Supercritical Fluid Polarity-Based Fractionation of Plant Products

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ABSTRACT

Fractionation of plant compounds to produce active purified fractions is a multi-step process, time-consuming and sometimes very expensive. This paper presents a single-step fractionation of tannins from a local plant, *Phyllanthus niruri* Linn. using supercritical fluid extraction (SFE) method. The effects of operating conditions (pressure, temperature, flow rate, cosolvent content and ethanol-water content in cosolvent) were investigated and these parameters were optimized by a response surface method using a face-centred central composite design for maximum yields. SFE produced fractionation between the less polar compounds (pre-extraction/pretreatment) and the more polar tannins (main extraction). At optimum conditions, higher contents could be obtained compared to a commercial *P. niruri* product, HEPAR-P^{TM}, in terms of gallic acid, corilagin, ellagic acid, and total flavonoids. Beside reducing the total liquid solvent consumption and fractionation time, the SFE method developed could enhance both the pre-extraction and main extraction yields, and produce fractionation of different polarity compound groups from the plant matrices.

INTRODUCTION

High-pressure extraction method such as the supercritical fluid extraction (SFE) has received wide interests for the extraction of natural products due to faster extraction time, better efficiency, minimal organic solvent consumption and lower operating temperature compared conventional extraction methods. However, extraction of solutes from plants is more complex since plants usually contain various components with different physicochemical properties and are trapped within complex solid matrices. Therefore the influence of extraction parameters is important to overcome the mass transfer limitations.

Fractionation by SFE is generally based on the molecular weights and the volatilities of the compounds within similar polarity group such as the fractionation of essential oils using adsorbents [1] and multiseparators [2]. Polar organic cosolvent or modifier such as methanol and ethanol, is usually added in small quantities to increase the extraction yield and selectivity of the more polar components [3]. Despite its high polarity, water as cosolvent was reported to be incapable of extracting very polar and hydrophilic (water-soluble) compounds, due to its low solubility in CO_2. The influence of water on the extraction behavior of plant materials by SFE is still not fully understood and conflicting results were obtained from previous studies [4-6]. So far, a single-step polarity-based fractionation has not been reported using SFE for different compound groups in plants.

Phyllanthus niruri Linn. or locally known as Dukung Anak in Malaysia, is a suitable plant for this study since it contains a wide range compound polarities. It is a medicinal plant consisting of alkaloids and lignans (non-polar), flavonoids (medium polar), and ellagitannins (polar/hydrolysable). Research has shown that these compounds might be responsible for anti-Hepatitis B, anti-HIV, liver protection, lipid lowering, antibacterial, and antioxidant properties [7-11]. A commercial HEPAR-P^{TM} extract has been chemically standardized for effective liver protection [12]. To date, however, the plant has yet to be extensively studied in terms of the efficient extraction of its active components, especially using SFE.

In this study, component fractionation of *P. niruri* was investigated using SFE by the addition of ethanol-water cosolvent mixtures in CO_2 at different operating conditions. Optimization by statistical experimental design was carried out for maximum extraction and component yields.

MATERIALS AND METHODS

Plant Material

Dried and ground *P. niruri* samples were obtained from Nova Laboratories Sdn. Bhd. (Malaysia). The sample contained stems and aerial parts of the plant and has been used for the commercial production of HEPAR-P^{TM}. The particle size distribution (% wt/wt) determined by sieving was in the range of 45 – 212 µm (8%), 212 – 600 µm (35%), 600 µm – 1.18 mm (43%) and 1.18 – 3.35 mm (14%).

Experimental Designed Supercritical Fluid Extraction

In this study, each run employed a 5 g (± 0.05 g) of *P. niruri* plant sample. The experiments were performed using an SFE system described in Markom et al. [13]. The influence of pressure (P), temperature (T), cosolvent concentration (c) and percent ethanol in aqueous cosolvent (r) were investigated and the low, center and high levels are shown in Table 1. An hour static extraction was allowed for the mixture to equilibrate at the temperature and pressure studied, followed by a dynamic extraction at a total solvent flow rate of 1.5 mL/min and 4 hours total extraction time. The extract fractions were collected every 30 minutes followed by drying in an air oven (Shel Lab, USA) at 70 °C for about 15 – 30 hours to remove the remaining cosolvent. All extracts were cooled at room temperature and placed in a desicator before weighing gravimetrically using analytical balance (± 0.0001 g) to determine the yields. The dried extracts were then stored in a cool room at freezing temperature (-4 ^{o}C) for later HPLC analysis.

Table 1. Selection of Operating Parameters.

Factor	Coded Factor	Level		
		Low (-)	Center (0)	High (+)
Pressure (bar)	P	100	200	300
Temperature (^{o}C)	T	40	60	80
Cosolvent Concentration (% v/v)	c	5	10	15
Ethanol Content (% v/v)	r	30	50	70

Optimization by experimental design was carried out using Design Expert® 6.0 software (Stat-Ease, USA). Using a Response Surface Method, a face-centered Central Composite Design (α=1) was selected. The optimization was conducted using four factors and three levels as shown in Table 1. The experiments consist of 16 factorial, 6 centered, and 8 axial runs (total of 30 runs). The responses selected for the optimization were pre-extraction yield (Y_{PRE}), main extraction yield (Y_{ME}), component contents of gallic acid (C_{GA}), corilagin (C_{CL}) and ellagic acid (C_{EA}), and component yields of gallic acid (Y_{GA}), corilagin (Y_{CL}) and ellagic acid (Y_{EA}).

Component Analysis

The identification and quantification of components were determined by High Performance Liquid Chromatography (HPLC) technique. The equipment was equipped with an autosampler and a UV/vis detector (Agilent Technologies, Germany). The column used for the analysis was a reverse-phase C18 Genesis with 250 x 4.6 mm i.d. and 4 μm particle diameter (Jones Chromatography, UK). The chromatographic separation was carried out using a mobile phase of 0.1% phosphoric acid in water (solvent A) and acetonitrile (solvent B) with a gradient of solvent B: 8-22% (35 minutes), 22-8% (10 minutes) at flow rate of 1 mL/min. The injection volume was set at 20 μL and the detection was in UV absorbance at 270 nm. Total flavonoid contents were determined based on the method of Yuan et al. [14].

RESULTS

Fractionation of P. niruri Compounds

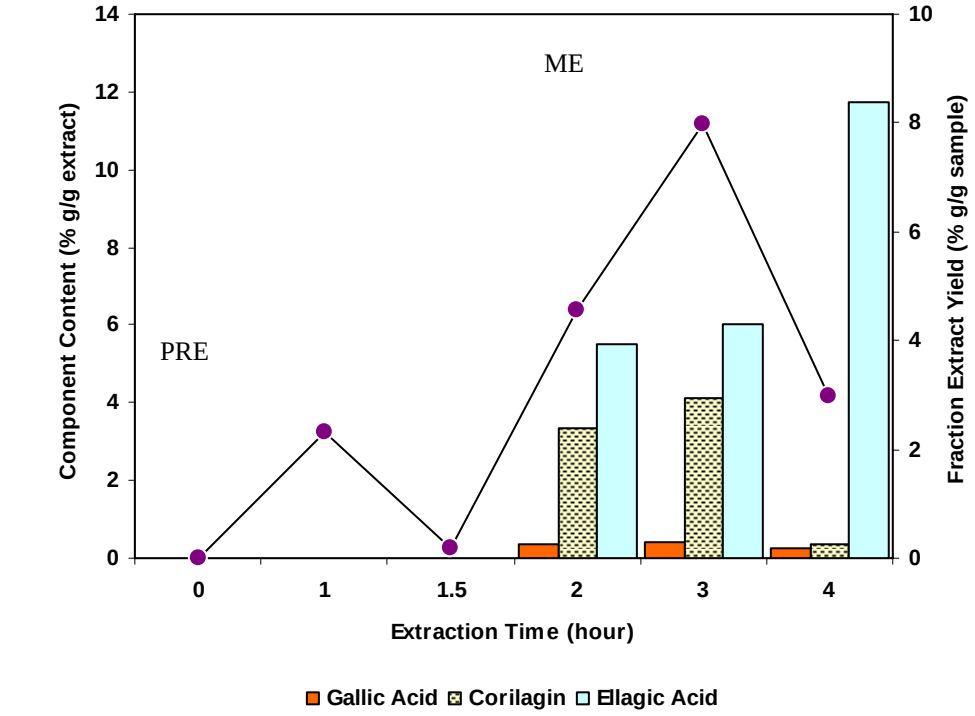
Preliminary result showed that SFE using pure CO_2 at 200 bar and 60^{o}C was incapable of extracting hydrolysable tannins from *P. niruri*. It produced whitish to yellowish fractions as a function of time. These wax-like fractions were completely soluble in n-hexane indicating the presence of non-polar components. These odorous fractions might contain free fatty acids [15].

Different cosolvents tested showed that organic cosolvents such as ethanol and methanol improved the extraction yields to 44 – 58%. This was accompanied by the appearance of intense yellowish to greenish fractions containing flavonoid compounds. This finding was similar to other organic cosolvent studies of plants [5,16]. The yellowish and greenish fractions were completely soluble in dichloromethane, indicating the presence of medium polar compounds. The green color might be caused by the presence of plant pigments such as chlorophylls. The HPLC analysis also showed the presence of flavonoids such as quercetin, (+)-catechin, (-)-epigallocatechin, (+)-gallocatechin, (-)-epicatechin and rutin in the fractions obtained.

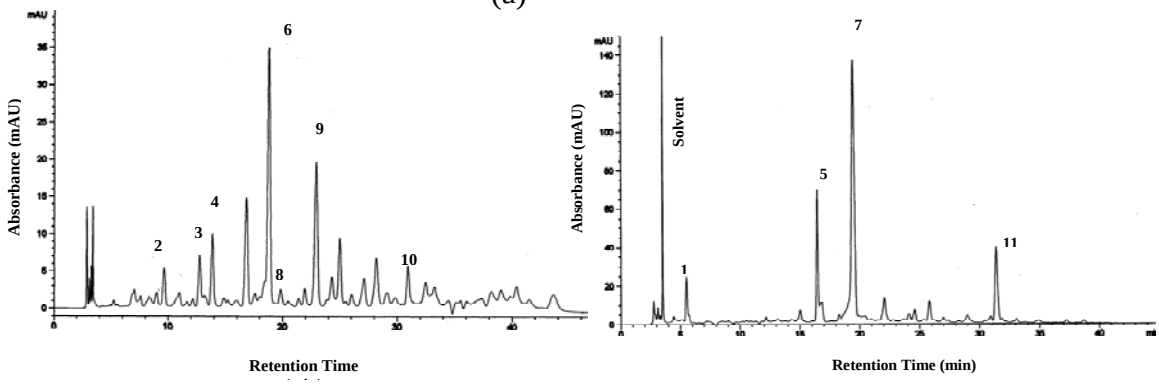
On the other hand, water cosolvent increased the extraction slightly without any appearance of greenish fraction (no flavonoid was detected). It was then followed by a fractionation to a second extraction (brownish fractions), where the presence of hydrolysable tannins (gallic acid, corilagin and ellagic acid) was observed. These fractions were soluble in 50% ethanol.

A 50% ethanol cosolvent gave interesting results since it improved the first extraction yield with flavonoids (pre-extraction or PRE), and also produced the second extraction containing hydrolysable tannins (main extraction or ME). Figure 1 shows the fractionation of the extracts from the *P. niruri* at 200 bar, 60^{o}C and 10% v/v of 50% ethanol-water cosolvent. The initial

fractions showed whitish to yellowish to greenish fractions and exhibited higher yield compared to either pure ethanol or water cosolvent. The fractions might also contain lignans based on the study of delignification of wood chips and sugar cane bagasse using SFE with ethanol-water mixture cosolvent [17]. The latter fractions were dark brownish liquid which contained the three hydrolysable tannins. The last fraction showed almost full selectivity for ellagic acid. The total extraction yield (PRE and ME) of 19.83% (±1.14) was comparable to Soxhlet extraction yield (22.5 ±1.90%).



(a)



(b) (c)

Figure1. *P. niruri* fractionation by CO_2-ethanol-water at 200 bar, 60°C and 10% v/v cosolvent. (a) Extraction profile, (b) PRE component profile and (c) ME component profile. Components detected by HPLC were gallic acid (1), quercetin (2), (–)-epigallocatechin (3), (+)catechin (4), unknown A (5), unknown B (6), corilagin (7), (–)epicatechin (8), (–)gallocatechin (9), and rutin (10) and ellagic acid (11).

Fractionation of different compound groups (non-polar, medium polar, and polar) could thus be obtained. The most important thing to consider is that the solubility parameter of the solvent or solvent mixture should reach the same value as that of the solute for any possible extraction to occur in either the PRE or ME step. The solubility parameter is not only dependent on the density of the fluid mixture but is also influenced by the electrostatic or intermolecular hydrophilic forces (dipole and hydrogen bonding) between the solvent and the solute [13].

The higher yield in the PRE could also be a result of the enhanced solvent power of the supercritical phase. Water might also affect the matrix structure and simultaneously enhance the component desorption. However, due to its limited solubility in CO_2, water is not expected to have significant interaction and chemical bonding with the non-polar or medium polar compounds in the PRE step. Because of the higher critical points of water, vapor and liquid phases might co-exist together at sub-critical condition of the CO_2-water mixtures. Thus, it was suspected that the extraction behavior of hydrophilic compounds in the ME step might be strongly governed by the water in the liquid phase. Due to the different phase extractions, component fractionation was possible. Even though several studies on the SCO_2 + alcohol + water have been published [17-20], the fractionation behavior as encountered in this study has never been reported before.

Optimization of SFE Parameters

The optimization was carried out by experimental design approach (central composite design and response surface method) using a Design Expert® 6.0 software. The experiments at the center were conducted in six replicates (n = 6) in order to estimate the repeatability and the experimental errors of the responses. The individual and interaction effects of the factors on the responses were systematically determined from the statistical analysis of the designed experiments. The ANOVA results of the selected quadratic models indicated good regression fits for all the models ($R^2 > 0.86$). Diagnostic tests to determine model adequacy were also generally satisfied in this study.

Pressure (P), temperature (T), and cosolvent concentration (c) significantly affected the PRE yield, whereas only cosolvent (c) and ethanol content (r) influenced the ME and tannin yields. Cosolvent concentration and water-methanol composition were also found to be the significant factors in the cocaine extraction from coca leaves [21]. Therefore, the optimization of these parameters was performed to determine the exact parameter combinations in order to obtain maximum yield within the shortest extraction time.

In order to optimize the operating conditions and determine the quality of the extracts, both the content and yield of corilagin (C_{CL} and Y_{CL}) were maximized. The response surface plot of corilagin content (C_{CL}) is shown in Figure 2 in square root. In HEPAR-P™, it was standardized to 4% g/g [12]. It is obvious from Figure 2 that this corilagin content or higher could be obtained at a region of 9 – 13% cosolvent content and 30 – 60% ethanol in water as cosolvent.

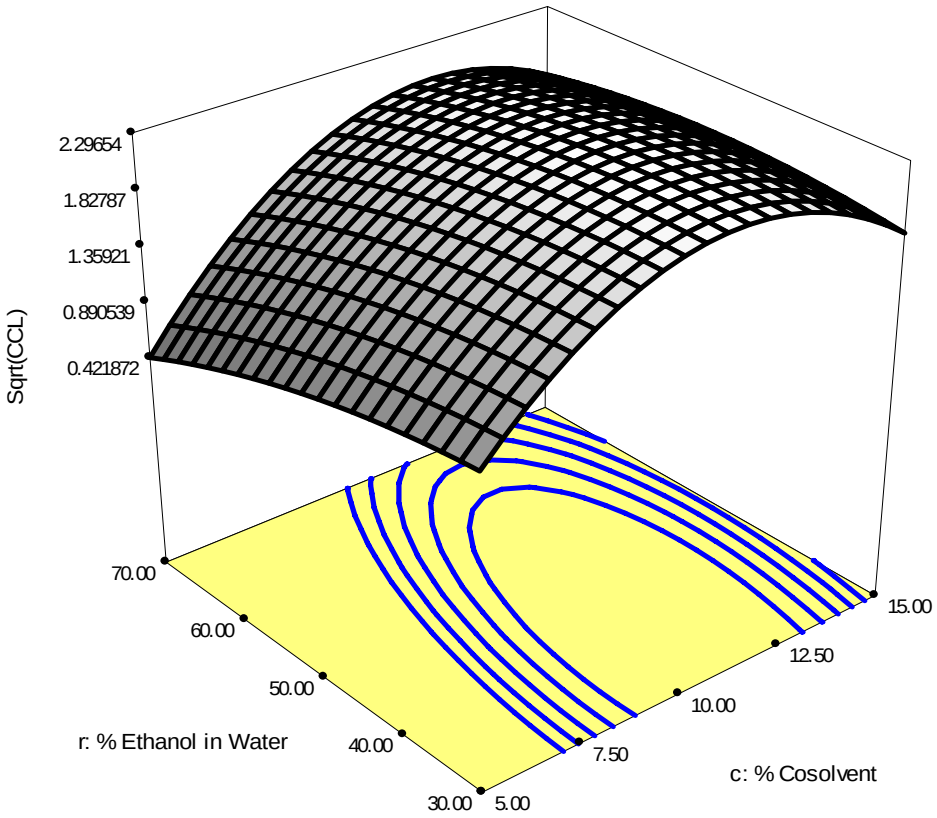


Figure 2. Response surface plot of corilagin content as function of significant factors.

In this study, two optimum conditions were predicted and verified by experiments. They were SFE_1 and SFE_2. The difference was that the yield of pre-extraction (Y_{PRE}) was minimized in SFE_1 but maximized in SFE_2. All experimental data were in good agreement with the predicted values since they fall within the 95% confidence intervals. The extract from SFE_2 run was further analyzed for its total flavonoid content in addition to the hydrolysable tannin contents. It was found that a single SFE run (SFE_1 or SFE_2) could remove the unwanted components in the PRE step and produce *P. niruri* extract that is comparable to the commercially available product (HEPAR-P^{TM}) in terms of component contents in the ME step as shown in Table 2.

It can be concluded that a single run of SFE in this study can do both the pre-treatment and the extraction of desired products from plant materials, compared to the two-step SFE suggested in other plant extraction studies [5,16]. It was also observed that there is no clear threshold extraction limit for the separation of waxes with chlorophylls/flavonoids in PRE. This should be resolved for an effective fractionation between the pretreatment/pre-extraction and the main extraction steps.

Table 2. Component Contents of Optimized *P. niruri* Extracts in Comparison to Commercial Standardized Extract

Product	Main Extraction Yield, Y_{ME} (%g/g sample)	Pre-extraction Yield, Y_{PRE} (%g/g sample)	Component Content (% g/g extract)			Total Flavonoid Content (% g/g extract)
			Gallic Acid, C_{GA}	Corilagin, C_{CL}	Ellagic Acid, C_{EA}	
SFE_1		0.37	0.59	3.96	9.38	-[nd]
SFE_2		1.81	0.57	2.82	8.12	16.18
HEPAR-P™	-	-	0.21	2.64 (4)	4.17	14.23 (18)

-[nd] not determined.
- values in brackets are the standardized contents in commercial extract.

CONCLUSION

The study found that ethanol-water cosolvent mixture employed in SFE was capable of simultaneously pre-treating and extracting the hydrolysable gallic acid, corilagin and ellagic acid from *P. niruri*. It was found that the presence of water in SFE enhanced both the desorption of the less polar compounds (waxes, lipids, lignans, flavonoids, chlorophylls) in the pre-extraction fractions, and the extraction of hydrolysable tannins in the main extraction fractions. A three-level optimization of pressure, temperature, cosolvent concentration in CO_2 and ethanol content in water by central composite design and response surface method showed that the pre-extraction yield was mainly influenced by pressure, temperature and cosolvent content while the main extraction and ellagitannin yields were significantly affected by cosolvent and ethanol-water contents. Therefore, a single-step polarity-based fractionation by SFE could reduce the plant processing steps and extraction time while producing high-quality yield, minimize the use of liquid solvent consumption and lower the operating temperature required.

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