Hiruntad et al.

PHARMACOGNOSTIC SPECIFICATION AND QUANTITATIVE ANALYSIS OF STRYCHNINE AND BRUCINE IN STRYCHNOS NUX-VOMICA SEEDS

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Abstract: Strychnos nux-vomica L. (Strychnaceae) dried seeds have been used as medicinal and poisonous drug. The seeds which are hard shell and dish shape contain two major alkaloids i.e. strychnine and brucine. The quality specification of S. nux-vomica seeds as well as its strychnine and brucine contents in Thailand needed to be established. Crude drug samples from 15 sources throughout Thailand were collected. The pharmacognostic specification was determined according to WHO guideline. The seeds were exhaustively extracted in ethanol by Soxhlet apparatus. The contents of strychnine and brucine in the extracts were quantified by TLCdensitometric method using silica gel 60 GF_{254} as stationary phase and toluene: ethyl acetate: diethylamine (7: 2: 1 v/v) as mobile phase. Quantitative analysis of strychnine and brucine were detected at wavelength of 257 and 302 nm at hRf of 50 and 32.5 respectively. The pharmacognostic specification of S. nux-vomica seeds was established in average contents of water content, loss on drying, total ash, acid insoluble ash, water extractive matters and ethanol extractive matters as 8.08 ± 0.99 , 8.80 ± 1.31 , 1.20 ± 0.09 , 0.15 ± 0.06 , 13.12 ± 2.92 and 3.90 ± 1.31 1.83 g/100g of dry weight respectively. The contents of strychnine and brucine in dried seeds were found to be 1.03 ± 0.51 and 0.46 ± 0.28 g/100g respectively. TLC-densitometric method was demonstrated for its validity in terms of specificity, linearity, accuracy, precision, LOD-LOQ and robustness.

Keywords: Strychnos nux-vomica seeds, Strychnine, Brucine, TLC-densitometry, Strychnaceae

บทคัดย่อ: เมล็ดแห้งจากต้นแสลงใจ (Strychnos nux-vomica L.) มีการใช้เป็นขารักษาและขาพิษมาขาวนาน เมล็ดเปลือกแข็งรูปกระคุมนี้มี สารประกอบหลักเป็นอัลกาลอยด์ซึ่งพบว่ามีสารสตริกนินและสารบรูซีน การทำข้อกำหนดทางเกสัชเวทของเมล็ดจากด้นแสลงใจและปริมาณสาร สตริกนินกับสารบรูซีนจำเป็นค้องมีการก้นกว้าวิจัยขึ้นในประเทศไทย จึงเก็บตัวอย่างสมุนไพร is แหล่งจากทั่วประเทศไทขจัดทำข้อกำหนดทาง เกสัชเวทและเตรียมสารสกัดเอทานอลของเมล็ดแห้งโดยใช้วิธีชื่อกเลต วิเกราะห์ปริมาณสารสกัดสตริกนินกับบรูซีนด้วยวิธีโครมาโทกราฟฟีชนิด แผ่นบางและเด็นซิโทเมทรีโดยใช้แผ่น ซิลิกาเจล 60 GF₂₅₄ เป็นวัฏภาคงที่และมีตัวทำละลายไทลูอีน เอทิลอะซีเทต และไดเอทิลเอมีน ในอัตราส่วน (7: 2: 1) เป็นวัฏภากเกลื่อนที่ วิเกราะห์ปริมาณสารสดริกนินกับบรูซีนที่ hRF 50 และ 32.5 โดยใช้ก่าการดูดกลืนแสงที่ 257 และ 302 นาโนเมตร ตามลำดับ การจัดทำข้อกำหนดทางเกสัชเวทของเมล็ดจากด้นแสลงใจได้ก่าเฉลี่ยปริมาณน้ำ ปริมาณน้ำหนักที่หายไปเมื่อทำให้แห้ง ปริมาณเถ้ารวม ปริมาณเถ้าที่ไม่ละลายในกรด ปริมาณสารสกจัดวัยน้ำ และปริมาณสารสกดด้วยเอทานอลเท่ากับร้อยละ 8.08 ± 0.99, 8.80 ± 1.31, 1.20 ± 0.09, 0.15 ± 0.06, 13.12 ± 2.92 และ 3.90 ± 1.83 โดยน้ำหนัก ตามลำดับ ปริมาณของสารสดร์นินกับสารบรูซีนในเมล็ดแห้งพบว่ามีอยู่ร้อยละ 1.03 ± 0.51 และ 0.46 ± 0.28 โดยน้ำหนัก ตามลำคับ วิธีโครมาโทกราฟฟีชนิดแผ่นบาง-เด็นซิโทเมทรีที่ใช้วิเกราะห์หาปริมาณมีกวามเชื่อถือได้ในด้านความจำเพาะ ความสัมพันธ์เชิงเส้น ความแม่นยำ ความเที่ยง จึดจำกัดในการตรวจสอบ ขีดจำกัดในการวัดเชิงปริมาณและความดงทน กำสำคัญ: เมล็ดแสลงใจ, สตริกนิน, บรูซิน, โครมาโทกราฟฟีชนิดแผ่นบาง-เด็นซิโทเมทรี สิกเวทาซีอี

INTRODUCTION

Strychnos nux-vomica L. belongs to family Strychnaceae. The seed is called as "Kodka-kling" and appeared in traditional Thai medicine remedy for a long time. According to traditional Thai medical textbook, S. nux-vomica seed has been used to treat central nervous system diseases, numbness, and paralysis; furthermore, it has been used in combination with kod-ka-kra (roots of Anacyclus pyrethrum (L.) DC.) and kod-num-tao (rhizome of Rheum *palmatum* L.) as a Pikhad-kodpisate remedy for the treatment of oral cavity and oropharynx diseases, fever, menstrual disorders, hemorrhoids and wound from insect bite. Another studies reported pharmacological properties of S. nux-vomica seeds such as analgesics, antiinflammatory and anti-tumor (Chen et al., 2012; Deng et al., 2006). However, the standardization of S. nux-vomica seed and crude drug in Thailand has never been established. In addition, strychnine and brucine, the major alkaloids in S. nux-vomica seeds have been reputed to be poisonous substances and used as rodenticide (Philippe et al., 2004). Strychnine has been highly toxic to humans and used to be a self-poisoning (Lambert et al., 1981; Wood et al., 2002). Several chromatographic techniques have been demonstrated to quantify these two compounds (Choi et al., 2004; Marques et al., 2000; Rathi et al., 2008). This research aimed to investigate the pharmacognostic specification of S. nux-vomica seed and quantify the contents of two compounds by TLC-densitometric method.

MATERIALS AND METHODS

Sample collection

Fifteen samples of *S. nux-vomica* seeds were collected throughout Thailand (Bangkok, Buengkhan, Khonkaen, Lumpang No. 1 and 2, Nakhon Pathom, Nonthaburi, Phra Nakhon Si Ayutthaya, Phrae, Phuket, Ranong, Songkhla, Surat Thani, Ubon Ratchathani and Uttaradit). The samples were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi, Chulalongkorn University. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University.

Chemicals and materials

Standard strychnine was given by Assoc. Prof. Dr. Nijsiri Ruangrungsi. Brucine was purchased from Sigma-Aldrich, USA. Analytical grade toluene, ethyl acetate and diethylamine were purchased from RCL Labscan, Bangkok, Thailand. TLC Silica gel 60 GF_{254} (10x10 cm, Merck) plate was used as stationary phase.

Instrumentation

Densitometry was performed by a CAMAG TLC scanner 4, Lambda Scientific, Switzerland and free software of WinCATS 1.4.9. Microscopy was performed by Imager A2, Carl Zeiss model, Axio Lab, Germany.

Pharmacognostic specification of Strychnos nux-vomica seeds

S. nux-vomica seeds were visually inspected for sorting out any foreign matters, cleaned and dried. The macroscopic characters were recorded and photographed. The seeds were transverse sectioned, pulverized and investigated using the light microscope attached with digital camera. Photography and drawing were done by one of the authors (Y.H.). The anatomical and histological characters of the seeds were illustrated by drawing. Physicochemical evaluation of the crude drugs from 15 different sources throughout Thailand were proceeded in triplicate (n=3) including water content by azeotropic distillation technique, loss on drying under 105 $^{\circ}$ C, total ash and acid insoluble ash contents by furnace under 500 $^{\circ}$ C;

solvent extractive matters (water and ethanol by maceration). Data were calculated for grand mean and pooled SD. The fingerprints of the ethanolic extracts were performed by qualitative TLC using toluene: ethyl acetate: diethylamine (7: 2: 1 v/v) as mobile phase.

Quantitative analysis of strychnine and brucine by TLC-densitometry

The standard solutions were prepared in 95 % ethanol to the concentration of 0.08, 0.16, 0.24, 0.32, 0.4 mg/ml for strychnine and 0.04, 0.08, 0.12. 0.16, 0.2 mg/ml for brucine. Five grams of each crude powder were exhaustively extracted with 250 ml of 95 % ethanol by Soxhlet apparatus. The ethanolic extract was filtered and evaporated to dryness. The extract solutions of 2 mg/ml and 2.5 mg/ml were prepared in ethanol for strychnine and brucine analyses respectively. Five microliters of standard and extract solutions were spotted on the same plates and developed on the mobile phase of toluene: ethyl acetate: diethylamine (7: 2: 1 v/v). Densitograms of strychnine and brucine were obtained under the wavelength of 257 and 302 nm respectively. Method validation including specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness was determined according to the ICH guideline (ICH Harmonized Tripartite Guideline, 2005).

RESULTS AND DISCUSSION

S. nux-vomica is a tree up to 15 m tall; simple leaf, shiny and dark green in color, opposite, ovate 8 x 15 cm in size with 3-5 veins from the base; cymose typed - inflorescence flower with greenish-white to white in color; fruit, 3-5 cm in diameter, globose and smooth with orange peel when matured; 3-5 seeds with dish shape, 1-3 cm in diameter, light silvery-gray in color and hard shell covering with hairs (Figure 1).



Figure 1. (a) Branch of *Stryhnos nux-vomica* L. (b) Fruit (c) Seed (d) Dried seeds

Microscopic evaluation

The anatomical and histological characters of the seeds were illustrated in Figure 2 and 3 respectively. The cross section of the seed showed bent and twisted of lignified unicellular trichomes. The single layer of epidermal cells was large thick walled. The collapsed parenchymas were present as two flat layers. The endosperms consisted of cellulosic parenchymatous cells with hemicellulose and aleurone grains in the cell walls, plasmodesma stained between the walls and oil globules as small oil droplets (fixed oil) in the endospermic cells. The powders showed brown color pigments and fragment tissues as aforementioned (Khandelwal, 2011).



Figure 2. Transverse section of seed of *Strychnos nux-vomica*(1)Testa (2) Cavity (3) Hilum (4) Endosperm (5) Lignified trichomes (6) Epidermal cells (7) Collapsed parenchyma (8) Sclerenchyma (9) Plasmodesma (10) Oil globules



Figure 3. Histological characters of powder of seeds of *Strychnos nux-vomica*(1) Oil globules (2) Endosperm containing fixed oil and aleurone grains (3) Collapsed parenchyma (4) Unicellular trichomes rod (5) Basal lignified rod (6) Sclerenchymatous epidermis of testa in surface view (7) Testa and pigments

Physico-chemical properties

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The constant numbers due to the quality of S. nux-vomica seed crude drug were established as shown in Table 1. Water content, loss on drying, total ash and acid insoluble ash should be not more than 8.1, 8.8, 1.2 and 0.2 % by weight respectively. Water extractive value and ethanol extractive value should be not less than 13.1 and 3.9 % by weight respectively. The water content was less than reported in China which was 13.0 % by weight (Chinese pharmacopia commission, 2010). The result of loss on drying in this study was similar to the content reported in India as 7.8 % by weight (Bhati et al., 2012). The content of total ashes in India and China were 4.2 and 2.0 % by weight respectively (Bhati et al., 2012; Chinese pharmacopia commission, 2010) which higher than this result.

Table 1. Physico-chemical properties of Strychnos nux-vomica dried seeds

Parameter	Content (% by weight)
Water	8.076 ± 0.988
Loss on drying	8.800 ± 1.310
Total ash	1.204 ± 0.091
Acid insoluble ash	0.152 ± 0.056
Water soluble extractive value	13.122 ± 2.924
Ethanol soluble extractive value	3.896 ± 1.827

TLC fingerprint of ethanolic extract of S. nux-vomica seeds was illustrated in Figure 4. The solvent system of toluene: ethyl acetate: diethylamine (7: 2: 1 v/v) could separate two compounds clearly at hRf of 50 and 32.5 which looked the same as another mobile phase of chloroform: ethyl acetate: diethylamine (0.5: 8.5: 1 v/v) resulting in hRf 55 and 42 for strychnine and brucine respectively (Rathi et al., 2008).



Figure 4. TLC fingerprint of *Strychnos nux-vomica* seeds ethanolic extract Detection I = detection under UV 254 nmII = detection under UV 365 nmIII = detection with Dragendorff's reagent

Strychnine and brucine contents by TLC-densitometry

TLC-chromatograms of strychnine and brucine performed using mobile phase of toluene: ethyl acetate: diethylamine (7: 2: 1 v/v) and visualized under UV 254 were shown in Figure 5. Their densitograms under λ max of 257 and 302 nm respectively were shown in Figure 6. The calibration curves of strychnine and brucine showed linear equations of y = 5320.7x + 1897.5, R² = 0.9915 for strychnine and equations of y = 10200x + 1719.5, R² = 0.9923 for brucine as shown in Figure 7. Specificity of the method represented by peak identity and peak purity was expressed in Figure 8 and 9. The λ max of these two compounds are similar to the previous report as 254 and 301 nm for strychnine and brucine respectively (Merck & Co., 1952).



(a) (b) Figure 5. TLC chromatograms of 15 samples and standard (a) strychnine, (b) brucine under 254 nm



Figure 6. TLC densitograms of 15 samples and standard (a) strychnine and (b) brucine under 257 and 302 nm respectively.



Figure 7. The calibration curves of (a) strychnine and (b) brucine



Figure 8. Absorbance spectra of (a) strychnine and (b) brucine among standard and sample spots representing peak identity



Figure 9. Absorbance spectra of strychnine (a) and brucine (b) among peak start, peak apex and peak end of sample spots representing peak purity

This TLC-densitometric method was demonstrated for its validity (Table 2). The accuracy at three levels of spiking standard showed recovery of 113.3 %, 117.6 %, 116.1 % for strychnine and 97.9 %, 95.6 %, 91.7 % for brucine. The precisions were shown on % RSD of repeatability and intermediate precision. The robustness of deliberate variations in mobile phase ratio was low than 5 % RSD. LOD and LOQ were calculated from standard deviation of regression line and its slope (ICH Harmonized Tripartite Guideline, 2005).

	Validity				
Parameter					
	Strychnine	Brucine			
Accuracy (% Recovery)	113.30 - 117.58	91.66 - 97.94			
Repeatability (% RSD)	0.93 - 2.51	0.62 - 2.77			
Intermediate precision (% RSD)	0.07 - 7.20	1.41 - 3.10			
Limit of detection (µg/spot)	0.22	0.11			
Limit of quantitation (µg/spot)	0.68	0.32			
Robustness (% RSD)	2.99	1.01			

The yield of ethanolic extracts of *S. nux-vomica* seeds were 8.45 ± 3.17 g/100g of dry weight (Table 3). The contents of strychnine and brucine in the extracts and the crude drugs were 0.12 ± 0.01 g/g and 1.03 ± 0.51 g/100g for strychnine and 0.05 ± 0.00 g/g and 0.46 ± 0.28 g/100g for brucine. The data were shown in Table 3. These contents of two compounds in *S. nux-vomica* seeds were much less than the previous report from India as 2.80 ± 0.02 and 0.90 ± 0.02 g/100g for strychnine and brucine respectively (Dhalwal et al., 2007).

Source	Ethanolic extract yield in dried crude drug (g/100g)	Strychnine content (g/g extract)	Strychnine in S. nux-vomica seeds) g/100g of dried crude drug)	Brucine content (g/g extract)	Brucine in S. nux-vomica seeds (g/100g of dried crude drug)
1	6.56	0.14	0.93	0.05	0.30
2	10.71	0.13	1.38	0.05	0.56
3	10.47	0.12	1.21	0.06	0.65
4	9.79	0.12	1.20	0.07	0.64
5	14.54	0.13	1.92	0.07	1.03
6	7.89	0.13	1.01	0.07	0.52
7	4.88	0.12	0.56	0.04	0.22
8	12.52	0.14	1.73	0.06	0.79
9	12.67	0.14	1.78	0.06	0.80
10	5.19	0.11	0.59	0.04	0.23
11	8.40	0.12	0.99	0.05	0.38
12	6.70	0.12	0.81	0.04	0.29
13	4.82	0.08	0.39	0.03	0.13
14	6.51	0.09	0.58	0.03	0.19
15	5.08	0.07	0.34	0.03	0.13
Average	8.45 ± 3.17	0.12 ± 0.01	1.03 ± 0.51	0.05 ± 0.00	0.46 ± 0.28

 Table 3. The percent of strychnine and brucine contents in Strychnos nux-vomica seeds by

CONCLUSION

The pharmacognostic specification of *S. nux-vomica* seeds in Thailand were established. Two major toxic alkaloids, strychnine and brucine, were quantified by TLC densitometry was demonstrated as a simple, precise, specific and reliable technique.

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