Phenolic Constituents from the Twigs of *Euonymus alatus* and Their Cytotoxic and Anti-inflammatory Activity

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Abstract

A bioassay-guided fractionation and chemical investigation of the MeOH extract of the twigs of *Euonymus alatus* resulted in the isolation and identification of five new phenolic compounds, named alatusols A–E (**1**–**5**), together with six known compounds (**6**–**11**). The structures of these new compounds were elucidated through spectral analysis, including extensive 2D-NMR data, and their absolute configurations were determined by the modified Mosher's method. Compounds **1**–**3** and **7**–**9** showed consistent cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines with IC_{50} values of 15.20–29.81 µM. In addition, compounds **5** and **7** inhibited nitric oxide production in lipopolysaccharide-activated BV-2 cells with IC_{50} values of 22.77 and 32.52 µM, respectively.

Key words

Euonymus alatus · Celastraceae · phenylglycerol derivatives · cytotoxicity · anti-neuroinflammation

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Euonymus alatus (Thunb.) Sieb. (Celastraceae), known as the winged euonymus in Korea, has been widely used in traditional medicine to prevent the development of atherosclerosis and to treat dysmenorrhea [1]. In particular, the cork cambium on the twigs of this tree, which is called "Gui-Jun Woo", has been used as an anticancer agent in Korean traditional medicine [1]. Recent pharmacological studies have revealed the potential of *E. alatus* as an anticancer agent using a variety of *in vivo* and *in vitro* models [2–5], which was also confirmed by our screening test in which an 80% methanolic extract of *E. alatus* twigs exhibited significant cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cells in a sulforhodamine B (SRB) bioassay. In our continuing search for bioactive constituents from Korean medicinal plants, we investigated the active constituents of *E. alatus* using bioactivity-guided isolation techniques. In the present study, we report the isolation and structural elucidation as well as the cytotoxic and anti-neuroinflammatory activities of five new phenolic compounds (**1**–**5**) (**Fig. 1**), together with six known compounds (**6**–**11**) from the active $CHCl_3$-soluble fraction.

Compounds **1** and **2** were obtained as a colorless gum. The molecular formula of **1** was determined to be $C_{13}H_{18}O_6$, which corresponded with that of **2** by their molecular ion peak $[M + Na]^+$ at *m/z* 293.2678 and *m/z* 293.2681 (calcd. for $C_{13}H_{18}NaO_6$, 293.2682) on HR-ESIMS, respectively. Their NMR spectral data (**Table 1**) were similar to those of (1′*R*,2′*R*)-guaiacyl glycerol (**6**) [6], with an apparent difference in the presence of additional signals for a methoxy group [δ_H 3.22 (s); δ_C 55.5 in **1** and δ_H 3.20 (s); δ_C 55.5 in **2**] and an acetyl group [δ_H 2.01 (s); δ_C 171.3, 19.2 in **1** and δ_H 2.03 (s); δ_C 171.3, 19.2 in **2**]. The location of the methoxy group was confirmed to be at C-7 by a correlation from the methoxy proton to C-7 in an HMBC experiment, in which the HMBC correlation between H-9 and the acetyl group also indicated that an acetoxyl group is located at C-9. Full assignments of the ^1H and ^{13}C NMR data of **1** and **2** were performed by ^1H-^1H COSY, HMQC, and HMBC spectra. The absolute configurations of C-7 and C-8 for **1** were established based on the modified Mosher's method in combination with the observed coupling constant [7,8]. Treatment of **1** with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*S*)-MTPA-Cl] and DMAP in pyridine gave the (*R*)-MTPA esters **1 r**. Similar treatment of **1** with (*R*)-(−)-MTPA-Cl afforded the (*S*)-MTPA esters **1 s**. Analysis of the ^1H NMR chemical shift differences ($\Delta\delta_{S-R}$) (Supporting Information) of the two

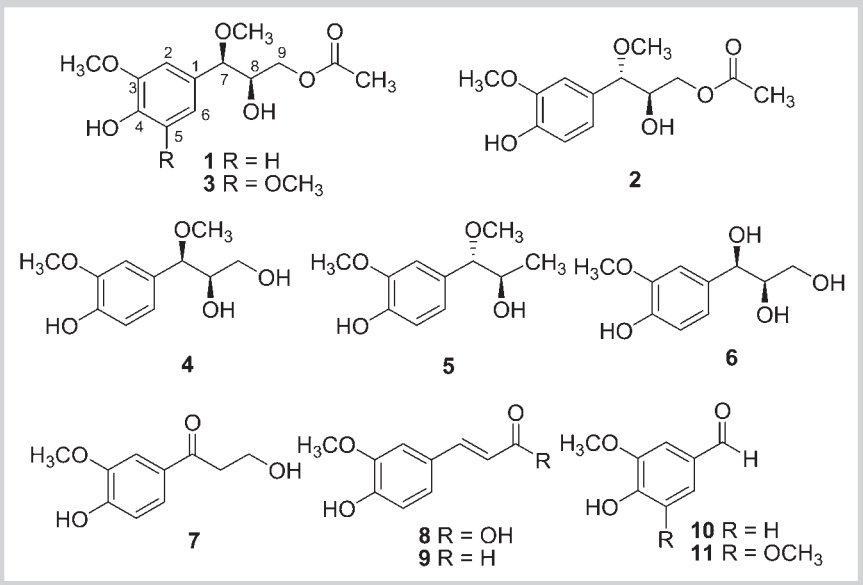

Fig. 1 Chemical structures of **1**–**11**.

Table 1 ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data of **1–3** in CD_3OD (δ in ppm).[a]

Position	1		2		3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		129.4		129.5		131.0
2	6.89 d (1.5)	110.2	6.92 d (1.5)	110.5	6.60 s	104.1
3		147.7		147.3		148.1
4		146.3		146.2		135.6
5	6.78 d (7.5)	114.6	6.80 d (7.5)	114.4		148.1
6	6.75 dd (7.5, 1.5)	120.1	6.76 dd (7.5, 1.5)	120.4	6.60 s	104.1
7	4.07 d (7.0)	84.5	3.75 d (4.5)	84.0	4.07 d (7.0)	84.7
8	3.82 ddd (7.0, 6.0, 3.5)	72.7	3.88 ddd (6.0, 4.5, 3.5)	72.1	3.79 m	72.8
9	3.91 dd (11.5, 3.5)	65.2	4.20 dd (11.5, 3.5)	65.4	3.98 dd (11.5, 3.5)	65.2
	3.79 dd (11.5, 6.0)		4.06 dd (11.5, 6.0)		3.82 m	
3-OCH_3	3.83 s	54.9	3.85 s	54.8	3.83 s	55.2
5-OCH_3					3.83 s	55.2
7-OCH_3	3.22 s	55.5	3.20 s	55.5	3.24 s	55.6
OAc		171.3		171.3		171.3
	2.01 s	19.2	2.03 s	19.2	2.01 s	19.3

[a] Assignments were based on 2D NMR including HMQC and HMBC. Well-resolved couplings are expressed with coupling patterns and coupling constants in Hz in parentheses

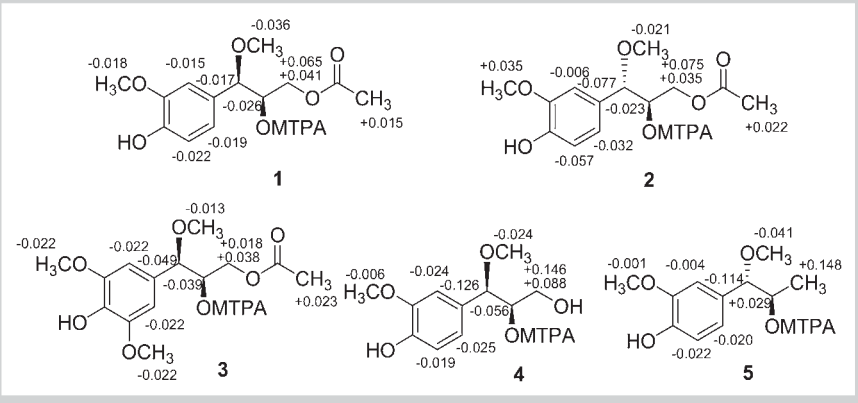

Fig. 2 $\Delta\delta$ Values ($\delta_S - \delta_R$) in ppm of the two MTPA esters derived from **1–5**.

MTPA esters allowed the assignment of the absolute configuration of C-8 as *R* (**Fig. 2**). The coupling constant (J = 7.0 Hz) between H-7 and H-8 of **1** suggested a *threo* isomer in the cases of guaiacylglycerol derivatives [8] and, thus, the absolute configuration at C-7 of **1** was assigned as *R*. The absolute configuration of C-8 for **2** was also determined using the modified Mosher's method [7], which demonstrated the *R*-configuration for C-8 (**Fig. 2**). In addition, the absolute configuration of C-7 in **2** was determined to be *S* based on the coupling constant (J = 4.5 Hz) between H-7 and H-8 of **2** suggesting an *erythro* isomer [8].

Compound **3** was isolated as a colorless gum, whose molecular formula was established to be $C_{14}H_{20}O_7$ from the $[M + Na]^+$ peak at *m/z* 323.1103 (calcd. for $C_{14}H_{20}NaO_7$, 323.1107) in the HR-ESIMS. The ^{1}H and ^{13}C NMR data of **3** (**Table 1**) were quite similar to those of **1**, with a noticeable difference being the chemical shifts assignable to the aromatic ring, which were consistent with those of syringoylglycerol derivatives [9]. Analysis of the 2D-NMR data of **3** (^{1}H-^{1}H COSY, HMQC, and HMBC) led to unambiguous ^{1}H and ^{13}C NMR assignments. The absolute configuration of C-7 and C-8 for **3** was determined by the modified Mosher's method in combination with the observed coupling constant (J = 7.0 Hz) between H-7 and H-8 [7,8], which demonstrated the *R*-configuration for C-7 and for C-8 (**Fig. 2**).

Compound **4**, a colorless gum, had a molecular formula of $C_{11}H_{16}O_5$ as established by the $[M + H]^+$ peak at *m/z* 229.1080 (calcd. for $C_{11}H_{17}O_5$, 229.1076) in the HR-ESIMS. The ^{1}H and ^{13}C NMR spectra (**Table 2**) were quite similar to those of 1-*O*-methylguaiacylglycerol, whose absolute configuration has not been established [10]. The structure of **4** was unambiguously confirmed by HMQC and HMBC experiments. The absolute configuration of C-8 for **4** was determined as *R* by the modified Mosher's method (**Fig. 2**). In addition, the coupling constant (J = 7.0 Hz) between H-7 and H-8 of **4** suggested the *threo* isomer and allowed assignment of the absolute configuration of C-7 also as *R* [8].

Compound **5**, a colorless gum, exhibited the molecular formula of $C_{11}H_{16}O_4$ as deduced by the $[M + H]^+$ peak at *m/z* 213.1121 (calcd. for $C_{11}H_{17}O_4$, 213.1127) in the HR-ESIMS. Inspection of the ^{1}H and ^{13}C NMR data (**Table 2**) revealed that the NMR data of **5** were nearly identical to those of 1-(4-hydroxy-3-methoxyphenyl)-1-methoxypropan-2-ol, with the only difference being the coupling constant (J = 3.5 Hz) between H-7 and H-8 in **5** [11]. The complete assignments of the ^{1}H and ^{13}C NMR data of **5** were determined by ^{1}H-^{1}H COSY, HMQC, and HMBC spectra. The absolute configuration of C-7 and C-8 for **5** was established by the modified Mosher's method in combination with the observed coupling constant ($J_{7,8}$ = 3.5 Hz) [7,8], which demonstrated the *S*-configuration for C-7 and *R*-configuration for C-8 (**Fig. 2**).

The six known compounds were identified as (1′*R*,2′*R*)-guaiacyl glycerol (**6**) [6], 3-hydroxy-1-(3-methoxy-4-hydroxyphenyl)

Position	4		5	
	δ_H	δ_C	δ_H	δ_C
1		130.0		130.2
2	6.90 d (1.5)	110.3	6.84 d (2.0)	110.2
3		147.7		147.7
4		146.1		146.1
5	6.78 d (8.0)	114.5	6.77 d (8.0)	114.5
6	6.75 dd (8.0, 1.5)	120.1	6.72 dd (8.0, 2.0)	120.3
7	4.07 d (7.0)	84.1	3.78 d (3.5)	89.1
8	3.67 ddd (7.0, 6.0, 3.5)	75.6	3.82 qd (6.0, 3.5)	70.7
9	3.42 dd (11.0, 3.5) 3.29 dd (11.0, 6.0)	62.5	0.90; 3H d (6.0)	17.7
3-OCH_3	3.85 s	54.8	3.84 s	54.8
7-OCH_3	3.21 s	55.4	3.20 s	55.4

Table 2 ^1H (500 MHz) and ^{13}C NMR (125 MHz) spectral data of **4**–**5** in CD_3OD (δ in ppm).[a]

[a] Assignments were based on 2D NMR including HMQC and HMBC. Well-resolved couplings are expressed with coupling patterns and coupling constants in Hz in parentheses

Compound	IC_{50} (μM)[a]			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	27.54	29.06	24.01	24.26
2	21.39	28.98	20.11	29.81
3	18.67	26.75	16.96	22.78
7	25.87	23.48	28.19	18.63
8	28.96	18.34	25.39	15.20
9	23.42	27.08	29.28	22.16
Doxorubicin[b]	0.002	0.012	0.001	0.129

Table 3 Cytotoxicity of compounds **1**–**3** and **7**–**9** against four cultured human tumor cell lines using the SRB bioassay *in vitro*.

[a] IC_{50} value of compounds against each tumor cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*; [b] Doxorubicin as a positive control

propan-1-one (**7**) [12], (*E*)-ferulic acid (**8**) [13], (*E*)-coniferyl aldehyde (**9**) [14], vanillin (**10**) [15], and syringaldehyde (**11**) [15] by comparison of their spectroscopic data with reported data.

The cytotoxic activities of the isolates (**1**–**11**) were evaluated by determining their inhibitory effects on human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-15) using the SRB bioassay [16]. The results (**Table 3**) showed that compounds **1**–**3** and **7**–**9** had consistent cytotoxicity against all cell lines tested with IC_{50} values of 15.20-29.81 μM. The other compounds were inactive (IC_{50} > 30 μM). Doxorubicin was used as a positive control (IC_{50}: 0.002, 0.012, 0.001, and 0.129 μM, respectively for the A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines). Interestingly, the presence of an acetoxyl group at C-9 in the arylglycerols seemed to increase cytotoxic activity against the tested cell lines based on the biological data of **1**–**6**.

The isolated phenolic compounds (**1**–**11**) were further tested for their inhibitory effect on lipopolysaccharide (LPS)-stimulated nitric oxide (NO) production in microglia cells. A recent report indicated that phenolic constituents (caffeic acid and syringin) isolated from *E. alatus* leaves and twigs have an anti-neuroinflammatory effect [2]. However, no reports are available on the anti-neuroinflammatory activity of the 11 phenolic constituents isolated from *E. alatus* in this study. The inhibitory activities of the prepared compounds on NO production are given in **Table 4**. Compounds **1, 3**–**7**, and **10**–**11** suppressed LPS-induced NO release from microglial cells without a cytotoxic effect. None of the other compounds had an anti-inflammatory effect (IC_{50} > 200 μM). Of the isolates, compounds **5** and **7** had lower IC_{50} values (22.77 and 32.52 μM) than those of other compounds. N^G-monomethyl-L-arginine, a well-known NOS inhibitor, was tested as a positive control (IC_{50}: 18.23 μM).

Materials and Methods

E. alatus twigs were collected from Chungju, Chungcheongbuk-do, Korea in March 2010. Samples of plant material were identified by one of the authors (K. R. Lee). A voucher specimen (SKKU 2010–3) has been deposited at the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea. The air-dried and finely chopped plant material (5.2 kg) was extracted twice (each 20 L × 4 h) with 80% aqueous MeOH under reflux. The resulting extract (220 g) was fractionated with *n*-hexane, $CHCl_3$, and *n*-BuOH, subsequently using H_2O. The active $CHCl_3$-soluble fraction (11.7 g) was applied to repeated column chromatography to purify compounds **1**–**11** (Supporting Information).

Isolates

Alatusol A (**1**): colorless gum; $[\alpha]_D^{25}$ −45.2 (*c* 0.30, MeOH); IR (KBr): ν_{max} = 3367, 2962, 2910, 2852, 1740, 1613, 1454 cm^{-1}; UV (MeOH): λ_{max} (log ε) 278 (1.4), 235 (3.7), 215 (4.2) nm; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see **Table 1**; ESIMS (positive-ion mode): *m/z* = 293 $[M + Na]^+$; HR-ESIMS (positive-ion mode): *m/z* = 293.2678 $[M + Na]^+$ (calcd. for $C_{13}H_{18}NaO_6$, 293.2682).

Alatusol B (**2**): colorless gum; $[\alpha]_D^{25}$ +28.4 (*c* 0.20, MeOH); IR (KBr): ν_{max} = 3368, 2960, 2910, 2851, 1740, 1613, 1454 cm^{-1}; UV (MeOH): λ_{max} (log ε) 278 (1.4), 235 (3.7), 215 (4.2) nm; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see **Table 1**; ESIMS (positive-ion mode): *m/z* = 293 $[M + Na]^+$; HR-ESIMS (positive-ion mode): *m/z* = 293.2681 $[M + Na]^+$ (calcd. for $C_{13}H_{18}NaO_6$, 293.2682).

Alatusol C (**3**): colorless gum; $[\alpha]_D^{25}$ −33.5 (*c* 0.20, MeOH); IR (KBr) ν_{max} = 3388, 2961, 2908, 2851, 1613, 1454 cm^{-1}; UV (MeOH): λ_{max} (log ε) 278 (1.5), 235 (3.7), 215 (4.2) nm; ^1H (500 MHz) and

Table 4 Inhibitory effect on NO production of compounds **1**–**11** in LPS-activated BV-2 cells

Compound	IC_{50} (μM)[a]	Cell viability (%)[b]	Compound	IC_{50} (μM)[a]	Cell viability (%)[b]
1	44.19	109.7.1 ± 5.7	**7**	32.52	103.4 ± 5.4
2	> 200	96.9 ± 3.1	**8**	> 200	106.3 ± 3.0
3	43.35	105.5 ± 3.5	**9**	> 200	101.3 ± 5.0
4	130.91	97.6 ± 2.8	**10**	52.71	101.6 ± 5.0
5	22.77	112.5 ± 4.8	**11**	42.96	103.9 ± 6.2
6	55.13	99.8 ± 5.3	NMMA[c]	18.23	101.2 ± 2.1

[a] IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells; [b] Cell viability after treatment with 20 μM of each compound was expressed as a percentage (%) of the LPS only treatment group. The results are averages of three independent experiments, and the data are expressed as mean ± SD. Statistical comparisons were performed using a one-way ANOVA test with Student's t-test; [c] NMMA as a positive control

^{13}C (125 MHz) NMR data, see **Table 1**; ESIMS (positive-ion mode): *m/z* = 323 $[M + Na]^+$; HR-ESIMS (positive-ion mode): *m/z* = 323.1103 $[M + Na]^+$ (calcd. for $C_{14}H_{20}NaO_7$, 323.1107).

Alatusol D (**4**): colorless gum; $[\alpha]_D^{25}$ − 42.1 (*c* 0.25, MeOH); IR (KBr) ν_{max} = 3383, 2960, 2910, 2851, 1615, 1454 cm^{-1}; UV (MeOH): λ_{max} (log ε) 280 (1.5), 243 (2.7), 226 (4.2) nm; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see **Table 2**; ESIMS (positive-ion mode): *m/z* = 229 $[M + H]^+$; HR-ESIMS (positive-ion mode): *m/z* = 229.1080 $[M + H]^+$ (calcd. for $C_{11}H_{17}O_5$, 229.1076).

Alatusol E (**5**): colorless gum; $[\alpha]_D^{25}$ + 32.4 (*c* 0.30, MeOH); IR (KBr) ν_{max} = 3385, 2962, 2910, 2851, 1612, 1454 cm^{-1}; UV (MeOH): λ_{max} (log ε) 282 (1.6), 242 (2.7), 226 (4.2) nm; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see **Table 2**; ESIMS (positive-ion mode): *m/z* = 213 $[M + H]^+$; HR-ESIMS (positive-ion mode): *m/z* = 213.1121 $[M + H]^+$ (calcd. for $C_{11}H_{17}O_4$, 213.1127).

A detailed description of the bioassays is available in Supporting Information. The positive controls, doxorubicin (purity ≥ 98%) and N^G-monomethyl-L-arginine (purity ≥ 98%), were purchased from Sigma Corporation. The tested compounds were demonstrated to be pure as evidenced by NMR and HPLC analyses (purity ≥ 95%).

Supporting information

The general experimental procedures, isolation details, bioassay protocol, and partial ^1H NMR data of the (*S*)- and (*R*)-MTPA esters of **1**–**5** are available as Supporting Information.

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Conflict of Interest

All authors declare that there are no conflicts of interest.

References

1 *Pack JH, Sung SH.* Medicinal plants. Seoul: Shinil Books Co.; 2007: 446–447
2 *Jeong EJ, Yang H, Kim SH, Kang SY, Sung SH, Kim YC.* Inhibitory constituents of *Euonymus alatus* leaves and twigs on nitric oxide production in BV2 microglia cells. Food Chem Toxicol 2011; 49: 1394–1398
3 *Lee TK, Kim DI, Han JY, Kim CH.* Inhibitory effects of *Scutellaria barbata* D. Don. and *Euonymus alatus* Sieb. on aromatase activity of human leiomyomal cells. Immunopharmacol Immunotoxicol 2004; 26: 315–328
4 *Parka WH, Kim SH, Kim CH.* A new matrix metalloproteinase-9 inhibitor 3, 4-dihydroxycinnamic acid (caffeic acid) from methanol extract of *Euonymus alatus*: isolation and structure determination. Toxicology 2005; 207: 383–390
5 *Chung TW, Moon SK, Chang YC, Ko JH, Lee YC, Cho G, Kim SH, Kim JG, Kim CH.* Novel and therapeutic effect of caffeic acid and caffeic acid phenyl ester on hepatocarcinoma cells: complete regression of hepatoma growth and metastasis by dual mechanism. FASEB J 2004; 18: 1670–1681
6 *Ishikawa T, Fujimatu E, Kitajima J.* Water-soluble constituents of anise: new glucosides of anethole glycol and its related compounds. Chem Pharm Bull 2002; 50: 1460–1466
7 *Kim KH, Choi SU, Kim YC, Lee KR.* Tirucallane triterpenoids from *Cornus walteri*. J Nat Prod 2011; 74: 54–59
8 *Yang XW, Zhao PJ, Ma YL, Xiao HT, Zuo YQ, He HP, Li L, Hao XJ.* Mixed lignan-neolignans from *Tarenna attenuate*. J Nat Prod 2007; 70: 521–525
9 *Otsuka H, Takeuchi M, Inoshiri S, Sato T, Yamasaki K.* Phenolic compounds from *Coix lachryma-jobi* var. *ma-yuen*. Phytochemistry 1989; 28: 883–886
10 *Huang Z, Zhang W, Lin S, Liu C, Huang D, Song T, Lu L, Pei Y.* Chemical constituents from roots of *Incarvillea mairei*. Zhongguo Zhongyao Zazhi 2009; 34: 1672–1675
11 *Sadhu SK, Khatun A, Ohtsuki T, Ishibashi M.* Constituents from *Hoya parasitica* and their cell growth inhibitory activity. Planta Med 2008; 74: 760–763
12 *Lin RC, Skaltsounis AL, Seguin E, Tillequin F, Koch M.* Phenolic constituents of *Selaginella doederleinii*. Planta Med 1994; 60: 168–170
13 *Kisiel W, Zielinska K.* Sesquiterpenoids and phenolics from *Lactuca perennis*. Fitoterapia 2000; 71: 86–87
14 *Barakat HH, Nawwar AM, Buddrus J, Linscheid M.* Niloticol, a phenolic glyceride and two phenolic aldehydes from the roots of *Tamarix nilotica*. Phytochemistry 1987; 26: 1837–1838
15 *Chen CY, Chang FR, Teng CM, Wu YC.* Cheritamine, a new N-fatty acyl tryptamine and other constituents from the stems of *Annona cherimola*. J Chin Chem Soc 1999; 46: 77–86
16 *Skehan P, Storeng R, Scudiero D, Monks A, Mcmahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR.* New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990; 82: 1107–1112

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Bibliography
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