Antioxidant Activity and Lipoxygenase 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 lipoxygenase 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 lipoxygenase 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 lipoxygenase and total flavonoid content by colorimetric methods AlCl3. **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 lipoxygenase 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 lipoxygenase inhibition activity, with ethyl acetate extract as the most active extract which contains total flavonoids.

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

**INTRODUCTION**

Free radicals can cause oxidative stress. Oxidative stress related to the pathogenesis of inflammatory mediators because it can induce the synthesis of inflammatory mediators. Lipoxygenase 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. Therefore, research on the compound to prevent the synthesis of inflammatory mediators through the lipoxygenase continue to do. An antioxidant is used as a therapeutic strategy to overcome oxidative stress and inflammatory diseases. 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. Every part of Garcinia like fruit, flowers, leaves, and stems are widely used to treat inflammation and oxidative stress 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-di-phenyl-2-picrylhydrazyl (DPPH) respectively 57.97; 59.18 and 55.67%. Garcinia also has anti-inflammatory activity on *Garcinia nervosa* leaves and stems extract showed the percentage lipoxygenase inhibition activity, respectively for 86.53 and 62.54%.

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. The objective of this research was to study antioxidant and lipoxygenase inhibition activity from *Garcinia kydia* Roxb. leaves extract.

**MATERIALS AND METHODS**

**Materials**

Methanol, ethyl acetate, and n-hexane leaf 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. 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.
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. The plates were sprayed with AlCl₃ at 5% and Rf values were counted.

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 - 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 was 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,

\[ TFC = \frac{(R \times DF \times V \times 100)}{W} \]

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

<p>| Table 1: Calibration curve and EC₅₀ values of baicalein and extract |</p>
<table>
<thead>
<tr>
<th>No</th>
<th>Sample</th>
<th>Calibration Curve</th>
<th>EC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Baicalein</td>
<td>17,663 + 27,756x, r² = 0.992</td>
<td>1,165</td>
</tr>
<tr>
<td>2</td>
<td>Methanol Extract</td>
<td>22,893 + 1,4694x, r² = 0.9789</td>
<td>18,448</td>
</tr>
<tr>
<td>3</td>
<td>Ethyl acetate extract</td>
<td>18,629 + 2,5321x, r² = 0.9855</td>
<td>12,389</td>
</tr>
<tr>
<td>4</td>
<td>n-Hexane extract</td>
<td>18,448 + 3,575x, r² = 0.9977</td>
<td>31,260</td>
</tr>
</tbody>
</table>

<p>| Table 2: Calibration curve and EC₅₀ values of baicalein and extracts |</p>
<table>
<thead>
<tr>
<th>No</th>
<th>Sample</th>
<th>Calibration Curve</th>
<th>IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Baicalein</td>
<td>5,9918 + 186x, r² = 0.992</td>
<td>0.237</td>
</tr>
<tr>
<td>2</td>
<td>Methanol Extract</td>
<td>13,545 + 114,33x, r² = 0.9756</td>
<td>0.556</td>
</tr>
<tr>
<td>3</td>
<td>Ethyl acetate extract</td>
<td>18,448 + 3,575x, r² = 0.9977</td>
<td>0.212</td>
</tr>
<tr>
<td>4</td>
<td>n-Hexane extract</td>
<td>18,448 + 3,575x, r² = 0.9977</td>
<td>3,575</td>
</tr>
</tbody>
</table>
was classified as a very strong antioxidant because the value of EC₅₀ < 50 µg/mL.³⁵

Antioxidant activity test showed that methanol, ethyl acetate, and n-hexane leaves extract of *Garcinia kydia* Roxb. had EC₅₀ respectively 18,448; 12,389 and 31,260 µg/mL. These results indicate that all extract has very strong antioxidant activity (Table 1).³⁵

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.³⁶ 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.³⁶ The largest group of phenolic compounds are flavonoid. Flavonoid has an OH group at the ortho position C 3', 4', 3, keto group at C 4, the double bond at the C2 and C3 which has high antioxidant capacity. OH group in the ortho position C 3', 4' have the highest impact on the antioxidant capacity of flavonoids because it can be used to donate H atoms in free radicals.³⁶ 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.³⁷ Then, the anthraquinone compounds are known to have antioxidant activity through reducing the hydroxyl radical.³⁷ Tetrapenten compounds such as terpenoids and carotenoids have antioxidant activity both in vitro and in vivo.³⁸ The main mechanism of carotenoids as antioxidants is reverse the singlet oxygen and donates electrons or hydrogen.³⁹ Based on these studies, it is known that secondary metabolites such as alkaloids, flavonoids, tannins, anthraquinone, and terpenoids, and phenolic compounds 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.⁴⁰ The product reaction between lipoxygenase and linoleic acid is hydroperoxycodadecadienoat (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 (Fe³⁺) 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 (Fe²⁺), under aerobic conditions these radicals react with dioxygen to a hydroperoxy radical. As a result of the oxidation of Fe²⁺ to Fe³⁺, fatty acids are formed into 13-hydroperoxy-9 (Z), 11 (E) -octadecadienoat (13(S)-HPOD).²⁰,²¹,²² The results showed baicalein IC₅₀ was 0.237 µg/mL, whereas in the previous study the value of IC₅₀ obtained was 0.324 µg/mL (Table 2).²² Baica-lein 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 IC₅₀ respectively 0.556; 0.212 and 3,575 µg/mL. These results indicate that the three extracts could inhibit lipoxygenase activity. Ethyl acetate extract was the most active extracts to form quinones and the enzyme can not react with the substrate to form leukotrienes.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. Therefore, it is thought that the presence of such compounds has a synergistic effect in lipoxygenase inhibition activity from ethyl acetate extract.²⁵

**Figure 1:** Chromatogram quercetin and Garcinia kydia Roxb leaves extract.
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* Roxb.TLC 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 with AlCl₃ will react with the keto group in the C4, C5, and OH group on ortho position to form yellow complex compounds. 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 AlCl₃ with quercetin as a positive control. AlCl₃ can form a stable complex with C4 keto group and a hydroxyl group at C3 and C5 of flavones and flavonols. In addition, AlCl₃ can form labile complexes with ortho-dihydroxyl group in ring A and B flavonoid. 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.

**REFERENCES**

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