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Short communication

Analysis of naphthoquinone derivatives in the Asian medicinal plant *Eleutherine americana* by RP-HPLC and LC–MS

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ABSTRACT

The first analytical procedure for the determination of a new naphthopyrone, eleutherinoside A, together with the known bioactive compounds eleuthoside B, isoeleutherin, eleutherin and eleutherol in *Eleutherine americana* was established. Optimum HPLC separation of these naphthoquinone derivatives was possible on RP-12 column material, using water and acetonitrile as mobile phase. Flow-rate, detection wavelength and temperature were adjusted to 1.0 mL/min, 254 nm and 40 °C, respectively. Validation results indicated that the HPLC method is well suited for the determination of naphthoquinone derivatives in the bulbs of *E. americana* with a good linearity ($r^2 > 0.9996$), precision (intra-day R.S.D. <4.70%, inter-day R.S.D. <5.68%) and recovery rates from 96.26 to 103.48%. Limit of detection (LOD) was found to be below 0.84 μg/mL for all five compounds. LC–MS analyses performed in positive and negative electrospray ionization mode assured peak purity and identity. The analysis of different *E. americana* samples from Thailand revealed that eleutherol (0.10–0.20%) was dominant in all specimens, followed by isoeleutherin and eleutherin. The new natural product 2,5-dimethyl-10-hydroxynaphthopyrone 8-*O*-β-D-glucopyranoside occurred in percentages of less than 0.05%.

1. Introduction

Eleutherine americana (Aubl.) Merr. originates from tropical America, where its elongated red roots have been used by the indigenous population for centuries as a herbal remedy. The plant, belonging to the Iridaceae family, is now widely cultivated in China for ornamental and medicinal purposes. The bulb is used for the treatment of coronary diseases [1], and as diuretic, emetic and purgative [2]. In Thailand, in pure form it is a traditional carminative, as mixture with galangal it is applied externally on the forehead to treat cold and nasal congestion in children [3]. Previous research on the chemical constituents of *E. americana* resulted in the isolation of several naphthalene derivatives (see Fig. 1) such as hongconin, elecanacin, eleuthoside B (**II**), isoeleutherin (**III**), eleutherin (**IV**) and eleutherol (**V**) [4,5]. Pharmacological studies indicated significant anti-HIV activity of isoeleutherin [5], and cardio protective activity of hongconin [6]. Eleutherin revealed inhibition of human topoisomerase II (anticancer activity) [7], and antibacterial activity against *Pycococcus aureus, Streptococcus haemolyticus A*, and *Bacillus subtilis* [8,9]. Furthermore, compounds **III** and **IV** inhibited the proliferation of K562 (human erythroleukemia) cells [10], and showed strong antifungal activity in the bioautography assay against *Cladosporium sphaerospermum* [11]. It also was reported that a mixture of **III**, **IV** and **V** isolated from the rhizome of this plant was beneficial for the treatment of heart diseases (angina pectoris) in clinical trials [12].

Because of the clinical use and interesting pharmacological properties of *E. americana* it is essential to assure quality of crude plant material and products thereof; but to the best of our knowledge no respective analytical methods have been reported so far. Thus, here we present an assay suitable for the separation and quantification of all major naphthoquinones in *E. americana* by means of reversed-phase HPLC and LC–MS. After method development and validation authentic herbal specimens purchased in Thailand were analyzed for their content of **I**–**V**.

2. Experimental

2.1. Herbal material

Ten samples (EA-1 to EA-10) of *E. americana* were purchased from various vendors in Thailand. The samples were identified at the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and

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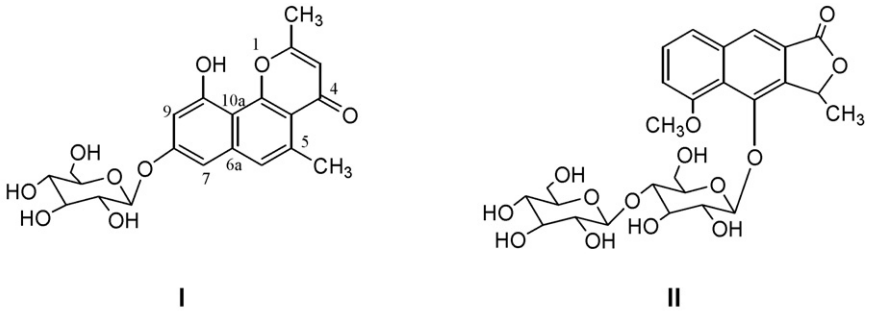
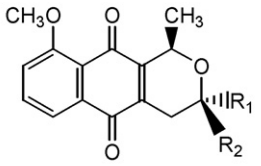
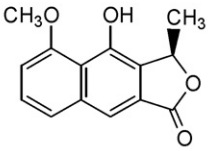


I

II



	R_1	R_2
III	CH_3	H
IV	H	CH_3



V

Fig. 1. Chemical structures of the determined naphthoqinones: (**I**) eleutherinoside A, (**II**) eleuthoside B, (**III**) isoeleutherin, (**IV**) eleutherin, and (**V**) eleutherol.

Environment, Bangkok; respective voucher specimens (no. WEA 03200701–WEA 03200710) are deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

2.2. Standards and reagents

2,5-Dimethyl-10-hydroxynaphthopyrone 8-*O*-β-D-glucopyranoside (**I**), eleuthoside B (**II**), isoeleutherin (**III**), eleutherin (**IV**), and eleutherol (**V**) were separated and purified from a crude *E. americana* extract by chromatographic methods (column chromatography using silica gel, Sephadex LH-20 and RP-18 material). Purity of the isolated compounds was assured by TLC and HPLC, and structures of **II**–**V** were identified by comparison of their ^{1}H NMR and ^{13}C NMR and MS data with those reported in literature [13–15]. For isolation and structural elucidation of **I**, see Supplementary information.

HPLC grade acetonitrile was purchased from Acros Organics (Geel, Belgium). HPLC grade methanol, formic acid and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Water for HPLC analysis was prepared using a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.3. HPLC sample preparation

The finely powdered plant material (0.100 g) was extracted three times with 3 mL of methanol by sonication (10 min each, at ambient temperature), and then centrifuged at 3000 rpm for 5 min. The extracts were combined in a 10 mL volumetric flask which was adjusted to the final volume with methanol. Prior to injection all solutions were filtered through a 0.45 μm nylon membrane filter (Phenex, Phenomenex, Torrance, CA, USA). Each sample solution was assayed in triplicate.

2.4. HPLC and HPLC–MS conditions

Chromatographic experiments were performed on a Hewlett Packard 1050 series HPLC instrument equipped with autosampler, column heater and diode array detector (Agilent, Waldbronn, Germany). For all experiments a C-12 column (Synergi Max-RP, 150 mm × 4.6 mm, 4 μm particle size) from Phenomenex was used as stationary phase, separation temperature and injection volume were set to 40 °C and 10 μL, respectively. The mobile phase composed of water (A) and acetonitrile (B), applying a gradient program starting from 15% B to 30 % B in 10 min, in further 10 min to 50% B, and in 10 min to 80% B. The column was cleaned with 20% A/80% B for 5 min, and then the system was equilibrated for 10 min with the starting conditions. Flow rate was 1 mL/min, and the detection wavelength adjusted to 254 nm.

LC–MS experiments were performed on a Bruker Esquire 3000 plus iontrap (Bruker-Daltronics, Bremen, Germany), which was connected to an Agilent 1100 series HPLC. The same separation conditions as described above were used, only 0.01% formic acid was added to solvent A. For optimum MS results ionization was accomplished in alternating ESI mode, with probe temperature, nebulizer (nitrogen) and dry gas (nitrogen) adjusted to 350 °C, 30 psi and 10 L/min, respectively. Mass analysis was carried out in full-scan mode from 100 to 900 amu, with a solvent split ratio of 1:3.

2.5. Validation of assay

A standard stock solution of I-V was prepared by dissolving each compound in methanol to obtain a concentration of 2 mg/mL. Six addition calibration levels were prepared by diluting this solution 1:1 with methanol, and each level was assayed in triplicate.

Extraction efficacy was investigated by comparing peak areas of the same sample extracted with different sonication times and volumes of methanol. Limit of detection is defined as concentration showing peak heights of three times baseline noise and the limit of quantitation as 10 times baseline noise; respective values were determined by consequently diluting standard solutions to appropriate concentrations. Peak purity was assured by evaluating available DAD data using the respective "peak purity" option within the Chemstation software (threshold value was set to 980), as well as LC–MS results. In all samples the peaks of interest were found to be pure and free of co-eluting compounds.

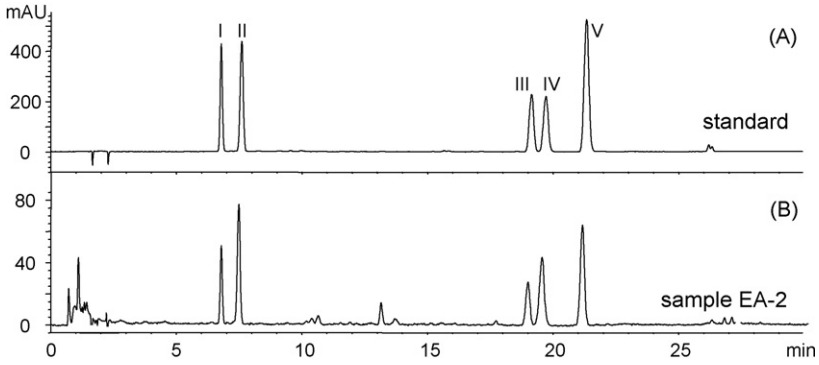


Fig. 2. HPLC chromatograms of a standard mixture (A) and one sample solution (B) obtained under optimized separation conditions (column: Synergi Max-RP, 150 mm × 4.6 mm, 4 μm; mobile phase: water (A) and acetonitrile (B); gradient: from 15% B in 10 min to 30% B, in 10 min to 50% B, in 10 min to 80% B; temperature: 40 °C; detection: 254 nm; flow rate: 1.0 mL/min).

Accuracy was determined by adding three individual concentrations of standard compounds (low, medium and high spike) to dry, powdered samples of EA-1, and extracting and analyzing the spiked material under optimum conditions. Repeatability of method was assured by observing relative standard deviations of multiple injections of the same sample solution. Precision of method was evaluated by running five replicate samples prepared independently on day 1; the same procedure was repeated on two more days [16].

3. Results and discussion

3.1. Structure elucidation of compound *I*

Isolation and structural elucidation of eleutherinoside A (**I**), a novel natural product, is described in Supplementary data.

3.2. Optimization of HPLC method

The chromatographic conditions were developed and optimized using a mixture of standard compounds and extracts of *E. americana*. Several stationary phases (C-8, C-12, C18, phenyl-hexyl and cyano) were initially screened, and the best resolution obtained with a Synergi Max-RP from Phenomenex. Regarding mobile phase, the use of water and acetonitrile showed optimum results, adding acids (TFA, formic acid, or acetic acid), buffers (sodium phosphate or ammonium acetate) or modifiers like tetrahydrofuran or methylbutyl ether was not advantageous. Only for LC–MS experiments 0.01% of formic acid was added to the aqueous phase in order to improve ionization of the analytes. The optimal wavelength for HPLC analysis was determined to be 254 nm, suitable for the sensitive detection of all compounds of interest. An elevated column temperature of 40 °C was beneficiary in order to reduce the required separation time, yet maintaining excellent resolution between the compounds of interest. Especially the separation of compounds **III** and **IV** was difficult to achieve, requiring a truly optimized setup, as otherwise both compounds merged (Fig. 2).

3.3. Method validation

Calibration data for eleutherinoside A, eleuthoside B, eleutherin, isoeleutherin and eleutherol is summarized in Table 1. Linearity of the detector signal was confirmed over a more than 30-fold concentration range (from approximately 6.5 to 230 μg/mL), with correlation coefficients of 0.9996 or higher. Detection limits from 0.39 to 0.84 μg/mL, and quantitation limits from 1.30 to 2.80 μg/mL indicated the methods sensitivity. By repeatedly assaying (extraction and analysis) one sample within 3 days intra- and inter-day precision were determined. The results presented in Table 2 show that the maximum deviations were reached for compound **I** on day

Table 1
Calibration data of compounds **I**–**V**, including regression equation (*Y* reflects peak area, *X* the amount in μg/mL), correlation coefficient (r^2), linear range (μg/mL), limit of detection (μg/mL) and limit of quantitation (μg/mL)

	Regression equation	r^2	LOD	LOQ	Range
I	$Y = 26.758X + 3.989$	1.0000	0.39	1.30	6.28–201
II	$Y = 32.556X + 53.882$	0.9999	0.44	1.46	7.09–227
III	$Y = 26.972X + 76.761$	0.9998	0.40	1.33	6.34–203
IV	$Y = 27.711X - 10.931$	1.0000	0.84	2.80	6.69–214
V	$Y = 57.651X + 121.17$	0.9996	0.46	1.53	7.28–233

Table 2
Intra- and inter-day precision of the HPLC assay using sample EA-1; results are based on peak area, relative standard deviation in parenthesis

	Intra-day			Inter-day
	Day 1	Day 2	Day 3	
I	305.7 (4.70)	297.1 (4.36)	298.6 (2.94)	300.5 (4.00)
II	842.1 (4.33)	853.9 (2.37)	841.4 (1.66)	845.8 (2.86)
III	341.0 (3.25)	310.0 (2.49)	306.0 (2.35)	319.0 (5.68)
IV	361.8 (3.64)	399.5 (1.98)	399.4 (1.89)	386.9 (5.31)
V	670.4 (2.18)	673.7 (2.11)	682.3 (2.16)	675.5 (2.13)

Table 3
Accuracy of the HPLC assay, based on recovery experiments (low, medium and high spike) utilizing sample EA-1; quantitative values in μg/mL

Level	Compound	Theoretical	Found	Recovery (%)
Low	**I**	10.05	10.40	103.48
	II	11.35	11.65	102.64
	III	10.15	9.77	96.26
	IV	10.70	10.97	102.52
	V	11.37	11.02	96.92
Medium	**I**	20.10	19.37	96.37
	II	22.70	22.34	98.41
	III	20.30	19.82	97.63
	IV	21.40	21.18	98.97
	V	22.83	22.09	96.76
High	**I**	40.20	38.94	96.86
	II	45.40	44.96	99.03
	III	40.60	40.79	100.47
	IV	42.80	41.56	97.10
	V	45.67	46.56	101.95

Table 4
Percentage of naphthoquinones in different *Eleutherine americana* samples from Thailand, relative standard deviation in parenthesis ($n = 3$)

Sample	Compound					
	I	**II**	**III**	**IV**	**V**	Total **I–V**
EA-1	0.03 (0.42)	0.12 (1.02)	0.04 (1.99)	0.04 (2.07)	0.10 (1.35)	0.33
EA-2	0.03 (1.99)	0.09 (1.04)	0.03 (0.19)	0.08 (0.53)	0.11 (0.61)	0.34
EA-3	n.d.	n.d.	0.04 (1.28)	0.06 (1.38)	0.15 (0.13)	0.25
EA-4	0.02 (2.32)	0.09 (1.09)	0.05 (0.68)	0.06 (2.13)	0.11 (0.66)	0.33
EA-5	0.05 (4.56)	0.14 (2.32)	0.05 (3.59)	0.10 (0.98)	0.15 (1.32)	0.49
EA-6	0.05 (0.72)	0.13 (1.51)	0.04 (2.11)	0.10 (1.60)	0.14 (0.87)	0.46
EA-7	0.02 (4.41)	0.08 (3.23)	0.05 (4.02)	0.07 (1.95)	0.12 (0.68)	0.34
EA-8	0.01 (0.69)	0.07 (1.36)	0.07 (1.14)	0.10 (4.08)	0.20 (1.28)	0.45
EA-9	n.d.	0.02 (2.70)	0.04 (3.00)	0.08 (1.68)	0.15 (3.01)	0.29
EA-10	0.03 (0.89)	0.11 (0.88)	0.04 (1.93)	0.07 (1.33)	0.13 (0.76)	0.38

n.d.: not detectable.

1 (R.S.D = 4.70%; intra-day) and for compound **III** (R.S.D. = 5.68%; inter-day); thus precision of the method can be assumed.

The methods accuracy was investigated in recovery experiments (Table 3). Plant material was spiked with three concentrations of compounds **I–V**, and after extraction and analysis the obtained values were compared with theoretical amounts (natively present plus spiked amount). With recovery rates of 96.26 to 103.48% at all three spiked levels the determined recoveries were well within the usually required limit of $100 \pm 5\%$. When assaying different plant extracts a maximum relative standard deviation of 4.6% for multiple injections was observed, indicating repeatability of the method (Table 4).

3.4. Quantitative analysis

Prior to analysis of actual *E. americana* samples the most suitable method for sample extraction was determined, and as using a sonicator for this purpose is a well established procedure we utilized this approach too. Extracting the plant material repeatedly with methanol showed to be very efficient, as after the third repetition no marker compounds were left in the matrix (results not shown in detail). Recovery rates close to 100% (see above) additionally indicated an exhaustive extraction procedure.

In Fig. 2 separation of a standard mixture and a plant extract under optimized HPLC conditions is presented; all five compounds are nicely separated within less than 25 min. Assignment of the markers was achieved by comparison of retention times and UV-spectra of standards and by LC–MS experiments. For the latter an iontrap mass spectrometer was used, applying the same chromatographic conditions as described before (except of the addition of acid to solvent A). Ionization was performed in positive and negative ESI mode, and all compounds were unambiguously identified by their molecular peaks ($[M+H]^+$ for **I**, **III**, **IV** and **V**; $[M-H]^-$ for **II**; Supplementary data). Quantitative results of ten different *E. americana* samples are compiled in Table 4. Even the composition of individual naphthoquinones varied from sample to sample, the total amounts of **I–V** were rather homogeneous ranging from 0.25% in sample EA-3 to 0.49% in EA-5. This indicates a rather consistent occurrence of these compounds in the plant, regardless of origin and harvest time. Most dominant in all samples was compound **V** (0.11–0.20%) except in EA-1, in which **II** was major (0.12%). Interestingly, the amount of the latter is quite variable, reaching from no detectable amounts in sample EA-3 to 0.14% in sample EA-5. As known from other plants like Senna there is a possibility that glycosides are converted to respective aglyca due to enzymatic activity during storage [17]. Therefore, compound **V** might actually be a degradation product of **II**, but further studies are necessary to clarify this question.

4. Conclusion

The first analytical procedure for the analysis of naphthoquinones in *E. americana* bulbs was established. After method development and validation (linearity, precision, accuracy and peak purity were confirmed), the assay showed to be well suited for the qualitative and quantitative determination of these bioactive constituents in herbal samples. Owing to the widespread use of *E. americana* in Asia and the interesting pharmacological properties of naphthoquinones in general, with this method an important step towards comprehensive characterization of *E. americana* and its products is made.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2008.04.005.

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