Anti-Inflammatory therapeutic potential of Bungur (*Lagerstroemia speciosa* L.) leaves ethanolic extract against LPS-activated macrophage through TLR4/MD2 signaling: a computational study

Nuraini Rosyadah[1], *Dinia Rizqi* Dwijayanti[1,2], *Yuslinda* Annisa[1], *Fairuz Sarah* Kamila[1], *Muhammad* Hermawan Widyananda[1], *Siti Mariyah* Ulfa[3], and *Nashi* Widodo[1*]

[1]Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia
[2] Research Center of Complementary Medicine and Functional Food, Universitas Brawijaya, Malang, Indonesia
[3]Chemistry Department, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia

Abstract. Excessive inflammation, driven by macrophage activation and nitric oxide (NO) production, underlies various diseases. This study investigates the anti-inflammatory potential of bungur (*Lagerstroemia speciosa* L.) leaf extract. We characterized its phytochemical profile and evaluated the effects using *in silico* approaches. Bungur leaves (*L. speciosa* L.) were extracted using MAE extraction and subjected to phenol, flavonoid, terpenoid, alkaloid content and antioxidant DPPH assay. The compound profile was analyzed using LC-HRMS. Identified compounds were screened *in silico* for drug-likeness, bioactivity, and membrane permeability. Molecular docking and molecular dynamics simulations were conducted to evaluate the interaction and stability of selected compounds with TLR4/MD2 complex. This study demonstrated that bungur leaf extract exhibited higher levels of phenolic and flavonoid compounds than terpenoids and alkaloids. This finding was confirmed by the LC-HRMS analysis, which revealed a dominance of phenolic and flavonoid compounds in the extract. The extract also showed excellent DPPH antioxidant activity, as evidenced by its low IC_{50} value. *In silico* studies identified luteolin, luteolin 7-sulfate, and quercetin as the three best compounds, which acted as potent competitive inhibitors of TLR4 activation. Therefore, compounds contained in bungur leaf exhibited promising anti-inflammatory activity through TLR4 inhibition.

1 Introduction

* Corresponding author: widodo@ub.ac.id

Inflammation is the body's response to various stimuli, including pathogens, damaged cells, and irritants. It is brought on by the production of cytokines and chemokines from mast cells and macrophages, and it can result in swelling, redness, discomfort, and heat [1]. Chronic inflammation is a growing issue due to unhealthy lifestyles and environments and can regulate carcinogenesis through mechanisms like accelerated cell proliferation, evasion of apoptosis, increased angiogenesis, and metastasis. Chronic inflammation can recruit and activate immune cells into the tumor microenvironment, causing tissue damage, DNA damage, and transcription factors at the convergence of oncogenic signaling pathways. Although it is thought to facilitate the development of cancer, it may also have tumour-suppressive properties and enhance immunotherapy responses [2, 3, 4]. Treatment for inflammation often involves NSAIDs, which have many side effects, including digestive disorders, cardiovascular disease risk, kidney disorders, blood pressure disorders, and cancer. Alternative treatments using traditional herbs are considered safer and have minimal side effects [4].

Macrophages are immune cells that initiate the body's immune response and can be divided into two main phenotypes: M1 and M2 macrophages. M1 macrophages have anti-inflammatory properties and M2 macrophages maintain immune response homeostasis by stimulating Th2 cell responses, mediating parasite clearance, and regulating extracellular matrix formation [1, 6]. Macrophage activation triggers the release of LPS, a gram-negative inflammatory molecule, which interacts with TLR4 and other adaptors, causing various responses, including glycosylation, MAP kinase activity, NF-κβ, and interferon production. TLR4, as the main LPS receptor also interacts with MD-2 as its co-activator must be inhibited to suppress its inflammation activity [7].

Bungur (*Lagerstroemia speciosa* L.) is a native plant found in Indonesia, known for its medicinal properties and cooking ingredients [8, 9]. The leaves as the most abundant part of bungur are used in traditional medicine, particularly for treating diabetes, obesity, high blood pressure, skin diseases, and malaria [10, 11]. The plant's potential lies in its bioactive compounds, including triterpenes, quercetin, isoquercetin, tannin, triterpenoids, corosolic acid, flavones, and glycosides [12]. Bungur leaf extraction uses ethanol solvent due to its wide extraction area from various compound groups [13].

The wide use of bungur leaves as a traditional medicinal material shows the potential of Bungur leaves as an anti-inflammatory agent, but research data that supports this activity against LPS-induced TLR4 activation has not been widely studied. Various tests need to be conducted such as phytochemical profile analysis of bungur leaf ethanol extract. In addition, the compound profile of bungur leaf ethanol extract needs to be identified to analyze the content of compounds that can act as anti-inflammatory agents, which are then supported by in silico data. Therefore, this research is very important to do to support data on the potential utilization of Bungur leaf ethanol extract as an alternative anti-inflammatory agent.

2 Material and methods

2.1 Bungur leaf extraction

Bungur leaf ethanol extract was prepared using the microwave-assisted extraction (MAE) method [14]. Bungur leaves were obtained from Balai Materia Medica, Batu, East Java, Indonesia (Batch No: 181018.BGR.F.R.008). The leaves were grounded into powder and was put into the vessel of the MAE equipment and mixed with 96% ethanol in a ratio of 1:10. The device was operated with a holding temperature protocol of 50°C, warming up 50°C for 5 minutes, holding time for 10 minutes, and 5 minutes cooling down. The 96% ethanol was

filtered with filter paper, and the solvent was evaporated on a rotary evaporator with a rotation of 50 rpm and a water bath temperature of 50°C [15].

2.2 Total Flavonoid Content (TFC)

The total flavonoid content of bungur leaf ethanol extract was calculated using the aluminium chloride colorimetric test method adapted from [16]. The standard solution used was quercetin dissolved in 96% ethanol with different concentration variations (1.5625 - 400 µg/mL). The ethanol extract of bungur leaves with a concentration of 100 µg/mL as much as 50 µL or quercetin was added to the 96-wellplate. 96% ethanol solution was used as a blank. A total of 10 µL $AlCl_3$ (10% w/v) was added in each well. $AlCl_3$ solution was dissolved in 96% ethanol. Then the mixture was added 150 µL of 96% ethanol. Next, 10 µL of 1 M CH_3COONa was added to the mixture and incubated for 40 minutes at room temperature under dark conditions. Absorbance measurements were measured at a wavelength of 405 using a MultiSkan SkyHigh Microplate Spectrophotometer microplate reader (ThermoFischer Scientific, USA). The total flavonoid content was calculated by calculating the equation obtained from the standard curve of 0-100 µg/mL quercetin. The unit used from the calculation was mgQE/g.

2.3 Total Phenolic Content (TPC)

The total phenol content of Bungur leaf ethanol extract was calculated using the Folin-Ciocalteu assay method modified from [16]. A total of 10 µL of extract (100 µg/mL) or standard in the form of gallic acid (1.5625 - 100 µg/mL) was added to a 96-well plate. The extract and standard were both dissolved in 96% ethanol. Folin-Ciocalteu 10% solution prepared in distilled water was added as 100 µL to each well. The microplate was incubated for 5 minutes. In all wells, 100 µL Na_2CO_3 (7.5% w/v) was added and incubated for 90 minutes at room temperature under dark conditions. The absorbance was then measured at a wavelength of 725 nm using a MultiSkan SkyHigh Microplate Spectrophotometer (ThermoFischer Scientific, USA). The value of total phenol content was calculated by calculating the equation obtained from the standard curve of 0-100 µg/mL gallic acid and the unit was mgGAE/g.

2.4 Total Terpenoid Content (TTC)

The total content of terpenoid compounds in the ethanol extract of bungur leaves was analyzed using a colorimetric-based method modified from [17]. The total terpenoid test standard uses linalool compounds to obtain a standard curve with a concentration variation of 0.1296 µM - 12.965 µM (1 mg/200 µL - 100 mg/200 µL). A total of 200 µL of extracts and standards were put into a microtube, then 1500 µL of chloroform was added and the mixture was homogenized with a vortex until mixed and then left for 3 minutes. 100 µL of concentrated sulfuric acid (H_2SO_4) was added to each mixture then incubated at room temperature and dark conditions for 1.5 to 2 hours. After incubation, a reddish-brown precipitate formed and the supernatant was slowly removed without changing the precipitate. The precipitate at the bottom of the microtube was added with 1500 µL of 95% methanol (vol/vol) and homogenized until the precipitate was completely dissolved. The mixture was then transferred into a cuvette and the absorbance was read at 538 nm wavelength using MultiSkan SkyHigh Microplate Spectrophotometer (ThermoFischer Scientific, USA). The total terpenoid concentration of the samples was analyzed using the linalool standard curve equation and calculated as linalool equivalent (mg linalool/g) [18].

2.5 Total Alkaloid Content (TAC)

The total alkaloid content of the ethanol extract of bungur leaves was analyzed based on the Dragendroff method modified from [19]. This test uses several reagents that need to be prepared first, such as bromocresol green (BCG) which is made by heating 69.8 mg of BCG in 3 mL of 2N NaOH and 5 mL of distilled water until dissolved. BCG solution was diluted in 1000 mL of distilled water. A standard curve was prepared using 1 mg of atropine compound dissolved in 10 mL of water, and concentration variations of 0.4, 0.6, 0.8, and 1.2 mL were made. Phosphate buffer saline pH 4.7 was made from 2M sodium phosphate (71.6 g Na_2HPO_4 dissolved in 1000 ml distilled water), then the pH was adjusted to 4.7 using citric acid. Extract samples were pretreated using 2N HCl and filtered and the pH was adjusted to neutral with 0.1 N NaOH. Samples or standards were placed in the separating funnel, then 5 mL of BCG was added. Next, 5 mL of PBS pH 4.7 was added and homogenized until a complex was formed. Rinse with chloroform as much as 1, 2, 3, and 4 mL gradually while shaking vigorously and accommodated in a flask tube. The mixture was transferred into a cuvette and the absorbance was measured at 470 nm using a MultiSkan SkyHigh Microplate Spectrophotometer (ThermoFischer Scientific, USA).

2.6 DPPH antioxidant assay

The antioxidant activity of the ethanol extract of bungur leaves was analyzed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. A total of 100 µL of extract (3.125 - 400 µg/mL) and standard ascorbic acid (0.3125 - 40 µg/mL) were added with 100 µL of 0.4 mM DPPH solution in a 96-well plate. Then the plate was incubated for 30 minutes at room temperature. The absorbance was calculated using a MultiSkan SkyHigh Microplate Spectrophotometer microplate reader (ThermoFischer Scientific, USA) at a wavelength of 517 nm. This test was performed in triplicate and the antioxidant activity was determined based on the IC_{50} value calculated from the equation obtained from the standard curve [20].

2.7 LC-HRMS analysis

The active compounds contained in the extract were identified using Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS) at the Metabolomics Laboratory, Bogor Agricultural University. A 5 mg sample of bungur extract was dissolved in 1 mL MeOH, then filtered with a µm Nylon membrane. The sample was then injected as much as 2 µL into the device. The LC-HRMS device used was a ThermoScientific Vanquish Tandem Q Exactive Plus Orbitrap HRMS UHPLC with Accucore C18 column, 100 x 2.1 mm, 1.5 µm (ThermoScientific) at 30°C. The ionization method used was negative ionization. The eluents used were H_2O + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Gradient 0-1 minute (5% B), 1-25 minutes (5-95% B), 25-28 minutes (95% B), 28-33 minutes (5% B). The analysis flow rate is 0.2 mL/min. Mass spectrum range used at 100-1500 m/z with negative ionization mode. The spectrum reading results were then analyzed using Compound Discoverer 3.2 (ThermoScientific) [21]. The mass accuracy of each identified compound were then calculated in mass error (ppm) using the web server https://warwick.ac.uk/fac/sci/chemistry/research/barrow/barrowgroup/calculators/mass_errors/ with mass error value ±10 ppm [22].

2.8 Drug-likeness

The compounds from bungur leaf extract using LC-HRMS are then selected based on drug-likeness parameters such as Lipinski, Ghose, Veber, Egan, and Muegge. This selection

process employed canonical SMILES data, which was subsequently used in the SWISS ADME web server (http://www.swissadme.ch/) to identify properties necessary for drug-likeness selection. Compounds that had no more than two violations were decided to proceed to the next step [23].

2.9 Membrane permeability

The ability of a compound to penetrate the cell membrane lipid bilayer was analyzed using PerMM server (https://permm.phar.umich.edu). The pdb file of compounds was uploaded and the physiological condition was set to temperature 310 K and pH 7.4. The energy transfer values of each compound were compared and visualized in 3D using Discovery Studio 2019 [24].

2.10 Bioactivity PASS Online

The prediction of the bioavailability of a compound can be done using the PASS Online web server (http://www.way2drug.com/passonline). The probable activity (Pa) values for each compound were assessed for various inflammation activities such as anti-inflammatory, non-steroidal inflammatory agent, antioxidant, free radical scavenger, nitrite reductase (NO-forming) inhibitor, nitric oxide antagonist, nitric oxide scavenger, antitoxic, transcription factor NF kappa A & B inhibitor, transcription factor inhibitor, and TNF expression inhibitor. Compounds with a mean Pa value above 0.5 were selected [15].

2.11 Molecular docking

Molecular docking was conducted between compounds and their respective target proteins. The three-dimensional protein structures of TLR4/MD2 complex was obtained from the PDB RCSB database using PDB ID: 3FXI (https://www.rcsb.org). Protein preparation involved using the Biovia Discovery Studio 2019 software to remove water molecules and unnecessary ligands in the docking process (Dassault Systemes Biovia, San Diego, CA, USA).

The three-dimensional structures of the selected bungur leaf compounds were obtained from the PubChem database and prepared using OpenBabel within the PyRx 0.9.5 software [25]. Molecular docking was performed specifically by utilizing grid-boxes and active sites from the control ligand for each protein, employing AutoDock Vina within the PyRx 0.9.5 software. The grid box was set at center X:25.7918, Y:-19.6631, Z:14.8296, and dimensions X: 32.3490, Y:32.0500, Z:27.9360Å The docking results were visualized using Biovia Discovery Studio 2019. Subsequently, the affinity values for each interaction and binding interactions were analyzed.

2.12 Molecular dynamics

Molecular dynamics simulation was carried out using the YASARA (Yet Another Scientific Artificial Reality Application) software, utilizing the AMBER14 force field. The system's conditions were adjusted to mimic the physiological conditions of cells, including temperature (37°C), pH (7.4), pressure (1 atm), and salt content (0.9%), for a duration of 20 nanoseconds. The macro programs employed included md_run for running simulations, md_analyze and md_analyzeres for assessing RMSD (Root Mean Square Deviation) and RMSF (Root Mean Square Fluctuation) [15].

3 Result and discussion

3.1 Phytochemical properties of bungur leaf extract

The phytochemical content of phenol and flavonoid groups in bungur leaf extract showed a high amount of phenol compounds of 423.595 ± 55.22 mgGAE/g, compared to the total flavonoid compounds of 205.248 ± 39.2 mgQE/g. The terpenoid and alkaloid content was calculated in 2.306 ± 0.218 mgLE/g and 2.414 ± 0.143 mgAE/g. Based on the IC_{50} DPPH value, bungur leaf extract was able to counteract DPPH by 50% at a concentration of 96.348 ± 3.468 µg/ml. The DPPH antioxidant ability of the extract is high, this is supported by the high content of phenol and flavonoid compounds. Compounds from the phenol and flavonoid groups act as good antioxidants by being involved in the transfer of hydrogen atoms, single electron transfers, sequential proton loss electron transfers, and transition metal chelation [26].

Table 1. The total phenol, flavonoid, terpenoid, alkaloid content and DPPH antioxidant activity of Bungur leaf extract.

TPC (mgGAE/g)	TFC (mgQE/g)	TTC (mgLE/g)	TAC (mgAE/g)	DPPH Antioxidant IC_{50} (µg/ml)
423.595 ± 55.22	205.248 ± 39.2	2.306 ± 0.218	2.414 ± 0.143	96.348 ± 3.468

3.2 Compound profile identification of bungur leaf by LC-HRMS analysis

LC-HRMS analysis aimed to identify compounds contained in Bungur leaf extract. One amino acid derivatives compound was found, N-acetyl-L-glutamic acid. Three carboxylic acid such as citric acid, gluconic acid, and DL-malic acid, also corchorifatty acid as fatty acid derivative. A total of five phenol compounds and three flavonoid compounds also identified in the extract (Table 2). Compounds from the phenol group include chlorogenic acid, D-(-)-quinic acid ellagic acid, and neochlorogenic acid, while luteolin, luteolin 7-sulfate, and quercetin are included in the flavonoid group. This identified phenol, flavonoid, terpenoid, and alkaloid compounds are also in accordance with the results of TPC, TFC, TTC, and TAC content analysis.

All confirmed compounds have a mass error value of ±10 ppm, indicating the mass accuracy between the calculated mass of the compound and the reference [27]. Both phenol and flavonoid compounds are characterized by aromatic rings with one or more hydroxyl (-OH) groups (Fig. 1) [28]. Amino acid-derived compounds are characterized by having hydrophobic chains attached to amino acid head groups through biodegradable ester bonds [29]. Carboxylic acid has a carbon (C) atom connected by a double bond to an oxygen (O) atom and a single bond to a hydroxyl group (OH). The carbon atom is joined to a hydrogen (H) atom or to another univalent combining group by a fourth bond [30]. The triterpenoid is characterized by 30 carbon atom, polymerized to form six isoprene units [31].

3.3 Drug-Likeness compound screening

Drug-likeness screening aims to determine compounds in bungur leaf extract that have drug-like characteristics when administered into the body. From 14 compounds, a total of seven compounds passed the drug-likeness screening using Lipinski, Ghose, Veber, Egan, and Muegge parameters, namely luteolin, luteolin 7-sulfate, quercetin, ellagic acid, D-(-)-quinic acid, N-acetyl-L-glutamic acid, and corchorifatty acid F (Fig 2.a). Other compounds could not be continued because they violated two or more rules and thus could not show good

character as a medicine [32]. Bioactivity analysis indicates the ability of a compound to engage in inflammation-related activities, particularly in the macrophage polarization process. Probable activity (Pa) values of more than 0.7 indicate a high ability of a compound on the related bioactivity [33]. Seven compounds from drug-likeness screening that showed good probability in bioactivity analysis were luteolin, luteolin 7-sulfate, quercetin, ellagic acid, D-(-)-quinic acid, and corchorifatty acid D. N-acetyl-L-glutamic acid could not continue to the next analysis because of its low Pa value in some activity (Fig. 2.b). The membrane permeability ability of a compound was analyzed through PerMM. Six compounds from the bioactivity analysis showed similar membrane penetration ability. All compounds had a low energy transfer pattern when passing through the lipid bilayer membrane (Fig. 2.c). This allows the compounds to enter the cell and interact with intracellular proteins [24].

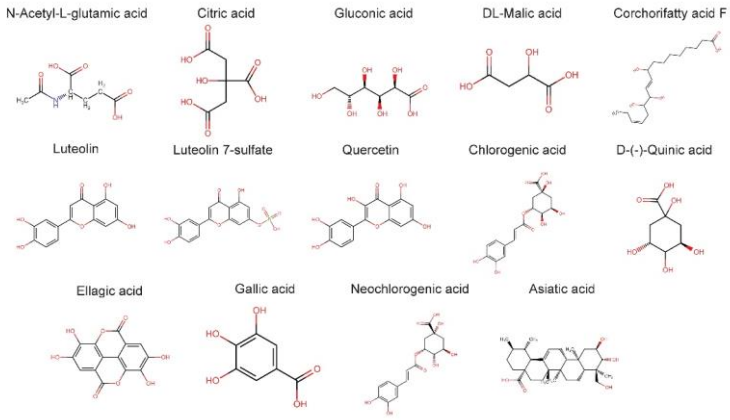

Fig. 1. Two dimensional structures of compounds of bungur leaf extract.

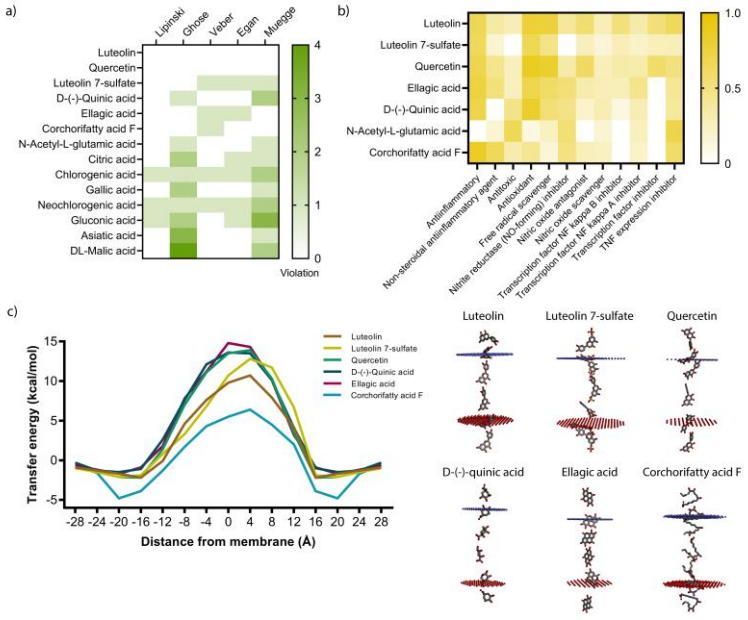

Fig. 2. a) Drug-likeness screening for 14 compounds of Bungur leaf, b) PASS Online bioactivity analysis, c) Membrane permeability analysis.

Table 2. Compounds identified in Bungur leaf extract from LC-HRMS analysis in negative ionization.

No.	Remark	RT (min)	Molecular Formula	m/z (Theo.)	m/z (Calc.)	m/z (Exp.)	Mass Error (Δppm)	MS/MS Fragments in $[M-H]^-$	Class
1	N-Acetyl-L-glutamic acid	1.458	C7 H11 N O5	189.06214	189.0637	189.06355	-0.793383	170.04500, 144.06561, 128.03419, 102.05491	Amino acid derivatives
2	Citric acid	1.228	C6 H8 O7	192.1189	192.027	192.0269	-0.52076	173.00845, 147.02896, 111.00770, 87.00764, 85.02838	Carboxylic acid
3	Gluconic acid	1.163	C6 H12 O7	196.06584	196.0583	196.05812	-0.918094	177.03928, 129.01826, 111.00741, 99.00761, 75.00761	Carboxylic acid
4	DL-Malic acid	1.187	C4 H6 O5	134.02205	134.0215	134.02107	-3.20844	133.01318, 115.00261, 71.01273	Dicarboxylic acid
5	Corchorifatty acid F	12.36	C18 H32 O5	328.22583	328.225	328.22525	0.761673	291.19717, 125.46147	Fatty acid derivative
6	Luteolin	12.828	C15 H10 O6	286.04767	286.0477	286.04815	1.573164	151.00262	Flavonoid
7	Luteolin 7-sulfate	8.91	C15 H10 O9 S	366.01494	366.0046	366.00476	0.437153	285.04010, 257.04477, 241.05069	Flavonoid
8	Quercetin	11.450	C15 H10 O7	302.0430	302.0427	302.0429	0.662158	273.03915, 257.04456, 229.04848, 178.99767, 151.00267, 149.02290, 121.02840, 107.01271, 65.00214	Flavonoid
9	Chlorogenic acid	6.093	C16 H18 O9	354.10394	354.0951	354.09543	0.931953	191.05527	Phenolic acid
10	D-(-)-Quinic acid	1	C7 H12 O6	192.508	192.0634	192.0632	-1.041323	173.04439, 111.04387, 93.03345	Phenolic acid
11	Ellagic acid	8.577	C14 H6 O8	302.0068	302.0063	302.00647	0.562902	283.99448, 229.01337, 185.02374, 173.02396, 145.02870	Phenolic acid
12	Gallic acid	1.917	C7 H6 O5	170.0219	170.0215	170.0212	-1.76	125.02335, 107.01274, 97.02847, 79.01772, 69.03335	Phenolic acid
13	Neochlorogenic acid	4.704	C16 H18 O9	354.09664	354.0951	354.09549	1.101399	191.05531	Polyphenol
14	Asiatic acid	16.098	C30 H48 O5	488.39784	488.3502	488.3520	3.68588	487.34415, 397.37091, 390.72577, 215.06105	Triterpenoid

RT = Retention time

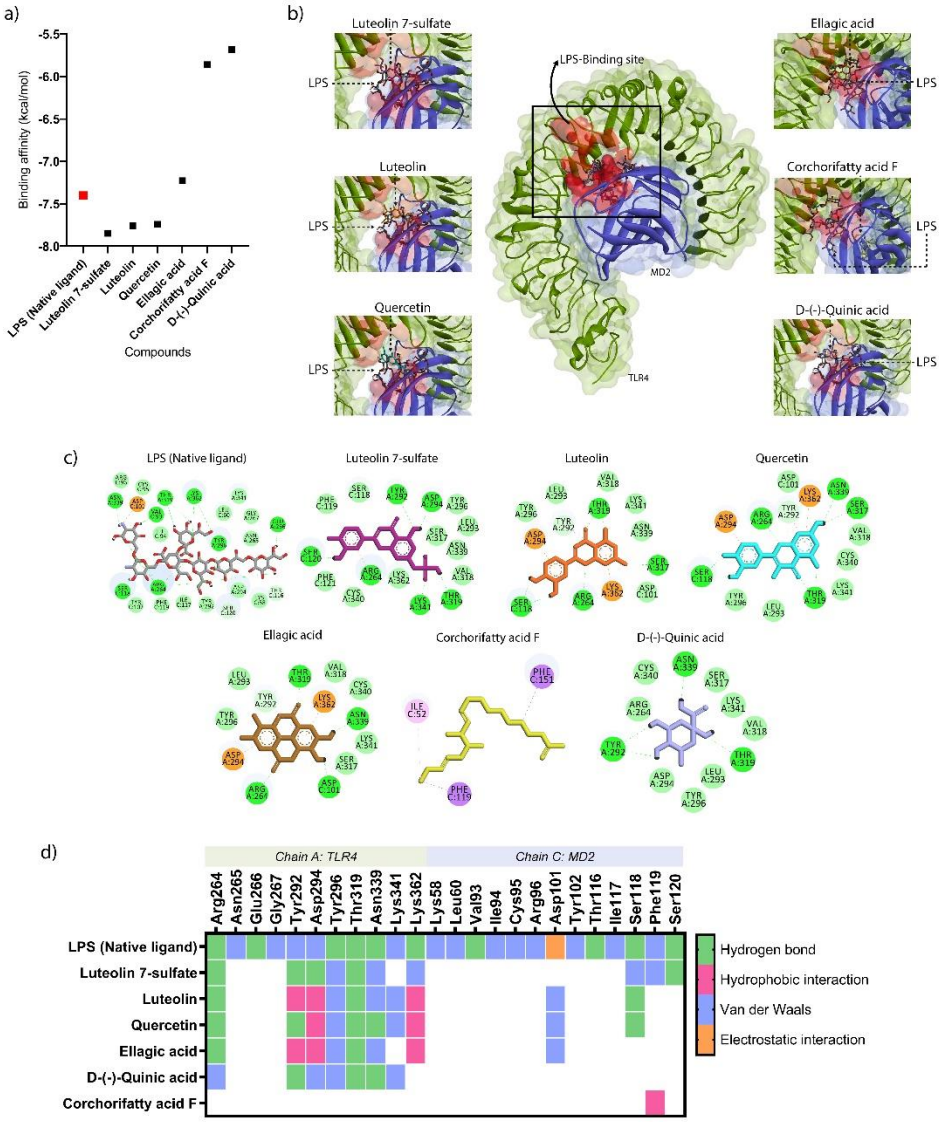

Fig. 3. TLR4/MD2 and compound interaction a) binding affinity value from molecular docking, b) ligand position in LPS-binding site after molecular docking, c-d) interacting residue of each key residue with ligands.

3.4 Molecular docking

Molecular docking was performed to observe the interaction ability between compounds with TLR4/MD2 protein. The binding affinity values are shown in Fig.3.a. Three compounds have more negative binding affinity compared to LPS as a negative ligand. LPS has a binding affinity value of -7.399 kcal/mol. The compounds luteolin 7-sulfate, luteolin, and quercetin have binding affinities of -7.847, -7.76, and -7.743 kcal/mol, respectively. Ellagic acid and D-(-)-quinic acid have weaker interactions, which can be seen from the higher binding affinity compared to LPS, namely at -7.228 and -5.682 kcal/mol. Corchorifatty acid F have

a binding affinity of -5.861 kcal/mol. Based on these values, luteolin 7-sulfate, luteolin, and quercetin are compounds with better interaction abilities compared to LPS.

TLR4/MD2 amino acids that interact with compounds indicate the interaction site and type of binding that occurs. All compounds that can interact with the TLR4/MD2 complex are able to bind to proteins in the LPS-binding site area (Fig. 3.b). Each compound shows different interactions with key amino acids in TLR4/MD2 (Fig. 3.c-d). Based on residue interactions, several residues of TLR4, such as Arg264, Tyr292, Asp294, Tyr296, Thr319, Lys341, and Lys362 were also found to interact with luteolin 7-sulfate, luteolin, quercetin, and ellagic acid in the form of hydrogen bonds, hydrophobic interactions, and van der Waals. Amino acids in MD2, such as Asp101 and Ser118, show hydrogen bonds and hydrophobic interactions with luteolin 7-sulfate, luteolin, and quercetin.

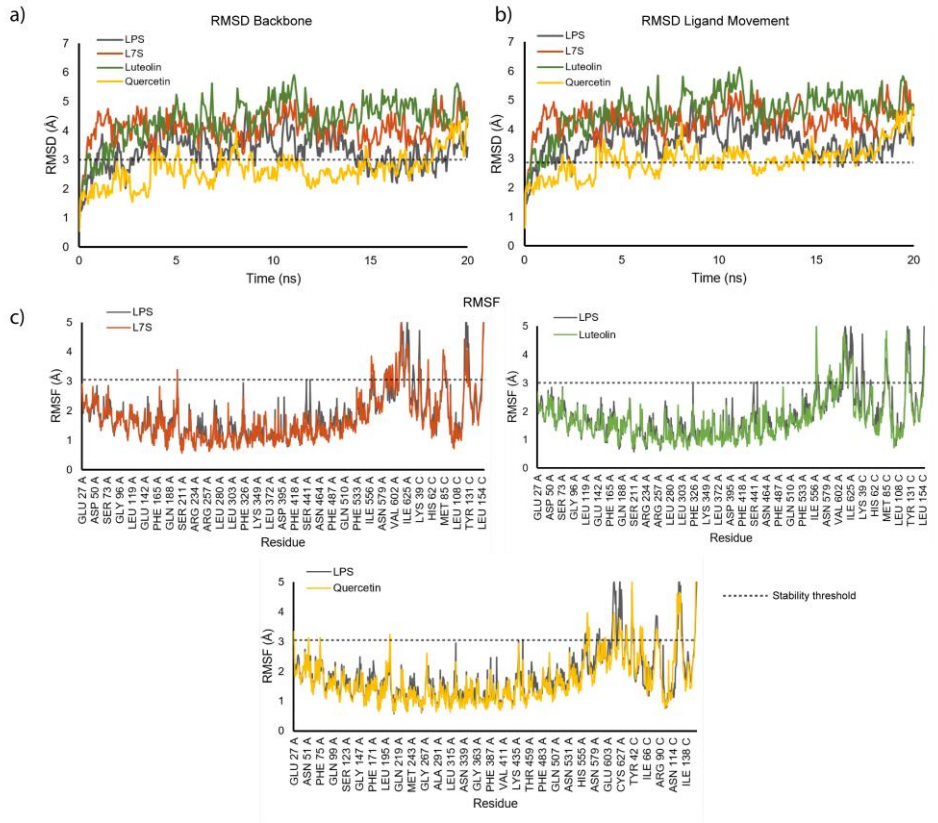

Fig. 4. TLR4/MD2 and compounds interaction stability a) RMSD Backbone protein, b) RMSD ligand movement, and c-e) RMSF.

The interaction of LPS on TLR4 and MD2 is crucial in initiating TLR4 activation. LPS binds to the binding site connected by TLR4 and MD2. The recognition and binding of LPS depend on MD-2, a secreted auxiliary molecule that physically binds to TLR4. The formation of an intracellular adaptor protein complex will be triggered by LPS-induced TLR4 homodimerization (TLR4/MD-2/LPS). The primary signaling adaptors Myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adaptor inducing interferon-β (TRIF) is then used by TLR4, along with the corresponding adaptor molecules MyD88-adaptor-like (MAL), also known as TIR-domain containing adaptor protein (TIRAP), and TRIF-related adaptor molecule (TRAM). This signaling process will continue

until the cells are able to provide a pro-inflammatory response [34], [35]. Disruption of the interaction between LPS on TLR4/MD2 is important in preventing LPS-mediated TLR4 activation. Compounds in Bungur leaves are able to be competitive inhibitors at the LPS-binding site and prevent TLR4 activation [7]. Inhibition of LPS binding to TLR4 by compounds was predicted to affect the activation of the NF-κB signaling pathway in in vitro studies, such as in vitro administration of Morroniside to RAW 264.7 cells [36].

3.5 Interaction stability between TLR4/MD2 and bungur leaf compounds

The stability of the interaction between TLR4/MD2 with three compounds with the best binding affinity, namely luteolin 7-sulfate (L7S), luteolin, and quercetin, is shown in Fig. 4. The Backbone RMSD parameter shows the level of protein stability during interaction with the three compounds, and is compared to LPS. Quercetin is the most stable compound compared to other compounds as seen from the minimal fluctuations in the Backbone RMSD and ligand movement RMSD. Luteolin and luteolin 7-sulfate show less stable interactions when viewed from the Backbone RMSD and ligand movement RMSD graphs (Fig 4. a-b). Backbone RMSD and ligand movement RMSD indicate the stability of an interaction if the value does not exceed 3 Å [37]. When viewed from the RMSF parameter, luteolin 7-sulfate is involved in the instability of residues Lys 615A, Asn 624A, Met 85C, and Phe 128C. TLR4/MD2 interaction showed a quite high fluctuation when interacting with luteolin at Lys 560A, Asp 614A, Asn 83C, and Lys 125C. The TLR4/MD2 complex experienced the least instability when interacting with quercetin, namely at residues Gln 565A, Lys 39C, and Lys 125C (Fig 4. c-e).

4 Conclusion

This study identified five phenol and three flavonoid compounds in Bungur leaf extract. These compounds are potentially predicted to be anti-inflammatory, especially by interfering with the activation of the TLR4/MD2 complex on its LPS-binding site. Quercetin becomes the compound that can act as a competitive inhibitor and the most stable compound during its interaction to interfere with the LPS binding to the TLR4/MD2, suppressing the pro-inflammatory signalling.

The authors thank the Animal Physiology Laboratory, Department of Biology, Universitas Brawijaya, for supporting this research. The authors also thank the 'High-Performance Computing (HPC) AI-Center UB, which has provided HPC facilities for this research. This research was also funded by DRTPM (Direktorat Jenderal Pendidikan Tinggi Kementerian Pendidikan dan Kebudayaan Republik Indonesia) through the scheme of Penelitian Fundamental Reguler (PFR) (Grant No. 045/E5/PG/02/00/PL/2024).

References

1. C. Franceschi and J. Campisi, Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences. **69**, S4–S9 (2014). doi: 10.1093/gerona/glu057.
2. A. Mantovani, P. Allavena, A. Sica, and F. Balkwill, Cancer-related inflammation, Nature. **454**, 436–444 (2008). doi: 10.1038/nature07205.
3. N. Michels, C. Van Aart, J. Morisse, A. Mullee, I. Huybrechts, Chronic inflammation towards cancer incidence: A systematic review and meta-analysis of epidemiological

studies. Critical Reviews in Oncology/Hematology. **157**, 103177 (2021). doi: 10.1016/j.critrevonc.2020.103177.
4. M. E. Lynch and C. P. N. Watson, The pharmacotherapy of chronic pain: a review. Pain Res Manag. **11**, 11–38 (2006). doi: 10.1155/2006/642568.
5. P. J. Delves, S. J. Martin, D. R. Burton, and I. M. Roitt, Roitt's essential immunology, 13th edition. Chichester, West Sussex; Hoboken, [NJ]: Wiley Blackwell, 2017.
6. Z. Shen, X. Liu, G. Fan, J. Na, Q. Liu, F. Liu, Z. Zhang, and L. Zhong, Improving the therapeutic efficacy of oncolytic viruses for cancer: targeting macrophages. J Transl Med. **21**, 842 (2023). doi: 10.1186/s12967-023-04709-z.
7. L. Pérez-Regidor et al., Small molecules as Toll-like receptor 4 modulators drug and in-house computational repurposing. Biomedicines. **10**, 2326 (2022). doi: 10.3390/biomedicines10092326.
8. S. J. Stohs, H. Miller, and G. R. Kaats, A review of the efficacy and safety of banaba (*Lagerstroemia speciosa* L.) and corosolic acid. Phytother Res. **26**, 317–324 (2012). doi: 10.1002/ptr.3664.
9. S. K. Tripathi, S. Behera, M. Panda, G. Zengin, and B. K. Biswal, A comprehensive review on pharmacology and toxicology of bioactive compounds of *Lagerstroemia speciosa* (L.) Pers., CTM. **7**, 504–513 (2021). doi: 10.2174/2215083806999201211213931.
10. Y. Bramasto et al., Trees of the city: Profil tanaman hutan untuk perkotaan wilayah Jawa Barat, Banten dan DKI Jakarta. Bogor: Balai Penelitian Teknologi Perbenihan Tanaman Hutan, 2015.
11. A. H. Karsono, O. M. Tandrasasmita, and R. R. Tjandrawinata, Bioactive fraction from *Lagerstroemia speciosa* leaves (DLBS3733) reduces fat droplet by inhibiting adipogenesis and lipogenesis. J Exp Pharmacol. **11**, 39–51 (2019). doi: 10.2147/JEP.S181642.
12. E. W. C. Chan, L. N. Tan, and S. K. Wong, Phytochemistry and pharmacology of *Lagerstroemia speciosa*: a natural remedy for diabetes. Int J Herb Med. **2**, 81–87 (2014).
13. N. E. H. Lezoul, M. Belkadi, F. Habibi, and F. Guillén, Extraction processes with several solvents on total bioactive compounds in different organs of three medicinal plants. Molecule. **25**, 4672 (2020). doi: 10.3390/molecules25204672.
14. S. B. Bagade and M. Patil, Recent advances in microwave assisted extraction of bioactive compounds from complex herbal samples: a review. Crit Rev Anal Chem. **51**, 138–149 (2021). doi: 10.1080/10408347.2019.1686966.
15. M. H. Widyananda, N. Rosyadah, L. Muflikhah, N. Widodo, D. Dwijayanti, and S. M. Ulfa, Lagerstroemin from *Lagerstroemia speciosa* as antibreast cancer candidate targeting AURKA, EGFR and SRC protein: a comprehensive computational study. Trends Sci. **21**, 8205 (2024). doi: 10.48048/tis.2024.8205.
16. E. N. Sembiring, B. Elya, and R. Sauriasari, Phytochemical screening, total flavonoid and total phenolic content and antioxidant activity of different parts of *Caesalpinia bonduc* (L.) Roxb. PJ. **10**, 123–127 (2017). doi: 10.5530/pj.2018.1.22.
17. N. Ghorai, S. Chakraborty, S. Guchhait, S. K. Saha, and S. Biswas, Estimation of total terpenoids concentration in plant tissues using a monoterpene, linalool as standard reagent. Protocol Exchange. (2012). doi: 10.1038/protex.2012.055.
18. A. Łukowski, R. Jagiełło, P. Robakowski, D. Adamczyk, and P. Karolewski, Adaptation of a simple method to determine the total terpenoid content in needles of coniferous trees. Plant Science. **314**, 111090 (2022). doi: 10.1016/j.plantsci.2021.111090.

19. P. Tan, The determination of total alkaloid, polyphenol, flavonoid and saponin contents of Pogang gan (*Curcuma* sp.). International Journal of Biology. **10**, 42 (2018). doi: 10.5539/ijb.v10n4p42.
20. D. R. Dwijayanti, S. Puspitarini, N. Widodo, *Piper betle* L. leaves extract potentially reduce the nitric oxide production on LPS-induced RAW 264.7 Cell Lines. JEL. **13**, 78–83, (2023). doi: 10.21776/ub.jels.2023.013.02.02.
21. R. Ningsih, M. Rafi, A. Tjahjoleksono, M. Bintang, and R. Megia, Ripe pulp metabolite profiling of ten Indonesian dessert banana cultivars using UHPLC-Q-Orbitrap HRMS. Eur Food Res Technol. **247**, 2821–2830 (2021). doi: 10.1007/s00217-021-03834-7.
22. A. Singh, V. Bajpai, S. Kumar, K. R. Sharma, and B. Kumar, Profiling of gallic and ellagic acid derivatives in different plant parts of *Terminalia arjuna* by HPLC-ESI-QTOF-MS/MS. Natural Product Communications. **11**, 1934578X1601100227 (2016). doi: 10.1177/1934578X1601100227.
23. A. Daina, O. Michielin, and V. Zoete, SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci Rep. **7**, 42717 (2017). doi: 10.1038/srep42717.
24. A. L. Lomize et al., PerMM: a web tool and database for ana lysis of passive membrane permeability and translocation pathways of bioactive molecules. J Chem Inf Model. **59**, 3094–3099 (2019). doi: 10.1021/acs.jcim.9b00225.
25. E. Susanti, In silico analysis of bioactive compounds of *Hibiscus sabdariffa* as potential agonists of LXR to inhibit the atherogenesis process, International Conference on Bioinformatics and Nano-Medicine from Natural Resources for Biomedical Research: 3rd Annual Scientific Meeting for Biomedical Sciences, Malang, Indonesia (2019) 020008. doi: 10.1063/1.5109983.
26. A. Zeb, Concept, mechanism, and applications of phenolic antioxidants in foods. J Food Biochem. **44**, (2020). doi: 10.1111/jfbc.13394.
27. E. N. Fung, Y. Xia, A.-F. Aubry, J. Zeng, T. Olah, and M. Jemal, Full-scan high resolution accurate mass spectrometry (HRMS) in regulated bioanalysis: LC–HRMS for the quantitation of prednisone and prednisolone in human plasma. Journal of Chromatography B. **879**, 2919–2927 (2011). doi: 10.1016/j.jchromb.2011.08.025.
28. D. Tungmunnithum, A. Thongboonyou, A. Pholboon, and A. Yangsabai, Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: an overview. Medicines (Basel). **5**, 93 (2018). doi: 10.3390/medicines5030093.
29. K. Ita, Chapter 5 - Chemical permeation enhancers, in Transdermal Drug Delivery, K. Ita, Ed., Academic Press. **2020**, 63–96. doi: 10.1016/B978-0-12-822550-9.00005-3.
30. J. DeRuiter, Carboxylic Acid Structure and Chemistry, 2005.
31. J.-R. Du, F.-Y. Long, and C. Chen, Chapter Six - Research Progress on Natural Triterpenoid Saponins in the Chemoprevention and Chemotherapy of Cancer, in The Enzymes, vol. 36, S. Z. Bathaie and F. Tamanoi, Eds., in Natural Products and Cancer Signaling: Isoprenoids, Polyphenols and Flavonoids, vol. 36. Academic Press (2014) 95–130. doi: 10.1016/B978-0-12-802215-3.00006-9.
32. C. J. Ononamadu and A. Ibrahim, Molecular docking and prediction of ADME/drug-likeness properties of potentially active antidiabetic compounds isolated from aqueous-methanol extracts of *Gymnema sylvestre* and *Combretum micranthum*. BioTechnologia (Pozn). **102**, 85–99 (2021). doi: 10.5114/bta.2021.103765.
33. D. A. Filimonov et al., Prediction of the biological activity spectra of organic compounds using the Pass online web resource. Chem Heterocycl Comp. **50**, 444–457 (2014). doi: 10.1007/s10593-014-1496-1.

34. P. Hankittichai et al., *Artocarpus lakoocha* extract inhibits LPS-induced inflammatory response in RAW 264.7 Macrophage Cells. Int J Mol Sci. **21**, 1355 (2020). doi: 10.3390/ijms21041355.
35. A. Zamyatina and H. Heine, Lipopolysaccharide recognition in the crossroads of TLR4 and Caspase-4/11 Mediated Inflammatory Pathways. Front Immunol. **11**, 585146 (2020). doi: 10.3389/fimmu.2020.585146.
36. C. Park, H.-J. Cha, H. Lee, G.-Y. Kim, and Y. H. Choi, The regulation of the TLR4/NF-κB and Nrf2/HO-1 signaling pathways is involved in the inhibition of lipopolysaccharide-induced inflammation and oxidative reactions by morroniside in RAW 264.7 macrophages. Archives of Biochemistry and Biophysics. **706**, 108926, (2021). doi: 10.1016/j.abb.2021.108926.
37. W. Nafisah et al., Potential of bioactive compound of *Cyperus rotundus* L. rhizome extract as inhibitor of PD-L1/PD-1 interaction: An in silico study. ANRES. **56** (2022). doi: 10.34044/j.anres.2022.56.4.09.