In the control group without LPS or compounds, NO accumulation increased approximately 9-fold after incubation for 24 hours. When compounds **3** and **7** were tested at concentrations of 1, 3, 10, and 30 μM, they reduced the nitrite accumulation in a dose-dependent manner following LPS-stimulated RAW 264.7 cells (Fig. 2). This finding further highlights the anti-inflammatory effect of compounds **3** and **7**, as they effectively inhibited NO production even in the presence of LPS-induced inflammatory stimulation.

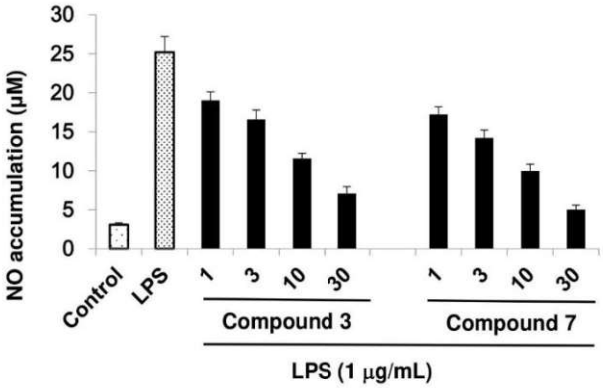

Fig. 2. Inhibition of LPS-induced NO production in RAW 264.7 cells by compounds **3** and **7**. Control values were recorded in the absence of both the compounds and LPS. The results are presented as the mean ± SD of triplicates (n = 3).

4. Conclusion

Seven compounds (**1**–**7**) were isolated from the fruiting body of *A. pantherina*. Their chemical structures were determined by NMR data and compared with the literature. The anti-inflammatory activity of the isolates (**1**–**7**) was evaluated by inhibiting LPS-induced NO production in macrophage RAW 264.7 cells. Compound **7** showed the most potent inhibitory activity on NO production with an IC_{50} value of 33.4 μM, followed by compound **3** with an IC_{50} value of 48.2 μM. Compounds **1**, **2**, and **4** showed IC_{50} values of 94.8, 64.9, and 62.4 μM, respectively, while **5** and **6** were inactive (IC_{50} > 100 μM). This is the first time compounds **3** and **7** have been evaluated for their inhibitory effects on NO production. In addition, compounds **2**, **3**, and **7** showed weak inhibitory activity against TNF-α with IC_{50} values of 83.9, 72.8, and 58.1 μM, respectively. The results suggested that *A. pantherina* and compounds **3** and **7** are promising candidates for further research and potential development as therapeutic agents targeting inflammatory processes.

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DETERMINATION OF STRYCHNINE AND BRUCINE BY HPLC FOR BETTER QUALITY CONTROL OF *STRYCHNI SEMEN*

Vu Ngan Binh[1], Pham Thi Thanh Ha[1], Dang Thi Ngoc Lan[1], Do Thi Bich Diep[1], Nguyen Quynh Chi[1], Do Quyen[1]*, Huynh Truc Thanh Ngoc[2], Nguyen Thi Ngoc Dung[2], Sysay Palamy[3], Chiobouaphony Phakeovilay[3]

[1]Hanoi University of Pharmacy, Vietnam; [2]Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam; [3]Faculty of Pharmacy, University of Health Sciences Laos

*Corresponding author: doquyen@hup.edu.vn

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Summary

Determination of Strychnine and Brucine by HPLC for Better Quality Control of *Strychni semen*

In the 5th Vietnamese pharmacopoeial monograph for *Strychni semen*, 19 species of the genus *Strychnos* are accepted as medicinal material, and the quality of *Strychni semen* varies significantly. In this study, a high-performance liquid chromatographic (HPLC) method is used to identify and quantify strychnine and brucine in 12 *Strychni semen* samples collected from different regions in Vietnam and Laos. Based on the alkaloid content in combination with the morphological properties of the analyzed samples, some comments on measures to improve quality control of *Strychni semen* were suggested for the Vietnam market.

Keywords: *Strychnine, Brucine, Strychni semen, HPLC, Quality control.*

1. Introduction

Strychni semen is a precious traditional medicine of Vietnam, which is included in Vietnamese pharmacopoeia (VP) [1]. This medicinal material was described in other pharmacopoeial monographs such as Pharmacopoeia of the People Republic of China 2020 [2], Hong Kong Chinese Materia Medica Standards - volume 10 (HK) [3], Taiwan Herbal Pharmacopoeia (TP) [4], Korean Pharmacopoeia X (KP) [5], European Pharmacopoeia 11.1 (EP) [6]. *Strychni semen* is made from ripe or dried seeds of the Strychni tree. However, both definitions and quality standards in the Vietnamese pharmacopoeia were different from the other pharmacopeias (Table 1). While in all other pharmacopoeial monographs, the definition of *Strychni semen* specified as seeds from only *Strychnos nux-vomica*, in Vietnamese pharmacopoeia, *Strychni semen* was defined as seeds from *Strychnos nux-vomica* and other *Strychnos* species. In Vietnam, there are 19 species of the genus *Strychnos*, including 16 species of vines trees, 1 species of a small tree, 2 species of *S. nux-incerica*; *S. nux-blanda*: large tree (stem diameter 20-40 cm). However, different species have significantly different strychnine and brucine contents, leading to variations in the quality of traditional medicine. This leads to difficulties in controlling the quality of *Strychni semen*.

Concerning quality standards, alkaloid content is the crucial criterion. Strychnine and brucine are the two most abundant alkaloids in *Strychni semen* and have an important influence on the effects and toxicity of this medicinal herb. The content limits of strychnine and brucine in different pharmacopoeias were listed in Table 1.

Table 1. Comparision of *Strychni semen* in different pharmacopoeias

Pharmacopoeia	Ref.	Strychnos species	Alkaloid content - analytical method
Vietnamese - VP	[1]	*Strychnos* species	≥ 1.2% total alkaloid (UV-Vis 262/300 nm)
Chinese - CP	[2]	*Strychnos nux-vomica*	1.20 - 2.20% strychnine; ≥ 0.8% brucine (HPLC)
Hongkong - HKS	[3]	*Strychnos nux-vomica*	1.20 - 2.20% strychnine; 0.69 - 1.60% brucine (HPLC)
Taiwan - TP	[4]	*Strychnos nux-vomica*	1.20 - 2.20% strychnine; ≥ 0.8% brucine (HPLC)
Korean - KP	[5]	*Strychnos nux-vomica*	≥1.05% strychnine (HPLC)
European - EP	[6]	*Strychnos nux-vomica*	≥1.5% (strychnine + brucine); 43-67% strychnine (HPLC)

Both strychnine and brucine content limits were mentioned in the quality standards of *Strychni semen* in Chinese, Hong Kong, Taiwan, and European Pharmacopoeias. In the Vietnamese and Korean Pharmacopoeias, only strychnine content was taken as quality control of this herbal medicine. Since the pharmacological effect and toxicity of strychnine and brucine are different, the

ratio between these two alkaloids was also important, and the upper content limit was also considered in some pharmacopoeias. In the Chinese, Hongkong, and Taiwan pharmacopoeias, the content of strychnine was controlled for both lower and upper limits, but in Vietnamese and Korean pharmacopoeias, only a lower limit was specified. Only the Hong Kong standards for medicinal material set both upper and lower limits for the two substances strychnine and brucine.

Another difference in Vietnamese pharmacopoeia compared to other pharmacopoeias was the analytical technique. High performance liquid chromatographic (HPLC) analysis was used for strychnine and brucine determination in these 5 pharmacopoeial monographs. Only in Vietnamese pharmacopoeia, UV-Vis spectrophotometric analysis was used. Although the dual-wavelength quantitative method in Vietnamese pharmacopoeia also enabled simultaneous determination of strychnine and brucine, the specificity and thus precision and accuracy of the UV-Vis method could not be compared to the HPLC method. On the other hand, due to the acceptance of many *Strychnos* species, the other components in *Strychnos* seeds might interfere with the results of the assay.

Because of these differences in *Strychni semen* specifications in Vietnamese pharmacopoeia compared to all other prestigious pharmacopoeias, it was suggested that the definition in Vietnamese pharmacopoeia of this herbal material should be revised in terms of *Strychnos* species specified and alkaloid content. However, the monograph can only be revised if more information was collected on the alkaloid content and ratio between strychnine and brucine content among different *Strychnos* species in Vietnam. In this study, strychnine and brucine content were determined in *Strychni semen* samples collected randomly on the market from different regions in Vietnam and compared to samples from China and Laos. Some interesting observations were obtained when comparing the alkaloid content among the *Strychni semen* samples from different species, and when correlating the alkaloid content with the morphological properties of the samples. Based on these observations, some suggestions were raised for better quality control of *Strychni semen*.

2. Materials and methods

2.1. Sample collection

Twelve samples were bought from the market in the north, center, and south of Vietnam, and one from Laos. A Chinese *Strychni semen* sample was bought from an official source (MS11), and a reference medicinal material (MS3) was obtained from the Vietnam National Institute of Drug Quality Control (NIDQC, Hanoi, Vietnam). Table 2 presented sample details and morphological characteristics, recorded with visual observation under normal daylight.

Table 2. Signs and shape characteristics of samples

Sample	Place	Sensory/ Color	Shape characteristics
MS1	DakLak, from market	white with brown patches	distorted, not round, ridged edges, concave inside
MS2	DakLak, collected on site		
MS3	Reference (*S. nux-vomica)*	grey, brown	evenly round, evenly concave
MS4	Nghe-An, from market	grey white, grey	evenly round, evenly concave
MS5	Hanoi, from company (*S. nux-vomica)*	grey white, grey	evenly round, evenly concave
MS6	Hochiminh, from company (*S. nux-vomica)*	many white seeds	round, clear edges
MS7	Hanoi, from market	grey, brown, white	round, oblong, irregular size
MS8	Binh-Dinh, from market	black, dark grey	small, only ¼ normal size
MS9	Lang-Son, from market	white, light brown	oblong seeds
MS10	Hanoi, from market	grey	round, quite even
MS11	China, from company (*S. nux-vomica)*	grey, white	evenly round, ridges around and concave in the middle
MS12	Laos (*S. blanda)*	white, light brown	oblong seeds

2.2. Chemicals and instrument

Strychnine reference standard (PRF1001259, 98.0%) was purchased from Biopurify Phytochemicals Ltd (Sichuan, P.R.China); brucine reference standard (CFS202102, 99.0%) was obtained from ChemFaces (Hubei, P.R.China). Acetonitrile and methanol (HPLC grade), as well as other chemicals at analytical

grade (sodium heptane sulfonate, potassium dihydro phosphate, phosphoric acid, chloroform), were purchased from Merck (Darmstadt, Germany). Analysis was done on the Agilent Technology 1200 series HPLC system with an DAD detector (Agilent, CA, USA).

2.3. Method for standard and sample preparation

Standard preparation

Stock reference standard solutions were prepared in methanol at 250 mg/L for brucine and 500 mg/L for strychnine. These stock solutions were diluted in methanol to obtain the necessary single and mixture standard solutions.

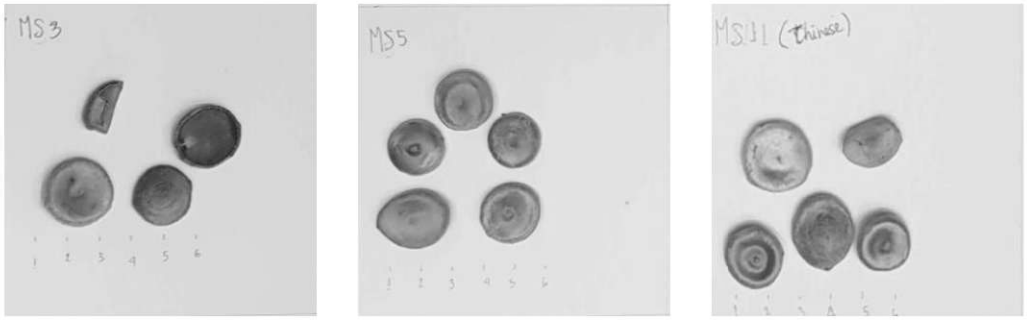
Sample preparation

About 0.6 g of Strychni semen powder was accurately weighed into a 50 mL conical flask with a ground stopper, followed by adding 3 mL of 0.1 M NaOH. After 30 minutes, 20 mL of chloroform was added to the conical flask. The entire conical flask was weighed (mass m1), put under reflux extraction for 2 hours and then weighed again. Chloroform was added to obtain the original mass (m1), if necessary. The obtained mixture was filtered through Whatman filter paper with anhydrous sodium sulfate, and the extract was collected into a 20 mL volumetric flask (A), and filled to volume with chloroform. Exactly 3 mL of the extract was transferred from the flask (A) into a 10 mL volumetric flask (B) and added to volume with methanol to obtain the test sample.

3. Research results

3.1. Morphological properties

All collected samples were identified as *Strychni semen* samples according to the morphological characteristics in Vietnamese pharmacopoeia. Since the samples were collected from the market, species could not be determined for all samples. Only samples bought from pharmaceutical companies or obtained from institutes were determined with species names, as shown in Table 2. Besides, the sample collected onsite from Daklak (MS2) was told to be from a different species than *S. nux-vomica*, but not specified which species. It was observed that samples from different species possessed some different morphological properties (colour, shape as shown in Table 2). Pictures of some typical samples were illustrated in Fig. 1 (the size was marked with a line and number in centimeters).



MS3- reference | MS5-Vietnamese *S. nux-vomica* (round shape) | MS11-Chinese

MS2-*unknown species* (not round) | MS8-*species unknown* (small size) | MS12-*S. blanda* - Laos (oblong)

Fig. 1. Pictures of some samples with different morphological properties

3.2. Strychnine and brucine determination by HPLC analysis

Different chromatographic conditions were experimented with, including those from Chinese pharmacopoeia (CP) [2], Hong Kong Chinese Materia Medica Standards (HKS) [3], and European pharmacopoeia (EP) [6]. The chromatographic conditions for the HPLC method in HKS and CP are similar, using a normal C18 column with an isocratic mobile phase containing ion-pairing reagent sodium heptane sulfonate. In the EP monograph, an end-capped ethylene-bridge C18 column with hybrid material was needed for the assay of strychnine and brucine. With the investigated results in terms of peak resolution, analysis time, and simplicity of the analytical procedure, the chromatographic conditions in Chinese pharmacopoeia were chosen.

Chromatographic conditions

An octadecylsilyl column (4.6 x 250 mm; 5 µm) was used as the stationary phase, with the mobile phase consisting of sodium heptane sulfonate 0.01 M in acetonitrile - potassium dihydrogen phosphate 0.02 M adjusted pH to 2.8 with 10% phosphoric acid (21 : 79, v/v) at a flow rate of 1 mL/min was used. Detection was done at 260 nm.

Method validation

The method was validated in terms of system suitability, selectivity, repeatability, and linearity before application to the collected samples. The national reference medicinal material (MS3) was used for method validation. The system suitability test was done according to the CP monograph. The number of theoretical plates calculated on the strychnine peak was 11000, higher than the required number according to CP (5000). For selectivity, in the MS3 sample, the peaks with retention time corresponding to strychnine and brucine in the reference standard solution were determined as strychnine and brucine peaks, with match ratio and peak purity of higher than 0.999 for both compounds, showing that the chromatographic separation was adequate for quantitation. For linearity, prepare standard solutions of strychnine and brucine in methanol. Carry out analysis with the selected conditions (results in Table 3). The results showed good linearity of strychnine and brucine peak area and concentration within the investigated range, with a correlation coefficient of higher than 0.999 for both compounds. For repeatability, one *Strychni semen* sample was analyzed 6 times separately, and the relative standard deviation (RSD%) was 1.91% and 2.10% for strychnine and brucine content in the sample, respectively. Therefore, the CP method was suitable for application in the *Strychni semen* samples in Vietnam.

Table 3. Results of calibration curve for strychnine and brucine determination

Linearity			
Strychnine		***Brucine***	
Concentration (µg/ml)	Peak area (mAU.min)	Concentration (µg/ml)	Peak area (mAU.min)
12.152	195.8	5.742	59.4
60.76	1033.5	28.71	328.5
121.52	2001.1	57.42	674.5
243.04	3949.3	114.84	1346.3
303.8	4871.2	143.55	1729.5
y = 16.005x + 37.378 (R = 0.9998)		y = 12.04x - 15.814 (R = 0.9996)	

Analysis of Strychni semen samples

The *Strychni semen* samples were prepared as described in section 2.3. Method for sample preparation and extract was analyzed using the selected chromatographic conditions. Results of strychnine and prucine content in the samples were presented in Table 4. These results were compared to Vietnamese pharmacopoeia (VP) limits, presented in Table 4 as complies (C) or not complies (NC) to pharmacopoeial standards. Samples were also compared to Chinese pharmacopoeia (CP), Hong Kong standard (HK), and Korean pharmacopoeia (KP) since the limits are different. Taiwan pharmacopoeia (TP) had the same limit as Chinese pharmacopoeia, and the European pharmacopoeia standard was meant for homoeopathic preparations, thus these two standards were not included. Since four samples (i.e. MS3, MS5, MS6, and MS11) were *S. nux-vomica*, the comparison to other pharmacopoeias was officially acceptable, and results were marked as capital letters C/NC in Table 4. Other samples (species unknown) were also compared to the other pharmacopoeia standards, but the unofficial results were noted in Table 4 in small letters, in italics. Sample MS12 specified as *S. blanda* was not evaluated according to other pharmacopoeias. To

look more closely at the alkaloid content, the ratio of strychnine/brucine content was also calculated in Table 4. Some typical complied and non-complied samples are presented in terms of chromatograms in Fig. 2, and as picture in Fig. 3, for better comparison.

Table 4. Strychnine and brucine content in *Strychni semen* samples

Sample	Total (%)	Strychnine (%)	Brucine (%)	Content ratio S/B	Complies to Pharmacopoeias			
					VP	CP	HK	KP
MS1	0.00270	0.00270	+	+	NC	*nc*	*nc*	*nc*
MS2	0.00274	0.00274	+	+	NC	*nc*	*nc*	*nc*
MS3	3.019	1.529	1.490	1.03	C	C	C	C
MS4	2.894	1.561	1.333	1.17	C	*c*	*c*	*c*
MS5	2.243	1.337	0.806	1.66	C	C	C	C
MS6	2.433	1.356	1.077	1.26	C	C	C	C
MS7	1.374	0.901	0.473	1.91	NC	*nc*	*nc*	*nc*
MS8	0.304	0.235	0.069	3.41	NC	*nc*	*nc*	*nc*
MS9	1.713	1.713	-	-	C	*nc*	*nc*	*c*
MS10	1.950	0.790	1.160	0.68	NC	*nc*	*nc*	*nc*
MS11	0.709	0.619	0.090	6.88	NC	NC	NC	NC
MS12	1.135	0.644	0.491	1.31	NC			
MS13	0.653	0.444	0.209	2.12	NC	*nc*	*nc*	*nc*

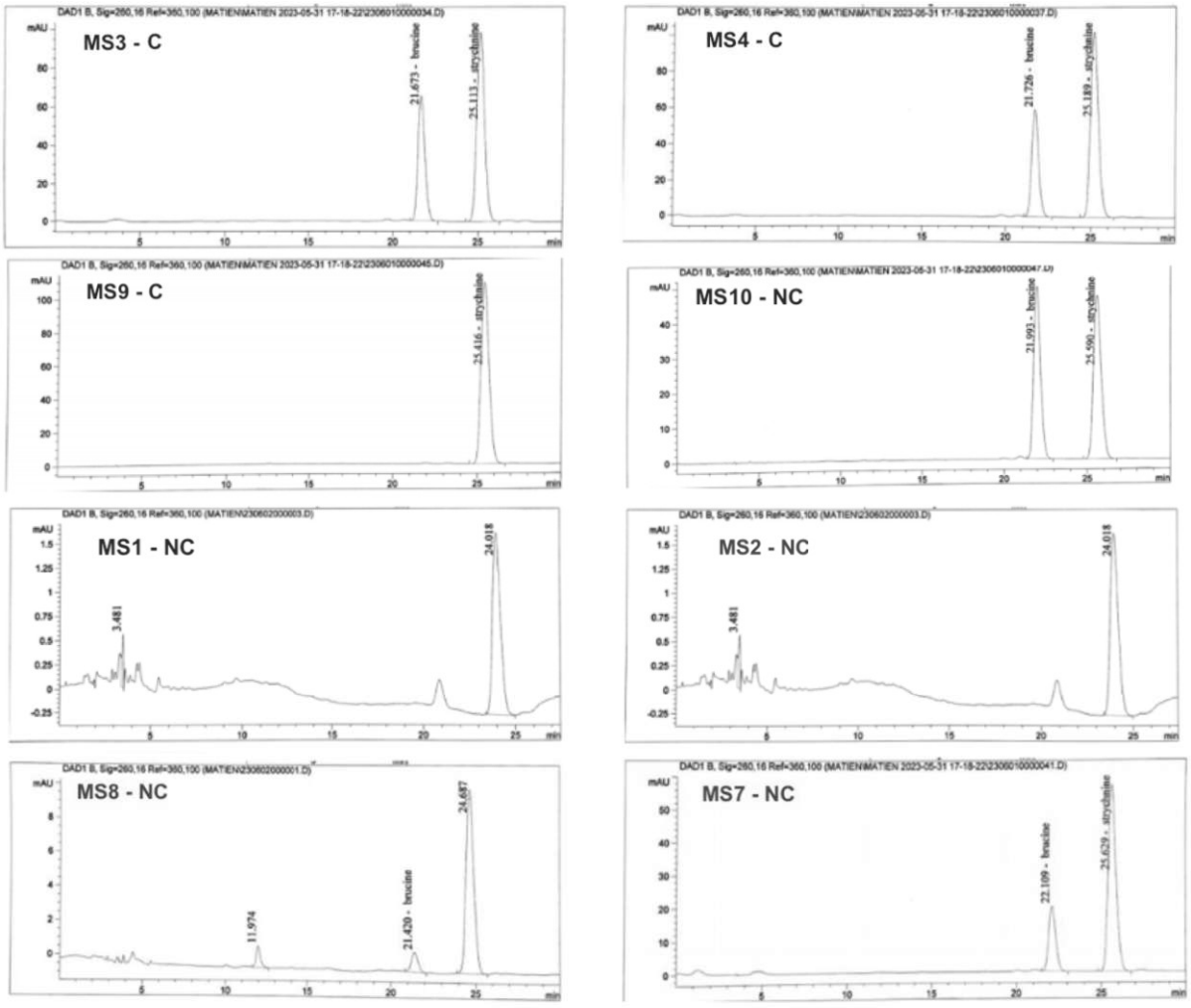


Fig. 2. Chromatograms of strychnine and brucine in *Strychni semen*

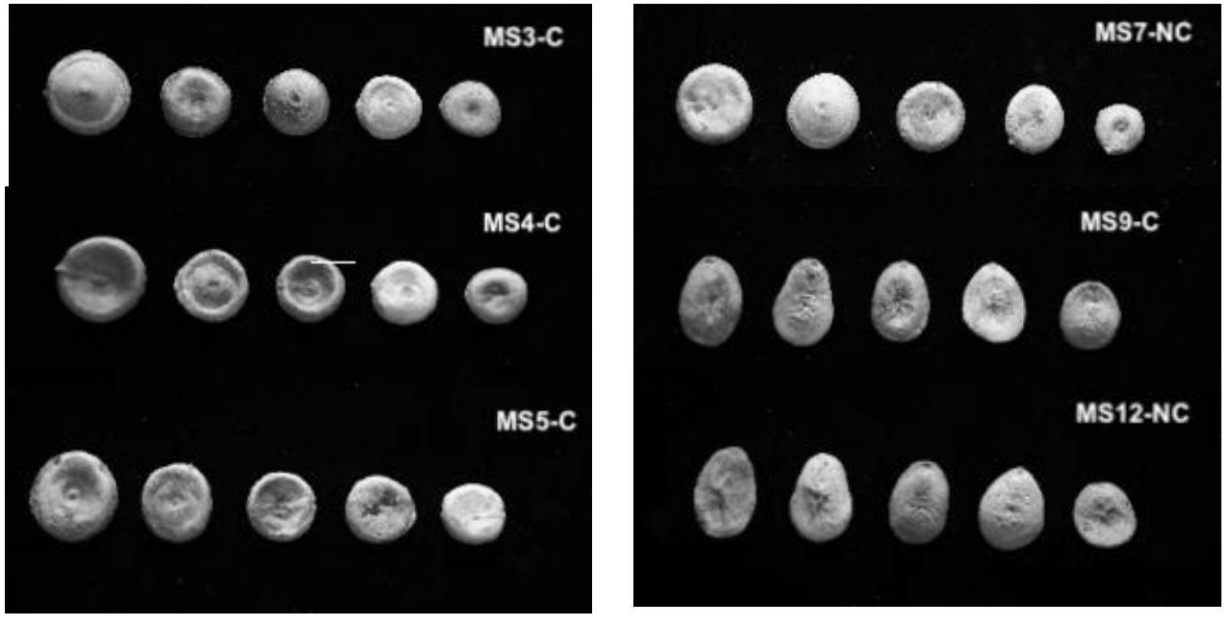


Fig. 3. Shape and size of some *Strychni semen* samples with alkaloid content complied (on the left) and non-complied (on the right) with standards

4. Discussion

Results in Table 4 showed that only 5/12 samples (5/10 Vietnamese samples) complied with Vietnamese Pharmacopoeia. These results again enforced the concern on quality control of *Strychni semen* samples on the market. In most cases, those complying with VP also complied with other pharmacopoeias and vice versa, except sample MS9, which will be discussed further later.

Comments on complied samples

Among 5 complied samples, 3 samples (MS3, MS5, and MS6) were specified as *S. nux-vomica*. Only one *S. nux-vomica* sample (MS11, from China) did not conform to the content limit, and this one also failed other pharmacopoeias' limits, too. Sample MS4 had the same morphological properties as the *S. nux-vomica* samples (similar size, shape, and colour of MS4 versus MS3 and MS5 in Fig. 3), and the strychnine/brucine content ratio was also similar to *S. nux-vomica* samples (chromatograms MS4 vs MS3 in Fig. 2, and values in Table 4). The last complied sample MS9 was not supposed to be *S. nux-vomica*. Firstly, it was the only sample that hardly contained brucine (Table 4). As observed in Fig. 2, the brucine peak was not detected on the chromatogram. Therefore, although the alkaloid content complied with VP and KP, but failed to comply with CP and HK (also failed with TP and EP). The morphological properties of MS9 were somewhat similar to MS12 (*S. blanda*), as shown in Fig. 3, but the S/B ratio was not similar. So MS9 might be from another *Strychnos* species.

Comments on non-complied samples

Some samples had very low alkaloid content: total content of strychnine and brucine < 0.5% for MS1, MS2, MS8, and brucine peaks in MS1 and MS2 were even not quantifiable. While collecting MS2 on-site in Daklak, it was even revealed that this *Strychni semen* sample was supposed to be from a different species than *S. nux-vomica*, and it usually possessed very low toxicity. Five samples showed very low total alkaloids (<1%), only <50% of the required total content for *S. nux-vomica* in CP, HK, and TP): MS1, MS2, MS8, MS11, and MS13. After processing, the alkaloid content would be even lower, efficacy could hardly be expected. Not only the total content was low, the S/B ratio for MS11 was much higher than the normal range for *S. nux-vomica* (0.64 to 1.56, calculated from the range of % strychnine 43-67% for *S. nux-vomica* stated in EP). These samples might be from other species.

Although MS11 was stated as *S. nux-vomica* (MS11), the assay result did not support this fact, because of the very high S/B ratio (6.88). This sample was imported from China from a pharmaceutical company. The appearance of this sample was totally fine (Fig. 1) with typical *S. nux-vomica* morphological properties. The low quality can only be discovered by an alkaloid content assay. This could be a typical example of the problem in Vietnam market has been facing for

many years: importing low-quality herbal materials.

Only 2 samples (MS7, MS10) with morphological properties similar to *S. vomica* (MS7 in Fig. 3) failed to meet the content requirement, but the total content of alkaloid was still higher than 1.2% (the limit for strychnine in VP), similarly to what observed with sample MS12 *S. blanda* (1.14%).

Comments on the Strychnus species mentioned in Vietnamese pharmacopoeia

Although the number of samples was very limited in this study, some observations could be obtained to support the suggestion that the diversified source of *Strychni semen* materials to all 19 *Strychnus* species in Vietnam had resulted in difficulties in controlling *Strychni semen* quality for users, buyers and even sellers of this herbal material. If the VP monograph limited the source of *Strychni semen* to only *S. nux-vomica* as other pharmacopoeias, it could be an economic loss, for sure. Because we could not exploit a variety of other *Strychnos* species that also contain a good amount of alkaloid. Therefore, it would be ideal forthe *Strychni semen* monograph to specify the names of the *Strychnos* species with high potential as herbal materials.

However, in order to reach this optimal solution, many other studies had to be performed to evaluate the economic as well as the scientific potential of the most important *Strychnos* species in Vietnam. Until that time, maybe the option of limiting the source would be a better choice for better quality control of *Strychni semen* as herbal material.

Comments on alkaloid content in Vietnamese pharmacopoeia

At present, all manufacturers of drugs and dietary supplements (that might contain *Strychni semen*) are required to run under GMP guidelines. The HPLC systems have become a common instrument in the laboratory of these manufacturers. Therefore, the UV-Vis method used in Vietnamese pharmacopoeia should be updated to the HPLC method. Apart from the HPLC methods from pharmacopoeias that were investigated in this study, other HPLC-DAD methods were also found in national [7] and international [8] publications. However, the HPLC method used in this study - the official method from Chinese pharmacopoeia - proved to be feasible for applications in Vietnam. It was a simple method, easy to perform, and provided repeatable results within an acceptable analysis time. The content limit was suggested to include both strychnine and brucine content in the requirements because the strychnine/brucine content ratio might bring along information on species.

5. Conclusion

In Vietnam, 19 *Strychnos* species are available throughout the country. Therefore the sources of materials for *Strychni semen* are diversified, and the quality of this herbal medicine also varies significantly. In the Vietnam Pharmacopoeia V, the monograph on *Strychni semen* only referred to the genus *Strychnos* in general. Hereby, we proposed to quality control only the *Strychnos nux-vomica* species in the Vietnamese Pharmacopoeia. In this study, 12 *Strychni semen* samples were collected from the north, the center, and south of Vietnam. The HPLC analysis of the strychnine and brucine content of the samples revealed the situation of low-quality samples on the market with a high ratio: of 5/10 Vietnamese *Strychni semen* samples did not comply with Vietnamese pharmacopoeial standards. Observations on the complied and non-complied samples also supported this proposal. However, since the number of samples was very limited in this study, this could only be considered as the first step for further studies supporting this proposal.

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