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Effect of solid-to-solvent ratio on phenolic content and antioxidant capacities of "*Dukung Anak*" (*Phyllanthus niruri*)

[1]Wong, B.Y., [2]Tan, C.P. and [1*]Ho, C.W.

[1]Department of Food Science and Nutrition, Faculty of Applied Sciences, UCSI University, No.1 Jalan Menara Gading, UCSI Heights, Cheras, 56000 Kuala Lumpur, Malaysia*
[2]Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

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Abstract

The objective of this study was to evaluate the effects of solid-to-solvent ratio (1:5. 1:10, 1:15 and 1:20) on the extraction of phenolic compounds (TPC and TFC) and antioxidant capacity (ABTS and DPPH radical scavenging capacity) of *P. niruri*. Solid-to-solvent ratio showed a significant effect for both phenolic compounds (TPC and TFC) and antioxidant capacity (ABTS and DPPH radical scavenging capacity) with 1:20 was the condition for extracting the highest of phenolic compounds (TPC and TFC) with a value of 5788.7 mg GAE/100 g DW and 1906.5 mg CE/100 g DW, respectively and exhibited high antioxidant capacities (ABTS and DPPH radical scavenging capacities) with a value of 0.820 mM and 1.598 mM, respectively among the four levels studied. TPC was positively and significantly correlated with ABTS and DPPH (r=0.999 and r=0.999) under the effects of solid-to-solvent ratio as compared to TFC, positively and strongly correlated (r=0.865 and r=0.868) with ABTS and DPPH.

Introduction

Phyllanthus niruri which known as "*Dukung Anak*" in Malaysia, is a small temperate herb widely distributed throughout different regions in the world such as India, South America and Malaysia (Rajeshkumar *et al*., 2002). According to Fang *et al*. (2008), *Phyllanthus niruri* which is one of the 500 temperate and tropical species of *Phyllanthus* (*Euphorbiaceae*) have been commonly used as folk medicine in most tropical countries. For the past decade, phenolic compounds have gained an intense focus of research due to their strong antioxidant activities and ability to scavenge free radicals (Chan *et al*., 2009). Studies conducted by Tan (2010) showed that phenolic compounds found naturally in *Dukung Anak* were suggested to be the major contributors to the antioxidant activities of the plant. Therefore, an optimum extraction method for phenolic compound is utmost important from the industrial point of view.

However, the extraction method of phenolic compounds differs from plant to plant and an ideal extraction method for a particular phenolic source has to be individually designed and optimized (Tan *et al*., 2011). According to Thoo *et al*. (2010), the extraction efficiency is influenced by various factors such as method of extraction, solvent type, solvent concentration, extraction time and temperature, particle size and solid-to-solvent ratio. An unpublished optimized condition for phenolic compound extraction from "*Dukung Anak*" had been studied and suggested that the optimum extraction temperature was 33.1°C, extraction time 40 min and solvent concentration 60.4% ethanol. This optimized condition was studied at fixed solid-to-solvent ratio at 1:10. Apart from that, it was reported that the phenolic content is affected by the extraction solvent volume being used (Tan *et al*., 2011). Therefore, the effect of solid-to-solvent ratio on the efficiency of extracting phenolic compounds from Phyllanthus niruri was further investigated.

Materials and Methods

Chemicals and plant materials

A total of 2 different batches of 1kg "*Dukung Anak*" (*Phyllanthus niruri*) dry powder with particle size 0.08 mm were purchased from Ethno Resources, Selangor, Malaysia. All solvents and chemicals used were of analytical grade. The de-ionized water

*Corresponding author.
Email: *cwho@ucsi.edu.my*

used throughout the experiments was obtained from Milli-Q water purification system (Milipore Corporation, USA).

Sample preparations

The ready-milled 0.08 mm sample was weighed using analytical balance (EL-4100S, Setra, USA) and vacuum packaged into nylon-linear low density polyethylene (nylon-LDPE) pouch (Flexoprint, Malaysia) using vacuum packaging machine (DZQ400/500, Clarity, China). The packaged samples were wrapped with aluminum foil and stored at room temperature in a dark environment until use.

Solvent extraction and experimental design

The solvent extraction procedure was carried out according to the extraction procedures described by Tan *et al*. (2011) with slight modifications. A required amount of *Dukung Anak* (*Phyllanthus niruri*) dry powder was weighed accurately using analytical balance (AB204-S, Mettler Toledo, Switzerland) and extracted with 4 different solid-to-solvent ratios (1:5, 1:10, 1:15 and 1:20) by using60.4% ethanol in conical flasks which were wrapped with parafilm (Pechiney plastic packaging, USA) and aluminum foil (Diamond, USA) to prevent spilling of mixture and light exposure, respectively. The mixture was then shaken at rotation level 8 using a temperature-controlled water bath shaker (WNB 7–45, Memmert, Germany) for 40 min at 33.1°C. After extraction, the *Dukung Anak* (*P. niruri*) extract underwent filtration using Whatman No.1 filter paper (Whatman International, England) to obtain a clear solution of crude extract. The crude extract was collected in a light-protected amber bottle for analysis without storage. All extractions were carried out in replicate.

The optimal solid-to-solvent ratio was selected according to the values of total phenolic content (TPC, mg GAE/100 g dry weight), total flavonoid content (TFC, mg CE/100g dry weight), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacity (mM) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity (mM).

Determination of total phenolic content (TPC)

TPC of *P. niruri* extracts was determined with Folin-Ciocalteu reagent, according to the method of Tan *et al*. (2011) with slight modifications. Crude extracts were diluted to 100 times with de-ionized water prior to analysis. An amount of 1 mL diluted crude extract was added to 1 mL of 1:10 diluted Folin-Ciocalteu reagent (FCR) in an aluminum foil-wrapped 15 mL test tube. After incubating the mixture at room temperature for 4 min, 0.8 mL of sodium carbonate (7.5%, w/v) solution was added into the mixture. The mouth of the test tube was covered with parafilm (Pechiney plastic packaging, USA) and aluminum foil (Diamond, USA). The mixture was then immediately vortex (VTX-3000L, Copens Scientific, Germany) for 10s and subsequently incubated in dark environment at room temperature for 2 h. The absorbance of the mixture was measured at 765 nm against the blank reagent using Uvi light spectrophotometer (UviLine 9400, Secomam, France). The blank was prepared by replacing 1 mL of *P. niruri* extract with 1 mL of de-ionized water. Gallic acid was used to calibrate the standard curve and the calibration equation for gallic acid was $y = 0.0396x$ ($R^2 = 0.9975$). The measurements were carried out in triplicate. The results were expressed as gallic acid equivalent (GAE)in mg per 100 g of dry weight (DW) of sample (mg GAE / 100 g DW).

Determination of total flavonoid content (TFC)

TFC was determined by using procedures described by Tan *et al*. (2011) with slight modifications. An amount of 1.25 mL de-ionized water followed by 75 µL of 5% (w/v) sodium nitrite ($NaNO_2$) solution was added to 0.25 mL of undiluted crude extract or water (blank) or Catechin (positive control) in an aluminum foil-wrapped 15 mL test tube. The mixture was allowed to stand for 6 min before adding 150 µL of 10% (w/v) aluminum chloride ($AlCl_3$) solution. The mixture was allowed to stand at room temperature for another 5 min before adding 0.5 mL of 1 M sodium hydroxide (NaOH) and 275 µL of de-ionized water. The mouth of the test tube was covered with parafilm (Pechiney plastic packaging, USA) and aluminum foil (Diamond, USA). The mixture was then immediately vortex (VTX-3000L, Copens Scientific, Germany) for 10 s. The absorbance of the mixture was measured at 510 nm against the blank reagent using Uvi light spectrophotometer (UviLine 9400, Secomam, France). (+)-Catechin was used to calibrate the standard curve and the calibration equation for (+)-Catechin was $y = 0.0033x$ ($R^2 = 0.9991$). The measurements were carried out in triplicate. The results were expressed as Catechin equivalent (CE) in mg per 100 g of dry weight (DW) of sample (mg CE / 100 g DW).

ABTS radical-scavenging capacity

The radical scavenging capacity of *P. niruri* extract against ABTS radical cation was measured by using the method of Tan *et al*. (2011) with slight modifications. An ABTS radical solution was prepared by mixing 7 mM ABTS solution and 2.45 mM potassium persulfate solution at a ratio 1:1 (v/v)

in a 250 mL amber volumetric flask. The mixture was vortex for 10s and then allowed to stand in dark condition at room temperature for 12-16 hrs to give a dark blue solution and it was used for analysis within 2 days. The absorbance of ABTS radical solution was equilibrated to an absorbance of 0.7 ± 0.02 at 734 nm by diluting with ethanol before used. 0.1 mL of *P. niruri* extract was mixed with 3.9 mL of ABTS radical solution. Blank was also prepared by replacing 0.1 mL de-ionized water with *P. niruri* extract. The absorbance of mixture was read immediately at 734 nm against blank after incubation at room temperature for 6 min.

Simultaneously, absorbance of negative control (3.9 mL of ABTS radical solution and 0.1 mL of ethanol) was also measured at 734 nm by using UVi spectrophotometer (UviLine 9400, Secomam, France). Absorbance of negative control was measured at each level of treatments and the values were averaged and used as a constant for calculation. The radical-scavenging capacity of ABTS (%) was calculated as [1- (A_s / A_c)] x 100% (A_s = Absorbance of sample at 734 nm; A_c = Absorbance of negative control at 734 nm). Trolox solution was used to calibrate the standard curve and the calibration equation for Trolox was y = 120.1142x (R^2 = 0.9984). Each crude extract was analyzed in triplicate and the results were expressed as µmol Trolox equivalent per 100 g dried weight (µmol TEAC / 100 g DW).

DPPH radical-scavenging capacity

DPPH assay was carried out as described by Tan *et al*. (2011) with slight modification. 100 µL of *P. niruri* extract was mixed with 3.9 mL of ethanolic DPPH (60 µM). Subsequently, the mixture was immediately vortex for 1 min and it was allowed to stand at room temperature for 30 min. Blank was prepared by replacing 100 µL of *P. niruri* extract with 100 µL de-ionized water. The absorbance of mixture was measured against blank at 517 nm by using UVi light spectrophotometer (UviLine 9400, Secomam, France).

Simultaneously, the absorbance of negative control (100 µL of ethanol and 3.9 mL of ethanolic DPPH) was measured at 517 nm. Absorbance of negative control was measured three times and the values were averaged and used as a constant for calculation. The DPPH solution scavenging activity (%) was calculated as [1- (A_s / A_c)] x 100% (A_s = Absorbance of sample at 517 nm; A_c = Absorbance of negative control at 517 nm). Trolox solution was used to calibrate the standard curve and the calibration equation for Trolox was y = 37.284x (R^2 = 0.9997). Each crude extract was analyzed in triplicate and the results were expressed as µmol Trolox equivalent per 100 g dried weight (µmol TEAC / 100 g DW).

Statistical analysis

All the experimental results were expressed as mean ± standard deviation of replicate solvent extraction and the triplicate of assays. All the results were analyzed by using Minitab software (Minitab Version 15.1.1.0) for One-way analysis of variance (ANOVA) with Tukey's test was used to determine the significant differences between the means at the 5% level. Pearson correlations between variables were also established using MINITAB software (Minitab Version 15.1.1.0) to access the correlation among the antioxidant compound assays and antioxidant capacity assays.

Results and Discussion

Effects of solid-to-solvent ratio on extraction of phenolic compounds

Solid-to-solvent ratio showed a significant effect ($p<0.05$) on total phenolic content (TPC) and total flavonoid content (TFC) as shown in Figure 1 and 2. Solid-to-solvent ratio of 1:20 (w/v) showed the highest amount of TPC and TFC with a value of 788.7 mg GAE/100 g DW and 1906.5 mg CE/100 g DW, respectively.

A high solid-to-solvent ratio was found to be favourable in extraction of phenolic compounds. These results were consistent with mass transfer principle where the driving force for mass transfer is considered to be the concentration gradient between the solid and the solvent. A high solid-to-solvent ratio could promote an increasing concentration gradient, resulting in an increase of diffusion rate that allows greater extraction of solids by solvent (Al-Farsi and Chang, 2007; Tan *et al*., 2011). This statement was in agreement with Zhang *et al*. (2007) which stated that the chance of bio-active components coming into contact with extracting solvent expanded with increase amount of extracting solvent and will not continue to increase once equilibrium is reached (Tan *et al*., 2011). Overall, the main effect of the solid-to-solvent ratio was to modify the solubility and equilibrium constant and thus increase the total phenolic and flavonoid yields to a maximum at the optimum solid-to-solvent ratio (Cacace and Mazza, 2003).

However, an equilibrium constant trend was not observed in current study indicating an insufficient amount of extracting solvent used in extracting the phenolic compounds from the *P. niruri*. Although amount of phenolic compounds generally increased

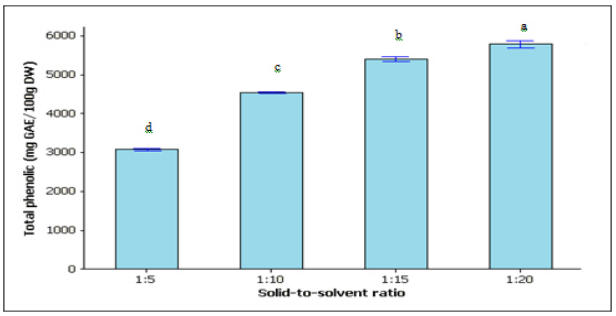

Figure 1. Effect of solid-to-solvent ratio on total phenolic content of *Dukung Anak* (*Phyllanthus niruri*). The columns are expressed as mean ± standard deviation of two replications (n=2). Columns marked with different letters indicate significance difference ($p<0.05$)

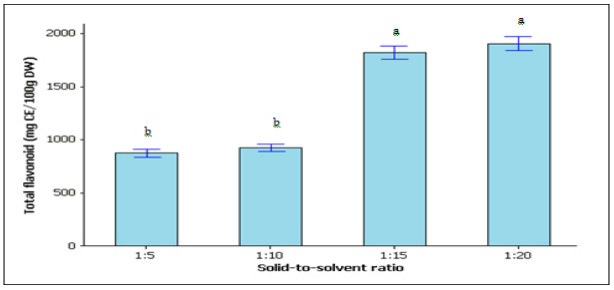

Figure 2. Effects of solid-to-solvent ratio on total flavonoid content of *Dukung Anak* (*Phyllanthus niruri*). The column are expressed as mean ± standard deviation of two replications (n=2). Columns marked with different letters indicate significance difference ($p<0.05$)

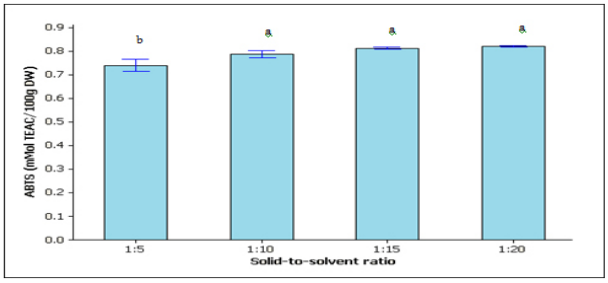

Figure 3. Effect of solid-to-solvent ratio on ABTS antioxidant capacity of *Dukung Anak* (*Phyllanthus niruri*). The columns are expressed as mean ± standard deviation of two replications (n=2). Columns marked with different letters indicate significant difference ($p<0.05$)

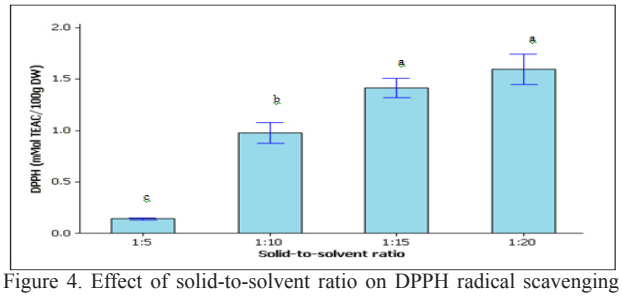

Figure 4. Effect of solid-to-solvent ratio on DPPH radical scavenging capacity of *Dukung Anak* (*Phyllanthus niruri*). The columns are expressed as mean ± standard deviation of two replications (n=2). Columns marked with different letters indicate significant difference ($p<0.05$)

with increase of solid-to-solvent ratio, the increase in yield of phenolic compounds may not be directly proportional. Thus, it is important to evaluate the influence of solid-to-solvent ratio at beyond 1:20 ratio. This approach will aid in efficient usage of solvent and solvent mixtures for extracting phytochemicals and avoidance of saturation effect, as well as reducing solvent waste disposal cost. Furthermore, use of high solid-to-solvent ratios would result in dilute solutions (Ho *et al*., 2008).

Effect of solid-to-solvent ratio on antioxidant capacities

Solid-to-solvent ratio showed a significant effect ($p<0.05$) for both ABTS and DPPH radical scavenging capacities as shown in Figure 3 and Figure 4, respectively. Solid-to-solvent ratio at 1:20 exhibited the highest ABTS (0.820 mM) and DPPH (1.598 mM) radical scavenging capacities, as well as extracted the highest total phenolic content (TPC) and total flavonoid content (TFC) (Figure 1 and 2). Coincidently, trend of the TPC and TFC yields were similar with the trend obtained in ABTS and DPPH radical scavenging capacity assays. The same trend was observed in a study of Pegaga (*Centella asiatica*) conducted by Tan *et al*. (2011) in which the phenolic compounds yields (TPC and TFC) and antioxidant capacities (ABTS and DPPH radical scavenging capacities) were increased gradually with the increase of the solid-to-solvent ratio and achieved the highest ABTS and DPPH radical scavenging capacities as well as having the highest TPC and TFC at 1:15. Thus, it can be deduced that antioxidant capacity increase with the increase of solid-to-solvent ratio until reaching an optimum level. Overall, this finding suggested that both phenolic and flavonoid compounds are potentially responsible for the antioxidant capacity in *P. niruri*.

Correlation between different methods for analyzing antioxidant compounds and antioxidant capacities

According to Table 1, TPC was positively and significantly ($p<0.05$) correlated with DPPH ($r=0.999$) and ABTS ($r=0.999$) as compared to TFC which was not significantly ($p>0.05$) correlated with DPPH and ABTS. This linear correlation suggested that the phenolic compounds in *P. niruri* largely accounted for its antioxidant capacity. Tan *et al*. (2009) reported that polyphenols in *P. niruri* extract contributed to its antioxidant activity with the correlation of $r=0.890$. However, a high TPC value can be explained by the presence of some non-polyphenolic reducing agents such as amino acids and proteins that can also react with Folin-Ciocalteu reagent (Meda *et al*., 2005). On the other hand, TFC was strongly correlated with DPPH ($r=0.868$) and ABTS ($r=0.865$) without significant difference ($p>0.05$). Although flavonoids are known for its antioxidant capacities, flavonoids exhibit a wide variation in radical scavenging activity. Some flavonoids have very strong radical

Table 1. Correlation coefficients (r) between different methods for analyzing phenolic compounds and antioxidant capacities

	TPC	TFC	ABTS
TFC	0.882		
ABTS	0.999*	0.865	
DPPH	0.999*	0.868	1.000*

*p<0.05

scavenging activity and others may seem to be inactive (Cai *et al*., 2006). The moderate correlation can also be explained by the choice of method used to estimate flavonoid content in the study. The aluminum chloride method which was used in this study is specific only for flavones and flavonols, as compared to flavanones and flavanonols react better with 2,4-dinitrophenylhydrazine method (Chang *et al*., 2002) and DMACA method for flavanol. This fact suggest that by using aluminum chloride method alone, one will underestimate the content of total flavonoid, which probably accounts for a not significant correlation between flavonoid content and antioxidant activity (Meda *et al*., 2005; Prasad *et al*., 2009). In addition, flavones detected by aluminum chloride method was reported to posses no radical scavenging capacity (Cai *et al*., 2006).

These results agree with Tan *et al*. (2011), Conforti *et al*. (2009) and Shahidi and Marian (2003) studies who reported that differences in antioxidant activities of plant extracts could be due to different qualitative and quantitative composition of phenolic constituents, ranging from phenolic acids to flavonoids and its derivatives. Furthermore, the antioxidant activity of a plant does not rely solely on phenolic compounds, but also on other non-polyphenolic substances such as caretenoids, vitamins and minerals (Ratnam *et al*., 2006). Synergistic effect may also take place between different types of antioxidants.

Conclusion

Solid-to-solvent ratio had significant effect ($p<0.05$) on extraction of antioxidant compounds (TPC and TFC) and capacities (ABTS and DPPH), and both results of antioxidant compounds and capacities showed a similar trend. The solid-to-solvent ratio 1:20 (w/v), yielded the highest amounts of TPC and TFC with a value of 5788.7 mg GAE/100 g DW and 1906.5 mg CE/100 g DW, respectively and exhibited high antioxidant capacities (ABTS and DPPH radical scavenging capacities) with a value of 0.820 mM and 1.598 mM, respectively. Total phenolic content (TPC) assay positively and significantly correlated (r=0.999, r=0.999) with both antioxidant capacities assays, namely ABTS and DPPH. Whereas, the flavonoid content determined using total flavonoid content (TFC) assay was not significantly correlated (r=0.865, r=0.868) with both ABTS and DPPH antioxidant capacities assays.

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