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The phenolic contents and antiradical activity of Indonesian *Phyllantus urinaria* L.

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Abstract

In this study, the 2,2-diphenyl-1-picrilhydrazil (DPPH) antiradical activity as well as the phenolics and flavonoid contents of ethanolic extract and its fraction of *Phyllantus urinaria* L. was evaluated. The ethanolic extract of *P. urinaria* L. was fractionated using vaccum column chromatography and gradient elution. Based on TLC profile, the fractination process yielded 6 fractions. The ethanolic extract of *P. urinaria* L. showed the high DPPH free radical scavenging with IC_{50} of 5.74 µg/mL. Among 6 fractions, fraction 6 had the highest DPPH free radical scavenging activity with IC_{50} of 4.22 µg/mL. Among ethanol extract and its fractions evaluated, fraction 5 had the highest phenolics and flavonoid contents. The correlation between DPPH free radical scavenging activities and phenolics contents existed with the equation of y = -43.454x + 827.67 with coefficient of determination (R^2) of 0.3475, while, the R^2 for the relationship between IC_{50} values and flavonoid contents of ethanolic extract and its fraction is 0.2325.

Introduction

The reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) induced by oxygen and nitrogen radicals, and other free radical-mediated reactions are involved in some degenerative or pathological processes such as aging (Ames, 1993; Yosefi *et al.*, 2010; Biswas *et al.*, 2011), neurodegenerative disorders, some kinds of cancer (Kupeli and Yesilada, 2007), atherosclerosis, gastric ulcer, and other conditions (Kumaran and Karunakaran, 2007). Some epidemiological studies revealed that an association between people who consume a diet rich in fresh fruits and vegetables and a decrease in the risk of cardiovascular diseases and other degenerative diseases (Rohman *et al*., 2010; Rababah *et al*., 2011). Several reports have focused on the antioxidant activities of natural compounds (Salah *et al*., 1995).

Plants, especially fruits and vegetables are the good source of natural antioxidants. Many antioxidant compounds, naturally occurring from plant sources, have been recognized as the scavengers of free radicals (Zheng and Wang, 2001). Currently, some scientists are interested to find naturally occurring antioxidants to be used in foods, medicinal materials and pharmaceutical products to replace synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which are suspected to cause carcinogenic (Chouhan and Singh, 2011). In addition, natural antioxidants have the capacity to improve food quality and stability and can also act as nutraceuticals to terminate free radical chain reactions in biological systems (Djeridane *et al*., 2006).

The plants of the genus *Phyllanthus* (Euphorbiaceae) are commonly distributed in most tropical and subtropical regions, and have long been widely used in folk medicine for the treatment of a broad spectrum of diseases, such as disturbances of the kidney and urinary bladder, intestinal infections, diabetes, and the hepatitis B virus (Kumaran and Karunakaran, 2007). The *Phyllanthus urinaria* L., also called ‘‘Meniran” in Indonesian medicine, has been reported that boiling water extracts of *P. urinaria* exhibited cytotoxic activity against Lewis lung carcinoma cells (Huang *et al*., 2003) and human cancer cells such as HL-60, Molt3, HT 1080, K-562, Hep G2, and NPC-BM1 (Huang *et al*., 2004). *P. urinaria* is also reported to have anti-hypertensive activities (Lin *et al*., 2008), cardioprotective effects against doxorubicin-induced cardiotoxicity (Chularojmontri *et al*., 2005), anti-inflammatory and antioxidative effects (Fang *et al*., 2008), antibacterial, anti-HIV-1

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reverse transcriptase (Eldeen *et al*., 2011), and effective in attenuating the acetaminophen induced hepatotoxicity, and the inhibition of cytochrome P450 CYP2E1 enzyme may be an important factor for its therapeutic mechanism (Hau et al., 2009).

Several natural compounds have been isolated from *P. urinaria* namely ellagic acid, a flavonoid (Shin *et al*., 2005), phyllanthin, phyltetralin, trimethyl-3,4-dehydrochebulate, methylgallate, rhamnocitrin, quercitrin, and rutin (Fang *et al.*, 2008), 5-demethoxyniranthin, urinatetralin, dextrobursehernin, urinaligran (Chang *et al*., 2003), and phyllurin (Ueda *et al*., 1998). In this study, the antiradical activities of ethanolic and its fractions of Indonesian *P. urinaria* L. were evaluated using stable 2,2-diphenyl-1-picrilhydrazil (DPPH). The antiradical activity was also correlated with total phenolics and flavonoids contents (Javanmardi *et al.*, 2003). Therefore, we also correlate the total phenolic and flavonoid contents of the evaluated extracts and fractions with its DPPH free radical scavenging activities.

Material and Methods

Materials

Plant material of *Phyllantus urinaria* L. was collected and identified in Research Center of medicinal plant and traditional medicine, Tawangmangu, Central Java, Indonesia. 2,2-diphenyl-1-picrylhidrazyl (DPPH), gallic acid and quercetin were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Silica gel F254, Folin-Ciocalteu, and other reagents and solvents were bought from E. Merck (Darmstadt, Germany).

Figure 1. *Phyllantus urinaria* L.

Preparation of ethanolic extract of P. urinaria *L.*

An approximately of 1 kg of plant material was powdered and then macerated with 10 L ethanol. The mixture was stand for 6 days and at least shaken vigorously twice a day. After that, the mixture was filtered, and the supernatant was evaporated to dryness using vacuum rotary evaporator at 60°C.

Fractination of ethanolic extract

Ethanolic extract of *P. urinaria* L. was fractionated as in Rohman *et al.* (2006) using vacuum column chromatography with stationary phase of silica gel and eluents were delivered in gradient manner in the order as shown in Table 1. Each eluate (100 mL) obtained was subsequently evaporated and subjected to TLC profiling using silica gel F254 with the mobile phase of chloroform: methanol (9:1 v/v). The eluates with the similar TLC profile were then pooled and designated with fraction. Each fraction was further used for DPPH free radical scavenging test, as well as for determination of total phenolic and flavonoid contents.

Table 1. The elution systems used during the fractionation of ethanolic extract of *Phyllantus urinaria*

No.	Eluent (mL)		
	Petroleum ether	Chloroform	Methanol
1.	300	-	-
2.	250	50	-
3.	100	20	-
5.	150	150	-
6.	150	160	-
7.	100	200	-
8.	50	250	-
9.	-	300	-
10.	-	250	50
11.	-	250	50
12.	-	200	100
13.	-	150	150
14.	-	100	200
15.	-	-	300
16.	-	-	300
17.	-	-	300

Determination of DPPH free radical scavenging activity

The scavenging activity of DPPH free radical of the ethanolic extract and its fraction of *P. urinaria*, was performed based on Kikuzaki *et al*. (2002). In this assay, a-50 µL test solutions (extract or fraction solutions with different levels) was added with 1.0 ml of 0.4 mM methanolic-DPPH and added with methanol in volumetric flask 5.0 mL. The mixture was shaken vigorously using vortex for 1 min and allowed to stand for 30 min at room temperature in a dark room. After that, the absorbance of solution was measured using spectrophotometer (Genesys 10) at 514 nm using methanol as blank. The radical scavenging activity was calculated using the following formula:

$$Radical\ scavenging\ activity\ (\%) = \frac{Ac - As}{Ac} x100$$

Where Ac is absorbance of control (DPPH free radical without the addition of test solution), As is sample absorbance (absorbance of DPPH free radical after the addition of test solutions).

Analysis of total phenolics content

The total phenolic contents (TPC) of ethanolic extract and its fraction of *P. urinaria* L. was determined using Folin-Ciocalteu spectrophotometrically according to method described by Chun *et al*. (2003). Briefly, an amount of volume from the certain levels of extract/fraction was added with 0.4 mL Folin-Ciocalteu Reagent (FCR) and allowed to stand for 5-8 min. After that, 4.0 mL sodium carbonate solution 7% (w/v) was added to the mixtures and diluted with bidistilled water to 10 mL. The mixtures were allowed for 2 hr at ambient temperature. The absorbance of mixtures was then measured at 765 nm, against the blank using all reagents without test solutions (extract/fraction). TPC was expressed as gallic acid equivalent (GAE) in g/100 g dry weight of extract/fraction.

Determination of flavonoid contents

The flavonoids content in the evaluated extract/fraction was determined as in Zou *et al.* (2004) using quercetin as a reference standard. An amount of volume from the certain concentration of extract/fraction in methanol was added with 4.0 mL aquadest and 0.3 mL sodium nitrite solution 10% (w/v). After 6 min, the mixture was added with 0.3 mL aluminium trichloride 10% (b/v) in aquadest and allowed to stand for 5 min. After that, 4 mL sodium hydroxide solution 10% was added to the mixture. Finally, the solution was then diluted with aquadest to 10 mL. After operating time of 15 min, the absorption of the solution was measured at 510 nm against blank. The blank samples were prepared from all reagents without test solutions. The absorption of standard quercetin solutions in ethanol was measured under the same conditions for making calibration curve. All determinations were carried out in triplicates. The amount of flavonoids contents in plant extract/fractions was expressed as g quercetin equivalents (QE)/100 g of dry extract/fraction.

Results and Discussion

DPPH free radical scavenging activity

DPPH is routinely used as a reagent for the assessment of free radical scavenging activity of natural antioxidants. This assay is considered as one of the standard and easy colorimetric techniques for the evaluation of antioxidant activity of pure compound and plant extract (Mishra *et al*., 2012). DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Kumaran and Karunakaran, 2007). The reduction capability of DPPH free radical is determined by the decrease in absorbance at 514 nm induced by antioxidants. In this study, we used vitamin C and vitamin E as standards, and methanol as DPPH solvent. The use of methanol has been recommended to be used in DPPH assay due to its capability to give the sensitive results (Sharma and Bhat, 2009).

Figure 2 exhibited the IC_{50} values (the amount of samples necessarys to decrease the DPPH absorbance of 50% of initial absorbance) of ethanolic extract and its fractions of *P. urinaria* L. The scavenging effect of ethanol extract, fractions and standards with the DPPH free radical is in the following order: vitamin C (IC_{50} 2.31 µg/mL), vitamin E (IC_{50} 7.96 µg/mL), fraction 6 (IC_{50} 4.22 µg/mL), fraction 5 (IC_{50} 5.41 µg/mL), ethanolic extract (IC_{50} 5.74 µg/mL), fraction 4 (IC_{50} 16.42 µg/mL), fraction 3 (IC_{50} 231.53 µg/mL), fraction 2 (IC_{50} 339.74 µg/mL), and fraction 1 (IC_{50} 1853.31 µg/mL). The experimental data showed that all fractions and ethanolic extract are likely to have the scavenging effect of DPPH free radical, except fraction 1 and fraction 2. This is not surprising because the main component of fraction 1 and fraction 2 is non-polar compounds such as fatty acids and sterols having weak radical scavenging activity. Furthermore, natural compounds contributing for DPPH radical scavenging in fraction 3, 4, 5, and fraction 6 as well as ethanolic extract of *P. urinaria* L. are mainly phenolics and flavonoids, due to its ability of both groups of compounds to donate hydrogen radical to neutralize DPPH free radical (Mansouri *et al*., 2005).

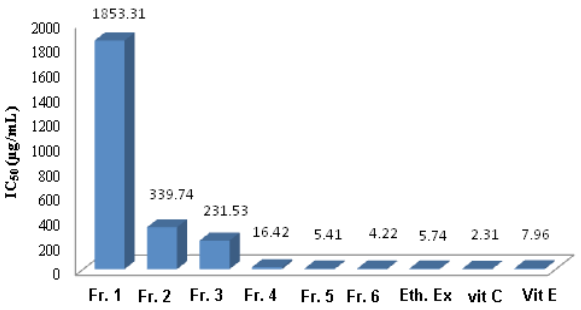

Figure 2. The radical scavenging activity of ethanolic extract (Eth. Ex) of *Phyllantus urinaria* L. and its fraction (Fr.) expressed with IC_{50}

Analysis of phenolics contents

Antiradical scavenging activity of natural compounds is usually correlated with the presence

of phenolics compounds (Spiridon *et al.*, 2011). The phenolics compounds contribute to radical scavenging activities mainly due to their redox properties (Amarowicz *et al.*, 2004). Determination of phenolics compounds in plant extract was assessed by Folin-Ciocalteau (F-C) assay using gallic acid as a standard, and expressed as gallic acid equivalent. The F-C assay for total phenolics contents is a fast and simple method, and can be useful in characterizing and standardizing botanical samples. F-C method is based on oxidation of phenolics by a molybdotungstate in F-C reagent to yield a colored product (molybdenum blue) having maximum wavelength of 745 – 750 nm (Prior *et al*., 2005).

Table 2 expressed the phenolic contents of ethanolic extract and its fraction of *P. urinaria* L. expressed as gram gallic acid equivalent (GAE)/100 gram dry extract/fraction. Among ethanolic extract and fractions evaluated, fraction 5 (22. 81% w/w GAE) and fraction 6 (20.82% w/w GAE) have the highest phenolics contents. Meanwhile, the highest antiradical activities were also observed in Fraction 5 and 6. Figure 3 showed the correlation between free radical scavenging activity expressed with IC_{50} with total phenolic contents. The coefficient of determination (R^2) obtained was 0.3475. These results indicated that phenolic compounds contributed of 34.75% toward the DPPH radical scavenging in the evaluated ethanolic extract and its fractions. In addition, the scavenging effect of extract/fractions is not limited to phenolics compounds. The activity may also come from the presence of other antioxidant secondary metabolites in the extracts such as volatile oils, carotenoids, and vitamins (Javanmardi *et al.*, 2003).

Table 2. The total phenolic contents of ethanolic extract of *Phyllantus urinaria* L. and its fraction

Sample	± SD (% w/w gallic acid equivalent)
Ethanolic extract	15.46 ± 0.21
Fraction 1	1.86 ± 0.02
Fraction 2	1.60 ± 0.01
Fraction 3	2.27 ± 0.06
Fraction 4	11.97 ± 0.19
Fraction 5	22.81 ± 0.54
Fraction 6	20.82 ± 0.10

Analysis of flavonoid contents

Flavonoids are the most common and widely distributed group of plant phenolic compounds, which are characterized by a benzo-pyrone structure, and are ubiquitous plants (Bakar *et al*., 2009). Total flavonoid can be determined in the sample extracts by reaction with sodium nitrite, followed by the development of coloured flavonoid-aluminum complex formation using aluminum chloride which can be monitored spectrophotometrically at 510 nm. The total flavonoid content was expressed as g quercetin equivalents (QE)/100 g sample.

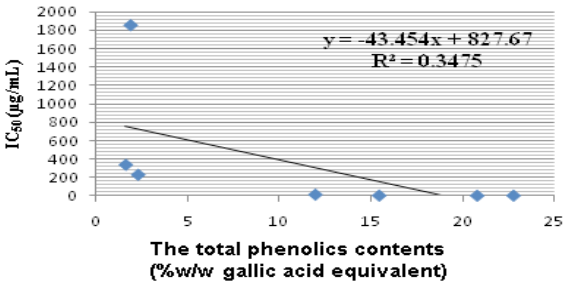

Figure 3. The correlation between IC50 and phenolic contents of ethanolic extract of *Phyllantus urinaria* L. and its fraction

Table 3 listed the flavonoid content of ethanolic extract and its fraction of *P. urinaria* L. The result showed that Fraction 5 (27.96% w/w EQ) had the highest flavonoid content compared to the other fractions. This is in agreement with the antiradical scavenging activity. Figure 4 exhibited the correlation between IC_{50} (Y) and total flavonoid contents (X) of ethanolic extract and its fraction with R^2 value of 0.2325. This result suggests that 23.25% of DPPH radical scavenging activity of test solutions was attributed from flavonoid compounds.

Table 3. The total flavonoid contents of ethanolic extract of *Phyllantus urinaria* L. and its fraction

Sample	± SD (% w/w quercetin equivalent)
Ethanolic extract	11.75 ± 0.2
Fraction 1	5.81 ± 0.42
Fraction 2	4.52 ± 0.24
Fraction 3	-
Fraction 4	11.28 ± 0.13
Fraction 5	27.96 ± 0.52
Fraction 6	13.27 ± 0.19

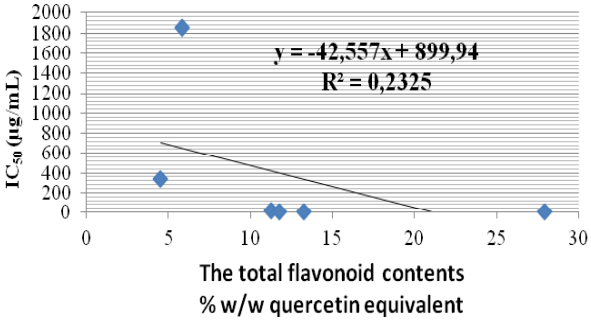

Figure 4. The relationship between IC_{50} and flavonoid contents of ethanolic extract of *Phyllantus urinaria* L and its fraction

Conclusion

Our study indicated that ethanolic extract of *Phyllantus urinaria* L. and its fractions are rich sources of antiradical scavenging, with significant high level of phenolic and flavonoid content. Therefore, the use of *P. urinaria* L. as sources of phytochemicals could offer diverse opportunities for nutraceutical and functional food applications.

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