

Highly pure alpha-hederin from *Hedera helix* leaves isolated by centrifugal partition chromatography

Tran Phan Huynh Nhu¹, Tran Chieu An¹, Pham Hong Ngoc¹, Doan Le Quynh Nhu², Pham Thi Thanh Lan¹, Le Ngoc Hung³, Nguyen Tuan Hiep⁴, Phung Van Trung^{1,3,*}



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¹Institute of Applied Materials Science, Vietnam Academy of Science and Technology (VAST), Ho Chi Minh City, 70000, Vietnam

²Hoang Hoa Tham High School, Ho Chi Minh City, 70000, Vietnam

³Center for Research and Technology Transfer, Vietnam Academy of Science and Technology (VAST), Ha Noi City, 100000, Vietnam

⁴National Institute of Medicinal Materials, Ha Noi City, 100000, Vietnam

Correspondence

Phung Van Trung, Institute of Applied Materials Science, Vietnam Academy of Science and Technology (VAST), Ho Chi Minh City, 70000, Vietnam

Center for Research and Technology Transfer, Vietnam Academy of Science and Technology (VAST), Ha Noi City, 100000, Vietnam

Email: pvtung@ict.vast.vn

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ABSTRACT

Introduction: *Hedera helix* is a species of the Araliaceae family, which contains flavonoids, coumarins, phenolic acids, sterols, alkaloids, anthocyanins, and triterpene saponins. α -Hederin is a saponin compound of the *H. helix* that possesses many potential biological activities. In previous studies, several methods were used to isolate and purify α -hederin from ivy leaves. However, the loss of α -hederin obtained during solid chromatography purification was relatively high, and it was difficult to obtain α -hederin with high purity. In this research, a centrifugal partition chromatography (CPC) method was established for the isolation of α -hederin directly from ethanol crude extract of *Hedera helix* leaves from Da Lat, Lam Dong province, Vietnam. **Methods:** The separation of α -hederin was carried out by applying centrifugal partition chromatography with a PLC 2250 system (Gilson Inc., France) coupled with an SCPC-250 column (Armen Instrument, France). In a CPC run, a two-phase solvent system consisting of n-hexane–ethyl acetate–methanol–water (2:3:2:3, v/v) in ascending mode (the lower phase was the stationary phase), with a rotation speed of 1600 rpm and a flow rate of 8.0 mL/min, was used. **Results:** The purity of the isolated α -hederin was 95.7%, as confirmed by HPLC-DAD at 210 nm. The structure of purified α -hederin was determined by ¹H-NMR and ¹³C-NMR. The yield of α -hederin was 12.7%. **Conclusion:** This investigation might be used as a reference for the large-scale separation of high-purity α -hederin from *H. helix* leaves. **Key words:** *Hedera helix*, α -hederin, CPC, HPLC-DAD

INTRODUCTION

Ivy (*Hedera helix* L.), one of 15 species in the genus *Hedera*, is an evergreen dioecious woody liana that belongs to the Araliaceae family¹. The chemical composition of *Hedera helix* leaves contains flavonoids, coumarins, phenolic acids, sterols, alkaloids, anthocyanins, and triterpene saponins¹⁻⁴. Saponins from ivy leaves exhibit anthelmintic, antifungal, antimutagenic, antileishmanial, antioxidant, and anti-inflammatory activities⁵⁻¹⁰. α -Hederin (Figure 1) is one of the effective saponins of *Hedera helix* leaves and has numerous bioactivities, including antileishmanial⁸, anti-inflammatory, antifungal, anticancer, antispasmodic, and hepatoprotective activities¹⁰⁻¹⁴. Centrifugal partition chromatography (CPC) is a liquid–liquid partition chromatographic technique that has the advantages of a faster separation time, no sample adsorption, and the ability to purify a wide range of phytochemicals using a variety of solvent systems. Because of these merits, CPC is widely used in the preparative separation of natural products¹⁵. The liquid stationary phase is immobilized in the CPC column, and the sample components are partitioned between the two phases and separated using partition

coefficient differences. Only a restricted number of solvents are employed in CPC. The most common solvents are n-hexane, ethyl acetate, methanol, and water¹⁶.

The present study aimed to establish an efficient CPC method for the purification of α -hederin from *Hedera helix* leaf extract.

MATERIALS AND METHODS

Chemicals and reagents

n-hexane, ethyl acetate (EtOAc), chloroform (CHCl₃), methanol (MeOH), acetonitrile (ACN) of HPLC grade, formic acid of analytical grade, dimethyl sulfoxide (DMSO) (Scharlau, Spain); deionized water for HPLC; sulfuric acid for analysis (Merck, Germany); ethanol absolute (Chemsol, Vietnam); α -hederin (Natpro, Vietnam).

Methods

Extraction of plant materials

Hedera helix leaves were collected from Da Lat, Lam Dong province, Vietnam. Approximately 1.0 kg of *H. helix* leaves were cut into small pieces and extracted in

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5.0 l of ethanol for 24 hours at room temperature. After vacuum evaporation of methanol using a rotavapor at 60°C, the crude extract was obtained and later used for CPC separation of α -hederin.

Thin layer chromatography (TLC)

TLC was performed on silica gel plates (Si60 F254, 100 x 200 mm, 0.2 mm layer thickness, Merck, Germany). The crude extract of ivy leaves and standard α -hederin were monitored on TLC plates. CHCl_3 -MeOH (4:1, v/v) was used as the mobile phase. After developing, the plate was sprayed with TLC reagent (H_2SO_4 in 10% ethanol) and heated at 105°C for a minute. Then, light purple α -hederin spots appeared.

Selection of a two-phase solvent system

The two-phase solvent system was chosen according to the partition coefficient (K) value of α -hederin. In this experiment, approximately 10 mg of the *H. helix* crude extract was dissolved in 4 mL of the pre-equilibrated two-phase solvent system consisting of n-hexane-ethyl acetate-methanol-water with three different ratios of Arizona systems: J (2:5:2:5), L (2:3:2:3), and N (1:1:1:1). Both the upper and lower phases were analyzed by TLC to determine the partition coefficient values of α -hederin. For efficient CPC separation, the appropriate K value was between 0.2 and 5.0 and was preferably 1.0^{17,18}.

CPC separation method

CPC separation of the ivy extract was performed with a system comprising an SCPC-250 column (Armen Instrument, France) coupled with a PLC 2250 system (Gilson Inc., France) built in with a quaternary pump, a diode array detector, and a fraction collector. The total column volume was 250 mL. Gilson Glider CPC Software V5.1d.01 (Gilson Inc., France) was used to control the instrumentation.

In this experiment, the CPC method was established with an optimized Arizona L biphasic solvent system in ascending mode. First, the CPC column was filled with the lower stationary phase (n-hexane-EtOAc-MeOH- H_2O , 0:16:32:52, v/v) at a flow rate of 30.0 mL/min with a rotation speed of 800 rpm. Then, the upper mobile phase (n-hexane-EtOAc-MeOH- H_2O , 45:48:6:1, v/v) was pumped into the column at a flow rate of 8.0 mL/min with a rotation speed of 1600 rpm. Equilibrium was reached when the mobile phase emerged from the CPC system.

Approximately 100 mg of *H. helix* extract was dissolved in 2 mL of the Arizona L system. The whole sample mixture was injected through a 2.0 mL loop

for α -hederin separation. UV detection was monitored at 210 nm. The fractions were collected in 32 mL tubes (5 mL for each fraction) in the collector.

HPLC analysis and quantification of α -hederin

H. helix crude extract and CPC fraction peaks were analyzed by HPLC-DAD using the method described in our previous study¹⁹. The Hewlett Packard 1050 HPLC system (Agilent Technologies, USA) with an Inertsil ODS-3 column (250 x 4.6 mm; 5 μm) (GL Sciences, Japan) was used at a flow rate of 1.0 mL/min, with a gradient solvent system of water containing 0.1% formic acid (A) and acetonitrile (B). The mobile phase gradient was performed as follows: 0-20 min: from 80% to 40% A in B; 20-24 min: from 40% to 0% A in B; 24-30 min: 100% B. The sample injection volume was 20.0 μL . The effluents were monitored at 210 nm. Data were analyzed using ChemStation for LC 3D software (Agilent Technologies, USA).

NMR analysis

NMR experiments of the isolated compound were carried out using a Bruker Avance III spectrometer with DMSO as the solvent and TMS as the internal standard.

RESULTS

Selection of a two-phase solvent system

The ivy leaf crude extract was dissolved in the Arizona system composed of n-hexane-EtOAc-methanol-water with three different ratios as follows: J (2:5:2:5), L (2:3:2:3), and N (1:1:1:1). TLC analyses of the sample in the upper and lower phases are presented in Figure 2. In comparison to Arizona systems J and N, the α -hederin spots in the upper and lower phases of Arizona L appeared with similar light purple color and band size, indicating that the concentration of α -hederin in the upper and lower phases of Arizona L is virtually equivalent. As a result, the K value of Arizona L was preferred, and this solvent system was considered acceptable for CPC separation of α -hederin.

CPC separation and HPLC analysis

After performing HPLC-DAD analysis, the *H. helix* crude extract containing α -hederin (RT = 21.88) was subjected to CPC separation. Figure 3 shows the HPLC-DAD analysis result of the ivy crude extract at a UV wavelength of 210 nm.

The crude extract of *H. helix* was separated by a single CPC run using a solvent system composed of n-hexane-EtOAc-methanol-water (2:3:2:3, v/v). Identification of the CPC peak fractions was performed

by HPLC-DAD analysis. After a one-step operation of CPC, a high purity of α -hederin was achieved at 95.7%. The isolated compound was a white powder, TLC Rf: 0.5. The eluted solvent system was CHCl_3 -MeOH (4:1, v/v). The chemical structure of the isolated compound was confirmed by NMR (Table 1). Figure 4 shows the HPLC-DAD analysis of the *H. helix* extract and the isolated α -hederin (Rt: 21.88 min). The yield reached 12.7% compared to the α -hederin content in the total extract.

DISCUSSION

NMR analysis of the isolated compound

The ^1H -NMR spectrum (DMSO- d_6 , 500 MHz) of the compound had three signals of the CH_3 group at δ_H 0.57(s); 1.07(s); 1.08(s), three CH_3 groups give 1 at δ_H 0.87(s), 1 oxymethylene H-23 group at δ_H 3.12 and 3.28, and 1 oxymethine H-3 group at δ_H 3.49 (1H, m). The ^1H -NMR spectrum also showed the presence of an olefin proton signal at δ_H 5.16, which is specific for H-12 in the structure of the 5-ring triterpenes.

The ^{13}C -NMR and DEPT spectra of the compound showed resonance signals of 41 carbons, 30 of which are consistent with published data for the structure of hederagenin consisting of 7 methyl group signals, 11 methylene group signals, 5 methine group signals, and 8 quaternary carbon signals, including the signal of C-12 olefin carbon at δ_C 121.58 for methine carbon and C-13 quaternary carbon at δ_C 143.86, also confirming the existence of a double bond in the ring at the C-12 position and a C-28 signal at δ_C 178.62 of the carboxyl group. In addition, the remaining 11 carbon signals showed the presence of 2 sugar units with resonance signals of 2 anomeric carbons C-1' at δ_C 102.97 and C-1'' at δ_C 99.94, 7 methine group signals, 1 C-5' methylene group signal, and 1 C-6'' methyl group signal at δ_C 17.77.

^1H -NMR and HSQC spectra also confirmed that two anomeric protons interacted with the above 2 anomeric carbons at δ_H 5.06 (1H, s) and δ_H 4.63 (1H, d, 6.0). On the ^1H -NMR spectrum, a doublet signal of the methyl group at δ_H 0.71 showed the existence of a deoxy sugar in the structure of the compound.

The protons in the ring of the sugar units were correctly assigned thanks to the COSY, HSQC, and HMBC spectra starting from the identified anomeric protons. The chemical shift of C-3 (δ_C 79.32) in the ^{13}C -NMR spectrum indicated that the sugar chains were attached to this site. Connecting to the sugar units was confirmed by HMBC interactions between the anomeric proton (H-1') of arabinose (δ_H 4.63) with C-3 (δ_H 79.32), H-1'' of rhamnose (δ_H 5.06),

and C-2' of arabinose (δ_H 74.28). By comparison to the reference²⁰, the isolated compound was determined to be α -hederin.

Advantages of CPC in α -hederin isolation and purification

Saponins are phytochemicals mostly found in plants and are also present in most vegetables, beans, and herbs. It is a serious challenge in saponin extraction and separation because of their unique structural characteristics. In addition, it is difficult to isolate saponins since there are a mixture of saponin compounds present in plants with similar structures and polarities. Some saponins may undergo enzymatic hydrolysis during water extraction, while esterification of acidic saponins may also occur at some stage in alcohol extraction thus, relatively mild conditions should be employed, and special attention conditions should be utilized when conducting the extraction. Therefore, different separation techniques, e.g., thin layer chromatography (TLC), column chromatography (CC), low pressure liquid chromatography (LPLC), medium pressure liquid chromatography (MPLC), and high-performance liquid chromatography (HPLC), are typically used to achieve pure saponin components²¹.

Centrifugal partition chromatography (CPC) is known as liquid-liquid partition chromatography without any sorbent, which increases sample recovery and eliminates peak tailing on solid supports²². Thanks to these two advances, CPC has been accomplished using the separation of several bioactive saponins. In 2011, Jing et al. applied centrifugal partition chromatography combined with evaporative light scattering detection (CPC-ELSD) for systematic separation and purification of four saponins, including notoginsenoside R₁, ginsenoside Rg₁, Re, and Rb₁, from the crude extract in a one-step separation, of which purities were over 98% from *Panax notoginseng*²³. In 2013, Wang et al. successfully isolated and purified two ginsenosides, Rb₁ (95.6%) and Rb₂ (97.8%), from the extract of *P. ginseng* by high-performance centrifugal partition chromatography (HPCPC)²⁴. In 2014, Bahrami et al. performed HPCPC-ELSD to purify the saponin mixture and to isolate saponin congeners and isomeric saponins from the extract of the viscera of sea cucumber *H. lessona*²⁵.

α -Hederin is a bioactive triterpene saponin of *Hedera helix* leaves. Therefore, the extraction and isolation of α -hederin by traditional methods presents many difficulties. In this research, by applying the CPC

technique, highly purified α -hederin (95.7%) was isolated in just one run, and the isolation yield compared to the crude extract was 12.7%. The results demonstrated that CPC was a feasible and efficient technique for the isolation and purification of α -hederin from *H. helix* leaves.

CONCLUSION

CPC proved to be an excellent method for separating α -hederin from *H. helix* leaves. In a single CPC run, a high purity of isolated α -hederin (95.7%) was obtained. This study could serve as a benchmark for the large-scale isolation of high-purity α -hederin from *H. helix* leaves.

LIST OF ABBREVIATIONS

ACN: acetonitrile
 CC: column chromatography
 CHCl₃: chloroform
 COSY: correlated spectroscopy
 DEPT: distortionless enhancement by polarization transfer
 DMSO: dimethyl sulfoxide
 ELSD: evaporative light scattering detection
 EtOAc: ethyl acetate
 HMBC: heteronuclear multiple bond correlation
 HPCPC: high-performance centrifugal partition chromatography
 HPLC-DAD: high-performance liquid chromatography-diode array detector
 HSQC: heteronuclear single quantum coherence
 LPLC: low pressure liquid chromatography
 MeOH: methanol
 MPLC: medium pressure liquid chromatography
 NMR: nuclear magnetic resonance
 TLC: thin layer chromatography

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHOR'S CONTRIBUTIONS

Tran Phan Huynh Nhu: analyzed the data and contributed to the writing of the manuscript. Tran Chieu An: performed HPLC analyses and contributed to the writing of the manuscript. Pham Hong Ngoc: synthesized documents, analyzed the results, and proposed the outline for the article. Doan Le Quynh Nhu: extracted the sample and determined α -hederin structure by NMR spectroscopy. Pham Thi Thanh Lan:

performed CPC run. Le Ngoc Hung: devised the project and the conceptual ideas. Nguyen Tuan Hiep: collected the sample. Phung Van Trung: devised the main conceptual ideas and developed the theoretical framework. All authors read and approved the final manuscript.

REFERENCES

1. Yulia L, Wiesława B, Irena M, Roman D. Hedera helix as a medicinal plant. *Herba Pol.* 2010;56(1):83-96.
2. Mahran GH, Hilal SH, El-Alfy TS. The isolation and characterization of emetine alkaloid from Hedera helix. *Planta Med.* 1975;27(2):127-32; PMID: 1121525. Available from: <https://doi.org/10.1055/s-0028-1097773>.
3. Crespin F, Elias R, Morice C, Ollivier E, Balansard G, Faure R. Identification of 3-O- β -D-glucopyranosyl hederagenin from the leaves of Hedera helix. *Fitoterapia.* 1995;66(5):477.
4. Trute A, Nahrstedt A. Identification and quantitative analysis of phenolic compounds from the dry extract of Hedera helix. *Planta Med.* 1997;63(2):177-9; PMID: 17252341. Available from: <https://doi.org/10.1055/s-2006-957639>.
5. Egualde T, Tilahun G, Debella A, Feleke A, Makonnen E. Haemonchus contortus: in vitro and in vivo anthelmintic activity of aqueous and hydroalcoholic extracts of Hedera helix. *Exp Parasitol.* 2007;116(4):340-5; PMID: 17382320. Available from: <https://doi.org/10.1016/j.exppara.2007.01.019>.
6. Timon-David P, Julien J, Gasquet M, Balansard G, Bernard P. Research of antifungal activity from several active principle extracts from climbing ivy: Hedera helix. *Ann Pharm Fr.* 1980;38(6):545-52; PMID: 7283353.
7. Villani P, Orsière T, Sari-Minodier I, Bouvenot G, Botta A. In vitro study of the antimutagenic activity of alpha-hederin. *Ann Biol Clin (Paris).* 2001;59(3):285-9; PMID: 11397676.
8. Delmas F, Di Giorgio C, Elias R, Gasquet M, Azas N, Mshvildadze V et al. Antileishmanial activity of three saponins isolated from ivy, α -hederin, β -hederin and hederacolchiside A1, as compared to their action on mammalian cells cultured in vitro. *Planta Med.* 2000;66(4):343-7; PMID: 10865451. Available from: <https://doi.org/10.1055/s-2000-8541>.
9. Gülçin I, Mshvildadze V, Gepdiremen A, Elias R. Antioxidant activity of saponins isolated from ivy: alpha-hederin, hederasaponin-C, hederacolchiside-E and hederacolchiside-F. *Planta Med.* 2004;70(6):561-3; PMID: 15241892. Available from: <https://doi.org/10.1055/s-2004-827158>.
10. Gepdiremen A, Mshvildadze V, Süleyman H, Elias R. Acute anti-inflammatory activity of four saponins isolated from ivy: alpha-hederin, hederasaponin-C, hederacolchiside-E and hederacolchiside-F in carrageenan-induced rat paw edema. *Phytomedicine.* 2005;12(6-7):440-4; PMID: 16008120. Available from: <https://doi.org/10.1016/j.phymed.2004.04.005>.
11. Favel A, Steinmetz MD, Regli P, Vidal-Ollivier E, Elias R, Balansard G. In vitro antifungal activity of triterpenoid saponins. *Planta Med.* 1994;60(1):50-3; PMID: 8134417. Available from: <https://doi.org/10.1055/s-2006-959407>.
12. Cheng L, Xia TS, Wang YF, Zhou W, Liang XQ, Xue JQ et al. The anticancer effect and mechanism of α -hederin on breast cancer cells. *Int J Oncol.* 2014;45(2):757-63; PMID: 24842044. Available from: <https://doi.org/10.3892/ijo.2014.2449>.
13. Trute A, Gross J, Mutschler E, Nahrstedt A. In vitro antispasmodic compounds of the dry extract obtained from Hedera helix. *Planta Med.* 1997;63(2):125-9; PMID: 9140224. Available from: <https://doi.org/10.1055/s-2006-957627>.
14. Liu J, Liu Y, Bullock P, Klaassen CD. Suppression of liver cytochrome P450 by alpha-hederin: relevance to hepatoprotection. *Toxicol Appl Pharmacol.* 1995;134(1):124-31; PMID: 7676446. Available from: <https://doi.org/10.1006/taap.1995.1175>.

15. Hopmann E, Frey A, Minceva M. A priori selection of the mobile and stationary phase in centrifugal partition chromatography and countercurrent chromatography. *J Chromatogr A*. 2012;1238:68-76;PMID: 22503586. Available from: <https://doi.org/10.1016/j.chroma.2012.03.035>.
16. Pauli GF, Pro SM, Friesen JB. Countercurrent separation of natural products. *J Nat Prod*. 2008;71(8):1489-508;PMID: 18666799. Available from: <https://doi.org/10.1021/np800144q>.
17. Oka H, Harada KI, Ito Y, Ito Y. Separation of antibiotics by countercurrent chromatography. *J Chromatogr A*. 1998;812(1-2):35-52;PMID: 9691308. Available from: [https://doi.org/10.1016/S0021-9673\(97\)01277-6](https://doi.org/10.1016/S0021-9673(97)01277-6).
18. Ito Y. Principles and instrumentation of countercurrent chromatography. In: Mandava NB, Ito Y, editors. *Countercurrent chromatography. Theory and practice*. New York: Marcel Dekker; 1988. p. 79-442;.
19. Mai Nth, Diep TT, Trung PV. 2016. "Simultaneous quantitation of hederacoside C and α -hederin from the leaves of *Hedera helix* L. by HPLC". *Journal of Medicinal Materials*;21(6):388-93;.
20. Parveen A, Farooq MA, Kyunn WW. A new oleanane type saponin from the aerial parts of *Nigella sativa* with antioxidant and antidiabetic potential. *Molecules*. 2020;25(9):2171;PMID: 32384790. Available from: <https://doi.org/10.3390/molecules25092171>.
21. Majinda RRT. Chapter 16: Extraction and isolation of saponins. In: Sarker SD, Nahar L, editors. *Natural products isolation, methods in molecular biology*. Vol. 864. Humana Press; 2012. p. 415-26;PMID: 22367906. Available from: https://doi.org/10.1007/978-1-61779-624-1_16.
22. Foucault AP, Chevolut L. Countercurrent chromatography: instrumentation, solvent selection and some recent applications to natural product purification. *J Chromatogr A*. 1998;808(1-2):3-22; Available from: [https://doi.org/10.1016/S0021-9673\(98\)00121-6](https://doi.org/10.1016/S0021-9673(98)00121-6).
23. Wang J, Liu CM, Li L, Bai HL. Isolation of four high-purity dammarane saponins from extract of *Panax notoginseng* by centrifugal partition chromatography coupled with evaporative light scattering detection in one operation. *Phytochem Anal*. 2011;22(3):263-7;PMID: 21259375. Available from: <https://doi.org/10.1002/pca.1274>.
24. Wang J, Yu J, Li L, Liu CM, Zhang Y, Wang Q. Isolation of high purity ginsenosides from plant extract of *panax ginseng* by high performance centrifugal partition chromatography coupled with evaporative light scattering detection. *Journal of Liquid Chromatography and Related Technologies*. 2013;36(5):583-90; Available from: <https://doi.org/10.1080/10826076.2012.668739>.
25. Bahrami Y, Zhang W, Franco C. Discovery of novel saponins from the viscera of the sea cucumber *Holothuria lessona*. *Mar Drugs*. 2014;12(5):2633-67;PMID: 24821624. Available from: <https://doi.org/10.3390/md12052633>.