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Antioxidant properties: Effects of solid-to-solvent ratio on antioxidant compounds and capacities of Pegaga (*Centella asiatica*)

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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 *C. asiatica*. Solid-to-solvent ratio 1:15 was the optimum condition for extraction of phenolic compounds (TPC and TFC) with a value of 967.2 mg GAE/100 g DW and 908.3 mg CE/100 g DW, respectively and exhibited high antioxidant capacities (ABTS and DPPH radical scavenging capacities) with a value of 0.8133 mM and 2.0945 mM, respectively. TPC was positively and strongly correlated with ABTS and DPPH (r=0.808 and r=0.859, respectively) under the effects of solid-to-solvent ratio as compared to TFC, positively and moderately correlated (r=0.590, r=0.663) with ABTS and DPPH.

Keywords: Pegaga (*Centella asiatica*), phenolic compounds, antioxidants, antioxidant capacities stability

Introduction

Pegaga (*Centella asiatica*) is a long-used medicinal herb found in many Asian countries including Malaysia. This plant is listed as one of the Traditional Chinese Medicine (TCM) in China and Ayurveda medicine in India (Gupta *et al*., 1999; Rafamantanana *et al*., 2009). Hence, Pegaga is being chosen studied under the Malaysia-MIT Biotechnology Partnership Programme apart from Tongkat Ali (Aziz, 2003).

For the past decade, phenolic compounds have gained an intense focus of research due to their strong *in vitro* and *in vivo* antioxidant activities and ability to scavenge free radicals (Chan *et al*., 2009). Experimental studies conducted by Jayashree *et al*. (2003) and Gnanapragasam *et al*. (2004) showed that pegaga possessed antioxidant activity and phenolic compounds found naturally in pegaga were suggested to be the major contributors to the antioxidant activities of the plant (Zainol *et al*., 2003). 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 (Silva *et al*. 2007). According to Pinelo *et al*. (2005), the extraction efficiency is influenced by various factors such as method of extraction, solvent type, solvent concentration, contact time, extraction temperature, solid to solvent ratio and particle size. An unpublished optimized condition for phenolic compound extraction from pegaga had been studied and suggested that the optimum extraction temperature was 56.8°C, extraction time 100 min and solvent concentration 63% ethanol. This optimized condition was study at fixed solvent to solid ratio at 1:10 and it was reported that the phenolic content is affected by the extraction solvent volume being used (Cacace and Mazza, 2003). Therefore, the effects of solid-to-solvent ratio to the extraction efficiency of phenolic compounds from pegaga was further investigated.

Materials and Methods

Chemical and plant materials

A total of 1 kg pegaga (*Centella asiatica*) 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 deionised water 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,

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Malaysia) using vacuum packaging machine (DZQ400/500, Clarity, China). The packaged samples were wrapped with aluminium foil and stored at room temperature in a dark environment until use.

Solvent extraction

The solvent extraction procedure was carried out according to the extraction procedures described by Khoo (2009) with slight modifications. A required amount of pegaga (*Centella asiatica*) dry powder according to experimental design was weighed accurately using analytical balance (AB204-S, Mettler Toledo, Switzerland) and mixed with 60 mL of ethanol in a conical flask which was wrapped with parafilm (Pechiney plastic packaging, USA) and aluminium 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 100 min at 56.8°C. After extraction, the pegaga (*C. asiatica*) 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.

Experimental design

The experimental design for this study was carried out using one-factor-at-a-time approach to obtain the optimal solid-to-solvent ratio for extraction of phenolic compound and antioxidant capacities from pegaga (*Centella asiatica*). The optimum range of solid-to-solvent ratio for phenolic extraction from pegaga (*C. asiatica*) was set at ratio 1:5, 1:10, 1:15 and 1:20 (w/v). The extraction procedures were repeated as described in solvent extraction section. 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/100 g 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 was determined using Folin-Ciocalteu (F-C) colorimetric method described by Khoo (2009) with slight modifications. Crude extract obtained from extraction was diluted 50 times before use. An amount of 1 mL diluted crude extract was added to 1 mL of 1:10 diluted Folin-Ciocalteu reagent (FCR) in an aluminium foil-wrapped 15 mL test tube. After 4 min, 800 µL of sodium carbonate (75%, w/v) was added. The mouth of the test tube was covered with parafilm (Pechiney plastic packaing, USA) and aluminium foil (Diamond, USA) then vortexed (VTX-3000L, Copens Scientific, Germany) for 10 s with subsequent 2 hours incubation at room temperature in a dark environment. The absorbance was measured at 765 nm against the blank reagent using Uvi light spectrophotometer (UviLine 9400, Secomam, France). The blank sample was prepared by replacing 1 mL of sample with 1 mL of deionised water. The measurements were carried out in triplicate. Gallic acid was used for calibration of a standard curve. The results were expressed as gallic acid equivalent (GAE) in mg per 100 g dry weight (DW) of sample.

Determination of total flavonoid content (TFC)

TFC was determined using procedures described by Khoo (2009). The crude extract was diluted 10 times. An amount of 1.25 mL deionised water followed by 75 µL of 5% sodium nitrite ($NaNO_2$) was added to 0.25 mL of diluted crude extract or water (blank) or catechin (positive control) in an aluminium foil-wrapped 15 mL test tube. The mixture was allowed to stand for 6 min before adding 150 µL of 10% (w/v) aluminium chloride ($AlCl_3$). The mixture was allowed to stand for another 5 min before adding 0.5 mL of 1 M sodium hydroxide (NaOH) and 275 µL of deionised water, accordingly. The mouth of the test tube was covered with parafilm (Pechiney plastic packaing, USA) and then mixed using vortex mixer (VTX-3000L, Copens Scientific, Germany) for approximately 10 s. Absorbance of the mixture was determined at 510 nm versus the prepared blank using Uvi light spectrophotometer (UviLine 9400, Secomam, France). Total flavonoid content was expressed as mg catechin equivalent (CE) per 100 g dry weight of sample (mg CE/100 g DW). Samples were measured in triplicate.

ABTS radical scavenging capacity

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacity assay was carried out using procedures described by Khoo (2009). An amount of 10 mL 7 mM ABTS solution and 10 mL of 2.45 mM potassium persulfate ($K_2S_2O_8$) solution were transferred into a 250 mL light protected amber bottle then mixed manually for 5 s and allowed to stand in a dark environment at room temperature for 12-16 hours to give a dark blue solution. The solution was diluted with 95% ethanol until the absorbance was equilibrated to 0.7(±0.02) abs at 734 nm. An amount of 3.9 mL ABTS solution with equilibrated absorbance of 0.700 ± 0.02 abs was added to 0.1 mL

of the undiluted crude extract in a 15 mL aluminium foil-wrapped test tube. Negative control was prepared by replacing 0.1 mL of undiluted crude extract with 0.1 mL of 95% ethanol. Blank was prepared by using 95% ethanol solely. The reaction was allowed to occur at room temperature for 6 min and the absorbance at 734 nm was immediately recorded against blank using Uvi light spectrophotometer (UviLine 9400, Secomam, France). Absorbance measurements of the crude extracts and negative controls were taken in triplicate. Trolox (0.2 – 1.0 mM) was used as standard. The results were expressed as % ABTS radical scavenging capacity and calculated as [1 - (A_s / A_c)] x 100% (A_s and A_c is the absorbance of crude extract and control, respectively, at 734 nm). The results were expressed in mM.

DPPH radical scavenging capacity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay of the extract was determined using methods described by Khoo (2009) with slight modification. An amount of 0.1 mL undiluted crude extract was added to 3.9 mL of ethanolic DPPH in a 15 mL aluminium foil-wrapped test tube. The mouth of the test tube was wrapped with parafilm (Pechiney plastic packaging, USA) and mixed using vortex mixer (VTX-3000L, Copens Scientific, Germany) for exactly 1 min. The mixture was allowed to stand at room temperature in dark environment for 30 min. Negative control was prepared by replacing 0.1 mL of undiluted crude extract with 0.1 mL of 95% ethanol. Blank was prepared by solely using absolute ethanol. Absorbance of blank, negative control and sample were measured at 517 nm using Uvi light spectrophotometer (UviLine 9400, Secomam, France). Absorbance measurements of the crude extracts and negative controls were taken in triplicate. Trolox (0.5 – 2.5 mM) was used as standard. The results were expressed as % DPPH radical scavenging capacity and calculated as [1 - (A_s / A_c)] x 100% (A_s and A_c is the absorbance of crude extract and control, respectively, at 517 nm). The results were expressed in mM.

Statistical analysis

All the experimental results were analysed using Minitab software (Minitab Version 15.1.10.). Every measurement of each assay was done in triplicate and every assay was replicated. All values were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's test was used to determine the significant differences (p<0.05) between means. Pearson correlation coefficient was also determined by using Minitab software (Minitab Version 15.1.10.).

Results and Discussion

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

The effects of solid-to-solvent ratio on total phenolic content (TPC) and total flavonoid content (TFC) are shown in Fig. 1. Solid-to-solvent ratio of 1:15 (w/v) showed high amount of TPC and TFC with a value of 967.2 mg gallic acid equivalent (GAE)/100 g dry weight (DW) and 908.3 mg catechin equivalent (CE)/100 g dry weight (DW), respectively. A further increase in solid-to-solvent ratio to 1:20 did not significantly (p>0.05) increase both values of TPC and TFC.

A high solid-to-solvent ratio was found to be favourable in extraction of phenolic compounds. These results were consistent with mass transfer principles 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 (Cacace and Mazza, 2003; Al-Farsi and Chang, 2007). In addition, the chance of bio-active components coming into contact with extracting solvent expanded with increase amount of extraction solvent, leading to higher leaching-out rates (Zhang *et al*., 2007). However, active component yields will not continue to increase once equilibrium is reached (Herodež *et al*., 2003). Hamdam (2008) stated that solid-to-solvent ratio could significantly affect the equilibrium constant and characterized the relationship between yield and solvent use as a steep exponential increase followed by a steady state to give the maximum yield.

A solid's solubility is affected by changes in the activity coefficient, which varies with the temperature and composition of the solution (Frank *et al*., 1999). Interactions of the compounds with solvent could have modified the activity coefficient and thus the solubility of the compounds to the solvent. 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 highest solid-to-solvent ratio (Cacace and Mazza, 2003). Although amount of phenolic compounds generally increased 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 during optimisation of extraction of phytochemicals from different plant materials. 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).

Effects of solid-to-solvent ratio on antioxidant capacities

Solid-to-solvent ratio of 1:15 obtained a high value for both ABTS and DPPH radical scavenging capacities with a value of 0.8133 mM and 2.0945 mM, respectively (Figure 2). A further increase in solid-to-solvent ratio to 1:20 did not significantly (p>0.05) increase both ABTS and DPPH radical scavenging capacities. Solid-to-solvent ratio 1:15 exhibited high ABTS and DPPH radical scavenging capacities, as well as having high total phenolic content (TPC) and total flavonoid content (TFC) (Figure 1). Limited studies were conducted on the effects of solid-to-solvent ratio on antioxidant capacity. However, as the result (Fig. 2) shows, it can be deduced that antioxidant capacity increases with the increase of solid-to-solvent ratio until reaching an optimum level. Trend of the total phenolic and flavonoid content yields were similar with the trend obtained in ABTS and DPPH radical scavenging capacity assays. This finding suggested that both phenolic and flavonoid compounds are potentially responsible for the antioxidant capacity.

Correlation between different methods for analysing antioxidant compounds and antioxidant capacities

Table 1 shows that total phenolic content (TPC) was significantly and positively correlated with total flavonoid content (TFC) (p<0.05, r=0.952). Since flavonoids are a group of plant phenolics, this finding suggested that the phenolic compounds found in pagaga (*Centella asiatica*) may be mainly flavonoids. The correlation between antioxidant compounds (by TPC and TFC assays) and antioxidant capacities (by ABTS and DPPH assays) were positively correlated but not significant (p>0.05).

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

	TPC	TFC	ABTS
TFC	0.952*		
ABTS	0.808	0.590	
DPPH	0.859	0.663	0.995*

*p<0.05

The relationship between antioxidant compounds and antioxidant capacities are often complicated. According to Khoo (2009), antioxidant capacity does not rely only on the amounts of antioxidants, but also on the structure and interactions among each other. The structure of phenolic compounds is a key determinant of the radical scavenging activity. Antioxidant activity depends on the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group (Balasundram *et al*., 2006). Therefore, high phenolic or flavonoid contents do not necessarily exhibit high antioxidant capacity. These results agree with Kähkönen *et al*. (1999), Shahidi and Marian (2003) and Conforti *et al*. (2009) studies who reported that differences in antioxidant activities of plant extracts could be due to different qualitative and quantitative compositions of phenolic constituents, from phenolic acids to flavonoids and the derivatives. Furthermore, the antioxidant activity of a plant does not rely solely on phenolic compounds, but also on other substances such as carotenoids, vitamins and minerals (Ratnam *et al*., 2006). Synergistic effects may also take place between different types of antioxidants.

According to Table 1, TPC was highly correlated with DPPH (r=0.859) and ABTS (r=0.808) but the correlation was not significant (p>0.05). This linear correlation suggested that the phenolic compounds in pegaga (*C. asiatica*) largely accounted for its antioxidant capacity. Zainol *et al*. (2003) reported that polyphenols in pegaga extract contributed to its antioxidative efficiency activity with the correlation of r^2=0.9. However, a high TPC value can also be explained by the presence of some 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 moderately correlated with DPPH (r=0.663) and ABTS (r=0.590) without significant difference (p>0.05). Although flavonoids are known for its antioxidant capacities, flavonoids exhibit a wide variation in radical scavenging activity. Some have very strong activity and others may seem to be inactive (Cai *et al*., 2006). The moderate correlations can also be explained by the choice of method used to estimate flavonoid content in the study. The aluminium chloride method which was used in this study is specific only for flavones and flavonols, while flavanones and flavanonols react better with 2,4-dinitrophenylhrdrazine method (Chang *et al*., 2002). This fact suggests that by using aluminium chloride method alone, one will underestimate the content of total flavonoids, which probably accounts for a lower correlation between flavonoid content and antioxidant activity (Meda *et al*., 2005; Prasad *et al*. 2009). In addition, flavones detected by aluminium chloride method was reported to posses no radical scavenging capacity (Cai *et al*., 2006).

As described in Figure 1 and Figure 2, solid-to-solvent ratio had significant effects (p<0.05) on the extraction of phenolic compounds and antioxidant

capacities, as well as having a similar trend. By taking economical considerations into account, solid-to-solvent ratio of 1:15 was chosen as the optimum solid-to-solvent ratio.

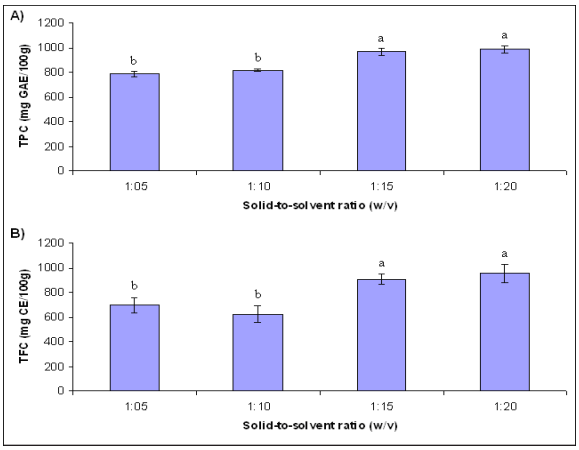

Figure 1. Effects of solid-to-solvent ratio on A) TPC and B) TFC of pegaga (*Centella asiatica*) (n=2)[x].

[x] Replication.
[a-b] Values shown with different letters are significantly different (p<0.05).
Note: Error bars represent standard deviation.

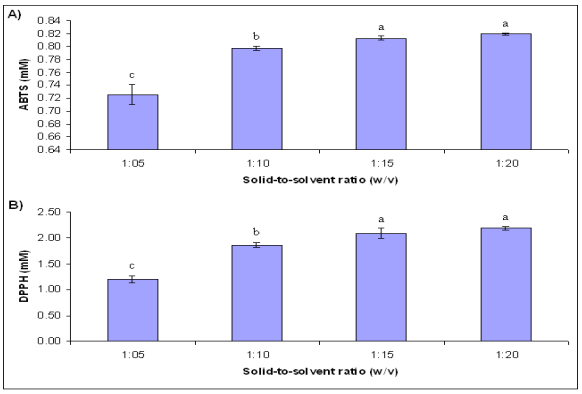

Figure 2. Effects of solid-to-solvent ratio on A) ABTS and B) DPPH radical scavenging capacities of pegaga (Centella asiatica) (n=2)[x].

[x] Replication.
[a-c] Values shown with different letters are significantly different (p<0.05).
Note: Error bars represent standard deviation.

Conclusion

Solid-to-solvent ratio had significant effects (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 similar trend. The optimum solid-to-solvent ratio was 1:15 (w/v), yielded high amounts of TPC and TFC with a value of 967.2 mg gallic acid equivalent (GAE)/100 g dry weight (DW) and 908.3 mg catechin equivalent (CE)/100 g DW, respectively. Solid-to-solvent ratio 1:15 also exhibited high ABTS and DPPH radical scavenging capacities with a value of 0.8133 mM and 2.0945 mM, respectively. Yields of antioxidant compounds and capacities did not significantly (p>0.05) increase with the increase of ratio up to 1:20. Total phenolic content (TPC) assay positively and strongly correlated (r=0.808, r=0.859) with both antioxidant capacities assays, ABTS and DPPH. On the other hand, total flavonoid content (TFC) assay positively and moderately correlated (r=0.590, r=0.663) with ABTS and DPPH.

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