INTRODUCTION

Inflammation is a protective response to exogenous or endogenous stimuli 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 in the identification of terpenoids and alkaloids, beta-sitosterol (Sigma-Aldrich-465 119, USA) as a standard in the identification of terpenoids and alkaloids, and quercetin (Sigma-Aldrich-Q4951, USA) as a standard to test the inhibitory activity of lipoxygenase.

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 IC₅₀ 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 (AlCl₃) as a reagent.

D I S C U S S I O N
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 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

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

| Material                                      | Volume (mL)                  |
|-----------------------------------------------|------------------------------|
| Blank                                         | Blank control                |
| Baicalein solution/extract solution           | 1700                         |
| 0.2 M borate buffer pH 8.5                    | 2000                         |
| Linoleic acid 300 μM                          | 1000                         |
| Incubated for 10 min at a temperature of 25°C | 1000                         |
| Lipoxgenase 1000 units/mL                     | 300                          |
| Incubated for 15 min at a temperature of 25°C | 1000                         |
| Methanol stop solution                        | 1000                         |
| Absorbance measured at 234 nm                 | 1000                         |
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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 which has very important roles in the inhibition of lipoxygenase activity [19]. Flavonoids, glycosides, and terpenoids in the Scutellaria baicalensis lipoxygenase substrate is being weakened [18]. Flavonoids, glycosides, and terpenoids 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 AlCl3 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 IC50 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 mg QE/g. This finding indicated that C. barbata provides a possible anti-inflammatory eff 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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