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Original Article

Antioxidant Activity of *Rubus fraxinifolius* Poir. and *Rubus rosifolius* J. Sm. Leaves

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ABSTRACT

Objective: *Rubus fraxinifolius* and *R. rosifolius* are found growing in mountainous areas of West Java and potentially act as an antioxidant. The objective of this research was to examine the antioxidant activity of *Rubus rosifolius* J. Sm. and *R. fraxinifolius* Poir. leaves. **Methods:** Dried leaves powder were extracted using Soxhlet apparatus with n-hexane, ethyl acetate, and methanol. The extracts were evaporated, and antioxidant activity was determined using DPPH free radical scavenger methods as well as FRAP reduction capability. Total phenolic contents from the extract gave the best antioxidant activity was measured by the Folin-Ciocalteu method. **Results:** IC_{50} of DPPH scavenging activity of n-hexane, ethyl acetate and methanolic extracts of *R. fraxinifolius* were >200; 140.97; and 4.48 ppm, while for *R. rosifolius* were >200; 29.67; and 5.17 ppm, respectively. Percent FRAP capacity of *R. fraxinifolius* in 25 ppm were 55.65; 89.47; and 97.65 %, while for *R. rosifolius* were 73.51; 82.83; and 96.5, respectively. Total phenolic content of the methanolic extract of *R. fraxinifolius* and *R. rosifolius* were 39.0 + 26.5 and 80.62+21.6 mg GAE/g extract. **Conclusion:** Both methanolic extracts showed a significant optimum trapping capability of free radicals and the hexane extracts did not show antioxidant activity. Thus, it may be concluded that the methanolic extracts of both leaves possess potent antioxidant properties and had high total phenolic content. This investigation provides promising results to emphasize the importance of antioxidant capabilities of both plants.

Key words: Antioxidant, *Rubus fraxinifolius, Rubus rosifolius*, Total phenolic content.

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INTRODUCTION

Indonesia is a tropical country and has numerous mountains with thousands of plants unexplored including chemical and biological activities. One of the genus that lives in the highlands of Indonesia is Rubus (Rosaceae). From the literature review, the collection of herbarium and exploration life collection of Indonesian Institute of Sciences (LIPI), it is known that there are 25 species of Rubus genus scattered across Indonesian mountain forests, including *R. alpestris, R. chrysophyllus, R. ellipticus, R. fraxinifolius, R. lineatus, R. moluccanus, R. pyrifolius,* dan *R. rosifolius*.[1] Fruit of *R. fraxinifolius Poir.* and *R. rosifolius J. Sm.* in the West Java area (Sundanese) are known as "beberetean" or "arben".[2-3] Both fruits are edible, has similarity in shape which is small, have a red color, and have a sweet rather than sour taste. Rubus genus is a part of berries plants, which are small, soft, commonly edible, colorful and have important nutritional content.[4]

Some Rubus were reported to have a potential activity of antioxidant, anti-bacteria, anti-elastase, anti-collagenase, anti-thrombotic, and potential for the treatment of radical generated disorders, mainly cancer, and other inflammatory diseases as well as a large content of polyphenols and flavonoids.[5-13] *R. fraxinifolius* Poir. had economic value due to its ability to produce fruit throughout the year.[14] Leaves of *R. rosifolius* J. Sm. were reported to have 5,7-dihydroxy-6,8,4'-trimethoxyflavonol, 5-hydroxy-3,6,7,8,4'-pentamethoxyflavone, and tormentic acid which had anti-proliferative activity against ovary cancer cell.[15] The main isolated compound from hexane extract of *R. rosifolius* herb was 28-methoxy-tormentic acid and was reported to have a potential analgesic activity.[16]

Fruit of *R. fraxinifolius* Poir. was reported have good antioxidant activity (FRAP method)[17] but only few studies had established this result. It has been reported in Rubus species that there are phenolic compounds such as ellagic acid (usually found as glycosylated glycosylation polymers), gallic acid, chlorogenic acid, and caffeic acid.[17]

This study was aimed to examine the antioxidant capacity of both leaves extract using DPPH scavenging and FRAP methods. DPPH can only be dissolved in organic media; this condition becomes an important limitation when it comes to interpreting the role of hydrophilic antioxidants. The ferric ion reducing antioxidant power (FRAP) method is based on the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$ to the intensely blue colored Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in low pH medium. Both methods can be measured the product using spectrophotometry instrument.

MATERIALS AND METHODS

Chemical and reagents

2,2- Diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu's phenol reagent, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), gallic acid were purchased from Sigma-Aldrich®, Methanol, HCl, $FeCl_3.6.H_2O$, aqua demineralisata.

Plant Material

Rubus rosifolius leave were collected from Jayagiri, Mount Tangkuban Perahu and *Rubus fraxinifolius* leaves were collected from Cibodas,

Mount Gede-Pangrango National Park in November 2016. Both plants was identified by Research Center for Biology, Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia. Fresh samples were cleaned, air dried and grounded into a fine powder by laboratory mill.

Extraction

Each dried powdered of leaves (20 g) were extracted using Soxhlet apparatus by n-hexane, ethyl acetate, and methanol. The organic solvents were evaporated using rotary vacuum evaporator, and then were dried using vacuum oven.

Determination of Total Antioxidant Capacity

The DPPH radical-scavenging activity was determined using the method proposed by Blois.[18] DPPH was dissolved in pure methanol (0.1 mM). The DPPH solution (180 µL ml) was added to 20 µL of extracts in methanol. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The decrease in absorbance of the reaction mixture was monitored at 517 nm at 96-well microplate reader (Versamac). All determinations were performed in triplicate. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%)= {[(Abs control – Abs sample)]/ (Abs control)}x 100 where Abs control is the absorbance of DPPH radical and methanol; Abs sample is the absorbance of DPPH radical + extract/ control.[8]

Ferric Reducing Antioxidant Potential (FRAP)

FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ (10:1:1). The reagent (270 ul) and sample solutions (30 ul) were added to each well and mixed thoroughly. The absorbance was taken at 593 nm after 30 min 96-well microplate reader (Versamac). All solutions were used on the same day of preparation. All determinations were performed in triplicates.[19] The antioxidant capacity was calculated using the formula FRAP capacity (%) = [(Abs sample – Abs control) / Abs sample] x 100, where FRAP reagent was used as a control.

Total Phenolic Content Assay

Total phenolic content of both methanol extract was assessed by using a modified Folin Ciocalteau method (Singelton and Rossi, 1965). Folin Ciocalteau reagent (FCR) is a mixture of phosphotungstate and phosphomolybdate, which oxidizes the phenolates to a blue complex. Diluted sample (20 µL) in 95% ethanol and 100µl FCR (1:4 in aquadest) were mixed in a 96-well plate, shake in 5 min, then add 75 µl of sodium carbonate (100 g/L). After a 120min incubation period at room temperature, the absorbance of the reaction mixture was measured at 765 nm with a microplate spectrophotometer (Versamac). The gallic acid was used as a reference (10-200 mg/L). All samples were performed in triplicate. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract.

RESULTS

Extraction

The dried extracts from both Rubus leaves were weighed and the yield of each extract was calculated. The extraction rendement of n-hexane, ethyl acetate and methanolic extracts of *R. fraxinifolius* were 1.76%; 5.85%; and 6.22%, while for *R. rosifolius* were 2.11%; 6.93%; and 6.32%, respectively.

Determination of Total Antioxidant Capacity

The DPPH scavenging activity of selected Rubus species and standard (Vitamin C) are depicted in Table 1. The result showed IC_{50} of DPPH scavenging activity of n-hexane, ethyl acetate and methanolic extracts of *R. fraxinifolius* were >200; 140.97; and 4.48 ppm, while for *R. rosifolius* were >200; 29.67; and 5.17 ppm, respectively. Both n-hexane fraction gave no activity with IC_{50} more than 200ppm, and methanol fraction of both leaves had strong activity to scavenging free radical DPPH. Vitamin C which serves as positive control has IC_{50} 2.24 ppm.

Table 1: Results of antioxidant activity testing of DPPH free radical scavenger

DPPH test	Fraction	IC_{50} (ppm)
Rubus fraxinifolius leaves	n-hexane	>200 ppm
	Ethyl acetate	140.97
	methanol	4.48
Rubus rosifolius leaves	n-hexane	>200 ppm
	Ethyl acetate	29.67
	methanol	5.17
Vitamin C		2.24

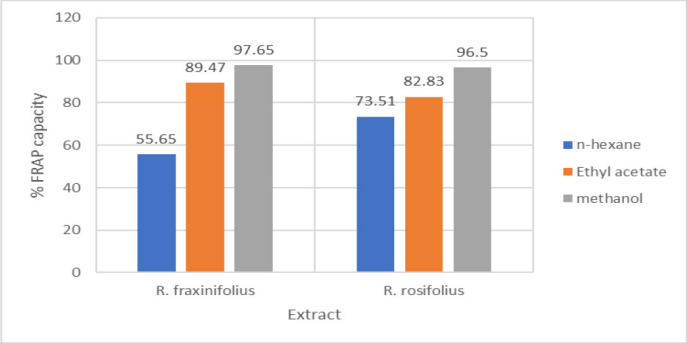

Figure 1: FRAP Capacity of Leaves Extract (25 ppm).

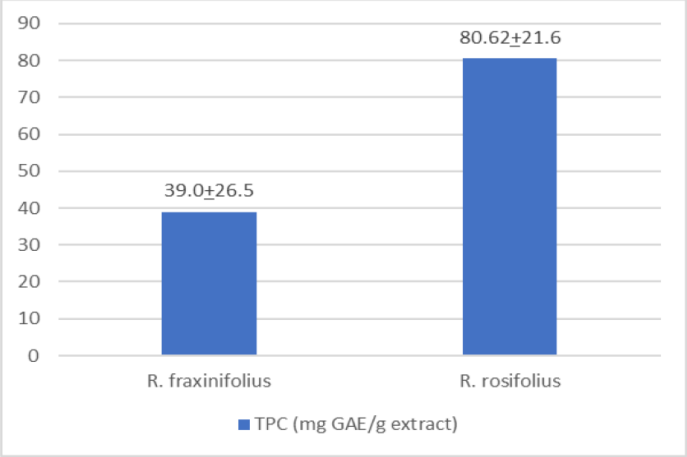

Figure 2: Total Phenolic Content of Methanolic Leaves Extract.

Ferric Reducing Antioxidant Potential (FRAP)

The FRAP capacity of both Rubus species are given in Figure 1. Percent FRAP capacity of *R. fraxinifolius* in 25 ppm were 55.65%; 89.47%; and 97.65%, while for *R. rosifolius* were 73.51%; 82.83%; and 96.50%, respectively. All fraction showed potential activity to reduction of the complex of ferric ion to the ferrous ion and correlated with antioxidant activity which involves Single Electron Transfer (SET) mechanism. All experi-

ments were triplicated and analyzed using ANOVA two-factor with replication using Microsoft Excel 2016 showed significant differences ($p < 0.05$) in the FRAP capacity between extracts and both species.

Total Phenolic Content Assay

The total phenolic content of both methanolic leaves extracts are shown in Figure 2. Total phenolic content of the methanolic extract of *R. fraxinifolius* and *R. rosifolius* were 39.0 ± 26.5 mg GAE/g extract and 80.62 ± 21.6 mg GAE/g extract. All results are presented as mean ± standard deviation (SD) of three replicates and were calculated using Microsoft Excel 2016.

DISCUSSION

The antioxidant activities of both Rubus species were investigated using two different *in vitro* antioxidant tests. DPPH (α-diphenyl-β-picrylhydrazyl) free radical scavenging method developed by Blois offers the first approach for evaluating the antioxidant potential of a compound, an extract or other biological sources. This method is the simplest, in which the compound or extract is mixed with DPPH solution and the absorbance is recorded after a certain period of time. (DPPH=$C_{18}H_{12}N_5O_6$, M=394.33).[20] The antioxidant capacity of extracts on DPPH free radical scavenging was due to its hydrogen-donating process. In the DPPH test, the purple color of DPPH solution turns yellow in the presence of antioxidant compounds.

FRAP method developed by Benzie and Strain, measured the antioxidants capacity in reducing ferric (Fe^{3+}). FRAP method was used to determine the antioxidant activity which involves Single Electron Transfer (SET) mechanism. It is based on the reduction of the complex of ferric ion to the ferrous ion (Fe^{2+}) at acidic condition. The latter form a blue complex (Fe^{2+}/TPTZ (2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-dienechloride)), which monitored by measuring the change in absorption at 593 nm, using a microplate reader.[21] The FRAP assay is cheap, reagents are easy to prepare, reproducible, and the procedure is simple and quick so it can be done in every laboratory and researcher who interested in antioxidant assay. Based on the result showed that all fraction of both leaves gave potential FRAP capacity. Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agent. An antioxidant is a compound that can contribute an electron or a hydrogen atom to reduce the reacting radical.

Both methanol fractions of *R. rosifolius* and *R. fraxinifolius* showed a significant optimum trapping capability of free radicals. However, the hexane fractions did not show any antioxidant activity. In the methanol extract contains polar compounds such as phenolic compounds. These phenolic compounds are known to have antioxidant activity, especially phenolic acids and flavonoids. Phenolic acid is the main class of phenolic compounds, widely found in plants especially in fruits and vegetables.[19] It has been reported in Rubus species that there are phenolic compounds such as ellagic acid (usually found as glycosylated glycosylation polymers), gallic acid, chlorogenic acid, and caffeic acid.[17]

Polyphenolic compounds are known to react strongly in scavenge free radicals by donating hydrogen atoms or electron chelating metal ions. The use of FCR is the most commonly used method for assessing TPC. In fact, this method may give a false positive reaction and do not provide the actual measurement of phenolic compounds due to the presence of reducing compounds such as ascorbic acid and other reducing sugars, which can also react with the FCR. Various modification attempts have been made to improve the specificity of the FCR based test originally developed by Singelton and Rossi (1965). The total phenolic content of methanolic extracts varied and methanol leave extract of *R. rosifolius* showed higher phenolic content than *R. fraxinifolius*. The Pearson correlation coefficient (r) was calculated to show correlation between TPC and FRAP activity, it was considered statistically extremely significant with r=0.9939.

CONCLUSION

Thus, it may be concluded that both methanol extracts showed better radical scavenging activity than other selected solvents and had high total phenolic content. This investigation gave the promising result to emphasize the importance of antioxidant activities of both leaves.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

DPPH: 2,2- Diphenyl-1-picrylhydrazyl; **TPTZ:** 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta- 1,4-dienechloride; **GAE:** Gallic Acid Equivalent; **FRAP:** Ferric Ion Reducing Antioxidant Power.

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