Effect of Differences Methanol Concentration and Extraction Time on the Antioxidant Capacity, Phenolics Content and Bioactive Constituents of Orthosiphon Stamineus Extracts

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Abstract: Bioactive compounds of *Orthosiphon stamineus* have been known to have the beneficial effects on health. Efficient extraction of these beneficial compounds is very important. The study was conducted to determine the efficiency of methanol as an extraction solvent for bioactive compound extraction of *O. stamineus* raw materials. Leaf sample was extracted in 25, 50 and 100% methanol and distilled water for 2, 4 and 8 hours at 40^0C. The free radical scavenging activity (FRSA) method was used to determine antioxidant capacity and Follin-Ciocalteu method was used to determine samples total phenolic compounds. The quantification of bioactive constituents by using the high performance liquid chromatography (HPLC) and the standard markers was used for this analysis such as rosmarinic acid (RA), sinensetin (SEN) and 3-hydroxy-5,6,7,4-tetramethoxyflavone (TMF). The current research shows that the yield of RA concentration was high in 50% methanol extracts at 2, 4 and 8 h of extraction. For the FRSA result showed that methanol at 50% and 100% was the most effective concentration levels inhibition between 45-55% at 8 hours' time extraction. For the total phenolic compound, the 50% methanol concentration in *O. stamineus* leaf had the highest value compared with 0%, 25% and 100% methanol concentration. But, it was observed that time extraction did not influence the TPC values for different concentrations of methanolic extracts. These indicated that time extraction had affected the total capacities of antioxidant and 50% methanolic extracts was the best concentration for indicated the TPC in the *O. Storminess* leaf.

1 Introduction

Orthosiphon stamineus (O.stamineus) contains 20 phenolic compounds, including two flavonol glycosides, nine lipophilic flavones and nine caffeic acid derivatives, such as

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rosmarinic acid and 2,3-dicaffeoyltartaric acid. The core components of *O.stamineus* plant are the pharmacologically active polyphenols (the polymethoxylated flavonoids and caffeic acid derivatives) [1]. These elements have been reported to be actual in reducing oxidative stress by inhibiting the formation of lipid peroxidation products in biological systems [2].

Phenolics and polyphenolics compounds are considered by an aromatic or phenolics ring structure. These compounds are including the flavonoids, phenolics acids and lignans. Phenolics compounds are located in vacuole and have a tendency to be soluble in water or organic solvents [3]. In this study, the antioxidant activity of phenolic compounds was found to be mainly due to their scavenging and redox properties through counteracting and quenching of free radicals. Nevertheless, the extraction yield and antioxidant activity of the extracts highly depend on the solvent polarity, which determines both quantitatively and qualitatively the extracted antioxidant compounds. Usually, the constancy of different extracts from the same material depends on the extraction solvent used for removal of the polyphenol compounds, and it is apparent that extracts from the same plant material may vary widely with respect to their antioxidant concentrations and activities [4].

Hence, the objective of this study was to study the effects of different methanol concentration and different time extraction on the regaining of total phenolic content (TPC), antioxidant capacity by FRSA method of *O.stamineus* leaf extracts. Besides that, the effects of the extraction were also measured of bioactive compounds by HPLC instrument.

2 Materials and methods

2.1 Chemicals and reagents

Tetrahydrofuran (THF) [HPLC grade] and methanol (HPLC grade) were obtained from AR Alatan (K) Sdn Bhd. Sinensetin (SEN), eupatorin (EUP), Rosmarinic acid (RA) and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF) were purchased from YKN Tech Ent. The other chemical such as Folin-ciocalteu reagent, caffeic acid, methanol (100% v/v), $NaNO_3$ and 2,2-diphenly-1- picrylhydrazyl (DPPH) were purchased from Sigma Aldrich.

2.2 Sample collection and preparation

In the early morning, the *O.stamineus* plant which grown at UniMAP Agrotechnology Research Station, Sg. Chucuh, Perlis was randomly harvested. Meanwhile, the parts of *O. stamineus* plant were dried at 40^0C for 5 days. The dried leaf of *O.stamineus* plant was balanced and stranded to powder form and used for the extraction process.

2.3 Extraction process

2.3.1 Differences of methanol concentration

Ten gram of *O.stamineus* dry powder was weighed and mixed with different methanol concentration. Actually, they are five methanol concentrations. For instance 0, 25, 50 and 100% methanol concentration. The methanol concentration was prepared by adjusting the composition of methanol and water. The extraction process was used in the conical flask, whereas the flask was closed with cotton wool and wrapped with aluminum foil to prevent spattering of mixture. Furthermore, the mixture was shaken at 150 rpm by incubator shaker at 40^0C.

2.3.2 Differences of time extraction

The samples with different methanol concentrations were extracted by using different time extraction for 2, 4 and 8 hours. Then, the temperature was fixing constantly at 40^0C. Furthermore, the extract was filtered into the storage bottle for further analysis. The extraction was conveyed out in replicates.

2.4 Free radical scavenging activity (FRSA)

The free radical scavenging activity (FRSA) method was used to determine the total antioxidant activity of O.stamineus leaf. The 1,1-diphenyl-2- picrylhydrazyl (DPPH) methanolic solution was prepared and two ml of the solution was mixed with 200 µl of sample. Meanwhile, the mixture was added with methanol solvent to make a final volume of 3 ml. Then, the fusions allowed to stance for 60 min, and the absorbance value was observed by using spectrophotometer at 517nm. The FRSA (%) of the samples were compared with a control. The formula for FRSA (%) as bellows:

$$\text{FRSA} = \left(\frac{(A_C - A_S)}{A_C} \times 100 \right)$$

Ac as an absorbance value for control; As as absorbance value for sample.

2.5 Total phenolic content (TPC) sssay

The total phenolic content (TPC) was determined by using Follin-Ciocalteu Reagent (FCR). The calibration curve was prepared with Caffeic Acid (CA) as a standard [5]. Nevertheless, the 0.2 ml of sample extract was mixed with 0.2 ml FCR. Then, the 1.58 ml water was added and mixed as a solution. Then, 1 ml of 20% $NaCo_3$ was added and allowed to stand at room temperature for two hours. Next, the mixture was restrained for the absorbance value at 760nm by spectrophotometer. The CA was used as a standard and the results were expressed as mg caffeic acid equivalent per 1 g of dry weight sample (CA mg/g DW).

2.6 Analysis of compounds by HPLC

The identification and quantification of bioactive compounds of *O.stamineus* leaf were determined by using HPLC instrument. Before that, the sample extract was filtered through a 0.45µm nylon membrane filter into a HPLC vial prior. The HPLC used was demoralized for analysis should equip with autosampler, column oven and UV/VIS detector. The HPLC column used was Merck Licrochart Purospher Start RP 18 column (250mm, 4.6 mm i.d, 5µm pore size). Then, the mobile phase used was prepared, whereas a mixture of water: methanol: tetrahydrofuran (50: 45: 5 v/v) (G. A. Akowuah et al.). The sample was evaluated at the mobile phase flow rate of 1 ml/min and detector wavelength of 340nm at 30^oC for 40 min. However, the calibration curve was prepared by exploiting standard marker (Rosmarinic acid, TMF and Sinensetin) compounds purchased from Chromadex for SEN and Sigma Aldrich for RA.

3 Results and discussions

3.1 Extraction by differences concentration solvent.

Extraction was done according to the method by [7]. Whereas, the combination of water and methanol were used for the extraction on this experiment. The characteristic of similar polarity solvents as the principle " like dissolve like" was suggested only extract those compounds which have same polarity [2]. We advocated that the methanol extract was found to be the good solvent for extraction of polyphenolic compounds from *O.stamineus* leaf and stem. Based on the situation of TPC and FRSA, methanolic extracts expressing good biological capacity. Besides that, in the extraction process, the polarity of solvents is very important to increase the solubility of antioxidant compounds [8]. Thus, it was significant to measure process to validate a good solvent in antioxidant compound extraction. This validation can be optimized to get the maximum antioxidant activity for a certain sample.

3.2 Free radical scavenging activity (FRSA)

Results of the FRSA of *O.stamineus* leaf extracts are presented in Figure 1. The results showed that 50 and 100% methanol concentration were the highest percentage of inhibition at different time extraction. However, the 0 and 25% methanol concentration were significantly declined the FRSA at 2 and 4 hours extraction.

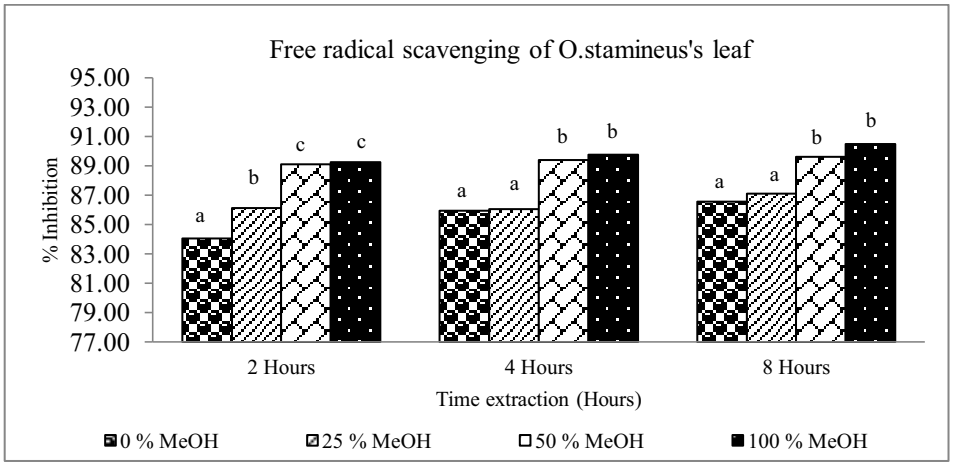

Fig. 1. Effect of different methanol extract at different time extraction on FRSA for *O.stamineus* leaf. Values with different letters are significantly different at $p < 0.05$.

Surprisingly, the FRSA for 100% methanol extract was slightly affected and not significantly different at 4 and 8 hours extraction. Based on previous studies, they were also indicated that sustained time extraction would enhance the amount of oxidation in extracting phenolic compound extract [6].

3.3 Total phenolic content (TPC)

The effects of different concentration of methanol extracts and different extraction time on TPC for *O.stamineus* leaf was presented in Figure 2. Results showed that the highest TPC was 50% methanol concentration extract and follows by 100% methanol concentration. However, the lowest value of TPC was 0% methanol concentration extract.

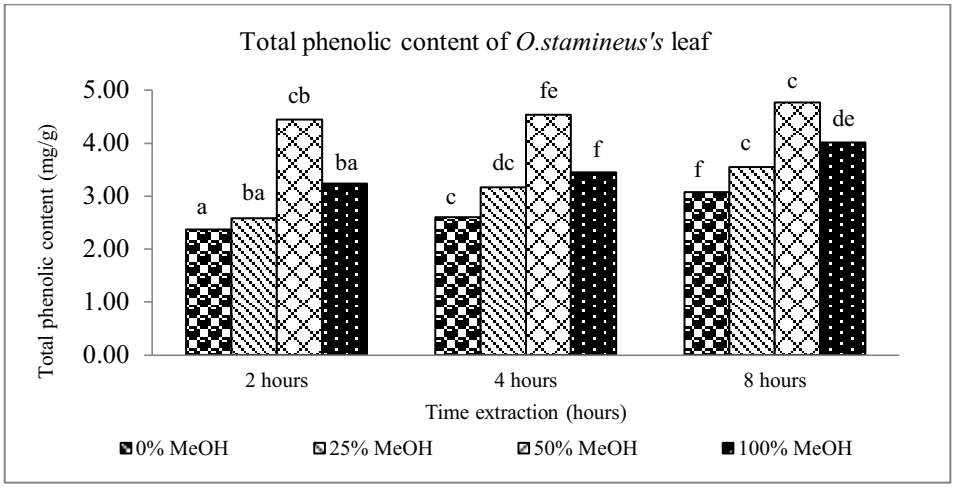

Fig. 2. Effects of different methanol extract at different time extraction on TPC for *O.stamineus* leaf. Values with different letters are significantly different at $p < 0.05$.

A similar situation was also observed in the previous study, it was reported that the long time extended for extraction would prime decreasing of phenolic compounds cause of the occurred extending and exposing to environment factors [9]. In our study, we suggested 50% methanol extract is the most suitable concentration extracts for the purpose of speed analysis.

3.4 HPLC Analysis

The results of the HPLC analysis are represented by its bioactive compound concentration (µg/g.dw). A result of the bioactive concentrations for O. stamineus leaf was presented in Table 1. The present study was indicated that the amount of RA, TMF and SEN concentration as the source of active constituents in *O.stamineus* leaf. Similarly to [2], they also indicated that the yield of RA was significantly affected in 50% methanol extract at 8 hours extraction. Despite the order of RA amounts were increased as 50% methanol extract> 100% methanol extract> 25% methanol extract> 0% methanol extract.

In contrast to TMF concentration, the TMF concentration was drastically increased after 8 hours extraction. It was observed that the 100% of methanol extract was significantly obtained high amount of TMF at 8 hours extraction. The sequence of TMF amounts were increased as 100% methanol extract>50% methanol extract>25% methanol extract>0% methanol extract. Similar situation was also observed for SEN concentration, whereas the amount of SEN compounds was significantly improved after 8 hours extraction in 100% methanol extract. Although, the SEN concentration increased in sequence as 100% methanol extract>50% methanol extract>25% methanol extract>0% methanol extract. Contrary to previous report, they were obtained 100% methanol extract did not influenced the TMF and SEN concentrations at different time extraction [7].

Table 1. The effects of different methanol extract at different time extraction on HPLC analysis of *O.stamineus* leaf.

Methanol	Time extraction	Concentration (µg/g.dw)		
Concentration (%)	(hrs)	RA	TMF	SEN
	2	4.799[a]	0.302[abc]	1.359[a]
0	4	5.755[a]	0.257[bc]	9.848[b]
	8	7.223[a]	0.350[abc]	1.681[c]
	2	9.261[a]	0.129[c]	0.701[d]
25	4	49.844[b]	0.586[a]	1.377[a]
	8	26.500[c]	0.451[ab]	1.674[c]
	2	208.764[d]	0.415[abc]	4.280[e]
50	4	240.155[e]	4.151[d]	5.195[f]
	8	235.153[ef]	2.925[e]	6.888[g]
	2	197.205[d]	4.653[f]	7.135[g]
100	4	225.379[f]	4.891[f]	7.946[h]
	8	235.773[ef]	5.307[g]	8.272[i]

Different letters for each treatment was stated as significant different values of means ($P < 0.05$).

4 Conclusions

As we can conclude that the *O. stamineus* leaf had the highest FRSA at 100% methanol extract and slightly changed at difference time extraction. Nevertheless, the 50% methanol extract was significantly influenced the TPC, but prolonged extraction did not significantly affected the TPC value. Therefore, the RA compounds was increased effectively in 50% methanol extract for four hours extraction. Differently with TMF and SEN compounds, it was significantly affected in 100% methanol extract at 8 hours time extraction.

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