# Cytotoxic Naphthoquinones from Alkanna cappadocica

**ARTICLE** *in* JOURNAL OF NATURAL PRODUCTS · MAY 2010

Impact Factor: 3.8 · DOI: 10.1021/np900778j · Source: PubMed



**5 AUTHORS**, INCLUDING:

Canan sevimli gür
Kocaeli University
**9** PUBLICATIONS **157** CITATIONS
SEE PROFILE

Hakki Akgun
Ege University
**9** PUBLICATIONS **130** CITATIONS
SEE PROFILE

Ismet Deliloglu Gurhan
Ege University
**35** PUBLICATIONS **188** CITATIONS
SEE PROFILE

Kemal Sami Korkmaz
Ege University
**47** PUBLICATIONS **954** CITATIONS
SEE PROFILE

# Cytotoxic Naphthoquinones from *Alkanna cappadocica*[⊥]

Canan Sevimli-Gur, Ismail H. Akgun, Ismet Deliloglu-Gurhan, Kemal S. Korkmaz,* and Erdal Bedir*

*Department of Bioengineering, Faculty of Engineering, Ege University, 35100 Bornova-İzmir, Turkey*

*Received December 8, 2009*

In a continuing program to discover new anticancer agents from plants, especially naphthoquinones from the *Alkanna* genus, *Alkanna cappadocica* was investigated. Bioassay-guided fractionation of a dichloromethane/methanol (1:1) extract of the roots led to the isolation of four new and four known naphthoquinones. The known compounds are 11-deoxyalkannin (**1**), *β*,*β*-dimethylacrylalkannin (**2**), 11-*O*-acetylalkannin (**3**), and alkannin (**4**). The new compounds 5-*O*-methyl-11-deoxyalkannin (**5**), 8-*O*-methyl-11-deoxyalkannin (**6**), 5-*O*-methyl-11-*O*-acetylalkannin (**7**), and 5-*O*-methyl-*β*,*β*-dimethylacrylalkannin (**8**) were characterized by spectroscopic analyses (LC-ESIMS, 1D and 2D NMR). Cytotoxicity of the isolated compounds was evaluated versus 12 human cancer cell lines, HT-29, MDA-MB-231, PC-3, AU565, Hep G2, LNCaP, MCF7, HeLa, SK-BR-3, DU 145, Saos-2, and Hep 3B together with two normal cell lines, VERO and 3T3, by using the MTT assay. Compound **7** showed remarkable cytotoxicity with $IC_{50}$ values between 0.09 and 14.07 $\mu$M. It was more potent than the other compounds in six out of 12 cancer cell lines and the positive controls doxorubicin and etoposide. The mono-*O*-methylated alkannin derivatives and their cytotoxicities are reported for the first time.

*Alkanna* Tausch, a member of the family Boraginaceae, is a small genus with about 50 species, originally from Europe, the Mediterranean, and western Asia. The *Alkanna* genus is represented by 34 species and 40 taxa in the flora of Turkey.[1] Since the entire genus is almost endemic (32 endemic species), one can assume that the Anatolian peninsula is the gene center of *Alkanna* species.

Among *Alkanna* species, the roots of *A. tinctoria* Tausch is a well-known dyestuff for fabrics dating back to centuries B.C. Perhaps the first recorded medicinal use of *A. tinctoria* roots is found in the work of Hippocrates (fourth and fifth centuries B.C.), who described their use for the treatment of ulcers.[2] In 1976, the wound-healing and antimicrobial properties of *A. tinctoria* root extracts were described by Papageorgiou and his co-workers, and alkannins were reported for the first time as the active components.[2] Phytochemical investigations performed on the *Alkanna* genus has led to the identification of various flavonoids,[3] fatty acids,[4] pyrolizidine alkaloids,[5] and alkannin derivatives.[6a–f]

Alkannins are naphthoquinones that have two ketone moieties at C-1 and C-4. 5,8-Dihydroxy derivatives of these classes of compounds are called hydroxynaphthoquinones (Figure 1). Alkannin and shikonin, which are enantiomers (*S* and *R*, respectively), are isohexenylnaphthazarine derivatives of hydroxynaphthoquinones and are present as ester derivatives in the outer surface of the roots of at least 150 species that belong to the genera *Alkanna*, *Lithospermum*, *Echium*, *Onosma*, *Anchusa,* and *Cynoglossum* of the Boraginaceae family.[7]

Isohexenylnaphthazarines are used as natural colorants for food, cosmetics, and textiles. Recently interest in the isohexenylnaphthazarines increased because of their potent anti-inflammatory,[8] antifungal,[9] antioxidant,[10a,b] cytotoxic,[1] radical scavenging,[1] and enzyme inhibitor properties.[13a,b] Beside these activities, acyl derivatives of isohexenylnaphthazarines have been investigated for topoisomerase I inhibition and proved to be potential anticancer agents.[14a–d]

During our continuing studies on the genus *Alkanna*,[15a,b] we screened the MeOH root extracts of 16 Anatolian *Alkanna* species versus Hep-3B, MCF-7, MDA-MB-231, and PC-3 cancer cell lines for their cytotoxic activities. *A. cappadocica* Boiss. & Bal was found to be a potent tumor inhibitor, with $IC_{50}$ values of $\leq 10$ $\mu$g/mL. Thus, a bioassay-guided fractionation study was undertaken to purify the active principles of *A. cappadocica*, and here we report the structure elucidation of four known (**1**−**4**) and four new (**5**−**8**) naphthoquinone derivatives together with their cytotoxic activities.

## Results and Discussion

Fractionation of the $CH_2Cl_2$/MeOH (1:1) root extract of *A. cappadocica* resulted in the localization of the cytotoxic activities in the apolar part. Column chromatography performed on silica gel, reversed-phase (RP) C-18, and Sephadex LH-20 together with preparative thin-layer chromatography (PTLC: silica gel and RP C-18) led to the isolation of eight naphthoquinones (**1**−**8**) (Figure 1), of which **1**−**4** were identified by comparison of their physical data with those previously reported.[14d,6c]

Compound **5** was obtained as reddish powder, and its thin-layer chromatogram demonstrated a typical naphthoquinone profile. The HR-MALDITOF mass spectrum of **5** (*m*/*z* 287.1287 $[M + H]^+$, calcd for $C_{17}H_{19}O_4$, 287.1283) supported a molecular formula of $C_{17}H_{18}O_4$.

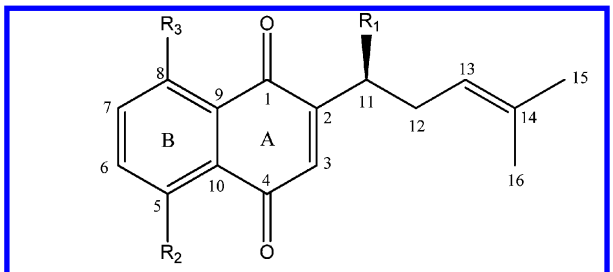
The $^1$H NMR spectrum of **5** exhibited a deshielded proton at $\delta$ 12.49 (s, *O*-H), two *ortho*-coupled and one singlet aromatic proton ($\delta$ 7.33, 1H, d, $J = 9.2$ Hz; $\delta$ 7.27, 1H, d, $J = 9.2$ Hz; $\delta$ 6.70, 1H, s), and an *O*-methyl at $\delta$ 3.97 (s, 3H). Inspection of the $^1$H and $^{13}$C NMR spectra of **5** (Table 1) showed characteristic signals for a 4-methylpent-3-enyl side chain, which was also present in compound **1** (11-deoxyalkannin) [$^1$H: $\delta$ 1.69 and $\delta$ 1.60, each s, each 3H, $H_3$-15 and $H_3$-16; $\delta$ 5.13, t, $J = 7.2$ Hz, H-13; $\delta$ 2.28, t, $J = 7.6$ Hz, 2H, H-11; $\delta$ 2.58, q, $J = 7.6$ Hz, 2H, H-12; $^{13}$C: 30.4, t, C-11; 26.8, t, C-12; 122.8, d, C-13; 133.6, s, C-14; 25.9, q, C-15; 18.0, q, C-16]. The presence of the deoxyalkannin nucleus was readily deduced from the proton and carbon chemical shifts.[14d,6c]

The full assignment of the proton and carbon signals of the naphthoquinone part of **5**, secured by HMQC and HMBC spectra and the comparison of these data with those of 11-deoxyalkannin (**1**), indicated the similarities of these compounds. The significant differences between the two compounds were (i) the presence of an O*H* signal in **5** instead of two O*H* signals in **1** ($\delta$ 12.5 and 12.6), (ii) the presence of an O-*CH*$_3$ signal ($\delta$ 3.97) in **5**, and (iii) two *ortho*-coupled aromatic protons (H-6 and H-7 in **5**) in place of a two-proton aromatic singlet in **1**. All of these differences in **5** could be attributed to the presence of a methoxy group, which resulted in destabilizing the symmetry of ring B and a missing hydrogen bond between 5-*OH* and C-4 carbonyl in, for example, **1**. Intrigu-

[⊥] In memory of Salahi Yıkıcı, whom we have lost to cancer (1990−2010, undergraduate student). As a bright student, he was planning to engage in anticancer natural products.

* To whom correspondence should be addressed. Tel: +90 232 388 4955. Fax: + 90 232 388 4955. E-mail: erdal.bedir@ege.edu.tr (E.B.); ks_korkmaz@yahoo.com (K.S.K.).

10.1021/np900778j 



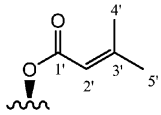
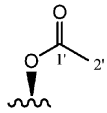
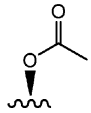
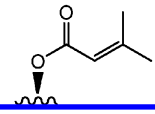

| Compound | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 1 | H | OH | OH |
| 2 |  | OH | OH |
| 3 |  | OH | OH |
| 4 | OH | OH | OH |
| 5 | H | $OCH_3$ | OH |
| 6 | H | OH | $OCH_3$ |
| 7 |  | $OCH_3$ | OH |
| 8 |  | $OCH_3$ | OH |

**Figure 1.** Structures of compounds **1**−**8**.

ingly, as a result of the missing symmetry and absence of the extra ring system formed by the hydrogen bond, compound **5** behaved more polar than **1** on thin-layer chromatography [$R_f$ values on silica gel: 0.27 for **5** and 0.77 for **1** (mobile phase: *n*-hexane/EtOAc/HOAc, 80:20:0.2)].

The position of the *O*-methyl group was unambiguously determined by the HMBC experiment (Figure 2). The carbonyl signal at $\delta$ 183.7 showed HMBC connectivities to $H_2$-11 ($\delta$ 2.28) and H-3 ($\delta$ 6.70), allowing it to be assigned to C-1. Thus, the carbonyl signal at $\delta$ 190.7 was readily assigned to C-4 (ring A). The chemical shift of C-4 requires a ca. 7−8 ppm downfield shift in the case of *O*-$CH_3$ substitution at C-5. Thus, the position of the methoxy group was deduced to be at C-5. On the other hand, long-range correlations from C-9 and C-7 ($\delta$ 115.2 and 126.6, respectively) to the *OH*-proton ($\delta$ 12.49, at C-8) and from C-10 ($\delta$ 118.2) to H-3 ($\delta$ 6.70) substantiated our assignments regarding the position of the *O*-$CH_3$ group.

On the basis of these results, the structure of **5**, a new natural product, was established as 8-hydroxy-5-methoxy-2-(4-methylpent-3-enyl)naphthalene-1,4-dione (5-*O*-methyldeoxyalkannin).

The HR-MALDITOF mass spectrum of **6** also showed a quasimolecular ion peak for $[M + H]^+$ at *m/z* 287.1280 (calcd for $C_{17}H_{19}O_4$, 287.1283), in agreement with the molecular formula $C_{17}H_{18}O_4$, as for **5**.

Examination of the 1D and 2D NMR spectra of **6** and comparison with those of **5** showed their considerable structural similarity: a 4-methylpent-3-enyl side chain, one missing *OH* proton, and the presence of an *O*-methyl group. The main difference between **5** and **6** was evident from the carbon signals of ring B, suggesting regioisomerism of the methoxy group (Table 1). Indirect confirmation based on the $^{13}C$ NMR differences together with direct evidence of the linkage site derived from the HMBC experiment revealed the location of the *O*-$CH_3$ group at C-8. On the basis of these results, the structure of **6** was elucidated as 5-hydroxy-8-methoxy-2-(4-methylpent-3-enyl)naphthalene-1,4-dione (8-*O*-methyldeoxyalkannin).

The HR-MALDITOF mass spectra of **7** and **8** showed ion peaks for $[M + H]^+$ at *m/z* 345.1332 and 385.1654, in agreement with the molecular formulas $C_{19}H_{20}O_6$ and $C_{22}H_{24}O_6$, respectively. Examination of the 1D NMR spectra of **7** and **8** and comparison with those of **5** showed their considerable structural similarity. The signals due to the naphthalene-1,4-dione moiety were superimposable. The differences consisted only in the signals of the side chain. $^1H$ and $^{13}C$ NMR spectra of **7** and **8** suggested the presence of a 4-methylpent-3-en-1-ol moiety instead of a 4-methylpent-3-enyl side chain of **5**.

The $^{13}C$ and DEPT spectra of **7**, excluding the signals ascribable to the naphthoquinone moiety, showed that the remaining resonances comprise three methyl ($\delta$ 18.2, 21.2 and 25.9), one methylene ($\delta$ 33.1), one oxygenated methine ($\delta$ 70.3), two olefinic ($\delta$ 118.2 and 136.1), and one carbonyl carbon ($\delta$ 169.9).

Comparison of the NMR spectra of **7** with those of **3** suggested that **7** and **3** had the same 4-methylpent-3-enyl acetate side chain.

In a similar fashion, assignment of the proton and carbon signals of **8**, secured by DQF-COSY and HMQC spectra and the comparison of these data with those of **2** ($\beta$,$\beta$-dimethylacrylalkannin),[14d,6c] indicated that both compounds possessed a 4-methylpent-3-enyl 3-methylbut-2-enoate side chain.

Having established the structures of **7** and **8**, the configuration of C-11 remained to be solved. The absolute configurations of compounds **7** and **8** were resolved using optical rotation data. Comparison of the specific rotation of **7** and **8** ($[\alpha]_D^{25}$ −50.0; *c* 0.02, $CHCl_3$ for both compounds) with alkannin (**4**) ($[\alpha]_D^{20}$ −150.5; *c* 0.05, benzene) and shikonin ($[\alpha]_D^{20}$ +135.6; *c* 0.05, benzene)[16] was consistent with **7** and **8** possessing an 11*S* absolute configuration.

Consequently, the structures of compounds **7** and **8** were established as 5-*O*-methylacetylalkannin [1-(8-hydroxy-5-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl acetate] and 5-*O*-methyl-$\beta$,$\beta$-dimethylacrylalkannin [1-(8-hydroxy-5-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 3-methylbut-2-enoate].

Compounds **5**−**8** represent the first examples of naphthoquinone-type compounds from the Boraginaceae family possessing a distinct mono-*O*-methyl unit at C-5 or C-8.

All of the tested compounds demonstrated cytotoxic activity against the entire cell lines (Table 2). Compounds **1**, **2**, **7**, and **8** exhibited superior activity than the other compounds and positive controls. Compounds **7** and **8** were effective versus HT-29, MDA-MB-231, PC-3, AU565, HepG2, HeLa, and SK-BR-3 lines with $IC_{50}$ values ranging between 0.09 and 0.97 $\mu$M. Compound **1** had greater activity toward MCF-7 and DU 145 cell lines ($IC_{50}$ = 0.79 and 1.24 $\mu$M, respectively), whereas **2** was the most active metabolite against Saos-2 (0.84 $\mu$M).

Among the cancer cell lines, MDA-MB-231, AU565, HeLa, and SK-BR-3 turned out to be the most susceptible cell lines inhibited by naphthoquinones **1**−**8**. Interestingly, three of the most sensitive cell lines (MDA-MB-231, AU565, and SK-BR-3) were breast cancers. Especially the new metabolite **7** showed a significant $IC_{50}$

**Table 1.** NMR Spectroscopic Data (400 MHz, $CDCl_3$) for Compounds **5**−**8**

| | **5** | | **6** | | **7** | | **8** | |
|---|---|---|---|---|---|---|---|---|
| position | $\delta_H$ (*J* in Hz) | $\delta_c$[a] | $\delta_H$ (*J* in Hz) | $\delta_c$[a] | $\delta_H$ (*J* in Hz) | $\delta_c$[a] | $\delta_H$ (*J* in Hz) | $\delta_c$[a] |
| 1 | | 183.7 s | | 191.0 s | | 182.1 s | | 182.1 s |
| 2 | | 154.6 s | | 153.8 s | | 152.2 s | | 152.6 s |
| 3 | 6.70 s | 133.2 d | 6.80 t (1.2) | 138.3 d | 6.76 d (1.2) | 131.6 d | 6.75 d (1.2) | 131.6 d |
| 4 | | 190.7 s | | 183.8 s | | 190.3 s | | 190.4 s |
| 5 | | 154.3 s | | 156.9 s | | 154.5 s | | 154.5 s |
| 6 | 7.27 d (9.2) | 123.2 d | 7.27 d (9.2) | 126.5 d | 7.28 d (9.2) | 123.6 d | 7.28 d (9.2) | 123.6 d |
| 7 | 7.33 d (9.2) | 126.6 d | 7.34 d (9.2) | 123.6 d | 7.35 d (9.2) | 127.0 d | 7.35 d (9.2) | 127.0 d |
| 8 | | 156.4 s | | 148.7 s | | 156.6 s | | 156.6 s |
| 9 | | 115.2 s | | 118.0 s | | 114.8 s | | 114.9 s |
| 10 | | 118.2 s | | 115.3 s | | 117.7 s | | 117.8 s |
| 11 | 2.28 t (7.6) | 30.4 t | 2.55 dt (7.6, 1.2) | 29.1 t | 5.95 ddd (1.2, 4.4, 5.6) | 70.3 d | 6.02 ddd (1.2, 3.4, 6.8) | 70.1 d |
| 12 | 2.58 q (7.6) | 26.8 t | 2.28 q (7.6) | 26.5 t | 2.43 dd (7.6, 15.2) | 33.1 t | 2.47 m | 33.2 t |
| | | | | | 2.61 dd (15.2, 5.6) | | 2.66 m | |
| 13 | 5.13 t (7.6) | 122.8 d | 5.13 t (7.6) | 122.7 d | 5.12 dt (7.6, 1.2) | 118.2 d | 5.15 t (7.6) | 118.4 d |
| 14 | | 133.6 s | | 133.7 s | | 136.1 s | | 135.9 s |
| 15 | 1.60 s | 25.9 q | 1.59 s | 25.8 q | 1.58 s | 25.9 q | 1.59 s | 26.0 q |
| 16 | 1.69 s | 18.0 q | 1.69 s | 18.0 q | 1.69 s | 18.2 q | 1.68 s | 18.2 q |
| O-*CH*$_3$ | 3.97 s | 57.1 q | 3.97 s | 57.1 | 3.98 s | 57.1 q | 3.98 s | 57.1 q |
| 1′ | | | | | | 169.9 s | | 166.6 s |
| 2′ | | | | | 2.12 s | 21.2 q | 6.15 dq (6.0, 1.2) | 127.5 d |
| 3′ | | | | | | | | 139.7 s |
| 4′ | | | | | | | 2.00 dd (1.6, 5.6) | 20.7 q |
| 5′ | | | | | | | 1.95 dd (1.2, 1.6) | 16.0 q |

[a] Multiplicities of carbon signals were determined by DEPT (135°) experiments.

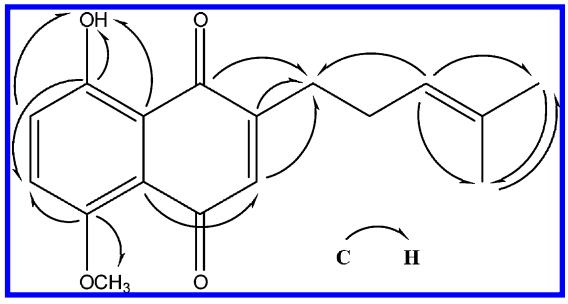



**Figure 2.** Key long-range correlations of compound **5**.

value (0.09 $\mu$M) against the estrogen-receptor negative cell line MDA-MB-231, for which new therapeutics urgently need to be developed.

Earlier studies indicated that acylation of C-11(*O*) potentiates the cytotoxicity due to the enhancing effect of acylation on the electrophlicity of the quinonoid moiety.[17] The most effective compounds identified in this study (**2**, **7**, and **8**) possessed C-11-*O*-acyl groups. Although the presence of acylation at C-11 seemed to be important, 11-*O*-acetylalkannin (**3**) was found to be 13- (HT-29), 26- (MDA-MB-231), 7- (Hep G2 and HeLa), 6- (Saos-2), and 10-fold (PC-3) less active than **2**, having a $\beta$,$\beta$-dimethylacryl unit.

Additionally it was intriguing to observe that the 5-*O*-methyl derivative of 11-*O*-acetylalkannin (**7**) was 17-, 15-, 14-, 9-, 10-, and 4-fold more active than the congener **3** against HT-29, MDA-MB-231, PC-3, Hep G2, HeLa, and SK-BR-3, respectively, whereas 5-*O*-methyl-$\beta$,$\beta$-dimethylacrylalkannin (**8**) and its congener $\beta$,$\beta$-dimethylacrylalkannin (**2**) were almost equally active versus most of the cancer cell lines, implying a complex relationship between structure and activity.

Compounds **1**−**8** and positive controls were also cytotoxic toward normal cell lines VERO and 3T3 (murine cell line) (Table 2). The distinctive mono-*O*-methylated alkannin derivatives **7** and **8** (5-*O*-methylated) are good candidates for semisynthesis studies to improve the therapeutic index and develop new chemical entities as drug leads for the treatment of cancer.

## Experimental Section

**General Experimental Procedures.** IR spectra were obtained on an ATI Mattson Genesis Series and Jasco 430 FT-IR spectrometers. The LC-MS spectrometric analysis was performed on an Agilent Series 1100 SL equipped with an ESI source. Exact masses were measured by a Voyager DE mass spectrometer. Samples were analyzed by MALDITOF mass spectrometry. Optical rotation measurements were done on an Autopol IV polarimeter in $CHCl_3$ at 25 °C. NMR spectra were recorded on a Varian Oxford AS400 NMR spectrometer. Proton and carbon chemical shifts are relative to the internal standard TMS. Multiplicity determinations (DEPT) and 2D NMR spectra (COSY, HMQC, HMBC) were run using standard pulse programs. Column chromatography was carried out on silica gel (Merck, 40 $\mu$m), Sephadex LH-20 (Amersham Biosciences, 17-0090-02), and RP (C-18, 40 $\mu$m) (Merck). TLC analyses were carried out on silica gel 60 $F_{254}$ (Merck) and RP-18 $F_{254s}$ (Merck) plates. Compounds were detected by UV and 30% $H_2SO_4$ spraying reagent followed by heating at 105 °C for 1−2 min.

**Plant Material.** *Alkanna cappadocica* was collected from Karapınar Village, Konya, Turkey, in June 2007, and identified by Serdar G. Senol (Deparment of Biology, Faculty of Sciences, Ege University, Izmir, Turkey). Voucher specimens (SG 3263) have been deposited at the herbarium of the Department of Botany, Faculty of Science, Ege University, Izmir, Turkey.

**Extraction and Isolation.** The air-dried, powdered roots (153.4 g) of *A. cappadocica* were extracted for four days with $CH_2Cl_2$/MeOH (1:1, 4 × 2.5 L) by stirring at room temperature, and the extraction unit was protected from light to prevent polymerization of the naphthoquinones. The solvent was removed by rotary evaporation at 45 °C, yielding 17.7 g of extract. The extract was subjected to VLC using silica gel (500 g, 70−230, 7 × 50 cm) as stationary phase and employing *n*-hexane (3 L), *n*-hexane/EtOAc gradient mixtures (8:2, 3 L; 6:4, 3 L; 4:6, 3 L; 2:8, 3 L), EtOAc (3 L), and MeOH (1 L) for elution to afford eight fractions (A1−A8). Fractions A1, A2, A3, A4, A5, and A6 were rich in naphthoquinones. Fractions A1−A6 showed the most potent cytotoxicity ($IC_{50}$ < 10 $\mu$g/mL). Fraction A1 (1.2 g) was subjected to open column chromatography (Si gel 60, 120 g, 2 × 60 cm) using *n*-hexane (750 mL), *n*-hexane/EtOAc [9.9:0.1, 9.5:0.5, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 (each 500 mL)], EtOAc (500 mL), and MeOH (500 mL), yielding five main fractions (A1-F1−A1-F5). Fractions A1-F1 and A1-F4 (301 and 420 mg, respectively) were further separately applied to Sephadex LH-20 (10 g, 2 × 50 cm) column chromatography and eluted with MeOH (500 mL), yielding compounds **1** and **2** (46.9 and 100.3 mg, respectively).

Fractions A3 and A4 (each 1.2 g) were combined and chromatographed on a Si gel column (200 g, 2 × 60 cm) eluted with *n*-hexane (1 L), *n*-hexane/EtOAc (9:1, 3 L; 8:2, 2 L; 7:3 2 L; 6:4, 2 L; 5:5, 2 L; 4:6, 2 L; 3:7, 500 mL; 2:8, 500 mL), EtOAc (500 mL), and MeOH (500 mL) to yield 10 fractions (B1−B10). Fractions B1 and B3 (10 and 577 mg, respectively) were further separately applied to Sephadex

**Table 2.** Cytotoxic Activities of Compounds **1**−**8**

| | tumorigenic cell lines $IC_{50}$ ($\mu$M)[a] | | | | | | | | | | | non-tumorigenic cell lines $IC_{50}$ ($\mu$M) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| compound | HT-29 | MDA-MB-231 | PC-3 | AU565 | Hep G2 | LNCaP | MCF7 | HeLa | SK-BR-3 | DU 145 | Saos-2 | Hep 3B | VERO | 3T3 |
| **1** | 5.12 (±0.099) | 5.08 (±0.185) | 4.29 (±0.008) | 0.92 (±0.020) | 0.90 (±0.065) | 12.21 (±0.70) | 0.79 (±0.081) | 0.85 (±0.010) | 0.69 (±0.017) | 1.24 (±0.031) | 0.85 (±0.061) | 6.02 (±0.530) | 0.66 (±0.015) | 5.84 (±0.142) |
| **2** | 0.80 (±0.040) | 0.32 (±0.037) | 1.10 (±0.03) | 0.62 (±0.059) | 1.00 (±0.056) | 11.26 (±0.24) | 5.15 (±0.307 | 0.96 (±0.076) | 0.68 (±0.017) | 4.81 (±0.250) | 0.84 (±0.026) | 4.09 (±0.540) | 0.32 (±0.083) | 0.77 (±0.008) |
| **3** | 10.46 (±0.081) | 8.47 (±0.230) | 11.03 (±0.504) | 0.88 (±0.025) | 7.41 (±0.220) | 11.53 (±0.40) | 7.05 (±0.170) | 6.75 (±0.220) | 1.66 (±0.012) | 6.65 (±0.130) | 4.93 (±0.110) | 6.06 (±0.570) | 0.76 (±0.015) | 7.73 (±0.310) |
| **4** | 4.03 (±0.046) | 4.27 (±0.079) | 3.25 (±0.121) | 0.90 (±0.016) | 3.80 (±0.105) | 10.74 (±0.79) | 3.06 (±0.197) | 0.91 (±0.024) | 0.78 (±0.155) | 5.02 (±0.221) | 2.31 (±0.147) | 3.84 (±0.350) | 0.70 (±0.054) | 5.21 (±0.278) |
| **5** | 5.09 (±0.037) | 1.19 (±0.023) | 6.81 (±0.533) | 0.88 (±0.061) | 7.64 (±0.484) | 10.92 (0.170) | 15.96 (0.530) | 0.91 (±0.028) | 2.20 (0.213) | 8.71 (0.666) | 20.41 (±0.210) | 15.77 (±0.252) | 0.98 (±0.015) | 5.18 (±0.079) |
| **6** | 5.98 (±0.248) | 5.11 (±0.125) | 7.48 (±0.326) | 7.08 (±0.197) | 8.05 (±0.343) | 11.08 (±0.173) | 11.89 (±0.686) | 6.38 (±0.710) | 6.52 (±0.292) | 12.05 (±0.441) | 12.51 (±0.420) | 24.49 (±0.230) | 6.39 (±0.330) | 6.57 (±0.020) |
| **7** | 0.62 (±0.029) | 0.09 (±0.004) | 0.78 (±0.024) | 0.59 (±0.013) | 0.87 (±0.076) | 10.99 (±0.20) | 14.07 (±0.412) | 0.66 (±0.026) | 0.44 (±0.054) | 7.41 (±0.639) | 13.81 (±0.178) | 10.19 (±0.111) | 0.59 (±0.014) | 0.66 (±0.006) |
| **8** | 0.59 (±0.034) | 0.26 (±0.023) | 0.42 (±0.016) | 0.75 (±0.053) | 0.97 (±0.014) | 11.40 (±0.58) | 14.83 (±0.405) | 0.82 (±0.007) | 0.83 (±0.051) | 8.55 (±0.716) | 14.48 (±0.171) | 11.77 (±0.160) | 0.79 (±0.097) | 4.67 (±0.083) |
| doxorubicin | 2.05 (±0.412) | 2.00 (±0.903) | 3.16 (±0.88) | | | | | | | | | | | 3.70 (±0.06) |
| etoposide | 30.38 (±5.233) | 23.22 (±3.051) | 69.54 (±3.43) | | | | | | | | | | | 62.97 (±4.23) |

[a] $IC_{50}$ values are means of three independent experiments; standard deviation is given in parentheses. Results are presented as mean ± SD of three assays.

LH-20 (10 g, 2 × 50 cm) column chromatography and eluted with MeOH (500 mL), yielding compounds **2** (52 mg) and **3** (319.3 mg), respectively. Fraction B6 (504 mg) was subjected to a Si gel column (100 g) using *n*-hexane (1 L), *n*-hexane/EtOAc mixtures (9:1, 1 L; 8:2, 1 L; 7:3, 1 L; 6:4, 1 L; 5:5, 500 mL; 4:6, 500 mL; 3:7, 500 mL; 2:8, 500 mL), EtOAc (500 mL), and MeOH (500 mL), yielding three fractions (B6-F1−B6-F3). B6-F3 was subjected to Sephadex LH-20 (10 g, 2 × 50 cm) column chromatography and eluted with MeOH (500 mL) to yield compound **4** (3.5 mg). Fr. B7 (500 mg) was subjected to column chromatography using Si gel (120 g) as stationary phase, eluting with *n*-hexane/EtOAc (9:1, 1 L; 8:2, 1 L; 7:3, 500 mL; 6:4, 500 mL; 5:5, 500 mL; 4:6, 500 mL; 3:7, 500 mL; 2:8, 500 mL), EtOAc (500 mL), and MeOH (500 mL) to give four fractions (B7-F1−B7-F4). Fraction B7-F1 was subjected to column chromatography using Sephadex LH-20 (10 g) as stationary phase, eluting with MeOH (500 mL), to give an additional sample of compound **4** (1.2 mg). Fraction B7-F2 (300 mg) was chromatographed on a Si gel (120 g, 2 × 60 cm) column. Elutions were carried out with *n*-hexane/EtOAc mixtures (9:1, 1 L; 8:2, 1 L; 7:3, 500 mL; 6:4, 500 mL; 5:5, 500 mL; 4:6, 500 mL; 3:7, 500 mL; 2:8, 500 mL) and EtOAc (500 mL), yielding three fractions (B7-F2-SC1 to B7-F2-SC3). Fr. B7-F2-SC2 was respectively applied to TLC (20 × 20 cm) on Si gel and reversed-phase material (C-18) using *n*-hexane/EtOAc (7:3 for silica gel) and $H_2O$/MeOH (3:7 for C-18) as a mobile phase, and then the recovered material from the C-18 plate was subjected to column chromatography using Sephadex LH-20 (10 g, 2 × 50 cm) as stationary phase, eluting with MeOH (500 mL), to give compound **5** (0.8 mg). Fraction A5 (500 mg) was chromatographed on reversed-phase material (C-18, 3 × 90 cm, 200 g) eluted with $H_2O$/MeOH (3:7, 500 mL; 2:8, 2 L) to yield four fractions (C1−C4). Fraction C2 (150 mg) was applied to column chromatography using reversed-phase material (C-18, 70 g, 2 × 60 cm). Elution was performed with $H_2O$/MeOH (3:7, 500 mL; 2:8, 2 L (8:2, 6:4)) to give two fractions (C2-F1 and C2-F2). Fraction C2-F2 (50 mg) was subjected to a Sephadex LH-20 column (10 g) using MeOH (1 L) to yield four fractions (C2-F2-SH1 to C2-F2-SH4). C2-F2-SH2 and C2-F3-SH3 were combined and respectively applied to preparative TLC on Si gel and reversed-phase material (C-18) using the above-mentioned methodology to obtain an additional portion of compound **5** (0.5 mg). Fraction C4 (200 mg) was chromatographed on reversed-phase material (C-18, 2 × 70 cm, 200 g) eluted with $H_2O$/MeOH (3:7, 500 mL; 2:8, 2 L) to yield three fractions (C4-F1−C4-F3). Fraction C4-F3 was applied to a VLC column using reversed-phase material (C-18, 200 g, 3 × 50 cm). Elution was performed with $H_2O$/MeOH (4:6, 500 mL; 3:7, 500 mL; 2:8, 2 L) and MeOH to give three fractions (fractions C4-F1-RP1 to C4-F1-RP3). Fr. C4-F1-RP3 was applied to Sephadex LH-20 (10 g) column chromatography. Elution was carried out with MeOH (500 mL), yielding compound **8** (1.9 mg). Active fraction A6 was subjected to open column chromatography (Si gel 60, 150 g, 60 × 2 cm) using *n*-hexane/EtOAc (9:1, 300 mL; 8:2, 2 L; 7:3, 2 L; 6:4, 600 mL; 5:5, 600 mL; 4:6, 600 mL), EtOAc (500 mL), and MeOH (500 mL) to afford seven fractions (A6-B1 to A6-B7). Fraction A6-B3 (435 mg) was subjected to Si gel (100 g, 2 × 60 cm) column chromatography. Elution was performed with *n*-hexane/EtOAc mixtures (9:1, 300 mL; 8:2, 2 L; 7:3, 2 L; 6:4, 300 mL; 5:5, 300 mL; 4:6, 200 mL), EtOAc (500 mL), and MeOH (500 mL) to yield seven fractions (A6-B3-SC1 to A6-B3-SC7). Fractions A6-B3-SC3 and A6-B3-SC6 were combined (273 mg) and subjected to column chromatography using reversed-phase material (70 g, 2 × 60 cm) as stationary phase, eluting with $H_2O$/MeOH (3:7, 500 mL; 2:8, 2 L), to give nine fractions (A6-B3-SC1-RP1 to A6-B3-SC1-RP9). Fractions A6-B3-SC1-RP2 and A6-B3-SC1-RP6 were further separately subjected to Sephadex LH-20 (10 g, 2 × 50 cm) column chromatography and eluted with MeOH (500 mL), yielding compounds **7** (27.6 mg) and **6** (7.7 mg), respectively.

**Cell Culture.** The eight pure compounds were dissolved in DMSO to a final concentration of 10 mM. Subsequent dilutions were made in culture medium. The same proportion of DMSO/culture medium was added to the controls. The final DMSO content was never above 0.1%. MTT [3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was purchased from Sigma Chemical Company (St. Louis, MO). Doxorubicin hydrochloride and etoposide were purchased from Pfizer Inc. (USA), Applichem GmbH (Germany, catalog number A 4361), and Sigma-Aldrich (USA, catalog number E1383), respectively.

Twelve human tumorigenic cell lines [PC-3 (human prostate adenocarcinoma), HT-29 (human colon colorectal adenocarcinoma), MDA-MB-231 (human breast adenocarcinoma), AU565 (human breast

adenocarcinoma), Hep G2 (human hepatocellular carcinoma), MCF7 (human breast adenocarcinoma), HeLa (human cervix adenocarcinoma), SK-BR-3 (human breast adenocarcinoma), DU 145 (human prostate carcinoma), Saos-2 (human osteosarcoma), Hep 3B (human hepatocellular carcinoma), LNCaP (human prostate carcinoma)] and two nontumorigenic cell lines [3T3 (Swiss albino mouse fibroblasts) and VERO (African green monkey kidney epithelial)] were used. The cells were cultured in RPMI 1640 or DMEM-Ham's F12 supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 $\mu$g/mL) and maintained at 37 °C with 5% $CO_2$ in a humidified atmosphere.

**Cytotoxic Activity Assay.** The cytotoxic activity of the compounds and doxorubicin and etoposide, which were used as positive controls on the various cancer cells, was measured by the MTT assay. Cells in the exponential growth phase were placed in 96-well plates to make 6000 cells/wells. After 24 h of incubation and adding sample solutions in concentrations ranging from 0.1 to 10 $\mu$M, respectively, in each well, they were incubated for 48 h.

Cell proliferation was determined by adding 0.5 mg/mL per well, prepared as a sterile stock solution of 5 mg/mL in Dulbecco's phosphate-buffered saline (Gibco, USA), diluted 1:10 with medium prior to use. Medium was removed 3 h later, and blue formazan crystals were dissolved in 200 $\mu$L of 100% DMSO per well. Quantities of blue formazan product were measured at 570−690 nm using a microplate reader (Versamax, tunable microplate reader, USA). For the cells, strong correlations between numbers of cells present and amounts of MTT formazan product were observed.

The data were obtained from three independent assays, using three sets of wells for each assay. Cytotoxic effects of the compounds were determined according to percent cell viability.

**Compound 5:** amorphous, reddish powder; IR (KBr) $\nu_{max}$ 3414, 2921, 2853, 1737, 1655, $cm^{-1}$; LC-ESIMS *m/z* 309.12 $[M + Na]^+$; HR-MALDITOFMS *m/z* 287.1287 $[M + H]^+$ (calcd for $C_{17}H_{19}O_4$, 287.1283); $^1H$ NMR ($CDCl_3$, 400 MHz) and $^{13}C$ NMR ($CDCl_3$, 100 MHz) data, see Table 1.

**Compound 6:** amorphous, reddish powder; IR (KBr) $\nu_{max}$ 3415, 2923, 2851, 1724, 1655 $cm^{-1}$; LC-ESIMS *m/z* 309.12 $[M + Na]^+$; HR-MALDITOFMS *m/z* 287.1287 $[M + H]^+$ (calcd for $C_{17}H_{19}O_4$, 287.1280); $^1H$ NMR ($CDCl_3$, 400 MHz) and $^{13}C$ NMR ($CDCl_3$, 100 MHz) data, see Table 1.

**Compound 7:** amorphous, reddish powder; $[\alpha]_D^{25}$ −50.0 (*c* 0.02, $CHCl_3$); IR (KBr) $\nu_{max}$ 3414, 2921, 1637, 1617 $cm^{-1}$; LC-ESIMS *m/z* 367.16 $[M + Na]^+$; HR-MALDITOFMS *m/z* 345.1332 $[M + H]^+$ (calcd for $C_{19}H_{21}O_6$, 345.1338); $^1H$ NMR ($CDCl_3$, 400 MHz) and $^{13}C$ NMR ($CDCl_3$, 100 MHz) data, see Table 1.

**Compound 8:** amorphous, reddish powder; $[\alpha]_D^{25}$ −50.0 (*c* 0.02, $CHCl_3$); IR (KBr) $\nu_{max}$ 3419, 2921, 2853, 1637, 1618 $cm^{-1}$; LC-ESIMS *m/z* 407.22 $[M + Na]^+$; HR-MALDITOFMS *m/z* 385.1654 $[M + H]^+$ (calcd for $C_{22}H_{25}O_6$, 385.1651); $^1H$ NMR ($CDCl_3$, 400 MHz) and $^{13}C$ NMR ($CDCl_3$, 100 MHz) data, see Table 1.

**Acknowledgment.** We thank Dr. Serdar Şenol for his valuable assistance in collection and identification of the plant material. We also thank Dr. Daniela Eletto for her assistance in obtaining HR-MALDITOFMS of the new compounds. This research was supported by TUBITAK (The Scientific and Technical Research Council of Turkey) (Project No. 105S101).

**Supporting Information Available:** This material is available free of charge via the Internet at http://pubs.acs.org.

## References and Notes

(1) Davis, P. H. *Flora of Turkey and the East Aegean Islands*; Edinburg University: Edinburgh, 1978; Vol. VI, p 414.
(2) Papageorgiou, V. P.; Assimopoulou, A. N.; Couladouros, E. A. *Angew. Chem., Int. Ed.* **1999**, 270–301.
(3) El Sohly, H. N.; El-Feraly, F. S.; Joshi, A. S.; Walker, L. A. *Planta Med.* **1997**, 384.
(4) Papageorgiou, V. P.; Assimopoulou, A. *Phytochem. Anal.* **2003**, 251–258.
(5) Roeder, E.; Wiedenfeld, H.; Schraut, R. *Phytochemistry* **1984**, 2125–2126.
(6) (a) Afzal, M.; Muhammad, N. *Agric. Biol. Chem.* **1983**, 411–412. (b) Papageorgiou, V. P. *Chem. Chron.* **1977**, 365–374. (c) Papageorgiou, V. P. *Planta Med.* **1979**, 185–187. (d) Papageorgiou, V. P. *Planta Med.* **1980**, 305–307. (e) Papageorgiou, V. P.; Digenis, G. A. *Planta Med.* **1980**, *39*, 181–184. (f) Papageorgiou, V. P. *Planta Med.* **1980**, 193–203.
(7) Assimopoulou, A. N.; Karapanagiotis, I.; Vasiliou, A.; Kokkini, S.; Papageorgiou, V. *P. Biomed. Chromatogr.* **2006**, 1359–1374.
(8) Singh, B.; Sharma, M. K.; Meghwal, P. R.; Sahu, P. M.; Singh, S. *Phytomedicine* **2003**, 375–380.
(9) Sasaki, K.; Abe, H.; Yoshizaki, F. *Biol. Pharm. Bull.* **2002**, 669–670.
(10) (a) Assimopoulou, A. N.; Boskou, D.; Papageorgiou, V. P. *Food Chem.* **2004**, 433–438. (b) Kourounakis, A. P.; Assimopoulou, A. N.; Papageorgiou, V. P.; Gavalasa, A.; Kourounakis, P. N. *Arch. Pharm.* **2002**, 262–266.
(11) Cui, X. R.; Tsukada, M.; Suzuki, N.; Shimamura, T.; Gao, L.; Koyanagi, J.; Komada, F.; Saito, S. *Eur. J. Med. Chem.* **2007**, 1–10.
(12) Assimopoulou, A. N.; Papageorgiou, V. P. *Phytother. Res.* **2005**, 141–147.
(13) (a) An, S.; Park, Y. D.; Paik, Y. K.; Jeonga, T. S.; Lee, W. S. *Bioorg. Med. Chem. Lett.* **2007**, 1112–1116. (b) Kajimoto, S.; Hori, M.; Manabe, H.; Masuda, Y.; Shibayama-Imazu, T.; Nakajo, S.; Gong, X. F.; Obama, T.; Itabe, H.; Nakaya, K. *Biochim. Biophys. Acta, Mol. Basis Dis.* **2008**, 241–250.
(14) (a) Ahn, B. Z.; Baik, K. U.; Kweon, G. R.; Lim, K.; Hwangl, B. D. *J. Med. Chem.* **1995**, 1044–1047. (b) Plyta, Z. F.; Li, T.; Papageorgiou, V. P.; Mellidis, A. S.; Assimopoulou, A. N.; Pitsinos, I. E. N.; Couladouros, E. A. *Bioorg. Med. Chem. Lett.* **1998**, 3385–3390. (c) Lu, Q.; Liu, W.; Ding, J.; Cai, J.; Duan, W. *Bioorg. Med. Chem. Lett.* **2002**, 1375–1378. (d) Fan Yang, F.; Yi Chen, Y.; Duan, W.; Zhang, C.; Hong Zhu, H.; Ding, J. *Int. J. Cancer* **2006**, 1184–1193.
(15) (a) Pekin, G.; Ganzera, M.; Senol, S.; Bedir, E.; Korkmaz, K.; Stuppner, H. *Planta Med.* **2007**, 267–72. (b) Akgun, I.; Ganzera, M.; Gur, C.; Senol, S.; Korkmaz, K.; Bedir, E. *Chromatographia* **2009**, 963–967.
(16) Zhang, J. G.; Lu, Q.; Wen, H. D.; Cai, J. C. *Chin. Chem. Lett.* **2005**, 465–467.
(17) Baik, K.; Song, G. Y.; Kim, Y.; Sok, D.; Ahn, B. *Arch. Pharm. Med. Chem.* **1997**, 377–382.

NP900778J