MINISTRY OF EDUCATION AND TRAINING

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STUDY ON CHEMICAL COMPOSITION AND STRUCTURE ELUCIDATION OF ISOLATED COMPOUNDS FROM *Pandanus tonkinensis* MART.EX B.STONE USING MODERN PHYSICOCHEMICAL METHODS

SUMMARY OF DISSERTATION ON ANALYTICAL CHEMISTRY

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LIST OF THE PUBLICATIONS RELATED TO THE DISSERTATION

1. **Dinh Thi Huyen Trang**, Pham Hung Viet, Duong Hong Anh, Bui Huu Tai, Ngo Quoc Anh, Nguyen Xuan Nhiem and Phan Van Kiem, 2022, Lignans and Other Compounds from the Roots of *Pandanus tonkinensis* with Their Lipid Peroxidation Inhibitory Activity, *Natural Product Communications*, 17(4), pp. 1-5.

2. **Dinh Thi Huyen Trang**, Duong Hong Anh, Quoc Anh Ngo, Pham Hung Viet, Bui Huu Tai, Nguyen Xuan Nhiem & Phan Van Kiem, 2022, Pandatonkinosides A and B: two new phenolic glycosides from the roots of *Pandanus tonkinensis* and their nitric oxide production inhibitory activities, *Natural Product Research*, 37(19), pp. 3253-3260.

3. **Dinh Thi Huyen Trang**, Pham Thu Trang, Do Minh Phuong, Duong Hong Anh, Ngo Quoc Anh, Phan Van Kiem, and Pham Hung Viet, 2023, The chemical composition from the fruits of *Pandanus tonkinensis* and their inhibitory NO production and lipid peroxidative inhibitory activities, *Vietnam Journal of Chemistry*, 61(special issue), pp. 1-7.

4. **Dinh Thi Huyen Trang**, Bui Van Trung, Ngo Quoc Anh, Duong Hong Anh and Pham Hung Viet, 2023, Determination of pinoresinol 4-O-beta-D-glucopyranoside and vladinol F as markers in *Pandanus tonkinensis* fruits by high performance liquid chromatography, VNU Journal of Science: Medical and Pharmaceutical Sciences, Vol. 39, No. 2, pp. 1-9.

INTRODUCTION

1. The urgency of the thesis

As one of the countries located in the tropical monsoon climate zone, Vietnam has a diverse and rich flora, with medicinal plants accounting for about 30%. Many research projects on medicinal plants of the Vietnamese flora have made great contributions to protecting human health. Plants that have medicinal effects on humans leave fewer side effects than drugs of synthetic origin. Therefore, with the increasing number of diseases such as cardiovascular disease, cancer, liver disease, etc., research on medicinal plants is of scientific and topical significance [1].

Pandanaceae is a family of flowering plants native to tropical and subtropical regions, distributed from West Africa to the Pacific. *Pandanus* is the largest and most important genus with about 600 species, which can be used as a food source and medicine. In Vietnam, the Pandanaceae family includes 23 species belonging to two genera: *Freycinetia* (3 species) and *Pandanus* (20 species). According to traditional medicine documents, there are 9 species of the *Pandanus* genus used as medicine in Vietnam, mainly effective in kidney diseases (diuretics, treatment of kidney stones, gallstones, urinary tract infections,...), liver diseases (hepatitis, cirrhosis and ascites), heat-clearing, fever reducing, skin diseases,...[2], [3].

Pandanus tonkinensis Mart. ex B. Stone, also known as Northern pineapple, is present from the Northern midland mountains to the Central region, Central Highlands, Binh Thuan, Long An. It is one of the 9 species mentioned above whose buds, leaves, roots and fruits can be used in medicine [2]. In the program of Science and Technology for Sustainable Development in the Northwest region, the results of investigation and research on the remedy for treating liver and gallbladder diseases were announced, in which the water extract of the remedy with two Stixis *suaveolens* and *Pandanus tonkinensis* have been shown to have good hepatoprotective effects, higher than sylimarin [4]. Currently, there is no

research on the chemical composition and pharmacological effects of *Pandanus tonkinensis* [5]. In order to obtain scientific evidence about ingredients, biological activities as well as quality control of medicinal herbs and preparation products in the direction of hepatoprotection, the thesis titled: "Study on chemical composition and structure elucidation of isolated compounds from *Pandanus tonkinensis* Mart.ex B.Stone using modern physicochemical methods" were proposed and implemented.

2. The objectives of the thesis

- Analyze the composition, chemical structure and hepatoprotective activity of compounds isolated from *Pandanus tonkinensis.*

- Identify markers for *Pandanus tonkinensis* in the direction of hepatoprotection and develop an analytical procedure for quantitation of markers in medicinal herbs to serve the quality control of *Pandanus tonkinensis* and products developed from thi medicinal herb.

3. The main contents of the thesis

- Using modern extraction, isolation techniques and physicochemical and biological methods to analyze the composition, chemical structure, and biological activity of compounds isolated from *Pandanus tonkinensis*.

- Selecting markers for *Pandanus tonkinensis* in the direction of hepatoprotection; Extract, purify, and analise the purity of the produced markers.

- Developing and validating the method for quantitative analysis of markers in the medicinal herb *Pandanus tonkinensis*. Applying the validated method to analyze the content of markers in medicinal herbs collected from difference provinces.

CHAPTER 1. OVERVIEW

Chapter 1 consists of 21 pages, presenting an overview of the literature on the genus *Pandanus,* research works on the chemical composition and biological activity of compounds isolated from the genus *Pandanus.*

Introduction to *Pandanus tonkinensis* Martelli ex BC Stone. Overview of physicochemical methods to extract, isolate, and elucidate the structure of substances isolated from medicinal herbs; overview of biological tests for evaluate hepaprotective activities, and introduction of chemical markers for medicinal herbs.

CHAPTER 2: RESEARCH METHODS AND EXPERIENCE

Chapter 2 includes 13 pages, detailing methods of isolation, structure determination, and biological activity assessment methods for antioxidant and anti-inflammatory properties of substances isolated from *Pandanus tonkinensis*. Methods for selecting markers, extracting and purifying markers from medicinal herbs, developing and validating procedures for quantitative analysis of markers in medicinal herbs using high-performance liquid chromatography.

CHAPTER 3 : RESULTS AND DISCUSSION

3.1. Isolation and identification of compounds from *Pandanus tonkinensis*

3.1.1. Procedure for isolating compounds from fruit Pandanus tonkinensis

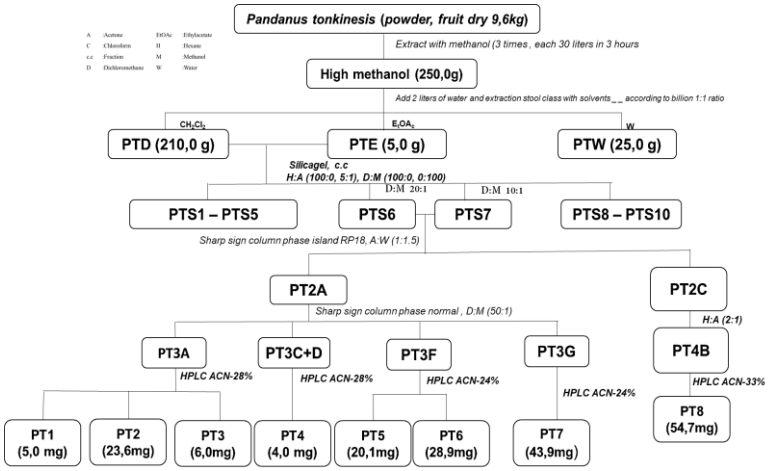


Figure 3.1. Diagram of isolation of compounds from *Pandanus tonkinensis* fruit

3.1.2. Isolation of compounds from Pandanus tonkinensis roots

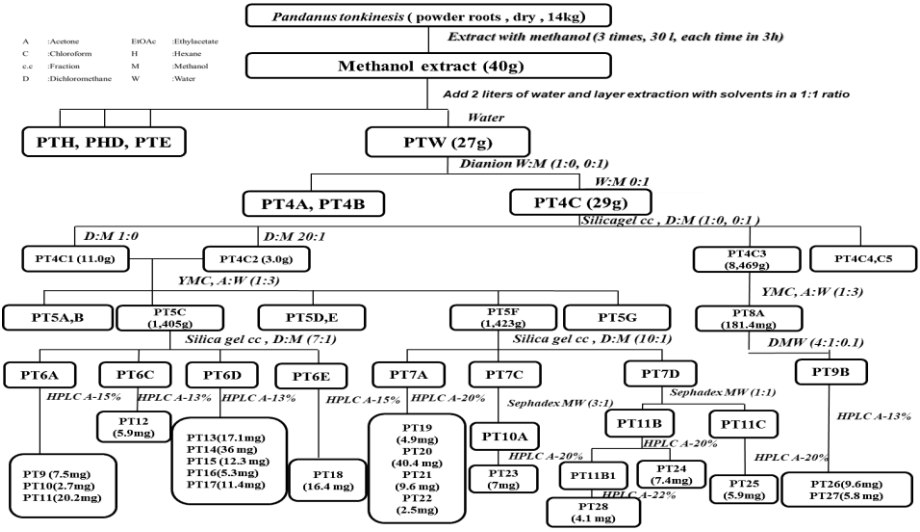
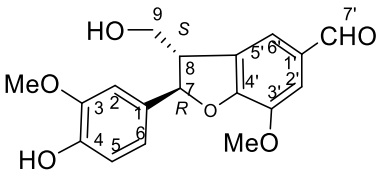


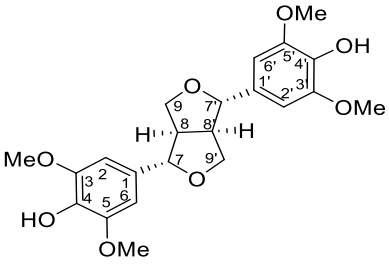
Figure 3.2. Diagram for isolating compounds from *Pandanus tonkinensis* roots

3.1.3. Identification of compounds isolated from Pandanus tonkinensis fruit

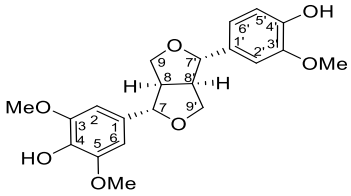
From *Pandanus tonkinensis* fruit, eight pure compounds were isolated, including: ficusal (**PT1**), syringaresinol (**PT2**), medioresinol (**PT3**), lariciresinol (**PT4**), secoisolariciresinol (**PT5**), vladinol F (**PT6**), luteoliflavan (**PT7**), isorhapontigenin (**PT8**).



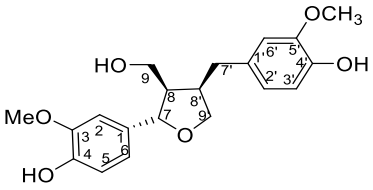
PT1 - ficusal



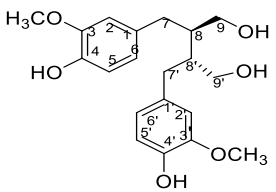
PT2 - syringaresinol



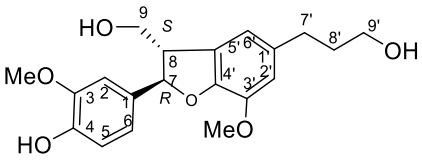
PT3 - medioresinol



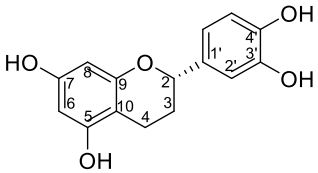
PT4 - lariciresinol



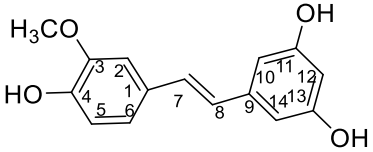
PT5 - secoisolariciresinol



PT6 - vladinol F



PT7 - luteoliflavan



PT8 - isorhapontigenin

Figure 3.3. Chemical structures of compounds isolated from *Pandanus tonkinensis* fruit

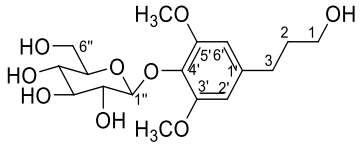
3.1.4. Identification of compounds isolated from Pandanus tonkinensis roots

From Pandanus tonkinensis roots, 20 compounds were isolated, including 3 new compounds and 17 known compounds.

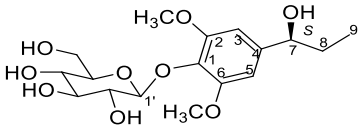
3 new compounds include: (7*S*)-2,6-dimethoxyphenyl-7-propanol-1-O-*β*-D-glucopyranoside **(PT10)**, *trans*-cinnamyl alcohol 9-O-(6′-O-*α*-L-arabinofuranosyl)-*β*-D-glucopyranoside **(PT25)**, 4-(3-hydroxypropyl)-2,6-dimethoxyphenol-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside **(PT26)**.

17 known compounds include: dihydrosyringin **(PT9)**, (6*S*,9*S*)-roseoside **(PT11)**, 1-O-*β*-Dglucopyranosyl-2-{2,6-dimethoxy-4-[1-(E)-propen-3-ol]phenoxyl}propan-3-ol **(PT12)**, 1-O-(*β*-D-glucopyranosyl)-2-

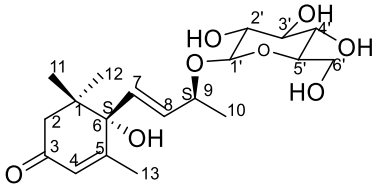
[2-methoxy-4-(*ω*-hydroxypropyl)-phenoxyl]-propan-3-ol **(PT13)**, benzyl O-*α* -L-arabinopyranosyl-(1→6)- *β* -D-glucopyranoside **(PT14)**, 1-O-(*β*-D-glucopyranosyl)-2-[2,6-dimethoxy-4-(*ω*-hydroxypropyl)-phenoxyl]-propan-3-ol **(PT15)**, (7*R*,8*R*)-threo-4,7,9,9′-tetrahydroxy-3,3′-dimethoxyl-8-O-4′-neolignan-4-O-*β*-D-glucopyranoside **(PT16)**, (7*S*,8*S*)-threo-4,7,9,9′-tetrahydroxy-3,3′-dimethoxyl-8-O-4′-neolignan-4-O-*β*-D-glucopyranoside **(PT17),** pinoresinol-4,4′-di-O-*β*-D-glucoside **(PT18)**, isoeucommin A **(PT19)**, pinoresinol 4'-O-*β*-D-glucopyranoside **(PT20)**, acanthoside B **(PT21)**, eucommin A **(PT22)**, rourinoside **(PT23)**, (7*S*,8*R*)-5-methoxydihydrodehydrodiconiferyl alcohol-4-O-*β*-D-glucopyranoside **(PT24)**, kelampayoside A **(PT27)**, urolignoside **(PT28)**.



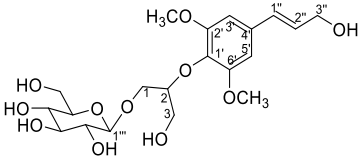
PT9 - dihydrosyringin



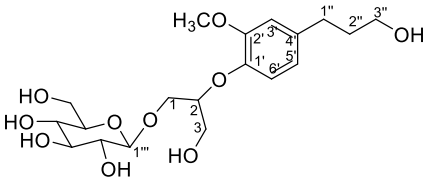
PT10 - (7*S*)-2,6-dimethoxyphenyl-7-propanol-1-O- *β* -D-glucopyranoside



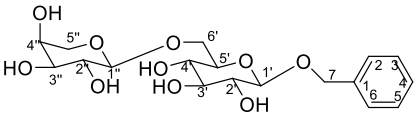
PT11 - (6*S*,9*S*)-roseoside



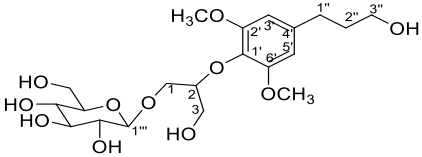
PT12 - 1-O-*β*-Dglucopyranosyl-2-{2,6-dimethoxy-4-[1-(E)- propen-3-ol]phenoxyl}propan-3-ol



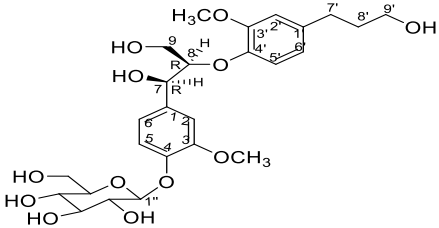
PT13 - 1-O-(*β* -D-glucopyranosyl)-2-[2-methoxy-4-(*ω*-hydroxypropyl)-phenoxyl]-propan-3-ol



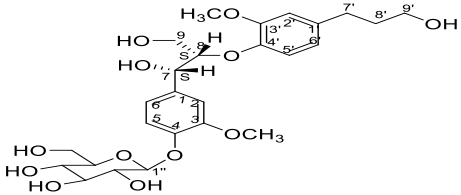
PT14 - benzyl-O- *α* -L-arabinopyranosyl-(1→6)-*β*-D-glucopyranoside



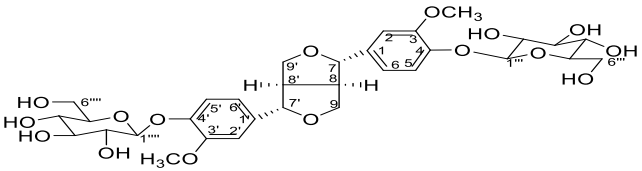
PT15 - 1-O-(*β*-D-glucopyranosyl)-2-[2,6-dimethoxy-4-(*ω*- hydroxypropyl)-phenoxyl]-propan-3-ol



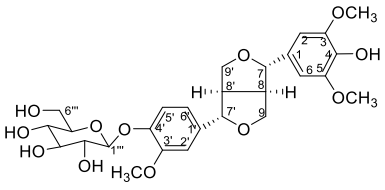
PT16 - (7*R*,8*R*)-threo-4,7,9,9′-tetrahydroxy-3,3′-dimethoxyl-8-O-4′-neolignan-4-O-*β*-D-glucopyranoside



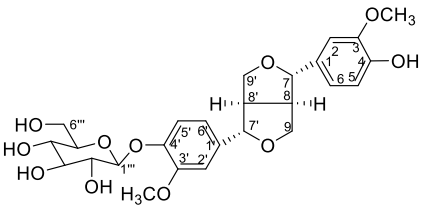
PT17 - (7*S*,8*S*)-threo-4,7,9,9′-tetrahydroxy-3,3′-dimethoxyl-8-O-4′-neolignan-4-O-*β*-D-glucopyranoside



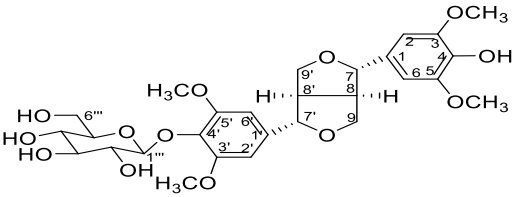
PT18 - pinoresinol-4,4′-di-O-*β*-D-glucoside



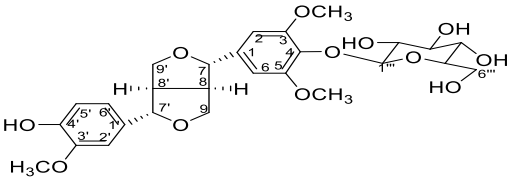
PT19 - isoeucommin A



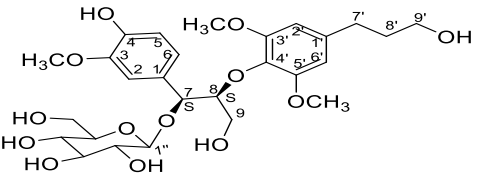
PT20 - pinoresinol 4'-O-*β*-D-glucopyranoside



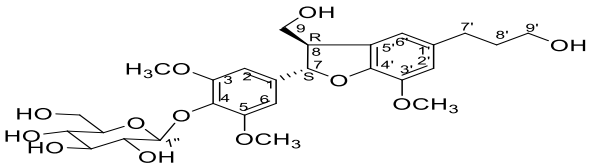
PT21 - acanthoside B



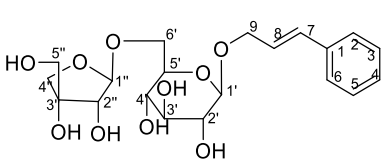
PT22 - eucommin A



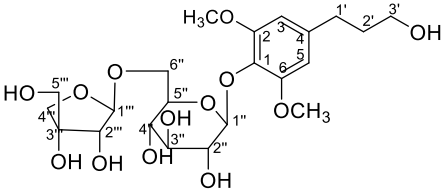
PT23 - rourinoside



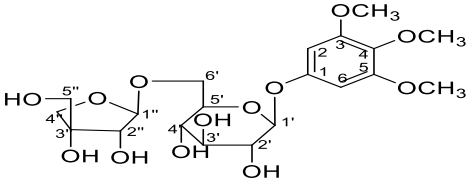
PT24 - (7*S*,8*R*)-5-methoxydihydrodehydrodiconiferyl alcohol-4-O-*β*-D-glucopyranoside



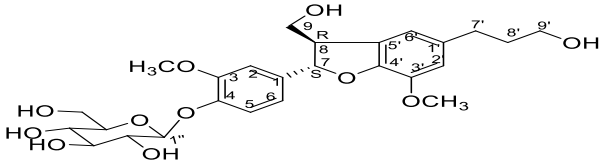
PT25 - *trans*-cinnamyl alcohol 9-O-(6′-O- *α* - L-arabinofuranosyl)-*β* -D-glucopyranoside



PT26 - 4-(3-hydroxypropyl)-2,6-dimethoxyphenol-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside



PT27 - (7*S*,8*R*)-5-methoxydihydrodehydrodiconiferyl alcohol-4-O-*β*-D-glucopyranoside



PT28 - urolignoside

Figure 3.4. Chemical structures of compounds isolated from *Pandanus tonkinensis* roots

3.2. Evaluation of biological activity of isolated compounds

3.2.1. Anti-inflammatory activity of isolated substances through inhibition of NO production on RAW 264.7 cells stimulated by LPS

The anti-inflammatory activity of the isolated compounds is presented in table 3.1.

Table 3.1. NO production inhibitory activity of substances isolated from *Pandanus tonkinensis*

Matter	IC_{50} (µM)	Matter	IC_{50} (µM)	Matter	IC_{50} (µM)
PT1	17.4 ± 1.94	**PT11**	28.5 ± 1.76	**PT21**	-
PT2	126 ± 7.65	**PT12**	-	**PT22**	32.2 ± 3.47
PT3	39.3 ± 3.30	**PT13**	44.4 ± 6.24	**PT23**	1.93 ± 0.23
PT4	5.25 ± 0.39	**PT14**	-	**PT24**	54.0 ± 2.03
PT5	23.0 ± 1.35	**PT15**	1.78 ± 0.13	**PT25**	83.3 ± 4.67
PT6	21.4 ± 2.08	**PT16**	34.4 ± 1.81	**PT26**	20.1 ± 2.07
PT7	7.08 ± 0.44	**PT17**	80.3 ± 7.26	**PT27**	5.84 ± 0.44
PT8	48.3 ± 5.11	**PT18**	-	**PT28**	-
PT9	37.0 ± 4.04	**PT19**	-	**L-NMMA**	37.8 ± 3.2
PT10	94.0 ± 10.3	**PT20**	24.7 ± 1.08		

The above test results showed that 22 substances **PT1** - **PT9**, **PT10**, **PT11**, **PT13**, **PT15** - **PT17**, **PT20**, **PT22** - **PT27** showed NO production inhibitory activity with IC_{50} values ranging from 1.78 to 125.83 µM, of

which 14 substances **PT1**, **PT4 - PT7**, **PT9**, **PT11**, **PT15**, **PT16**, **PT20**, **PT22**, **PT23**, **PT26**, **PT27** showed better anti-inflammatory ability through IC_{50} results than the positive control.

3.2.2. Antioxidant activity of isolated substances through inhibition of cell membrane lipid peroxidation

The results of determining the antioxidant activity of compounds isolated from *Pandanus tonkinensis* wild pineapple are presented in table 3.2.

Table 3.2. Membrane lipid peroxidation inhibitory activity of substances isolated from *Pandanus tonkinensis*

Matter	IC_{50} (µM)	Matter	IC_{50} (µM)	Matter	IC_{50} (µM)
PT1	-	**PT11**	-	**PT21**	27.5 ± 2.76
PT2	126 ± 5.57	**PT12**	-	**PT22**	54.2 ± 3.54
PT3	-	**PT13**	-	**PT23**	-
PT4	32.2 ± 1.42	**PT14**	-	**PT24**	-
PT5	20.2 ± 1.71	**PT15**	-	**PT25**	-
PT6	84.8 ± 6.69	**PT16**	-	**PT26**	-
PT7	26.3 ± 3.57	**PT17**	-	**PT27**	-
PT8	23.3 ± 1.67	**PT18**	-	**PT28**	-
PT9	-	**PT19**	57.5 ± 5.53	**Trolox**	31.4 ± 2.20
PT10	-	**PT20**	10.4 ± 0.71		

In the test, the positive control trolox performed stably giving an IC_{50} value of 31.4 ± 2.2 µM. 10 compounds including **PT2**, **PT4 - PT8**, **PT19 - PT22** showed inhibitory activity on cell membrane lipid peroxidation with IC_{50} values ranging from 10.36 to 126.39 µM. These 10 compounds show anti-oxidant ability, of which 5 substances **PT5**, **PT7**, **PT8**, **PT20**, **PT21** have better anti-oxidant ability than the positive control.

3.3. Markers

In the marker selection step, *Pandanus tonkinensis* are extracted with 50% methanol. The extract was preliminarily analyzed by HPLC- DAD. 06 pure compounds with the highest amount isolated from medicinal herb including **PT8**, **PT7**, **PT6** and **PT20**, **PT15**, **PT11** were dissolved in 50% methanol and analysed by HPLC using the same conditions. The retention time of peaks occured in the chromatogram of medicinal herb analysis and their UV spectra were then compared to those of analysis of 6 mentioned compounds. The results showed that in the chromatogram for medicinal herb analysis, there were two peaks with retention times equal to the retention times of **PT20** and **PT6**. Adding **PT20** and **PT6** standards to the medicinal herb sample extract matrix confirms the presence of **PT20** and **PT6** in the medicinal herb sample extract. Based on these results, two **PT20** and **PT6** were selected as markers for the *Pandanus tonkinensis* medicinal herb. A noteworthy point is that these compounds have both anti-inflammatory and antioxidant activities according to *in vitro* screening tests.

Several spectral data including: mass spectrum, nuclear magnetic resonance spectrum, UV-Vis spectrum, infrared spectrum of two markers were measured. Their structures were determined to be as pinoresinol 4'-O-β-D-glucopyranoside (PT2B1A or **PT20**) and vladinol F (PT2D1A or **PT6)** by comparison of their spectral data with those reported in the literature. The purity of the two extracted markers was determined through HPLC analysis, giving a corresponding result of 98.0% for pinoresinol 4'-O-β-D-glucopyranoside and 98.7% for vladinol F.

3.4. Developing and validating the analytical procedure for quantifying pinoresinol 4'-O-*β*-D-glucopyranoside and vladinol F in *Pandanus tonkinensis* medicinal herb

3.4.1. Survey the sample treatment before analysis

Using 50% methanol or 50% ethanol solvents to extract the analytes (**PT20** and **PT6**) from the medicinal herbs at a ratio of 10g of medicinal powder/100 ml of solvent, with each solvent extracted 3 times. Ultrasound for 30 minutes each time, then combine the extract, evaporate and redissolve it in 5 mL of solvent to collect the first final extract. Repeat the above extraction process to collect the second final extract. Analyze the solutions, the 1st and 2nd final extraction of each solvent by high-performance liquid chormatography (HPLC), compare the results to select the solvent and number of extraction times to be able to extract the analytes most effectively. Survey results showed that the selected sample treatment conditions were 50% methanol and 1- time extraction.

3.4.2. Analytical procedure for quantifying pinoresinol 4'-O-β-D-glucopyranoside and vladinol F in Pandanus tonkinensis medicinal herb

Through preliminary investigation, the following conditions were used for analysis: HPLC using a diode array detector (DAD), C18 column (250 mm × 4.6 mm; 5µm); separation column temperature: 40^{o}C; mobile phase A (acetonitrile) and mobile phase B (0.1% acetic acid solution) with solvent gradient program: starting from mobile phase ratio A : B = 10 : 90 (v : v), increasing to 30 : 70 (v : v) for 30 minutes, increased to 90 : 10 (v : v) for the next 10 minutes, returned to the original 10 : 90 ratio for 10 minutes of column stabilization; mobile phase flow rate: 1.0 mL/min; detection wavelength at 228 nm.`

Experimental solutions were prepared as follows: i) *Stock standard solutions **PT20** and **PT6**:* prepared separately by accurately weighing about 5 mg of standard into a 5 mL volumetric flask, adding about 3 ml of 50%

methanol, ultrasonic shaking to dissolve, then adjusting the volume to just the right amount and shaking well; ii) *Mixture standard solution* : accurately pipet 1.0 ml of **PT20** stock standard solution and 1.0 mL of **PT6** stock standard solution into a 20 ml volumetric flask, add enough 50% methanol to the mark, shake well ; iii) *Test solution*: Accurately weigh about 5.0 g of medicinal herbs into a 100 mL conical flask, add exactly 50.0 mL of 50% methanol, sonicate for 30 minutes (extract 3 times), combine the resulting extract, then rotary evaporate until dry. Dissolve it in 5.0 mL of methanol to obtain the test solution.

Samples of the mixture standard solution and test solution were injected into the chromatography column with a volume of 10µl, the chromatogram was determined, and the retention time and area of the **PT20** and PT6 signals were determined. The concentrations of **PT20** and **PT6** in the solution injected into the chromatograph were determined according to the standard curve method. The content of **PT20**, **PT6** in medicinal herbs is calculated according to the concentration of **PT20**, **PT6** in the injection solution into the machine and the parameters of the sample processing process such as the volume of injected solution (5ml) and the amount of medicinal herbs (calculated in dry medicinal herbs) according to the formula:

$$C_{\textbf{PT20, PT6 in medicinal herb}}\ (\text{mg/g}) = \frac{C_{PT20,PT6\ injected\ solution}\ \left(\frac{mg}{ml}\right) x\ 5\ (ml)}{m_{medicine}\ (g)\ x\ (1-\text{m.moisture})}$$

3.4.3. Evaluation of the analytical procedure for quantifying pinoresinol 4'-O- β -D-glucopyranoside and vladinol F in Pandanus tonkinensis medicinal herb

The analytical procedure was validated according to the guidelines of the Association of Official Analytical Chemists (AOAC) and the International Conference on Harmonization of Procedures for Registration

of Pharmaceuticals for Human Use (ICH) with criteria: specificity, system suitability, linearity, precision (repeatability and reproducibility), precision, limit of detection and limit of quantification.

3.4.3.1. Specificity

Experiments show that on the chromatogram of the test solution, peaks with retention times of 20.230 minutes and 27.045 minutes appear, corresponding to peak **PT20** (20.247 minutes) and peak **PT6** (27.122 minutes) on the chromatogram of the mixture standard solution. The UV spectrum of the peak with a retention time of 20.230 minutes obtained on the chromatogram of the test solution corresponds to the UV spectrum of the **PT20** peak obtained on the chromatogram of the mixture standard solution with λ_{max} = 228.2 nm and 280.0 nm.

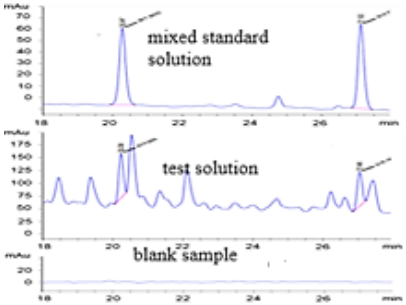


Figure 3.5 . Chromatogram of mixture standard solution, test solution and blank sample .

The UV spectrum of the peak with a retention time of 27.045 minutes obtained on the chromatogram of the test solution corresponds to the UV spectrum of the **PT6** peak obtained on the chromatogram of the mixed standard solution with λ_{max} = 280.0 nm. The peaks **PT20** and **PT6** on the chromatogram of the test solution and the mixed standard solution are pure. The blank sample (methanol solvent) does not affect the analysis results.

These results show that the test procedure meets the requirements for specificity.

3.4.3.2. System suitability

The results of evaluating the suitability of the system are presented in table 3.3. The relative standard deviation values of the retention time of peaks **PT20** and **PT6** when analyzing repeatedly (n=6) mixture standard solutions at a concentration level of 0.12% and 0.07%, respectively, met the requirements requirement ≤ 1.0%, the relative standard deviation value of peak area of **PT20** and **PT6** peaks are 1.31% and 1.35% respectively, meeting the requirement ≤ 2.0%. The similarity coefficients of **PT20** and **PT6** between two concentration levels analyzed in duplicate are RF = 1.01 and RF = 1.00. The chromatographic conditions chosen for repeatable results in terms of retention time and peak area using the high-performance liquid chromatography equipment system are suitable to ensure the stability of the analysis.

Table 3.3. Results of evaluating the suitability of the system

	PT20 peak retention time (minute)	Area of peak PT20 (mAU.s)	PT6 peak retention time (minute)	PT6 peak area (mAU.s)
Average (n=6)	20,293	878.87132	27,115	899.61777
RSD (%)	0.12	1.31	0.07	1.35
Similarity coefficient RF = 1.01 RF = 1.00				

3.4.3.3. Range of calibration curves

The evaluation of the calibration curves of two markers are presented in table 3.4 and figure 3.7. In the concentration range from 25.5 x 10^{-3} to 101.9 x 10^{-3} mg/mL for **PT20**, there is a linear dependence between peak area and **PT20** concentration with a linear correlation coefficient R^2 = 0.9983. In the concentration range from 26.0 x 10^{-3} to 103.9 x 10^{-3} mg/mL for **PT6**, there is a linear dependence between peak area and **PT6**

concentration with linear correlation coefficient $R^2 = 0.9974$. The calibration curves have good linearity for quantitative analysis of **PT20** and **PT6**.

Table 3.4. Calibration curves to quantify **PT20** and **PT6**

Marker	**PT20**		**PT6**	
	Concentration (mg/mL)	Peak area (mAU.s)	Concentration (mg/mL)	Peak area (mAU.s)
Calibration range range	25.5 x 10^{-3}	436.59933	26.0 x 10^{-3}	411.81412
	40.8 x 10^{-3}	708.55096	41.6 x 10^{-3}	749.87158
	51.0 x 10^{-3}	878.87132	51.9 x 10^{-3}	899.61777
	61.2 x 10^{-3}	1028.60651	62.3 x 10^{-3}	1071.64014
	76.4 x 10^{-3}	1345.65894	77.9 x 10^{-3}	1318,18884
	101.9 x 10^{-3}	1733.94653	103.9 x 10^{-3}	1799.81677
Regression equation	y = 17084x + 6.3385		y = 17376x - 11.091	
Correlation coefficients	$R^2 = 0.9983 > 0.99$		$R^2 = 0.9974 > 0.99$	
%Y	0.72% (< 2.0%)		1.23% (< 2.0%)	

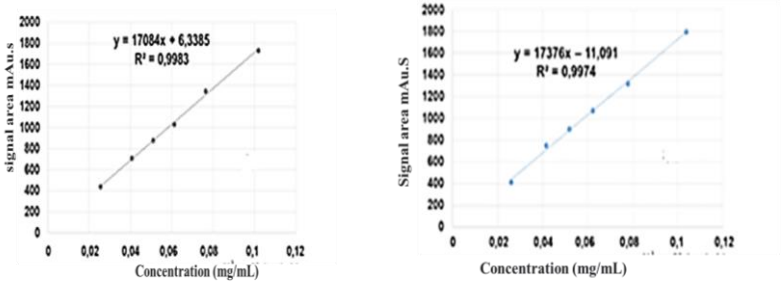


Figure 3.6 . Calibration curves for quantification of pinoresinol 4'-O-*β*-D-glucopyranoside (**PT20**) and vladinol F (**PT6**).

3.4.3.4. Accuracy

The results obtained when analyzing 6 independent test samples on 2 different days, with 2 different testers, were used to evaluate the precision (repeatability and reproducibility). Specific results in table 3.5 and table 3.6

show that the developed method has repeatability when the RSD (n = 6) for **PT20** and **PT6** are both less than 5.3% and the RSD (n = 12) is repeatable. For **PT20** and **PT6**, both are less than 8%, meeting the requirements according to AOAC guidelines with test samples containing quantity from 0.001% to 0.01%.

Table 3.5. Results of repeatability survey

Sample	Quantity weigh (g)	Peak area **PT20** _ (mAU.s)	Peak area **PT6** _ (mAU.s)	Content of **PT20** (mg/g)	Content of **PT6** (mg/g)
1	5.10042	674.06952	616.18457	0.0404	0.0367
2	5.32086	704.51483	643.13428	0.0404	0.0368
3	5.52441	724.00456	665,42011	0.0400	0.0366
4	5.31051	706.41534	628.91473	0.0406	0.0360
5	5.20041	700.22448	632.86042	0.0411	0.0370
6	5.18079	699.01453	631.08778	0.0412	0.0371
			Average	0.0405	0.0366
			RSD (%)	1.13	1.02

Table 3.6. Results of evaluating the reproducibility of the method

	Day 1- tester 1 (n = 6)	Day 2- tester 2 (n = 6)	Two days (n = 12)
Average content of PT20 (µg/g)	40.5	41.2	40.9
RSD (%)	1.13	1.43	1.54
Average content of PT6 (µg/g)	36.6	36.7	36.7
RSD (%)	1.02	1.67	1.32

3.4.3.5. Accuracy and working range

The results of evaluating the accuracy of the method are presented in table 3.7.

Table 3.7. Results of evaluating the accuracy of the method

Quantitative concentration level (% compared to test sample)	Average amount of added standard (µg)	Average amount of found standard (µg)	Average recovery efficiency (%)	RSD (%) (n = 3)
PT20: 50%	6.29	6.38	101	0.06
100%	12.58	12.66	101	0.82
150%	25.17	25.03	99	1.41
PT6: 50%	6.41	6.44	101	0.96
100%	12.82	13.01	101	0.55
150%	25.64	25.76	100	1.31

The average value of **PT20** recovery efficiency at all three addition levels ranges from 99% to 101% with relative standard deviation from 0.06% to 1.41%; With **PT6**, the average recovery efficiency at 3 concentration levels ranges from 101% to 102% and the relative standard deviation ranges from 0.55% to 1.31%. According to AOAC requirements, with samples with concentrations from 0.001% to 0.01%, the average sample recovery must reach from 90% to 107%, thus the analytical method ensures accuracy for quantifying **PT20** and **PT6**.

3.4.3.6 . Limit of detection and limit of quantification:

Gradually dilute the mixturre standard solution and inject it into the HPLC. At the concentration of 0.76 µg/ml PT20 and 0.78 µg/ml PT6, the obtained height PT20 and PT6 peaks were about 3 times of the baseline noise. Dilute the above solution twice, inject it into the HPLC, almost no peak response appears on the chromatogram. Thus, the instrument detection limit (IDL) with the standard solution is about 0.76 µg/ml **PT20** and 0.78 µg/ml **PT6**. According to calculations, the instrument quantification limit (IQL) of the standard solution will be about 3.3 times the instrumant

detection limit, corresponding to concentrations of 2.55 µg/ml **PT20** and 2.60 µg/ml **PT6**.

Prepare the mixture standard solutions ($IQL_{standard\ solution}$) and the spike solution based on medicinal extract ($IQL_{spike\ solution}$) with **PT20** and **PT6** concentrations of 2.55 µg/ml and 2.60 µg/ml, respectively. Chromatographic analysis of each solution was repeated 6 times. On the chromatogram of the $IQL_{solution}$ and IQL_{spike} the **PT20** and **PT6** peaks appear clearly. The RSD (n = 6) of the area of the **PT20** and **PT6** peaks when analyzing the IQL_{spike} is 2.87% and 2.31% is less than the 7.3% required by AOAC. Thus, it can be confirmed that the limits of quantification for **PT20** and **PT6** are 2.55 µg/ml and 2.60 µg/ml, respectively, for the test solution.
Using parameters of sample treatment process (5.0 g of medicinal material transferred to 5.0 ml of test solution), the method detection limit (MDL) is calculated as 0.76 µg/g **PT20** and 0.78 µg/g **PT6** in dried medicinal herbs; The method quantification limit (MQL) is 2.55 µg/g **PT20** dry medicinal herb and 2.60 µg/g **PT6** in dry medicinal herb.

Thus, the appraisal results in section 3.4.3 show that the process of simultaneously analyzing **PT20** and **PT6** in *P.tonkinensis* medicinal herbs using the HPLC method meets the requirements of specificity, system suitability, and linearity, precision, accuracy (repeatability and intermediate precision), suitable for applying simultaneous qualitative and quantitative testing of **PT20** and **PT6** in medicinal materials.

3.5. Quantification of pinoresinol 4'-O-*β*-D-glucopyranoside (PT20) and vladinol F (PT6) in *Pandanus tonkinensis* collected at some localities

Using the analytical procedure validated above, the average content of two markers in *Pandanus tonkinensis* species collected in Thanh Hoa, Hoa Binh, and Thai Nguyen was determined in the range of 25.0 ± 0.4 to 43,5 ± 0.4 µg/g dry herb with pinoresinol 4-O-*β*-D-glucopyranoside and 24.3 ± 0.5 to 37.1 ± 0.5 µg/g dry herb with vladinol F.

Table 3.8. Quantitative results of pinoresinol 4'-O-β-D-glucopyranoside (**PT20**) and vladinol F (**PT6**) in *Pandanus tonkinensis* collected in some localities

	Muong Lat - Thanh Hoa	Cam Thuy - Thanh Hoa	Luong Son - Hoa Binh	Dinh Hoa - Thai Nguyen
PT20: Content (µg/g)	40.9	43.5	25.0	38.6
RSD %	1.54	0.9	1.62	0.37
PT6: Content (µg/g)	36.7	37.1	24.3	30.7
RSD %	1.32	1.44	1.97	1.96

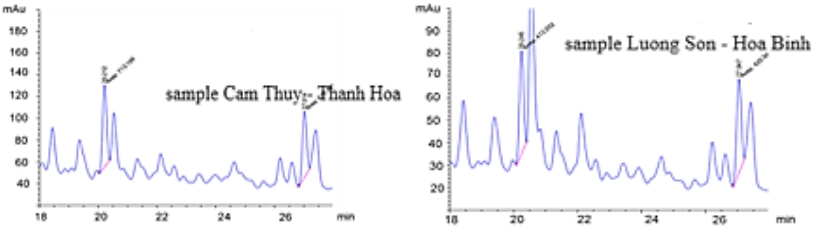


Figure 3.7 . Chromatogram analysis of pinoresinol 4'-O-*β*-D-glucopyranoside (**PT20**) and vladinol F (**PT6**) in *Pandanus tonkinensis*.

CONCLUSIONS

1. Research on chemical composition of *Pandanus tonkinensis*

Using a combination of chromatographic methods and modern spectroscopic methods, 28 compounds were isolated and determined from the fruits and roots of *Pandanus tonkinensis,* including 3 new compounds.

- From the fruit of *P.tonkinensis* 8 known compounds were isolate including: ficusal (**PT1**), syringaresinol (**PT2**), medioresinol (**PT3**), lariciresinol (**PT4**), secoisolariciresinol (**PT5**), vladinol F (**PT6**), luteoliflavan (**PT7**), isorhapontigenin (**PT8**).

- From the roots of *P.tonkinensis,* 20 compounds were isolated. There are 3 new compounds: (7*S*)-2,6-dimethoxyphenyl-7-propaniol-1-O-*β*-D-glucopyranoside (**PT10**); *Trans*-cinnamyl alcohol 9-O-(6′-O-*α*-L-arabinofuranosyl)- *β* -D-glucopyranoside (**PT25**); 4-(3-hydroxypropyl)-2,6 dimethoxyphenol-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (**PT26**) and 17 known compounds dihydrosyringin (**PT9**); (6*S*,9*S*)-roseoside (**PT11**); 1-O-*β*-Dglucopyranosyl-2-{2,6-dimethoxy-4-[1-(E)-propen-3-ol]phenoxyl}propan-3-ol(**PT12**); 1-O-(*β*-D-glucopyranosyl)-2-[2-methoxy-4-(*ω*-hydroxypropyl)-phenoxyl]-propan-3-ol (**PT13**); benzyl O-*α*-L-arabinopyranosyl-(1→6)-*β*-D-glucopyranoside (**PT14**); 1-O-(*β*-D-glucopyranosyl)-2-[2,6-dimethoxy-4-(*ω*-hydroxypropyl)-phenoxyl]-propan-3-ol (**PT15**); (7*R*,8*R*)-threo-4,7,9,9′-tetrahydroxy-3,3′-dimethoxyl-8-O-4′-neolignan-4-O-*β*-D-glucopyranoside (**PT16**); (7*S*,8*S*)-threo-4,7,9,9′-tetrahydroxy-3,3′-dimethoxyl-8-O-4′-neolignan-4-O- *β* -D -glucopyranoside (**PT17**); pinoresinol-4,4′-di-O-*β*-D-glucoside (**PT18**); isoeucommin A (**PT19**); pinoresinol 4'-O- *β* -D-glucopyranoside (**PT20**); acanthoside B (**PT21**); eucommin A (**PT22**); rourinoside (**PT23**); (7*S*,8*R*)-5-methoxydihydrodehydrodiconiferyl alcohol-4-O-*β*-D-glucopyranoside (**PT24**); kelampayoside A (**PT27**); urolignoside (**PT28**).

2. Research on biological activities of isolated compounds

Anti-inflammatory activity and antioxidant activity of 28 isolated compounds were evaluated by inhibition of NO production on RAW 264.7 cells, induction with LPS, and inhibition of membrane lipid peroxidation tests. The results show that:

- 22 compounds **PT1 - PT11**, **PT13**, **PT15 - PT17**, **PT20**, **PT22 - PT27** have anti-inflammatory activities, of which 14 compounds **PT1**, **PT4 - PT6**, **PT7**, **PT9**, **PT11**, **PT15**, **PT16**, **PT20**, **PT22**, **PT23**, **PT26**, **PT27** have IC_{50} values from 1.78 - 37.03 indicating that they possess better anti-inflammatory abilities than the positive control L-NMMA (IC_{50} 37.8) according to IC_{50} results.

- 10 compounds including **PT2**, **PT4 - PT8**, and **PT19 - PT22** showed antioxidant abilities, of which 5 substances **PT5**, **PT7**, **PT8**, **PT20**, and **PT21** have IC_{50} values ranging from 10.36 - 27.45 indicating that they possess better antioxidant abilities than the positive control trolox (IC_{50} 31.4).

3. Research on markers

- Two markers for *P. tonkinensis*, pinoresinol 4'-O-*β*-D-glucopyranoside and vladinol F were extracted and purified from this medicinal her with the purities higher than 98%.

- The analytical procedure for quantitative these two markers in *P. tonkinensis* using the HPLC-DAD method was developed. The validation results show that the analytical method is specific, with a linear standard curve range from 25.5 to 101.9 μg/ml (R^2 = 0.9983) and from 26.0 to 103.9 μg/ml (R^2 = 0.9974) for the two markers, with good precision (RSD < 2%), high precision (recovery efficiency between 99.4 and 101.5%), within quantification with pinoresinol 4'-O-*β*-D-glucopyranoside and vladinol F was 2.55 μg/g and 2.60 μg/g dry medicinal material, respectively.

RECOMMENDATIONS

The two extracted and purified compounds: pinoresinol 4'-O-*β*-D-glucopyranoside and vladinol F together with the validated analytical method can be used for the purpose of quality control of medicinal materials and research on production of hepaprotective products from *P.tonkinensis* medicinal herb.