Development of a high-performance liquid chromatography method for simultaneously analysis of saponins and flavonoid in materials and dietary supplements containing Hedera helix extracts

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Abstract

English Ivy or Hedera Helix is a multi-medicinal functioned plant in nature. Most cough-medicines in Vietnam were extracted from Ivy leaves because of its 5 main active components, in which 4 Saponins included Hederacoside C (predominance), α-Hederin, Hederacoside D, Hederasaponin B are responsible for eliminating congestion (breaking up the phlegm and mucus) and Flavonoid: Kaempferol 3-rutinoside plays the role of reducing inflammations [1]. This study aimed to develop a HPLC-PDA method to simultaneously and fast analyze these 5 compounds in materials and dietary supplements containing hedera helix extract in Vietnam market. After the simple preparation procedure, the analytes were separated by using a C18 column (150 mm × 4.6mm, 5 µm) as stationary phase, and a mixture of 0.1% phosphoric acid and acetonitrile as mobile phase. The detection and quantification were in PDA detector at 205 nm. The method validation followed AOAC criteria. The calibration curves in the range of 0.5 - 200 mg/L for 4 saponins and 0.1 - 100 mg/L for the flavonoid with high correlation coefficient ($R^2 > 0.9999$). The MDL (0.03 - 0.15 mg/kg) and MQL (0.15 - 0.50 mg/kg); RSD_r (%) for repeatability (1.01 - 3.90%) and RSD_R reproducibility (1.25 - 6.89%); recoveries (91.3 - 106%) for 5 compounds satisfied the AOAC requirements. The method was applied successfully for determining the content of the analytes in 10 real samples including dried ivy extract powder, dried leaves, and some cough relief products purchased from markets in Hanoi. The levels of the 5 analytes were different in each sample in which Hederacoside C and α-Hederin account for the main proportions.

Keywords: *Hedera Helix, Saponins, Flavonoid, HPLC-PDA.*

1. INTRODUCTION

Ivy is the common name of an entire genus of plants called Hedera, which is primarily found throughout Europe, Asia, Northern Africa, and parts of the Pacific [2]. Vietnam, a

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humid tropical monsoon climate area is considered as a favorable environment for the Ivy development. The ivy plants are not only for ornamental purposes but also for medicinal purposes. The leaves can be used directly or the leave extract is commonly used as a supplement in herbal treatment. Ivy leaves are commonly used to eliminate respiratory tract congestion and inflammation. They are considered as an expectorant which can break up the phlegm and mucus in the bronchial system. By eliminating these breeding grounds for pathogens and bacteria, they can improve your overall health and shorten the recovery time. It also plays important roles in reducing the inflammation of allergic reactions and asthma [1, 3].

For its chemical constituents, triterpene saponins, flavonoids, polyacetylenes and some phenolic compounds have been isolated from Ivy plant [1, 4]. Because of its β2-adrenergic actions, α-hederin, a triterpene saponin, has been discovered for the therapeutic activity in ivy leaf extract, which can help spasmolytic, bronchodilatory, mucolytic, and expectorant action [4]. Hederacoside C, another triterpene saponin predominant substance, can be converted into the active form inducing the effect of α-hederin in the body [4]. Although α-hederin and hederacoside C have been identified as active ingredients in ivy leaf extract or its pharmaceutical preparations, it is impossible to rule out the possibility of other chemicals contributing to efficacy or toxicity.

Previous studies using thin layer chromatography [5], high-performance liquid chromatography (HPLC) with a photodiode array detector (PDA) [1, 2, 4, 6], and high-performance liquid chromatography (HPLC) tandem mass spectrophotometry [3, 7] were conducted to determine 5 compounds in supplements. However, the PDA method were evaluated for the selectivity, rapidity, and economy. Therefore, this study aimed to develop HPLC-PDA method for simultaneously, fast analyzing saponins and flavonoids in ingredients and dietary supplements containing hedera helix extracts in Hanoi market.

2. MATERIAL AND METHOD

2.1. Apparatus

HPLC system (Shimadzu, model: 20A) was equipped with high-pressure pump, autosampler, PDA detector and Sunfire C18 column (150 mm x 4.6 mm, 5 µm); a pH meter 744 (Mettler Toledo); Hermle Z383K centrifuge; analytical weigh (Mettler Toledo), Ultrasonic bath (Germany).

2.2. Chemicals and materials

Standards: Hederacoside C (HC, Code: 97151, Lot: BCCF4212), α-Hederin (HE, Code: 07512, Lot: BCCG7208) from Sigma Aldrich, Hederacoside D (HD, Code: BP0709, Lot: PRE10012641), Kaempferol 3-rutinoside (KE, Code: BP0823, Lot: PRE20071321), Hederasaponin B (HB, Code: BP0711, Lot: PRE20072421) from Biopurify Phytochemicals. Other reagents at HPLC analytical grade including acetonitrile (ACN), methanol (MeOH), ethanol (EtOH) and ortho-phosphoric acid 85% (AA), ammonium acetate (AMA),

triethylamine (TEA) were from Merck. Ultra-pure water was prepared using a Milli-Q water system (Millipore, Billerica, MA, USA).

The stock standard solutions were prepared separately in methanol.

Samples including dried ivy leaves, hedera helix extract and some pharmaceutical cough relief products in the form of syrup, soft capsules, hard capsules and granules were collected from local markets in Hanoi, and stored under room temperature. Samples were homogenized before analysis. The dried ivy leaves and hedera helix extract samples were dried at 105°C for 2h before extraction.

2.3. Method

2.3.1. Sample preparation

Approximately 0.1 - 0.2 g dried Ivy extract; 2 - 3 g dietary supplement soild and 5 - 6 grams syrups of the homogenized samples were placed into 50 mL polypropylene centrifuge tubes. Add 30 mL of 80% methanol in water to the tubes. Shake horizontally by a mechanical shaker and put in ultrasonic vibarate without temperature for 20 min and centrifuged at 6,000 rpm for 5 min. The aliquot of the extract was transferred into 50 mL volumetric flask. Reduplicate the previous extraction with 15 mL of 80% methanol in water. Combine the aliquot, and dilute to 50 mL. The extract was filtered through a 0.2 µm PTFE syringe filter before being analyzed by HPLC-PDA.

2.3.2. Analysis by HPLC

Saponins and flavonoids can absorb at wavelengths in the ultraviolet region, therefore PDA detector was chosen in coupling with the HPLC system. The following experimental conditions for the detection and quantification of Saponins and Flavonoids in supplements by HPLC-PDA system were selected [1, 2, 4]:

- Detector: PDA at wavelength 205 nm
- Chromatographic Column C18 (150mm × 5mm, 4.6 µm)
- Flow rate: 1.0 mL/min
- Sample injection volume: 20 µL

2.3.3. Method validation

This method was validated for linearity, method quantification limit (MDL), method quantification limit (MQL), repeatability, reproducibility, recovery, and measurement uncertainty. Working standards at concentrations of 0.5 - 200 mg/L for 4 saponins and 0.1 - 100 mg/L for the flavonoid were prepared by diluting the stock standard solutions with methanol into 10 mL volumetric flasks. Spike standards with decreasing concentrations to the blank sample. MDL and MQL was determined at concentration in which the signal-to-noise ratio was equal to 3 (for MDL) and 10 (for MQL).

To evaluate the precision (intra-day repeatability RSD_r % and inter-day reproducibility RSD_R %), the measurement was repeated 6 times (n = 6) with the same sample, equipment and operator in a day. For reproducibility, n = 10 for two operators on different days. The recoveries were performed by adding the known amounts at one level of 5 compounds into

the sample matrices with 6 replicates. The measurement uncertainty was also estimated for every analyte in ingredients and dietary supplements using the combined standard uncertainty. To obtain measurement uncertainty (U), a coverage factor of ~ 95%, where k = 2, was used [8].

3. RESULTS AND DISCUSSION

3.1. Mobile phase

The mobile phase is a decisive factor in chromatographic separation efficiency. In general, the mobile phase can affect the selectivity of the phase system, retention time of solutes, efficiency of separation columns (Nef quantity), resolution of analytes and width of chromatographic peaks.

According to [1, 2, 4], 3 mobile phases were selected for investigation: ACN-H_3PO_4 0.1% with gradient in table 1; ACN-H_2O, 40 : 60 (v/v) and AMA (pH 8.5 by TEA)-ACN, 70 : 30 (v/v). It shows that when using the ACN-H_2O mobile phase with C18 column, the chromatogram shows only 4 analytes. With the ACN- AMA pH 8.5 as mobile phase, 5 peaks appear quite early, with large width and unbalanced shape. Therefore, in this study, ACN- H_3PO_4 0.1% mobile phase was selected due to its narrow peak width, good analyte signal, low background noise signal and full detection of 5 analytes (Figure 1).

The mobile phase concentration has a great influence on the separation efficiency and signal of the analytes. In this study, H_3PO_4 mobile phase was investigated with the concentrations of 0.05; 0.1; 0.15 and 0.2% with gradient 2 in Table 1. At concentrations of 0.1 and 0.15% H_3PO_4, the analyte signal is the highest. Due to the fact that high concentration of H_3PO_4 (high acidity) may damage the column and analyte, in this study, the concentration of 0.1% H_3PO_4 was selected for further investigations.

Analytes HDC, HD, HE, KE and HB are substances with different properties and molecular weights. Besides, in dried ivy, health supplements, the medicinal sample matrices are quite complicated that makes diffifcult for chromatographic separation. Therefore, gradient program was conducted with mobile phase concentration of 0.1% orthophosphoric acid and ACN. The increase of ACN proportion in the mobile phase composition leads to the decrease of retention time, thus the analytes come out earlier. At gradient mode 1 in Table 1, due to the lower concentration of ACN, the analysis time is very long (about 26 minutes). On the contrary, at gradient mode 3 in table 1, due to high ACN concentration, the first substance came out very soon (about 5 minutes) making the analytes possibly affected by interferences. Therefore, gradient mode 2 was chosen to analyze in subsequent studies. This study is highly evaluated for the short analysis time (33 minutes) comparing with the previous research about 75 minutes [4].

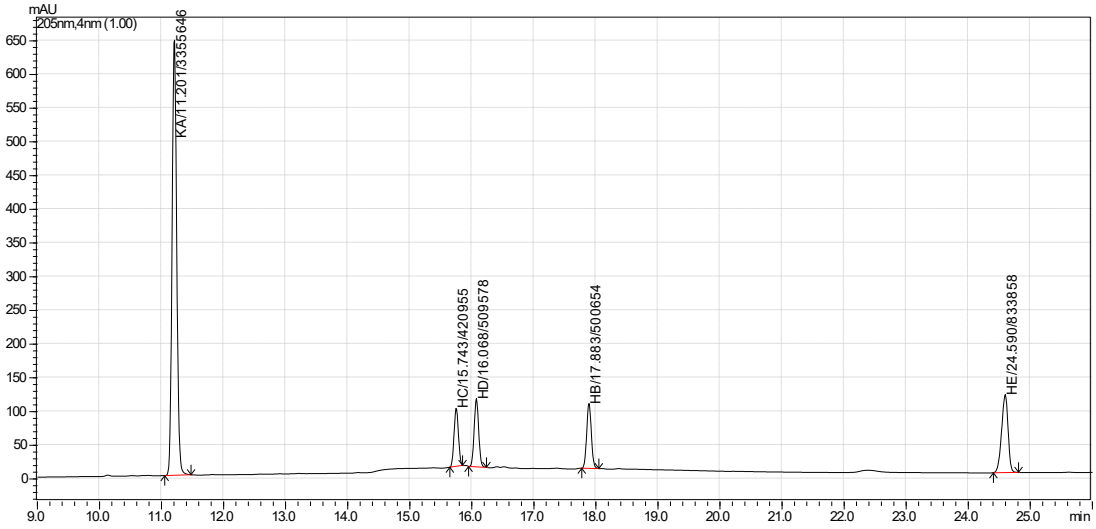

Figure 1. *Chromatograms of 5 compounds in 0.1% H_3PO_4-ACN gradient 2 in Table 1*

In summary, HPLC conditions for analyzing 5 compounds include the use of C18 Column (150 mm × 4.6 mm, 5 µm), the mobile phase of 0.1% H_3PO_4 and ACN with gradient condition as in Table 1. The flow rate was 1 mL/min, the sample injection volume was 20 µL and the detection wavelength was 205 nm.

Table 1. *Gradient Programs*

Time (min)	***H_3PO_4 0.1%***	***ACN***	***H_3PO_4 0.1%***	***ACN***	***H_3PO_4 0.1%***	***ACN***
	Gradient 1		***Gradient 2***		***Gradient 3***	
0.01	100	0	90	10	80	20
25.0	40	60	40	60	40	60
26.0	60	40	60	40	60	40
28.0	100	0	90	10	80	20
33.0	100	0	90	10	80	20

3.2. Sample preparation

Investigation of different solvents for sample extraction was carried out with dried ivy sample since the association of analytes in the natural matrix seems more complicated than other products. After homogeneousness and being dried at 105°C for 2h, approximately 0.1 - 0.2 grams dried Ivy sample was weight for extraction solvent study. According to the following references [1, 2, 4] and the soluble property in organic solvents such as EtOH and MeOH of some analytes, EtOH and MeOH were studies for extraction. The obtained results are shown in Figure 2. It illustrates that ethanol solvent gives lower extraction efficiency than MeOH solvent. For that reason, MeOH solvent is selected for further investigations.

On the same dried ivy sample, extraction was carried out at different MeOH concentrations from 20 - 100%. From the obtained results in Figure 3, it can be seen that with increasing the proportion of MeOH from 20 - 100%, the total content of 5 substances raises gradually. It is not clear difference in the extraction efficiency at the MeOH concentration of 80 and 100%, therefore, the 80% MeOH was chosen to reduce the volatility of the solvent during analysis.

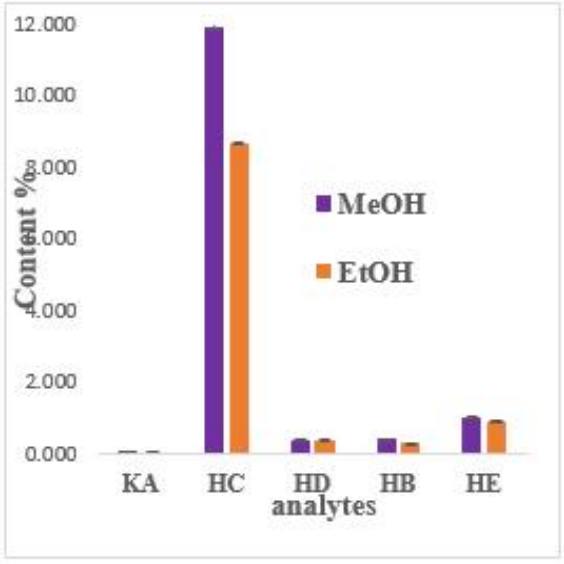

Figure 2. *Content (%) of saponins and flavonoid obtained with different extraction solvents*

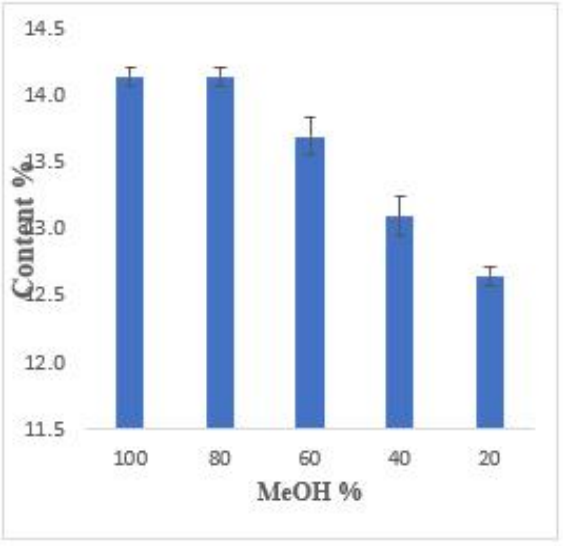

Figure 3. *Total content of saponins and flavonoid at different methanol concentrations*

The sample was weighed into a 50 mL centrifuge tube, 80% MeOH was added as solvent extraction and then put into the ultrasonic vibration bath at room temperature. Different extraction times of 10, 20, 30 and 40 minutes were investigated. The obtained results indicated that with increasing the extraction time from 10 to 30 minutes, the amount of saponins obtained also increased. However, the content of saponins drop down slightly after 40 minutes of ultrasonic extraction. There is no significant difference between extraction time of 20 minutes and 30 minutes, therefore, in order to shorten the time and reduce the energy, the optimal 20 minutes ultrasonic vibration extraction time was selected.

3.3. Method validation

Specification: Blank sample (syrup sample without containing hedera helix extract), working standards, samples were injected into HPLC. There was no peak appearing in the retention time of 5 compounds in blank chromatography (Figure 4A). The spiked sample had signals at retention times close to that of the standard (difference < 5%) [8] (Figure 4B, 4C).

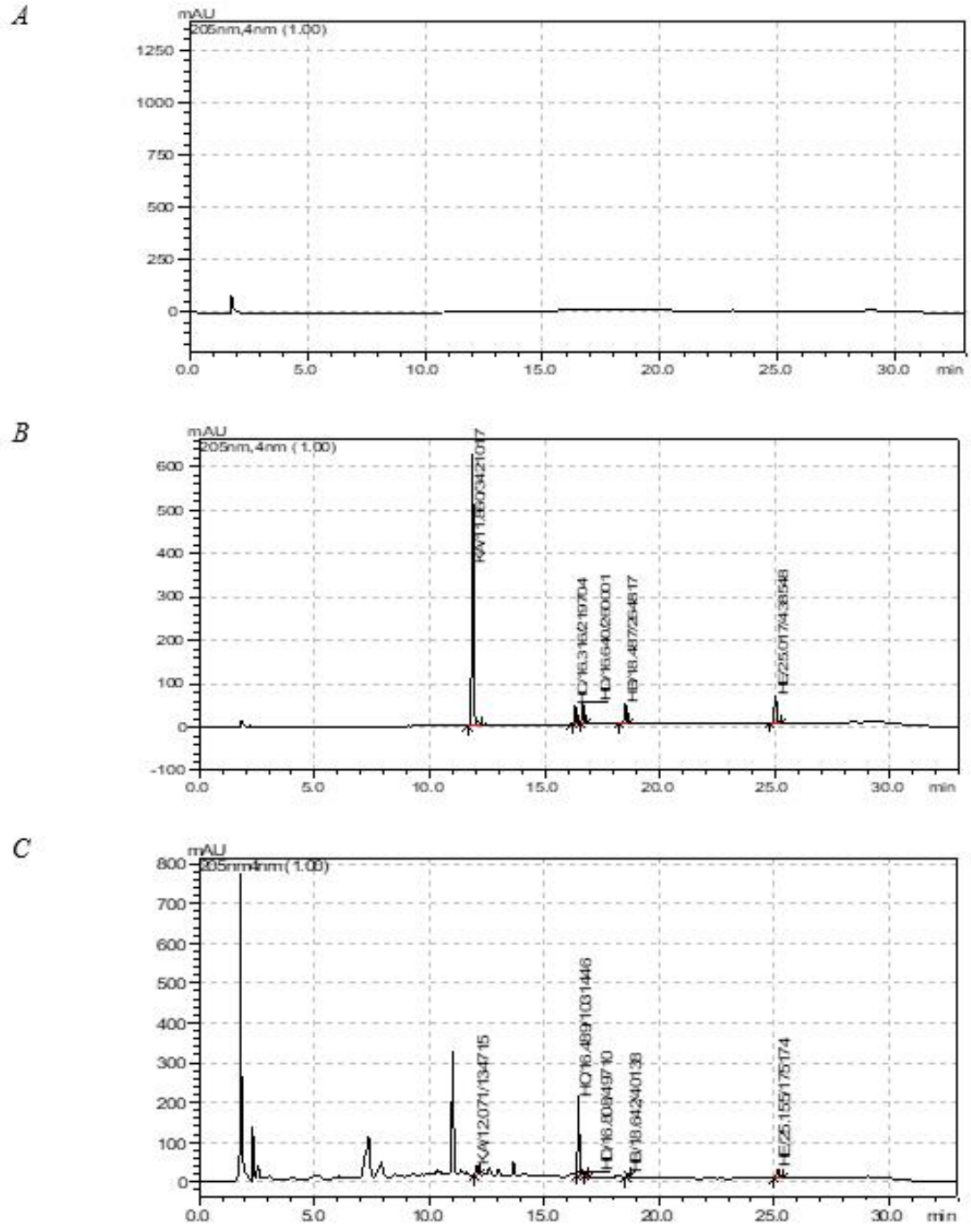

Figure 4. *Chromatograms of blank sample (A), standard (B), dried ivy extract sample (C)*

Working standards at concentrations of 0.5 - 200 mg/L for 4 saponins and 0.1 - 100 mg/L for the flavonoid with bias values were satisfied lower than 15% for all compounds. The results of the standard curve equations, correlation coefficients, method detection limit (MDL) and method quantification limit (MQL), repeatability (RSD_r), reproducibility (RSD_R), recovery (R) and uncertainty (U) of the analytes are presented in Table 2, 3.

Table 2. *The standard curve equations, MDL and MQL of 5 compounds*

Analytes	*Calibration equation*	*Bias (%)*	R^2	*MDL (mg/kg)*	*MQL (mg/kg)*
KE	y = 68484x - 3171	0.01 - 14.3	1.0000	0.03	0.1
HDC	y = 4460.4x - 178.53	0.09 - 8.71	1.0000	0.15	0.50
HD	y = 5179.1x + 202.15	0.08 - 3.81	1.0000		
HB	y = 5224.2x - 186.36	0.29 - 5.18	0.9999		
HE	y = 8721.6x + 776.03	0.04 - 9.26	1.0000		

Table 3. *Summary of method evaluation results*

Analytes	*Parameter*	*Dried Ivy extract*	*Dietary supplement*			
			Syrups	*Soft capsules*	*Hard capsules*	*Granules*
KE	RSD_r (%)	3.58	2.09	1.44	1.62	2.34
	RSD_R (%)	2.79	3.21	2.05	1.48	2.17
	R (%)	101 - 105	94.2 - 99.5	92.8 - 99.1	94.4 - 103	94.7 - 106
	U (%)	7.19	8.87	8.86	5.13	7.00
HDC	RSD_r (%)	1.01	2.01	3.00	2.20	2.16
	RSD_R (%)	1.30	1.64	3.48	2.22	2.89
	R (%)	98.1 - 101	96.5 - 103	94.9 - 101	97.7 - 102	99.7 - 104
	U (%)	2.99	4.86	8.73	5.11	6.40
HD	RSD_r (%)	2.88	3.90	1.61	2.18	2.50
	RSD_R (%)	4.81	4.40	2.47	1.97	3.39
	R (%)	98.3 - 105	96.7 - 103	95.8 - 102	98.7 - 102	94.0 - 105
	U (%)	10.3	9.70	6.80	4.64	9.15
HB	RSD_r (%)	2.73	2.28	2.19	1.58	3.34
	RSD_R (%)	5.81	2.93	2.84	2.15	6.89
	R (%)	99.2 - 104	95.6 - 105	94.1 - 96.9	95.5 - 101	93.6 - 103
	U (%)	12.2	7.70	9.19	5.31	14.46
HE	RSD_r (%)	1.31	3.49	1.70	1.61	2.13
	RSD_R (%)	1.31	4.73	2.73	1.25	2.64
	R (%)	97.5 - 103	97.8 - 103	94.7 - 103	92.8 - 97.1	93.0 - 102
	U (%)	4.75	9.87	8.18	7.83	9.03

The results qualified according to AOAC guidelines. The obtained results proved that the analytical method is suitable for the determination of 5 compounds in ingredients and dietary supplements containing hedera helix extracts.

3.4. Analysis of real sample

The developed method was applied on real samples from different countries collected from markets in Hanoi at different times. The obtained results are shown in Table 4 and Figure 5.

Table 4. *Analytical results of dried ivy extract and dried ivy leaves*

Samples	*KE (%)*	*HC (%)*	*HD (%)*	*HB (%)*	*HE (%)*	*Total (%)*
Dried ivy extract 1	0.015 ± 0.00	2.70 ± 0.01	0.33 ± 0.00	0.25 ± 0.00	0.29 ± 0.00	3.59
Dried ivy extract 2	0.10 ± 0.00	11.8 ± 0.02	0.39 ± 0.01	0.37 ± 0.01	0.41 ± 0.00	13.1
Dried ivy extract 3	0.012 ± 0.00	2.36 ± 0.01	0.32 ± 0.00	0.25 ± 0.01	0.21 ± 0.00	3.15
Dried ivy extract 4	0.11 ± 0.00	11.9 ± 0.04	0.48 ± 0.01	0.42 ± 0.02	1.03 ± 0.02	13.9
Dried ivy extract 5	0.11 ± 0.00	11.6 ± 0.03	0.48 ± 0.02	0.40 ± 0.02	1.01 ± 0.01	13.6
Dried leaves	0.012 ± 0.00	3.48 ± 0.01	0.058 ± 0.00	0.23 ± 0.01	0.33 ± 0.00	4.11

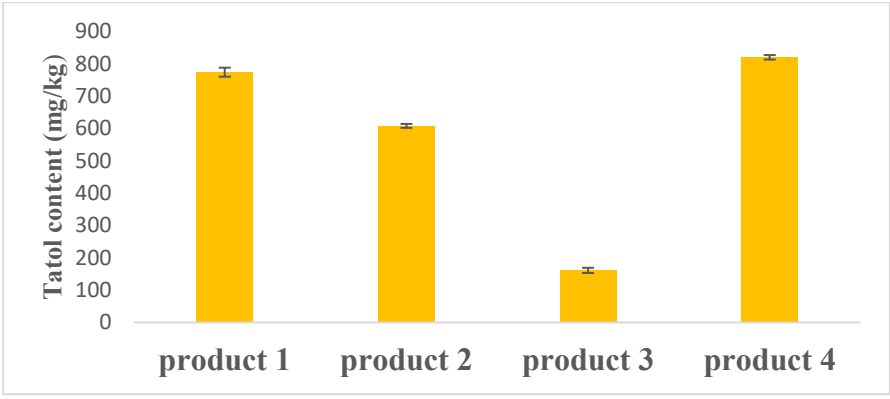

Figure 5*. Analysis results of dietary supplement samples*

For 10 actual samples, the saponins and flavonoid contents in the dried samples were high different, possibly due to the cultivation conditions and the harvest season. The total contents of 5 analytes in the dried ivy extract sample ranged from 3.15 to 13.9%. In hard capsules (product-1), soft capsules (product-2), granules (product-3) and syrup (product-4), the total content ranged from 161 - 820 mg/kg. The content in the dried leaf samples was the lowest. HDC, HE are the main components in all samples consistent with previous studies on saponins and flavonoid content in the dried samples, which account for about 81 - 93% for HE and accounts for mainly 75 - 85% for HC.

These results are consistent with previous studies on the composition of saponins and flavonoid in ivy samples [2]. In which, the content of HDC in the extract was 15.6%, which satisfied the requirement at least 10% of HDC in dried Ivy extract. All 10 ivy products showed good resolution separation of the tested compounds. However, there were some differences in the contents of other compounds even among the products with the same specification. Figure 6 show chromatograms of soft capsules sample.

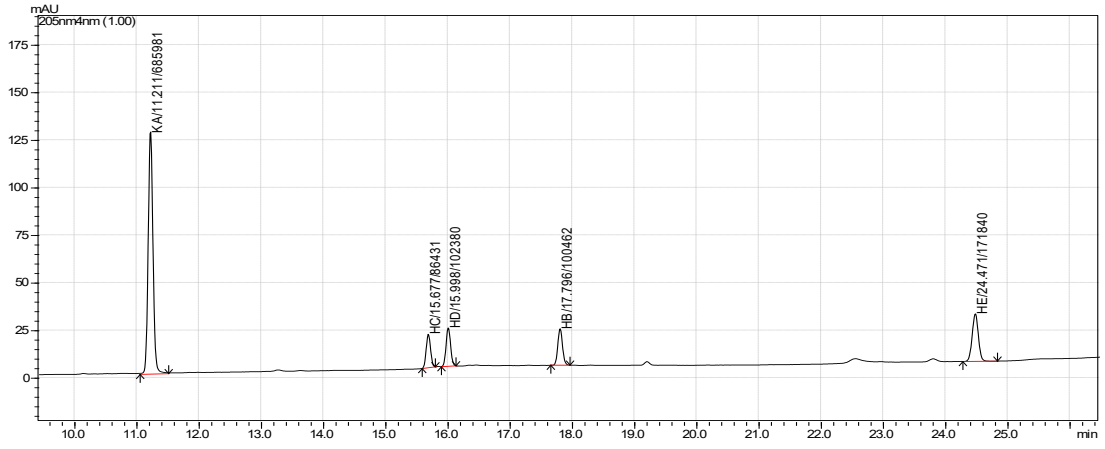

Figure 6. *Chromatograms of soft capsules sample*

4. CONCLUSION

A method to determine simultaneously Hederacoside C, α-Hederin, Hederacoside D, Hederasaponin B and Kaempferol 3-rutinoside was developed and validated following AOAC criteria including specification, linearity, precision, accuracy, MDL, MQL and uncertainty. Samples preparation procedure was fast and simple. The method was applied successfully to analyze 5 groups of matrices with 10 products. Therfore, it can be used as a routine method for analyzing 5 compounds in ingredients and dietary supplements containing hedera helix extracts.

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Xây dựng phương pháp HPLC phân tích đồng thời một số saponins và flavonoid trong nguyên liệu và thực phẩm bảo vệ sức khỏe chứa cao khô thường xuân

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Tóm tắt

Cây thường xuân (Ivy hay Hedera Helix) là cây có nhiều tác dụng chữa bệnh trong tự nhiên. Hầu hết các loại thuốc ho ở Việt Nam đều chiết xuất từ lá cây thường xuân vì có 5 hoạt chất chính, trong đó 4 chất thuộc nhóm Saponin: Hederacoside C (hoạt chất chính), α-Hederin, Hederacoside D, Hederasaponin B có tác dụng tiêu trừ tắc nghẽn (làm tan đờm và chất nhầy) và 1 chất thuộc nhóm flavonoid: Kaempferol 3-rutinoside giúp giảm viêm. Nghiên cứu được thực hiện với mục tiêu xác định đồng thời 5 hợp chất bằng phương pháp HPLC-PDA trong nguyên liệu và thực phẩm bảo vệ sức khỏe chứa cao khô thường xuân. Các điều kiện HPLC đã được nghiên cứu tối ưu bao gồm: cột C18 (150 mm × 4,6 mm x 5 µm); pha động gồm 0,1% acid orthophosphoric và acetonitril chế độ gradient, bước sóng phát hiện 205nm. Phương pháp đã được thẩm định đạt theo các tiêu chí của AOAC. Đường chuẩn của 5 chất phân tích xây dựng trong khoảng nồng độ 0,5 - 200 mg/L đối với 4 saponin và 0,1 - 100 mg/L đối với flavonoid có hệ số tương quan ($R^2 > 0,999$); MDL (0,03 - 0,15 mg/kg) và MQL (0,15 - 0,50 mg/kg); RSD_r cho độ lặp lại (1,01 - 3,90%) và RSD_R độ tái lập (1,25 - 6,89%); độ thu hồi (91,3 - 106%). Qui trình phân tích đã được áp dụng để phân tích 10 mẫu bao gồm cao thường xuân khô, lá thường xuân khô và một số mẫu thực phẩm bảo vệ sức khỏe có chứa cao khô thường xuân tại Hà Nội. Hàm lượng chất phân tích có sự khác nhau giữa các mẫu, trong đó Hederacoside C và α-Hederin chiếm tỷ lệ chính.

Từ khóa: *Cây thường xuân, Saponins, Flavonoid, HPLC-PDA.*