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FRACTIONATION OF ROSMARINIC ACID FROM CRUDE EXTRACT OF *ORTHOSIPHON STAMINEUS* BY SOLID PHASE EXTRACTION

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Abstract

Rosmarinic acid (RA) which is the major compound of *Orthosiphon stamineus* possesses various pharmaceutical properties. It is about 5-10% w/w of rosmarinic acid in the ethanolic extract of the plant. In the present study, solid phase extraction (SPE) was used to fractionate the phytochemical from the crude extract of *O. stamineus*. The effect of different elution solvents was investigated in this paper. A C18 column with the dimension of 10mm diameter 15mm height was used for the fractionation. The solvent system of water – acetonitrile mixture showed a better separation of rosmarinic acid from *O. stamineus* crude extract. The results showed that the content of rosmarinic acid was increased from 10 to 27% w/w after fractionation by SPE. The finding of this study could be used to further up-scaling of rosmarinic acid fractionation in the future study.

Keywords: *Orthosiphon stamineus*, Rosmarinic acid, Fractionation, Solid phase extraction.

1. Introduction

Rosmarinic acid is the main constituent present in *Orthosiphon stamineus*. It is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid as presented in Fig. 1. Akowuah et al [1] found that *O. stamineus* extracts contained approximately 5.3% w/w of rosmarinic acid. Interestingly, *O. stamineus* contains the highest amount of rosmarinic acid compared to other medicinal plant from the family of Lamiaceae such as *Rosmarinus officinalis* (1.03% w/w), *Salvia officinalis* (1.04% w/w),

Nomenclatures

Fr	Fraction
ΔH_v	Enthalpy of vaporization (kJ/mol)
$\%R$	Recovery percentage (%)
R	Ideal gas constant
T	Absolute temperature (K)
V_m	Molar volume (m^3/mol)
x_i	Composition of solvents in the mixture

Greek Symbols

δ	Hildebrand solubility parameter ($MPa^{1/2}$)
δ_i	Hildebrand solubility parameter of solvent
$\delta_{mixture}$	Hildebrand solubility parameter of mixture

Abbreviations

ACN	Acetonitrile
FA	Formic acid
HPLC	High performance liquid chromatography
MeOH	Methanol
MS/MS	Tandem mass spectrometry
RA	Rosmarinic acid
SPE	Solid phase extraction
UPLC	Ultra-performance liquid chromatography

Thymus vulgaris (0.66% w/w) and *Melissa officinalis* (1.33% w/w) [2-4]. Rosmarinic acid has high medicinal value due to its biological and pharmacological properties such as antioxidant activity, anti-inflammatory activity, antibacterial activity, antiviral property and antimutagen activity [5-7]. Previous studies also reported that rosmarinic acid exhibited anti-HIV activities [8-10]. Previous studies found that plant extract supplements with high content of rosmarinic acid were consumed to manage osteoarthritis and rheumatoid arthritis [11]. Because of the beneficial effects of rosmarinic acid, the present study was carried out to fractionate the phenolic compound from the ethanolic crude extract of *O. stamineus*. To the best of our knowledge, the separation and fractionation of rosmarinic acid from *O. stamineus* using solid phase extraction (SPE) method have not been reported till to date.

SPE is a solid-liquid extraction method which is widely used for sample clean-up and purification of target compounds from plant extract. This method is considered as a cost effective technique since the usage of solvent is significantly reduced compared to liquid-liquid extraction method. Furthermore, the solvent system used for elution can be optimized according to the polarity of target compound and packing material of packed bed. Several studies by previous investigators reported the high efficiency of SPE (up to 100% w/w of recovery yield) in fractionation and purification of phenolic compounds from plant samples [12-14]. Therefore, the present study investigated the high recovery of rosmarinic acid fractionation from ethanolic crude extract of *O. stamineus* using C18 SPE column by varying the polarity of solvent system. The effect of elution solvents on the quality (purity) and quantity (yield) of rosmarinic acid was also investigated in this paper.

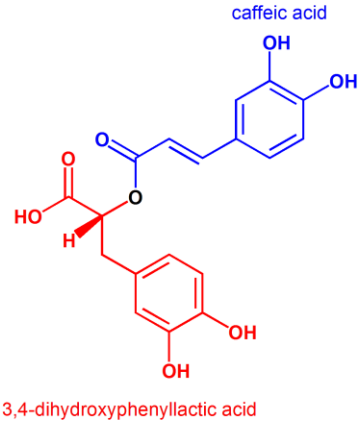

Fig. 1. Chemical Structure of Rosmarinic Acid.

2. Materials and Methods

2.1. Chemicals

Ethanol (95%) was purchased from Fisher Scientific Co. (Fair Lawn, NJ). HPLC grade of methanol (MeOH), acetonitrile (ACN) and formic acid (FA) were purchased from Merck (Darmstadt, Germany). 18.2 MΩ-cm water was produced from Barnstead NANOpure Diamond water purification system (Thermo, Waltham, MA). The standard chemical of rosmarinic acid (RA) with 96% purity was purchased from Sigma-Aldrich (St. Louis, MO, USA). The plant materials (*O. stamineus*) were purchased from local supplier (Fidea Resources, Selangor, Malaysia).

2.2. Sample preparation

The plant materials (273.5 g) were extracted with 70% ethanol (2500 ml) at 60°C for 3 hours. The crude extracts were then filtered and concentrated by drying under vacuum at 60°C using a rotary evaporator (Heidolph, Laborota 4000, Germany). The crude extracts were then kept in the oven at 50°C until completely dried. The crude extracts were stored at -4°C until further analysis.

2.3. Solid phase extraction (SPE)

SPE was performed by using Chromabond C18 ec SPE cartridges (6 ml/1000mg, Macherey-Nagel, Düren, Germany). The SPE cartridge was fixed to the port of SPE vacuum manifold. The SPE cartridge was first conditioned with methanol (12 ml), and then equilibrated by water with 0.5% formic acid (6 ml). The crude extract (0.15 g) was dissolved with 6 ml of 60% MeOH and 1 ml of crude extract sample was loaded onto the SPE column.

The SPE was performed in gradient elution. Fraction 1 was collected by eluting 6 ml of deionized water. The next fractions were collected by eluting 20% acetonitrile, followed by 40%, 60%, 80% and 100% acetonitrile. The elution

volume was 6 ml (1 column volume) for each fraction. A total of 6 fractions were collected for each run. The fractionation was repeated by using water – methanol mixture and methanol – acetronitrile mixture as eluting solvents. Table 1 shows the solvent ratio of SPE for each run. The recovery percentage (%*R*) of rosmarinic acid in each fraction was determined gravimetrically (Eq. 1) and the concentration of rosmarinic acid in each fraction was determined by UPLC -MS/MS analysis. The fractions with the highest content of rosmarinic acid were dried using IR concentrator coupled with cold trap (Micro-Cenvac NB 503CIR, N-BIOTEK Co., Ltd., Korea) at 40°C, 1700 rpm. The yield of rosmarinic acid (RA) was expressed by Eq. (2).

$$\%R = \frac{Mass\ of\ RA\ in\ fraction}{Mass\ of\ RA\ in\ crude\ extact} \times 100 \quad (1)$$

$$Yield\ (w/w\%) = \frac{Mass\ of\ RA\ in\ fraction}{Mass\ of\ dry\ fraction} \times 100 \quad (2)$$

where, Mass = concentration of rosmarinic acid in the sample × volume of sample (elution volume)

Table 1. Elution Solvent Ratio (%) for Run 1 to 4.

Fr.	Solvent ratio (%)							
	Run 1		Run 2		Run 3		Run 4	
	Water	ACN	Water	MeOH	ACN	MeOH	MeOH	ACN
1	100	0	100	0	100	0	100	0
2	80	20	80	20	80	20	80	20
3	60	40	60	40	60	40	60	40
4	40	60	40	60	40	60	40	60
5	20	80	20	80	20	80	20	80
6	0	100	0	100	0	100	0	100

2.4. Quantification of rosmarinic acid by UPLC-MS/MS

The quantification of rosmarinic acid in the fractions and extract of *O. stamineus* were performed by an ultra-performance liquid chromatography (UPLC, Waters Acquity; Milford, MA) system coupled with a triple quadrupole-linear ion trap tandem mass spectrometer (Applied Biosystems 4000 QTRAP; Life Technologies Corporation, Carlsbad, CA), and a C18 reversed phase Acquity column (2.1×150 mm, 1.7 μm). The negative mode of multiple reactions monitoring method with 3 transition ions, namely m/z 359-179, m/z 359-161 and m/z 359-197 was used for the quantitation.

The mixture of 0.1% formic acid in water (A) and acetonitrile (B) was used as mobile phase. The separation was performed in gradient elution with the following proportions (v/v) of solvent A: 0-10 min, 90%; 10–12 min, 10%; 12–14 min, 10–90%; 14–15 min, 90%, at the flow rate of 0.2 mL/min and the injection volume of 5 μl. The standard solution was prepared by dissolving 5 mg of rosmarinic acid in 5 mL of methanol-water (6:4). The stock standard solution was diluted with different dilution factors to prepare a serial concentration of standard

solutions. Syringe filters from Membrane Solutions (Dallas, TX, USA) with 0.22µm pore size were used to filter the samples before injection.

2.5. Chromatographic fingerprinting of the plant fractions and extract

The fingerprinting of phytochemicals in the fractions and crude extract of *O. stamineus* were performed by an analytical scale liquid chromatography (Ultimate 3000) system coupled with a diode array detector (Dionex, Thermo Scientific; MA, USA), and a C18 reversed phase XSelect High Strength Silica (HSS) column (2.1×100 mm, 2.5µm, Waters; Milford, MA). The mixture of 0.1% formic acid in water (A) and acetonitrile (B) was used as mobile phase. The separation was performed in gradient elution with the following proportions (v/v) of solvent B: 0-10 min, 10%; 10–25 min, 10–80%; 25–30 min, 80%; 30–35 min, 10%, at the flow rate of 150µL/min. Syringe filters from Membrane Solutions (Dallas, TX, USA) with 0.22µm pore size were used to filter the samples before injection.

3. Results and Discussion

3.1. Separation of rosmarinic acid by solid phase extraction (SPE)

Solvent plays an important role in the fractionation or isolation of phenolic compounds from the crude extract. The solubility of the phenolic compounds into the eluting solvent is highly influenced by the polarity and elutropic values of the solvent against the stationary phase in the packed column. The degree of interaction (solubility) between materials can be numerically estimated by Hildebrand solubility parameter, Eq. (3). The materials with similar Hildebrand solubility parameter tend to be miscible [15].

$$\delta = \sqrt{\frac{\Delta H_v - RT}{V_m}} \tag{3}$$

In this study, a reversed phase C18 SPE column was used for the separation of rosmarinic acid from the crude extract of *O. stamineus*. Rosmarinic acid (δ=21.2 $MPa^{1/2}$) is relatively polar, therefore it tend to be eluted from the non-polar C18 column when polar solvent is used as mobile phase. The challenge of fractionation is to separate rosmarinic acid from the other plant metabolites, including compounds with almost similar polarity to rosmarinic acid. Therefore, four different solvent systems were used to fractionate rosmarinic acid from the highly complex crude extract of the plant.

The bi-solvent systems used in this study included water–acetonitrile, water–methanol, acetonitrile-methanol and methanol–acetonitrile. The solubility parameters of the solvent mixtures as presented in Table 2 are calculated by Eq. 4.

$$\delta_{mixture} = \frac{\sum x_i \delta_i}{\sum x_i} \tag{4}$$

The table shows that Run 4 gave the highest total recovery of rosmarinic acid (approximately 85%), followed by Run 1 (60%) and Run 2 (38.5%). Run 3 gave the lowest recovery of rosmarinic acid (approximately 4%). According to Hildebrand solubility parameter approach, the compounds with the difference in solubility value not more than 3.4 units are likely to be miscible [16]. Since the difference in solubility parameter between methanol (δ= 29.7) and rosmarinic

acid is lesser than in water (δ= 48). Thus, rosmarinic acid is more dissolvable in methanol than water.

In Run 3, the addition of acetronitrile as the initial solvent into the SPE column after sample loading caused solidification of less polar compounds on the top of column bed. This is because the crude extract of the plant was prepared from ethanolic aqueous solvent. The precipitate had affected the elution of rosmarinic acid, and thus the first fraction in Run 3 contained no rosmarinic acid. A slight increase of methanol (20%) in the solvent system had increased the elution strength and rosmarinic acid was detected in the fraction 2 (Run 3) as presented in Table 2. Significantly, Run 3 produced lower content of rosmarinic acid as a result of the use of less polar solvent, namely acetonitrile as the starting eluting solvent for ethanolic aqueous extract of *O. staminues*.

Table 2. The Solubility of Solvent Mixture ($\delta_{mixture}$) and Recovery Percentage (R) of Rosmarinic Acid in the SPE Fractions for Run 1 to 4.

Fr.	Run 1		Run 2		Run 3		Run 4	
	$\delta_{mixture}$ ($MPa^{1/2}$)	$\%R$ (%)	$\delta_{mixture}$ ($MPa^{1/2}$)	$\%R$ (%)	$\delta_{mixture}$ ($MPa^{1/2}$)	$\%R$ (%)	$\delta_{mixture}$ ($MPa^{1/2}$)	$\%R$ (%)
1	48.00	21.59	48.00	37.35	23.90	0	29.70	80.21
2	43.18	13.94	44.34	0.6	25.06	3.61	28.54	4.14
3	38.36	24.64	40.68	0.55	26.22	0.1	27.38	0.17
4	33.54	0.17	37.02	0.005	27.38	0.03	26.22	0.01
5	28.72	0	33.36	0	28.54	0	25.06	0
6	23.90	0	29.70	0	29.70	0	23.90	0

Figures 2 and 3 illustrate the chromatograms of fractions obtained from Run 1 and Run 4, respectively. The figures show that water–acetonitrile solvent system (Fig. 2) separated the phenolic compounds in *O. stamineus* better than methanol–acetonitrile system (Fig. 3). In Run 1, the increase of acetronitrile percent in water would reduce the polarity of solvent. Hence, fraction 1 to 3 contained more polar phenolic compounds including rosmarinic acid, and fraction 4-6 contained less polar phenolic compounds where no rosmarinic acid was detected.

The chromatogram of Run 4 shows a poor separation of phenolic compounds as almost all the phenolic compounds were eluted out in fraction 1. Hence, the rosmarinic acid contained in fraction 1 from Run 4 was low in purity. According to Brown [17], the separation selectivity for early-eluting compounds is better with water–acetonitrile mixture; water–methanol mixture elutes late-eluting compounds better. Therefore, the water–acetonitrile mixture is likely to be more efficient than water–methanol mixture as the mobile phase for the separation of rosmarinic acid at same solvent ratio with higher purity. Besides that, a combination of tri-solvent system such as water–methanol–acetonitrile can be used as an elution solvent in future study in order to improve the efficiency of rosmarinic acid separation.

This study clearly showed that Run 1 produced better rosmarinic acid separation, in term of the yield and purity than the other three runs. The yield of rosmarinic acid was increased from 10% in the crude extract to 27% in the fraction 3 of Run 1. However, the performance of Run 2 and Run 4 was almost

similar which was only increased to 14% of rosmarinic acid in the fraction 1 for both Run 2 and Run 4.

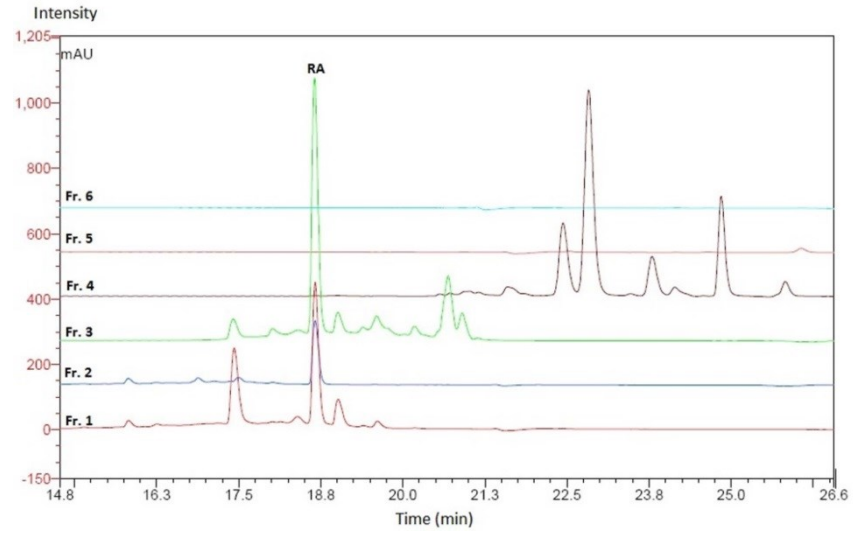

Fig. 2. Chromatogram of *O. stamineus* Fractions (Run 1).

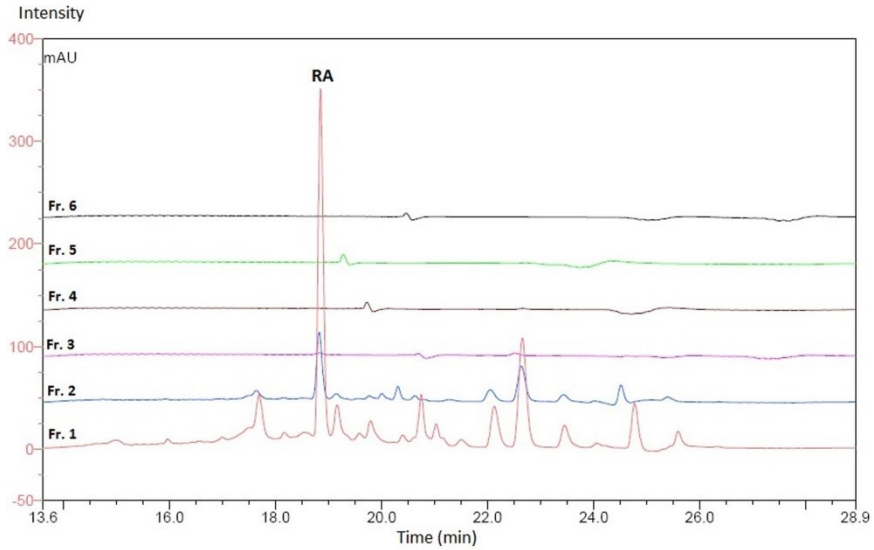

Fig. 3. Chromatogram of *O. stamineus* Fractions (Run 4).

4. Conclusion

In this study, a C18 SPE column was applied to fractionate rosmarinic acid from the crude extract of *O. stamineus* by using different ratios of bi-solvent system as eluting solvent. The fractionation of rosmarinic acid using methanol–acetonitrile solvent system showed the highest content of rosmarinic acid, but lower in purity.

On the other hand, the fractionation of rosmarinic acid using water–acetonitrile solvent system showed a good separation in term of recovery, yield and purity. The content of rosmarinic acid was increased from 10 to 27% w/w after fractionation by SPE. The finding of this study could be used to further up-scaling of rosmarinic acid fractionation in the future study.

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