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Research Article

ANTIOXIDANT ACTIVITY TEST OF FRACTIONS FROM STAR FRUIT LEAVES (*AVERRHOA CARAMBOLA* L.) FROM THREE REGIONS IN WEST JAVA

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ABSTRACT

Objectives: Previous *in vitro* research showed that ethyl acetate fractions of star fruit leaves exhibit strong antioxidant activities toward 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (IC_{50} = 90 µg/ml). This study's aim was to evaluate antioxidant activity of fractions from leaf extracts from star fruit sourced from three different regions in Indonesia (Depok, Sukabumi, and Subang) and, for the most active fraction (that with the highest antioxidant activity), determine whether there was any correlation with phenolic and flavonoid content.

Methods: Liquid–liquid partitioning, fractions were evaluated for *in vitro* antioxidant activity using DPPH radical scavenging and ferric reducing ability of plasma (FRAP) assay.

Results: The ethyl acetate fraction from the Subang region exhibited the strongest radical scavenging activity with both the DPPH assay (IC_{50} = 96 µg/ml) and the FRAP assay (FeEAC value = 1405 µmol/g).

Conclusion: Ethyl acetate fractions from star fruit leaves could be used as natural antioxidants. No correlation was found between antioxidant activity and phenolic or flavonoid content.

Keywords: Antioxidant, 2,2-diphenyl-1-picrylhydrazyl, Fractionation, Ferric reducing ability of plasma, Star fruit.

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INTRODUCTION

Star fruit is distributed around the world, especially in tropical countries such as India, Malaysia, Indonesia, and Philippines. Star fruit belongs to the genus *Averrhoa,* containing five species; *Averrhoa bilimbi*, *Averrhoa carambola*, *Averrhoa dolichocarpa*, *Averrhoa leucopetala*, and *Averrhoa microphylla*. *A. carambola* is the species most widely produced on a commercial scale and is, therefore, considered the most important. It is cultivated in the Asia Pacific region on a large scale and is also popular in the American and Australian markets [1]. Previous research demonstrated that ethyl acetate and n-butanol fractions of *A. carambola* ethanolic extract have high antioxidant activity, as measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and iron (Fe) reduction methods [2].

Among the chemical compounds present in *A. carambola* are apigenin, quercetin, anisaldehyde, gallic acid, cyanidin, epigallocatechin, beta-sitosterol, beta-amyrin, proanthocyanidin, lupeol, rutin, and Vitamin C [3]. Results from quantitative analyses have shown that star fruit leaves fulfill Farmakope Herbal Indonesia requirements with respect to flavonoid levels; they contain apigenin (6.37%) and quercetin (4.49%) in the ethyl acetate fraction [4]. This study's aims were to determine antioxidant activity in leaf fractions from *A. carambola* from three different regions in West Java (Depok, Sukabumi, and Subang), to determine total phenolic and flavonoid content in relation to antioxidant activity and to detect any regional differences. West Java was chosen as the sampling site since it is a tropical area with high rainfall (2000–4000 mm per annum) and varied geography, with mountainous areas in the central and southern parts and lower-lying areas in the northern part [5].

The ferric reducing ability of plasma (FRAP) antioxidant capacity assay was used to reduce a ferric tripyridyltriazine (TPTZ) complex to the ferrous form, resulting in an intense blue color whose absorbance can be measured at 593 nm [6]. While the FRAP method is based on a single electron transfer mechanism, the principle underlying the DPPH method is delocalization of free electrons, giving rise to a violet color whose absorbance is measured at 515 nm. The reaction between DPPH and a hydrogen donor reduces the violet color intensity due to the reduction of DPPH to DPPH–H [7].

METHODS

The research was carried out in the Phytochemistry and Pharmacognosy Laboratory at Universitas Indonesia, Depok, from January 2018 to May 2018. Dried *A. carambola* leaves from three different regions (Depok, Sukabumi, and Subang) were used. Extraction was done by maceration in 70% ethanol, with a sample: solvent ratio of 1:10 (w/v). Fractionation was performed by liquid–liquid partitioning using three solvents of different polarity; ethyl acetate, hexane, and distilled water. A water bath and rotary vacuum evaporator were used to obtain the viscous extract for the subsequent assay [8].

Preliminary DPPH antioxidant activity tests were performed on all fractions (ethyl acetate, hexane, and water) in a round-bottomed microplate with a 1:1 ratio of DPPH and sample (100 µL DPPH solution [200 µg/ml] and 100 µL sample solution). The plate was incubated in the dark for 30 min at room temperature. The absorbance was read at 515 nm [9]. In this DPPH test, sample concentrations need to be the same to determine the most active fraction; in this study, the sample concentration for all fractions tested was 1000 µg/ml. Then, the most active fraction from preliminary testing was further tested to obtain its half-maximal inhibitory concentration (IC_{50}). The scavenging capacity (%) was calculated with the formula:

$$\frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of methanol and DPPH solution, and A_{sample} is the absorbance of sample and DPPH solution [10].

The FRAP antioxidant activity assay was carried out as previously described. The FRAP working solution was a mixture of acetate buffer (300 mM, pH 3.6), TPTZ (10 mM dissolved in HCL 40 mM), and $FeCl_3$ (20 mM dissolved in distilled water). Using a micropipette, 20 µL of either the sample or ferrous ammonium sulfate (AFS; a standard solution for producing a standard curve) and 280 µL of FRAP working solution were added to the microplate wells. The mixture was shaken and incubated for 30 min at 37°C before its absorbance at 593 nm was recorded. All assays were done in triplicate [11].

Total phenolic content was measured by ultraviolet (UV)–visible spectrophotometry based on Farmakope Herbal Indonesia methods with gallic acid as a standard. Five milliliters Folin–Ciocalteu reagent (7.5% in distilled water) was added to each 1-ml tube of sample or diluted standard solution. The mixture was incubated for 8 min before 4 ml of 1% NaOH was added. Then, the mixture was further incubated for 1 h before its absorbance was read at 730 nm [12].

The total flavonoid content was measured by UV–visible spectrophotometry with rutin as standard. The sample contained 5 ml of extract solution dissolved in methanol and 5 ml of 2% $AlCl_3$ (b/v in methanol). The sample was incubated for 10 min. Methanolic 2% $AlCl_3$ was used as a blank. Absorbance was read at 430 nm [13].

Before the detailed quantitative analyses, we carried out preliminary qualitative tests using thin-layer chromatography (TLC) and DPPH spray reagent; increasing intensity of yellow coloration developing on the plate indicated increasing antioxidant concentration.

Flavonoid identification was done using TLC with chloroform:acetone:formic acid (10:2:1) as the mobile phase and 5% $AlCl_3$ spray reagent, with quercetin as the positive control.

RESULTS AND DISCUSSION

Qualitative analysis results showed that the ethyl acetate fraction of *A. carambola* leaves exhibited the greatest concentration of antioxidants, while the concentration in the hexane fraction was low. A possible explanation could be that star fruit contains polyphenolic compounds as the major component which is still extracted by less polar solvents. Preliminary DPPH quantitative testing was done using quercetin as positive control (Fig. 1). Preliminary DPPH quantitative antioxidant testing highlighted the ethyl acetate fraction of *A. carambola* leaves from Subang as the most active (Fig. 2), for which the IC_{50} value was 96 µg/ml (Fig. 3 and Table 1). It could be that environmental (e.g., geography and climate) parameters were optimum for *A. carambola* in Subang. Chemical compounds that may have a role in antioxidant activity are polyphenols (flavonoids, tannins, etc.). A previous study showed that antioxidant activity in the ethyl acetate fraction of *A. carambola* leaves was significantly correlated with total phenolic content (r^2=0.997), though such a relationship was not evident in this study. It will be discussed further in the next section.

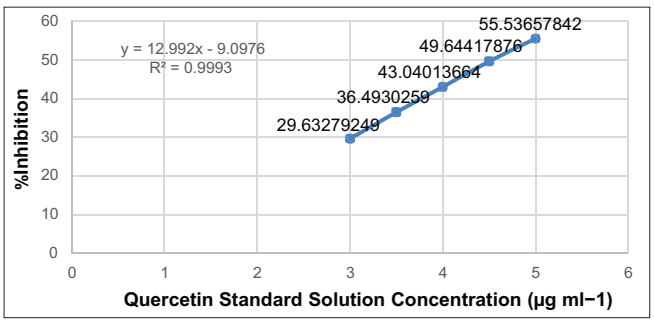

Fig. 1: IC_{50} plot for quercetin as a positive control

TLC analysis for flavonoids showed that the ethyl acetate fraction of *A. carambola* leaves contained flavonoids, as indicated by yellowish fluorescent bands in the UV–visible spectrograph at 366 nm (Fig. 4). The bands occurred not only in same the retention zone as quercetin but also elsewhere as well, indicating the presence of flavonoids other than quercetin (Fig. 4, left panel). Using the same mobile phase, the antioxidant content was analyzed, with gallic acid as the positive control and DPPH solution at 100 µg/ml as the spray reagent. The lower, higher polarity area of the TLC plate indicated good antioxidant activity (the yellow spot on the violet background; Fig. 4, right panel).

The FRAP antioxidant assay results showed that the ethyl acetate fraction of *A. carambola* leaves from the Subang region gave the highest value (1405 µmol/g). The complete data are shown in Fig. 5 and Table 2. This was lower than for the standard (gallic acid) which, at a concentration of 25 ppm, gave a value of 26,863 µmol/g. These results reflected the purity of the standard versus the variety of compounds in the sample. Gallic acid was used as the standard to allow comparison with the determination of total phenolic content using the Folin–Ciocalteu method (both this method and the FRAP method are classified as indirect methods of antioxidant assay not involving chemical radicals). Ferrous ammonium sulfate standard was

Table 1: IC_{50} data for ethyl acetate fraction of *Averrhoa carambola* leaves from Subang, the most active fraction

Concentration	% Inhibition
50	29.62
75	39.29
100	51.39
125	66.21
150	72.29

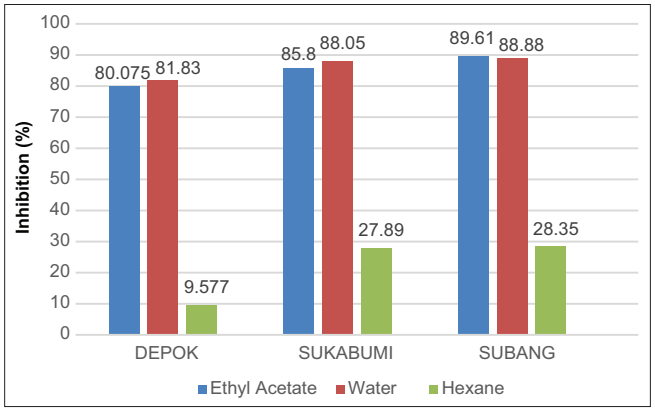

Fig. 2: Antioxidant levels for leaf fractions of *Averrhoa carambola* from three regions in West Java as shown by preliminary DPPH testing

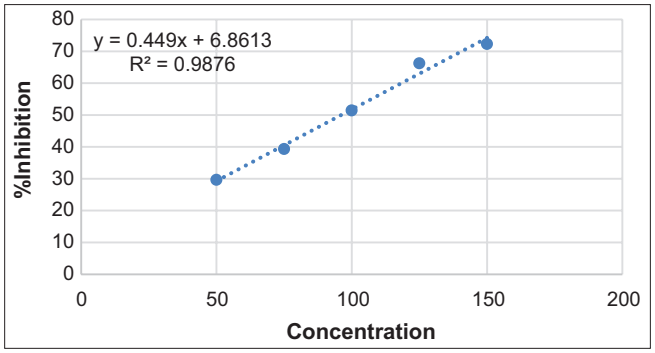

Fig. 3: IC_{50} curve for ethyl acetate fraction of *Averrhoa carambola* leaves from Subang, the most active fraction

Table 2: FeEAC values in *Averrhoa carambola* leaf fractions from three regions in West Java (Depok, Sukabumi, and Subang)

Sample	FeEAC (µmol/g)
Ethyl acetate fraction from Depok	1203
Ethyl acetate fraction from Sukabumi	1277
Ethyl acetate fraction from Subang	1405
Water fraction from Depok	793
Water fraction from Sukabumi	886
Water fraction from Subang	1022
Hexane fraction from Depok	670
Hexane fraction from Sukabumi	701
Hexane fraction from Subang	936

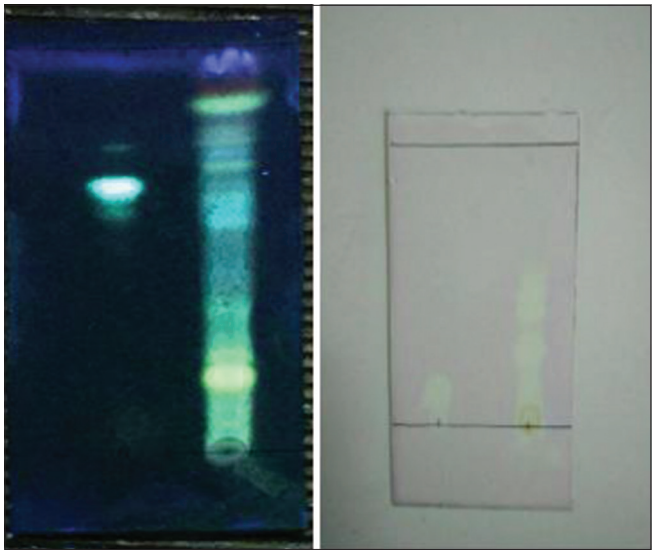

Fig. 4: Flavonoid identification and antioxidant spot sprayed by DPPH on ethyl acetate fraction of *Averrhoa carambola* leaves from Subang, the most active fraction

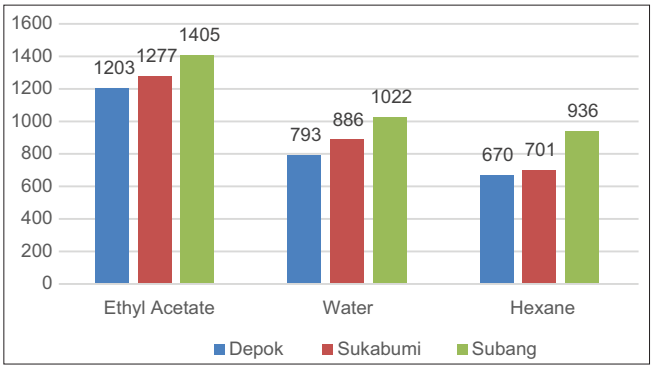

Fig. 5: FeEAC values for leaf fractions of *Averrhoa carambola* from three regions in West Java (Depok, Sukabumi, and Subang)

used as a calibration curve. The absorbance data are shown in Table 3 and Fig. 6.

In this study, the total phenolic and flavonoid content in *A. carambola* leaves was determined using the most active fraction, obtained using ethyl acetate, with phenolic content expressed as gallic acid equivalent and flavonoid content expressed as rutin equivalent. The gallic acid calibration curve is shown in Table 4 and Fig. 7. The rutin calibration curve is shown in Fig. 8 and Table 5. Phenolic contents for leaf fractions of *A. carambola* from three regions in West Java (Depok, Sukabumi, and Subang) are shown in Table 6. Flavonoid concentrations for leaf fractions of *A. carambola* from three regions in West Java (Depok, Sukabumi, and Subang) are shown in Table 7. Levels of both were highest in leaves from the Sukabumi region followed by Subang and Depok.

Table 3: Absorbance data for the ferrous ammonium sulfate standard

Concentration (µM)	Absorbance
26.67	0.204
33.33	0.316
40	0.393
46.67	0.521
53.33	0.611
60	0.717

Table 4: Gallic acid calibration curve

Concentration (µg/ml)	Absorbance
1	0.242
1.5	0.351
2	0.436
2.5	0.539
3	0.632
3.5	0.752

Table 5: Rutin calibration curve

Concentration	Absorbance
10	0.28
12.5	0.387
15	0.473
17.5	0.575
20	0.662
22.5	0.747

Table 6: Total phenolic content of ethyl acetate fraction of *Averrhoa carambola* leaves from three locations

Sample	Phenolic content (mg GAE/g)
Ethyl acetate fraction from Sukabumi	76.7
Ethyl acetate fraction from Subang	61.3
Ethyl acetate fraction from Depok	56.3

mg GAE/g: mg gallic acid equivalent per g

Table 7: Total flavonoid content of the ethyl acetate fraction of *Averrhoa carambola* leaves from different locations

Sample	Flavonoid content (µg rutin equivalent/g sample)
Ethyl acetate fraction from Sukabumi	2609
Ethyl acetate fraction from Subang	2491
Ethyl acetate fraction from Depok	673

One disadvantage of the antioxidant assay with the DPPH method is steric stability. Low molecular weight molecules have a greater chance of reacting with radicals, causing high total antioxidant capacity readings [14]. Another disadvantage is that radicals can react with reductants that do not possess antioxidant activity. For example, DPPH can be reduced to DPPH–H by H_2O_2 which is obviously not an antioxidant. In polar protic solvents (such as ethanol and methanol: DPPH can be dissolved in water), the reaction between DPPH and phenolic compounds can be accelerated by stepwise proton-transfer electron-transfer mechanism [15].

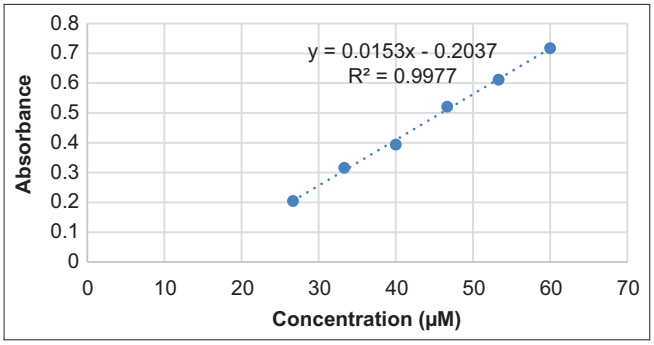

Fig. 6: Calibration curve for the FAS standard

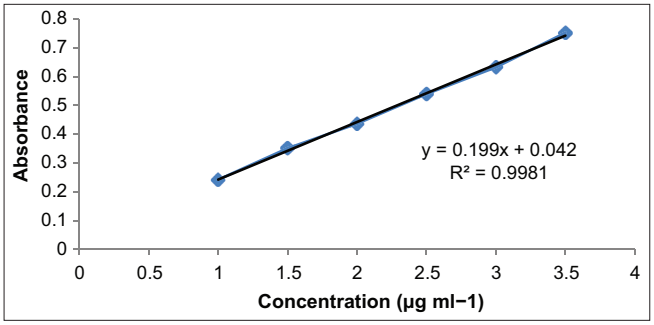

Fig. 7: Gallic acid calibration curve

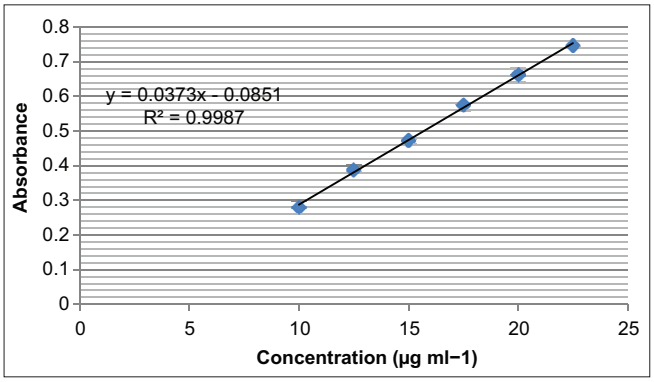

Fig. 8: Rutin calibration curve

A disadvantage of antioxidant assay with the FRAP method is that values can vary depending on the analysis time-scale. Fast-reacting phenols that can bond with Fe or break into less reactive components may be analyzed satisfactorily with a short incubation time. In one study involving a FRAP assay on dietary polyphenols in water and methanol, absorbance increased slowly for caffeic acid, tannic acid, ferulic acid, ascorbic acid, and quercetin although the incubation period was over some hours. This shows that absorption at a single time point may not represent a complete reaction [16].

CONCLUSION

Based on DPPH and FRAP assays of 70% ethanolic extracts, the highest antioxidant activity detected in ethyl acetate fractions was from *A. carambola* leaves from the Subang region (IC_{50} = 96.077 µg/ml, with FeEAC value of 1405 µmol/g). In this fraction, the total phenolic content was 61.32 mg gallic acid equivalent/g and total flavonoid content was 2491 µg rutin equivalent/g. There was no correlation between antioxidant activity and total phenolic or flavonoid content from the ethyl acetate fraction across three different regions (Depok, Sukabumi, and Subang). These results indicate that ethyl acetate leaf fractions of *A. carambola* would be valuable for antioxidant activity; however, further assay testing is required to find the correlation between antioxidant activity and total phenolic or flavonoid content.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors report that they have no conflicts of interest.

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