Vietnam Journal of Science and Technology ***64*** *(2026)*

doi:10.15625/2525-2518/21503

Comparison of chemical and molecular characteristics of *Phyllanthus amarus* and *Phyllanthus urinaria* species

Huynh Thi Thu Hue[1, 2, *], Le Tat Thanh[1, 2], Do Thi Thanh Trung[1], Nguyen Thi Bich Ngoc[1]

[1]*Institute of Genome Research, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Ha Noi, Viet Nam*

[2]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Ha Noi, Viet Nam*

[*]Email: hthue@igr.ac.vn

Received: 11 September 2024; Accepted for publication: 18 December 2024

Abstract. *Phyllanthus amarus* and *Phyllanthus urinaria* belonging to *Phyllanthaceae* are well-studied for their precious extracted compounds. Phyllanthin and hypophyllanthin provide a variety of potential health benefits for humans. Phyllanthin and hypophyllanthin biosynthesis pathway is different between *P. amarus* and *P. urinaria*. In our study, with High-performance liquid chromatography (HPLC) to quantify hypophyllanthin and phyllanthin concentration extracted from leaf and stem tissue of these two species, these compounds significantly varied between *P. amarus* .TN and *P. urinaria* .TN. Combined with quantitative PCR (qRT-PCR) used to measure the expression of six genes related to the biosynthesis pathway, the expression of these genes was stronger in *P. amarus* than *P. urinaria*. DNA barcoding markers are also used for divergence analysis and phylogenetic construction. Thereby, *ITS* sequence was expected to be one of standard DNA barcoding markers due to its high conserved site percentage for variations analysis and phylogenetic construction.

Keywords: *P. amarus, P. urinaria*, hypophyllanthin, phyllanthin, DNA barcoding

Classification numbers: 1.3.2; 1.3.4

1. INTRODUCTION

The genus *Phyllanthus* (*Phyllanthaceae)* consists of approximately 1000 species, spread over the American, African, Australian and Asian continents [1, 2]. It is a large genus and widely distributed in tropical and subtropical zones like tropical Africa, tropical America, Asia and Oceania [1]. Phyllanthus has been used as a traditional herbal remedy for many years in countries like China, India, Brazil, and various Southeast Asian nations. Traditional medicine systems have long utilized *Phyllanthus* species for conditions such as cancer, diabetes, and liver diseases [2 - 4]. The most abundant species are used in India and have a beneficial role in Ayurveda for treatmenting digestive, genitourinary, respiratory and skin diseases [1, 5]. In China, herbs and their prescriptions are used to treat hepatitis B, hypertension, dropsy and sore throat [1]. These herbal drugs are employed by local inhabitants of Thailand, Latin America, Brazil and Africa to cure jaundice, renal calculus and malaria [1]. Plants of the *Phyllanthus* genus contain

metabolites in the form of alkaloid, terpenoid and polyphenolic compounds such as flavonoid, phenolic acid, stilbene, anthocyanin, coumarin and lignin [5]; more than 510 compounds have been isolated from *Phyllanthus* [1]. *Phyllanthus urinara* L. and *Phyllanthus amarus* Schum. & Thonn are two different species within the *Phyllanthus* genus of *Phyllanthaceae* family.

The extracts from different parts of *Phyllanthus* address conditions like wounds, urinary tract disorders, sexually transmitted diseases, hypertension [2, 4, 6]. Phyllanthin and hypophyllanthin found in various *Phyllanthus* species, offer a range of potential health benefits based on scientific research. Their molecular formulae have been shown as C24H34O6 and C24H30O7, respectively. The structure of phyllanthin has been demonstrated by NMR spectra to be (+)3,4,3′,4′,9,9′-hexamethoxy-8:8′-butyrolignan (IV) [7 - 9] (Fig .1). Many studies suggest that hypophyllanthin and phyllanthin have anti-inflammatory [10], immuno-modulating properties [11], anticancer effects [9] and antiviral activity [10 - 13]. Phyllanthin and hypophyllanthin also have been investigated for their antidiabetic and chemoprotective effects [14 - 15]. These compounds have shown potential cytotoxicity and chemo-modulatory effects, particularly in synergizing with anticancer properties against resistant breast cancer cells [13]. They have been found to interfere with cell cycle progression, induce apoptosis and inhibit tumor cell migration and invasion, showcasing their promising anticancer activities [13]. Additionally, recent studies suggest that phyllanthin and hypophyllathin may have activity against COVID-19 by inhibiting specific viral proteins, indicating a broader spectrum of potential pharmacological effects [16]. No direct toxicity and side effects were reported from these two plant-derived lignans [17]. In Vietnam, Phien H. H. and Men T. T. (2023) applied bioactive compounds from *Phyllanthus* for the treatment of urinary stones [18]. Additionally, a study on the value chain of *Phyllanthus* medicinal plants conducted by Huynh Bao Tuan *et al*. (2013) focused on their application in pharmaceutical production [19]. However, *P. amarus* and *P. urinaria* are widely used in traditional medicine in which *P. amarus* tends to be favored and trusted more, particularly for its perceived stronger medicinal properties [19]. Acttualy, the two species cannot easily be distinguished on the basis of whole plant but it becomes very difficult when the materials are in dried and matted.

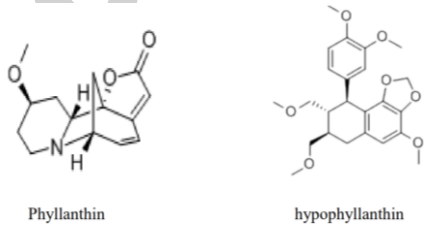

Figure 1. The structure of phyllanthin and hypophyllanthin [20].

The biosynthesis pathway of phyllanthin and hypophyllanthin from *Phyllanthus* is involved in the dimerization of cinnamic acid via the shikimate, phenylpropanoid,and flavonoid pathways [10 - 21]. Several important enzymes that are responsible for these biochemical processes include phenylalanine ammonia-lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*), pinoresinol-lariciresinol reductase (*PLR*), secoisolariciresinol dehydrogenase (*SLD*), chalcone isomerase (*CHI*), chalcone synthase (*CHS*) [22 - 23]. Besides several genes serving as important catalysts, a variety of genes that are probably related to these pathways. *PAL2* is involved in the phenylpropanoid pathway and is responsible for the biosynthesis of flavonoids, lignins, and other phenolic compounds [22 - 24]. *CHI* catalyzes the conversion of chalcones to flavanones,

which are precursors to flavonoids [25]. *CHS* initiates the phenylpropanoid pathway by converting phenylalanine to chalcones, which are then converted to flavonoids by *CHI* [26]. *CHS13* (a specific isoform of *CHS*) is involved in the biosynthesis of flavonoids in *Phyllanthus* species [26]. *PrR* is involved in the regulation of phenylalanine metabolism and phenolic compound biosynthesis [27]. *PCBER* is involved in phenylalanine's catabolism, which is a precursor to phenolic compounds [27 - 28]. These genes play crucial roles in the biosynthesis of flavonoids and other secondary metabolites in *Phyllanthus urinaria* and *Phyllanthus amarus*, known for their various biological and pharmacological activities [27 - 29]. According to Mazumdar, 2016, the transcriptome analysis of *P. amarus* shown several genes were predicted to be involved in the synthesis of lignan, phenylpropanoid and flavonoid such as *PCBER*, *PAL*, *CHS*, *CHI*, etc [6]. PLR is also one of the key enzymes of the lignan biosynthetic pathway which responsible for catalyzing the formation of secoisolariciresinol from pinoresinol, the precursor of phyllanthin and hypophyllanthin [30]. There was a high similarity in sequence between *PCBER* and *PLR* (pinoresinol/lariciresinol reductase) [31]. The phyllanthin biosynthetic pathway in *Phyllanthus* species has not been fully characterized to date. Therefore, at the genetic level, these genes are mainly involved in the phenylpropanoid pathway, which plays an important role in the formation of precursors such as p-coumaric acid and specific enzymes involved in the synthesis of lignans from these precursors. Moreover, the number of annotated genes and sequences that are involved in the phyllanthin biosynthetic pathway is very limited [6, 26, 32].

DNA barcoding markers use specific regions of DNA in order to identify species [33]. As a research tool for taxonomists, it assists in identification by expanding the ability to diagnose species by including all life history stages of an organism. As a biodiversity discovery tool, it helps flag species potentially new to science [34]. The process entails two basic steps: (1) building the DNA barcode library of known species and (2) matching the barcode sequence of the unknown sample against the barcode library for identification. Furthermore, DNA barcoding has been extensively used to identify and discriminate *Phyllanthaceae* species. Chloroplast DNA sequences, particularly *rbc*L and *mat*K, are commonly used as barcodes for *Phyllanthaceae* species identification and phylogenetic analysis [35 - 39]. Phylogenetic trees using chloroplast protein-coding genes and polymorphic loci show clear separation of *Phyllanthus* species [37]. It has been successfully applied to identify *Phyllanthus* species used medicinally in Brazil [35] and to assess product adulteration and species admixtures in the raw drug trade of *Phyllanthus* in India [36]. In a study of flowering plants in Indonesia, *rbc*L had higher amplification and sequencing success compared to *mat*K for *Phyllanthaceae* species [38]. However, using both markers together provides more reliable identification. The genetic diversity and phylogenetic relatedness of *Phyllanthus* herbs are also being studied for future conservation strategies [2, 40]. Therefore, the research was conducted to have an overview between the two *P. amarus* and *P. urinaria* species which often to be confused in folklore in Viet Nam.

2. MATERIAL AND METHODS

2.1. Plant materials and sample preparation

The plants were grown under the same conditions in the botanical garden in Thai Nguyen, Viet Nam, were collected and deposited at Institute of Genome Research, Vietnam Academy of Science and Technology. The samples notation are *P.amarus* .TN and *P.urinaria* .TN. For DNA barcode sequencing, three different samples were collected in each species, store in silica gel beads. The leaf and stem tissues were collected for the RNA extraction procedure and stored in

the RNA-later stabilization reagent (Qiagen, Germany) under -80°C. The whole plants were dried for chemical extraction.

2.2. Determine the phyllanthin and hypophyllanthin content

Dried crushed *P. amarus* and *P. urinaria* (10 g) were successively and exhaustively extracted by a hot Soxhlet process with ethanol 100 %. The solvents were removed under reduced pressure to give *P. amarus* extract (2.13 g, yield 21.3 %) and *P. urinaria* extract (1.92 g, yield 19.2 %). All the extracts were stored at 4 °C for further work.

The HPLC analysis was carried out on HPLC Alliance™ e2695 - Waters system with autosampler, XBridge BEH C18 column (250 × 4.6 mm, 5 µm) and UV detector, at 28 °C with acid phosphoric 0.1 % during 30 min. The detection wavelength was 230 nm and the injection volume was 10 µl with a flow rate of 1 mL/ min. The extract was dissolved to give a final concentration at 1 mg/mL. The was phyllanthin and hypophyllanthin were evaluated on the basis of calibration curve y = 17711x + 1365,6; R^2 = 1 and y = 9619,6x – 113,7; R^2 = 1, respectively.

2.3. Molecular markers

We utilized a nuclear marker (*ITS*) and two chloroplast markers (*rpo*C1 and *psb*A-*trn*H) to evaluate their effectiveness as DNA barcodes across all samples. Consequently, the *rpo*C1 and *psb*A-*trn*H markers were consolidated into a chloroplast DNA (cpDNA) dataset after successful amplification. Based on the conserved regions at both ends of each marker and the reference sequences of *P. amarus* and *P. urinaria*, specific primer pairs were designed to amplify these markers in the selected samples (Table 1).

Table 1. The designed primers for amplification of the DNA barcoding markers.

Region	Primer name	Sequence (5' → 3')	Length (bp)
ITS1	ITS1 F	CCTTATCAYTTAGAGGAAGGAG	22
	ITS1 R	GCCRAGATATCCGTTGCCGAG	21
ITS2	ITS2 F	YGACTCTCGGCAACGGATA	19
	ITS2 R	CCGCTTAKTGATATGCTTAAA	21
*rpo*C1	*rpo*C1 F	GGCAAAGAGGGAAGATTTCG	20
	*rpo*C1 R	CCATAAGCATATCTTGAGTTGG	22
*psb*A-*trn*H	*psb*A-*trn*H F	GTTATGCATGAACGTAATGCTC	22
	*psb*A-*trn*H R	ATGGTGGATTCACAATCC	18

2.4. DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was extracted using a modified version of the Doyle and Doyle (1990) protocol [36, 41]. Specifically, 0.1 g of fresh leaf tissue was ground in 2 ml of preheated CTAB buffer at 60 °C and incubated for 30 minutes. The DNA was subsequently extracted twice with a chloroform-isoamyl alcohol mixture (24:1). Nucleic acids were precipitated with 100 % ethanol in the presence of 5 mM sodium acetate (1/10 volume). DNA concentration was measured using a Nanodrop Spectrophotometer 2000 (Thermo Fisher Scientific, USA).

For PCR amplification, each 25 µL reaction contained 12.5 µL of 2X DreamTaq PCR Master Mix (Thermo Fisher Scientific), 2.5 µL of template DNA (25 - 50 ng/µL), 2.5 µL of primers (10 pmol each), and 7.5 µL of distilled water. The PCR protocol included an initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of 95 °C for 30 seconds, 1 minute at the annealing temperature, and 72 °C for 5 minutes, with a final hold at 4 °C. The amplified

products were analyzed via 1 % agarose gel electrophoresis and purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific). Sequencing of the purified PCR products was performed using the Sanger method by 1st BASE service, and the sequences were verified through the BLAST tool (NCBI).

2.5. Sequence analysis

The amplified DNA regions were aligned with reference sequences using the ClustalW tool in MEGAvX software. Sequence variations and other indicators were then identified using DNasp software. Genetic distances within and between species were assessed using the Kimura 2-Parameter (K2P) distance matrix in MEGAvX software. The nucleotide sequences of each barcode region from each sample, sequenced three times, were nearly identical. Consequently, the representative sequences for each marker were submitted to GENBANK with the assigned accession numbers.

2.6. Phylogenetic construction

Nucleotide sequences of four markers from other *Phyllanthus* species and the Phyllanthaceae family were obtained from GENBANK for phylogenetic analysis. For each species, a sequence matrix was created individually for two nuclear markers and two chloroplast markers. The concatenated sequence datasets were edited and aligned using the ClustalW algorithm in MEGAvX software. Separate phylogenetic trees for *ITS*, *rpo*C1, and *psb*A-*trn*H datasets were constructed using the Maximum Likelihood (ML) approach with a bootstrap value of 1000. The best-fit evolutionary model was determined to be the Kimura 2-Parameter model with a discrete Gamma distribution.

2.7. Total RNA extraction, cDNA synthesis and qRT-PCR amplification

To compare the expression patterns of genes involved in the synthesis of phyllanthin and hypophyllanthin, total RNA was extracted from leaf and stem tissues using Trizol reagent (Invitrogen). The quantity and integrity of the RNA were assessed with High Sensitivity RNA ScreenTape (Agilent Technology). For RNA extraction from the plant material, 0.1 g of leaf and stem tissue was ground in liquid nitrogen with 2 % PVP, mixed with 2 ml of preheated EB buffer (comprising 1% SDS, 25 mM EDTA, 100 mM Tris-HCl pH 8.0, and 1 % β-mercaptoethanol), and then vortexed with 3 ml of acid phenol: chloroform. This mixture was kept at room temperature for 10 minutes and extracted twice with acid phenol: chloroform to ensure complete dissociation of nucleoprotein complexes. The nucleic acids were then recovered using 0.1 volume of 3M sodium acetate and an equal volume of isopropanol [42].

The total RNA was reverse-transcribed into cDNA using the Reverse Aid Transcriptase Kit (Thermo Fisher Scientific). Six primer pairs were designed to amplify key genes in the phyllanthin and hypophyllanthin synthesis pathway. For qRT-PCR, the reaction mixture included 10 µL of Luna Universal qPCR Master Mix, 20 ng of cDNA template, and 0.5 µL of each 10 µM primer, in a total volume of 20 µL. Amplification was performed in the LightCycler 96 System (Roche, Switzerland) with initial denaturation at 95°C for 3 minutes, followed by 45 cycles of 95 °C for 20 seconds and 60 °C for 30 seconds. Melting curves were obtained using the default settings of the LightCycler 96 system. Each assay was conducted in triplicate, with biological replicates for each tissue type (leaf or stem). The *EF1α* gene served as an endogenous control in the experiment. Statistical analysis followed the comparative method [43]. Primer details and amplification conditions are provided in Table 2.

Table 2. qRT-PCR primers to validate the gene expression

Primer name	Sequence (5'-3')	Length (bp)
PAL2 F	ATTGCTGTGAAAACCAAATCGAAC	273
PAL2 R	GAAAACGAGAAGAATGCTAGCAC	
PCBER F	TCCAACATAGTTTAAAATAGGAGAG	265
PCBER R	TTTGTACCATTTCTGGAGTCAAGA	
PrR F	AGGTATTTGGACTTTGTCTGGAT	250
PrR R	ATTTCTTCTTGTTAGAGACACTGC	
CHI F	ACAGTGACCATGATCTTGCC	122
CHI R	TTGTCAAGAGCTTCGCCTTC	
CHS F	CACAGCGGGTTATGCTATAC	156
CHS R	GTCTATCTGCACTTGGTGG	
CHS13 F	GAAGCGATACATATGCACCTGA	180
CHS 13 R	TGCGTGATCTTTGACTTTGG	
EF1α F	CACAGCGGGTTATGCTATAC	117
EF1α R	GTCTATCTGCACTTGGTGG	

3. RESULTS

3.1. Hypophyllanthin and phyllanthin content

The yield of extract from *P. amarus* and *P. urinaria* were 21.3 % and 19.2 %, respectively. By HPLC, the retention time of phyllanthin and hypophyllanthin of analyte samples were similar when compared to retention time of standard compounds. In the *P. amarus* .TN extract, the content of phyllanthin and hypophyllanthin were 0.008 and 0.403 %. While in the *P. urinaria*. TN extract, the content of phyllanthin and hypophyllanthin were 0.015 and 0.944 %.

Additionally, in 100 g dry-weight of *P. amarus* .TN, there are 85.9 mg of hypophyllanthin and 201.1 mg of phyllanthin, equivalent to 0.0859 and 0.2011 %. In 100 g dry-weight of *P. urinaria*. TN, there are 1.53 mg of hypophyllanthin 2.97 mg of phyllanthin, equivalent to 0.00153 and 0.00297 %.

The *P. amarus* .TN could provide a higher content of these two lignans than *P. urinaria* .TN. The extracted phyllanthin content was higher than hypophyllanthin. HPLC analysis showed the concentration of phyllanthin and hypophyllanthin significantly varied between *P. amarus* .TN and *P. urinaria* .TN. (Fig. 2).

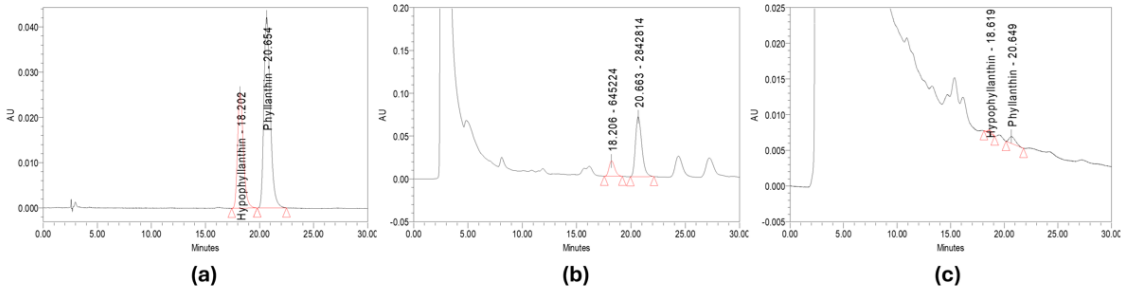

Figure 2. A chromatogram of the mixture of the standard compounds (a) and a chromatogram of *P. amarus* .TN (b) and *P. urinaria* .TN extracts (c)

3.2. The efficiency of marker amplification

It was found that the primers of *rpo*C1, *psb*A-*trn*H provided a quality amplicon while those of *ITS*1, *ITS*2 were optimized in *P.urinaria* and *P.amarus*. After trimming and alignment, the whole sequenced amplicon of each marker was 675 bp for *ITS*, 480 bp for *rpo*C1 and 302 bp for *psb*A-*trn*H. *ITS* amplified region had the highest GC% content with 52.85 % followed by *rpo*C1 with 41.8 % and *psb*A-*trn*H with 26 % (as shown in Table 3).

Several nucleotide differenes in each alignment set for *P.urinaria* TN and *P.amarus* TN were indicated in Table 4 and Fig.3. Overall, *psb*A-*trn*H has the shortest amplicon length (302 bp) while it provided the highest variable sites and parsimony-informative (PI) sites (9 sites). The mean pairwise distance according was the highest number for *psb*A-*trn*H (0.39). However, the higher numbers of variable sites and pairwise distance do not correlate with the high classification ability of marker [44].

Table 3. DNA marker dataset statistics

	ITS	***rpo*C1**	***psb*A-*trn*H**
Length range (bp)	705	500	350
Aligned/analysis length (bp)	675	480	302
Average %GC content	52.85	41.8	26
Conserve site (%)	99.7	99.2	92.09
Variable site	3	5	9
Parsimony informative (PI) site	2	6	9
Gap/ missing site (%)	0	1	15
Mean pairwise distance	0.17	0.2	0.39
Intraspecific distance (min-max/ mean)	0.00 – 0.03	0.00 – 0.34	0.01 –- 0.44
Interspecific distance (min-max/ mean)	0.0078 – 0.58	0.008 – 0.26	0.012 – 0.433

After combining and aligning the two amplified gene regions *ITS*1 and *ITS*2, the complete *ITS* fragment obtained has a size of 675 bp. Average %GC content of *ITS* was the highest (52.85 %), so conserve site was the highest (99.7 %). Gap/missing site of *ITS* sequence was 0. Mean pairwise distance of *ITS* was the lowest (0.17) compared to 0.2 (*rpo*C1) and 0.39 (*psb*A-*trn*H). Intraspecific distance of *ITS* was the narrowest distance range (0.00 - 0.03). However, interspecific distance of *ITS* was the largest distance range (0.009 - 0.58). These results indicate that intraspecies distance was expected to be as low as possible and interspecies distance was expected to be as high as possible for accurate classification. As a result, *ITS* sequence seems to be the standard marker among DNA barcoding markers for the speices.

In the present study, intraspecific and interspecific distance values overlapped in the discrimination power of separated barcodes. This overlap might be due to the diversity in the *Phyllanthus* genus and the similarity in phenotypic characteristics of the *Phyllanthaceae* family. This dataset was used for the phylogenetic construction. There were numerous variations among *Phyllanthus* genus (Table 5).

Table 4. Nucleotide of DNA sequence comparision between *P.urinaria* .TN and *P.amarus* .TN

ITS			**rpoC1**			**psbA-trnH**		
Site	***P. urinaria*. TN**	***P. amarus*. TN**	**Site**	***P. urinaria*. TN**	***P. amarus*. TN**	**Site**	***P. urinaria*. TN**	***P. amarus*. TN**
11..15	GGAAT	-GGAT	**42**	C	T	**45**	T	C
25	T	A	**159**	T	C	**53..54**	TG	CA
30..39	TTA-ACAGA	GC.TGGC..C	**339**	T	C	**66**	T	G
46	-	C	**438**	C	G	**74..76**	CTA	T--
55..71	TTCTATTCACT GTGGAT	G.T...C...G.CC..A	**455**	C	G	**97..101**	GAGAG	CTCTC
79..93	T_A_T_C	C..G..C..A				**122..128**	T_A	-_C
103..115	GAGATGCTAT ACG	AG.G..T...G.C				**136..148**	C_T	A_C
118..120	CAC	-				**153..154**	AG	TT
125..127	AGT	TTG				**176..181**	A_C	G_T
132..134	TGT	C.-				**196..201**	TTCTAA	AGAA.G
130	C	A				**209**	-	T
155	T	A				**229**	A	G
167..177	AACAAACAAA A	C..G...GT.T				**240**	A	T
188..195	TCTACATT	C.GT.GAA				**241..243**	TTC	AGT
200..201	TC	CT				**254..259**	G_G	A_A
209..229	ACGTGTGTTTG TAAGTTGATT	GT.C...C.C.AT..GC. T. G				**288..291**	TAT	-
239..244	TAACCA	A..T-G				**295_297**	T_G	G_A
275..282	-	AACGGATA						
290	T	-						
297..298	-	TT						
357..420..426	T_T_T	C_A_C						
440..462	CATTTGGGATT GCGAAATTAG CGA	GT..G...C.C.T..G..T. .T G						
481..493	GAGTATTTACA TT	T.TCCC.G.GT.A						
503..515..518	T_C_G	C_T_A						
524..530..540	T_C_T	C_T_A						

551..552	AA	T-						
557..568	CTTACAACGCCT	T.C.G..T…G						
579..580	AT	TC						
586..602	TAAAT-TGGTCCTCAAA	A..G.A….T…G.C						
610	A	T						

The dot "." Indicates the same nucleotide as the reference sequence, the dash "-" indicates the aligned gap.

However, in each barcode marker, in the same species, there are less divergence. For example, in *ITS* region, there are less divergence when comparing between *P.urinaria* reference sequence AB550081 and *P.urinaria*.TN sequence. Similarly, there are less variations between *P.amarus* reference sequence LS975764 and *P.amarus*. TN sequence. Outgroup sequence was used to ensure the accuracy of dataset of sequences, and *rpo*C1 variations were mainly single nucleotide variations.

3.3. Phylogenetic tree construction

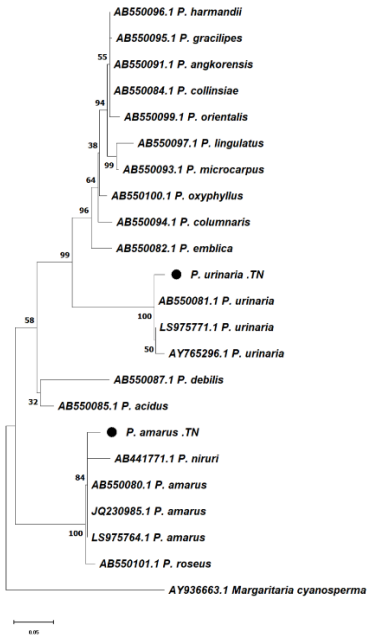

Figure 4. The maximum likelihood tree of the ITS sequence

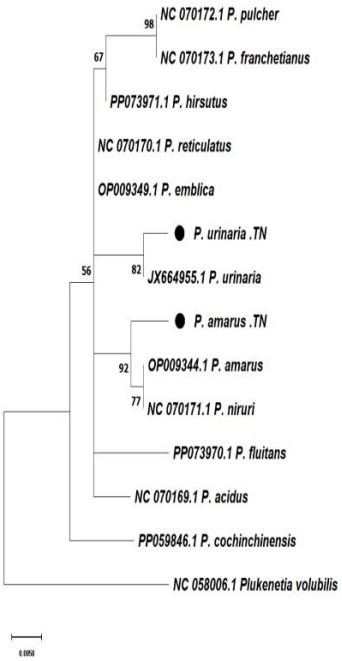

Figure 5. The maximum likelihood tree of the *rpo*C1 sequence

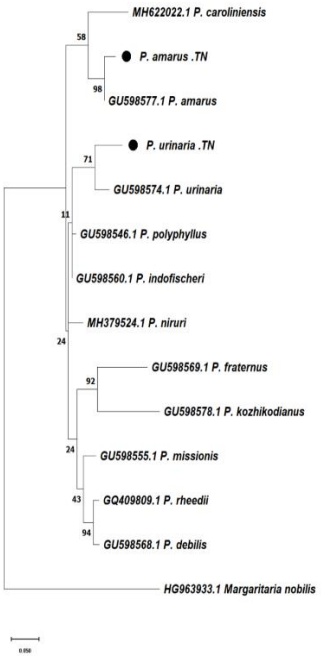

Figure 6. The maximum likelihood tree of the *psb*A-*trn*H sequence

To clarify the classification of aboriginal cultivars, we identified the optimal model and parameters before constructing the phylogenetic tree. The analysis revealed differences in the branching patterns between trees based on the ITS region and those based on concatenated cpDNA markers. In the *ITS* tree, *P. amarus* TN cultivars were grouped together with the reference sequences for *P. amarus*, forming a distinct cluster separate from the cluster containing *P. urinaria* and related species (Figures 4, 5, and 6). A similar pattern was observed in trees

constructed using other individual cpDNA markers. Notably, the two plants were also positioned in their own branch, situated between *P. amarus* .TN and *P. urinaria* .TN.

3.4. Gene expression pattern among samples

The research focuses on the expression of genes involved in the lignin and flavonoid biosynthesis pathways in *P. amarus* .TN and *P. urinaria* .TN. To validate gene expression, qRT-PCR was conducted successfully on 6 genes (*PAL2, PCBER, PrR, CHI, CHS,* and *CHS13*) in both leaves and stem tissue. Figure 7 showed the different expression patterns between two organs of the species. In general, the expression of these genes was stronger in *P.amarus* than *P. urinaria*. In detail, the *CHS* and *CHS13* genes were the most consistently expressed in all tested tissues, while the *CHI* and *PCBER* genes tended to be more strongly expressed in leaves than in stems. On the other hand, the *PAL* gene was strongly expressed in the *P. amarus* stems.

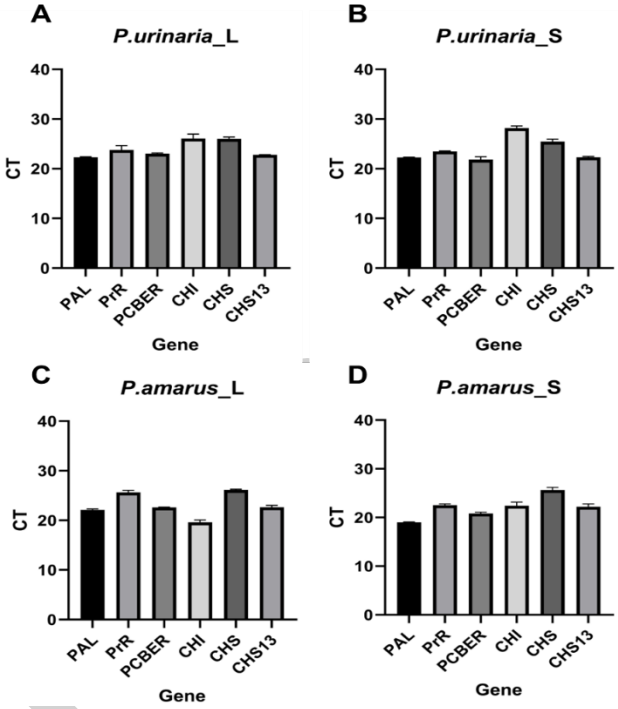

Figure 7. Expression of the genes function in hypophyllanthin and phyllanthin bio synthesis pathway in the leaf (L) and stem tissue (S) of *P.amarus* .TN and *P.urinaria* .TN

4. DISCUSSION

High-Performance Liquid Chromatography (HPLC) analysis showed that phyllanthin and hypophyllanthin concentrations varied between *P. amarus* .TN and *P. urinaria* .TN. The analysis revealed that *P. amarus* .TN contains significantly higher amounts of hypophyllanthin and phyllanthin compared to *P. urinaria* .TN. When analyzing the retention time, they were similar, indicating accurate identification. However, additional interference peaks were observed in the chromatograms of *P. amarus* .TN and *P. urinaria* .TN extracts, suggesting the presence of other compounds. In another research, phyllanthin and hypophyllanthin content in the whole plant of *P. amarus* collected from various locations were ranged from 0.102 to 0.394 and 0.033

to 0.149% w/w, respectively [45]. Thus, the phyllanthin (0.2011 %) and hypophyllanthin (0.0859%) content of *P. amarus*.TN was consistent with other studies.

The consistency of these findings across different studies and methodologies underscores the potential of *P. amarus* .TN as a valuable source of hypophyllanthin and phyllanthin. The study highlights that the peak areas for phyllanthin and hypophyllanthin in *P. amarus* .TN were larger than those in *P. urinaria* .TN, confirming the higher concentration in *P. amarus* .TN. Previous studies support these findings, Tripathi *et al.* (2006) developed an HPTLC method for quantifying these lignans across various *Phyllanthus* species, finding *P. amarus* to have the highest concentrations [46]. Murugaiyah & Chan (2007) used reverse-phase HPLC with fluorescence detection to analyze lignans in *P. niruri*, highlighting that leaves contain higher concentrations, with phyllanthin being the most prevalent [47]. Ilangkovan *et al*. (2015) also quantified major compounds in *P. amarus* extracts, reporting higher hypophyllanthin levels than phyllanthin [48]. Srivastava et al. (2008) used chiral TLC to quantify lignans in various *Phyllanthus* species, again finding the highest hypophyllanthin content in *P. amarus*, which was also supported by qPCR results for the *PAL, PCBER*, and *PrR* genes [49]. This indicates that the results from HPLC and qPCR showed similar trends. In conclusion, *P. amarus* .TN demonstrates a significantly higher concentration of hypophyllanthin and phyllanthin than *P. urinaria* .TN, validating its potential for pharmacological applications.

The overlap in intraspecific and interspecific distances in the discrimination power of separated barcodes may be due to the diversity within the *Phyllanthus* genus and the phenotypic similarities within the Phyllanthaceae family. Despite this overlap, the current dataset provides a robust basis for phylogenetic construction and highlights the distinct chemical profiles of these species. The comparison of marker efficiency in the present study highlights some key observations when contrasted with previous research findings. The analysis revealed that the primers for *rpo*C1 and *psb*A-*trn*H produced high-quality amplicons, with *rpo*C1 yielding a 480 bp fragment and *psb*A-*trn*H a 302 bp fragment, while *ITS*1 and *ITS*2 were optimized to produce a complete *ITS* fragment of 675 bp. Among these markers, *ITS* exhibited the highest GC content (57%) and the lowest mean pairwise distance (0.17), indicating its high conservation and effectiveness in distinguishing between species. Conversely, *psb*A-*trn*H, despite having the shortest amplicon (302 bp), showed the highest number of variable and parsimony-informative sites but did not necessarily correlate with better classification ability. These findings align with the broader literature, which suggests that *ITS* is often regarded as the most effective marker among DNA barcoding options due to its high percentage of conserved sites and its capability to differentiate species. For instance, research has shown that most genetic variation (87.5% to 92.44%) occurs within populations rather than between them, indicating minimal genetic differentiation among populations [22, 50]. This supports the utility of *ITS* as a robust marker for accurate species identification. Additionally, *ITS* has been successfully employed in conjunction with other markers such as *mat*K, *rbc*L, and *psb*A-*trn*H for the identification of species and quality control in herbal products [45, 51]. This reinforces the *ITS* sequence's effectiveness in DNA barcoding, particularly for ensuring the safety and reliability of commercial herbal products. The overlap of intraspecific and interspecific distances observed in this study may be attributed to the diverse nature of the *Phyllanthus* genus and the phenotypic similarities within the Phyllanthaceae family, underscoring the need for a multi-marker approach to enhance classification precision.

The *PAL2* and *PCBER* genes were more active in stem tissues, with *P. amarus* .TN showing the lowest Ct values. Similarly, the *PrR* gene was more expressed in the stems of both species. These genes, which are crucial for lignin biosynthesis, were found to have higher expression

levels in the stem tissues of *P. amarus* .TN. This is consistent with research in other plants, such as *Arabidopsis thaliana*, where lignin-related genes are often upregulated in response to biotic stress, leading to increased lignin production as a defense mechanism. For example, a study by Fraser & Chapple (2011) demonstrated that *PAL* genes, which initiate the lignin biosynthesis pathway, play a significant role in reinforcing cell walls through lignin deposition during pathogen attacks [52].

The study highlighted the expression of *CHI*, *CHS*, and *CHS13* genes, with *P. amarus* .TN generally showing higher expression levels, particularly in leaf tissues. However, *CHS* gene expression was slightly higher in *P. urinaria* .TN. Flavonoids, including compounds such as isoflavones and anthocyanins, are essential for protecting plants from UV radiation and other environmental stresses. The role of *CHS* (Chalcone Synthase) in flavonoid production has been well documented in various plant species. For instance, a study by Yin *et al*. (2019) [25] on *Glycine max* (soybean) found that *CHS* genes were predominantly expressed in leaves, mirroring the expression pattern observed in *P. amarus* .TN.

Overall, the higher expression of lignin and flavonoid biosynthesis genes in *P. amarus* .TN compared to *P. urinaria* .TN suggests stronger metabolic activity in these pathways, potentially linked to the plant's environmental adaptation or defensive strategies. This aligns with the broader understanding that secondary metabolite production in plants is closely tied to environmental factors and the plant's specific needs in its habitat. The slight increase in *CHS* expression in *P. urinaria* .TN could indicate a variation in metabolic focus, reflecting different ecological adaptations between the two species.

Previously study of Kiran *et al.* 2021 conducted DNA barcoding analysis (*Rpo*C1) for discrimination of *Phyllanthus* species and chemical analysis (HPLC) however, in our research combining chemical analysis (HPLC) with gene expression analysis (qRT-PCR) and DNA barcoding (*rpo*C1, *psb*A-*trn*H, *ITS*) when comparing two related species thus the approach is more comprehensive [36]. These two species are Vietnamese variants (TN strains), so it is a potential novel contribution.

5. CONCLUSION

The research based on DNA markers – *ITS1, ITS2*, *rpo*C1, *psb*A-*trn*H showed efficiency in resolving at subspecies level of *P. amarus* and *P. urinaria*. The *ITS* sequence was expected to be one of standard DNA barcoding markers due to its high conserved site percentage for variation analysis and phylogenetic construction. In contrast, *rpo*C1 variations were mainly single nucleotide variations. In addition, HPLC coupled with qRT-PCR provided detailed information about the hypophyllanthin and phyllanthin contents and their biosynthesis route. The qRT-PCR was performed to evaluate biosynthesis gene expressions in leaf and stem tissue of *P.amarus* .TN and *P.urinaria* .TN collected in Viet Nam of six genes - *PAL2, PCBER, PrR, CHI, CHS*, and *CHS13* were examined for the first time in the two species. This research focused on the expression of genes involved in the lignin and flavonoid biosynthesis pathways of *P. amarus* .TN and *P. urinaria* .TN.

Acknowledgement. This work was funded by Vietnam Academy of Science and Technology (VAST) under grant number CSCL40.01/23-24.

Authors's contribution. All the authors have contributed to the study.

Declaration of interest's statement.The authors declare there are no competing interests.

REFERENCES

1. Mao X., Wu L. F., Guo H.L., Chen W.J., Cui Y.P., Qi Q., Li S., Liang S.W.Y., Yang G.H., Shao Y.Y., Zhu D., She G.M., You Y. and Zhang L.Z. - The Genus *Phyllanthus*: An Ethnopharmacological, phytochemical, and Pharmacological Review. Evidence-based Complementary and Alternative Medicine. (2016) 1-36.
2. Sarin B., Verma N., Martín J. P., Mohanty A. - An Overview of Important Ethnomedicinal Herbs of *Phyllanthus* Species: Present Status and Future Prospects. The Scientific World Journal/The Scientific World journal. (2014) 1-12.
3. Ghosh A. B. M., Banerjee A., Chattopadhyay S. - An insight into the potent medicinal plant *Phyllanthus amarus* Schum. and Thonn. Nucleus/the Nucleus. **65**(3) (2022)437-472.
4. Joujeh R., Joujeh D. - Genus *Phyllanthus*: traditional uses and biological activities. Zagazig Journal of Pharmaceutical Sciences/Zagazig Journal of Pharmaceutical Science. (2023).
5. Husnunnisa H., Hartati R., Mauludin R., Insanu M. - A review of the Phyllanthus genus plants: Their phytochemistry, traditional uses, and potential inhibition of xanthine oxidase. Pharmacia. **69**(3) (2022) 681-687.
6. Bose M.A., Chattopadhyay S. - Sequencing, De novo Assembly, Functional Annotation and Analysis of *Phyllanthus amarus* Leaf Transcriptome Using the Illumina Platform. Front Plant Sci. **28**(6) (2016) 1199.
7. Row R. L., Srinivasulu C.-Crystalline constituents of euphorbiaceae-V: New lignans from *Phyllanthus niruri* Linn. - The constitution of phyllanthin. Tetrahedron. **22** (1966) 2899-908.
8. Abdel-Sattar O. E., Rasha M. A., Ahmed M.Al-Abd, Ali M.El-Halawany, Ahmed M. EL-Desoky, Shanaz O.M., Sherouk H.S., Mohammad K., Essam Abdel-Sattar, and Meselhy R.M. - Hypophyllanthin and Phyllanthin from Phyllanthus niruri Synergize Doxorubicin Anticancer Properties against Resistant Breast Cancer Cells. ACS Omega. (2023) 28563-28576.
9. Xin J., Sheng L. - Quantitative Determination of Four Lignans in *Phyllanthus niruri* L. by HPLC Publication Event LCGC International. **1**(2) (2024) 38-44.
10. Wan S., Jantan W.A., Wahab I.A., Jalil S. M., Said J.M., Yusoff M., Husain S.D. - Pharmacological activities and mechanisms of action of hypophyllanthin: A review. Frontiers in Pharmacology. **9**(13) (2023) 1070557.
11. Ilangkovan M., Jantan I., Bukhari S. N. A. - Phyllanthin from *Phyllanthus amarus* inhibits cellular and humoral immune responses in Balb/C mice. Phytomedicine. **23**(12) (2016) 1441-1450.
12. Salim K., Al-Qurainy F., Ram M., Ahmad S., Abdin M.Z. - Phyllanthin biosynthesis in *Phyllanthus amarus*: Schum and Thonn growing at different altitudes. J Med Plants Res. **4**(1) (2009) 041-048.
13. Abdel-Sattar O.E., Allam R.M., Al-Abd A.M., El-Halawany A.M., El-Desoky A.M., Mohamed S.O., Sweilam S.H., Khalid M., Abdel-Sattar E., Meselhy M.R. - Hypophyllanthin and Phyllanthin from *Phyllanthus niruri* Synergize Doxorubicin Anticancer Properties against Resistant Breast Cancer Cells. ACS Omega. **8**(31) (2023) 28563-28576.

14. Chopade A.R., Pol R.P., Patil P.A., Dharanguttikar V.R., Naikwade N.S., Dias R.J. and Mali S.N. - An Insight into the Anxiolytic Effects of Lignans (Phyllanthin and Hypophyllanthin) and Tannin (Corilagin) Rich Extracts of *Phyllanthus amarus*: An *In-Silico* and *In-vivo* approaches. Comb Chem High Throughput Screen. **24**(3) (2021) 415-422.
15. Bhushan V., Bharti S.K., Krishnan S. and Kumar A. - Antidiabetic effectiveness of *Phyllanthus niruri* bioactive compounds *via* targeting DPP-IV. Nat Prod Res. **9** (2024) 1-7
16. Marhaeny H.D., Widyawaruyanti A., Widiandani T., Fuad Hafid A. and Wahyuni T.S. - Phyllanthin and hypophyllanthin, the isolated compounds of *Phyllanthus niruri* inhibit protein receptor of corona virus (COVID-19) through *in silico* approach. J Basic Clin Physiol Pharmacol. **32**(4) (2021) 809-815.
17. Sylvester I.O., Delport J., Kangw T.S., Rahman Z., Hussein A.A., Lorke D.E. Ekpo O.E. - An update on the bioactivities and health benefits of two plant-derived lignans, phyllanthin and hypophyllanthin. Advances in traditional medicine. (2024).
18. Phien H.H., Men T.T. - *Phyllanthus amarus* ameliorates calcium oxalate-induced nephrolithiasis via antioxidant and anti-inflammatory. TNU Journal of Science and Technology. **228**(05) (2023) 262-269.
19. Tuan B.H., Ho H.P., Tran C.T., Nguyen C.N.K. - The research of pharmaceutical value chain - Diep Ha Chau (*Phyllanthus amarus*). Science and Technology Development Journal. **16**(2) (2013) 37-45.
20. Danladi S., Idris M. A., Umar II. - Review on pharmacological activities and phytochemical constituents of P*hyllanthus niruri* (Amarus). The Journal of Phytopharmacology. **7**(3) (2018) 341-348.
21. Mendoza N., Silva E.M.E. - Introduction to phytochemicals: Secondary metabolites from plants with active principles for pharmacological importance. Phytochemicals - source of antioxidants and role in disease prevention. (2018) 25-47.
22. Liu, W., Feng Y., Yu S., Fan Z., Li X., Li J. and Yin H. - The Flavonoid Biosynthesis Network in Plants. Int J Mol Sci. **22**(23) (2021) 12824.
23. Nawfetrias W., Devy L., Esyanti R. R., Faizal A. - *Phyllanthus* Lignans: A Review of Biological Activity and Elicitation. Horticulturae. **10**(2024) 95.
24. Moriwaki M., Yamakawa T., Washino T., Kodama T., Igarashi Y. - Suppressed phenylalanine ammonia-lyase activity after heat shock in transgenic *Nicotiana plumbaginifolia* containing an *Arabidopsis* HSP18.2-parsley *PAL2* chimera gene. J Biosci Bioeng. **87**(5) (1999) 588-93.
25. Yin Y.C., Zhang X.D., Gao Z.Q., Hu T., Liu Y. - The Research Progress of Chalcone Isomerase (*CHI*) in Plants. Mol Biotechnol. **61**(1) (2019) 32-52.
26. Kumar A., Sharma M., Chaubey S.N., Kumar A. - Homology modeling and molecular dynamics-based insights into *Chalcone synthase* and *Chalcone isomerase* in *Phyllanthus emblica* L. Biotech. **10**(8) (2016) 373.
27. Buddhachat K., Osathanunkul M., Madesis P., Chomdej S., Ongchai S. - Authenticity analyses of *Phyllanthus amarus* using barcoding coupled with HRM analysis to control its quality for medicinal plant product. Gene. **573**(1) (2015) 84-90.
28. Inglis P.W., Mata L.R., da Silva M.J., Vieira R.F., Alves R.B.N., Silva D.B., Azevedo V.C.R. - DNA Barcoding for the Identification of *Phyllanthus* Taxa Used Medicinally in Brazil. Planta Med. **84**(17) (2018) 1300-1310.

29. Jantan I., Yuandani M.I., Mohamad H.F. - Correlation between the major components of *Phyllanthus amarus* and *Phyllanthus urinaria* and their inhibitory effects on phagocytic activity of human neutrophils. BMC Complement Altern Med. **1**(14) (2014) 429.
30. Von Heimendahl C.B.I., Schafer K.M., Eklund P., Sjoholm R., Schmidt T.J., Fuss E. - Pinoresinol-lariciresinol reductases with different stereospecificity from *Linum album* and *Linum usitatissimum*. Phytochem. **66**(2005) 1254-1263.
31. Vander M.K., Beeckman H., De Rycke R., Van M.M., Engler G., Boerjan W. - Phenylcoumaran benzylic ether reductase, a prominent poplar xylem protein, is strongly associated with phenylpropanoid biosynthesis in lignifying cells. Planta. **211**(2000) 502-509.
32. Anindita B., Sharmila C. - Effect of over-expression of *Linum usitatissimum* pinoresinol lariciresinol reductase (LuPLR) gene in transgenic *Phyllanthus amarus*. Plant Cell Tissue and Organ Culture. **103**(3) (2023) 315-323.
33. De Vere, N., Rich T. C. G., Trinder S. A., Long C. - DNA barcoding for plants. In Methods in molecular biology. (2014) 101-118.
34. Kress W.J., Erickson D.L. - DNA barcodes: methods and protocols. In Methods in molecular biology. (2012) 3-8.
35. Fang H., Dai G., Liao B., P. Zhou, Liu Y. - Application of chloroplast genome in the identification of *Phyllanthus urinaria* and its common adulterants. Frontiers in Plant Science. (2023)13.
36. Kiran K. R., Swathy P. S., Paul B., Prasada K.S., Rao M.R., Joshi M.B., Rai P.S., Satyamoorthy K., Muthusamy A. - Untargeted metabolomics and DNA barcoding for discrimination of *Phyllanthus* species. Journal of Ethnopharmacology. **273** (2021) 113928.
37. Rehman, U., Sultana N., Abdullah A., Jamal M., Muzaffar, Poczai P. - Comparative chloroplast genomics in Phyllanthaceae species. Diversity. **13**(9) (2021) 403.
38. Amandita F. Y., Rembold K., Vornam B., Rahayu S., Siregar I. Z., Kreft H., Finkeldey R. - DNA barcoding of flowering plants in Sumatra, Indonesia. Ecology and Evolution. **9(**4) (2019) 1858-1868.
39. Cahyaningsih R., Compton L.J., Rahayu S., Brehm J. M. and Maxted N. - DNA Barcoding Medicinal Plant Species from Indonesia. Plants. **11**(10) (2022) 1375.
40. Geethangili M., Ding S.T. - A Review of the Phytochemistry and Pharmacology of *Phyllanthus urinaria* L. Frontiers in Pharmacology. **1**(9) (2018) 1109.
41. Doyle J.J., Doyle J.L. - Isolation of plant DNA from fresh tissue. Focus. **12**(1) (1990) 13-15.
42. Deepa K., Sheeja T.E., Santhi R., Sasikumar B., Anu C., Deepesh P.V., Prasath D. - A simple and efficient protocol for isolation of highquality functional RNA from different tissues of turmeric (*Curcuma longa* L.). Physiol Mol Biol Plants. **20**(2) (2014) 263-271.
43. Thomas D.S., Kenneth J.L. - Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. **3**(6) (2008) 1101-8.
44. Rakhi K., Dilawar U., Anshu S., Sheetal A. - Simultaneous Quantification of Three Bioactive Lignans, viz., Phyllanthin, Hypophyllanthin, and Niranthin from *Phyllanthus amarus* Using High-Performance Thin-Layer Chromatography. Journal of Planar Chromatography. **27**(4) (2014) 281-286
45. Manzanilla V., Kool A., Nguyen N.L., Nong H.V., Le H.T.H. and de Boer H.J. - Phylogenomics and barcoding of Panax: toward the identification of ginseng species. BMC Evol. Biol. **18**(1) (2018) 44.

46. Tripathi A.K., Verma R.K., Gupta A.K., Gupta M.M. - Quantitative determination of phyllanthin and hypophyllanthin in *Phyllanthus* species by high-performance thin layer chromatography. Phytochem Anal. **17**(6) (2006) 394-7.
47. Murugaiyah V., Kit-Lam C. - Chan Determination of four lignans in *Phyllanthus niruri* L. by a simple high-performance liquid chromatography method with fluorescence detection. J Chromatogr A. **1154**(1-2) (2007) 198-204.
48. Ilangkovan M., Jantan I., Mesaik M.A., Bukhari S.N. - Immunosuppressive effects of the standardized extract of Phyllanthus amarus on cellular immune responses in Wistar-Kyoto rats. Drug Des Devel Ther. **26**(9) (2015) 4917-4930.
49. Srivastava V., Singh M., Malasoni R., Shanke K., Verma R.K., Gupta M.M., Gupta A.K., Khanuja S.P. - Separation and quantification of lignans in *Phyllanthus* species by a simple chiral densitometric method. J Sep Sci.
50. Padmini S., Rao M.N., Ganeshaiah K.N., Shaanker R. U. - Genetic diversity of *Phyllanthus emblica* in tropical forests of south india: impact of anthropogenic pressures. Journal of Tropical Forest Science. **13**(2) (2001) 297-310.
51. Intharuksa, A., Masashi K., Nichakan P., Wannaree C., Hirokazu A., Yohei S., Panee S. A. - Evaluation of white Kwao Krua (*Pueraria candollei* Grah. ex Benth.) products sold in Thailand by molecular, chemical, and microscopic analyses. J. Nat. Med. **74**(1) (2020) 106-118.
52. Christopher M.F., Clint C. - The phenylpropanoid pathway in *Arabidopsi*s. Arabidopsis Book. **9** (2011) e0152.