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RESEARCH ARTICLE

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Large-scale purification of unstable, water-soluble secologanic acid using centrifugal partition chromatography

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Abstract

Introduction: Secologanic acid, a major secoiridoid in the flower buds of *Lonicera japonica*, is a fragile, highly polar compound that readily changes to epivogeloside or vogeloside after being dissolved in methanol. Thus, it is very difficult to obtain secologanic acid on a large-scale.

Objective: To develop a centrifugal partition chromatography (CPC) method for large-scale purification of secologanic acid with high purity from the flower buds of *L. japonica*.

Methods: After fractionation with Diaion HP-20 macroporous resin, 30% methanol eluent was purified by CPC with a ternary biphasic solvent system with ethyl acetate/isopropanol/water (6:4:10, *v*/*v*/*v*). CPC was performed separately twice with the same solvent system, first in descending mode and second in ascending mode.

Results: After the first CPC operation, a secologanic acid enriched fraction (586 mg) was obtained from 3 g of crude extract, and secologanic acid (206 mg) was isolated with a purity over 93% in the subsequent ascending mode with the same solvent system from a 586 mg enriched fraction. In addition, it was confirmed that epivogeloside and vogeloside were reversely converted to secologanic acid in an aqueous acidic solution.

Conclusion: These results demonstrate that CPC is a simple, effective, and rapid method for the purification of secologanic acid in the flower buds of *L. japonica*.

KEYWORDS

centrifugal partition chromatography (CPC), epivogeloside, *Lonicera japonica* L, secologanic acid, vogeloside

1 | INTRODUCTION

The flower buds of *Lonicera japonica* Thunb. (Caprifoliaceae) are traditionally used as a herbal medicine in the treatment of various diseases, including acute fever, pharyngodynia, respiratory infection, infections, sores, swelling, arthritis and diabetes mellitus. The chemical constituents of *L. japonica* have been identified as caffeoylquinic acids, secoiridoids, homosecoiridoids, nitrogen containing iridoids, flavonoids, saponins and cerebrosides.[1-6] Among these compounds, secologanic acid is a major component but the biological activities of secologanic acid have been only reported to be for the inhibition of nitric oxide production in lipopolysaccharide (LPS)-activated macrophages.[7,8] Vogeloside and epivogeloside, unwanted products of secologanic acid, were found to exert a weak inhibitory effect on the growth of influenza A/PR/8/34.[9,10] Recently, HS-23, a Lonicerae Flos extract, improved sepsis-induced mortality via inhibition of IL-1 receptor-associated kinase 4,[11] suppression of toll-like receptor 4 signalling pathways[12] and inhibition of lymphocyte apoptosis.[13] In HS-23, caffeoylquinic acids were identified but other major constituents were not identified.[13] Pharmacological studies and quality control of herbal medicines require a significant amount of purified compounds. In this respect, secologanic acid (**1**) has a disadvantage in that it is very difficult to separate. Secologanic acid is highly polar and is easily converted to epivogeloside (**1a**) or vogeloside (**1b**) in a methanolic solution (Figure 1).[10] Secologanic acid reacts with acidic substances such as silica gel, which is widely used for separation and purification, to produce surplus products. CPC can be used for mass separation and purification of secologanic acid as a method to reduce remnant production. In addition, since secologanic

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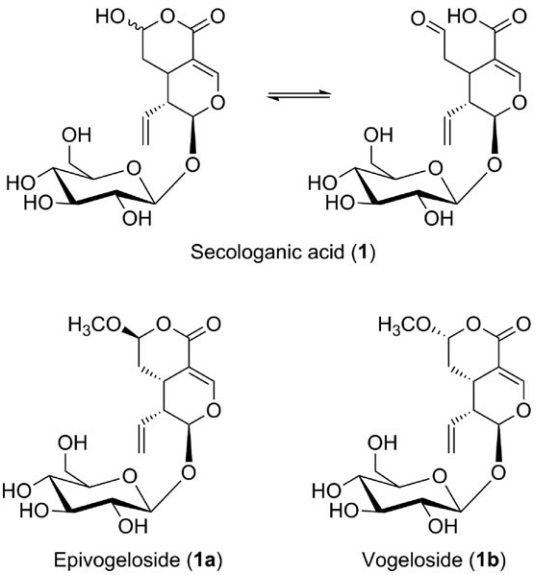

FIGURE 1 Chemical structures of secologanic acid (**1**) and its unwanted products, epivogeloside (**1a**) and vogeloside (**1b**)

acid has a high polarity, it is easy to produce unwanted products because only methanol can be used with the use of silica gel or silica-based ODS column to separate it. To overcome these disadvantages, the CPC method was introduced. CPC, a solid free separation based on liquid–liquid partition, possesses many advantages such as a higher loading capacity and the absence of sample loss or irreversible sample absorption to a solid column. Moreover, CPC has the ability to separate fragile compounds because there is no chemical reaction,[14] and it is also applicable to various types of compounds with a flexible operation mode and the choice of a wide range of solvent systems.[15-20]

In this study, we develop the CPC methods for fragile and highly polar natural secologanic acid (**1**). Furthermore, it was confirmed that secologanic acid (**1**) converted to epivogeloside (**1a**) or vogeloside (**1b**), unwanted products of secologanic acid in methanolic solution, and reversed in an aqueous solution.

2 | EXPERIMENTAL

2.1 | Apparatus

CPC was performed on an Armen fully integrated SCPC-1000 CPC spot instrument (Armen Instrument, St-Ave, France). This instrument is a fully automated system consisting of a CPC column compartment, pump, injector, UV-vis detector, fraction collector, digital screen flat PC, and Armen Glider CPC software (Armen Instrument). The Agilent 1260 HPLC system (Agilent Technologies, Palo Alto, CA, USA) consists of a G1312C binary pump, G1329B autosampler, G1315D DAD detector, G1316A column oven and ChemStation software.

2.2 | Reagents and materials

All HPLC-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). The organic solvents used for extraction and CPC operation were obtained from Daejung (Gyeonggi-do, Korea).

2.3 | Preparation of the crude sample

The flower buds of *L. japonica* were purchased from the Kyungdong oriental herbal market, Seoul, Korea, in November 2013. A voucher specimen was deposited in the Herbarium of the College of Pharmacy, Hanyang University (HYUP-LJF-001). The crude extract of *L. japonica* was obtained by a previously described method.[11] Briefly, flower buds of *L. japonica* were extracted with hot water (10 L × 2) for 3 h at 100°C. The hot water extract was partitioned with ethyl acetate. The aqueous partition was then chromatographed over a Diaion HP-20 gel column with 100% water and 30% methanol in water, and 30% methanol eluent was used as the crude sample.

2.4 | HPLC analysis of crude extract and CPC peak fractions

Crude extract and CPC peak fractions were analysed by high-performance liquid chromatography (HPLC) using an Agilent 1260 HPLC system equipped with an Inno C18 column (250 mm × 4.6 mm, 5 μm, YoungJin Biochrom Co., Ltd, Seongnam, Korea). The mobile phase was a linear gradient of acetonitrile with 0.1% formic acid (A) and water with 0.1% formic acid (B): 0–2 min, 10% A; 2–20 min, 10–60% A. The flow rate was 1 mL/min while the injection volume was 10 μL. The diode array detector (DAD) measured the UV spectrum over a range from 210 to 400 nm and the chromatogram of the effluents was recorded at 280 nm.

2.5 | Selection of a ternary biphasic solvent system

The partition coefficient of secologanic acid in various ternary biphasic solvent systems was determined by HPLC. As secologanic acid is polar, polar ternary biphasic conditions were tested to find a suitable solvent system. Briefly, 10–15 mg of crude sample was transferred to a 2 mL tube and dissolved with 1.8 mL of the respective phase (1:1, *v*/*v*) of two-phased immiscible solvent systems. The tube was then shaken, followed by thorough equilibration with a centrifuge. After 1 min of centrifugation, the equal volume of the upper and lower phases were analysed separately by HPLC to examine the applicability of the two-phase solvent system. The *K* values of the secologanic acid were calculated by the following equation: *K* = the peak areas of the upper phase in HPLC/the peak areas of the lower phase in HPLC.

2.6 | CPC operation

According to the appropriate *K* value, CPC separation was performed with a ternary biphasic system consisting of ethyl acetate/isopropanol/water with a volume ratio of 6:4:10 (*v*/*v*/*v*). The upper and lower phase solvents were obtained after the three solvents were mixed vigorously and equilibrium was reached. In the first separation, the CPC rotor was first entirely filled with the upper organic phase as the stationary phase at a flow rate of 5 mL/min with 300 rpm rotation. Then, the rotor was rotated at a speed of 1200 rpm, and the lower aqueous phase was pumped into the column in descending mode at a flow rate of 10 mL/min. After equilibrium was reached as indicated by the mobile phase eluting from the outlet (stationary

retention volume: 550 mL, operation pressure: 29 bar), the sample solution (3 g of crude sample in 20 mL of mixed lower and upper phase) was injected. The effluent from the outlet was monitored at 254 and 280 nm with a UV detector. The respective peaks from the resulting chromatogram were gathered and concentrated. After HPLC analysis, the secologanic acid rich fraction (**Fr. I**) was further purified by the second CPC operation, which was carried out using the same two-phase solvent system and the same operation method. The only difference was a switch of the mobile phase and stationary phase. In the second separation, the upper organic phase was used as the mobile phase and the lower aqueous phase was used as the stationary phase. The second CPC conditions were as follows: flow rate, 10 mL/min; rotation speed, 1200 rpm; sample solution, 586 mg of **Fr. I** in 18 mL of lower phases; UV detector, 254 and 280 nm. After equilibrium was reached as indicated by the mobile phase eluting from the outlet (stationary retention volume: 720 mL, operation pressure: 31 bar)

2.7 | Structural elucidation

Isolated compounds were identified by their ^{1}H-NMR, ^{13}C-NMR and electrospray ionisation mass spectrometry (ESI-MS) spectral data. NMR spectra were obtained in dimethyl sulfoxide (DMSO-d_6) using an AVANCE III 400 spectrophotometer (Bruker, Germany). ^{1}H-NMR and ^{13}C-NMR spectra were processed using the MestReNova program (Mestrelab Research, Santiago de Compostela, Spain; version 6.0.3). ESI-MS was performed with an Advion Expression CMS system (New York, USA). The ESI-MS spectra conditions were as follows: negative ion mode; mass range, *m/z* 100–1200; capillary temperature, 200°C; capillary voltage, 150 V; source voltage offset, 30; source voltage span, 10; source gas temperature, 250°C; ESI voltage, 3500 V.

2.8 | Stability of secologanic acid in acetonitrile or methanol

Secologanic acid (**1**) was dissolved in 100% methanol, 50% methanol, 100% acetonitrile, 50% acetonitrile or water, and incubated for 7 h. Each sample was analysed by an Agilent 1260 HPLC system with an Inno C18 column (150 mm × 2.0 mm, 5 μm, YoungjinBiochrome Co., Seongnam, Korea). The mobile phase was a linear gradient of acetonitrile with 0.1% formic acid (A) and water with 0.1% formic acid (B): 0–3 min, 10% A; 3–20 min, 10–20% A. The flow rate was 0.3 mL/min while the injection volume was 1 μL. The UV wavelength was at 254 nm.

2.9 | Conversion of epivogeloside (1a) or vogeloside (1b) into secologanic acid (1) in an acidic aqueous condition

Mixtures of epivogeloside (**1a**) and vogeloside (**1b**) were dissolved in acidic methanol and acidic water containing 5% acetic acid. After incubation of the indicated time, each sample was analysed by HPLC. The HPLC conditions were same as mentioned earlier.

3 | RESULTS AND DISCUSSION

3.1 | Selection of a ternary biphasic solvent system

Successful separation of the target compounds by CPC requires the choice of an appropriate biphasic solvent system. Secologanic acid (**1**) is very polar, so a biphasic solvent system was composed for polar conditions. Ternary biphasic solvent systems for polar natural products used ethyl acetate or *n*-butanol as less-polar solvent, and water used as more-polar solvent. Tetrahydrofuran (THF), acetonitrile, isopropanol, ethanol or methanol were used as best solvent to get appropriate *K* values.[21-24] Several variations of ternary biphasic solvent systems, each composed of ethyl acetate/isopropanol/water (*v*/*v*/*v*), ethyl acetate/acetonitrile/water (*v*/*v*/*v*), or ethyl acetate/*n*-butanol/water (*v*/*v*/*v*) in a different ratio, were tested to determine the appropriate partition coefficients (*K* values) The *K* values for the various solvent ratios are summarised in Table 1. In addition to more propanol, acetonitrile or *n*-butanol in the ternary biphasic solvent system increased the *K* values, but the setting time of the solvent system was not satisfied. Thus, ethyl acetate/isopropanol/water (6:4:10, v/v/v) was chosen for CPC operation.

3.2 | CPC operation

HPLC analysis showed that secologanic acid (**1**) was one of the main peaks in the crude sample (30% methanol eluent in Diaion HP-20 macroporous resin) (Figure 2a). Initially, according to the *K* value, the ethyl acetate/isopropanol/water (6:4:10, *v*/*v*/*v*) system was used to purify secologanic acid in the descending mode; lower aqueous solution was used as the mobile phase. The secologanic acid (**1**) enriched fraction was obtained at 1.14–1.42 h (Figure 2b,d). In Table 2, the expected retention time of secologanic acid (**1**) where the retention time was calculated as the partition coefficient = 0.33, stationary retention volume = *c*. 550 mL (retention of stationary: 52.3%).[25] After evaporation, the secologanic acid rich fraction (peak I in Figure 2d) was analysed by HPLC, which revealed that peak fraction I contained some impurities (Figure 2b). In particular, two peaks (**1a** and **1b**) around 13 min were observed in the HPLC result (Figure 2b). These two peaks seemed to be unwanted products of secologanic acid where the UV

TABLE 1 The partition coefficient (*K*) values of secologanic acid (**1**) in various solvent systems

Two-phase solvent systems (*v*/*v*/*v*)	*K* value for secologanic acid
ethyl acetate/isopropanol/water 8:2:10	0.13
ethyl acetate/isopropanol/water 7:3:10	0.23
ethyl acetate/isopropanol/water 6:4:10	0.33
ethyl acetate/acetonitrile/water 9:1:10	0.01
ethyl acetate/acetonitrile/water 8:2:10	0.02
ethyl acetate/acetonitrile/water 7:3:10	0.03
ethyl acetate/*n*-butanol/water 9:1:10	0.03
ethyl acetate/*n*-butanol/water 8:2:10	0.07
ethyl acetate/*n*-butanol/water 7:3:10	0.10

The *K* values of the secologanic acid were calculated by the following equation: *K* = the peak areas of the upper phase in HPLC/the peak areas of the lower phase in HPLC.

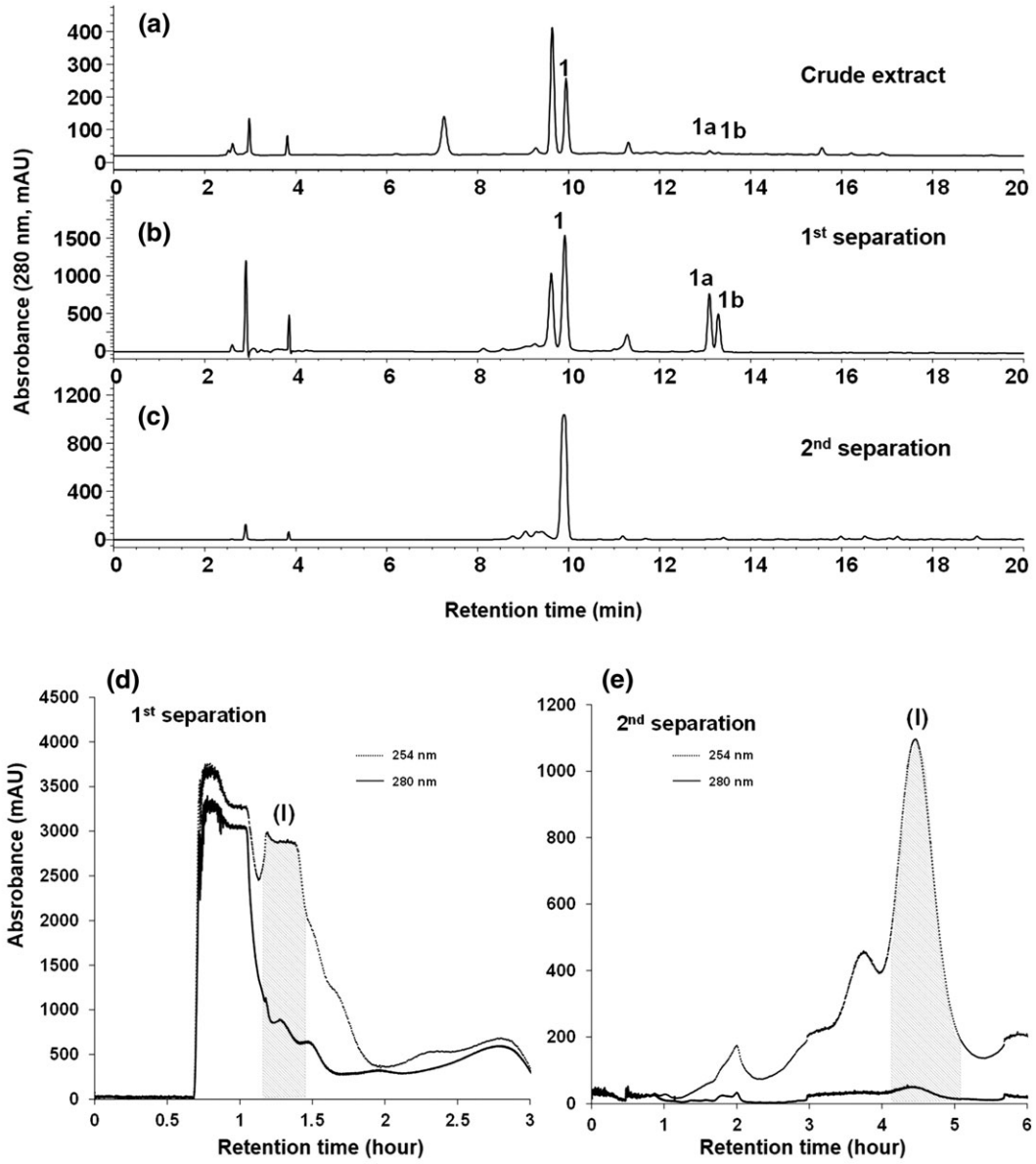

FIGURE 2 HPLC (a–c) and CPC (d–e) chromatograms of crude sample from the flower buds of *Lonicera japonica*. (a) Crude sample of 30% methanol eluent in Diaion HP-20 macroporous resin; (b) peak fraction I after first CPC operation (ascending mode); (c) peak fraction I after second CPC operation (ascending mode); (d) CPC chromatogram of first CPC operation; (e) CPC chromatogram of second CPC operation. Peaks: secologanic acid (**1**); epivogeloside (**1a**); vogeloside (**1b**). HPLC and CPC conditions were described in the Experimental section

TABLE 2 The retention time of secologanic acid (**1**) in normal phase or reversed phase CPC operations

Operation mode	Partition coefficient (*K*)	Stationary retention volume (V_S) (mL)	Mobile phase volume (V_M) (mL)	Calculated retention volume (V_R) and calculated retention time (T_R)	Experimental retention time (T_R) (h)
Descending mode (reversed phase)	0.33	550	500	681.5 mL/1.14 h	1.14–1.42
Ascending mode (normal phase)	3.03	720	330	2511.6 mL/4.19 h	4.03–5.02

Note: Retention times calculated as an equation $V_R = V_M + K\,V_S$, where

V_R is the retention volume of solute, V_C the column volume (in this experiment, 1050 mL), V_M the mobile phase volume, V_S the stationary volume, K is the partition coefficient (peak area of stationary phase/peak area of mobile phase), the mobile phase flow rate = 10 mL/min.

absorption were similar to that of the secologanic acid (**1**). During evaporation, methanol was used to collect the samples in each test tube. Thus, the second CPC was carried out in ascending mode with the same solvent system (Figure 2e). The *K* value was 3.03 (in ascending mode), and the retention of stationary phase was 68.6% (720 mL). The secologanic acid was expected to elute at around 4.19 h (Table 2). In ascending CPC, the secologanic acid was eluted after 4 h (Figure 2e) with high purity in HPLC analysis (Figure 2c). To confirm

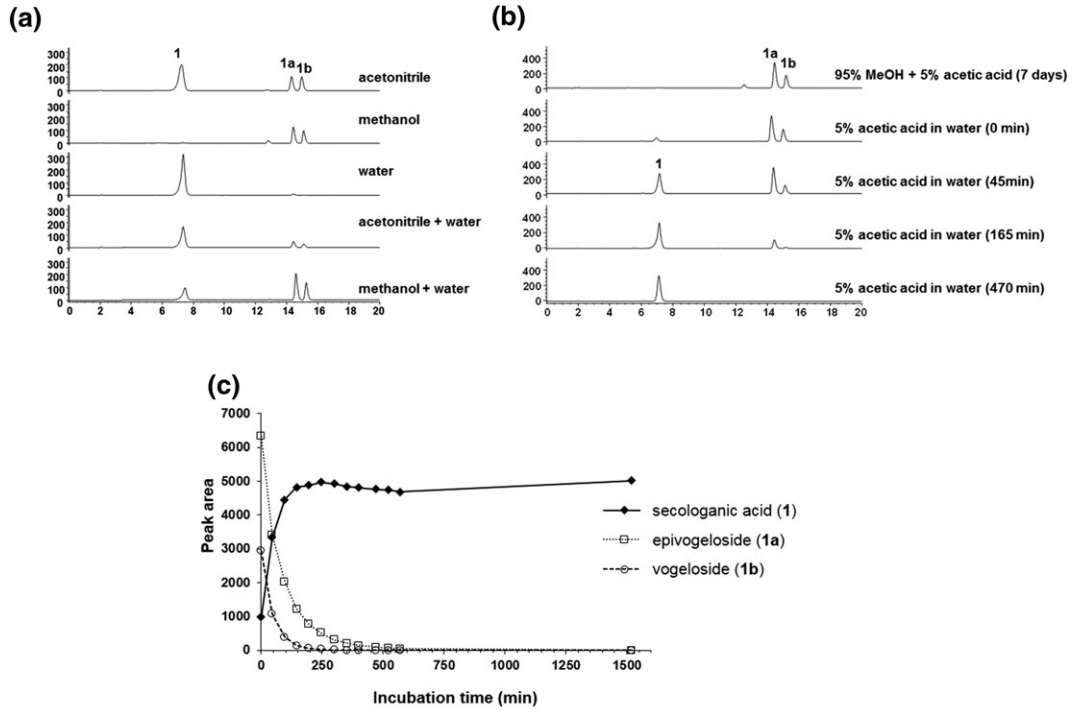

FIGURE 3 Conversion of secologanic acid (**1**) into epivogeloside (**1a**) or vogeloside (**1b**) or vice versa. (a) Conversion of secologanic acid (**1**) into epivogeloside (**1a**) or vogeloside (**1b**) in acetonitrile or methanol solution. (b) Conversion of epivogeloside (**1a**) or vogeloside (**1b**) into secologanic acid (**1**) in an acidic aqueous solution. (c) Conversion rate of epivogeloside (**1a**) or vogeloside (**1b**) into secologanic acid (**1**) in aqueous solution correspondence to the incubation time

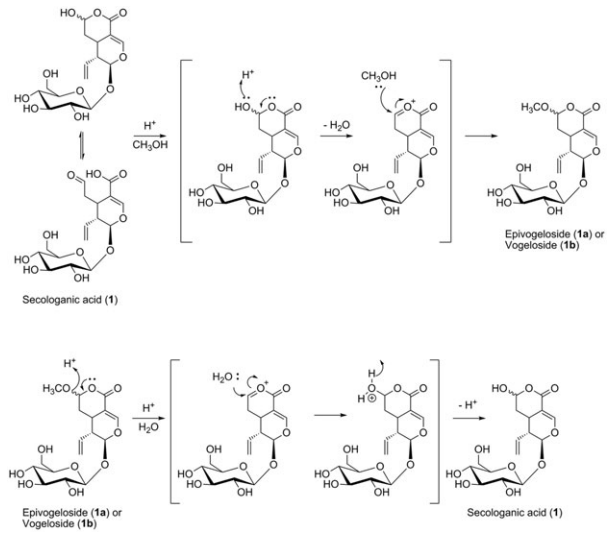

FIGURE 4 Proposed mechanism of (a) conversion of secologanic acid (**1**) into epivogeloside (**1a**) or vogeloside (**1b**), or (b) vice versa

the chemical structures of the two products (**1a** and **1b**) around 13 min, semi-prep HPLC was further performed.

3.3 | Structural identification of the isolated compounds

Structural identification of the CPC fraction peaks was accomplished with ^{1}H-NMR, ^{13}C-NMR and ESI-MS. The DMSO-d_6 peak was used as the reference peak at 2.50 in ^{1}H-NMR by comparison with previously published spectra.[26] The purified compounds were identified as secologanic acid (**1**), epivogeloside (**1a**) and vogeloside (**1b**).

3.4 | Conversion of secologanic acid into vogeloside or epivogeloside in methanol and vice versa in an acidic aqueous solution

Secologanic acid (**1**) was dissolved in 100% methanol, 50% methanol, 100% acetonitrile, 50% acetonitrile or water, and incubated for 7 h. After incubation in each indicated solution, the secologanic acid (**1**) was converted to epivogeloside (**1a**) or vogeloside (**1b**). In 100% methanol, almost all the secologanic acid (**1**) was converted to epivogeloside (**1a**) or vogeloside (**1b**). However, in 100% water, all the secologanic acid (**1**) remained (Figure 3a).

Transformation of epivogeloside (**1a**) and vogeloside (**1b**) into secologanic acid (**1**) was also tested. After seven days of incubation in methanol containing 5% acetic acid, the secologanic acid was completely converted into epivogeloside (**1a**) and vogeloside (**1b**). This solution was lyophilised and redissolved in water containing 5% acetic acid and incubated at room temperate. HPLC analysis revealed that epivogeloside (**1a**) and vogeloside (**1b**) were changed into secologanic acid (**1**) in an acid aqueous solution. After 7 h, only the secologanic acid (**1**) was found (Figure 3b). The proposed mechanism of transformation of secologanic acid (**1**) to epivogeloside (**1a**) or vogeloside (**1b**) or vice versa is depicted in Figure 4.

In this study, secologanic acid (**1**) was successfully purified from crude extract of flower buds of *L. japonica* by a CPC operation conducted twice with the same ternary biphasic solvent system composed of ethyl acetate/isopropanol/water (6:4:10, *v*/*v*/*v*) in descending mode

followed by ascending mode operation. The first operation was fast and suitable to obtain a secologanic acid enriched fraction, and then, the second CPC operation was performed for a longer time (*K* value = 3.03) to obtain pure secologanic acid. Since separation factors between secologanic acid and other unknown impurities was too small, a relative long time CPC operation was required. Furthermore, secologanic acid converted to products, epivogeloside or vogeloside in methanolic solution that was also reversed by the addition of acidic aqueous solution.

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