provide a good source of minerals and antioxidants. This is the first report on the chemical basis of these species, which provides new insight into the possibility to use these plants as food supplements.

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STABILITY STUDY OF LIGNAN CONTENT IN *HERBA PHYLLANTHI AMARI* AND IN PREPARATIONS THEREOF

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Summary

Stability Study of Lignan Content in *Herba Phyllanthi amari* and in Preparations Thereof

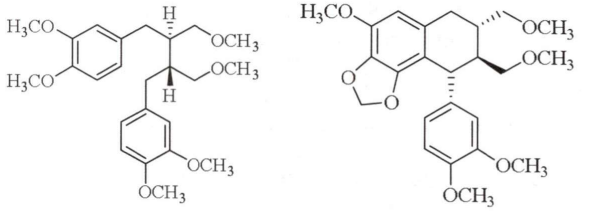
The study was conducted with the aim of evaluating the stability of lignans content including phyllanthin and hypophyllanthin - major active compounds in crude plant materials of *Phyllanthus amarus* and in preparations thereof in several conditions by HPLC. Quantification of phytomarkers was based on USP 41 by using an elution system including Acetonitrile (A)/0.14 g KH_2PO_4 and 500 µL H_3PO_4 in 1000 mL water (B) (A/B = 4/6) equipped with InertSustain C_{18} column (250 x 4.6 mm; 5 µm) from GL science and guard column (Phenomenex) at a flow rate 1.5 mL/min and 10 µL of injection volume with UV detection at 230 nm for HPLC analysis. The results indicated a significant reduction of lignan contents in raw materials of *Phyllanthus amarus*, but it was a slight in extract and tablet in the conditions of investigations. These may have implications for shelf-life determination of medicinal herbs, and the preparation of dosage forms from *Phyllanthus amarus*.

Keywords: *Phyllanthus amarus, Phyllanthin, Hypophyllanthin, Stability.*

1. Introduction

Phyllanthus amarus (Schum. et Thonn), belonging to the Euphorbiaceae family, is a medicinal herb with abundant chemical constituents including lignans, flavonoids, hydrolysable tannins, polyphenols, triterpenes, sterols, alkaloids and exhibits a wide range of pharmacological properties such as antiviral, antibacterial, antiplasmodial, anti-inflammatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective, nephroprotective and diurectic properties [1]. It has been utilized in folk remidies in quite a few tropical countries to effectively cure various ailments involving aundice, constipation, diarrhea, kidney ailments, ringworm, ulcers, malaria, genito - urinary infections, hemorrhoids and gonorrhea [2].

Specifically, according to previous reports, phyllanthin (**Fig. 1.**) which is one of the crucial lignans in *Phyllanthus amarus,* protected against carbon tetrachloride and galactosamine - induced cytotoxicity in primary cultured rat hepatocytes [3],[4]. It is also reported to inactivate Hepatitis-B, both *in vitro* and *in vivo* [4]. Hence, it can be considered as a potential active compound for treating liver dysfunctions and diseases. Phyllanthin and hypophyllanthin are considered as phytomarkers for the quality evaluation of *Phyllanthus amarus* in several documents [5],[6].



(1) **(2)**

Fig. 1. Chemical structure of phyllanthin **(1)** and hypophyllanthin **(2)** in *Phyllanthus amarus*

Recently, P. Srivastava et al. have revealed that concentration of phyllanthin in crude materials decreased to 58% within one-month period, and the quantity of lignans remained was only 8% after six months in the accelerated study sample. In case of real-time samples, there was a 50% loss of these lignans within three-month period. The quantity of phyllanthin reduced to 41.82% after six-month period, and the lignans completely vanished in nine-month period by using HPLC analysis [7]. The deterioration of the bioactive compounds can lead to the reduction of drug quality. Thus, it is necessary to evaluate the content of active substances in the period of storage, process, extraction, and preparation owing to chemical complexity in the matrix. Further, botanical drugs are now considered as active substances in their entirety and there may be challenges in the selection of the assay or of the parameters and protocols that profile their stability characteristics [8]. Although there were various publications investigating on pharmacological activities and chemical components of lignans, the reports regarding to the stability of phyllanthin and hypophyllanthin in herb, extract and dosage form have been limited. Therefore, the objective of this paper was to assess the content of two major lignans in raw material and preparation thereof.

2. Materials and Methods

2.1. Materials

Fresh whole aerial plants of *Phyllanthus amarus* were collected from Research Centre for Cultivation and Processing of Medicinal Plants, National Institute of Medicinal Material (NIMM) in June 2020. Leaves were separated, cleaned, sun dried and then ground into powder for further studies. Powder material samples were divided into 3 parts: One part was dried at 60°C in the oven to constant weight (48h) (**RM - 1**), another part (**RM - 2**) was processed at high temperature (100 - 150°C) for drying and the rest part was initial material without processing (**RM - 3).** Then, samples mentioned were kept in plastic bottle for storage.

Dried powder of ethanol extract (EE) from *Herba Phyllanthi amari* was a product of the project "***Preparation of lignans - rich extract from Phyllanthus amarus***" (No. YD.19.VDL.20-21). It was manufactured at scale of 100kg batch. Briefly, one kilogram of dried crude materials was boiled with 10 L distilled water for 1 h. In the next step, the water was squeezed out of the plant material before it was extracted with 10L of 50% (v/v) ethanol in water for 1 h. Solvent was recovered in low vacuum distillation apparatus, then dried in a vacuum oven at 60°C. Dried powder of EE was ground into powder and packaged in aluminum bag for storage.

PA dried extract tablets were prepared containing dried powder of ethanol extract (EE) with excipients and achieved standard quality grade. Tablets were produced at scale of 10.000 tablets batch, packaged in brown glass bottle with 1gram-silicagel bag and aluminum seal. Three batches 01,02,03 were manufactured on 17,18,19 November 2020, respectively.

2.2. Chemicals and instruments

Chemicals: Reference standard phyllanthin (CAS: 1536509, LOT FOK 152, purity 99,6%), Powdered *Phyllanthus amarus* Extract reference standard (CAS: 1536510; LOT FOK 140) was purchased from Sigma-Aldrich; acetonitrile, ortho phosphoric acid, potassium dihydrogen phosphate (KH_2PO_4) used for HPLC analysis were obtained from Merck, distilled water (for HPLC).

Instrument: MRC Ultrasonic Cleaner Bath (Germany), High Performance Liquid Chromatography (Shimazu, Japan), analytical balance Sartorius CPA224S (Switzerland), Moisture content meter Precisa Gravimetrics AG (Switzerland), Oven Nabertherm GmbH (Germany), R100 Buchi vacuum rotary distillation apparatus (Switzerland) and other necessary equipments for experiments.

2.3. Methods

Stability stude of lignans (phyllanthin and hypophyllanthin) in samples.

Stability studies were conducted according to

ICH guidelines including long term (LT) storage condition (30°C ± 2°C, 75% ± 5% relative humidity) and 40°C ± 2°C, 75% ± 5% relative humidity for accelerated storage condition (AS) [9]. Samples were periodically quantified lignans content including phyllanthin and hypophyllanthin at the interval of 1, 3 and 6 months by HPLC for AS and at 3, 6, 9, 12 for LT, respectively.

Based on the stability monitoring data under normal conditions for extrapolating the shelf life (90% of initial content) using MINITAB software.

All experiments were repeated in triplicate. The data were presented as the means ± SD (standard derivation).

Preparation of standard solutions and extraction of samples for analysis.

Standard solution A: 0.1 mg/mL of Phyllanthin RS in methanol

Standard solution B: 10 mg/mL of Powdered *Phyllanthus amarus* extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution:

Transfer about 3.0 g of *Phyllanthus amarus*, finely powdered and accurately weighed, to a 250 mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux for about 20 min, allow to settle, and decant the supernatant. Repeat until the extract is colorless. Combine the extracts, concentrate under vacuum, transfer quantitatively into a 100 - mL volumetric flask, and adjust to volume with methanol. Before injection, pass through a membrane filter of 0.45-µm, discarding the first few mL of the filtrate.

0.1 g of dried powder of ethanol extract from *Phyllanthus amarus* or a tablet was precisely weighed and transferred to a 100-mL conical flask. A 25 mL of methanol was added, and the mixture was sonicated for 30 minutes for the extraction and repeated 2 times. The solution collected was transferred to a 50 mL volumetric flask, replenished with MeOH for sufficient. The solution is filtered through a 0.45 µm filter prior to HPLC analysis.

HPLC protocol for analysis

Mobile phase: Acetonitrile and *Solution A* (4:6) (Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas). Chromatography system: InertSustain Column C_{18} (4.6 x 250 nm, 5 µm), guard column (Phenomenex); flow rate: 1.5 mL/min; Injection volume: 10 µL; Detector: UV 230 nm. Identify the retention times of the peaks corresponding to phyllanthin and hypophyllanthin, respectively. The percentages were calculated the samples taken:

Lignans content (phyllanthin, hypophyllanthin) = $(r_U/r_S) \times C_S \times (V/W) \times F \times 100$

Where: r_U is peak area of the relevant analyte from the *Sample solution;* r_S is peak area of phyllanthin from *Standard solution A,* C_S is concentration of Phyllanthin RS in *Standard solution A* (mg/mL); V is volume of the *Sample solution* (mL); W is weight of *sample* taken to prepare the *Sample solution* (mg); F is conversion factor: 1.00 for phyllanthin; 0.75 for hypophyllanthin

3. Results and discussion

3.1. Validation of analytical methods

Analytical methods were validated according to ICH guidelines and referenced by AOAC including system suitability, calibration curve, repeatability, limits of detection (LOD), limit of quantification (LOQ) and recovery. Results summaried in **Table 1** indicated that HPLC method established for analyzing phyllanthin contents in samples has been suitable for the determination. **Fig. 2.** illustrates HPLC chromatogram of phyllanthin, hypophyllanthin reference standards and sample.

Table 1. Validation of analytical methods

Criteria of validation	Phyllanthin
System suitability	t_R : RSD = 0.016 % S peak: RSD = 0.76 %
Linear range Calibration curve Correlation coefficient (r^2)	6.09 - 97.51 mg/mL y = 14033.30x - 204.42 r^2 = 0.9996
Repeatablity	RSD = 0.346 %
Limits of detection (LOD)	0.61 µg/mL
Limit of quantification (LOQ)	2.01 µg/mL
Recovery	95.66% - 99.32% RSD = 1.92 %

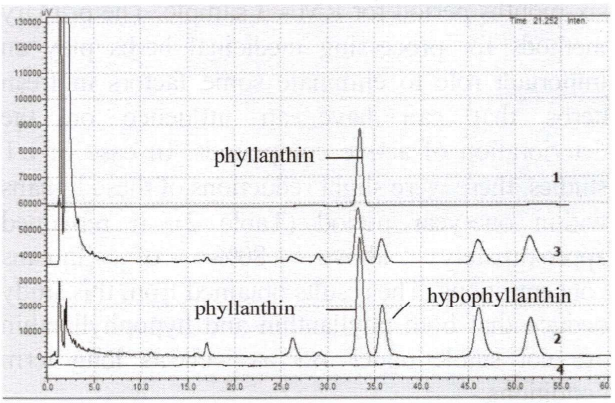


Fig. 2. HPLC chromatogram of Standard solution A (**1**); Standard solution B (**2**); Sample solution (**3**) and blank sample (**4**)

3.2. Evaluation of phyllanthin and hypophyllanthin content in Herba Phyllanthi amari samples under storage conditions

Determination of phyllanthin and hypophyllanthin contents (% w/w) were shown in **Table 2.**

Table 2. Content of phyllanthin (% w/w, means ± SD) in RMs

Sample code	Storage condition	Phyllanthin content (%)	% RC(*) of phyllanthin	Hypophyllanthin content (%)	% RC(*) of hypophyllanthin
RM - 1	***0 month***	***1.03***	***100.00***	***0.38***	***100.00***
	AS (1 month)	0.93	90.83	0.35	91.79
	AS (3 months)	0.94	91.93	0.32	83.70
	AS (6 months)	0.81	78.81	0.26	69.04
	LT (3 months)	0.99	96.11	0.37	97.36
	LT (6 months)	0.94	91.26	0.35	91.79
	LT (9 months)	0.91	88.35	0.34	89.47
	LT (12 months)	0.89	86.41	0.33	86.84
RM - 2	***0 month***	***0.84***	***100.00***	***0.31***	***100.00***
	AS (1 month)	0.87	103.251	0.34	108.78
	AS (3 months)	0.81	96.18	0.31	100.00
	AS (6 months)	0.72	85.68	0.29	92.56
	LT (3 months)	0.84	100.00	0.245	79.03
	LT (6 months)	0.76	90.48	0.28	90.32
	LT (9 months)	0.77	91.67	0.26	83.87
	LT (12 months)	0.72	85.71	0.25	80.65
RM - 3	***0 month***	***0.51***	***100.00***	***0.24***	***100.00***
	AS (1 month)	0.51	100.00	0.23	100.00
	AS (3 months)	0.49	96.04	0.23	100.00
	AS (6 months)	0.34	65.37	0.15	63.49
	LT (3 months)	0.50	98.04	0.23	95.83
	LT (6 months)	0.46	90.20	0.21	87.50
	LT (9 months)	0.42	82.35	0.20	83.33
	LT (12 months)	0.40	78.43	0.19	79.17

(*) *RC* - the relative concentrations of phyllanthin or hypophyllanthin in the experiments calculated by ratio of content at time testing compared to baseline.

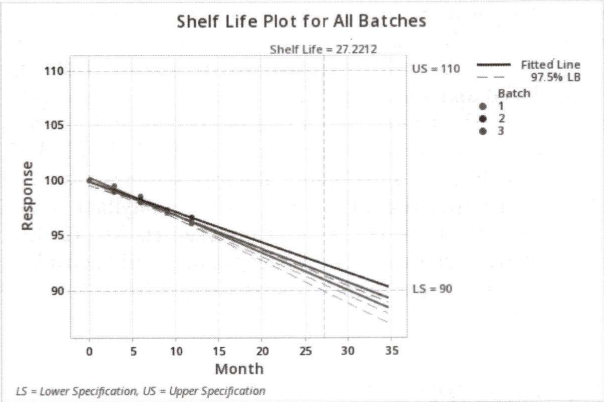
In terms of AS sample, there were no difference in content of phytomarkers in comparison with baseline for each method of process after one-month of storage, but the quantity of phyllanthin and hypophyllanthin reduced to 78.81% and 69.04%, respectively for RM - 1 in 6 months and it significantly reduced to 65.37% and 63.49% within six-months period for RM - 3 sample. The primary methods for processing medicinal herbs play an important role to eliminate some factors in fresh herbs that can have an influence on the deterioration of active compounds. In case of LT studies, there were slight reductions of these lignans within one-year period (Table 2). It remained approximately above 80% of lignans concentrations. The results obtained from this study deduce that both phyllanthin and hypophyllanthin are not stable under AS as well as long term conditions.

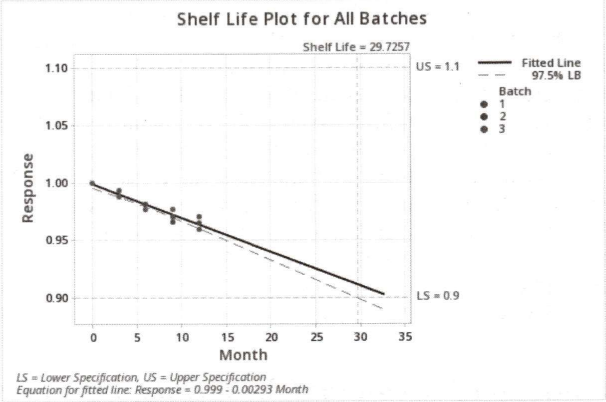
3.3. Evaluation of lignan content in dried powder of ethanol extract from Phyllanthus amarus under storage conditions

The results of quantitative analysis of lignans content in EE powder were presented in **Table 3**.

Table 3. Contents of lignans (% w/w, n=3) in dried powder of ethanol extract samples

Sample code	Storage condition	Lignans content (%)	% RC
Lot. No. 1	***0 month***	***4.41 ± 0.02***	***100.00***
	AS (1 month)	4.40 ± 0.08	99.77
	AS (3 months)	4.38 ± 0.10	96.15
	AS (6 months)	4.15 ± 0.03	94.10
	LT (3 months)	4.39 ± 0.05	99.54
	LT (6 months)	4.35 ± 0.01	98.63
	LT (9 months)	4.28 ± 0.01	97.05
	LT (12 months)	4.24 ± 0.04	96.41
Lot. No. 2	***0 month***	***4.24 ± 0.02***	***100.00***
	AS (1 month)	4.20 ± 0.05	99.05
	AS (3 months)	4.25 ± 0.06	96.81
	AS (6 months)	4.13 ± 0.03	94.70
	LT (3 months)	4.20 ± 0.02	99.06
	LT (6 months)	4.17 ± 0.07	98.34
	LT (9 months)	4.13 ± 0.04	97.41
	LT (12 months)	4.10 ± 0.03	96.70
Lot. No. 3	***0 month***	***4.04 ± 0.05***	***100.00***
	AS (1 month)	4.01 ± 0.06	99.26
	AS (3 months)	4.08 ± 0.04	96.91
	AS (6 months)	3.92 ± 0.11	93.11
	LT (3 months)	4.01 ± 0.08	99.25
	LT (6 months)	3.97 ± 0.01	98.26
	LT (9 months)	3.94 ± 0.01	97.52
	LT (12 months)	3.90 ± 0.03	96.53





The lignan contents tended to decrease during the period of investigation. Based on the decline, the interim extrapolated shelf-life of extract was justified about 27.2 months by minitab software.

3.4. Evaluation of lignan content in tablets prepared from dried powder of ethanol extract from Phyllanthus amarus under storage conditions

Table 4. Contents of lignans in tablet preparations

Sample code	Storage condition	Lignans content (mg per unit)	% RC
Lot. No. 1	***0 month***	***17.4 ± 0.15***	***100.00***
	AS (1 month)	17.0 ± 0.23	97.70
	AS (3 months)	17.0 ± 0.20	97.70
	AS (6 months)	16.7 ± 0.32	95.97
	LT (3 months)	17.3 ± 0.17	99.40
	LT (6 months)	17.1 ± 0.25	98.28
	LT (9 months)	17.0 ± 0.14	97.70
	LT (12 months)	16.9 ± 0.33	97.12
Lot. No. 2	***0 month***	***17.2 ± 0.29***	***100.00***
	AS (1 month)	17.0 ± 0.30	98.84
	AS (3 months)	16.9 ± 0.25	98.26
	AS (6 months)	16.4 ± 0.18	95.35
	LT (3 months)	17.0 ± 0.22	98.83
	LT (6 months)	16.9 ± 0.25	98.25
	LT (9 months)	16.7 ± 0.35	97.01
	LT (12 months)	16.6 ± 0.23	96.50
Lot. No. 3	**0 month**	**17.8 ± 0.30**	**100.00**
	AS (1 month)	17.2 ± 0.28	96.63
	AS (3 months)	16.4 ± 0.25	95.35
	AS (6 months)	16.9 ± 0.32	94.94
	LT (3 months)	17.6 ± 0.20	98.88
	LT (6 months)	17.4 ± 0.29	97.75
	LT (9 months)	17.2 ± 0.40	96.63
	LT (12 months)	17.1 ± 0.23	96.01

In case of LT condition, phytomarker contents did not change significantly after 12 months. The lignan contents decreased by about 3% compared with the average content. For stability under accelerated study conditions, there was unsignificant alteration after 6 months. The lignans content was reduced by about 5% compared to baseline content. Lignans content data under LT condition was used to predict the interim extrapolated shelf-life of the product. Normally, it was the time bioactive marker content low to 90% of initial content. For this product, it was 29,7 months by using minitab software.

4. Conclusion

This study was evaluated the content of phyllanthin and hypophyllanthin - major lignans in crude materials and preparation thereof in several conditions. We found a significant change in lignans content in the *Herba Phyllanthin amari* in long term storage condition and accelerated storage condition. The lignans content in unprocessed samples were strongly lowered to 80% and 70% in LT and AS after 12 and 6 months. These results may have suggestion for the heat processing could preserve lignans in *Herba Phyllanthin amari*. Both ethanol extract and tablet showed the slight decrease of lignans content below 5% during LT and AS. This plays the most important role in determining shelf-life of herbal medicine preparation from *Phyllanthus amarus*.

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HEPATOPROTECTIVE EFFECTS AND SAFETY EVALUATION OF METHANOLIC EXTRACT FROM *SEDUM SARMENTOSUM* BUNGE

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Summary

Hepatoprotective Effects and Safety Evaluation of Methanolic Extract from *Sedum sarmentosum* Bunge

The aim of this study was first to investigate the hepatoprotective activity of methanol extract from *Sedum sarmentosum* Bunge (SSE) in *Swiss albino* mice submitted to paracetamol-induced liver injury. Secondly, acute and subchronic oral toxicity assays of SSE were carried out in *Swiss albino* mice and *Wistar* rats. The results showed that SSE (at the doses of 0.5 and 1.0 g/kg; p.o) attenuated both acute and chronic paracetamol-induced liver intoxication as indicated by reduced level of serum liver enzymes, aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT). Additionally, the LD_{50} of SSE in mice was 25.56 g/kg body weight for single dose oral administration. In the subacute toxicity study, rats were administered SSE doses of 275 and 825 mg/kg (p.o) for 30 days. After 15 and 30 days, blood and tissue samples were taken for determination of hematological, biochemical and histopathological parameters. All parameters in SSE groups did not significantly change as compared to respective control.

Keywords: *Sedum sarmentosum, Acute and subacute toxicities, Paracetamol-induced liver injury, Hepatoprotective effect.*

1. Introduction

Sedum sarmentosum Bunge (Crassulaceae) is a perennial plant distributed throughout mountainous areas in China, Korea, Vietnam and Thailand where it has been used for hepatoprotection and other benefits [1],[2]. Principal chemical components of *Sedum sarmentosum* (*S. sarmentosum*) with hepatoprotective effects and inhibition of lipid accumulation in the liver are megastigman and flavonoids [2],[3]. Few studies of the chemical composition and biological activities of *S. sarmentosum* have been made in Vietnam [4], for which the age-adjusted death rate due to liver disease is 23.34 per 100,000 population [5]. The objective of this study was to evaluate the acute, subacute toxicity and hepatoprotective effects of *S. sarmentosum* collected in Vietnam.

2. Materials and Methods

2.1. Materials

✓ Preparation of *S. sarmentosum* Bunge extract:

Whole plant parts of *S. sarmentosum* Bunge were collected at Sa Pa, Lao Cai province, Vietnam in June 2015 and identified by Department of Resource Medicinal Materials, National Institute of Medicinal Materials, Hanoi, Vietnam (NIMM). The voucher specimens have been deposited in the Herbarium of Military Institute of Traditional Medicine (voucher specimens DL-300615). *S. sarmentosum* extract (SSE) was prepared by cutting aerial parts into small pieces and drying at 55°C. Plant material (9 kg) was extracted with methanol (3 times x 15 L). The organic layer was evaporated under vacuum to yield the dried crude extract (400 gr).

✓ Chemicals and reference drug:

- Paracetamol (Efferalgan® 500 mg), Bristol-Myers Squibb, French.
- Silymarin (Legalon® 54 mg), Madaus, Germany.