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LPS로 자극된 RAW 264.7 대식세포에서의 고광나무 메탄올 추출물의 항염증 활성

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Anti-inflammatory Activity of *Philadelphus schrenkii* Methanol Extract in Lipopolysaccharide-stimulated RAW 264.7 Cells

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ABSTRACT

Background: *Philadelphus schrenkii* Rupr. is a widespread in East Asia and has historically been used as a medicinal herb in Korea to reduce heat, swelling, and pain. This study investigated the anti-inflammatory effects of *P. schrenkii* methanol extract (PSE) on lipopolysaccharide-stimulated RAW 264.7 macrophage cells.

Methods and Results: PSE inhibited the expression of nitric oxide, prostaglandin E_2, inducible nitric oxide synthase, and cyclooxygenase-2 at concentrations that did not affect cell viability as determined by the tetrazolium salt (WST-1) assay. The quantitative reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay measured that pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-1β, IL-6, and monocyte chemoattracted protein-1, were decreased in PSE-treated groups. Western blotting confirmed that PSE inhibited the phosphorylation of nuclear factor kappa B and mitogen-activated protein kinases. Gas chromatography-mass spectrometry analysis revealed the presence of active anti-inflammatory compounds in PSE.

Conclusions: This study demonstrated that PSE has potent anti-inflammatory activity *in vitro* and provides a reasonable basis for the traditional use of *P. schrenkii* in treating inflammation-related diseases.

Key Words: *Philadelphus schrenkii* Rupr., Anti-inflammatory, Mitogen Activated Protein Kinase, Nuclear Factor Kappa B, RAW 264.7 Cells

INTRODUCTION

Inflammation is a necessary defense mechanism for living organisms to protect themselves against tissue injuries, infections, and harmful external stimuli. Inflammation can be triggered by damage-associated molecular patterns, foreign nucleic acids, or pathogen-associated molecular patterns such as lipopolysaccharide (LPS) (Newton and Dixit, 2012). During the inflammatory response, macrophages receive stimuli such as LPS into the cells and produce nitric oxide (NO), prostaglandin E_2 (PGE_2), and pro-inflammatory cytokines (Yang *et al.*, 2012; Kim *et al.*, 2019). And the production of these inflammatory factors requires the activation of the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways (Liu *et al.*, 2018; Cao *et al.*, 2021).

Although inflammation is generally a beneficial mechanism, chronic inflammation can lead to the development of various diseases, including rheumatoid arthritis, metabolic syndromes, and cancer (Chippada *et al.*, 2011; Kim *et al.*, 2014; Dong *et al.*, 2017). Aspirin, metformin, and diclofenac, which are inhibitors of inflammation-related enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2),

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are anti-inflammatory agents that can help prevent the development of various degenerative diseases (Munn, 2017; Chen *et al.*, 2018; Nore *et al.*, 2020; Chakrabarti and Mukherjee, 2021). Therefore, reducing inflammation is essential to preventing a number of degenerative diseases.

Approximately 49% of newly approved medicines worldwide in the last 39 years (1981 - 2019) were either natural products or derived from natural sources. Notably, 25% of pharmaceutical products are derived from plants. Natural products are an important source of new drug development because of their chemical and structural variety, which often results in better bioactivity than synthesized molecules (Newman and Cragg, 2020). For these reasons, numerous studies have recently been reported on research into natural anti-inflammatory agents (Nunes *et al.*, 2020).

The genus *Philadelphus* L., which has 75 species, is found in East Asia, North and South America, and the Himalayas (Park *et al.*, 2005). The flowers and fruits of *P. schrenkii* are traditionally used as herbal remedies in South Korea to reduce fever, swelling, and pain (Xiao, 1989). The *Hydrangeaceae* family, to which *Philadelphus schrenkii* Rupr. belongs, has been reported to have antibacterial, anti-inflammatory, anti-obesity, antidiabetic, and hepatoprotective activities (Nakamura *et al.*, 2011; Shi *et al.*, 2015; Myung *et al.*, 2020; Sung *et al.*, 2023).

In the case of *P. schrenkii*, it has antibacterial and antioxidant activity, as reported in a study by Shin *et al.* (1997), Lee *et al.* (1998), and Kim *et al.* (2022). In particular, the NO inhibitory ability was confirmed to analyze the correlation between the phenolic acid content and the physiological activity of *P. schrenkii* extract in the study of Kim *et al.* (2021). However, the molecular mechanism of the anti-inflammatory activity of *P. schrenkii* has not been studied to date.

Therefore, this study aims to evaluate the anti-inflammatory activity of *P. schrenkii* methanol extract (PSE) by measuring the inhibitory effect of NO production in LPS-activated RAW 264.7 cells. Additionally, the study seeks to understand the anti-inflammatory mechanism of PSE by examining the expression of various proteins and inflammatory factors, including NF-κB.

MATERIALS AND METHODS

1. Preparation of Plant Extract

The *P. schrenkii* methanol extract (KPM033-007) used in this research was obtained from the Natural Product Central Bank at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The plant was collected from Inje-gun, Gangwon-do, Korea in 2008. A voucher specimen (KRIB 0017867) is kept in the herbarium of the Korea Research Institute of Bioscience and Biotechnology.

The plant (69 g) dried in the shade and powdered was added to 1 ℓ of methyl alcohol 99.9% (HPLC grade) and extracted through 30 cycles (40 ㎑, 1,500 W, 15 min. Ultrasonication - 120 min. standing per cycle) at room temperature using an ultrasonic extractor (SDN-900H, SD-ULTRASONIC Co., Ltd, Seoul, Korea). After filtration (Qualitative Filter No.100, HYUNDAI MICRO Co., Ltd, Seoul, Korea) and drying under reduced pressure, *P. schrenkii* extract (6.2 g) was obtained.

The extract was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Co., Saint Louis, MO, USA) at a concentration of 100 ㎎/㎖ and kept at 4℃.

2. Cell Culture

Murine macrophage RAW 264.7 cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM, Corning™, Corning, NY, USA) with 10% heat-inactivated fetal bovine serum (FBS, Corning™, Corning, NY, USA) and a 1% mixture of penicillin and streptomycin (Corning™, Corning, NY, USA). Cells were incubated at 37℃ under humidified conditions with 5% CO_2.

3. Cell Viability assay

To determine the cytotoxicity of PSE, RAW 264.7 cells were seeded in a 96-well cell culture plate with a density of 1×10^4 cells per well and incubated for 24 h. The cells were treated with various concentrations (50, 100, 150, and 200 ㎍/㎖) of PSE for 24 h.

After treatment, the medium was replaced with a fresh one, and 10 ㎕ of EZ-cytox Cell Viability Assay Solution WST-1 (Daeil Lab Service, Seoul, Korea) was added to each well. Then, the cells were further incubated for 90 minutes at 37℃. The absorbance was measured at 460 ㎚ using a VersaMax™ microplate reader (Molecular Devices, San Jose, CA, USA).

4. Nitric oxide (NO) assay

Following preincubation of RAW 264.7 cells (5×10^4 cells/well) in 24-well cell culture plates for 22 h, the medium was replaced with a fresh medium.

Then, various concentrations (50, 100, 150, and 200 ㎍/㎖)

of PSE were treated for 2 h. Afterward, 1 ㎍/㎖ of LPS derived from *Escherichia coli* O111:B4 (Sigma-Aldrich Co., Saint Louis, MO, USA) was added to each well and further incubated for 24 h.

To analyze NO production, 100 ㎕ of the culture supernatant was transferred to 96-well plates, and the same amount of Griess reagent (Sigma-Aldrich Co., Saint Louis, MO, USA) was added. The plates were incubated for 15 min at room temperature in dark conditions.

The absorbance was determined by the VersaMax™ microplate reader (Molecular Devices, San Jose, CA, USA) at a wavelength of 540 ㎚. A standard curve of $NaNO_2$ was used for the calculation of the nitrite level.

5. Prostaglandin E_2 (PGE_2) Quantification Assay

RAW 264.7 cells were cultured in 24-well plates for 24 h, and then treated with 100 and 200 ㎍/㎖ of PSE for 2 h. Afterward, the culture supernatant was collected to quantify the amount of PGE_2 secreted by RAW 264.7 cells that were activated by exposure to 1 ㎍/㎖ of LPS for 22 h.

The investigation of PGE_2 production levels was performed following the protocol provided by the manufacturer of the PGE_2 Parameter Assay Kit (R&D Systems Inc., Minneapolis, MN, USA). The absorbance was measured at 450 ㎚ using the VersaMax™ microplate reader (Molecular Devices, San Jose, CA, USA).

6. Enzyme-Linked Immunosorbent Assay (ELISA)

The culture supernatant was collected after treating the RAW 264.7 cells with PSE for 2 h and stimulating them with LPS for 22 h.

The levels of tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) secreted by LPS-stimulated RAW 264.7 cells were quantified using the TNF-α Mouse ELISA Kit and the MCP-1 Mouse ELISA Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Absorbance measurements were taken using the VersaMax™ microplate reader (Molecular Devices, San Jose, CA, USA) at a wavelength of 450 ㎚.

7. Reverse Transcription Quantitative Real-time Polymerase Chain Reaction (RT-qPCR)

The mRNA expression levels of inflammatory proteins, including iNOS and COX-2, along with inflammatory cytokines such as TNF-α, interleukin (IL)-1β, IL-6, and MCP-1, were investigated by RT-qPCR. The PSE was incubated with RAW 264.7 cells for 2 h, followed by the addition of LPS for 22 h.

The cells were then harvested, and total mRNA was isolated using the standard protocol of the RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany). The isolated mRNA was assessed for purity and quantified at 260 ㎚ and 280 ㎚ wavelengths using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific™, Waltham, MA, USA). RT-PCR was performed using AccuPower® CycleScript™ RT PreMix (dT20) (Bioneer, Daejeon, Korea) to synthesize 1 ㎍ of mRNA into cDNA. The primer sequences utilized in qPCR are listed in Table 1.

The qPCR reaction mixture was prepared by adding 10 ㎕ of AccuPower® 2x GreenStar™ qPCR Master Mix (Bioneer) containing SYBR green dye, 1 ㎕ of forward primer (10 pmol), 1 ㎕ of reverse primer (10 pmol), 1 ㎕ of synthesized cDNA, and distilled water to a final volume of 20 ㎕.

qPCR was performed using the QuantStudio™ 6 Flex System (Thermo Fisher Scientific™). The qPCR conditions are as follows: pre-denaturation for 10 min at 95℃, denaturation for 15 sec at 95℃, annealing for 30 sec at 60℃, extension for 30 sec at 72℃, repeated for 40 cycles, and final extension for 5

Table 1. Primer sequences of mRNA expression levels of inflammation-related genes by treatment of *P. schrenkii* methanol extract (PSE) in quantitative real-time polymerase chain reaction.

Protein	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)
iNOS	CAGATCGAGCCCTGGAAGAC	CTGGTCCATGCAGACAACCT
COX-2	CACTCTATCACTGGCACCCC	TTGGCACATTTCTTCCCCCA
TNF-α	AAGAGGCACTCCCCCAAAAG	ATCCCTTTGGGGACCGATCA
IL-1β	GCTACCTGTGTCTTTCCCGT	CATCTCGGAGCCTGTAGTGC
IL-6	TCCGGAGAGGAGACTTCACA	TTCTGCAAGTGCATCATCGT
MCP-1	CCTGCTGCTACTCATTCACCA	ATTCCTTCTTGGGGTCAGCA
GAPDH	GAAGGTCGGTGTGAACGGAT	ACTGTGCCGTTGAATTTGCC

min at 72℃. After completion of the reaction, the qPCR reaction mixture was subjected to denaturation at 95℃ for 15 sec, followed by annealing at 60℃ for 1 minute. The fluorescence signal for melt curve analysis was measured as the temperature was increased from 60℃ to 95℃ at a rate of 0.05℃/s.

The amount of total mRNA was normalized using GAPDH, a housekeeping gene. The comparative Ct ($_{\Delta\Delta}$Ct) approach was then used to calculate the relative mRNA expression levels of each target gene.

8. Western Blot Analysis

RAW 264.7 cells (1.5×10^6 cells/㎖) were cultured for 24 h and pre-treated with PSE (100 and 200 ㎍/㎖) for 2 h. After adding LPS (1 ㎍/㎖), the cells were incubated for 30 min or 22 h.

Whole-cell lysates were prepared by washing and harvesting the cells with ice-cold phosphate-buffered saline (PBS, Biosesang, Seongnam, Korea), and lysing them with ice-cold PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Seongnam, Korea) in ice for 30 min.

The supernatant was obtained by performing centrifugation at 13,200 rpm for 20 min at 4℃. To prepare nuclear and cytoplasmic protein extracts, RAW 264.7 cells were treated with PSE for 2 h. The cells were then collected 30 min after LPS stimulation.

The nuclear and cytoplasmic fractions were separated and extracted following the manufacturer's instructions for NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific™, Waltham, MA, USA).

The protein concentration was quantified using the Bradford reagent (Biosesang) and calculated with the BSA standard. An equal concentration of protein was prepared with Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA) and then boiled for 5 min.

Protein samples were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and subsequently transferred onto nitrocellulose membranes (PALL Life Sciences, Port Washington, NY, USA). The membrane was blocked for 1 h in PBST (PBS with 0.1% Tween 20) containing 5% skim milk (BioShop, Ontario, Canada). After blocking, the membrane was incubated with the primary antibodies overnight at 4℃.

The primary antibodies used in this experiment included iNOS, COX-2, IKKα, IKKβ, p-IKKα/β (Ser176/180), IκBα, p-IκBα (Ser32), NF-κB, p-NF-κB p65 (Ser536), ERK 1/2, p-ERK 1/2 (Thr202/Tyr204), SAPK/JNK, p-SAPK/JNK (Thr183/Tyr185), p38, p-p38 (Thr180/Tyr182), β-actin, and Histone H3 (Cell Signaling Technology Inc., Danvers, MA, USA).

The membrane was washed three times with PBST buffer and then incubated with a horseradish peroxidase-conjugated secondary antibody (either anti-rabbit IgG or anti-mouse IgG, Cell Signaling Technology Inc., Denver, MA, USA) for 1 h at room temperature.

Afterward, the membrane was rewashed three times. Enhanced chemiluminescence (ECL) solution (AbFrontier, Seoul, Korea) was used for protein development. The signals were detected with iBright™ CL1500 Imaging System (Thermo Fisher Scientific™, Waltham, MA, USA).

9. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The compounds of PSE were analyzed using a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan).

The PSE sample solution dissolved in methanol was injected at a flow rate of 1 ㎖/min and a split ratio of 10 : 1 with splitless injection mode into the Agilent DB-5ms Ultra Inert Columns (30 m × 0.25 ㎜ × 0.25 ㎛) (Agilent Technologies, Santa Clara, CA, USA) using helium as a carrier gas. The injection temperature was 250℃, and the temperature program was set to 2 min at 50℃, 5 ℃/min from 50℃ to 250℃, 10 ℃/min from 250℃ to 320℃, and hold for 11 min at 320℃.

MS analysis conditions were electron ionization at 70 eV, an ion source temperature of 200℃, a solvent cut time of 5 min, and a scanning interval of 0.3 sec at a 40 to 600 *m/z* scan range.

10. Statistical Analysis

Data from all experiments are reported as mean values ± standard deviation (SD) based on at least three replicates. Statistical analysis was conducted using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA).

Statistical significance was evaluated with One-way ANOVA, followed by Dunnett's Multiple Comparison Test to analyze the differences between each group. When $p < 0.05$ was achieved, differences were considered statistically significant.

RESULTS AND DISCUSSION

1. Effects of *P. schrenkii* methanol extract (PSE) on cell viability

To investigate the cytotoxic effect, the PSE was treated at

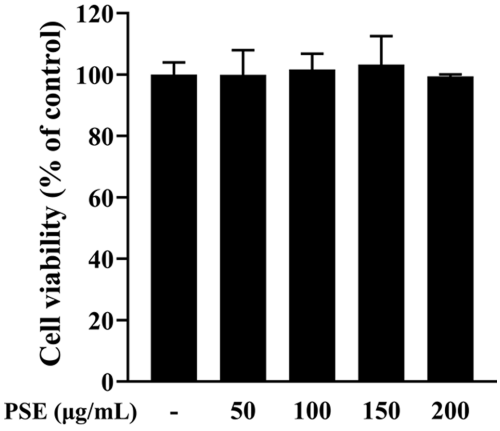

Fig. 1. Effects of *P. schrenkii* methanol extract (PSE) on cell viability in RAW 264.7 cells.

various concentrations (50, 100, 150, and 200 ㎍/㎖) on RAW 264.7 cells and measured using the WST-1 assay.

RAW 264.7 cells are mice-derived macrophages that produce a higher amount of inflammatory mediators, such as NO and PGE_2, and promote phagocytosis when stimulated by LPS (Taciak *et al.*, 2018). Therefore, the discovery of an agent that can regulate macrophage activation and inhibit the secretion of inflammatory mediators is considered an effective way to develop a new anti-inflammatory drug (Konishi *et al.*, 2008; Marasinghe *et al.*, 2022; Seo *et al.*, 2022).

Cell viability assay results of PSE did not show any cytotoxic effects at concentrations up to 200 ㎍/㎖ compared to the control (Fig. 1). Therefore, the PSE was used up to a concentration of 200 ㎍/㎖ in the study.

2. Effects of *P. schrenkii* methanol extract (PSE) on the inflammatory response

In order to investigate the anti-inflammatory capacity of PSE, the NO and PGE_2 production in LPS-induced RAW 264.7 cells were examined using a non-toxic dose range of the PSE. NO is a signaling molecule that plays vital roles in several human physiological systems (Vuolteenaho *et al.*, 2007).

NO is produced when inflammation is induced by various factors, such as stimulation of exposure, and is an essential molecule for the immune response as it has the ability to eliminate bacteria and viruses (Wink *et al.*, 2011). However, excessive production of NO can act as a pro-inflammatory mediator and cause tissue damage and inflammation. Therefore, inhibition of NO production is crucial in modulating the inflammatory response (Sharma *et al.*, 2007).

PGE_2 causes edema and redness by dilating arteries and increasing vascular permeability in inflammation. It also affects the nervous system to cause pain (Funk, 2001). Therefore, the anti-inflammatory effect of PSE was confirmed by measuring the production of NO and PGE_2, which are inflammatory mediators.

The results showed that, compared to the control group treated only with LPS, the levels of NO and PGE_2 production were remarkably reduced to 19.1% and 15.04%, respectively, in the group treated with PSE at a concentration of 200 ㎍/㎖ (Fig. 2A and B). These results indicated that the PSE could suppress inflammatory responses through the inhibition of NO and PGE_2 production.

RT-qPCR and western blot analysis were performed to verify the protein and mRNA expression levels of two major inflammatory enzymes, iNOS and COX-2, in order to examine the effects of PSE on NO production further. The iNOS is one of the isoforms of nitric oxide synthase that synthesizes L-arginine into NO. The expression of NOS is induced by LPS or cytokines such as IL-1 and TNF-α (Miyasaka and Hirata, 1997; Habib and Ali, 2011).

Similarly, COX is also considered a key enzyme in inflammatory reactions and has two isoforms, COX-1 and COX-2. After being stimulated by pro-inflammatory factors, COX-2 produces one of the major inflammatory mediators, particularly PGE_2, by converting arachidonic acid (Simon, 1999).

Results indicated that, compared to the LPS-only treatment group, the mRNA expression of iNOS and COX-2 in the PSE treatment group was suppressed by up to 7.83% and 14.37%, respectively (Fig. 2C).

Furthermore, protein expression was suppressed by up to 43.84% for iNOS and up to 35.84% for COX-2 when PSE was added (Fig. 2D and 2E). This indicated that the PSE treatment effectively suppressed both the mRNA and protein expressions of iNOS and COX-2. These results demonstrated that the PSE reduced NO and PGE_2 production by preventing the expression of iNOS and COX-2 in LPS-induced RAW 264.7 cells.

3. Effects of *P. schrenkii* methanol extract (PSE) on the expression of pro-inflammatory cytokines

To evaluate the anti-inflammatory effects of PSE, the production and mRNA expression levels of inflammation-related cytokines, including TNF-α, IL-1β, IL-6, and MCP-1, were measured using ELISA and RT-qPCR.

TNF-α maintains the nuclear translocation of NF-κB, which

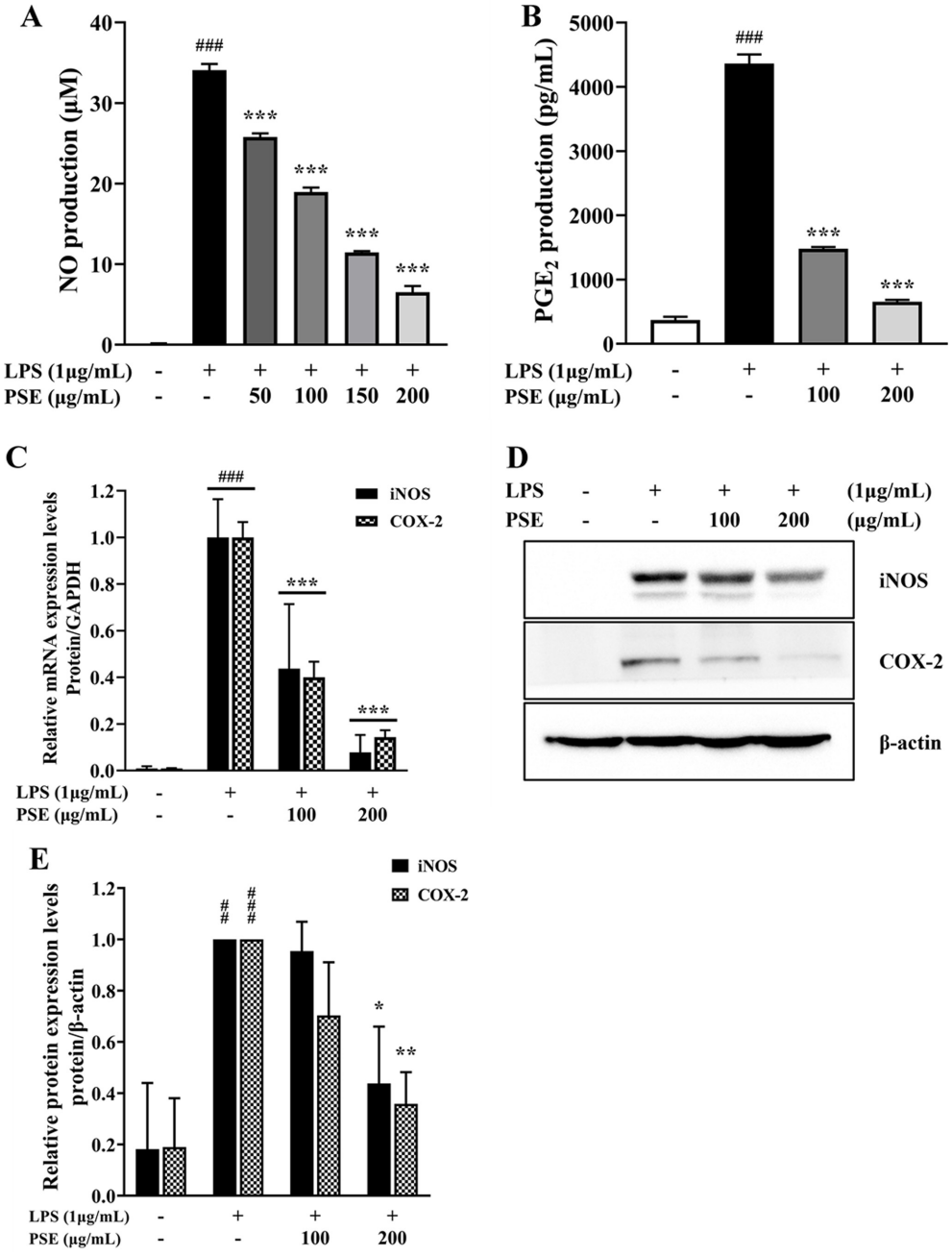

Fig. 2. Effects of *P. schrenkii* methanol extract (PSE) on the inflammatory response in LPS-treated RAW 264.7 cells. (A) The NO production was measured in the cell supernatant using the Griess reagent. (B) The PGE_2 synthesis level was analyzed using the PGE_2 assay kit. (C) The mRNA expression levels of iNOS and COX-2 in LPS-activated RAW 264.7 cells were determined using RT-qPCR. The results were normalized to the Ct value of GAPDH and analyzed by the comparative Ct method. (D) The western blot assay result, and protein level of iNOS and COX-2 protein expression in whole-cell lysates. β-actin was used as a loading control. (E) The intensity of iNOS and COX-2 proteins was quantified relative to the β-actin using ImageJ. Data represent the means ± SD (n = 3). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to the non-treated group, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the LPS-only treated cells.

activates the expression of inflammation-associated genes such as iNOS and COX-2 (Dinarello, 2000). MCP-1 is an inflammatory cytokine that is transcriptionally upregulated by NF-κB during the inflammation response and strongly attracts monocytes (Yadav *et al.*, 2010).

In addition, the continuous release of pro-inflammatory

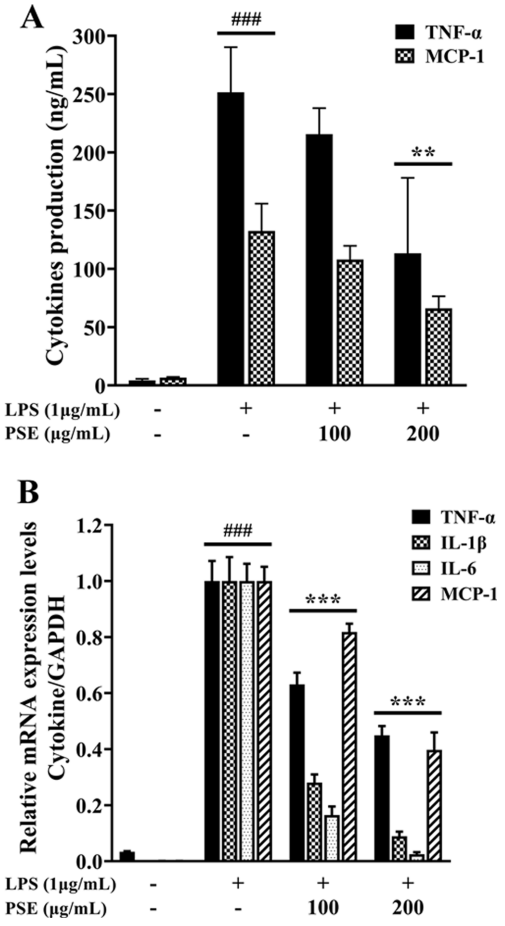

Fig. 3. Effects of *P. schrenkii* methanol extract (PSE) on the production of pro-inflammatory cytokines. (A) The ELISA kits were used to measure the release levels of TNF-α and MCP-1 secreted into the supernatant. (B) The mRNA expression levels of TNF-α, IL-1β, IL-6, and MCP-1 were estimated using RT-qPCR. The results were normalized to the Ct value of GAPDH and analyzed by the comparative Ct method. Data represent the means ± SD (n = 3). $^{###}p < 0.001$ compared to the non-treated group, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the LPS-only treated cells.

cytokines can lead to the development of chronic inflammatory diseases. IL-1β acts as an important inflammatory mediator and causes inflammatory pain hypersensitivity. IL-6 plays an essential role in the transition from acute to chronic inflammation (Gabay, 2006).

As shown in Fig. 3A, in comparison to the LPS control, the PSE demonstrated inhibition of TNF-α and MCP-1 levels to 49.9% and 45.06%, respectively. The PSE also showed inhibitory activities against the mRNA expressions of inflammatory cytokines in LPS-induced RAW 264.7 cells. The mRNA expression levels of TNF-α, IL-1β, IL-6, and MCP-1 treated with the PSE of 200 ㎍/㎖ were considerably reduced to 44.94%, 8.94%, 2.54%, and 49.75%, respectively, compared to the group treated with LPS alone (Fig. 3B).

These results suggested that PSE effectively prevented the development of chronic inflammation by reducing the synthesis and mRNA expression of pro-inflammatory cytokines.

4. Inhibitory effects of *P. schrenkii* methanol extract (PSE) on the NF-κB in LPS-stimulated RAW 264.7 cells

Western blot analysis was performed to determine the phosphorylation levels of proteins in the NF-κB and MAPK signaling pathways.

Inflammatory responses are known to be significantly regulated by the activation of the NF-κB and MAPK signaling pathways (Jayawardena *et al.*, 2021). NF-κB is a crucial transcriptional regulatory factor that affects inflammation, cell proliferation, and apoptosis (Viatour *et al.*, 2005). In the quiescent state, NF-κB exists in the cytoplasm as an inactive form combined with its suppressor, NF-κB inhibitor alpha (IκBα). When the cells are exposed to LPS, the IκB kinase (IKK) complex phosphorylates IκBα and causes the degradation of IκBα. As a result, NF-κB, liberated from IκBα, is activated and translocated to the nucleus (Jayawardena *et al.*, 2021). Nuclear translocation of NF-κB initiates the transcription of genes that encode pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and inflammatory mediators, such as iNOS and COX-2 (Kim *et al.*, 2018; Asanka Sanjeewa *et al.*, 2019).

According to the results shown in Fig. 4A and 4C, PSE did not affect the total expression of IKKα/β and NF-κB. In addition, the phosphorylation levels of IKKα/β and NF-κB compared to their total expression levels were suppressed by 19.57% and 3.24%, respectively, in the 200 ㎍/㎖ PSE-treated group. In the LPS-only treated group, total IκBα protein expression was decreased; in the PSE-treated group, it was not downregulated. But PSE at 200 ㎍/㎖ reduced LPS-induced IκBα phosphorylation to 38.97%. Our results indicated that the expression of total IκBα was suppressed by LPS, and PSE reduced the LPS-induced phosphorylation of IκBα. This also means that PSE inhibits the phosphorylation of IKKα/β and IκBα, thereby interfering with the activation of NF-κB.

Afterwards, we verified the translocation of NF-κB from the cytoplasm to the nucleus. Activated NF-κB is necessary for inducing responses to several immunological stimuli by binding to the promoter/enhancer regions of pro-inflammatory cytokine/chemokine genes (Smale, 2011). In other words, targeting NF-

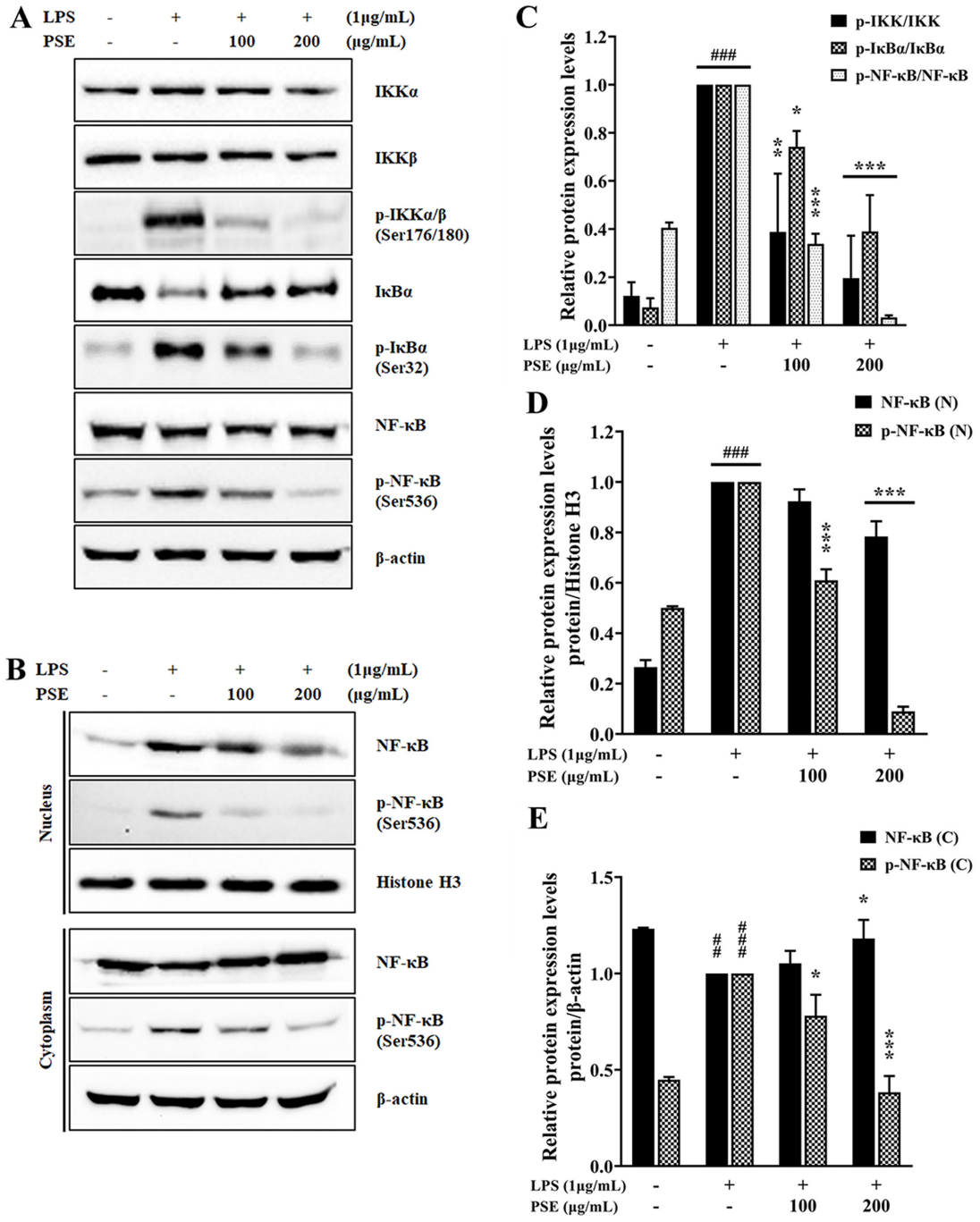

Fig. 4. Effects of *P. schrenkii* methanol extract (PSE) on the NF-κB signaling pathway in LPS-induced RAW 264.7 cells. The expression levels of total and phosphorylated forms of (A) IKKα/β, IκBα, and NF-κB in whole-cell lysates, and (B) NF-κB in the nuclear and cytoplasmic fractions were determined by western blot analysis. β-actin and Histone H3 were used as loading controls. The relative protein expression levels of (C) p-IKK/IKK, p-IκBα/IκBα, and p-NF-κB/NF-κB, (D) NF-κB/Histone H3 and p-NF-κB/Histone H3 in nuclear fraction, (E) NF-κB/Histone H3 and p-NF-κB/Histone H3 in cytoplasmic fraction. The intensity of proteins was measured using ImageJ. Data represent the means ± SD (n = 3). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to the non-treated group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the LPS-only treated cells.

κB signaling could be a useful therapeutic strategy for inflammatory diseases (Ben Neriah and Karin, 2011). Regulation of NF-κB signaling can be achieved through modulation of several steps between receptor activation and initiation of gene transcription, nuclear translocation, DNA binding, or interference with transcription initiation of NF-κB target genes

(Ramadass *et al.*, 2020).

As shown in Figures 4B and 4D, the expression of total and phosphorylated NF-κB in the nuclear fraction gradually reduced in the PSE-treated group. In the cytoplasmic fraction, NF-κB phosphorylation increased upon LPS stimulation and decreased with increasing PSE concentration (Fig. 4B and 4E). Conversely, although total cytoplasmic NF-κB was decreased by LPS, its expression was not decreased in the PSE-treated group. Thus, the result of inhibition of nuclear translocation of NF-κB in Fig. 4B indicates that PSE can be helpful in suppressing inflammation.

These results showed that PSE effectively interrupted the activation and nuclear translocation of NF-κB in LPS-activated RAW 264.7 cells by modulating IκBα and IKKα/β phosphorylation levels.

5. Effects of *P. schrenkii* methanol extract (PSE) on the MAPK pathway

The phosphorylation of MAPK was examined for further investigation into the anti-inflammatory activities of PSE. MAPK, which consists of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, regulates biological activities such as cell growth, differentiation, and apoptosis. They are also involved in inflammation, innate immune systems, and cancer (Wang *et al.*, 2023). MAPK proteins are activated by mitogenic stimuli, cytokines, various growth factors, and pro-inflammatory or stressful responses (Kim *et al.*, 2005; Zhang *et al.*, 2019). When MAPK proteins are phosphorylated by LPS stimulation, they can enhance the transcription of inflammatory genes by inducing the activation of downstream transcription factors, especially NF-κB (Kim *et al.*, 2018).

In the case of the MAPK pathway, the phosphorylation levels of ERK, JNK, and p38 were reduced to 36.06%, 44.64%, and 57.75%, respectively, without significant alteration of total protein expressions in 200 ㎍/㎖ PSE-treated groups compared to those in the LPS-stimulated group (Fig. 5A and 5B). These results demonstrate that the anti-inflammatory activity of PSE results from inhibiting NF-κB activity by interfering with the phosphorylation of NF-κB upstream proteins, including the IKK complex, IκBα, and MAPK family.

6. GC-MS analysis of the *P. schrenkii* methanol extract (PSE)

The ingredients of the PSE were investigated by GC-MS to identify the compounds that enable it to have an anti-inflammatory effect. A total of 79 compounds were detected in the GC-MS analysis of PSE (Fig. 6), and 38 compounds (their peak area accounted for 80.54% of the entire peak area of the

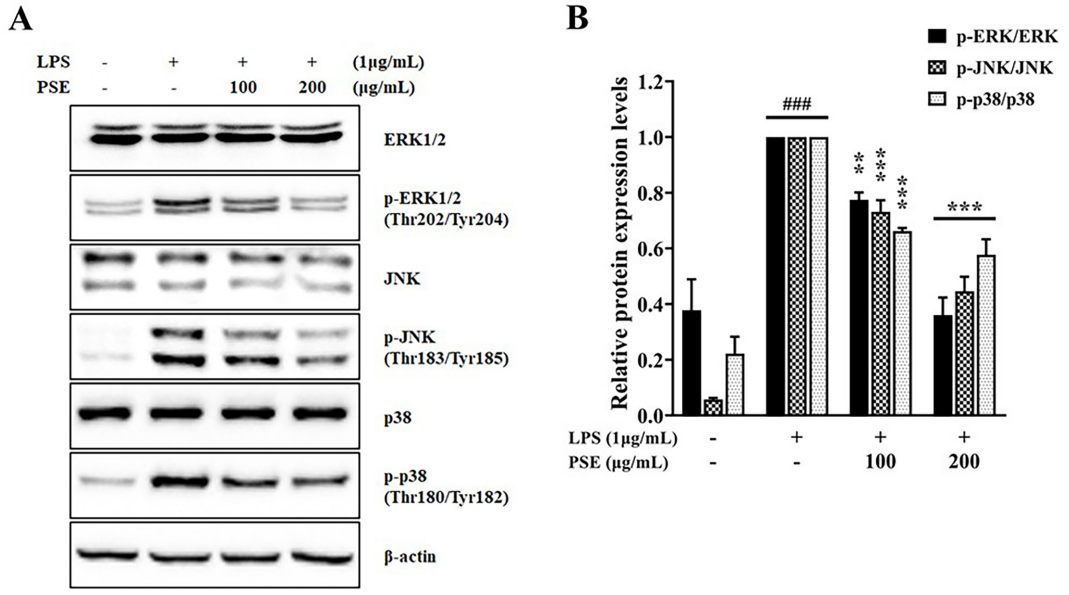

Fig. 5. Effects of *P. schrenkii* methanol extract (PSE) on the MAPK signaling pathway in LPS-induced RAW 264.7 cells. The expression levels of total and phosphorylated forms of (A) ERK, JNK, and p38 were determined by western blot analysis in whole-cell lysates. β-actin was used as a loading control. The relative protein expression levels of (B) p-ERK/ERK, p-JNK/JNK, and p-p38/p38. The intensity of proteins was measured using ImageJ. Data represent the means ± SD (n = 3). $^{###}p < 0.001$ compared to the non-treated group, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the LPS-only treated cells.

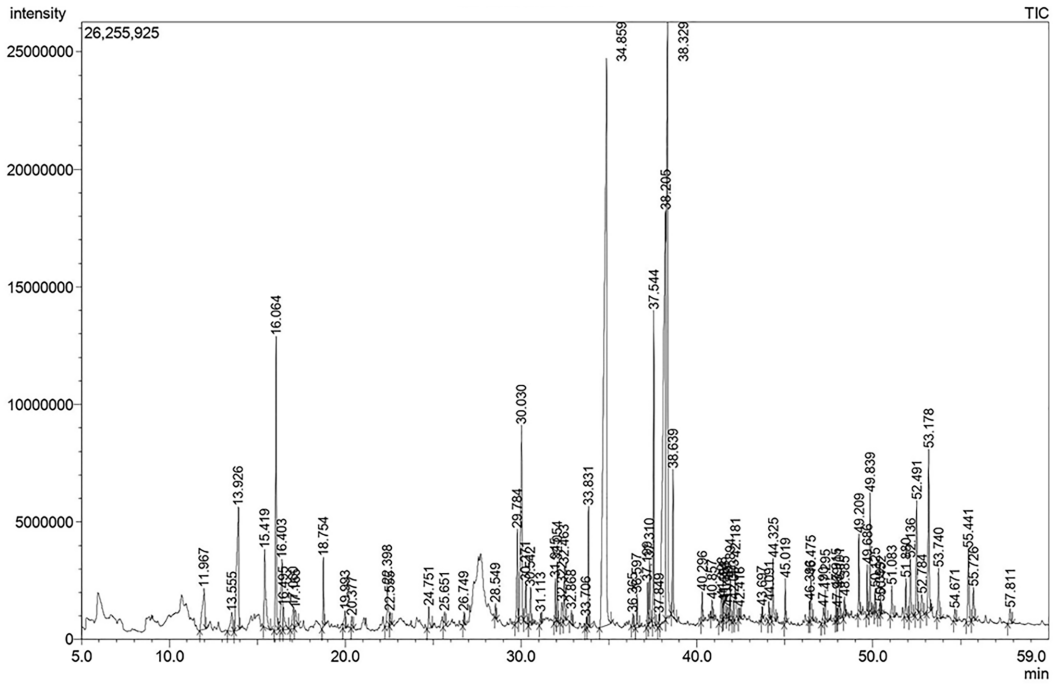

Fig. 6. Chromatogram of the *P. schrenkii* methanol extract (PSE) components as elucidated by GC-MS.

chromatogram) were clarified as a result of comparing the mass spectrum of the compound with the National Institute of Standards and Technology (NIST)11 and Wiley9 mass spectral databases (Table 2).

An overview of the identified compounds is shown in Table 2, and 25 have been reported to have anti-inflammatory activity. These compounds accounted for 73.69% of the total peak area in the chromatogram.

Among the identified compositions, the most abundant compound in the PSE was n-Hexadecanoic acid (Retention time 34.859 min), well known as palmitic acid, accounting for 20.31%. n-hexadecanoic acid has anti-inflammatory activity by reducing the synthesis of inflammatory mediators such as NO, PGE_2, TNF-α, IL-1β, and IL-6 (Aparna *et al*., 2012).

The second most abundant compound in PSE is 9,12-Octadecadienoic acid (Z,Z)- (Retention time 38.205 min),

Table 2. GC-MS data of the *P. schrenkii* methanol extract (PSE).

Retention Time (min)	Area (%)	Names	Structure formula	Molecular weight	Anti-inflammatory activity
13.926	3.44	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	C_6H_8O	144.1253	(Shalini and Ilango, 2021)
15.419	1.71	Catechol	$C_6H_6O_2$	110.1106	(Chang *et al.*, 2014)
16.064	3.82	Benzofuran, 2,3-dihydro-	C_8H_8O	120.1485	(Closse *et al.*, 1981)
16.403	1.46	5-Hydroxymethylfurfural	$C_6H_6O_3$	126.11	(Insuan *et al.*, 2022)
18.754	0.75	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.1745	(Vadivel and Gopalakrishnan, 2011; Al-Marzoqi *et al.*, 2016)
19.993	0.33	Neric acid	$C_{10}H_{16}O_2$	168.2328	
22.555	0.2	Phenol, 2-methoxy-4-(1-propenyl)-, (Z)-	$C_{10}H_{12}O_2$	164.2011	(Mohammed *et al.*, 2016)
24.751	0.33	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	$C_{11}H_{16}O_2$	180.2435	(Chuang *et al.*, 2020)

Table 2. Continued.

Retention Time (min)	Area (%)	Names	Structure formula	Molecular weight	Anti-inflammatory activity
26.749	0.13	3-Hydroxy-β-damascone	$C_{13}H_{20}O_2$	208.2967	
29.784	1.29	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180.2005	(Vadivel and Gopalakrishnan, 2011; Al-Marzoqi *et al.*, 2016)
30.271	0.6	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.3709	(Alonso-Castro *et al.*, 2022)
30.542	0.5	(-)-Loliolide	$C_{11}H_{16}O_3$	196.2429	(Jayawardena *et al.*, 2021)
32.054	0.6	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	268.4778	
32.322	0.38	7-Hydroxycoumarin	$C_9H_6O_3$	162.1421	(Mazimba, 2017)
32.463	0.75	Pentadecanoic acid	$C_{15}H_{30}O_2$	242.3975	(Venn-Watson and Butterworth, 2022)
33.831	1.14	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.4507	(Hamed *et al.*, 2020)
34.859	20.31	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4241	(Aparna *et al.*, 2012)
36.597	0.38	Heptadecanoic acid	$C_{17}H_{34}O_2$	270.4507	(Okur *et al.*, 2020)
37.199	0.42	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294.4721	
37.31	0.68	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292.4562	
37.544	3.3	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	$C_{20}H_{40}O$	296.531	(Islam *et al.*, 2018)
37.849	0.12	Methyl stearate	$C_{19}H_{38}O_2$	298.5038	(Olukanni, 2020)
38.205	14.44	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280.4455	(Omeje *et al.*, 2018; Chinnadurai *et al.*, 2019)
38.329	11.97	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278.4296	(Kumar *et al.*, 2010; Jananie *et al.*, 2011)
38.639	1.8	Octadecanoic acid	$C_{18}H_{36}O_2$	284.4772	(Aparna *et al.*, 2012; Rajeswari *et al.*, 2012)
41.894	0.42	4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	324.5411	
42.181	0.93	Eicosanoic acid	$C_{20}H_{40}O_2$	312.5304	(Das, 2020)
44.325	0.74	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330.5026	
45.019	0.46	Docosanoic acid	$C_{22}H_{44}O_2$	340.5836	(da Silva *et al.*, 2019)
46.396	0.19	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{21}H_{38}O_4$	354.524	
47.295	0.31	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281.4766	(Ameamsri *et al.*, 2021; Moon *et al.*, 2018)
48.101	0.46	α-Tocospiro B	$C_{29}H_{50}O_4$	462.7049	
49.686	0.45	(R)-6-Methoxy-2,8-dimethyl-2-((4R,8R)-4,8,12-trimethyltridecyl) chroman	$C_{28}H_{48}O_2$	416.6795	
52.491	1.57	Stigmasta-7,22-dien-3-ol, (3β,5α,22E,24R)-	$C_{29}H_{48}O$	412.6908	
53.178	2.47	β-Amyrin	$C_{30}H_{50}O$	426.7174	(Holanda Pinto *et al.*, 2008)
53.74	0.83	Lupeol	$C_{30}H_{50}O$	426.7174	(Tsai *et al.*, 2016)
55.726	0.56	Friedelan-3-one	$C_{30}H_{50}O$	426.7174	
57.811	0.3	Urs-12-en-28-al, 3-(acetyloxy)-, (3β)-	$C_{32}H_{50}O_3$	482.7376	

which accounts for 14.44%. The third major ingredient in PSE was 9,12,15-Octadecatrienoic acid (Z,Z,Z)- (Retention time 38.329 min), accounting for 11.97%. Both 9,12-Octadecadienoic acid (Z,Z)- and 9,12,15-Octadecatrienoic acid (Z,Z,Z)- have been reported to reduce the production of NO, and in particular, 9,12-Octadecadienoic acid (Z,Z)- was found to suppress the production of TNF-α, IL-1β, and IL-6 (Lee *et al.*, 2011).

Furthermore, it was established that numerous compounds contained in PSE have anti-inflammatory activities, as Table 2 indicates. Therefore, it can be inferred that the anti-inflammatory activity of PSE demonstrated in the previous experiments may be caused by the presence of these compounds.

This study investigated the anti-inflammatory effects of PSE on LPS-stimulated RAW 264.7 cells. PSE effectively reduced LPS-induced inflammation through the regulation of NF-κB and MAPK signaling pathways. Additionally, PSE inhibited the production of key inflammatory mediators and pro-inflammatory cytokines. Finally, GC-MS analysis was used to determine the chemical composition of PSE, and among the 38 compounds identified, 25 were reported to have anti-inflammatory activity. The results of this study demonstrated the anti-inflammatory activity of *P. schrenkii* at the molecular level, suggesting the possibility of using *P. schrenkii* to develop as an anti-inflammatory agent.

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