

Antioxidant Activity and Lipoyxygenase Inhibition Test with Total Flavonoid Content from *Garcinia kydia* Roxburgh Leaves Extract

Nur Laily Putri, Berna Elya*, Nuraini Puspitasari

ABSTRACT

Introduction: Antioxidant is one of the therapeutic strategies to overcome oxidative stress and inhibit synthesis of inflammatory mediators through lipoyxygenase pathway. *Garcinia* is the largest of Clusiaceae family which has been proven to provide antioxidant and anti-inflammatory activity. *Garcinia kydia* Roxburgh is one of the plants of this genus which is known to have antioxidant activity but lipoyxygenase inhibition activity from this plant was unknown. **Methods:** This study aimed to test antioxidant activity of the methanol, ethyl acetate and n-hexane extract from *Garcinia kydia* Roxburgh leaves by FRAP (Ferric Reducing Antioxidant Power) method, anti-inflammatory activity was tested by inhibiting lipoyxygenase and total flavonoid content by colorimetric methods AICI3. **Results:** The results showed an antioxidant activity of methanol extract, ethyl acetate and n-hexane leaves of *Garcinia kydia* Roxburgh have EC₅₀ value, respectively 18,448; 12,389 and 31,260 µg/mL, and the lipoyxygenase inhibition activity have IC₅₀ value, respectively 0,556; 0,212 and 3,575 µg/mL. Ethyl acetate extract of *Garcinia kydia* Roxburgh leaves was the most active extract in this study which has total flavonoid content, 30,650 mgQE/gram extract. **Conclusion:** The conclusion, *Garcinia kydia* Roxburgh has antioxidant and lipoyxygenase inhibition activity, with ethyl acetate extract as the most active extract which contains total flavonoids.

Key words: Antioxidant, Flavonoid content, FRAP, *Garcinia kydia* Roxburgh, Lipoyxygenase.

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INTRODUCTION

Free radicals can cause oxidative stress. Oxidative stress related to the pathogenesis of inflammatory because it can induce the synthesis of inflammatory mediators.¹ Lipoyxygenase is an enzyme which involved in the biosynthesis of inflammatory mediators such as leukotrienes which play an important role in the development of inflammatory diseases such as asthma.² Therefore, research on the compound to prevent the synthesis of inflammatory mediators through the lipoyxygenase continue to do. An antioxidant is used as a therapeutic strategy to overcome oxidative stress and inflammatory diseases.^{3,4} Some plants are known to have antioxidant activity, such as *Garcinia*. *Garcinia* is the largest genus of Clusiaceae which has 400 species and 77 species among them are found in Indonesia.⁵ Every part of *Garcinia* like fruit, flowers, leaves, and stems are widely used to treat inflammation and oxidative stress⁶ and also *Garcinia* mostly contain xanton, benzophenone, and mangosteen which has antibacterial, antioxidant and anti-inflammatory activity, so Indonesia has a big chance to explore the plant *Garcinia* as herbal medicine.

Previous antioxidant study on *Garcinia* showed that methanol extract, ethyl acetate and n-hexane leaves of *Garcinia atroviridis* could scavenge radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) respectively 57,97; 59,18 and 55,67%.⁷ *Garcinia* also has anti-inflammatory activity. Study about anti-inflammatory activity on *Garcinia nervosa* leaves and stems extract showed

the percentage lipoyxygenase inhibition activity, respectively for 86.53 and 62,54%.⁸

This study aimed to investigate *Garcinia kydia* Roxburgh. The leaves of this plant are used traditionally as antitumor and anti-inflammatory. The previous study showed that leaves of *Garcinia kydia* Roxb. using DPPH method on methanol extract, ethyl acetate, and n-hexane have antioxidant activity. The most-active extracts from these studies were ethyl acetate extract with IC₅₀ 11,06 µg/ML.¹⁰ The objective of this research was to study antioxidant and lipoyxygenase inhibition activity from *Garcinia kydia* Roxb. leaves extract.

MATERIALS AND METHODS

Materials

Methanol, ethyl acetate, and n-hexane leave extract of *Garcinia kydia* Roxb. Are obtained from Laboratory of Phytochemistry and Pharmacognosy, Faculty of Pharmacy, University of Indonesia.

Ferric Reducing Antioxidant Power(FRAP) Assay

The ferric reducing power of *Garcinia kydia* extracts was performed using a modified version of FRAP assay.¹¹ This reduction was measured using Spectrophotometer UV-Vis (PG Instruments Ltd T80) at 596 nm. The stock FRAP reagent was prepared daily by mixing 1 volume 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, with 1 volume mL

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20 mM FeCl₃.6H₂O solution and 10 volume 300 mM acetate buffer pH 3,6. Baicalein used as positive control. For the test, 0,2 mL sample test solution were allowed to react with 3,8 mL of the FRAP solution for 30 minutes at 37°C in the incubator. For the control, the sample contained 0,2 mL ethanol and 3,8 mL FRAP solutions. Readings the absorbance of colored product (Fe²⁺-TPTZ complex) at 596 nm. Antioxidant capacity was evaluated by calculating the production of Fe²⁺-TPTZ complex. % antioxidant capacity = (1-Ts) x 100%. Where Ts is transmittance, As = -logTs and As is derived from sample absorbance - control sample absorbance.

Lipoxygenase Inhibition Test

Enzyme activity was measured using Spectrophotometer UV-Vis (PG Instruments Ltd T80) at 235 nm and 25°C after addition of boric acid 200 mM pH 8,5, lipoxygenase (375U/mL, final concentration), using linoleic acid (225 µM, final concentration) as a substrate, and methanol as stop solution. Baicalein was employed as a positive control. For the test, 1,69 mL boric acid and 1 mL linoleic acid was preincubated with 0,01 mL sample test solution for 10 minutes at 25°C, followed by addition of 0,3 mL lipoxygenase and was incubated again for 15 minutes at 25°C. Enzyme reaction was terminated by the addition of 1 mL methanol to the final volume of 4 mL and absorbance of product reaction HPOD was measured at 235 nm. For the sample control, only boric acid, linoleic acid, boric acid and methanol. Blanks contained boric acid, linoleic acid, lipoxygenase, and methanol. For the blanks, control contained boric acid, linoleic acid, and methanol were pipetted into the cuvette. The lipoxygenase inhibitory activity was evaluated by calculating the percentage of the inhibition of HPOD production from the changes in absorbance values. % inhibition = [(A_b-A_{bc})-(A_c-A_{cc})] x 100/(A_b-A_{bc}). Where A_b is the absorbance of control, A_{bc} is the blanks control, A_c is the absorbance of sample and A_{cc} is the absorbance of sample control.

Thin Layer Chromatography (TLC) Profile

To activate the plates, silica gel 60 F₂₅₄ 20x20 cm was heated at 105°C for 30 minutes in the oven before used. Chamber was saturated using mobile phase of each extract. An optimum mobile phase used for methanol, ethyl acetate, and n-hexane leaves extract of *Garcinia kydia* Roxb. respectively ethyl acetate - formic acid (20: 1), toluene - ethyl acetate - formic acid (61: 30: 9) and n-hexane - ethyl acetate (6: 4). For the test, 10 mg/mL sample test solution was spotted on TLC plates using 1 µL microcapillary tube. The plates were eluted by mobile phase after elution was completed the plates were dried in the air and extract spots were observed with

visible light of short wavelength ultraviolet (254 nm) and longwave (366 nm). Then the plates were sprayed with AlCl₃ to 5% and Rf values were counted.

Flavonoid Content

The assay was performed according to a previously described procedure.¹² Flavonoid in methanol, ethyl acetate and n-hexane leaves extract of *Garcinia kydia* Roxb. were estimated as Quercetin equivalent. Quercetin was used to make the calibration curve. For the test, 0,5 mL sample test solution were mixed with 1,5 mL methanol, 0,1 mL aluminium chloride 10%, 0,1 mL sodium acetate 1 M and 2,8 mL distilled water. The volume of aluminium chloride was substituted by the same volume of distilled water in blank. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 435 nm. From the calibration curve of quercetin, concentration values of all extracts and total flavonoid content was calculated by using formula, TFC = (R x DF x V x 100)/W. Where R is the result obtained from standard curve (mg/mL), DF is dilution factor, V is volume of stock solution (mL), and W is weight of extract used in sample test (gram).¹³

Statistical analysis All values obtained were analyzed statistically using Microsoft Office Excel 2013 and Graphpad Prism 7.

RESULTS AND DISCUSSION

Antioxidant Activity

The FRAP assay is the only assay that directly measures antioxidants or reductants in a sample. The other assays are more indirect because they measure the inhibition of reactive species (free radicals) generated in the reaction mixture, and these results also depend strongly on the type of reactive species used.¹⁴

The principle of the test using FRAP method measures the ability of an antioxidant to reduce Fe³⁺ in the complex TPTZ to Fe²⁺. The reduction reaction is shown by baicalein solution and extracts were initially yellowish colored clear solution when mixed with FRAP, the mixture solution became blue and blue more intense after incubation. If the sample reduces Fe³⁺ to Fe²⁺, at the same time sample will be oxidized, so sample can act as an antioxidant.¹⁵

Baicalein was used as a positive control because it has catecholic alcohol to ligate the iron of lipoxygenase and cause an inner sphere reduction on the active site iron, with baicalein undergoing oxidation to its quinone form. The result showed EC₅₀ baicalein was 1,165 µg/mL. So, baicalein

Table 1: Calibration curve and EC₅₀ values of baicalein and extract

| No | Sample | Calibration Curve | EC ₅₀ (µg/mL) |
|----|-----------------------|--|--------------------------|
| 1 | Baicalein | y = 17,663 + 27,756x, r ² = 0,992 | 1,165 |
| 2 | Methanol Extract | y = 22,893 + 1,4694x, r ² = 0,9789 | 18,448 |
| 3 | Ethyl acetate extract | y = 18,629 + 2,5321x, r ² = 0,9855 | 12,389 |
| 4 | n-Hexane extract | $y = \frac{100}{(1+10^{(1,495-X)+1,784})}$, r ² = 0,9897 | 31,260 |

Table 2: Calibration curve and EC₅₀ values of baicalein and extracts

| No | Sample | Calibration Curve | IC ₅₀ (µg/mL) |
|----|-----------------------|--|--------------------------|
| 1. | Baicalein | y = 5,9918 + 186x, r ² = 0992 | 0,237 |
| 2. | Methanol Extract | y = -13,545 + 114,33x, r ² = 0,9756 | 0,556 |
| 3. | Ethyl acetate extract | $y = \frac{100}{(1+10^{(-0,6728-X)+3,954})}$, r ² = 0,9868 | 0,212 |
| 4. | n-Hexane extract | $y = \frac{100}{(1+10^{(0,5582-X)+2,287})}$, r ² = 0,9773 | 3,575 |

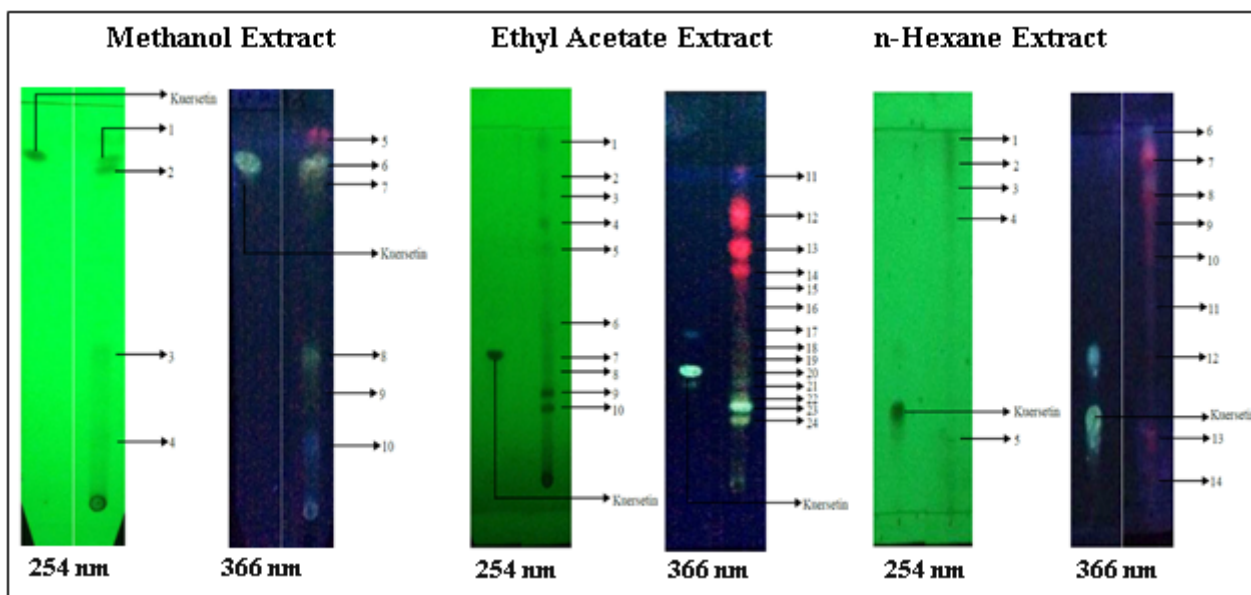


Figure 1: Chromatogram quercetin and Garcinia kydia Roxb leaves extract.

was classified as a very strong antioxidant because the value of $EC_{50} < 50 \mu\text{g/mL}$.¹⁵

Antioxidant activity test showed that methanol, ethyl acetate, and n-hexane leaves extract of *Garcinia kydia* Roxb. had EC_{50} respectively 18,448; 12,389 and 31,260 $\mu\text{g/mL}$. These results indicate that all extract has very strong antioxidant activity (Table 1).¹⁵

Based on phytochemical screening conducted on *Garcinia kydia* Roxb. leaves extract showed that methanol extract contained several secondary metabolites, such as alkaloids, flavonoids, tannins, anthraquinone, and saponin; ethyl acetate extract contained alkaloids, flavonoids, and terpenoids; as well as n-hexane extract contained terpenoids.¹⁰ Flavonoids and tannins classified into phenolic compounds. In general, phenolic compounds having one or more aromatic rings with one or more hydroxyl groups. Their antioxidant activity will increase if they have more free hydroxyl groups and bond conjugation in the aromatic ring. Antioxidant potency of phenolic compounds is associated with the ability to donate electrons, reduce and chelate metal ions.¹⁶ The largest group of phenolic compounds are flavonoid. Flavonoid has an OH group at the ortho position C 3', 4', 3, keto group at C4, the double bond at the C2 and C3 which has high antioxidant capacity. OH group in the ortho position C3' - C4' have the highest impact on the antioxidant capacity of flavonoids because it can be used to donate H atoms in free radicals.¹⁵ In addition to phenolic compounds, indole alkaloids such groups also have antioxidant activity. Alkaloid compounds can stop free radical chain by donating H atoms in free radicals.¹⁷ Then, the anthraquinone compounds are known to have antioxidant activity through reducing the hydroxyl radical.¹⁸ Tetraterpen compounds such as terpenoids and carotenoids have antioxidant activity both in vitro and in vivo.¹⁶ The main mechanism of carotenoids as antioxidants is reverse the singlet oxygen and donates electrons or hydrogen.¹⁹ Based on these studies, it is known that secondary metabolites such as alkaloids, flavonoids, tannins, anthraquinone, and terpenoids have antioxidant activity. Therefore, it can be estimated that compounds contribute to an antioxidant activity held *Garcinia kydia* Roxb. leaves extract.

Lipoxygenase Inhibition

The principle of this study was inhibited lipoxygenase activity to form hydroperoxide compound which plays important roles in the development of acute inflammation.²⁰ The product reaction between lipoxygen-

ase and linoleic acid is hydroperoxyoctadecadienoat (HPOD). Iron in lipoxygenase is involved in electron transfer during the process of insertion of oxygen into unsaturated fatty acids containing cis, cis-1,4-pentadiene. Lipoxygenase in the oxidized form (Fe^{3+}) can catalyze the release of the hydrogen atoms of the C-11 methylene group in linoleic acid to form a radical pentadiene and reduced lipoxygenase (Fe^{2+}), under aerobic conditions these radicals react with dioxygen to a hydroperoxy radical. As a result of the oxidation of Fe^{2+} to Fe^{3+} , fatty acids are formed into 13-hydroperoxy-9 (Z), 11 (E) -octadecadienoat (13(S)-HPOD).^{20,21,22}

The results showed baicalein IC_{50} was 0.237 $\mu\text{g/mL}$, whereas in the previous study the value of IC_{50} obtained was 0.324 $\mu\text{g/mL}$ (Table 2).²³ Baicalein acted as an inhibitor which inhibits the oxidation of linoleic acid into HPOD. Baicalein have a catechol group that can bind iron and lead reduction in the active site of iron lipoxygenase so baicalein undergo oxidation to form quinones and the enzyme can not react with the substrate to form leukotrienes.²⁴

The test results showed that methanol, ethyl acetate, and n-hexane leaves extract of *Garcinia kydia* Roxb. have IC_{50} respectively 0,556; 0,212 and 3,575 $\mu\text{g/mL}$. These results indicate that the three extracts could inhibit lipoxygenase activity. Ethyl acetate extract was the most active extracts that have the lowest IC_{50} , 0,212 $\mu\text{g/mL}$. Phytochemical screening ever conducted, showed that the ethyl acetate extract contains alkaloids, flavonoids, and terpenoids. Therefore, it is thought that the presence of such compounds has a synergistic effect in lipoxygenase inhibition activity from ethyl acetate extract.²⁵

Based on phytochemical screening conducted on *Garcinia kydia* Roxb. leaves extract showed that methanol extract contained secondary metabolites such as alkaloids, flavonoids, tannins, anthraquinone, and saponin; ethyl acetate extract contains alkaloids, flavonoids, and terpenoids; as well as n-hexane extract contains terpenoids.¹⁰ Based Chedea and Jisaka, polyphenol compounds such as flavonoids and tannins can inhibit lipoxygenase activity. Flavonoids such as flavonols have a catechol group that can form complexes with Fe^{3+} on an enzyme. Other flavonoids such as isoflavones can inhibit lipoxygenase activity with donating electrons. This causes the electron donor Fe^{3+} on enzyme become reduced to its inactive form (Fe^{2+}).²⁶ Terpenoids compounds such as carotenoids also inhibit lipoxygenase activity by keeping the iron in the enzyme in its inactive form (Fe^{2+}). In addition to these compounds, it is known that the alkaloid and anthraquinone can also inhibit lipoxygenase activity.^{27,28}

Based on these studies, it is known that secondary metabolites such as alkaloids, flavonoids, tannins, anthraquinone, and terpenoids can inhibit lipoxygenase activity. Therefore, it can be estimated that compounds contribute to the inhibition of lipoxygenase activity which is owned by the leaf extract of *Garcinia kydia* RoxbTLC Profile (Figure 1).

TLC profile aimed to determine the most flavonoid content on extracts qualitatively and supports antioxidant and lipoxygenase inhibition activity result. Chromatogram has sprayed by AlCl_3 will react with the keto group in the C4, C5 and OH group on ortho position to form yellow complex compounds.²⁹ The results showed that methanol and ethyl acetate extract have a yellow spot. The conclusion, both extracts contained flavonoid and ethyl acetate extract has more yellow spots than methanol extract. So, flavonoid content on ethyl acetate extract would be counted quantitatively.

Total Flavonoid Content

Flavonoid content assay was performed to determine total flavonoids content in the most active extract. The most-active extract is an extract which has the highest antioxidant and lipoxygenase inhibition activity. Most active extracts obtained from the study was ethyl acetate extract. Total flavonoid assay was tested by colorimetric methods AlCl_3 with quercetin as a positive control. AlCl_3 can form a stable complex with C4 keto group and a hydroxyl group at C3 and C5 of flavones and flavonols. In addition, AlCl_3 can form labile complexes with ortho-dihydroxyl group in ring A and B flavonoid.³⁰ Based on the results, quercetin calibration curve, $y = 0.1393 + 0.0434x$, $R^2 = 0.9957$ and 1 gram of ethyl acetate extract contain 30,65 mg quercetin equivalents.

CONCLUSION

Garcinia kydia Roxburgh leaves extract has antioxidant and lipoxygenase inhibition activity, with ethyl acetate extract as the most active extract which contains a total flavonoid.

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CONFLICT OF INTEREST

No conflict of interest are declared.

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