A Simple Isocratic HPLC Method for Simultaneous Determination of Shikonin Derivatives in Some *Echium* Species Wild Growing in Turkey

INTRODUCTION

Species of *Echium* are occurs abundantly as flowering plant native to North Africa, mainland Europe and the Macaronesian islands, but have also become invasive in southern Africa and Australia. Out of the 60 species of this genus, nine are found in Turkey: *E. angustifolium, E. arenarium, E. italicum, E. glomeratum, E. oriantale, E. parviflorum, E. plantagineum, E. russicum*, *and E. vulgare.*

Based on the traditional documents on folk medicine, *Echium sp.* have been employed as diaphoretic, diuretic, expectorant, febrifuge, sedative, analgesic, vulnerary, anxiolytic, and in the treatment of upper respiratory tract infections (1-4). Pharmacological studies demonstrate that species of *Echium* possesses antioxidant (5-7), anti-inflammatory (8), antibacterial (9-10), antiviral (11), antiproliferative (12-14), analgesic (15) and antidepressant (16) activities. It is clinically proved as a useful and safe drug in the patient of obsessive compulsive disorders (16-18). It is also reported that various extracts of *E.amoenum* have good antianxiety activity on *in vivo* models (19-23). *Echium* species contain a variety of phytochemicals, including naphthoquinones, flavonoids, pyrrolizidine alkaloids, steroids, anthocyanins, fatty acids, amino acids, and essential oils. Many shikonin derivate have been isolated from the roots of the *Echium* plants. Naphthoquinones are a series of shikonin derivative such as shikonin (S), acetylshikonin (AS), deoxyshikonin (DS), β, β-dimethylacrylshikonin (DMAS), isobutrylshikonin (IBS), isovalerylshikonin (IVS), and 2-methyl butrylshikonin (MBS) (24).

Shikonin, R enantiomer of Alkannins, are lipophilic red pigments commonly known as isohexenylnaphthazarins. They are mostly found in more than 150 genus such as *Lithospermum*, *Echium*, *Onosma*, *Anchusa,* and *Cynoglossum* of the Boraginaceae family. Historically they were used as dyes by ancient Greeks and Romans. Shikonin contains two parts structurally: the naphthazarine moiety (5, 8-dihydroxy-1, 4 naphthoquinone) and the chiral six-carbon side chain. It is readily sensitive to polymerize following treatment with acids, bases, heat, or light due to the naphthazarine core, and easily to oxidation by exposure to light or air just for the high chemical reactivity. Most of the shikonin derivatives are present as ester derivatives linked with the hydroxyl group of the side chain, maintaining the naphthazarine moiety (25). Shikonin derivatives have attracted attention of many researchers due to their several potential pharmacological activities such as antimicrobial, antitumor, wound healing, and antioxidants. Shikonin extracts obtained from *Lithospermum erythrorhizon* induced apoptosis against human colorectal carcinoma cells, HL60 cells, and HeLa cells by mechanism of tumor suppression via signaling pathways that possibly involve reactive oxygen species, p53, p27, Bcl-2, caspase, and inhibition of DNA topoisomerase I/II and telomerase (26-28). A clinical study showed that shikonin derivatives were efficacious in treatment of later-stage lung cancer patients who were inapt for other treatment (29). Furthermore, the cream Helixderm® made from shikonin and derivatives has been evaluated for wound healing activity in clinical trials undertaken at the Freie University showed good results with granulation and epithelization (30).

In terms of toxicological effect of naphthoquinone compounds, it was reported that shikonin derivatives (ShD) did not induce any hematological toxicity in animal model which indicate that ShD may be safe for usage *in vivo* (31), while in another report demonstrated toxicity in mice by intraperitoneal administration at a dose of 20 mg/kg for shikonin, 41.0/22.75 mg/kg for acetylshikonin and, 48mg/kg for 3,3-dimethylacrylshikonin. In addition, toxicity also has been observed during the *in vivo* testing for antitumor effects of shikonin derivatives (32).

Herbals and herbal drugs contain active ingredients, present in the naturally occurring plant source, in certain quantity and the proportion between different constituents or active principles is a key quality parameter for the efficacy of the product. It is in this regard that the modern tools

and techniques of analysis provide vital support and required evidence. According to our knowledge, there was no report related to the phytochemical analyses of naphthoquinone derivatives in *Echium sp*. by HPLC quantitatively. Thus, the aims of the present study are to quantify biologically active naphthoquinone derivatives in the ethanol extracts of the roots of selected four *Echium* species wild growing in Turkey.

EXPERIMENTAL

HPLC Analysis of Shikonin Derivatives in Different Samples

Plant material and chemicals

E.italicum L., *E.vulgare* L., plant procured from North of Ankara 56.km, and *E.angustifolium* Miller., *E.parviflorum* Moench collected from seaside of Side, Antalya province of Turkey, identified and authenticated by Dr. Gülderen Yılmaz, Assist. Prefessor, Department of pharmaceutical Botany, Faculty of Pharmacy, Ankara University. A voucher specimen has been deposited in Herbarium of Gazi University Faculty of Pharmacy (GUE2991, GUE2992) & in herbarium of Ankara University Faculty of Pharmacy (AEF26023, AEF26024). The reference compounds were purchased from *Pureone biotechnology Co. Ltd*, China. Chemicals and reagents used in the study were of HPLC grade or analytical grade.

Extraction of plant samples

Extraction of crude plant extracts

The extraction procedure was performed as below: the shade dried and coarsely powdered roots of four *Echium* sp. (100 g) at a water bath with temperature of 40°C were extracted with 96% alcohol (2×750 mL) for 48 h. After cooling, the material was filtered and the residue was further extracted with ethanol (500 mL) two times. Following this procedure, all the extracts were pooled together, were concentrated under vacuum using rotavapor (Büchi, Switzerland) to afford the alcoholic extract, and then were freeze-dried.

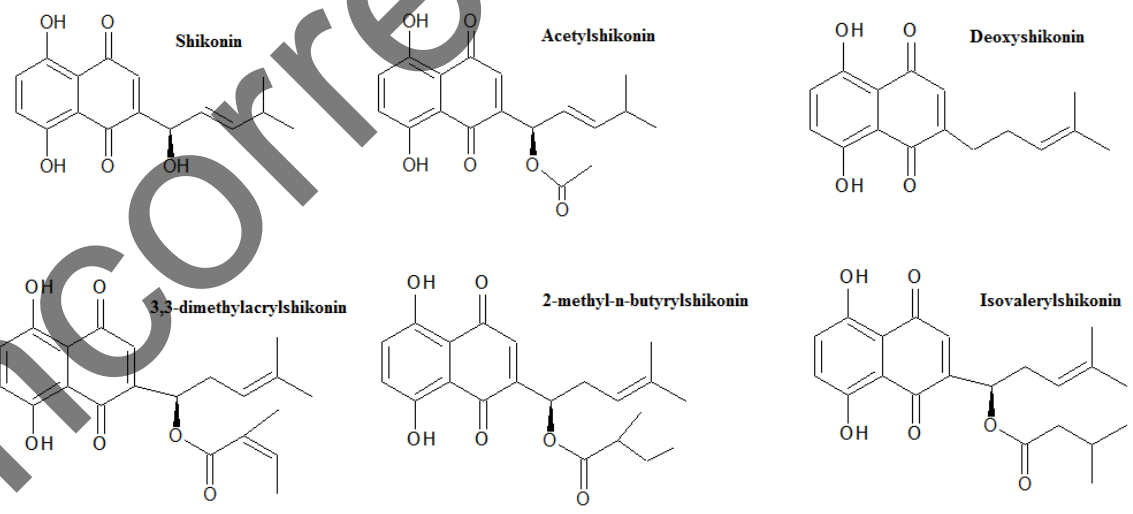

Figure 1. The structures of standard compounds.

Identification of shikonin derivatives by thin-layer chromatography

Fifty mg of the ethanol extracts made from roots of four *Echium* species were taken into a set of 10 mL volumetric flasks separately and the volumes were completed to the mark with methanol. A 10-µL aliquot of test samples of different extracts was applied to the TLC plate (aluminum foil backed TLC plate coated with silica gel 60 F254, Merck, 0.2 mm layer thickness) separately. It was positioned at 10 mm from the bottom of the plate, and then the plate was developed to a distance of about 9.5 cm using petroleum ether: ethyl acetate: acetic acid (85:15: 1 v/v) as the mobile phase. The plates were then removed, air-dried, and the spots were visualized under a UV lamp at 254 nm. After spraying of 5% sulphuric acid reagent on the plate and then heated at 105 °C for about 5 minutes until the bands are clearly visible. The chromatogram obtained with test solution shows a band corresponding to those shikonin derivatives (Figure 2).

High performance liquid chromatography

Instrumentation

An Agilent 1200 LC system (equipped with a G1311A pump, G1328B manual injector, G1322A degasser, G1316A column heater, and G1314B variable wavelength UV-detector) was employed (Agilent Technologies -Santa Clara, CA).

Chromatographic conditions

An ACE 5 C18 (150 mm × 4.6 mm, 5 µm) (Scotland) reversed-phase column was used as the stationary phase. Isocratic elution program is applied for chromatographic analysis. The mobile phase was a mixture of acetonitrile: 0.1 M acetic acid contained water = 70:30 (pH = 2.82) with isocratic elution, filtered through a 0.45 µm membrane filter (Millipore) and degassed by sonication for 30 min prior to use. The analysis was carried out at a flow-rate of 1.0 mL/min. Injection volume was adjusted to 10 µL. The temperature of column was set to 25°C. The analysis was performed at 520 nm due to maximum UV absorbance for the analytes were obtained at this wavelength.

Preparation of test solution

Dry extracted samples weighed and finely powdered. Approximately 100 mg of grinded powder weighed carefully to the vials, then the sample was ultrasonically extracted with 15 mL methanol and 1 mL DMSO for about 30 min. After cooling, the volume was made up to 50 ml with the same solvent. Prior to analysis, the extraction solutions were filtered through a 0.45-μm filter membrane (Merck, Millipore) and 10-μL aliquot was injected into the HPLC system.

Preparation of Standard Solution

Stock standard solution of S, AS, DS, DMAS, MBS, and IVS were prepared by dissolving 1.0 mg of each compound in 10 mL of acetonitrile in a volumetric flask. For determination of limit of detection (LOD) and limit of quantification (LOQ), working standard solutions were prepared by further dilution of this stock solution with aqueous methanol.

Calibration Curve

Linear calibration plots were obtained for the related standard compounds at six different concentration levels. After filtering through a 0.45-µm membrane filter, 10 μL of each concentration of the standard solution was injected into the HPLC system for analysis in triplicates. Regression equation and coefficient of correlation was given in Table 1.

Validation of Method

The limit of detection (LOD) and limit of quantification (LOQ) were determined experimentally based on signal to noise ratio until the average responses were approximately three (S/N = 3) and ten times (S/N = 10) of the blank responses, respectively.

Accuracy of the method was ascertained by spiking the pre-analyzed samples with known amount of standard solution prepared at three level concentrations (50, 100, and 150 ppm) in triplicates. The average percentage recovery was estimated by applying values of peak area to the regression equations of the calibration graph.

Precision test: The mixtures composed of S, DS, AS, DMAS, MBS, and IVS were taken and repeatedly injected six times to measure the peak areas respectively. The results are expressed as relative standard deviation (RSD).

The precision of the method (inter-day and intra-day variations of replicate determinations) was checked by injecting standard solution at different concentrations (12.5, 50, and 100 ppm) in triplicates on the same day and on five consecutive days. The results are reported in terms of RSD.

RESULTS AND DISCUSSION

Identification of active compounds by thin-layer chromatography

The structures of reference compounds analyzed with TLC technique were shown in Figure 1. The separation of four *Echium* species root and aerial part ethanol extract with TLC technique were presented in Figure 2. A comparative chromatographic assessment showed the presence of shikonin derivatives in alcoholic extracts of roots of *E. italicum* L. *E. vulgare* L. and *E. angustifolium* Miller. The identification of the bands of shikonin derivatives (R_f=0.7~0.3) in the sample extract was confirmed by overlaying their absorption with those of the standard compounds at 254 nm and 366 nm.

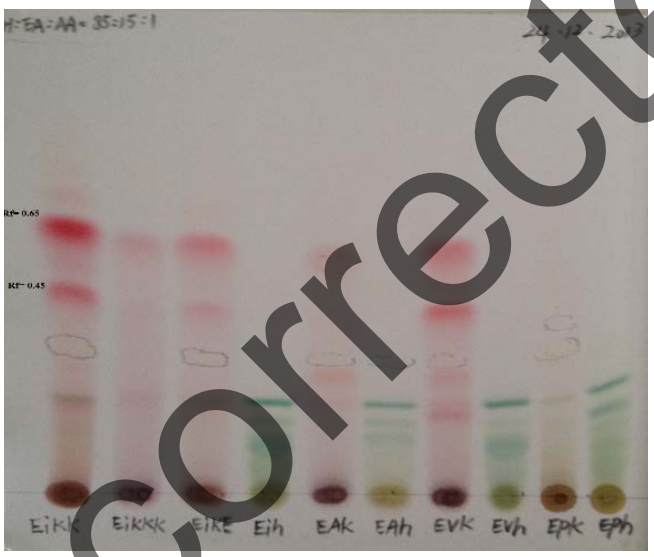

Figure 2. Thin layer chromatogram of four *Echium* species ethanol extracts from roots and aerial parts.

Note: EIKK: Chloroform extract of *E.italicum* roots; EIKKK: Chloroform extract of *E.italicum* root barks; EIKE: Ethanol extract of *E.italicum* roots; EIH: Ethanol extract of *E.italicum* aerial parts; EAK: Ethanol extract of *E.angustifolium* roots; EAH: Ethanol extract of *E.angustifolium* aerial parts; EVK: Ethanol extract of *E.vulgare* roots; EVH: Ethanol extract of *E.vulgare* aerial parts; EPK: Ethanol extract of *E.parviflorum* roots; and EPH: Ethanol extract of *E.parviflorum* aerial parts.

Optimization of HPLC Method

The critical aspects of developing a method in liquid chromatography are to provide a good resolution between studied compounds at a least analysis time. Under the current optimized conditions of HPLC with C18 column and UV detector at 520 nm using isocratic mixture of acetonitrile and water as mobile phase achieved best-resolved symmetric peaks for shikonin derivatives. It was observed that the retention time of reference standards was 4.176~16.58 minutes (Figure 3). The total run time of Shikonin derivatives was found to be 20 minutes and the shikonin derivatives in each extract could be observed on HPLC-UV chromatogram (Figure 4-7). This indicates that the present HPLC method is fast, specific, and convenient. The average retention time ± SD for AS, S, DS, DMAS, MBS, and IVS was found to be 4.176 ± 0.003, 8.752 ± 0.005, 11.006 ± 0.004, 15.059 ± 0.003, 15.672 ± 0.004 and 16.584 ± 0.003, respectively, for six replicates.

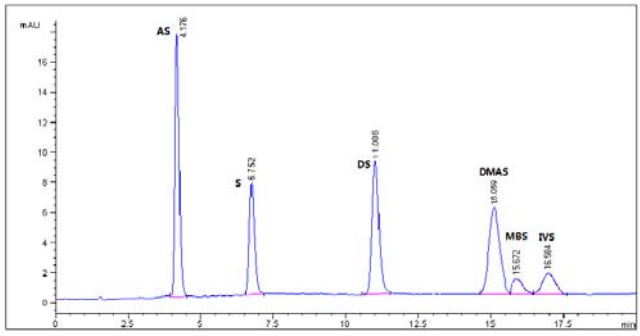

Figure 3. HPLC-UV chromatogram of reference standard mixtures prepared of 50 ppm

(AS, acetylshikonin; S: shikonin, DS: deoxyshikonin, DMAS: β, β-dimethylacryshikonin, IVS: isovalerylshikonin, MBS: 2-methyl butrylshikonin)

Validation of Method

The calibration graphs were generated by plotting the linear regression of peak area versus the corresponding analyte concentration; the calibration curve for each analyte was obtained using six concentration in the range of 2 - 500 ppm. The linear correlation coefficient (r^2) for all calibration curves was showing best linearity. The method was validated in terms of precision, accuracy and other validation method parameters. The repeatability of the HPLC method and intermediate precisions for intra-day and inter-day variations were given in Table 1. The LOD value was found to be 0.19~2.01 ppm, which is the concentration that yields a signal-to-noise (S/N) ratio of 3:1. The LOQ value under the described conditions was 0.57~6.03 ppm with an S/N ratio of 10:1. This confirmed the sensitivity for quantification of compounds.

Table 1. Validation parameters of the HPLC method for determination of shikonin derivatives.

Validation Parameters	S	AS	DS	DMAS	MBS	IVS
Calibration range (ppm)	2-100	1-20	2-50	2-25	6.25-500	6.25-500
Correlation coefficient (r)	0.999	0.998	0.999	0.9996	1	0.999
Regression equation	Y = 5.761x + 0.333	Y = 9.516x - 0.430	Y = 7.509x -3.424	Y = 10.2x - 5.073	Y = 2.920x -1.794	Y = 4.417x – 0.108
Limit of detection (ppm)	0.55	0.19	0.65	0.52	1.98	2.01
limit of quantitation (ppm)	1.65	0.57	1.95	1.55	5.94	6.03
Method precision (RSD %)	1.12	2.012	1.464	1.87	1.58	2.72

Intermediate precision (RSD%)						
Interday (%)	1.11	1.25	0.98	1.28	1.76	0.87
Intraday (%)	1.26	2.36	1.45	2.11	2.95	1.93
Accuracy (RSD %)	2.67	2.08	3.03	4.24	2.62	2.96
S: Shikonin; AS: Acetylshikonin; DS: Deoxyshikonin; DMAS: 3,3-dimethylacrylshikonin; MBS: 2-methyl-n-butyrylshikonin; IVS: Isovalerylshikonin;						

HPLC Analysis of Shikonin Derivatives in Different Samples

Concentrations of shikonin derivatives in ethanol extracts of different *Echium* species were determined from the peak-area ratios using the equations of linear regression obtained from the calibration curves. Data for concentration of shikonin derivatives in ethanol extracts obtained from different *Echium* species wild growing in Turkey are presented in Table 2. As can be observed, the content of shikonin derivatives was the highest in *E.italicum* L. compared to the other species.

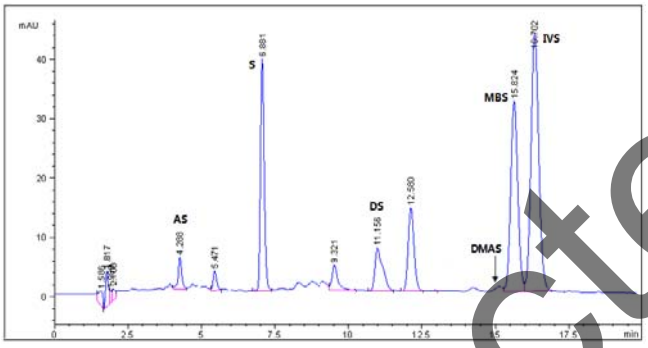

Figure 4. HPLC -UV chromatogram of ethanol extract of *E. italicum* L. root

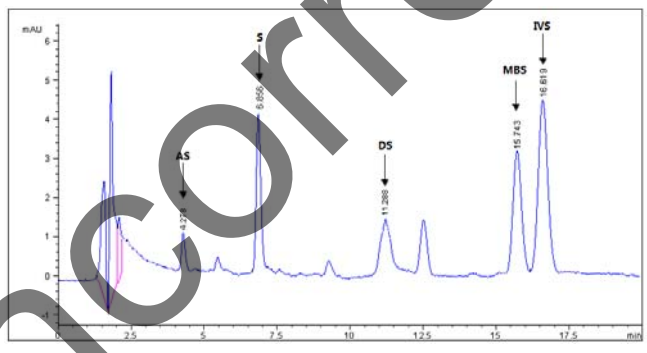

Figure 5. HPLC-UV chromatogram of ethanol extract of *E. vulgare* L. root

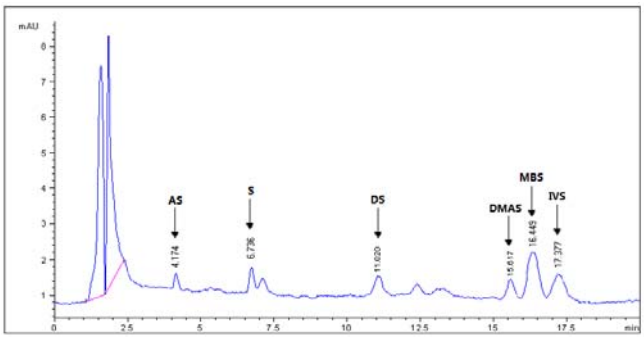

Figure 6. HPLC-UV chromatogram of ethanol extract of *E. angustifolium* Miller root

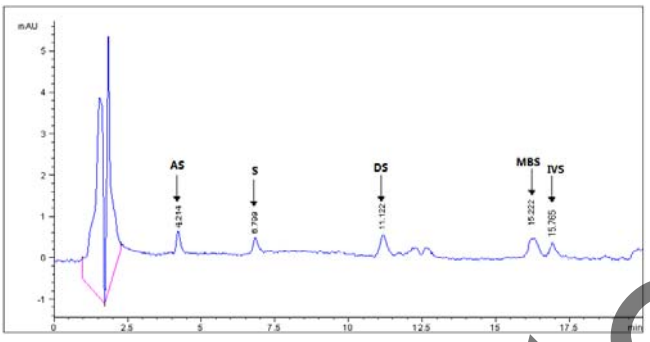

Figure 7. HPLC-UV chromatogram of ethanol extract of *E. parviflorum* Moench root

Table 2. Quantitative determination of shikonin derivatives in each *Echium* species

Content (w/w %)	S	AS	DS	DMAS	MBS	IVS
E.italicum L. root alcoholic Extract	0.391	0.037	0.105	0.015	1.147	1.185
E.vulgare L. root alcoholic Extract	0.053	0.040	0.022	0.004	0.162	0.121
E.angustifolium Millter root alcoholic Extract	0.007	0.003	0.009	0.010	0.074	0.028
E.parviflorum Moench root alcoholic Extract	0.007	0.003	0.008	n.d.*	0.023	0.015

*n.d.: Not detected

CONCLUSIONS

Shikonin and derivatives naturally occurring isohexenylnaphthazarine compounds, not only used as red dye in drugs, cosmetics and textile industry in the Far East and Europe (33), but also comprise the active ingredients of several pharmaceutical preparations due to exerting potent biological activities such as wound healing, antimicrobial, cytotoxic and antioxidant. However, these bioactive constituents become expensive due to their physical instability and tedious separation process. Therefore, it is important to develop a simple, fast, and sensitive method to evaluate shikonin compounds in drugs, extracts, and pharmaceutical preparations. The implications of current study would be important regarding to the first-time standardization of raw materials and preparations of the studied four *Echium sp*. roots. To the best of our knowledge,

the amount of shikonin and shikonin derivatives in four *Echium* sp. was determined for the first time in this study. We developed a simple, accurate, fast, and reproducible HPLC method to quantify the active principles in *Echium* species. This method can be used for routine quality control analysis of this species. Although shikonin derivatives are the main constituents of *Echium sp.* roots, the results of current study also indicate that the maximum shikonin derivative content in *Echium species* can be found in *Echium italicum* L. extract as compared to other studied extracts. Since most of the biological activities such as antimicrobial, antiproliferative, antioxidant, analgesic, and antidepressant activities of *Echium sp.* roots can be attributed to shikonin derivatives, the quantification of such compounds in the roots of these plants need to be considered as very important for future usage of preparations of these plants in the several areas of clinical medicine. By using the optimized separation method described above, shikonin derivatives can be detected in herbals or formulation prepared from Boraginaceae family plants, which are rich with naphthoquinone derivatives. We have demonstrate that the method is suitable for application in quality control of herbal formulations.

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