PHARMACIA

Pharmacia 72: 1–14
DOI 10.3897/pharmacia.72.e153095

Research Article

Exploring the inflammatory pathway modulation of *Phaleria macrocarpa*: evidence from *in vitro* and *in silico* studies

Assa'idatus Shofiah[1], Yuyun Ika Christina[2,3], Dinia Rizqi Dwijayanti[2,3,4], Aries Soewondo[4], Nashi Widodo[2,3,4], Muhammad Sasmito Djati[2,3,4]

1 *Magister Program, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, 65145, East Java, Indonesia*
2 *Dewan Jamu Indonesia East Java Region, Malang, 65145, East Java, Indonesia*
3 *Innovation Center of Integrative Jamu and Eco-pharmaca, Brawijaya University, Malang 65145, East Java, Indonesia*
4 *Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, 65145, East Java, Indonesia*

Corresponding author: Muhammad Sasmito Djati (msdjati@ub.ac.id)

Received 16 March 2025 ♦ **Accepted** 16 June 2025 ♦ **Published** 4 August 2025

Citation: Shofiah A, Christina YI, Dwijayanti DR, Soewondo A, Widodo N, Djati MS (2025) Exploring the inflammatory pathway modulation of *Phaleria macrocarpa*: evidence from *in vitro* and *in silico* studies. Pharmacia 72: 1–14. https://doi.org/10.3897/pharmacia.72.e153095

Abstract

Phaleria macrocarpa has been traditionally used in alternative medicine, yet its molecular anti-inflammatory mechanisms remain underexplored. This study investigates the anti-inflammatory potential of *P. macrocarpa* leaf ethanol extract in LPS-stimulated RAW 264.7 macrophages and elucidates its anti-inflammatory mechanisms through *in silico* studies. Cells were treated with the extract at 15, 30, and 60 µg/mL following LPS induction. Nitric oxide (NO) production and cell viability were assessed using the Griess and WST-1 assays, respectively. Flow cytometry quantified inducible nitric oxide synthase (iNOS) and interleukin (IL)-1β expression. Molecular docking and molecular dynamics simulations were performed to evaluate binding affinity and stability. The extract significantly reduced NO production, with an IC_{50} value of 18.4 ± 3.1 µg/mL, while maintaining cell viability above 80%. Furthermore, treatment with *P. macrocarpa* extract significantly suppressed iNOS and IL-1β expression in a dose-dependent manner. Computational studies revealed strong interactions between extract-derived compounds and inflammation-related targets (AKT1, MMP9, PI3KCA, PI3KCG, and PTGS2), with stable molecular dynamics. These findings suggest that *P. macrocarpa* leaf extract exerts potent anti-inflammatory effects, highlighting its potential as a promising therapeutic candidate.

Keywords

AKT-1, inflammation, iNOS, nitric oxide, *Phaleria macrocarpa*

Introduction

Inflammation is a complex biological response triggered by infection, cellular damage, or exposure to toxic agents, functioning as a crucial defense mechanism to maintain homeostasis and facilitate tissue repair (Chen et al. 2018). This immune response involves the activation of various signaling pathways and the release of pro-inflammatory mediators, including nitric oxide (NO) (Dong et al. 2017), tumor necrosis factor-alpha (TNF-α) (Wiart et al. 2024), prostaglandin E_2 (PGE_2) (Dong et al. 2017), cyclooxygenase-2 (COX-2) (Zhao et al. 2021), and interleukin-1 beta

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(IL-1β) (Kang et al. 2015). These mediators play a pivotal role in modulating vascular permeability, recruiting immune cells, and restoring the structural and functional integrity of affected tissues. However, when the inflammatory response becomes dysregulated or persists beyond its physiological necessity, it transitions into chronic inflammation, a key contributor to the pathogenesis of various non-communicable diseases, including cancer (Singh et al. 2019), diabetes mellitus (Dwijayanti et al. 2019), cardiovascular disorders (Bansal et al. 2023), and neurodegenerative conditions such as Alzheimer's disease (Kinney et al. 2018). The global burden of chronic inflammation-related diseases is substantial, accounting for approximately 71% of all deaths worldwide, with an estimated 41 million fatalities recorded in 2020 (Gracia-Aranda et al. 2020).

Currently, non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed pharmacological agents for managing inflammation. These drugs exert their effects primarily by inhibiting cyclooxygenase enzymes (COX-1 and COX-2), thereby reducing the synthesis of pro-inflammatory prostaglandins. However, prolonged or excessive use of NSAIDs is associated with a range of adverse effects, including gastrointestinal complications, nephrotoxicity, increased risk of cardiovascular events such as stroke and myocardial infarction, and potential reproductive toxicity leading to male infertility (Brune and Patrignani 2015; Chang et al. 2019). Given these limitations, there has been growing interest in the development of safer and more effective anti-inflammatory alternatives. Herbal-based therapeutics, derived from bioactive compounds with anti-inflammatory properties, have emerged as promising candidates due to their relatively lower toxicity profiles and multifaceted mechanisms of action.

Herbal therapies as anti-inflammatory agents frequently target multiple inflammatory signaling pathways, particularly those involved in the production of pro-inflammatory mediators. Pro-inflammatory mediator production engages several pathways, including mitogen-activated protein kinase (MAPKs), nuclear factor kappa B (NF-κB), Janus kinase (JAK)–signal transducer and activator of transcription (STAT) (Chen et al. 2018), and PI3K/AKT/mTOR (Vergadi et al. 2017). The MAPK pathway plays a crucial role in maintaining the stability of mRNA and proteins related to inflammation, such as COX-2, TNF-α, IL-6, and IL-1β (Zhao et al. 2021). The NF-κB pathway is essential for inflammatory and immune responses, as well as apoptosis. Phosphorylation of IKK leads to degradation of the inhibitor protein (IκB), allowing NF-κB to dissociate. NF-κB then translocates to the nucleus, where it binds to target genes and induces the transcription of pro-inflammatory proteins (Dong et al. 2017). On the other hand, the JAK–STAT pathway regulates inflammatory gene expression via extracellular factors. Upon activation, JAK receptors are phosphorylated and bind to STATs, which, after dimerization, translocate to the nucleus to regulate transcription of inflammatory genes (Chen et al. 2018). The PI3K/AKT/mTOR pathway enhances the production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β. However, it also plays a dual role by limiting the expression of inflammatory mediators and promoting anti-inflammatory responses (Vergadi et al. 2017).

One of the medicinal plants with potential anti-inflammatory activity is *Phaleria macrocarpa* (Scheff.) Boerl. *P. macrocarpa* is a plant native to Indonesia and commonly found in various regions. It is widely used as a traditional alternative medicine in several Asian countries. The plant has several beneficial parts, including stems, leaves, fruits, and seeds (Christina et al. 2021a, 2021b; Ahmad et al. 2023). Its leaves are often used to treat high blood pressure, elevated blood sugar levels, and hemorrhoids, which are attributed to their high antioxidant activity. Antioxidants play a role in protecting the body from excessive and uncontrolled oxidative stress (Ahmad et al. 2023). The leaf extract of *P. macrocarpa* has been reported to exhibit high antioxidant activity, containing high levels of phenolic and flavonoid compounds (Christina et al. 2021b, 2021c). The high antioxidant content and abundance of phenolic and flavonoid compounds in *P. macrocarpa* leaf extract suggest its potential as an anti-inflammatory agent.

Pharmacological studies on *P. macrocarpa* have been conducted extensively, particularly regarding its anti-cancer effects. However, research on its potential as an anti-inflammatory agent and its underlying mechanisms remains limited. Therefore, this study aimed to investigate the anti-inflammatory activity of ethanol extracts from the leaves of *P. macrocarpa* in LPS-induced RAW 264.7 cells *in vitro* by monitoring nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) protein. Additionally, *in silico* studies were conducted to analyze the molecular mechanisms of *P. macrocarpa* extract related to its anti-inflammatory effects.

Materials and methods

P. macrocarpa leaves extraction

The extraction procedure followed the method described by Christina et al. (2022). *P. macrocarpa* leaves (sourced from the UPT Laboratory of Herbal Materia Medica Batu, Malang, Indonesia) were identified, cleaned, and dried at 50 °C for 2 days. The dried leaves were ground into powder. Two hundred grams of powdered extract were macerated with 2 L of 95% ethanol at room temperature for 24 h under dark conditions. The mixture was filtered using filter paper and concentrated using a rotary evaporator (IKA® RV 10, IKA Works (Asia) Sdn Bhd, Malaysia) at 50 °C. The sample was labeled and stored in sealed tubes at −20 °C until further analysis.

RAW 264.7 cell culture

RAW 264.7 mouse macrophage cells, obtained from Elabscience® (Catalog No.: CL-0190, Elabscience Biotechnology

Inc., USA), were cultured in complete medium containing high-glucose Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, Co., Merck KGaA, Darmstadt, Germany), 10% fetal bovine serum (Gibco™, Thermo Fisher Scientific, USA), and 1% 10,000 U/mL penicillin-streptomycin (Gibco™, Thermo Fisher Scientific). RAW 264.7 cells with passage numbers ranging from 19 to 30 were used.

Preparation of the extract treatment

Forty milligrams of *P. macrocarpa* leaf extract were dissolved in 1 mL of dimethyl sulfoxide (EMSURE®, Merck KGaA, Darmstadt, Germany) to obtain a stock solution (40 mg/mL). The stock solution was diluted in complete medium to prepare three treatment concentrations: 15, 30, and 60 µg/mL.

Nitric oxide (NO) assay

Nitric oxide production was measured based on the procedure by Djati et al. (2024), with slight modifications. The modified Griess reagent (Sigma-Aldrich, Co., St. Louis, MO, USA) was used to assess NO production. RAW 264.7 cells were seeded in 24-well plates at a density of 1×10^5 cells/well and incubated at 37 °C in a 5% CO_2 incubator for 24 h. Cells were treated with *P. macrocarpa* leaf extract at 15, 30, and 60 µg/mL for 24 h. To induce inflammation, 5 µg/mL of lipopolysaccharides (LPS) from *E. coli* (L2630-10MG, Sigma-Aldrich, Co., St. Louis, MO, USA) were added to the culture. Non-LPS-treated cells served as the negative control. After 24 h, 75 µL of the culture medium was mixed with 75 µL of Griess reagent in a 96-well plate. Absorbance was measured at 540 nm using a microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific, USA). The inhibitory concentration (IC_{50}) value of the extract against NO production was determined using a sodium nitrite standard curve.

Cell viability assay

Cell viability was determined using the WST-1 assay (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate). After treatment, the medium was replaced with 120 µL of culture medium containing 5% WST-1 reagent per well. The plate was incubated in a 5% CO_2 incubator at 37 °C for 30 min. Absorbance was measured at 450 nm using a microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific, USA). Cell viability was calculated using the formula:

$$\%\text{Cell viability} = \frac{\text{Absorbance}_{\text{treatment}} - \text{Absorbance}_{\text{medium}}}{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{medium}}} \times 100\% \quad (1)$$

Flow cytometry analysis

RAW 264.7 cells (1×10^5 cells/well) were seeded in 24-well plates and incubated at 37 °C in a 5% CO_2 incubator for 24 h. Cells were then treated with *P. macrocarpa* leaf extract at three concentrations corresponding to 1/2× IC_{50}, IC_{50}, and 2× IC_{50} for 24 h. After incubation, 75 µL of 0.25% trypsin-EDTA (Gibco™, Thermo Fisher Scientific) was added to detach the cells. The protocol followed Djati et al. (2024) with minor modifications. Cells were washed with 500 µL phosphate-buffered saline (PBS), centrifuged at 2,500 rpm for 5 min, and resuspended in 50 µL of intracellular fixation buffer (eBioscience™, Invitrogen, Thermo Fisher Scientific, USA). Samples were incubated at 4 °C for 20 min, followed by permeabilization using 500 µL permeabilization buffer (Invitrogen, Thermo Fisher Scientific) and centrifugation. The cell pellet was stained with 50 µL of FITC-conjugated IL-1β antibody and 50 µL of PE-conjugated inducible nitric oxide synthase (iNOS) antibody (1:100 dilution). Fluorescence levels of IL-1β and iNOS were measured using a flow cytometer (BD FACSCalibur™, San Jose, CA, USA) and analyzed using BD CellQuest™ Pro software.

Identification of bioactive compounds using LC-HRMS

Liquid chromatography–high-resolution mass spectrometry (LC-HRMS) was carried out following Christina et al. (2021a). A 100 µL sample was injected into a Hypersil GOLD™ aQ C18 column (50 × 1 mm, 1.9 µm; Thermo Fisher Scientific Inc., USA) maintained at 30 °C. The mobile phases consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The flow rate was set at 40 µL/min for a total run time of 30 min. The analysis was conducted in positive ion mode over an m/z range of 50–500. Detected ion products were identified by comparing spectra with entries in the Food Database (https://foodb.ca/).

In silico study

Compound bioactivity screening

The obtained compounds of *P. macrocarpa* leaf extract predicted its anti-inflammatory properties using the PASS Online webserver (https://www.way2drug.com/passonline/) (Christina et al. 2021a). The canonical SMILES for each compound were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The parameters assessed included anti-inflammatory, antioxidant, nitric oxide scavenger, NF kappa B transcription factor inhibitor, TNF expression inhibitor, free radical scavenger, MMP9 expression inhibitor, immunosuppressant, JAK2 expression inhibitor, and NOS2 expression inhibitor. Compounds with a Pa value ≥ 0.5 were selected for target protein prediction.

Target protein prediction

Target proteins were directly predicted using the SWISS Target Prediction database (http://www.swisstargetprediction.ch/). SWISS Target Prediction is a database capable of accurately predicting protein targets based on compound structural similarity with previously reported compounds. Indirect target proteins were obtained from direct targets using the STRING database, with a confidence level of 0.4 and a maximum of five interactions (Widyananda et al. 2022). Visualization of target protein analysis was performed using Cytoscape software (Cytoscape Consortium, US) (Lotia et al. 2013).

Molecular docking

Molecular docking was conducted on compounds and their target proteins based on SWISS Target Prediction. The 3D structures of the target proteins were obtained from the RCSB PDB database (https://www.rcsb.org/). Target proteins were prepared with BIOVIA Discovery Studio 2019 software (Dassault Systèmes Biovia, San Diego, CA, USA) by removing water molecules and contaminants. The 3D structures of the compounds were obtained from the PubChem database and prepared with OpenBabel, integrated with PyRx 0.8 software (Molecular Graphics Laboratory, La Jolla, CA, USA). Specific docking was then conducted between each compound and the active site of each protein using AutoDock Vina. Docking results were visualized using BIOVIA Discovery Studio 2019 (Dassault Systèmes Biovia, San Diego, CA, USA).

Molecular dynamics simulation

Molecular dynamics simulations were performed with YASARA (Yet Another Scientific Artificial Reality Application) (YASARA Biosciences GmbH, Vienna, Austria) software using the AMBER 14 force field. Simulations were conducted under natural physiological conditions with a pH of 7.4, an NaCl concentration of 0.9%, a water density of 0.997 g/mL, and a temperature of 310 K for 20 ns. Post-simulation analysis was performed using *md_analyze* for Root-Mean-Square Deviation (RMSD) analysis and *md_analyzeres* for Root-Mean-Square Fluctuation (RMSF) analysis.

Statistical analysis

The absorbance values obtained from the NO assay and the relative number from flow cytometry analysis were presented as mean values ± standard deviation. Nitric oxide (NO) data were analyzed using Student's *t*-test, with significance levels at $p < 0.05$ and $p < 0.01$. The relative number of iNOS and IL-1β was analyzed using one-way ANOVA (analysis of variance), followed by Duncan's post hoc test with $p < 0.05$.

Results

Effects of *P. macrocarpa* leaf extract on NO production and cell viability

The NO assay demonstrated that LPS stimulation significantly ($p < 0.01$) increased NO production in RAW 264.7 cells, reaching 54 ± 1.01 µM, compared to unstimulated RAW 264.7 cells (0 ± 18.68 µM). This confirms that LPS successfully induced an inflammatory response in RAW 264.7 cells, as indicated by the substantial increase in NO production. RAW 264.7 cell lines stimulated with LPS were used as positive controls to compare NO production and cell viability under inflammatory conditions with normal conditions and inflammatory conditions without extract administration. Meanwhile, cell lines with no LPS stimulation were used as negative controls, and NO production was not observed in these cells.

The results also showed that administration of LPS could increase cell viability compared to negative controls. Interestingly, treatment with the ethanol extract of *P. macrocarpa* leaves resulted in a dose-dependent suppression of NO production. NO levels were significantly reduced to 27.29 ± 2.21 µM at 15 µg/mL, 21.98 ± 2.93 µM at 30 µg/mL, and 8.84 ± 1.16 µM at 60 µg/mL (Fig. 1A). The calculated IC_{50} value of the extract was 18.4 ± 3.1 µg/mL, indicating a potent NO inhibitory effect.

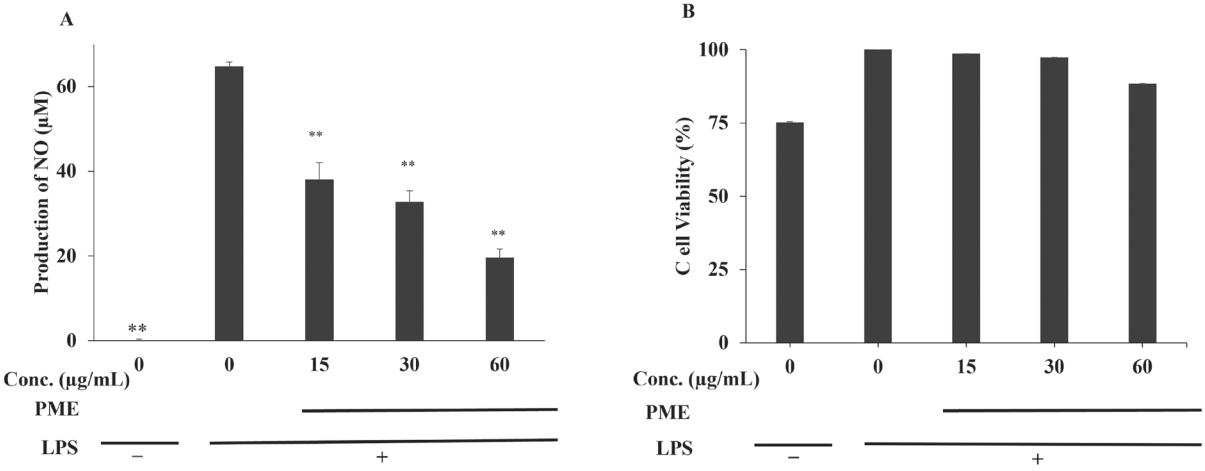


Figure 1. The levels of nitric oxide (NO) production (**A**) and cell viability (**B**) of LPS-stimulated RAW 264.7 cells after being treated with *P. macrocarpa* leaf ethanol extract. -: unstimulated RAW 264.7 cells (no LPS); +: LPS-stimulated RAW 264.7 cells. PME: LPS-stimulated RAW 264.7 cells treated with *P. macrocarpa* leaf ethanol extract at 15, 30, and 60 µg/mL. * $p < 0.05$ and ** $p < 0.01$ versus LPS alone.

To determine whether the reduction in NO levels was due to cytotoxicity or an anti-inflammatory mechanism, cell viability was assessed using the WST-1 assay. The results showed that the ethanol extract of *P. macrocarpa* leaves did not compromise cell viability at concentrations of 15–60 µg/mL, with survival rates ranging from 88% to 99% (Fig. 1B). These findings confirm that the observed NO inhibition was not due to cell death but rather the anti-inflammatory activity of the extract. These results suggest that *P. macrocarpa* leaf extract effectively suppresses inflammatory responses in RAW 264.7 macrophages by inhibiting NO production without inducing cytotoxic effects, highlighting its potential as a natural anti-inflammatory agent.

Effect of *P. macrocarpa* leaf extract on iNOS expression and IL-1β levels

The results showed that *P. macrocarpa* leaf extract effectively suppressed the expression of iNOS and IL-1β in LPS-induced RAW 264.7 cells (Fig. 2A). Cells stimulated with LPS were used as a positive control to compare protein expression in cells under inflammatory conditions with cells not stimulated with LPS (negative control) and inflamed cells without extract administration. LPS stimulation (positive control) significantly ($p < 0.05$) increased iNOS protein expression to 61.4 ± 4.79%, compared to unstimulated RAW 264.7 cells (negative control) (17.38 ± 0.71%). This increase in iNOS expression correlated with elevated NO production, confirming a strong inflammatory response. Notably, treatment with *P. macrocarpa* leaf extract in LPS-induced RAW 264.7 cells significantly reduced iNOS expression at 18 µg/mL (33.57 ± 4.45%) and 36 µg/mL (33.80 ± 4.01%), demonstrating a potent inhibitory effect (Fig. 2A).

Similarly, LPS stimulation (positive control) markedly ($p < 0.05$) increased IL-1β levels to 53.69 ± 2.12%, compared to unstimulated RAW 264.7 cells (negative control) (16.59 ± 3.2%). Treatment with *P. macrocarpa* leaf extract resulted in a significant decrease in IL-1β levels to 41.71 ± 3.82% (18 µg/mL) and 45.08 ± 2.27% (36 µg/mL) (Fig. 2B). However, no significant reduction in iNOS expression (55.97 ± 6.26%) or IL-1β cytokine levels (51.77 ± 2.6%) was observed at a concentration of 9 µg/mL. These findings suggest that *P. macrocarpa* leaf extract effectively attenuates inflammatory responses by downregulating iNOS expression and IL-1β production, potentially contributing to its anti-inflammatory activity. Further studies are needed to explore its molecular mechanisms, possibly involving PI3K/AKT pathway modulation.

Identification of bioactive compound in *P. macrocarpa* leaf extract

The LC-HRMS analysis identified 12 bioactive compounds in the *P. macrocarpa* leaf extract, including flavonoids, alkaloids, terpenoids, and coumarins. Each compound exhibited a specific ion fragmentation pattern. The ion fragmentation pattern of compounds can be used as confirmation of their molecular structure (Table 1 and Fig. 3). Furthermore, a comparison of the measured *m/z* values in this study showed consistency with theoretical values reported in the literature.

Anti-inflammatory properties screening

A bioactivity prediction screening was conducted to assess the anti-inflammatory potential of the identified compounds in *P. macrocarpa* leaf extract. Among the 12

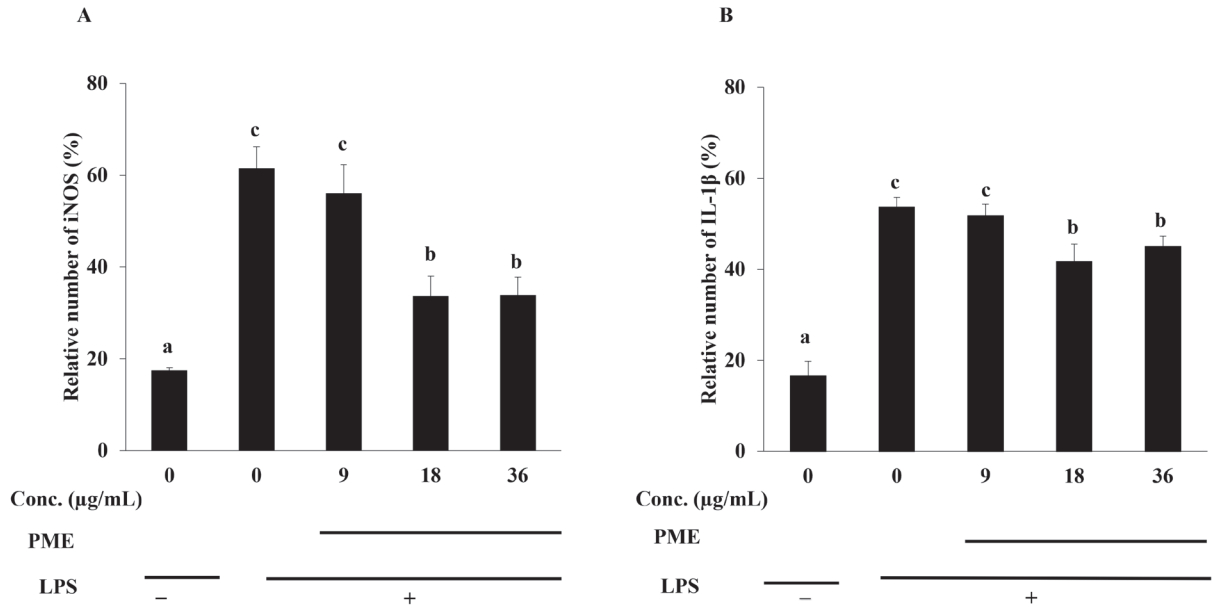


Figure 2. The effect of *P. macrocarpa* leaf extract on iNOS expression (**A**) and IL-1β levels (**B**) in LPS-stimulated RAW 264.7 cells. -: unstimulated RAW 264.7 cells (no LPS); +: LPS-stimulated RAW 264.7 cells. PME: LPS-stimulated RAW 264.7 cells treated with *P. macrocarpa* leaf ethanol extract at 9 µg/mL (1/2× IC_{50}), 18 µg/mL (IC_{50}), and 36 µg/mL (2× IC_{50}). Different notation indicates a significant difference between groups ($p < 0.05$).

Table 1. The identification of bioactive compounds of *P. macrocarpa* leaf extract using LC-HRMS analysis.

No	Compound	Formula	RT (min)	m/z [M⁺H⁺ (theo)	Observed m/z [M⁺H]⁺ (Exp)	Mass Error (ppm)	MS/MS Fragmentation pos mode [M⁺H]⁺ (m/z)	References
1	Trigonelline	$C_7H_7NO_2$	0.90	138.05	138.05	0.00	116.07 70.07 56.05	Wang et al. (2024)
2	Sterubin	$C_{16}H_{14}O_6$	3.67	303.09	303.09	0.00	167.03	Aldana-Meija et al. (2024)
3	Corymboside	$C_{26}H_{28}O_{14}$	4.50	565.15	565.15	0.00	503.19 437.11 393.08 341.14 261.08 181.12 131.05	Zhou et al. (2023)
4	Apigenin	$C_{15}H_{10}O_5$	5.34	271.06	271.06	0.00	149.03 83.06	Aldana-Meija et al. (2024)
5	Glycitein	$C_{16}H_{12}O_5$	5.95	285.06	285.06	0.00	214.18 136.02	Chen et al. (2024)
6	Sakuranetin	$C_{16}H_{14}O_5$	6.32	287.09	287.09	0.00	261.08 149.02	Zhao et al. (2020)
7	Carvone	$C_{10}H_{14}O$	8.41	151.11	151.11	0.00	136.02 111.04 94.05 83.06	Lopes et al. (2020)
8	Cafestol	$C_{20}H_{28}O_3$	11.50	317.21	317.21	0.00	235.17 219.17 149.02	Brand et al. (2024)
9	Nootkatone	$C_{15}H_{22}O$	13.71	219.17	219.17	0.00	149.02 136.02 94.05 83.06	Zhao et al. (2025)
10	Betulin	$C_{30}H_{50}O_2$	18.69	443.38	443.38	0.00	342.34 283.26 149.02 83.06	Rasanen et al. (2019)
11	Matairasenol	$C_{20}H_{22}O_6$	5.03	359.15	359.15	0.00	341.14 127.04 83.06	Huh et al. (2019)
12	Fraxetin	$C_{10}H_8O_5$	1.63	209.04	209.04	0.00	205.10 123.04 82.54 73.07	Sisó-Terraza et al. (2016)

compounds, five showed the most promising anti-inflammatory activity, with a Pa value ≥ 0.5. These included apigenin, glycitein, sakuranetin, sterubin, and fraxetin (Fig. 4). These compounds were selected for further analysis in molecular docking studies to explore their potential interactions with inflammatory targets.

Target protein prediction

The SWISS Target Prediction results revealed 31 inflammation-related proteins, consisting of five direct target proteins and 26 indirect target proteins (Fig. 5). The direct target proteins for apigenin included prostaglandin-endoperoxide synthase 2 (PTGS2), matrix metalloproteinase-9 (MMP9), and AKT serine/threonine kinase 1 (AKT1). The direct target protein for glycitein was prostaglandin-endoperoxide synthase 2 (PTGS2). The direct target protein for glycitein was PTGS2. The direct target proteins for sakuranetin and sterubin were MMP9. Meanwhile, the direct target proteins for fraxetin included MMP9, AKT1, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA), and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (PI3KCG).

Molecular docking analysis

Molecular docking was performed on the five compounds with their respective direct target proteins. The results are presented in Table 2. The compound with the most negative

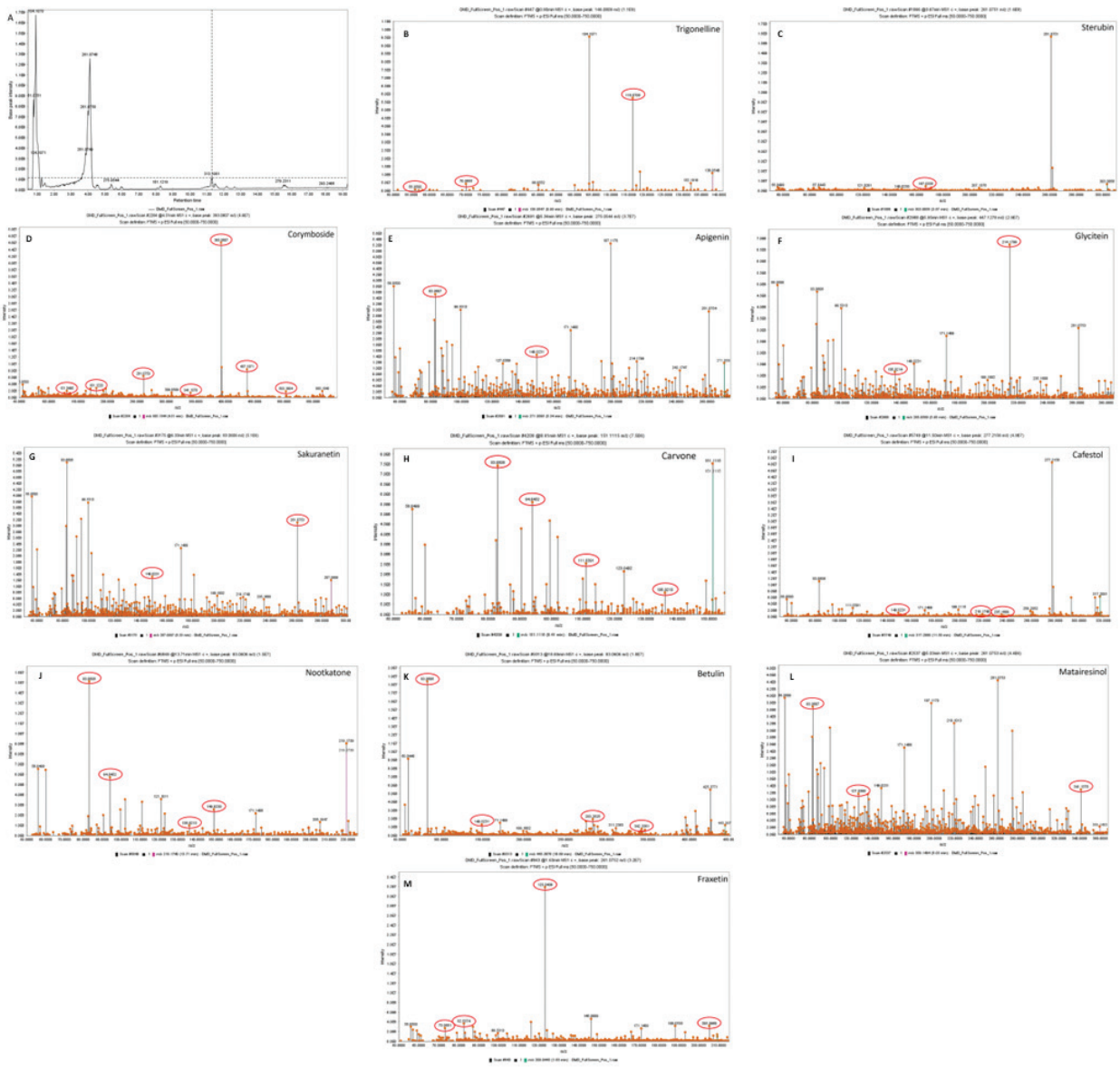


Figure 3. Chromatography and ion fragmentation patterns of bioactive compounds in *P. macrocarpa* leaf extract. Red circles indicate product ions identified as specific fragments of the corresponding compounds, confirmed through comparative analysis with literature studies.

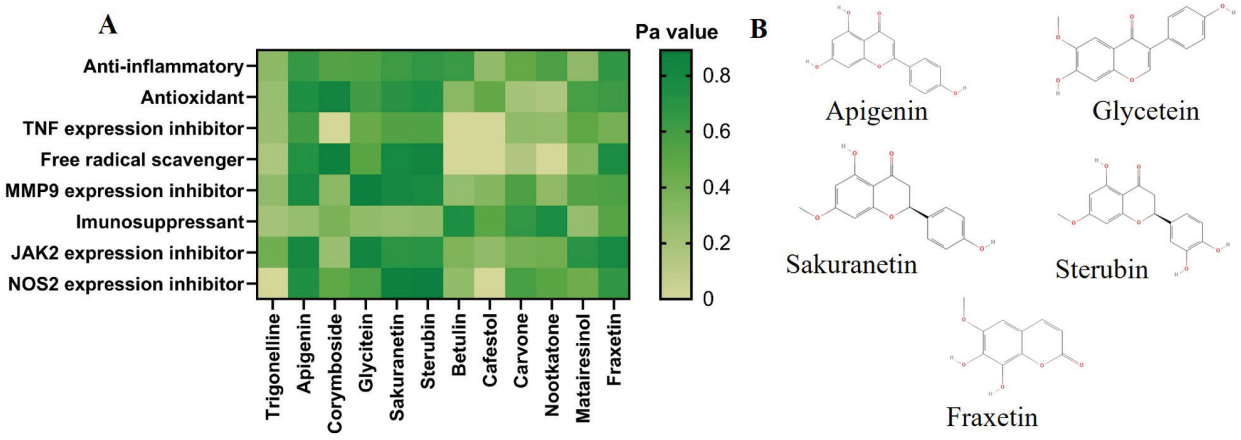


Figure 4. Compound bioactivity related to anti-inflammatory properties screening. **A.** PASS online screening; **B.** 2D structures of compounds that passed the screening.

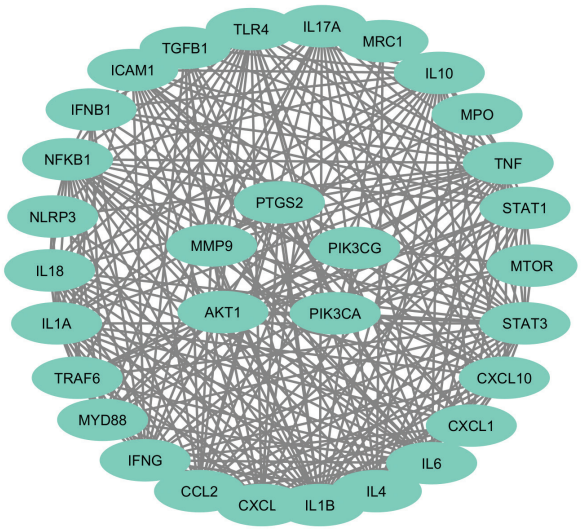


Figure 5. Prediction of inflammation-related target proteins. The outer circle indicates indirect targets, while the inner circle indicates direct targets.

binding affinity was selected for further molecular dynamics simulations. Apigenin exhibited the most negative binding affinity compared to fraxetin and approached the binding affinity value of the control for the AKT1 protein. Sterubin displayed a more negative binding affinity than the other bioactive compounds and the control for the MMP9 protein. Glycitein's binding affinity was close to that of the control for the PTGS2 protein. In contrast, fraxetin demonstrated a significantly different binding affinity compared to the control for the PI3KCA and PI3KCG proteins.

The docking results indicated that the five bioactive compounds from *P. macrocarpa* leaves interact at the same sites as the control. The molecular docking visualization results demonstrate that the compounds with the most negative binding affinity interact with the protein at several residues shared with the control (Fig. 6). These bioactive compounds exhibit similar activity to that of the control. Apigenin binds to the active site of the AKT1 protein, forming four hydrogen bonds and two hydrophobic interactions. Apigenin interacts with the same residues as

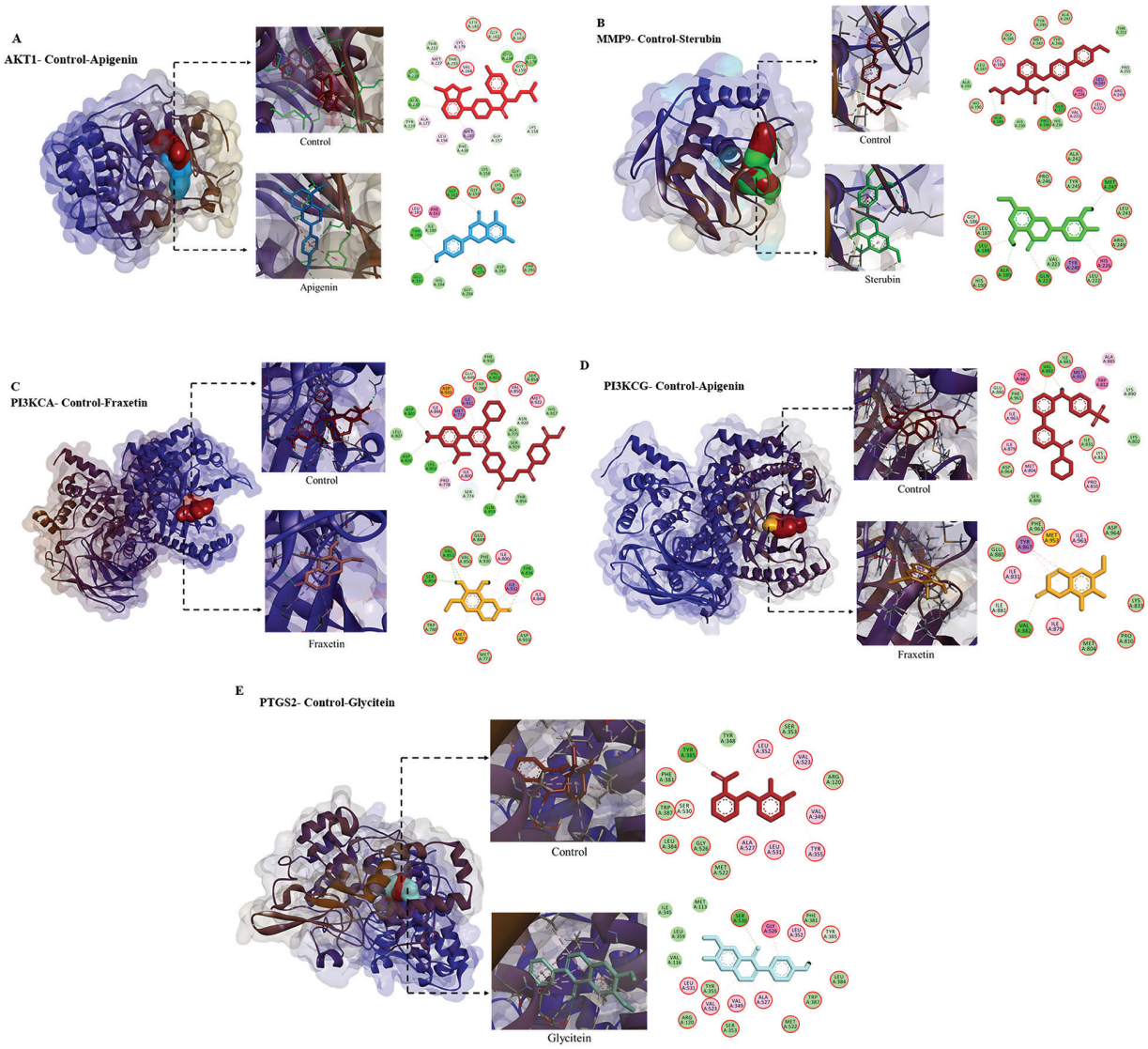


Figure 6. Visualization results of molecular docking simulation. **A.** AKT1-control-apigenin; **B.** MMP9-control-sterubin; **C.** PI3KCA-control-fraxetin; **D.** PI3KCG-control-fraxetin; **E.** PTGS2-control-glycitein. Red circles indicate identical residues between the control and the compound.

Table 2. Result of molecular docking simulation between the targeted protein and the bioactive compound of *P. macrocarpa* leaves.

No	Target Protein	PDB ID	Compound	Binding Affinity (kcal/mol)
1	AKT1	4EKL	Control (CDC0068)	-9.358
			Apigenin	-8.515*
			Fraxetin	-6.839
2	MMP9	6ESM	Control (BE4)	-10.175
			Sterubin	-10.259*
			Sakuranetin	-10.060
			Apigenin	-9.856
			Fraxetin	-9.013
3	PI3KCA	7R9V	Control (2QZ)	-9.576
			Fraxetin	-6.954*
4	PI3KCG	4FUL	Kontrol (0VU)	-10.547
			Fraxetin	-6.830*
5	PTGS2	5IKR	Control (Mefenamic acid)	-8.498
			Fraxetin	-8.255*

* Compounds with the most negative binding affinity

the control, including Gly162, Lys179, Glu191, Leu181, and Phe161. Sterubin binds to the active site of the MMP9 protein, forming four hydrogen bonds and two hydrophobic interactions. Sterubin interacts with several residues that overlap with the control, namely Leu188, Ala189, Gln227, Met247, Tyr248, and His226.

Fraxetin can target two proteins, PI3KCA and PI3KCG. Fraxetin binds to the active site of the PI3KCA protein, forming three hydrogen bonds and three hydrophobic interactions. This compound interacts with the same residues as the control, namely Ser854, Val851, Ile800, Ile848, and Ile932. At the active site of the PI3KCG protein, fraxetin forms one hydrogen bond and four hydrophobic interactions. Some overlapping residues with the control include Val882, Ile831, Ile963, Ile879, and Tyr867. In the active site of the PTGS2 protein, glycitein forms one hydrogen bond and six hydrophobic interactions, sharing residues with the control such as Ser530, Val349, Val523, Leu531, Ala527, Gly526, and Leu352.

Molecular dynamics simulation

Molecular dynamics simulations were conducted to analyze the stability of the bonds formed between the proteins and the bioactive compounds of *P. macrocarpa* leaves. The parameters used included root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF). The RMSD results indicate that nearly all compounds from *P. macrocarpa* leaves exhibit stability comparable to the control (Fig. 7A). Apigenin demonstrates stability similar to that of the control, with RMSD values over 20 ns remaining below 2.2 Å. Sterubin shows a more stable interaction compared to the control, with RMSD values lower than 2.2 Å. The MMP9–Sterubin complex is highly stable due to its more negative binding affinity than the control. Fraxetin exhibits interaction stability generally similar to that of the control. It experiences fluctuations from approximately 0 ns to ~5 ns at the target protein PI3KCA. However, fraxetin tends to be more stable when interacting with the PI3KCG protein compared to the control. The RMSF results demonstrate the fluctuations of amino acid residues in the proteins with the ligands. The PTGS2–glycitein complex has a binding affinity value close to that of the control. The RMSD results also indicate that the PTGS2–glycitein complex maintains a stable interaction over time, similar to the control. The RMSF results suggest that the four bioactive compounds from *P. macrocarpa* leaves are generally more stable and less fluctuating compared to the control (Fig. 7B).

Discussion

Inflammation is a crucial biological response that prevents pathogen spread and facilitates tissue repair. Pharmacological studies frequently utilize lipopolysaccharide (LPS)-induced RAW 264.7 macrophages as an in vitro inflammation model due to their stability (passage 10–30) and responsiveness to pro-inflammatory stimuli. LPS, a component of the outer membrane of Gram-negative bacteria, can induce the release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Stimulation of RAW 264.7 cells with LPS enhances nitric oxide (NO) production and phagocytosis (Chang et al. 2019). Dong et al. (2017) reported that LPS triggers the production of pro-inflammatory cytokines and NO. These mediators are produced through various molecular signaling pathways, underscoring the importance of developing therapeutic agents that inhibit their production via specific molecular mechanisms (Zhao et al. 2021).

Previous pharmacological studies on *P. macrocarpa* have mainly focused on single compounds from the fruit extract. The fruit extract is known for its high total phenolic and flavonoid content and strong antioxidant activity (Hendra et al. 2011). Yuliana et al. (2022) demonstrated that Predimenol isolated from *P. macrocarpa* (DLBS1442) suppresses inflammation by inhibiting iNOS and COX-2 expression and reducing levels of inflammatory markers, including NO, TNF-α, IL-1β, IL-2, and IL-6 in LPS-activated RAW 264.7 cells. Tandrasasmita et al. (2015) also reported that DLBS1442 alleviates endometriosis symptoms through antiangiogenic, anti-inflammatory, and proapop-

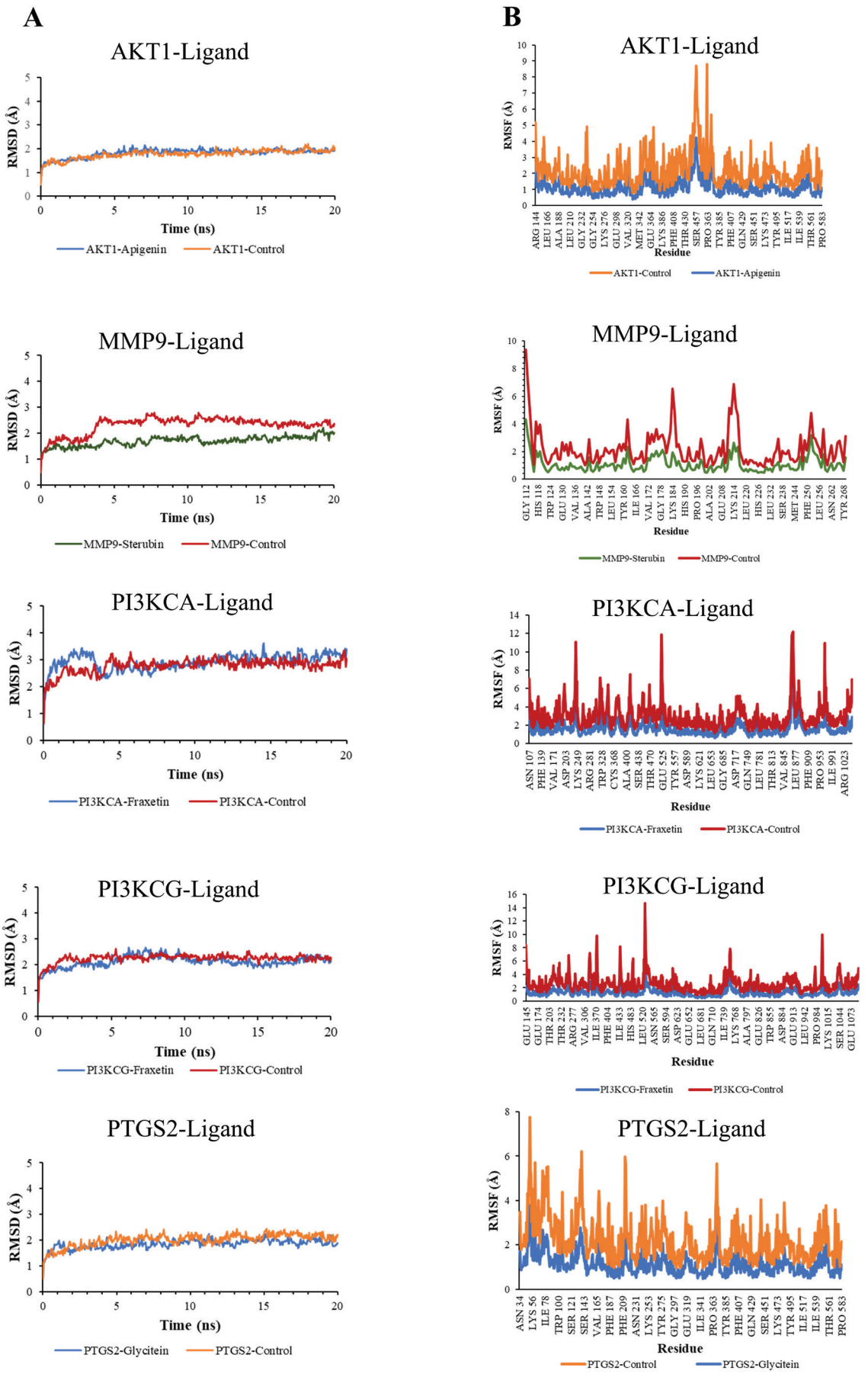


Figure 7. Results of protein-ligand complex molecular dynamics simulation. **A.** Root-mean-square deviation (RMSD) of protein-ligand complex; **B.** Root-mean-square fluctuation (RMSF) of protein-ligand complex.

totic activities by reducing NF-κB and iNOS. Hendra et al. (2011) further showed that the pericarp and mesocarp of *P. macrocarpa* fruit possess anti-inflammatory activity through iNOS inhibition in LPS/IFN-γ-induced RAW 264.7 cells. In our study, we used crude ethanol extract of *P. macrocarpa* leaves, based on prior findings indicating stronger antioxidant and anticancer activities compared to other plant parts (Christina et al. 2021b, 2021c). Suprapti et al. (2014) also found that *P. macrocarpa* leaves reduce inflammation in mice exposed to chemical carcinogens (azoxymethane and dextran sodium sulfate). Our results suggest that whole compounds from ethanol extract of *P. macrocarpa* leaves exhibit comparable anti-inflammatory potential to Predimenol (DLBS1442).

This study demonstrated that *P. macrocarpa* leaf extract significantly reduces NO production (Fig. 1), iNOS expression (Fig. 2A), and IL-1β levels (Fig. 2B), while maintaining high cell viability. NO, synthesized by inducible nitric oxide synthase (iNOS), is cytotoxic and can lead to DNA damage, inhibition of cellular respiration, and cell death (Napoli et al. 2013). iNOS expression is regulated by cytokines such as IL-1, TNF-α, and IFN-γ (Dong et al. 2017; Dwijayanti et al. 2019). LPS binds to macrophage receptors CD14, TLR-4, and CR3, initiating signaling cascades that activate NF-κB and increase iNOS expression and NO production (Chang et al. 2019). LPS also induces IL-1β expression (Djati et al. 2024), a cytokine implicated in inflammatory diseases and tumor progression (Kaneko et al. 2019). However, lower concentrations of *P. macrocarpa* leaf extract did not significantly inhibit iNOS and IL-1β expression, suggesting that its bioactive compounds may act via secondary pathways such as PI3K/AKT rather than directly targeting NF-κB.

LC-HRMS analysis identified several bioactive compounds in the ethanol extract, including trigonelline, sterubin, corymboside, apigenin, glycitein, sakuranetin, carvone, cafestol, nootkatone, betulin, matairesinol, and fraxetin. Trigonelline, an alkaloid, has been shown to reduce apoptosis and inflammation by modulating the MAPK/NF-κB pathway and lowering IL-6 levels (Uslu et al. 2025). Flavonoids such as apigenin and corymboside have been reported to suppress iNOS and COX-2 expression in LPS-induced RAW 264.7 cells (Kim et al. 2024). Sterubin exhibits strong anti-inflammatory and neuroprotective effects via Nrf2 induction in neural and microglial cells, making it a candidate for Alzheimer's disease therapy (Liang and Maher 2022). Glycitein, an isoflavone, displays antioxidant activity by suppressing free radicals and apoptosis (Mittal et al. 2024). Sakuranetin from *Prunus yedoensis* reduces iNOS, COX-2, TNF-α, IL-6, and IL-12 expression in LPS/IFN-γ-stimulated cells, confirming its role as a potent anti-inflammatory flavonoid (Kim and Kang 2016). Betulin, a terpenoid, has been shown to reduce inflammation and COX-2 activity in macrophages (Szlasa et al. 2023). Carvone, a monoterpene, possesses neuroprotective properties (Bouyahya et al. 2021), while cafestol, commonly found in coffee, has antitumor and anti-inflammatory effects (Silva et al. 2022). Nootkatone inhibits IL-1β and COX-2 in mice with leg edema (Dantas et al. 2020). Fraxetin, a coumarin, reduces pro-inflammatory mediators via the TLR4/MyD88/NF-κB pathway in osteoarthritic mice (Wang et al. 2020).

Computational analysis revealed that several compounds inhibit key inflammatory proteins such as AKT1, MMP9, PI3KCA, PI3KCG, and PTGS2. Apigenin binds to Thr291 of AKT1, a crucial phosphorylation site, destabilizing AKT1 structure and reducing its activation (Kashyap et al. 2013). Since AKT1 activation promotes iNOS expression and cytokine secretion through NF-κB, its inhibition leads to reduced inflammation. Fraxetin interacts with Val851 in PI3KCA and residues in PI3KCG (Val882, Ile831, Ile963, Tyr867), similar to known inhibitors (Jamieson et al. 2011). Because PI3K/AKT signaling drives iNOS expression and NF-κB translocation (Bhowmick and Girotti 2013), fraxetin-mediated PI3K inhibition likely attenuates inflammation.

Sterubin binds to Arg249 and His226, key residues for MMP9 activity (Lin et al. 2023). These residues are frequently targeted by MMP-9 inhibitors for enhanced ligand-protein stability, essential for therapeutic efficacy (Sanapalli et al. 2021; Malekipour et al. 2023). MMP-9 facilitates extracellular matrix degradation and generates superoxide (O_2^-) and peroxynitrite ($ONOO^-$), potent iNOS activators (O'Sullivan et al. 2014). It also promotes IL-1β maturation via NLRP3 inflammasome activation. Thus, sterubin's inhibition of MMP-9 likely contributes to reduced iNOS and IL-1β expression. Glycitein interacts with Tyr385 and Ser530 of PTGS2 (COX-2), the same sites targeted by aspirin's acetylation (Orlando and Malkowski 2016; Rouzer and Marnett 2020). As COX-2 catalyzes prostaglandin E2 (PGE2) production, which enhances NF-κB activation and IL-1β secretion, PTGS2 inhibition reduces these pro-inflammatory mediators (Hua et al. 2014).

The PI3K/AKT pathway has both pro- and anti-inflammatory effects. PI3K isoforms PIK3CA (p110α) and PIK3CG (p110γ) generate PIP3, activating AKT and leading to IKK phosphorylation and NF-κB activation (Hawkins and Stephens 2015). However, PI3K/AKT signaling may also suppress TLR4 expression and promote M2 macrophage polarization, thus limiting inflammation (He et al. 2022). Therefore, targeting PI3K/AKT may reduce inflammation but may also disrupt regulatory immune balance.

This study highlights the anti-inflammatory potential of *P. macrocarpa* leaf extract, particularly via reduction of NO, iNOS, and IL-1β, mediated by inhibition of key inflammatory proteins. The bioactive compounds may act through specific molecular pathways, such as PI3K/AKT and MMP9, rather than directly targeting primary inflammatory regulators like NF-κB. However, the study is limited to *in vitro* and *in silico* analyses. Future research should evaluate other inflammatory markers (e.g., NF-κB, TNF-α, IFN-γ), perform *in vivo* validation of efficacy and safety, and explore bioavailability and pharmacokinetics of active compounds. Overall, this research supports the potential of *P. macrocarpa* as a therapeutic candidate for inflammation-related diseases and encourages further exploration in drug development.

Conclusion

The ethanol extract of *Phaleria macrocarpa* leaves demonstrated significant anti-inflammatory activity by reducing the production of nitric oxide (NO), a key pro-inflammatory mediator, while maintaining high cell viability. The extract effectively downregulated the expression of pro-inflammatory proteins, including inducible nitric oxide synthase (iNOS) and interleukin-1β (IL-1β), particularly at concentrations of 18 and 36 μg/mL. Bioactive compounds identified in the extract may exert anti-inflammatory effects by targeting key signaling proteins such as AKT1, MMP9, PI3KCA, PI3KCG, and PTGS2, which are involved in molecular inflammation pathways. However, further studies are needed to assess the extract's effects on other inflammatory mediators and anti-inflammatory cytokines. *In vivo* experiments should be conducted to validate these findings and to evaluate the pharmacokinetics and bioavailability of the active compounds. These results support the therapeutic potential of *P. macrocarpa* leaves in the development of anti-inflammatory agents.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

The authors declared that no experiments on animals were performed for the present study.

Use of commercially available immortalised human and animal cell lines: RAW 264.7 mouse macrophage cells, obtained from Elabscience® (Catalog No.: CL-0190, Elabscience Biotechnology Inc., USA).

Use of AI

No use of AI was reported.

Funding

The authors express their sincere gratitude to Universitas Brawijaya for supporting this research through the 2024 Excellent Basic Research Program (Research Contract No: 00140.13/UN10.A0501/B/PT.01.03.2/2024).

Author contributions

A.S. carried out the experiment and drafted the manuscript. Y.I.C. and D.R.D analyzed the data and drafted the manuscript. A.S. and N.W. interpreted the data. MSD conceived and designed the study and supervised the project.

Author ORCIDs

Assa'idatus Shofiah https://orcid.org/0009-0003-8785-5668
Yuyun Ika Christina https://orcid.org/0000-0002-9185-1147
Dinia Rizqi Dwijayanti https://orcid.org/0009-0009-1971-0284
Nashi Widodo https://orcid.org/0000-0002-1126-498X
Muhammad Sasmito Djati https://orcid.org/0000-0002-1245-2528

Data availability

All of the data that support the findings of this study are available in the main text.

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