LIPOXYGENASE INHIBITORY ASSAY OF ETHYL ACETATE FRACTION FROM STAR FRUIT LEAVES (AVERRHOA CARAMBOLA L.) FROM THREE REGIONS IN WEST JAVA

DEFI SUCIANA SUBARDINI, BERNAA ELYA*, ARIKADIA NOVIANI

Faculty of Pharmacy, Universitas Indonesia, Depok 16424, Indonesia. Email: berna.elya@farmasi.ui.ac.id

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INTRODUCTION

Star fruit (Averrhoa carambola L.) leaves are used as traditional medicines for the treatment of inflammatory skin disorders such as eczema, boils, and pyoderma or skin infections caused by bacteria, viruses (smallpox), or fungi (ringworm) [1-3]. Lipoxygenase is an enzyme that is involved in the biosynthesis of inflammatory mediators such as leukotrienes, which plays an important role in the pathogenesis of inflammatory diseases such as asthma [4-6]. Natural chemicals in the form of flavonoids in A. carambola plants, particularly flavones, exhibit potential anti-inflammatory activity [7]. The previous studies have reported an IC\textsubscript{50} inhibition value of apigenin against lipoxygenase activity of 2.08 ppm and an IC\textsubscript{50} value of 7.84 ppm from the star fruit ethyl acetate fraction; therefore, it was considered to prevent skin edema and has been used in cosmetic preparations as a skin-soothing agent [8]. The aim of the present study was to obtain data on lipoxygenase inhibition activity of the ethyl acetate fraction of A. carambola L. leaves from three regions in West Java (Depok, Subang, and Sukabumi) and to examine the correlation between the total flavonoid and total phenol contents based on IC\textsubscript{50} values.

MATERIALS AND METHODS

Chemicals and materials

A. carambola L. leaves were obtained from three regions in West Java (Depok, Subang, and Sukabumi). All other chemical and reagents were commercially sourced, including 5-lipoxygenase from soybean (Sigma-Aldrich-L7395-15MU, USA), linoleic acid (Sigma-Aldrich-L2376, USA), methanol, n-hexane, ethanol, ethyl acetate, sodium hydroxide, boric acid, and potassium chloride (Merck, Germany), aqua distilled (Brataco, Indonesia), aqua demineralization (Brataco, Indonesia), sodium acetate (Brataco, Indonesia), and Folin–Ciocalteu concentrated solution reagent (Merck, Germany). Apigenin (Sigma-Aldrich-01760595-10MG) was used as the standard in the inhibition test of lipoxygenase activity, whereas quercetin (Sigma-Aldrich-Q4951, USA) and gallic acid (Sigma-Aldrich, USA) were used for the determination of total flavonoid and total phenol contents, respectively.

Preparation of A. carambola leaf extracts

A. carambola leaves from three regions in West Java were collected in December 2017; they were identified by the Center for Plant Conservation of Indonesian Institute of Sciences (LIPI), Bogor, West Java, Indonesia.

Extraction and fractionation

Dried powdered leaves (700 g) were extracted using the maceration method with 70% ethanol at room temperature for 1 day and then the solvent was evaporated using a rotary vacuum evaporator and a water bath (50°C). Ethanol extracts were fractionated using the liquid-liquid partition method with n-hexane, ethyl acetate, and water as the solvents. The ethyl acetate fraction was reconcentrated using a rotary vacuum evaporator (100 rpm) at 40°C [8].

Lipoxygenase activity inhibitor test

Enzyme activity was measured using an ultraviolet-visible (UV-Vis) spectrophotometer at 234 nm and 25°C with apigenin as a positive control. The lipoxygenase assay was performed by reacting 50 µl of the ethyl acetate fraction sample solution of five different concentrations with apigenin as a positive control, 1690 µl of 0.2 M borate buffer (pH 9), 1000 µl of 900 µM linoleic acid substrate (Sigma-Aldrich), and then incubated for 10 min at 25°C. Subsequently, 300 µL of 300 U/mL lipoxigenase solution was added and the mixture incubated for 15 min at 25°C. The enzyme reaction was terminated by adding 1 mL methanol to obtain a final volume of 4 mL and absorbance of product is an hydroperoxy-octadecadienoic acid reaction [8].

Keywords: Averrhoa carambola, Inflammation, Lipoxygenase, Determination of flavonoid content, Determination of phenol content.

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The inhibition of lipoygenase activity was calculated using the following equation:

\[
\% \text{ lipoygenase inhibition} = \left(\frac{(A - B) - (C - D)}{(A - B)}\right) \times 100\%
\]

A = Absorbance of reference solution with enzyme
B = Absorbance of reference solution without enzyme
C = Absorbance of standard or sample solution with enzyme
D = Absorbance of standard or sample solution without enzyme.

### Determination of total flavonoid content

The total flavonoid content was determined using a UV-Vis spectrophotometer at 730 nm and gallic acid was used as the standard and to plot the calibration curve. The total flavonoid content was reported as total quercetin equivalent per g of extract (mg QE/g extract). For the test, take 0.5 mL sample test solution or the standard was mixed with 1.5 mL ethanol concentrated solution, 0.1 mL of 10% AlCl$_3$ solution, 0.1 mL of 1 M sodium acetate solution, and 2.8 mL distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 730 nm [9].

### Determination of total phenol content

The total phenol content was determined using a UV-Vis spectrophotometer at 730 nm and gallic acid was used as the standard and to plot the calibration curve. The total phenol content was reported as total gallic acid equivalent per g of extract (mg GAE/g extract). For the test, 1 mL standard or sample solution was mixed with 5 mL of 8% Folin-Ciocalteu reagent, incubated for 5 min and 4 mL of 1% NaOH added followed by incubation for 1 h. The absorbance of the reaction mixture was measured at 730 nm [10].

### Statistical analysis

Statistical analyses were conducted to determine the IC$_{50}$ values with the total flavonoid and phenol contents in the ethyl acetate fraction of the leaves obtained from the three regions. The analyses were performed using IBM SPSS Statistics 22 (IBM Corp, Armonk, NY, US). First, the data were tested for normality and then a correlation test was performed. The results of the normality test facilitated the determination of the appropriate correlation test.

### RESULTS

#### Extraction and fractionation

Extraction is a method for separating the active compound from the plant using a selective solvent. Extracts of A. carambola leaves were obtained using the maceration method. The yields obtained from 700 g leaf samples were 225.55 g (32.33%) from the Depok samples, 246.59 g (35.22%) from the Subang samples, and 165.79 g (23.68%) from the Sukabumi samples. Fractionation is a method of separating the components of a sample based on their solubility. Based on the results of obtained fraction, the highest amount of the ethyl acetate fraction was observed in A. carambola leaves from Subang (15.24 g [6.18%]) followed by Depok at 16.85 g (4.19%) and the lowest amount observed was 6.3 g from Sukabumi (3.79%).

#### Lipoygenase activity inhibition

The inhibition of lipoygenase activity by the ethyl acetate fractions of the leaves obtained from the three study regions in West Java was evaluated using a colorimetric method with quercetin as the positive control. A. carambola leaves were macerated with linoleic acid as the substrate were compared using standard apigenin. The inhibition IC$_{50}$ value of the ethyl acetate fraction of leaves samples from Depok, Subang, and Sukabumi was 19.38, 16.65, and 15.07 ppm, respectively.

#### Total flavonoid contents

Based on the standard quercetin calculation curve, there was a linear correlation between absorbance and concentration, with the following linear regression equation: y = 0.0829x + 0.0067 (r = 0.9994). The equation was used to estimate the total flavonoid content in the extracts. The total flavonoid contents in the ethyl acetate fraction of the leaf samples from Depok, Subang, and Sukabumi were 14.875, 16.884, and 22.276 mg QE, respectively, with pure quercetin used as the standard.

#### Total phenol contents

Based on the standard gallic acid calculation curve, there was a linear correlation between absorbance and concentration, with the following linear regression equation: y = 0.1998x + 0.0425 (r = 0.9981). The equation was used to estimate the total phenol contents in the extracts. The total phenol contents in the ethyl acetate fraction of the leaf samples from Depok, Subang, and Sukabumi were 54.102, 61.066, and 72.180 mg GAE with pure gallic acid used as the standard.

#### Statistical analysis

The results of the correlation test between IC$_{50}$ values and flavonoid content yielded a correlation coefficient with a value of -0.917, whereas that of the test between IC$_{50}$ values and phenol content yielded a value of -0.960.

### DISCUSSION

#### Extraction and fractionation

Extraction using the maceration method with 70% ethanol at room temperature could minimize challenges associated with metabolic processes and the degradation of metabolites. About 70% ethanol was selected because it provided higher yield extracts from A. carambola L. leaves [8]. The extraction of each solvent was performed thrice to maximize yield [11]. Using solvents with different polarities could facilitate the extraction of chemical compounds based on their polarity; for example, semi-polar compounds such as isoflavones, flavones, and flavonols are extracted using ethyl acetate solvents, and it can be used to inhibit lipoygenase activity. The highest ethyl acetate fraction yield was obtained from A. carambola L. leaves from Subang followed by Depok and Sukabumi.

#### Lipoygenase activity inhibition

Based on the results, the ethyl acetate fraction obtained from A. carambola L. leaves of the Sukabumi region exhibited the highest lipoygenase inhibition activity with an IC$_{50}$ value of 15.07 ppm followed by those from the Subang and Depok regions with IC$_{50}$ values of 16.65 ppm and 19.38 ppm, respectively. Based on the previous study, the IC$_{50}$ value of the ethyl acetate fraction of A. carambola leaves from Depok was 7.84 ppm [8]; however, in the present study, which collected numerous samples, the IC$_{50}$ value was 19.38 ppm. We concluded that the regions did not influence the lipoygenase inhibition activity; however, other factors such as time of harvesting of the tree could have influenced the activity.

#### Total flavonoid content

The total flavonoid assay was performed using the AlCl$_3$ colorimetric method with quercetin as a positive control. AlCl$_3$ can form a stable complex with the C$_3$-keto group and a hydroxyl group at C$_6$ and C$_7$ of flavones and flavonols. In addition, AlCl$_3$ can form labile complexes with ortho-dihydroxyl groups in ring A and B flavonoids [9]. Flavonoids present in an extract may contribute to the inhibition of lipoygenase activity. Based on the results, the highest total flavonoid content was noted in the ethyl acetate fractions obtained from A. carambola L. leaves of the Sukabumi region followed by those of the Subang and Depok regions.

#### Determination of total flavonoid content

Determination of the total flavonol content was performed by a colorimetric method using standard gallic acid and Folin–Ciocalteu reagent, which relies on the transfer of electrons in the alkaline medium from phenol compounds to phosphomolybdic-phosphotungstic complexes [10]. According to the results, the highest total flavonol content in the ethyl acetate fraction was observed in A. carambola leaves of the Sukabumi region followed by those of the Subang and Depok regions.

#### Statistical analysis

The purpose of a correlation test is to determine the correlation level between the variables stated based on a correlation coefficient.
Total flavonoid and phenol contents were highly correlated and inversely proportional to the IC$_{50}$ values of the extracts of the leaves from three regions, with correlation coefficients of −0.917 and −0.960, respectively, which indicate that the higher the total flavonoid and total phenol contents, the lower the IC$_{50}$ values.

CONCLUSION

The total flavonoid contents and IC$_{50}$ values of the extracts of the leaves from the three regions were highly correlated and inversely proportional, with a correlation coefficient of −0.917. In addition, the total phenol contents in the ethyl acetate fraction from the regions sampled, including Depok, Subang, and Sukabumi, were highly correlated and inversely proportional to the IC$_{50}$ values, with a correlation coefficient of −0.960. According to the results, the higher the total flavonoid and total phenol contents, the lower the IC$_{50}$ values.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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