INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
24–0118/2024/32–1–71–78
DOI: 10.17957/IJAB/15.2178
http://www.fspublishers.org

Full Length Article

In Silico and *In Vitro* Anti-Inflammatory Studies on Lipoxygenase Inhibitor from *Tetracera indica* Leaves

Vera Ladeska[1,2], Berna Elya[1*], Muhammad Hanafi[3,4], Kusmardi[5,6,7], Rini Prastiwi[2], Ema Dewanti[2] and Ahmad Zuhdi Firmansyah[2]

[1]*Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, University of Indonesia, Depok 16424, Indonesia*
[2]*Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy and Science Muhammadiyah Prof. Dr. Hamka, Jakarta 13460, Indonesia*
[3]*Research Centre for Chemistry, Indonesian National Research and Innovation Institute, Serpong, Indonesia*
[4]*Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy University of Pancasila, Jakarta, Indonesia*
[5]*Department of Anatomic Pathology, Faculty of Medicine, University of Indonesia, Jakarta 10430, Indonesia*
[6]*Drug Development Research Cluster, Indonesia Medical Educational and Research Institute, Jakarta 10340, Indonesia*
[7]*Human Cancer Research Cluster, Indonesia Medical Educational and Research Institute, Jakarta 10340, Indonesia*
[*]For correspondence: berna.elya@farmasi.ui.ac.id
Received 03 March 2024; Accepted 29 March 2024; Published 26 May 2024

Abstract

Inflammation is the body's natural immune response to infection or injury. If the inflammation continues, it will cause organ function failure due to activating inflammatory mediators, including lipoxygenase (LOX). This study aimed to predict the affinity of hydroxychromone and quercetin compounds found in *T. indica* leaves in inhibiting 5-LOX *in silico* and *In vitro*. *In silico* prediction compares Autodock Tools-1.5.7 software with zileuton. *In silico* results were used to base *In vitro* experiments using RAW 264.7 cells induced by lipopolysaccharide. A viability assay was carried out on RAW 264.7 cells using the MTT method. Based on the binding energy and inhibition constant, hydroxychromone and quercetin compounds showed a higher affinity for 5-LOX than zileuton. These results are in line with *In vitro* research, which shows that ethyl acetate extract of *T. indica* leaves has a lower LOX activity value (6,763 ± 0.819 mU/mL), which indicates that this extract has potential as an inhibitor of 5-LOX enzyme activity. This extract contained hydroxychromone compounds based on LC-MS/MS analysis from previous research. The viability test shows that a dose of 0-62.5 ppm is safe for RAW 264.7 cells with a viability percentage above 80%. This study can be expanded to hunt for novel natural anti-inflammatory sources that inhibit the 5-LOX enzyme's action.

Keywords: Anti-inflammatory; *In silico*; Lipoxygenase; *Tetracera indica*; RAW 264.7 cells

Introduction

The prevalence of diseases involving inflammatory processes in the body in Indonesia is relatively high. According to Health Research and Development Agency (2019), the national prevalence of diabetes mellitus is 1.5%, hypertension is 8.36%, and joint disease is 7.3%. The world prevalence of diabetes in adults was 6.4% in 2010 and will increase by 7.7% in 2030. This increase is higher in developing countries than in developed countries (Adesoba and Brown 2023). All chronic diseases begin with the reaction of several inflammatory mediators within them.

Inflammation is the body's immune response to a microbial attack or physical trauma. Inflammation aims to eliminate attacking factors and restore the structure and physiological function of the body. The inflammatory response that occurs continuously damages body tissue, causing organ dysfunction (Hu and Ma 2018). Conventional drugs that are often used to treat inflammation are non-steroidal anti-inflammatory drugs (NSAIDs). Although these anti-inflammatory drugs can help treat many inflammatory diseases, they have many adverse side effects. NSAIDs cause gastrointestinal bleeding, hypertension, stroke and kidney disorders (Wongrakpanich *et al.* 2018). This occurs because the mechanism of action for most NSAIDs is based on inhibiting prostaglandin synthesis, where both types of cyclooxygenases (COX-1 and COX-2) are blocked. The ideal NSAID is expected only to inhibit COX-2 (inflammation) and not COX-1 (protection of the gastric mucosa) and also inhibit lipoxygenase (leukotriene formation) (Trevor and Katzung 2002; Wallace 2019). Currently, a lot of research is being conducted to develop

To cite this paper: Ladeska V, B Elya, M Hanafi, Kusmardi, R Prastiwi, E Dewanti, AZ Firmansyah (2024). *In Silico* and *In vitro* anti-inflammatory studies on lipoxygenase inhibitor from *Tetracera indica* leaves. *Intl J Agric Biol* 32:71–78

inflammation treatments that inhibit lipoxygenase (LOX) activity to minimize the side effects of COX inhibition.

The LOX is a non-heme iron compound containing an enzyme that catalyzes polyunsaturated fatty acid dioxygenase. LOX converts arachidonic acid, which is a component of membrane phospholipids, into inflammatory mediators called leukotrienes (Abbas *et al.* 2019). Leukotrienes are potent molecules. They are involved in various disease states such as asthma, osteoporosis, cancer and atherosclerosis (Perera *et al.* 2018). One of the isoforms of LOX that is widely developed is 5-5-lipoxygenase (5-LOX). By inhibiting LOX, neutrophil activation that induces cysteinyl LTs does not occur, so vascular permeability remains stable. This protects the gastric mucosa and prevents ulceration (Hu and Ma 2018; Wallace 2019).

Natural compounds that provide a lot of pharmacological activity are phenols, flavonoids and triterpenoids. Flavonoid compounds such as campesterol, quercetin, morin and myricetin were found to be 5-LOX inhibitors and less active against 12-LOX (Laughton *et al*. 1991; Kim *et al*. 1998; Borbulevych *et al*. 2004). Prenylated flavonoids such as artonin are the most potent 5-LOX inhibitors with an IC_{50} of 0.36–4.3 µM. A plant that has the potential to be developed as an anti-inflammatory is *Tetracera indica* (Christm. and Panz.) Merr because it contains high phenolic and flavonoid compounds (Ladeska *et al*. 2023). Scientifically, *T. indica* contains wogonin, norwogonin, quercetin, tectocrisin, 5,7-dihydroxyflavone-O- 8-sulfate, kaemferol, and quercetin (Abdullah *et al*. 2013; Hasan *et al*. 2017; Alhassan *et al*. 2019). In Malaysia, empirically, the *T. indica* plant is used to treat fever, flu, sinus symptoms, red skin, diarrhoea, insect bites and diabetes (Hasan *et al*. 2017). In Indonesia, the people of South Sumatra especially use *T. indica* bark, which is used to treat kidney stones and gout, while the leaves are used to treat hypercholesterolemia (Samitra and Rozi 2017; Muharni *et al*. 2018).

Experimentally, the ethyl acetate fraction of *T. indica* stems has the potential to inhibit the xanthin oxidase enzyme with an IC_{50} of 21.14 µg/mL, while the methanol extract has an IC_{50} of 42.02 µg/mL (Abdullah *et al*. 2013). Meanwhile, Hasan *et al.* (2017) reported wogonin, norwogonin, and tectocrisin from *T. indica* have the potential to be antidiabetics (Hasan *et al*. 2017). *T. indica* shows inhibition of the enzyme acetylcholinesterase to improve symptoms associated with Alzheimer's *In vitro* (Rawa *et al.* 2019). *T. indica* is also reported to have anti-HIV, antidiabetic, antibacterial and anti-inflammatory activity (Ahmed *et al*. 2012; Lima *et al*. 2014; Ragesh *et al*. 2016; Hasan *et al*. 2017). Wogonin from *T. indica* is active as an antibacterial, and this compound is also found in *T. Indica* fruit extracts (Lima *et al*. 2014). Isolate 5,8-dihydroxy-7-methoxyflavone from the stem bark of *T. indica* has potent antioxidant activity with IC_{50} 8.25 µg/mL (Hasan *et al*. 2017).

Research on anti-inflammatory activity through inhibiting LOX activity from *T. indica* plants has yet to be available. Based on the chemical content and pharmacological effects of several previous studies, the chemical content of *T. indica* plant is a candidate for anti-inflammatory compounds. This research develops inflammation treatment towards inhibiting LOX activity to minimize the side effects of cyclooxygenase (COX) inhibition. This is new research on the *T. indica* plant as an anti-inflammatory in reducing leukotriene formation through inhibiting LOX activity. The discovery of this active anti-inflammatory compound has also never been reported. Inhibition of the LOX pathway causes leukotrienes not to be formed so that inflammation does not occur. Before testing *In vitro*, the affinity of the compounds contained in *T. indica* was predicted *in silico* using the Autodock tool with zileuton as a comparison. Development of *In vitro* testing methods using the RAW 264.7 macrophage cell model. Lipopolysaccharide induces cells first to stimulate inflammation and release inflammatory mediators such as LOX. This LOX formation pathway will be inhibited by administering *T. indica* leaf extract.

Materials and Methods

Chemicals

The chemicals used during the study were of analytical standard. Chloroform, methanol, n-hexane, ethyl acetate, were obtained from Merck (Darmstadt, Germany), DMEM HG, Phospat Buffer Saline (PBS), trypsin-EDTA, Pen-Strep solution, zileuton (Solarbio Science and Technology, Beijing), Fetal Bovine Serum (FBS) were obtained from Biosera (South America), lipopolysaccharida from E. coli 0111(St. Louis, MO, USA), RAW cell 264.7 from BRIN Serpong, quercetin, bovine serum albumin and MAK363-1KT LOX assay Kit were purchased from Sigma Aldrich (St. Louis, MO, USA).

Plant material

T. indica plants were obtained from the Tropical Biopharmaceutical Study Center-LPPM Bogor Agricultural Institute (IPB), Bogor, West Java. The species was authenticated by the plant taxonomist of the Biology Research Center, Indonesian National Research and Innovation Institute, Cibinong, Indonesia, with collection number B-689/V/D1.05.07/11/2021.

Preparation of extract

The extract was made using 6 kg of fresh *T. indica* leaves. The leaves are sorted, washed with running water and dried. The leaves were powdered and macerated using solvents with increased polarity, namely n-hexane, ethyl acetate and methanol. All extracts were evaporated using a rotary evaporator (Eyela 05B-2100, China) at a temperature of 50˚C and thickened over a water bath to obtain thick n-

hexane extract, ethyl acetate extract and methanol extract. However, the research on *n*-hexane extract (DI1) was not continued due to its low phenol and flavonoid content (Ladeska *et al*. 2022).

***In silico* assay**

The database as research material refers to the Protein Data Bank (PDB) https://www.rcsb.org/ and Pub-Chem (https://PubChem.ncbi.nlm.nih.gov), the software used is Chimera (https://www.cgl.ucsf.edu/chimera/download.htmL), Biovia Discovery Studio (https://discover.3ds.com/discovery-studio-visualizer-dowmLoad). *In silico* assay was carried out using Autodock Tools (http://autodock.scripps.edu), Vina (https://vina.scripps.edu/download/), PyRx (pyrx.sourceforge.io), and Pymol (pymol.org/edu). Docking was carried out on flavonoids compounds contained in *T. indica* plants based on LC-MS/MS data, namely quercetin and hydroxy-3,4-hydroxybenzylidene-chromanone (Ladeska *et al*. 2023). For comparison zileuton (Benzothiophene N-hydroxyurea) ALOX5 inhibitors were used.

The crystallographic structure of the LOX protein was selected by utilizing the UNIPROT database. This protein has been validated experimentally and by text mining. The UNIPROT database is integrated with the RCSB Protein Data Bank database (https://www.rcsb.org) and the alpha fold. Protein crystallography depicts human LOX bound to X-ray diffraction NDGA (PDB: 6N2W) at 2.71 Å resolution. Ligand-protein docking was carried out using the Autodock Tools program. The discovery studio visualizer program (https://discover.3ds.com/discovery-studio-visualizer-dowmLoad/Biovia) was used to visualize ligand and receptor interactions. The ligand structure downloaded in PubChem is then entered into Marvinsketch to prepare it to become an active 3D shape. The search algorithm used is the Larmarckian Genetic Algorithm (LGA) to create a stable conformation between the ligand and the receptor's active site with 100x docking. Optimization of test ligands is produced by applying Marvinskecth, energy minimization, to find the most stable position of the atoms in the test compound. The results were analyzed by looking at the inhibition constant value, RMSD value, and the free energy produced.

Cell viability assay

Cell viability tests were carried out to determine safe concentrations for LOX activity tests. Cell toxicity tests were performed using the MTT assay method on RAW 264.7 cells (Zheng *et al*. 2017, with slight modifications). The condition of the cells was observed, and the cells were harvested when they reached 80% confluence. The culture medium was gently removed, and then the cells were washed with PBS, adding EDTA trypsin. Incubate in a 5% CO_2 incubator at 37˚C for 3 min. To inactivate the trypsin, 5 mL of DMEM medium was added. Cells were resuspended using a pipette and transferred to a new sterile conical tube. Cells were centrifuged at 1200 rpm for 5 min. The supernatant was discarded, and 1 mL of culture medium was added to the remaining pellet. Cells were counted using a haemocytometer and trypan blue to determine the number of cells available in 1 mL of media. Cells are diluted with culture medium until the desired number of cells is reached. Transfer the cells to a 96-well plate with a volume of 100 µL per well. Cells were incubated in a 5% CO_2 incubator at 37ºC overnight.

Test sample preparation involved weighing 10 mg of the sample (DI2 and DI3) and adding DMSO to get 50,000 µg/mL for the sample stock concentration. Sample concentrations of 1000, 250, 125, 62.5, 31.25 and 15.625 µg/mL were created. Each sample was carried out in triplicate. The culture medium in the wells was disposed of after 24 h. Place 100 µL of diluted samples containing culture media into each well (each concentration is prepared in triplicate), and place just the culture medium in the wells designated for control cells. Following another 24-h incubation period, the cells were documented. After 24 h, the medium was again discarded, and the cells were washed using PBS. MTT reagent, which had been diluted with 100 µL of the medium, was added to the wells; then the cells were incubated again in the incubator for 4 h. After 4 h, adding 100 µL of 10% SDS, wrap the plate with aluminium foil and incubate while shaking in an incubator at room temperature overnight. After overnight, the plate was read for absorption using an ELISA Reader at a maximum wavelength of 573 nm (Wu *et al*. 2016; Soonthornsit *et al*. 2017; Muniandy *et al*. 2018).

$$\text{Viability cell (\%)} = \frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control} - \text{Abs blank}} \text{x } 100$$

Determination protein concentration

Protein concentration was determined using the Bovine Serum Albumin method (BSA). Standard series (0.8, 0.6, 0.4, 0.2, 0 mg/mL with distilled water) were prepared from 1 mg/mL BSA stock solution. Absorbance of standards was measured using a spectrophotometer (Shimadzu UV 1900, Japan) with λ= 280 nm. Protein concentration from the line equation obtained were calculated ranging between 1-10 µg/µL. Dilute the sample if needed using LOX Assay Buffer (Esfahlan *et al*. 2019; Bulletin LOX Kit 2021).

Standard curve preparation of LOX assay

Oxidized Probe LOX standard curve was made with 0, 2, 6, 8, and 10 pmol/well concentration series. Stock solution (1 µM Oxidized Probe) was prepared by diluting 100 µM aliquots 100-fold with DMSO and read using plate reader ready at λEx=500 nm/λEm=536 nm (Thermo scientific, Variouskan flash, Finland) (Bulletin LOX Kit 2021). Then Immediately started recording fluorescence (RFU) at 10 and 40 min.

LOX assay standard curve

A LOX standard curve was prepared by preparing 1 µM oxidized Probe Premix by diluting a 100 µM oxidized probe standard aliquot with DMSO. Then pipette 10; 8; 6; 4; 2; 0 µL/well add to LOX Assay buffer to 100 µL to obtain a concentration of 10; 8; 6; 4; 2; 0 pmol/well. Absorbance was measured with a spectrofluorometer (RFU) with T1 (10 min) and T2 (40 min) at room temperature. From the 10-min LOX standard curve, the regression equation $y = 4.913x–2.617$ with $R^2 = 0.9812$ and the 40-min LOX standard curve $y = 3.927x–1.618$ with $R^2 = 0.9882$.

LOX activity assay

The LOX activity inhibition test was conducted using RAW 264.7 macrophage cells induced by inflammation with lipopolysaccharide (LPS). RAW 264.7 cells were taken from the liquid nitrogen tank and thawed in an ultrasonic cleaner at 37ºC for 2 min. Cells were supplemented with DMEM HG 10% FBS and 1% penicillin-streptomycin, then centrifuged for 5 min at 1600 rpm. Cells were harvested after reaching 80-90% confluent. Cells were counted using a haemocytometer and trypan blue to determine the number of cells available in 1 mL of media.

The LOX activity assay was carried out based on the procedure stated in the Lipoxygenase Activity Assay Kit with (Catalogue number MAK363, Sigma-Aldrich, USA). LOX activity testing was divided into six groups, namely extract group (RM2 and RM3), positive control (quercetin and zileuton), normal group, and negative group. Cells were seeded at density of 4×10^5 RAW per well added with 200 µL of 25 µg/mL test solution, incubated for 2 h. Then 200 µL LPS was added to each well and incubated at 37ºC in a moisture-saturated atmosphere containing 5% CO_2 for 24 h. Then 100 µL LOX Lysis buffer was added and kept on ice for 10 min. Cells (4×10^5 cells) were homogenized with 100 µL of ice-cold LOX Lysis Buffer and kept on ice for 10 min. Centrifuged this homogenate at 10,000 × g for 15 min at 4ºC and collected the supernatant. Then prepared three wells for each sample and labelled with sample background control (BC), sample (S) and sample plus inhibitor (SI). Then added 1 µL sample into each of the three wells. For SI wells, added 70 µL SI mix, 27 µL LOX assay buffer, and 2 µL of LOX inhibitor in addition to the sample. For S wells, added 70 µL S mix and 29 µL LOX assay buffer in addition to the sample, while for BC wells, added 70 µL BC mix and 29 µL LOX assay buffer in addition to the sample. Immediately started recording fluorescence (RFU) at λ_{Ex}=500 nm/λ_{Em}=536 nm (Thermo scientific, Variouskan flash, microplate reader, Finland). The LOX activity was calculated using the following equations:

$$\text{Lox activity in sample (mU/mL)} = \frac{\Delta M \times DF}{\Delta M \times V}$$

Specific LOX activity in sample is the difference between activity in sample was detected and activity in sample plus inhibitor was detected. Where ΔM is change in amount of oxidized probe between time T1 and T2, DF is dilution factor of sample, V is sample protein content added to well (mg) and ΔT is T2-T1 (min).

Data analysis

The results were shown as mean (n=3) ± SD. The LOX activity was statistically analyzed by using the one-way ANOVA test with 95% confidence (α = 0.05) by SPSS version 26. Tukey test was applied to observe a significant difference among the treatments.

Results

Leaf extractions

The results of extraction of *T. indica* leaves showed that yield of ethyl acetate extract (DI2) from *T. indica* leaves was 1.862%, and methanol extract (DI3) was 3.17%. The research on hexane extract was not done due to its low phenol and flavonoid content.

***In silico* assay**

The results of native ligand validation of the ALOX5 protein, namely on grids 40, 50 and 60, respectively, the Root Mean Square Deviation (RMSD) values are 2.1 Å, 1.85 Å and 1.98 Å. A small RMSD value in docking validation showed that the native ligand can pose and interact optimally and produce significant biological effects. The ALOX5 protein was then docked with three compounds, namely quercetin, hydroxychromone and zileuton. It was found that in the ALOX5 protein, the compounds effective in causing biological effects were hydroxychromone, quercetin and zileuton, respectively (Table 1). The active sites of ALOX5 included PHE359, HIS432, TRP599, and ARG596. The results of 2D and 3D visualization of molecular docking and ligand interactions with the target protein ALOX5 (6N2W) are presented in (Fig. 1–3). The active site was determined from the intersection of amino acid residues in the three compounds docked to the target protein (Table 2).

Viability assay

The viability test showed that a dose of 0–62.5 ppm is safe for RAW 264.7 cells with a viability percentage above 80%,

Table 1: Docking results of *T. indica* flavonoid compounds against ALOX5

Compounds	ALOX5	
	Binding energy (kcal/mol)	Inhibition constant (µM)
Zileuton	-5.93	44.95
Hydroxychromone	-7.48	3.30
Quercetin	-6.38	21.13

Table 2: Result of amino acid residues

Protein	Compounds	Amino acid residue
ALOX5	Quercetin	PHE359, GLN363, THR364, HIS432, TRP599, ALA603, ARG596, HIS600
	Hydroxychromone	PHE359, GLN363, HIS432, LEU368, HIS367, ARG596
	Zileuton	PHE359, HIS360, THR364, HIS432, TRP599, ALA603, VAL604, ARG596, HIS600

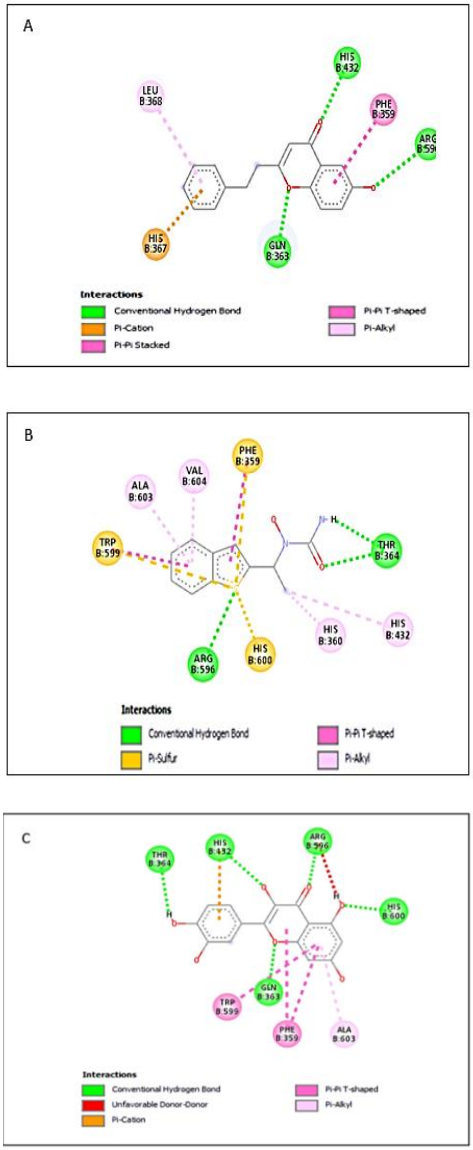

Fig. 1: The 2D molecular docking visualization results and ligand interactions with the target protein ALOX5 (6N2W), A. Hydroxychromone, B. Zileuton, C. Quercetin

while doses of 125, 250 and 1000 ppm produced a viability below 80%. From a linear equation y = -10.168x+93.806, the maximum dose produced a viability percentage above 80%. The value 80 was entered as *y* to obtain an antilog x value of 22.803 ppm. This dose was the maximum. DM3 was not toxic to RAW 264.7 cells because its viability reached 100% (Fig. 4).

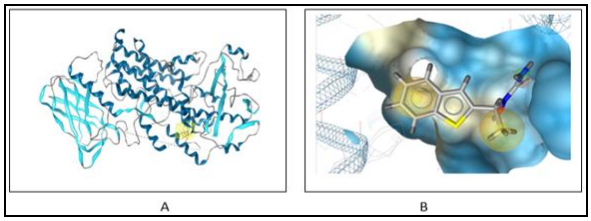

Fig. 2: Complex binding of ALOX5 with zileuton. The complete structure of ALOX5 with zileuton (A) and interaction of ALOX5 with zileuton (B)

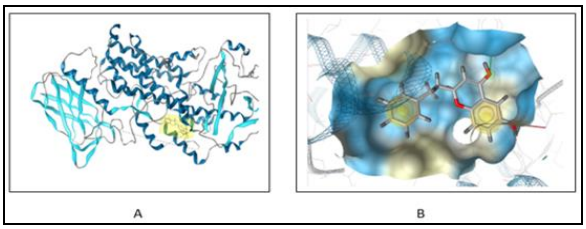

Fig. 3: Complex binding of ALOX5 with hydroxychromone. The complete structure of ALOX5 with hydroxychromone (A) and interaction of ALOX5 with hydroxychromone (B)

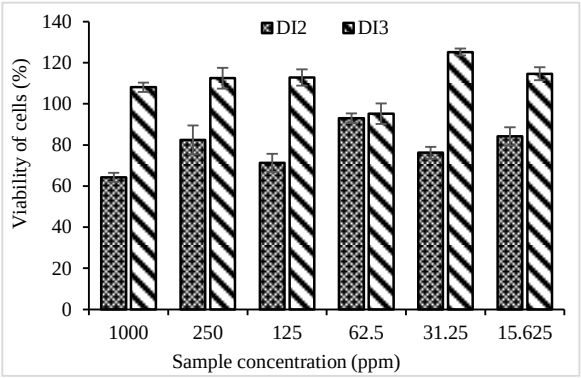

Fig. 4: Percent cell viability RAW 264.7 against DI2 (ethyl acetate extract) and DI3 (methanol extract) of leave *T. indica*. Data represent mean ± SD (n=3). The graph shows that the average viability is above 80%, indicating that the extract is not toxic to RAW cells.

Determination of protein concentration and

Before the LOX activity test, the sample's protein concentration was measured to find that LOX enzyme activity is particular for the unit weight of the protein. The linear regression equation obtained from the protein standard curve was y = 0.5649x – 0.0003, R^2= 0.9996. The measured protein concentrations in the ethyl acetate (DI2) and methanol extracts (DI3) of *T. indica* leaves were 1.553

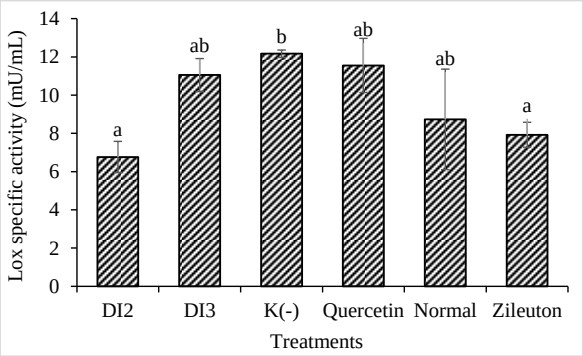

Fig. 5: Specific LOX activity of ethyl acetate (DI2) and methanol (DI3) extracts of *T. indica* leaves, negative control group K(-), normal group and positive controls quercetin and zileuton. Data are presented as mean ± SD. One-way ANOVA testing was followed by Tukey's test ($P < 0.05$). Different letters indicate significant differences ($P < 0.05$)

and 3.770 µg/µL.

LOX activity assay

The LOX enzyme activity test was carried out on ethyl acetate extract (DI2) and methanol extract of *T. indica* leaves (DI3). Quercetin from natural ingredients and zileuton as a 5-LOX inhibitor were used as positive controls. From the average specific LOX activity results, the methanol extract of *T. indica* leaves was not significantly different from the positive controls of quercetin and zileuton. However, it was significantly (P<0.05) different from the negative control. Ethyl acetate extract of *T. indica* leaves had smaller specific LOX activity than DI3, quercetin and zileuton, significantly differing from negative and normal controls (Fig. 5).

Discussion

In silico and *In vitro* assay of *T. indica* leaf extract on anti-inflammatory effects are reported in this study for the first time. According to previous research, ethyl acetate (DI2) and methanol extracts (DI3) of *T. indica* twigs contain phenolic and flavonoid compounds (Ladeska *et al*. 2022). This compound is essential for pharmacological effects, especially antioxidant and anti-inflammatory (Zeb 2020). Based on previous research, LC-MS/MS qualitative analysis of ethyl acetate and methanol extract of *T. indica* leaves contained quercetin and 5,7-dihydroxy 3-4 hydroxylbenzyl chromone compounds. This compound is also found in *T. macrophylla*, which comes also from the genus *T. indica*. This compound is found in the ethyl acetate extract of *T. macrophylla* twigs, which provides an antioxidant effect comparable to quercetin (Ladeska *et al*. 2023). The potent antioxidant effect also provides good anti-inflammatory activity.

Docking simulations were carried out for the two compounds above *in silico* with the positive control zileuton (Wu *et al*. 2019; Dwita *et al*. 2021). Zileuton has been demonstrated to be an anti-inflammatory agent due to its well-known ability to inhibit 5-LOX. Zileuton is the only 5-LOX inhibitor marketed as a treatment for asthma, and is often utilized as a selective tool to evaluate the role of 5-LOX and leukotrienes (Wu *et al*. 2019). The lowest energy score and smallest inhibition constant required for ligand interaction with the protein is the best conformation. The first stage was protein preparation by downloading the structure of the LOX target protein (PDB code: 6N2W) and preparing it to use autodocktools. The autodocktools program adds charges to ligands and receptors (removing water molecules and adding polar hydrogen), performs docking and determines grid boxes. Grid box dimensions were 40×50×60 for x, y and z.

In silico assay were carried out at this research stage to see the potential activity and interaction between the ligand molecule (native ligand) and the receptor (Meng *et al.* 2011) This research aims to predict compounds with anti-inflammatory properties with a strong affinity for ALOX5. This test was also carried out on the comparison drug zileuton as a LOX inhibitor. The ALOX5 protein has a native ligand so that the size of the active site can be revalidated, whereas ALOX5 has a relatively large active site. Revalidation was performed to determine whether the molecular docking system was close to the ligand conformation resulting from crystallography (Atmajani *et al*. 2019). The validation aims to guarantee the reliability of the native ligand docking approach as a docking reference. This validation is carried out by re-attaching the native ligand to the original protein that has been prepared. The method is said to be valid if it has a Root Mean Square Deviation (RMSD) value < 2 Å (Henever *et al.* 2009). On validation, the grid box is set using AutoDock Tools, where the grid box is the space interaction of the ligand with the amino acid residues of the target protein. Based on the validation process, a valid docking method was obtained where the RMSD value was small (1.85 Å) in order for proteins to undergo molecular docking under the same conditions as validation.

Flavonoid bioactive compounds that bind to proteins can be used as positive controls for validation using advanced molecular dynamics simulations. From the molecular docking results, it can be concluded that hydroxychromone had the highest potential to produce biological effects on the ALOX5 protein. Hydroxychromone indicated the lowest energy score and the smallest inhibition constant, so it has the highest inhibitory effect on the LOX activity (Table 1).

The RAW 264.7 cell viability test by administering DI2 and DI3 extracts showed a decrease in the number of live cells as the dose increased (Fig. 5). The maximum dose that is safe and can be used in LOX studies is 22,803 ppm. But in this study, we refer to the procedure stated in the MAX363 Kit where the dose used is much smaller than the safe dose above. LOX enzyme metabolizes arachidonic acid and produces leukotrine, which is inflammatory mediators

(Wisastra and Dekker 2014). The activity of this enzyme needs to be inhibited so that inflammation does not last long. The ethyl acetate extract (DI2) can inhibit LOX enzyme activity through oxidation of the LOX probe. In testing LOX activity using the Max 363 kit, the LOX enzyme will convert the LOX substrate into an intermediate, reacting with the LOX assay probe to produce a fluorescent product. The increase in fluorescence signal is directly proportional to LOX activity (MAK363). LOX inhibitors ultimately inhibit LOX activity, expressed as specific LOX activity in samples. As a comparison for LOX activity, zileuton and quercetin were used. Zileuton has been established as an inhibitor of 5-LOX (Wu *et al*. 2019; Dwita *et al*. 2021). Quercetin belongs to the group of natural catecholic compounds and is known as a potent, competitive inhibitor of LOX (Borbulevych *et al*. 2004). The LOX inhibitory activity of DI2 extract can be related to the content of hydrochromone compounds detected via LC-MS/MS. *In silico*, this compound has the lowest energy score and the smallest inhibition constant, so it has the highest inhibitory effect on LOX activity.

Conclusion

From the results of this research, it can be concluded that ethyl acetate extract of *T. indica* leaves has the potential to inhibit LOX activity in RAW 264.7 cells induced by lipopolysaccharide. This inhibitory activity is by the predictions of *In silico* tests that the hydroxychromone compound contained in the ethyl acetate extract plays a role in inhibiting this activity. Hydroxychromone has the lowest energy score and the smallest inhibition constant, so it has the highest inhibitory effect on LOX activity.

Acknowledgments

The authors are grateful for the Funding and Implementation of the 2024 Muhammadiyah Batch VII National Research Grants with Number: 0258.022/I.3/D/2024. The authors acknowledge the facilities and scientific and technical support from Advanced Characterization Laboratories Serpong, the National Research and Innovation Institute through E-Science Services, and the National Research and Innovation Agency (BRIN).

Author Contributions

The experimental designs, monitoring, evaluation, and interpretation of the research results are done by BE, MH, KK, and VL. BE and VL also did the write-up. VL conducted lab work and statistically analyzed the data, in *silico* assay by VL, RP, ED, AZF and analysis was made.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this study.

References

Abbas A, A Lichtman, S Pillai (2019). *Basic Immunology E-book: Functions and Disorders of the Immune System*. Elsevier Health Sciences, Beijing, China

Abdullah F, NH Ismail, F Jamaluddin, NS Hashim (2013). Xanthine oxidase inhibitory activity of *Tetracera Indica*. *Open Conf Proc J* 4:93–94

Adesoba TP, CC Brown (2023). Trends in the Prevalence of Lean Diabetes Among U.S. Adults, 2015–2020. *Diabetes Care* 46:885–889

Ahmed QU, BBS Dogarai, MZ Amiroudine, M Taher, J Latip, A Umar, BY Muhammad (2012). Antidiabetic activity of the leaves of *Tetracera indica* Merr (Dilleniaceae) *in vivo* and *in vitro*. *J Med Plants Res* 6:5915–5918

Alhassan AM, QU Ahmed, J Latip, SAA Shah (2019). A new sulphated flavone and other phytoconstituents from the leaves of *Tetracera indica* Merr and their alpha-glucosidase inhibitory activity. *Nat Prod Res* 33:1–8

Atmajani W, AB Kurniawan, R Hapsari, B Santoso (2019). Kajian *in silico* agonis PPAR gamma-receptor protein (5Y2O) sebagai antihiperglikemia menggunakan dock6. *In*: *The 9th University Research Colloqium*, Vol. 9, pp:193–200. Held at Universitas Muhammadiyah Purworejo, Indonesia

Borbulevych OY, J Jankun, SH Selman, E Skrzypczak-Jankun (2004). Lipoxygenase interactions with natural flavonoid, quercetin, reveal a complex with protocatechuic acid in its X-ray structure at 2.1 A resolution. *Proteins* 54:13–19

Dwita LP, Supandi, Yeni (2021). *In vitro* study of *Kaempferia galanga* L. compound, 3-carene against 5-lipoxygenase. *Adv Health Sci Res* 33:443–444

Esfahlan AJ, A Ostadrahimi, RJ Esfahlan, L Roufegarinejad, M Tabibiazar, R Amarowicz (2019). Recent developments in the detection of bovine serum albumin. *Intl J Biol Macromol* 138:602–617

Hasan MM, QU Ahmed, ZMS Siti, J Latip, M Taher, MFS Tengku, MS Nazira, AM Alhassan, ZA Zakaria (2017). Flavonoids from *Tetracera indica* Merr. induce adipogenesis and exert glucose uptake activities in 3T3-L1 adipocyte cells. *BMC Complement Altern Med* 17:431

Health Research and Development Agency (2019). *RISKESDAS National Report 2018*. Publishing Institution BALITBANGKES, Ministry of Health of the Republic of Indonesia, Jakarta, Indonesia

Hu C, S Ma (2018). Recent development of lipoxygenase inhibitors as anti-inflammatory agents. *Medchemcomm* 9:212–225

Henever KE, W Zhao, DM Ball, K Babaoglu, J Qi, SW White (2009). Validation of molecular docking programs for virtual screening againts dihydropteroate synthase. *J Chem Inf Model* 49.2:444–460

Kim HP, I Mani, L Iversen, VA Ziboh (1998). Effects of naturally-occurring flavonoids and biflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. *Prostaglandins Leukot Essent Fatty Acids* 58:17–24

Ladeska V, B Elya, M Hanafi, Kusmardi (2023). Pharmacognostic evaluation and antioxidant capacity of *Tetracera macrophylla* Hook. F. & Thoms twigs. *J Pharm Pharmacogn Res* 11:523–536

Ladeska V, S Saudah, D Inggrid (2022). Potensi antioksidan, kadar fenolat dan flavonoid total ranting *Tetracera indica* serta uji toksisitas terhadap sel RAW 264,7. *J Sains Farm Klin* 9:95–104

Laughton MJ, PJ Evans, MA Moroney, JRS Hoult, B Halliwell (1991). Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. *Biochem Pharmacol* 42:1673–1681

Lima CC, RPL Lemos, LM Conserva (2014). Dilleniaceae family: An overview of its ethnomedicinal uses, biological and phytochemical profile. *J Pharmacog Phytochem* 3:183–196

Meng XY, HX Zhang, M Mezei, M Cui (2011). Molecular docking: A powerful approach for structure-based drug discovery. *Curr Comput Aided-Drug* 7:146–157

Muharni, Elfita, R Adillah, H Yohandini, Julinar (2018). Flavon compound from the ethyl acetat extract of the stem of supit (*Tetracera indica* Merr), *Molekul* 13:38–47

Muniandy K, S Gothai, KMH Badran, SS Kumar, NM Esa, P Arulselvan (2018). Suppression of proinflammatory cytokines and mediators in LPS-induced RAW 264.7 macrophages by stem extract of *Alternanthera sessilis* via the inhibition of the NF-*κ*B pathway. *J Immunol Res* 2018:3430684

Perera HDSM, JKRR Samarasekera, SM Handunnetti, OVDSJ Weerasena, HD Weeratunga, A Jabeen, MI Choudhary (2018). *In vitro* pro-inflammatory enzyme inhibition and antioxidant potential of selected Sri Lankan medicinal plants. *BMC Complement Altern Med* 18:271

Ragesh RN, SR Suja, V Vilash, AL Aneeshkumar, S Rajasekharan (2016). Pharmacognostic standardisation and phytochemical analysis of *Tetracera akara* (Burm. f.) Merr. *J Tradit Folk Practices* 4:79–95

Rawa MSA, Z Hassan, V Murugaiyah, T Nogawa, HA Wahab (2019). Anticholinesterase potential of diverse botanical families from Malaysia: Evaluation of crude extracts and fractions from liquid-liquid extraction and acid-base fractionation. *J Ethnopharmacol* 245:112160

Samitra D, ZF Rozi (2017). Pengaruh air rebusan daun mempelas (*Tetracera indica* Merr.) terhadap kadar kolesterol darah mencit. *Scripta Biol* 4:197–199

Soonthornsit N, C Pitaksutheepong, W Hemstapat, P Utaisincharoen, T Pitaksuteepong (2017). *In vitro* anti-inflammatory activity of *Morus alba* L. stem extract in LPS-stimulated RAW 264.7 cells. *Evid Based Complement Alternat Med* 23:928956

Trevor AJ, BG Katzung (2002). *Master S.B. Katzung and Trevor's Pharmacology*, 6th edn. Lange Medical Books/McGraw-Hill, New York, USA

Wallace JL (2019). Eicosanoids in the gastrointestinal tract. *Brit J Pharmacol* 176:1000–1008

Wisastra R, FJ Dekker (2014). Inflammation, cancer and oxidative lipoxygenase activity are intimately linked. *Cancers* 6:1500–1521

Wongrakpanich S, A Wongrakpanich, K Melhado, J Rangaswami (2018). Comprehensive Review of Non-Steroidal Anti-Inflammatory Drug Use in The Elderly. *Aging Dis* 9:143

Wu J, K Liu, X Shi (2016). The anti-inflammatory activity of several flavonoids isolated from *Murraya paniculata* on murine macrophage cell line and gastric epithelial cell (GES-1). *Pharm Biol* 54:868–881

Wu Q, W Deng, Y Xiao, JJ Chen, C Liu, J Wang, Y Guo, M Duan, Z Cai, S Xie, Y Yuan, Q Tang (2019). The 5-lipoxygenase inhibitor zileuton protects pressure overload-induced cardiac remodeling via activating PPAR*α*. *Oxidative Med Cell Longevity* 2019:7536803

Zeb A (2020). Concept, mechanism, and applications of phenolic antioxidants in foods. *J Food Biochem* 44:e13394

Zheng L, M Wang, Y Peng, X Li (2017). Physicochemical characterization of polysaccharides with macrophage immunomodulatory activities isolated from red ginseng (*Panax ginseng* C.A. Meyer). *J Chem* 2017:3276430