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Development of a pre-purification process for homoharringtonine

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Abstract: A novel pre-purification method was developed for producing homoharringtonine from *Cephalotaxus koreana*, giving high purity and yield. The simple, efficient procedure involved biomass extraction, liquid–liquid extraction, and synthetic adsorbent treatment, followed by low-pressure chromatography. The use of active clay treatment and silica gel low-pressure chromatography in the pre-purification process allowed for the rapid, efficient separation of homoharringtonine from interfering compounds and, compared with alternative processes, increased the yield and purity of crude homoharringtonine for subsequent high-performance liquid chromatography (HPLC) purification. Homoharringtonine of over 52% purity could be obtained simply with high yield from biomass using this pre-purification method, while minimizing solvent use and the scale and complexity of HPLC operations for homoharringtonine purification.

Keywords: homoharringtonine (HHT); active clay treatment; silica low-pressure chromatography; pre-purification

INTRODUCTION

Homoharringtonine (HHT) (Fig 1), an alkaloid isolated from the genus *Cephalotaxus*, is an alkyl-substituted succinic acid ester of cephalotaxine,[1,2] it possesses antileukemic activity and is a potent myelosuppressive agent.[3–6] Several researchers have investigated the antineoplastic mechanism of HHT and related alkaloids; all have concluded that these drugs inhibit protein biosynthesis in the cell. HHT and congeners cause the breakdown of polyribosomes to monosomes, the release of completed globin chains, and delayed inhibition of the initiation of protein synthesis without affecting chain elongation. HHT has been tested clinically in advanced breast cancer, acute myelogenous leukemia, myelodysplastic syndrome (MDS), and MDS evolving to acute myeloid leukemia.[7–11] Although the chemical synthesis of cephalotaxine and its esters has been reported, extraction from plants is still the major source of HHT.[12,13]

There are few reports of procedures for isolating and purifying HHT that are directly applicable to commercial-scale operations.[14] Existing purification methods using solvent extraction and chromatography procedures primarily aim to obtain crude HHT of low purity (1–4%) and provide a mixture of HHT and related compounds, such as terpenoids, lipids, chlorophyll, and phenols.[15] Consequently, high-purity HHT has not been obtained, even with multiple chromatographic columns and large solvent volumes, moreover there is a heavy impurity load on the chromatography columns used. As the solubility of HHT in organic solvents is very low, the purity and yield during chromatography are not easily controlled, and crystallization steps are essential to obtain high-purity HHT. Since the existing purification methods are impractical, there remains a need for a method of isolating high-purity HHT in a simple, economical manner.

This work developed a new pre-purification method for industrial production of HHT from *C koreana*. The method is rapid, simple and efficient, yielding HHT with high purity and yield. The pre-purification process serves to minimize solvent usage and the scale and complexity of the high-performance liquid chromatography (HPLC) equipment needed for HHT purification.

MATERIALS AND METHODS

Plant material

The bark and needles were collected from Korean plum yew (*C koreana*) growing on Mt Kyeryong, South Korea, in November 2002. The bark and needle samples were freeze-dried and ground in a mortar and pestle. The dried powder (bark/needle = 1/4, w/w) was used for all the subsequent process development work.

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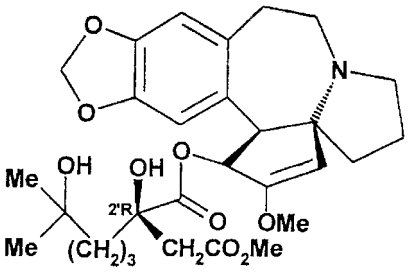

Figure 1. Structure of homoharringtonine.

Analysis of HHT

An HPLC system (Waters, Milford, USA) was used for the analytical characterization of the intermediate and finished products. A C18 column (4.6 × 250 mm, 5 μm, Shiseido, Tokyo, Japan) was eluted with a methanol/0.1 mol ammonium formate gradient from 25:75 (v/v) to 45:55 (v/v) at a flow rate of 1.0 mL min^{-1}. The injection volume was 20 μL, and the effluent was monitored at 290 nm with a UV detector. The dried residue was redissolved in methanol and used for the quantitative analysis of HHT. Authentic HHT (purity: 98.6%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as a standard.

Biomass extraction and liquid–liquid extraction of HHT from biomass

Biomass extraction

The plant biomass was mixed with methanol and stirred at room temperature for 30 min. This mixture was filtered through filter paper in a Buchner funnel under vacuum. The biomass was added to methanol at a ratio of 1:8(w/v). The extraction was repeated at least three times. Each methanol extract was collected, pooled, and concentrated at 40 °C under reduced pressure to decrease the volume of the methanol extract to 20% of the original.

Liquid–liquid extraction

The concentrated methanol extract was added to organic solvents (methylene chloride, chloroform, diethylether, and hexane) at a volume ratio of 4:1 for liquid–liquid extraction, and this was extracted at room temperature for 30 min. The extraction was repeated at least four times, and the crude extracts were pooled and dried at room temperature under reduced pressure.

Adsorbent treatment of the crude extract

The dried crude extract from the liquid–liquid extraction was dissolved in methanol at a ratio of 20 of dried crude extract (v/w), and several synthetic adsorbents were added and tested individually, including the active clays P-1 and P-1G (Mizukalife Chemical Co, Tokyo, Japan), the activated carbons CA-1 and SX-PLUS (Norit, Amersfoort, The Netherlands), and charcoal (Merck, Frankfurt, Germany) at a ratio of 50% (w/w) of dried crude extract. The mixtures were stirred at room temperature for 30 min and filtered with the appropriate synthetic adsorbent to obtain the filtration solution. The adsorbent cake thus obtained was washed several times with chloroform/ethanol (1/2, v/v) and washings were combined with the filtration solution. The solution was dried at 40 °C under a reduced pressure for the chromatography.

Silica gel low-pressure chromatography

The dried crude extract obtained after the adsorbent treatment was dissolved in methanol at a ratio of 10 of extract (v/w) and then filtered through diatomaceous earth (Fuji Silysia Chemical Ltd, Tokyo, Japan). The filter aid was washed five times with methanol, and the washings were combined. The resulting solution was applied to a 25 × 400 mm column packed with silica gel 60N (Merck, Germany), which was equilibrated with methylene chloride/methanol (80/20, v/v). The column was eluted using an isocratic method with the same solution. The fractions containing HHT were collected and dried by rotary evaporation.

RESULTS AND DISCUSSION

Biomass extraction

Several solvents or combinations of solvents have been tested for biomass extraction. As shown in Table 1, methanol gave the highest HHT recovery with the least amount of solvent and was chosen for all the subsequent work. Moreover, at least three extractions were required to obtain a high yield (>99%) from the biomass (Fig 2). Several different modes of extracting biomass are possible, and these have a marked effect on solvent usage.[16,17] We used a batch extraction process in which biomass was extracted sequentially with fresh solvent at each of three extraction stages. In the batch mode of operation, equilibrium (ie the ratio of HHT in biomass to HHT in the extraction solvent) was reached within 20 min, and neither the equilibrium nor the extraction time were greatly affected by temperature (5–50 °C) (data not shown). After three biomass extractions with methanol, the methanol extract was concentrated in rotary evaporators at a vacuum of 635 mm Hg

Table 1. Effect of solvents on biomass extraction (5 g biomass/40 mL solvent, room temperature, 30 min reaction)

Solvent	Polar impurity[a]	HHT amount	Nonpolar impurity
Methanol	+++	++++	−
Diethylether	−	−	−
Propyl alcohol	++	++	−
Acetone	+	+	−
Chloroform	−	−	−
Chloroform/ethanol (9/1)	+	+	−
Chloroform/ethanol (8/2)	++	++	−
Methylene chloride	−	−	−
Methylene chloride/ethanol (9/1)	+	+	−
Methylene chloride/ethanol (8/2)	++	++	−

[a] +: High extraction efficiency, −:low extraction efficiency.

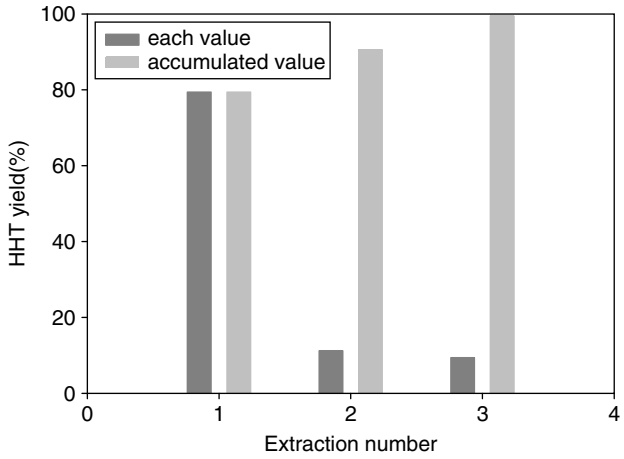

Figure 2. Effect of extraction number on homoharringtonine recovery from biomass.

and 40 °C. This crude extract was used for the subsequent processes to optimize the pre-purification of HHT.

Liquid–liquid extraction

The methanol extract was reduced to 20% of the original volume. With excessive concentration of the extract, a precipitate formed in the solution, which had a negative influence on the subsequent procedures. To eliminate polar impurities, the efficiency of liquid–liquid extraction was assessed using methylene chloride, chloroform, diethylether, and hexane/methylene chloride in a batch process. Of these, the best result was obtained with chloroform (data not shown). At least four liquid–liquid extractions were needed to obtain a high yield (>90%), as shown in Fig 3. At the liquid–liquid extraction step, the polar impurities were removed from the lighter phase (methanol and water solution) efficiently, as shown in Fig 4(A and B). The pH, an important process variable in liquid–liquid extraction, of the lighter phase was optimized in terms of the yield of HHT. The highest yield (>90%) of HHT was obtained from the heavier phase (chloroform phase) at pH 5.0 (Fig 5). The yield and purity of the dried extract were 90% and 8.0%, respectively.

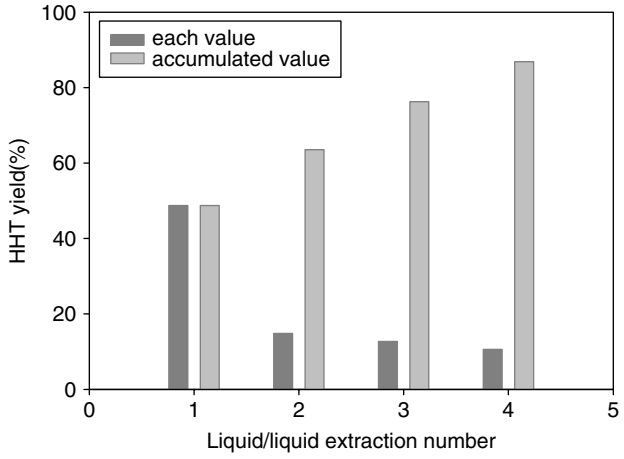

Figure 3. Effect of liquid–liquid extraction number on homoharringtonine recovery from the methanol extract.

Adsorbent treatment

To further remove impurities, which were a deeply colored, tar-like, insoluble material in chloroform, the crude extract was treated with various synthetic adsorbents, such as active clay (P-1, P-1G), activated carbon (CA-1, SX-PLUS), and charcoal. Of these, active clay P-1 was the most efficient in terms of yield, purity, and removal of impurities (Table 2, Fig 4(C)). Conversely, activated carbon and charcoal were more efficient than active clay in terms of removing the color from the crude HHT. Generally, activated carbon is usually used as an absorbent and has been used broadly to decolorize natural products and water, as well as to remove impurities from the crude product.[18–20]

Nair has also reported the use of charcoal to remove the color from a crude extract of paclitaxel.[21] The optimal amount of active clay P-1 was 50% (w/w) at room temperature for 30 min (data not shown). The washing solvent was optimized by testing various solvents [ethanol; methylene chloride; chloroform; methylene chloride/ethanol, 2/1 (v/v); and chloroform/ethanol, 1/2 and 1/1 (v/v)]. The highest yield was obtained by washing with chloroform/ethanol (1/2, v/v), as shown in Table 3. The amount of washing solution was adjusted to 20 times the volume of the dried extract, because additional washing solution reduced the purity of the resulting solution. Although this step seemed to improve the purity only slightly, this treatment had a great effect on the convenience and feasibility of the following steps by removing waxy substances. The

Table 2. Comparison of active clays and charcoals in the adsorbent treatment step

	Color[a]	Purity (%)	Yield (%)
Starting	10	8.0	—
P-1	4	10.0	90
P-1G	4	9.5	75
CA-1	2	8.9	20
SX-PLUS	2	8.5	30
Charcoal	2	8.4	45

[a] Color; deep(10)–light(1).

Table 3. Effect of the washing solvent on active clay (P-1) treatment

Solvent	Polar impurity[a]	HHT amount	Nonpolar impurity
Ethanol	++++	++++	–
Methylene chloride	–	–	–
Chloroform	–	–	–
Methylene chloride/ethanol (2/1)	++	++	–
Chloroform/ethanol (1/2)	+++	++++	–
Chloroform/ethanol (1/1)	++	+++	–

[a] +: High extraction efficiency, –:low extraction efficiency.

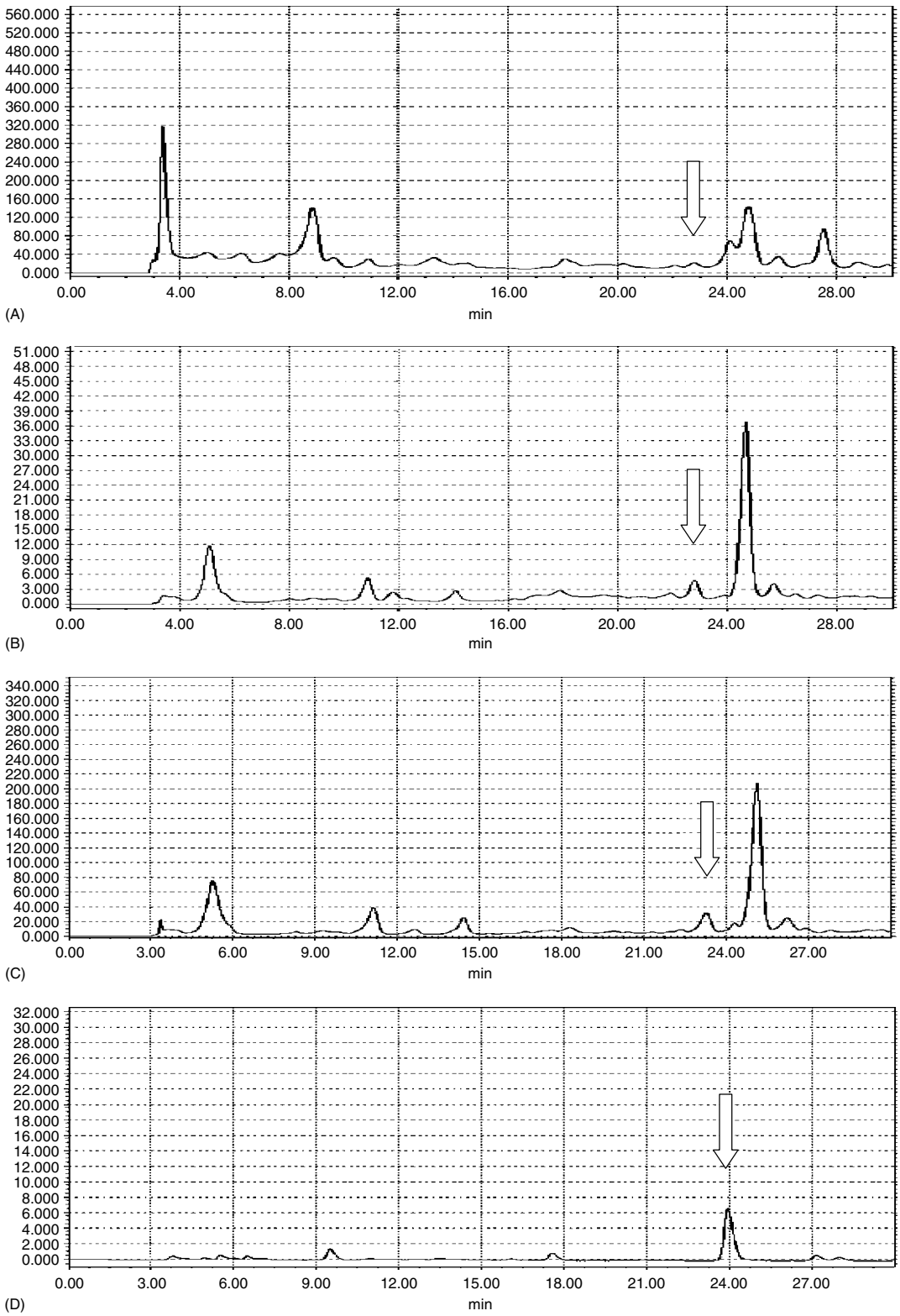

Figure 4. Chromatogram of the purification steps analyzed using RP-HPLC: biomass extraction with methanol (A), liquid–liquid extraction with chloroform (B), adsorbent treatment with active clay (P-1) (C), and low-pressure chromatography with silica gel (D). The arrow indicates the position of HHT.

yield and purity of HHT in the adsorbent treatment step were 90% and 10.0%, respectively.

Low-pressure chromatography

To optimize the low-pressure chromatography conditions, experiments were carried out using an isocratic method, which is useful owing to its advantages of simple equipment and operation, and good resolution.[22] The eluting solvents used for chromatography were methylene chloride and methanol. Under isocratic conditions for silica gel low-pressure chromatography, the efficiency of purity and yield were compared for

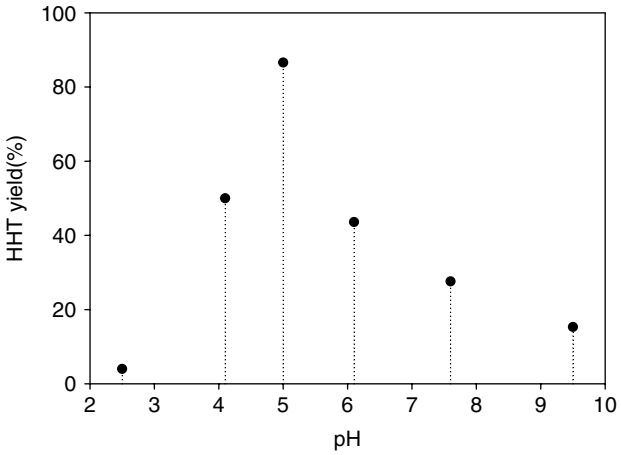

Figure 5. Effect of lighter phase pH value on homoharringtonine yield in liquid–liquid extraction.

different solvent ratios. On elution with methylene chloride/methanol (80/20, v/v), the extract was purified to greater than 52% with an 85% step yield (Fig 4(D)). The extracts from biomass were pre-purified efficiently, and the purity increased from 10% to over 52% with low-pressure chromatography under isocratic conditions. A schematic showing the major elements of the pre-purification process and the results obtained for our pre-purification of HHT from biomass are summarized in Fig 6 and Table 4. The purity (52%) of the crude HHT ensures that a minimum of material enters the HPLC purification process, thereby minimizing costs. The method described here is a simple, efficient procedure for isolating and pre-purifying HHT from *C koreana* biomass. The procedure consists of biomass extraction, liquid–liquid extraction, and active clay treatment, followed by silica gel low-pressure chromatography. Compared with alternative processes,[14,15] the use of active clay treatment and silica gel low-pressure chromatography in the pre-purification process allowed the rapid separation of HHT from interfering compounds. This pre-purification process serves to minimize the size and complexity of the HPLC operations for HHT purification.

Table 4. Summary of the pre-purification of homoharringtonine from biomass (16 g)

	HHT (g)	Purity (%)	Step yield (%)	Yield (%)
Biomass	0.055	—	100.0	100.0
Biomass extraction	0.055	0.5	99.0	99.0
Liquid–liquid extraction	0.049	8.0	90.0	89.1
Adsorbent treatment	0.044	10.0	90.0	80.2
Low-pressure chromatography	0.038	52.0	85.0	68.2

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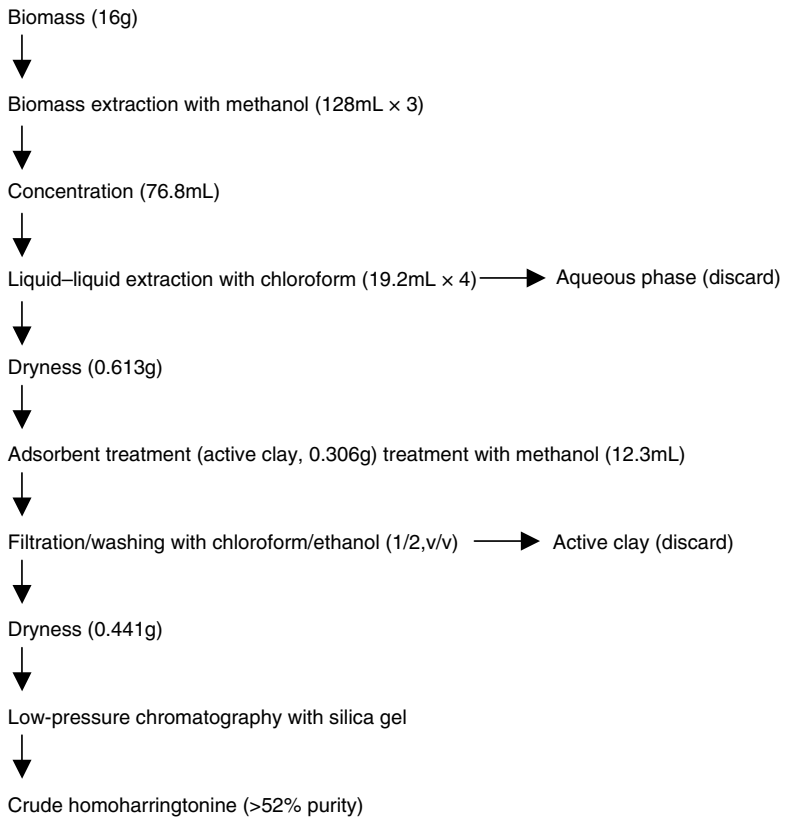

Figure 6. Schematic of recovery/pre-purification process.

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