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ANTI-INFLAMMATORY ACTIVITY OF PIPER LONGUM METHANOLIC EXTRACT IN LPS-STIMULATED RAW 264.7 MACROPHAGES

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Abstract

Piper longum L. is traditionally recognized for its broad therapeutic applications; however, its cellular anti-inflammatory mechanisms remain insufficiently characterized. The present study investigated the anti-inflammatory potential of the methanolic stem extract of *Piper longum* in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages. The extract exhibited a clear concentration-dependent suppression of key inflammatory enzymes, including cyclooxygenase (COX), lipoxygenase (LOX), myeloperoxidase (MPO), and inducible nitric oxide synthase (iNOS). Maximum inhibition was observed at 100 µg/mL, with IC_{50} values of 110 µg/mL (COX), 107.49 µg/mL (LOX), and 99.26 µg/mL (iNOS), indicating substantial enzyme inhibitory potency. Furthermore, intracellular nitrite levels were significantly reduced from 792.00 µg in the LPS control to 492.03 µg at 100 µg/mL, confirming effective suppression of nitric oxide production. These findings demonstrate that the methanolic stem extract of *Piper longum* attenuates LPS-induced inflammatory responses through multitarget modulation of enzymatic pathways, supporting its potential as a natural antiinflammatory therapeutic candidate.

1. INTRODUCTION

Inflammation is a complex physiological response triggered by harmful stimuli, including pathogens, tissue injury, and toxic agents. Although acute inflammation is protective, persistent or dysregulated inflammatory responses contribute to the pathogenesis of

chronic disorders such as arthritis, cardiovascular diseases, diabetes, neurodegeneration, and cancer [1,2].

Macrophages play a central regulatory role in inflammatory processes. Upon activation, these cells release pro-inflammatory mediators, including nitric oxide (NO), prostaglandins, and cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) [3]. The production of these mediators is regulated by enzymes such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and lipoxygenases (LOX), which amplify and sustain inflammatory signaling cascades [4,5].

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is widely used to induce inflammatory responses *in vitro*. LPS activates macrophages through Toll-like receptor-mediated signaling pathways, leading to increased expression of iNOS and COX-2 and enhanced production of nitric oxide and pro-inflammatory cytokines [6,7]. The murine macrophage cell line RAW 264.7 represents a well-established and reproducible *in vitro* model for evaluating antiinflammatory agents [8,9].

Piper longum L. (family *Piperaceae*) is extensively used in traditional medicine systems and is rich in bioactive phytoconstituents, including alkaloids (notably piperine), phenolics, flavonoids, terpenoids, and essential oils. These compounds have been reported to exhibit antioxidant, antimicrobial, anticancer, and anti-inflammatory properties [10]. However, systematic investigations evaluating its multitarget antiinflammatory effects in macrophage-based models remain limited.

Therefore, the present study aimed to evaluate the anti-inflammatory efficacy of the methanolic stem extract of *P. longum* in LPS-stimulated RAW 264.7 macrophages, with particular emphasis on modulation of nitric oxide production and key inflammatory enzymes, including COX, LOX, MPO, and iNOS.

2 MATERIALS AND METHODS

2.1 Plant Material and Extraction

Stems of *Piper longum* L. were collected

from Mulagumoodu (8°16′03.00″ N, 77°17′28.20″ E), Kanyakumari District, Tamil Nadu, India. The plant material was identified and authenticated by Dr. R. Subitha Shajini, Department of Botany, Women's Christian College, Nagercoil, Tamil Nadu, India. The collected stems were shade-dried, pulverized into coarse powder, and 20 g of the powdered material was subjected to Soxhlet extraction with methanol for 24 hours. The extract was filtered and concentrated under reduced pressure at 40–45°C using a rotary evaporator to obtain a semi-solid residue, which was stored at 4°C until further experimental use.

2.2 Cell Culture and Treatment

RAW 264.7 murine macrophages were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum, L-glutamine, sodium bicarbonate, and antibiotic solution (penicillin 100 U/mL, streptomycin 100 µg/mL, amphotericin B 2.5 µg/mL). Cells were maintained at 37°C in a humidified 5% CO_2 incubator. Upon reaching approximately 60% confluency, cells were stimulated with LPS

(1 µg/mL) and treated with methanolic stem extract of *Piper longum* at concentrations of 25, 50, and 100 µg/mL for 24 hours. Cell lysates were subsequently prepared for enzymatic and nitric oxide assays [11].

2.3 Enzyme Inhibition Assays

2.3.1 Cyclooxygenase (COX) Activity

To assess COX activity [12], a 100 µL aliquot of cell lysate was incubated with Tris-HCl buffer (pH 8), glutathione (5 mM/L), hemoglobin (5 mM/L), and arachidonic acid. The mixture was incubated for 1 min at 25°C, and the reaction was initiated by adding arachidonic acid (200 mM/L). After 20 min at 37°C, the reaction was terminated with 200 µL of 10% trichloroacetic acid in 1 N HCl. The tubes were centrifuged, followed by addition of 200 µL of 1% thiobarbituric acid. Samples were boiled for 20 min, cooled, and centrifuged again. Absorbance was measured at 632 nm. Percentage COX inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{(A\text{control} - A\text{test})}{A\text{control}} \times 100$$

2.3.2 LIPOXYGENASE (LOX) ACTIVITY

To determine LOX activity [13], the

reaction mixture (final volume 2 mL) contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and 200 µL of sodium linoleate as substrate. The increase in absorbance at 234 nm was recorded using an Agilent Cary

60 UV-Vis spectrophotometer, corresponding to the formation of 5-

hydroxyeicosatetraenoic acid. Percentage LOX inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{(A\text{control} - A\text{test})}{A\text{control}} \times 100$$

2.3.3 MYELOPEROXIDASE (MPO) ACTIVITY

Cell lysates were homogenized in a solution containing 50 mM potassium phosphate buffer and 0.57% hexadecyltrimethylammonium bromide (HTAB) and centrifuged at 2000 g for 30 min at 4°C. MPO activity was assayed by incubating the supernatant with 50 mM phosphate buffer (pH 6) containing guaiacol (1.67 mg/mL) and hydrogen peroxide (0.0005%). The change in absorbance at 460 nm was recorded. One unit of MPO activity was defined as the amount degrading 1 µM peroxide per minute at

25°C [14]. MPO activity was calculated using:

$$U = \frac{\Delta OD \times 4 \times V_t \times \text{dilution factor}}{L \times \varepsilon_{470} \times \Delta t \times V_s}$$

where:

V_t = 1.1 mL(total volume)

L = 1 cm(light path)

ε_{470} = 26.6 $mM^{-1}cm^{-1}$(molar extinction coefficient)

V_S = sample volume

Δt = time interval (min).

2.3.4 INDUCIBLE NITRIC OXIDE SYNTHASE (INOS) ACTIVITY

iNOS activity was quantified using the assay mixture containing HEPES buffer, L-arginine (2 µmol/L), manganese chloride (4 µmol/L), dithiothreitol (10 mmol/L), NADPH (1 mmol/L), tetrahydrobiopterin (4 µmol/L), oxygenated hemoglobin (10 µmol/L), and 0.1 mL cell lysate. The increase in absorbance at 401 nm was recorded [15], and enzyme inhibition was calculated as:

$$\% \text{ Inhibition} = \left(\frac{A\text{control} - A\text{test}}{A\text{control}}\right) \times 100$$

2.3.5 Nitric oxide (NO) estimation

Nitrite levels were estimated by mixing cell lysate (0.5 mL) with 0.1 mL of 3% sulphosalicylic acid and vortexed for 30 min,

followed by centrifugation at 5000 rpm for 15 min. The protein-free supernatant (200 µL) was treated with 30 µL of 10% NaOH and 300 µL of Tris-HCl buffer, followed by 530 µL of freshly prepared Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-(1-naphthyl) ethylene diamine dihydrochloride). After 10-15 min incubation in the dark, absorbance was measured at 540 nm [16]. Sodium nitrite was used as the standard, and nitrite concentration was determined from the calibration curve.

3. RESULT AND DISCUSSION

Treatment with methanolic stem extract of *P. longum* significantly attenuated LPS-induced inflammatory responses in RAW 264.7 macrophages in a concentrationdependent manner.

3.1 Modulation of Cyclooxygenase and Lipoxygenase

The extract effectively inhibited both branches of the arachidonic acid cascade. COX inhibition increased from 17.50% at 25 µg/mL to 47.50% at 100 µg/mL (Table 1), while LOX inhibition ranged from 21.71% to 48.48% across the tested concentrations (Table 2). The IC_{50} values of 110 µg/mL (COX) and 107.49 µg/mL (LOX) indicate substantial interference with prostaglandin and leukotriene biosynthesis.

Table 1. Effect of *Piper longum* on COX activity in LPS-stimulated RAW 264.7 macrophages

Concentration (µg/ml)	OD at 632 nm	Percentage inhibition
LPS	0.04	0.000
25	0.033	17.500
50	0.026	35.000
100	0.021	47.500

Table 2. Effect of *Piper longum* on LOX activity in LPS-stimulated RAW 264.7 macrophages

Concentration (µg/ml)	OD at 234 nm	Percentage inhibition
LPS	0.198	0.000
25	0.155	21.717
50	0.122	38.384
100	0.102	48.485

Dual inhibition of COX and LOX is particularly significant, as simultaneous modulation of both pathways may prevent compensatory inflammatory signaling often observed with selective COX inhibitors. These findings are consistent with previous reports attributing anti-inflammatory effects of *P. longum* to alkaloids and phenolic constituents capable of regulating inflammatory pathways [17,18,19].

3.2 SUPPRESSION OF MYELOPEROXIDASE ACTIVITY

A pronounced reduction in MPO activity was observed following extract treatment. MPO activity decreased from 0.1535 U/mL in LPS-stimulated cells to 0.0181 U/mL at 100 µg/mL (Table 3). This substantial decline indicates attenuation of neutrophil-associated oxidative processes and reduced generation of reactive oxygen species.

Given the established antioxidant properties of *P. longum* phytoconstituents, the observed MPO inhibition likely reflects peroxidase-quenching and ROS-scavenging mechanisms, contributing to suppression of oxidative amplification during inflammation [20,21,22].

Table 3. Effect of *Piper longum* on MPO activity in LPS-stimulated RAW 264.7 macrophages

Concentration (µg/ml)	ΔOD	Enzyme Activity (U/ml)
LPS	0.0093	0.1535
25	0.0040	0.0660
50	0.0016	0.0264
100	0.0011	0.0181

3.3 Inhibition of iNOS activity and nitric oxide production

The extract significantly downregulated iNOS activity, achieving 50.18% inhibition at 100 µg/mL (IC_{50} = 99.26 µg/mL) (Table 4). Correspondingly, intracellular nitrite levels decreased markedly from 792.00 µg in LPS-treated cells to 492.03 µg at 100 µg/mL (Table 5).

Table 4. Effect of *Piper longum* on iNOS activity in LPS-stimulated RAW 264.7 macrophages

Concentration (µg/ml)	Δ OD	Percentage of Inhibition
LPS	0.0265	0.0000

25	0.0199	24.9057
50	0.0166	37.3585
100	0.0132	50.1887

Table 5. Effect of *Piper longum* on cellular nitrate levels in LPS-stimulated RAW 264.7 macrophages

Concentration (µg/ml)	OD	Concentration of Nitrite (µg)
LPS	0.160	792.00
25	0.157	778.14
50	0.126	625.19
100	0.099	492.03

Nitric oxide overproduction is a hallmark of macrophage-driven inflammation. Therefore, suppression of the iNOS–NO axis suggests effective modulation of reactive nitrogen species and inflammatory amplification. These findings corroborate earlier reports demonstrating that *P. longum* extracts inhibit LPS-induced NO release and iNOS expression in macrophages [18].

Thus, the methanolic stem extract of *P. longum* exhibited a multitarget antiinflammatory profile, simultaneously modulating COX, LOX, MPO, and iNOS pathways. Such broad-spectrum regulation enhances therapeutic relevance and supports its traditional medicinal application.

4. CONCLUSION

The methanolic stem extract of *Piper longum L.* demonstrated significant antiinflammatory activity in LPS-stimulated RAW 264.7 macrophages through concentration-dependent inhibition of COX,

LOX, MPO, and iNOS pathways. The marked reduction in nitrite accumulation further confirmed effective suppression of nitric oxide synthesis.

The multitarget enzymatic modulation observed in this study emphasizes the pharmacological relevance of *P. longum* as a natural anti-inflammatory agent. Future investigations involving molecular docking, gene expression profiling, and *in vivo* validation are warranted to further elucidate its mechanisms and translational potential.

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DECLARATION

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

CONFLICT OF INTERESTS

The authors report no potential conflicts of interest regarding this work.

REFERENCES

[1] Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., Zhao, L., 2017. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, 9(6), 7204-7218. https://doi.org/10.18632/oncotarget.23208

[2] Chavda, V.P., Feehan, J., Apostolopoulos, V., 2024. Inflammation: the cause of all diseases. *Cells*, 13(22), 01-07.

[3] Arango Duque, G., Descoteaux, A., 2014. Macrophage cytokines: Involvement in immunity and infectious diseases. *Frontiers in Immunology*, 5(491), 1-12. https://doi.org/10.3389/fimmu.2014.00491

[4] Choi, Y., Lee, M.K., Lim, S.Y., Sung, S.H., Kim, Y.C., 2009. Inhibition of inducible NO synthase, cyclooxygenase-2 and interleukin-1beta by torilin is mediated by mitogen-activated protein kinases in microglial BV2 cells. *British Journal of Pharmacology*, 156(6), 933-40.

[5] Wautier, J.L., Wautier, M.P., 2023. Pro- and anti-inflammatory prostaglandins and cytokines in humans: a mini review. *International Journal of Molecular Sciences*, 24(11), 01-13.

[6] Mazgaeen, L., Gurung, P., 2020. Recent advances in lipopolysaccharide recognition systems. *International Journal of Molecular Sciences*, 21(2), 1-18.

[7] Skrzypczak-Wiercioch, A., Salat, K., 2022. Lipopolysaccharide-Induced Model of Neuroinflammation: Mechanisms of Action, Research Application and Future Directions for Its Use. *Molecules*, 27(17), 01-22.

[8] Luo, R., Yao, Y., Chen, Z., Sun, X., 2025. An examination of the LPS-TLR4 immune response through the analysis of molecular structures and protein-protein interactions. *Cell Communication and Signaling*, 23(142), 1-15.

[9] Zhou, J.T., Ren, K.D., Hou, J., Chen, J., Yang, G., 2021. α-rhamnrtin 3 α-rhamnoside exerts anti-inflammatory effects on lipopolysaccharide-stimulated RAW264.7 cells by abrogating NF-κB and activating the Nrf2 signaling pathway. *Molecular Medicine Reports*, 24(799), 1-11. https://doi.org/10.3892/mmr.2021.12439

[10] Mgbeahuruike, E.E., Yrjonen, T., Vuorela, H., Holm, Y., 2017. Bioactive compounds from medicinal plants: Focus on *Piper* species. *South African Journal of Botany*, 112, 54–69.

[11] Korambayil, P.M., Varkey, P., Tharakan, M., 2023. Anti-inflammatory and cytotoxicity assay of cardamom-based nutraceuticals in comparison with 1,8-cineol. *Journal of Drug Delivery and Therapeutics*, 13(10), 88-93.

[12] Walker, M.C., Gierse, J.K., 2010. *In vitro* assays for cyclooxygenase activity and inhibitor characterization. *Methods in Molecular Biology*, 644, 131-144. https://doi.org/10.1007/978-1-59745-364-6_11

[13] Axelrod, B., Cheesbrough, T.M., Laakso, S., 1981. Lipoxygenase in soyabeans: EC 1.13.11.12 linoleate: Oxygen oxidoreductase. *Methods in Enzymology*, 71, 441-451. https://doi.org/10.1016/0076-6879(81)71055-3

[14] Chandran, S.S.R., Tharalakshmi, S., Gowri, M., 2022. *In vitro* anti-inflammatory

activity of Pachanamrutham kashayam. *International Journal of Novel Research and Development*, 7(7), 1331-1337.

[15] Salter, M., Duffy, C., Garthwaite, J., Strijbos, P.J., 1996. *Ex vivo* measurement of brain tissue nitrite and nitrate accurately reflects nitric oxide synthase activity *in vivo*.

Journal of Neurochemistry, 66(4), 1683-1690. https://doi.org/10.1046/j.1471-4159.1996.66041683.x

[16] Lepoivre, M., Chenais, B., Yapo, A., Lemaire, G., Thelander, L., Tenu, J. P., 1990. Alterations of ribonucleotide reductase activity following induction of the nitritegenerating pathway in adenocarcinoma cells. *The Journal of Biological Chemistry*, 265(24), 14143-14149.

[17] Kaushal, N., Dahiya, D.P., Kumari, C., Sharma, N., 2025. A review: phytochemical and pharmacological properties of *Piper longum*. *International Journal of Pharmaceutical Sciences*, 3(10), 3385-3393.

[18] Phan, U.T.T., Nguyen, H.D., Nguyen, T.K.O., Tran, T.H., Le, T.H., Tran, T.T.P., 2024. Anti-inflammatory effect of *Piper longum* L. fruit methanolic extract on lipopolysaccharide-treated RAW 264.7 murine macrophages. *Heliyon*, 10(4), 01-14.

[19] Rudrapal, M., Eltayeb, W.A., Rakshit, G., El-Arabey, A.A., Khan, J., Aldosari, S. M., Alshehri, B., Abdalla, M., 2023. Dual synergistic inhibition of COX and LOX by potential chemicals from Indian daily spices investigated through detailed computational studies. *Scientific Reports*, 13, 1-27. https://doi.org/10.1038/s41598023-35161-0

[20] Biswas, P., Pandey, D.K., Shekhawat, M.S., Dey, A., Malik, T., 2024. Tissuespecific variations of piperine in ten populations of *Piper longum* L.: Bioactivities and toxicological profile. *Scientific Reports*, 14(1), 1–14.

[21] Carsono, N., Tumilaar, S.G., Kurnia, D., Latipudin, D., Satari, M.H., 2022. A review

of bioactive compounds and antioxidant activity properties of *Piper* species.

Molecules, 27(19), 1–22.

[22] Kumar, S., Malhotra, S., Prasad, A.K., Van der Eycken, E.V., Bracke, M.E., StetlerStevenson, W.G., Parmar, V.S., Ghosh, B., 2015. Anti-inflammatory and antioxidant properties of *Piper* species: a perspective from screening to molecular mechanisms.

Current Topics in Medicinal Chemistry, 15(9):886-93.