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DEVELOPMENT AND VALIDATION OF QUANTITATIVE ANALYSIS OF L-DOPA FROM *MUCUNA PRURIENS*

Le Thi Loan[1], Dang Minh Tu[1], Dang Viet Hau[1], Nguyen Thi Thu Trang[1], Luong Thi Lan[1], Ha Van Oanh[2], Nguyen Van Tai[1,*]

[1]*National Institute of Medicinal Materials (NIMM), Hanoi, Vietnam;*

[2]*Hanoi University of Pharmacy, Vietnam*

*Corresponding author: nguyenvantai1111@gmail.com

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Summary

Development and Validation of Quantitative Analysis of L-Dopa from *Mucuna pruriens*

Mucuna pruriens is an important medicinal plant used for the treatment of Parkinson's disease and many others in ancient traditional Indian medicine. This study reports a simple and rapid high performance liquid chromatography (HPLC) method for the quantification of L-dopa in *Mucuna pruriens* seeds. Methanol/water/hydrochloric acid (70:30:0.1) was used as the extraction solvent. The chromatographic condition was performed on the Agilent Zorbax Eclipse plus C_{18} column (250 x 4.6 mm, 5 µm), the mobile phase was 0.1% acetic acid/methanol (99:1), flow rate 0.8 mL/min, and the ultraviolet (UV) detector was set at 280 nm. The method has been validated, showing that it is selective, specific, accurate, and suitable for the quantification of L-dopa in *Mucuna* seeds. The recovery was 99.89 - 101.49%, and relative standard deviation (RSD) values of the repeatability and intermediate precision were less than 2%. The limit of quantification of L-dopa was 0.12 µg/mL, corresponding to 0.07 mg/g in samples. This method was used to determine the L-dopa content in 5 samples of *Mucuna pruriens* seeds and showed the L-dopa content ranging from 5.20 ± 0.02% to 5.92 ± 0.10% by weight of dried medicinal herbs.

Keywords: *Mucuna pruriens, L-dopa, Levodopa, HPLC.*

1. Introduction

Mucuna pruriens (L.) DC. is a climbing legume distributed across Africa and Asia. The seeds of the plant contain a high amount (3- 6%) of L-dopa [1],[2]. L-dopa is also known as L-3,4-dihydroxyphenylalanine, an aromatic amino acid, which is biosynthesized via L-tyrosine. It is a precursor molecule of the neurotransmitters dopamine, norepinephrine, and epinephrine. Nowadays, the compound is used for the clinical treatment of Parkinson's disease, a neurodegenerative disorder, which substantiates its prolonged use in traditional Indian medicine. The control of crucial human body functions can be affected by a lack or excess of L-dopa and its metabolites. Treatment of L-dopa in Parkinson's patients is often individualized [3], the initial dose is 125 mg x 2 times/day, may be increased by 100-750 mg every 3-7 days, maximum 8 g/day [4]. Consequently, it is necessary to monitor the concentration of L-dopa in *Mucuna* seeds as well as in all plant matrices destined for human consumption.

There have been several publications on methods to quantify L-dopa in *Mucuna* seeds and products containing it. L-dopa's low molecular weight and polar nature generally make its determination by reversed-phase liquid chromatography challenging. In addition, L-dopa aqueous solutions are unstable and degrade naturally over time, so the extraction procedure also requires special attention. Vachani et al. [5] and Sundaram et al. [6] reported the HPTLC methods for quantitation of L-dopa in polyherbal formulations containing *M. pruriens* seeds. Pulikkalpura et al. [7] reported studies on quantification and degradation of L-dopa in *M. pruriens* seeds by HPTLC. Soumyanath et al. [8] quantified L-dopa in *M. pruriens* seeds and its formulations by HPLC. British Pharmacopoeia and the United States Pharmacopeia describe methods for L-dopa determination in tablets or capsules [9],[10].

In Vietnam, *M. pruriens* has also been reported to appear in some places. However, up to now, there have been no studies on the L-dopa content in *Mucuna* seeds in Vietnam. Here, we report a validated HPLC method according to the International Conference on Harmonization guidelines for the quantification of L-dopa content in *M. pruriens* seeds collected in Vietnam.

2. Materials and methods

2.1. Plant material

Mucuna pruriens were collected in Bac Giang province. The scientific name of the samples was identified by Master Dang Minh Tu - Center of Medicinal Material Resources, Institute of Medicinal Materials, based on botanical

characteristics and compared with the taxonomic keys of the genus *Mucuna* [11]. Specimens were kept at the Center of Medicinal Material Resources - Institute of Medicinal Materials. Fresh samples were dried at 50ºC, ground, and stored in sealed plastic bags until use.

Table 1. Samples of information

No.	Sample name	Collection location	Time collection
1	DM1	Son Dong, Bac Giang	12/2021
2	DM2	Luc Nam, Bac Giang	11/2022
3	DM3	Luc Nam, Bac Giang	12/2022
4	DM4	Luc Nam, Bac Giang	10/2023
5	DM5	Luc Nam, Bac Giang	12/2023

2.2. General experimental procedures

Standard material: Levodopa (Chengdu Biopurify), Lot No. 14022406, purity 98,0%.

Solvents, reagents: methanol, acetic acid, phosphoric acid, hydrochloric acid (HPLC grade)

Instruments: HPLC analysis was performed on a Shimadzu system, consisting of a binary pump LC-20AD, a SIL-20A autosampler, a CTO-20A column oven, and a UV-Vis detector SPD-20A.

2.3. Methods

Moisture of samples was determined according to the method of Vietnam Pharmacopoeia V, Appendix 9.6.

The method of sample extraction and chromatographic conditions were investigated and modified based on the methods of P. Siddhuraju and the United States Pharmacopeia [10], [12].

Sample treatment:

Sample preparation: Standard stock solution of L-dopa was prepared by dissolving it in methanol: water: hydrochloric acid (70: 30: 0.1, v/v/v), then diluted to establish calibration curves in ranges of 19.88 - 397.60 µg/mL. The stock and working solutions were stored at 4°C.

Sample extraction: sample preparation factors were investigated including extraction solvents (0.1% HCl solution, methanol, 70% methanol, 50% methanol, 0.1% HCl in methanol, 0.1% HCl in 50% methanol, 0.1% HCl in 70% methanol and 1.0% HCl in 70% methanol), extraction method (ultrasonic and heat reflux methods), extraction time (1 - 3 hours), the ratio of the mass of the sample to the volume of the solvent (g/mL) (1/125, 1/250, 1/500). Select the test sample preparation method to get the highest content of L-dopa.

HPLC condition: The separation of the compound was performed on an Agilent Zorbax Eclipse plus C_{18} column (250 x 4.6 mm, 5 µm). The column temperature was maintained at 40°C. The mobile phase was a mixture of methanol and 0.1% acetic acid in water (1: 99, v/v). The flow rate was kept at 0.8 mL/min. The injection volume was 5 µL. Detection was set at a wavelength of 280 nm.

Method validation: the method was validated according to ICH guidelines [13].

Application: samples were treated and analysed according to the developed method. The concentration of analytes in the samples is calculated based on the calibration curve.

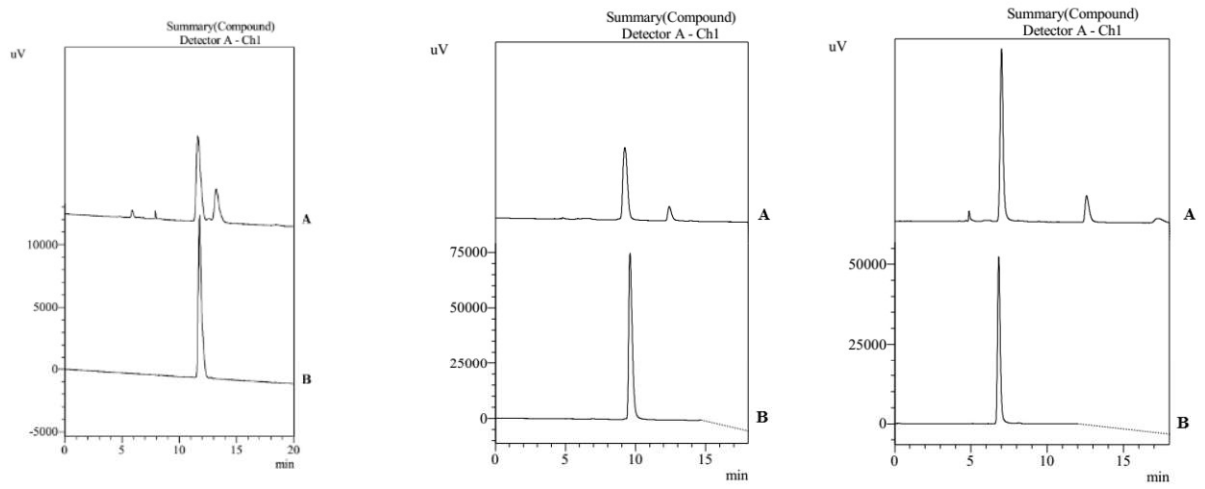
3. Results and Discussion

3.1. Method development

The chromatographic conditions were performed on the Agilent Zorbax Eclipse plus C_{18} column (250 x 4.6 mm, 5 µm). The column temperature was maintained at 40°C. The detection was set at 280 nm.

Selection of mobile phase programs

Three mobile phase program including: (1) methanol and 0.1% trifluoroacetic acid in water (1: 99, v/v) at flow rate of 0.8 mL/min; (2) methanol and 0.1% phosphoric acid in water (1: 99, v/v) at flow rate of 0.6 mL/min; (3) methanol and 0.1% acetic acid in water (1: 99, v/v) at flow rate of 0.8 mL/min were tested. The results showed that with the mobile phase program 1 and the mobile phase program 2, the peaks of L-dopa were broadened (Fig. 1). With the mobile phase consisting of methanol and 0.1% acetic acid in water, the peaks were symmetric and sharp.



(1) methanol and 0.1% trifluoroacetic acid in water (1: 99, v/v) at a flow rate of 0.8 mL/min

(2) methanol and 0.1% phosphoric acid in water (1: 99, v/v) at a flow rate of 0.6 mL/min

(3) methanol and 0.1% acetic acid in water (1: 99, v/v) at a flow rate of 0.8 mL/min

Fig. 1. Chromatograms for selection of mobile phase programs (A: sample solution; B: standard)

Selection of injection volume

Simultaneously, 2 levels of sample injection volume were also investigated. The results showed that when the injection volume was 5 µl, the peaks shape had a better shape than when the injection volume was 10 µl.

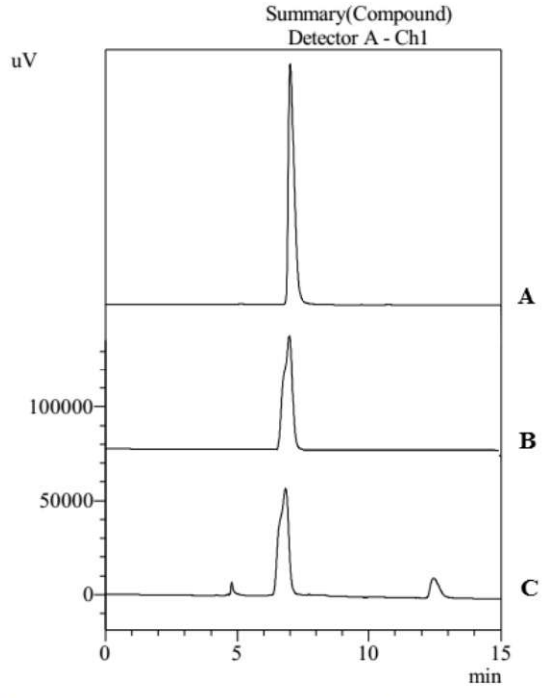


Fig. 2. Chromatograms for selection of injection volume (A: standard solution 5 µl; B: standard solution 10 µl; C: sample solution 10 µl)

Therefore, the selected chromatographic conditions analysis of L-dopa in *Mucuna* seeds were: Agilent Zorbax Eclipse plus C_{18} column (250 x 4.6 mm, 5 µm); the mobile phase consisted of methanol and 0.1% acetic acid in water (1: 99, v/v) at flow rate of 0.8 mL/min; the column temperature was maintained at 40°C; the detection was set at 280 nm; the injection volume was 5 µl.

3.2. Optimization of extraction conditions

The results are presented in Table 2. showed that acidic solvents (methanol, 70% methanol containing 0.1% HCl) have higher L-dopa extraction efficiency than corresponding neutral pH solvents (methanol, 70% methanol). Furthermore, the experimental process showed that when using neutral pH solvents, the extract is not stable and quickly changes color, even when stored in cold conditions (2-8°C) for 3 days. Meanwhile, using an acidic extraction solvent, the color of the extract is more stable, even when stored at room temperature for up to 7 days. This result is consistent with the literature [12].

The solvents 70% methanol containing 0.1% HCl and 70% methanol containing 1.0% HCl were shown to be better extraction solvents, reaching 5.21 ± 0.02% and 5.20 ± 0.2% respectively. There was no significant difference in the efficiency of extracting L-dopa from medicinal herbs when

increasing the concentration of HCl in the extraction solvent from 0.1% to 1.0%. Besides, at both levels of HCl concentration used, the medicinal extracts are color stable under normal or cold conditions.

This result is consistent with published results on the method of quantifying L-dopa in medicinal herbs 0.1 M HCl, MeOH: 0.1 M HCl (70:30) [14],[15],[16], 0.1 M HCl : EtOH (1:1) [17], HCOOH : EtOH (1:1) [12]. So, 70% methanol containing 0.1% HCl was chosen as the extraction solvent from the *Mucuna* seeds.

Table 2. The results of extraction conditions optimization (n = 3)

Extraction solvents (m= 0.2000 g, 100 mL solvents, reflux in 90 min.)		**Methods and times of extraction** (m= 0.2000 g, 100 mL of 0.1% HCl in 70% methanol)	
Solvents	**L-dopa (%)**	**Conditions**	**L-dopa (%)**
0.1% HCl solution	5.02 ± 0.02	Sonication 10 min.	4.71 ± 0.05
50% Methanol	5.05 ± 0.02	Sonication 15 min.	4.98 ± 0.06
0.1% HCl in 50% methanol	5.11 ± 0.09	Sonication 30 min.	5.09 ± 0.03
70% Methanol	5.11 ± 0.02	Sonication 60 min.	5.02 ± 0.03
0.1% HCl in 70% methanol	**5.21 ± 0.02**	Reflux 60 min.	5.05 ± 0.01
Methanol	4.89 ± 0.09	**Reflux 90 min.**	**5.21 ± 0.03**
0.1% HCl in methanol	5.09 ± 0.03	Reflux 120 min.	5.21 ± 0.01
1.0 % HCl in 70% methanol	5.20 ± 0.02	Reflux 150 min.	5.21 ± 0.02
		Reflux 180 min.	5.17 ± 0.03
Ratio of sample/solvent (m/v, g/mL) (m= 0.2000 g, 0.1% HCl in 70% methanol, reflux in 90 min.)			
Conditions	**L-dopa (%)**	**Conditions**	**L-dopa (%)**
0.2000 g/25 mL	4.90 ± 0.05	**0.2000 g/100 mL**	**5.20 ± 0.02**
0.2000 g/50 mL	5.11 ± 0.04	0.2000 g/50 mL, 2 times	5.21 ± 0.02

Other factors of sample preparation conditions, including extraction methods, extraction time, and ratio of sample/solvent, were also investigated.

From the results, the sample preparation procedure was selected as follows: weigh accurately 0.2000g of seed powder, add 100mL 0.1% HCl in 70% methanol, reflux for 1.5 hours, cool to room temperature, filter and transfer the filtrate to a 100-mL volumetric flask, make up to the mark with 0.1% HCl in 70% methanol. The solution was filtered through a 0.45 µm membrane filter before HPLC analysis.

3.3. Method validation

Specification

The experiment was performed with the blank, standard, and sample solutions according to the analytical procedure. The obtained chromatograms are shown in Fig. 3.

The results showed that there was no peak in the blank sample at the retention times of the targeted peaks in the standard solution. The obtained chromatograms showed clear separation peaks and low background noise. Therefore, the analytical method was specific for the determination of the targeted marker.

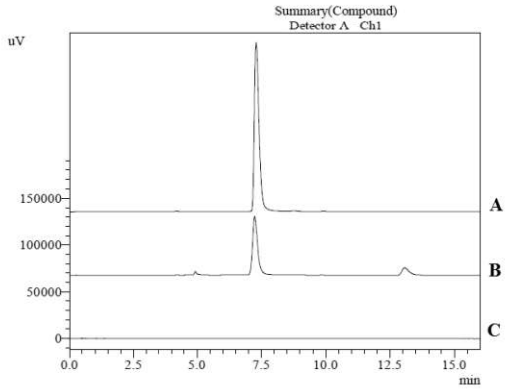


Fig. 3. Chromatograms for specificity study (A: standard; B: sample solution; C: blank)

System suitability

Table 3. System suitability testing

No	Retention time (min.)	Peak area (mAu.s)
1	7.437	666416
2	7.497	664118
3	7.422	661140
4	7.453	656679
5	7.298	665378
6	7.416	655072
7	7.525	652013
Mean	7.435	660116.6
SD	0.073	5588.0
RSD (%)	0.98	0.85

Table 3 shows that the RSD % of retention time and peak area were all under 2%. These indicated that the developed method conforms to system suitability criteria.

Linearity

Different concentrations of L-dopa were prepared and injected into the HPLC system. The calibration curve was constructed by plotting the peak areas versus the concentrations of the analyte. The result showed satisfactory linearity in the concentration ranges of 19.88 - 397.60 µg/mL for L-dopa. The typical calibration curve equation of L-dopa was y = 6747x -17272, with the P-value of y-intercept less than 0.05. Linear correlation value $R^2 = 0.9999$. Therefore, it can be concluded that this developed method conforms the linearity.

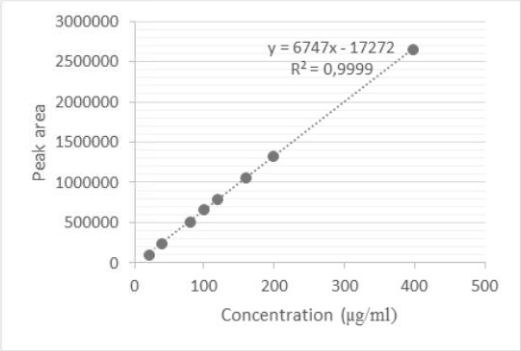


Fig. 4. Illustrating the linearity between L-dopa concentration and its corresponding peak area *Precision*

The results of the repeatability and intermediate precision study are presented in Table 4. The average content of L-dopa is 5.20% and the RSD all under 2.0%. The result was in good agreement with acceptable values for validation of the analytical procedure.

Table 4. The precision results

No.	L-dopa content (%)		
	Day 1	Day 2	
1.	5.22	5.20	
2.	5.19	5.21	
3.	5.25	5.18	
4.	5.19	5.17	**Average**: 5.20%
5.	5.21	5.22	**RSD**: 0.40%
6.	5.19	5.19	
Average (%)	**5.21**	**5.20**	
SD (%)	**0.02**	**0.02**	
RSD (%)	**0.44**	**0.36**	

Accuracy

Weigh accurately approximately 1.00 mg, 2.00 mg, and 3.00 mg of L-dopa, then dissolved each in 50 mL of 0.1% HCl in 70% methanol to obtain solutions with a corresponding concentration of approximately 20.00, 40.00, and 60.00 µg/mL. These solutions were added to 0.1000 g of the known samples, then extraction and analysis were performed. The results in Table 5 show that recovery rates of L-dopa were in the range of 95-105%. The recovery values were from 99.34 to 100.74%, indicating that the accuracy of the method was acceptable.

Table 5. The accuracy testing results

Amount added (µg/mL)	Amount found (µg/mL)	Recovery (%)	M ± SD (%)
19.80	20.04	101.21	100.74 ± 0.81
20.20	20.44	101.20	
20.20	20.16	99.80	
40.00	39.65	99.12	99.81 ± 0.60
40.20	40.28	100.20	
40.40	40.44	100.11	
61.00	60.45	99.10	99.34 ± 0.46
60.60	60.52	99.87	
60.80	60.22	99.05	

LOD and LOQ

The limits of detection (LOD) and the limits 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 0.128 µg/mL and 0.4224 µg/mL for L-dopa, corresponding to 0.07 mg/g and 0.23 mg/g in samples.

The analytical method for the determination of L-dopa in *Mucuna* seeds met the requirements of systematic suitability, selectivity, linearity, precision, and accuracy according to ICH criteria. Therefore, this method could be used to analyze L-dopa in *Mucuna* seeds.

3.4. Applications

The proposed method was applied for the determination of L-dopa in 5 samples *Mucuna* seeds. Each sample was analyzed in triplicate to determine the meant content (%). The results are summarized in Table 6.

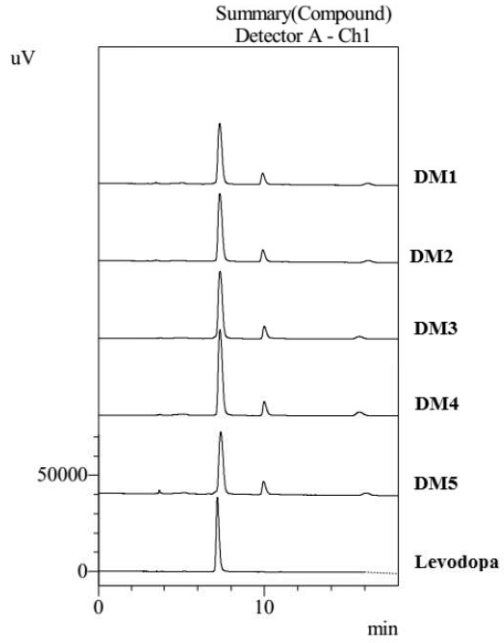


Fig. 5. Chromatograms of samples

Table 6. L-dopa content in the *Mucuna pruriens* seeds

No.	Sample name	Moisture (%)	Weight (g)	Peak area (mAu.s)	Concentration (µg/mL)	Content (%)	M ± SD (%)
1	DM1	9.08	0.2154	672562	101.61	5.19	5.20 ± 0.01
			0.2198	689422	104.05	5.21	
			0.2188	684372	103.32	5.19	
2	DM2	9.79	0.2137	757923	113.96	5.91	5.80 ± 0.22
			0.2078	739813	111.34	5.94	
			0.2152	714330	107.65	5.55	
3	DM3	10.03	0.2102	740915	111.50	5.90	5.94 ± 0.06
			0.2009	720446	108.54	6.00	
			0.2055	724797	109.17	5.90	
4	DM4	9.12	0.2186	790182	118.63	5.97	5.92 ± 0.10
			0.2114	764344	114.89	5.98	
			0.2070	724737	109.16	5.80	
5	DM5	9.85	0.2105	741043	111.52	5.88	5.82 ± 0.07
			0.2124	743934	111.94	5.85	
			0.2305	795228	119.35	5.74	

Discussion

The validation results showed that the developed method is selective, specific, accurate, and suitable for the quantification of L-dopa in *Mucuna* seeds. According to published studies, the LOD of L-dopa ranges from approximately 10 ng/mL to 15 µg/mL [18]. The analytical method of Kasture and colleagues published in 2014 has a LOD/LOQ of 0.115/0.348 µg/mL [17]. Meanwhile, Baranowska's method has a lower LOD/LOQ, 10 and 30 ng/mL, respectively [19]. Thus, the developed method has a relatively low detection limit and quantitation limit, suitable for quantifying L-dopa in *Mucuna* seeds.

Quantitative results show that the studiedsamples have L-dopa marker content ranging from 5.20 ± 0.02% to 5.92 ± 0.10% by weight of dried seeds.

This result is consistent with published literature. Modi et al. [2], Behara et al. [20], and Raina et al. [1] estimated 5.60, 4.83, and 3.29-5.44%, respectively of L-dopa in *M. pruriens* seeds by HPTLC. In 2003, L. Capo-Chichi and colleagues researched and announced that the L-

dopa content in *Mucuna cochinchinensis* seeds in Colombia was 5.4% [21]. Research by Perumal Siddhuraju in 2001 showed that the L-dopa content in the seeds of *Mucuna pruriens* var. *utilis* was 4.96% [22]. Bhumika G. Rathod and colleagues in 2014 developed a method and determined the L-dopa content in *Mucuna pruriens* from 3.84 - 5.095% [15]. In 2016, author Deokar G. and his colleagues researched and published the L-dopa content in *Mucuna pruriens* seeds quantified by the HPLC method with a content ranging from 3.9 - 6.2% [23]. According to Patil, the L-dopa content in *Mucuna sanjappae* seeds reached 7.3% [24]. A study by Fernandez-Pastor and colleagues in 2019 showed that the amount of L-dopa in the seeds of *Mucuna pruriens* ranged from 3.0-3.2% [25].

The L-dopa content in *Mucuna* seeds depends on the species and collection area. However, our results showed that *Mucuna pruriens* seeds in Vietnam have quite high concentrations, up to more than 5%. This is interesting information, suggesting that *Mucuna pruriens* seeds in Vietnam can be a potential source of raw materials to provide L-dopa naturally for humans. These can be considered initial results, providing important suggestions for further research to develop the medicinal source of *Mucuna pruriens* seeds in Vietnam.

4. Conclusion

In this study, the method for quantifying L-dopa in *Mucuna* seeds by HPLC has been developed and validated, showing that the method is selective, specific, accurate, and suitable for the quantification of L-dopa in *Mucuna* seeds. In addition, the quantitative results showed that the *Mucuna pruriens* seeds samples collected at Bac Giang province had L-dopa content ranging from 5.20 ± 0.02% to 5.92 ± 0.10% by weight of dried.

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ANTI-AGGREGATORY AND ANTI-COAGULANT ACTIVITIES OF *POLYGONUM CUSPIDATUM* EXTRACTS

Le Hong Luyen*, Nguyen Thi Van Anh

University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology, Hanoi, Vietnam

*Corresponding author: le-hong.luyen@usth.edu.vn

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Summary

Anti-Aggregatory and Anti-Coagulant Activities of *Polygonum cuspidatum* Extracts

Polygonum cuspidatum, also called Cốt khí củ in Vietnam, is a well-known traditional plant with many approved pharmacological effects. In the current study, various extracts from *P. cuspidatum* rhizomes were tested for their anti-thrombotic activity on human blood for the first time. The platelet aggregation triggered by collagen was measured in human platelet-rich plasma. The blood clotting time was determined using three factors of the coagulation system: activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT). The results showed that the ethyl acetate extract (PC-EA) displayed the most potent inhibitory effect against collagen-induced platelet aggregation, with the percentage of inhibition increasing from 28.03% at 0.2 mg/mL to 82.38% at 0.8 mg/mL. Moreover, this extract also expressed a more potent anti-coagulant activity than the *n*-hexane and ethanol extracts ($p < 0.05$). In addition, PC-EA was the only sample that prolonged the blood clotting time in a dose-dependent manner for both APTT and TT assays. However, this extract could slightly extend the PT at 0.8 mg/mL with 13.57 ± 0.48 s compared to 12.13 ± 0.17 s of DMSO 0.1% used as the negative control ($p < 0.05$). The ethanol fraction from 0.4 mg/mL possessed considerably higher APTT and TT values than the negative control, meanwhile, the *n*-hexane display no effects on the blood coagulation by any pathways. In conclusion, the ethyl acetate was the most active fraction from *P. cuspidatum*. Therefore, this extract could provide a source of promising phytoconstituents used in further prevention and treatment of thrombosis-associated diseases.

Keywords: *Polygonum cuspidatum, Anti-aggregatory activity, Anti-coagulant activity, Thrombosis.*

1. Introduction

The World Health Organization (WHO) stated that cardiovascular diseases (CVD) are the leading cause of death globally [1]. Thrombosis, or the formation of blood clots in blood arteries, is the main source of CVD [2]. The main antithrombotics include antiplatelet drugs aiming to reduce platelet aggregation and anticoagulant agents focusing on the suppression of blood clot development. Conventional antithrombotic medications are powerful, however, also linked to several negative consequences [3]. Therefore, seeking new effective antithrombotic agents from natural resources with fewer side effects is required for further treatment of CDV.

Polygonum cuspidatum Sieb. *et* Zucc (*P. cuspidatum*), belonging to the family Polygonaceae, is a well-known medicinal plant largely growing in many Asia countries. In the literature, many pharmacological effects *in vitro* and *in vivo* of this plant have been approved including the anti-inflammatory effect [4], anticancer activity [5], neuroprotective effect [6], and hepatoprotective effect [7]. Moreover, previous studies documented that several polyphenolic compounds such as resveratrol, polydatin, quercetin, emodin, and their derivatives were the key active phytoconstituents in *P. cuspidatum* [8]. A few reports on the cardioprotective action of polydatin have been published, justifying its ability to prevent heart failure and cardiac hypertrophy caused by