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Antioxidant activity, total phenolic, and total flavaonoid of extracts and fractions of red fruit (*Pandanus conoideus* Lam)

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Abstract: Red fruit (*Pandanus conoideus* Lam) is one of the fruits commonly consumed as diet by societies, especially in Papua, Indonesia. Preliminary research revealed that among three extracts of red fruit evaluated, ethyl acetate extract had the highest antiradical activities; therefore, this research was directed to fractionate ethyl acetate extract, evaluate antioxidant activities using *in vitro* methods of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, reducing power, and metal chelating activity, and to determine the phenolics and flavonoid contents of ethyl acetate extract and its fractions. Ethyl acetate extract and its fractions can strongly scavenge DPPH radical with IC_{50} value ranged from 5.25 to 53.47µg/ml. Its antiradical scavenging activity revealed a moderate relationship with the total phenolics content ($r^2 = 0.645$) and with flavonoid content (r2 = 0.709). Ethyl acetate extract and its fractions also had a capability to reduce Fe^{3+} to Fe^{2+} with reduction activity ranged 91.26 – 682.18 µg ascorbic acid equivalent/g extract or fraction. These extract and its fractions had metal chelating activity lower than that of EDTA (IC_{50} 18.19 µg/ml). Red fruit can be used as natural antioxidant source to prevent diseases associated with free radical.

Keywords: antioxidant, red fruit (*Pandanus conoideus* Lam), extract, fraction.

Introduction

The active nitrogen and oxygen species may induce some damage to the human body. Over production of various forms of activated oxygen species, such as oxygen radicals and non-free radical species is considered to be the main contributor to oxidative stress, which has been linked to several diseases like atherosclerosis, cancer, and tissue damage in rheumatoid arthritis (McDonald *et al*., 2001; Jang *et al*., 2007). Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress (Ozsoy *et al*., 2008).

Recently, there have been great efforts to find safe and potent natural antioxidants from various plant sources. As harmless sources of antioxidants, edible fruits have been investigated for their antioxidant properties, for example Noni (*Morinda citrifolia*, L) (Rohman *et al*., 2006; Zin *et al*., 2004); Mango (*Mangifera indica* L.) (Riberio *et al*., 2008); Bitter gourd (*Momordica charantia* L.) (Kubola and Siriamornpun, 2008); and blueberry (*Vaccinium corymbosum* L.) (Castrejon *et al*., 2008). Fruits have been also associated inversely with morbidity and mortality from degenerative and coronary heart diseases (Verzelloni *et al*., 2007).

Numerous crude extracts and pure natural compounds from fruits were reported to have antioxidant and radical-scavenging activities. Within the antioxidant compounds, flavonoids and phenolics, with a large distribution in nature, have been studied (Li *et al*., 2009). Phenolics or polyphenols, including flavonoids have received considerable attention because of their physiological functions such as antioxidant, antimutagenic and antitumor activities (Othman *et al*., 2007).

The growing interest in the antioxidant properties of the phenolics compounds in vegetables and fruits derives from their strong activity and low toxicity compared with those of synthetic phenolics antioxidant such as BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole), and propyl gallate (Cailet *et al*., 2006).

Plants have been used for several years as a source of traditional medicine to treat various diseases and

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conditions (Razali *et al*., 2008). *P. conoideus* is an indigenous plant from Papua Province, Indonesia. For local communities, it is believed that fruit of *P. conoideus* can treat several degenerative diseases such as cancer, arteriosclerosis, rheumatoid arthritis, and stroke (Budi and Paimin, 2004).

Mun'im *et al*. (2006) have investigated the effect of water extract of *P.conoideues*. The result showed that extract at dose 0.21 ml/200g bw can inhibit lung carcinogenesis rat female Sprague-Dawley induced by 7,12-dimethylbenz[a]anthrasene (DMBA).

Literature showed that no information exists regarding the utilization of *P. conoideus* fruit as a source of natural antioxidant. Therefore, the objectives of this study were to evaluate *P. conoideus* as a source of natural antioxidants using different extracting solvents to determine their antioxidant capacities. Because of the important roles of the total phenolics and total flavonoids as antioxidants, the amounts of total phenolics and total flavonoids in the extracts/fractions were also determined.

Materials and Methods

Plant materials

Fruit of *P. conoideus* was collected from Papua Province, Indonesia. Botanical identification was performed by Department of Biological Pharmacy, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia.

Plant extract

P. conoideus Lam fruit was macerated by methanol and partitioned successively using chloroform and ethyl acetate to afford extracts of chloroform and ethyl acetate respectively. Among these extracts, ethyl acetate extract showed the highest antioxidant activity; therefore it was subject to fractionation using vacuum liquid column chromatography (Figure 1).

Fractionation of ethyl acetate extract

Ethyl acetate extract was fractionated using flash column using stationary phase of silica gel and mobile phase delivered in gradient manner in the order: 1.5 l PE; mixt.150 ml PE, 50 $CHCl_3$; mixt.200 ml PE, 200 $CHCl_3$; mixt.125 ml PE, 375 ml $CHCl_3$; 250 ml $CHCl_3$; mixt. 450 ml $CHCl_3$, 50 ml EtOAc; mixt.400 ml $CHCl_3$, 100 ml EtOAc; mixt.350 ml $CHCl_3$, 150 ml EtOAc; mixt. 300 ml $CHCl_3$, 200 ml EtOAc; mixt.125 ml $CHCl_3$, 125 ml EtOAc; mixt.150 ml $CHCl_3$, 100 ml EtOAc; mixt.50 ml $CHCl_3$, 200 ml EtOAc; 250 ml EtOAc; mixt. 125 ml EtOAc, 125 ml MeOH ; and 1.5 l MeOH. Eluates were then pooled to fraction based on the similarity of TLC profile (Figure 1). Each fraction was evaluated for its total phenolic and total flavonoid contents, its DPPH radical scavenging activity, its reducing power and its metal chelating ability.

Chemicals

2,2-diphenyl-1-picrylhidrazyl (DPPH), ferrozine, gallic acid, trichloracetic acid (TCA), rutin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Silica gel GF_{254}, silica gel 7736, ethanol, methanol, chloroform, petroleum ether, hexane, aluminum hydroxide, Folin-Ciocalteu reagent, sodium nitrite, sodium carbonate, potassium ferricyanide, feri chloride, fero chloride, phosphate buffer, ethylene diamine tetraacetate (EDTA) were purchased from E.Merck (Darmstadt, Germany). Bidistilled water was obtained from Ikapharmindo (Indonesia).

Determination of total phenolic content

Folin Ciocalteu reagent was used for analysis of total phenolics content (TPC) according to Chun *et al*. (2003). In a-10 ml volumetric flask, a-0.2 ml aliquot of the extracts and fractions in methanol (1.0 mg/ml) was mixed with 0.4 ml of Folin-Ciocalteu reagent. The solution was allowed to stand at 25°C for 5-8 min before adding 0.2 ml of 4.0 ml of sodium carbonate solution 7.0 % and made to 10 .0 ml with bidistilled water. The mixture was allowed to stand for 2 h before its absorbance was measured at 725 nm. Gallic acid was used as standard for the calibration curve. TPC was expressed as mg gallic acid equivalents (GAE) per gram of sample (mg/g).

Determination of total flavonoid content

The total flavonoid contents were measured by a colorimetric assay (Zhishen *et al*. 1999; Zou *et al*., 2004). A-100.0 µl aliquot of extracts or fractions in methanol was added to a 10 ml volumetric flask containing 4 ml of distilled water. At zero time, 0.3 ml 5% sodium nitrite was added to the flask. After 5 min, 0.3 ml of 10% aluminium chloride was added. At 6 min, 2 ml of 1 M sodium hydroxide was added to the mixture. Immediately, the mixture was diluted to volume with the addition of 2.4 ml distilled water and thoroughly mixed. Absorbance of the mixture, pink in color, was determined at 510 nm versus a blank containing all reagents except samples of extracts or fractions. Rutin was used as standard for the calibration curve. Total flavonoid content of the extracts and fractions were expressed as mg rutin equivalents (RE) per gram of sample (mg/g).

Measurement of DPPH-radical scavenging activity

DPPH radical scavenging activity was assessed according to Kikuzaki *et al*. (2002). In this assay, a-50 µl of extract or fraction solutions with different concentrations was added with 1.0 ml of 0.4 mM methanolic-DPPH and added with methanol to 5.0 ml. The mixture was shaken vigorously using vortex and left to stand for 30 min at room temperature in a dark room. The scavenging effect on the DPPH radical was read using spectrophotometer (Genesys-10) at 517 nm.

The radical scavenging activity was expressed as the radical scavenging percentage using the following equation:

Percentage (%) of DPPH radical scavenging =

$$(1-\frac{As}{Ac})\,x100$$

Where; A_C =absorbance of control and A_S = absorbance of sample solution.

The DPPH solution without sample solution was used as control. IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the plotted graph of radical scavenging activity against the concentration of extracts or fractions. Ascorbic acid and tocopherol were used as positive control.

Reducing power assay

The reducing power of different extracts or fractions were measured according the method used by Hinneburg *et al*. (2006). One milliliter of extracts or fractions with different concentrations was mixed with 2.5 ml of phosphate buffer (200 mM; pH 6.6) and 2.5 ml of potassium ferricyanide1% and incubated at 50°C for 20 min. The mixture was added with 2.5 ml of 10% TCA and centrifuged at 3000 rpm for 10 min. A-2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%) and the absorbance was measured spectrophotometrically at 700 nm. Increase in absorbance of the reaction mixture was interpreted as increase in reducing activity of the extract and the results were compared with ascorbic acid which was used as a positive control. The percentage of reduction of the sample as compared to standard (ascorbic acid) was calculated using the formula:

Percentage (%) of reduction power =

$$[1-(1-\frac{As}{Ac})]\,x100$$

A_C = absorbance of standard at maximum concentration tested and A_S = absorbance of sample.

Metal ion-chelating assay

The ferrous ion-chelating activity of extracts and fractions was measured according to the method of Su *et al.* (2008). The absorbance of the ferrous iron-Ferrozine complex at 562 nm was measured to determine the Fe^{2+} -chelating ability of the extract or fraction. The reaction mixture, containing of extracts or fractions with different concentrations, $FeCl_2$ (2 mM), and ferrozine (5 mM), was adjusted to 5 ml with bidistilled water. The mixture was shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against a blank. EDTA was used as positive control. The ability of extracts to chelate ferrous ion was calculated using the following equation:

Chelating activity (%) = $(1-\frac{As}{Ac})\,x100$

IC_{50} value (mg/ml) is the concentration at which the chelating activity was 50%.

Statistical Analysis

All data are presented as means ± SD for at least four replications for each prepared sample. Statistical analysis was performed based on one way Analysis of variance (ANOVA) at confidence level 95% using SPSS (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) version 12.0 for Windows. Significances of differences were conducted with a Tukey-HSD. Linear regression to correlate between total phenolics as well as total flavonoid with antioxidant activity was carried using Excel 2003.

Results and Discussions

Determination of total phenolic contents

Phenolics or polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Many of the phenolics have been shown to contain high levels of antioxidant activities (Razali *et al*., 2008).

Phenolic compounds contribute to the overall antioxidant activities of plants mainly due to their redox properties. Generally, the mechanisms of phenolic compounds for antioxidant activity are neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals (Javanmardi *et al*., 2003; Li *et al*., 2009).

TPC of extracts and ethyl acetate fractions was determined by Folin-Ciocalteau (F-C) assay using Gallic acid as a standard phenolic compound. The F-C assay for total phenolics contents is a fast and simple method and can be useful in characterizing and

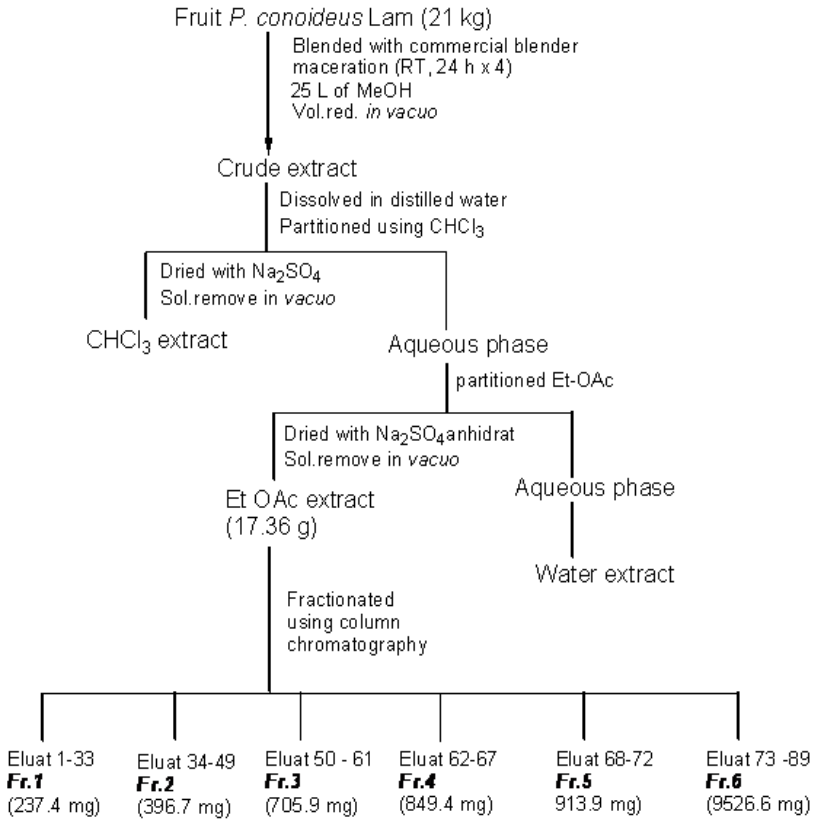


Figure 1. Extraction and fractionation scheme for production of extract and fractions of *P.conoideus* fruit. PE = petroleum eter; mixt = mixture; RT = room temperature; vol. = volume; red. = reduced; Fr. = fraction

standardizing botanical samples. F-C method is based on oxidation of phenolics by a molybdotungstate in F-C reagent to yield a colored product with λ_{max} 745 – 750 nm (Prior *et al*., 2005).

A linear calibration curve of Gallic acid, in the range of 25–700 µg/ml with coefficient of determination (r^2) value of 0.998, was obtained (Fig. 2). TPC of the three extracts of *P. conoideus* and ethyl acetate fractions is demonstrated in Table 1. The ethyl acetate extract of *P. conoideus* revealed the highest total phenolic content at 627.52 ± 13.65 mg GAE/g, approximately 8 fold more than the methanol extract and 12 fold more than the chloroform extract. Fractions of 3, 4, and 5 showed no significance difference of TPC values (P >0.05).

F-C assay gives a crude estimate of the total phenolic compounds present in an extract/fraction. It is not specific to polyphenols, but many interfering compounds may react with the reagent, giving elevated apparent phenolic concentrations (Prior *et al*., 2005). Moreover, various phenolic compounds respond differently in this assay, depending on the number of phenolic groups they have and total phenolics content does not incorporate necessarily all

Table 1. Total phenolic contents of extracts and ethyl acetate fractions from *P.conoideus* fruit.

Extracts	Phenolics content (mg GAE/g extract or fraction)
Methanol	80.27 ± 3.21
Chloroform	49.72 ± 2.24
Ethyl acetate	627.52 ± 13.65
Fractions (Fr)	
Fr. 1	-
Fr.2	550.23 ± 23.43
Fr.3	751.21 ± 12.21
Fr.4	753.64 ± 16.05
Fr.5	725.08 ± 9.33
Fr.6	148.44 ± 5.12

Values are means ± SD of four determinations.

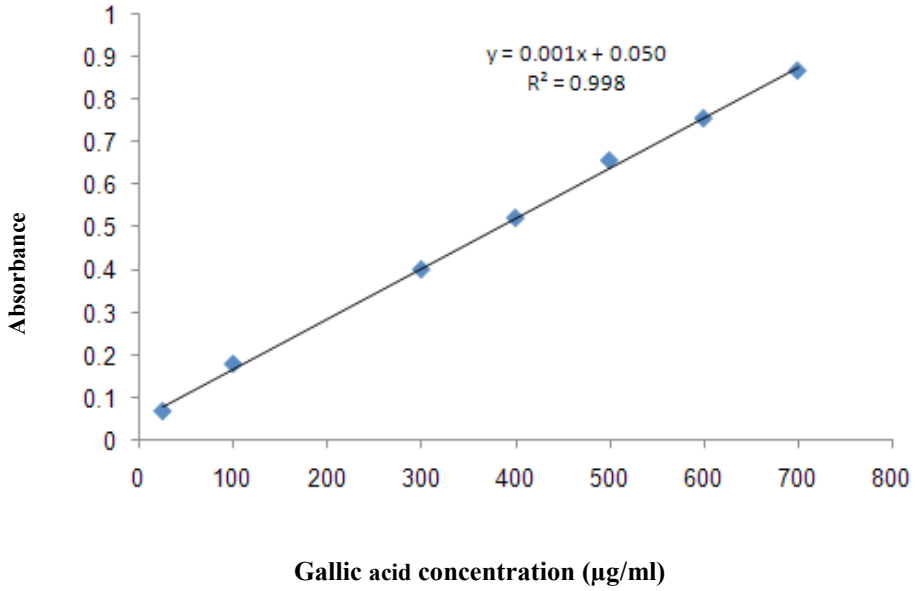


Figure 2. Calibration curve of standard gallic acid for determination of total phenolics content

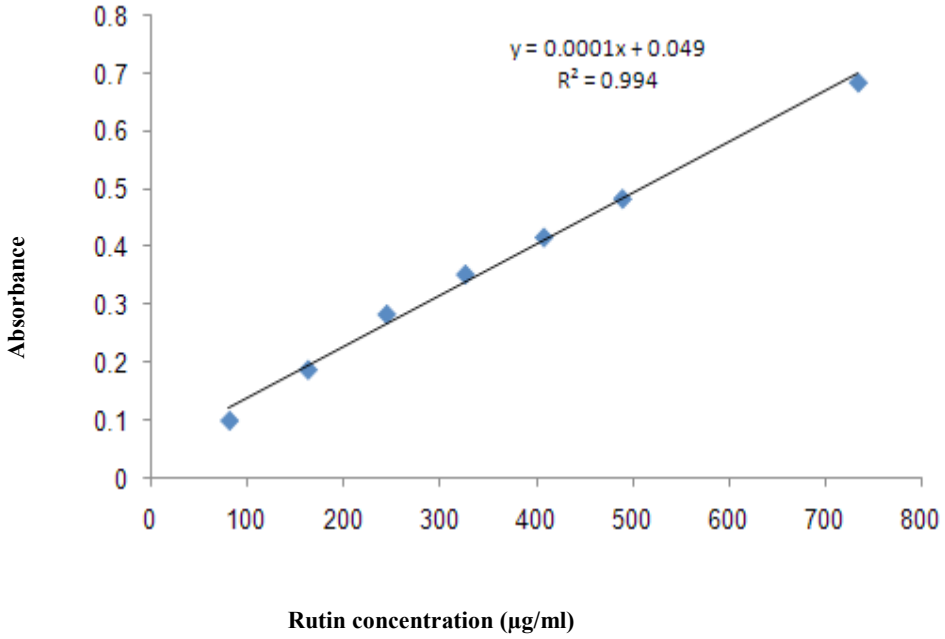


Figure 3. Calibration curve of standard rutin for determination of total flavonoid content

the antioxidants that may be present in an extract or fraction (Tawaha *et al*., 2007).

Determination of total flavonoid contents

Flavonoids are the most common and widely distributed group of plant phenolic compounds, characterized by a benzo-γ-pyrone structure. It is ubiquitous in fruits and vegetables. Total flavonoid contents can be determined in the sample extracts/ fractions by reaction with sodium nitrite, followed by the development of coloured flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm (Abu Bakar *et al*., 2009).

The total flavonoid content was expressed as rutin equivalents (RE) in milligram per gram dry material of extracts and fractions. The calibration curve of rutin to determine flavonoid content was shown in Fig. 3. Total flavonoid content of ethyl acetate extract and its fraction was compiled in Table 2. The result showed that Fr. 4 had the highest flavonoid content compared to that of other fractions.

Scavenging activity on 2,2-diphenyl-1-picrylhidrazyl (DPPH) radical

The measurement of the scavenging of DPPH radical allows one to determine exclusively the intrinsic ability of substance to donate hydrogen atom or electrons to this reactive species in a homogenous system. The method is based on the reduction of methanolic-DPPH solution because of the presence of antioxidant substances having hydrogen donating groups (RH) such as phenolics and flavonoids compounds due to the formation of non radical DPPH-H form (Paixao *et al*., 2007). The primary reaction which takes place is the formation of free radical R· and the reduced form of DPPH· (Fig. 4). The free radical produced can undergo further reactions which control the number of the molecules of DPPH reduced by one molecule of the reductant.

The DPPH radical-scavenging capacity in the studies was reported after 30 min reaction time for all samples evaluated. The parameter used to measure the radical scavenging activity of extracts and fractions evaluated is IC_{50} value, defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals in this specified time period. The smaller IC_{50} value, the higher antioxidant activity of the plant extract/fraction (Maisuthisaku *et al*., 2007). The IC_{50} value of various plant extracts/fractions and positive controls was shown in Table 3.

Some fractions of ethyl acetate extract revealed the higher activity compared than that of ethyl acetate extract itself in the order Fr. 4 (IC_{50} = 5.25 ± 0.05 µg/ml) > Fr. 3 (IC_{50} = 6.06 ± 0.15 µg/ml) > Fr. 5 (IC_{50} = 7.10 ± 0.23 µg/ml). Two other fractions i.e Fr. 2 (IC_{50} = 40.10 ± 0.74 µg/ml) and Fr. 6 (IC_{50} = 53.47 ± 0.88 µg/ml) have the lower DPPH radical scavenging activity than that of corresponding ethyl acetate extract before being partitioned.

The phytochemicals which might be responsible for the scavenging activity in this species is phenolic and flavonoid constituents. Analysis of correlation between free radical scavenging activity with total phenolic contents as well as with total flavonoid contents was shown in Fig. 5.

Table 2. Total flavonoid contents of extracts and ethyl acetate fractions from P.conoideus fruit.

Extracts	Flavonoid content (mg RE/g extract or fraction)
Methanol	260.03 ± 11.13
Chloroform	31.21 ± 8.64
Ethyl acetate	697.12 ± 1.08
Fractions (Fr)	
Fr. 1	-
Fr.2	463.03 ± 4.92
Fr.3	586.92 ± 9.49
Fr.4	825.14 ± 25.92
Fr.5	725.07 ± 9.34
Fr.6	299.61 ± 0.72

Values are means ± SD of four determinations.

Table 3. IC_{50} value of some extracts/fractions and positive controls

Extracts/fractions	IC_{50} ± SD (µg/ml)
Methanol	392.06 ± 8.33
Chloroform	605.68 ± 9.32
Ethyl acetate	10.35 ± 0.86
Fractions (Fr)	
Fr. 1	-
Fr.2	40.10 ± 0.74
Fr.3	6.06 ± 0.15
Fr.4	5.25 ± 0.05
Fr.5	7.10 ± 0.23
Fr.6	53.47 ± 0.88
Positive controls	
Ascorbic acid	4.95 ± 0.12
Tocopherol	8.23 ± 0.21

Values are means ± SD of four determinations.

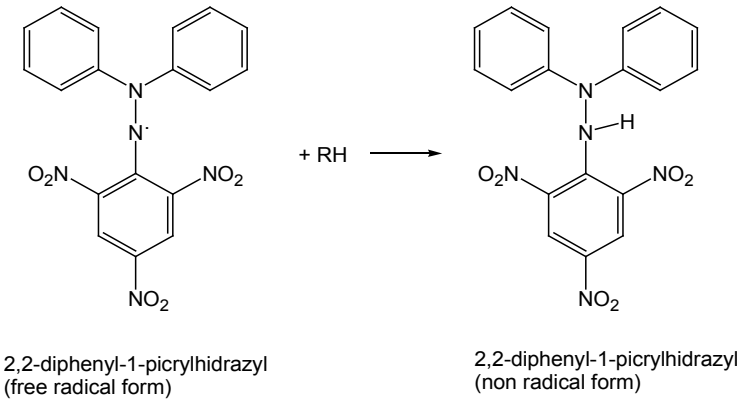
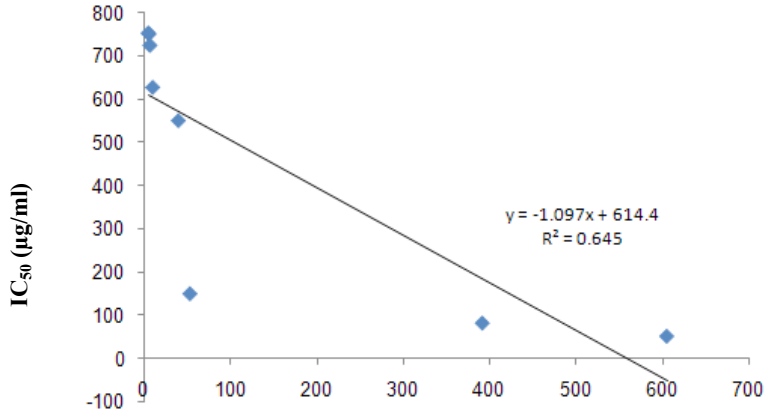


Figure 4. Structure of DPPH and its reduction form by the antioxidant RH



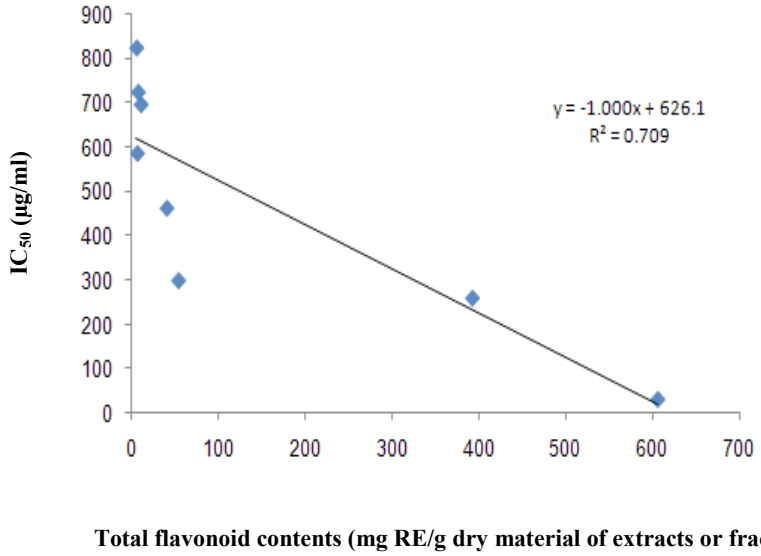


Figure 5. Correlation between free DPPH radical scavenging activity (IC_{50}) and total phenolic contents (A) and with total flavonoid contents (B)

Table 4. The reducing power of ethyl acetate extract and its fractions

Sample	Reducing power (µg ascorbic acid equivalent per gram extract/fractions)
Ethyl acetate extract	311.28 ± 12.68
Fr. 1	-
Fr. 2	415.98 ± 6.44
Fr. 3	569.62 ± 6.44
Fr. 4	682.18 ± 2.77
Fr. 5	311.65 ± 10.91
Fr.6	91.26 ± 1.42

Values are means ± SD of four determinations.

The relationship between free radical scavenging activity (Y) with total phenolic contents (X) revealed coefficient of determination (R^2) of 0.645, whereas with total flavonoid content (X) has R^2 of 0.709. These results suggested that phenolic compounds and flavonoid compounds contributed of 64.5 % and 70.9 % to free DPPH radical scavenging of the extracts and ethyl acetate fractions from *P. conoideus* fruit. Also, it can be stated that scavenging effect of extracts/ fractions is not limited to phenolics and flavonoid 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).

Reducing power

The reducing power (RP) of the extracts and ethyl acetate fractions was determined by direct electron donation in the reduction of ferri cyanide $[Fe(CN)_6]^{3-}$ to ferro cyanide $[Fe(CN)6]^{4-}$. The product was visualized by addition of free Fe^{3+} ions after the reduction reaction, by forming the intense Prussian blue colour complex, $(Fe^{3+})_4[Fe^{2+}(CN^-)_6]^3$, and quantified by absorbance measurement at 700 nm (Riberio *et al*., 2008).

The reducing power of ethyl acetate extract and its fractions was expressed as µg ascorbic acid equivalent per gram extract/fractions and its results were shown in Table 4. Among fractions evaluated, Fr. 4 has the highest reducing power, which was in agreement with the total phenolics content and total flavonoid content.

The correlation between reducing power activity of fractions and its phenolic contents revealed an equation y = 0.8834x + 241.97 and R^2 = 0.6373; meanwhile with the flavonoid content showed: y = 0.602x + 360.5; R^2 = 0.4303. Taking into account of R^2 values, these results suggested that phenolics compounds more likely contribute to its reducing activity than do of flavonoid compounds.

Table 5. IC_{50} of ethyl acetate extract and its fraction

Sample	IC_{50} ± SD (µg/ml)
Etyl acetate extract	8559.09 ± 86.18
Fr. 1	-
Fr.2	490.10 ± 8.32
Fr.3	1293.74 ± 14.84
Fr.4	65.98 ± 7.24
Fr.5	282.93 ± 7.23
Fr.6	1053.47 ± 13.88
EDTA	18.19 ± 0.30

Values are means ± SD of four determinations.

Metal ion-chelating assay

Ferrozine can quantitatively form complexes with Fe^{2+}. The complex formation can be disrupted by the presence of other complexing agents which cause a decrease in the red colour intensity of complexes. Substances or samples that can reduce its colour intensity can be considered as antioxidant through the mechanism of inhibition of heavy metal. It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Kumaran and Karunakaran, 2006). Table 5 list IC_{50} value of ethyl acetate extract and its fraction obtained from measurement of metal chelating activity. The smaller IC_{50} value, the higher metal chelating activity.

Compared to positive control of EDTA, there is no fraction having IC_{50} which is smaller than that of EDTA. It means that all evaluated fractions are weak metal chelating agent.

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