Original Article

Quantitative analysis of a phenanthrene from *Eulophia* species by TLC-image analysis method

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Abstract

A phenanthrene constituent in *Eulophia macrobulbon* (E.C.Parish & Rchb.f.) Hook.f., 1-(4'-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (**1**) was previously reported as a phosphodiesterase 5 inhibitor. A simple and rapid thin layer chromatographic (TLC)-image analysis method was developed to quantify this compound in plant samples. Chromatographic separation of the phenanthrene was completed on silica gel TLC using dichloromethane–methanol (96:4, v/v) as a mobile phase. Image analysis of the scanned TLC plate was performed by Photoshop CS6 to quantify the amount of phenanthrenes. The area under the spot corresponding to **1** was integrated and used for quantification. The method was validated by the detection limit, calibration curve, repeatability, reproducibility, and recovery. The results showed that our digital imaging method was applicable for quantification of **1** on TLC. The method was convenient, efficient, and moderately accurate for the quantitative analysis of **1** in plant samples.

Keywords: TLC-image analysis, phenanthrenes, PDE5 inhibitors, *Eulophia macrobulbon*

1. Introduction

Erectile dysfunction (ED) or impotence is the persistent inability to attain and maintain an erection sufficient to permit satisfactory sexual performance (Hatzimouratidis & Hatzichristou, 2010). ED has been recognized as a serious problem in men worldwide which has prompted the search for medicines and especially for alternative treatments based on herbal plants in many countries including Thailand (Wylie & Kenney, 2010). Phosphodiesterase 5 inhibitors are considered to be the first line of therapy in the management of ED. However, it may produce many side effects such as nausea,

headache, and cutaneous flushing (Hatzimouratidis & Hatzichristou, 2007). Thus, the interest in natural substances that cause fewer side effects is increasing (Gurib-Fakim, 2006).

Eulophia macrobulbon (E.C.Parish & Rchb.f.) Hook.f. is a plant belonging to Orchidaceae family. It was recently reported to exhibit PDE5 inhibitory activity. A PDE5 inhibitor was isolated and identified as 1-(4'-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (**1**) (Temkitthawon *et al.,* 2017) (Figure 1). For further research and development of *E. macrobulbon* or other *Eulophia* spp., an analytical method to quantify **1** is needed. Several methods to quantify phenanthrene compounds, including HPLC-DAD, (Yan *et al.,* 2016) and LC-MS (Tóth *et al.,* 2016) have been described. Even though these methods claimed to have high sensitivity and specificity, the analytical instruments are quite costly (Phattanawasin *et al.,* 2012). Recently, thin-layer chromato-

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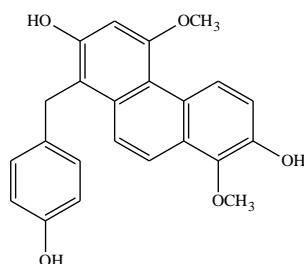*Corresponding author
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Figure 1.   Structure of 1-(4'-hydroxybenzyl)-4,8-dimethoxyphenan-threne-2,7-diol (compound **1**).

graphy (TLC) has been applied as a simple, inexpensive, and rapid method for quantitative analysis (Phattanawasin *et al.,* 2012) in combination with a simple digital camera and image analysis software. The TLC-image analysis method can be used for quantitative assays with good accuracy and precision (Hong, 2008). The aim of this study was to develop an accurate, reproducible and convenient TLC-image analysis method for a rapid determination and quantitative analysis of compound **1** in *E. macrobulbon* and other *Eulophia* spp. The proposed method was validated based on the ICH Q2(R1) guidelines (ICH Guideline. 2005).

2. Materials and Methods

2.1 Plant materials

Four *Eulophia* spp. were collected from Prachinburi Province, Thailand. Voucher herbarium specimen numbers of *E. andamanensis* Rchb.f. (PNU 4300), *E. graminea* Lindl. (PNU 4301), *E. macrobulbon* (PNU 4302), and *E. spectabilis* (Dennst.), Suresh (PNU 4303) were identified by Asst. Prof. Dr Anupan Kongbangkerd, Faculty of Science, Naresuan University and deposited in the Herbarium of the Department of Biology in the Faculty of Science, Naresuan University, Phitsanulok, Thailand.

2.2 Chemicals and reagents

Methanol and dichloromethane were purchased from Carlo Erba, France. All solvents were analytical grade purity. A standard phenanthrene, **1**, was isolated and purified from *E. macrobulbon* (Temkitthawon *et al.,* 2017). The structure of the compound was elucidated by several spectroscopic methods and the purity was proved by ^{1}H-NMR (Temkitthawon *et al.,* 2017). Its wavelength of maximum absorbance (λmax) was 260 nm.

2.3 Preparation of standard solution

A standard solution of 10 mg/mL was prepared by dissolving **1** in methanol (AR grade, 99.5%). The solution was diluted to various concentrations for method validation and establishing the calibration curve.

2.4 Extraction and sample preparation

Samples of the powder of each plant part were selected, one sample of leaf powder, eleven samples of tuber powder, and one of root powder. Each sample (100 mg) was extracted with 3 mL of methanol (100%) in an ultrasonic water bath (35 W, 47 KHz) for 15 min. The extracts were filtered with Whatman No. 1 filter paper and the residues were re-extracted three times with fresh solvent following the same procedure. The combined filtrates were evaporated under reduced pressure at 60 °C and the volume was adjusted with methanol in a 5 mL-volumetric flask to yield a stock solution of 100 mg/5 mL. The extraction was done in triplicate. The solution was filtered through a 0.25 μm nylon membrane (Merck Millipore, Darmstadt, Germany) before analysis.

2.5 Chromatographic condition

Sample solutions (2 μL) were spotted onto pre-coated TLC sheets (ALUGRAM® Xtra SIL G/UV$_{254}$ plates [0.20 mm thick]; MACHEREY-NAGEL, Duren, Germany). Three TLC plates were needed to accommodate all 13 samples. The distance between each spot of sample was 1 cm. The TLC plates were developed to a distance of 180 mm with dichloromethane: methanol (96:4, v/v) in a TLC chamber previously saturated with the mobile phase for 30 min.

2.6 TLC-image analysis method

After development, the TLC plates were dried at room temperature. An image of the TLC chromatogram on each plate was taken using a Canon EOS 1100D digital camera under a visualizer (UVITEC Cambridge, France) at 254 nm. The image files were each opened with the Adobe Photoshop CS6 program. The digital color photos were converted into grayscale digital images which were resized and cropped to the plate dimensions of 20x10 cm and saved at a resolution of 50 pixels cm^{-1} for image analysis. The spots of **1** in the samples were indicated by comparison with the reference standards. The gray values of **1** at the area of 40x40 pixels were measured. The quantities of **1** in the samples were calculated using the calibration curve of the reference standards applied in the same TLC plate.

2.7 Calibration curve

Reference standard solutions of **1** were prepared in methanol. Aliquots (2 μL) of each solution were spotted onto a TLC plate to obtain the concentrations of **1** at 0.25, 0.5, 1, 2, 4, 6, and 8 μg/spot. The TLC plates were developed and analyzed using the method mentioned above. The calibration curve between the grayscale intensity and the ln function of the concentration range of the standard (μg/spot) was constructed and the calibration data were subjected to least-squares regression analysis.

2.8 Method validation

The specificity of the TLC method was determined in relation to the interferences from other components in the samples by a comparison with an R$_f$ value of **1** in a sample of the reference standard. The limit of detection (LOD) and limit of quantification (LOQ) were determined by considering the signal-to-noise ratio (S/N). LOD was considered as S/N 3:1, while LOQ was S/N 10:1. The repeatability (intraday precision) and the intermediate precision (inter-day precision) were determined by analyzing the amount of **1** at three different

concentration levels (0.5, 2, and 6 μg/spot), which was done in triplicate. Intermediate precision was determined on three consecutive days. Precision was expressed as relative standard deviation (%RSD) values. The accuracy was expressed in terms of recovery studies using a standard addition method. A sample (100 μg/spot) was spiked with a known amount of **1** to give additional concentrations of 0.5, 2, and 6 μg/spot and then analyzed by the proposed methods. The experiments were done in triplicate. The percentage recovery was calculated.

2.9 Analysis of compound 1 in *Eulophia* spp.

Two μL of each *Eulophia* samples (200 mg/mL) was applied to a TLC plate to obtain ≈400 μg/spots of the solution which were analyzed by the methods described above. The amount of **1** in the samples was analyzed by the TLC-image analysis. The contents of **1** in the dried samples were calculated as mg/g of dried samples. Each sample was analyzed in triplicate.

3. Results and Discussion

3.1 Method validation

A series of mobile phases were tested by TLC analysis for **1**. The results indicated dichloromethane: methanol (96:4, v/v) gave a narrow band of **1** at an R_f value of 0.22±0.02 with the band well separated from the other components in the samples. The specificity of the TLC method was ensured by comparing the R_f value of **1** in the samples that were spiked with standard **1**. The chromatographic condition described in Section 2.5 gave reproducible operation, appropriate separation, uniform visualization, and sensitive detection. These are prerequisites to high quality imaging and accurate image analysis.

The method for digital imaging was developed using the camera set to operate in auto mode using the timer instead of pressing the shutter button. This avoided shaking during the relatively long exposure time. To minimize background noise, images were taken in a dark room using a light to eliminate heterogeneous light.

The linearity of the calibration curve was investigated at six concentration levels using the working standards (Figure 2). Aliquots of 2 μL from each solution were applied in triplicate and analyzed by the procedure previously mentioned (Figure 3). A linear relationship was found between the ln function of the concentration range of 0.25–8 μg/spot for **1** (regression equation y = −29.144x + 101.76; R^2 = 0.9989). The LOD value was 0.125 μg/spot with the LOQ value equal to 0.25 μg/spot. The precision of the TLC method was found to be satisfactory as the RSD values implied repeatability and in the intermediated precision studies were less than 5% (Table 1). The accuracy of the method was investigated by spiking a sample with three different concentrations of standard **1**. The results were found to be satisfactory and indicated good mean recovery that ranged from 95.14% to 103.99%. Finally, the validated TLC method was applied to evaluate the contents of **1** in thirteen samples.
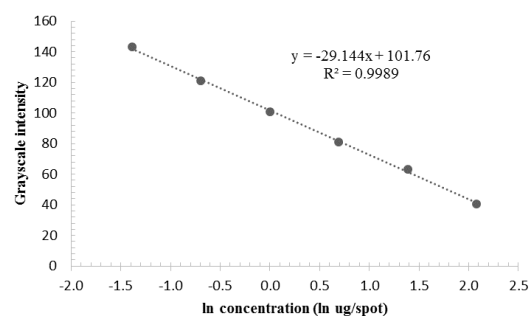

Figure 2. Calibration curve between grayscale intensity and ln concentration (ln μg/spot) of 1-(4'-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (**1**), n=5.
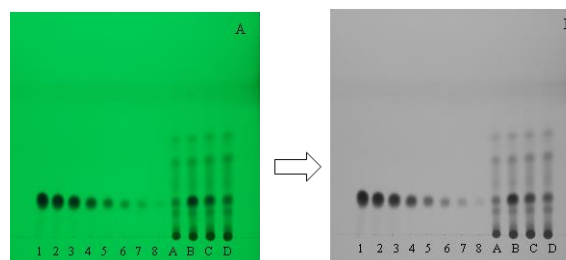

Figure 3. TLC chromatogram (A) under UV₂₅₄ and TLC image analysis (B) obtained by photoshop CS6 in gray scale (Tracks 1-8: standard 1 at 8, 6, 4, 2, 1, 0.5, 0.25, and 0.125 μg/spot, respectively. Tracks A: *E. macrobulbon* extract at 100 μg/spot, Tracks B: A adding standard **1** at 6 μg/spot, Tracks C: A adding standard **1** at 2 μg/spot, Tracks D: A adding standard **1** at 0.25 μg/spot.
1=1-(4'-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol

Table 1. Precision (intraday and interday) and accuracy (recovery) of the TLC-image analysis method developed.

Standard 1 concentration (μg/spot)	TLC image analysis		
	Intraday precision[a]	Interday precision[b]	Recovery[c] (%)
6	2.52	1.92	101.07±4.92
2	4.29	4.54	103.99±8.21
0.25	4.35	3.93	95.14±12.38

Relative standard deviation (%RSD, n=3)[a], (%RSD, n=5)[b], Recovery (n=5)[c]

3.2 Analysis of other *Eulophia* species

TLC-image analysis was applied for detection and quantification of compound **1** in 13 samples of *Eulophia* spp. (Table 2). The presence of compound **1** was clearly observed in the TLC chromatograms of the samples (Figure 4). From a comparison of **1** content in the different parts of *E. macrobulbon*, it was clear that the tubers contained the highest level of **1**. The contents of **1** in the tubers of *E. macrobulbon* varied from trace amount to 1.70 mg/g of dried plant powder. To

Table 2.   Content of **1** in *Eulophia* spp. analysed by TLC- image analysis using the method described in Experimental (n=3).

| Scientific name	Part of used	Code	Collection period	Content of **1** in dried samples (mg/g) (mean±SD)
E. macrobulbon	tubers	E	July 2010	1.70±0.031
		F	December 2010	0.92±0. 013
		G	February 2012	1.41±0.018
		H	March 2013	0.82±0.014
		I	April 2013	trace amount*
		J	April 2011	0.94±0.010
		K	April 2013	0.91±0.006
		L	January 2014	trace amount*
	leaves	N	July 2010	trace amount*
	roots	O	July 2010	trace amount*
E. spectabilis	tubers	P	March 2013	0.97±0.034
E. graminea		R	March 2013	trace amount*
E. andamanensis		S	April 2013	trace amount*

* below LOQ
1=1-(4'-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol
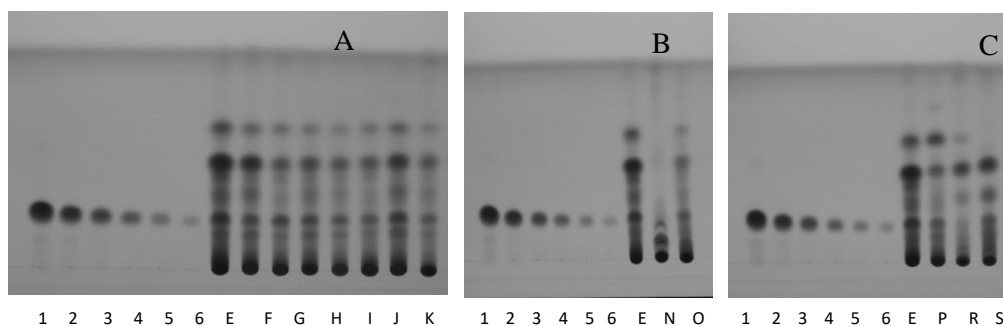
Figure 4.   TLC chromatograms obtained by photoshop CS6 in gray scale (Tracks 1-6: standard **1** at 8, 4, 2, 1, 0.5, and 0.25 μg/spot, respectively. (A): Tubers of *E. macrobulbon* from different collection periods (tracks E-L), (B): Different parts of *E. macrobulbon* (tracks E=tuber, N=leaf, O=root), (C): Tubers of 4 *Eulophia* spp. (tracks E=*E. macrobulbon*, P=*E. spectabilis*, R=*E. graminea*, S=*E. andamanensis*).

standardize the quality of the plant raw material, good agriculture and collection practice of this plant should be further studied. In addition, we found that apart from the tubers of *E. macrobulbon*, the tubers of *E. spectabilis* contained a high amount of **1**. *E. spectabilis* might be considered as an alternative source of this compound.

4. Conclusions

This is the first report of a validated TLC-image analysis method using common computer technology for detection and quantification of **1**. This method was successfully applied for the quantitative analysis of **1** content in *Eulophia* spp. The results indicated that tubers of *E. macrobulbon* contained the highest level of **1**. This method can be used as a routine method for the quality control of herbal materials with the advantages of low-cost, convenience of use, and it is an accurate method of analysis.

Acknowledgements

References

Gurib-Fakim, A. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine, 27*(1), 1-93.

Hatzimouratidis, K., Amar, E., Eardley, I., Giuliano, F., Hatzichristou, D., Montorsi, F., & European Association of Urology. (2010). Guidelines on male sexual dysfunction: Erectile dysfunction and premature ejaculation. *European Association of Urology, 57*(5), 804-814.

Hatzimouratidis, K., & Hatzichristou, D. (2007). Phospho-diesterase type 5 inhibitors: the day after. *European Association of Urology, 51*(1), 75-89.

Hong, T. X. (2008). An image analysis system for thin-layer chromatography quantification and its validation. *Journal of Chromatographic Science, 46*, 560-564.

ICH Expert Working Group. (1994). ICH harmonised tripartite guideline: Validation of analytical procedures: Text and methodology, Q2 (R1), Current step 4 version parent guideline dated 27 October 1994 (Complementary guideline on methodology dated 6 November 1996 incorporated in November 2005). http://www.ich.org/LOB/media/MEDIA417.pdf.2005.

Phattanawasin, P., Sotanaphun, U., Sukwattanasinit, T., Akkarawaranthorn, J., & Kitchaiya, S. (2012). Quantitative determination of sibutramine in adulterated herbal slimming formulations by TLC-image analysis method. *Forensic Science International, 219*(1–3), 96-100.

Temkitthawon, P., Changwichit, K., Khorana, N., & Ingkaninan, K. (2017). Phenanthrenes from *Eulophia macrobulbon* as novel phosphodiesterase-5 inhibitors. *Natural Product Communications, 12*(1), 79-82.

Tóth, B., Liktor-Busa, E., Urbán, E., Csorba, A., Jakab, G., Hohmann, J., & Vasas, A. (2016). Antibacterial screening of Juncaceae species native to the Carpathian Basin against resistant strains and LC-MS investigation of phenanthrenes responsible for the effect. *Fitoterapia, 115*, 69-73.

Wylie, K., & Kenney, G. (2010). Sexual dysfunction and the ageing male. *Maturitas, 65*(1), 23-27.

Yan, H. G., Zhao, H. R., Hu, J., Lu, A. M., Fu, X. M., Jia, B., & Yang, M. H. (2016). Determination of phenanthrenes and stilbenoid in the ethyl acetate extract of *Thunia alba* (Lindl) by HPLC-DAD. *Analytical Methods, 8*(24), 4867-4871.