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Chemical Profiling and Quantification of Tannins in *Phyllanthus niruri* Linn. Fractionated by SFE Method

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Chemical profiles or fingerprints of polyphenolic compounds (condensed and hydrolyzable tannins) in various fractions of *Phyllanthus niruri* Linn extracted using supercritical carbon dioxide and various polar cosolvents, namely water, methanol, and ethanol are presented. Chemical analysis of the extracted fractions was undertaken using High Performance Liquid Chromatography (HPLC) with the in-house method. Good peak reproducibility of intra-day (R.S.D range 0.01–0.21 min) and inter-day (R.S.D range 0.5–0.8 min) was obtained for the detection of ellagitannins (hydrolyzable tannins) and flavonoids (condensed tannins). Fractions extracted using ethanol-water mixtures as cosolvent at 200 bar and 60°C exhibited an appealing behavior whereby non-polar compounds and flavonoids were able to be fractionated before the extraction of ellagitannins. Contents of three major ellagitannins, namely gallic acid (0.39–0.48% g/g), corilagin (2.42–3.00% g/g), and ellagic acid (5.94–6.48% g/g), were relatively higher compared to the commercial HEPAR-P^{TM} standardized extract (0.21, 2.64, and 4.17% g/g, respectively). The study shows that the supercritical fluid extraction (SFE) method with the use of appropriate cosolvents is able to produce *P. niruri* fractions with improved yields and different chemical characteristic, which thus can be used as a rapid preparative tool for further downstream processing of plant samples.

Keywords chemical analysis; fractionation; *Phyllanthus niruri*; polar cosolvents; supercritical carbon dioxide

INTRODUCTION

Phyllanthus niruri Linn. (Euphorbiacea) plant was scientifically found to exhibit hepatoprotective, antinoniceptive, antiviral, antitumor, antioxidant, and antiplasmodial properties (1–6). The medicinal properties of *P. niruri* have been attributed to the presence of secondary metobolites. Active constituents that have been identified in *P. niruri* are phenolic acids, ellagitannins, flavonoids, lignans, alkaloids, terpenes, steroids, glycosides, as well as some conjugates and derivatives (6–9).

Most research on *P. niruri* has been on chemical screening and identification, isolation of active components, biological assay, and pharmacological studies (3,7,10). However, no analysis method has so far been developed to not only identify but quantify the compounds as well. The identification of chemical structures by Neutron Magnetic Resonance (NMR) technique needs many isolation steps for obtaining pure compounds. Most often, elution by the chromatographic technique is required for component isolation. Ueno et al. (10) used Sephadex L-20 to isolate ellagic acid, geraniin, and gallic acid and these compounds were identified by using the ^{13}C NMR method. Ishimaru et al. (7) and Qian-Cutrone (8) showed that different groups of compounds could be isolated using different types of column packings such as MCL-gel CHP-20P, Sephadex L-20 and Bondapak C18 Porasil B.

De Souza et al. (11) quantified the amount of gallic acid in the water extract of *P. niruri* using the high performance liquid chromatography (HPLC) technique. However, other chromatogram peaks were not identified but were calibrated and quantified with gallic acid as a reference. Ishimaru et al. (7), on the other hand, were able to identify a range of components such as gallic acid and some flavonoids namely (−)-epicatechin, (+)-catechin, (+)-gallocatechin, (−)-epigallocatechin, (−)-epicatechin 3-*O*-gallate and (−)-epigallocatechin 3-*O*-gallate, but no quantification had been carried out. Nova Laboratories Sdn. Bhd (12). has published a chemical fingerprint for *P. niruri* based on a capsule developed called HEPAR-P^{TM}, which contains 250 mg of standardized *P. niruri*. HEPAR-P^{TM} is commercially

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produced by solvent extraction followed by a series of chromatographic purification steps. The HPLC chromatogram identifies four components, namely gallic acid, brevifolin carboxylic acid, corilagin, and rutin. However, the analysis method has not been published.

In order to establish a reliable chemical fingerprint for the identification and quantification of hydrolysable and condensed tannins using HPLC, the selection of an appropriate working solvent for the preparation of standard and extract solutions, and the optimization of HPLC conditions such as the mobile phase and the UV wavelength, are very important (13,14). Hydrolysable and condensed tannins belong to a polyphenol group, which has more than one phenol ring in their chemical structures.

In this study, various fractions of *P. niruri* were obtained by extraction using carbon dioxide with polar cosolvent and polar cosolvent mixtures at conditions above the critical point of carbon dioxide which is referred to as the supercritical fluid extraction (SFE) method. Extraction at supercritical conditions usually employs carbon dioxide (CO_2) as the choice solvent operating above 31°C and 73 bar, which are the critical point of CO_2. Cosolvents (polar and non-polar) are often added in order to enhance the solvating power of the solvent. The solvating power of a supercritical fluid can also be easily manipulated by its density (a function of pressure and temperature). A comparison of the SFE method with other solvents and extraction methods (Soxhlet, ultrasonic, pressurized water), in terms of extraction yield and efficiency, but not the chemical profiles, had been previously reported and discussed by Markom et al. (15).

This paper focuses on the chemical profiles obtained by the SFE method. An HPLC method has been developed for the identification and quantification of ellagitannins (gallic acid, corilagin, and ellagic acid) in *P. niruri*. The identification of condensed tannins (flavonoids) was carried out using a similar method. Chemical profiles of extracts without the use of a cosolvent and with the addition of different organic and aqueous cosolvents were obtained and compared. In addition, the content of ellagitannins extracted was also compared to *P. niruri* standardized extract (HEPAR-P^{TM}).

MATERIALS AND METHODS

Plant Material

Dried and ground *P. niruri* samples were obtained from Nova Laboratories Sdn. Bhd. (Malaysia). The plant is being cultivated and standardized for the commercial production of HEPAR-P^{TM}, a *P. niruri* health supplement product. The particle size distribution (% wt/wt) of sample (stem and aerial parts), 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%).

Reference Standards and Chemicals

The reference standards (gallic acid and ellagic acid) were both purchased from Sigma Chemicals (USA) at 98% purity. Flavonoid standards such as quercetin dehydrate, (+)-catechin, (−)-epicatechin, (+)-gallocatechin, and (−)-epigallocatechin (all at 98% purity) were also obtained from Sigma Chemicals (USA). Rutin (97% purity) was purchased from Acros Chemical, UK. Commercial standard of geraniin was not available and was sourced by the courtesy of Prof. H. Wagner (University of Munich, Germany). The identification of corilagin was carried out by Nova Laboratories Sdn. Bhd. (Sepang, Malaysia) using their isolated standard (purity of 98%). The HEPAR-P^{TM} standardized extract was obtained from the same company in order to compare their sample with that of this study. This product has been standardized to 4% corilagin and 18% total flavonoid content.

All chemical reagents for the solvent extraction and component analysis (ethanol, methanol, acetone, acetonitrile, and phosphoric acid) were of analytical grades. Ultra-pure water of 18 MΩ-cm obtained using ultrafiltration system (USF ELGA, UK) was utilized for the aqueous solvents, cosolvents, and HPLC mobile phase. Industrial grade liquid CO_2 (99.7–99.8%) was obtained from Gas Pantai Timur (Kuala Lumpur, Malaysia).

Supercritical Fluid Extraction (SFE)

The detailed equipment setup and experimental procedures have been previously described by Markom et al. (15). In this semi-continuous extraction process, 5 g (±0.05 g) *P. niruri* samples were used. Carbon dioxide was the main solvent used and the extraction conditions investigated were at 60°C and pressure of 200 bar. Extraction with the addition of 10% v/v cosolvent, namely ethanol, methanol, 70% ethanol (v/v in water), and 50% ethanol, were also studied. An hour of 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 (CO_2 and cosolvent) of 1.5 mL/min for 4 hours of extraction time. Therefore, a total amount of solvent required for the dynamic extraction was 72 mL solvent per g of plant sample used. 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 traces of cosolvent. All extracts were cooled at room temperature and placed in a dessicator before weighing gravimetrically using analytical balance (±0.0001 g) to determine the extract yields.

HPLC Analysis

Component analysis was carried out using High Performance Liquid Chromatography (HPLC) technique equipped with an auto sampler and a UV/vis detector (Agilent Technologies, Germany). The analysis method

(column, mobile phase and gradient program, flow rate, and UV absorbance) used was modified and developed in-house, and described in detail by Markom et al. (15).

Chromatographic peaks of gallic acid, ellagic acid, and flavonoids were identified by comparing the retention times with external standards. In order to quantify these phenolic acids, linear calibrations (peak area versus concentration) of standard solutions for gallic acid and ellagic acid were first prepared. Gallic acid dissolved in water was prepared with a concentration ranging between 0.75 μg/mL to 1.2 mg/mL. Ellagic acid was dissolved in 50% ethanol at concentrations ranging from 2.5 μg/mL to 1 mg/mL.

For corilagin sample preparation, HPLC analysis and identification were done by the Nova Laboratories Sdn. Bhd. (Malaysia) using their own purified standard. The corilagin standard was diluted in methanol at 0.5 mg/mL concentration. The spiking method was carried out by the company for the identification and quantification of corilagin in HEPAR-P™ and several *P. niruri* extracts.

The flavonoids were each dissolved either in water or 50% ethanol depending on their solubilities in the respective solvents. In addition, purified geraniin was also dissolved in 50% ethanol for analysis. HEPAR-P™ and *P. niruri* extracts were all dissolved in 50% ethanol at concentrations of 1–5 mg/mL.

All of the standards and extract solutions were ultra-sonicated at 60°C for 30 minutes in order to remove air bubbles and to ensure that all solids were completely dissolved before analysis by HPLC. Prior to injection, all sample solutions were pre-filtered using nylon micro membranes (0.45 μm).

A single injection of solvent (blank) was also carried out to determine the solvent retention time. To determine reproducibility, repeat analysis of the standards was undertaken on alternate extracts.

RESULTS AND DISCUSSION

Chemical Profiles and Identification

Hydrolysable Tannins (Ellagitannins)

The HPLC chromatogram of the HEPAR-P™ standardized extract is shown in Fig. 1. This chemical fingerprint consisted of gallic acid, unknown A, corilagin, and ellagic acid. Their retention times are presented in Table 1 and their identities were confirmed based on the injected standards. The chemical profile in Fig. 1 is almost similar to the one obtained by Nova Laboratories Sdn. Bhd. (12).

Gallic acid was also the first component to be eluted in *P. niruri* extracts analyzed by other HPLC methods (7,11). When geraniin standard was injected, no single major peak was observed. This is probably caused by the hydrolysis of this compound to simpler compounds, such as corilagin and phenolic acids, inappropriate HPLC conditions, low absorbance of the component in the UV wavelength used, or the use of an overly diluted sample. Geraniin also consists of a dehydrohexahydroxydiphenoyl (DHHP) that is always in equilibrium with its hydrated hemiacetal ring in aqueous solution (16). This condition might also be a factor for the difficulty in detecting geraniin in the extracts. Due to the very small amount of standard available and an inconsistent appearance of a single major peak, geraniin was not quantified in the *P. niruri* extracts. Based on the result of this study, it is suggested that for geraniin detection and quantification by HPLC, the hydrolysis process should be prevented or minimized. This can be done by selecting an appropriate dilution solvent and sample preparation prior to its analysis.

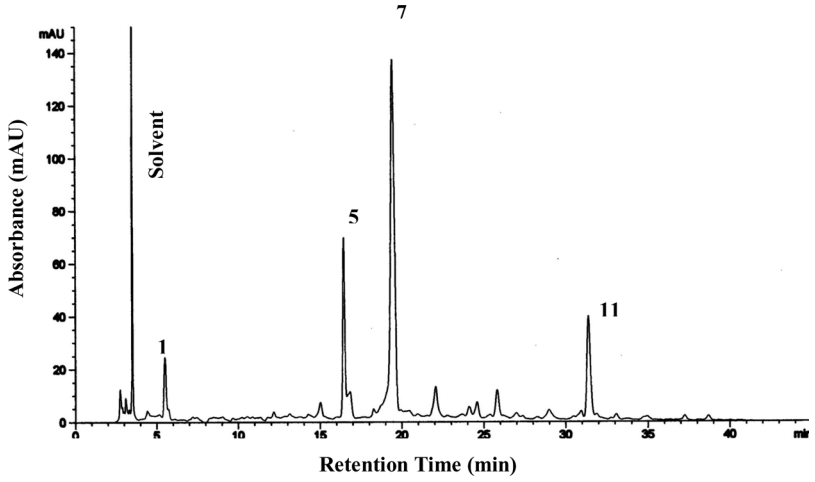


FIG. 1. HPLC chromatogram of HEPAR-P™. The column used was a reverse-phase C18 Genesis 250 × 4.6 mm i.d., 4 μm particle diameter. A mobile phase consisted of 0.1% phosphoric acid in water (solvent A) and acetonitrile (solvent B) with a gradient of solvent B: 8–22% (35 minutes), 22–8% (10 minutes) at flow rate of 1 mL/min. The injection volume was set at 20 μL and the detection was in UV absorbance at 270 nm. Components detected were gallic acid (1), component A (5), corilagin (7) and ellagic acid (11).

TABLE 1
Identification and reproducibility of hydrolysable and condensed tannins in *P. niruri* extracts using HPLC method

Peak no.	Component	Retention time (min) ± R.S.D[b]	Inter-day reproducibility (% R.S.D)[b]
1	Gallic Acid	5.39 ± 0.01	0.7
2	Quercetin	9.75 ± 0.42	–
3	(−)-Epigallocatechin	12.38 ± 0.04	0.5
4	(+)-Catechin	14.54 ± 0.04	0.5
5	Component A	16.54 ± 0.01	–
6	Component B	18.80 ± 0.07	–
7	Corilagin	18.86 ± 0.21[a]	–
8	(−)-Epicatechin	20.03 ± 0.06	0.8
9	(+)-Gallocatechin	23.16 ± 0.06	0.7
10	Rutin	31.10 ± 0.05	–
11	Ellagic Acid	31.21 ± 0.04	0.8

–not determined.
[a]Determined from retention times of corilagin peak in the extracts and not of the standard.
[b]Relative standard deviation of repeat analysis of the standards (n = 3).

In the HPLC analysis, ellagic acid was also detected when the UV range was varied between 210 to 360 nm. The ellagic acid peak gave significant absorbance at 270 nm. This is the first finding which showed the presence of this component in the *P. niruri* using the HPLC method. Previously, it was only determined by an NMR method for this plant (7). In raspberry fruits, ellagic acid had been detected using a gradient of 8–21% acetonitrile-water (with 1% formic acid) in HPLC-MS with DAD at 365 nm (17). In that study, a higher UV wavelength was required because the formic acid used has a higher baseline absorbance compared to that of phosphoric acid used in this study.

The detection limit for the HPLC analysis was determined to be at 0.75 μg/mL for gallic acid and at 2.5 μg/mL for ellagic acid.

Condensed Tannins (Flavonoids)

Several flavonoids were detected in the SFE extracts when methanol or ethanol cosolvents were used. They were from group flavonols (quercetin and rutin), and flavon-3-ol ((+)-catechin, (−)-epicatechin, (+)-gallocatechin, and (−)-epigallocatechin). The retention time of Unknown B (the highest peak) was close to corilagin's, but it was not identified in this study due to a commercial unavailability of corilagin standard. The chemical fingerprint for the flavonoid-rich fraction is shown in Fig. 2. The retention times of the components confer with those of the reference standards and are reported in Table 1.

Interestingly, the flavonoids were selectively detected in the initial fractions of SFE containing 50% ethanol or 70% ethanol as cosolvent. The latter fractions were found to be

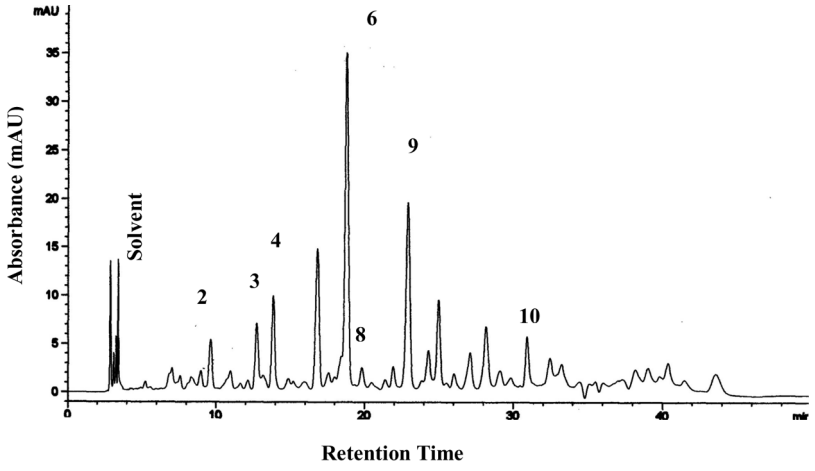


FIG. 2. HPLC chromatogram of initial fraction in SFE extract. HPLC conditions were similar to Fig. 1. Components detected were quercetin (2), (−)-epigallocatechin (3), (+)-catechin (4), component B (6), (−)-epicatechin (8), (−)-gallocatechin (9), and rutin (10).

rich in the hydrolysable tannins. These flavonoids were not selectively extracted by the Soxhlet solvent extraction (15), even though most of them had been detected in the same sequence by Ishimaru et al. (7) from the methanol extract of *P. niruri*. This is expected since their extract had been further fractionated and isolated prior to HPLC analysis. The flavonoids identified in this study are also commonly found in the green tea extracts (18).

It was also noted that the retention times of ellagic acid (ellagitannin) and rutin (flavonoid) peaks were very similar and difficult to differentiate. The presence of more than one peak and peaks that overlap can be detected by an HPLC with a diode array detector (DAD). In this study, due to the unavailability of DAD, the spiking method was used to confirm the component peak. Ellagic acid was found to be present in the hydrolyzable fractions of both Soxhlet (15) and SFE used in this study, while rutin exists in the less polar fraction of the SFE method together with other flavonoids. This might be due to the different functional groups of the two compounds. Rutin is a condensed tannin and absorbs less in the UV wavelength used compared to ellagic acid. It might be possible that the rutin peak (which has lower UV absorbance) overlapped with the ellagic acid peak. The presence of rutin was only reported by Nova Laboratories Sdn. Bhd (12). and Qian-Cutrone (8), whereas other researchers did not detect rutin in all of the alcoholic or aqueous *P. niruri* extracts obtained (3,7,10).

Reproducibility

Table 1 shows good reproducibility for the component detection (retention times and peak areas). The intra-day accuracy was determined by the relative standard deviation (R.S.D) of component retention times of different extract samples. The deviation of peak areas at different days (inter-day) for selected components was found to be less than 1% R.S.D.

Quantification of Ellagitannins in SFE Extracts

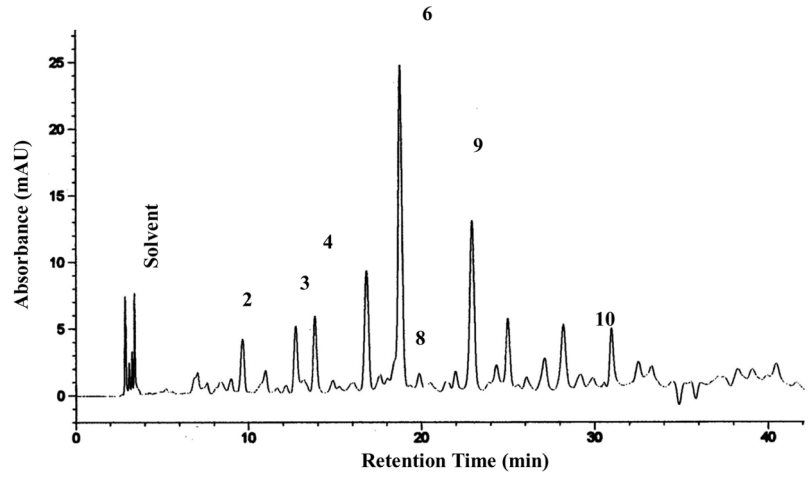
It was observed that the SFE method was able to fractionate the condensed and hydrolysable tannins at a specific extraction time as shown by the HPLC chromatograms in Fig. 3(a)–(c) at 60°C and 200 bar with addition of 10% of 70% ethanol as cosolvent. Initially, the extracts contains flavonoid-rich fractions (at 0.5 and 1.5 hours) followed by the ellagitannin-rich fraction. The appearance of ellagitannins occurred at a specific extraction time (in this case at 1.5 hours and above) and was very much dependent on the cosolvent composition. This is a very useful finding since a single-step fractionation can reduce the processing steps required for extraction or component purification.

From the standard calibration of HPLC peak area versus concentration, quantification of various SFE extracts has been obtained and is shown in Table 2. It can be seen that the total extract yield was slightly higher with the addition of alcohol cosolvent ethanol (1.57%) or methanol (1.73%) compared to SFE with pure CO_2 (1.09%). Flavonoids were detected in all extracts containing alcohol cosolvents, which is consistent with previous works (19,20). Ethanol and methanol have been used as cosolvents in SFE to extract polyphenols from ginkgo biloba (21,22). However, in this study, no ellagitannins were detected using these cosolvents since ellagitannins are relatively more polar than flavonoids. The polarity index of all cosolvents and cosolvent mixtures used in this study is shown in Table 2.

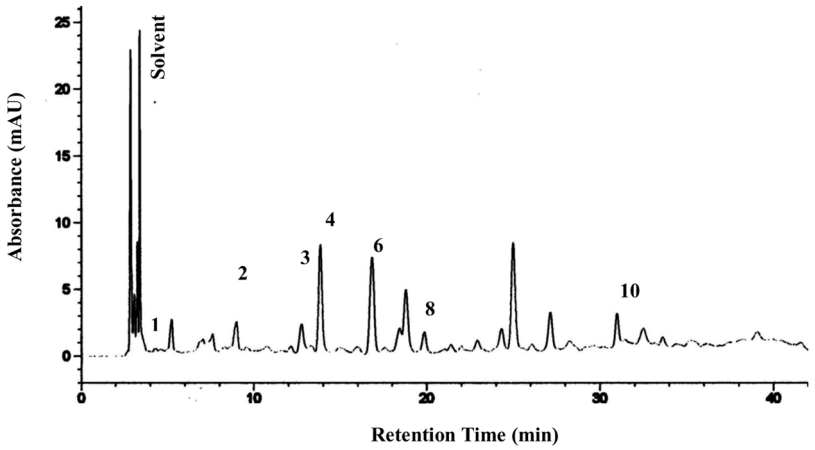
On the other hand, water (very polar) as cosolvent exhibited different effects compared to ethanol and methanol, where two completely different fractions were obtained in sequence. It is presumed that water enhanced the desorption of less polar components extracted by the CO_2 in the first fraction, and then was subsequently followed by extracts of more polar and hydrophilic compounds in the second fraction. However, the presence of flavonoid was not significantly detected in both fractions since no medium polar cosolvent such as ethanol or methanol was present. The total yield of these two fractions was significantly higher (17.78%) than the SFE method with ethanol or methanol as cosolvent. This indicates that most of the compounds present in *P. niruri* are hydrophilic or polar compounds. Due to a low solubility of water in CO_2, two phases (gas and liquid) might co-exist at sub-critical condition. This might result in a component fractionation, whereby the initial fractions were governed by the supercritical CO_2 and the latter fractions were governed by the pressurized water.

Extracts obtained by the SFE method with and without the cosolvent were compared to the commercial HEPAR-P^{TM} in terms of ellagitannin contents (Table 2). It can be seen that the use of water or ethanol/water cosolvent is able to produce comparable or higher contents of gallic acid (0.39–0.48%), corilagin (2.42–3.00%) and ellagic acid (5.94–6.48%) than HEPAR-P^{TM} (0.21%, 2.64%, and 4.17%, respectively). However, a comparison to the total extract yield could not be made since the information was not reported for HEPAR-P^{TM}.

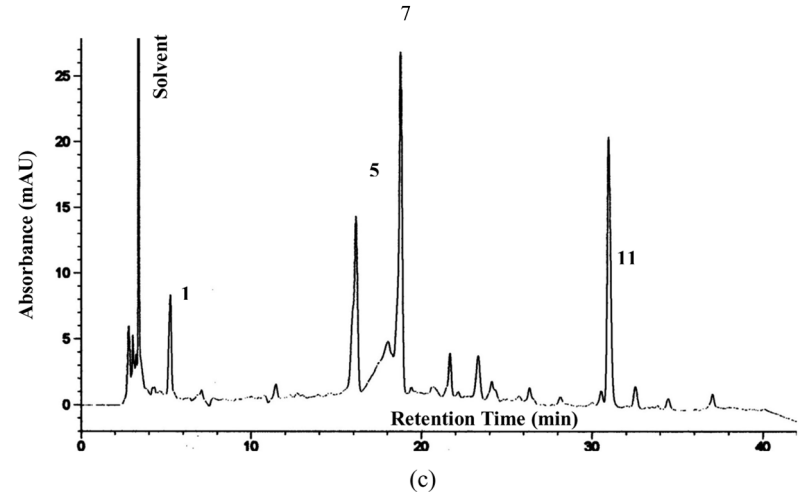
A cosolvent mixture of 50% or 70% v/v ethanol in water showed an interesting behavior where the flavonoids were profoundly detected in the first fractions while the ellagitannin contents in the second fractions were comparably higher than those of the HEPAR-P^{TM} product. In the Soxhlet extraction by Markom et al. (15), water was found to be the best solvent in terms of extracting gallic and ellagic acid but the optimum addition of ethanol in water could increase the corilagin content. It was also reported by Notka et al. (3) that the plant extract obtained using 50% ethanol solvent was the most active in the inhibition



(a)



(b)



(c)

FIG. 3. Chemical profiles of *P. niruri* extracts by SFE at 200 bar, 60°C and 10% v/v of 70% ethanol at different extraction times: a) 0.5 hr b) 1.5 hr c) 3.5 hr. HPLC conditions were similar to Fig. 1.

TABLE 2
Quantification of ellagitannins in *P. niruri* extracts

Extraction method	Snyder's polarity index (for cosolvent)[a]	Total extract yield (% w/w sample)	Presence of flavonoids	Ellagitannin content (% w/w extract)		
				Gallic acid	Corilagin	Ellagic acid
SFE[b]:						
CO_2	–	1.09	nd	nd	nd	nd
CO_2 + ethanol	5.2	1.57	Yes	nd	nd	nd
CO_2 + methanol	6.6	1.73	Yes	nd	nd	nd
CO_2 + water	9.0	17.78	nd	0.48	2.42	6.48
CO_2 + 70% v/v ethanol/water	8.2	8.50	Yes (Fractionated)	0.45	2.82	5.94
CO_2 + 50% v/v ethanol/water	7.9	19.83	Yes (Fractionated)	0.39	3.00	5.97
HEPAR-P™		na	Yes	0.21	2.64	4.17

[a]Cited from Markom et al. (15).
[b]SFE conditions at 200 bar; 60°C; 10% v/v cosolvent in CO_2 if added.
na – information not available.
nd – not detected.

of HIV-1 RT strain replications compared to the water or methanol extracts due to the higher contents of geraniin and corilagin.

Based on the chemical profiles obtained in this study, the bioactive compound such as corilagin along with the simpler gallic and ellagic acids could be fractionated by the SFE method with ethanol-water cosolvent in relatively higher contents. Furthermore, the flavonoid-rich fraction could also be obtained in a single run. Thus, it is possible to produce an SFE extract that can be tailored to specific applications depending upon its chemical profiles and compositions.

CONCLUSIONS

A reliable HPLC method for the identification and quantification of gallic acid, corilagin, and ellagic acid in *P. niruri* has been developed. The component profile showing the three components can be used as a chemical fingerprint for the plant extract. Good reproducibility was achieved for all components analyzed.

The condensed tannins (flavonoids) have been detected and were found rich in the earlier fractions while the hydrolyzable tannins (ellagitannins) were detected to be rich in the latter fractions of the SFE method. This finding has shown that the SFE method is able to successfully extract and fractionate different groups of *P. niruri* compounds, and can be further employed for sample preparation or tailoring the end products from plants. Future study should look into the optimization of the SFE operating parameters on the *P. niruri* extraction to produce extracts with desired components.

ACKNOWLEDGEMENTS

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