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A QUANTITATIVE METHOD FOR DETERMINING CALYCOSIN-7-*O*-*β*-D-GLUCOSIDE AND FREE ASTRAGALOSIDE IV IN RAW AND HONEY-PROCESSED *ASTRAGALI RADIX* BY HPLC-DAD

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Summary

A Quantitative Method for Determining Calycosin-7-*O*-*β*-D-Glucoside and Free Astragaloside IV in Raw and Honey-Processed *Astragali radix* by HPLC-DAD

An HPLC-DAD method was developed to quantify calycosin-7-*O*-*β*-D-glucoside and free astragaloside IV in raw and honey-processed *Astragali Radix* samples. The HPLC analysis was performed on an Agilent C_{18} column (4.6 × 250 mm, 5 µm particle size), with a mobile phase of acetonitrile-0.2% formic acid, the flow rate of 1.0 mL/min, and detection wavelengths of 260 nm for calycosin-7-*O*-*β*-D-glucoside and 203 nm for astragaloside IV. All calibration curves displayed a good linear relationship (R^2=0.999). The method was fully validated concerning the specificity, linearity, accuracy, and recovery according to the ICH guideline. The raw *Astragali Radix* samples had varying levels of calycosin-7-*O*-*β*-D-glucoside and free astragaloside IV, ranging from 0.016 - 0.032 % and 0.016-0.066 %, respectively. In the honey-processed *Astragali Radix* samples, the contents of free astragaloside IV and calycosin-7-*O*-*β*-D-glucoside were 0.018 - 0.052 % and 0.010 - 0.022 %, respectively.

Keywords: *Astragali Radix, HPLC-DAD, Calycosin-7-O-β-D-glucoside, Free astragaloside IV.*

1. Introduction

The dried root of *Astragalus membranaceous* (Fisch.) Bge. var. *mongolicus* (Bge.) Hsiao. is known as *Radix Astragali.* In Chinese traditional medicine, this plant has been utilized as a tonic, analgesic, and antisudorific [1],[2]. It has been shown to have well-researched and reviewed hepatoprotective, antioxidative, antiviral, antihypertensive, and immunostimulant properties [1],[2],[3]. Additionally, honey-processed *Astragali Radix* is a product of *Astragali Radix* mixed with honey by a traditional Chinese medicine processing method, which strengthens the tonic effect. Phytochemical studies have shown that *Astragali Radix* contains various active compounds, including isoflavonoids, saponins, and polysaccharides [1],[2],[3]. Two components (calycosin-7-*O*-*β*-D-glucoside and astragaloside IV) have been regarded as the main active constituents and were

markers for quality control of *Astragali Radix* in Chinese Pharmacopoeia 2020, Hong Kong Chinese Materia Medica Standards and Vietnamese Pharmacopoeia V [2],[4],[5]. In Chinese Pharmacopoeia 2020 and Vietnamese Pharmacopoeia V, monographs of *Astragali Radix* used HPLC-UV for the quantification of calycosin-*7-O-β*-D-glucoside and HPLC-ELSD for the quantification of the total content of astragaloside IV because of the weak chromophoric functionality of astragaloside IV in the 196-208 nm region [6],[7]. These pharmacopeias suggest using an ammonia solution during sample preparation to increase the level of astragaloside IV in the extract because hydrolysis of other astragalosides into astragaloside IV, and we might not determine the concentration of free astragaloside IV in *Astragali Radix* samples. In this study, we developed an HPLC-DAD method for the quantification of calycosin-*7-O-β*-D-glucoside and free astragaloside IV in *Astragali Radix*. The developed method was validated according to the corresponding official documents (ICH) and applied to evaluate the content of these compounds in ten samples of raw and four samples of honey-processed *Astragali Radix*.

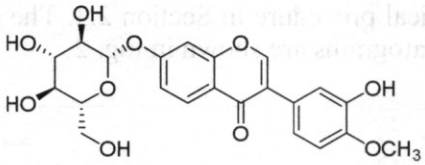
2. Experimental

2.1. Materials

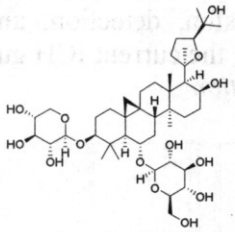
The *Astragali Radix* sample used in this study was supplied by the Indochina Company (Vietnam) (**HK1**). One sample of honey-processed *Astragali Radix* (**HKCM1**) was processed from the HK1 sample. Nine samples of *Astragali Radix* (**HK2 → HK10**) and three samples of honey-processed *Astragali Radix* (**HKCM2 → HKCM4**) were purchased at different stores on Lan Ong Street, Hanoi.

2.2. Chemicals

Calycosin-*7-O-β*-D-glucoside (CAS: 20633-67-4, Lot CFS202202, ≥98%) and astragaloside IV (CAS: 84687-43-4, Lot CFS202301, ≥98%) were purchased from ChemFaces, China (Fig. 1). Acetonitrile and formic acid (HPLC grade) were purchased from Merck (Germany). All other solvents were of analytical grade.



calycosin-*7-O-β*-D-glucoside



astragaloside IV

Fig.1. The structures of calycosin-*7-O-β*-D-glucoside and astragaloside IV

Standard solution calycosin-7-O-β-D-glucoside: weigh accurately 10 mg of astragaloside IV and dissolve in 10 mL of methanol to produce a stock standard solution calycosin-*7-O-β*-D-glucoside 1.0 mg/mL. Measure accurately the volume of calycosin-*7-O-β*-D-glucoside Std-Stock solution, dilute with methanol to produce a series of solutions of 0.00625, 0.0125, 0.025, 0.0625, 0.125 and 0.25 mg/mL for calycosin-*7-O-β*-D-glucoside.

Standard solution astragaloside IV: weigh accurately 12.5 mg of astragaloside IV and dissolve in 5 mL of methanol to produce a stock standard solution astragaloside IV 2.5 mg/mL. Measure accurately the volume of astragaloside IV Std-Stock solution, dilute with methanol to produce a series of solutions of 0.125, 0.25, 0.625, 1.25, 2.5 mg/mL for astragaloside IV.

2.3. HPLC-DAD method for determination of calycosin-7-O-β-D-glucoside and free astragaloside IV in raw and honey-processed Astragali Radix samples

Sample preparation: weigh accurately 1.0 g of the powdered sample to a stoppered flask, accurately add 50 mL of methanol, and weigh. Heat under reflux for 4 hours, cool, and weigh again, replenish the loss of solvent with methanol, mix well, and filter. Measure accurately 25 mL of the successive filtrate and evaporate to dryness. Dissolve the residue in methanol and transfer it to a 5 mL volumetric flask, dilute with methanol to volume, mix well, filter, and use the successive filtrate as the test solution [4].

HPLC-DAD conditions: The HPLC system (Shimadzu, Japan) used for the analysis consisted of an LC-30AD pump, SIL-30AC auto-sampler, CTO20AC column oven, and SPD-M20A Photodiode Array Detector (Nexera X2, Shimazu, Kyoto, Japan) with Mass spectrometry

8045 Detector. Quantitative estimation was performed with the LabSolutions software program. The separation of compounds was performed on an Agilent C18 column (4.6 × 250 mm, 5 µm particle size). The mobile phase was a mixture of acetonitrile (A) and 0.2 % formic acid (B) using a gradient elution of 20 - 40%A at 0 - 20 min, 40%A at 20 - 25 min, 40-95%A at 25 - 30 min, 95%A at 30 - 35 min, 95-20%A at 35 - 37 min, 20%A at 37-45 min. Detector DAD was set at a wavelength of 260 nm for calycosin-*7-O-β*-D-glucoside and 203 nm for astragaloside IV [4],[6],[7]. The run time was 45 min. The injection volume was 10 µL, and the flow rate was 1.0 ml/min.

This study used the HPLC-DAD-MS system and compared the MS spectra of the substance peaks to evaluate the specificity. The mass range was set at *m/z* of 100-1200 in both positive (ESI^+) and negative (ESI^-) ionization modes and the centroid mode was utilized. Nitrogen was used as cone gas and argon was kept as collision gas.

2.4. Method validation

The analytical method was validated for system suitability, specificity, calibration curve, accuracy, precision, detection, and quantitation limits following the current ICH guidelines [8].

2.5. Calculation

Calculate the content of calycosin-*7-O-β*-D-glucoside and free astragaloside IV in *Astragali Radix* and honey-processed *Astragali Radix* samples taken:

$$X(\%) = \frac{C_t \times V \times 100}{m \times (100 - a)} \times 100$$

X: the content of calycosin-*7-O-β*-D-glucoside and free astragaloside IV in samples (%);

C_t: the concentration of analyzed compounds in the sample solution from the calibration curve equation (mg/mL);

V: volume of the sample solution (mL);

m: weight of sample taken to prepare the sample solution (g);

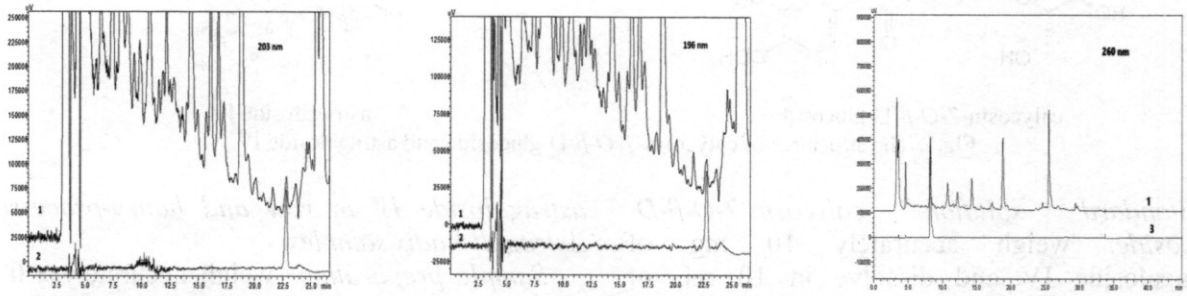
P: the purity of standards (P=0.98 for calycosin-*7-O-β*-D-glucoside and astragaloside IV);

a: the moisture of sample (%).

3. Results and Discussion

3.1. Analysis of free astragaloside IV and calycosin-7-O-β-D-glucoside in Radix Astragali by HPLC-DAD

The experiment was performed with the astragaloside IV standard, calycosin-*7-O-β*-D-glucoside, and sample solutions according to the analytical procedure in Section 2.3. The obtained chromatograms are shown in Fig. 2.



A-203 nm B-196 nm C-260 nm

Fig.2. HPLC-DAD chromatograms for analysis of free astragaloside IV and calycosin-*7-O-β*-D-glucoside in the *Radix Astragali* sample
(1- *Astragali Radix* sample; 2- astragaloside IV 1.25 mg/mL; 3- calycosin-*7-O-β*-D-glucoside 0.125 mg/mL)

The obtained results showed that astragaloside IV and calycosin-*7-O-β*-D-glucoside in the *Astragali Radix* sample might be analyzed using the HPLC-DAD method. For HPLC-DAD, the peaks of astragaloside IV and calycosin-*7-O-β*-D-glucoside appeared at 22.7 min and 8.20 min, respectively. The peak signal of astragaloside IV observed at 203 nm was sharper than that observed at 196 nm. To quantify the two compounds free astragaloside IV and calycosin-*7-O-β*-D-glucoside in the *Astragali Radix* sample, the HPLC-DAD method (at 203 nm for astragaloside IV and 260 nm for calycosin-*7-O-β*-D-glucoside) was selected in this study.

3.2. Validation method

3.2.1. Specificity:

The experiment was performed with the standard solutions and sample solutions according to the analytical procedure (section 2.3). The obtained chromatograms are shown in Fig. 3.

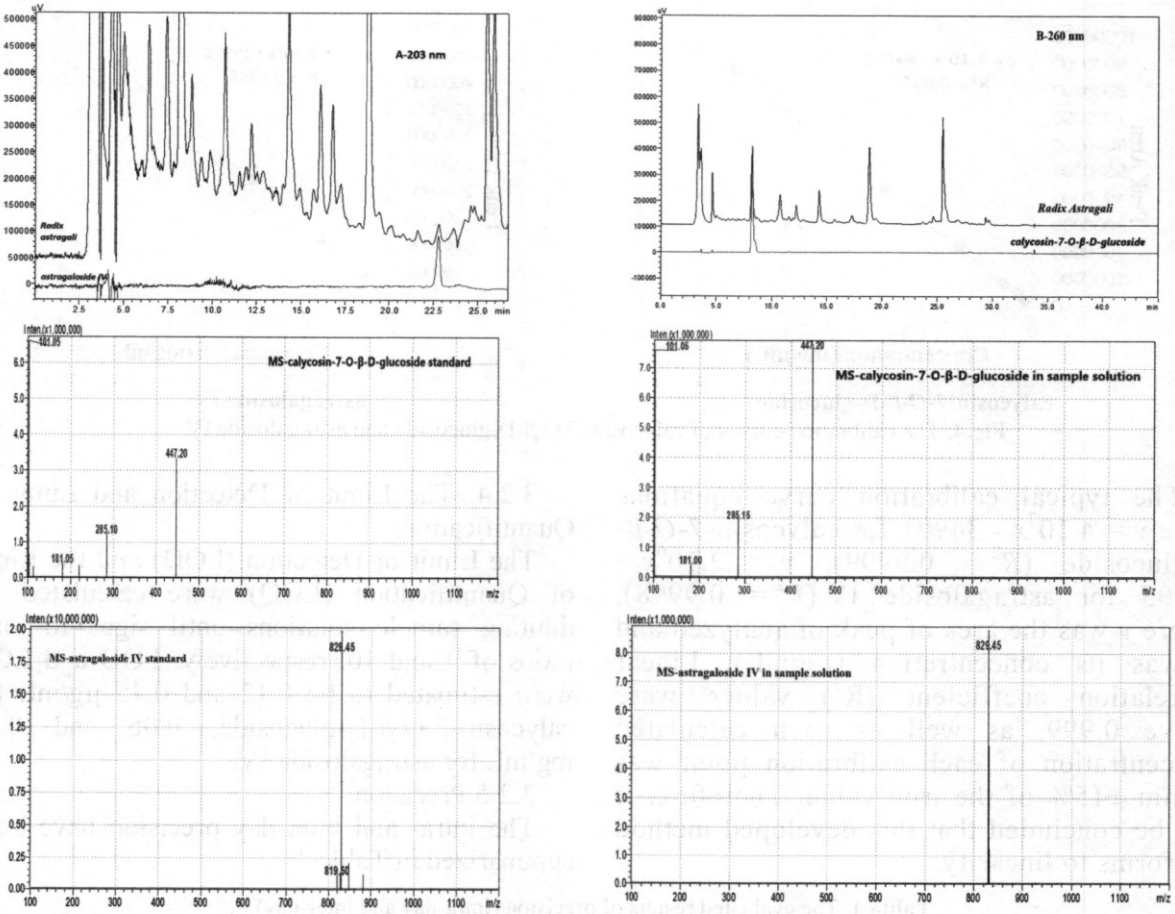


Fig.3. Chromatograms for specificity study

Table 2. Peak purity of two analyzed compounds by comparing UV spectrums

Compound	calycosin-*7-O-β*-D-glucoside	astragaloside IV
Matching ratio	0.9999	0.9987

The retention times of calycosin-*7-O-β*-D-glucoside and astragaloside IV were 8.20 min and 22.77 min, respectively. The obtained chromatograms showed clear separation peaks and low background noise. Additionally, the MS spectra of the peaks of calycosin-*7-O-β*-D-glucoside and astragaloside IV in the test and standard solutions were similar. The matching ratios of UV spectrums were above 0.99 (Table 2). These demonstrated the specificity of this method.

3.2.2. System suitability:

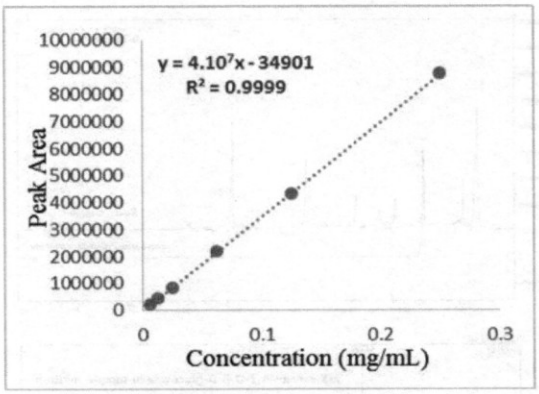
It can be observed from Table 3 that the RSD% of retention time, and peak area were all under 3%. These indicate that the developed method conforms to system suitability criteria [8].

Table 3. System suitability testing (n = 6)

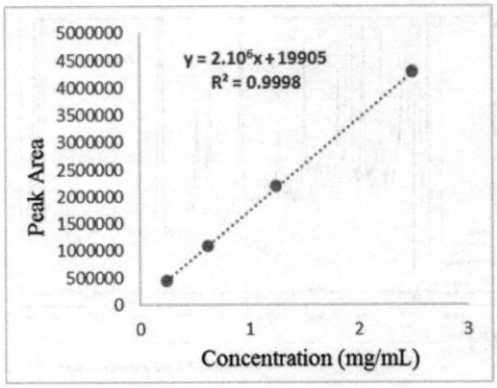
Parameter	**calycosin-*7-O-β*-D-glucoside 0.125 mg/mL**		**astragaloside IV 1.25 mg/mL**	
	Mean	RSD(%)	Mean	RSD(%)
Retention time, t_R (min)	8.20	0.34	22.77	0.04
Peak area (mAu.s)	4380784	1.29	222811	2.91

3.2.3. Calibration curve:

Different concentrations of calycosin-*7-O-β*-D-glucoside and astragaloside IV standard solutions were analyzed to validate the linearity criteria. The result showed satisfactory linearity in the concentration ranges of 0.00625 - 0.25 mg/mL for calycosin-*7-O-β*-D-glucoside and 0.25 - 2.5 mg/mL for astragaloside IV (Fig. 4).



calycosin-7-*O*-*β*-D-glucoside



astragaloside IV

Fig.4. The calibration curves of calycosin-7-*O*-*β*-D-glucoside and astragaloside IV

The typical calibration curve equations were $y = 4.10^7x - 34901$ for calycosin-7-*O*-*β*-D-glucoside ($R^2 = 0.9999$), $y = 2.10^6x + 19905$ for astragaloside IV ($R^2 = 0.9998$), where y was the area of peak of analyzed and x was its concentration (μg/mL). Linear correlation coefficient (R^2) values were above 0.999, as well as each calculated concentration of each calibration point was within ±15% of the true value. Therefore, it can be concluded that this developed method conforms to linearity.

3.2.4. The Limit of Detection and Limit of Quantification:

The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were calculated by diluting sample solutions until signal-to-noise ratios of 3 and 10, respectively. LOD and LOQ were estimated to be 0.12 and 0.45 μg/mL for calycosin-7-*O*-*β*-D-glucoside, 0.06 and 0.20 mg/mL for astragaloside IV.

3.2.5 Precision:

The intra- and inter-day precision have been summarized in Table 4.

Table 4. The evaluated results of precision (intra-day and inter-day)

Time	Code	Weight of sample (g)	Content of calycosin-7-*O*-*β*-D-glucoside (%)	Content of free astragaloside IV (%)
Day 1	DL-HK1.1	2.0056	0.020	0.066
	DL-HK1.2	2.0012	0.020	0.067
	DL-HK1.3	2.0011	0.020	0.066
	DL-HK1.4	2.0007	0.020	0.064
	DL-HK1.5	2.0903	0.019	0.064
	DL-HK1.6	2.1002	0.020	0.062
	Mean (n=6)		**0.020**	**0.065**
	RSD% (n=6)		**2.494**	**2.967**
Day 2	DL-HK1.7	2.0008	0.020	0.064
	DL-HK1.8	2.0104	0.020	0.063
	DL-HK1.9	2.0902	0.019	0.062
	DL-HK1.10	2.1003	0.019	0.063
	DL-HK1.11	2.1101	0.019	0.061
	DL-HK1.12	2.0035	0.020	0.064
	Mean (n=12)		**0.020**	**0.064**
	RSD% (n=12)		**2.879**	**2.934**

The evaluation was performed by calculating the relative standard deviation (RSD%). The criterion of acceptance was RSD% <3.7% for the mean contents of two compounds calycosin-7-*O*-*β*-D-glucoside and astragaloside IV, respectively. The results showed that the method had good precision.

3.2.6. Accuracy:

The accuracy was evaluated by adding a known amount of two standards at three different levels (50%, 100%, and 150%) to the sample. The obtained results are shown in Table 5.

The results in Table 5 showed that the recovery rates of the two compounds were in the range of 94-103%. The recovery values were 94.58-96.96% for calycosin-7-*O*-*β*-D-glucoside and 96.10-102.66% for free astragaloside IV. The results indicated that the accuracy of the developed method was acceptable.

Table 5. The results of evaluating the accuracy

Compound	Level	Mean recovery rate (%)	RSD (%)
calycosin-7-*O*-β-D-glucoside	50% spiked	96.89	0.11
	100% spiked	94.58	0.08
	150% spiked	96.96	0.09
astragaloside IV	50% spiked	102.66	0.24
	100% spiked	96.10	0.17
	150% spiked	100.40	0.15

3.3. Evaluating the contents of calycosin-7-O-β-D-glucoside and free astragaloside IV in the raw and honey-processed Astragali Radix samples

The proposed method was applied to quantitatively determine the contents of calycosin-7-*O*-β-D-glucoside and free astragaloside IV in ten samples of *Astragali Radix* and four samples of honey-processed *Astragali Radix*. The results are summarized in Table 6.

Table 6. Contents of calycosin-7-*O*-β-D-glucoside and free astragaloside IV in the *Astragali Radix* samples (HK1-HK10) and honey-processed *Astragali Radix* samples (HKCM1-HKCM4)

No.	Code	Content[a] of calycosin-7-*O*-β-D-glucoside (%)	Content[a] of free astragaloside IV (%)
1	HK1	0.022	0.066
2	HK2	0.026	0.052
3	HK3	0.032	0.032
4	HK4	0.022	0.018
5	HK5	0.030	0.055
6	HK6	0.016	0.020
7	HK7	0.028	0.039
8	HK8	0.024	0.028
9	HK9	0.027	0.016
10	HK10	0.021	0.019
11	HKCM1	0.019	0.052
12	HKCM2	0.018	0.021
13	HKCM3	0.010	0.018
14	HKCM4	0.022	0.024
The limits specified in the Vietnamese Pharmacopoeia V		≥ 0.02	≥ 0.04

[a] *Data are mean from three separate experiments;*

The contents of free astragaloside IV and calycosin-7-*O*-β-D-glucoside in the raw and honey-processed *Astragali Radix* samples have been determined using the calibration curves of the standard compounds. In the raw *Astragali Radix* samples, the contents of free astragaloside IV and calycosin-7-*O*-β-D-glucoside were 0.016 - 0.066 % and 0.016 - 0.032 %, respectively. In the honey-processed *Astragali Radix* samples, the contents of free astragaloside IV and calycosin-7-*O*-β-D-glucoside were 0.018 - 0.052 % and 0.010 - 0.022 %, respectively. The contents of free astragaloside IV and calycosin-7-*O*-β-D-glucoside in the raw crude *Astragali Radix* sample (**HK1**) were higher than those in the honey-processed sample (**HKCM1**).

Many researchers have reported that flavonoids and saponins are the main active components in *Astragali Radix* responsible for its biological activities. Therefore, techniques for the quantitative and qualitative analysis of these compounds in *Astragali Radix* are of great importance. Pharmacopoeia of some countries such as China, Hong Kong, and Vietnam all use two separate methods to quantify each substance calycosin-7-*O*-β-D-glucoside and astragaloside IV [2,4,5]. According to the Chinese Pharmacopoeia (2015 edition) and Vietnamese Pharmacopoeia V, the content of astragaloside IV in *Astragali Radix* should be more than 0.04%. However, the recent edition of Chinese Pharmacopoeia 2020 stated, that the threshold of astragaloside IV must not be less than 0.08%. These pharmacopoeias also advise that the assays should use ammonia during extraction to increase the level of astragaloside IV in the extract and the true concentration of astragaloside IV naturally present in *Astragali Radix* was not measured. This occurs by hydrolysis of several other astragaloside compounds into astragaloside IV, and then quantifying the content of total astragaloside IV by HPLC-ELSD. Similar hydrolysis may not occur in the human digestive tract when astragaloside IV is taken orally, so determining free astragaloside IV content is necessary and meaningful in researching the quality of medicinal herbs, pharmacokinetics and product development from *Astragalus* herb [9]. This study was conducted to develop an HPLC-DAD method to quantify calycosin-7-*O*-β-D-glucoside and free astragaloside IV from *Astragali Radix*. The sample preparation only uses methanol as the extraction solvent, so the astragaloside IV content determined is the free form that exists in the *Astragali Radix* sample. The developed HPLC-DAD method has several

advantages including simple, popular equipment and rapid sample preparation.

4. Conclusion

In this study, we developed and validated a simple and reliable HPLC-DAD method for quantitative analysis of calycosin-*7-O-β*-D-glucoside and free astragaloside IV in the raw and honey-processed *Astragali Radix* samples. The developed HPLC-DAD method has met the criteria of the current ICH guidelines: specificity, linearity, accuracy, and recovery. Utilizing this HPLC method, we quantified the contents of calycosin-*7-O-β*-D-glucoside and free astragaloside IV in 10 samples of *Astragali Radix* and four samples of honey-processed *Astragali Radix*. The results showed that the calycosin-*7-O-β*-D-glucoside content varied from 0.016 - 0.032% in the *Astragali Radix* samples and 0.010 - 0.022% in the honey-processed *Astragali Radix* samples. The free astragaloside IV content varied from 0.016 - 0.066% in the *Astragali Radix* samples and 0.018 - 0.052% in the honey-processed *Astragali Radix* samples. However, the total content of astragaloside IV in *Astragali Radix* has not been determined by this method, so the analysis results of astragaloside IV in *Astragali Radix* samples in this study cannot be compared with regulations in Vietnamese Pharmacopoeia V ($\geq$ 0.04%). In the future, the developed HPLC-DAD method will be tested and applied to analyse the total content of astragaloside IV in *Astragali Radix* using an ammonia solution during sample preparation to apply in quality standardization of *Astragali Radix*.

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INVESTIGATION OF THE ANTITHROMBOTIC, ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL CONTENT OF THE AERIAL PART OF *OXALIS DEBILIS* KUNTH

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Summary

Investigation of the Antithrombotic, Antioxidant Activity and Phytochemical Content of the Aerial Part of *Oxalis debilis* Kunth

Oxalis debilis Kunth (OD) is traditionally used to treat various ailments, including blood stasis syndrome, despite the lack of scientific evidence. This study investigated the antiplatelet, anticoagulant, and antioxidant properties, as well as the phytochemical profile of OD aerial extracts. The aerial part of OD was extracted with methanol, and fractionated with *n*-hexane, ethyl acetate (EtOAc), and water. The antiplatelet and anticoagulant effects were tested with human blood samples. DPPH and ABTS assays were performed to examine the antioxidant capacity. The total flavonoid content (TFC) and total phenolic content (TPC) were quantified. The study indicated that the methanol extract and all fractions had potent antiplatelet effects. The water fraction had the highest antiaggregatory effect induced by adenosine diphosphate among fractions. In addition, the EtOAc and water fraction had anticoagulant effects. Free radical scavenging activities against both DPPH and ABTS were also observed for the MeOH extract, EtOAc, and water fraction. Moreover, the aerial OD was rich in TPC and TFC with the highest values observed in the methanol extract and the water fraction. These novel findings indicate the