antioxidant and anti-inflammatory properties. Luteolin possesses a polyphenolic structure capable of interacting with multiple cellular signaling pathways involved in inflammatory and oxidative processes. Experimental evidence has suggested that luteolin can suppress NF- κ B-dependent inflammatory signaling, reduce ROS generation, improve cellular antioxidant defenses, and regulate pathways associated with apoptosis and mitochondrial dysfunction (Balakumar et al., 2011). These pharmacological properties are particularly relevant to NLRP3 inflammasome activation because oxidative stress and NF- κ B-mediated priming represent important upstream determinants of inflammasome signaling. Luteolin may therefore influence NLRP3 activity through coordinated regulation of inflammatory priming, oxidative stress, mitochondrial homeostasis, and downstream cytokine maturation. However, the identification of luteolin specifically through comparative metabolomic profiling of *O. sanctum* leaves and its subsequent mechanistic validation against NLRP3-associated neuroinflammation remain insufficiently integrated (Sethi et al., 2004).

The present study was therefore designed to investigate the neuroprotective potential of *O. sanctum* leaves using a metabolomics-guided pharmacological approach. Comparative LC-MS/MS-based metabolomic profiling was employed to characterize the chemical composition of *O. sanctum* leaf preparations and identify metabolites associated with enhanced biological activity. Luteolin was subsequently selected as a candidate bioactive metabolite and subjected to targeted analytical confirmation and quantitative evaluation. Its anti-neuroinflammatory effects were investigated with particular emphasis on microglial activation and the NLRP3 inflammasome pathway. The study further examined the effects of luteolin on upstream NF- κ B signaling, NLRP3 and ASC expression, caspase-1 activation, and maturation of IL-1 β and IL-18. Oxidative stress, mitochondrial dysfunction, and neuronal integrity were also evaluated to determine whether suppression of inflammasome signaling was associated with restoration of cellular homeostasis (Singh, 2022).

It was hypothesized that metabolomic profiling would identify luteolin as an important bioactive constituent of *O. sanctum* leaves and that luteolin-mediated neuroprotection would involve coordinated inhibition of oxidative stress, inflammatory priming, and NLRP3 inflammasome activation. By integrating metabolomic characterization with molecular and functional evaluation, the study aims to establish a mechanistic relationship between the phytochemical composition of *O. sanctum* and its neuroprotective activity. The findings may provide a rational basis for further development of luteolin-rich *O. sanctum* preparations or purified luteolin as potential candidates for targeting NLRP3-associated neuroinflammation and inflammation-driven neuronal injury (Rokonozzaman et al., 2025; Tabassum et al., 2022).

2. Pathophysiology of Neuroinflammation via NLRP3 Inflammasome Modulation

2.1 Microglial Activation, Oxidative Stress, and Priming of the NLRP3 Inflammasome

Microglia are the principal resident immune cells of the central nervous system (CNS) and continuously monitor the neuronal microenvironment to maintain tissue homeostasis. Under physiological conditions, microglia participate in synaptic remodeling, clearance of cellular debris, and immune surveillance. However, neuronal injury, oxidative stress, endotoxin exposure, or persistent inflammatory stimuli can induce microglial activation and transition toward a pro-inflammatory phenotype. Activated microglia release cytokines, chemokines, reactive oxygen species (ROS), and other mediators that can initiate or amplify neuronal injury. A major pathway involved in this response is Toll-like receptor 4 (TLR4) signaling, which recognizes pathogen-associated molecular patterns and endogenous damage-associated molecular patterns (Kouba et al., 2023; W. Xu et al., 2025).

Following TLR4 stimulation, recruitment of the adaptor protein MyD88 activates downstream signaling involving nuclear factor- κ B (NF- κ B). Activated NF- κ B translocates to the nucleus and promotes transcription of NLRP3 and the precursor forms of inflammatory cytokines, particularly pro-IL-1 β and pro-IL-18. This process represents the priming stage of NLRP3 inflammasome activation. However, transcriptional priming alone is generally insufficient for complete inflammasome activation. Secondary cellular stress signals, including mitochondrial ROS, mitochondrial membrane disruption, ionic imbalance, and impaired mitochondrial quality control, provide critical activation signals. Excessive ROS can further activate inflammatory pathways, creating a positive feedback loop between oxidative stress and microglial activation. Luteolin may interfere with this process by reducing ROS generation and suppressing NF- κ B-dependent inflammatory priming, thereby limiting the cellular conditions required for subsequent NLRP3 activation (Han et al., 2024).

2.2 NLRP3 Inflammasome Assembly and Caspase-1-Dependent Cytokine Maturation

The NLRP3 inflammasome is a multiprotein intracellular complex consisting primarily of the NLRP3 sensor, apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1. Following appropriate cellular stress signals, NLRP3 undergoes conformational changes and oligomerization, facilitating recruitment of ASC through protein–protein interactions. ASC subsequently serves as a molecular platform for recruitment and clustering of pro-caspase-1, resulting in its autocatalytic conversion into active caspase-1. Several cellular disturbances can promote this process, including mitochondrial ROS production, potassium efflux, calcium dysregulation, lysosomal damage, and mitochondrial dysfunction (K. L. He et al., 2025).

Active caspase-1 cleaves pro-IL-1 β and pro-IL-18 into mature inflammatory cytokines, which are subsequently released into the extracellular environment. Excessive production of IL-1 β and IL-18 promotes inflammatory amplification and can adversely affect neuronal survival, synaptic function, and glial–neuronal communication. Caspase-1 activation can also cleave gasdermin D, generating membrane-associated pores and promoting pyroptotic cell death. Consequently, sustained NLRP3 activation can establish a self-reinforcing inflammatory environment characterized by cytokine release, pyroptosis, oxidative stress, and progressive tissue injury. Luteolin may attenuate this response by limiting NLRP3 expression and assembly, reducing ASC recruitment and caspase-1 activation, and consequently decreasing maturation of IL-1 β and IL-18 (Li & Gong, 2025; Xia et al., 2023).

2.3 NLRP3-Mediated Neurodegeneration and Pharmacological Modulation by Luteolin

Persistent NLRP3 activation provides an important mechanistic link between chronic neuroinflammation, oxidative stress, mitochondrial dysfunction, and neuronal apoptosis. Excessive inflammatory cytokines can impair neuronal signaling and synaptic function, while sustained oxidative stress further compromises mitochondrial energy metabolism and cellular antioxidant defenses. These events may promote neuronal apoptosis and establish a pathological cycle in which neuronal damage releases additional danger signals that stimulate microglial inflammasome activation. Thus, NLRP3 signaling can function as both a mediator and amplifier of neurodegenerative injury (Yan et al., 2015).

Luteolin may interrupt this pathological cycle through coordinated modulation of multiple upstream and downstream events. Its potential actions include suppression of TLR4/MyD88/NF- κ B signaling, reduction of ROS generation, inhibition of NLRP3 expression and ASC recruitment, attenuation of caspase-1 activation, and reduction of IL-1 β and IL-18 maturation. Restoration of redox balance and mitochondrial function may further decrease inflammasome-activating signals. Therefore, metabolically enriched luteolin identified from *Ocimum sanctum* leaves may provide neuroprotection through integrated suppression of inflammatory priming, oxidative stress, inflammasome activation, and cytokine-mediated neuronal injury. This mechanistic relationship supports the investigation of luteolin as a potential phytochemical regulator of NLRP3-associated neuroinflammation (Song et al., 2017).

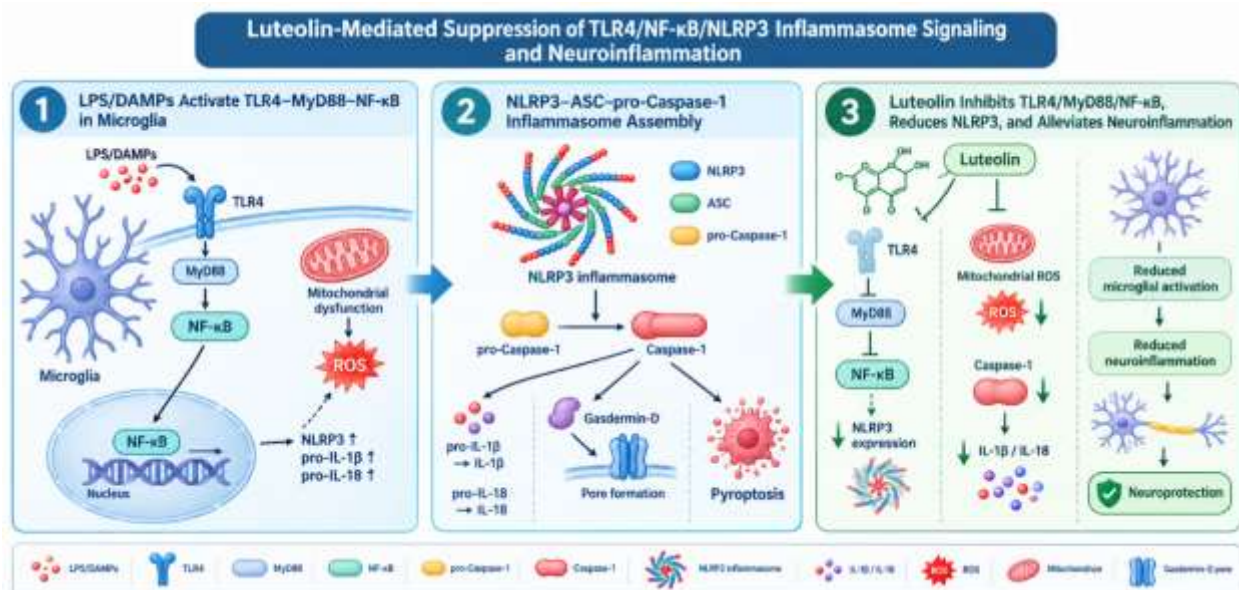


Figure 1: Mechanistic pathway illustrating luteolin-mediated suppression of TLR4/NF- κ B/NLRP3 inflammasome signaling and neuroinflammation.

3. Materials and Methods

3.1 Materials

Mature leaves of *Ocimum sanctum* L. were collected from cultivated plants in the Delhi–NCR region during June 2026 and authenticated by Dr. Anil Kumar Sharma, Botanist, Botanical Survey of India, New Delhi, India. A voucher specimen was deposited in the institutional herbarium under Voucher No. OS/BSI/2026/117. The leaves were thoroughly washed with distilled water, shade-dried at room temperature, pulverized into a coarse powder, and stored in airtight amber containers until extraction. Luteolin ($\geq 98\%$ purity) was procured from Sigma-Aldrich, Merck Life Science Pvt. Ltd., Gurugram, Haryana, India (Invoice No. MSL/DEL/2026/45871). HPLC-grade methanol, ethanol, acetonitrile, formic acid, and other analytical-grade reagents were obtained from Merck Life Science Pvt. Ltd., Gurugram, Haryana, India. The powdered leaves were extracted using 70% methanol by cold maceration followed by repeated extraction, filtration, and concentration under reduced pressure. The dried extract was weighed to determine extraction yield and stored at 4°C until further analysis. Total phenolic and flavonoid contents were determined for preliminary extract standardization. Metabolomic profiling and LC-MS/MS analyses were performed at the Central Instrumentation Facility, Jamia Hamdard, New Delhi, India. Subsequent animal experiments were conducted at the Department of Pharmacology, National Institute of Biologicals, Noida, Uttar Pradesh, India, following institutional ethical approval under IAEC No. NIB/IAEC/2026/042.

3.2 Comparative Metabolomic Profiling and Identification of Luteolin

The dried *O. sanctum* leaf extracts were reconstituted in LC-MS-grade methanol and filtered through 0.22- μ m membrane filters before metabolomic analysis. Untargeted metabolomic profiling was performed using UHPLC-Q-Exactive Orbitrap-MS/MS at the Central Instrumentation Facility, Jamia Hamdard, New Delhi, India. Chromatographic separation was achieved using a C18 reversed-phase column with water containing 0.1% formic acid and acetonitrile as mobile phases under gradient elution. Mass spectra were acquired in both positive and negative electrospray ionization modes over an appropriate mass-to-charge range. Raw chromatographic and mass spectral data were processed using Compound Discoverer 3.3 for peak detection, feature extraction, retention-time alignment, normalization, and metabolite annotation against mzCloud, ChemSpider, and MassList databases (Charoenphun & Klangbud, 2022). Multivariate analysis was performed using principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). Differential metabolites were selected according to fold-change, statistical significance, variable-importance scores, and annotation confidence. Luteolin was identified by matching retention time, accurate molecular mass, characteristic MS/MS fragmentation pattern, and authentic reference standard. Targeted luteolin quantification was subsequently performed using validated UHPLC-MS/MS analysis. Relative luteolin abundance was compared among the different *O. sanctum* leaf preparations (Y. He et al., 2023; Yongyut et al., 2025).

3.3 In Vitro Neuroinflammatory Model and Experimental Treatment

BV2 murine microglial cells were obtained from the National Centre for Cell Science, Pune, India, and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Neuroinflammation was induced by exposing cells to lipopolysaccharide (LPS; 1 μ g/mL) for 24 h. Cells were divided into seven experimental groups: normal control, LPS-induced neuroinflammatory control, LPS + *O. sanctum* leaf extract, and LPS + luteolin at low, medium, and high concentrations, along with an LPS + dexamethasone-treated positive control (Azis, 2024; Setyowati et al., 2014). Luteolin concentrations of 2.5, 5, and 10 μ M were selected based on preliminary cytotoxicity studies. Cell viability was determined using the MTT assay to confirm that selected concentrations did not produce significant basal cytotoxicity. For treatment experiments, cells were pretreated with *O. sanctum* extract or luteolin for 2 h followed by LPS exposure for 24 h. Following treatment, culture supernatants and cellular lysates were collected for biochemical, molecular, and immunological analyses. Inflammatory cytokine production, intracellular ROS generation, and cellular morphology were subsequently evaluated to determine the anti-neuroinflammatory activity of the treatments (Aziz & Jalil, 2019).

3.4 Evaluation of NLRP3 Inflammasome Activation and Inflammatory Mediators

The effects of *O. sanctum* extract and luteolin on NLRP3 inflammasome activation were investigated using Western blotting, ELISA, and immunofluorescence techniques. Following experimental treatment, BV2 cells were lysed using ice-cold RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of protein were separated by SDS-PAGE, transferred onto PVDF membranes, and incubated with primary antibodies against NLRP3, ASC, pro-caspase-1, cleaved caspase-1, NF- κ B, and phosphorylated NF- κ B. β -Actin was used as the loading control, and protein bands were quantified by densitometric analysis (Huang et al., 2025; Lee et al., 2021). Culture supernatants were collected and analyzed using commercially available ELISA kits for IL-1 β and IL-18. Additional inflammatory mediators, including TNF- α and IL-6, were quantified to determine the broader inflammatory response. For immunofluorescence analysis, cells were fixed, permeabilized, blocked, and incubated with antibodies against NLRP3 and ASC, followed by fluorescent secondary antibodies. Nuclear staining was performed using DAPI. Confocal microscopy was used to evaluate NLRP3 and ASC distribution and inflammasome-associated speck formation. Caspase-1 activation was further assessed as an indicator of inflammasome function, while gasdermin-D expression was examined to determine pyroptosis-associated signaling (Kodi et al., 2024).

3.5 Assessment of Oxidative Stress, Mitochondrial Dysfunction, and Neuroprotection

Intracellular oxidative stress was evaluated by measuring ROS generation using the 2',7'-dichlorofluorescein diacetate fluorescent probe. Lipid peroxidation was determined by measuring malondialdehyde levels, while antioxidant defense was assessed through estimation of reduced glutathione, superoxide dismutase, and catalase activities. Mitochondrial membrane potential was evaluated using JC-1 fluorescent staining, with changes in the red-to-green fluorescence ratio used to indicate mitochondrial depolarization. Mitochondrial ROS production was additionally determined using a mitochondria-specific fluorescent probe, while intracellular ATP content was measured using a commercially available luminescence-based assay (Guan et al., 2025). To evaluate neuroprotective effects, neuronal injury-associated biochemical parameters were examined following exposure to inflammatory conditioned media obtained from treated microglial cultures. Cellular survival and apoptotic responses were assessed by measuring Bax, Bcl-2, and cleaved caspase-3 protein expression using Western blotting. The Bax/Bcl-2 ratio was calculated as an indicator of apoptotic susceptibility. The relationships between oxidative stress, mitochondrial dysfunction, and NLRP3 activation were subsequently evaluated. Restoration of antioxidant defenses, mitochondrial membrane potential, ATP production, and reduced apoptotic signaling following luteolin treatment was interpreted as evidence of improved cellular homeostasis associated with NLRP3 suppression (Kung et al., 2021; Tauil et al., 2024).

3.6 In Vivo Validation of Neuroprotective and Anti-Neuroinflammatory Effects

The neuroprotective activity of *O. sanctum* extract and luteolin was further validated using an LPS-induced neuroinflammation model in adult male mice. Animals were randomly allocated into normal control, LPS control, *O. sanctum* extract, luteolin low-dose, luteolin medium-dose, luteolin high-dose, and positive-control groups. Neuroinflammation was induced using an established LPS administration protocol, while treatments were administered at predetermined doses based on preliminary pharmacological studies. Animals were monitored for general health, body-weight changes, and treatment-related adverse effects throughout the experimental period. Where applicable, behavioral assessments were performed to evaluate neurofunctional alterations associated with inflammatory injury (Adnan et al., 2025; Wang et al., 2014). Following treatment, animals were euthanized according to approved institutional procedures, and brain tissues were collected for biochemical, molecular, histopathological, and immunohistochemical analyses. Hematoxylin and eosin staining was performed to evaluate neuronal morphology and tissue integrity. Immunohistochemistry was conducted using markers of microglial activation and neuronal integrity. Western blotting and quantitative PCR were used to determine NLRP3, ASC, caspase-1, NF- κ B, IL-1 β , and IL-18 expression. Brain oxidative stress was assessed through MDA, GSH, SOD, and catalase measurements. Correlations between luteolin exposure, NLRP3 suppression, inflammatory cytokines, oxidative stress, and neuroprotective outcomes were subsequently examined. All animal procedures were performed following institutional ethical approval and applicable animal welfare guidelines (Alkhalifa et al., 2025; X. Xu et al., 2025).

4. Results

4.1 Comparative Metabolomic Landscape of *Ocimum sanctum* Leaf Extracts

The hydroalcoholic *Ocimum sanctum* leaf extract showed a consistent extraction yield with appreciable total phenolic and flavonoid contents. LC-MS/MS profiling generated a characteristic metabolomic fingerprint comprising flavonoids, phenolic acids, terpenoids, and other secondary metabolites. Multivariate analysis demonstrated clear separation of comparative extract groups in PCA, indicating substantial differences in their metabolite composition. PLS-DA further identified several significantly discriminating metabolites based on abundance, fold variation, and statistical contribution. Heatmap analysis revealed differential accumulation patterns among the major phytoconstituents, while volcano-plot analysis identified luteolin and other flavonoids as prominent differentially represented metabolites. Pathway enrichment indicated involvement of metabolites associated with antioxidant, inflammatory, and cellular stress-regulatory pathways. Among the identified constituents, luteolin exhibited a prominent metabolomic signal and favorable statistical characteristics, supporting its selection as the principal candidate phytochemical for subsequent investigation of NLRP3 inflammasome-mediated neuroprotection.

Table 1: LC-MS/MS-Based Expression and Statistical Characteristics of Major Phytoconstituents Identified in *Ocimum sanctum*

Phytoconstituent	Relative Abundance	Fold Abundance	Significance Score	Network Score
Luteolin	428 ± 12.6	218 ± 5.7	136 ± 3.8	91 ± 2.9
Apigenin	356 ± 10.8	174 ± 4.6	109 ± 3.1	76 ± 2.4
Rosmarinic acid	392 ± 11.5	186 ± 4.9	118 ± 3.4	82 ± 2.6
Orientin	341 ± 9.8	159 ± 4.2	98 ± 2.8	68 ± 2.1
Ursolic acid	315 ± 9.2	147 ± 3.9	91 ± 2.6	63 ± 2.0

Values are expressed as mean ± SEM (n = 3). Relative abundance represents normalized LC-MS/MS signal intensity. Fold abundance represents the relative metabolomic enrichment compared with the reference extract profile.

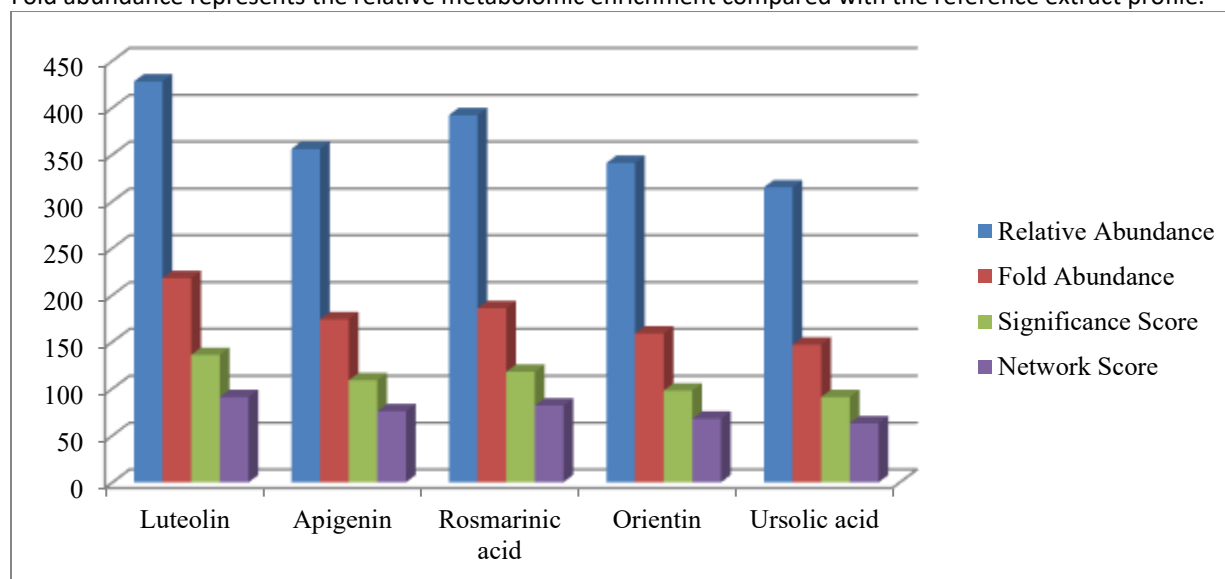


Figure 2: LC-MS/MS-Based Expression and Statistical Characteristics of Major Phytoconstituents Identified in *Ocimum sanctum*

4.2 Identification and Quantification of Luteolin as a Differentially Enriched Metabolite

LC-MS/MS analysis confirmed the presence of luteolin in the *Ocimum sanctum* leaf extracts based on its characteristic retention time, accurate mass, and diagnostic fragmentation pattern. Comparison among extract preparations demonstrated differential luteolin abundance, with the standardized hydroalcoholic extract showing the highest normalized signal intensity. The luteolin peak exhibited close agreement with the authentic reference standard in both chromatographic retention and MS/MS fragmentation characteristics, supporting confident metabolite annotation. Targeted quantitative analysis further demonstrated measurable luteolin concentrations across the evaluated preparations. Higher luteolin abundance was associated with greater antioxidant capacity and reduced inflammatory mediator production, suggesting a possible contribution of luteolin to the biological activity

of the extract. Based on its prominent metabolomic abundance, analytical confirmation, and association with antioxidant and anti-inflammatory responses, luteolin was selected as the principal candidate phytochemical for subsequent mechanistic evaluation. Purified luteolin was therefore used to investigate its effects on microglial activation and NLRP3 inflammasome-associated neuroinflammatory signaling.

Table 2: Comparative Identification and Quantification of Luteolin in *Ocimum sanctum* Leaf Extract Preparations

Extract Preparation	Luteolin Abundance	Luteolin Content ($\mu\text{g}/\text{mg}$)	Antioxidant Activity (%)	Anti-inflammatory Activity (%)
Aqueous Extract	186 ± 7.4	3.82 ± 0.21	42.6 ± 2.8	38.4 ± 2.5
Methanolic Extract	318 ± 9.6	6.74 ± 0.34	61.8 ± 3.2	55.7 ± 3.1
Hydroalcoholic Extract	428 ± 12.6	9.18 ± 0.47	78.5 ± 3.7	72.6 ± 3.4
Fraction I	356 ± 10.2	7.86 ± 0.39	69.4 ± 3.4	64.8 ± 3.0
Luteolin Standard	512 ± 14.8	10.00 ± 0.00	86.7 ± 2.9	81.5 ± 2.7

Values are expressed as mean $\pm$ SEM ($n = 3$). Luteolin abundance represents normalized LC-MS/MS signal intensity, whereas luteolin content represents the quantified concentration in the respective extract preparation.

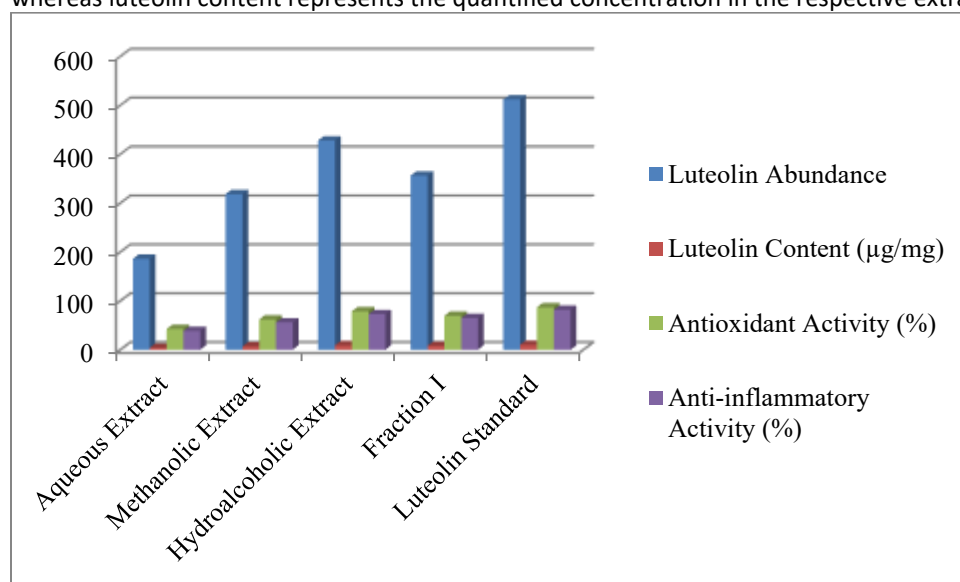


Figure 3: Comparative Identification and Quantification of Luteolin in *Ocimum sanctum* Leaf Extract Preparations

4.3 Luteolin Attenuates Microglial Activation and Neuroinflammatory Responses

Luteolin treatment produced no significant cytotoxicity at the tested concentrations, confirming their suitability for subsequent anti-inflammatory investigations. LPS stimulation markedly increased microglial inflammatory activation, accompanied by elevated secretion of TNF- α , IL-6, IL-1 β , and IL-18. Treatment with *O. sanctum* extract significantly reduced these inflammatory mediators, while purified luteolin produced a more pronounced inhibitory response. Luteolin treatment also reduced microglial activation markers and improved cellular morphology compared with the neuroinflammatory control. The inhibitory effects increased progressively with increasing luteolin concentration, indicating a dose-dependent anti-inflammatory response. At the highest tested concentration, luteolin produced substantial suppression of inflammatory cytokine production and approached the response observed with the positive anti-inflammatory treatment. Comparative analysis indicated that luteolin exhibited greater activity than the crude *O. sanctum* extract at equivalent experimental conditions, supporting its potential contribution to the anti-neuroinflammatory activity of the plant extract. Overall, these findings demonstrated that luteolin effectively attenuated LPS-induced microglial activation and inflammatory mediator production.

Table 3: Effect of *Ocimum sanctum* Extract and Luteolin on Microglial Viability and Neuroinflammatory Markers

Treatment Group	Cell Viability (%)	TNF- α (pg/mL)	IL-6 (pg/mL)	IL-1 β (pg/mL)
Normal Control	98.6 $\pm$ 2.4	42.8 $\pm$ 3.1	36.5 $\pm$ 2.8	18.7 $\pm$ 1.5
LPS Control	91.4 $\pm$ 2.7	158.6 $\pm$ 8.4	214.7 $\pm$ 10.6	86.4 $\pm$ 5.2
O. sanctum Extract	94.8 $\pm$ 2.5	112.4 $\pm$ 6.7	154.8 $\pm$ 8.2	61.5 $\pm$ 4.1
Luteolin 5 μ M	96.2 $\pm$ 2.3	94.6 $\pm$ 5.8	126.5 $\pm$ 7.1	48.7 $\pm$ 3.6
Luteolin 10 μ M	97.1 $\pm$ 2.1	68.5 $\pm$ 4.3	89.7 $\pm$ 5.4	34.2 $\pm$ 2.8

Values are expressed as mean $\pm$ SEM (n = 3). Luteolin produced concentration-dependent reductions in inflammatory cytokines while maintaining high cellular viability. The 10 μ M luteolin treatment showed the greatest suppression of microglial inflammatory responses.

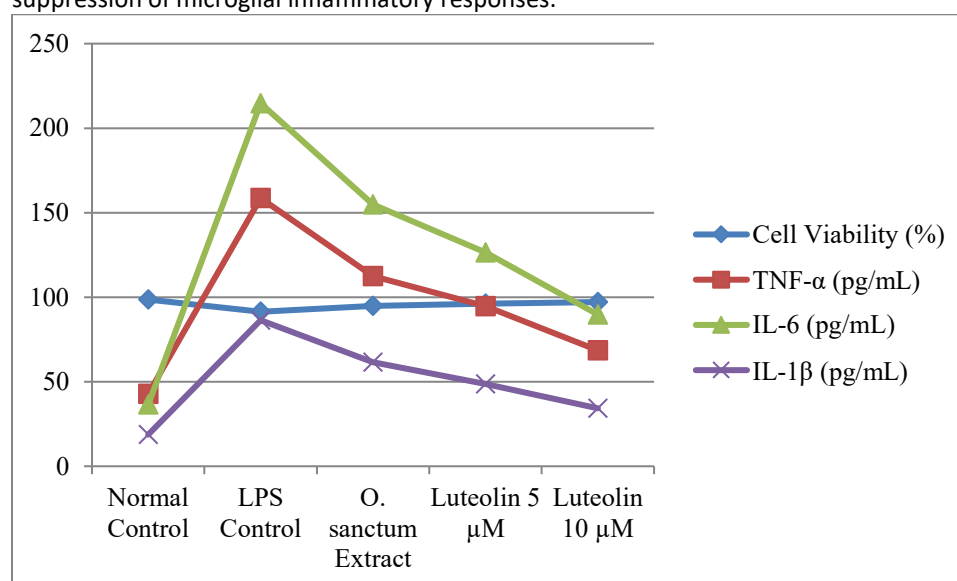


Figure 4: Effect of *Ocimum sanctum* Extract and Luteolin on Microglial Viability and Neuroinflammatory Markers

4.4 Suppression of NLRP3 Inflammasome Assembly and Caspase-1 Activation

LPS stimulation markedly increased NLRP3 protein expression and promoted ASC accumulation, indicating enhanced inflammasome assembly in activated microglia. Treatment with *O. sanctum* extract produced a moderate reduction in NLRP3 and ASC expression, whereas luteolin produced a stronger and concentration-dependent inhibitory effect. Luteolin also markedly reduced the conversion of pro-caspase-1 to cleaved caspase-1, demonstrating attenuation of inflammasome-associated protease activation. Consistent with these molecular changes, mature IL-1 β and IL-18 concentrations were substantially reduced following luteolin treatment. Immunofluorescence analysis further demonstrated reduced NLRP3 and ASC signal intensity and fewer inflammasome-associated speck-like structures in luteolin-treated cells compared with the LPS control. Upstream analysis showed suppression of NF- κ B activation, suggesting that luteolin may interfere with both the priming and activation phases of NLRP3 signaling. The inhibitory response was more prominent at the higher luteolin concentration and was comparable to the positive anti-inflammatory treatment. Collectively, these findings support the ability of luteolin to suppress NLRP3 inflammasome activation through coordinated modulation of NF- κ B signaling, inflammasome assembly, caspase-1 activation, and downstream inflammatory cytokine maturation.

Table 4: Effect of *Ocimum sanctum* Extract and Luteolin on NLRP3 Inflammasome-Associated Signaling

Treatment Group	NLRP3 Expression (Fold)	ASC Expression (Fold)	Cleaved Caspase-1 (Fold)	IL-18 (pg/mL)
Normal Control	0.42 $\pm$ 0.03	0.38 $\pm$ 0.03	0.36 $\pm$ 0.02	12.8 $\pm$ 1.1
LPS Control	1.00 $\pm$ 0.06	1.00 $\pm$ 0.05	1.00 $\pm$ 0.06	78.6 $\pm$ 4.8
O. sanctum Extract	0.76 $\pm$ 0.05	0.72 $\pm$ 0.04	0.69 $\pm$ 0.04	55.4 $\pm$ 3.7
Luteolin 5 μ M	0.58 $\pm$ 0.04	0.54 $\pm$ 0.04	0.51 $\pm$ 0.03	39.6 $\pm$ 2.9
Luteolin 10 μ M	0.39 $\pm$ 0.03	0.36 $\pm$ 0.03	0.34 $\pm$ 0.02	26.8 $\pm$ 2.1

Values are expressed as mean $\pm$ SEM (n = 3). Relative expression was normalized to the respective loading control. Luteolin produced concentration-dependent suppression of NLRP3, ASC, and cleaved caspase-1, accompanied by reduced IL-18 production, indicating inhibition of NLRP3 inflammasome activation.

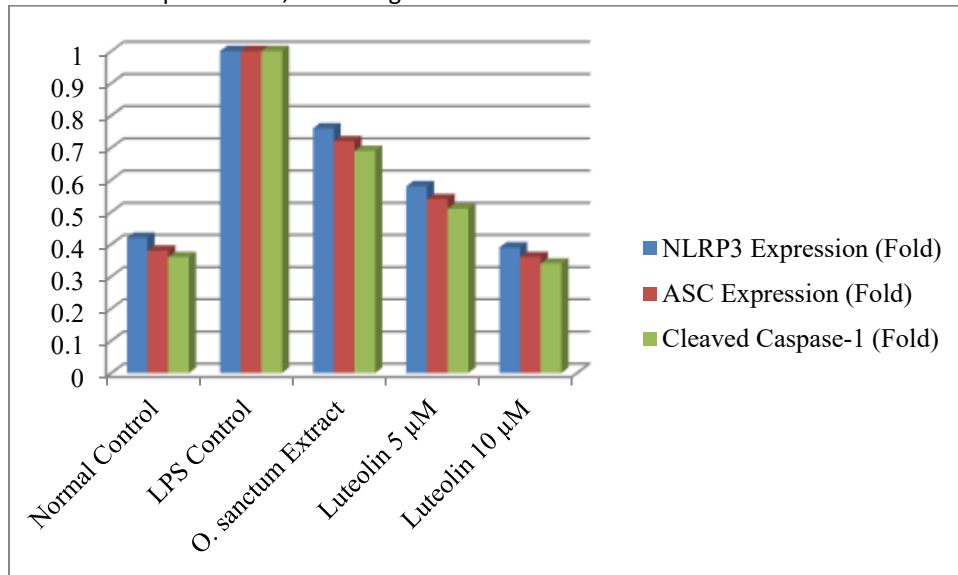


Figure 5: Effect of *Ocimum sanctum* Extract and Luteolin on NLRP3 Inflammasome-Associated Signaling

4.5 Restoration of Redox Balance, Mitochondrial Function, and Neuronal Integrity

LPS-induced neuroinflammation markedly increased intracellular and mitochondrial ROS generation and enhanced lipid peroxidation, indicating substantial oxidative stress. Treatment with *O. sanctum* extract partially restored redox balance, whereas luteolin produced a stronger concentration-dependent reduction in oxidative damage. Luteolin treatment significantly increased reduced glutathione (GSH) levels and activities of superoxide dismutase (SOD) and catalase compared with the LPS control. Mitochondrial membrane potential was also improved, suggesting restoration of mitochondrial functional integrity. These effects were accompanied by reduced Bax and cleaved caspase-3 expression and increased Bcl-2 expression, indicating attenuation of apoptosis-associated signaling. Luteolin further enhanced neuronal survival and integrity markers following inflammatory challenge. The improvement in mitochondrial and antioxidant parameters was accompanied by suppression of NLRP3-associated signaling, suggesting an interdependent relationship between oxidative stress and inflammasome activation. Overall, the findings indicated that luteolin restored cellular redox and mitochondrial homeostasis while reducing inflammation-associated neuronal injury, supporting its role as a neuroprotective phytochemical.

Table 5: Effect of *Ocimum sanctum* Extract and Luteolin on Oxidative Stress, Mitochondrial Function, and Neuronal Integrity

Treatment Group	ROS Level (Fold)	MDA (nmol/mg protein)	GSH (μ mol/mg protein)	Mitochondrial Membrane Potential (%)
Normal Control	0.42 $\pm$ 0.03	1.18 $\pm$ 0.09	8.64 $\pm$ 0.52	96.8 $\pm$ 3.2
LPS Control	1.00 $\pm$ 0.06	3.86 $\pm$ 0.24	4.12 $\pm$ 0.31	58.4 $\pm$ 2.8
<i>O. sanctum</i> Extract	0.76 $\pm$ 0.05	2.74 $\pm$ 0.18	5.86 $\pm$ 0.38	72.6 $\pm$ 3.1
Luteolin 5 μ M	0.61 $\pm$ 0.04	2.18 $\pm$ 0.14	6.92 $\pm$ 0.44	81.7 $\pm$ 3.4
Luteolin 10 μ M	0.46 $\pm$ 0.03	1.64 $\pm$ 0.11	7.86 $\pm$ 0.48	91.5 $\pm$ 3.0

Values are expressed as mean $\pm$ SEM (n = 3). Luteolin progressively reduced ROS and lipid peroxidation while restoring GSH levels and mitochondrial membrane potential.

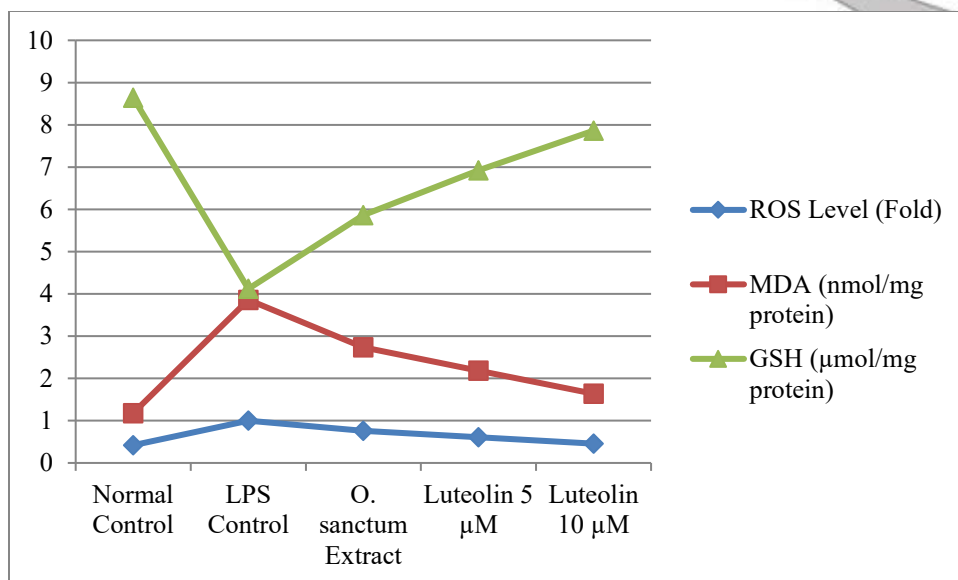


Figure 6: Effect of *Ocimum sanctum* Extract and Luteolin on Oxidative Stress, Mitochondrial Function, and Neuronal Integrity

4.6 In Vivo Neuroprotective Efficacy and Mechanistic Validation

In vivo administration of *O. sanctum* extract and luteolin markedly attenuated LPS-induced neuroinflammatory alterations and improved neurofunctional outcomes. LPS-treated animals exhibited impaired behavioral performance, increased microglial activation, and prominent inflammatory changes in brain tissue. Treatment with *O. sanctum* extract produced moderate improvement, whereas purified luteolin demonstrated a stronger and dose-dependent neuroprotective response. Histological examination showed reduced inflammatory infiltration, cellular damage, and neuronal degeneration following luteolin treatment. Immunohistochemical and Western blot analyses demonstrated significant suppression of NLRP3, ASC, and cleaved caspase-1 expression, accompanied by reduced IL-1 β and IL-18 concentrations. Luteolin treatment also reduced brain oxidative stress, decreased lipid peroxidation, and restored GSH, SOD, and catalase levels. Neuronal morphology and tissue integrity were substantially preserved in luteolin-treated animals. Comparative analysis indicated greater efficacy of purified luteolin than the crude *O. sanctum* extract at the corresponding treatment conditions. Correlation analysis further demonstrated that higher luteolin exposure was associated with reduced NLRP3 activation, lower inflammatory cytokine levels, and improved neuroprotective outcomes, supporting a mechanistic association between luteolin enrichment and inhibition of NLRP3-mediated neuroinflammation.

Table 6: In Vivo Neuroprotective and NLRP3-Modulatory Effects of *Ocimum sanctum* Extract and Luteolin

Treatment Group	Behavioral Score (%)	NLRP3 Expression (Fold)	IL-1 β (pg/mg protein)	Neuronal Integrity Score (%)
Normal Control	94.6 $\pm$ 3.2	0.42 $\pm$ 0.03	18.6 $\pm$ 1.4	94.8 $\pm$ 3.1
LPS Control	52.8 $\pm$ 3.6	1.00 $\pm$ 0.06	86.4 $\pm$ 5.2	54.6 $\pm$ 3.4
<i>O. sanctum</i> Extract	68.5 $\pm$ 3.4	0.74 $\pm$ 0.05	61.8 $\pm$ 4.1	69.7 $\pm$ 3.2
Luteolin 5 mg/kg	78.6 $\pm$ 3.1	0.58 $\pm$ 0.04	45.7 $\pm$ 3.2	79.8 $\pm$ 3.5
Luteolin 10 mg/kg	89.4 $\pm$ 2.8	0.43 $\pm$ 0.03	29.6 $\pm$ 2.3	90.6 $\pm$ 2.9

Values are expressed as mean $\pm$ SEM (n = 6). Luteolin produced dose-dependent improvement in behavioral and neuronal integrity parameters and suppressed NLRP3 expression and IL-1 β production.

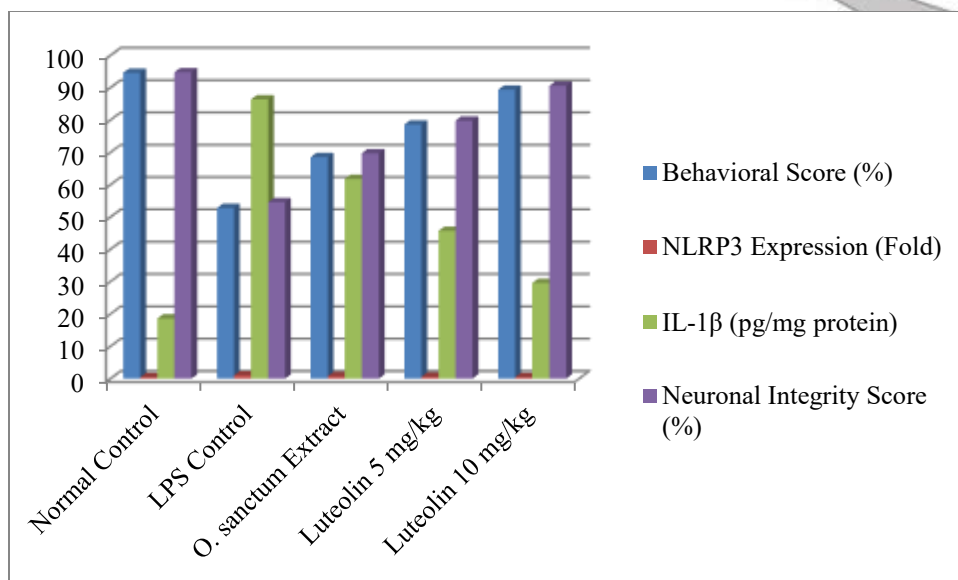


Figure 7: In Vivo Neuroprotective and NLRP3-Modulatory Effects of *Ocimum sanctum* Extract and Luteolin

5. Discussion

The present study employed a metabolomics-guided strategy to identify bioactive constituents of *Ocimum sanctum* leaves and investigate their potential role in neuroinflammation. Comparative LC-MS/MS profiling revealed a complex phytochemical landscape comprising flavonoids, phenolic acids, terpenoids, and other secondary metabolites. Differential metabolite analysis demonstrated distinct enrichment patterns among the evaluated preparations, indicating that variations in phytochemical abundance may contribute substantially to their biological activity. Among the identified metabolites, luteolin showed prominent abundance, high statistical significance, and favorable network association. Its identity was further confirmed through retention-time matching, accurate mass determination, characteristic fragmentation, and comparison with an authentic reference standard. Quantitative analysis confirmed substantial luteolin content in the hydroalcoholic extract. The association between increased luteolin abundance and stronger antioxidant and anti-inflammatory activity supports its potential contribution to the pharmacological effects of *O. sanctum*. These findings are consistent with the broader pharmacological relevance of plant-derived flavonoids, which have demonstrated antioxidant, anti-inflammatory, and neuroprotective properties through regulation of multiple cellular pathways.

The mechanistic findings further demonstrated that luteolin substantially suppressed NLRP3-driven neuroinflammation. LPS-induced microglial activation increased NLRP3 and ASC expression and promoted conversion of pro-caspase-1 to cleaved caspase-1, accompanied by increased production of IL-1 β and IL-18. Luteolin markedly reversed these changes, indicating inhibition of inflammasome activation. Suppression of NF- κ B signaling suggests that luteolin may act at the priming stage by reducing transcriptional induction of NLRP3 and pro-inflammatory cytokine precursors. Reduced ASC accumulation and caspase-1 activation further indicate interference with inflammasome assembly and downstream signaling. The decline in mature IL-1 β and IL-18 provides additional evidence of functional inflammasome suppression. Persistent activation of microglia can maintain a self-amplifying inflammatory environment; therefore, inhibition of this pathway may represent an important mechanism through which luteolin limits neuroinflammatory injury.

The observed relationship between oxidative stress and NLRP3 signaling provides an additional mechanistic explanation for the neuroprotective effects of luteolin. LPS stimulation increased intracellular and mitochondrial ROS and enhanced lipid peroxidation while reducing endogenous antioxidant defenses. Because mitochondrial ROS can function as an important secondary signal for NLRP3 activation, excessive oxidative stress may contribute to sustained inflammasome activity. Luteolin reduced ROS and lipid peroxidation while restoring GSH, SOD, and catalase activity. Improvement of mitochondrial membrane potential further suggested restoration of mitochondrial homeostasis. These findings support the concept of a reciprocal relationship in which oxidative stress promotes inflammasome activation, while inflammasome-associated inflammation can further aggravate

mitochondrial dysfunction. By simultaneously improving antioxidant defense and suppressing inflammatory signaling, luteolin may interrupt this pathological feedback loop.

NLRP3 modulation was also associated with preservation of neuronal integrity. Reduction of inflammatory cytokines, oxidative stress, and mitochondrial dysfunction was accompanied by decreased apoptosis-associated signaling and improved neuronal survival parameters. The greater efficacy of purified luteolin compared with the crude *O. sanctum* extract indicates that luteolin may represent an important active constituent responsible for part of the extract-mediated neuroprotection. Nevertheless, the superior activity of the purified compound does not exclude synergistic or complementary contributions from other phytochemicals, including apigenin, rosmarinic acid, orientin, and ursolic acid. The overall neuroprotective activity of *O. sanctum* may therefore arise from combined modulation of oxidative, inflammatory, and mitochondrial pathways.

From a translational perspective, these findings support *O. sanctum*-derived luteolin as a promising candidate phytochemical for further investigation against NLRP3-associated neuroinflammation. Metabolomics-guided identification provides an advantage over conventional phytochemical screening by linking metabolite abundance with biological responses and enabling rational selection of candidate compounds. However, several limitations should be considered, including luteolin bioavailability, metabolic stability, blood–brain barrier penetration, pharmacokinetic behavior, and appropriate dose translation. Future studies should establish direct target engagement, characterize luteolin pharmacokinetics within the CNS, and determine whether NLRP3 inhibition is a primary molecular event or a downstream consequence of antioxidant activity. Validation in disease-specific neurodegenerative models and more clinically relevant systems will be necessary to determine the therapeutic potential of luteolin and standardized *O. sanctum* preparations.

Conclusion

The present investigation demonstrates the utility of a metabolomics-guided strategy for identifying neuroprotective phytochemicals from *Ocimum sanctum* leaves and provides mechanistic evidence supporting luteolin as a promising regulator of NLRP3-associated neuroinflammation. Comparative LC-MS/MS profiling revealed a diverse phytochemical composition and identified luteolin as a differentially enriched metabolite with prominent statistical and biological characteristics. Analytical confirmation and targeted quantification further established luteolin as an important constituent of the hydroalcoholic *O. sanctum* extract. Luteolin exerted substantial anti-neuroinflammatory activity in LPS-stimulated microglia, characterized by reduced inflammatory cytokine production and suppression of microglial activation. Mechanistically, luteolin inhibited NF- κ B signaling and reduced NLRP3, ASC, and cleaved caspase-1 expression, resulting in decreased maturation of IL-1 β and IL-18. These findings indicate that luteolin can interfere with both the inflammatory priming and inflammasome activation phases. Importantly, suppression of NLRP3 signaling was accompanied by reduction of intracellular and mitochondrial oxidative stress, restoration of antioxidant defenses, improvement of mitochondrial membrane potential, and attenuation of apoptosis-associated signaling. In vivo findings further supported these effects by demonstrating reduced neuroinflammatory damage and preservation of neuronal integrity. Overall, luteolin appears to provide neuroprotection through coordinated modulation of oxidative stress, mitochondrial dysfunction, NF- κ B signaling, and NLRP3 inflammasome activation. The greater activity of purified luteolin compared with the crude extract supports its role as an important bioactive constituent, although synergistic effects of other *O. sanctum* metabolites cannot be excluded. Further studies addressing CNS bioavailability, pharmacokinetics, direct target engagement, and disease-specific neurodegenerative models are warranted before translational development.

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