that hydroxychavicol is the main ingredient in *Piper betle*, with a wide range of content ranging from 4.55-6.88% in *Piper betle* leaves and 0.57-1.68% in *Piper betle* branches. In addition, the hydroxychavicol content in *Piper betle* samples collected in winter is higher than in other seasons. The results obtained are intended to contribute to the development of standards for the *Piper betle*.

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PRELIMINARY RESEARCH ON ESTABLISHING THE QUALITY STANDARDS FOR ALCOHOL-PROCESSED *RADIX ACHYRANTHIS BIDENTATAE*

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Summary

Preliminary Research on Establishing the Quality Standards for Alcohol-Processed *Radix Achyranthis bidentatae*

In this study, a foundational standard for the herbal medicine *Achyranthes bidentata* Blume, commonly known as "Hoai nguu tat," processed with alcohol, is developed. The evaluation criteria encompass qualitative organoleptic assessment, moisture content, total ash content, and extractable substances in the herbal material, which collectively contribute to establishing a quality benchmark for *Achyranthes bidentata* products available in the market. The study results also provide recommendations for upgrading the monograph of *Radix Achyranthes bidentata* in the Vietnamese Pharmacopoeia. The quantitative analysis specifies that the content should be no less than 2% for oleanolic acid and 0.038% for β-ecdysterone, with a moisture content not exceeding 7%, total ash content not exceeding 8%, and extractable substances not less than 7%.

Keywords: *Oleanolic acid, Achyranthes bidentata* Blume, *β-ecdysterone.*

1. Introduction

Traditional medicine is currently experiencing significant growth and being favored due to its proven therapeutic efficacy. The processing methods of medicinal herbs and traditional medicines greatly influence their chemical compositions and therapeutic effects. Thus, applying standards meant for unprocessed herbs to traditionally processed medicines is not entirely appropriate. Consequently, standardization of traditional medicinal materials has been addressed in several national pharmacopoeias. However, in the Vietnamese Pharmacopoeia V, only specific processed herbal monographs like "Dang sam" and "Che thuc dia" are presented, while others are lacking. Therefore, standardizing the raw materials is the most fundamental step.

Achyranthes bidentata Blume, also known as "hoai nguu tat" in traditional medicine, is characterized by its neutral property and bitter-sour

taste, primarily affecting the liver and kidneys [1],[2],[3]. The dried root is used mainly as the medicinal part [2]. Current market products of *A.bidentata* have been studied for their anti-inflammatory properties, cholesterol reduction, blood pressure lowering, and analgesic effects [2],[3]. The therapeutic effects are enhanced when the herb is processed with alcohol [4],[5]. However, this processing can alter the compositions and standards of *A.bidentata* [6]. In Vietnam, research on the standards of alcohol-processed *Radix Achyranthis bidentatae* is limited, leading to insufficient quality in management and assurance. Hence, establishing standards for wine-processed *Radix Achyranthis bidentatae* is imperative.

Research Objective:

This study aims to establish foundational standards for alcohol-processed *Radix Achyranthis bidentatae*, following the standards of Vietnamese Pharmacopoeia V.

2. Materials and Methods

2.1. Materials, Chemicals, and Equipment

Materials:

The *Radix Achyranthis bidentatae* sample used in this study was supplied by Indochina Company Vietnam (DL) and satisfied the quality requirements according to the standards of the Vietnamese Pharmacopoeia V. The alcohol-processed sample was derived from the DL sample.

Processing Method: *Radix Achyranthis bidentatae* is cleaned, drained, and cut into slices 4-6 mm thick. Approximately 1 kg of *Radix Achyranthis bidentatae* is soaked with 150 mL of alcohol, then roasted over low heat until the alcohol aroma is released and the slices turn light brown. The slices are then cooled and packaged.

Radix Achyranthis bidentatae processed with alcohol was prepared at the Center of Science and Technology Applications on Pharmaceuticals - Institute of Medicinal Materials, according to the guidelines specified in Circular 30/2017/TT-BYT [4] on the methods for processing traditional medicines. The large roots of *A.bidentata* were sliced, while the smaller roots were cut into 3-5 cm segments, dried, and then ground into a coarse powder.

Chemicals and Reagents:

All solvents and chemicals used met the standards for analytical reagent grade (PA). The solvents used for chromatographic analysis (TLC, HPLC) were purchased from Merck (or equivalent). The reference substances included oleanolic acid, batch number CFN98800 with a purity of 98%, and *β*-ecdysterone (20-Hydroxyecdysone), batch number CFN98873 with a purity of 99%, supplied by Chemfaces, China.

2.2. Research Methods

The standard for alcohol-processed *Radix Achyranthis bidentatae* was developed based on the general criteria specified in the Vietnamese Pharmacopoeia V and National Standard TCVN 11776:2017 for processed herbal materials, including description, moisture content, total ash content, qualitative and quantitative analysis, and other relevant parameters.

- **Description**: Organoleptic evaluation of the shape, texture, color, smell, and taste of the herbal material.
- **Moisture Content**: Measured using the weighing method as outlined in Vietnamese Pharmacopoeia V, Appendix 9.6.
- **Total Ash Content**: Tested according to Vietnamese Pharmacopoeia V, Appendix 9.8.
- **Extractable Substances in the Herbal Material**: Conducted as per Vietnamese Pharmacopoeia V, Appendix 12.10, using the hot extraction method with *n*-butanol saturated with water.
- **Qualitative Analysis**

A. Chemical Reaction Requirements

Two grams of powdered herbal material were weighed, and 50 mL of 1% sodium chloride solution was added. The mixture was then gently boiled and filtered, and the filtrate was transferred into a test tube. It was shaken vigorously until stable foam appeared.

B. Thin-Layer Chromatography (TLC)

Method 1: Comparison with the reference substance, oleanolic acid [7]

- **Plate**: *Silica gel* G
- **Developing Solvent**: Chloroform - methanol /(40:1, v/v)
- **Sample Solution**: 2 g of powdered herbal material was added to 20 ml ethanol, refluxed for 40 minutes, and left to stand. From this solution, 10 mL was taken, 10 mL HCl was added and refluxed for 1 hour. The extract was concentrated to about 5 mLl, then 5 mL water was added and extracted with 20 mL chloroform. The chloroform extract was evaporated to dryness, and the residue was dissolved in 2 mL ethanol to obtain the test solution.
- **Reference Solution**: 0.1% ethanol solution of oleanolic acid.
- **Procedure**: 10 µL each of the reference solution and test solution were separately spotted onto the TLC plate. After development, the plate was air-dried and sprayed with 5%

phosphomolybdic acid in ethanol, then heated at 120°C for 5 minutes. The chromatogram was observed under normal light; the sample solution should show spots with the same color and R_f value as the oleanolic acid reference solution.

Method 2: Comparison with the reference substance, β-ecdysterone [8],[9]

- **Plate**: *Silica gel* G
- **Developing Solvent**: Dichloromethane: methanol (85:15, v/v)
- **Sample Solution**: 4 g of powdered herbal material was added to 50 mL of 80% methanol, refluxed for 3 hours, cooled, and filtered. The solvent was recovered and the filtrate concentrated to dryness. The residue was dissolved in 15 mL water and transferred to a column packed with D101 resin, sequentially eluted with 100 mL water, 100 mL 20% ethanol, and 100 mL 80% ethanol. The 80% ethanol fraction was evaporated to dryness, and the residue was dissolved in 1 mL of 80% methanol for TLC analysis.
- **Reference Solution**: β-ecdysterone standard dissolved separately in methanol to obtain two different solutions with concentrations of approximately 1 mg/mL.
- **Procedure**: 4 µL of each solution was spotted separately on the TLC plate. After development, the plate was air-dried, sprayed with 5% vanillin in sulfuric acid, and heated at 105°C until spots were clearly visible. The chromatogram was observed under normal light; the sample solution should show spots with the same color and R_f value as the β-ecdysterone reference solution.

• **Quantitative Analysis**:

High-Performance Liquid Chromatography (HPLC) for Oleanolic Acid [9]:

- **HPLC System and Conditions**: The HPLC system (Shimadzu, Japan) included an LC-20AD pump, SIL-20AHT auto-sampler, UV-VIS detector, and Labsolution software for data recording. Excel software was used for result calculation.
- Analysis conditions: Shimpack C_{18} column (250 mm x 4.6 mm; 5 µm), UV-VIS detector (wavelength 210 nm), mobile phase acetonitrile - formic acid 0.1% (20:80, v/v) in 20 minutes, isocratic elution mode, flow rate 1.0 mL/min, injection volume 10 µL.
- **Standard Solution**: Oleanolic acid standard dissolved in methanol to obtain solutions with concentrations of 300, 200, 100, 50, 25, 10, and 5 µg/mL.
- **Sample Solution**: Precisely weighed 1 g of powdered herbal material was Soxhlet extracted three times, each with 50 mL of ethanol at 50°C for 1 hour. The extract was concentrated and adjusted to 100 mL with ethanol at 50°C. 5 mL of the filtrate was taken, 2.5 mL 10% HCl was added, and hydrolyzed for 2 hours at 90°C. After cooling, the precipitate was filtered, dissolved in MeOH, and adjusted to 10 mL. This solution was filtered through a 0.45 µm membrane and analyzed by HPLC.

High-Performance Liquid Chromatography (HPLC) for β-ecdysterone [10]:

- **HPLC System and Conditions**: The HPLC system (Shimadzu, Japan) included an LC-20AD pump, SIL-20AHT auto-sampler, UV-VIS detector, and Labsolution software for data recording. Excel software was used for result calculation.
- Analysis conditions: Shimpack C_{18} column (250 mm x 4.6 mm; 5 µm), UV-VIS detector (wavelength 321 nm), mobile phase acetonitrile - formic acid 0.1% (15:85, v/v) in 35 minutes, isocratic elution mode, flow rate 1.0 mL/min, injection volume 10 µL.
- **Standard Solution**: β-ecdysterone standard dissolved in methanol to obtain solutions with concentrations of 300, 200, 100, 50, 25, and 10 µg/mL.
- **Sample Solution**: Precisely weighed 1 g of powdered herbal material was placed in a capped container, added with 30 mL of butanol saturated with water, and soaked overnight. The mixture was sonicated for 30 minutes (300 W, 40 kHz), filtered, and the container rinsed with 10 mL MeOH. The filtrate and washings were combined and evaporated to dryness. The residue was dissolved in MeOH, transferred to a 5 mL volumetric flask, and adjusted to volume with MeOH.
- **Formula for calculating content (X%):**

$$X(\%) = \frac{Ct \times V \times 100 \times 100 \times P}{m \times 1000 \times 100 \times (100 - a)} = \frac{Ct \times V \times P}{m \times 10 \times (100 - a)}$$

where Ct: The concentration of analyte in sample solution (µg/mL);

V: The volume of the test solution (mL).

m: The mass of medicinal materials (mg)

a: The sample moisture (%);

P: The purity of standard (%).

3. Results and Discussion

3.1. Description of alcohol-processed Radix Achyranthis bidentatae

Fig. 1. Alcohol-processed *Radix Achyranthis bidentatae*

Long cross-section or cylindrical slice, 3-5 cm in length, with a hard texture that becomes pliable when moistened. It has a light brown or yellowish-gray exterior. The outer surface is yellow-brown with many small vertical wrinkles. The medicinal substance has a mild fragrance, a slightly bitter-sweet taste, and a little acrid.

3.2. Moisture and ash content

The results of the moisture content and total ash content of the alcohol-processed *Radix Achyranthis bidentatae* are presented in the following table:

Table 1. Moisture and ash content in alcohol-processed *Radix Achyranthis bidentatae* samples

No.	Sample Name	Moisture (%)	Total Ash (%)
1	VT_NT_1	5.92 ± 0.03	5.79 ± 0.11
2	VT_NT_2	6.01 ± 0.06	5.25 ± 0.07
3	VT_NT_3	5.77 ± 0.10	5.81 ± 0.14
Mean		5.90	5.62

(The number of samples tested in replicate, n=3).

Proposed Standards: Moisture not exceeding 10 %, total ash not exceeding 8 %.

3.3. Extractable Substances

The results of the extractable substances of the alcohol-processed *Radix Achyranthis bidentatae* are presented in the following table:

Table 2. Percentage of extractable substances in alcohol-processed *Radix Achyranthis bidentatae* samples

No.	Sample Name	Moisture (%)	Extractable Substances (%)	Mean (%)
1	VT_NT_1	5.92	7.429 ± 0.012	7.37
2	VT_NT_2	6.01	7.171 ± 0.025	
3	VT_NT_3	5.77	7.523 ± 0.107	

(The number of samples tested in replicate, n=3).

Proposed Standards: Extractable substances not less than 4%.

3.4. Qualitative analysis

A. Chemical reaction requirements

Result: The qualitative test for saponins showed the presence of foam, which remained stable for 10 minutes after shaking.

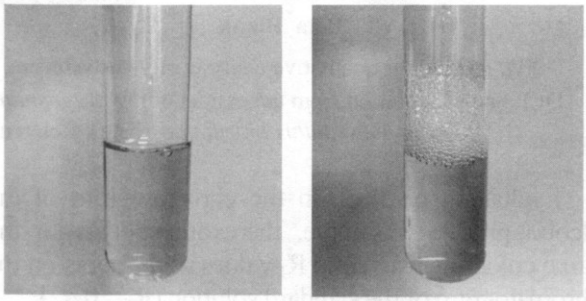

Fig. 2. Foam formation reaction

B. Chromatographic method

Qualitative analysis for oleanolic acid

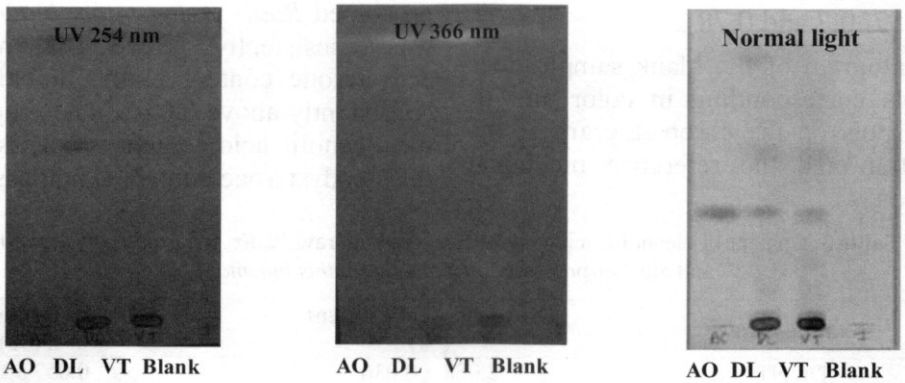

Fig. 3. TLC for qualitative analysis of oleanolic acid in raw and alcohol-processed *Radix Achyranthis bidentatae* (DL): Sample solution from the extract of raw *A.bidentata*, (VT): Sample solution from the extract of alcohol-processed *Radix Achyranthis bidentatae*, (AO): Reference standard solution (oleanolic acid).

Evaluation results: On the chromatogram of the alcohol-processed sample, there are spots with the same color, intensity, and R_f values as the spots on the chromatogram of the standard solution ($R_f = 0.62$).

On the chromatogram of the alcohol-processed sample, there are spots corresponding in color and R_f values to the spots on the chromatogram of the reference medicinal material solution ($R_f = 0.41$; 0.55; and 0.62).

The chromatogram of the blank sample does not show spots corresponding in color and R_f values to the spots on the chromatogram of the standard solution and the reference medicinal material solution.

Qualitative analysis for *β*-ecdysterone

Result: When observed under UV light at 254 nm, both the standard solution and the sample solutions exhibited spots. After spraying the plate with vanillin-sulfuric reagent and heating, the sample solutions showed spots with the same color and an R_f value of 0.42 as the standard solution.

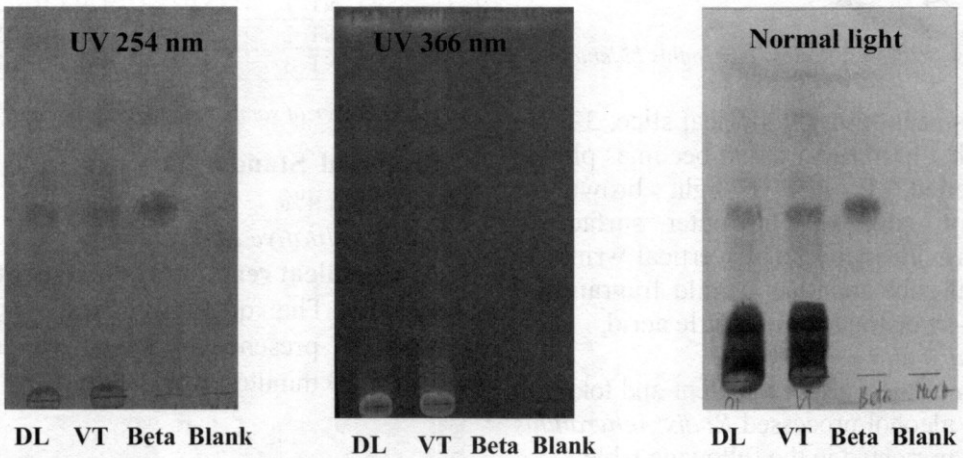

Fig. 4. TLC for qualitative analysis of ***β*-ecdysterone** in raw and alcohol-processed *Radix Achyranthis bidentatae* (DL): Sample solution from the extract of raw *A.bidentata*, (VT): Sample solution from the extract of alcohol-processed *Radix Achyranthis bidentatae*, (beta): Reference standard solution (*β*-ecdysterone), Blank: MeOH

Evaluation results: On the chromatogram of the alcohol-processed sample, there are spots with the same color, intensity, and R_f values as the spots on the chromatogram of the standard solution ($R_f = 0.42$).

On the chromatogram of the alcohol-processed sample, there are spots corresponding in color and Rf values to the spots on the chromatogram of the reference medicinal material solution (R_f = 0.14; 0.42; 0.0.5; 0.57; 0.7 and 0.78).

The chromatogram of the blank sample does not show spots corresponding in color and R_f values to the spots on the chromatogram of the standard solution and the reference medicinal material solution.

3.5. Content of oleanolic acid and β-ecdysterone in raw Radix Achyranthis bidentatae and alcohol-processed Radix Achyranthis bidentatae

The quantitative analysis of oleanolic acid and *β*-ecdysterone showed that the HPLC chromatograms of the test samples had peaks with the same retention times as the standard samples. The content of oleanolic acid in alcohol-processed *Radix Achyranthis bidentatae* samples was consistently above 2%, while the *β*-ecdysterone content in the herbal samples was consistently above 0.03%. The proposed standard for oleanolic acid content is not less than 2% and for *β*-ecdysterone content is not less than 0.03%.

Table 3. Content of oleanolic acid and *β*-ecdysterone in raw *Radix Achyranthes bidentata* and alcohol-processed *Radix Achyranthis bidentatae*

No.	Sample Name	Oleanolic Acid Content (%)	*β*-ecdysterone Content (%)
1	DL NT	2.058 ± 0.036	0.057 ± 0.018
2	VT_NT_1	2.113 ± 0.025	0.048 ± 0.026
3	VT_NT_2	2.103 ± 0.031	0.051 ± 0.027
4	VT_NT_3	2.075 ± 0.033	0.047 ± 0.032

(The number of samples tested in replicate, n3).

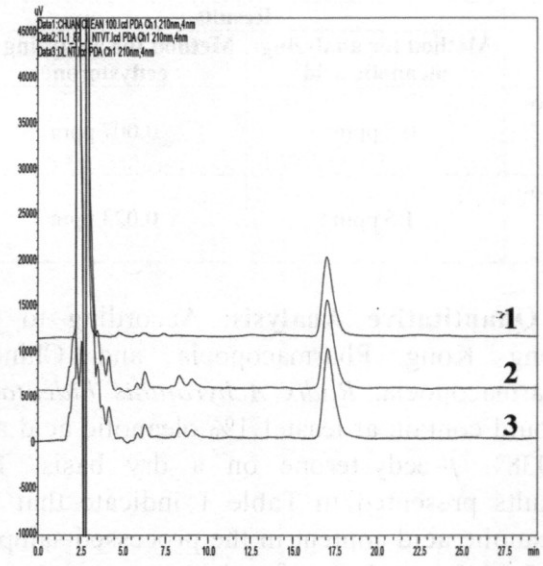

Fig. 4. HPLC chromatogram for quantitative analysis of oleanolic acid in *Radix Achyranthis bidentatae* samples (1: raw *Radix Achyranthes bidentata*, 2: Alcohol-processed *Radix Achyranthis bidentatae*, 3: Oleanolic acid standard)

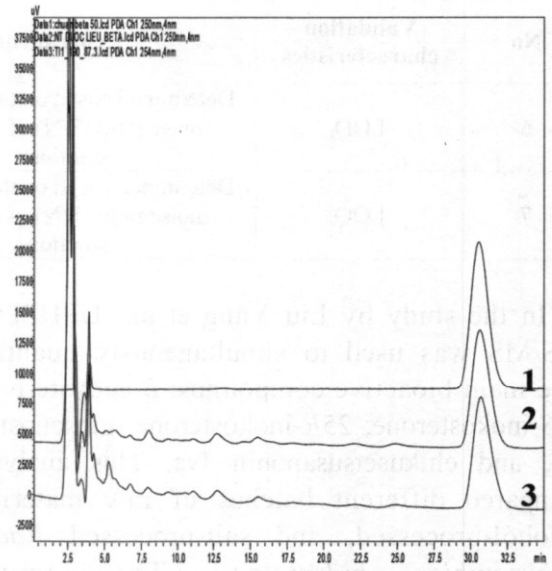

Fig. 5. HPLC chromatogram for quantitative analysis of *β*-ecdysterone in *Radix Achyranthis bidentatae* samples (1: *β*-ecdysterone standard, 2: raw *Radix Achyranthes bidentata*, 3: alcohol-processed *Radix Achyranthis bidentatae*)

The validation of the analytical method was conducted according to ICH guidelines and AOAC [11],[12]. The results obtained are presented in Table 4.

Table 4. The result of the validation of the analytical method for oleanolic acid and *β*-ecdysterone in alcohol-processed *Radix Achyranthis bidentatae*

No	Validation characteristics	Experiment	Results	
			Method for analyzing oleanolic acid	**Method for analyzing β-ecdysterone**
1	System Suitability	Repeat analysis of the standard sample 6 times on the HPLC system	RSD (t_R) = 0.52% RSD (S) = 1.10%	RSD (t_R) = 0.46% RSD (S) = 1.26%
2	Selectivity (Specificity)	Analysis of blank sample (Methanol), standard sample, and test sample	High specificity t_R (AO) = 17.5	High specificity t_R (ES) = 31.2
3	Linearity	Analysis of standard solutions of different concentrations	Linear range: 5- 300 µg/mL Calibration curve: y =3975.7x - 4308.7 R^2=0.999; x is the concentration of AO (µg/mL); y is the peak area (mAu)	Linear range: 10- 300 µg/mL Calibration curve: Y=22342.X - 33598 R^2=0,999; x is the concentration of *β*-ecdysterone (µg/mL); y is the peak area (mAu)
4	Precision	Repeat analysis of a test sample within a day and on different days (6 times/day), The concentrations of oleanolic acid and *β*-ecdysterone in standard solution were 108.352, and 42.389 µg/mL, respectively.	RSD (Content % AO) = 0.45%	RSD (Content % ES) = 2.54%
5	Accuracy	Standard addition method (The accuracy was evaluated by adding standards to the sample at 3 levels 80%, 100%, and 120%).The concentrations of oleanoic acid and *β*-ecdysterone in the alcohol-processed *Radix Achyranthis bidentatae sample* were 108.575 and 42.951 µg/mL, respectively.	Recovery rate (%) = 101.1%	Recovery rate (%) = 99.8 %

No	Validation characteristics	Experiment	Results	
			Method for analyzing oleanolic acid	Method for analyzing β-ecdysterone
6	LOD	Determined based on the signal-to-noise ratio (S/N) of standard solutions.	0.5 ppm	0.007 ppm
7	LOQ	Determined based on the signal-to-noise ratio (S/N) of standard solutions.	1.5 ppm	0.023 ppm

In the study by Liu Yang et al., UPHPLC-MS/MS was used to simultaneously quantify five main bioactive compounds: *β*-ecdysterone, 25*S*-inokosterone, 25*R*-inokosterone, ginsenoside Ro, and chikusetsusaponin Iva. This analysis compared different batches of raw material, alcohol-processed, and salt-processed *Radix Achyranthis bidentatae*. The results demonstrated significant differences in the active ingredient content between materials processed with alcohol and salt [13]. Another study indicated that alcohol processing reduces the phytoecdysteroid content, whereas salt processing and sulfur fumigation do not alter the phytoecdysteroid content [13].

4. Discussion

Currently, the standardization of traditional medicinal herbs has received attention and has been addressed in the pharmacopeias of several countries worldwide. Among the 103 herbs regulated by Circular 30/2017/TT-BYT, the Vietnamese Pharmacopoeia V currently specifies monographs for only three processed herbs: Thuc dia, Dang sam and Ha thu o do. Therefore, establishing foundational standards for processed herbs is essential.

Description: The alcohol-processed *Radix Achyranthis bidentatae* has a light brown color and becomes pliable when moistened. It has a mild aromatic odor, initially a sweet taste, followed by a slightly bitter and astringent aftertaste. In contrast, the raw *Radix Achyranthis bidentatae* is cylindrical, 20 to 30 cm long, with a diameter of 0.5 to 1.0 cm. The upper end bears traces of the stem base, while the lower end tapers. The outer surface is yellow-brown with many small vertical wrinkles and traces of fine roots.

Qualitative Analysis: The results are consistent with the qualitative reactions described for *Radix Achyranthis bidentatae* in the Vietnamese Pharmacopoeia V.

Quantitative Analysis: According to the Hong Kong Pharmacopoeia and Chinese Pharmacopoeia, *Radix Achyranthis bidentatae* should contain at least 1.1% oleanolic acid and 0.038% *β*-ecdysterone on a dry basis. The results presented in Table 1 indicate that the oleanolic acid content in the processed samples is 2.074%, and the *β*-ecdysterone content is 0.050%. Nguyen Thi Phuong et al. (2016) reported that the total oleanolic acid content in samples of *Achyranthis bidentatae* grown in Vietnam ranged from 0.78% to 2.02%, while the *β*-ecdysterone content ranged from 0.008% to 0.029% [15]. Therefore, we propose that the quantitative standards for alcohol-processed *Radix Achyranthis bidentatae* should be no less than 2% for oleanolic acid and 0.038% for *β*-ecdysterone.

Moisture Content: The Vietnamese Pharmacopoeia V requires that the moisture content of *Radix Achyranthis bidentatae* should not exceed 15%. The moisture content in the processed samples was determined to be 5.95%. The results are consistent with those determined by Pushpendra Kumar Shukla and colleagues, where the moisture content of *Radix Achyranthis bidentatae* samples was 5% [16]. Hence, we propose that the moisture content for alcohol-processed *Radix Achyranthis bidentatae* should not exceed 10%.

Total Ash Content: According to the Vietnamese Pharmacopoeia V, The total ash content in the raw herbal material of *Radix Achyranthis bidentatae* was found to be 9%, while in the processed samples, it was 5.97%. The results are consistent with those determined by Wang and colleagues, where the total ash content of *Radix Achyranthis bidentatae* samples ranged from 5.04% to 6.43% [17]. Therefore, we propose that the total ash content should not exceed 8%.

Extractable Substances: The Vietnamese Pharmacopoeia V requires that the extractable substances in herbal material should not be less than 6%. The extractable content in the raw herbal material was 6.844%, and in the processed samples, it was 7.351%. Wang et al. reported that the extractable substance content of ethanol varies from 6.57% to 11.12% [16]. It is evident that substances extracted with butanol are more selective compared to those extracted with ethanol. Thus, we propose that the standard for extractable substances in alcohol-processed *Radix Achyranthis bidentatae* should not be less than 4 %.

5. Conclusion

The quality standards for alcohol-processed *Radix Achyranthis bidentatae* (include descriptive criteria, qualitative analysis by the foam test, thin-layer chromatography (TLC) for oleanolic acid with a solvent system of chloroform - methanol (40:1, v/v), and β-ecdysterone with a solvent system of Dichloromethane : Methanol (85:15, v/v). The quantitative analysis specifies that the content should be no less than 2% for oleanolic acid and 0.038% for β-ecdysterone, with a moisture content not exceeding 7%, total ash content not exceeding 8%, and extractable substances not less than 7%. Additionally, we propose to quantify other significant markers to further differentiate between the raw herbal material and the alcohol-processed *Radix Achyranthis bidentatae*, providing a more accurate basis for establishing comprehensive standards. The preliminary results of this study aid in evaluating the quality of processed herbal materials and lay the groundwork for further research about the chemical composition and pharmacological effects of processed herbal medicine.

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