HPLC–Electrospray Ionization–MS–MS Analysis of *Cephalotaxus harringtonia* Leaves and Enhancement of the Extraction Efficiency of Alkaloids Therein by SFE

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Abstract

A high-performance liquid chromatographic method is developed for use in the electrospray mass spectrometric (MS)–MS analysis of alkaloids contained in *Cephalotaxus harringtonia* leaves. Nine alkaloids having ester groups can be separated and detected with good sensitivity. The MS and MS–MS spectra obtained provides information on their chemical structures. Supercritical fluid extraction is also applied in order to improve the extraction efficiency of Cephalotaxus alkaloids such as cephalotaxine, harringtonine, homoharringtonine, and isoharringtonine. When carbon dioxide–methanol–water (80:18:2, v/v) is used the extraction yield is found to be higher than that using the other supercritical solvents evaluated and conventional organic solvent extraction.

Introduction

Cephalotaxus alkaloids such as cephalotaxine, harringtonine, and homoharringtonine (Figure 1) are a family of cytotoxic alkaloids synthesized by the genus *Cephalotaxus*, some of which have shown potent antileukemic activity when intraperitoneally injected in mice (1). Recent clinical studies of these alkaloids have shown that they have certain effects on various types of acute leukemia when intravenously administered (2). For example, homoharringtonine inhibits protein synthesis in a dose- and time-dependant manner by acting on the ribosomes of cancer cells. It blocks the progression of cells from the G1 phase to the S phase and from the G2 phase to the M phase. The alkaloid has a synergistic effect when administered *in vitro* with cytarabine (AraC), amsacrine, actinomycin D, and dexamethasone (3). Recently, these antitumor alkaloids have been isolated on a large scale from plants for clinical use (4) because their chemical synthesis has not yet been fully developed (5,6). However, previously

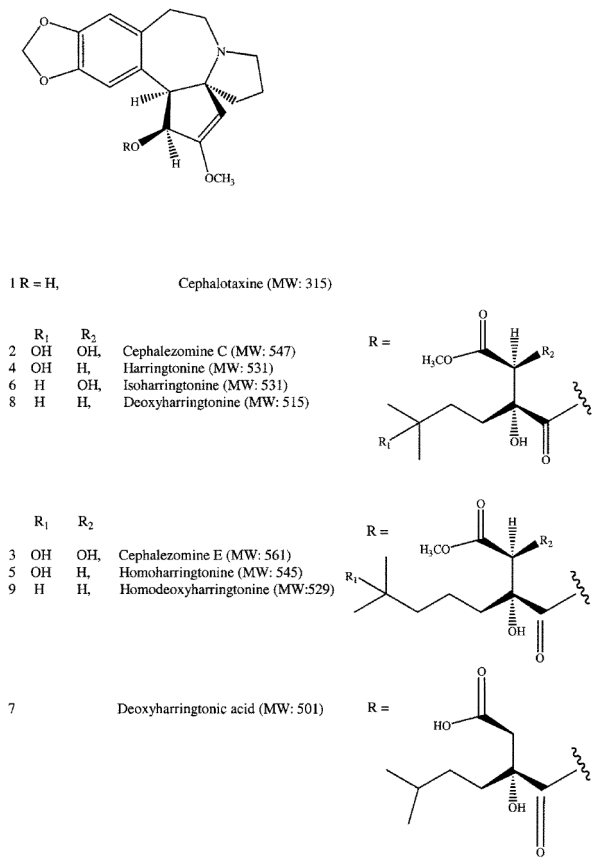

Figure 1. Structures and molecular weights of the *C. harringtonia* constituents evaluated in this study. MW = molecular weight.

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developed extraction methods involve aqueous or alcohol extraction followed by pH control and re-extraction with an organic solvent. Such procedures are long and tedious and involve the use of large quantities of toxic organic solvents. In addition, the analytical method currently in use, high-performance liquid chromatography (HPLC)–UV or gas chromatography (GC)–mass spectrometry (MS), requires a cleanup or other reaction procedures in order to enhance sensitivity and remove compounds that interfere with the detection of the target compounds (7,8). Therefore, an alternative method for the extraction and analysis of Cephalotaxus alkaloids from Cephalotaxus species would be highly desirable.

In the past few years, HPLC–MS has been successfully applied to the online analysis of natural products (9–12). HPLC combined with frit-fast atom bombardment and thermospray interfaces are in widespread use in the quantitative analysis of natural products. Of all the HPLC–MS techniques, electrospray ionization (ESI) is recognized as one of the most important ionization techniques for the online coupling of liquid-phase separation methods with MS (13). It is a simple and robust method capable of handling both small and large molecules, operates at atmospheric pressure and at a moderate temperature, and is probably the most gentle ionization technique currently available for MS. In this study, the analysis of alkaloids from the leaves of *Cephalotaxus harringtonia* using this HPLC–ESI–MS technique is described and the results compared with one another. For a more accurate quantitative analysis, the MS–MS technique was applied to the analysis instead of a simple MS one.

Cephalotaxine, harringtonine, homoharringtonine, and isoharringtonine were extracted by supercritical fluid extraction (SFE) in an attempt to enhance the yield and selectivity of that obtained using a conventional organic solvent extraction. In view of the increasing environmental and health concerns for the use of organic solvents in the extraction of natural products, a growing interest in using supercritical fluids has developed.

SFE has been demonstrated to be a valuable alternative in that it requires less solvent, a short extraction time, and is capable of extracting thermally labile compounds under mild conditions. In addition, by selecting the fluid polarity or density, the solvating power of the fluid can be adjusted in order to achieve selective extraction, and the extraction fluids can be removed from the fractions by decompression into a suitable collection device. SFE has been applied to a wide range of biologically active constituents from natural products, including essential oils, other flavor and fragrance compounds, as well as medicinal compounds (14–16).

We also report on our studies on the use of SFE for extracting the cytotoxic alkaloids from the leaves of *C. harringtonia*.

Experimental

Plant material

Samples of the stem and leaves of *C. harringtonia* (Knight) K. Koch were collected at the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University, Korea in May of 2000. All samples were freeze-dried and pulverized.

Reagents

All solvents used were HPLC grade and purchased from Fisher Scientific Korea (Seoul, Korea). The purity of the carbon dioxide, purchased from Daesung Gas (Seoul, Korea), was 99.9% at a minimum. The reference compounds cephalotaxine, harringtonine, and homoharringtonine were purchased from Sigma (St. Louis, MO).

Organic solvent extraction

A pulverized sample of plant material (100 mg) was ultrasonicated with methanol for 60 min and evaporated in vacuum to dryness. Each dried extract was dissolved in 10 mL of methanol for HPLC–ESI–MS analysis.

SFE

SFE was performed using an ISCO (Lincoln, NE) Model SFX 3560 supercritical-fluid extractor with CO_2 at 40ºC, 60ºC, or 80ºC and pressures of 20.4 or 34 MPa. In order to determine the optimum volume percent of methanol to be used as a modifier, 5%, 10%, or 20% methanol (v/v); methanol–diethylamine (90:10, v/v); methanol–triethylamine (90:10, v/v); and methanol–water (90:10, v/v) were supplied by a cosolvent pump. In each experiment, the plant material (100 mg) was loaded in a 10-mL extraction cell and the remaining volume filled with a glass wool. The restrictor was kept at 80ºC, and the static extraction time was set at 15 min. The flow rate was 1.0 mL/min under each set of experimental conditions, and dynamic extraction was performed for 30 min. In each extraction step the extract was collected in methanol. The preparation of the sample solution for HPLC–ESI–MS analysis was the same as that for organic solvent extraction.

HPLC–ESI–MS–MS analysis

An Agilent (Waldbronn, Germany) 1100 series HPLC system equipped with an autosampler, a photodiode-array detector, a column oven, a binary pump, and a degasser was used. Separations were performed using an XDB-C_8 (150 × 4.6 mm, 5-mm particle size) (Agilent) at 40ºC. The mobile phase was a gradient of acetonitrile–0.1% trifluoroacetic acid (0–8 min, 10:90; 15 min, 30:70; 30 min, 50:50; 45 min, 85:15; and 60 min, 85:15). The flow rate of the mobile phase was 0.4 mL/min, and 5 mL of the sample solution was injected into the HPLC system via the autosampler. ESI–MS analysis was performed using an Agilent 1100LC/MSD ion-trap MS (Agilent) equipped with an ESI interface. Nitrogen was used as a nebulizing gas at a pressure of 50 psi at 10 L/min, a temperature of 350ºC, and a capillary voltage of –4 kV. HPLC–ESI–MS analyses were carried out in the positive-ion mode with the scan range *m/z* 50–1500.

Results and Discussion

HPLC–ESI–MS–MS analysis of the extract obtained from *C. harringtonia* leaves

A good chromatographic separation of the constituents of *C. harringtonia* leaves was achieved on the reversed-phase column using a linear gradient of 0.1% trifluoroacetic acid and acetoni-

trile. The reference compounds cephalotaxine, harringtonine, and homoharringtonine were analyzed by loop injection in order to optimize the MS conditions. MS detection was then operated in the positive-ion mode in the scan range *m/z* 50 to 1500. Figure 2 shows the EIC targeted at each $[M+H]^+$ ion of cephalotaxine, harringtonine, and homoharringtonine, of which the ESI–MS spectra shows the $[M+H]^+$ ion as the base peak at *m/z* 316, 532, and 546.

The total ion current together with the UV at 254 nm chromatogram (TIC) of the extract of *C. harringtonia* showed a series of complex peaks that could not fully be identified. Thus, MS–MS analysis was carried out in order to analyze the Cephalotaxus alkaloids in the plant extract. The reference compounds such as cephalotaxine, harringtonine, and homoharringtonine displayed a characteristic peak as the base peak in the MS–MS spectra of each $[M+H]^+$ ion. $[M+H–CH_4O]^+$ was at *m/z* 284 of cephalotaxine and *m/z* 298 of harringtonine and homoharringtonine, which might be produced from the loss of an ester chain (Figure 3). On the basis of these MS–MS results, the plant extract was reanalyzed using the extracted ion current chromatogram (EIC) of *m/z* 284 and 298 in the MS–MS of all peaks showing a defined intensity (auto-MS–MS). As a result, the analogues of cephalotaxine, homoharringtonine, and homoharringtonine could be successfully identified from the complex constituents of the plant extracts (Figure 4). As shown in Figure 5, the MS and MS–MS analysis allows the assignment of peak 1 to cephalotaxine because of the presence of *m/z* 284 and the others (peaks 2–9) to the analogues of harringtonine or homoharringtonine. The peaks 4 and 5 were identified as harringtonine and homoharringtonine, respectively, by comparison of the MS and MS–MS spectra with those of the reference compound. Peaks 2, 3, 6, 7, 8, and 9 exhibited *m/z* 548, 562, 532, 502, 516, and 530, respectively, as $[M+H]^+$. All showed *m/z* 298, thus indicating that they contain a common cephalotaxine moiety such as harringtonine and homoharringtonine. The peaks were tentatively identified as cephalezomine C, cephalezomine E, isoharringtonine, dexoxyharringtonic acid, deoxyharringtonine, and homodeoxyharringtonine, respectively, from the MS and MS–MS spectra (Figure 1).

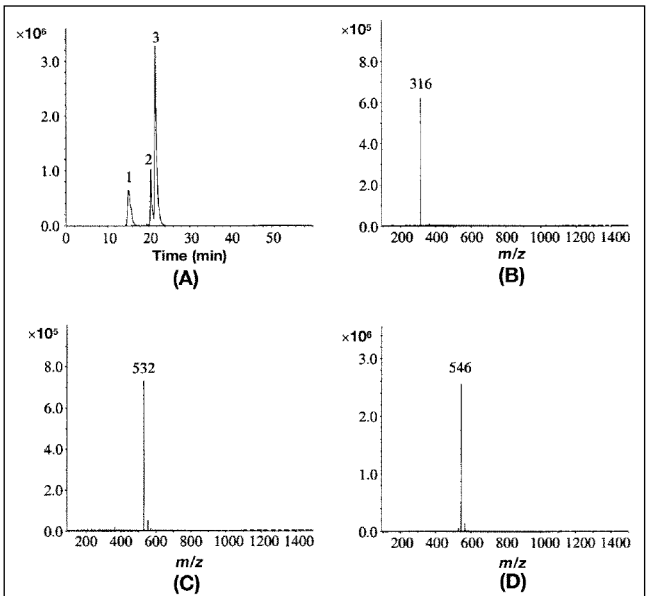

Figure 2. EIC of a standard mixture (2 mg/mL) of cephalotaxine, harringtonine, and homoharringtonine and their ESI–MS spectra: (A) EIC of the standard mixture, (B) ESI–MS spectra of cephalotaxine, (C) ESI–MS spectra of harringtonine, (D) ESI–MS spectra of homoharringtonine, (1) cephalotaxine, (2) harringtonine, and (3) homoharringtonine.

SFE of cephalotaxine, harringtonine, homoharringtonine, and isoharringtonine from *C. harringtonia* leaves

Among the many types of alkaloids isolated from Cephalotaxus species, cephalotaxine, harringtonine, homoharringtonine, and isoharringtonine are the major constituents. Although the synthesis of cephalotaxine and its esters has been reported, extraction from plants is still the major source of the alkaloids. Unfortunately, a reliable method for their quantitative analysis from plant samples has not been developed because of the complexity of the extract even though numerous trials have been attempted using HPLC–UV or GC–MS. Thus, the contents of the major Cephalotaxus alkaloids such as cephalotaxine, harringtonine, homoharringtonine, and isoharringtonine in *C. harringtonia* leaves were evaluated by HPLC–ESI–MS–MS analysis developed in this study. For this MS–MS method, an EIC was used targeting *m/z* 284 and 298 of the MS–MS spectra of each compound, for which only 10 mg of the plant sample was required for the quantitative analysis. The linear coefficients (r^2) of the calibration curve were determined to be above 0.994 (for isoharringtonine the calibration curve of harringtonine was used). As a

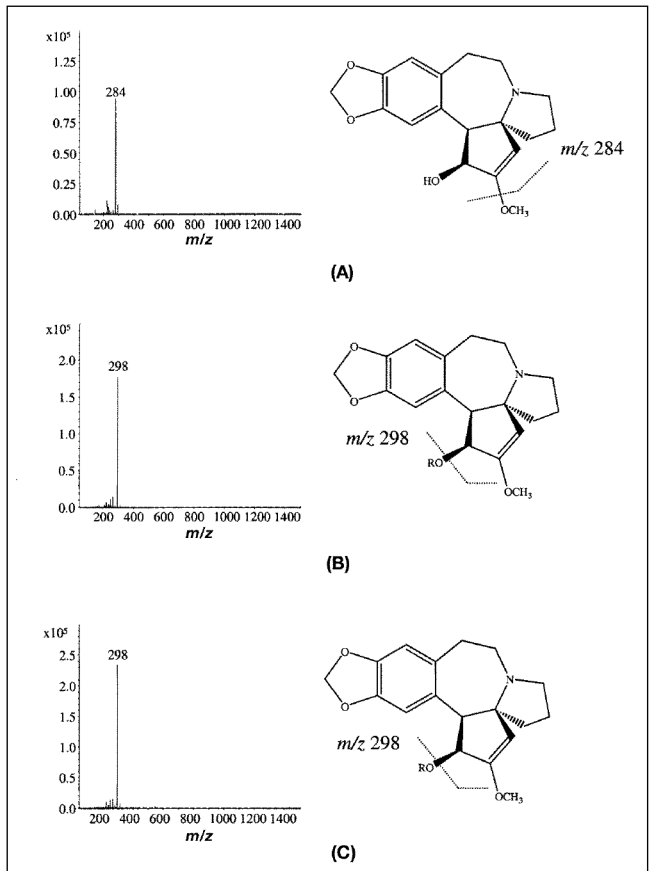

Figure 3. ESI–MS–MS spectra of $[M+H]^+$ of cephalotaxine, harringtonine, and homoharringtonine: (A) ESI–MS–MS spectra of $[M+H]^+$ of cephalotaxine, (B) ESI–MS–MS spectra of $[M+H]^+$ of harringtonine, and (C) ESI–MS–MS spectra of $[M+H]^+$ of homoharringtonine.

result, the amounts of cephalotaxine, harringtonine, homoharringtonine, and isoharringtonine were determined to be 307.8 (± 18.5) mg/g, 304.5 (± 19.5) mg/g, 304.9 (± 12.7) mg/g, and 459.7 (± 23.4) mg/g in the leaves. Figure 6 shows the TIC of MS and EIC of MS–MS targeting *m/z* 284 and 298 for a methanol extract of *C. harringtonia* leaves.

SFE technology was next applied in order to develop an alternative extraction method in an attempt to reduce the consumption of toxic organic solvents and enhance the extraction yield of the Cephalotaxus alkaloids such as cephalotaxine, harringtonine, homoharringtonine, and isoharringtonine. However, pure supercritical carbon dioxide failed to extract the Cephalotaxus alkaloids at any of the conditions of temperature and pressure evaluated (40–80ºC and 20.4–34.0 MPa). Therefore, a cosolvent was incorporated into the carbon dioxide (1–20%, v/v) in order to increase the polarity of the supercritical solvent and thus improve the extraction yield. Our previous studies showed that the addition of a small amount of a basic modifier could dramatically enhance the SFE yields of some alkaloids such as ephedrine, hyoscyamine, and scopolamine (17–19). Thus, the effects of cosolvents such as methanol, methanol–diethylamine (90:10), methanol–triethylamine (90:10), and methanol–water (90:10) were evaluated and compared with each other. As shown in Table I, SFE using carbon dioxide–methanol–water (80:18:2) at 80ºC and 34.0 MPa resulted in higher yields than any of the other SFE solvents evaluated in this study. Differently from some alkaloids such as ephedrine, scopolamine, and hyoscyamine, the basic modifiers including methanol–diethylamine and methanol–triethylamine had little effect on improving the SFE yields of Cephalotaxus alkaloids when compared with neat methanol. Otherwise, methanol–water would largely enhance the SFE yields of the alkaloids. This may be because of the fact that water aids in releasing the target compounds from the plant matrix. A recent report on the SFE of natural products concluded that the addition of a small amount of water greatly enhanced the extraction yield from plants

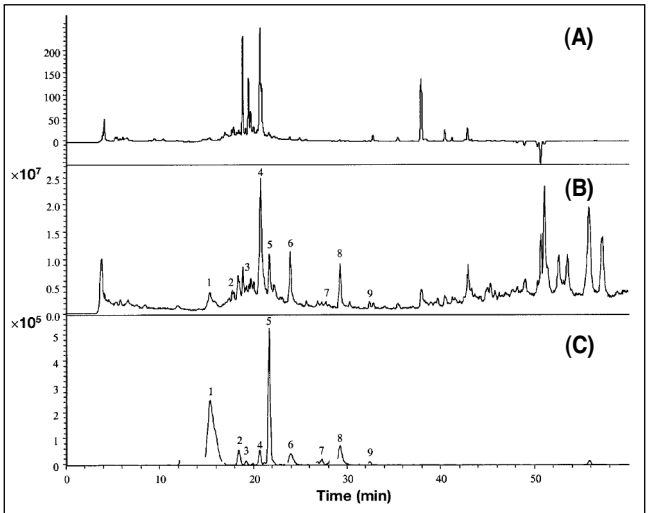

Figure 4. UV absorbed at 254 nm, A; TIC of MS, B; and EIC of MS–MS targeting at *m/z* 284 and 298, C, chromatograms of *C. harringtonia* extract: (1) cephalotaxine, (2) cephalezomine C, (3) cephalezomine E, (4) harringtonine, (5) homoharringtonine, (6) isoharringtonine, (7) dexoxyharringtonic acid, (8) deoxyharringtonine, and (9) homodeoxyharringtonine.

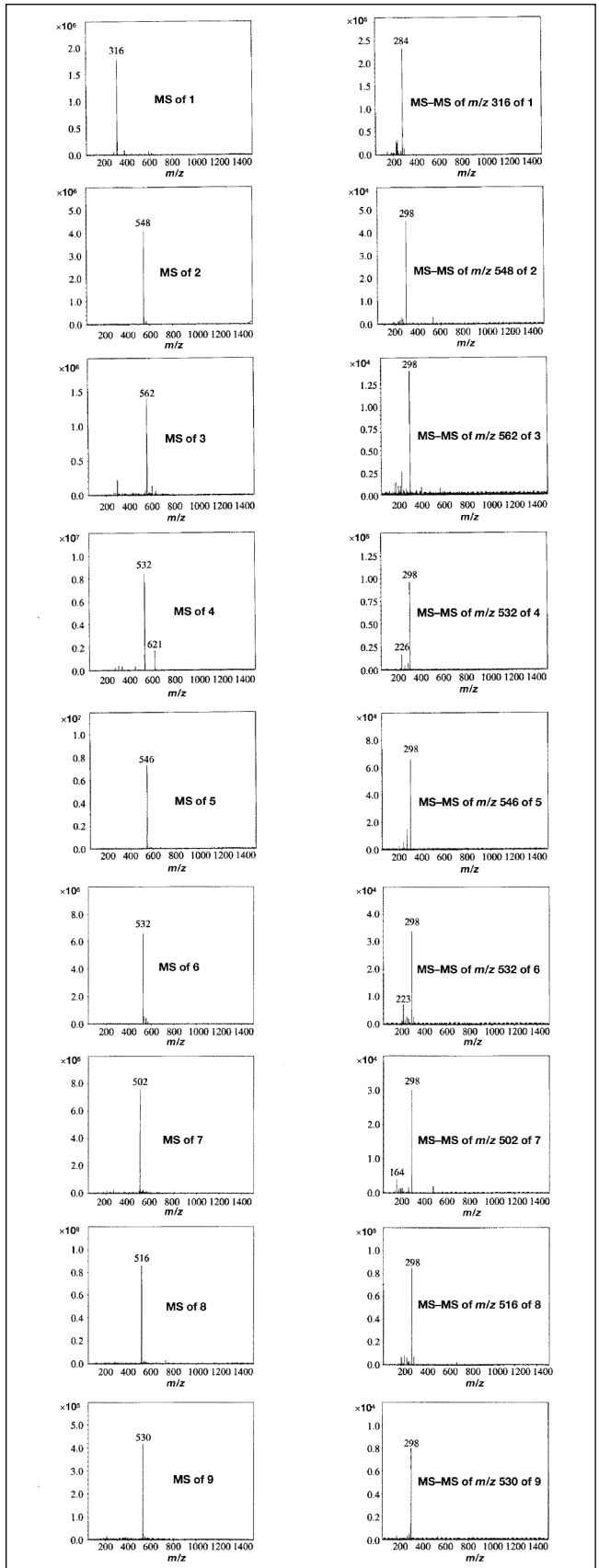

Figure 5. ESI–MS spectra of compounds 1–10 and the MS–MS spectra of each $[M+H]^+$: (1) cephalotaxine, (2) cephalezomine C, (3) cephalezomine E, (4) harringtonine, (5) homoharringtonine, (6) isoharringtonine, (7) dexoxyharringtonic acid, (8) deoxyharringtonine, and (9) homodeoxyharringtonine.

[e.g., baicalin, baicalein, and wogonin from Scutelariae Radix (20), cocaine from hair matrix (21), and stevioside from Stevia leaves (22)]. Therefore, these results suggest that the Cephalotaxus alkaloids may be freely desorbed from plant matrix by SFE solvents that contain water. SFE using a mixture of carbon dioxide–methanol–water (80:18:2) led to an increase in the yields of Cephalotaxus alkaloids by 10–33% when compared with the methanol extraction.

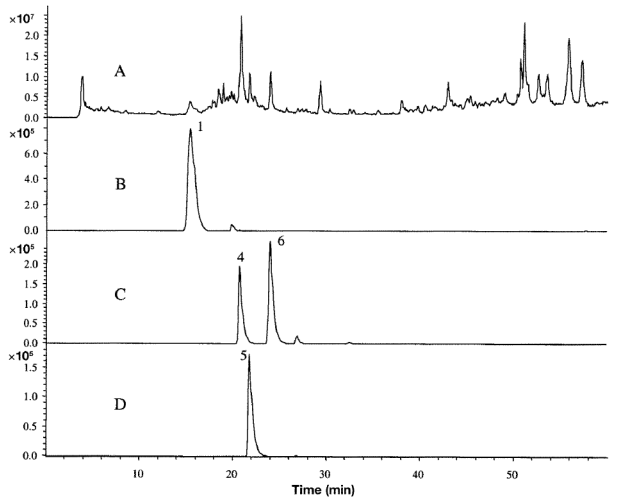

Figure 6. TIC of MS (A); EIC at *m/z* 284 in MS–MS of *m/z* 316 (B); EIC at *m/z* 298 in MS–MS of *m/z* 532 (C); and EIC at *m/z* 298 in MS–MS of *m/z* 546 (D), of methanol extracts of *C. harringtonia* leaves.

Table I. Extraction Yield* of Cephalotaxine, Harringtonine, Homoharringtonine, and Isoharringtonine from the Stems of *C. harringtonia* Obtained by SFE at 80°C and 34.0 MPa and Methanol Extraction†

Extraction method SFE		Compound (µg/g)			
Kind of cosolvent	% of cosolvent	Cephalotaxine	Harringtonine	Homoharringtonine	Isoharringtonine
Methanol	1%	20.5 ± 4.2	45.6 ± 6.3	74.7 ± 10.8	153.6 ± 9.7
	5%	51.5 ± 10.6	80.2 ± 16.1	107.0 ± 19.3	203.3 ± 25.6
	10%	172.7 ± 19.0	135.1 ± 14.1	163.8 ± 14.8	288.0 ± 26.3
	20%	251.1 ± 25.2	187.1 ± 13.5	227.5 ± 23.3	402.7 ± 33.0
Methanol–diethylamine (9:1)	1%	–‡	80.3 ± 19.0	112.8 ± 20.5	219.4 ± 40.2
	5%	193.5 ± 41.9	183.3 ± 44.7	225.2 ± 45.1	374.8 ± 46.3
	10%	234.0 ± 22.1	220.6 ± 19.8	300.1 ± 24.2	485.6 ± 43.1
	20%	273.6 ± 9.6	236.4 ± 10.9	298.6 ± 12.5	474.2 ± 38.7
Methanol–triethylamine (9:1)	1%	–	–	–	–
	5%	73.2 ± 18.9	48.7 ± 6.8	84.6 ± 9.8	143.9 ± 13.3
	10%	153.1 ± 12.7	80.3 ± 17.2	115.5 ± 20.1	241.2 ± 43.3
	20%	253.0 ± 29.0	129.2 ± 10.7	183.5 ± 15.3	362.8 ± 35.1
Methanol–water (9:1)	1%	–	–		– –
	5%	84.6 ± 3.7	220.2 ± 18.4	280.4 ± 22.5	428.0 ± 23.7
	10%	181.2 ± 38.6	282.1 ± 18.0	354.8 ± 23.5	495.2 ± 34.9
	20%	338.4 ± 52.9	348.8 ± 16.5	405.0 ± 27.8	575.2 ± 31.1
Methanol extraction		307.8 ± 18.5	304.5 ± 19.5	304.9 ± 12.7	459.7 ± 23.4

* µg/g ± standard deviation.
† Based on triplicate.
‡ Trace amount (< 10 µg/g).

Conclusion

The purpose of this study was to develop a reliable quantitative analysis and method for extracting the antileukemic alkaloids from *C. harringtonia* leaves using HPLC–ESI–MS–MS and SFE. HPLC–ESI–MS–MS in the positive-ion mode proved to be a highly sensitive analytical method for the detection of the alkaloids that are impossible to detect using conventional HPLC–UV or GC–MS. This HPLC–ESI–MS–MS technique is also thought to be a promising analytical method for detecting alkaloids from any other plant resource in addition to the plant source *C. harringtonia* used in this study.

For developing an alternative extraction method of Cephalotaxus alkaloids such as cephalotaxine, harringtonine, homoharringtonine, and isoharringtonine, SFE efficiency was evaluated with temperature, pressure, type, and percent of modifier as variables. As a result, increasing the temperature and pressure had little effect on the SFE efficiency of the alkaloid because of its higher polarity. Therefore, a polar modifier was introduced into the CO_2. Among the modifiers evaluated, a mixture of methanol and water showed a greater extraction efficiency than any other mixture tested.

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