

CYCLEA BARBATA LEAF EXTRACT: LIPOXYGENASE INHIBITORY ACTIVITY AND PHYTOCHEMICAL SCREENING

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ABSTRACT

Objective: The aim of this study was to test the anti-inflammatory activity of methanol, ethyl acetate, and n-hexane *Cyclea barbata* Miers. leaf extracts using a lipoxygenase inhibition method.

Methods: Enzyme activity assay was performed using colorimetric method with lipoxygenase from soybean and linoleic acid as a substrate. Absorbance was measured at 234 nm. The total flavonoid content of the most active extract was determined using a colorimetric method with $AlCl_3$ and phytochemical screening.

Results: The ethyl acetate extract had the highest lipoxygenase inhibiting activity, with an IC_{50} value of 0.267 μ g/mL. In addition, this extract had a total flavonoid content of 21.62 mgQE/g and contained flavonoids, glycosides, and terpenoids.

Conclusion: This finding indicated that *C. barbata* provides a possible anti-inflammatory effect through the inhibition of lipoxygenase. However, further research is still needed to strengthen this result.

Keywords: Anti-inflammation, *Cyclea barbata* Miers. leaves, Flavonoid content, Lipoxygenase, Phytochemical screening.

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INTRODUCTION

Inflammation is a protective response to exogenous or endogenous stimulus that occurs to eliminate the initial cause of cell injury and to remove necrotic cells and tissues caused by cell damage [1]. The inflammatory response involves microvasculature damage, increased capillary permeability, and leukocyte migration into inflamed tissues. Localized symptoms of inflammation include redness (rubor), heat (calor), pain (dolor), and swelling (tumor).

Cyclooxygenase and lipoxygenase are enzymes that play the key roles in inflammation. Although both enzymes have the same substrate, namely arachidonic acid, each has its own metabolic pathway and products. Leukotrienes are products formed by the lipoxygenase pathway in response to immunological stimuli and non-immunological pathway [2].

The World Health Organization has stated that the use of traditional herbal medicine is very important in the maintenance of health and the prevention and treatment of diseases, especially chronic diseases [3]. One herb that has been shown empirically to have anti-inflammatory properties is *Cyclea barbata* Miers. *C. barbata* leaves are often used to treat diarrhea, abdominal pain, fever, inflammation, hypertension, and oral ulceration [4,5].

According to previous research, *C. barbata* leaves contain secondary metabolites, such as flavonoids, saponins, tannins, and steroids [6,7]. In medicine, flavonoid compounds have been proven to have many benefits, including antioxidant, hepatoprotective, antibacterial, anti-inflammatory, anticancer, and antiviral properties. Some flavonoids, such as hesperidin, apigenin, luteolin, quercetin, and baicalein, are reported to have anti-inflammatory and analgesic effects [8,9]. Flavonoids have the ability to inhibit the expression of isoforms which induce nitric oxide, cyclooxygenase, and lipoxygenase that are responsible for the production of nitrate oxide, prostanoids,

leukotrienes, and other mediators of the inflammatory process, such as cytokines and chemokines. Therefore, the flavonoid compounds found in *C. barbata* leaves are predicted to inhibit leukotriene formation and thus have anti-inflammatory effects. Research on the ability of *C. barbata* extracts to inhibit lipoxygenase activity is still lacking. Therefore, the aim of the present study was to test the lipoxygenase inhibitory activity of methanol, ethyl acetate, and n-hexane *C. barbata* extracts and assay the total flavonoids and phytochemicals in the most active extract.

MATERIALS AND METHODS

Materials

C. barbata used in this study originated from Purwokerto and was obtained from the Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Indonesia. Reference materials were baicalein (Sigma Aldrich-465 119, USA) as a standard to test the inhibitory activity of lipoxygenase, quercetin (Sigma-Aldrich-Q4951, USA) as a standard in the identification and assay of total flavonoids, beta-sitosterol (Sigma-Aldrich, USA) as a standard in the identification of terpenoids, and boldine (Sigma-Aldrich, USA) as a standard in the identification of terpenoids and alkaloids.

Extract preparation

The extract was prepared from 200 g *C. barbata* leaf powder by leveling extraction using a reflux method with a solvent ratio of 1:17. Three extracts were produced with a non-polar solvent (n-hexane), semi-polar solvent (ethyl acetate), and polar solvent (methanol), respectively. The reflux time per cycle was 1 h. This extraction was performed in triplicate. Then, each extract solution was evaporated in a vacuum rotary evaporator at 60°C, the viscous extract was weighed, and the yield was calculated according to the following formula:

$$\% \text{ yield} = \frac{\text{Final extract weight}}{\text{Weight of powder}} \times 100$$

Lipoxygenase activity test

First, standard solutions of baicalein and *C. barbata* extract (n-hexane, ethyl acetate, methanol, and *C. barbata* leaves at concentrations of 60, 70, 80, 100, 120, and 140 µg/mL) were prepared. The effect of baicalein on lipoxygenase activity in three extracts was assessed according to the method of Choironi (2014), with some modifications [10]. Inhibition of lipoxygenase assay is shown in Table 1.

Phytochemical screening

Phytochemical screening aimed to determine the content of the compounds contained in *C. barbata* leaf extracts with the smallest IC_{50} values. Identification was performed using color reagents to test for the presence of alkaloids, flavonoids, terpenoids, tannins, saponins, anthraquinone, and glycosides.

Determination of total flavonoid content

Finally, a total flavonoid assay was conducted on the most active extract: Ethyl acetate. Total flavonoid content was measured using a colorimetric method, using aluminum chloride ($AlCl_3$) as a reagent. Tests were carried out to samples with or without $AlCl_3$, and the results were compared with those of a standard solution of quercetin with or without $AlCl_3$. The results were plotted on a graph to obtain a calibration curve for quercetin ($y = a + bx$) which could then be used to determine the levels of flavonoids in the leaf extract. The value of x indicates the concentration of the sample (µg/mL) and y represents the absorption of the sample at 434 nm. Then, the flavonoid levels can be calculated by dividing $CxDFxV$ with mass [11]. C is the concentration of samples obtained from the calibration curve (mg/mL); DF is dilution factor; V is volume of stock solution (mL); M is mass of extract used in the test (g). Levels of total flavonoid were expressed as an equivalent of the amount of quercetin in a 1 g sample (QE).

RESULTS

Extraction

The yield obtained from the extraction process was 1.215% in the n-hexane extract, 1.905% in the ethyl acetate extract, and 8.365% in the methanol extract.

Lipoxygenase activity test

Preliminary test

In the preliminary test, the optimization results indicated that pH 8.5 is the optimum condition for lipoxygenase to catalyze reaction, thus showing the highest absorption (Fig. 1).

A preliminary test of substrate concentration showed that absorption increased in line with increased linoleic acid concentrations. Significant increases occurred at the concentrations of 200 and 300 µM and absorption began to decline at 400 µM (Fig. 2).

Inhibition of lipoxygenase activity by baicalein and extracts

The IC_{50} value obtained for baicalein was 0.153 mg/mL (Fig. 3a). Lipoxygenase activity assay was performed using three extracts at a concentration of 100 mg/mL. Ethyl acetate extract had the

highest percent inhibition, amounting to 53.76%, so that IC_{50} values obtained ethyl acetate extract of *C. barbata* leaf are 0.267 mg/mL (Fig. 3b).

Phytochemical screening

Ethyl acetate extract of *C. barbata* leaves contained flavonoids, glycosides, and terpenoids.

Total flavonoid content

Based on calculation as described before, 1 g of the ethyl acetate extract contains 21.62 mg of quercetin equivalents.

DISCUSSION

Lipoxygenase activity

Preliminary test

A preliminary test was carried out to obtain the optimum conditions for enzyme activity, including pH, temperature, and concentration of the substrate [2]. Large changes in pH can alter the enzyme active site as well as the enzyme formation such that the bond between enzyme and substrate will be broken, which causes the reaction to slow down or cease altogether. In some cases, it can cause enzyme denaturation [2]. pH optimization is, therefore, important before testing and storage of an enzyme. Our optimization results showed that pH 8.5 provided optimum results, i.e., caused the linoleic

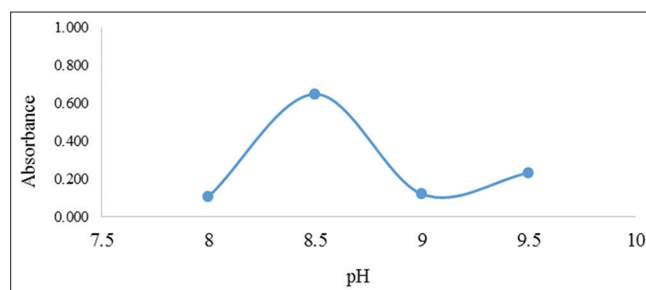


Fig. 1: Optimization of pH condition

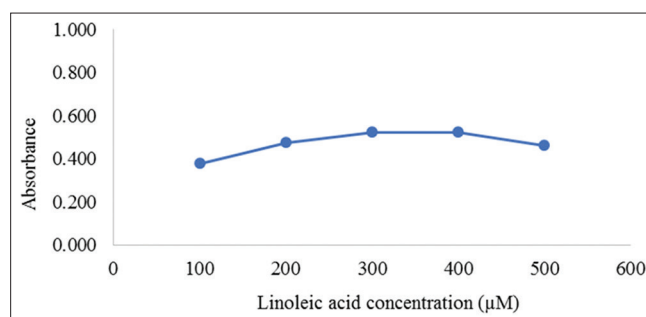


Fig. 2: Optimization of substrate concentration linoleic acid

Table 1: Inhibition of lipoxygenase assay in a baicalein (as positive control) and sample extract

Material	Volume (mL)			
	Blank	Blank control	Sample	Control sample
Baicalein solution/extract solution	-	-	10	10
0.2 M borate buffer pH 8.5	1700	2000	1700	2000
Linoleic acid 300 µM	1000	1000	1000	1000
Incubated for 10 min at a temperature of 25°C				
Lipoxygenase 1000 units/mL	300	-	300	-
Incubated for 15 min at a temperature of 25°C				
Methanol stop solution	1000	1000	1000	1000
Absorbance measured at 234 nm				

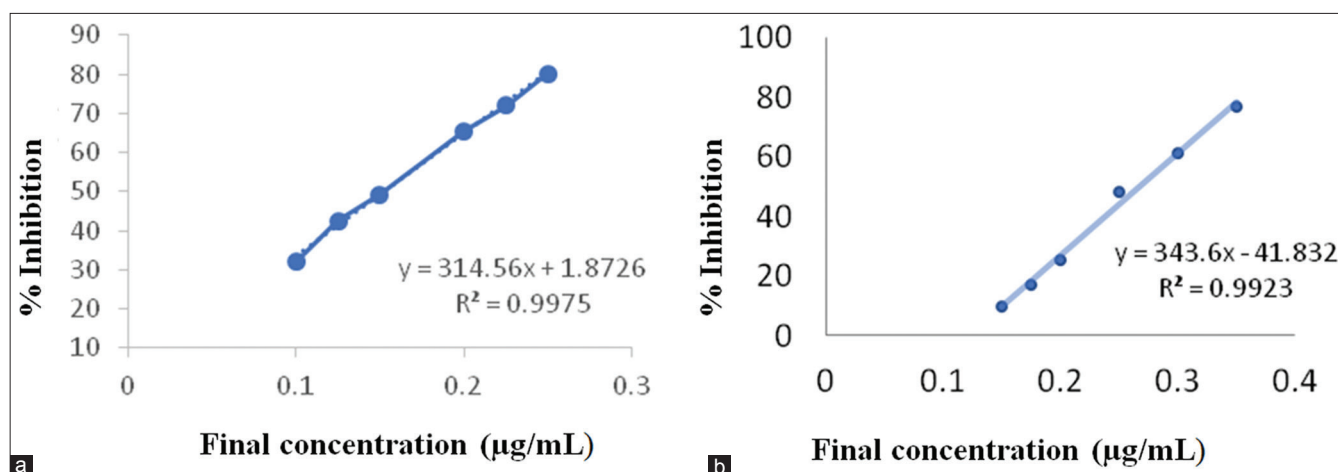


Fig. 3: (a and b) Calibration curves and effect of baicalein and ethyl acetate on lipoxygenase activity

acid substrate and lipoxygenase enzymes to produce the highest absorption (Fig. 1).

The concentration of linoleic acid substrate was also optimized. Various concentrations were tested: 100, 200, 300, 400, and 500 µM (final concentrations 25, 50, 75, 100, and 125 µM). Selection of the substrate concentration was based on previous research. The absorption of linoleic acid increased in line with increased linoleic acid concentrations (Fig. 2). Significant increases occurred at concentrations of 200 µM and 300 µM and absorption began to decline at 400 µM because the enzyme active sites were then filled with substrate so that there was no free enzyme remaining to form additional complexes. Therefore, further increases in linoleic acid concentration will neither increase the reaction rate nor significantly affect absorption [2]. Thus, it can be concluded that the optimum substrate concentration is 300 µM (final concentration 75 µM).

Lipoxygenase concentrations were also optimized. Various enzyme concentrations were tested: 400, 800, and 1000 units/mL (final concentrations 30, 60, and 75 units/mL). The enzyme reaction rate is directly proportional to the concentration of the enzyme: The higher the concentration of enzyme, the faster the reaction and the higher the resulting absorption, because when more enzyme active sites are present, more substrate can bind to the enzyme and form a complex. An increased absorption indicates that more products were produced [15]. The enzyme used in this assay is 1000 units/mL. Selection of a concentration of 1000 units/mL is also supported by data from a previous study [12,13,14,16,17].

Optimization of the stop methanol solution was performed to ensure that the reaction between linoleic acid and lipoxygenase was completely stopped on addition of methanol. Various incubation times were tested: 0, 5, and 10 min. No significant changes in absorption were noted with variations in incubation time. At 0 min, absorption was measured at 0.249; at 5 min, absorbance was measured at 0.240; and at 10 min, absorption was measured at 0.228. This indicates that methanol is effective in stopping the lipoxygenase reaction. Researchers used methanol in stop solutions in similar experiments in a previous study [13,14,20,21].

Inhibition of lipoxygenase activity by baicalein

Baicalein is a natural flavonoid derived from *Scutellaria baicalensis* roots. It is a lipoxygenase inhibitor that possesses a catechol group that chokes the iron on the lipoxygenase and reduces its active site, preventing the substrate and enzyme from reacting [9]. The IC_{50} value obtained for baicalein in the present study was 0.153 µg/mL (Fig. 3a), whereas it was 0.237 in a previous study [14]. Different IC_{50} results can occur due to differences in measurement conditions, such as temperature, incubation time, and instruments used.

In the lipoxygenase inhibition test, it was found that the ethyl acetate extract had the highest percentage inhibition (53.76%), so IC_{50} values were obtained for the ethyl acetate extract (0.267 µg/mL; Fig. 3b). When compared to the positive control, baicalein was shown to have an IC_{50} value lower than that of the ethyl acetate extract. This shows that baicalein is more able to inhibit the formation of lipoxygenase products with a linoleic acid substrate.

Based on previous research, the *C. barbata* ethyl acetate extract contains some secondary metabolites, such as flavonoids, glycosides, and terpenoids [6,7]. Flavonoid compounds are able to inhibit lipoxygenase activity by donating electrons from their OH groups to reduce the active site of the lipoxygenase so that the bond between the lipoxygenase substrate is being weakened [18]. Flavonoids, glycosides, and terpenoids in the *C. barbata* ethyl acetate extract also have very important roles in the inhibition of lipoxygenase activity [19].

Phytochemical screening

Phytochemical screening was performed on the most active extract, the ethyl acetate extract, which was shown to contain flavonoids, glycosides, and terpenoids.

Determination of total flavonoid content

The extract used for this test was ethyl acetate as it was shown to be the most active of all extracts produced in the present study. Determination of total flavonoid content was done by colorimetric methods using $AlCl_3$ and quercetin as a standard. Standard solution of quercetin measured with various concentrations of 3.03, 4.04, 5.05, 6.06, 7.07, and 8.08 µg/mL measured at a wavelength of 434 nm. Absorbance values of the ethyl acetate extract quercetin plotted against a standard curve which has been obtained and calculated the total flavonoid content. The content of total flavonoids of ethyl acetate extract is 21.62 mg QE/g.

CONCLUSION

Ethyl acetate extracts of *C. barbata* leaves have the highest lipoxygenase inhibitory activity of all extracts tested with an IC_{50} of 0.267 µg/mL. Secondary metabolites contained in the ethyl acetate extract were flavonoids, glycosides, and terpenoids. The level of flavonoids in the ethyl acetate extract was 21.62 mgQE/g. This finding indicated that *C. barbata* provides a possible anti-inflammatory effect through inhibition of lipoxygenase. However, further research is still needed to strengthen this result.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest in this research.

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