HAYATI
Journal of Biosciences

Vol. 32 No. 2, March 2025 287-299
DOI:10.4308/hjb.32.2.287-299
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

Anti-Inflammatory Activity of *Phyllanthus niruri* L. Leaf Extract: *In Vitro* Study on RAW 264.7 Macrophage Cells and *In Silico* Analysis

Ngurah Intan Wiratmini[1], Made Pharmawati[1*], Anak Agung Sagung Alit Sukmaningsih[1], Sentot Joko Raharjo[2], Wahyu Widowati[3]

[1]Biology Study Program, Faculty of Mathematics and Natural Sciences, Udayana University, Jimbaran, Kuta Selatan, Badung, Bali, Indonesia
[2]Diploma of Food and Pharmacy Analyst, Polytechnic of Health of Putra Indonesia Malang, Malang, Indonesia
[3]Faculty of Medicine, Maranatha Christian University, Bandung 40164, Indonesia

ARTICLE INFO

Article history:
Received June 4, 2024
Received in revised form July 8, 2024
Accepted September 30, 2024

KEYWORDS:
anti-inflammatory,
COX-2,
cytokines,
IL-1β,
RAW 264.7 cells,
TNF-α

ABSTRACT

Phyllanthus niruri L. is a medicinal plant recognized for its wide range of therapeutic benefits, particularly its anti-inflammatory properties. This research focused on assessing the impact of *P. niruri* leaf extract on pro-inflammatory cytokines *in vitro* using RAW 264.7 macrophage cells, analyze its phytochemical composition, and validate its potential anti-inflammatory mechanisms through molecular docking studies. The extract was analyzed for its phytochemical composition using LC-MS, revealing the presence of sesquiterpene glycosides, flavonoid glycosides, and tannins among the identified compounds. Cell viability tests showed that the extract was not toxic to the cells at concentrations up to 50 µg/ml. Treatment with the extract significantly reduced the levels of pro-inflammatory cytokines TNF-α and IL-1β in LPS-induced RAW 264.7 cells, with the most effective concentration being 50 µg/ml. Molecular docking studies further supported the anti-inflammatory potential of the extract by demonstrating its ability to inhibit COX-2 and TNF-alpha. These findings suggest that *P. niruri* leaf extract has promising anti-inflammatory properties and needs further investigation as a potential treatment for inflammatory diseases. The *in silico* study provided insights into the molecular interactions between the extract's compounds and inflammatory targets, supporting its anti-inflammatory mechanism of action.

1. Introduction

The body's defense mechanism reacts to harmful agents like pathogens, dead cells, and toxic substances, by initiating the inflammatory process. This response attracts leukocytes in the blood, activates tissue macrophages, and produces a series of mediators (Megha *et al.* 2021). Macrophages play a crucial role in the natural immune system during the inflammatory response. The main functions include engulfing foreign agents, eliminating apoptotic cells, and developing and resolving inflammation (Sieweke and Allen 2013). Uncontrolled acute inflammation gradually develops into numerous persistent inflammatory diseases such as neurodegenerative, cancer, and cardiovascular diseases (Zhou *et al.* 2016).

Lipopolysaccharide (LPS), present in the outer membrane of Gram-negative bacteria, stimulates host's immune reaction to inflammation. This leads to the immune system producing elevated levels of chemokines, cytokines, and pro-inflammatory mediators (Noailles *et al.* 2018). During persistent inflammation, activated macrophages secrete elevated levels of chemokines such as interleukin-8 [IL-8], CC-chemokine ligand 5 [CCL 5], and eotaxin; cytokines such as tumor

* Corresponding Author
E-mail Address: made_pharmawati@unud.ac.id

necrosis factor-alpha [TNF-α], IL-1α, IL-1β, IL-6, and granulocyte-macrophage colony-stimulating factor [GM-CSF]. Additionally, they release pro-inflammatory mediators like nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE2) (Abdulkhaleq *et al.* 2018; Laveti *et al.* 2013). Macrophages subjected to LPS during microbial infection activate the immune system to release cytokines and chemokines, leading to inflammatory responses. Therefore, a primary focus of therapeutic strategies for treating inflammatory diseases is the inhibition of macrophage activation caused by LPS (Shin *et al.* 2019; Diao *et al.* 2019; Jeong *et al.* 2019).

Synthetic chemical drugs have been widely used as an anti-inflammatory in the treatment of inflammatory diseases (Vishal *et al.* 2014). However, these drugs have many limitations including serious side effects, expensive, and limited availability in many countries (Sostres *et al.* 2013). As an alternative, herbal medicines offer a valuable and significant alternative for the prevention and treatment of inflammatory diseases (Ghasemian *et al.* 2016; Gessner *et al.* 2017). Anti-inflammatory herbal medicines are plants that have demonstrated anti-inflammatory properties in traditional applications, clinical trials, or experimental evaluations (Adegbola *et al.* 2017). Common anti-inflammatory herbal plants are *Ribes nigrum, Harpagophytum procumbens, Salvia officinalis, Persea americana, Uncaria tomentosa, Elaeagnus angustifolia, Curcuma longa, Zingiber officinale, Rosmarinus officinalis, Borago officinalis, Oenothera biennis, Urtica dioica*, etc. These plants typically contain compounds such as steroids, flavonoids, alkaloids, polyphenols, glycosides, terpenoids, curcumin, GLA, linear aliphatic alcohols (eg. tetracosanol), harpagoside, phenolic diterpenes, which have anti-inflammatory properties (Yatoo *et al.* 2018).

A plant widely used as a medicine is *Phyllanthus niruri* L, an annual plant from the Euphorbiaceae family (Tharakan 2012). *Phyllanthus niruri* has been reported to have various medicinal properties, including antiviral activity against hepatitis B, hepatoprotective, antimicrobial, anticancer, and hypocalcemic effects (Narendra *et al.* 2012; Ullah *et al.* 2020). Additionally, it inhibits the protein receptor of COVID-19 (Marhaeny *et al.* 2021). According to Bagalkotkar *et al.* (2016), *P. niruri* has phytochemical activity such as flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins, and saponins. Flavonoids in plants have been extensively researched for their anti-inflammatory, antioxidant, and analgesic properties (Zhang *et al.* 2013; Choy *et al.* 2019; Ferraz *et al.* 2020).

The objectives of this study were to determine the effectiveness of *P. niruri* leaf extract in reducing inflammation in in LPS-induced RAW 264.7 macrophage cells *in vitro* and analyzing the interaction between its compounds and target proteins, and to analyze the interaction between compounds found in *P. niruri* leaf extract and target proteins. It is expected that *P. niruri* extract can be an alternative medicine for reducing inflammation issues.

2. Materials and Methods

2.1. Plant Collection and Extraction of *P. niruri*

Fresh *P. niruri* leaves were obtained from Cianjur Regency, West Java, Indonesia (7°12'12.54" S; 107°50'38.38" E). The plant identification was based on Flora of Java (Backer *et al.* 1968). The dried leaves were chopped and then macerated using 70% ethanol (Merck). The extraction process was carried out at Borobudur Natural Herbal Industry, Semarang, to obtain Good Manufacturing Practice (GMP) standard herbal extracts (https://jamuborobudur.co.id/; Arif *et al.* 2024)

2.2. Identification of Active Compounds in *P. niruri* Leaf Extract using LC-MS

Active compound identification using Liquid Chromatography-Mass Spectrometry (LC-MS – Shimadzu 8040 LC/MS) was carried out on the 70% ethanol extract of leaves. A total of 0.5 grams of 70% ethanol extract was dissolved in 50 ml methanol (Merck). The solution was filtered using a 0.22-micron syringe filter, put into a 2 ml vial, and 1µL was injected into the LC-MS (Tukiran *et al.* 2021).

2.3. Cell Culture and Viability Test

This research used RAW 264.7 cells (ATCC® TIB-71™) obtained from Aretha Medica Utama, Center for Biomolecular and Biomedical Research, Bandung, Indonesia. The RAW 264.7 cells were grown in Dulbecco's Modified Eagle's Medium-High Glucose/DMEM-High Glucose (Biowest L0103) supplemented with 10% Fetal Bovine Serum (FBS) (Bowest, S181B-500) and 1% Antibiotic-Antimyotic (ABAM) (Biowest, L0010 -100), 0.1% Gentamicin (Gibco, 15750060) and 1% Amphotericin B (Biowest, L0009-050). The cells were incubated at 37°C and 5%

CO_2. The growth medium was changed every 2-3 days during cell treatment. Cells that have grown in a flask were examined under an inverted microscope until the cells reach about 70-80% confluence. The cells were subsequently rinsed with Phosphate Buffer Saline (PBS) and collected using a scraper (Widowati *et al.* 2016).

Cell viability testing was carried out using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Elabscience, E-CK_A362). The cells were counted using a hemocytometer. Cells were grown at a density of 1 $\times 10^4$ cells/well in 96 well plates and incubated for 24 h at 37°C with 5% CO_2. After that, cells were induced by LPS by replacing 180 µL of fresh culture medium containing 1 µg/ml LPS. The cells were incubated for 18 h. As much as 20 µL EDP (Elastin-derived peptide) 200; 150; 100; 50; 25; 12.5; 6.25 mg/ml was added in each well. The culture was incubated for 24 h at 37°C with 5% CO_2. After 24 h, 20 µL of CCK-8 (Cell Counting Kit-8) solution was included to each well, and incubated for 2 h at 37°C with 5% CO_2. Absorbance was determined with spectrophotometry (Multiskan GO Thermo Scientific 51119300) at a reading wavelength of 450 nm (Widowati *et al.* 2016; Zhang *et al.* 2020).

2.4. Measurement of TNF-α and IL-1β Concentrations

Cytokine testing was carried out by quantifying the supernatant resulting from the secretion of TNF-α and IL-1β from culture cells with the Mouse TNF-α ELISA Kit (Elabscience, E-EL-M0049) and IL-1β ELISA Kit (Elabscience, E-EL-M0037). The steps follow the manufacturer's protocol. RAW 264.7 cells were grown in 6-well plates at a density of 1 × 10^5 cells per well and then incubated until confluence cell was around 80% (for 48 h). Cells were induced using 1µg/ml LPS and incubated for 18 h. Cells that had been induced by LPS were given *P. niruri* extract (50; 12.5; and 3,125 µg/ml) and incubated for 24 h. Supernatants were collected to detect IL-1β and TNF-alpha levels (Widowati *et al.* 2019).

2.5. *In Silico* Analysis

2.5.1. Searching for Amino Acid Sequence

Molecular docking analysis was conducted to assess the interaction between the active flavonoid compounds in *P. niruri* leaves against the Cyclooxygenase-2/COX-2 and TNF-alpha. Amino acid sequences comprising COX-2 and TNF-alpha were retrieved from The Research Collaboratory for Structural Bioinformatics Protein Data Bank (https://www.rcsb.org). The 3D structure of the protein was downloaded for COX-2 (ID PDB: 6COX) and TNF-alpha (ID PDB: 2az5).

2.5.2. Preparation of Ligand Compounds

The ligands in this study were compounds found in *P. niruri* leaf extract. The ligands were then interacted with a receptor, and their chemical structures were obtained by accessing PubChem. The PubChem Open Chemistry Database was used to obtain the 3D structures of 20 compounds from *P. niruri* leaf extract. The 3D structure of the compounds in the *.sdf file format was then transformed into *.pdb files using Avodgadro software ver 1.2.0.

2.5.3. Molecular Docking of Ligand with Protein Target

The COX-2 (ID PDB: 6COX) and TNF-alpha (ID PDB: 2az5) three-dimensional structure was prepared individually by eliminating water molecules, ions, and cofactors present in the protein. The protein was modified by incorporating hydrogen atoms and then assigned Gasteiger charges. A grid box was configured to interact with the active site residues of the protein, measuring of (40 × 40 × 40) and centered at coordinates (0.449; 0.063; 0.016) (Ahmad *et al.* 2020). Protein preparation was performed using AutoDock Tools 1.5.7 software. The files were then saved in *pdbqt format. The software was also used to prepare *P. niruri* leaf extract active compound. The ligand files were then saved in *pdbqt format for molecular docking purposes.

Docking simulations involving the compounds from *P. niruri* leaf extract and target proteins were conducted using AutoDock Vina v1.2.3 software. After preparing the receptor and ligand, the docking operation was initiated with the command prompt. The results of the docking were subsequently visualized using Discovery Studio 4.1 software.

The interaction between protein and ligand from the docking results were then examined and visualized with BIOVIA Discovery Studio software. The results were analysed by determining the ligand conformation that had the best binding affinity value and analysing binding interactions based on amino acid residues in 2D and 3D form. The binding affinity value was determined based on the most negative value and compared to the native ligand.

2.6. Data Analysis

The *in vitro* studies used three replications. The data were expressed as mean ± standard deviation and analyzed using One-Way ANOVA. Non-homogeneously distributed data was followed by the Dunnet T3 Post hoc Test and Tukey's HSD Post-hoc Test for homogeneously distributed data. Data processing uses SPSS software (Version 21.0).

3. Results

3.1. Identification of Compounds in *P. niruri* Leaf Extract by LC-MS

The LC/MS-MS analysis produces a chromatogram that shows the heights and weights of molecules in the extract. The chromatogram (Figure 1) shows the results for a 70% ethanol extract of *P. niruri* leaves. Out of 107 identified compounds, 20 main components were analyzed using LC-MS in the 70% ethanol extract of *P. niruri* leaves (Table 1). Those 20 compounds constitute the major components, while others compounds are considered minor components, based on their percentage content in the LC-MS analysis results.

3.2. Cell Viability

The cell viability was tested to make sure that the cells were healthy before the bioactive test and that the extract concentrations were not toxic. The results showed a significant decrease in the viability of RAW 264.7 cells ($P<0.05$) in the positive control and DMSO control groups compared to the negative control and the groups of cells that received *P. niruri* leaves extract at concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 µg/ml. This shows that LPS caused the cells to release harmful inflammatory substances. There was a significant drop in cell viability ($P<0.05$) at the highest concentration of 200 µg/ml (Figure 2). The reduction in cell viability was accompanied by a notable increase in cell death ($P<0.05$), as shown in Figure 3. The test results showed that *P. niruri* leaves extract at concentrations of 50, 25, 12.5, 6.25, and 3.125 µg/ml were safe for LPS-induced RAW 264.7 cells because they had viability values above 80%. Based on these results, doses of 50, 12.5, and 3.13 µg/ml of *P. niruri* leaves extract were used for the pro-inflammatory test.

3.3. Effects of *P. niruri* Leaf Extract on the LPS-stimulated IL-1β

The ELISA results showed that the treatment group receiving 50 µg/ml of *P. niruri* leaves extract had the greatest reduction in IL-1β cytokine levels. The decrease in IL-1β levels increased with higher concentrations of the extract (Figure 4).

3.4. Effects of *P. niruri* Leaf Extract on the LPS-stimulated TNF-alpha

The analysis of IL-1β cytokine levels using ELISA showed that the treatment group most effective in reducing TNF-alpha levels was the one treated with 50 µg/ml of *P. niruri* leaves extract. This aligns with the group that was most effective in reducing IL-1β levels, as the decrease in TNF-alpha levels matched the increase in the concentration of *P. niruri* leaf extract administered (Figure 5).

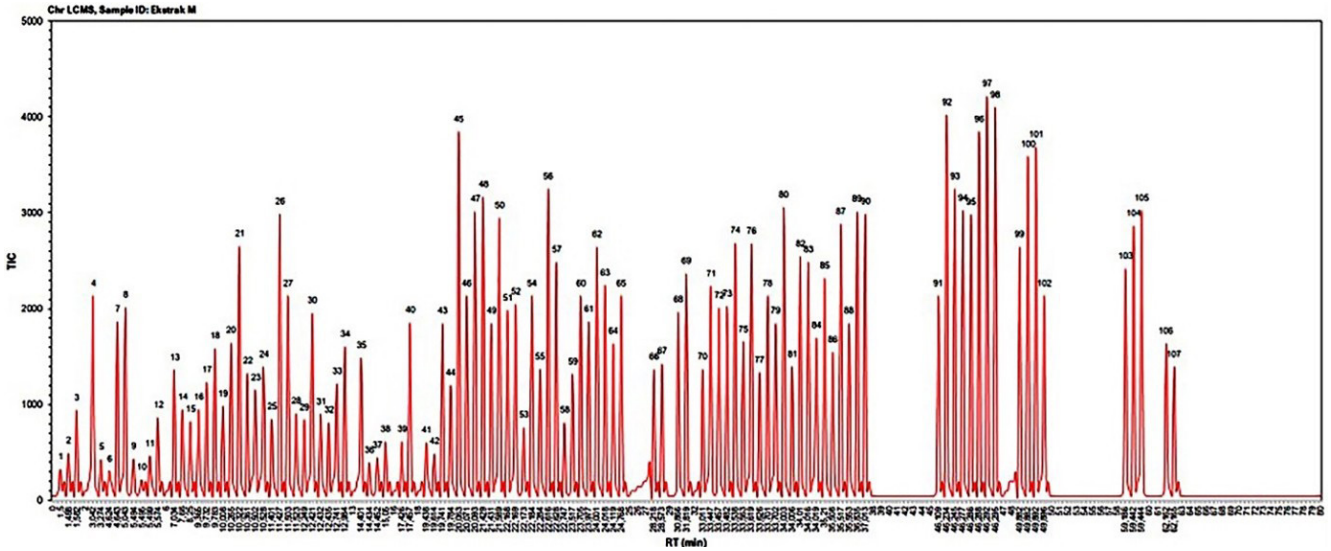


Figure 1. Chromatogram of *P. niruri* leaf extract identification with LC-MS

Table 1. Active compounds in *P. niruri* leaf extract

Peak number tR	RT (min)	Composition (%)	Compound	Classification
26	11,427	1.49353	quercetin	flavonoid
45	20,063	1.92375	kaempferol-4'-rhamnoside	flavonoid glycoside
47	20,093	1.50648	apigenin-7-O-glucoside	flavonoid glycoside
48	21,429	1.58317	kaempferol-3-O-rhamnoside	flavonoid glycoside
56	22,616	1.62624	quercetin-3-O-rhamnoside	flavonoid glycoside
80	34,003	1.52971	kaempferol-7-rhamnoside-4'glucoside	flavonoid glycoside
87	35,517	1.44251	rutin	flavonoid glycoside
89	36,835	1.50649	quercetin-3-gentiobioside	flavonoid glycoside
90	37,013	1.49365	corilagin	hydrolyzable tannins
92	46,234	2.01118	phyllanthostatin 6	sesquiterpene glycoside
93	46,245	1.62596	apigenin-7-rhamnoside-4'rutinoside	flavonoid glycoside
94	46,277	1.51365	epigallocatechin-(4β8)-epigallocatechin-3-O-gallate ester	tannin
95	46,286	1.49143	kaempferol-3-glucoside-2"rhamnoside-7-rhamnoside	flavonoid glycoside
96	46,288	1.92375	apigenin-7-rutinoside-4'-transcaffeate	flavonoid glycoside
97	46,292	2.10735	phyllanthusol B	sesquiterpene glycoside
98	46,295	2.04984	phyllanthusol A	sesquiterpene glycoside
100	49,882	1.79375	phyllanthostatin 1	sesquiterpene glycoside
101	49,892	1.84321	quercetin-3,7,4'-triglucoside	flavonoid glycoside
104	59,442	1.43201	phyllanthostatin 2	sesquiterpene glycoside
105	59,444	1.51321	(+)-phyllanthostatin 3	sesquiterpene glycoside

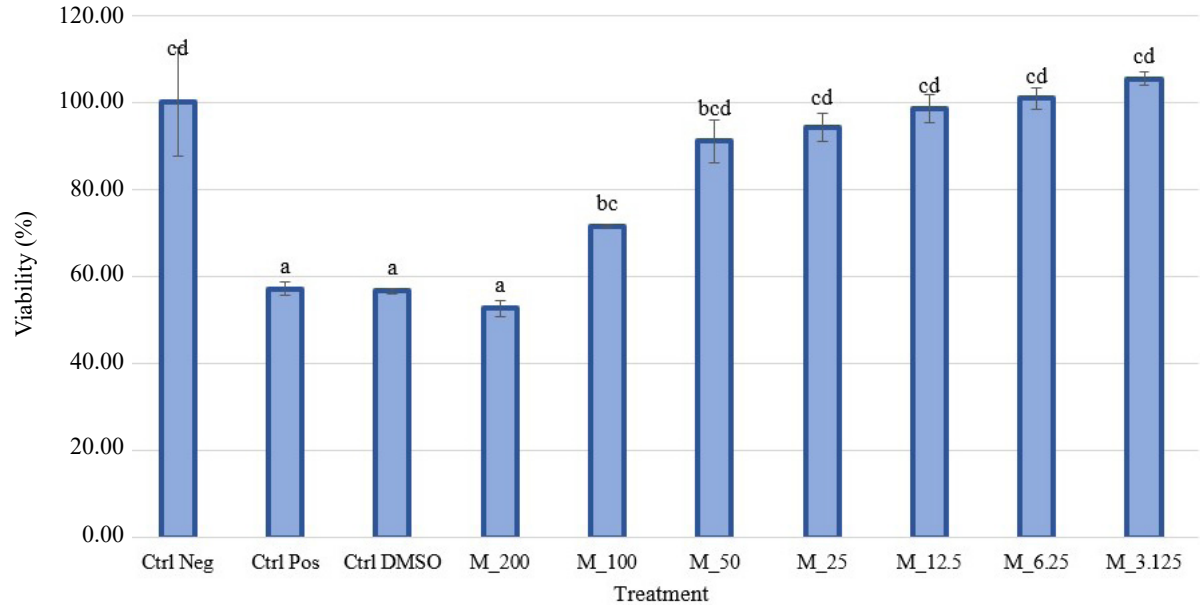


Figure 2. Cell viability of LPS-induced RAW 264.7 cells post *P. niruri* leaf extract administration. Ctrl Neg: Negative Control; Ctrl Pos: Positive Control; Ctrl DMSO: Positive Control + 10% DMSO; M_200: 200 µg/ml *P. niruri* leaves extract; M_100: 100 µg/ml extract; M_50: 50 µg/ml extract; M_12.5: 12.5 µg/ml extract; M_3.125: 3.125 µg/ml extract. Data presented as mean ± SD. Different superscript indicates significant difference ($p<0.05$) based on post hoc Dunnet T3 test

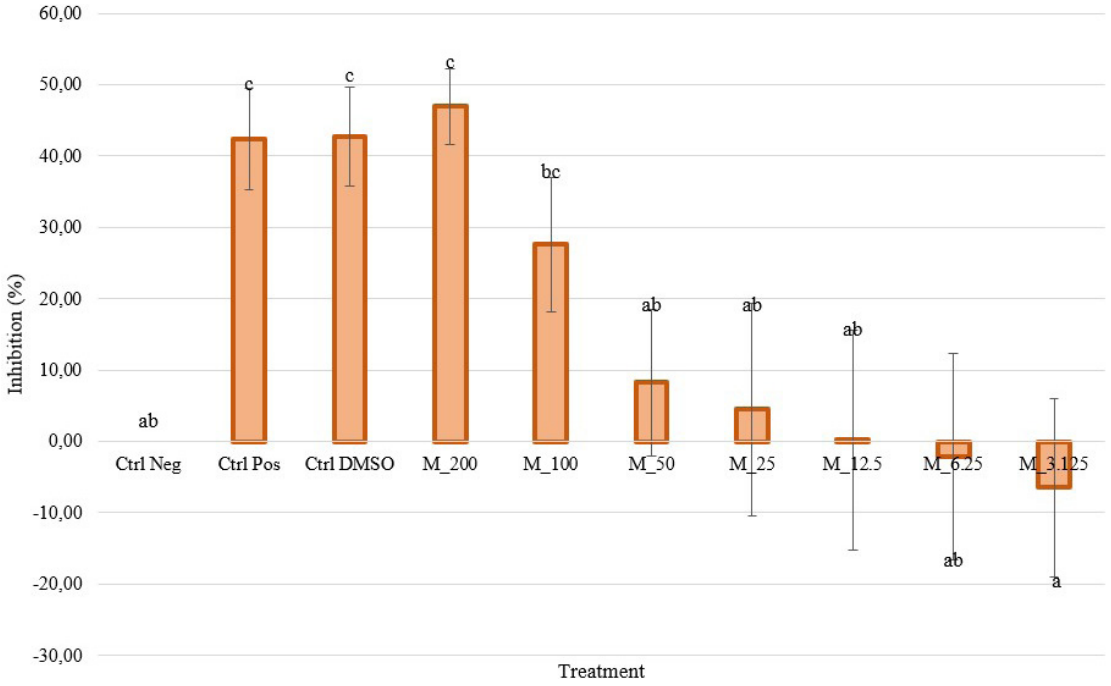


Figure 3. Cellular inhibition of LPS-induced RAW 264.7 cells post *P. niruri* leaf extract administration. Ctrl Neg: Negative Control; Ctrl Pos: Positive Control; Ctrl DMSO: Positive Control + 10% DMSO; M_200: *P. niruri* leaves extract 200 µg/ml; M_100: 100 µg/ml extract; M_50: 50 µg/ml extract; M_12.5: 12.5 µg/ml extract; M_3.125: 3.125 µg/ml extract. Data are presented as mean ± SD. Different superscript marks indicate significant differences ($p<0.05$) using Dunnet's T3 post hoc test

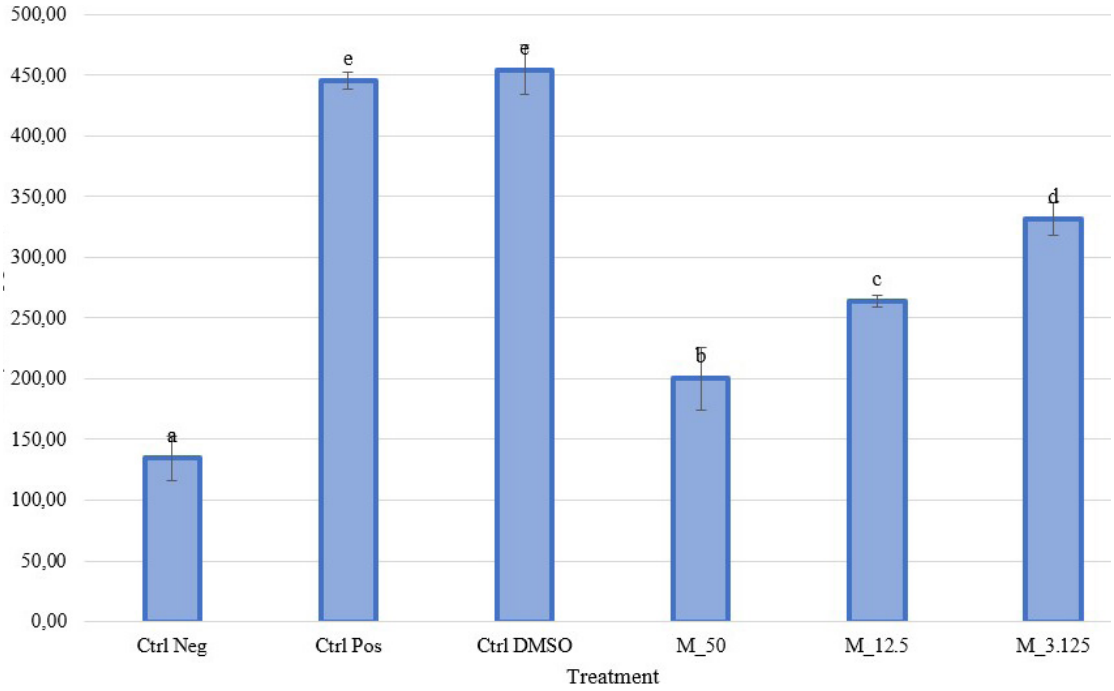


Figure 4. Graph of IL-1β levels in RAW 264.7 cells against LPS-induced *P. niruri* extract. Ctrl Neg: Negative Control; Ctrl Pos: Positive Control; Ctrl DMSO: Positive Control + DMSO 10%; M_50: 50 µg/ml *P. niruri* extract; M_12.5: 12.5 µg/ml extract; M_3.125: 3.125 µg/ml extract. Data are presented as mean ± SD. Different superscript letters indicate significant differences for each treatment based on the Tukey HSD ANOVA test with $p<0.05$

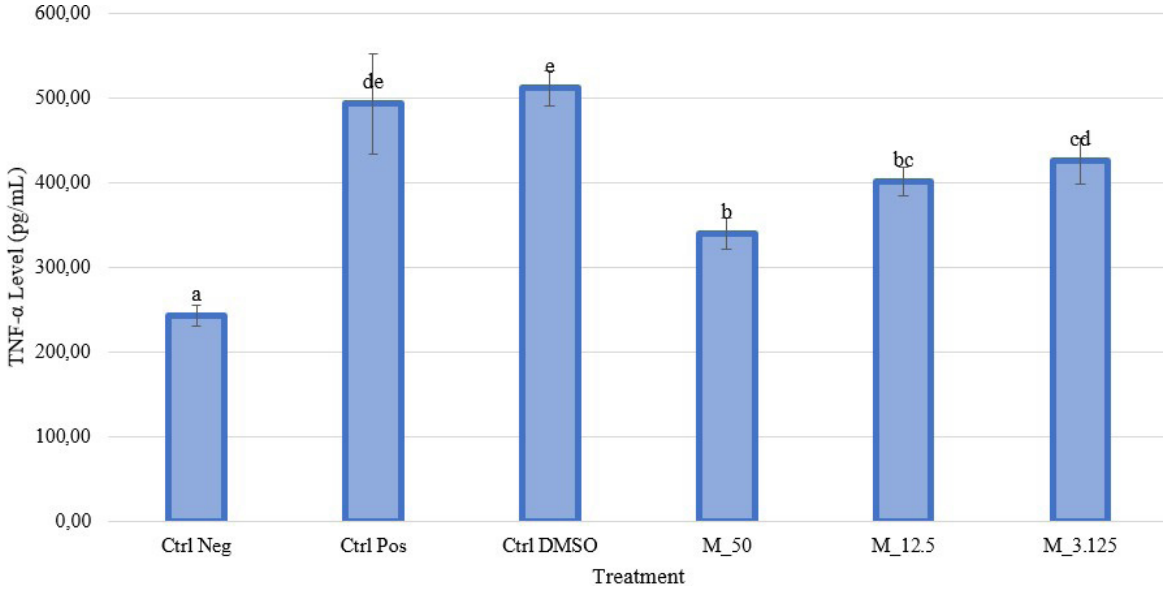


Figure 5. Graph TNF-α levels in RAW 264.7 cells treated with LPS-induced *P. niruri* extract. Ctrl Neg: Negative Control; Ctrl Pos: Positive Control; Ctrl DMSO: Positive Control + 10% DMSO; M_50: 50 µg/ml *P. niruri* extract; M_12.5: 12.5 µg/ml extract; M_3.125: 3.125 µg/ml extract. Data presented as mean ± SD. Different superscript letters indicate significant differences between each treatment based on ANOVA Tukey HSD test with $p<0.05$

3.5. Binding Energy of Docking Selected Compounds from *P. niruri* Extract to COX-2 and TNF-alpha Protein

The binding energy, which represents the strength of interaction between selected compounds from *P. niruri* leaves extract and the protein target (COX-2 and TNF-alpha), is presented in Table 2. The results showed that the compounds exhibited varying degrees of binding affinity to the proteins. A lower binding energy (more negative) indicates a stronger interaction between the compound and the protein. The compound with the strongest interaction with COX-2 and TNF-alpha was phylostantin-2. Docking, binding pattern, and energy values of phyllanthostatin 2 to TNF-alpha and phyllanthostatin 2 to COX-2 are shown in Figures 6 and 7.

4. Discussion

The phytochemical analysis of *P. niruri* leaf extract in this study differs from that reported by Rusmana *et al.* (2017), where no alkaloid and terpenoid content was found. The LC-MS analysis revealed 20 major compounds out of 107 identified compounds. Some of these compounds belong to the group of secondary metabolites, including sesquiterpene glycosides, flavonoid glycosides/flavonoids, and tannins/hydrolyzable tannins.

Leaf extract of *P. niruri* was tested on LPS-induced RAW 264.7 cells. The highest concentration of extract (200 µg/ml) was toxic to the cells, while lower concentrations (50, 25, 12.5, 6.25, and 3.125 µg/ml) did not affected cell viability. During inflammation caused by LPS, RAW 264.7 cells release PGE2 and pro-inflammatory cytokines such as IL-1β and TNF-alpha (Widowati *et al.* 2019). The measurement of TNF-alpha secretion through supernatants of cell culture can be seen in Figure 5. The concentration of TNF-alpha in cell cultures induced by LPS (positive control and DMSO control) significantly increased ($P<0.05$) compared to the TNF-alpha concentration in cell cultures treated with *P. niruri* leaves extract. This means that inflammation induction using LPS has been shown to trigger inflammation. The treatment group that most effectively reduced TNF-alpha levels was the group treated with 50 µg/ml of *P. niruri* leaf extract.

The decrease in TNF-alpha levels in RAW 264.7 cells induced by LPS was caused by the secondary metabolites sesquiterpenoid glycoside, sesquiterpene glycoside, and tannin contained in *P. niruri* leaves extract. The phytochemical test of *P. niruri* leaf extract found the presence of saponins, phenols, steroids, terpenoids, alkaloids, flavonoids, and tannins. Gardi *et al.* (2015) reported that the flavonoids contained in red betel leaves extract can inhibit the expression of TNF-alpha by regulating nuclear factor kappa B (NF-κB), which is

Table 2. Binding energy of docking selected compounds from *P. niruri* extract to COX-2 and TNF-alpha protein

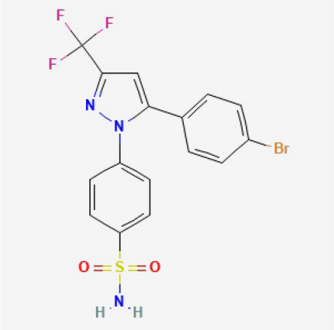
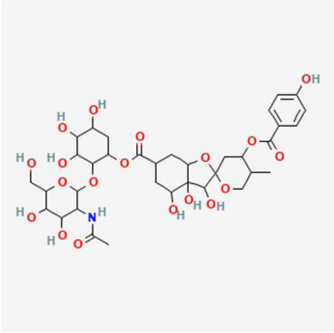
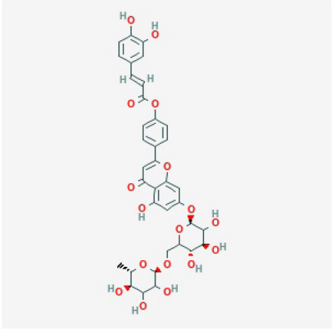
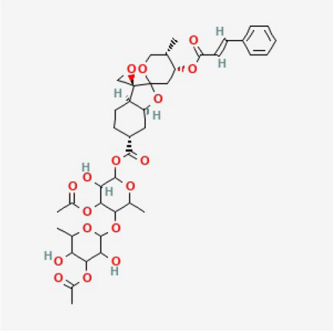
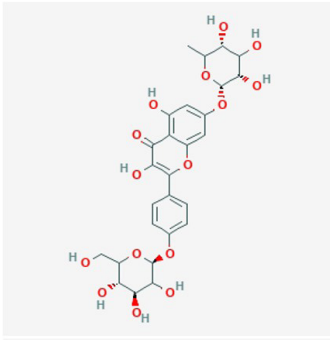
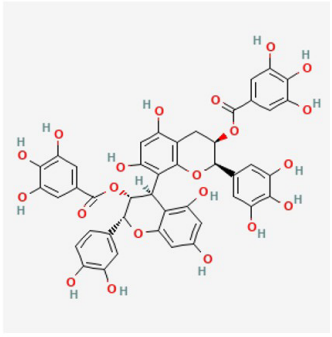
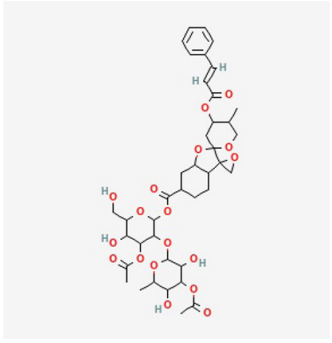
Compound	Structure	Energy	
		6 COX-Cyclooxgenase-2	2az5_tnf_alpha
Native: 1-Phenylsulfonamide-3-trifluoromethyl-5-parabromophenylpyrazole SC-558		10.78 kcal/mol	
Phyllanthusol A		-6.531 kcal/mol	-8.162 kcal/mol
Apigenin-7-rutinoside-4'-transcaffeate		-6.72 kcal/mol	-8.29 kcal/mol
Phyllanthostatin 1		-5.337 kcal/mol	-7.588 kcal/mol

Table 2. Continued

Compound	Structure	Energy	
		6 COX-Cyclooxgenase-2	2az5_tnf_alpha
Kaempferol-7-rhamnoside-4'glucoside		-4.763 kcal/mol	-8.364 kcal/mol
Epigallocatechin-(4β8)-epigallocatechin-3-O-gallate ester		-5.812 kcal/mol	-9.267 kcal/mol
Phyllnthostatin 2		-6.546 kcal/mol	-8.94 kcal/mol

a transcription factor for the expression of TNF-alpha. Activation of nuclear factor kappa B (NF-κB) can also cause upregulation of COX-2 (Cyclooxygenase-2) expression (Yang *et al.* 2021). COX-2 is an enzyme that leads to inflammation. The COX-2 protein catalyses the biosynthesis of prostaglandins from arachidonic acid. Prostaglandins produced by COX-2 contribute to the inflammatory process by increasing vasodilation, enhancing blood vessel permeability, sensitizing pain receptors, and other effects (Ricciotti and FitzGerald 2011). The expression of the COX-2 gene increases in human oral mucosa after inflammation induction (Khan *et al.* 2007).

Flavonoid compounds can directly inhibit the activity of COX and lipoxygenase enzymes which causes inhibition of prostaglandin biosynthesis which is the end product of the COX and lipoxygenase pathway. Inhibition of prostaglandin biosynthesis can inhibit leukocyte

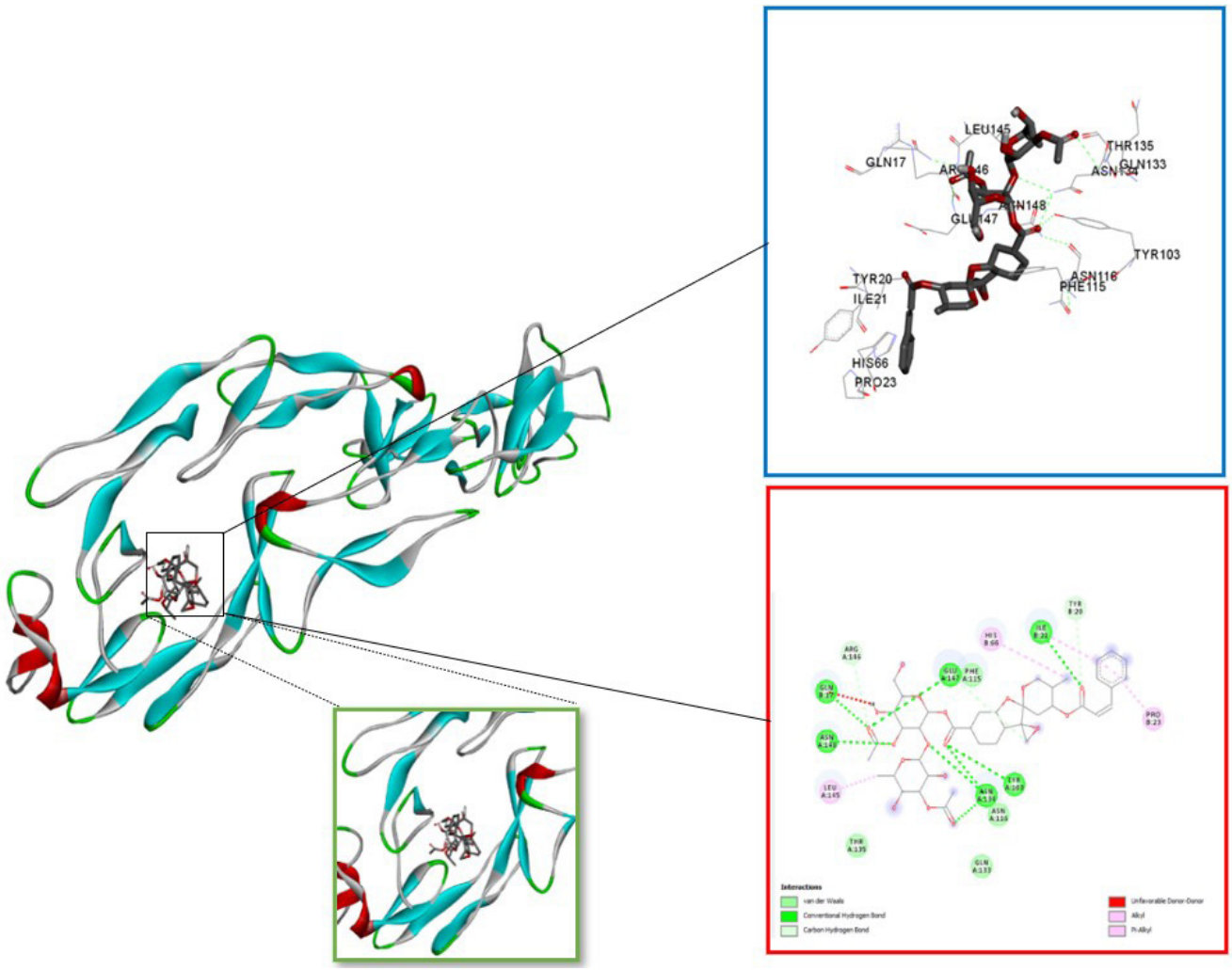


Figure 6. Interaction of Phyllanthostatin 2 to TNF-alpha (ID PDB : 2az5): -8.94 kcal/mol

accumulation and neutrophil degranulation, thereby directly reducing the release of arachidonic acid by neutrophils, as well as inhibiting histamine release. Under normal circumstances, leukocytes travel unrestricted along the endothelial wall. However, during inflammation, various mediators produced by the endothelium and complement factors trigger leukocyte adhesion to the endothelial surface. Flavonoid administration can reduce leukocyte levels and suppress complement activation, thereby decreasing leukocyte adhesion to the endothelium and diminishing the body's inflammatory response (Nijveldt *et al.* 2001).

Ju *et al.* (2022) noted that numerous natural compounds, such as natural phenols, flavonoids, terpenoids, alkaloids, and other hybrids, have been recognized for their COX-2 inhibitory properties or identified as COX-2 inhibitors. The extract from the *Phyllanthus amarus* plant has been shown to suppress COX-2 protein expression and reduce TNF-alpha production in LPS-stimulated human U937 macrophages (Harikrishnan *et al.* 2018).

In line with the results of measuring TNF-alpha levels, IL-1β levels in the positive control and DMSO control were also significantly higher $P<0.05$ compared to culture cells treated with *P. niruri* extract. The treatment group that best reduced IL-1β levels was the treatment group that was given the addition of 50 µg/ml extract. The decrease in IL-1β levels corresponds to the increase in the concentration of *P. niruri* leaves extract given. In a study by Hou *et al.* (2019), the targets of flavonoids in reducing inflammation and inhibiting recurrent colitis and colorectal cancer were TNF-alpha, and IL-1β.

Medicinal substances sourced from natural ingredients have the ability to interact with one or more receptors (Siswandono 2000). The interaction between substances and receptors can be assessed through docking studies. Such studies have been shown to evaluate the effectiveness

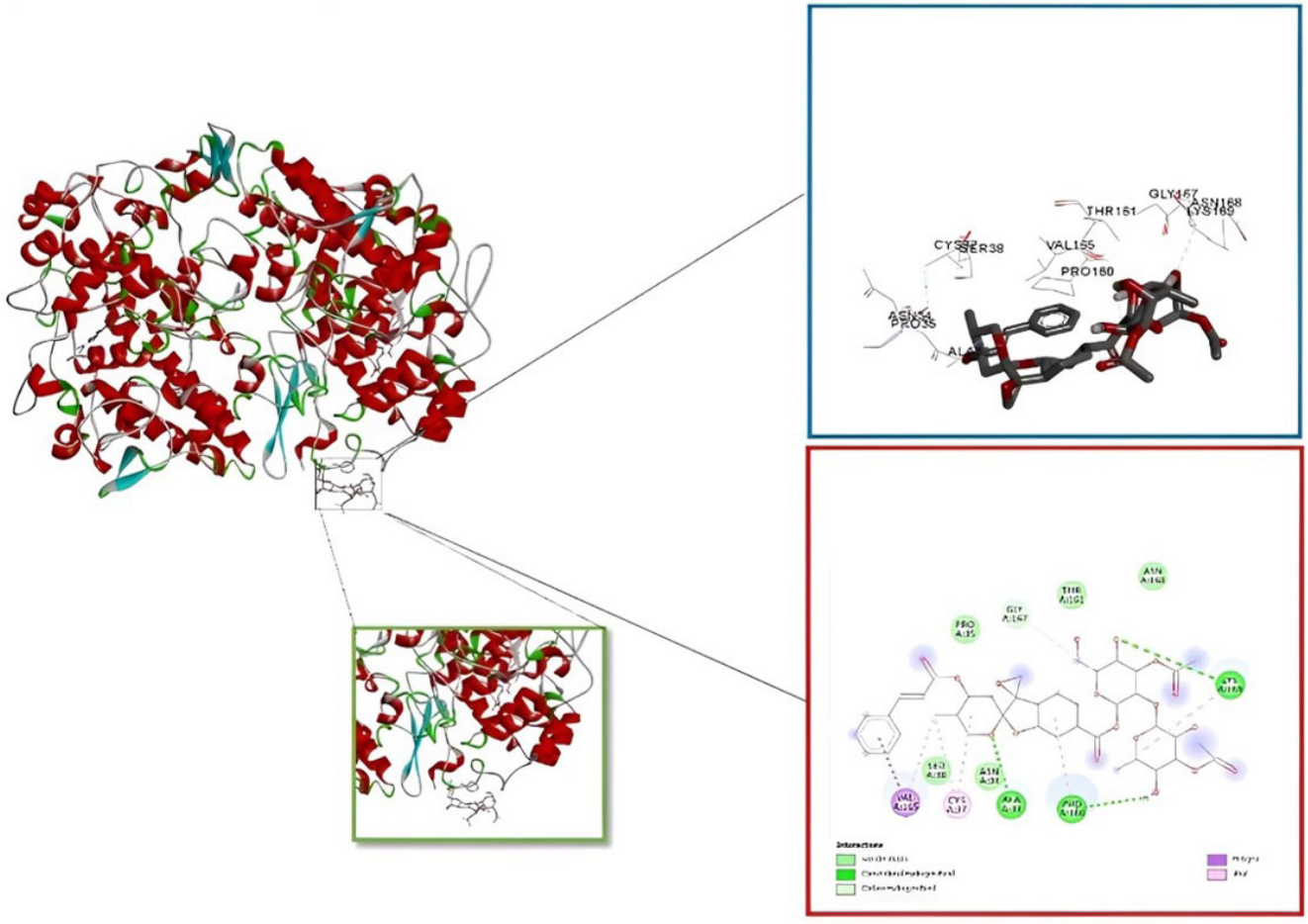


Figure 7. Interaction of Phyllanthostatin 2 to COX-2 (ID PDB : 6COX) : -6.546 kcal/mol

of a compound with a receptor by analyzing its binding energy and bond types (Yanuar 2012). The molecular docking showed the ability of the sesquiterpenoid glycoside, flavonoid glycoside, and tannin compound groups to inhibit the COX-2 and TNF-alpha enzymes.

The binding energy of the compound phyllanthostatin-2 (sesquitepenoid glucoside) shows the ability to inhibit the COX-2 enzyme (binding energy = -6.546 kcal/mol) approaching the comparison inhibitor compound SC-558 (binding energy = -10.78 kcal/mol). Likewise, the compound apigenin-7-rutinoside-4'-transcaffeate (flavonoid glycoside) has a binding energy of -6.72 kcal/mol. Phyllanthostatin-2 epigallocatechin-(4β8)-epigallocatechin-3-O-gallate ester (tannin) also has a relatively better inhibitor compared to other compounds from *P. niruri* leaves extract analysed by LC-MS.

Phylostantin-2 inhibits the COX-2 enzyme's activity. This leads to a decrease in prostaglandin synthesis, thereby reducing inflammation. This mechanism similar to inhibition of COX by flavonoid compounds (Nijveldt *et al.* 2001). Phylostantin-2 also binds to TNF-alpha and inhibits its activity. This prevents TNF-alpha from interacting with its receptors on cell surfaces, thereby blocking the downstream signalling pathways that result in the generation of additional pro-inflammatory cytokines and the activation of inflammatory cells (Muth *et al.* 2023).

The results of this molecular docking analysis are also in accordance with other natural compounds that inhibit COX-2 and TNF-alpha, such as carnosic acid (CA) and carnosol (CS) compounds derived from *Rosmarinus officinalis* L. Results from molecular docking showed the capacity of extract to inhibit COX-2 and TNF-alpha enzymes, which are consistent with the invitro assessment of IL-1β and TNF-alpha in RAW 264.7 cells stimulated by LPS.

In conclusion, ethanol extract of *P. niruri* leaves contains sesquiterpene glycosides, flavonoids, flavonoid glycosides, and tannins. Quantitative ELISA analysis of IL-1β and TNF-alpha showed that the most effective treatment for reducing inflammatory markers was *P. niruri* extract at a concentration of 50 µg/ml. The extract works by inhibiting COX-2 and TNF-alpha.

Acknowledgements

We acknowledge Udayana University for funding this research with grant number B/78.621/UN14.4.A/PT.01.03/2022. We also thank Aretha Medika Utama for providing the research facility.

References

Abdulkhaleq, L.A., Assi, M.A., Abdullah, R., Zamri-Saad, M., Taufiq-Yap, Y.H., Hezmee, M.N.M., 2018. The crucial roles of inflammatory mediators in inflammation: a review. *Veter. World.* 11, 627–635.

Adegbola. P., Aderibigbe, I., Hammed, W., Omotayo, T., 2017. Antioxidant and anti-inflammatory medicinal plants have a potential role in the treatment of cardiovascular disease: a review. *Am. J. Cardiovasc. Dis.* 7, 19-32.

Ahmad, J., Ikram, S., Ahmad, F., Rehman, I.U., Mushtaq, M., 2020. SARS-CoV-2 RNA Dependent RNA polymerase (RdRp) - A drug repurposing study. *Heliyon.* 6, e04502. DOI:10.1016/j.heliyon.2020.e04502

Arif, J., Nasution, A.N., Nasution, S.W., Widowati, W., Dewi, N.S.M., Kusuma, H.S.W., 2024. Antioxidant activities and potential wound healing effects of meniran (*Phyllanthus niruri*) extract gel. *Folia Medica Indonesiana.* 60, 69-77.

Bagalkotkar, G., Sagineedu, S.R., Saad, M.S., Stanlas, J., 2006. Phytochemicals from *Phyllanthus niruri* Linn. and their pharmacological properties: a review. *J. Pharm. Pharmacol.* 58, 1559-70.

Backer, C.A., Bakhuisen van den Brink, R.C., 1968. Flora of Java (Spermatophytes Only). Vol. III Wolters-Noordhoff, N.V. – Groningen-The Netherlands.

Choy, K.W., Murugan, D., Fang Leong, X., Abas, R., Alias, A., Mustafa, M.R., 2019. Flavonoids as natural anti-inflammatory agents targeting Nuclear Factor-Kappa B (NFκB) signaling in cardiovascular diseases: a mini review. *Front. Pharmacol*. 10, 1295. DOI:10.3389/fphar.2019.01295

Diao, J., Chi, Z., Guo, Z., Zhang, L., 2019. Mung bean protein hydrolysate modulates the immune response through NF-κB pathway in lipopolysaccharide-stimulated RAW 264.7 macrophages. *J. Food Sci.* 84, 2652–2657.

Ferraz, C.R., Carvalho, T.T., Manchope, M.F., Artero, N.A., Rasquel-Oliveira, F.S., Fattori, V., Casagrande, R., Verri, W.A., 2020. Therapeutic potential of flavonoids in pain and inflammation: Mechanisms of action, pre-clinical and clinical data, and pharmaceutical development. *Molecules*. 25, 1-35.

Gardi, C., Bauerova, K., Stringa, B., Kuncirova, V., Slovak, L., Ponist, S., Drafi, F., Bezakova, L., Tedesco, I., Acquaviva, A., Bilotto, S., Russo, G.L. 2015. Quercetin reduced inflammation and increased antioxidant defense in rat adjuvant arthritis. *Arch. Biochem. Biophys.* 583, 150–157.

Ghasemian, M., Owlia, S., Owlia, M.B. 2016. Review of anti-inflammatory herbal medicines. *Adv. Pharmacol. Sci.* 2016, 9130979.

Gessner, D.K., Ringseis. R., Eder, K. 2017. Potential of plant polyphenols to combat oxidative stress and inflammatory processes in farm animals. *J. Anim. Physiol. Anim. Nutri*. 101, 605-28.

Harikrishnan, H., Jantan, I., Haque, MA., and Kumolosasi, E. 2018. Anti-inflammatory effects of *Phyllanthus amarus* Schum. & Thonn. through inhibition of NF-κB, MAPK, and PI3K-Akt signaling pathways in LPS-induced human macrophages. *BMC Complement Altern. Med.* 18, 224. DOI:10.1186/s12906-018-2289-3

Hou, S., Yuan, Q., Yu, N., Liu, B., Huang, G., Yuan, X. 2019. Cardamonin attenuates chronic inflammation and tumorigenesis in colon. *Cell Cycle.* 18, 3275–3287.

Jeong, Y.H., Oh, Y.C., Cho, W.K., Yim, N.H., Ma, J.Y., 2019. Hoveniae semen Seu Fructus ethanol extract exhibits anti-inflammatory activity via MAPK, AP-1, and STAT aignaling pathways in LPS-stimulated RAW 264.7 and mouse peritoneal macrophages. *Mediators Inflamm.* 2019, 9184769.

Ju, Z., Xu, J., Howell, D.C., Li, Z., Chen, F.E., 2022. Recent development on COX-2 inhibitors as promising anti-inflammatory agents: The past 10 years. *Acta Pharm Sin B.* 12, 790-2807.

Khan, A.A., Iadarola, M., Yang, H.Y.T., Dionne, R.A., 2007. Expression of COX −1 and −2 in a clinical model of acute inflammation. *J. Pain.* 8, 349–354.

Laveti, D., Kumar, M., Hemalatha, R., Sistla, R., Naidu, V.G., Talla, V., Nagpal, R., 2013. Anti-inflammatory treatments for chronic diseases: a review. *Inflamm. Allergy Drug. Targets.* 12, 349-361.

Marhaeny, H.D., Widyawaruyanti, A., Widiandani, T., Hafid, A.F. and Wahyuni, T.S., 2021. Phyllanthin and hypophyllanthin, the isolated compounds of *Phyllanthus niruri* inhibit protein receptor of corona virus (COVID-19) through *in silico* approach. *J. Basic Clin. Physiol. Pharmacol.* 32, 809–815.

Megha, K.B., Joseph, X., Akhil, V., Mohanan, P.V., 2021. Cascade of immune mechanism and consequences of inflammatory disorders. *Phytomedicine.* 91, 153712. DOI:10.1016/j.phymed.2021.153712

Muth, K.N., Rech, J., Losch, F.O., Hoerning, A., 2023. Reversing the inflammatory process-25 years of tumor necrosis factor-α inhibitors. *J. Clin. Med.* 12, 5039. https://doi.org/10.3390/jcm12155039

Narendra, K., Swathi, J., Sowjanya, K.M., Satya, A.K., 2012. *Phyllanthus niruri*: a review on its ethno botanical, phytochemical and pharmacological profile. *J. Pharm. Res.* 5, 4681-4691.

Nijveldt, R.J., Nood, E.V., Hoorn, D.E.C.,Boelens P.G., Norren K.V., danLeeuwen, P.A.M. 2001. Flavonoids: a review of probable mechanismsof action and potential application. *Am. J. Clin. Nutr.* 74, 418-425.

Noailles, A., Maneu, V., Campello, L., Lax, P., Cuenca, N., 2018. Systemic inflammation induced by lipopolysaccharide aggravates inherited retinal dystrophy. *Cell Death Dis.* 9, 1–18.

Ricciotti, E., Fitzgerald, G.A., 2011. Prostaglandins and inflammation. *Arterioscler. Thromb. Vasc. Biol.* 31, 986-1000.

Rusmana, D., Wahyudianingsih, R., Elisabeth, M., Balqis, Maesaroh, Widowati, W. 2017. Antioxidant activity of *Phyllanthus niruri* extract, rutin and quercetin. *Indones. Biomed. J.* 9, 84-90.

Shin, W.B., Dong, X., Kim, Y.S., Park, J.S., Kim, S.J., Go, E.A., Kim, E.K., Park, P.J., 2019. Anti-inflammatory effects of *Batillaria multiformis* water extracts via NF-êB and MAPK signaling pathways in LPS-induced RAW 264.7 cells. *Adv. Exp. Med. Biol.* 1155, 1001–1014.

Sostres, C., Gargallo, C.J., Lanas, A., 2013. Nonsteroidal anti-inflammatory drugs and upper and lower gastrointestinal mucosal damage. *Arthritis Res. Ther.* 15, S3.

Sieweke, M.H., Allen, J.E., 2013. Beyond stem cells: self-renewal of differentiated macrophages. *Science.* 342, 1242974.

Siswandono, B., 2000, *Kimia Medisinal* 1. Airlangga University Press, Surabaya.

Tharakan, S.T., 2012. Taxonomy of the genus *Phyllanthus*, in: Kutthan, R., Harikumar, K.B. (Eds.), *Phyllanthus Species: Scientific Evaluation and Medicinal Application*. CRC Press, Taylor and Francis Group, New York, pp. 3-21.

Tukiran, Suyatno, Frisca Nadya Safitri, F.N. 2021. Identification of the chemical constituents of the selected fraction of the dichloromethane extract of *Syzygium samarangense* stem bark using LC-ESI-MS and evaluation its potential as antifungal agent. *Indones. J. Chem.* 21, 340-349.

Ullah, A., Munir, S., Badshah, S.L., Khan, N., Ghani, L., Poulson, B.G., Emwas, A.H., Jaremko, M. 2020. Important flavonoids and their role as a therapeutic agent. *Molecules*, 25, 5243.

Vishal, V., Sharma, G.N., Mukesh, G., Ranjan, B. 2014. A review on some plants having anti-inflammatory activity. *J. Phytopharmacol*. 3, 214-221.

Widowati, W., Darsono, L., Suherman, J., Fauziah, N., Maesaroh, M., Erawijantari, P.P. 2016 Anti- inflammatory effect of mangosteen (*Garcinia mangostana* L.) peel extract and its compounds in LPS-induced RAW264.7 cells. *Nat. Prod. Sci*. 22, 147-53

Widowati, W., Prahastuti, S, Ekayanti, N. L. W., Munshy, U.Z., H S W Kusuma, H. S. W., Wibowo, S. H. B., Amalia, A.,Widodo, W. S., and Rizal, R. 2019. Anti-inflammation assay of black soybean extract and its compounds on lipopolysaccharide-induced RAW 264.7 cell. *J. Phys. Conf. Ser.* 1374 012052.,1-10.

Yatoo, M.I., Gopalakrishnan, A., Saxena, A., Parray O.R., Tufani, N.A., Chakraborty, S., Tiwari, R., Dhama, K., and Iqbal, H.M.N., 2018. Anti-inflammatory drugs and herbs with special emphasis on herbal medicines for countering inflammatory diseases and disorders - a review. *Recent Pat. Inflamm. Allergy. Drug Discov.* 12, 39-58.

Yanuar, A., 2012. *Penambatan Molekuler. Praktek dan Aplikasi pada Virtual Scrining*. Fakultas Farmasi Universitas Indonesia, Depok.

Yang, C.M., Yang, C.C., Hsiao, L.D., Yu, C.Y., Tseng, H.C., Hsu, C.K., Situmorang, J.H., 2021. Upregulation of COX-2 and PGE2 induced by TNF-α mediated through TNFR1/MitoROS/PKCα/P38 MAPK, JNK1/2/FoxO1 cascade in human cardiac fibroblasts. *J. Inflamm. Res.* 14, 2807-2824.

Zhang, Y.R., Tang, T., Huang, Y., Li, Y., Xie, L., Zhan, X., Tang, Z.H., Tang, J.T., 2013. Study on anti-oxidation and anti-inflammatory activity of *Capsicum annuum* leaves polyphenols. *J. Food Sci. Technol.* 8, 346–349.

Zhang, L., Haddouti, E.M., Beckert, H., Biehl, R., Pariyar, S., Rüwald, J.M., Li, X., Jaenisch, M., Burger, C., Wirtz, D.C., Kabir, K., Schildberg, F.A., 2020. Investigation of cytotoxicity, oxidative stress, and inflammatory responses of tantalum nanoparticles in THP-1- derived macrophages. *Mediators Inflamm.* 2020, 3824593. DOI:10.1155/2020/3824593

Zhou, Y., Hong, Y., Huang, H., 2016. Triptolide attenuates inflammatory response in membranous glomerulo-nephritis rat via downregulation of NF-κB signaling. *Kidney Blood Press Res.* 41, 901-910.