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Anti-inflammatory activity of *Leonotis nepetifolia* leaf essential oil in LPS-stimulated RAW 264.7 cells and its molecular mechanism of action

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The anti-inflammatory activity and the underlying molecular mechanisms of action of the leaf essential oil of *Leonotis nepetifolia* (LNLEO), traditionally used in folk and ethnomedicine against inflammatory diseases, are evaluated in this study through an in vitro LPS-induced RAW 264.7 macrophage model. GC–MS analysis revealed the presence of 44 constituents in LNLEO, germacrene D, β-caryophyllene, α-humulene, and phytol being the predominant terpenoids. Based on the MTT assay, the non-toxic doses of 12.5 and 50 µg/mL of LNLEO were used for in vitro studies. Incubation of LNLEO significantly reduced the expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6), inflammatory enzymes (iNOS, COX-2), and intracellular ROS, while enhancing the levels of endogenous antioxidant enzymes (SOD, GSH, GPx, and CAT). LNLEO alleviated oxidative stress by attenuating Keap1 and upregulating Nrf2, HO-1 expression in a concentration-dependent manner. The RT-qPCR analysis revealed that LNLEO treatment inhibited TLR4 and MyD88 expression. Confocal microscopy and RT-qPCR analysis showed a considerable reduction in both nuclear localization and transcriptional activity of NF-κB p65 in LPS-activated macrophages. ELISA studies also indicated that LNLEO suppressed inflammation by limiting phosphorylation of MAPKs that are implicated in the activation of NF-κB and other transcription factors. Based on these results, it can be concluded that LNLEO has the potential to modulate several key signaling pathways, which are intricately involved in the inflammatory response and oxidative stress, and they can be effectively targeted for therapeutic benefits. However, in vivo studies, as well as clinical trials, will be helpful in validating the results for future applications.

Keywords TLR4, NF-κB, MAPK, Keap1/Nrf2/HO-1, Pro-inflammatory cytokines, iNOS and COX-2, ROS

The mint family Lamiaceae (Labiatae) contains about 225 genera and 7,000 species[1] and it is widely distributed throughout the world with greater diversity in the Mediterranean region and SW Asia. Many species of the family are aromatic in nature, contain essential oils, and are important to humans as folk medicine, culinary herbs, flavor, and fragrance. They are also widely used in food, pharmaceutical, cosmetic, and healthcare products. Most of the species are known to possess antioxidant, anti-inflammatory, anti-asthmatic, antimicrobial, insecticidal, antidiabetic, antirheumatic, immunoregulatory, antiallergic, and other therapeutic properties, and these bioactivities are often attributed to the presence of the essential oils in the plants[2].

The genus *Leonotis* (Lamiaceae) is represented by nine species in the world, native to Africa and the Indian Subcontinent. Of these, *Leonotis nepetaefolia* is the most widespread species, growing as a weed in wastelands, agricultural fields, and open forests in the Indian subcontinent and Africa. The species is also naturalized in China, Southeast Asia, North and South America. The plant has a long history of traditional use in ethnomedicine worldwide, especially in India, Madagascar, Brazil, Canada, Kenya, and many African countries. The infusions, decoctions, pastes, and juices prepared from different plant parts are widely employed to treat inflammatory conditions, such as joint pain, rheumatism, arthritis, diabetes mellitus, bronchial asthma, pulmonary infections, burns, and wound-healing[2–4].

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Roots of *L. nepetifolia,* referred to as "Granthiparni" in the Ayurvedic system of medicine are used for the preparation of various formulations like Brihat Guduchi Taila, Himasagara Taila, and Mritasanjeevani Sura in India to treat bronchitis, asthma, inflammation, fever, and other toxic conditions[5,6]. In India, the plant is reported to be used by different tribal and rural communities for curing wounds, burns, breast swelling, eczema, and other chronic inflammatory skin diseases[7].

Several in vivo studies have demonstrated significant anti-inflammatory and anti-rheumatic[8–10], wound healing[11], antioxidative[12], and anti-asthmatic activities[13] of the decoction and extracts of *L. nepetifolia.* Such findings validated the traditional claim pertaining to the local use of the species for the treatment of rheumatism, osteogenesis, and chronic inflammatory conditions. The anti-inflammatory effect of its extracts has been attributed to the presence of some bioactive constituents such as leonotinin, stigmasterol, and certain flavonoids[8,14]. As regards the underlying mechanisms of action, it has been reported that the extract exhibits anti-inflammatory and wound-healing activity by suppressing the expression of pro-inflammatory cytokines, bringing down the reactive oxygen species (ROS) levels, and consequently offering protection against oxidative damage[15].

Though there are several publications on the bioactivities of the extracts of *L. nepetifolia,* including their anti-inflammatory properties[8,9,11–13], the research done so far on the essential oil of the species is restricted to chemical characterization, antibacterial, antifungal, and cytotoxic activities[16,17]. There is no published report on the anti-inflammatory and wound-healing activities of the essential oil of *L. nepetifolia*. The present work, therefore, aims at fulfilling the existing gap by evaluating its in vitro anti-inflammatory activity with an LPS-induced murine macrophage cell line (RAW 264.7) model, and to unravel the underlying molecular mechanism of action, thereby providing a comprehensive scientific validation of its effectiveness against inflammatory conditions.

Results

Yield and composition of phytoconstituents of *L. nepetifolia* leaf essential oil (LNLEO)

Hydro-distillation of 350 g of dried *L. nepetifolia* leaves yielded 180 µL of dark yellow essential oil with a yield percentage of 0.048% (w/w) on a dry weight basis. Gas Chromatography-Mass Spectrometry (GC–MS) analysis of the essential oil revealed the presence of a total of 44 compounds accounting for 91.19% of the total essential oil (Table 1).

Sesquiterpene hydrocarbons were the most prevailing chemical group (69.28%), followed by oxygenated sesquiterpenes (18.07%) and others (3.12%). Sesquiterpene hydrocarbons such as germacrene D (32.17%), β-caryophyllene (16.53%), α-humulene (7.36%), and δ-cadinene (4.16%) were the major phytoconstituents in LNLEO. Among the oxygenated sesquiterpenes, phytol (6.67%) and α-muurolol (3.00%) were of quantitative importance. In addition, the essential oil also contained small amounts of monoterpene hydrocarbons (0.72%) and other minor constituents (3.12%).

Effect of LNLEO on viability and morphology of RAW 264.7 cells

The cytotoxic effects of LNLEO on RAW 264.7 macrophage cells were tested by MTT assay at the concentrations of 12.5, 25.0, 50.0, and 100.0 µg/mL. The results demonstrated that treatment with LPS at a concentration of 1 µg/mL significantly reduced cell viability to 33.79%, confirming its cytotoxic effect. In contrast, LNLEO exhibited no significant cytotoxicity (cell viability > 90%) and did not induce any noticeable morphological changes in RAW 264.7 macrophage cells up to a concentration of 50 µg/mL (Fig. 1). However, at the highest concentration (100 µg/mL) of LNLEO, the cell viability receded to below 85%. Based on the above finding, the lowest dose of 12.5 µg/mL and the highest dose of 50 µg/mL of LNLEO were fixed for subsequent in vitro experiments.

Role of LNLEO on TLR4/MyD88/NF-κB signaling pathways

Toll-like receptor 4 (TLR4), a pattern recognition receptor, plays a crucial role in initiating and mediating inflammatory responses by activating downstream signaling pathways primarily through the adaptor protein myeloid differentiation factor 88 (MyD88)[19]. The TLR4 and MyD88 activities in LPS-stimulated RAW 264.7 cells were validated by real-time quantitative PCR (RT-qPCR) analysis using the $2^{-\Delta\Delta CT}$ method. TLR4 and MyD88 mRNA expression sharply increased by 9.8-fold and 8.9-fold, respectively in the LPS-alone treated groups compared to untreated cells. However, prolonged incubation of the pre-LPS sensitized cells with LNLEO resulted in a concentration-driven reduction in TLR4 and MyD88 expression (Fig. 2A and B). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a protein complex, plays a crucial role in regulating the immune response and inflammation. It acts as a transcription factor, thus controlling the expression of genes involved in these processes[20]. In consideration of its critical role, the effect of LNLEO on the NF-κB signaling pathway was explored by analyzing the translocation of nuclear factor-κB p65 (NF-κB p65) to the nucleus by confocal microscopy and quantifying mRNA expression levels using RT-qPCR. Confocal microscopy analysis revealed that 24 h exposure to LPS induced nuclear translocation of NF-κB dimers (Fig. 2C). This was accompanied by phosphorylation of NF-κB p65, resulting in a significant increase in the mean fluorescence intensity. The proportion of RAW 264.7 cells emitting NF-κB p65 fluorescence increased drastically by 98.90% in LPS-treated groups compared to the untreated control. Further, the immunofluorescence levels got reduced by 71.51–89.77% when the pre-LPS modulated (1 µg/mL, 2 h) macrophage groups were co-treated with LNLEO at the concentrations of 12.5 and 50 µg/mL (Fig. 2D). RT-qPCR analysis showed that 24 h LPS treatment resulted in an 11.6-fold increase in NF-κB p65 levels in the LPS-only treated groups. However, overnight exposure to LNLEO at 12.5 and 50 µg/mL concentrations effectively mitigated the adverse effects associated with LPS. Specifically, LNLEO reduced the elevated NF-κB p65 mRNA levels in groups pre-treated with LPS in a concentration-dependent manner (Fig. 2E). These results demonstrated that LNLEO inhibits the recruitment

S. No	Compound[a]	Ri[b]	Ri[c]	Peak Area %
1	1-Octen-3-ol	971	979	0.18 ± 0.01
2	(Z)-β-Ocimene	1026	1037	0.17 ± 0.01
3	(E)-β-Ocimene	1037	1050	0.20 ± 0.01
4	Terpinolene	1093	1088	0.34 ± 0.02
5	γ-Terpineol	1187	1199	0.13 ± 0.01
6	Pulegone	1232	1237	0.35 ± 0.01
7	Geraniol	1253	1252	0.14 ± 0.01
8	α-Cubebene	1336	1351	0.63 ± 0.03
9	α-Copaene	1364	1376	0.30 ± 0.01
10	β-Bourbonene	1372	1388	0.94 ± 0.03
11	β-Elemene	1378	1390	1.47 ± 0.05
12	β-Longipinene	1392	1400	0.23 ± 0.01
13	β-caryophyllene	1409	1419	16.53 ± 0.55
14	β-Copaene	1417	1432	0.69 ± 0.03
15	trans-α-Bergamotene	1421	1434	0.35 ± 0.01
16	α-Humulene	1444	1454	7.36 ± 0.20
17	cis-Muurola-4(14),5-diene	1449	1466	0.18 ± 0.01
18	Germacrene D	1473	1481	32.17 ± 1.07
19	β-Selinene	1476	1490	0.13 ± 0.01
20	cis-β-Guaiene	1483	1493	1.88 ± 0.07
21	α-Muurolene	1486	1500	0.83 ± 0.02
22	Bicyclogermacrene	1493	1500	0.76 ± 0.02
23	Germacrene A	1495	1509	0.17 ± 0.01
24	δ-Cadinene	1506	1523	4.16 ± 0.14
25	trans-Cadina-1,4-diene	1518	1534	0.23 ± 0.01
26	α-Cadinene	1523	1538	0.25 ± 0.01
27	Isocaryophyllene oxide	1537	1555	0.17 ± 0.01
28	Spathulenol	1568	1578	1.88 ± 0.08
29	Viridiflorol	1579	1592	0.26 ± 0.01
30	β-Atlantol	1595	1608	0.65 ± 0.02
31	10-epi-γ-Eudesmol	1606	1623	0.40 ± 0.01
32	1-epi-Cubenol	1613	1628	0.26 ± 0.01
33	epi-α-Cadinol	1628	1640	0.99 ± 0.04
34	Cubenol	1630	1646	1.60 ± 0.03
35	α-Muurolol	1642	1646	3.00 ± 0.14
36	α-Cadinol	1658	1658	0.23 ± 0.01
37	7-epi-α-Eudesmol	1679	1663	0.55 ± 0.02
38	Mint sulfide	1719	1741	1.32 ± 0.06
39	Hexahydrofarnesyl acetone	1827	1836	0.23 ± 0.01
40	(2E,6E)-Farnesyl acetate	1834	1846	0.48 ± 0.02
41	Manoyl oxide	1988	1987	0.35 ± 0.02
42	13-epi-Manoyl oxide	2009	2010	1.23 ± 0.05
43	Abietatriene	2034	2056	0.12 ± 0.01
44	Phytol	2108	2114	6.67 ± 0.21
		Monoterpene hydrocarbons		0.72 ± 0.03

Continued

S. No	Compound[a]	Ri[b]	Ri[c]	Peak Area %
		Sesquiterpene hydrocarbons		69.28 ± 2.26
		Oxygenated diterpenes		18.07 ± 0.65
		Others		3.12 ± 0.13
		Total identified		91.19 ± 3.07

Table 1. Chemical composition of *Leonotis nepetifolia* leaf essential oil (LNLEO). Data are represented as average ± SD (n = 3). [a]Compounds are listed in order of elution on the Elite-5 MS column. [b]RI experimentally determined using straight chain n-alkane series (C_8-C_{20}, C_{21}-C_{40}). [c]RI retrieved from the literature[18].

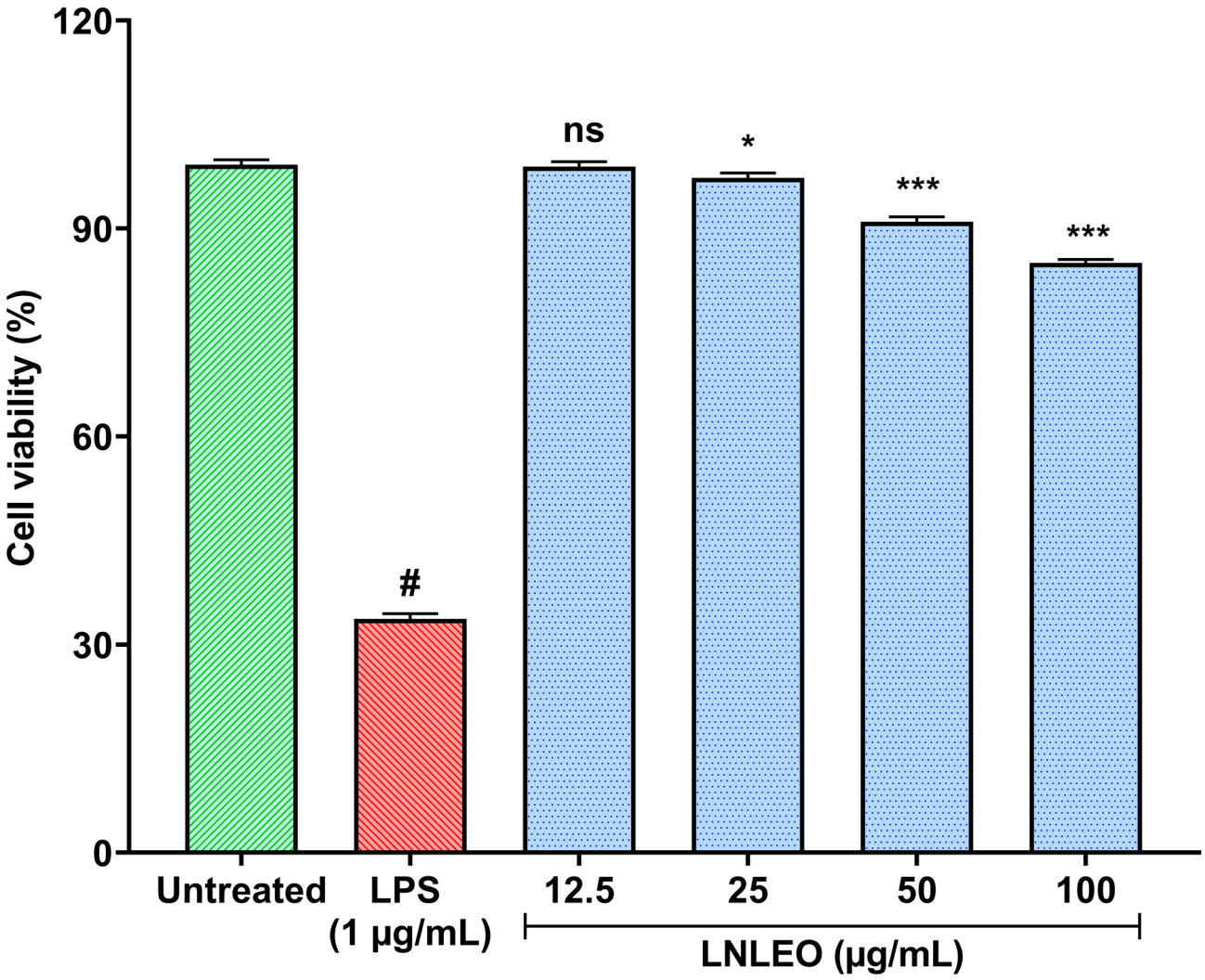

Fig. 1. Impact of *L. nepetifolia* leaf essential oil (LNLEO) on viability and morphology of RAW 264.7 cells. RAW 264.7 cells (2×10^5 cells/mL) propagated in a 96-well plate were exposed to varying doses of LNLEO (12.5, 25, 50, and 100 µg/mL) for 24 h, and the cytotoxicity levels of LNLEO were assessed by MTT assay. Each experimental value is shown as the mean ± SD from three independent trials (n = 3). The statistical significance of data across the groups was assessed employing One-way ANOVA followed by Tukey's post hoc multiple comparison tests. Significance levels are indicated as $^{\#}p < 0.001$ for comparisons between the untreated and LPS-treated group, and $^{ns}p > 0.5$, $^{*}p < 0.05$, $^{***}p < 0.001$ for comparison between the LPS-treated and LNLEO-treated group.

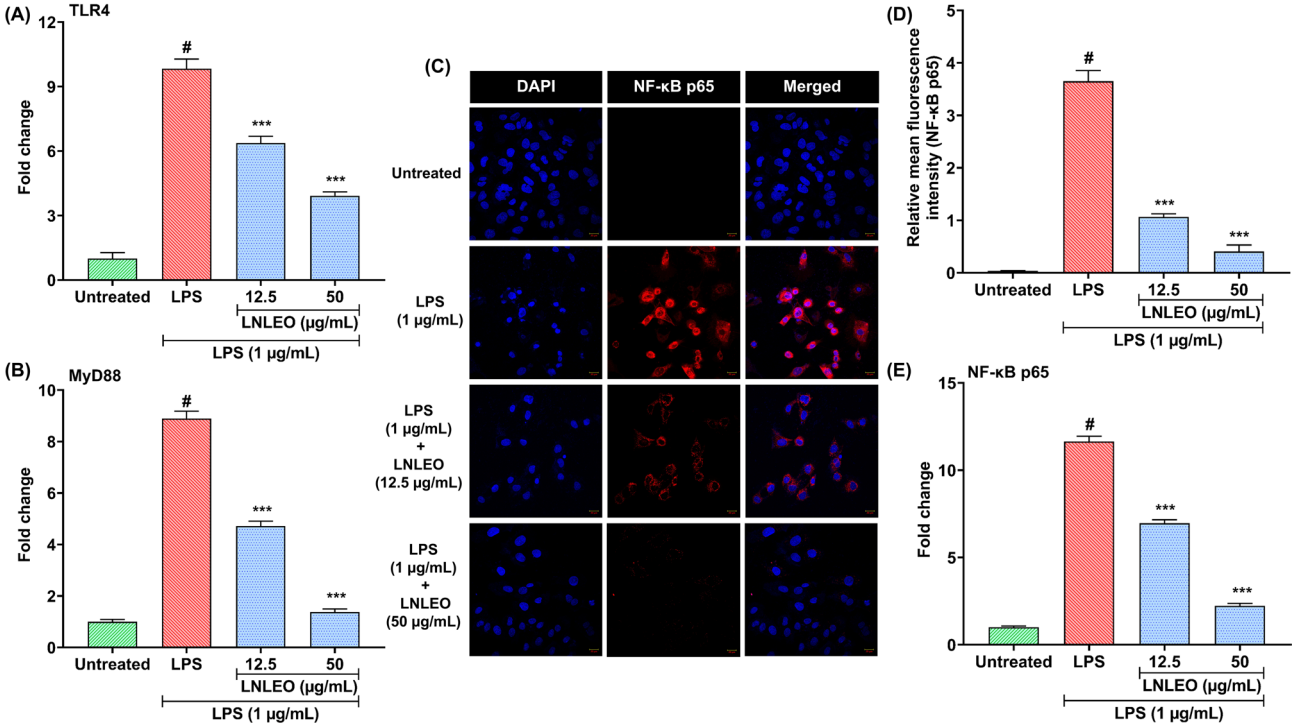

Fig. 2. Impact of *L. nepetifolia* leaf essential oil (LNLEO) on MyD88-dependent TLR4 signaling pathway, NF-κB nuclear localization, and NF-κB p65 gene expression in LPS-modulated RAW 264.7 cells. The LPS-activated RAW 264.7 cells were inoculated in vitro at a 2×10^5 cells/mL density were subjected to LNLEO (i.e., 12.5 and 50 µg/mL) treatment for 24h. Bar graphs represent the mRNA expression levels of (A) TLR4 and (B) MyD88 were analyzed using RT-qPCR. (C) The cells were fluorescently tagged with PE-conjugated mouse anti-NF-κB p65 antibody, and the nuclei were counterstained with DAPI solution after overnight incubation. The fluorescence levels and nuclear localization of NF-κB p65 were assessed using a Confocal Microscope (scale bar = 20 µm). (D) Bar graphs illustrating the relative mean immunofluorescence intensity, and (E) mRNA expression levels of NF-κB p65 in Untreated, LPS-alone, and LPS+LNLEO treated murine RAW 264.7 cells. RT-qPCR results were quantified and normalized by the $2^{-\Delta\Delta CT}$ method, with β-actin serving as the internal housekeeping reference gene. Each experimental value is shown as mean ± SD from three independent trials (n = 3). The statistical significance of data across the groups was assessed employing One-way ANOVA followed by Tukey's post hoc multiple comparison test. Significance levels are indicated as $^{\#}p < 0.001$ for comparisons between the untreated and LPS-treated group, and $^{***}p < 0.001$ for comparisons between the LPS-treated and LNLEO-treated group.

of TLR4 and MyD88 genes and interferes with the activation of NF-κB by inhibiting its translocation from the cytoplasm to the nucleus.

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Effect of LNLEO on phosphorylation of MAP kinases genes and expression of pro-inflammatory mediators

Mitogen-activated protein kinases (MAPKs) like extracellular signal-regulated kinase-2 (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK play a crucial role in inflammation by mediating cellular responses to various stimuli. They act as signal transducers, relaying information from the cell's exterior to its interior, ultimately influencing gene expression and cellular behavior related to inflammation. LPS treatment phosphorylates MAPKs, thereby activating the signaling pathways and triggering the production of various pro-inflammatory mediators[21–23]. To evaluate the anti-inflammatory effect of LNLEO on these MAPKs, the ratio of phosphorylated MAPKs to total MAPKs was determined by ELISA. LPS exposure elevated the ratios of phosphorylated ERK (pERK)/total ERK, phosphorylated JNK (pJNK)/total JNK, and phosphorylated p38 (phospho-p38 MAPK)/total p38 MAPK to a large extent in both treated and control groups. On the other hand, when the LPS-exposed cells were maintained overnight with LNLEO at 12.5 and 50 µg/mL, the phosphorylated MAPKs, such as pERK (Fig. 3A), pJNK (Fig. 3B), and phospho-p38 MAPK (Fig. 3C), were markedly inhibited.

Similarly, the inhibitory potential of LNLEO on pro-inflammatory cytokines, namely tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) levels in LPS-stimulated macrophage cells was evaluated by ELISA. The LPS-induced pro-inflammatory cytokine levels were reduced in a concentration-specific manner when treated with 12.5 and 50 µg/mL of LNLEO. As depicted in Figs. 3D-F, at a dose of 50 µg/mL, LNLEO brought down the escalated levels of TNF-α, IL-1β, and IL-6 by 3.2-fold, 3.1-fold, and 4.2-fold, respectively. In this experiment, the effect of LNLEO on the mRNA expression levels of inducible nitric oxide

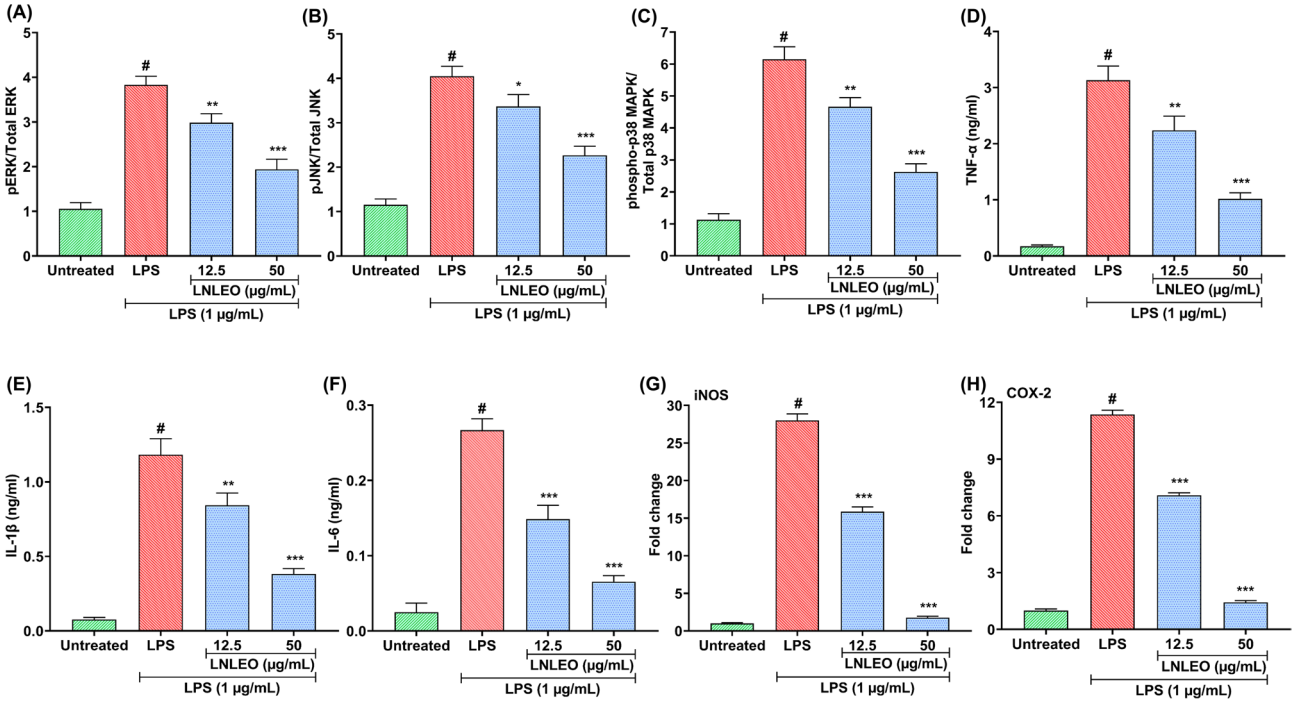


Fig. 3. Impact of *L. nepetifolia* leaf essential oil (LNLEO) on the expression levels of MAPK proteins, proinflammatory cytokines, and inflammatory mediators in LPS-elicited RAW 264.7 cells. Murine RAW 264.7 cells (2×10^5 cells/mL) grown in 96-well plates were initially exposed to LPS, 1 μg/mL for 2 h to trigger inflammation and then co-treated with LNLEO at two doses set at 12.5 and 50 μg/mL for 24 h. Bar graphs representing the ratios of phosphorylated to total MAPKs i.e., (A) ERK, (B) JNK, (C) p38 MAPK, and secreted pro-inflammatory cytokine levels like (D) TNF-α, (E) IL-1β, and (F) IL-6 in the macrophage cells, spectrophotometrically measured using specialized ELISA Development kits. The mRNA expression levels of inflammatory mediators such as (G) iNOS and (H) COX-2 were analyzed using RT-qPCR. Results were quantified and normalized by the $2^{-\Delta\Delta CT}$ method, with β-actin serving as the internal housekeeping reference gene. Each experimental value is shown as mean ± SD from three independent trials (n = 3). The statistical significance of data across the groups was assessed employing One-way ANOVA followed by Tukey's post hoc multiple comparison tests. Significance levels are indicated as $^{\#}p < 0.001$ for comparison between the untreated and LPS-treated group, and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for comparisons between the LPS-treated and LNLEO-treated group.

synthase (iNOS) and cyclooxygenase-2 (COX-2) in LPS-triggered RAW 264.7 cells were determined by RT-qPCR. Compared to the non-stimulated macrophage cells, LPS exposure markedly upregulated iNOS and COX-2 expression by manyfold as determined from the $2^{-\Delta\Delta CT}$ method. Despite that, healing the pre-LPS-induced cells (1 μg/mL for 2 h) with LNLEO at 12.5 and 50 μg/mL for 24 h substantially attenuated the expression of iNOS (Fig. 3G) and COX-2 (Fig. 3H) in a concentration-specific manner as opposed to cells treated with LPS alone. Particularly, overnight nurturing with 50 μg/mL of LNLEO showed a marked reduction in the expression levels of iNOS and COX-2 from 28-fold and 11.4-fold in LPS-stimulated cells to 1.8-fold and 1.4-fold, bringing them close to the baseline level.

These findings suggest that LNLEO can modulate the MAPK signaling cascade by inhibiting the phosphorylation of key proteins, leading to a subsequent decrease in the expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and inflammatory mediators (iNOS and COX-2).

Effect of LNLEO on the Keap1-Nrf2-HO-1 antioxidative pathway

The Kelch-like ECH-associated protein 1 (Keap1) and nuclear factor erythroid 2-related factor 2 (Nrf2) pathway is the principal protective response to oxidative and electrophilic stresses. Under homeostatic conditions, Keap1 forms part of an E3 ubiquitin ligase, which tightly regulates the activity of the transcription factor Nrf2 by targeting it for ubiquitination and proteasome-dependent degradation[24]. In this study, ROS levels and the antioxidant activities of the enzymes, i.e., superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx), and catalase (CAT), were quantitatively assessed by flow cytometry with H_2DCFDA (2′, 7′-dichlorodihydrofluorescein diacetate) dye and ELISA. As illustrated in Figs. 4A and B, the proportion of macrophage cells emitting DCF (2′, 7′-dichlorofluorescein) fluorescence escalated from 1.06 to 74.89% after 24 h of LPS-driven stimulation. This rise in ROS levels led to manyfold downregulation of SOD, GSH, GPx, and CAT (Fig. 4C-F) in the LPS-stimulated cells compared to the untreated controls. Then, the ability of LNLEO to reduce ROS levels and enhance antioxidant enzyme activities was evaluated by treating pre-stimulated cells overnight with 12.5 and 50 μg/mL of essential oil. Overnight incubation with LNLEO at both concentrations not

only lowered the ROS level but also substantially restored and enhanced the SOD, GSH, GPx, and CAT enzyme levels in a dose-dependent manner. Administration of LNLEO at 50 µg/mL managed to alleviate the ROS levels and brought about manyfold increase in the level of endogenous antioxidant enzymes compared to the LPS-only treated groups, thereby creating a more enduring cellular environment.

Through this experiment, the influence of LNLEO on oxidative and antioxidative genes like Keap1, Nrf2, and heme oxygenase 1 (HO-1) in LPS-alone stimulated RAW 264.7 cells was investigated by RT-qPCR. 24 h LPS stimulation significantly amplified Keap1 expression (Fig. 4G), while restraining Nrf2 (Fig. 4H) and HO-1 (Fig. 4I) levels to nearly baseline compared to untreated cells. However, these detrimental effects were substantially reverted in the endotoxin-stimulated (LPS, 1 µg/mL for 2 h) macrophage cells upon co-culturing with LNLEO at 12.5 and 50 µg/mL for 24 h. These results indicate that LNLEO exerts a protective effect in LPS-treated RAW 264.7 cells by reducing ROS levels, enhancing the release of endogenous antioxidant enzymes, and activating the Keap1-Nrf2-HO-1 antioxidative pathway.

In summary, findings of the in vitro experiments suggest a possible mode of action underlying the anti-inflammatory mechanism of the leaf essential oil of *Leonotis nepetifolia* (LNLEO), involving the mitigation of pro-inflammatory cytokines, endogenous antioxidant enzymes, and ROS by regulating Keap1/Nrf2/HO-1, TLR4/MyD88, NF-κB, and MAPK signaling pathways, as depicted in the proposed graphical representation of the mechanism of action (Fig. 5).

Discussion

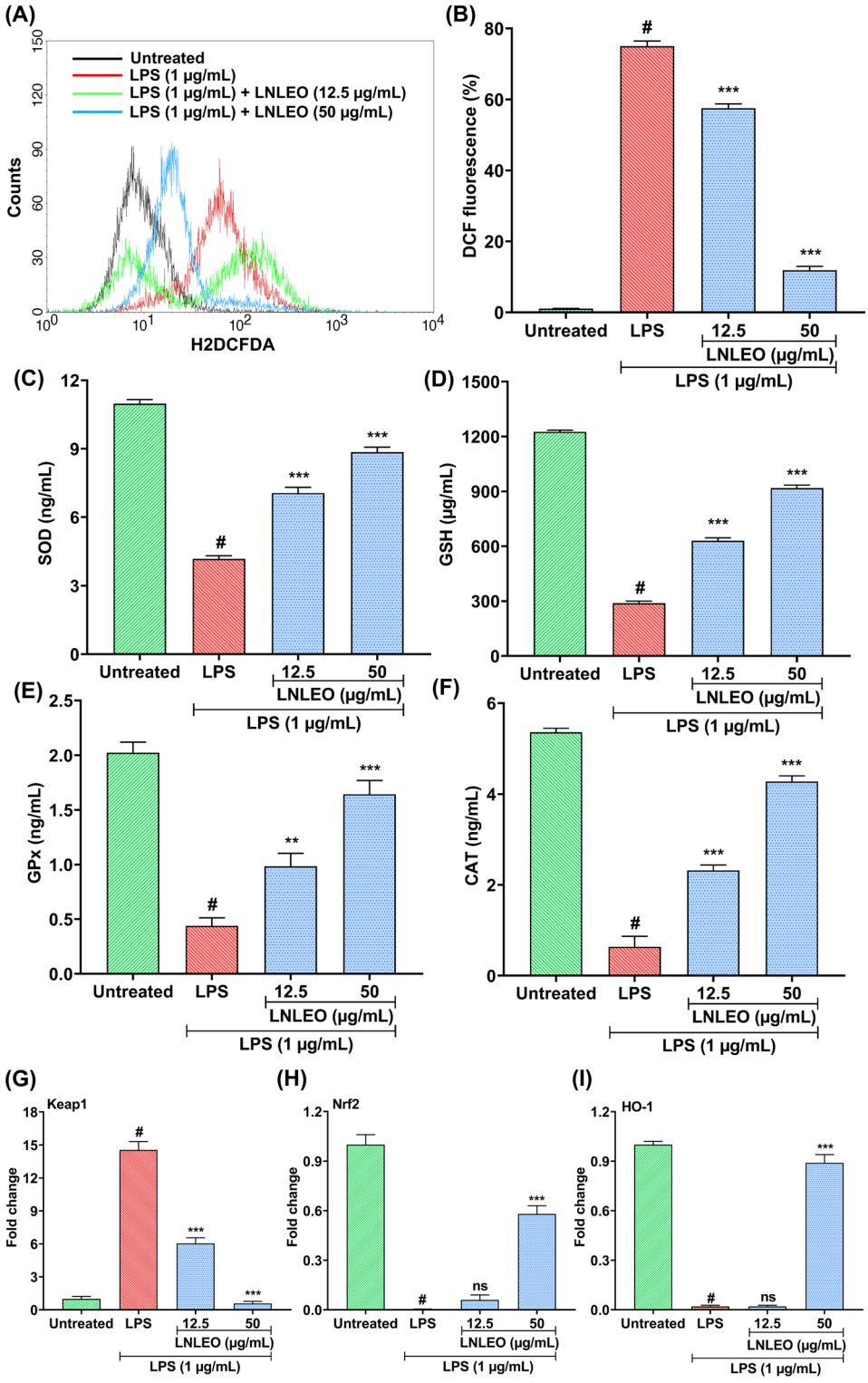
Inflammation is a highly complex immune response considered to be the primary defense mechanism triggered to protect organisms from harmful stimuli such as injury, trauma, bacterial and viral infections, toxic chemicals, irradiation, etc., and it acts by removing the inflammatory factors damaging the tissues and initiating the healing process[25]. However, excessive and uncontrolled inflammation may become chronic over time and be the major cause of several human diseases like stroke, rheumatoid arthritis, type-2 diabetes, pulmonary, neurodegenerative, and cardiovascular diseases, obesity, inflammatory bowel syndrome, and cancer[26]. Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for treating several inflammatory conditions like rheumatism, arthritis, and body pain. However, prolonged usage of these drugs is reported to have adverse side effects, resulting in gastrointestinal, cardiovascular, hepatic, renal, cerebral, and pulmonary complications, thus limiting their application. In consideration of this, the usage of anti-inflammatory agents with better efficacy and minimal side effects has been emphasized[27] and the world's attention has now been diverted towards natural compounds, such as herbal products and dietary supplements, which may offer a safer and alternative treatment to get relief from pain and for other inflammatory conditions, especially for long-term use[28].

Essential oils, the complex mixture of volatile secondary metabolites present in different plant parts, have been used for a long time in many different traditional healing systems all over the world and have gained global recognition for their potential biological activities, especially in treating inflammatory disorders[29,30]. Essential oils and anti-inflammatory drugs such as corticosteroids and NSAIDs differ significantly in their mechanisms of action. NSAIDs primarily target cyclooxygenase (COX) enzymes to inhibit the production of inflammatory mediators such as prostaglandins, leading to a decrease in pain and inflammation, but prolonged use may lead to gastrointestinal, renal, and cardiovascular complications[31–35]. But corticosteroids broadly suppress the immune system and inhibit multiple inflammatory genes via glucocorticoid receptor-mediated repression of NF-κB and AP-1[36]. While highly effective, long-term use of corticosteroids is associated with serious side effects, including immunosuppression, osteoporosis, metabolic disturbances, and adrenal insufficiency[37]. On the other hand, the essential oils can influence multiple inflammatory pathways, including cytokines such as TNF-α, IL-1β, and IL-6, and signalling molecules like NF-κB, MAPK, and AKT[30,38,39]. In addition, essential oils can act as antioxidants, scavenging free radicals and reducing oxidative stress, and also modulate the immune response, potentially by influencing the activity of immune cells like macrophages and microglia. In essence, essential oils offer a broader, more natural approach to managing inflammation, with a potentially lower risk of serious side effects.

In vitro anti-inflammatory activity of the essential oils of several species of *Ocimum, Origanum, Mesosphaerum, Lavandula, Pogostemon, Rosmarinus, Stachys, Salvia, Scutellaria, Perilla, Thymus,* etc., belonging to the Lamiaceae family has been attributed to one or more factors, like suppression of NO and iNOS production; inhibition of TNF-α-induced NF-κB activation; decreased IL-6, IL-1β, and IL-8 expression; reduction of ROS, intercellular adhesion molecule 1 (ICAM-1) and COX-2; increased level of interleukin 10 (IL-10) expression, etc[40,41].

Out of the nine known species of *Leonotis* in the world, the chemical composition, antioxidant, cytotoxic, and antimicrobial activities of the essential oils of *L. nepetifolia, L. leonurus,* and *L. ocymifolia,* have been reported from different geographical regions[16,17,42,43]. However, to date, there is no report on the anti-inflammatory activity of the essential oil of *L. nepetifolia* and its mechanism of action. In view of this, the present research is aimed at scientifically validating the in vitro anti-inflammatory actions of LNLEO, employing the LPS-induced RAW 264.7 macrophage cells, and also shedding light on the molecular mechanisms underlying signaling cascades.

In the present study, LNLEO was found to possess a strong aroma, and the oil yield was 0.048% (w/w) on a dry weight basis. The oil yield was comparatively higher than those reported in the same species from Brazil (0.033%), Nigeria (0.03%), and sub-Saharan Africa (0.004%)[16,44]. GC–MS analysis of LNLEO revealed the presence of a total of 44 constituents representing 91.19% of the total essential oil. Terpenoids such as germacrene D (32.17%), β-caryophyllene (16.53%), α-humulene (7.36%), and phytol (6.67%) were the major compounds. Earlier studies have reported the predominance of germacrene D, β-caryophyllene, and α-humulene in the leaf essential oil of *L. nepetifolia* from Brazil[16], and the leaf and flower essential oils of *L. leonurus* and *L. ocymifolia*[43]. Germacrene D-rich essential oils of many other plant species, like *Cordia verbenacea*, *Seseli* species, *Aster spathulifolius*, and *Vitex rotundifolia,* have shown anti-inflammatory, antioxidant, and cytotoxic effects[45–47]. The anti-inflammatory effects of germacrene D, β-caryophyllene, phytol, and α-humulene have been established through in vitro and



in vivo experiments[46,48–50]. The anti-inflammatory action of LNLEO may be attributed to the presence of these major constituents or the synergistic interaction of some minor and major compounds.

The toxicity of LNLEO on LPS-stimulated RAW 264.7 cells was checked in vitro using the MTT assay. While 1 µg/mL of LPS alone reduced cell viability to approximately 33.79% and proved cytotoxic, the essential oil was non-toxic up to a concentration of 50 µg/mL, maintaining the cell viability of more than 90%. However, the viability of RAW 264.7 cells dropped below 85% with the treatment of 100 µg/mL of LNLEO, indicating minimal toxicity. Therefore, the subsequent assays were performed using two non-toxic concentrations of LNLEO, one low dose of 12.5 µg/mL and one higher dose of 50 µg/mL. Similar mild cytotoxicity was observed with *Cryptocarya amygdalina* essential oil, where exposure to 100 µg/mL reduced RAW 264.7 cell viability to 72.61%[51].

Cytokines play a crucial role in regulating inflammation, but their prolonged overexpression above the threshold results in chronic inflammatory diseases like osteoporosis and rheumatoid arthritis[52]. LPS stimulation

◀**Fig. 4**. Impact of *L. nepetifolia* leaf essential oil (LNLEO) on the intracellular ROS levels, endogenous antioxidant enzyme concentrations, and Keap1-Nrf2-HO-1 antioxidative pathway in the LPS-exposed RAW 264.7 cells. The macrophage cells growing at a density of 2×10^5 cells/mL were inflamed with LPS (1 µg/mL) for 2 h to induce inflammation and subsequently applied with LNLEO (i.e., 12.5 and 50 µg/mL) for 24 h. (**A**) Overlay histogram plot displaying ROS production, and (**B**) bar graph depicting mean DCF fluorescence intensity in untreated, LPS-alone, and LPS + LNLEO-treated cells assessed by flow cytometry with H_2DCFDA dye. Bar diagrams indicating the activities of endogenous antioxidant enzymes, i.e., (**C**) SOD, (**D**) GSH, (**E**) GPx, and (**F**) CAT quantitatively estimated using mouse immunoassay ELISA kits. RT-qPCR validation of Keap1-Nrf2-HO-1 pathway, with bar plots showing the mRNA expression levels of (**G**) Keap1, (**H**) Nrf2, and (**I**) HO-1 genes. RT-qPCR results were quantified and normalized by the $2^{-\Delta\Delta CT}$ method, with β-actin serving as the internal housekeeping reference gene. Each experimental value is shown as mean ± SD from three independent trials (n = 3). The statistical significance of data across the groups was assessed employing One-way ANOVA followed by Tukey's post hoc multiple comparison tests. Significance levels are indicated as $^{\#}p < 0.001$ for comparisons between the untreated and LPS-treated group, and $^{ns}p > 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$ for comparison between the LPS-treated and LNLEO-treated group.

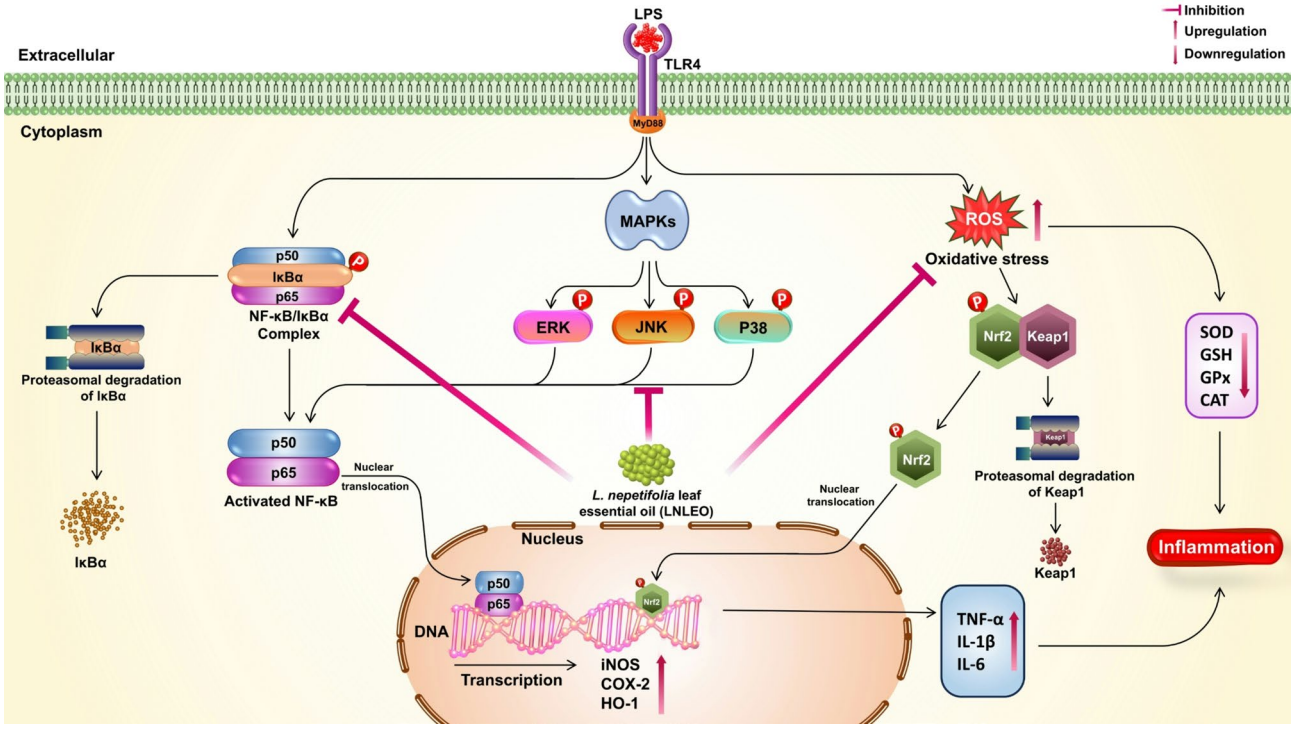


Fig. 5. Proposed scheme of anti-inflammatory mechanism of LNLEO in LPS-stimulated RAW 264.7 cells. LNLEO suppressed inflammation by inhibiting TLR4/MyD88 mRNA expression, downregulating MAPK phosphorylation and NF-κB signaling pathway, and enhancing the antioxidative Keap1/Nrf2/HO-1 pathway. These series of events limited the expression of pro-inflammatory targets like cytokines (TNF-α, IL-6, IL-1β), enzymes (iNOS, COX-2), and other mediators (ROS) while upregulating the expression of endogenous antioxidant enzymes (SOD, GSH, GPx, and CAT). Illustration created with Microsoft PowerPoint 2019 (Microsoft Corp., USA) with elements taken from the icon library of open-source project, "Reactome", https://reactome.org/ (Accessed on 13th November, 2024).

of RAW 264.7 macrophages may lead to a significant increase in the production of pro-inflammatory cytokines. This enhanced cytokine expression level has been attributed to the activation of TLR4 and associated intracellular signaling pathways[53,54]. In the present study, LNLEO was found to have significant inhibitory effects on the production of inflammatory cytokines like TNF-α, IL-1β, and IL-6 in a dose-dependent manner. Downregulation of pro-inflammatory cytokines directly suppresses immune cell activation and inflammatory signaling[55]. These cytokines are major mediators of tissue damage and immune cell recruitment in inflammatory diseases. Therefore, their inhibition implies functional benefits such as decreased inflammatory cell infiltration, lower tissue damage, and restoration of homeostasis. Similar pro-inflammatory cytokine inhibitory activity of the leaf and root extracts of *L. nepetifolia* has been reported[15,56]. Our results are also in agreement with the findings of earlier studies, where the essential oils of several species of *Citrus*, *Artemisia*, *Chimonanthus*, *Myrciaria*, *Eucalyptus*, *Verbesina*, *Lavandula*, *Melaleuca*, *Cinnamomum*, *Neocinnamomum*, *Cymbopogon* and *Mesosphaerum* have been reported to suppress the transcription of TNF-α, IL-1β, and IL-6 by limiting their mRNA expression and production of the

proinflammatory cytokines[39,41,57,58]. The major constituents in LNLEO, such as germacrene D, β-caryophyllene, and phytol are known to inhibit TNF-α, IL-1β, and IL-6 production[45,50,59,60], and its anti-inflammatory action could be due to the predominance of these compounds in its essential oil.

ROS can act as signaling molecules, influencing cellular processes such as cell division, gene expression, and host protection by perpetuating inflammatory responses through the release of pro-inflammatory molecules and the activation of macrophages. Under normal physiological conditions, the endogenous antioxidant enzymes like SOD, GSH, GPx, and CAT neutralize reactive oxygen species and protect cells from oxidative damage, thereby providing a first-line defense against free radicals. During pathogenic conditions such as LPS stimulation, both mitochondrial and cytoplasmic ROS levels get drastically enhanced in the RAW 264.7 cells, triggering the activation of NF-κB, MAPKs, and pro-inflammatory cytokines[61]. In the present experiment, the co-culturing of LPS-triggered cells with LNLEO regulated the over-production of ROS and restored the expression levels and functionality of endogenous antioxidant enzymes in a dose-dependent manner. The findings of the current study is in conformity with the observations that the root extracts of *L. nepetifolia* can effectively reduce ROS levels and restore the endogenous antioxidant enzymes[40,62]. The essential oils of *Origanum vulgare, Cymbopogon winterianus, Neocinnamomum caudatum*, and *Mesosphaerum suaveolens* have been shown to reduce oxidative stress by increasing the activities of antioxidant enzymes like CAT, SOD, GPx, and GSH[41,57,63,64].

The effect of LNLEO on the levels of iNOS and COX-2 was examined in LPS-triggered RAW 264.7 cells using RT-qPCR. Treatment with LNLEO suppressed iNOS and COX-2 expression to a large extent. This may be attributed to the presence of the sesquiterpenes such as β-caryophyllene, phytol, and α-humulene, which are known to inhibit iNOS and COX-2 enzyme production under inflammatory conditions[48,49]. Additionally, germacrene D-rich essential oils of *Seseli gummiferum* and *Seseli corymbosum* have also been reported to regulate the expression of iNOS[14,65]. The essential oils of several species of Lamiaceae, such as *Glechoma hederacea, Origanum vulgare, Thymus carnosus*, and *Thymus camphoratus* are known to suppress the inflammatory responses by regulating the expression of inflammation-related enzymes[66].

Keap1 acts as an inhibitor of Nrf2, a transcription factor that regulates the expression of genes involved in cellular defence against oxidative stress and other harmful stimuli. In LPS-elicited RAW 264.7 cells, Keap1 upregulation leads to Nrf2 inactivation and subsequent decrease in the expression of genes like HO-1involved in antioxidant defense and anti-inflammatory responses[67]. In the present study, it was found that LNLEO treatment reversed the adverse conditions by silencing the repressor protein Keap1, thereby activating the master regulator Nrf2 and the antioxidant enzyme HO-1. The augmentation of HO-1 levels triggered the breakdown of various biomolecules (i.e., heme, carbon monoxide, and biliverdin) and prevented the expression of iNOS, COX-2, and proinflammatory cytokines, resulting in a reduced cellular environment[67]. The phytoconstituents such as nepetaefuran and leonotinin isolated from the extracts of *L. nepetifolia* are reportedly responsible for the anti-inflammatory activity, which potently inhibits the LPS-induced activation of NF-κB[14]. Similarly, β-caryophyllene and phytol, two predominant compounds in the essential oil of *L. nepetifolia*, have been shown to regulate Nrf2 activation[68,69]. Our findings suggest that LNLEO can act as an anti-inflammatory agent by targeting and restraining pro-inflammatory mediators and modulating the Keap1-Nrf2-HO-1 antioxidative pathway, ultimately aiming at resolving inflammation and restoring tissue homeostasis.

TLRs can recognize both invading pathogens and endogenous danger molecules released from dying cells and damaged tissues, and play a key role in linking innate and adaptive immunity[70]. TLR4 along with MyD88 protein recognizes LPS and mediates inflammatory responses through both MyD88-dependent and MyD88-independent pathways. The MyD88-dependent pathway subsequently activates a series of signaling pathways, including MAPKs and NF-κB[71]. These conjoint events trigger the synthesis and activation of pro-inflammatory cytokines, enzymes, ROS, and PGE2, which collectively promote inflammation, vasodilation, and cell survival[72]. In consideration of the critical role of TLR4 in inflammatory signaling, we investigated the molecular mechanisms involved in the anti-inflammatory action of LNLEO by analyzing the expression of TLR4 and its associated gene MyD88 using RT-qPCR. LPS-induced inflammation enhanced the expression of TLR4 and MyD88, but the LNLEO administration reduced their levels in a concentration-dependent manner. Reduced levels of TLR4 and MyD88 in LNLEO-treated cells compared to LPS-only stimulated cells suggest that LNLEO exerts anti-inflammatory action by restricting LPS tethered to its receptor on the cell surface. Further, β-caryophyllene, the predominant component of LNLEO, has been found to regulate inflammation by influencing the TLR pathway in another Lamiaceae species, *Agastache rugosa*[73]. Our results indicate that β-caryophyllene may have a protective effect against inflammation through the downregulation of TLR4[74].

NF-κB activation leads to the upregulation of various genes, including those involved in inflammation, such as TNF-α, IL-6, and IL-8, and the translocation of the NF-κB p65 subunit from the cytoplasm to the nucleus. In the current investigation, the role of LNLEO in the NF-κB signaling pathway was evaluated using confocal microscopy and RT-qPCR analysis. NF-κB is typically sequestered in the cytosol in the basal state, attached to its suppressor inhibitor of κB (IκB). However, with LPS-mediated NF-κB activation, IκB is released from the core complex, permitting the translocation of the NF-κB p65 subunit from cytosol to nucleus[75]. This nuclear translocation suddenly increased the levels of cytokines (TNF-α, IL-1β, IL-6) as well as inflammatory mediators (iNOS, COX-2). Previous studies have shown that, through a feedback loop mechanism, certain stimuli can activate NF-κB, leading to sustained and amplified inflammatory response, potentially causing chronic inflammation and contributing to various diseases[22]. The confocal microscopy revealed that LPS treatment exacerbated the inflammation, as a notable surge was observed in the proportion of RAW 264.7 cells emitting NF-κB p65 fluorescence in LPS-treated groups compared to the untreated control. This increase could be due to the ubiquitin-mediated proteasomal degradation of IκB and the subsequent translocation of NF-κB p65. In contrast, co-treatment with LNLEO significantly reduced the immunofluorescence levels in the pre-LPS-triggered macrophages, suggesting that LNLEO abates inflammation by limiting nuclear translocation of NF-κB p65 through activation of IκB, thus suppressing the NF-κB signaling pathway. Additionally, RT-qPCR results

revealed that LNLEO also suppressed NF-κB gene expression, leading to the inhibition of the NF-κB signaling pathway and subsequent reduction in the expression of proinflammatory cytokines and other mediators at the transcriptional level. These findings align with the results reported for *Mesosphaerum suaveolens* and various other essential oils exhibiting similar bioactivities[39,41]. The essential oils of six species of Lamiaceae, namely *Pogostemon cablin, Mentha haplocalyx, Rosmarinus officinalis, Lavandula angustifolia,* and *Scutellaria baicalensis,* evaluated through TPA-induced mice models exhibited strong anti-inflammatory actions marked by decreased production of TNF-α, NF-κB, IL-6, and COX-2[76]. Germacrene D, β-caryophyllene, and phytol, the major compounds in LNLEO, reportedly ameliorate inflammation through the inhibition of the NF-κB signal pathway[49,68], and the present bioactivity of LNLEO could be due to synergistic or additive interactions among major and minor constituents. Although the present study does not include direct experimental validation of the anti-inflammatory activities of major constituents, our attribution of bioactivity to these compounds is based on their high relative abundance in the essential oil and strong reported bioactivities in previous studies[60,77–79]. However, such conclusions remain correlative rather than causative without single-compound assays or inhibition studies.

MAPK pathways are crucial signaling cascades involved in mediating cellular responses to inflammation, including the production of inflammatory cytokines and other mediators. Key MAPK pathways implicated in inflammation include p38 MAPK, JNK, and ERK, which can be targeted for anti-inflammatory therapies[39,80]. TLR4 plays a crucial role in inflammation by prompting the activation of MAPKs by phosphorylation. Activated MAPKs then transmit the signal to the nucleus, catalyzing the activation of NF-κB and other transcriptional factors that escalate inflammation[23]. In the present investigation, the effect of LNLEO on MAPKs was validated in vitro by comparing the ratios of phosphorylated MAPKs to total MAPKs in the macrophage cells. When LPS-exposed RAW 264.7 cells were maintained overnight with LNLEO at 12.5 and 50 µg/mL, the phosphorylated MAPKs levels of pERK, pJNK, and phospho-p38 MAPK were significantly inhibited. Earlier studies have experimentally demonstrated that inhibition of ERK, JNK, and p38 MAPKs leads to suppression of TNF-α, IL-1β, iNOS, and COX-2 expression[23]. Our findings are in agreement with the above observations, pointing at the therapeutic potential of LNLEO to curb inflammation through the inhibition of the MAPK pathway by suppressing the production of inflammatory mediators and cytokines[22,23]. Essential oils of *Citrus aurantium*, *Eucalyptus* species, *Hibiscus sabdariffa, Abies holophylla, Foeniculum vulgare, Schisandra chinensis,* etc., are reported to inhibit the activation and nuclear translocation of NF-κB, as well as phosphorylation of JNK and p38 MAPK proteins to alleviate LPS-induced inflammation of RAW 264.7 cells[39].

Although the current study offers strong in vitro support for LNLEO's anti-inflammatory properties in LPS-stimulated RAW 264.7 macrophages, in vivo investigations are necessary to validate these results under different physiological conditions. Appropriate in vivo models that allow evaluation of cytokine modulation, oxidative stress biomarkers, and histopathological alterations include the LPS-induced murine endotoxemia model for systemic inflammatory responses and the carrageenan-induced paw edema model for acute inflammation. Additionally, models of chronic inflammation, like collagen-induced arthritis, could throw light on the effectiveness of long-term treatments. To bridge the gap between efficient cell-based concentrations and feasible systemic levels in a living organism, a substantial pharmacokinetic study would also be necessary for translation from in vitro to in vivo settings. Metabolic stability and identification of primary metabolic pathways, tissue distribution to determine target-site bioavailability, evaluation of absorption dynamics following various administration routes (e.g., oral, intraperitoneal, topical), and excretion profiles via renal or biliary routes need to be assessed. The half-life of bioactive components, possible tissue accumulation, and metabolic conversion to active or inactive metabolites are to be elucidated using ADME profiling. Integrating these pharmacokinetic insights with pharmacodynamic endpoints would enable the establishment of scientifically grounded dosing regimens that ensure both efficacy and safety in in vivo conditions. These investigations will be helpful for validating the observed NF-κB, MAPK, and Nrf2 pathway modulations in vivo and to evaluate the safety and bioavailability of LNLEO for potential therapeutic use in the future.

Materials and methods

Cell culture and maintenance

RAW 264.7 murine macrophage cells obtained from the National Cell Repository of cell lines of the National Centre for Cell Science (NCCS), Pune, Maharashtra, India, were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic–antimycotic solution, and 1% L-glutamine (200 mM). The cells were maintained in a CO_2 incubator set at 37 °C, 18–20% O_2, and 5% CO_2, with a passage time of 3–4 days.

Plant sample collection and essential oil extraction

Mature and healthy leaves of *Leonotis nepetifolia* were collected from the wastelands of Andharua, Bhubaneswar, Khordha district, Odisha, India (20° 21′ 6.2568″ N, 85° 46′ 27.7752″ E; Alt. 39 m MSL) in late November 2023. The identity of the species was confirmed by Prof. Pratap Chandra Panda, Plant Taxonomist, and voucher herbarium specimens (2554/CBT, Dt. 24.11.2023) were deposited in the Herbarium of the Centre for Biotechnology, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar. The fresh leaves of *L. nepetifolia* were thoroughly washed, air-dried for 7–8 days, and ground into fine powders. The powdered leaf samples weighing 350 g were placed in a 5 L round-bottom flask equipped with a Clevenger-type apparatus and subjected to hydro-distillation for 8 h for essential oil extraction. The yield percentage of *L. nepetifolia* leaf essential oil (LNLEO) was determined on a dry weight basis (v/w) and subsequently dehydrated with anhydrous sodium sulfate (Na_2SO_4) to eliminate any residual water in the essential oil and kept at 4 °C for subsequent analysis.

GC–MS analysis of *L. nepetifolia* leaf essential oil (LNLEO)

The chemical composition of the extracted leaf essential oil was determined by injecting 0.1 µL of undiluted essential oil into the Clarus 580 Gas Chromatograph equipped with SQ-8MS detector (both manufactured by Perkin Elmer, USA) and a non-polar Elite-5 MS capillary column (30 m × 0.25 mm inner diameter × 0.25 µm film thickness). The operational conditions were set as follows: Helium (flow rate 1 mL/min) as carrier gas; split ratio 1:19; electron ionization energy 70 eV; source and injector temperatures held at 150 °C and 250 °C respectively; and oven temperature, 60 °C to 220 °C ramped at 3 °C/min, finally maintained at 220 °C temperature for 7 min.

The volatile constituents present in LNLEO were qualitatively identified by computer-based matching of their mass spectral fragmentation patterns against the pre-installed commercial database (NIST). This identification was further validated by comparing the calculated Kovats retention index (RI) values with the experimental RIs published in bibliographic literature[18]. The RIs were determined relative to the *n*-alkane series (C_8-C_{20}) injected under identical conditions, following the Van den Dool & Kratz Eq. [81].

Cell viability assay

The cytotoxicity of *L. nepetifolia* leaf essential oil (LNLEO) was quantitatively measured through MTT assay, a universal colorimetric method that relies on the principle of conversion of MTT reagents (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) into purple colored formazan crystals in the viable cells[82]. In this experiment, RAW 264.7 cells were seeded in triplicate at a density of 2×10^5 cells/mL in a 96 well-plate and then exposed to varying doses of LNLEO (12.5, 25, 50, and 100 µg/mL), diluted in 0.1% DMSO (v/v) and incubated for 24 h at 37 °C in a 5% CO_2 environment. Afterward, the used media was discarded from each well, washed with phosphate-buffered saline (PBS), and incubated with 100 µL of MTT reagent (0.5 mg/mL) for another 3 h. Then, MTT reagent-containing supernatants were aspirated, and the formazan crystals formed in viable cells were dissolved using 100 µL of DMSO. The absorbance of the purple formazan solution was measured at 540 nm using a microplate reader (ELX-800, BioTek, USA), and the cell viability percentage was calculated using the mathematical expression: Cell viability (%) = (OD of the LNLEO-treated cells / OD of the untreated cells) × 100. The optical density (OD) of untreated cells was set as 100% and used as a reference to determine the cytotoxicity of LNLEO-treated cells. Changes in the morphology of cells due to LNLEO exposure were also observed and recorded using an inverted microscope (CKX415F, Olympus, Tokyo, Japan), with a reference scale bar of 100 µm.

Measurement of intracellular pro-inflammatory cytokine levels

RAW 264.7 macrophage cells (2×10^5 cells/mL) were initially sensitized with lipopolysaccharide (LPS, 1 µg/mL) for 2 h and then propagated with LNLEO at a baseline concentration of 12.5 µg/mL and a top concentration of 50 µg/mL, respectively for another 24 h in a humidified incubator set at 37 °C and 5% CO_2. After that, the culture medium was collected from each well and centrifuged at 2000 rpm for 10 min at 4 °C. The supernatants were then collected carefully to assess the levels of pro-inflammatory cytokines. The concentrations of cytokines such as TNF-α, IL-1β, and IL-6 were measured spectrophotometrically in triplicate using ELISA Development Kits (Ray Biotech Life, Inc., USA). For this assay, 100 µL of the supernatants were dispensed into 96-well plates pre-coated with primary mouse antibodies specific to the cytokines. Subsequently, the assay was performed according to the manufacturer's instructions, which included the addition of a secondary biotinylated antibody, horseradish peroxidase (HRP)-conjugated streptavidin solution, tetramethylbenzidine (TMB), and a stop solution.

Assessment of endogenous antioxidant enzyme level

Similar to the procedure of the pro-inflammatory cytokine assay, the pre-LPS stimulated RAW 264.7 cells plated at a density of 2×10^5 cells/mL were incubated overnight with two concentrations of LNLEO (i.e., 12.5 and 50 µg/mL) at 37 °C in a 5% CO_2 environment. After 24 h, cellular lysates were collected from each well using RIPA buffer. The activities of these endogenous antioxidant enzymes, i.e., CAT (Krishgen Biosystems, USA), GSH (Krishgen Biosystems, USA), GPx (Elabscience, USA), and SOD (BT Lab, China), were estimated using mouse immunoassay ELISA kits. The collected cellular lysates were added to 96-well plates pre-coated with primary mouse antibodies specific to the antioxidant enzymes. Subsequently, the assay was completed by adding secondary biotinylated antibodies, HRP-conjugated streptavidin solution, tetramethylbenzidine (TMB), and a stop solution according to the manufacturer's protocol. The expression levels of SOD, GSH, GPx, and CAT were then spectrophotometrically quantified at an OD of 450 nm, with each measurement performed in triplicate.

Estimation of intracellular ROS level

The intracellular ROS levels were measured using a Flow Cytometer with H_2DCFDA (2′, 7′-dichlorodihydrofluorescein diacetate) dye[83]. RAW 264.7 cells (2×10^5 cells/mL) cultivated in a 24-well plate were initially stimulated with LPS (1 µg/mL) for 2 h to induce ROS overexpression, and subsequently, the cells were treated overnight with LNLEO (12.5 and 50 µg/mL) at 37 °C and 5% CO_2. After 24 h of essential oil treatment, the culture media from each well were discarded and gently rinsed twice with 1X PBS. The cells were then incubated in the dark at 37 °C for an additional 30 min with 10 µM H_2DCFDA. Post-incubation, the dye was discarded, the cells were washed twice with PBS, trypsinized, centrifuged, and the resulting pellets were resuspended in PBS. H_2DCFDA (2′, 7′-dichlorodihydrofluorescein diacetate) is a non-fluorescent cell-permeable compound, and once inside the cell, the cellular esterase cleaves the acetate groups, converting them into H_2DCF (2′, 7′-dichlorodihydrofluorescein), which remains non-fluorescent. However, in the presence of ROS, H_2DCF is oxidized to DCF (2′, 7′-dichlorofluorescein) in the viable cells, which is highly fluorescent, and fluorescence intensity was measured using a flow cytometer (BD FACScan, BD Bioscience, USA) at an excitation wavelength and emission wavelength of 488 nm and 535 nm, respectively.

Gene expression analysis by RNA isolation and RT-qPCR

The levels of expression of genes such as iNOS, COX-2, TLR4, MyD88, keap1, Nrf2, HO-1, and NF-κB p65 linked with various signaling pathways were checked using RT-qPCR. As stated earlier in the proinflammatory cytokine assay, the pre-LPS sensitized RAW 264.7 cells (2×10^5 cells/mL) were administered with LNLEO at an initial dose of 12.5 µg/mL and a final dose of 50 µg/mL, and were cultured in vitro for 24 h in a humidified incubator maintained at 37 °C and 5% CO_2. On completion of the incubation period, the cells were retrieved, and the total RNA was extracted using the Qiagen RNeasy kit (Hilden, Germany) as per the manufacturer's protocol. DNAse treatment was done to annihilate the possibility of genomic contaminations, and the RNA purity was verified spectrophotometrically by taking the OD ratio at 260/280 nm. Then, total RNA concentration (ng) was determined using the QiaExpert system (Hilden, Germany), and complementary DNA (cDNA) was synthesized from it by reverse transcription using IScript cDNA synthesis kit (Bio-Rad). Each PCR reaction master mix was carefully prepared with a total volume of 25 µL, including cDNA (20 ng/µL)—1 µL, Sensifast SYBR HiRoxkit Master Mix (Bioline, USA) (2X)—12.5 µL, forward and reverse primers (10 µM)—1 µL each (Eurofins Genomic India, Bangalore, India), and nuclease-free water—9.5 µL.

The cDNA amplification was then performed using gene-specific oligonucleotide primers of the aforementioned genes (Table S1) and β-actin in an RT-qPCR (Qiagen Rotor-Gene Q 5plex HRM, Qiagen, Germany). The RT-qPCR temperature program was set as follows: initial denaturation phase at 95 °C for 5 min, then 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and an extension phase at 72 °C for 20 s. In the end, the gene expression levels were quantified and normalized by the $2^{-\Delta\Delta CT}$ method, with β-actin serving as the reference gene to calculate the fold-change values.

Measurement of NF-κB immunofluorescence and nuclear translocation by confocal microscopy

RAW 264.7 macrophage cells (2×10^5 cells/mL) propagating overnight in a 24-well plate were inflamed by endotoxin (LPS, 1 µg/mL) for 2 h to facilitate prolonged NF-κB nuclear translocation. These cells were later grown with two aliquots of LNLEO i.e., 12.5 µg/mL and 50 µg/mL, for 24 h at 37 °C in a 5% CO_2 environment. Post-overnight treatment, the media was removed, washed twice with PBS (1X), and 0.5 mL BD Cytofix/Cytoperm solution (BD Biosciences, 554,714) was added to each well to fix the cells. After fixation, the cells were again washed with PBS solution containing bovine (0.5%) and sodium azide (0.1%). The cells were then stained with 10 µL of PE (phycoerythrin fluorochrome) conjugated mouse anti-NF-κB p65 antibody for 30 min, and the cell nuclei were counterstained with 1 µg/mL of DAPI solution (100 µL) for 10 min in the dark. In the end, fluorescence live imaging was performed using a ZEISS LSM 880 Confocal Microscope (Germany), with excitation and emission settings of 341 nm and 452 nm for DAPI, and 496 nm and 578 nm for PE channels. Images were analyzed using ZEN Blue Software, and NF-κB p65 expression was quantified with Image J software.

Estimating the relative expression levels of proteins in the MAPK signaling pathway

As described earlier, RAW 264.7 cells (2×10^5 cells/mL) grown in 96-well plates were first subjected to LPS, 1 µg/mL for 2 h to trigger the phosphorylation of ERK, JNK, and p38 MAPK proteins associated with the MAPK signaling pathway. Then, cells were co-treated with LNLEO at two concentrations i.e., 12.5 µg/mL and 50 µg/mL for 24 h. After the incubation, the levels of phosphorylated ERK (pERK), total ERK, phosphorylated JNK (pJNK), total JNK, phosphorylated p38 MAPK, and total p38 MAPK were quantified using specific ELISA kits (Ray Biotech Life, Inc., USA) following the instructions provided in the operational guidelines. Absorbance was measured at 450 nm after adding the biotinylated antibodies, HRP-conjugated streptavidin, and TMB substrate. The impact of LNLEO treatment on the activation of the MAPK signaling pathway was assessed by comparing the ratios of pERK/total ERK, pJNK/total JNK, and phospho-p38 MAPK/total p38 MAPK between treated and control groups.

Statistical analysis

The data obtained from each experiment are represented as mean ± SD (standard deviation) from three independent experiments (n = 3), each performed in technical triplicate to ensure reproducibility. The statistical significance of data between the groups was assessed using GraphPad Prism software v8.0.2 (GraphPad Software Inc., USA) employing One-way ANOVA followed by Tukey's post hoc multiple comparison test. A probability value of $p < 0.05$ was considered statistically significant. Exact p values and the specific comparisons evaluated are indicated in the respective figure legends.

Conclusion

The findings of this present investigation, in conjunction with earlier research, provide insight into the multifaceted molecular mechanisms behind the anti-inflammatory potential of *Leonotis nepetifolia* leaf essential oil (LNLEO). LNLEO exhibited anti-inflammatory activity by targeting and curbing TLR4/MyD88 mRNA expression, MAPK phosphorylation, and subsequent inhibition of the NF-κB signaling pathway. Additionally, LNLEO elevated the antioxidative Keap1/Nrf2/HO-1 pathway in the pre-LPS elicited RAW 264.7 cells, assisting in the restoration of cellular homeostasis. These cascades of events attenuated pro-inflammatory targets like cytokines (TNF-α, IL-6, IL-1β), enzymes (iNOS, COX-2), and other mediators (ROS) while upregulating the expression of endogenous antioxidant enzymes (SOD, GSH, GPx, and CAT). This establishes LNLEO as a promising and novel natural therapeutic agent for alleviating inflammation. In terms of therapeutic applications, LNLEO has the potential to be incorporated into topical formulations such as creams, gels, or ointments for the management of inflammatory skin conditions. However, in vivo experiments and studies on formulation development using this essential oil will be necessary to support its clinical application.

Data availability

All data generated in this research are available from the corresponding author on reasonable request.

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O.M.: All experiments, data collection and formal analysis, original draft manuscript writing. P.K.D: Plant collection, identification, GC-MS analysis. S.S.P: Formal analysis, Investigation, Methodology, Validation. S.J., A.S., and A.R.: Investigation, Analysis, Data interpretation, Manuscript correction, and editing. P.C.P. and S.N: Conceptualization, Supervision, review and editing. All authors read and approved the final manuscript.

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