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RESEARCH ARTICLE

Curcuma comosa Diarylheptanoids Attenuate LPS-induced Neuroinflammatory Responses via NF-κB Signaling in Microglial Cells: Comparison with Genistein and Estrogen

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Abstract

Microglial activation is a hallmark of neuroinflammation in several neurodegenerative disorders, including Parkinson's disease (PD), Alzheimer's disease (AD), and AIDS dementia complex. Inhibition of excessive production of pro-inflammatory mediators represents a promising strategy for mitigating neuroinflammatory responses. In menopausal women, declining estrogen levels are associated with increased neuroinflammation and a higher risk of neurodegenerative disorders. Although estrogen replacement therapy exerts anti-inflammatory effects, concerns regarding adverse events have prompted interest in alternative interventions. This study investigated the anti-inflammatory mechanisms of two diarylheptanoids isolated from *Curcuma comosa* (compounds 049 and 092) in comparison with genistein and estrogen in an LPS-induced microglial activation model. Compounds 049 and 092, as well as genistein and estrogen, significantly reduced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production, accompanied by downregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at both mRNA and protein levels. These effects were associated with reduced phosphorylation of IKKα/IKKβ, IκBα, and NF-κB p65. Collectively, the findings indicate that *C. comosa* diarylheptanoids attenuate microglial inflammatory responses through modulation of NF-κB-associated signaling pathways. Under the experimental conditions employed, compounds 049 and 092 exhibited anti-inflammatory effects comparable to those observed with genistein and estrogen, supporting further investigation of their neuroprotective potential.

Keywords: *Curcuma comosa*, Diarylheptanoid, Compound 049, Compound 092, Estrogen, Genistein, iNOS, COX-2, NF-κB, Microglial activation

Introduction

Microglia, the resident macrophages of the brain, play essential roles in maintaining homeostasis and mediating defense against injury. Upon activation by mitogens or circulating immune stimuli, microglia secrete a range of pro-inflammatory factors, including cytokines, chemokines, nitric oxide (NO), prostaglandin E_2 (PGE_2), and reactive oxygen species (ROS) [1,2]. Although microglial activation can be protective against pathogens and injury, sustained activation and excessive production of pro-inflammatory mediators may lead to cellular damage and exacerbate the initial insult.

Microglial activation has been implicated in the pathogenesis of Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), AIDS dementia complex, and cerebral ischemia [3-6]. Recent studies suggest that signaling molecules released from damaged

neurons can activate microglia, creating a self-perpetuating cycle in which neurodegeneration promotes neuroinflammation and sustained microglial activation further exacerbates neuronal injury [7-12]. Several studies have demonstrated that suppression of microglial-derived inflammatory mediators alleviates disease severity and reduces neuronal damage [13-15]. Accordingly, pharmacological agents and medicinal plants that inhibit the production of NO, PGE_2, and other pro-inflammatory mediators have shown neuroprotective effects in experimental models [16-18].

NF-κB is a key transcription factor that regulates inflammatory responses and plays a central role in microglial activation [19-22]. Following stimulation by inflammatory signals such as lipopolysaccharide (LPS), activation of the IKK complex leads to phosphorylation and degradation of IκB proteins, allowing NF-κB to translocate into the nucleus and induce the expression of pro-inflammatory mediators, including iNOS, COX-2, TNF-α, and IL-1β [23-28]. Numerous studies have demonstrated that natural anti-inflammatory compounds suppress NO and PGE_2 production, as well as iNOS and COX-2 expression, through inhibition of NF-κB signaling [29-32]. Therefore, NF-κB represents an important therapeutic target in neuroinflammatory disorders.

The investigation of anti-neuroinflammatory compounds is particularly relevant in the context of menopause. Declining estrogen levels during menopause are associated with increased neuroinflammation and a higher risk of neurodegenerative disorders, including Parkinson's disease [33]. Although estrogen replacement therapy (ERT) has demonstrated neuroprotective and anti-inflammatory effects through modulation of microglial activation and NF-κB signaling, its clinical use may be limited by adverse events such as venous thromboembolism [34]. Consequently, phytoestrogens, plant-derived compounds with structural similarity to 17β-estradiol, have attracted considerable interest as potential alternatives because they exhibit estrogen-like biological activities and may offer certain safety advantages over conventional estrogen therapy [35].

Turning to potential interventions, *Curcuma comosa*, an indigenous plant of Thailand known locally as Waan Chak Modluk, contains phytoestrogens and has a long history of use in traditional medicine [36,37]. The rhizome of *C. comosa* Roxb. (Zingiberaceae) has been widely used as an anti-inflammatory agent for treatment of postpartum uterine bleeding, peri-menopausal bleeding, and uterine inflammation. Previous studies have demonstrated the anti-inflammatory properties of *C. comosa* extracts and several diarylheptanoid constituents in microglial models [38-40]. However, direct comparisons between structurally distinct diarylheptanoids and established phytoestrogenic compounds under identical experimental conditions have not been reported. In particular, the relative anti-inflammatory efficacy of compounds 049 and 092 compared with genistein and estrogen, as well as their effects on key components of the NF-κB signaling pathway, remain insufficiently characterized.

Estrogen and genistein were selected as reference compounds because both have been reported to exert anti-inflammatory and neuroprotective effects in microglial cells. Estrogen represents the endogenous hormone whose decline during menopause is associated with increased neuroinflammation [33,34], whereas genistein is a well-characterized phytoestrogen that suppresses microglial activation and inflammatory signaling pathways, including TLR4/NF-κB and GPER/IGF-1R-mediated signaling [41-44]. Therefore, these compounds provide biologically relevant comparators for evaluating the anti-inflammatory activities of diarylheptanoids isolated from *C. comosa*.

Among the diarylheptanoids isolated from *C. comosa*, compounds 049 and 092 were selected for the present study because they represent structurally distinct diarylheptanoids with different substitution patterns and have previously exhibited distinct estrogenic and anti-inflammatory properties. Their selection enabled direct comparison of representative non-phenolic and phenolic diarylheptanoids under identical experimental conditions.

In light of these gaps, this study employed lipopolysaccharide (LPS), a widely used immunostimulant of microglia, to establish an *in vitro* model of neuroinflammation. Using this model, we investigated the anti-inflammatory effects of two purified compounds isolated from the hexane (compound 049) and methanol (compound 092) extracts of *C. comosa*, in comparison with genistein, a phytoestrogen abundant in soybeans, and estrogen. Microglial activation was assessed by measuring NO

and PGE_2 production, and the underlying molecular mechanisms were further evaluated. These findings are expected to provide insight into the anti-inflammatory mechanisms of phytoestrogens and to support future investigations in more physiologically relevant models of neuroinflammation.

Materials and methods

Chemicals and reagents

Cell culture reagents included Dulbecco's Modified Eagle's Medium (DMEM), Trypsin-EDTA, Trypan Blue (Gibco BRL, Grand Island, NY, USA), fetal bovine serum (FBS, PAA Laboratories, Morningside, Queensland, Australia), and Hank's Balanced Salts (HBSS, Sigma, St. Louis, MO, USA). Experimental and assay reagents included protease inhibitor cocktail, MTT, LPS (*Escherichia coli* serotype 026: B6), and a PGE_2 competitive EIA kit (Assay Designs, Ann Arbor, MI, USA; Sigma, St. Louis, MO, USA). Molecular biology reagents were GenElute Mammalian Total RNA Miniprep Kit, Enhanced Avian RT First Strand Synthesis Kit, JumpStartTM REDTaq® ReadyMixTM PCR Reaction Mix, PCR 100 bp Low Ladder, SYBR® GREEN I, Gel Loading Buffer (Sigma, St. Louis, MO, USA), and SYBR® Safe DNA gel stain (Invitrogen, Eugene, OR, USA). Western blotting and immunodetection reagents included mouse monoclonal anti-β-actin antibody (Sigma, St. Louis, MO, USA), rabbit polyclonal anti-iNOS antibody (Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-COX-2 antibody (Abcam, Cambridge, MA, USA), rabbit polyclonal anti-phospho-IKKα (Ser180)/IKKβ (Ser181), anti-phospho-NF-κB p65 (Ser536) antibody, mouse monoclonal anti-phospho-IκBα (Ser32/36) (5A5) antibody, biotinylated protein ladder (Cell Signaling Technology, Danvers, MA, USA), HRP-conjugated goat anti-rabbit Ig (H+L) (Zymed Laboratories, Carlsbad, CA, USA), and HRP-conjugated sheep anti-mouse IgG antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Visualization reagents included nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and ECL detection agent (Pierce, Rockford, IL, USA). Small molecules and controls consisted of genistein and estrogen (Sigma, St. Louis, MO, USA), 1400W (dihydrochloride); N-((3-(aminomethyl)phenyl)methyl)-ethanimidamide dihydrochloride (Cayman Chemical, Ann Arbor, MI, USA), and celecoxib; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonamide (LC Laboratories, Woburn, MA, USA).

Cell culture

Highly aggressive proliferation-immortalized (HAPI) microglial cells were obtained from Dr. James R. Connor (Penn State Hershey Medical Center, Hershey, PA, USA), the laboratory that originally established this cell line. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Before each experiment, cells were seeded and incubated for 24 h to achieve approximately 80% confluence.

Plant materials

Rhizomes of *C. comosa* Roxb. were collected and identified as previously described by Suksamrarn *et al*. [45], and a voucher specimen was deposited at the Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand (SCMU No. 300). Compounds 049 [(3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol] and 092 [(3*S*)-7-(3,4-dihydroxyphenyl)-1-phenyl-(1*E*)-1-hepten-3-ol] (**Figure 1**) were isolated from the rhizomes of *C. comosa* as previously described by Suksamrarn *et al*. [45]. Structural identification was established using spectroscopic analyses, including IR, HR-MS, ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC techniques, while absolute stereochemistry was confirmed using the modified Mosher's method [45]. Both compounds were dissolved in DMSO to prepare 10 mM stock solutions and further diluted for cell treatment. The final DMSO concentration in all experiments was maintained at 0.1%. The concentrations used in this study were selected based on previous studies demonstrating anti-inflammatory activity within the tested concentration range without detectable cytotoxicity in HAPI microglial cells [40,41,46,47].

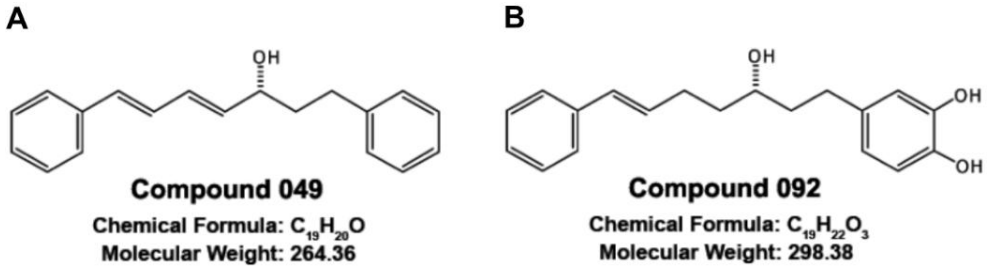


Figure 1 Chemical structures of the diarylheptanoids examined in this study: Compound 049 (A), (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol; and compound 092 (B), (3*S*)-7-(3,4-dihydroxyphenyl)-1-phenyl-(1*E*)-1-hepten-3-ol.

Assessment of cell viability

Cell viability was assessed using a quantitative colorimetric MTT assay, which measures cellular metabolic activity. HAPI cells were seeded at 2×10^4 cells per well in 96-well plates. After seeding, cells were incubated with compound 049, compound 092, genistein, or estrogen at the indicated concentrations for 1 h. Cells were then exposed to LPS (0.1 μg/mL) for 24 h. Following treatment, the medium was removed, and 50 μL of 1 mg/mL MTT in HBSS was added to each well. The cultures were incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2. The supernatant was then removed, and the purple formazan crystals were solubilized in 100 μL DMSO. Absorbance was measured at 570 and 620 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Assessment of NO production

Nitrite (NO_2^-), a stable and nonvolatile breakdown product of nitric oxide (NO), was measured using the Griess reagent method. HAPI cells were seeded at 5×10^5 cells per well in 6-well plates and treated with compound 049, compound 092, genistein, estrogen, or 1400W at the indicated concentrations for 1 h. Cells were then stimulated with LPS (0.1 μg/mL) for 24 h. Following treatment, 100 μL of each culture supernatant was transferred to a 96-well plate, mixed 1:1 with Griess reagent, and incubated at room temperature for 5 - 10 min. Absorbance was measured at 545 and 665 nm within 30 min using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Sodium nitrite standards (0 - 100 μM) were prepared in culture media.

Assessment of PGE_2 production

PGE_2 production was measured using a commercial competitive enzyme immunoassay kit (Assay Designs). HAPI cells were seeded in 24-well plates at 1×10^5 cells per well in DMEM with 5% FBS. Cells were pretreated with compound 049, compound 092, genistein, estrogen, or celecoxib at the indicated concentrations for 1 h prior to stimulation with LPS (0.1 μg/mL) for 24 h. Following treatment, the culture medium was collected and centrifuged at 10,000× g for 10 min. A 100 μL aliquot of each supernatant and standard was added to goat anti-mouse IgG-coated microwells. Mouse anti-PGE_2 antibody and alkaline phosphatase-conjugated PGE_2 were then added, and the plates were incubated at room temperature with shaking (500 rpm) for 2 h. Wells were washed, and p-nitrophenyl phosphate (pNPP) substrate was added. After a 45-min incubation, the reaction was terminated by the addition of trisodium phosphate (Na_3PO_4). Absorbance was measured at 405 and 590 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Standards ranged from 39 to 2500 pg/mL.

Semi-quantitative PCR analysis of iNOS and COX-2

HAPI cells were seeded in 21 cm^2 dishes at a density of 1×10^6 cells per dish and incubated for 24 h. Following incubation, cells were pretreated for 1 h with compound 049, compound 092, genistein, or estrogen at the indicated concentrations, and subsequently stimulated with LPS (0.1 μg/mL). Total RNA was isolated 6 h post-stimulation using the GenElute Mammalian Total RNA Miniprep Kit. One microgram of total RNA was reverse-transcribed into cDNA using the Enhanced Avian RT First Strand Synthesis Kit, yielding a final volume of 20 μL. For each semi-quantitative PCR reaction, 2 μL of cDNA was used with specific primers for iNOS, COX-2, and GAPDH, as described below.

The sequences of the primers were as follows: iNOS forward, 5′-GCAGAATGTGACCATCATGG-3′; iNOS reverse, 5′-ACAACCTTGGTGTTGAAGGC-3′; COX-2 forward, 5′-TGCGATGCTCTTCCGAGCTGTGCT-3′; COX-2 reverse, 5′-TCAGGAAGTTCCTTATTTCCTTTC-3′; GAPDH forward, 5′-TCCCTCAAGATTGTCAGCAA-3′; and GAPDH reverse, 5′-AGATCCACAACGGATACATT-3′.

PCR amplification of iNOS, COX-2, and GAPDH was initiated with an initial denaturation at 95 °C for 15 min, followed by 25 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 10 min. PCR products were separated on a 2% agarose gel and visualized using SYBR Green I. GAPDH served as an internal control for sample loading and mRNA integrity. Band intensities were quantified using Scion Image software (Scion, Frederick, MD, USA), and densitometric analyses were performed using data from three independent biological experiments. Relative mRNA expression levels were normalized to the corresponding GAPDH loading controls.

Western blot analysis of iNOS, COX-2, phospho-NF-κB p65, phospho-IκBα, and phospho-IKKα/IKKβ expression

HAPI cells were seeded at 5×10^5 cells per well in 6-well plates and incubated for 24 h prior to experimentation. Cells were pretreated with compound 049, compound 092, genistein, or estrogen at the indicated concentrations for 1 h, followed by stimulation with LPS (0.1 μg/mL). Cells were harvested at 6 h post-stimulation for analysis of iNOS, COX-2, and phospho-NF-κB p65; at 1 h for phospho-IκBα expression; and at 30 min for phospho-IKKα/IKKβ. Cells were washed three times with PBS and lysed in RIPA buffer. The lysates were collected and centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentrations were determined using the Bradford assay. Equal amounts of protein (20 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in TBST and incubated with primary antibodies at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibodies (1:10000) for 1 h at room temperature, according to the manufacturer's instructions. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) reagents and autoradiography film. Primary antibodies against iNOS, COX-2, phospho-NF-κB p65, phospho-IκBα, and phospho-IKKα/IKKβ were used at a dilution of 1:1000. Protein expression levels were normalized to β-actin (1:5000). Densitometric analyses were performed using data from three independent biological experiments, and band intensities were quantified and normalized to the corresponding β-actin loading controls.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Quantitative data are presented as mean ± SEM from three independent biological experiments unless otherwise specified. Measurements within each independent experiment were performed in technical triplicates and averaged prior to statistical analysis. Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A $p < 0.05$ was considered statistically significant.

Results and discussion

Effects of compound 049, compound 092, genistein, and estrogen on cell viability

The cytotoxic effects of compound 049, compound 092, genistein, and estrogen on microglial cells were assessed using HAPI cells seeded at 2×10^4 cells per well in 96-well plates. Cells were pretreated with the respective compounds for 1 h prior to exposure to LPS (0.1 μg/mL) for 24 h. Cell viability was quantified using the MTT assay. Importantly, none of the compounds reduced cell viability at any tested concentration, indicating a lack of cytotoxicity under these conditions. Furthermore, treatment of microglia with varying concentrations of compounds 049, 092, genistein, or estrogen, with or without LPS for 24 h, did not alter cell number relative to the LPS-treated control (**Figure 2**).

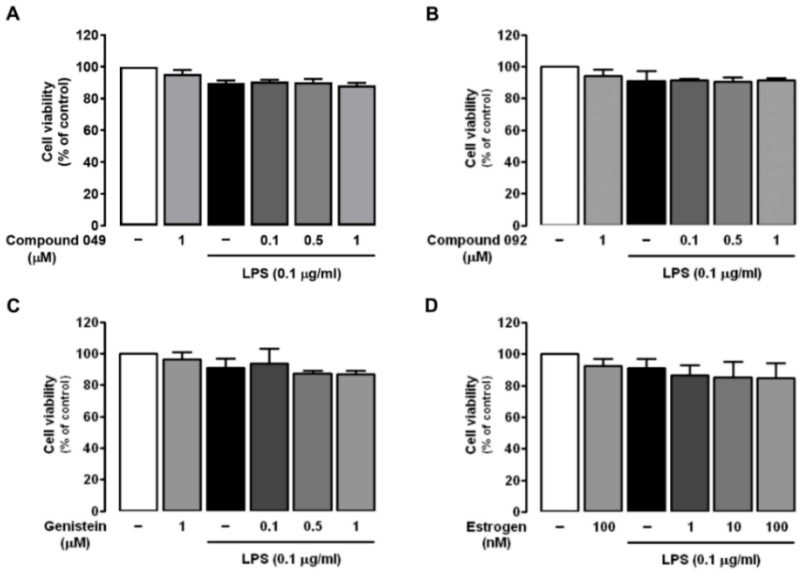


Figure 2 Effects of compound 049 (A), compound 092 (B), genistein (C), and estrogen (D) on HAPI microglial cell viability. Cells were treated with the indicated concentrations of each compound in the presence or absence of LPS. Cell viability was assessed using the MTT assay. Data are expressed as a percentage of the untreated control (100%) or the LPS-treated group. Results are presented as mean ± SEM from three independent biological experiments (n = 3).

Effects of compound 049, compound 092, genistein, estrogen, and 1400W dihydrochloride (1400W) on LPS-induced NO production

To investigate the effects of compound 049, compound 092, genistein, and estrogen on nitric oxide (NO) production in LPS-activated microglial cells, HAPI cells (5×10^5 cells per well) were cultured in 6-well plates and treated with the indicated concentrations of each compound for 1 h prior to stimulation with LPS (0.1 μg/mL) for 24 h. Culture media were subsequently collected and subjected to the Griess assay.

As shown in **Figure 3**, all tested compounds reduced LPS-induced NO production. Compound 049 exhibited a noticeable inhibitory effect at 0.5 μM, reducing NO levels to 61.10 ± 5.87% relative to the LPS-treated control. Greater inhibition was observed at 1 μM, where NO levels were further reduced to 35.34 ± 6.88% of the LPS-treated control (**Figure 3(A)**). Similarly, compound 092 inhibited NO production, with the effect becoming more pronounced at higher concentrations. NO levels were reduced to 60.32 ± 7.81% and 39.24 ± 5.90% of the LPS-treated control at 0.5 and 1 μM, respectively (**Figure 3(B)**). Genistein also attenuated LPS-induced NO production in a concentration-dependent manner, with a significant effect observed at concentrations as low as 0.01 μM (84.06 ± 1.80%), and further reduced NO levels to 41.72 ± 2.72% at 10 μM (**Figure 3(C)**). Estrogen exhibited a similar concentration-dependent inhibitory effect, with significant suppression observed at 0.1 nM (76.97 ± 3.29%) and a further reduction to 19.13 ± 3.76% at 1000 nM (**Figure 3(D)**). Basal NO production remained minimal in unstimulated cells and in all treatment groups without LPS.

A comparative analysis of anti-inflammatory activity, based on percentage reduction in NO production, demonstrated that all compounds significantly inhibited NO production in LPS-stimulated HAPI cells. Estrogen (1 nM) reduced NO production to 79.60 ± 2.50% relative to the LPS-treated control. At 0.5 μM, compound 049 markedly decreased NO production to 49.31 ± 6.24%, while compound 092 and genistein reduced NO levels to 50.32 ± 4.64% and 70.98 ± 4.68%, respectively. Notably, compounds 049 and 092 exhibited stronger inhibitory effects than genistein at the tested concentrations. Importantly, 1400W (0.5 μM) reduced NO production to 49.27 ± 4.96%, a level comparable to those observed with compounds 049 and 092, supporting the involvement of iNOS in mediating these inhibitory effects. In contrast, NO production was negligible in non-stimulated HAPI cells (**Figure 4**).

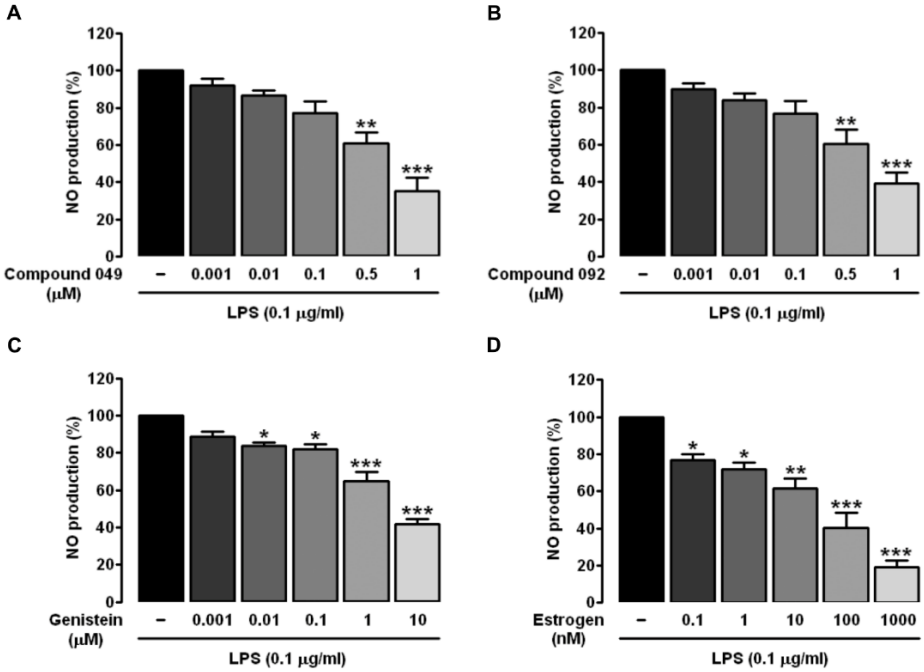


Figure 3 Effects of estrogen, genistein, compound 049, and compound 092 on LPS-induced NO production in HAPI microglial cells. Cells were pretreated with the indicated concentrations of each compound before LPS stimulation. NO production was measured using the Griess assay. Results are expressed as mean ± SEM from three independent biological experiments (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the LPS-treated group.

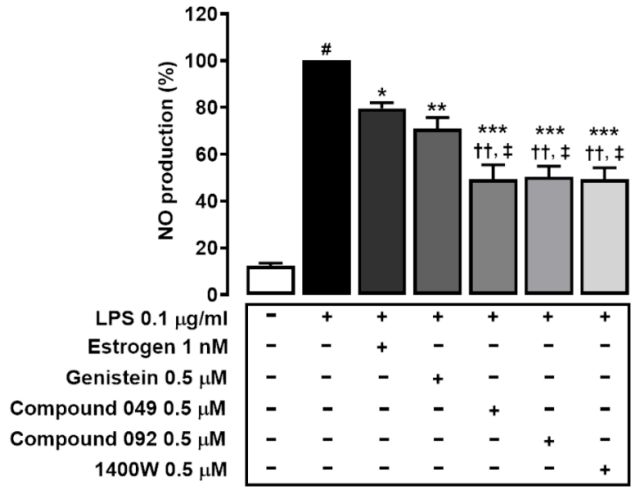


Figure 4 Effects of compound 049 (0.5 μM), compound 092 (0.5 μM), genistein (0.5 μM), estrogen (1 nM), and 1400W dihydrochloride (1400W, 0.5 μM) on LPS-induced NO production in HAPI microglial cells. Cells were pretreated with the indicated compounds prior to LPS stimulation. NO production was measured using the Griess assay. Results are expressed as mean ± SEM from three independent biological experiments (n = 3). #$p < 0.001$ vs. control; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the LPS-treated group; ††$p < 0.01$ vs. estrogen; ‡ $p < 0.05$ vs. genistein.

Effects of compound 049, compound 092, genistein, estrogen, and celecoxib on LPS-induced PGE_2 production

To investigate the effects of compound 049, compound 092, genistein, and estrogen on prostaglandin E_2 (PGE_2) production in LPS-activated microglial cells, HAPI cells (5×10^5 cells/well) were cultured in 6-well plates and treated with the indicated concentrations of each compound for 1 h prior to stimulation with LPS (0.1 μg/mL) for 24 h. Culture media were subsequently collected and analyzed using an ELISA.

As shown in **Figure 5**, all tested compounds reduced LPS-induced PGE_2 production. For compound 049, the inhibitory effect became more pronounced with increasing concentration, reducing PGE_2 levels to 66.33 ± 8.55% and 44.52 ± 6.59% of the LPS-treated control at 0.5 and 1 μM, respectively (**Figure 5(A)**). Similarly, compound 092 exhibited an inhibitory effect on PGE_2 production at 0.5 μM, reducing PGE_2 levels to 59.68 ± 9.59% of the LPS-treated control. The effect became more pronounced at 1 μM, where PGE_2 levels were further reduced to 41.61 ± 6.94% (**Figure 5(B)**). Genistein also attenuated LPS-induced PGE_2 production in a concentration-dependent manner, with a significant effect observed at concentrations as low as 0.01 μM (76.66 ± 4.11% of the LPS-treated control), and further reduced PGE_2 levels to 40.07 ± 6.03% at 10 μM (**Figure 5(C)**). Estrogen exhibited a similar concentration-dependent inhibitory effect, with significant suppression observed at concentrations as low as 0.1 nM (76.13 ± 3.55%), and a further reduction to 24.49 ± 5.33% at 1000 nM (**Figure 5(D)**). Basal PGE_2 production was negligible in unstimulated HAPI cells.

A comparative analysis of anti-inflammatory activity, based on percentage reduction in PGE_2 production, demonstrated that all compounds significantly inhibited PGE_2 production in LPS-stimulated HAPI cells. Estrogen (1 nM) reduced PGE_2 production to 73.59 ± 5.20% relative to the LPS-treated control. At 0.5 μM, compounds 049 and 092 markedly decreased PGE_2 levels to 44.14 ± 8.42% and 43.07 ± 4.43%, respectively, whereas genistein reduced PGE_2 production to 68.49 ± 4.67%. Notably, compounds 049 and 092 exhibited stronger inhibitory effects than genistein at the tested concentrations. Importantly, celecoxib (0.5 μM) reduced PGE_2 production to 44.07 ± 3.22%, a level comparable to those observed with compounds 049 and 092, supporting the involvement of COX-2 in mediating these inhibitory effects. In contrast, PGE_2 production was negligible in non-stimulated HAPI cells (**Figure 6**).

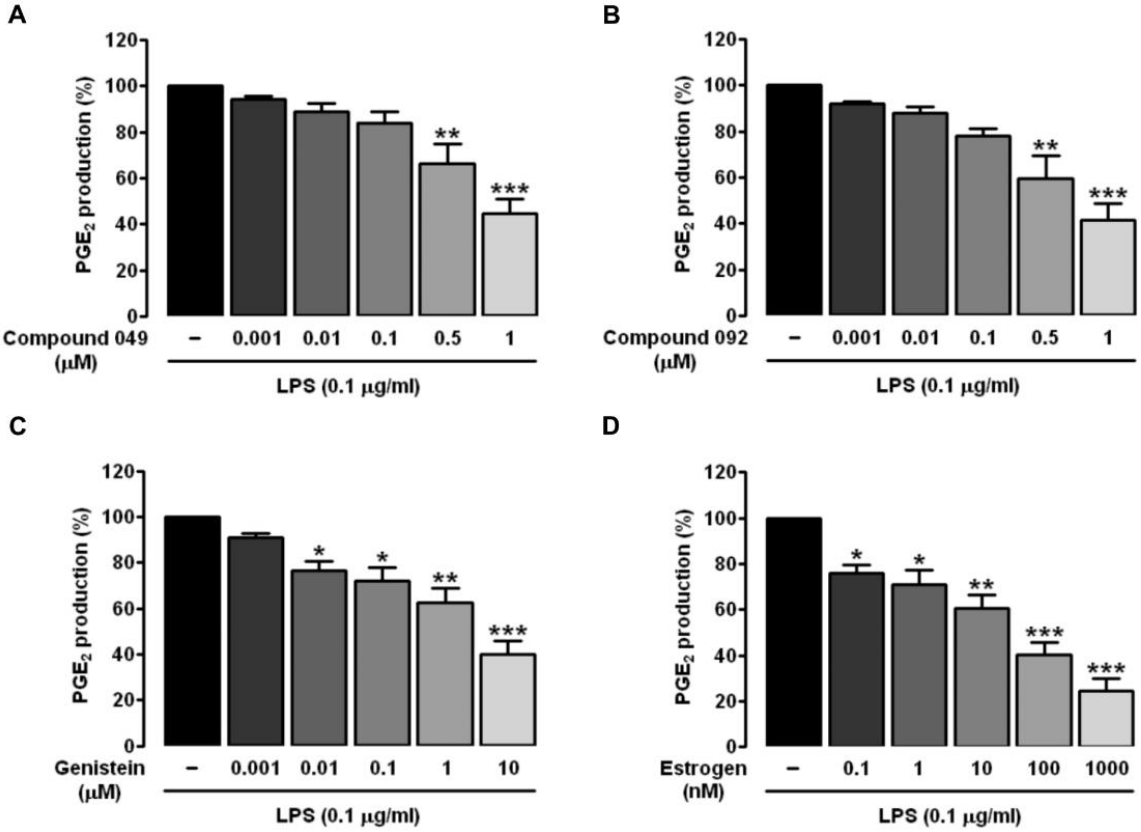


Figure 5 Effects of estrogen, genistein, compound 049, and compound 092 on LPS-induced PGE_2 production in HAPI microglial cells. Cells were pretreated with the indicated concentrations of each compound prior to LPS stimulation. PGE_2 production was measured by ELISA. Results are expressed as mean ± SEM from three independent biological experiments (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the LPS-treated group.

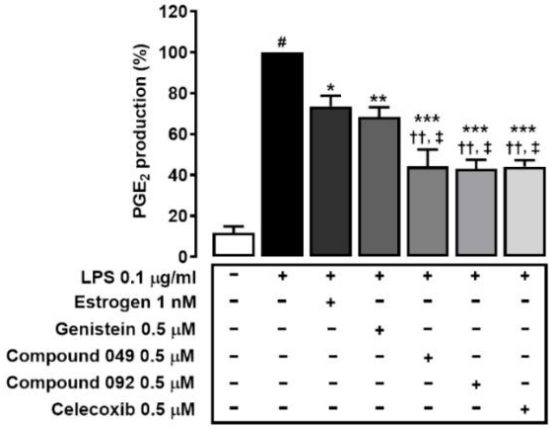


Figure 6 Effects of compound 049 (0.5 μM), compound 092 (0.5 μM), genistein (0.5 μM), estrogen (1 nM), and celecoxib (0.5 μM) on LPS-induced PGE_2 production in HAPI microglial cells. Cells were pretreated with the indicated compounds prior to LPS stimulation. PGE_2 production was measured by ELISA. Results are expressed as mean ± SEM from three independent biological experiments (n = 3). #$p < 0.001$ vs. control; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the LPS-treated group; ††$p < 0.01$ vs. estrogen; ‡ $p < 0.05$ vs. genistein.

Effects of compound 049, compound 092, genistein, and estrogen on iNOS protein and mRNA expression

This study examined whether the inhibitory effects of compound 049, compound 092, genistein, and estrogen on NO production are associated with reduced iNOS expression. HAPI cells (5×10^5 cells per well) were seeded in 6-well plates and pretreated with compound 049 (0.5 μM), compound 092 (0.5 μM), genistein (0.5 μM), or estrogen (1 nM) for 1 h prior to stimulation with LPS (0.1 μg/mL) for 6 h. iNOS protein and mRNA expression were quantified using Western blot analysis and RT-PCR, respectively. iNOS protein expression was barely detectable in untreated cells but was markedly increased following LPS stimulation (**Figure 7(A)**). This LPS-induced increase in iNOS protein expression was strongly attenuated by compounds 049 and 092, as well as by genistein and estrogen at the tested concentrations. Similarly, a significant reduction in iNOS mRNA expression was observed following compound pretreatment (**Figure 7(B)**). In contrast, the expression levels of β-actin protein and GAPDH mRNA remained unchanged regardless of LPS or compound treatment.

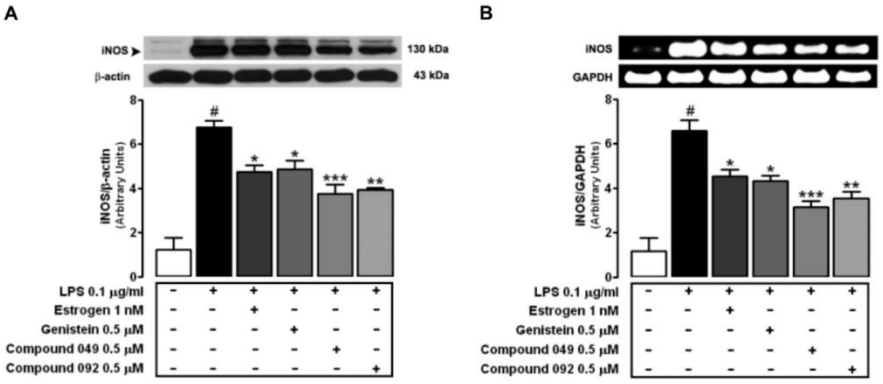


Figure 7 Effects of compound 049, compound 092, genistein, and estrogen on iNOS protein (A) and mRNA (B) expression in LPS-stimulated HAPI microglial cells. iNOS protein and mRNA expression were evaluated by Western blot analysis and RT-PCR, respectively. Representative Western blots and RT-PCR gels from three independent biological experiments are shown. Band intensities were quantified by densitometric analysis and normalized to the corresponding β-actin (protein) or GAPDH (mRNA) loading controls. Quantitative data are expressed as mean ± SEM from three independent biological experiments (n = 3). #$p < 0.001$ vs. control; *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ vs. the LPS-treated group.

Effects of compound 049, compound 092, genistein, and estrogen on COX-2 protein and mRNA expression

The relationship between the ability of compound 049, compound 092, genistein, and estrogen to inhibit PGE_2 production and their effects on COX-2 protein and mRNA expression was examined. HAPI cells (5×10^5 cells per well) were seeded in 6-well plates and pretreated with compound 049 (0.5 μM), compound 092 (0.5 μM), genistein (0.5 μM), or estrogen (1 nM) for 1 h prior to stimulation with LPS (0.1 μg/mL) for 6 h. COX-2 protein and mRNA levels were quantified using Western blot analysis and RT-PCR, respectively. As shown in **Figure 8(A)**, COX-2 protein expression was minimal in unstimulated cells but increased markedly following LPS stimulation. This increase was strongly attenuated by compounds 049 and 092, as well as by genistein and estrogen at the tested concentrations. Consistent with these findings, COX-2 mRNA expression was also significantly reduced following compound pretreatment (**Figure 8(B)**). In contrast, β-actin protein and GAPDH mRNA remained unchanged regardless of LPS or compound treatment.

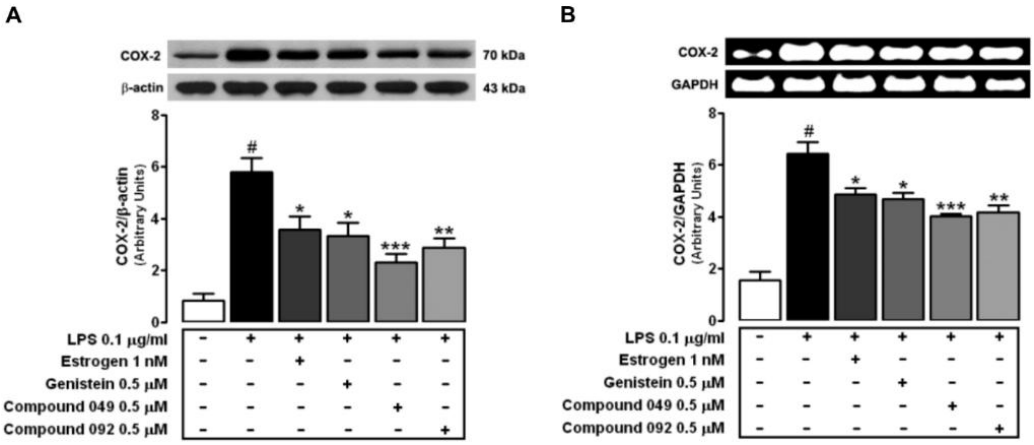


Figure 8 Effects of compound 049, compound 092, genistein, and estrogen on COX-2 protein (A) and mRNA (B) expression in LPS-stimulated HAPI microglial cells. COX-2 protein and mRNA expression were determined by Western blot analysis and RT-PCR, respectively. Representative Western blots and RT-PCR gels from three independent biological experiments are shown. Band intensities were quantified by densitometric analysis and normalized to the corresponding β-actin (protein) or GAPDH (mRNA) loading controls. Quantitative data are expressed as mean ± SEM from three independent biological experiments (n = 3). #$p < 0.001$ vs. control; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. the LPS-treated group.

Effects of compound 049, compound 092, genistein, and estrogen on phosphorylation of NF-κB p65, IκBα, and IKKα/IKKβ

The effects of compound 049, compound 092, genistein, and estrogen on the phosphorylation of NF-κB p65, IκBα, and IKKα/IKKβ in LPS-stimulated microglial cells were investigated. HAPI cells (5×10^5 cells/well) were seeded in 6-well plates and pretreated with compound 049 (0.5 μM), compound 092 (0.5 μM), genistein (0.5 μM), or estrogen (1 nM) for 1 h prior to stimulation with LPS (0.1 μg/mL). Cells were harvested at 6 h for analysis of phospho-NF-κB p65, at 1 h for phospho-IκBα, and at 30 min for phospho-IKKα/IKKβ. The phosphorylation levels of NF-κB p65, IκBα, and IKKα/IKKβ were determined by Western blot analysis. LPS stimulation markedly increased the phosphorylation of NF-κB p65 (**Figure 9(A)**), IκBα (**Figure 9(B)**), and IKKα/IKKβ (**Figure 9(C)**), whereas pretreatment with compounds 049 and 092, as well as genistein and estrogen, significantly attenuated LPS-induced phosphorylation of these proteins. In non-stimulated cells, phosphorylated NF-κB p65, IκBα, and IKKα/IKKβ were barely detectable. The expression level of β-actin remained unchanged following LPS or compound treatment.

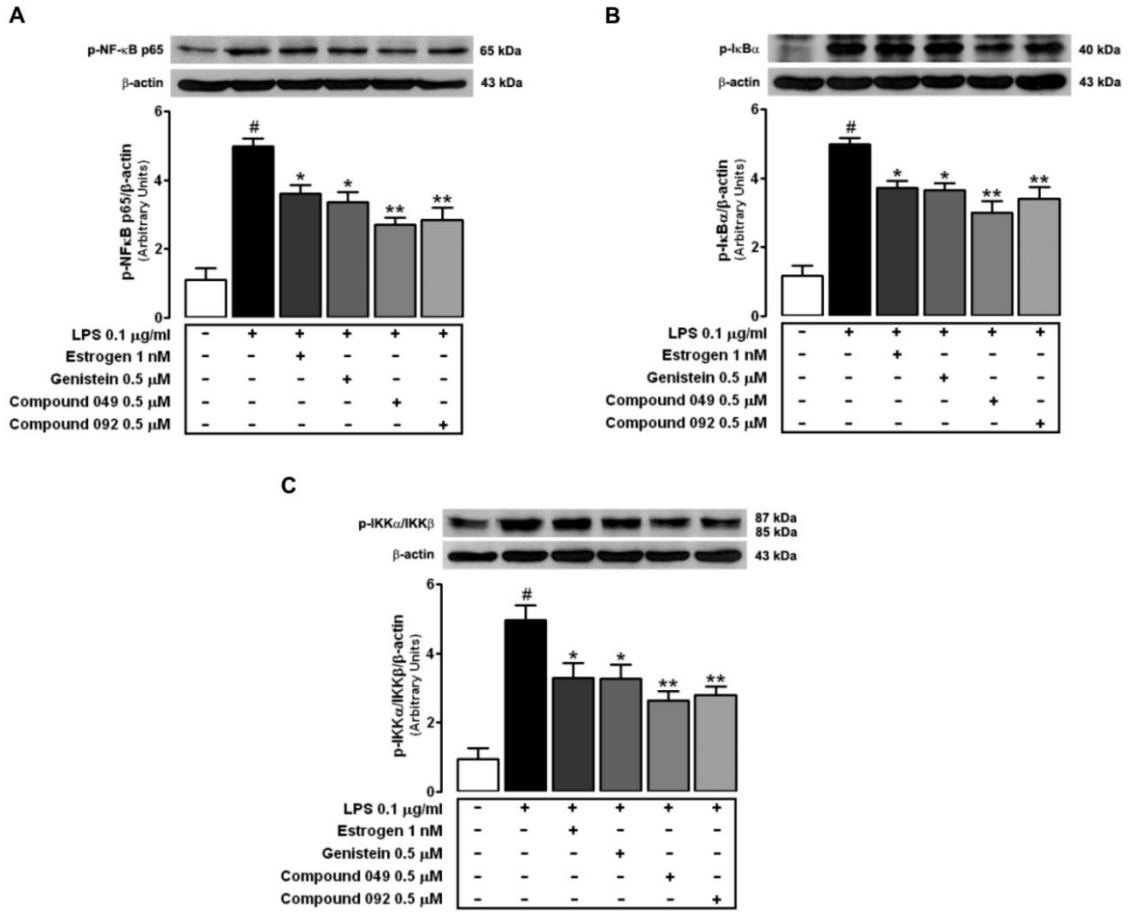


Figure 9 Effects of compound 049, compound 092, genistein, and estrogen on the phosphorylation of NF-κB p65 (A), IκBα (B), and IKKα/IKKβ (C) in LPS-stimulated HAPI microglial cells. Protein expression levels were determined by Western blot analysis. Representative blots from three independent biological experiments are shown. Band intensities were quantified by densitometric analysis and normalized to the corresponding β-actin loading controls. Quantitative data are expressed as mean ± SEM from three independent biological experiments (n = 3). #$p < 0.001$ vs. control; *$p < 0.05$ and **$p < 0.01$ vs. the LPS-treated group.

The anti-inflammatory activities of two *C. comosa* diarylheptanoids (compounds 049 and 092) were systematically compared with genistein and estrogen using LPS-stimulated HAPI microglial cells as a model of neuroinflammation. All four compounds significantly suppressed NO and PGE_2 production, accompanied by reduced iNOS and COX-2 expression. These effects were likely associated with inhibition of the NF-κB pathway, suggesting coordinated suppression of inflammatory mediator production via NF-κB-dependent signaling. Importantly, none of the tested concentrations induced cytotoxicity, indicating that the observed effects were pharmacological rather than secondary to reduced cell viability.

Genistein, a well-characterized phytoestrogen, has been consistently shown to exert neuroprotective and anti-inflammatory effects, primarily through estrogen receptor (ER)-dependent mechanisms in microglial cells [42]. These effects have been linked to modulation of TLR4/NF-κB and ER/IGF-1 signaling pathways [43,44,48]. Similarly, diarylheptanoids from *C. comosa* have been reported to modulate ERα-ERE-dependent pathways and exhibit selective estrogen receptor modulator (SERM)-like activity, with concentration-dependent agonistic or antagonistic effects [49,50]. In the present study, we compared the effects of purified compounds 049 and 092 with those of genistein and estrogen.

Although the anti-inflammatory profiles of compounds 049 and 092 were broadly comparable to those of genistein and estrogen, the present study was not designed to directly evaluate estrogen receptor

involvement. Nevertheless, previous studies have demonstrated that diarylheptanoids isolated from *C. comosa* possess phytoestrogenic properties and can activate ERα-dependent signaling pathways. In particular, compound 049 has been reported to exhibit potent estrogenic activity, whereas several related diarylheptanoids display selective estrogen receptor modulator (SERM)-like characteristics. Because estrogen and genistein are known to attenuate microglial inflammatory responses through ER-associated signaling mechanisms, the parallel inhibitory profiles observed in the present study raise the possibility that ER-related pathways may contribute to the actions of compounds 049 and 092. However, direct evidence of receptor involvement was not obtained, and future studies employing ER antagonists, receptor knockdown approaches, or receptor-specific reporter assays will be required to confirm this hypothesis.

All tested compounds effectively suppressed NO production. Notably, compounds 049 and 092 exhibited stronger inhibitory effects than genistein, while demonstrating comparable efficacy across other measured parameters. The efficacy observed in the estrogen-treated group should be interpreted with caution, as the concentration of estrogen was substantially lower than that of genistein and the diarylheptanoids (nM vs. μM range). Consistent with this, previous studies have shown that higher concentrations of estrogen exert greater inhibitory effects on NO production [41].

The present findings are consistent with previous reports demonstrating that crude hexane extracts of *C. comosa* reduce NO production and suppress iNOS mRNA and protein expression in LPS-stimulated HAPI microglial cells [38]. Analysis of seven naturally occurring diarylheptanoids from *C. comosa* extracts revealed that (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (compound 049) exhibits the highest estrogenic activity [51]. Structure-activity relationship analyses suggest that the activity of compound 049 may be related to its dienol structure and the absence of aromatic hydroxyl groups. However, the contribution of estrogen receptor signaling to the anti-inflammatory effects observed in the present study was not directly investigated and therefore remains to be established.

In parallel with our study, previous investigations have reported that compound 049 (0.1, 0.5, and 1 μM) significantly inhibits NO and PGE_2 production, as well as iNOS and COX-2 mRNA and protein expression in LPS-activated HAPI microglial cells in a concentration-dependent manner [40]. However, that study did not include comparisons with compound 092, genistein, or estrogen. The present comparative approach therefore provides new insight into the relative potency of these compounds within a unified experimental framework. Consistent with these findings, previous studies have demonstrated that genistein exerts dose-dependent inhibitory effects on NO production and iNOS expression in LPS-stimulated microglia via the IRF-1/pSTAT1 signaling pathway [41].

LPS stimulation robustly induced phosphorylation of IKKα/IKKβ at Ser180/181, IκBα at Ser32/36, and NF-κB p65 at Ser536, which are key events required for NF-κB activation and downstream inflammatory gene expression [52]. Pretreatment with compounds 049 and 092 markedly attenuated these phosphorylation events along the NF-κB pathway in a manner comparable to estrogen and genistein, suggesting that suppression of NF-κB activation is a key mechanism underlying their anti-inflammatory effects. Furthermore, previous studies have shown that compound 092 suppresses intracellular ROS accumulation and attenuates p38 MAPK phosphorylation in LPS-stimulated microglia [47]. It also exerts antioxidant effects through activation of the Nrf2 pathway in hydrogen peroxide-induced neurotoxicity models [46]. Although oxidative stress-related endpoints were not examined in the present study, the reported effects of compound 092 on ROS accumulation and Nrf2 activation suggest that antioxidant mechanisms may also contribute to the observed anti-inflammatory responses. Furthermore, estrogen has been reported to exert antioxidant and neuroprotective effects through modulation of oxidative stress pathways [53]. Collectively, the available evidence suggests that multiple signaling pathways may be involved in the regulation of microglial activation by diarylheptanoids. Future studies incorporating ROS measurements and Nrf2 pathway analyses will be valuable for further elucidating the contribution of oxidative stress-related mechanisms to the anti-inflammatory effects of *C. comosa* diarylheptanoids. In conclusion, given the postmenopausal increase in neuroinflammatory tone [33], compounds 049 and 092 may represent promising candidates for the

development of neuroprotective interventions. However, their safety profile relative to conventional estrogen therapy remains to be determined and warrants further investigation.

Importantly, several limitations should be acknowledged. First, this study was conducted using an immortalized microglial cell line, which may not fully recapitulate the complexity and heterogeneity of primary microglia or *in vivo* neuroinflammatory environments. Second, although compounds 049 and 092 have previously been reported to possess estrogenic activity, the involvement of estrogen receptor signaling in their anti-inflammatory effects was not directly evaluated in the present study. Therefore, the contribution of estrogen receptor-mediated pathways remains speculative and will require confirmation using receptor antagonism, gene-silencing, or other receptor-specific approaches. Third, differences in potency between estrogen and phytoestrogens limit direct comparisons of their concentrations. Future studies incorporating primary microglial cultures, *in vivo* models, receptor-specific mechanistic validation, and oxidative stress-related analyses will be essential to further delineate the underlying signaling pathways and confirm translational relevance.

Conclusions

This study presents the first direct comparison of estrogen, genistein, and purified diarylheptanoids from *C. comosa* (compounds 049 and 092) in a neuroinflammatory model. The results indicate that these compounds exert comparable anti-inflammatory effects by regulating iNOS and COX-2 expression as well as NO and PGE_2 production, potentially through modulation of NF-κB signaling. Further studies using primary microglial cultures and *in vivo* models are required to determine their potential relevance to neuroinflammatory and neurodegenerative diseases.

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Declaration of generative AI in scientific writing

No artificial intelligence was employed in content generation or data interpretation. The author assumes sole responsibility for the content and conclusions presented.

CRediT author statement

Busarat Karachot: Investigation; Data Curation; Writing-Original Draft. **Anusorn Thampithak**: Formal analysis; Writing-Original Draft; Writing - Review & Editing; Visualization. **Natttinee Jantaratnotai**: Methodology; Writing-Original Draft; Writing - Review & Editing. **Apichart Suksamrarn**: Resources. **Pimtip Sanvarinda**: Conceptualization; Writing-Original Draft; Writing - Review & Editing; Project Administration; Supervision.

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