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Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds and antioxidant capacity of *Orthosiphon stamineus* extracts

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Abstract: Optimization of phenolic extraction from *Orthosiphon stamineus* was carried out in present study by investigating the effects of ethanol concentration (0-100%, v/v), extraction time (60-300 min) and extraction temperature (25-65°C) on the phenolic recovery using single factor experiment. Total phenolic content (TPC), total flavonoid content (TFC) and condensed tannins content (CTC) were used for determination of phenolic compounds while 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical-scavenging capacity and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity were used for evaluating the antioxidant capacities of crude extract. Experimental results showed that all extraction parameters had significant (p<0.05) effect on phenolic contents (TPC, TFC and ABTS) and antioxidant capacities (ABTS and DPPH) of crude extract. The optimized conditions for phenolic extraction were 40% ethanol for 120 min at 65°C with values of 2003.4 mg GAE/100 g DW for TPC, 1611.9 mg CE/100 g DW for TFC, 202.4 mg CE/100 g DW for CTC, 765.4 µmol TEAC/100 g DW for ABTS and 2180.9 µmol TEAC/100 g DW for DPPH. DPPH was found to be positively significantly correlated with TPC but negatively significantly correlated with CTC under the influence of ethanol concentration. As a function of extraction temperature, all antioxidant compounds assays (TPC, TFC and CTC) were negatively correlated with antioxidant capacities (ABTS and DPPH).

Keywords: Misai Kucing *(Orthosiphon stamineus)*, total phenolic content (TPC), total flavonoid content (TFC), condensed tannin content (CTC), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical-scavenging capacity, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity

Introduction

Interest has been expressed in medicinal plants as natural sources of antioxidants in recent years, as most of the bioactive compounds from plant extracts have been discovered to exhibit antioxidant activity. *Orthosiphon stamineus* or locally known as misai kucing is one of the well known traditional medicinal plants which has been found to consist of several bioactive compounds, such as essential oils, terpenoids, phenolic compounds and inositol (Tezuka *et al*., 2000; Akowuah, 2004; Chan and Loo, 2006). Among these various bioactive compounds, phenolic compounds are one of the most important bioactive compounds presented in *O. stamineus* (Akowuah, 2004). These bioactive compounds have been found to play an important role in the antioxidant activity of *O. stamineus* crude extract (Khamsah *et al*., 2006). Based on the research done by Akowuah *et al*. (2004), there are 20 phenolic compounds have been isolated from this medicinal plants, including 9 lipophilic flavones, 2 flavonol glycosides and 9 caffeic acid deriavatives.

In order to recover bioactive compounds from plant raw materials, extraction is widely used and it is the first important step. However, there is no single universal extraction method applicable for extraction of phenolic compounds from plant materials because of the complexity of phenolic compounds and its interaction with other bioactive compounds presented in the plant matrices (Naczk and Shahidi, 2006; Contini, 2008). There are several factors can influence the extraction efficiency, including extraction method, solvent type and concentration, particle size of plant materials, extraction time and temperature, solvent to solid ratio and extraction pH (Chirinos *et al*., 2007).

Optimization of extraction process can be generally achieved based on two approaches: one-factor-one time approach and response surface methodology (RSM). One-factor-one time approach or also known as single factor experiment is a classical method, in which only one factor is variable at one time while all others are kept constant (Juntachote *et al*., 2006;

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Silva *et al*., 2007). This approach was used in present study to optimize the phenolic extraction from *O. staminues*, despite it has several drawbacks, such as time consuming, inability to determine interaction between the variables, costly and less effective (Sin *et al*., 2006). However, the results from present study can be used as preliminary test for purification of phenolic compounds in future study. Besides that, this approach can also be used to determine the range of the factor that has a significant effect on the recovery of phenolic compounds and served as key information in RSM for the generation of a central composite rotatable design (CCRD).

The availability of phenolic compounds in *O. staminues* as antioxidant source is ensured. However, there is no optimal protocol has been established for phenolic extraction from *O. staminues*. Hence, the aim of this study was to optimize the phenolic extraction from *O. staminues* by studying the effects of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds (total phenolic content, TPC; total flavonoid content, TFC; and condensed tannin content, CTC) and the free radical-scavenging capacity of crude extracts from *O. staminues* for radicals generated by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Materials and Methods

Plant materials

Powdered form of misai kucing (*O. staminues*), including stems and leaves with a particle size of 40 meshes were purchased from a local herb supplier (Ethno Resources, Sungai Buloh, Selangor, Malaysia). All the samples were vacuum packaged into a nylon-linear low density polyethylene pouch (nylon-LDPE) (Flexoprint, Malaysia) upon arrival at laboratory. The packaged samples were wrapped with dark coloured paper and stored at room temperature in a dark environment until they were used.

Chemical reagents

Folin-Ciocalteu reagent, sodium carbonate (≥99.9% purity) and concentrated hydrochloric acid (37% purity) were purchased from Merck (Darmstadt, Germany). Gallic acid (98% purity), vanillin (99% purity) and trolox (97% purity) were purchased from Acros Organics (New Jersey, USA). Methanol (≥99.8% purity), sodium nitrite, (+)-catechin hydrate (≥98% purity), potassium persulphate (≥98% purity), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS, ≈98% purity) and 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95% purity) were purchased from Sigma–Aldrich (Steinheim, Germany). Absolute ethanol (≥99.4% v/v), denatured ethanol (absolute ethanol 19: methanol 1), aluminium chloride-6-hydrate (>99% purity) and sodium hydroxide were purchased from Fisher Scientific Co. (Leicestershire, UK). All the chemical reagents used were of analytical grade and the deionized water used throughout the experiment was obtained from Milli-Q water purification system (Millipore Corporation, USA).

Solvent extraction procedure

Three grams of *O. stamineus* dry powder was accurately weighed into 100 mL conical flask and mixed with 30 mL of ethanol. The conical flask was then sealed with parafilm (Pechiney plastic packaging, USA) and wrapped with aluminium foil (Diamond, USA) to prevent spilling of mixture and light exposure, respectively. Subsequently, the mixture was shaken at 150 rpm by using a shaker (Green SSerikar, Vision, Korea) or at level 8 by using a temperature controlled water bath shaker (WNB 7-45, Memmert, Germany) for a particular duration at a specific temperature. The ranges for ethanol concentration, extraction time and extraction temperature were set according to the experimental design. After extraction, the extract was subjected to filtration using Whatman No. 1 filter paper (Whatman International, England) into amber bottle for analysis without storage overnight. All the extractions were carried out in replicates.

Experimental design

Single factor experiment was used to optimized the recovery of phenolic compounds from *O. stamineus* by studying the effects of three factors, namely ethanol concentration, extraction time and extraction temperature. After solvent extraction, the crude extract was subjected to antioxidant compounds assays, which are total phenolic content (TPC), total flavonoid content (TFC) and condensed tannins content (CTC) assay and antioxidant capacity assays, including 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assays. The levels for each independent variable were chosen based on the results of five responses.

(a)Ethanol concentration

By fixing extraction time and extraction temperature at 180 min and 25°C, respectively, samples were extracted using binary solvent of ethanol and water. Five different concentration of ethanol (20, 40, 60, 80, 100%, v/v) were used by adjusting the composition of ethanol and water in

extraction solvent. The best ethanol concentration was selected based on the values of five responses.

(b)Extraction time

Samples were extracted using the optimal ethanol concentration as determined in first step for 60, 120, 180, 240 and 300 min by fixing the extraction temperature constant at 25°C. The optimal extraction time was selected based on the values of five responses.

(c)Extraction temperature

By using the optimal ethanol concentration and extraction time as determined in first step and second step, respectively, samples were extracted at different temperatures, which were 25, 35, 45, 55, and 65°C. The best extraction temperature was selected according to the values of five responses.

Total phenolic content (TPC) assay

Total phenolic content (TPC) of crude extract was determined using Folin-Ciocalteu colorimetric method according to Li *et al*. (2008) and Wong *et al*. (2006) with slight modification.1 mL of 50 times diluted (v/v) crude extract was mixed with 1 ml of 1:10 (v/v, in deionized water) diluted Folin-Ciocalteu reagent (FCR). After 4 min, 800 µL of sodium carbonate solution (7.5%, w/v) was added into the mixture. Then, the mixture was vortexed for 5 sec and stored at room temperature in dark environment for 2 hours. Blank was also prepared by replacing 1 mL of diluted crude extract with 1 mL of deionized water. The absorbance of mixture was measured at 765 nm against blank using Uv light spectrometer (Model XTD 5, Secomam, France). The measurements were carried out in triplicate and gallic acid was used for calibration of standard curve. Results were expressed as mg gallic acid equivalent per 100 g of dry weight sample (mg GAE/100 g DW). The calibration equation for gallic acid was y = 0.0396x (R^2=0.9975).

Total flavonoid content (TFC) assay

Total flavonoid content (TFC) of crude extract was estimated using procedures described by Karadeniz *et al.* (2005) and Ozsoy *et al.* (2007). 1.25 mL of deionized water was added into 0.25 mL of undiluted crude extract, followed by addition of 75 µL of 5% (w/v) sodium nitrite solution. The mixture was allowed to stand for 6 min and 150 µL of 10% (w/v) aluminium chloride solution was then added. The mixture was allowed to stand for another 5 min and 0.5 mL of 1 M sodium hydroxide solution and 275 µL of deionized water were then added, accordingly. Subsequently, the mixture was vortexed for 5 sec and its absorbance was determined at 510 nm against blank using Uv light spectrometer (Model XTD 5, Secomam, France). Blank was prepared by replacing 0.25 mL of undiluted crude extract with 0.25 mL of deionized water. The measurements were done in triplicate and catechin was used for calibration of standard curve. The results were expressed as mg catechin equivalent per 100 g of dry weight sample (mg CE/100 g DW). The calibration equation for catechin was y = 0.0033x (R^2 = 0.9991).

Condensed tannins content (CTC) assay

The condensed tannins content assay was performed according to the method described by Makkar and Becker (1993). 0.5 mL undiluted crude extract was firstly mixed with 3 mL of vanillin reagent (4%, w/v, in absolute methanol), followed by addition of 1.5 mL of concentrated HCl (37%). Subsequently, the mixture was stored in dark environment at room temperature for 15 min. Blank was prepared by replacing 0.5 mL of undiluted crude extract with 0.5 of deionized water. The absorbance of mixture was measured at 500 nm against blank using Uv light spectrometer (Model XTD 5, Secomam, France). Each undiluted crude extract was measured in triplicate. Catechin was used for calibration of standard curve and the results were expressed as mg catechin equivalent per 100 g dry weight sample (mg CE/100 g DW). The calibration equation for catechin was 0.002x (R^2 = 0.9922).

ABTS radical scavenging capacity assay

ABTS radical scavenging capacity assay was carried out according to the procedures described by Cai *et al*. (2006), Wetwitayaklung *et al*. (2006), Guimarães *et al*. (2007) and Surveswaran *et al*. (2007). ABTS radical solution was firstly prepared by mixing 10 mL of 7mM ABTS solution with 10 mL of 2.45 mM potassium persulphate solution in a 250 mL amber bottle. Subsequently, the ABTS radical solution was allowed to stand in a dark environment at room temperature for 12-16 hours to give a dark blue solution. The ABTS radical solution was diluted with denatured ethanol until its absorbance was equilibrated to 0.7 ± 0.02 at 734 nm before usage.

3.9 mL of ABTS radical solution was firstly mixed with 0.1 mL of undiluted crude extract or ethanol (as control) and they were allowed to store in dark environment at room temperature for 6 min. Subsequently, the absorbance of crude extract and control was measured against ethanol (as blank) at 734 nm using Uv light spectrophotometer (Model XTD 5, Secomam, France). The absorbance measurements of crude extract and control were done in triplicate.

The percentage of ABTS radical scavenging capacity was calculated using this formula: $[1 – (A_s / A_c)] \times 100\%$ (A_s = absorbance of crude extract at 734 nm; A_c = absorbance of control at 734 nm). Trolox was used for calibration of standard curve and the results were expressed as µmol trolox equivalent antioxidant capacity (TEAC) per 100 g dry weight sample (µmol TEAC/100 g DW). The calibration equation for trolox was y = 120.1142x (R^2 = 0.9984).

DPPH radical scavenging capacity

The DPPH radical scavenging capacity of crude extract was determined based on the method described by Miliauskas *et al*. (2004), Saha *et al*. (2004) and Cai *et al*. (2006) with slight modification. 3.9 mL of ethanolic DPPH (60 µM) was firstly mixed with 0.1 mL of undiluted crude extract or ethanol (as control) and they were stored in dark environment at room temperature for 30 min. Subsequently, the absorbance of crude extract and control was measured against ethanol (as blank) at 517 nm using Uvi light spectrometer (Model XTD 5, Secomam, France). Absorbance measurements of crude extract and control were done in triplicate. The percentage of DPPH radical scavenging capacity was calculated using this equation: $[1 – (A_s / A_c)] \times 100\%$ (A_s = absorbance of crude extract at 517 nm; A_c = absorbance of control at 517 nm). Trolox was used for calibration of standard curve and the results were expressed and µmol trolox equivalent antioxidant capacity per 100 g dry weight sample (µmol TEAC/ 100 g DW). The calibration equation for trolox was y = 37.284x (R^2 = 0.9997).

Statistical analysis

All the experimental results were analyzed using Minitab software (Minitab Version 15.1.10.). All values were expressed as mean ± standard deviation (SD) of replicate solvent extraction and triplicate of assays. One-way analysis of variance (ANOVA) with Tukey's test was used to determine the significant differences (p<0.05) between the means. Besides that, Pearson correlations between assays were also established to access the correlation among the antioxidant compound assays and antioxidant capacity assays.

Results and Discussions

Extraction solvent concentration evaluation

Ethanol and water were used as the extraction solvents in this study due to that they are safer in handling as compared to other organic solvents, such as methanol and acetone and more importantly, they are acceptable for human consumption. As described in Figure 1a-e, ethanol concentration showed significant effect (p<0.05) on both phenolic contents (TPC, TFC and CTC) and antioxidant capacities (ABTS and DPPH) of crude extract. All TPC, TFC, ABTS and DPPH were increased as the ethanol concentration was increased up to 40% ethanol for both TPC and TFC and 80% ethanol for both ABTS and DPPH. Beyond these ethanol concentrations, TPC, TFC and DPPH were significantly (p<0.05) decreased while ABTS was not significantly (p>0.05) decreased with increasing of ethanol concentration. Conversely, with increasing of ethanol concentration, CTC was decreased to minimum at 60% ethanol and it was then increased until reaching a maximum at 100% ethanol.

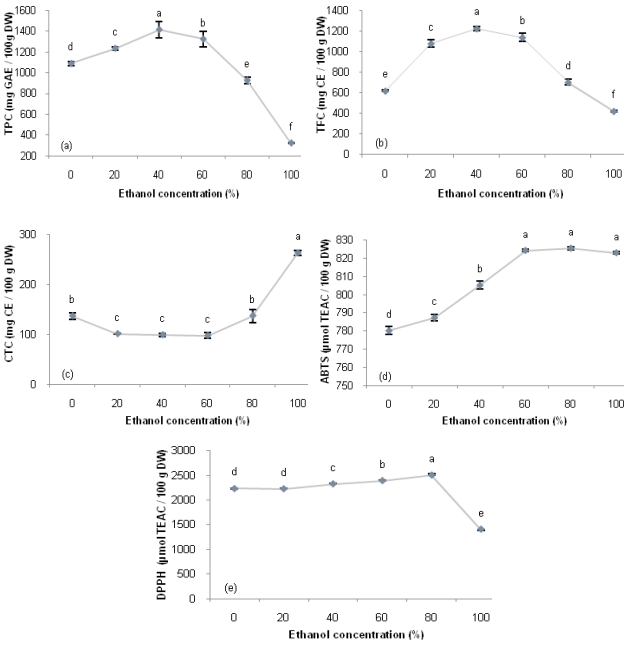

Figure 1. Effect of ethanol concentration on (a) TPC, (b) TFC, (c) CTC, (d) ABTS and (e) DPPH assays from *O. stamineus* (n = 2)*. Values are presented as means ± standard deviation of six measurements. Values marked by different lower case letters (a-f) are significantly different (p<0.05). *Replication of solvent extractions. Note: Error bars represent the standard deviation.

The experimental results of TPC and TFC were in accordance with previous studies, which reported that binary-solvent system was more useful and favorable in the extraction of phenolic compounds from plant samples as compared to mono-solvent system (Nawaz *et al*., 2006; Turkmen *et al*., 2006; Kim *et al*., 2007; Yang and Zhang, 2008). However, the optimized ethanol concentration for CTC was absolutely different with TPC and TFC, in which it was optimized at 100% ethanol (mono-solvent system). This circumstance could be due to most tannins were more soluble in weak polar or non-polar extraction medium (Tian *et al.*, 2009) and therefore CTC was optimized by using a relative non-polar

extraction solvent – pure ethanol.

According to the principle of "like dissolve like", solvents would only extract those compounds which have similar polarity with the solvents (Spigno *et al*., 2007; Zhang, 2007; Yang and Zhang, 2008). In other word, the phenolic compounds extracted from *O. stamineus* would have same polarity with the extraction solvent. Based on the results of TPC, TFC and CTC, no single ethanol concentration was found to give the highest values for all of these three responses. Hence, we suggested that *O. stamineus* consisted of diverse phenolic compounds with different polarities. However, based on the result of TPC, it was optimized at 40%, and thus we suggested that most of the phenolic compounds presented in *O. stamineus* had a moderately polar characteristic.

The antioxidant capacities of crude extracted were also found to be sensitive to ethanol concentration. Increasing of ethanol concentration up to 80% was associated with increasing of antioxidant capacities (ABTS and DPPH) of crude extract. Based on these experimental results, it was believed that highly active phenolic compounds presented in *O. stamineus* were moderately polar. However, further increased the ethanol concentration to 100% caused a significant (p<0.05) decrement in DPPH but no significant (p<0.05) effect on ABTS. It was believed that this observation was due to extraction of different molecular weight of phenolic compounds. Previous study reported that DPPH assay is more favourable to react with low molecular weight phenolic compounds (Paixão *et al*., 2007). Hence, we proposed that 100% ethanol was less efficient to extract low molecular weight phenolic compounds with high antioxidant capacity from *O. stamineus* as compared to 80% ethanol.

By taking into consideration the industrial requirements for extraction, economic parameter was primarily emphasized before detailed study of their antioxidant potential. Therefore, 40% ethanol was chosen for the subsequent experiments in determining optimum extraction time and temperature.

Extraction time evaluation

Extraction time is crucial in solvent extraction of phenolic compounds as appropriate extraction time can result in time and cost saving. The effects of extraction time on the phenolic contents (TPC, TFC and CTC) and antioxidant capacities (ABTS and DPPH) of crude extract are showed in Figure 2a-e. Overall, extraction time had significant (p<0.05) effect on TPC, TFC, ABTS and DPPH but not on TFC.

In term of antioxidant compound assays, TPC

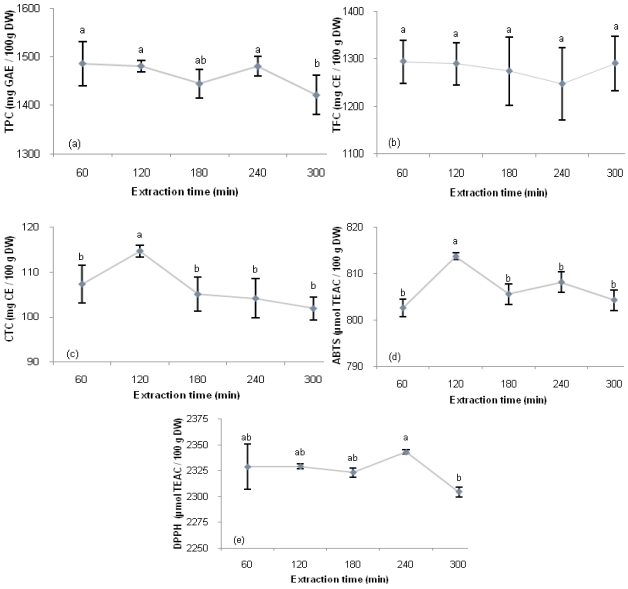

Figure 2. Effect of extraction time on (a) TPC, (b) TFC, (c) CTC, (d) ABTS and (e) DPPH assays from *O. stamineus* (n = 2)*. Values are presented as means ± standard deviation of six measurements. Values marked by different lower case letters (a-b) are significantly different (p<0.05). *Replication of solvent extractions. Note: Error bars represent the standard deviation.

and CTC were significantly (p<0.05) decreased when the extraction time was longer than 240 and 120 min, respectively. Previous study reported that prolonged extraction would lead to a decrease in the phenolic content of crude extract as oxidation of phenolic compounds was possible to be occurred by prolonging the exposure to environment factors such as light and oxygen (Juntachote *et al*., 2006; Chirinos *et al*., 2007; Chan *et al*., 2009). On the other hand, it was also observed that TPC, TFC and CTC were not significantly changed in the first 240 min, throughout the 300 min and after 180 min extraction time, respectively. These circumstances could be well explained by Fick's second law of diffusion, which predicts that after a certain time, there will be a final equilibrium between the solute in the solid matrix (plant sample) and in the bulk solution (extraction solvent) (Silva *et al*. 2007). Hence, excessive extraction time was no longer useful to extract more phenolic compounds from *O. stamineus*.

It was also observed that the antioxidant capacities (ABTS and DPPH) of crude extract were significantly (p<0.05) decreased after ABTS and DPPH reached maximum value at 120 and 240 min, respectively. As discussed before, prolonged extraction would increase the chance for the occurrence of oxidation of extracted phenolic compounds in crude extract and thus the ABTS and DPPH free radical scavenging ability of crude extract were decreased with prolonged extraction.

From the economical point of view and also taking into consideration the yield of phenolic compounds and antioxidant capacities of crude extract, 120

min was chosen as the optimal extraction time, as all responses at this extraction time had the highest value (CTC and ABTS) or had a value which was not significant different (p<0.05) with the value at optimized extraction time (60 min for both TPC and TFC and 240 min for DPPH).

Extraction temperature evaluation

As depicted in Figure 3a-e, extraction temperature demonstrated a significant (p<0.05) effect on both phenolic contents and antioxidant capacities of crude extract. Generally, positive linear relationships were obtained between all antioxidant compounds assays (TPC, TFC and CTC) and extraction temperature. All TPC, TFC and CTC were optimized by using the highest extraction temperature, which was 65°C. In contrast, the antioxidant capacities (ABTS and DPPH) of crude extract exhibited totally different tendency with phenolic contents, in which both ABTS and DPPH were linearly decreased with increasing of extraction temperature, reaching minimum at 65°C.

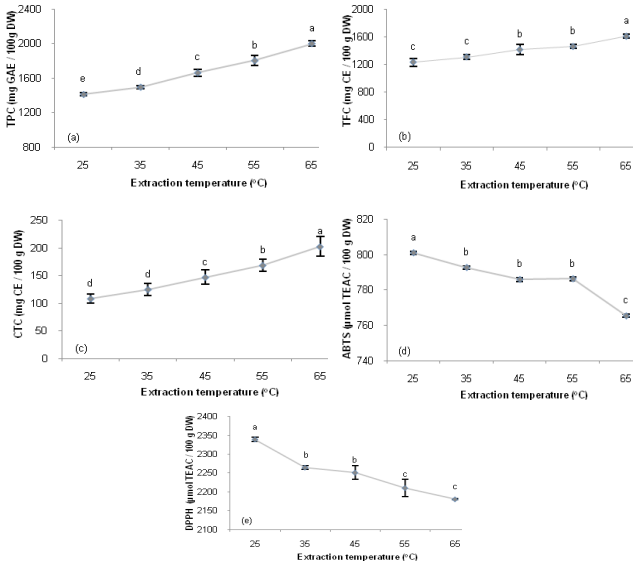

Figure 3. Effect of extraction temperature on (a) TPC, (b) TFC, (c) CTC, (d) ABTS and (e) DPPH assays from *O. stamineus* (n = 2)*. Values are presented as means ± standard deviation of six measurements. Values marked by different lower case letters (a-e) are significantly different (p<0.05). *Replication of solvent extractions. Note: Error bars represent the standard deviation.

The increment of TPC, TFC and DPPH as a function of temperature were in agreement with previous studies which reported that the yield of phenolic compounds would be increased with increasing of extraction temperature (Liyana-Pathirana and Shahidi, 2005; Pinelo *et al*., 2005; Cacace and Mazza, 2006). Heat could enhance the recovery of phenolic compounds from plant materials by increasing the diffusivity of extraction solvent into plant cells and also enhancing the solubility of phenolic compounds in extraction solvent (Cacace and Mazza, 2003; Vongsangnak *et al*., 2004; Kim *et al*., 2007). Besides that, Juntachote *et al*. (2006) also reported that elevated extraction temperature would increase the mass transfer of phenolic compounds and also reduce the solvent viscosity and surface tension and hence to promote the extraction of phenolic compounds. In addition, mild heating was also found to have the ability to soften the plant tissues, weaken the cell wall integrity and thus favored the release of bound phenolic compounds (Junctachote *et al.*, 2006; Spigno *et al*., 2007).

Table 1. Correlation coefficients between assays under the influence of ethanol concentration

Assay	TPC	TFC	CTC	ABTS
TFC	0.893*			
CTC	-0.976*	-0.850*		
ABTS	-0.366	-0.113	0.333	
DPPH	0.825*	0.620	-0.905*	-0.133

*p<0.05

Although the yield of phenolic compounds was increased with increasing of extraction temperature, both ABTS and DPPH free radical scavenging capacities of crude extract were linearly reduced with increasing of extraction temperature. The primary reason for this outcome could be due to detrimental effects of relatively high temperature on the bioavailability of bioactive components. Increase in extraction temperature would favour extraction by enhancing both solubility of solute and diffusion coefficient, but at the same time, thermal destruction of phenolic compounds can also be taken place and caused a reduction in the antioxidant capacities of crude extract (Vongsangnak *et al*., 2004; Spigno *et al.*, 2007). Based on the results obtained in this section, we deduced that the phenolic compounds with high antioxidant capacity presented in *O. stamineus* were heat sensitive. As the extraction temperature increased up to 65°C, these heat sensitive phenolic compounds would be destroyed and caused a reduction in the antioxidant capacities of crude extract.

High yield of phenolic compounds not necessary accompanies with high antioxidant capacity, as the antioxidant activity of crude extract can also be influenced by the structure and interaction between extracted phenolic compounds (Huang *et al*. 2005). Hence, further study should be carried out to identify the predominant phenolic compounds in *O. stamineus* extract which are extracted at different temperature with respect to their antioxidant mechanisms and synergistic effects.

As depicted in Figure 3a-c, the highest values for TPC, TFC and CTC were obtained at extraction temperature of 65°C. Hence, 65°C was selected as the optimal extraction temperature for the recovery of phenolic compounds from *O. stamineus* in this study.

Person correlation analysis

Correlations among the antioxidant assays were also analyzed in this study in order to make better understanding on the interrelationship between the phenolic contents and antioxidant capacities of crude extract under different extraction parameters. Under the influence of ethanol concentration (Table 1), DPPH was found to be positively significantly correlated ($p<0.05$, r = 0.825) with TPC but negatively significantly correlated ($p<0.05$, r = -0.905) with CTC. This correlation suggested that the phenolic compounds presented in *O. stamineus* may responsible for the DPPH radical scavenging ability of crude extract under the influence of ethanol concentration. On the other hand, the negative correlation between CTC and DPPH could be well explained in term of molecular weight. Condensed tannins are high molecular weight polymers (Dlamini *et al*., 2009) and therefore, they were less responsive to DPPH radical scavenging capacity assay, as DPPH free radicals are more effectively to be scavenged by low molecular weight antioxidants (Paixão *et al.*, 2007).

With regards to extraction time (Table 2), no significant ($p>0.05$) correlation was obtained among all antioxidant assays. All antioxidant assays were either weakly or moderately correlated with each other except for TPC and DPPH. It was found that TPC was highly correlated with DPPH (r = 0.860). This strong positive relationship again suggested that phenolic compounds might be the major contributors for DPPH radicals scavenging capacities of crude extract under the effect of extraction time.

Table 2. Correlation coefficients between assays under the influence of extraction time

Assay	TPC	TFC	CTC	ABTS
TFC	-0.180			
CTC	0.617	0.337		
ABTS	0.361	-0.164	0.743	
DPPH	0.860	-0.645	0.334	0.374

However, all antioxidant compounds assays were found to be negatively and strongly correlated ($-0.973 < r < -0.942$) at significant level ($p<0.05$) with antioxidant capacities assays under the influence of extraction temperature (Table 3). This negative relationship also suggested that increased temperature would enhance the recovery of phenolic compounds but at the same time, antioxidant capacities would be reduced or vice versa for reducing extraction temperature. It's believed that this negative correlation was due the phenolic compounds with high antioxidant capacity presented in *O. stamineus* were thermal sensitive and thus degraded as a function of temperature. As a result, these phenolic compounds might lose its abilities to scavenge the free radicals. Similarly, Durling *et al*. (2007) also reported that an increase in extraction temperature would lead to degradation of phenolic compounds and caused the antioxidant capacity of dried sage to be reduced.

Table 3. Correlation coefficients between assays under the influence of extraction temperature

Assay	TPC	TFC	CTC	ABTS
TFC	0.993*			
CTC	0.999**	0.994**		
ABTS	-0.947*	-0.973*	-0.958*	
DPPH	-0.942*	-0.948*	-0.949*	0.898*

* $p<0.05$
** $p<0.001$.

Conclusion

All the extraction parameters (ethanol concentration, extraction time and extraction temperature) showed significant ($p<0.05$) effect on the extraction efficiency of phenolic compounds and antioxidant capacities of *O. stamineus* crude extract. The optimal conditions for phenolic extraction from *O. stamineus* were 40% ethanol with extraction time of 120 min at extraction temperature of 65°C, with values of 2003.4 mg GAE/100 g DW for TPC, 1611.9 mg CE/100 g DW for TFC, 202.4 mg CE/100 g DW for CTC, 765.4 µmol TEAC/100 g DW for ABTS and 2180.9 µmol TEAC/100 g DW for DPPH. Under the influence of ethanol concentration, DPPH showed strong positive correlation with TPC ($p<0.05$, r=0.825) but strong negative correlation with CTC ($p<0.05$, r=-0.905). As a function of extraction temperature, all antioxidant compounds assays (TPC, TFC and CTC) were negatively correlated with antioxidant capacities (ABTS and DPPH).

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