



Effects of Bilobol from the Fruit Pulp of *Ginkgo biloba* on Cell Viability

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Abstract

Cytotoxic activity profiling of the n-hexane extract from the fruit pulp of *Ginkgo biloba* L. (Gingkoaceae) led to the purification of 8 compounds. HPLC based extract profiling identified a cytotoxic alkenylresorcinol. The extract was further separated by time-based fractionation in a gradient HPLC condition, and cytotoxicity of each fraction was evaluated using 293, B16F10, BJAB, and HCT116 cancer cells *in vitro*. Bilobol was isolated as an active constituent by semi-preparative HPLC and showed significant cytotoxic activities against the cancer cells in a dose-dependent manner at the concentrations of 15.0 ~ 50 µg/mL. The western blotting analysis showed that this compound increased expression of active caspase-3 and active caspase-8 in HCT116 human colon cancer cells, indicating that this compound causes apoptotic cell death in a dose-dependent manner. The results suggest that bilobol is the active component from fruit pulp of *Ginkgo Biloba* L. that can induce apoptotic cell death in various cancer cells.

Keywords: bilobol; cell viability; HPLC; fruit pulp; caspase-3,8.

Practical Application: Research about anti-cancerous natural products from *Ginkgo Biloba*.

1 Introduction

Cancer is the second cause of death all over the world that reached 9.6 million deaths in 2018 (World Health Organization, 2019). The incidence and mortality rate of the ten most common cancers (the cancer types include lung, stomach, liver, colorectum, esophagus etc) will increase by the year 2030 (Sankaranarayanan et al., 2014). The conventional cancer treatments such as surgery, chemotherapy and radiotherapy may offer toxicity, and multi-drug resistance and other side effects thus are often supplemented by other alternative therapies (Bell, 2010). Natural active substances from many herbs have begun to draw increasing scientific attention. Natural compounds have been used in the treatment of various cancers for a long period of time and their anticancer properties have been scientifically evaluated both *in vitro* and *in vivo* (Bonham et al., 2002; Gong et al., 2005). Many studies reported extracts from some herbal medicines that can inhibit cancer cell proliferation *in vitro* and *in vivo* (Itokawa et al., 1989).

Ginkgo biloba, also known as *Salisburia adiantifolia*, *Salisburia macrophylla*, and *Pterophylla salisburiensis*, is among the most sold medicinal plants. It belongs to the plant division Ginkgophyta whose all other species have extinct (Jacobs & Browner, 2000) The ginko tree has survived unchanged in the forests for over 200 million and hence it is also referred as a “living fossil” (Kozubek & Tyman, 1999; Gong et al., 2008). This plant has been used as herbal remedy for centuries in China and Korea, and now their extracts are currently used extensively in therapy for various diseases like arteriosclerosis or rheumatism. Many bioactive compounds, including flavonol and flavones glycosides,

terpenoids and ginkgolides (Mahadevan & Park, 2008; Ihl, 2013) has been isolated from the plant displaying several physiological actions and attributed to these compounds, including anoxia and ischemia of brain, heart and eye, arteriosclerosis, rheumatism, inhibition of platelet activating factor and neuro-transmitter modulation. *G. biloba* is one of the most studied medicinal plants (Matsumoto et al., 1990) However, the chemical constituents in *Ginkgo biloba* that are responsible for anti-cancerous activities *in vitro* remain to be explored.

In the course of investigating anti-cancerous natural products, we studied *Ginkgo Biloba*, and significant and dose dependent *in vitro* apoptotic activity of isolated constituent has been found. HPLC-based profiling was applied to an *n*-hexane extract from the fruit pulp of the plant. The extract was separated by time-based fractionation in a gradient HPLC condition, and cytotoxicity of each fraction was evaluated using 293, B16F10, BJAB and HCT116 cancer cells *in vitro*. Bilobol was isolated as an active constituent by semi-preparative HPLC. The isolates were structurally identified by spectral studies of NMR. The western blotting and cytotoxicity assay were used to describe apoptotic activities of the constituent.

2 Materials and methods

2.1 Plant material

The sarcotesta of *Ginkgo biloba* L. (Ginkgoaceae) was collected at Yecheon in Gyeongsangbukdo province, Korea in 2010.

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2.2 Extraction and isolation

The ripe fleshy seed coat of *G. biloba* (250 g) was exhaustively extracted three times with 2 L of MeOH. The filtered MeOH layer was concentrated at reduced pressure to obtain an extract. This MeOH extract (100.0 g) was partitioned with 400 mL of *n*-hexane and 300 mL of H₂O to afford an *n*-hexane extract (53.84 g). An aliquot of this extract (25 mg per injection, total 9.8 g) was repeatedly separated by HPLC with a linear gradient solvent system [H₂O containing 0.1% formic acid-MeOH (10:90) to MeOH (100%) for 70 min, flow rate 4.0 mL/min] using semi-prep Atlantis™ column under 280 nm. The active compound bilobol (**1**) (*t_R* 12.19 min, 31.0 mg) was isolated as yellow oil.

2.3 Bilobol

¹H NMR (MeOH-*d*₄, 600 MHz): δ 6.12 (2H, d, *J* = 2.4 Hz, H-4/6), 6.08 (1H, d, *J* = 2.4 Hz, H-2), 5.34 (2H, m, H-8'/9'), 2.43 (2H, t, *J* = 7.5 Hz, H-1'), 2.03 (4H, m, H-7'/10'), 1.56 (2H, quintet, *J* = 7.5 Hz, H-2'), 1.26 – 1.36 (12H, m, -CH₂-)^A, 1.31 (2H, m, H-2'), 1.29 (2H, m, H-13'), 0.90 (3H, t, *J* = 7.2 Hz, H-15'); ¹³C NMR (MeOH-*d*₄, 125 MHz): δ 159.40 (C-1/3), 146.43 (C-5), 130.98 and 130.96 (C-8' and 9'), 108.03 (C-4/6), 101.07 (C-2), 37.51 (C-1'), 33.09 (C-13'), 32.60 (C-2'), 30.99^A, 30.65^A, 30.51^A, 30.44^A, 30.19^A, 28.29 and 28.26 (C-7' and 10'), 23.88 (C-14'), 14.61 (C-15'). ^ANMR signals for methylenes in alkyl group, which were overlapped; HR-APCI-MS *m/z* 317.2476 [M-H]⁻ (317.2480, calcd for C₂₁H₃₃O₂).

2.4 Cell culture

CT26, B16F10 and HCT116 cells were purchased from the Korean Cell Line Bank (Seoul, Korea), and BJAB cells were a generous gift from Genentech, Inc. (South San Francisco, USA). CT26 and B16F10 cells was cultured in DMEM, BJAB in RPMI 1640, and HCT116 in McCoy's 5A (all media contained 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin) at 37 °C in a humidified 5% CO₂ atmosphere.

2.5 XTT assay

Cells were cultured in 96 well plates (2 x 10⁵/well or 1 x 10⁴/well) overnight and treated with indicated amounts of bilobol for 24 hours. Cell viabilities were assessed using XTT-based colorimetric assay kit, according to the manufacturer's instructions (Promega, WI, USA) and absorbances at 493 nm were monitored by TECAN Infinite M200 monochromator (Tecan, Switzerland). Data are representative of three independent experiments and are reported as means ± SDs.

2.6 Western blot analysis

HCT116 cells were treated with bilobol (50 µg/mL) for indicated time and lysed in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol tetraacetate, 1% Triton X-100, 25mM sodium pyrophosphate, 1 mM NaF, 1mM b-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mg/mL leupeptin and 10 mg/mL aprotinin. Twenty micrograms of total cell lysate was separated on sodium dodecyl

sulfate– polyacrylamide gel electrophoresis gel and transferred onto Immobilon-P membranes (Millipore, Etobicoke, Ontario, Canada). Membranes were blocked with 5% skim milk and then incubated with the indicated antibodies at room temperature for 1 h. Signals were detected using an enhanced chemiluminescence Western Blotting Kit (Amersham, Quebec, Canada). Anti-caspase-3 and anti-caspase-8 were commercially purchased (Santa Cruz, California, USA).

3 Results and discussion

3.1 Isolation and identification of active compound

To identify the anti-cancerous molecules from *G. biloba*, we extracted *n*-hexane soluble substances from the fruit pulp of sarcotesta of *G. biloba*. The *n*-hexane extract was subjected to partition and subsequent repeated HPLC column chromatography. The fractions were obtained and further purified by HPLC using reverse phase C18 column. Methanol: water solvent system was found to be the best solvent to extract fractions from the *G. biloba*. We isolated 8 fractions from the fruit pulp of *G. biloba* through bioassay-guided separation of the fractionated Methanol: water extract using repeated column chromatography (Figure 1). A linear gradient solvent condition applied to HPLC separation was H₂O containing 0.1% formic acid-MeOH (10:90) to MeOH 100% for 70 min, with flow rate 4.0 mL/min, at 280 nm. Of eight fractions evaluated, fraction **b** exhibited significant cytotoxic activity, and bilobol (**1**, *t_R* 12.19 min) was identified as a major component of this fraction.

Structure of isolated compound was further elucidated by spectroscopic interpretation including NMR and HR-MS. The ¹H NMR spectra of **1** showed three aromatic *para* protons at δ 6.12 (2H, d, *J* = 2.4 Hz, H-4/6) and 6.08 (1H, d, *J* = 2.4 Hz, H-2) suggesting a resorcinol moiety with C-5 substitution. This substituted moiety was identified as a pentadecenyl group including a *cis*-olefin at δ 5.34 (2H, m, H-8'/9'), twelve aliphatic methylenes at δ 2.43 (2H, t, *J* = 7.5 Hz, H-1'), 2.03 (4H, m, H-7'/10'), 1.56 (2H, quintet, *J* = 7.5 Hz, H-2'), 1.26 – 1.36 (12H,

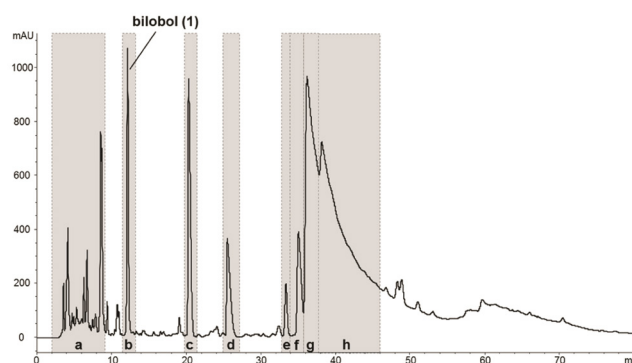


Figure 1. HPLC profile and peak-based fractions (a-h) of *n*-hexane extract from ginkgo fleshy seed coat. A linear gradient solvent condition was applied to HPLC separation; H₂O containing 0.1% formic acid-MeOH 10:90 to MeOH 100% for 70 min, flow rate 4.0 mL/min, 280 nm. Of eight fractions evaluated, fraction **b** exhibited significant biological activity, and bilobol (**1**, *t_R* 12.19 min) was identified as a major component in this fraction.

m), 1.31 (2H, m, H-2'), 1.29 (2H, m, H-13'), and a methyl group at δ 0.90 (3H, t, $J = 7.2$ Hz, H-15'). Based on full 1D- and 2D-NMR spectral analyses, the structure of **1** was assigned as 1,3-dihydroxy-5-[8'(Z)-pentadecenyl] benzene (bilobol). It showed m/z 317.2476 $[M-H]^-$ peak (calculated for $C_{21}H_{33}O_2$, 317.2480) in the HR-APCI-MS spectrum, indicating molecular formula of $C_{21}H_{34}O_2$. Bilobol was obtained as yellow oil (Figure 2). Bilobol, an alkenylresorcinol, and its derivatives have been demonstrated in different plants and microbes (Gohil et al., 2000), showing skin irritation (McKenna et al., 2001) and plant stress response (Papadopoulos et al., 2000).

3.2 Cancer cell growth inhibition assay

To evaluate the cytotoxic effects of isolated compound on tumor cells, we treated CT26 cells with different concentrations of bilobol (3.125 $\mu\text{g/mL}$, 6.25 $\mu\text{g/mL}$, 12.5 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$) for 24 hours. It showed dose dependent cytotoxic effects of the compound suppressing more than 50% of cell viability with highest concentration compared to that of control group (Figure 3).

The cytotoxic effects of bilobol on other tumor cells were further determined by XTT based colourimetric assay using 293, B16F10, BJAB, and HCT116. As shown in Figure 4, bilobol exhibited considerable inhibition of cancer cell growth in a dose dependent manner. 293 cells showed the highest sensitivity against bilobol (Figure 4). These results support the previous

anti-tumor studies of bilobol where administration of bilobol in a dosage of 40 mg/kg per day for 4 days after injection of tumor cells in mouse caused inhibition of the tumor cell growth (Risberg et al., 1998). Our results suggested that bilobol may have inhibiting effects on different human cancers and may be used in future tumor treatments.

3.3 Western blot assay

We evaluated the mode of cell death by measuring expression level of caspase-3 and caspase-8 in HCT116 cells. Activation of caspase-3 and caspase-8 are considered to be key events for induction of apoptotic cell death (Schwartzmann et al., 2002) HCT116 cells were treated with 50 $\mu\text{g/mL}$ bilobol for 0, 2, and 4 hrs and activation of caspases were determined by western blotting. Bilobol significantly augmented caspase-3 activation as reflected by production of cleaved caspase-3 in a dose-dependent manner (Figure 5). Also, caspase-8 activation by bilobol was observed.

Detailed molecular mechanism by which bilobol causes caspase activation is not clear. Bilobol could directly damage the mitochondria to release cytochrome c or possibly changes gene expression levels of death-inducing machinery proteins as indicated by Gohil et al. (2000) and Papadopoulos et al. (2000), showing that Ginkgo leaf extract changes the transcriptional level of genes involved in cell proliferation, cell differentiation and apoptosis in breast and cancer cell lines (Tanaka et al., 2011; van Beek & Montoro, 2009).

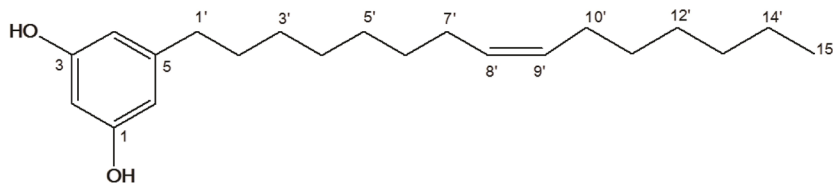


Figure 2. The structure of bilobol (**1**) isolated from the sarcotesta of *G. biloba*.

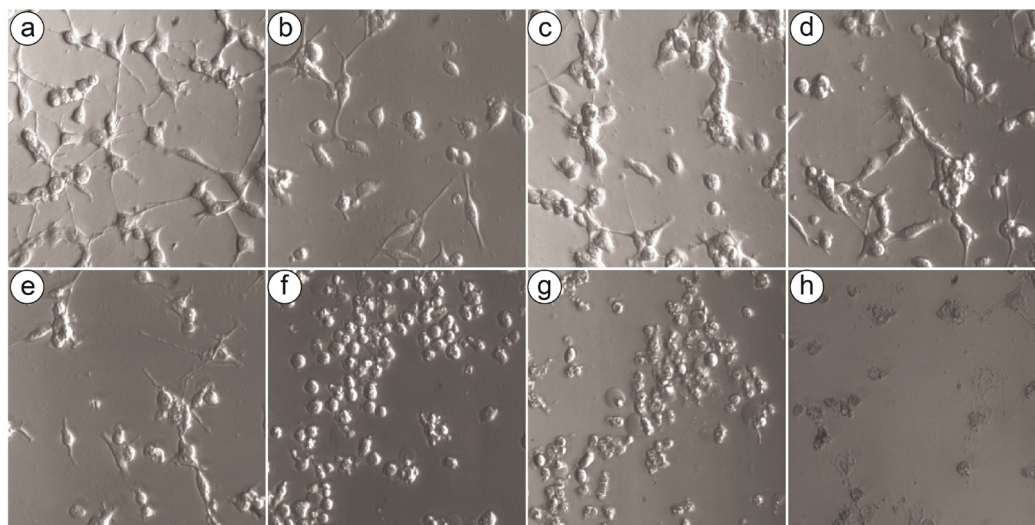


Figure 3. CT26 cells were treated with media alone (a), DMSO (b), or various amounts of bilobol (c-h) (c; 3.125 $\mu\text{g/mL}$, d; 6.25 $\mu\text{g/mL}$, e; 12.5 $\mu\text{g/mL}$, f; 25 $\mu\text{g/mL}$, g; 50 $\mu\text{g/mL}$, h; 100 $\mu\text{g/mL}$). Images (x 40) were taken under the microscope.

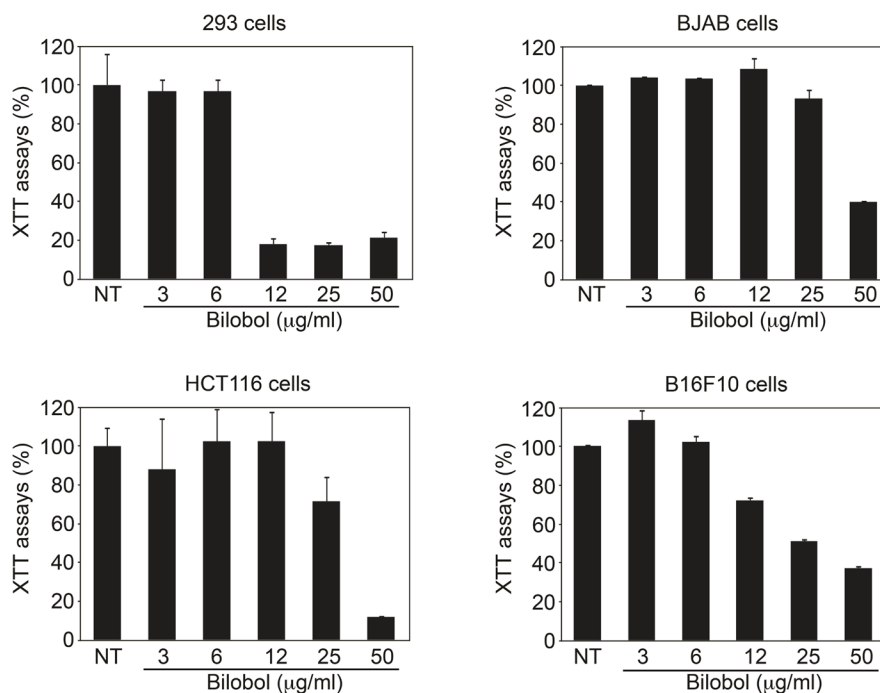


Figure 4. 293 cells, B16F10 cells, BJAB cells or HCT116 cells were treated with indicated amounts of bilobol for 24 hours. XTT assays were carried out to measure viabilities.

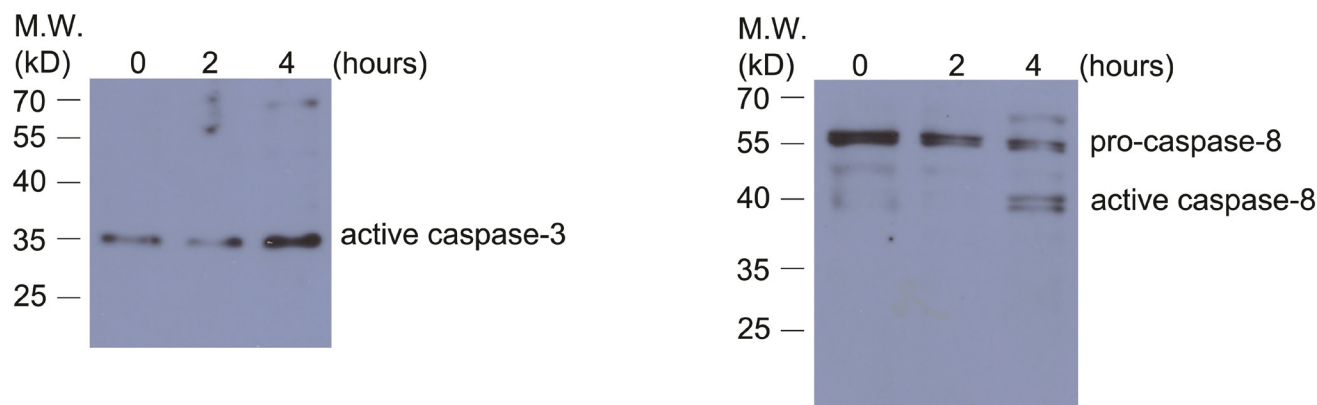


Figure 5. HCT116 cells were treated with bilobol (50 µg/mL) for indicated time, and cell lysates were harvested for Western blot analysis using anti-caspase-3 (right panel) or anti-caspase-8 (left panel) antibodies.

However, we hypothesize that bilobol may directly act on caspase activation through mitochondrial damage or activation of apoptotic pathways rather than through transcriptional gene expression, because it can activate caspase-3 and -8 within 4 hours after treatment.

4 Conclusions

In this study, the fast and effective structural screening was successfully accomplished by employing HPLC based extract profiling for the identification of a known alkenylresorcinol,

bilobol (1) with a single injection of the *n*-hexane extract of the fruit pulp of *Ginkgo biloba* L. (Gingkoaceae) without subsequent isolation procedures. We determined cytotoxic profiles of the fractions obtained from the *n*-hexane extract of *Ginkgo biloba* L. (Gingkoaceae) using XTT assay. The activity-guided isolation of compounds using HPLC provided a known compound, bilobol from the active fraction b, exhibiting dose-dependent cytotoxic effects on human colon cancer cells (CT26, B16F10, BJAB and HCT116 cells). However, further studies would be desirable to elucidate the signaling pathway for bilobol induced apoptosis as well as therapeutic potential to cancer using *in vivo* tumor model.

Conflict of interest

The authors declare that they have no conflict of interest.

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