Rev Anal Chem 30 (2011): 59–63 © 2011 by Walter de Gruyter • Berlin • Boston. DOI 10.1515/REVAC.2011.014

Quantitative determination of shikonin derivatives with UV-Vis spectrophotometric methods in the roots of *Onosma nigricaule*

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Abstract

The genus *Onosma* is represented by 97 species, 4 varieties, 1 hybrid species in the flora of Turkey. Some *Onosma* species with roots containing naphthoquinones (alkannin, shikonin and alkannin/shikonin derivatives) are known as "Havaciva" in Turkey. *Onosma nigricaule* is an endemic species for Turkey. The roots of *O. nigricaule* are used for wounds and burns in Erzurum (Turkey). In this study, quantitative determination of shikonin derivatives (deoxyshikonin, *β,β*-dimethylacrylshikonin and acetyl shikonin) isolated from the roots of *O. nigricaule* was performed using a UV-Vis spectrophotometric method. The isolated shikonin derivatives have shown three maxima at 493 nm, 523 nm and 562 nm. The mean absorption coefficient of the three shikonin derivatives (deoxyshikonin, *β,β*-dimethylacrylshikonin and acetyl shikonin) was determined at 523 nm and 562 nm as 15.27 l/g cm and 9.39 l/g cm, respectively. The total content of shikonin derivatives were determined as 214.6 mg/100 g root at 523 nm and 220.6 mg/100 g root at 562 nm by using mean absorption coefficient of the three shikonin derivatives (deoxyshikonin, *β,β*-dimethylacrylshikonin and acetyl shikonin).

Keywords: Boraginaceae; *Onosma*; *Onosma nigricaule*; shikonin derivatives; UV-Vis spectrophotometry.

Introduction

The Boraginaceae family contains about 100 genus and 2000 species in the world (Evans 1989). The genus *Onosma* (Boraginaceae) is known to include more than 150 species (El-Shazly et al. 2003). The genus *Onosma* is represented by 97 species, 4 varieties, 1 hybrid species (102 taxa) in the flora of Turkey, 50 species and 1 variety of which are endemic, and the rate of endemism is 50% (Riedl 1978, Binzet and Orcan 2009). The roots of some *Onosma* species contain naphthoquinones (alkannin, shikonin and alkannin/shikonin derivatives) (Romanova et al. 1967, Tareeva et al. 1970, Shcherbanovskii 1971, 1972a,b, Sherbanivs'kii 1971, Sung et al. 1980, Romanova et al. 1981, Kagramanyan and Mnatsakanyan 1985, Mellidis and Papageorgiou 1987, Ai et al. 1989, Khajuria and Jain 1993, Özgen et al. 2004, Sagratini et al. 2008). In previous studies, it has been found that alkannin, shikonin and alkannin/shikonin derivatives show antimicrobial (Shukla et al. 1969, Papageorgiou et al. 1999, Ozgen et al. 2003, Naz et al. 2006, Ahmad et al. 2009), anti-inflammatory (Kundakovic et al. 2006, Tosun et al. 2008), antitumor (Papageorgiou et al. 1999), wound-healing (Ozgen et al. 2006) and other effects (Özgen et al. 2004, Cadirci et al. 2007).

The roots of some *Onosma* species (*O. armeniacum*, *O. nigricaule*, *O. obtusifolium*, *O. sericeum*, *O. microcarpum*) are used in treatment of wounds and burns in Eastern Anatolia in Turkey (Sezik et al. 1997, Özgen and Coşkun 2000). *O. nigricaule* is an endemic species for Turkey (Riedl 1978) and its roots are also used for wound-healing and burns in Erzurum Province (Turkey).

In this study, we aimed to isolate the naphthoquinones and to analyze the quantitative determination of total naphthoquinones in the roots of *O. nigricaule* by using UV-Vis spectrophotometry.

Materials and methods

Materials

Plant materials The roots of *O. nigricaule* were collected from Narman District (Erzurum Province, Turkey) in 2009 and were identified by Prof. Dr. M. Koyuncu. A voucher specimen was deposited in the Herbarium of Ankara University Faculty of Pharmacy (AEF 25874).

Chemicals Silica gel 60 (0.063–0.2 mm, Merck, Darmstadt, Germany) and Sephadex LH-20 (Sigma-Aldrich, Steinheim, Germany) were used for column chromatography and silica gel 60 F254 (Merck 5554, Darmstadt, Germany) for thin layer chromatography (TLC). *n*-Hexane, dichloromethane, ethyl acetate (EtOAC) and acetic acid (Riedel-de Haen, Seelze, Germany) were used for TLC and as extraction solvents. Chloroform (Merck, Darmstadt, Germany) was used for UV-Vis spectrophotometric measurements.

Instruments and spectral analysis

^{1}H-NMR and ^{13}C-NMR spectra of isolated compounds were recorded with a Varian Mercury plus spectrometer at 400 MHz and 100 MHz in $CDCl_3$, respectively. A Thermo Spectronic double-beam UV spectrophotometer (HEλIOS- β,

USA) with a data processing system was used for obtaining the UV-Vis spectra of *n*-hexane: dichloromethane extract of the roots and the isolated shikonin derivatives. The spectra of isolated compounds were recorded in 1 cm quartz cells at a scan range of 350–750 nm fixed slit width of 2 nm. The absorption maxima and absorption coefficient were established by using isolated shikonin derivatives (deoxyshikonin, *β*,*β*-dimethylacrylshikonin and acetyl shikonin) that were diluted with chloroform used as a blank solution in determination of the absorption coefficient.

Extraction and isolation

The roots of *O. nigricaule* (590 g) were powdered and extracted with a *n*-hexane: dichloromethane mixture (1:1) (3000 ml×3) under reflux for 3 h for each extraction on a mantle. The combined extracts were evaporated under reduced pressure to give concentrated extract (11.5 g; yield: 1.9%). The extract (11 g) was subjected to silica gel column chromatography using *n*-hexane:EtOAc with gradient elution (95:5→0:100). Similar fractions determined by TLC were combined. *n*-Hexane:EtOAc (95:5) (fraction 1–15) gave compound (**1**), deoxyshikonin (10 mg). *n*-Hexane:EtOAc (90:10→80:20) (fraction 16–38) gave compound (**2**), *β*,*β*-dimethylacrylshikonin (20 mg). *n*-Hexane:EtOAc (80:20) (fraction 47–70) gave compound (**3**), acetyl shikonin (15 mg) (Figure 1). The final purification of the compounds was performed by preparative TLC [solvent system: *n*-hexane: EtOAc:HOAc (80:20:2)] and by Sephadex LH-20 column chromatography using $CHCl_3$.

Deoxyshikonin [2-(4-methyl-pent-3-enyl)-5,8-dihydroxy-naphthalene-1,4-dione] (**1**): (10 mg). Red oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 12.63 (s, 1H, phenolic OH), 12.47 (s, 1H, phenolic OH), 7.21 (s, 2H, H-6 and H-7), 6.84 (t, 1H, H-3, *J*=1.1 Hz), 5.13 (m, 1H, H-3′), 2.64 (bt, 2H, H-1′, *J*=7.2), 2.30 (m, 2H, H-2′), 1.69 (s, 3H, H-5′), 1.60 (s, 3H, H-6′). ^{1}H-NMR data are in agreement with the data given in the literature (Özgen et al. 2004).

***β*,*β*-Dimethylacrylshikonin** (5,8-Dihydroxy-2-[1-(β,β-dimethylacryloyloxy)-4-methyl-3-pentenyl]-1,4-naphthalenedione) (**2**): (20 mg). Dark red solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 12.59 (s, 1H, phenolic OH), 12.43 (s, 1H, phenolic OH), 7.18 (s, 2H, H-6 and H-7), 6.98 (s, 1H, H-3), 6.01 (m, 1H, H-3′), 5.78 (m, 1H, H-1″), 5.15 (tm, 1H, H-1′, *J*=7.3 Hz), 2.64–2.40 (m, 2H, H-2′), 2.16 (d, 3H, *J*=1.1 Hz), 1.94 (d, 3H, *J*=1.5 Hz), 1.68 (s, 3H), 1.58 (s, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 178.9 (C-4), 177.5 (C-1), 166.8 (C-8), 166.3 (C-5), 165.3 (C-1″), 158.9 (C-3″), 149.0 (C-2), 135.8 (C-4′), 132.6 (C-6), 132.5 (C-7), 131.6 (C-3), 118.0 (C-3′), 115.3 (C-2″), 111.9 (C-9), 111.6 (C-10), 68.7 (C-1′), 32.9 (C-2′), 27.6 (C-5″), 25.7 (C-5′), 20.3 (C-4″), 17.9 (C-6′). ^{1}H-NMR and ^{13}C-NMR data are in agreement with the data given in the literature (Han et al. 2008).

Acetyl shikonin [(+)-Acetic acid 1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydro-naphthalen-2-yl)-4-methyl-pent-3-enyl ester] (**3**): (15 mg). Dark red solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 12.58 (s, 1H, phenolic OH), 12.43 (s, 1H, phenolic OH), 7.18 (s, 2H, H-6 and H-7), 6.99 (d, 1H, H-3, *J*=0.8 Hz), 6.01 (ddd, 1H, H-1′, *J*=7.0, 4.6, 1.0 Hz), 5.12 (tm, 1H, H-3′, *J*=6.0 Hz), 2.64–2.45 (m, 2H, H-2′), 2.14 (s, 3H, H-2″), 1.69 (s, 3H, H-5′), 1.58 (s, 3H, H-6″). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 178.3 (C-4), 176.8 (C-1), 169.9 (C-1″), 167.8 (C-8), 167.2 (C-5), 148.4 (C-2), 136.3 (C-4′), 133.1 (C-6), 133.0 (C-7) 131.7 (C-3), 117.9 (C-3′), 112.1 (C-9), 111.8 (C-10), 69.7 (C-1′), 33.1 (C-2′), 25.9 (C-5′), 21.1 (C-2″), 18.1 (C-6′). ^{1}H-NMR and ^{13}C-NMR data are in agreement with the data given in the literature (Özgen et al. 2004).

Determination of total shikonin derivatives

A small aliquot of filtered extracts was accurately weighed and then diluted with chloroform to yield absorbance measurements within the optimum range of the instruments. The obtained solutions were stored in dark glass flasks, to protect them from light. The shikonin derivatives [deoxyshikonin (**1**), *β*,*β*-dimethylacrylshikonin (**2**) and acetyl shikonin (**3**)] showed three maxima at 493 nm, 523 nm and 562 nm. The total content of shikonin derivatives were determined at 523 nm and 562 nm and calculated by the help of the appropriate weight, volume, dilution factors and average absorption coefficient for the shikonin derivatives.

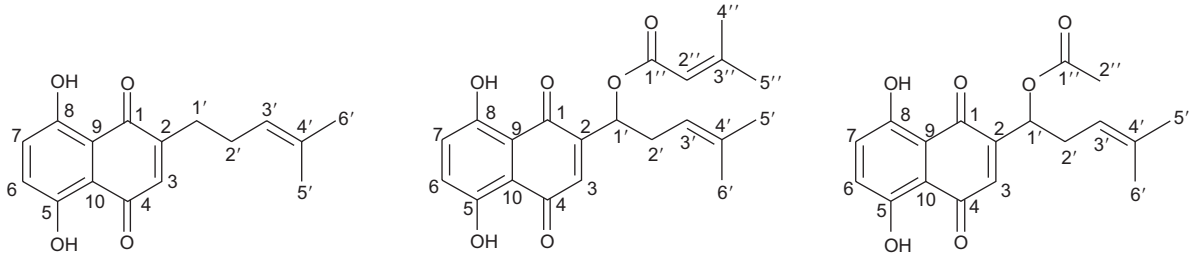

Figure 1 Compounds isolated from *O. nigricaule* roots.

Construction of the absorption coefficient

The purified shikonin derivatives were isolated from the extract of the roots of *O. nigricaule*. The stock solutions of individual shikonin derivatives were prepared with chloroform. Each concentration of them was 1 mg/ml and 0.2 ml aliquots were diluted to 4 ml with the same solvent (0.05 g/l). The absorbance values of the prepared shikonin derivative solutions were measured three times at the absorbance maxima by using a 1-cm cuvette.

Results and discussion

Evaluation of isolated shikonin derivatives

n-Hexane:dichloromethane extract of the roots of *O. nigricaule* was subjected to chromatographic separation by a gradient elution with *n*-hexane:EtOAc. Similar fractions were collected and combined. As a result of repeated TLC experiments, compounds **1–3** were purified. The chemical structure confirmation of these compounds was accomplished by extensive NMR (^{1}H-NMR and ^{13}C-NMR spectral data) studies. Their structures are shown in Figure 1.

Determination of the total shikonin derivatives

Absorbance measurements and absorption coefficient calculation All spectrophotometric measurements were carried out by using UV-Vis spectrophotometer. This type of apparatus does not allow changing measurement parameters, so the working conditions of the spectrophotometer were integration time 1 s, spectral band with 2 nm and spectrum scan 0.1 s. Optimal wavelength range should be chosen since the broad peaks become sharper, the ratio of signal/noise elevates and the sensitivity of the method increases by controlling the degree of low passes filtering or smoothing. Optimum results were obtained in the measuring wavelength range 350–750 nm for dilute shikonin derivatives solutions and Figure 2A,B and C show the spectra of deoxyshikonin, β,β-dimethylacrylshikonin and acetyl shikonin when each concentration equal to 0.05 g/l in chloroform, respectively.

In chloroform media, the absorbance spectrum of each shikonin derivative gives three peaks (493 nm, 523 nm and 562 nm) in the visible range. The absorbance measurements were carried out at 523 nm and 562 nm, having a sharper peak. The absorption coefficients of each shikonin derivative solution (0.05 g/l) at 523 nm and 562 nm were calculated (n=3). Differences between the absorption coefficients of three shikonin derivatives were rather small in both wavelengths. The obtained results are shown in Table 1.

Calculation of the total shikonin derivatives In the roots of *O. nigricaule*, the ratio of individual shikonin derivatives was not always known or determined with the spectrophotometric method. Thus, the mean absorption coefficient of the three shikonin derivatives (deoxyshikonin, β,β-dimethylacrylshikonin and acetyl shikonin) at 523 nm and 562 nm (Table 1) were used in the calculation of total shikonin derivatives. The total absorbance (TA) was calculated first. Through this way, the absorbance measured

Table 1 Results of regression analysis of the proposed methods.

Shikonin derivatives	Maximum (nm)	Absorption coefficients (l/g·cm)	SD
Deoxyshikonin	520	14.22	0.015
	560	8.61	0.017
β,β-Dimethylacrylshikonin	523	16.29	0.017
	562	10.04	0.012
Acetyl shikonin	523	15.28	0.017
	563	9.53	0.013
Mean	523	15.27	1.038
	563	9.39	0.724

SD, standard deviation of three replicate determinations.

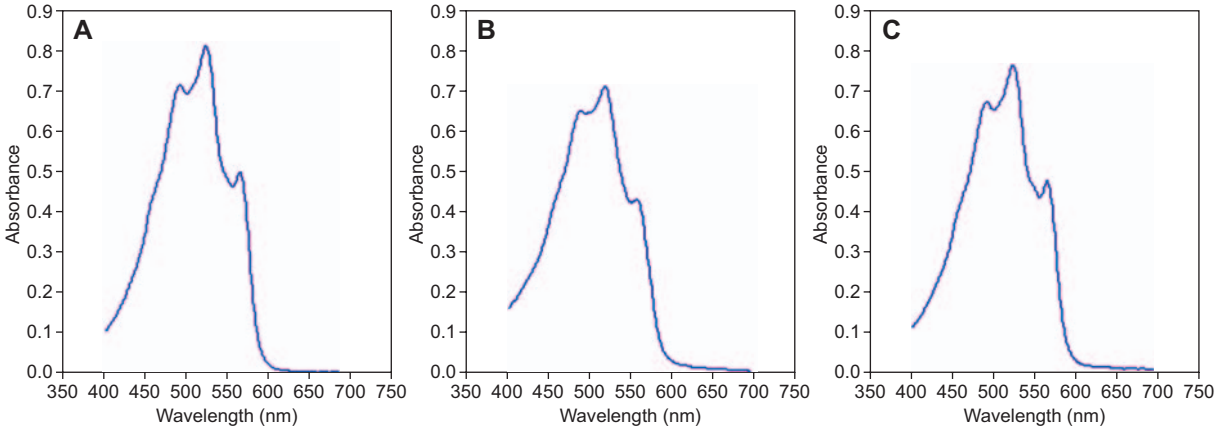

Figure 2 Spectra obtained from the dilute shikonin derivative solutions: (A) deoxyshikonin, (B) β,β-dimethylacrylshikonin, (C) acetyl shikonin.

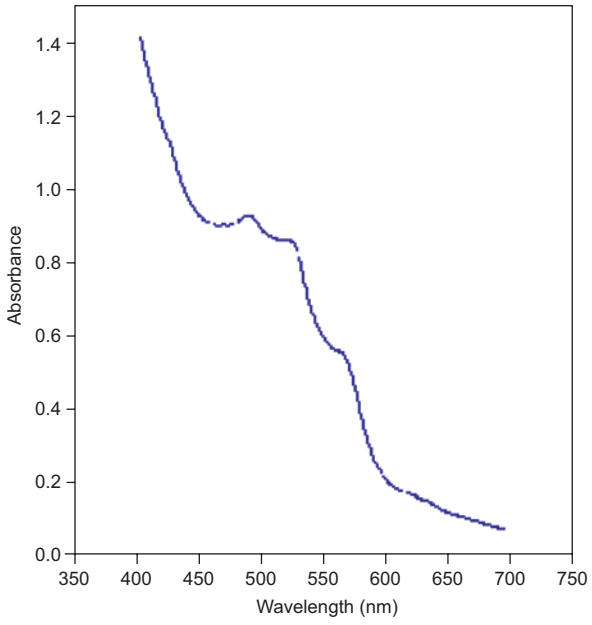

Figure 3 Spectrum obtained from the extract of the roots of *O. nigricaule*.

on small aliquot of the diluted extract was applied to certain quantity (0.1 g extract) of the root of *O. nigricaule* (n=5). The TA obtained was transformed to mg of shikonin derivatives with the aid of the absorbance coefficient (a). The TA for extract was calculated using the following equations:

$A=a\times b\times C$

Total shikonin derivatives (mg/0.1 g extract)$=TA\times DF/a_{523}^{0.05\%}$

Total shikonin derivatives [mg/100 g root (1.9 g extract)] $=(TA\times DF/a_{523}^{0.05\%})\times(1.9/0.1)$

Total shikonin derivatives (mg/0.1 g extract)$=TA\times DF/a_{562}^{0.05\%}$

Total shikonin derivatives [mg/100 g root (1.9 g extract)] $=(TA\times DF/a_{562}^{0.05\%})\times(1.9/0.1)$,

where TA is total absorbance and DF is dilution factor.

Total shikonin derivatives level of the roots of *O. nigricaule* was found as 214.6 mg/100 g root for 523 nm and 220.6 mg/100 g root for 562 nm.

The absorbance spectrum obtained from the extract of the roots of *O. nigricaule* is shown in Figure 3. In conclusion, it might be thought that the proposed method in our study could be applied to determination of total content of alkannin/shikonin derivatives in other *Onosma* species

Acknowledgments

The authors would like to thank Prof. Dr. Mehmet Koyuncu, Ankara University for identification of the plant material and Prof. Dr. Hasan Seçen, Atatürk University for structure elucidation of the compounds.

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