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STANDARDIZATION OF *NELUMBO NUCIFERA* PLUMULE AND DETERMINATION OF NEFERINE CONTENT BY TLC-DENSITOMETRY AND TLC-IMAGE ANALYSIS

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Abstract: Plumule of *Nelumbo nucifera* Gaertn., a traditional Thai medicine, is primarily used for nervous disorder and high fevers. Neferine is the main alkaloid component in *N. nucifera* plumule and has been reported to possess several pharmacological properties. Standardization of plant materials is necessary for an assurance of quality, efficacy and safety based on various parameters. This study aimed to investigate the pharmacognostic specification of *N. nucifera* plumule and determine the neferine content using TLC-densitometry and TLC-image analysis. Transverse section of this crude drug showed the anatomical characteristics of epidermis, air chamber, parenchyma and vascular bundle. Additionally, the histological study of powder showed anomocytic stomata, parenchyma with starch granule and layer of cotyledons. TLC fingerprint and physicochemical constants were also established. The results of total ash, acid insoluble ash, loss on drying, moisture content, ethanol and water extractive values were found to be 3.86 ± 0.17, 0.54 ± 0.04, 9.91 ± 0.09, 10.02 ± 0.72, 16.54 ± 1.34 and 28.27 ± 2.01 g%, respectively. Moreover, TLC-densitometry and TLC-image analysis methods were validated for determination of neferine content in *N. nucifera* plumule. The calibration range was between 0.25-3.0 µg/spot and exhibited suitable accuracy, precision and robustness in both methods. Neferine content in *N. nucifera* plumule were 0.495 ± 0.186 g% by TLC-densitometry and 0.497 ± 0.189 g% by TLC-image analysis. Information obtained from this study can be used for the identification and standardization of *N. nucifera* plumule and also toward monograph development on this plant.

Keywords: *Nelumbo nucifera* plumule, Nelumbonaceae, Neferine, TLC-densitometry, TLC-image analysis

บทคัดย่อ: ดีบัวในเมล็ดบัวหลวงเป็นเครื่องยาสมุนไพรไทย ที่ใช้ในการรักษาความผิดปกติของระบบประสาทและภาวะมีไข้สูง เนเฟอรีน เป็นสารอัลคาลอยด์หลักที่พบในดีบัว และมีการรายงานว่ามีฤทธิ์ทางเภสัชเวทหลายอย่าง การกำหนดมาตรฐานพืชสมุนไพรมีความจำเป็นสำหรับการควบคุมคุณภาพ ประสิทธิศักย์ และความปลอดภัย การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อที่จะจัดทำข้อกำหนดทางเภสัชเวทของดีบัวและการประเมินปริมาณสารเนเฟอรีนด้วยวิธีทินเลเยอร์โครมาโทกราฟี-เดนซิโทเมทรี และการวิเคราะห์เชิงภาพโดยใช้โปรแกรม ImageJ การศึกษาภาคตัดขวางของเครื่องยาดีบัวพบลักษณะที่สำคัญได้แก่ เซลล์ผิว ช่องอากาศ พาเรนไคมา และท่อลำเลียง นอกจากนี้การศึกษาจุลกายวิภาคศาสตร์ของเนื้อเยื่อของผงยาดีบัวพบปากใบชนิด อะโนโมไซติก พาเรนไคมาที่มีเม็ดแป้ง ในการศึกษาได้มีการจัดทำลายพิมพ์องค์ประกอบทางเคมีและค่าคงที่ทางเคมีและกายภาพ ผลการศึกษาพบว่ามีปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด น้ำหนักที่หายไปเมื่อทำแห้ง ปริมาณน้ำ ปริมาณสารสกัดด้วยเอทานอลและสารสกัดน้ำมีค่าเท่ากับร้อยละ 3.86 ± 0.17, 0.54 ± 0.04, 9.91 ± 0.09, 10.02 ± 0.72, 16.54 ± 1.34 และ 28.27 ± 2.01 ของน้ำหนักแห้ง ตามลำดับ นอกจากนี้ได้มีการตรวจสอบความถูกต้องของวิธีการหาปริมานสารเนเฟอรีนในดีบัวด้วยวิธีทินเลเยอร์โครมาโทกราฟี-เดนซิโทเมทรี และวิธีการวิเคราะห์เชิงภาพโดยใช้โปรแกรม ImageJ โดยผลการทดสอบของทั้งสองวิธีพบว่าค่าความเป็นเส้นตรงของความเข้มข้นอยู่ระหว่าง 0.25-3.0 ไมโครกรัมต่อสปอต และ มีค่าความถูกต้อง ความเที่ยงตรง และความคงทน ที่อยู่ในเกณฑ์เหมาะสม การวิเคราะห์ปริมาณเนเฟอรีนด้วยวิธีทินเลเยอร์โครมาโทกราฟี-เดนซิโทเมทรีมีค่าเท่ากับ 0.495 ± 0.186 กรัมต่อ100 กรัม และด้วยวิธี การวิเคราะห์เชิงภาพโดยใช้โปรแกรม ImageJ มีค่าเท่ากับ 0.497 ± 0.189 กรัมต่อ100 กรัม ข้อมูลที่ได้จากการศึกษาในครั้งนี้ สามารถนำไปใช้ในการตรวจเอกลักษณ์และการกำหนดมาตรฐานของดีบัว และการพัฒนาข้อกำหนดในโมโนกราฟของพืชชนิดนี้ต่อไปในอนาคต

คำสำคัญ: ดีบัว, Nelumbonaceae, เนเฟอรีน, ทินเลเยอร์โครมาโทกราฟี-เดนซิโทเมทรี, การวิเคราะห์เชิงภาพโดยใช้โปรแกรม ImageJ

INTRODUCTION

Nelumbo nucifera Gaertn. is an aquatic plant and the one of only two species in the family Nelumbonaceae (Figure 1). It has many common names such as Indian lotus, Chinese water lily and sacred lotus, and synonyms such as *Nelumbium nelumbo*, *N. speciosa*, *N. speciosum* and *Nymphaea nelumbo* (Mehta, Patel, Patani and Shah, 2013) which commonly known as Bua-luang in Thailand. Different parts (leaves, flowers, rhizomes, fruits and seeds) of this plant has been reported to possess various medicinal values to treat a variety of conditions, including diarrhea, abnormal bleeding, poor digestion, fever, and insomnia (Dhanarasu and Al-Hazimi, 2013).

Figure 1. *Nelumbo nucifera* Gaertn.

The one important part of *N. nucifera* is seed which has been used in folk medicine for the treatment of tissue inflammation, cancer, skin diseases, leprosy, poison antidote and generally prescribed to children as diuretic and refrigerant (Pal and Dey, 2013). The seed of *N. nucifera* is composed of three parts including integuments (3.74%), plumule (3.03%) and cotyledons (93.23%) (Mehta, Patel, Patani and Shah, 2013). Plumule is found only in seed of *N. nucifera* and reported to have antioxidant activities (Dhanarasu and Al-Hazimi, 2013) and recognized as a cooling food with anti-inflammatory activities (Lin, Wu, Liu and Lai, 2006). Many studies revealed the major active constituents in plumule of *N. nucifera* and their biological activities such as several major alkaloid compounds (e.g. liensinine, isoliensinine and neferine) which showed a significant cytoprotective effect against oxidative stress (Xie, Guo, Zeng, Zhang, Zhang and Zheng, 2013). Two major compositions (neferine and isoliensinine) have extensive cardiovascular activity; antiarrhythmic, antithrombic and antihypertensive (Yang and Zhou, 2004), four alkaloids (lotusine, liensinine, isoliensinine and neferine) were determined in *N. nucifera* plumule and reported as major bioactive alkaloids (Yang, Li and Pan, 2012). As previous reported, neferine (Figure 2) is one of the major bisbenzylisoquinoline alkaloid in *N. nucifera* plumule which was used as an importance medicinal plants.

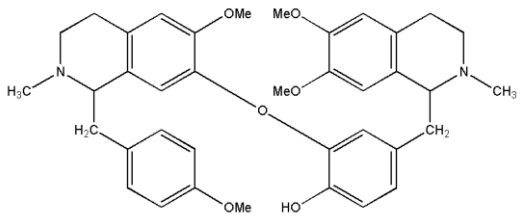

Figure 2. Chemical structure of neferine

Neferine content determination also has been reported by various methods such as TLC-scaning (Hu, Guo, Zhou, Luo and Cai, 1997), NMR spectroscopy (Yang and Zhou, 2004), LC–ESI-MS/MS (Chen, Fan, Wu, Wu and Mitchell, 2007), high-speed counter-current chromatography (Liu, Li, Liang, Qi and Wang, 2009). Many methods have been reported for determination of active constituent content in plant samples such as HPLC, TLC, GC, TLC-densitometer, TLC-image analysis. Among different methods, TLC-densitometry and TLC-image analysis have many advantages as low cost, rapid, easy (Tie-xin and Hong, 2008). In spite of the several medicinal health benefits of *N. nucifera* plumule but the basic information of the standardization parameters of this plant part is still limited. Therefore, the objective of this study is to investigate the standardization parameters of *N. nucifera* plumule and to determine the neferine content using TLC-densitometry and TLC-image analysis.

MATERIALS AND METHODS

Plant materials

Plant samples of *N. nucifera* plumule were collected from 15 various sources throughout Thailand and were authenticated by one of the authors (N.R.). A voucher specimen was deposited at the College of Public Health Sciences, Chulalongkorn University. All samples were air dried and pulverized after removal of foreign matters.

Chemicals

All chemicals used in this study were of analytical grade and standard neferine was purchased from Sigma-Aldrich, USA.

Morphological study

Morphological characters of *N. nucifera* plumule like shape, size, color, odor, taste and other visible properties were observed.

Microscopic study

Transverse section of *N. nucifera* plumule was observed under a microscope (Axio Image A2; Zeiss, Germany) to determine the anatomical characteristics. A small amount of *N. nucifera* plumule powders was heated with chloral hydrate and mounted over the glass slide in 50% glycerine and then observed its histological characteristics under alike microscope (Axio Image A2; Zeiss, Germany).

Physicochemical study

The physicochemical parameters including ethanol soluble extractives, water soluble extractives, moisture content, loss on drying, total ash and acid insoluble ash were evaluated from 15 samples of *N. nucifera* plumule powders in triplicate according to the WHO guideline on quality control method for medicinal plants materials with some modification (Yukongphan, Thitikornpong, Palanuvej and Ruangrungsi, 2013).

Thin layer chromatographic fingerprint

Five grams of *N. nucifera* plumule powders from each 15 samples were ethanolic extracted and then 20 ml was filtered and evaporated to dryness and redissolved in 1 ml of ethanol. Three microliters of ethanolic extract was applied on the silica gel G60 F_{254} TLC plate (20x10 cm; Merck, Germany). The plate was developed in a solvent system of toluene: ethyl acetate: diethylamine (7:2:1). The profile of TLC fingerprint was visualized under daylight, UV (254 nm and 365 nm) and sprayed with Dragendorff reagent.

Quantitative determination of neferine contents by TLC-densitometry and TLC-image analysis

N. nucifera plumule powders (5 g) were exhaustively extracted with 95 % ethanol using soxhlet apparatus. The extract was filtered and concentrated under rotary evaporator to dryness. The ethanolic extract was dissolved in ethanol to get the concentration of 40 mg/ml. The standard solutions of neferine were prepared in ethanol to obtain the concentration of 1 mg/ml. Three microliters of the extract and standard solutions (0.25, 0.5, 1.0, 2.0, 3.0 µg/spot) were applied on silica gel G 60 F_{254} TLC plates and developed in toluene: ethyl acetate: diethylamine (7:2:1). The migration distance was allowed up to 80 mm. Densitometric measurements were performed at 285 nm with CAMAG scanner III using WinCATS standard program. TLC-image analysis were carried out using ImageJ software.

Method validation

TLC-densitometry and TLC-image analysis methods for quantitative determination of neferine in *N. nucifera* plumule were developed and validated with respect to specificity, accuracy, precision, limits of detection (LOD), limits of quantification (LOQ), calibration range and robustness according to the ICH guideline (ICH, 2005).

RESULTS AND DISCUSSION

Standardization of plant materials by evaluation of pharmacognostic parameters including microscopic characteristics, microscopic characteristics, physicochemical constants and TLC fingerprint is important for identification, authentication, detection of adulteration and also assurance of quality control of crude drugs.

The plumule of *N. nucifera* is useful in traditional medicine for the treatment of some ailments, so it is important to standardize this plant part for safety and efficacy use as a medicinal plant material. In this study, microscopic evaluation including transverse section of *N. nucifera* plumule and powder study revealed the presence of various important characteristics of this plant. On the basis of microscopic aspects, the presence of these characters can serve as useful parameters for the identification of *N. nucifera* plumule. Microscopic study of medicinal plants materials is necessary for the identification of the broken and powdered materials (Bele and Khale, 2011).

Morphological study

Plumule of *N. nucifera* is the green germ of a mature seed, located between of two cotyledons, 1-1.4 cm long, from its radicle stretching two unequal clavate arms forming an arrow-shaped structure (Figure 3).

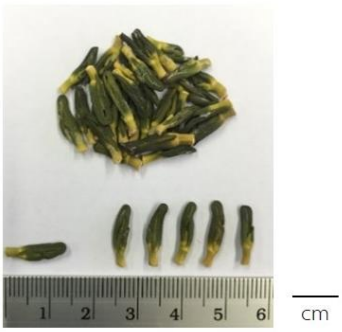

Figure 3. Dried plumule of *N. nucifera*

Microscopic study

Transverse section of *N. nucifera* plumule showed an important characteristics as present in Figure 4.

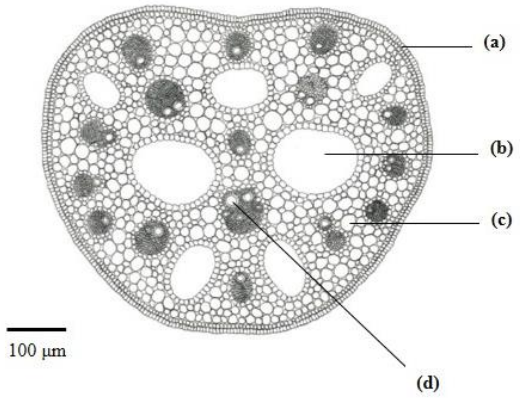

Figure 4. Transverse section of *N. nucifera* plumule transverse section: Epidermis **(a)**; Air Chamber **(b)**; Parenchyma **(c)**; Vascular bundle **(d)**

Powder study

The powder microscopy showed the present of anomocytic type stomata, parenchyma surface with starch granule, starch granule (round shape), starch granule (hilum shape), layer of cotyledons in surface view (Figure 5).

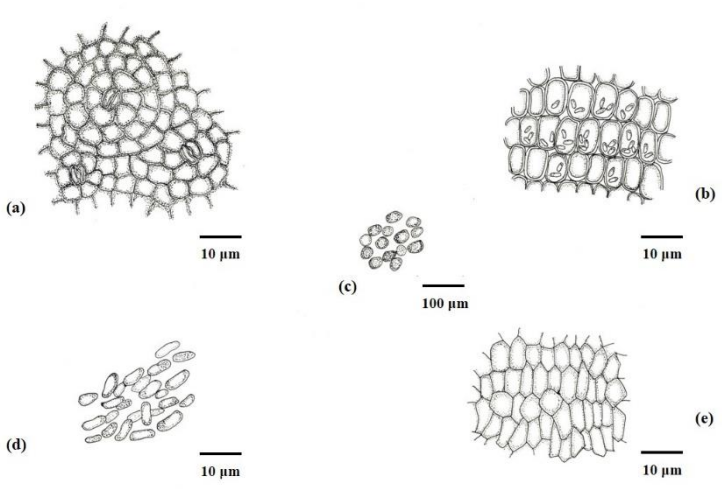

Figure 5. Histological character of *N. nucifera* plumule powders: anomocytic type stomata **(a)**; Parenchyma surface with starch granule **(b)**; Starch granule (round shape) **(c)**; Starch granule (with hilum) **(d)**; Layer of cotyledons in surface view **(e)**

Physicochemical study

Physicochemical parameters could be useful for the quality control and quality assurance of plant materials. The total ash is particularly important in the evaluation of purity of plant sample. Alcohol and water soluble extractive values were determined to find out the amount of water and alcohol soluble components. In present study, the moisture content of *N. nucifera* plumule is not too high (10.02 ± 0.72%w/w), thus it could discourage bacterial,

fungi or yeast growth, as the general requirement for moisture content in crude is not more than 14%w/w (Chumbhale and Upasani, 2012). In previous studies, physicochemical properties of the other parts of *N. nucifera* such as moisture content (10.50 %w/w) of *N. nucifera* seed (Pal and Dey, 2013), total ash (9.03±0.02); acid insoluble ash (1.28±0.23); alcohol extractive value (2.12±0.04); water soluble extractive value (16.45±0.54) of *N. nucifera* leaves (Srivastava, Lal and Pant, 2012) were also reported. The physicochemical parameters of *N. nucifera* plumule were presented in Table 1.

Table 1. Physicochemical specifications of *N. nucifera* plumule

Parameter	**Contents* (%w/w) Mean ± SD***
Total ash	3.86 ± 0.17
Acid insoluble ash	0.54 ± 0.04
Loss on drying	9.91 ± 0.09
Water soluble extractives	28.27 ± 2.01
Ethanol soluble extractives	16.54 ± 1.34
Moisture	10.02 ± 0.72

*Results were expressed as grand mean ± pooled SD from 15 samples in triplicate

Thin layer chromatographic fingerprint

The TLC fingerprint profile of *N. nucifera* plumule using toluene: ethyl acetate: diethylamine (7:2:1) was shown in Figure 6. The TLC fingerprint profile of compounds present in plant materials is useful in standard setting up for evaluating the quality and purity as well as screening analysis of plant materials. The advantages of TLC are its simplicity, versatility, high velocity, specific sensitivity and simple sample preparation. Thus, TLC is a convenient method of determination the quality and possible adulteration of plant materials (Mauji, Abdin, Khan Mather and Prabhakar, 2011). In this study, TLC was used to construct the fingerprint of *N. nucifera* plumule which exhibited clear pattern of compounds in all investigations; under visible light, UV 254 nm, UV 365 nm and after spraying with Dragendorff reagent.

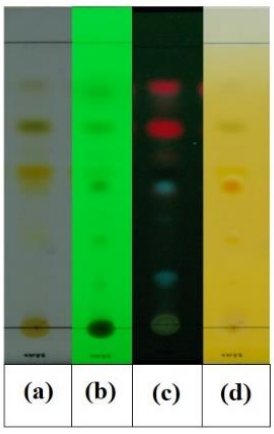

Figure 6. TLC fingerprint of ethanol extract of *N. nucifera* plumule under daylight **(a)**, UV 254 nm **(b)**, UV 365 nm **(c)**, under daylight after spraying with Dragendorff reagent **(d)**

Quantitative determination of neferine content by TLC-densitometry and TLC-image analysis

The yield of ethanolic extract by soxhlet extraction and neferine contents determination by 2 methods (TLC-densitometry and TLC-image analysis) of 15 samples of *N. nucifera* plumule from different locations in Thailand were presented in Table 2 and TLC chromatograms were shown in Figure 7. At present, several studies have been reported on the determination of neferine in both quantitative and qualitative aspects such as TLC, HPLC and GC-MS. Until now, the TLC densitometric method and TLC image analysis method have not been reported of the method validation for determination of neferine.

Table 2. The amount of neferine in *N. nucifera* plumule by TLC-densitometry and TLC-image analysis

Source	Extract yield (g% of dried crude drug)	Neferine content by TLC-densitometry (g% of dried crude drug)	Neferine content by TLC-Image analysis (g% of dried crude drug)
1	44.12	0.69 ± 0.01	0.76 ± 0.06
2	38.30	0.62 ± 0.04	0.61 ± 0.09
3	39.47	0.52 ± 0.04	0.51 ± 0.06
4	40.97	0.84 ± 0.03	0.85 ± 0.05
5	41.55	0.71 ± 0.05	0.66 ± 0.09
6	40.27	0.57 ± 0.03	0.51 ± 0.06
7	39.08	0.25 ± 0.03	0.29 ± 0.04
8	36.14	0.25 ± 0.02	0.29 ± 0.01
9	38.53	0.50 ± 0.01	0.55 ± 0.11
10	41.88	0.64 ± 0.04	0.69 ± 0.06
11	38.56	0.38 ± 0.01	0.37 ± 0.02
12	37.49	0.40 ± 0.02	0.37 ± 0.02
13	36.61	0.30 ± 0.04	0.27 ± 0.07
14	44.71	0.51 ± 0.05	0.47 ± 0.06
15	41.39	0.25 ± 0.01	0.25 ± 0.01
Average		0.495 ± 0.186	0.497 ± 0.189

*Samples were from 15 different sources throughout Thailand. Each sample was performed in triplicate.

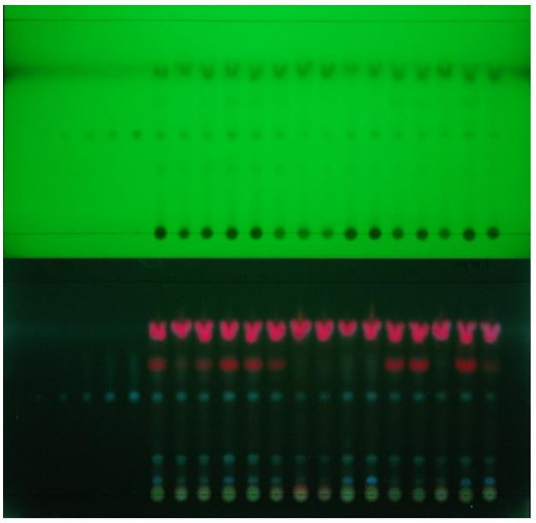

Figure 7. TLC chromatogram developed with toluene: ethyl acetate: diethylamine (7:2:1)
(a) detection under UV 254 nm
(b) detection under UV 365 nm

Method validation

The λmax of neferine was found at 285 nm. Method validity with respect to specificity, accuracy, precision, LOD, LOQ, calibration range and robustness according to the ICH guideline were shown in Table 3. In this study, TLC-densitometric method and TLC-image analysis method were developed and validated for the identification and quantification of neferine content in *N. nucifera* plumule samples. The overall results of the accuracy of TLC-densitometry and image analysis showed best recoveries (99.61 ± 5.34 and 103.74 ± 7.19 %), indicated that the methods were accurate. The results of repeatability (9.48 ± 3.61 and 2.99 ± 1.22%RSD), intermediate precision (8.52 ± 2.81 and 2.63 ± 1.15%RSD) and robustness for mobile phase variation (5.52 and 7.44%RSD) indicated that the method were precise and robust. These results can indicated that the propose methods are precise, accurate and robust for determination of neferine content in *N. nucifera* plumule. Moreover the methods are simple, rapid and economic which can be used for analysis in quality control of *N. nucifera* plumule.

Table 3. Method validation of TLC-densitometry and TLC-image analysis of neferine in *N. nucifera*

Parameter	TLC-densitometry	TLC-image analysis
λmax (nm)	285	285
Range (μg/spot)	0.25 - 3.0	0.25 - 3.0
Regression equation	$Y = -1137.3x^2 + 8041.2x + 1155.9$	$Y = -1214.8x^2 + 21048x - 2452.2$
R^2	0.9992	0.9997
LOD (μg/spot)	0.07	0.07
LOQ (μg/spot)	0.20	0.22
Accuracy (% Recovery)	99.61 ± 5.34	103.74 ± 7.19
Precision Repeatability(%RSD) Intermediate precision (%RSD)	 9.48 ± 3.61 8.52 ± 2.81	 2.99 ± 1.22 2.63 ± 1.15
Robustness (%RSD)	5.52	7.44

CONCLUSION

The present study was undertaken with an aim of pharmacognostic standardization of *N. nucifera* plumule and method validation of TLC-densitometry and TLC-image analysis for determination of neferine content in this plant. The finding generated from this study would be useful in quality assurance and also for preparation of monograph on the *N. nucifera* plumule.

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REFERENCES

Bele AA, Khale A. 2011. Standardization of herbal drugs: An overview. *IRJP*. 2(12): 56-60.

Chen Y, Fan G, Wu H, Wu Y, Mitchell A. 2007. Separation, identification and rapid determination of liensine, isoliensinine and neferine from embryo of the seed of *Nelumbo nucifera* GAERTN. by liquid chromatography coupled to diode array detector and tandem mass spectrometry. *J Pharm Biomed Anal.* 43: 99-104.

Chumbhale DS, Upasani CD. 2012. Pharmcognostic standardization of stems of *Thespesia lampas* (Cav.) Dalz&Gibs. *Asian Pac J Trop Biomed.* 2(5): 357-363.

Dhanarasu S, Al-Hazimi A. 2013. Phytochemistry, Phamacological andtherapeutic applications on *Nelumbo nucifera*. *AJPCR.* 1(2): 123 - 136.

Hu X, Guo Z, Zhou B, Luo S, Cai H. 1997. Quantitative determination of neferine in Plumula Nelumbinis by thin layer chromatography scanning. *Zhongguo Zhong Yao Za Zhi.* 22(1): 41-42.

ICH Harmonised Tripartite Guideline 2005. *Validation of analytical procedures: text and methodology Q2 (R1).* Geneva: ICH Press.

Lin JY, Wu AR, Liu CJ, Lai YS. 2006. Suppressive Effects of Lotus Plumule (*Nelumbo nucifera* Geartn.) Supplementation on LPS-Induced Systemic Inflammation in a BALB/c Mouse Model. *JFDA.* 14(3): 273-278.

Liu S, Li XZ, Liang YZ, Qi LF, Wang B. 2009. Preparative separation and purification of liensinine, isoliensinine and neferine from seed embryo of *Nelumbo nucifera* GAERTN using high-speed counter-current chromatography. *J Sep Sci*. 32(14): 2476-2481.

Mauji R, Abdin M.Z, Khan Mather A, Prabhakar J. 2011. *HPTLC fingerprint analysis: A quality control of authentication of herbal phytochemicals*. Springer Verlag Berlin Heidelberg.

Mehta NR, Patel EP, Patani PV, Shah B. 2013. *Nelumbo Nucifera* (Lotus): A review on ethanobotany, phytochemistry and pharmacology. *IJPBR.* 1(4): 152-167.

Pal I, Dey P. 2013. A review on lotus (*Nelumbo nucifera*) seed. *IJSR*. 4(7): 1659-1666.

Srivastava S, Lal VK, Pant KK. 2012. Preliminary screening of some of the emerging antidiabetic plants for quality control. *Int J Pharm Pharm Sci.* 4(3): 331-333.

Tie-xin T, Hong W. 2008. An image analysis system for thin-layer chromatography quantification and its validation. *J Chromatogr Sci.* 46: 560-564.

Xie Y, Guo ZB, Zeng SX, Zhang LT, Zhang Y, Zheng BD. 2013. Protective effects of alkaloid compounds from Nelumbinis Plumula on tert-butyl hydroperoxide-induced oxidative stress. *Molecules*. 18(9): 10285-10300.

Yang G, Li W, Pan Y. 2012. Rapid simultaneous detection of four alkaloids in lotus plumule by CZE with ephedrine hydrochloride as an internal standard. *Chromatographia*. 75: 1295-1300.

Yang J, Zhou K. 2004. NMR spectroscopic analysis of neferine and isoliensinine. *Magn Reson Chem.* 42: 994-997.

Yukongphan P, Thitikornpong W, Palanuvej C, Ruangrungsi N. 2013. The pharmacognostic specification of *Ardisia elliptica* fruits and their embelin contents by TLC image analysis compared to TLC densitometry. *BHST*. 11: 21-28.