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BUI VAN TRUNG

STUDY ON CHEMICAL COMPOSITION, STRUCTURE ELUCIDATION AND QUANTIFICATION OF ISOLATED COMPOUNDS FROM *HELICIOPSIS TERMINALIS* AND *HELICIOPSIS LOBATA* BEING ORIENTATED FOR HEPATOPROTECTION

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Scientific supervisor:

1: Prof. Dr. Pham Hung Viet

2: Assoc. Prof. Dr. Duong Hong Anh

Reviewer 1: …

Reviewer 2: …

Reviewer 3: ….

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INTRODUCTION

1. Urgency of the thesis

The "Bàn tay ma" remedy, which was researched by Professor Pham Hung Viet in the Northwest Science and Technology Development Program from 2017 to 2019, consists of three dried medicinal components: wood of *Heliciopsis terminalis*, the aerial part of *Gynostemma pentaphyllum*, and bark of *Solanum procumbens* (Cà gai leo). Among them, *Gynostemma pentaphyllum* and *Solanum procumbens* have been proven to have good hepatoprotective effects and have also been extensively researched for their chemical composition towards liver-protecting activities. The largest component in the remedy is *Heliciopsis terminalis*, namely "Bàn tay ma" plant, which is considered the main ingredient of the remedy, but has not been extensively researched for its liver-protecting effects. Research on this medicinal plants has so far focused mainly on their chemical composition without studying on its hepatoprotective activites.

Currently, there are two medicinal plants with the same name "Bàn tay ma": Bàn tay ma đỏ (*Heliciopsis lobata* (Merr.) Sleumer) and Bàn tay ma trắng (*Heliciopsis terminalis* (Kurz.) Sleumer), which are being used to treat liver diseases in folk medicine. The morphology of these two medicinal plants is quite similar, and the rate of recognition and differentiation of these two types of medicinal plants in folk medicine is still low, so the use of these two types of medicinal plants is still happening interchangeably. If these medicinal plants are not proven to have liver-protecting effects, such use may not only fail to cure the disease but also pose a risk to the health of patients.

To clarify the liver-protecting effects of "Bàn tay ma" as well as its role in the "Bàn tay ma" remedy, I conducted the topic ***"Study on chemical composition, structure elucidation and quantification of isolated compounds from Heliciopsis terminalis and Heliciopsis lobata being orientated for hepatoprotection".***

2. Research objectives of the thesis

(i) To determine the chemical composition and structure of organic compounds isolated from *Heliciopsis terminalis* and *Heliciopsis lobata* using modern physicochemical analysis methods;

(ii) To evaluate the liver-protecting effects of *Heliciopsis terminalis* and *Heliciopsis lobata*;

(iii) To develop a qualitative and quantitative procedure for identifying marker compounds with liver-protecting effects of *Heliciopsis terminalis* and *Heliciopsis lobata* with high accuracy and reliability in order to apply this procedure in practice.

CHAPTER 1. OVERVIEW

1.1. Overview of *Heliciopsis lobata* and *Heliciopsis terminalis*

Two species *Heliciopsis terminalis* (Kurz) Sleumer and *Heliciopsis lobata* (Merr.) Sleumer are two endemic species of the genus *Heliciopsis* Sleum (Protaceace family) growing in Vietnam. Both species *H. terminalis* and *H. lobata* are called by many different names. Recently, the name "Bàn tay ma" has been most commonly used. The reason for the name "Bàn tay ma" is because according to the legend of the Tay people (Cao Bang, Bac Kan), the tree grows in sacred forests, where the dead are buried, the leaves are split like giant hands, guarding the resting place of the deceased. The morphology of these two species is quite similar, so it is still difficult to distinguish these two species among the people.

In practice, both *H. lobata* and *H. terminalis* are used as medicine and share the same name “Bàn tay ma”. Recently, many places have recognized the difference between these two species and named them with different folk names as “Bàn tay ma đỏ” and “Bàn tay ma trắng” respectively through botanical characteristics. The botanical characteristics of these two species in many documents have not yet been consistently described to distinguish them, and the therapeutic effects of these two species have not been proven.

It is necessary to distinguish these two species both in terms of botanical characteristics and therapeutic effects. In a recent study by Associate Professor Tran Van On and his colleagues at Hanoi University of Pharmacy, these two medicinal plants have been botanically identified and the authors have pointed out some important characteristics to identify and distinguish these two species as *H. lobata* has covering feathers on its branches, buds, and leaves, while this part disappears in *H. terminalis*.

The most characteristic functional group of both species is polyphenol, expressed through the characteristic chemical reaction of phenyl glycosid groups. However, there is no overlap in the substances published by other authors in these two species.

Published results show that *H. terminalis* has a main group of phenyl glycoside and macrocyclic glycoside compounds. Some compounds in this group have the effect of enhancing glucose tolerance. While *H. lobata* also has a main group of phenyl glycosides, but typically arbutin derivatives. The biological effects of compounds isolated from this plant have not been widely published, mostly only related to antioxidant ability.

Further research is needed on liver-protecting effects as well as identifying chemical components related to liver-protecting effects to evaluate the effectiveness of using these two species in folk medicine.

1.2. Some methods for evaluating hepatoprotective effects

In vivo hepatoprotective effects are usually performed on rat or mouse models with liver toxicity induced by CCl_4 or high-dose paracetamol. Liver-protecting ability is evaluated through gross liver images, microscopic images, and liver enzyme indices ALT, AST; in addition, there are also TBARs, GSH indices.

In vitro models, liver-protecting effects are evaluated through some effects related to liver-protecting mechanisms such as antioxidant (TBARs, DPPH), anti-inflammatory, especially the ability to protect liver cells under toxic agents such as CCl_4 or paracetamol.

Medicinal plants or substances with hepatoprotective effects will also be evaluated for safety on acute toxicity models *in vivo*; cell-related toxicity.

1.3. Marker substances

According to the World Health Organization, chemical markers of medicinal plants are reference substances that have been identified in terms of the chemical composition of that medicinal plant. The criteria for selecting a substance as a chemical marker for a medicinal plant include:

- Being a component with a large enough content in the medicinal plant;
- If it is a quantitative marker, it must have a characteristic effect for the therapeutic effect of the medicinal plant;
- If it is a qualitative marker for the medicinal plant, it must be characteristic of that medicinal plant or species of that medicinal plant.

If the marker is both qualitative and quantitative, it will be most suitable, otherwise, a more characteristic marker can be chosen for qualitative identification.

- Chemical markers should be substances that can be evaluated by common methods such as TLC, HPTLC, HPLC, or GC.
- Different markers can be selected for the same medicinal plant when it is prepared in different ways or according to different therapeutic effects of that medicinal plant.
- The marker for a medicinal plant can be either a single substance or a group of characteristic substances of the medicinal plant. Appropriate qualitative and quantitative methods will be developed for marker compounds of medicinal plants. These methods must be fully validated according to the guidelines of ICH, AOAC international.

CHAPTER 2. RESEARCH MATERIALS AND METHODS

2.1. Research objects

The wood (branches and trunk) of *H. lobata* and *H. terminalis* were collected in Bach Thong district, Bac Kan province in 2019 and 2021. Two samples were identified by Dr. Nghiem Duc Trong, Hanoi University of Pharmacy, before being dried, and crushed to powder for use.

2.2. Research methods

2.2.1. Applying modern physicochemical analysis methods to determine the structures of several compounds from the two species of Heliciopsis Sleum genus

The method used is to ultrasound the medicinal plant and methanol with a medicinal plant-solvent ratio of 1:3 at a temperature not exceeding 50 ^{o}C. Repeat extraction 03 times, collect the ultrasound solution to evaporate the solvent to obtain methanol extract.

The methanol extract is then added with water to form a mixture in the aqueous phase, this mixture is successively extracted with less polar solvents such as n-hexane, dichloromethane, and ethyl acetate. Evaporate the solvent in the solvent fractions to obtain extracts in the phases.

Extracts obtained in the fractions are isolated and purified on combined normal and reverse phase chromatography systems (including both column chromatography and preparative liquid chromatography). The fractions are detected by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC).

Compounds after being purified to an appropriate purity are measured by mass spectrometry, nuclear magnetic resonance spectroscopy, CD spectroscopy, IR spectroscopy to determine their structure. The structure of the compounds is determined from reference materials or from the spectral data obtained.

2.2.2. Evaluating the hepatoprotective effects of the two species of Heliciopsis Sleum genus

2.2.2.1. In vivo evaluating the hepatoprotective effects of water extracts of two species of Heliciopsis Sleum genus

Medicinal plant samples (stem and branches) of both *H. terminalis* and *H. lobata* were ground, moisture content determined, extracted with boiling water 3 times, combined extract solution and vacuum rotary evaporated to obtain concentrated extract and used to test liver-protecting effects at high doses corresponding to doses of 60 g; 120 g and 240 g medicinal plant for human/day. Swiss albino test mice, liver

toxicity induced by paracetamol, positive control is Loganin.

Evaluation parameters include gross liver images, liver enzyme indices ALT, AST; antioxidant ability TBARs.

2.2.2.2. In vitro evaluation of the hepatocyte protection of the isolated compound

On an in vitro model, the liver cell-protecting effect of compounds isolated from medicinal plants was tested on HepG2 liver cancer cell line when induced by CCl_4 toxicity. Positive control is quercetin.

2.2.2.3. Evaluating the antioxidant activities

Antioxidant effects are evaluated based on the ability to scavenge DPPH free radicals and counteract CCl_4 impacts.

2.2.2.4. Evaluating the anti-inflammatory ability

Anti-inflammatory ability is performed on samples that have been determined to have liver-protecting effects and have high safety on in vitro models. This test is performed on a toxic model of *RAW264.7* macrophage cell line. Evaluation parameters are inhibition of NO production and recovery ability of cytokine indices IL-6, IL-10, and TNF-α.

2.2.2.5. Evaluating the cytotoxicity in vitro model

The cell toxicity evaluation method is performed by exposing the research substance to the test cell line which is human embryonic kidney cells with code *HEK-293A.* Toxicity is determined through the amount of living cells by Sulforhodamin B staining.

2.2.2.6. Evaluating the acute toxicity of aqueous extract of two species

Acute toxicity was assessed according to current regulations of the Ministry of Health and Dr. Do Trung Dam.

2.2.3. Development of qualitative and quantitative methods for hepatoprotective markers of the two species of Heliciopsis Sleum genus

The marker compound is identified according to the guidelines of the EMA, including the criteria: being the main compound, representative of the research sample; being a compound with liver-protecting activity and relatively high safety; feasible for qualitative and quantitative determination by routine methods.

In a complex medicinal plant matrix, the marker compound is expected to be qualitatively and quantitatively determined by HPLC method. Validation according to the criteria of AOAC guidelines, including: Specificity, System suitability, Linearity, Limit of quantification, Accuracy, and Repeatability.

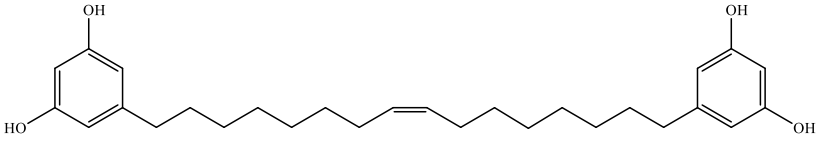
CHAPTER 3. RESULTS AND DISCUSSION

3.1. Isolation of the compounds from the two species of *Heliciopsis* Sleum genus

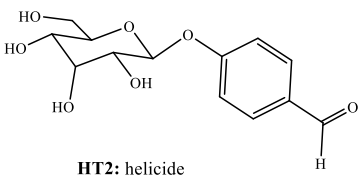
The purified compounds after isolation and purification were tested for morphology, determined HR-ESI-MS and NMR spectral parameters to determine their structure.

3.1.1. The compounds isolated from Heliciopsis terminalis

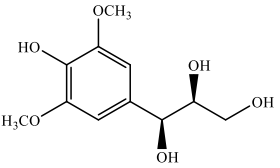
This study identified 10 compounds from *H. terminalis* species. Among them, there is a new compound (HT9, named helitermioside) and 09 known compounds, but these are the first compounds isolated from the *Heliciopsis* Sleum genus.



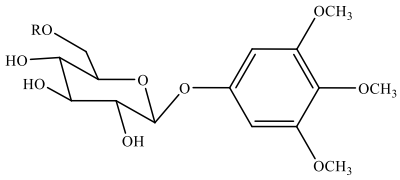
HT1: (Z)-5,5'-(hexadec-8-ene-1,16-diyl)bis(benzene-1,3-diol)

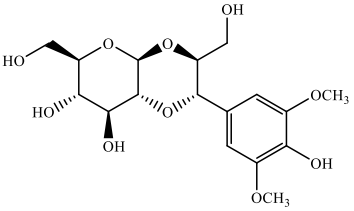


HT2: helicide



HT3: *threo*-syringylglycerol



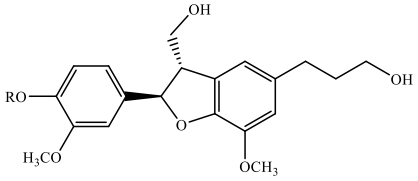


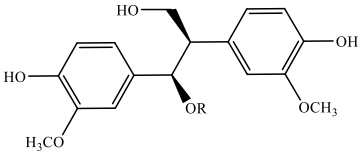
HT5 (R=H): 3,4,5-trimethoxyphenyl-*β*-D-glucopyranoside

HT4: ficuscarpanoside B

HT7 (R = Ara(f)): rhyncoside C

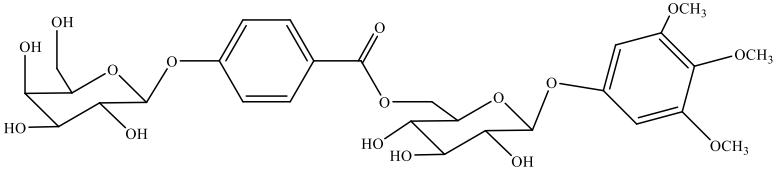
HT8 (R=Api): 3,4,5-trimethoxyphenyl-*β*-D-apiofuranosyl-(1″-6′)-*β*-D-glucopyranoside





HT6 (R=Glu): hovetrichoside A

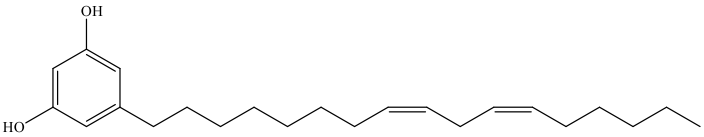
HT10: *7R,8S*-dihydrodehydrodiconeferyl alcohol 4-O-*β*-D-glucopyranoside



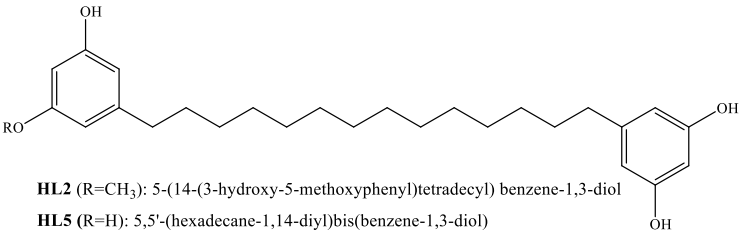
HT9: helitermioside

3.1.2. The compounds isolated from Heliciopsis lobata

This study identified 14 compounds from *H. lobata*. Among them, there is one new compound (HL12, named helilobatoside A) and 13 known compounds, but these are the first compounds isolated from the genus Heliciopsis sleum. Among the 13 known compounds, compound HL6 overlaps with compound HT1 in *H. terminalis* plant. These compounds include:

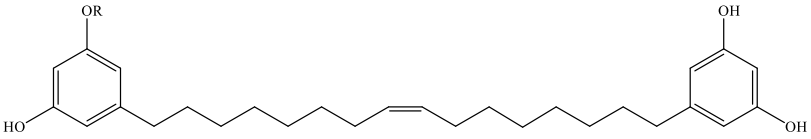


HL1: (8′*Z*,11′*Z*)-5-(heptadeca-8',11'-dienyl)benzene-l,3-diol



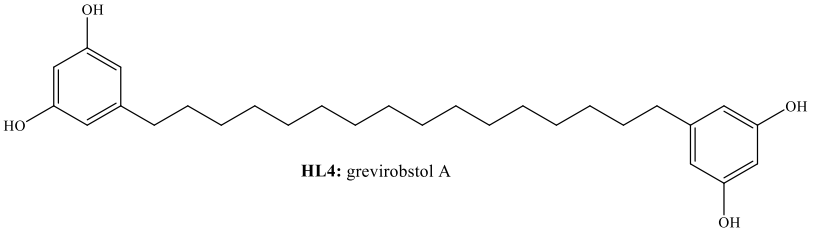
HL2 (R=CH_3): 5-(14-(3-hydroxy-5-methoxyphenyl)tetradecyl) benzene-1,3-diol

HL5 (R=H): 5,5'-(hexadecane-1,14-diyl)bis(benzene-1,3-diol)

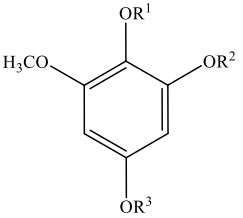


HL3 (R=CH_3): *(Z)*-5-(16-(3-hydroxy-5-methoxyphenyl)hexade-8-en-1-yl)

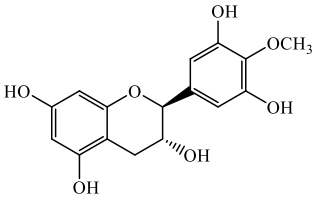
HL6 (R=H): *(Z)*-5,5'-(hexadec-8-ene-1,16-diyl)bis(benzene-1,3-diol)



HL4: grevirobstol A



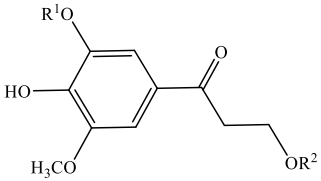
HL9 (R^1=Glu, R^2=H, R^3=H): isotachioside

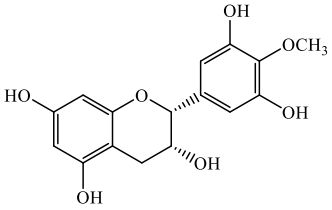


HL7: 4'-*O*-methyl-ent-gallocatechin

HL10 (R^1=H, R^2=H, R^3=Glu): tachioside

HL11 (R^1=H, R^2=CH_3, R^3=Glu): 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside

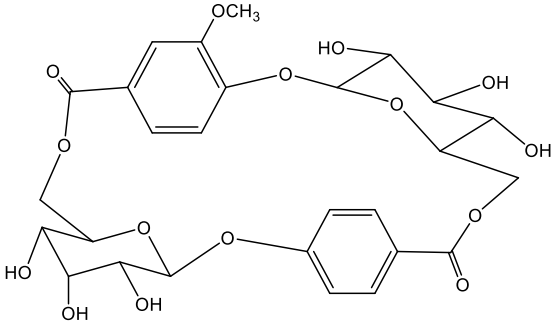




HL8: (-)-4'-*O*-methyl-epi-gallocatechin

HL13 (R^1=H, R^2=Glu): *β*-hydroxypropiovanillone 3-O-*β*-*D*-glucopyranosid

HL14 (R^1=CH_3, R^2=Glu): 3′,5′-dimethoxy-3,4′-dihydroxypropiophenone 3-O-β-D-*gluc*oside



HL12: Helilobatoside A

3.2. Hepatoprotective activities of the two species of *Heliciopsis* Sleum genus

The medicinal plants were independently evaluated for liver-protecting effects on an in vivo model, while the compounds isolated

from the two species were also screened for this effect on an in vitro model. Compounds with good and characteristic effects for medicinal plants will be selected as markers.

3.2.1. Hepatoprotective effects of two species of Heliciopsis Sleum in vivo model

After the experiment, the mice in the normal control group, the positive control group and the group treated with the dosage of *H. lobata* extract equivalent to 120 g of herbal medicine/person/day did not die. The number of mice that died in the toxic model group was 30 %, which was higher than the number in the groups that administrated with *H. terminalis* extracts but was lower than the number in the group taken *H. lobata* equivalent to 240 g of medicinal herbs/person/day (mice died by 50 %).

All the parameters would be evaluated on the number of live mice in each group.

The level of liver enzymes ALT and AST in mice taking silymarin (Loganin), an aqueous extract of *H. lobata* was significantly reduced compared with this parameter of the mice toxic control group. The lowest dosage and middle dosage of the *H. lobata* extract showed the best effect. In contrast, water extract of *H. terminalis* was not affected by these liver enzymes. Similar trends were also found on the level of TBARs and histopathology images of the mouse livers in corresponding groups.

It can be seen that *H. lobata* extract exhibited a quite good hepatoprotective effect, while its *H. terminalis* has not shown a hepatoprotective effect at the experimental dosages.

3.2.2. Hepatoprotective activities of the compounds isolated from H. terminalis and H. lobata

In general, the substances isolated from the *H. terminalis* material showed less toxicity but almost no protective effect on to *HepG2* cell line under CCl_4 induce. For *H. lobata* material, some high polar compounds have significant hepatoprotective activity, while some less polar substances are cytotoxic to this cell line.

After the screening process for hepatoprotective effects on *in vivo* and *in vitro* models, it can be confirmed that the *H. terminalis* material has not shown a hepatoprotective effect, while the *H. lobata* material has shown a relatively good liver protective effect.

For the above reasons, *H. terminalis* material and its isolated compounds would no longer be studied. Among the active compounds isolated from these two herbal materials, only compound HL11 showed the best hepatoprotective effect with the EC50 = 95.68 µg/ml.

In terms of antioxidant activity, compound HL11 has a significantly reduced effect of DPPH equivalent to positive control, ascorbic acid, and against lipid peroxidation with the IC50s for two models are 6.07 ± 0.17 µg/ml and 89.55 ± 8.26 µg/ml respectively. These activities were consistent with the antioxidant activity of the extract of *H. lobata* material.

In terms of anti-inflammatory, compound HL11 was not cytotoxic to the testing cell line, *RAW 264.7* macrophage. This compound showed the ability to inhibit NO-producing macrophages with IC50 of 76.49 ± 2.46 µg/ml. At the concentrations of 20 µg/ml and 100 µg/ml, it significantly reduced IL-6 production, increased IL-10 production and TNF-α production after 24 hours and nearly had no effect after 48 hours tested.

3.2.3. Evaluation of the toxicity of H. lobata

3.2.3.1. Acute toxicity of H. lobata extract

The highest dosage that can be given to mice was 32.60 g/kg calculated via extract weight per mouse weight (14 times higher than the highest dosage tested for hepatoprotective effects), the extract has not shown acute toxicity. It could be seen that the LD0 dosage (if available) would be greater than or equal to 32.60 g/kg based on the weight of the extract on the body weight of the tested mouse.

3.2.3.2. Cytotoxicity of some compounds isolated from the aqueous phase of H. lobata

Toxicity was evaluated on the *HEK-293A* cell line, the testing samples were selected from the compound isolated from the aqueous phase of *H. lobata* material (from HL9 – HL14).

The compound HL11, HL13, and HL14 showed cytotoxicity to HEK-293A cell line with IC50 in the range of 16.58 - 68.29 µg/mL; while the remaining compound did not show toxic activity at the tested concentrations.

According to the guideline of the US National Cancer Institute (NCI), the extract is considered to have good activity if IC50 ≤ 20 µg/ml, while the purity compound is considered to have strong toxic activity if IC50 ≤ 5 µM. Among the studied samples, the HL11 sample showed the strongest cytotoxicity with IC50 of 16.58 ± 1,986 µg/mL, corresponding to 49.94 ± 5.98 µM. The concentration of this value is still much higher than the reference value, so compounds HL11, HL13 and HL14 were toxic to the tested normal cell line but were not approached to the warning level.

3.3. Developing analytical procedure for identification and quantification of hepatoprotective markers of *Heliciopsis lobata* material

3.3.1. Select markers with hepatoprotective effects from H. lobata

HL11 (3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside) is the compound isolated in the greatest amount from the aqueous phase of *H. lobata*. HL11 has hepatoprotective activities at appropriate concentrations involving anti-inflammatory and antioxidant activities. At high concentrations, compound HL11 showed toxicity in hepatocytes and normal cell lines. Meanwhile, the aqueous extract of *H. lobata* also contained a relatively large amount of this compound in comparison with the other compounds, which could protect the liver from the toxic of paracetamol but a high dosage of this compound may be caused toxic when using combined with a high dosage of paracetamol. The other compounds in the aqueous phase did not show the same trend. The compounds HL9, HL10, HL12, HL13 and HL14 although were low toxic on tested cell lines, did not show any hepatoprotective effect.

It was clear that the compound HL11 was the specific compound for the hepatoprotective effect of *H. lobata* material. The structure of the compound HL11 contained an aromatic ring, so it could absorb UV radiation at a maximum of about 278 nm and would be detected by a UV detector. Furthermore, HL11 was a fairly polar compound that could be analyzed by the HPLC method, an effective and common tool for the analysis of herbal materials. For all these mentioned reasons, the compound HL11 would be selected as a hepatoprotective marker of the *H. lobata* material.

3.3.2. Procedure for indentification of 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside in Helicopsis lobata.

The HPLC procedure used for quantification of 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside would be used for identifying this

compound in *H. lobata* material. In the assay test, the retention time of the principal peak in the chromatogram of the sample solution corresponds to that of the peak due to 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside in the chromatogram of the standard sample.

The qualitative method has been evaluated in term of specificity in the below result of the validation quantitative method for this compound in *H. lobata* material.

3.3.3. Procedure for quantification of 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside in Heliciopsis lobata.

About 5 mg of HL11 sample after drying was placed into separate suitable test tubes, adding increasing volumes of research solvents, ultrasonic for 30 minutes. The results showed that the mixture of ethanol - water with a ratio of 50 - 50 had the best ability to dissolve the research sample. This solvent mixture will be selected to extract HL11 compound from the research medicinal plant sample, as well as used as a solvent to prepare samples during quantification.

After studying the properties of compound HL11, it is expected to choose a reverse phase liquid chromatography program with a stationary phase of C18 column (250 x 4.6 mm; 5 µm) and the solvent will be selected as the mobile phase is a combination of 0.1 % phosphoric acid solution (solution A) and acetonitrile (solution B) with appropriate mobile phase programs.

Compounds HL9, HL10, and HL11 have similar structures, and when surveyed, compounds HL10 and HL11 eluted closest together so compound HL10 was chosen as the compound to evaluate system resolution. The injection volume of 5 µl was chosen to ensure resolution of the compounds. Compounds HL10 and HL11 both have maximum absorption around 278 nm, so the detection wavelength is set at 278 nm.

The sample was prepared by ultrasonic medicinal plant with 50% ethanol in water. After surveying, the appropriate time for ultrasonic

is 30 minutes. The ultrasonic process was repeated 03 times, collecting the extract solution to ensure complete extraction of HL11 in medicinal plant. Perform additional extraction 03 more times to check the amount of extracted substance, then the result shows that the extract after that contains very small amount of HL11. Since HL11 is insoluble in ethyl acetate, use this solvent to extract the solution after combining and evaporating ethanol. Accordingly, less polar impurities were removed, ensuring safety for the column after analysis as well as reducing sample analysis time due to washing impurities.

After surveying and building methods, the procedure for quantifying HL11 has been built as follows: Liquid chromatography method (DĐVN V, Appendix 5.3).

- *Chromatography conditions:*

+ Column C18 (250 x 4.6 mm; 5µm).

+ Mobile phase: simultaneous combination of two solutions including 0.1% phosphoric acid (solution A) and acetonitrile (solution B) according to the following program (Table 3.8):

Table 3.8. Mobile phase program for quantifying HL11 in *H. lobata* material

Time (min)	**% solution A**	**% solution B**
0	95.0 %	5.0 %
15	85.0 %	15.0 %
20	20.0 %	80.0 %
30	20.0 %	80.0 %
31	95.0 %	5.0 %
35	95.0 %	5.0 %

+ Flow rate: 1.0 ml/min.

+ Detector: PDA set at wavelength of 278 nm.

+ Injection volume: 5 µl.

- *Sample preparation:*

+ Standard solutions: Dissolve and dilute 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside reference standard in 50 % of ethanol to obtain the solutions with accurate concentraion about 0.075 mg/ml.

+ Ressolution solution: Solution containing tachioside and 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside with accurate concentrations about 0.5 mg/ml in ethanol 50 %.

+ Sample solution: Accurately weigh about 10 g of *H. lobata* material powder into a suitable conical flask. Add 100 ml of 50 % ethanol, ultrasonic for 30 minutes. Filter out extract solution, continue adding another 100 ml of ethanol for another two times. Combine extract solution from three times, vacuum rotary evaporate at 60 ^{o}C until about 20 ml remains. Transfer to a separatory funnel, rinse and add enough water about 50 ml. Extract three times with ethyl acetate, discard ethyl acetate part. The aqueous phase solution is transferred to a rotary evaporator flask, rinsed with ethanol at 50 %. Vacuum rotary evaporate until dryness, let cool down. Add 5 ml of 50% ethanol, ultrasonic for 15 minutes, transfer the solution after ultrasonic into a 25 ml volumetric flask. Repeat the process twice with the same volume of 50 % ethanol. Combine the extract solution into a 25 ml volumetric flask, ultrasonic for another 15 minutes. Let cool down, add 50 % ethanol to the mark, filter through a 0.45 µm membrane to obtain the test solution.

Perform chromatography on the above solutions, record chromatograms and chromatographic parameters.

- *Result evaluation:*

+ Systematic suitability: The resolution between peaks of tachioside and 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside in the chromatogram of system suitability solution should not be less than 1.5.

+ The retention time of the principal peak in the chromatogram

of the sample solution corresponds to that of the peak due to 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside on the chromatogram of the standard sample.

+ The content of 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside (%, w/w) in the sample material is calculated as follows:

$$\text{HL (\%, w/w)} = \frac{C_{std} \text{ x } 25 \text{ x } 100 \text{ x } 100}{m_{spl} \text{ x } (100 - L)}$$

Where: + L is the loss on drying of the herbal material (%)

+ C_{std} is the concentration of the standard sample (mg/ml)

+ m_{spl} is the weight of the herbal sample (mg)

3.3.3. Preliminary determination of 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside content in purified HL11 material

The remaining amount of 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside (HL11) after being isolated and purified from *H. lobata* was finely ground and dried for 24 hours in a vacuum drying cabinet with P_2O_5 surface. After drying time, 283 mg of HL11 compound was obtained for testing. At this point, it is considered that the raw material has been dried to constant weight and exists in anhydrous form. The content of compound 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside will be evaluated by area normalization method. Calculated on the average value of 03 test samples, the content of compound 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside in HL11 sample after preparation is 96.4 % based on anhydrous product.

3.4.4. Evaluation of qualitative and quantitative procedure of 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside in H. lobata material

The method for the quantification of 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside in *H. lobata* material would be validated according to the guidelines of AOAC international.

Targets	Requirement	Result
Specificity	+ The chromatogram of the sample solution shows a principal peak that has a retention time corresponding to that of the peak due to 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside in the chromatogram of the standard sample. + The chromatogram of the blank solution does not show a principal peak that has a retention time corresponding to that of the peak due to 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside in the chromatogram of the standard sample.	Conforms
System suitability	+ The resolution of the HL10 peak and L11 peak ≥ 1.5; + RSD_area of standard peak (n=6) ≤ 2.0%	+) RS resolution = 2.2 +) RSD = 0.65 %
Linearity	R ≥ 0,998	Conforms (R = 0,99995)
Repeatability	RSD content of HL11 in test sample (n = 6) ≤ 3,7 %	Conforms (RSD = 3,1 %)
Accuracy	Recovery from 95 % to 105 %	Conforms (97,1 % - 97,9 %)
Working range	Determined based on linearity and accuracy results	0,0375 mg/ml - 0,15 mg/ml
Intermediate accuracy	RSD content of L11 in test sample (n = 12) ≤ 6 %	Conforms (3,2 %)
Limit of quantification	Determined based on the experiment	0,66 µg/ml

3.3.5. Determination of 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside in water extract of H. lobata

Apply the quantitative method for compound 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside in *H. lobata* material that has been developed to quantify this compound in water extract of this material. The content of this compound in water extract is 0.136 %, from which it can be calculated that the extraction efficiency by traditional extraction method is about 20 %.

CONCLUSIONS

4.1. Conclusions

This is the first study to simultaneously investigate *H. terminalis* and *H. lobata* to identify heptatoprotective compounds of these two species using modern physicochemical and biological analysis methods. From there, a suitable qualitative and quantitative method has been developed to help control the quality of *H. lobata* material and related products when used by the people. Specifically, the thesis obtained the following main results:

- In terms of chemical compositions of the two herbal material: This study was the first time isolation of the 10 compounds from *H. terminalis* and 14 compounds from *H. lobata*. Among them, the two species have an identical compound of 5,5'-(hexadecane-1,16-diyl)bis(benzene-1,3-diol). In particular, among the isolated compound, one new compound was found from each species, named helitermioside (isolated from *H. terminalis*) and helobatoside A (isolated from *H. lobata*).

- In terms of the hepatoprotective activities: The study also for the first time evaluated the hepatoprotective activities of both species on *in vivo* and *in vitro* models. Through this, it has been proven that

both water extract and compounds isolated from *H. terminalis* material have not shown liver-protecting effect at experimental dose, while water extract of *H. lobata* material has good liver-protecting effect at a dose equivalent to 60 g and 120 g of medicinal plant for human use per day. At the same time, from the compounds isolated from this medicinal plant, compound 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside (HL11) isolated from *H. lobata* was also screened as the main compound with liver cell protection effect, anti-inflammatory and antioxidant effects. Subsequently, this study also proved that water extract of *H. lobata* material and some compounds isolated from its water phase have relatively high safety. This result provides scientific evidence for the liver-protecting effect of *H. terminalis* and *H. lobata* currently used in Vietnam, contributing to helping doctors and people use these two species more effectively and correctly.

- Development of qualitative and quantitative methods for identifying liver-protecting marker compounds: From the hepatoprotective effect of the two studied species as well as the chemical components that have been isolated, this study has screened compound 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside to be used as a marker for liver protection effect of *H. lobata* material. From there, a qualitative and quantitative method for HL11 compound in *H. lobata* medicinal plant has been developed using HPLC-DAD detector method. This method met all requirements according to ICH and AOAC international guidelines. Applying the method to check HL11 content in *H. lobata* material and in water extract of this material, the result showed that the material contained about 0.02 % and the water extract contained about 0.136% HL11 based on anhydrous products.

Accordingly, traditional boiling water extraction method achieved about 20 % if calculated by HL11 extraction efficiency from H. lobata material. Through this, the study has provided an effective tool for quality control of *H. lobata* material as well as related products when circulating on the market.

4.2. Proposal

- The results of the thesis have provided scientific evidence proving that *H. terminalis* material has not shown liver protection effect while *H. lobata* material has shown relatively good liver protection effect on both in vivo and in vitro models. Therefore, it is necessary to distinguish these two medicinal plants when using them.

- Continue to build standards for medicinal plants, extracts and establish standard compound 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside for quality control of *H. lobata* material.

- *H. lobata* material has liver-protecting effects, and compound 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside is very characteristic of both the hepatoprotective effects and the side effects of this medicinal plant. Further research is needed on the toxicity of Bàn tay ma đỏ medicinal plant and compound 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside for therapeutic use.

- Continue to research ways to use compound 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside as a substitute for *H. lobata* material.

- Research extraction methods to obtain extracts with stable and economically efficient treatment efficiency based on marker compound 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside. Attention should be paid to evaluating toxicity and adjusting the appropriate dose when changing the extraction method because this medicinal plant contains some toxic substances.

NEW CONTRIBUTIONS OF THE DISSERTATION

- For the first time, the hepatoprotective effects of *H. terminalis* material and *H. lobata* material (materials are dried wood parts of these two species) were evaluated simultaneously and it was proven that *H. lobata* material has good liver protection effect while the remaining material has not shown liver protection effect at experimental doses.

- One new compound was isolated from *H. terminalis* species and one new compound from *H. lobata* species. At the same time, 22 other known compounds were also isolated for the first time from *Heliciopsis* Sleum genus.

- For the first time, the activities realated to hepatoprotection of compound 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside were evaluated and identified. This compound was selected as a chemical marker for hepatoprotective effect of *H. lobata* material.

- A new, common method has been developed to qualitatively and quantitatively determine compound 3,5-dimethoxy-4-hydroxy-1-*β*-D-glucopyranoside in *H. lobata* material to serve quality control of this material and its related products.

LIST OF PUBLICATIONS

1. Phan Minh Giang, Do Thi Thao, Nguyen Thi Nga, Bui Van Trung, Duong Hong Anh, and Pham Hung Viet, 2019, *Evaluation of the antioxidant, hepatoprotective, and anti-inflammatory activities of bisresorcinol isolated from trunk of Heliciopsis terminalis*, Pharmaceutical Chemistry Journal, 52(7).

2. Bui Van Trung, Do Thi Thao, Duong Hong Anh, Phan Van Kiem, and Pham Hung Viet, 2020, *Antioxidant and Hepatoprotective Activity of Phenyl Glycosides Isolated From Heliciopsis lobata*, Natural Product Communications, 15(8), pp. 1-7.

3. Bui Van Trung, Nguyen Thu Hang, Duong Hong Anh, Pham Hung Viet, 2022, *Evaluation of the Hepatoprotective Activity of the Wood of Heliciopsis lobata (Merr.) Sleumer*. VNU Journal of Science: Medical and Pharmaceutical Sciences, 38(4).

4. Bui Van Trung, Duong Hong Anh, Pham Hung Viet, Phan Van Kiem, 2023, *Hepatoprotective and Antioxidant Activities of Phenolic Compounds from Heliciopsis terminalis*. Natural Product Communications, *18(5), pp. 1-4.*

5. Bui Van Trung, Ha Thi Thu Thuy, Duong Hong Anh, Pham Hung Viet, 2023, *Identification and determination of 3,5-dimethoxy-4-hydroxy-1-β-D-glucopyranoside in wood of Heliciopsis lobata*, Vietnamese Journal of Drug Quality Control, 21(79), pp. 21-27.