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Original article

Botanical, genetic characteristics and preliminary screening of the phytochemical constituents of *Hydnophytum formicarum* Jack. in Phu Quoc forest, Vietnam

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Abstract: In Vietnamese folk medicine, Bi ky nam (*H. formicarum*) tuber has been widely used to treat rheumatism, liver and intestinal diseases. This work aimed to study botanical, genetic characteristics and screening of the phytochemical constituents of wild *H. formicarum* of Phu Quoc Island, Vietnam. Anatomical characteristics of the plant material were described. Fresh leaves were used to analyze DNA barcodes based on *rbc*L region amplified by PCR. Sequences of DNA products were identified by Sanger method and BioEdit 7.0.5 software, then compared to the control *rbc*L sequences published in GenBank by BLAST. The tuber powder was studied for pharmacognostic parameters, preliminary phytochemical screening and total phenolic contents by Folin-Ciocalteu method. Results showed that the similarity between the *rbc*L sequences of *H. formicarum* leaves collected in Phu Quoc and the control one published in Genbank was 99%. Moisture content, total ash value and acid insoluble ash value of dried tuber powder were 11.06%, 9.60% and 0.70%, respectively. Raw material contained carotenoids, triterpenoids, flavonoids, phenolics, tannins, saponins, reducing substances and amino acids. Total phenolics content was about 58.847 mg pyrrogallol equivalent/g dried powder weight. Our results provided information about botanical, genetic and preliminary phytochemical characteristics of *H. formicarum* growing on Phu Quoc Island. This could be useful for the authentication of *H. formicarum* as a medicinal material.

Keywords: *Hydnophytum formicarum* Jack., botanical characteristic, DNA barcodes, phytochemical study, phenolics.

1. INTRODUCTION

In recent years, there has been a remarkable increase in the medical uses of plant-derived products [1]. Approximate 80% of the world population relied on traditional medicine for their health care [2]. *Hydnophytum* is a genus of epiphytic myrmecophytes (ant plants) belonging to the Rubiaceae family. Many species of the genus *Hydnophytum* are native to Southeast Asia. *Hydnophytum formicarum* Jack. is one of the species of *Hydnophytum*, growing on Phu Quoc Island, South of Vietnam. The tuber of *H. formicarum* has traditionally been used for the treatment of rheumatism, arthritis, liver and

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intestinal diseases [3]. In Asia, some studies showed that extracts of this medicinal plant have anti-oxidative, anti-bacterial, cytotoxic and hypoglycemic effects [4, 5].

Our study has been performed to evaluate the botanical, pharmacognostic, phytochemical and DNA features of *H. formicarum* collected from Phu Quoc Island which could serve as criteria of identification and authentication.

2. MATERIALS AND METHODS

Plant material

The fresh plants of *H. formicarum* with a uniform structure were collected from Phu Quoc, Kien Giang, Vietnam on July 2018 for the study on DNA barcodes and on February 2014 for the others. The voucher specimens were kept at the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam.

Chemicals and reagents

The following chemicals and reagents were used: absolute ethanol, ether, ferric chloride, sodium hydroxide, lead acetate, sodium carbonate purchased from Chemsol Company, Vietnam. Folin - Ciocalteu reagent and isopropanol were obtained from Merck & Co., Inc, Germany; pyrogallol was purchased from Sigma-Aldrich Co., USA. CTAB buffer (CTAB 2%, Tris-HCl 100 mM pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl), β-mercaptoethanol, chloroform: isoamyl alcohol (24:1), Rnase and agarose enzymes was obtained from Bio Basic Inc., Canada; PCR Mix was provided by NEXproTM, Genes Labs Inc., Korea; GelRed dye, TAE 1X cushion, loading dye 6x, 1 kb plus ladder and TE pH 8.0 from Promega Co., USA.

Botanical study

Macroscopic and microscopic features

The fresh plants were washed thoroughly with distilled water and air-dried according to the instructions of herbarium technique standards. The tubers were then cut and ground to powder stored in airtight bags for further uses.

The macroscopic features of stems, roots, leaves and tubers were described. For the anatomical study, cross sections of the fresh plant organs were prepared and stained with iodine green-carmine then washed with water. The sections were then observed through a microscope. The microscopic characteristics of the tuber powder were also observed. The photographs were taken via an optical microscope and a Canon digital camera.

Genetic study

All the investigated fresh leaves of the plant were frozen, dried and ground to a fine powder with liquid nitrogen prior to DNA extraction.

DNA extraction

DNA was extracted from 100 mg of fresh leaf using the CTAB protocol described by Doyle and Doyle (1990) [6]. DNA was submitted to electrophoresis on 1% agarose gel containing 1 µl GelRed to check the integrity and purity. The electrophoresis result was photographed under UV light. The extracted DNA samples were then stored at -20°C for future use.

Polymerase chain reaction (PCR)

PCR amplification was performed in PCR GeneAmp PCR System 2700 thermocycler (Amplied Biosystems, Singapore) using PCR kit (NEXpro^{TM} Diagnostics). PCR was carried out using 50 µl of reaction mixture containing 10X e-Taq buffer, 10 mM dNTP, e-Taq DNA polymerase, primers *rbc*L and distilled water. The thermocycler was programmed for 35 cycles, each cycle included 5 mins at 95°C for initial strand separation, 30 secs at 95°C for denaturation, 30 secs for primer annealing at 60°C, 30 secs for primer elongation at 72°C followed by 5 mins of final primer extension at 72°C. PCR products were separated on 1% agarose gel by electrophoresis.

Purification and sequencing

The PCR products were purified by Wizard SV Gel and PCR Clean-up System kit (Promega Co., USA), then sequenced by Sanger method [9] at Phu Sa Biochem Company in Vinh Long, Vietnam. The size and molecular weight of DNA bands were calculated by GelAnalyzer software [10]. The sequence result was stored in FASTA and analyzed by BioEdit 7.0.5 [11]. The sequences were compared to GenBank using the BLAST algorithm (Basic Local Alignment Research tool) [10]

Phytochemical study

Preliminary phytochemical screening

The tuber powder was extracted with ether, ethanol and water respectively by maceration. The extracts were then concentrated. The extracts were then concentrated and used for preliminary phytochemical tests to identify alkaloids, saponins, flavonoids, coumarins, carbohydrates, tannins, steroids, triterpenoids, cardiac glycosides, fats, reducing compounds and amino acids by the improved Ciuley method [12].

Pharmacognostic evaluation

Pharmacognostic parameters of tuber powder (moisture content, total ash value and acid insoluble ash of dried powder) were determined according to methods described in the 4th and 5th Vietnamese Pharmacopoeia (2014, 2017) [12, 13].

Identification of phenolic compounds

Five grams of tuber powder were extracted with 50 mL of 50% ethanol at 90°C for 2 hours. After filtration, this extract was used for histochemical tests to identify the presence of phenolic compounds with 2% ferric chloride, 20% sodium hydroxide and 1% lead acetate.

Quantification of total phenolics content

One gram of tuber powder was extracted with 50% ethanol solution until the filtrate did not change its color in reaction with ferric chloride solution. The filtrate was concentrated to obtain the crude extract. This crude extract was totally dissolved in water using a sonicator and then filtrated through a paper filter for total phenolics content determination.

The total phenolic content was determined by using the Folin-Ciocalteu assay [15]. The extract and the standard pyrogallol solutions at different concentrations (10-50 µg/ml) were taken to test tubes before 0.5 mL of Folin-Ciocalteu

reagent (10%; w/v) was added. After 5 mins, 1.5 mL of sodium carbonate solution (29%; w/v) and 2.5 mL of distilled water were added to the mixtures and shaken. The mixtures were then incubated in 30 mins at room temperature under dark condition. After 5 mins of centrifugation at 5000 rpm to precipitate sodium carbonate crystals, the absorbances of solutions were measured at 760 nm with a UV-Visible spectrophotometer. The blank sample including Folin-Ciocalteu reagent, sodium carbonate solution and distilled water was carried out at the same time. The total phenolic content was expressed as mg of pyrogallol equivalents/g of dried tuber powder.

Statistical analysis

The data were analyzed using Microsoft excel 2013 software and obtained mean ± SD (n = 3).

3. RESULTS

Botanical characteristics

Macroscopic features: H. formicarum grows on trees in Phu Quoc Forest (Figure 1A). It is an epiphytic shrub with a tuber at its base also known as a caudex which is spineless and has two to four stems that reach about 60 cm long (Figure 1B). Slender stems, on which the leaves and flowers are born, grow out from the tip of the caudex. In contrast, leaves, which grow 4-15 cm long and 2-7 cm wide, are elliptical and have a leathery texture (Figure 1C and D). Swollen main stem (or caudex) is slightly bumpy, brown, 15-20 cm across and has many holes on the surface which are linked by numerous tunnel made by ants inhabit there (Figure 1E and F).

These characteristics have been described by Vo (1997). Because we could not collect the flowers, fruits and seeds of *H. formicarum* from Phu Quoc Forest, it was difficult to obtain fully macroscopic features for accurate identification of this species. Therefore, we added the study on DNA barcodes of *H. formicarum* from Phu Quoc Forest in this work.

Microscopic features:

Stem: The stem has a circular outline. The outermost zone consists of radial bands of rectangular, tangentially elongated, thin-walled cork cells in 5-10 rows. The cortical zone comprises heterogeneous cells of parenchyma and big nests of sclerenchyma. The xylem and phloem are continuously arranged with a ring of fibers outside. The secondary phloem is composed of phloem fibers in small patches with thin-walled parenchyma in between. The vascular cylinder is composed of dense solid secondary xylem with small vessels. At the inner end of the secondary xylem, a ring of thickened and pitched cells is located. The parenchymatous pith presents in the center.

Root: Cross section of the root shows circular or oval shape in outline with no fissures. Cork consists of thin-walled, regularly arranged cork cells. Thin phelloderm is composed of tangentially elongated, slightly thickened cells. Inside phelloderm, there are many layers of parenchyma with a big nest of sclerenchyma and aerenchyma. Secondary xylem is narrow and parenchymatous. Secondary xylem is dominated by thickened, pitted fibers.

Leaf: Epidermis of the upper and lower side is covered with papillae, trichomes are absent. Cross section of leaf blade is bifacial with a single row of large hypodermis under the upper epidermis. Mesophyll cells are differentiated into palisade and spongy parenchyma. Palisade parenchyma below Vhypodermis consists of three vertically elongated cell layers. Spongy parenchyma lies below palisade parenchyma and is loosely arranged. Midrib has 34 layers of collenchyma under the epidermis. Vascular bundles are of the open type with a ring of sclereids outside.

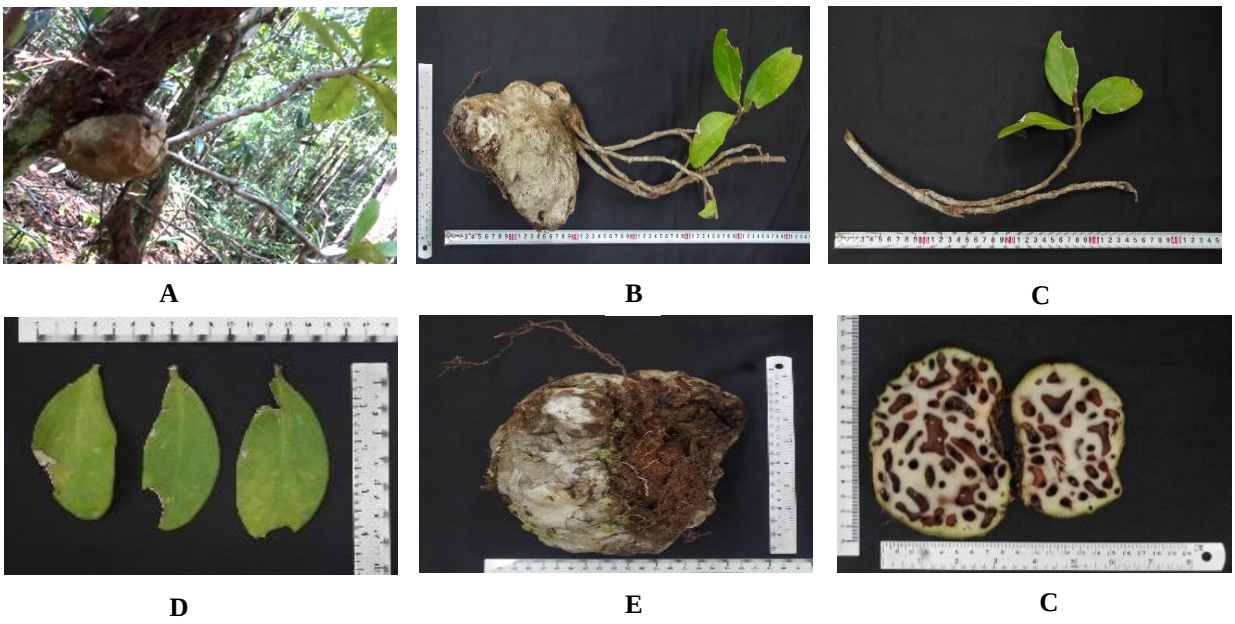


Figure 1. *H. formicarum* (A-F). (A) Photograph of the plant in Phu Quoc Forest. (B) Whole plant. (C) Stems with leaves. (D) Leaves. (E) Tuber outer surface. (F) Tuber inner.

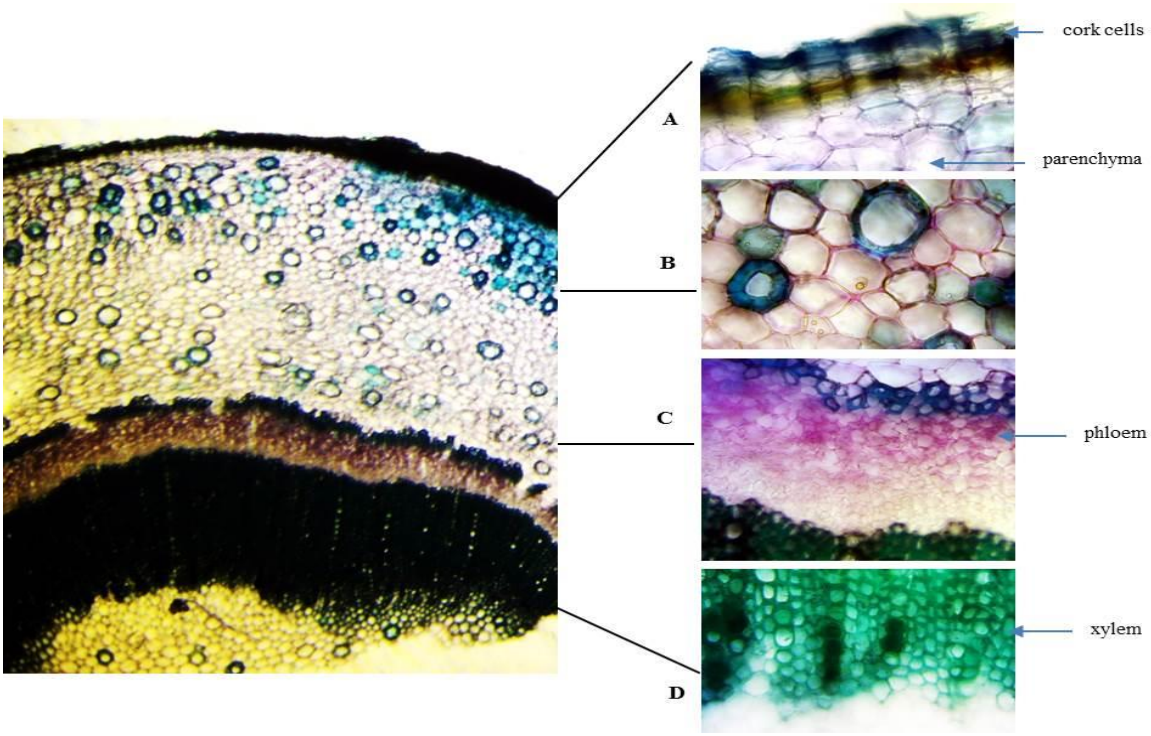


Figure 2. Stems of *H. formicarum.* (A) cork, (B) cortical parenchyma, (C) primary and secondary phloem, (D) secondary and primary xylem.

Powder characteristics: The dried tuber powder is yellowish, odorless and tasteless (Figure 5A). Under the microscope (Figure 5B) the powder shows acicular calcium oxalate crystals (1), groups of sclereids composed of elongated cells with thick wall (2), individual fibers (3), numerous fragments of cork with conspicuous granules (4), numerous fragments of thin-walled parenchyma (5) and elements of xylem (6).

Genetics characteristics

The electrophoresis image (Figure 6A) shows that the integrity of DNA extracted from fresh leaf was intact. Therefore, we used this DNA to amplify with designed *rbc*L primers. The *rbc*L primer successfully amplified the *rbc*L segment; the PCR product has a size of 600 bp (Figure 6B).

The data of DNA sequencing of *H. formicaum* was analyzed by BioEdit 7.0.5: GTAAAA-TCCAGTCCACCACGAAGGCATTCATAAACTGGCTCT ACCGTAGTTTTTAGCGGATAAACCTAATTTAGGTTT AATAGTACATCCCAACAGGGGACGACCATACTTGT TCAATTTATCTCTCTCGACTTGAATGCCGTGAGGCG GACCTTGGAAGGTTTTAACATACGCAATGGGAATTC GCAAATCTTCCAGACGCAGAGCACGCAGGGCTTTG AACCCAAATACATTACCTACAATAGAAGTAAACAT GTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGG GGTAAGCTACATAAGCAATATATTGATTTTCTTCTC CGGCAACTGGCTCAATATGGTAGCATCGCCCTTTGT AACGATCAAGACTGGTAAGCCCATCCGTCCATACA GTTGTCCATGTACCAGTAGAAGACTCGGCAGCTACC GCGGCCCCAGCTTCTTCCGGCGGAACTCCAGGTTGG GGAGTTACTCGGAATGCTGCCAAGATATCAGTATCT TTGGTTTGGTATTCAGGAGTATAATAAGTCAATTTG TACTCTTTAACACCAGCTTTGAACCCAACACTTGCT TTAGTCTCTGTTTTGGGGTGACATA.

Compared to the sequence of the chloroplast *rbc*L gene of *H. formicarum* published on Genbank [15], the result is shown in Table 1.

The similarity between the sequence of the *rbc*L fragment of the tested *H. formicarum* leaf sample and the control one (code X81099.1) [16] on Genbank was 99%. This result confirmed that plant samples collected from Phu Quoc Forest in Kien Giang, Vietnam were *Hydnophytum formicarum* Jack. Therefore, we could use these plant samples for further phytochemical, pharmacognostic and pharmacological studies.

Phytochemical characteristics

Preliminary phytochemical screening

Preliminary phytochemical screening is an important step in the bioactive analysis procedure that gives a brief summary of the phytochemical characteristics of the targeted plant. The results showed that there was the presence of carotenoids, triterpenoids, flavonoids, phenolics, tannins, saponins, reducing compounds and amino acids. Alkaloid, glycoside, coumarin and fat were not detected in *H. formicarum* tuber (Table 2).

Pharmacognostic parameters

The average of moisture content, total ash value and acid insoluble ash value of tuber powder were 11.06 ± 0.01 (%), 9.60 ± 0.70 (%) and 0.70 ± 0.06 (%), respectively. These results met the requirements of Vietnamese Pharmacopoeia for raw medicinal materials.

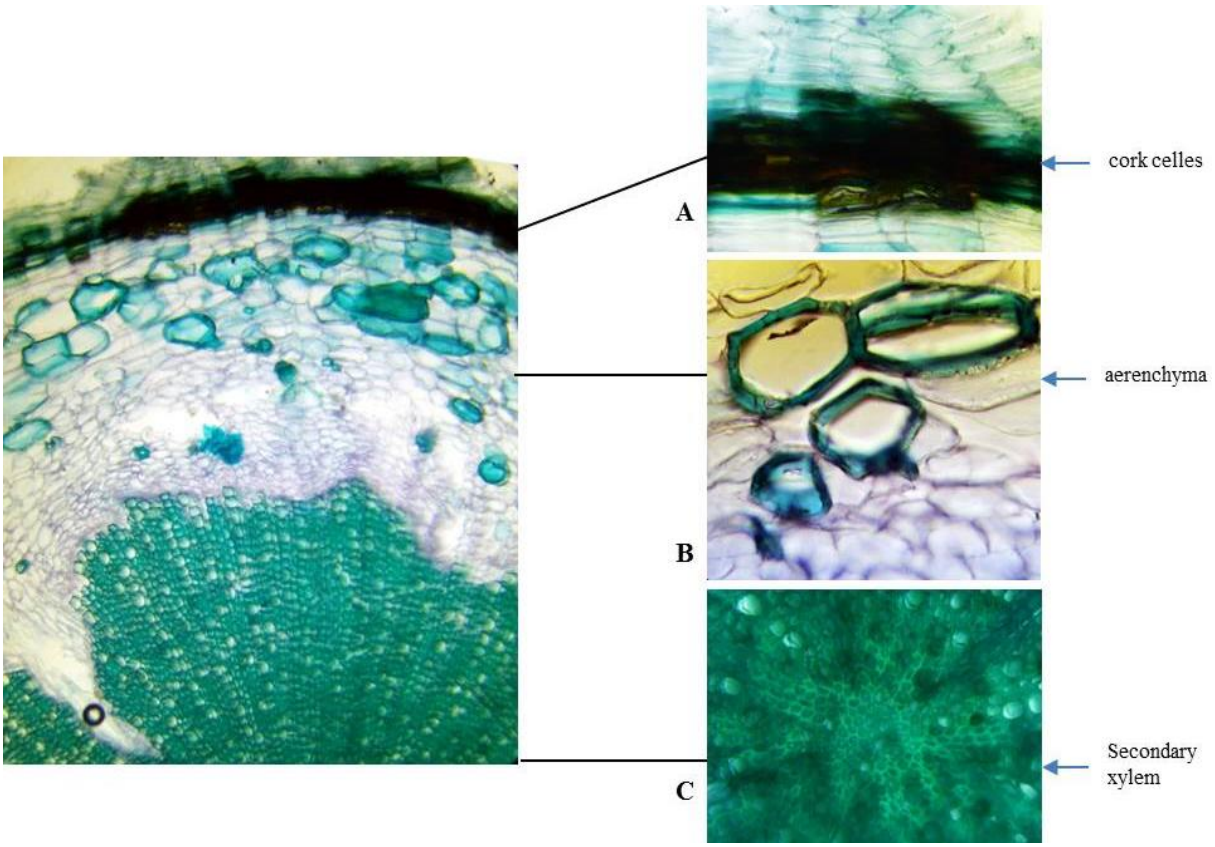


Figure 3. Roots of *H. formicarum*. (A) cork, (B) parenchyma with sclerenchyma, (C) secondary xylem

Table 1. The similarity in ***rbc*L gene sequences between** BLAST data and NCBI one

Sample	**Comparison of BLAST data with NCBI one**			Reference
	Similar species	Number code	% similarity	
BKN	*Hydnophytum formicarum* Jack.	X81099.1	99	Manen and Natali, 1995

Table 2. Preliminary phytochemical screening of *H. formicarum* tuber

Chemical constituents	**Name of the tests**	**Ether extract**	**Ethanolic extract**	**Water extract**
Alkaloids	Mayer's test, Dragendoff's test	-	-	-
Amino acids	Na_2CO_3 test	+	+	+
Cardiac glycosides	Raymond's test, Xanthydrol test	-	-	-
Coumarins	Fluorescence test	-	-	-
Fats	Stain test	-	-	-
Flavonoids	Shinoda test	+	+	+
Reducing compounds	Fehling's test	+	+	+
Saponins	Foam test	+	+	+
Tannins	Gelatin test, Ferric chloride test	-	+	+
Triterpenoid	Salkowski test	+	-	-

Result: + refers presence and - refers absence

Identification of phenolic compounds

The present study qualified and quantified phenolic compounds in *H. formicarum* tuber. The phenolic compound's presence shows by the color changes after the extract was mixed with 2% $FeCl_3$, 20% NaOH or 1% $(CH_3COO)_2Pb$ (Figure 7). All identification reactions were positive; thus, there was the presence of phenolic compounds in the *H. formicarum* tuber.

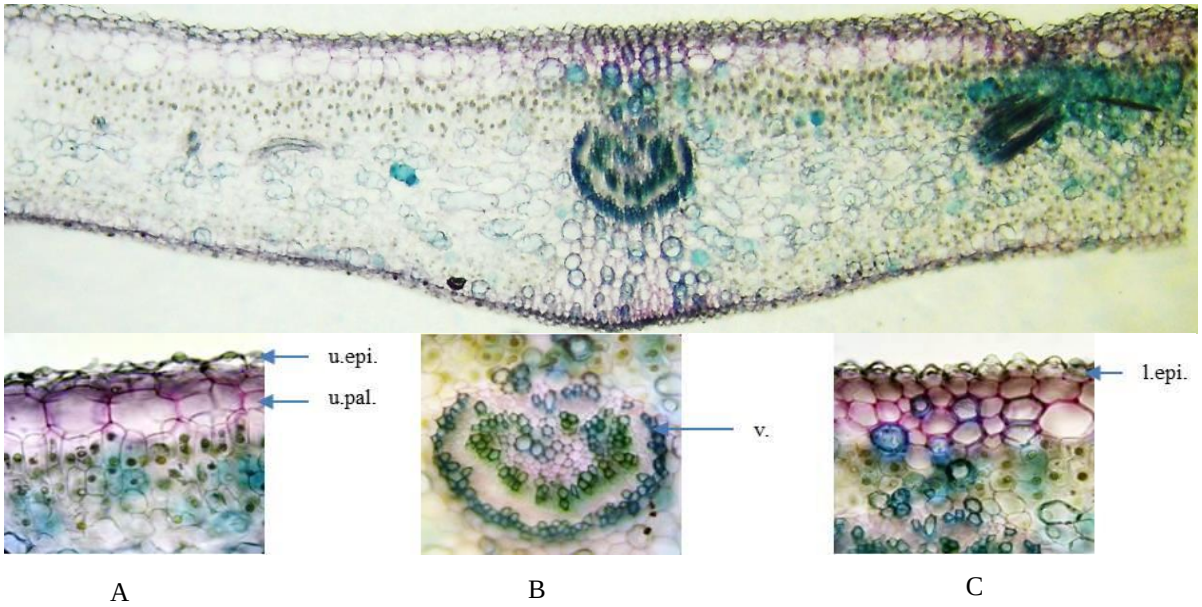


Figure 4. Leaf of *Hydnophytum formicarum* Jack. (A) upper epidermis and palisade parenchyma

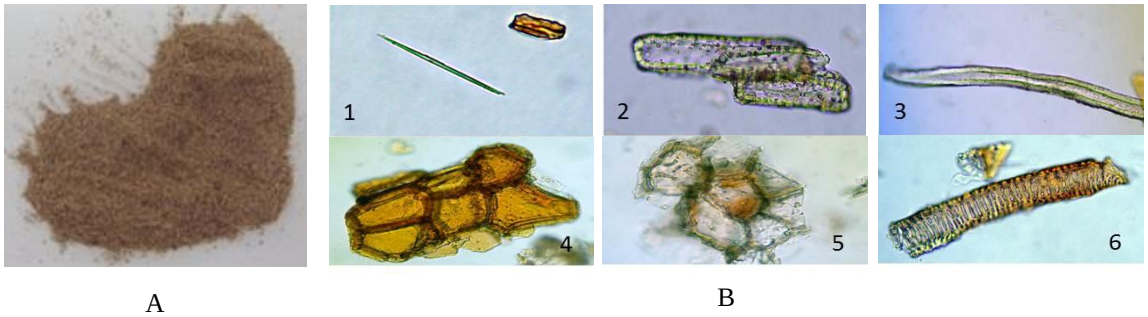


Figure 5. Tuber powder of *H. formicarum*

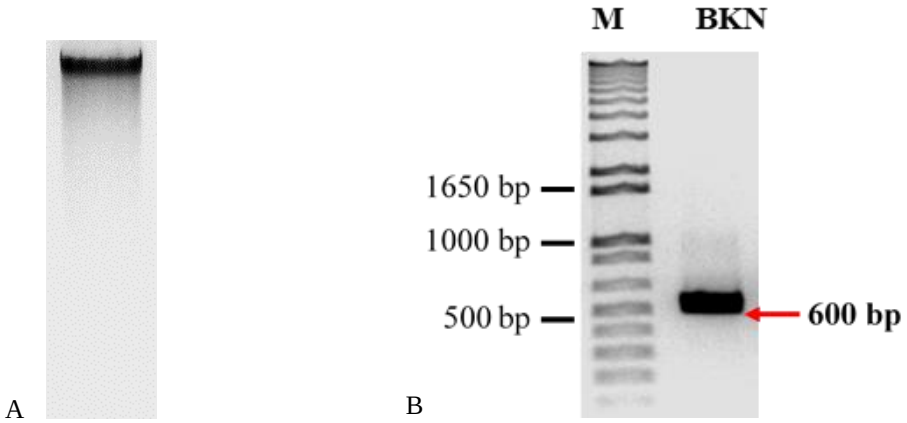


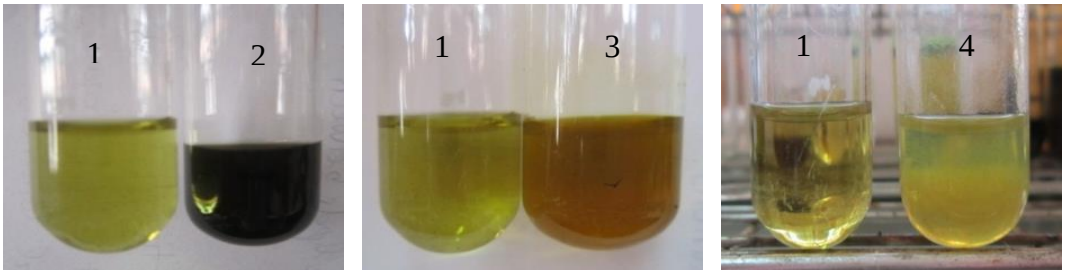
Figure 6. Electrophoresis image of DNA from fresh leaf (A); PCR products of *rbc*L segment (BKN) and 1 kb plus DNA ladder (M) (B).

Determination of total phenolics content

According to the calibration curve of pyrogallol standard ($y = 0.0177x - 0.0394$, $R^2 = 0.9984$), the total phenolic content in the dried tuber powder of *H. formicarum* was 58.847 ± 1.697 mg/g as pyrogallol equivalent, corresponding to about 6% (w/w).

4. DISCUSSION

The macroscopic characteristics of *H. formicarum* grown in Phu Quoc Forest are similar to those described by Vo et al. [3]. The identification of medicinal plants based on botanical characteristics is difficult, especially for species with high similarities in morphological characteristics. Therefore, DNA-based approaches have been used for the quality control of herbs



(1): Extract from *H. formicarum* tuber, (2) Extract with 2% $FeCl_3$,
(3) Extract with 20% NaOH, (4) Extract with 1% $(CH_3COO)_2Pb$

Figure 7. Identification of phenolic compounds in *H. formicarum* tuber by chemical tests

and herbal products. DNA barcoding which is based upon short and standardized gene regions known as barcodes is a quick, cos-effective and species-level technique to identify herbal materials [16]. Moreover, for wild *H. formicarum* in Phu Quoc forest, it is usually found and/or collected without flowers, fruits and seeds. Thus, this work studied its DNA fingerprint to identify the plant. The similarity between the sequence of the rbcL fragment of the tested *H. formicarum* leaf sample and the control one (code X81099.1) [16] on Genbank was 99%. This result confirmed that plant samples collected from Phu Quoc Forest in Kien Giang, Vietnam were *H. formicarum* Jack. Therefore, DNA-based approaches based on the rbcL fragment can be used for the identification of different species of Hydnophytum distributed throughout Vietnam.

The preliminary phytochemical screening indicated the presence of carotenoids, triterpenoids, flavonoids, phenolics, tannins, saponins, reducing compounds and amino acids. These phytoconstituents may contribute to the various medicinal as well as nutritional properties of the plant. These results corresponded to the previous report of Prachayasittikul et al. (2008) in which some flavonoid and phenolic compounds in the extracts from H. formicarum tuber expressed the cytotoxic, antioxidative and antimicrobial activities [4]. Senawong et al. (2013) reported that ethanolic and phenolic-rich extracts from *H. formicarum* tuber possessed histone deacetylase (HDAC) inhibitory activity to inhibit proliferation of 5 human cancer cell lines mediated by induction of apoptosis [5]. The present study indicated phenolic compounds are present in the *H. formicarum* tuber with the total content of 58.847 ± 1.697 mg/g dried tuber powder as pyrogallol equivalent, corresponding to about 6% (w/w). Phenolics are responsible for a wide set of pharmacological properties; thus these compositions are present in most drugs used in phytotherapy [17]. This suggests that *H. formicarum* tuber could be a medicinal plant rich in potent biological agents to use for further studies on developing novel functional foods or new drugs.

5. CONCLUSION

The present study provided information about the botanical, genetic and phytochemical characteristics of *H. formicarum* growing on Phu Quoc Island, Vietnam. Its tuber contains phenolics, flavonoids, tannins, triterpenoids, saponins, amino acids and reducing compounds. These results could be useful for the authentication of *H. formicarum* as medicinal material used in diseases prevention and treatment.

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