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ORIGINAL ARTICLE

Identification, Preliminary Genetic, and Biochemical Analyses the *Hedera* Plants Which Naturally Distribute in Vietnam

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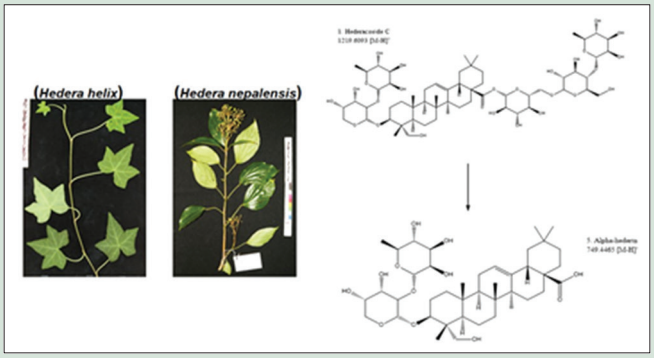
ABSTRACT

Background: *Hedera* is a genus of 12–15 species of evergreen climbing woody plants in the family Araliaceae that are considered as traditional medicinal plants. **Objectives:** The objectives were to classify *Hedera* species collected from different areas in Vietnam and characterize some chemical compounds of these species. **Materials and Methods:** In this study, the DNA-based technique was used to classify and analyze the genetic source of 21 *Hedera* plant samples found in Vietnam. In addition, high-performance liquid chromatography (HPLC) is also employed to assess the chemical compounds of these plants. **Results:** The collected plants mainly distribute into three groups (named as N1, N2, and N3). Based on the phenotypic identification and genetic analysis, the N1 group is indicated as *Hedera nepalensis* (*H. nepalensis*), while N3 is *Hedera helix* (*H. helix*). The HPLC analysis indicates that most of them contained more hederacoside C than α-hederin. Moreover, N1 plants are able to produce hederacoside C as N3 even the accumulation levels in N1 are moderately lower than those in N3 plants. **Conclusion:** These results provide important information about the local *Hedera* plants and help us to select the good medicinal sources for further applications in biomedical sciences.

Key words: *Hedera* plants, hederacoside C, high-performance liquid chromatography detection, *ITS* gene, *matK* gene, α-hederin

SUMMARY

- *Hedera* plants which were distributed naturally in several northern mountainous provinces of Vietnam were identified as *H. nepalensis* var. *sinensis*
- *matK* and *ITS* markers were performed
- Hederacoside C and alpha hederin, the active compounds with promising effects, were found in *Hedera nepalensis*



Abbreviations Used: ASE: Accelerated solvent extraction, BLAST: Basic local alignment search tool, GBSSI: Granule bound starch synthase, HPLC: *High-performance* liquid chromatography, ITS: Internal transcribed spacer, matK: Megakaryocyte-associated tyrosine kinase, PCR: Polymerase chain reaction.

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DOI: 10.4103/pr.pr_65_20

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INTRODUCTION

Hedera is a genus of the *Araliaceae* family that has 15 registered species. In terms of pharmacology, two species including *Hedera helix* and *Hedera nepalensis* are the most widely used and studied. *H. helix* is mainly found in Europe and parts of South Asia, while *H. nepalensis* distributes in several countries in Europe, the Himalayas, and some high mountain areas of Vietnam.[1,2] The extracts and products of *H. helix* and *H. nepalensis* have been found to positively function in antifungal, treat respiratory diseases, and support diabetes treatment. Most pharmacological studies involving *H. nepalensis* showed that this species has similar medicinal effects to *H. helix*. For example, Uddin *et al*. found that ethyl acetate extracts of *H. nepalensis* leaf also have antibacterial effects.[3] Besides, the ethyl acetate fraction of *H. nepalensis* has been documented to work as an antioxidant corresponding to the high content of flavonoid and phenolic compounds.[1] A previous study revealed that the *Hedera* genus contains various natural compounds such as triterpene saponins, flavonoids, polyacetylenes, and phenolic compounds.[4] In particular, triterpene saponins (most of which are hederacoside C and α-hederin) have been shown to work in bronchospasm, anti-inflammatory, anti-infection, liver protection, antioxidant, and hypoglycemia.[5] Few studies have investigated the classification and genetic variation of *H. helix*;[6] although this species has not been found in nature in Vietnam, this species has been imported to some localities such as Da Lat and Lam Dong. Meanwhile, in the last few years, *H. nepalensis* has been discovered in mountainous areas of northern Vietnam. Some preliminary assessments of the chemical and pharmacological properties suggest that *H. nepalensis* has a correlation and much potential to replace the imported *H. helix*. At the DNA level, the variety of *Hedera* plant species can be identified and evaluated by molecular markers.[6]

In the present work, twenty-one samples of *Hedera* plants which are naturally distributed in Vietnam were collected and these samples were then classified based on phenotypic characterizations as well as genetic

Cite this article as: Hiep NT, Nhung PT, Nguyen NH, Linh DT, Duong HT, Hang DT, *et al*. Identification, preliminary genetic, and biochemical analyses the *Hedera plants* which naturally distribute in Vietnam. Phcog Res 2020;12:450-4.

Submitted: 07-Jul-2020 **Revised:** 03-Sep-2020
Accepted: 18-Sep-2020 **Published:** 23-Jan-2021

information. The HPLC method was used to quantitatively measure the hederacoside C and α-hederin in these *Hedera* leaf samples. Taken together, we identified the correlation between the content of the main active ingredients and the genotypes, contributing more information to help for the selection of excellent genetic resources for exploitation and further applications in biomedical sciences.

MATERIALS AND METHODS

Plant materials

Twenty-one *Hedera* plant samples were identified and collected by the Vietnam National Institute of Medicinal Materials from different places in Vietnam as indicated in Table 1. Based on the phenotypic characterization, these plants were firstly divided to be two groups including *H. helix* (TL1_180_20 and 21) and *H. nepalensis var. sinensis* (TL1_180_1-19) [Figure 1]. Fresh leaves from the 21 plants were harvested randomly. Each sample was divided into two parts. One was stored in a deep-freezer (−80°C) and used for DNA extraction, whereas the other was dried in an oven at 60°C and ground for HPLC analysis.

DNA extraction, polymerase chain reaction, and sequencing

Partial *matK* was amplified by polymerase chain reactions (PCRs) with the primers matK-390F and matK-1326R.[7] The *ITS* region was amplified by PCR with ITS-17SE and ITS-28SE.[8] The primers were synthesized by PhuSa Biochem Ltd., (Vietnam). Amplification was of 25 µL containing 1 x HF buffer, 0.2 mM dNTPs mix, 0.02 u/µl Phusion DNA polymerase (Thermo Fisher Scientific, USA), 0.3 µM each of two primers and 10–25 ng DNA template. For 2 genes PCR, cycling conditions were: 98°C for 30 s; followed by 35 cycles of 98°C for 10 s 55.1°C for 30 s and 72°C for 30 s and a final extension at 72°C for 10 min. The amplified products were separated in 1.5% (w/v) agarose gels in 1x Tris-acetate-EDTA buffer. The PCR samples were purified and sequenced at First Base Laboratories (Malaysia).

Phylogenetic analysis

DNA sequences tested, refined through Sequencher versatile 5.4.6 and alignment with BioEdit version 7.2.6.1 software. The trimmed sequence was compared (in NCBI-BLAST) with other available *Hedera-ITS* and *Hedera-matK* sequences in the GenBank.

Phylogenetic trees of 21 *ITS* and *matK* sequences were constructed using MEGAX software based on maximum parsimony and maximum likelihood methods. The bootstrap method or statistically sampling method was chosen for assigning measures of accuracy of phylogenetic tree. Bootstrap analysis with 1000 replicates was conducted to estimate nodal support.

High-performance liquid chromatography analysis

Reference solution

Methanol was added to a certain quantity of hederacoside C and α-hederin standards (98%, Sigma-Aldrich, St Louis, MO) to obtain a range of standard solutions with the concentrations of 25, 50, 100, 200, 500, and 1000 µg/ml.

Test solution preparation

An accelerated solvent extraction (ASE) 350 system from Dionex Corporation (Sunnyvale, CA, USA) was used for the extractions. About

Figure 1: Sample pictures (a) TL1_180_21 (*Hedera helix*); (b) TL1_180_19 (*Hedera nepalensis*)

Table 1: List of 21 *Hedera* plants which were harvested in different locations in Vietnam and used for the current study

Number	Code	Area of collection	GPS
1	TL1_180_1	Pho Bang, Dong Van, Ha Giang	23°14'49.81"N - 105°11'36.53"E
2	TL1_180_2	Pho Bang, Dong Van, Ha Giang	23°14'46.36"N - 105°11'30.49"E
3	TL1_180_3	Pho Bang, Dong Van, Ha Giang	23°15'01.61"N - 05°11'24.46"E
4	TL1_180_4	Pho La, Dong Van, Ha Giang	23°15'48.33"N - 05°09'56.85"E
5	TL1_180_5	Pho La, Dong Van, Ha Giang	23°15'20.97"N - 105°10'29.61"E
6	TL1_180_6	Sung La, Dong Van, Ha Giang	23°14'39.08"N - 105°12'48.68"E
7	TL1_180_7	Sung La, Dong Van, Ha Giang	23°14'37.25"N - 105°47'6.49"E
8	TL1_180_8	Pho Cao, Dong Van, Ha Giang	23°12'49.49"N - 103°47'6.49"E
9	TL1_180_9	Pho Cao, Dong Van, Ha Giang	23°13'00.07"N - 105°10'31.06"E
10	TL1_180_10	Pho Cao, Dong Van, Ha Giang	23°14'39.08"N - 105°10'38.25"E
11	TL1_180_11	Ho Thau, Hoang Su Phi, Ha Giang	22°39'18.38"N - 104°35'45.13"E
12	TL1_180_12	Ho Thau, Hoang Su Phi, Ha Giang	22°39'43.50"N - 104°36'11.35"E
13	TL1_180_13	Thu Ta, Xi Man, Ha Giang	22°39'01.08"N - 104°35'31.20"E
14	TL1_180_14	Ho Thau, Hoang Su Phi, Ha Giang	22°39'28.47"N - 104°35'57.14"E
15	TL1_180_15	Sung La, Dong Van, Ha Giang	23°14'47.62"N - 105°12'43.66"E
16	TL1_180_16	Ho Thau, Hoang Su Phi, Ha Giang	22°39'31.37"N - 104°36'11.12"E
17	TL1_180_17	Ban Khoang, Sa Pa, Lao Cai	22°22'59.25"N - 103°47'6.49"E
18	TL1_180_18	O Qui Ho, Sa Pa, Lao Cai	22°22'51.15"N - 103°47'45.99"E
19	TL1_180_19	O Qui Ho, Sa Pa, Lao Cai	22°22'37.53"N - 103°47'31.76"E
20	TL1_180_20	Sa Pa, Lao Cai	22°21'10.29"N - 103°51'36.04"E
21	TL1_180_21	Da Lat, Lam Dong	11°56'11.56"N - 108°27'28.12"E

GPS: Global Positioning System

2 g of each powered *Hedera* leaves was homogeneously mixed with 4 g of diatomaceous earth and placed in a 100 ml stainless extraction cell. A stainless steel frit and a cellulose filter (Dionex) were placed at the bottom of the extraction cell to prevent the penetration of fine powder in the collection bottle. The extraction cell was arranged in the cell tray and the samples were extracted under some specific conditions: pressure (1500 psi), fixed volume (100 ml), nitrogen purge time (100 s), and static time (10 min). After extraction, the samples were centrifuged for 10 min at 4000 rpm. The supernatant was collected and filtered through a 0.45 μm nylon filter membrane before injection into the HPLC system. Three replicates of each sample were prepared and detected.

High-performance liquid chromatography conditions

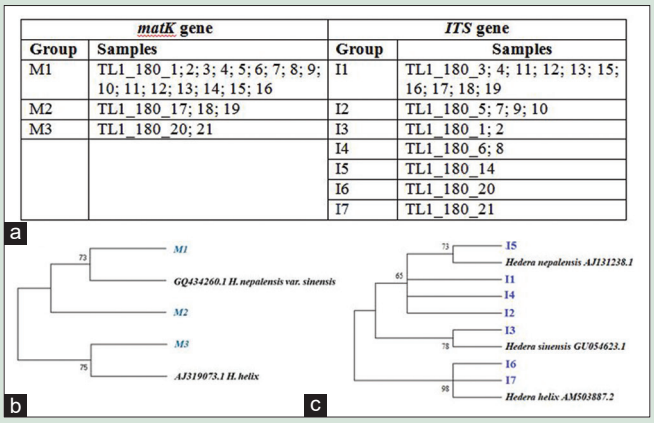
The Shimadzu SPD-20A system (C_{18} column; 250 mm × 4.6 mm, 5 μm) (Shimadzu Co., Ltd.) was used for HPLC analysis. A mixture of acetonitrile – 0.02% phosphoric acid solution was used as the mobile phase. The elution mode was a binary, high-pressure gradient system and the elution gradients were: 0–25 min, 20%–60% acetonitrile; 25–30 min, 60%–100% acetonitrile. Other running conditions include the detection wavelength (210 nm), the flow rate (1 ml/min), the injection volume (20 ml), and the column temperature (25°C).

RESULTS

Genetic analysis based on *matK* gene

The DNA sequences were analyzed using a BLAST search and divided into groups with 100% homogeneous samples. Based on the sequencing results, the plant samples were divided to be three groups (haplotypes) with *matK* (haplotypes M1 to M3) and seven groups with *ITS* (haplotypes I1 to I7) [Figure 2a]. To compute the phylogenetic tree from the *matK* gene, *H. helix* (AJ319073.1) and *H. nepalensis var. sinensis* (GQ424360.1) were chosen as reference sequences. Using maximum likelihood methods, *matK* phylogeny with the highest log likelihood (-1086.95) is shown in Figure 2b. The initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. Figure 2b shows that 16 samples of haplotype M1 are in the same group as *H. nepalensis var. sinensis,* while haplotype M3 is *H. helix*. On the other hand, 3 samples of haplotype M2 seem to be close to group M1.

The maximum parsimony tree with *ITS* gene in Figure 2c was obtained using the subtree-pruning-regrafting algorithm. Reference sequences for *ITS* are *H. helix* (AM503887.2), *H. sinensis* GU054623.1), and *H. nepalensis var. sinensis* (AJ131238.1). *ITS* phylogeny showed that haplotypes I1, I2, I4, and I5 have high similarities with the *H. nepalensis* reference sequence, haplotype I3 is more similar to *H. sinensis*. Meanwhile, I6 and I7 haplotypes are separated into the same group as *H. helix*. Thus, genetic analysis based on *ITS* and *matK* genes showed that they could help distinguish *H. helix* and *H. nepalensis*.



matK gene		*ITS* gene	
Group	Samples	Group	Samples
M1	TL1_180_1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11; 12; 13; 14; 15; 16	I1	TL1_180_3; 4; 11; 12; 13; 15; 16; 17; 18; 19
M2	TL1_180_17; 18; 19	I2	TL1_180_5; 7; 9; 10
M3	TL1_180_20; 21	I3	TL1_180_1; 2
		I4	TL1_180_6; 8
		I5	TL1_180_14
		I6	TL1_180_20
		I7	TL1_180_21

Figure 2: Group of 100% homogeneous samples (a), *matK* phylogeny using maximum likelihood methods (b) and *ITS* phylogeny using maximum parsimony method (c). (b) The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. (c) The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

Hederacoside C and α-hederin analysis by high-performance liquid chromatography

First, the hederacoside C and α-hederin standards were diluted to six gradients (25, 50, 100, 200, 500, and 1000 μg/ml) and detected by HPLC with described conditions. Next, the linear regression equations were formulated as Y = 2356X + 17365 (r^2 = 0.9998) (for hederacoside C) and Y = 5875X + 2620 (r^2 = 0.9998) (for α-hederin) (where X = the concentration and Y = the peak area).

The *Hedera* extracts were also analyzed by the same HPLC system and conditions. Based on the chromatograms, the peak areas of hederacoside C and α-hederin as well as the formulated linear regression equations, the quantities of these two compounds in 21 *Hedera extracts* were calculated and shown in Figure 3. The endogenous contents of both hederacoside C and α-hederin were found to be different among the analyzed *Hedera* samples and the hederacoside C levels were more abundant than α-hederin.

DISCUSSION

Hedera plants have been recognized as a traditional medicine for a long time and are widely used in many countries as potential medical herbal for various diseases. Hashmi *et al.* found lupeol compound from crude extract of *H. nepalensis* which showed beneficial effects on antidiabetogenic and anti-Alzheimeric rats via antioxidant defense system.[9] Recently, hederagenin such as sapogenin or saponins received many attentions of scientists due to multiple actions on various diseases pathways such as antitumor, anti-inflammatory, or antiviral infection, as well as neuron and cardiovascular protection.[10] Our study combined both genetic and chemical analysis as core criteria to identify *Hedera* species. The *matK* gene has been used for species identification and studies in plant molecular systematic and evolution in various plant species.[11] DNA gene analysis techniques have been developed and applied in studying the genetic diversity of *Hedera* plants. Ackerfield and Wen detected low levels of nucleotide sequence divergence when they use chloroplast DNA and *ITS* data.[12] Among *Hedera* species, *H. helix* is a well-known plant

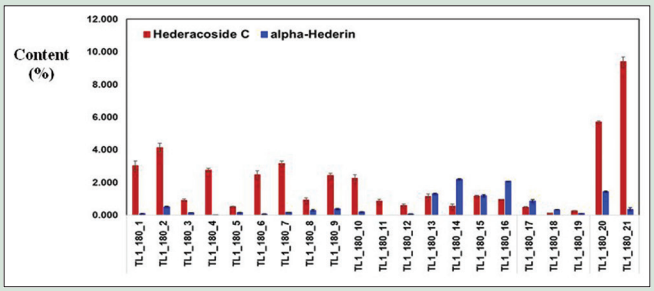


Figure 3: Hederacoside C and α-hederin contents of 21 *Hedera* species

distributed in Europe as well as Asia, however, *H. nepalensis* var. *sinensis* is mostly found in Asia including Vietnam. Out of 21 samples collected, 19 samples in our study were classified into *H. nepalensis* var. *sinensis* with *matK* gene. From the analysis of GBSSI (granule-bound starch synthase) marker, a previous study identified that the ivy plants which naturally grow and distribute in several northern mountainous provinces of Vietnam belong to the *H. nepalensis* K. Koch species.[13] In line with our study, Wu *et al*. analyzed 13 species in Araliaceae in China and using whole genome sequencing, it is clearly showed that *H. nepalensis* var. *sinensis* closed to *Fatsia japonica*.[13] However, this study did not identify the active compound in *H. nepalensis* var. *sinensis*.

In this study, it is found that *Hedera* species in Vietnam contain rich of hederacoside C and alpha-hederin compounds which are known to have beneficial effect on anti-inflammatory, especially for acute and chronic respiratory inflammation.[14] Using the HPLC method, extracted from leaves of *H. helix* grown in Poland and the Czech Republic provided lot of alpha-hederin and hederacoside C in the range from 0.7 to 2.3 mg/100 g extract and 13.9–18.4 mg per 100 g extract, respectively.[15] The concentration of hederacoside C was varied between *H. nepalensis* haplotypes and reached the highest concentration in haplotype I3 (TL1_180_1 and 2). Besides, the data analysis indicates that *H. nepalensis* samples are capable of producing hederacoside. Among those groups, groups were identified as *H. helix* which showed richest contents of hederacoside C with range from 5.7% to 9.4% and alpha-hederin with range from 0.4% to 1.4%. The HPLC results showed that the amount of those active compounds in this study was similar with *H. helix* grown in Europe. According to the European Pharmacopoeia, dried leaves contain at least 3% of the primary saponin, hederacoside C.[16] Similar *H. helix* content, hederacoside C in *H. nepalensis* var. *sinensis* from sample 1 to sample 12 was at higher level than alpha-hederin obtained from fresh leaf extract. Interestingly, from sample 13 to sample 18, alpha-hederin content was similar or even higher than hederacoside content. Alpha-hederin content in haplotype I5 (TL1_180_14) is more than 1.5 times higher than in the *H. helix* samples. Furthermore, it is known that hederacoside C is actively metabolized and produces the effect of α-hederin in the body.[17] Recently, the new analysis strategy has been found to discover the metabolic pathway of hederacoside C and the α-hederin biotransformation was observed through deglycosylation reactions by removal of sugar moieties [Figure 4].[18] An interesting paper published by Sieben *et al*. found that alpha-hederin but not hederacoside C or hederagenin had beneficial effects on cardiovascular disease via beta-2-adrenergic receptors.[19] Recently, alpha-hederin was also proved to have anticancer activities performed in cancer cell line.[18,20] These findings could be valuable suggestions for using *H. nepalensis* var. *sinensis* cultivated from sample 13 to sample 18 in group M1 which is abundant distributed in North Vietnam.

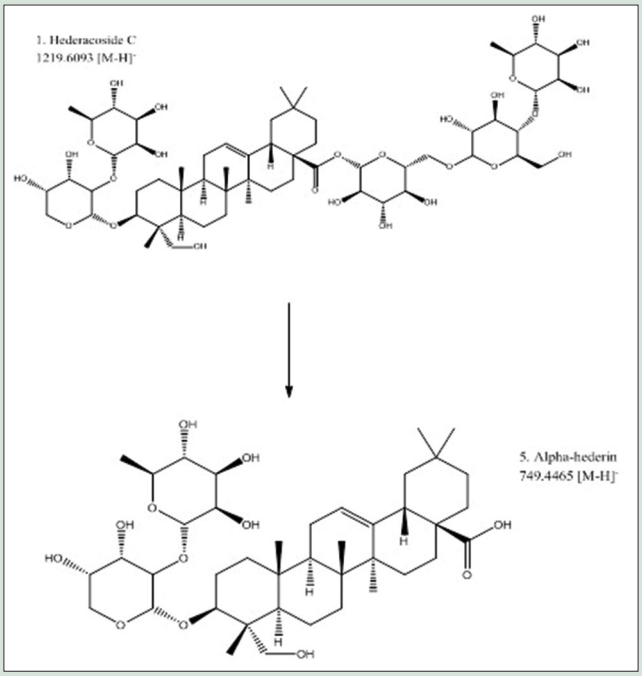


Figure 4: Hederacoside C metabolic pathway through biotransformation

CONCLUSION

In conclusion, local *Hedera* in Vietnam was recognized as *H. nepalensis* var. *sinensis*, which primarily distributed in Northern areas in Vietnam. By combining the data from genetic indicators with the analysis of the chemical composition, we can obtain essential information on local *Hedera* plants. From these results, we could recommend combine *matK* and *ITS* genes and hederacoside C analysis in classification of *Hedera* species in Vietnam. These are fundamental data, contributing to the construction and standardization of medical materials in Vietnam.

Acknowledgements

The study was funded by the Vietnam Ministry of Science and Technology (project code NC-2018/02). The authors sincerely thank Mr. Phan Van Truong (National Institute of Medicinal Materials) for assisting in taking samples for research purposes.

Financial support and sponsorship

The study was funded by the Vietnam Ministry of Science and Technology (Project code NC-2018/02).

Conflicts of interest

There are no conflicts of interest.

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