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Phytochemical Constituents and Evaluation of Lipoxygenase Activity of *Tetracera macrophylla* Twigs Wall.ex Hook.f.& Thoms

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ABSTRACT: Lipoxygenase is an enzyme that catalyzes the formation of leukotriene, which is involved in various diseases such as asthma, osteoporosis and atherosclerosis. Inhibition of this enzyme can overcome inflammatory diseases. *Tetracera macrophylla* Wall.ex Hook. f.& Thoms plant has shown various biological activities, especially as an anti-inflammatory. This study aims to analyze phytochemical constituents and evaluate the lipoxygenase activity of *Tetracera macrophylla* twig extract. The sample was gradually macerated using n-hexane, ethyl acetate, and methanol. Phytochemical analysis of the extract was carried out using the LC-MS/MS method. Lipoxygenase assay was performed in vitro using RAW 264.7 macrophage cells induced with lipopolysaccharide using spectrofluorometry. As a comparison, zileuton, which has been established as a 5-LOX inhibitor and quercetin, which has the potential as a 5-LOX inhibitor. The results showed that methanol extract of *Tetracera macrophylla* twigs (RM3) had lower specific LOX activity (0.350 mU/mL) than standard zileuton (7.927 mU/mL) and quercetin (12.837 mU/mL). Decreased LOX activity can lessen the generation of leukotrienes, which cause inflammation. The study showed that methanol extract of *Tetracera macrophylla* twigs contains active compounds that can be developed as lipoxygenase inhibitors.

Keywords: ***Tetracera macrophylla*; 5-lipoxygenase; raw cell 264.7; phenolic compounds.**

Introduction

Indonesia is the largest user of medicinal plants in the world [1]. One of them that is used traditionally to treat various diseases is *Tetracera macrophylla* Hook. F. & Thoms, belongs to the Dilleniaceae family. This plant has pharmacological activities such as antidiarrhea, antidysentery, antidiabetic, antihyperuricemia and to blood pressure [2-6]. *Tetracera macrophylla* has a high phenol and flavonoid content, which has potential radical scavenging activity [7]. Antioxidant activity has an important role in inflammatory mechanisms. Inflammation is known as the basic mechanism underlying various chronic diseases. The growing role of inflammation in disease pathophysiology makes inflammatory mediators targets for the development of new anti-inflammatory drugs. Currently, research on the development of 5-LOX inhibitors for anti-inflammatory indications is increasingly popular.

Lipoxygenases (LOX) are non-heme iron containing dioxygenases. These enzymes convert unsaturated fatty acids to epoxides; for example, the synthesis of leukotrienes from arachidonic acid is mediated by lipoxygenases [8]. They play an important role in cell proliferation, differentiation, and inflammation and have been implicated in inflammation and hyperproliferation-mediated diseases such as asthma, rheumatoid arthritis, and cancer [9]. The ethyl acetate fraction of the ethanol extract of *T. macrophylla* leaves contains the compounds 5, 7-dihydroxy-8-methoxy flavone (wogonin), betulinic acid, kaempferol, quercetin, and norwogonin [7]. Wogonin's constituents were found to show anti-inflammatory activity in several animal models of inflammation. However, no clear cellular mechanism was demonstrated until the down-regulating capacity of proinflammatory molecules was discovered [10]. Previous studies from LC-MS/MS data

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of ethyl acetate extract of *T. macrophylla* twigs contained the compounds 6-hydroxy-2-(2-phenylethyl) chromone, d-catechin, isorhamnetin, and epicatechin gallate [11]. The results of docking compounds from *T. macrophylla* twig extracts also show that the compound 6-hydroxy-2-(2-phenylethyl) chromone has a strong affinity for 5-LOX. Therefore, this research was continued to the stage of testing lipoxygenase activity in vitro.

Methods

Materials

The chemicals used during the study were of analytical standard. Chloroform, methanol, n-hexane, ethyl obtained from Merck (Darmstadt, Germany), DMEM HG, Phospate Buffer Saline (PBS), Trypsin-EDTA, Pen-Strep solution, zileuton (Solarbio Science & 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 and MAK363-1KIT LOX assay kit were purchased from Sigma Aldrich (St. Louis, MO, USA).

Collection and Authentication of Plant Material

T. macrophylla was obtained from the protected forest of Teweh Baru District, North Barito Regency, Central Kalimantan (114°27'00" – 115°49'00" East Longitude and 0°58'30" North Latitude – 1°26'00" South Latitude).). The Biology Research Centre plant taxonomist, Indonesian National Research and Innovation Institute, Cibinong, Indonesia, authenticated it with collection number B-644/V/DI.05.07/11/2021. The leaves and twigs of the plant collected were washed in running water, shade-dried, powdered, passed through a 40 mesh sieve, and stored in an airtight container for further use.

Extraction

Twigs of *T. macrophylla* (6 kg) were dried and powdered, extracted by gradual maceration using n-hexane, ethyl acetate, and methanol [12]. Twig powder (1.8 kg) was soaked with n-hexane at a ratio of 1 g powder in 10 mL solvent. The maceration was carried out at room temperature for 1 × 24 hours and filtered. The residue was remacerated with the same solvent until a clear filtrate was obtained. The residue was then macerated with ethyl acetate and methanol solvent, respectively, in the same way as with n-hexane solvent. The solvent was evaporated with a rotary vacuum evaporator at 50°C (Eyela 05B-2100, China) and dried over a water bath to obtain n-hexane extract (RM1), ethyl acetate extract (RM2), and methanol extract (RM3). The dry extract was stored at 2–8°C before phytochemical analysis with LC-MS/MS.

Identification of Compounds by LC-MS/MS

Qualitative analysis of the compounds in the active extract was carried out using Liquid chromatography withTandem Mass Spectrometry(LC-MS/MS), referring to several references [6,13,15]. The measurements were obtained using a Waters Acquity UPLC I-Class equipped with XEVO G2-XS QTof mass spectrometry (Water Corp, Milford, MA, USA). The samples were separated using a column type ACQUITY UPLC® BEH C18 (1.7 µm × 2.1 mm × 50 mm) with injection volume 1 µL and full scan m/z 100 – 1200 (mode ESI). The mobile phase

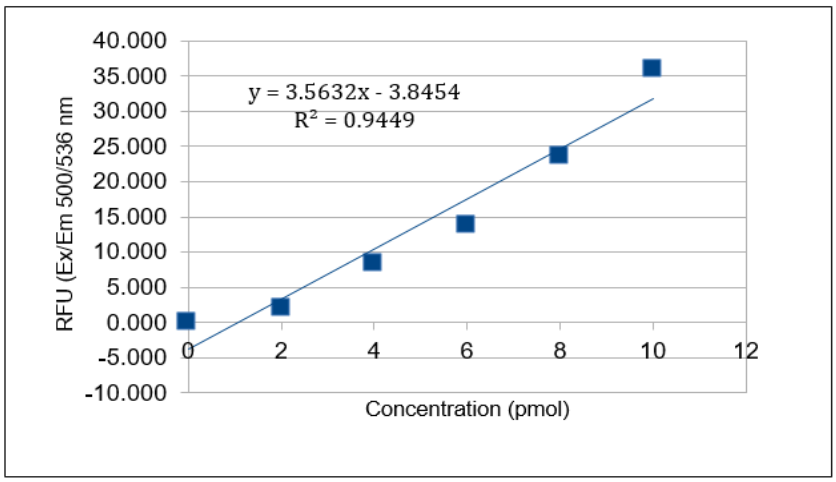

Figure 1. Graphic plot of linear regression analysis of standard lipoxygenase (LOX) at an incubation time of 10 minutes

used solvent A (H_2O + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid). The sample was prepared by dissolving 5 mg of solid and filtering through an 0.22 µm nylon filter. Mass fragmentation of compounds was detected using the instrument application's spectrum database of organic chemicals.

Determination Protein Concentration

Determine protein concentration using the Bovine Serum Albumin method (BSA). Make a standard solution from 1 mg/mL BSA stock. Make a dilution series, 0.8, 0.6, 0.4, 0.2, 0 mg/mL with distilled water. Measure the absorbance of standards and samples using a spectrophotometer (Shimadzu UV 1900, Japan**)** with λ= 280 nm. Calculate the protein concentration from the line equation obtained. Protein concentration should range between 1 and 10 µg/µL. Dilute the sample if needed using LOX Assay Buffer [16,17].

Standard Curve Preparation of LOX Assay

A new standard curve should be generated each day. Thaw one aliquot of the 100 µM oxidized probe standard at a time before performing the assay. Prepare a 1 µM oxidized probe premix solution by diluting the thawed 100µM aliquot 100-fold with DMSO. Prepare Oxidized Probe Standards in desired wells of a white 96-well plate with concentrations of 10,8,6,4,2,0 pmol/well. Have the plate reader ready at λEx=500 nm/λEm=536 nm (Thermo scientific, Variouskan flash, Finland)). Immediately start recording fluorescence (RFU) at 10 minutes and 40 minutes. LOX standard curves for incubation times of 10 and 40 minutes are in Figure 1 and Figure 2.

RAW 264.7 Cell Culture Preparation

RAW 264.7 Cells were in complete medium DMEM HG+FBS 10%+ Penicillin Streptomycin 1%. Cells were harvested when they had reached 80-90% confluence—detach cells using 0.05% trypsin in PBS as much as 1 mL. Add 5 ml of complete medium, then centrifuge for 5 minutes at 1200 rpm. Discard the supernatant, then add 1 mL of medium and suspend. Cells were counted using a hemocytometer and $4x10^5$ trypan blue per well.

LOX Activity Assay

The LOX activity assay was carried out based on the procedure stated in the Lipoxygenase Activity Assay Kit with catalogue number MAK363 [16] (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 x 10^5 RAW per well added with 200 µL of 25 µg/mL test solution, incubated for 2 hours. Then 200 µL LPS was added to each well, and incubated at 37°C in a moisture-saturated atmosphere containing 5% CO_2 for 24 hours. Then add 100 µL LOX Lysis buffer (keep on ice) for 10 minutes. Homogenize cells ($4x10^5$ cells) with 100 µL of ice-cold LOX Lysis Buffer and keep on ice for 10 minutes. Centrifuge at 10,000 x g for 15 minutes at 4°C. Collect the supernatant. Prepare three wells for each sample labelled, sample background control (BC), sample (S) and sample plus inhibitor (SI). Add 1 µL sample into each of the three wells. For SI wells, add 70 µL SI mix, 27 µL LOX assay buffer, and 2 µL of LOX inhibitor in addition to the sample. For S wells, add 70 µL S mix and 29 µL LOX assay buffer in addition to the sample, while for BC wells, add 70

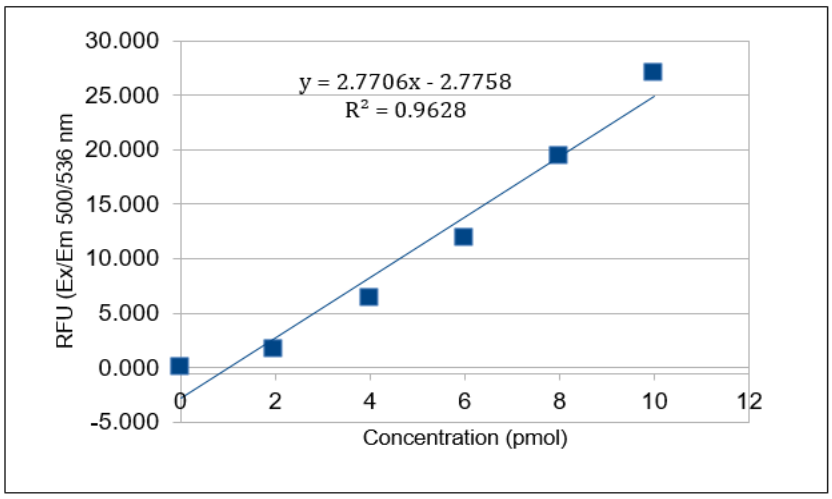

Figure 2. Graphic plot linear regression analysis of standard lipoxygenase (LOX) at an incubation time of 40 minutes

μL BC mix and 29 μL LOX assay buffer in addition to the sample. Immediately start recording fluorescence (RFU) at λ_{Ex}=500 nm/λ_{Em}=536 nm (Thermo scientific, Variouskan flash, microplate reader, Finland). LOX activity may be calculated using the following equations:

LOX activity in sample (mU/mL)=(ΔM x DF)/(ΔT x V)

ΔM = Change in amount of oxidized probe between time T1 and T2
DF = Dilution factor of Sample
V = Sample protein content added to well (mg)
ΔT = T2-T1 (minutes)

Statistical analysis

Tests were presented as mean ± SD. One-way analysis of variance (ANOVA $p<0.05$) was conducted using SPSS version 25 to assess the significant difference between the mean values, followed by the Tukey test.

Result and Discussion

Extraction of *T. macrophylla* twigs produced a dry extract of n-hexane (15.17g), ethyl acetate extract (31.33 g) and methanol extract (218.9 g). The results of protein determination showed protein concentrations ranging from 1-10 μg/μL. As positive controls, 5-LOX inhibitors were used zileuton and quercetin. Both of these compounds have been reported to reduce the formation of 5-LOX eicosanoids in vitro [18,19]. LC-MS/MS analyzed the chemical contents of the three extracts. The n-hexane extract contains triterpenoid compounds and fatty acids such as 2,4,7-trimethoxy-9,10-dihydrophenan-threne, ambronal, ergost-5-en-3-ol, samarcandone and taraxerone, while ethyl acetate and methanol extracts contain phenolic compounds and flavonoids such as 6-hydroxy-2-(2-phenylethyl) chromone, d-catechin, isorhamnetin and epicatechin gallate (Table 1). The n-hexane extract does not contain phenolic compounds, so it is not continued to the LOX test stage. Based on the above mentioned, different extraction solvents aim to separate compounds based on their polarity. Moreover, several reports show greater LOX inhibitory activity of isolated compounds themselves than extracts on the whole [20]. Isolating active extracts from this plant is the next research target.

From the LOX standard curve with an incubation time of 10 minutes, the regression equation y=3.5632x–3.8454 with R^2=0.9449 and the LOX standard curve with an incubation time of 40 minutes obtained the regression equation y=2.7706x–2.7758 with R^2 = 0.9628 (Figure 1 and Figure 2). The standard curve estimates the amount of oxidized probe in each sample reaction. The results of this study showed that the methanol extract of *T. macrophylla* twigs had a specific LOX activity of 0.350 ± 1.363 mU/mL, which was smaller than the positive controls of quercetin (12.837 ± 1.576 mU/mL) and zileuton (7,927 ± 0.655 mU/mL). Meanwhile, ethyl acetate extract had a specific LOX value of 14.990 ± 0.909 mU/mL (Figure 3). The smaller the specific LOX value, the weaker the LOX activity in converting unsaturated fatty acids into epoxides; for example, the synthesis of leukotrienes from

Table 1. Results identification of the methanol and ethyl acetate extract of twigs *T. Macrophylla* by LC-MS/MS

No	Extract	Observed m/z	Observed RT	Component Name	Formula
1	n-Hexane	271.13	6.68	2,4,7-Trimethoxy-9,10-dihydrophenan-threne	$C_{17}H_{18}O_3$
		439.35	9.13	Ambronal	$C_{30}H_{46}O_2$
		423.36	10.66	Ergost-5-en-3-ol	$C_{28}H_{48}O$
		421.20	9.38	Samarcandone	$C_{24}H_{30}O_5$
		425.37	9.29	Taraxerone	$C_{30}H_{48}O$
2	Ethyl acetate extract	267.10	4.54	6-hydroxy-2-(2-phenylethyl) chromone	$C_{17}H_{14}O_3$
		291.08	2.40	d-catechin	$C_{15}H_{14}O_6$
		317.06	5.25	Isorhamnetin	$C_{16}H_{12}O_7$
		443.09	3.27	Epicatechin gallate	$C_{22}H_{18}O_{10}$
3	Methanol extract	443.09	3.27	Epicatechin gallate	$C_{22}H_{18}O_{10}$

Note: RT = Retention Time, m/z = mass number/charge number

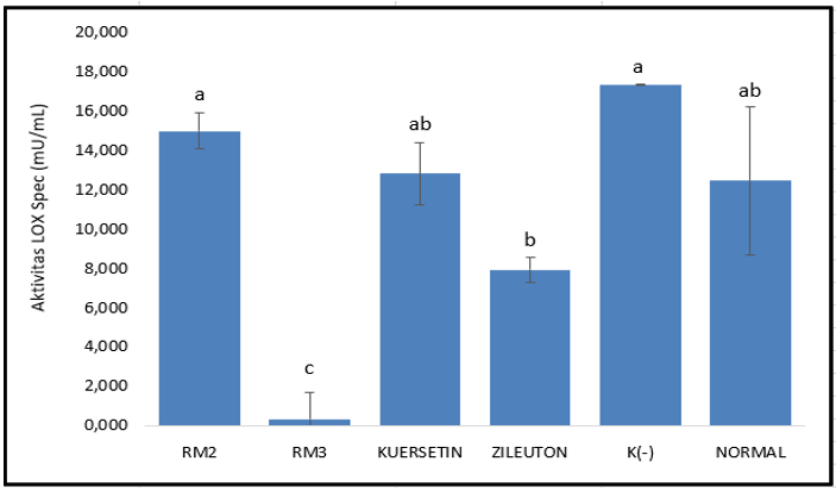

Figure 3. LOX activity specific of Twig *T. macrophylla Extracts* (RM2=Ethyl Acetate Extract, RM3= Methanol Extract). Significance value=p 0.05, with ANOVA followed by Tukey test. The letters difference is significant at the 0.05 level

arachidonic acid is mediated by lipoxygenases weak LOX activity results in the formation of pro-inflammatory mediators such as leukotrienes which are also impaired. As a result, the inflammatory response does not occur [21,22].

LOX inhibitory activity is thought to be related to the content of phenolic and flavonoid compounds found in *T. macrophylla* twigs. According to Kim et al 2004 [10] phenolic and flavonoid compounds have shown anti-inflammatory activity in vitro and in vivo. One important mechanism is inhibiting the LOX enzyme, thereby reducing leukotriene concentrations. Reduction of lipoxygenase ferric to ferrous ions, which prevents the activation of the enzyme and inhibits its activity [23]. The inhibitory activity of LOX is related to the high antioxidant activity of phenolic compounds [24]. The antioxidant activity of phenolic compounds is probably due to their ability to scavenge free radicals. Flavonoids also have antioxidant activity, inhibiting the expression of mast cells, macrophages, eosinophils and neutrophils, which are markers of inflammation. Inhibition of the expression of proinflammatory mediators causes the formation of inflammation to be reduced [25]. Generally, the groups that provide anti-inflammatory activity from flavonoids are unsaturated double bonds, hydroxyl groups and carbonyl groups [21].

Previous research shows that quercetin and catechin (epigalocatechin gallate) are natural flavonoids in *T. macrophylla* twigs. These two compounds have inhibition of dioxygenase and lipoxygenase in the prevention and treatment of disease [26]. Wogonin (5-7-dihydroxy-8-methoxy flavone), a flavonoid compound, inhibits COX-2 expression and COX-2 activity selectively without affecting COX-1 and LOX [10]. Flavonols, including kaempferol, quercetin, myricetin, were found to be potential 5-LOX inhibitors. Quercetin has been reported to reduce the formation of 5-LOX eicosanoids in vitro [27,28].

Conclusion

T. macrophylla twigs contain phenolic and flavonoid compounds. This compound has anti-inflammatory activity and has the potential to be developed as a LOX inhibitor. The methanol extract has the lowest particular LOX activity of 0.350 mU/mL, which means this extract can potentially reduce LOX activity. Meanwhile, this research showed that *T. macrophylla* contains active compounds that could be developed as LOX inhibitors.

Conflict of Interest

The authors have no conflicts of interest regarding this investigation

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