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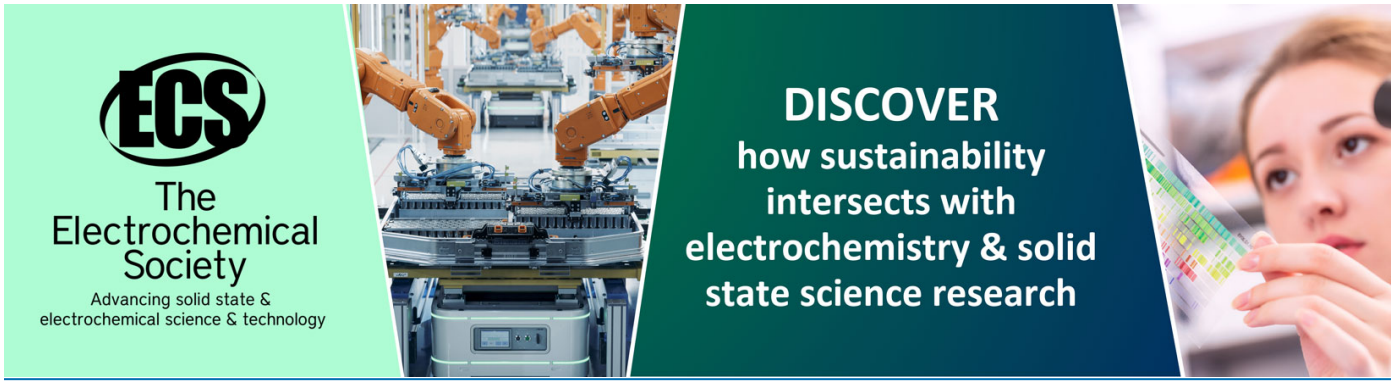

Quantitative determination of quercitrin and myricitrin in three different parts of *Euphorbia hirta* as bioflavonoid source for functional food

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Abstract. *Euphorbia hirta* L. is one of the potential sources for functional food material due to its sufficient biological activity coming from its active flavonoid. Quantitative analysis of its active substances using an adequate method and determining the plant part which possesses the highest yield are essential. However, there was no study reported such that information. Therefore, this study was aimed to analyze flavonoid content (quercitrin and myricitrin) of the three parts (leaf, flower, and stem) of *E. hirta* in order to determine the part with the highest productivity for production of bioflavonoid-rich extract. A precise and accurate HPLC quantitative analysis was performed to quantify the content of quercitrin and myricitrin in the ethanol extracts of leaves, flowers, and stems of *E. hirta*. The HPLC analysis showed that quercitrin and myricitrin was the most dominant flavonoid in the leaf, flower, and stem of *E. hirta*. The leaves exhibited the highest yield of quercitrin and myricitrin (27.55 mg/g and 8.43 mg/g, respectively). It was more than two times of the content of those substances in the flowers and stems. Thus, it can be determined that leaf was the prominent part of *E. hirta*, especially for the production of bioflavonoid-rich extract which can be used as a functional food active substance.

Keywords: *Flavonoid, HPLC analysis, myricitrin, patikan kebo, quercitrin.*

1. Introduction

Euphorbia hirta L. is a tropical weed included to the family of Euphorbiaceae. In Indonesia, *E. hirta* has several local names, *patikan kebo* or *patikan kerbau* (Java), *daun biji kacang* (Sumatra), sosonongan (Maluku). This plant usually grows in open grasslands, roadsides, and also between the paving blocks. It can be differentiated from the other weeds by its hairy leaves and its stem that produces white latex. The flowers are unisexual and found in axillary cymes at each leaf node. Traditionally, the herb of *E. hirta* has been used to relieve some diseases like asthma, cough, and mouth lesions [1]. This herb is also registered in Chinese Pharmacopoeia as a medicine to treat fever, diuretic, anti-toxic, and also to boost breast milk production.

Many modern studies have reported that *E. hirta* is an herb with broad biological effects due to its strong antioxidant activity [2], such as anti-inflammatory [3], anti-diabetes [4], and sedative effect [5]. Other pharmacological activities of *E. hirta* have been also reported, i.e., anti-malaria [6], anti-dengue [7], anti-tumor [8], anti-bacterial [9], anti-alergi [10], diuretic [11], anti-diarrhea [12], anti-asthma [13] and also immunity stimulant [14]. Investigation of the active substances of *E. hirta* has been also reported. Kumar *et al* [15] found that flavonoids and terpenoids are the essential secondary substances of *E. hirta.* Kandakar *et al* [16] reported that the main flavonoids of *E. hirta* are quercitrin, myricitrin, and afzelin. In our preliminary study, we isolated quercitrin and myricitrin from the aerial parts of this plant.

Quercitrin and myricitrin have been known as strong flavonoid antioxidants and possess antioxidative-related pharmacological effects [17]. As a group of hydroxylated phenolics, flavonoids have been widely studied for their potent free radical scavengers. Many degenerative diseases, such as inflammatory joint diseases, diabetes mellitus, atherosclerosis, stroke, cancer, dementia, asthma, and degenerative eye diseases are stimulated by the existence of free radicals [18]. Production of free radicals is stimulated by several factors, such as diet, stress, pollution, and lifestyle [16]. Consumption of food with an adequate content of antioxidant sources is required to attain a healthy life. However, modern peoples seem far from the practice of a healthy lifestyle due to the high pressure of their jobs, pollution, less body movement, and also a low fiber diet. Therefore, the presence of functional foods with the ability to supply the deficiency of antioxidant sources in the daily diet is important in this modern era.

As described, *E. hirta* has high potential as resources of antioxidants due to its flavonoids as the main compounds through individual or collaboration work with other substances. It is essential to understand the content of its flavonoid in different parts of *E. hirta* especially for the optimization and development of some pharmaceuticals or functional foods. Industry always concerns with the efficiency aspects. Information related to the content of active substances is essential in order to select the most productive and economic parts of the plant material for the industrial process. Therefore, this study was aimed to analyze the content of quercitrin and myricitrin as the main flavonoid of *E. hirta* in its three different parts (leaf, flower, and stem). The results of this study may be used by pharmaceutical or functional food industries as the guidance to select the proper material in terms of efficiency, productivity, and functionality.

2. Materials and methods

2.1. Materials

Aerial parts of *E. hirta* were collected in Banjarbaru, South Kalimantan, Indonesia. The collected samples were authenticated at the Department of Biology, Faculty of Natural Sciences, Lambung Mangkurat University, and a voucher specimen (EH180705) weree deposited in the Laboratory of Natural Product, Department of Agroindustrial Technology. Three different parts of the plant materials consisting of leaves, flowers, and stems were separated and dried in a dark room at room temperature for four days. To obtain dried materials with constant weight, the airy dried samples were continually dried using an oven dryer at 40°C for 12 hours. Soon, after pulverized and screened on an 18-mesh sieve the plant materials were saved in an air-tight box and stored in a refrigerator. Each part of the samples was measured for their water and ash contents.

2.2. Extraction

Extraction under reflux was employed following the extraction method of flavonoids from *C. papaya* as described by Nugroho *et al* [19]. Twenty grams of each dried plant part were extracted using 200 ml of 70% ethanol for 4 hours at 80°C. After extraction, the extract solution was filtered using filter paper and evaporated under vacuum using a rotary evaporator. Evaporation was stopped until there was no solvent evaporated and condensed, and a semi-solid state of the extract achieved. The obtained extract was weighed to measure the yield percentage of each sample. Replication was performed three times for each sample to obtain consistent results.

2.3. Quantitative analysis of the active substances

The concentration of quercitrin and myricitrin, as the active substances of *E. hirta*, was measured by HPLC quantitative analysis. Prior to HPLC analysis, the semi-solid extracts were intensively dried on a freeze-dryer for 24 hours in order to remove disturbing components during quantification, including solvent and water. HPLC analysis was performed on a Varian HPLC system consisted of a Prostar 325 UV-Vis detector, two Prostar 210 pumps, and a C18 Shiseido column (4.6 mm × 250 mm, Tokyo, Japan) with 5 μm of particle diameter. This HPLC method was carried out following the HPLC analysis method of Nugroho *et al* [19] with some adjustments to achieve the optimum method.

A combination of two types of solvents (methanol and water) were employed as the mobile phase of the HPLC with a linear gradient elution model. All solvents were HPLC grade purchased from J.T. Baker (NJ, USA). Water added with 0.05% of acetic acid was set as solvent A, while the methanol was set as solvent B. The gradient elution of the solvents was programmed following the time factor as follows, session 1: 0–20 minute (20% to 65% of solvent B); session 2: 20–21 minute (65% to 100% of solvent B); session 3 (column washing): 21–25 minute (100% solvent B); session 4: 25–27 minute (100 → 20% of solvent B); and session 5 (reconditioning): 27–30 minute (20% of solvent B). The flow rate of gradient elution was set constantly at 1.0 mL/min and the column temperature was 40°C.

Each sample analysis required 20μl of 5000 μg/ml extract solution. The extract solution was made by dissolving 25 mg of extract in 5 ml of methanol and ultrasonicated for well extract dissolution. Prior to injection to the HPLC, each of extract solution was filtered with a syringe filter (Whatman GmbH., Germany). The recording of the absorbance of the eluted sample was set at 254 nm for each sample.

2.4. Standard compounds for quantitative analysis

Two standard compounds, quercitrin, and myricitrin, were used for quantitative analysis. These two compounds were selected because their presence in the ethanol extract has been detected clearly in our preliminary study using a TLC detection with a standard compound comparison method. A standard compound of quercitrin and myricitrin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) with more than 95% of compound purity as stated by the producer. Chemical structures of the two compounds were presented in figure 1. Each compound was prepared as standard compounds at 1000 μg/ml. The calibration curves of each compound were produced by plotting the peak areas of the diluted compounds at six different concentrations.

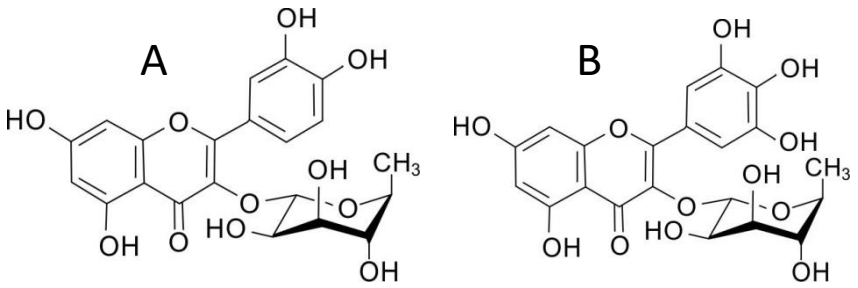

Figure 1. Chemical structures of quercitrin (A) and myricitrin (B).

3. Results and discussion

This study determined the contents of quercitrin and myricitrin, the main active substance of *E. hirta*, in its three different parts, namely leaf, flower, and stem. These three parts were selected due to their different morphological characteristics, growth rate, and also productivity. Prior to the content analysis, optimization of the HPLC method was carried out to meet the requirements of quantitative analysis and to produce a good separation of the targeted compounds in the chromatogram.

3.1. HPLC method optimization

HPLC method optimization was carried out by testing several parameters that may affect the quality of the analysis. Quality of analysis was determined by some indicators, such as linearity, sensitivity,

accuracy, recovery, robustness, and others [18]. Four parameters including mobile phase composition, gradient elution, a flow rate of eluent, and wavelength of the UV detection were examined to produce an optimum method. The optimum method was determined by considering the total time of elution and also the resolution of each analyte.

Finally, we decided an optimum method with a combination of two solvents as mobile phase, i.e., methanol and water. Methanol was set as solvent B. Water, as solvent A, was added with 0.05% of acetic acid to increase the resolution. The appropriate gradient elution of the combined solvents was programmed as follows, 0 – 20 min (20–65% B); 20 – 21 min (65–100% B); 21–25 min (100% B); 25–27 min (100 → 20% B); and 27–30 min (20% B). The flow rate of gradient elution was set constantly at 1.0 mL/min and the column temperature was 40°C. UV wavelength at 254 nm was selected due to its higher sensitivity compared to another wavelength.

Simultaneous quantification of quercitrin and myricitrin in three extracts was carried out by using the selected HPLC method. The targeted peaks were known by referencing the retention time to those of the standard compounds obtained from Sigma Aldrich, USA. Linear calibration equations of the standards were produced by plotting six consecutive concentrations of each compound with their peaks area shown in the HPLC chromatograms. As shown in table 1, the linear equations of the two compounds were presented together with the values of R^2, LOD, and LOQ. The value of R^2 indicates the linearity of the HPLC method. Each compound showed R^2 value more than 0.999 indicating a good linearity of the HPLC method. The values of LODs were also relatively low, less then 0.5 µg/ml signing that the optimized HPLC method had a good sensitivity.

Table 1. Linearity of standard curves and the LOD and LOQ.

Standard	t_R (min)	Calibration equation (linear model) [a]	Linear range (µg/ml)	R^2 [b]	LOD[c] (µg/ml)	LOQ[d] (µg/ml)
Myricitrin	14.15	$y = 459.63x + 101.12$	3.91–125.0	0.999	0.45	1.50
Quercitrin	16.68	$y = 998.98x + 135.26$	3.91–125.0	0.999	0.16	0.55

Note: [a] y, peak area at 254nm; x, concentration of the standard (µg/ml); [b] R^2, correlation coefficient for 6 data points in the calibration curves (n=3); [c] LOD, limit of detection (S/N = 3); [d] LOQ, limit of quantification (S/N = 10).

3.2. Content of quercitrin and myricitrin in extracts and dried plant materials

A total of nine sample solutions (5000 µg/ml) consisted of three different extracts with three replications of each extract were analysed using the optimized HPLC method. Based on the areas of the referenced peaks, concentration of the quercitrin and myricitrin in the samples were determined. Table 2 shows the concentration of quercitrin and myricitrin in the extracts of leaves, flowers, and stems of *E. hirta*.

Table 2. Content of quercitrin and myricitrin in extracts and dried plant materials.

Plant part	In extracts (mg/g)			In dried plant materials (mg/g)		
	Quercitrin	Myricitrin	Total	Quercitrin	Myricitrin	Total
Leaves	27.55[c]	8.43[c]	35.98	6.87[c]	2.11[c]	8.98
Flowers	12.91[b]	6.43[b]	19.34	2.23[b]	1.11[b]	3.34
Stems	10.19[a]	2.90[a]	13.09	1.65[a]	0.47[a]	2.12

In all extracts, the content of quercitrin was higher than myricitrin (table 2). Quercitrin is one of the famous types of flavonol glycoside. Quercitrin is quercetin with the addition of rhamnose at the position no. 3 of the C-ring (figure 1). Quercitrin and myricitrin are flavonol glycoside with the same sugar moieties but different from their aglycones. The aglycone of quercitrin is quercetin, while the

myricitrin's is myricetin, which has one more hydroxyl at atom C no. 5 on the B-ring. Hence, the retention time of myricitrin was shorter than quercitrin (figure 2).

Table 3. Water and ash content of the dried plant materials.

Plant part	Water content (%)	Ash content (%)	Yield (mg/g)
Leaves	10.89 ± 0.42	5.60 ± 0.37	24.88^{b} ± 0.71
Flowers	12.37 ± 0.08	9.90 ± 0.11	15.17^{a} ± 0.45
Stems	7.42 ± 0.36	10.34 ± 0.09	15.65^{a} ± 0.46

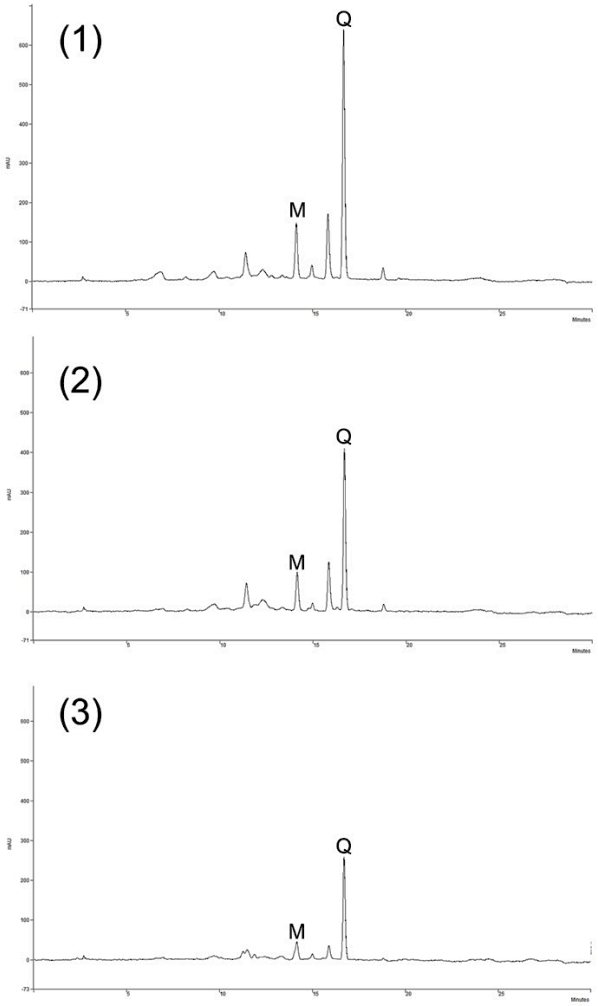

Figure 2. HPLC chromatograms of three extracts (1: leaf, 2: flower, 3: stem, M: myricitrin, Q: quercitrin).

As shown in table 2, concentrations of the two flavonol glycosides in the dried plant materials were calculated based on the yield of the extraction process (table 3). The total content of quercitrin and myricitrin in the dried leaves was 8.98 mg/g. It was equivalent to 0.898% of the dried leaves. The concentration of active compounds in the dried material depending on the yield of extract produced from the extraction process. The higher yield of extract resulting a higher percentage of active compounds in

the dried materials. Therefore, it is important to choose the appropriate extraction method to produce the optimum yield of extraction.

4. Conclusion

Comparing to the flower and stem, leaf of *E. hirta* exhibits the highest level of quercitrin and myricitrin. The total of quercitrin and myricitrin in the leaves was more than two times the content of the same compounds in both flower and stem. Thus, it can be concluded that the leaf was an adequate part of *E. hirta*, especially for the production of bioflavonoid-rich extract which can be used for functional food active substance.

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