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Cytotoxic and Antioxidant Compounds Isolated from the Cork of *Euonymus alatus* Sieb.

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Abstract – Seventeen compounds (**1 - 17**), *β*-sitosterone (**1**), lupenone (**2**), arborinone (**3**), *β*-sitosterol (**4**), lupeol (**5**), *epi*-lupeol (**6**), taraxerol (**7**), betulinic acid (**8**), 24*R*-methyllophenol (**9**), germanicol (**10**), hexatriacontane (**11**), nonacosan-1-ol (**12**), benzoic acid (**13**), tetradecyl(*E*)-ferulate (**14**), di(2-ethylhexyl) phthalate (**15**), trilinolein (**16**) and monopalmitin (**17**), were isolated from the methylene chloride-soluble fraction of the cork of *Euonymus alatus* Sieb. The structures of these compounds were elucidated on the basis of spectroscopic evidence. Compounds **6**, **11**, **13** and **14** were isolated for the first time from this plant. Compound **4** showed moderate cytotoxic activity with an IC_{50} value of 6.22 μM in HL-60 cell line. Compound **9** exhibited moderate cytotoxic activity with IC_{50} values of 63.31, 15.45, 15.14 and 21.72 μM in four kinds of human cancer cell lines, Jurkat T, HeLa, HL-60 and MCF-7, respectively. Compound **17** showed moderate cytotoxic activity with an IC_{50} value of 70.71 μM in Jurkat T cell line. In addition, compounds **2**, **3**, **14** and **16** exhibited weak antioxidant activity with IC_{50} values of 151.76, 170.79, 137.46 and 139.37 μM, respectively.
Keywords – *Euonymus alatus* Sieb., Celastraceae, Cytotoxicity, Antioxidant

Introduction

Euonymus alatus Sieb. (Celastraceae) is commonly known as winged euonymus in Korea and has been widely used in traditional medicine to regulate blood circulation, relieve pain, eliminate stagnant blood and treat dysmenorrhea. The young leaves of this tree are edible kitchen herbs, and the cork cambium on the twigs which is called 'Gui-Jun Woo' has been traditionally used to treat cancer in Korean traditional medicine (Park *et al*., 2007). Biological studies have revealed that this plant possesses numerous biological effects such as anti-tumor (Lee *et al*., 1993), anti-inflammatory (Oh *et al*., 2011), anti-hyperglycemic, anti-hyperlipidemic (Park *et al*., 2005), nitric oxide scavenging (Jeong *et al*., 2004) and cytotoxic activities (Cha *et al*., 2003).

Recently, pharmacological studies have reported potential of *E. alatus* Sieb. as an anticancer agent using a variety of *in vivo* and *in vitro* models, which was also confirmed cytotoxicity in A549, SK-OV-3, SK-MEL-2, and HCT15 cancer cell lines (Kim *et al*., 2013). So, we investigated the active constituents of *E. alatus* Sieb. using bioactivity-guided isolation techniques on the undetermined cancer cell lines (Jurkat T, HeLa, HL-60 and MCF-7) in previous reports.

Activity-directed isolation of the methylene chloride fraction resulted in the identification of seventeen known compounds (**1 - 17**) by silica gel column chromatography. In this paper, we report the isolation and structural elucidation of these compounds and their antioxidant and cytotoxic activities against Jurkat T, HeLa, HL-60 and MCF-7 cell lines.

Experimental

General – Melting points were determined on a Yanaco micro melting point apparatus and were uncorrected. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. IR spectra were measured on a Mattson Polaris FT/IR-300E spectrophotometer. UV spectra were measured on a Thermo 9423AQA2200E UV spectrophotometer. NMR spectra were measured on a Varian Unity INOVA-400 spectrometer (USA), and chemical shifts are expressed as δ values using TMS as an internal standard. Low- and high-resolution EI-MS and

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FAB-MS data were collected on a Quattro II spectrometer. Open column chromatography was performed using silica gel (Kieselgel 60, 70 - 230 mesh and 230 - 400 mesh, Merck). TLC tests were performed on Merck precoated silica gel 60 F_{254} (EM 5717) and/or RP-18 F_{254s} glass plates (0.25 mm), and spots were visualized by spraying with 10% H_2SO_4 and subsequent heating. All other chemicals and solvents were of analytical grade and used without further purification.

Plant material – The cork of *E. alatus* Sieb. was collected in July 2006 from the Palgong mountain in Gyeongsangbuk-Do, Republic of Korea. These materials were confirmed taxonomically by Professor Byung Sun Min, College of Pharmacy, Catholic University of Daegu, Korea. A voucher specimen (CUDP 200602) has been deposited at the College of Pharmacy, Catholic University of Daegu, Korea.

Extraction and isolation – The cork of *Euonymus alatus* Sieb. (3.5 kg) was extracted four times with MeOH under reflux for 8 hours. The MeOH extract was concentrated under reduced pressure to yield a black syrup (211.4 g). The concentrated MeOH extract was suspended in H_2O (2.2 L) and partitioned successively with CH_2Cl_2 (5 × 2 L, 103.5 g), EtOAc (5 × 2 L, 24.6 g), *n*-BuOH (5 × 2 L, 28.4 g) and H_2O-soluble fraction (31.5 g), respectively. The MeOH extract, CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O-soluble fractions were assayed for cytotoxic activities against Jurkat T, HeLa, HL-60 and MCF-7 cell lines (data not shown).

The most cytotoxic methylene chloride fraction (103.5 g) was chromatographed on a silica gel column (15 × 35 cm) and was eluted with *n*-hexane-CH_2Cl_2 (100 : 0 to 0 : 100) and CH_2Cl_2-MeOH-H_2O (100 : 0 : 0.1 to 0 : 100 : 0.1) gradient. Fractions (M1 to M27) were collected and pooled according to their similar TLC patterns. Fraction M6 (208.2 mg) was chromatographed on a normal phase column (3.5 × 15 cm) using the *n*-hexane-CH_2Cl_2 mixture as a solvent and eluted with a stepwise gradient (50 : 1 to 10 : 1) to yield compounds **2** (98.1 mg) and **3** (75.2 mg). Fraction M8 (1.25 g) was chromatographed on a normal phase column (4.5 × 15 cm) using the *n*-hexane-CH_2Cl_2 mixture as a solvent and eluted with a stepwise gradient (10 : 1 to 3 : 1) to yield compounds **4** (169.6 mg) and **7** (9.5 mg). Fraction M12 (216.0 mg) was chromatographed on a normal phase column (3.5 × 15 cm) using the *n*-hexane-CH_2Cl_2 mixture as a solvent and eluted with a stepwise gradient (40 : 1 to 5 : 1) to yield compounds **13** (12.5 mg) and **5** (73.7 mg). Fraction M15 (525.3 g) was chromatographed on a normal phase column (4.5 × 15 cm) using the *n*-hexane-CH_2Cl_2 mixture as a solvent and eluted with a stepwise gradient (50 : 1 to 4 : 1) to yield compounds **1** (267.9 mg) and **14** (13.1 mg). Fraction M18 (146.2 mg) was chromatographed on a normal phase column (3.5 × 15 cm) using the *n*-hexane-CH_2Cl_2 mixture as a solvent and eluted with a stepwise gradient (40 : 1 to 3 : 1) to yield compounds **8** (7.9 mg), **11** (10.5 mg) and **15** (41.6 mg). Fraction M20 (184.1 mg) was chromatographed on a normal phase column (3.5 × 15 cm) using the *n*-hexane-CH_2Cl_2 mixture as a solvent and eluted with a stepwise gradient (20 : 1 to 2 : 1) to yield compounds **9** (29.9 mg) and **10** (59.2 mg). Fraction M26 (1.03 g) was chromatographed on a normal phase column (4.5 × 15 cm) using the *n*-hexane-CH_2Cl_2 mixture as a solvent and eluted with a stepwise gradient (10 : 1 to 1 : 1) to yield compounds **6** (29.7 mg), **12** (19.4 mg), **16** (21.3 mg) and **17** (75.2 mg).

***β*-Sitosterone (1)** – White powder; m.p. 77 - 80 ºC; IR (KBr) cm^{-1} 3019, 3959, 3938, 3874, 1663, 1614, 1467, 1378, 1232; EI-MS *m/z* 412 $[M]^+$; The spectral data were identical with those reported in the literature (Gaspar *et al*., 1993).

Lupenone (2) – White powder; m.p. 171 - 172 ºC; IR (KBr) cm^{-1} 3076, 1732, 1454, 1365, 1250, 1026, 978, 877; EI-MS *m/z* 424 $[M]^+$; The spectral data were identical with those reported in the literature (Da *et al*., 1996).

Arborinone (3) – Colorless needles, m.p. 217 - 219 ºC; IR (KBr) cm^{-1} 3400, 2930, 2980, 1701, 1655, 1270; EI-MS *m/z* 424 $[M]^+$; The spectral data were identical with those reported in the literature (Akihisa *et al*., 1992).

***β*-Sitosterol (4)** – White powder; m.p. 135 - 139 ºC; IR (KBr) cm^{-1} 3420, 2935, 2864, 1457, 1375, 1052; EI-MS *m/z* 414 $[M]^+$; The spectral data were identical with those reported in the literature (Su *et al*., 2009).

Lupeol (5) – Colorless needles; m.p. 202 - 205 ºC; IR (KBr) cm^{-1} 3320, 2930, 1632, 1445, 1372, 1040, 880; EI-MS *m/z* 426 $[M]^+$; The spectral data were identical with those reported in the literature (Fuchino *et al*., 1995).

***epi*-Lupeol (6)** – Colorless needles; m.p. 202 - 205 ºC; IR (KBr) cm^{-1} 3466, 2924, 1560; EI-MS *m/z* 426 $[M]^+$; The spectral data were identical with those reported in the literature (De Souza *et al*., 2001).

Taraxerol (7) – Colorless crystal; m.p. 280 - 282 ºC; IR (KBr) cm^{-1} 3480, 1642, 813; EI-MS *m/z* 426 $[M]^+$; The spectral data were identical with those reported in the literature (Lee *et al*., 1992).

Betulinic acid (8) – White powder; m.p. 275 - 278 ºC; IR (KBr) cm^{-1} 3060, 1630, 880; EI-MS *m/z* 438 $[M]^+$; The spectral data were identical with those reported in the literature (Haque *et al*., 2000).

24*R*-Methyllophenol (9) – White powder; m.p. 140 - 146 ºC; IR (KBr) cm^{-1} 3421, 2930, 2878, 1450; EI-MS *m/z* 414 $[M]^+$; The spectral data were identical with those reported in the literature (Akihisa *et al*., 1981).

Germanicol (10) – White powder; m.p. 174 - 175 ºC; IR (KBr) cm^{-1} 3600, 3030, 2940, 2850, 1630, 1450, 1360, 1040, 855; EI-MS *m/z* 426 $[M]^+$; The spectral data were identical with those reported in the literature (Koul *et al*., 2000).

Hexatriacontane (11) – White powder; IR (KBr) cm^{-1} 2952; EI-MS *m/z* 506 $[M]^+$; The spectral data were identical with those reported in the literature (Turner *et al*., 1980).

Nonacosan-1-ol (12) – White powder; IR (KBr) cm^{-1} 3429, 2967; EI-MS *m/z* 424 $[M]^+$; The spectral data were identical with those reported in the literature (Kokpol *et al*., 1993).

Benzoic acid (13) – White powder; m.p. 122 - 122 ºC; IR (KBr) cm^{-1} 3202, 2363, 1675, 1246; EI-MS *m/z* 122 $[M]^+$; The spectral data were identical with those reported in the literature (Araora *et al*., 2005).

Tetradecyl(*E*)-ferulate (14) – White powder; m.p. 65.4 - 66.3 ºC; IR (KBr) cm^{-1} 3429, 1696, 1618, 1583, 1499; FAB-MS *m/z* 413 $[M + Na]^+$; The spectral data were identical with those reported in the literature (Das *et al*., 1997).

Di(2-ethylhexyl) phthalate (15) – Colorless syrup; IR (KBr) cm^{-1} 3030, 2960, 2930, 2880, 1730, 1600, 1585, 1470, 1385, 1080; EI-MS *m/z* 390 $[M]^+$; The spectral data were identical with those reported in the literature (Rao *et al*., 2000).

Trilinolein (16) – White powder; IR (KBr) cm^{-1} 1732, 1452, 1435, 990; EI-MS *m/z* 878 $[M]^+$; The spectral data were identical with those reported in the literature (Morelli *et al*., 2006).

Monopalmitin (17) – White powder; IR (KBr) cm^{-1} 3432, 1709, 1472, 1463, 1410, 1300, 930; EI-MS *m/z* 330 $[M]^+$; The spectral data were identical with those reported in the literature (Tsuzuki *et al*., 1995).

Cytotoxicity – Cytotoxicity was measured using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Jurkat T cells (2.5×10^4 cells/well), HeLa cells (5.0×10^3 cells/well), HL-60 cells (1.0×10^4 cells/well) and MCF-7 cells (3.0×10^3 cells/well) were seeded on 96-well microplates and precultured for 36 h. MTT solution (1.1 mg/mL) was added to each

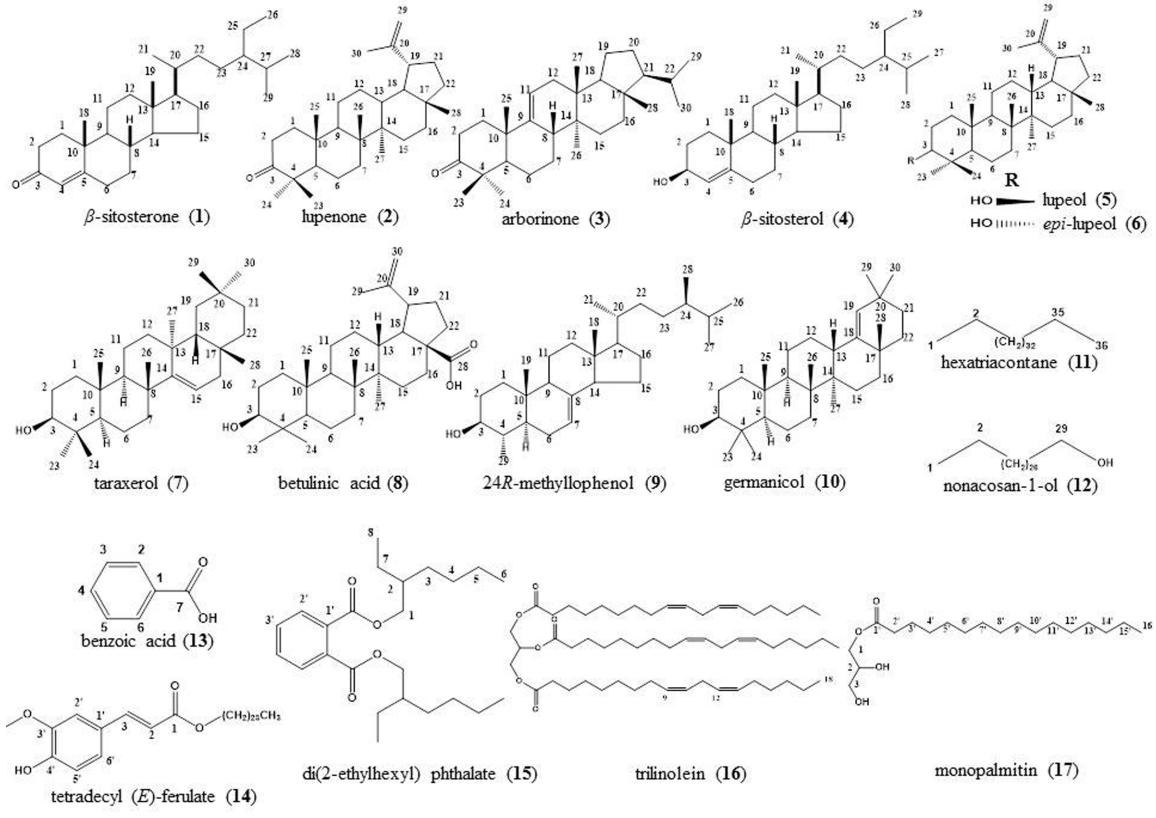

Fig. 1. Structures of compounds **1 - 17** isolated from the cork of *E. alatus* Sieb.

Table 1. MTT cytotoxic activity of **1 - 17** against cancer cell lines (Jurkat T, Hela, HL-60 and MCF-7) and their DPPH radical scavenging activity

Compound	Cytotoxic activity IC50 (μM)				Antioxidant activity IC_{50} (μM)
	Jurkat T[d]	HeLa[e]	HL-60[f]	MCF-7[g]	
β-sitosterone (**1**)	> 100	72.89	> 100	> 100	> 250.00
lupenone (**2**)	> 100	> 100	> 100	> 100	151.76
arborinone (**3**)	> 100	> 100	95.27	> 100	170.79
β-sitosterol (**4**)	> 100	42.88	6.22	89.70	> 250.00
lupeol (**5**)	> 100	33.64	38.11	59.17	> 250.00
epi-lupeol (**6**)	> 100	49.92	62.86	84.66	> 250.00
taraxerol (**7**)	> 100	> 100	76.18	> 100	243.23
betulinic acid (**8**)	> 100	44.31	42.82	62.77	> 250.00
24*R*-methyllophenol (**9**)	63.61	15.45	15.14	21.72	> 250.00
germanicol (**10**)	> 100	58.72	> 100	50.19	> 250.00
hexatriacontane (**11**)	> 100	36.57	45.46	> 100	> 250.00
nonacosan-1-ol (**12**)	> 100	> 100	> 100	> 100	> 250.00
benzoic acid (**13**)	> 100	> 100	> 100	> 100	> 250.00
tetradecyl(*E*)-ferulate (**14**)	> 100	65.57	84.68	> 100	137.46
di(2-ethylhexyl) phthalate (**15**)	> 100	> 100	> 100	> 100	> 250.00
trilinolein (**16**)	> 100	> 100	> 100	> 100	139.37
monopalmitin (**17**)	70.71	> 100	> 100	> 100	> 250.00
auraptene [a]	55.36	–	–	–	–
adriamycin[b]	–	0.31	0.07	17.2	
L-ascorbic acid [c]	–	–	–	–	25.21

[a] Positive control for cytotoxicity for Jurkat T cell-lines.
[b] Positive control for cytotoxicity for HeLa, HL-60 and MCF-7 cell-lines.
[c] Positive control for antioxidant activity
[d] Human T-lymphocyte cells
[e] Human cervical cancer cell-line.
[f] Human promyelocytic leukemia cells
[g] Human breast cancer cell-line.

well and incubated for an additional 4 h. The colored MTT formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The optical density (OD) values of the solutions were measured at 540 nm using a plate reader. All cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea).

DPPH radical scavenging activity – DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was measured using the method described by Tagashira et al (Tagashira *et al*., 1998). Briefly, 10 μL of each sample dissolved in EtOH was prepared in a 96-well microplate, and then 200 μL of 100 μM methanolic DPPH solution was added. After mixing and left standing at room temperature for 10 min, the absorbance of the reaction mixture was measured at 517 nm. L-Ascorbic acid (Sigma-Aldrich; purity: > 99%) was used as the positive control for DPPH radical scavenging activity.

Results and Discussion

This study was conducted to identify the bio-active compounds in this plant using bioactivity-guided isolation techniques. The cork of *E. alatus* Sieb. was extracted with methanol, and the extract was concentrated and fractionated into four parts; methylene chloride, ethyl acetate, *n*-butanol and water fractions. The cytotoxic activities of the methanol extract and its fractions were examined by MTT assay. Among the samples tested, the methylene chloride fraction showed cytotoxic activity against cancer cell lines (data not shown).

Seventeen compounds (**1 - 17**) were isolated from the methylene chloride fraction of cork of *E. alatus* Sieb. by repetitive column chromatography on silica gel. Compounds **1 - 17** were identified as *β*-sitosterone (**1**), lupenone (**2**), arborinone (**3**), *β*-sitosterol (**4**), lupeol (**5**), *epi*-lupeol (**6**), taraxerol (**7**), betulinic acid (**8**), 24*R*-methyllophenol (**9**),

germanicol (**10**), hexatriacontane (**11**), nonacosan-1-ol (**12**), benzoic acid (**13**), tetradecyl(*E*)-ferulate (**14**), di(2-ethylhexyl) phthalate (**15**), trilinolein (**16**) and monopalmitin (**17**), by spectroscopic methods and by comparing their data with the literature values. To the best of our knowledge, compounds **6**, **11**, **13** and **14** were isolated for the first time from this plant (Fig. 1).

The seventeen compounds isolated from *E. alatus* Sieb. were evaluated for their cytotoxic activity against Jurkat T, HeLa, HL-60 and MCF-7 cell lines using the MTT assay (Zhao *et al*., 2013). Compound **4** exhibited a moderate cytotoxicity against the HL-60 cell line (IC_{50}: 6.22 μM). But, compound **4** was essentially weak cytotoxic against the other tested HeLa and MCF-7 cell lines. Compound **9** exhibited significant moderate cytotoxic activity against the Jurkat T, HeLa, HL-60 and MCF-7 cell lines (IC_{50}: 63.31, 15.45, 15.14 and 21.72 μM, respectively). Compound **17** also showed moderate cytotoxic activity with an IC_{50} value of 70.71 μM in Jurkat T cell line (Table 1).

24*R*-methyllophenol (**9**) was previously found to have an anti-hyperglycemic effect. However, there is no report on the cytotoxic effect of compound **9** (Tanaka *et al*., 2006). Li and Xu (Li and Xu, 2012) had reported that monopalmitin (**17**) exhibited moderate molluscicidal activity against *Pomacea canaliculata* (Lamarck) and nematicidal activity against *Meloidogyne incognita* (Kofoid and White).

The protective effect of natural plants with respect to anticancer activity is assumed to be associated mainly the antioxidant activities of either individual or interacting bioactive components present in the natural plants (Balasubramanian and Ragunathan, 2012). Further, free radical reactions can produce deleterious modifications in membranes, proteins, enzymes and DNA, increasing the risk of diseases such as cancer and Alzheimer disease (Ahn *et al*., 2011). So, we simultaneously determined for antioxidant activity.

The radical scavenging effects of seventeen compounds from *E. alatus* Sieb. were evaluated by the DPPH radical scavenging assay (Table 1). The positive control, ascorbic acid, had a DPPH radical scavenging effect with an established IC_{50} value of 25.21 μM. Compounds **2**, **3**, **14** and **16** exhibited weak scavenging activities on DPPH with IC_{50} values of 151.76, 170.79, 137.46 and 139.37 μM, respectively. However, the other compounds had no scavenging activity compared to ascorbic acid.

In this study, the methylene chloride-soluble fraction of the cork of *E. alatus* Sieb. was found to exhibit significant cytotoxic activity against Jurkat T cells. Consequently, 24*R*-methyllophenol (**9**) and monopalmitin (**17**) showed moderate cytotoxicity. In addition, lupenone (**2**), arborinone (**3**), tetradecyl(*E*)-ferulate (**14**) and trilinolein (**16**) showed weak antioxidant activity.

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