igMin Research STEM ISSN: 2995-8067 A Multidisciplinary Open Access Journal

SCIENCE SUBJECT
Spectroscopy | Molecular Biology | Biochemistry | TOPIC(S)

Article Information

Submitted: June 27, 2024
Approved: July 08, 2024
Published: July 10, 2024

How to cite this article: Huong NT, Thuy DN, van Trung P, Hung LN, van Nam M. DNA Genetics and UHPLC-Q-TOF-MS Analysis of Phytochemicals for *Asparagus racemosus* Roots. IgMin Res. July 10, 2024; 2(7): 558-563. IgMin ID: igmin217; DOI: 10.61927/igmin217; Available at: igmin.link/p217

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Keywords: *Asparagus racemosus*; Wild; DNA genetics; Water extract; UHPLC-Q-TOF

Research Article

DNA Genetics and UHPLC-Q-TOF-MS Analysis of Phytochemicals for *Asparagus racemosus* Roots

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Abstract

The medicinal herb *Asparagus racemosus* has been used in traditional medicine to treat various diseases such as cough, diarrhoea, diabetes, gastric issues, gonorrhoea, headaches, piles, rheumatism, and even lactation enhancement. This study explores the genetic information and phytochemicals of the species native to Gia Lai province, Vietnam before its conservation and cultivation. Five species of *A. racemosus* were analyzed using the trnL-e/trnL-f regions sequence. *A. racemosus* roots were extracted by water then Ultra-High-Performance Liquid Chromatography coupled with Quadrupole/Time-Of-Flight Mass Spectrometry (UHPLC-Q-TOF-MS) spectroscopy was used to screen for their phytochemicals. We have confirmed the DNA genetics of *A. racemosus* species collected in Gia Lai, Vietnam. In water extract of *A. racemosus* roots, UHPLC-Q-TOF-MS tentatively identified two flavonoids (Quercetin-3-glucuronide, Rutin), five steroidal saponins (Shatavarin I, Shatavarin IV, Shatavarin IX, Asparacoside, Asparanin A), and two steroids (β-sitosterol, Daucosterol). The experimental findings confirm the *A. racemosus* species for conservation and cultivation in Vietnam and contribute the benefits to the chemical literature of Vietnamese natural flora. *A. racemosus* should be further studied for pharmaceutical activities.

Introduction

Asparagus racemosus willd is a well-known medicinal plant belonging to the family Liliaceace [1]. Generally, *A. racemosus* plants are found in India, some regions of the Himalayas, and all over the region of Sri Lanka, Asia, Australia, etc. In Vietnam, *A. racemosus* is found to be distributed in Gia Lai province [2]. Its tuberous roots are used for bronchitis, constipation, dementia, diabetes, diarrhea, diuretic, dyspepsia, emollient, galactogogue, nervine tonic, rejuvenating, stomachic activities, etc [3]. The whole plant of *A. racemosus* including its leaves and roots is especially useful in traditional medicine. As the demand for *A. racemosus* is constantly on the rise; the supply is inadequate and now considered ‘endangered’ in its natural habitat. It is crucial to carry out its conservation, especially in Vietnam [3,4].

DNA barcoding is considered an efficient and accurate tool for global species identification [5]. DNA barcodes that are utilized for the classification of plants mostly belong either to the Internal Transcribed Spacer (ITS)–rDNA region in the nuclear genome, or the rbcL, MatK, psbA–trnH, and atpF–atpH regions in the chloroplast genome [6,7].

Chemical constituents from the plant roots include several acids such as ferulic acid, isoferulic acid, malic acid, citric acid, asparagusic acid, caffeic acid, and fumaric acid; steroidal glycosides (Shatavarin I-IV), asparaginine, and 9,10-dihydrophenanthrene derivative [8]. Flowers and mature fruits contain quercetin, rutin, and hyperoside. The phytochemical evaluation of the *Asparagus racemosus* leaves was performed and showed excessive amounts of steroids and triterpenoids, flavonoids such as diosgenin and quercetin-3-glucuronide [9,10].

Ultra-High-Performance Liquid Chromatography coupled with Quadrupole/Time-Of-Flight Mass Spectrometry (UHPLC-QTOF-MS/MS) is capable of accurately measuring molecular mass by giving the elemental composition of obtained ions and is widely used in analyzing complex samples due to the high resolution and sensitivity. UHPLC-QTOF-MS was applied to characterize chemical constituents and metabolites in medicinal herbs and obtained considerable results. HPLC-Q-TOF analysis of *A. adscendens* roots tentatively assigned the possible presence of saponins and spirostanol [11]. Xue, et al. (2022) have successfully applied the HPLC-Q-TOF-MS/MS to simultaneously quantify five saponin glycosides, asparacoside, shatavarin IX, shatavarin IV, asparanin A and shatavarin V in *A. racemosus* roots [12].

In this research, our first step for the conservation of the plant before its cultivation in the appropriate lands is to confirm the genetics of wild *A. racemosus* by sequencing psbA-trnH, and ITS1 nuclear rDNA regions. We also study the phytochemistry of *A. racemosus* roots by UHPLC-Q-TOF-MS spectroscopy.

Methodology

Chemicals and reagents

All reagents were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO., USA). Deionized water for HPLC; HPLC grade acetonitrile, methanol, and analytical grade formic acid (≥ 98%) were obtained from Fisher (USA).

Plant material

The roots of *A. racemosus* from the wild were collected in five different places of Gia Lai Province, Vietnam in October 2023: 1rst set at Kon Lo Khong commune, Kbang district, around point $14^0$05070N, $108^0$567090E (abbreviation 1); 2nd set at Ia Sao commune, IaGrai district, around point $14^0$048287N, $107^0$943662E (abbreviation 2); 3rd set at Kon Chieng commune, Mang Yang district, around point $13^0$791426N, $108^0$276529E (abbreviation 3); 4th set at Yang Nam commune, Krong Chro district, around point $13^0$679035N, $108^0$512821E (abbreviation 5); and 5th set at IaALe commune, Chu Pu district, around point $13^0$45311N, $107^0$07201E (abbreviation 5). Coordinate was measured by Magellan GPS 315 equipment.

Samples were primarily recognized based on the morphology in the field, then preserved in silica gel-filled bags, and transferred to the Vietnam Academy of Science and Technology for final identification by Dr. Nguyen Sinh Khang, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST) before preservation at -30 ^{0}C. The voucher specimens have been lodged at the Center for High Technology Development (CHTD, VAST).

All fresh plants were delivered for DNA analysis. All fresh roots were cut off from the plants, dried, powdered, and stored in a fridge for further water extract and HPLC-QTOF chemical analysis.

DNA extraction, PCR amplification

Total genomic DNA was extracted according to the method of Doyle and Doyle under the local laboratory conditions. Total DNA was extracted from about 100 mg of the plant sample following the CTAB extraction method. The extracted DNA was resuspended in 30 µl miliQ water and a standard 50 ng of the DNA was used for amplification. trnL-e/trnL-f region was amplified using universal primers [13]. PCR was performed in 25 µL of reaction system containing 7 µL deionized H_2O, 12.5 µL of PCR Master mix kit (2×), 1.25 µL of each primer (10 pmol/µL), and 1 µL of DNA template (50 ng). The PCR reaction was performed using PCR Model 9700 (GeneAmp PCR System 9700) for 3 min at 94 °C for denaturation, 35 amplification cycles (45 s at 94 °C for denaturation, 30 s at 55 °C annealing, and 30 s at 72 °C for extension), then 10 min at 72 °C for extension and then held at 4 °C.

Sequence analysis and alignment

PCR products were screened by electrophoresis on 1% agarose gel, and then sequenced at FirstBase Co. Ltd. Raw sequences obtained were assembled and edited by Chromas-Pro 2.1.6 (Technelysium Pty Ltd). All the sequences were then aligned on BLAST, Genbank (http:www.ncbi.nlm.nih.gov/BLAST). Pairwise distance was determined using Mega 7.0 (Kumar, 2016). The phylogenetic trees were constructed using Maximum likelihood and Bayesian inference with a bootstrap value of 1000.

Water extraction

The fresh roots of *A. racemosus* are washed, sliced, and dried for 5 days in an oven at 40 ^{0}C and ground into powder. Dried powder of the roots of *A. racemosus* was extracted with water (3 times) at room temperature in an ultrasonic 20 kHz and 1 kW extractor. The combined extract was concentrated to dryness under reduced pressure, yielding a residue (brown solid). The residue of the water extract was flushed with nitrogen gas and stored at -20 ^{0}C for future use.

UHPLC-Q-TOF-MS analysis

Sample analysis by the UHPLC-Q-TOF-MS instrument was investigated with the procedure described previously [14]. 100.0 (mg) of the extract was accurately weighed into a tube with a cover, and 2.0 (mL) methanol-water (8:2, v/v) solvent was added. The sample was ultrasonicated for 10 min. The sample was filtrated through a 0.45 (µm) filter membrane before injecting for UHPLC-Q-TOF analysis. Sample analysis was performed on an Exion LCTM UHPLC system (AB SCIEX,

USA) consisting of an Exion LC degasser, AC pumps, AC autosampler, controller, and AC column oven. Samples were analyzed on a Hypersil GOLD C18 column (150 x 2.1 mm, 3µ) (Thermo Fisher Scientific, USA). The mobile phase, water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B), was run at a flow rate of 0.4 (mL/min) at room temperature. The gradient programming was as follows: 0–4 min, 2% - 20% B; 4-30 min, 20% - 68% B; 30-32 min, 68% - 98% B; 32-40min, 98% B. Sample injection volume was 5.0 (µL).

An AB SCIEX X500R QTOF mass spectrometer (AB SCIEX, USA) with a Turbo V ion source was coupled with the UHPLC system. Mass data were acquired in both negative and positive Electrospray Ionization (ESI) modes. The ESI-MS conditions were set as follows: the ion source temperature, 500 °C; curtain gas, 30 psi; nebulizer gas (GS 1), 45 psi; heater gas (GS 2), 45 psi. For the TOF MS scan, the mass range was set at m/z 70–2000. For the TOF MS/MS scan, the mass range was set at m/z 50–1500. For the negative mode, the ion spray voltage was set at −4.5 kV, the declustering potential (DP) was −70 V, the collision energy (CE) was performed at −20 eV and the Collision Energy Spread (CES) was 10 eV. For the positive mode, the ion spray voltage was set at 5.5 kV, the DP was 80 V, the CE was 20 eV and the CES was 10 eV. All the obtained data were processed by SCIEX OS software version 1.2.0.4122 (AB SCIEX, USA).

Results and discussions

Total DNA extraction and PCR amplification

For the analysis of plant genetics, at first, its DNA was extracted following the CTAB extraction method. The total genomic DNA of five samples was successfully extracted. Then, the extracted DNA was amplified by PCR technique with appropriate primers. Total DNA extracts were used as the templates to amplify the trnL-e/trnL-f regions (480 bp). The samples gave 100% efficiency for trnL-e/trnL-f - PCR amplification. Electrophoresis of the PCR product from samples showed clear bands with lengths of about 480 bp for trnL-e/trnL-f (Figure 1).

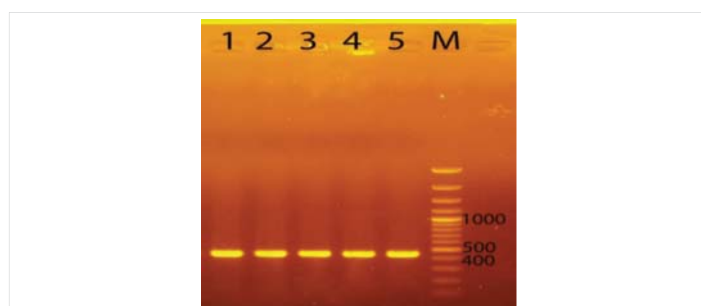

Figure 1: PCR product of DNA regions checked on 1% agarose gel. Abbreviations: 1, 2, 3, 4, 5, M: Marker 100bp plus DNA ladder, trnL-e/trnL-f (GGTTCAAGTCCCTCTATCCC/ATTTGAACTGGTGACACGAG).

PCR products were directly sequenced and then the obtained sequences were checked for the accuracy of the obtained PCR products by the Blast tool. The sequences were analyzed using Mega 10. The results showed trnL-e/trnL-f regions with 480 bp.

From Table 1 and Figure 2, the molecular phylogenetic tree of five *A. racemosus* samples and three *Asparagus* taxa was constructed by maximum likelihood analysis of the conserved regions. The stability of each tree node was tested by bootstrap analysis. Using trnL intron sequence, phylogenetic analysis of genus *Asparagus* species of Turkey revealed two clades [15]. In the trnL-e/trnL-f sequence diagram tree, five *A. racemosus* samples, taxa *Asparagus schoberioides* JN102165 were classified into two different genealogical branches. The analyzed samples grouped in four branches with similarity between 98,52% - 99,00%, taxa *Asparagus racemosus* NC047472.1 were closely classified in two different branches. All the referenced taxa are published in GenBank. More phylogenetic data of the Vietnamese *A. racemosus* species will be exposed when the number of analyzed samples increases. The data will be useful for DNA barcoding based on the authentication of this species.

UHPLC-Q-TOF-MS qualitative analysis

The total ion chromatograms (TICs) of *A. racemosus* water extract in HPLC-ESI-Q-TOF-MS negative mode are shown in Figure 3.

Some parent and fragment ions were observed in the mass spectra and summarized in Table 2.

Compound (1), at T_R = 7.32, in the ESI-QTOF negative mode, yielded a parent ion $[M-H]^-$ at m/z 477.1616, and provided fragment ions at m/z 59.0136, m/z 71.0121, m/z 89.0238, m/z 168.0412, and m/z 233.0669. Xue, et al. (2021) found the same compound in the roots of *A. racemosus* and determined its structure as Quercetin-3-glucuronide [16].

Table 1: Similarity of the *A. racemosus* samples.

Sample	Places	Scientific name	Similarity
1	Kon Lo Khong, Kbang	*Asparagus racemosus* Willd	98,78%
2	Ia Sao, IaGrai	*Asparagus racemosus* Willd	98,74%
3	Kon Chieng, Mang Yang	*Asparagus racemosus* Willd	99,00%
4	Yang Nam, Krong Chro	*Asparagus racemosus* Willd	98,78%
5	IaALe, Chu Pu	*Asparagus racemosus* Willd	98,52%

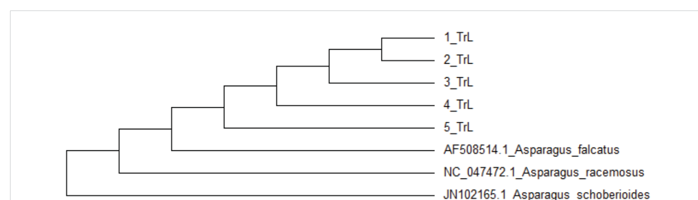

Figure 2: Tree diagram of the *A. racemosus* samples and references.

ISSN 2995-8067

DOI: 10.61927/igmin217

Table 2: HPLC-ESI-Q-TOF NEG assignments of compounds in *A. racemosus* roots.

RT	m/z and compound	MS spectrum	MS/MS spectrum
7.32	477.1616 Quercetin-3-glucuronide		
8.27	739.2095 Asparanin A		
8.47	609.1457 Rutin		
9.75	1066.5489 Shatavarin I		
11.14	901.4792 Shatavarin IX		
12.79	1003.5094 Asparacoside		
14.06	885.4821 Shatavarin IV		
18.93	397.2275 β-sitosterol		
21.08	397.3324 Daucosterol		

ISSN 2995-8067 DOI: 10.61927/igmin217

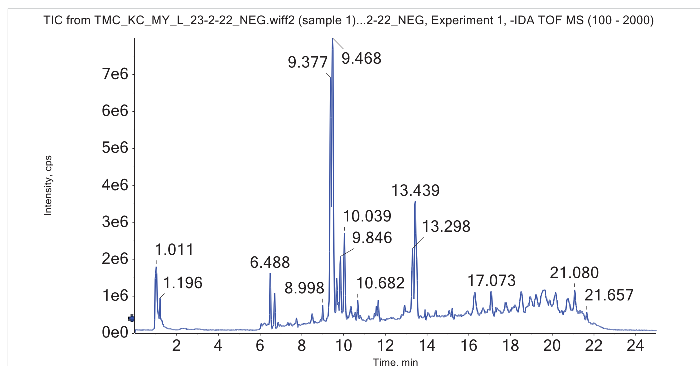

Figure 3: TIC of HPLC-ESI-Q-TOF-MS NEG mode of water extract of wild *A. racemosus* roots.

Thus, compound (1) was tentatively defined as Quercetin-3-glucuronide. Compound (2), at T_R = 8.27, in the ESI-QTOF negative mode, yielded a parent ion $[M-H]^-$ at m/z 739.2095, and provided major fragment ion at m/z 284.0315. Its structure is determined as Asparanin A [17]. At T_R = 8.47, in the ESI-QTOF negative mode, compound (3) yielded a parent ion at m/z 609.1457 and provided two fragment ions at m/z 112.9856 and m/z 301.0348. Saxena, et al. (2001) found the same compound in *A. racemosus* and determined its structure as Rutin [18]. The MS spectra of compound (4) (T_R = 9.75 min), yielded a parent ion $[M-H]^-$ at m/z 1066.5489 in the negative mode, without any fragmented ion. Compound (4) was determined as Shatavarin I [19]. Compound 5 (T_R = 11.14 min), exhibited parent ion $[M-H]^-$ at m/z 901.4792 without any fragmented ion in the ESI-QTOF negative mode. It was assigned to Shatavarin IX [19,20]. At T_R = 12.79, in the ESI-QTOF negative mode, compound (6) yielded a parent ion at m/z 1003.5094 and provided two fragment ions at m/z 112.9856 and m/z 301.0348. Onlom, et al. (2001) found the same compound in *A. racemosus* and determined its structure as Asparacoside [20]. Compound (7), at T_R = 14.06, in the ESI-QTOF negative mode, yielded a parent ion $[M-H]^-$ at m/z 885.4821, without any fragmented ion. Its structure is determined as Shatavarin IV [20,21]. At T_R = 18.93, in the ESI-QTOF negative mode, compound (8) yielded a parent ion at m/z 397.2275 and provided one fragment ion at m/z 96.9595. Ariful (2014) found the same compound in *A. racemosus* and determined its structure as β-sitosterol [22]. The MS spectra of compound (9) (T_R = 21.08 min), yielded a parent ion $[M-H]^-$ at m/z 397.3324 in the negative mode, and provided two fragment ions at m/z 335.3299, m/z 336.3342, and m/z 378.3200. Compound (10) was determined as Daucosterol [22]. In this study, ultrahigh-resolution liquid chromatography (UPLC) was used to effectively separate the phytoconstituents. A high-resolution mass spectroscopy (Q-TOF-MS/MS) detector was used to determine the parent ion and its fragments which can be compared with databases and literature for the structure of phytoconstituents without the use of their expensive standard compounds. With the use of standard compounds, respective constituents could be quantified by less expensive HPLC-DAD equipment.

In conclusion, from the water extract of *A. racemosus* roots, we have tentatively identified two flavonoids (Quercetin-3-glucuronide, Rutin), five steroidal saponins (Shatavarin I, Shatavarin IV, Shatavarin IX, Asparacoside, Asparanin A), and two steroids (β-sitosterol, Daucosterol) in the roots of *A. racemosus* by HPLC-Q-TOF-MS spectroscopy.

Conclusion

This study showed the DNA genetics and trnL-e/trnL-f sequence diagram tree of the Vietnamese plant *A. racemosus* samples from Gia Lai province. The chemical investigation of their roots was analyzed by UHPLC-Q-TOF-MS spectroscopy with the identification of nine known compounds. More samples collected from different wild forests should be genetically analyzed to show their DNA genetic relationships. And, quantitative analysis of the assigned compounds might be carried out to assess the quality of *A. racemosus* species. It might be of interest to further investigate the *A. racemosus* species in isolation of its chemical constituents and pharmaceutical activities.

Acknowledgements and source of funding

The project was supported by the grant KHGL-01-22.

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How to cite this article: Huong NT, Thuy DN, van Trung P, Hung LN, van Nam M. DNA Genetics and UHPLC-Q-TOF-MS Analysis of Phytochemicals for *Asparagus racemosus* Roots. IgMin Res. July 10, 2024; 2(7): 558-563. IgMin ID: igmin217; DOI: 10.61927/igmin217; Available at: igmin.link/p217