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ORIGINAL ARTICLE

Anwulignan from the Fruits of *Schisandra chinensis* and its Cytotoxicity against Human Cancer Cell Lines

Yeong-Geun Lee, Rong Bo Wang, Hyoung-Geun Kim, Dae-Young Lee[1], Yeon-Ju Kim, Nam-In Baek

Department of Oriental Medicine Biotechnology, Kyung Hee University, Yongin-Si, [1]Herbal Science, RDA, Eumseong-gun, Korea

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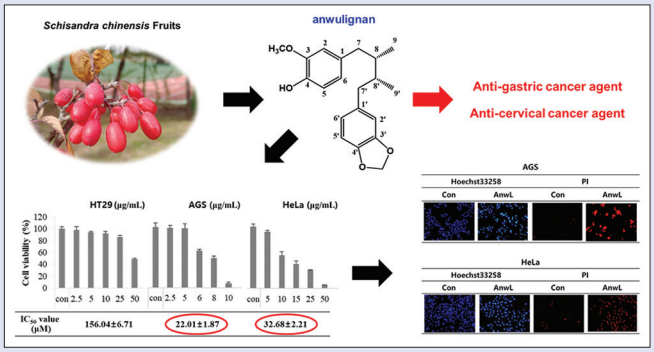
ABSTRACT

Background: The fruits of *Schisandra chinensis* have been used traditionally as the medicinal materials in East Asia. Their extracts have been reported to be cytotoxic to several cancer cells and phytochemical research has identified numerous cytotoxic lignans. **Objectives:** We reviewed for the effects of cytotoxic lignans against various human cancer cells. **Materials and Methods:** Solvent extraction of fruits in aqueous methanol and fractionation of the extract into H_2O, *n*-BuOH, and EtOAc fractions (Fr) were carried out. Column chromatography (CC) of the non-polar EtOAc Fr, which indicated cytotoxicity to cancer cells, was performed to isolate the cytotoxic lignin. They were identified on the basis of the intensive spectroscopic interpretation of IR, 1D-, 2D-NMR, and mass spectrometry data. The cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and cell staining using Hoechst 32258 and propidium iodide. **Results:** Anwulignan was isolated and identified as a cytotoxic principal component. The IC_{50} value was calculated to be 22.01 ± 1.87, 156.04 ± 6.71, and 32.68 ± 2.21 μM for human stomach adenocarcinoma, human colon cancer (HT29), and human cervical cancer cells, respectively. **Conclusion:** Solvent extraction, systematic fractionation, and repeated CC for *S. chinensis* fruits led to the isolation of a lignin identified to be anwulignan based on various spectroscopic analyses. Anwulignan demonstrated very high cytotoxicity to most human cancer cell lines, and cell death was induced by apoptosis.

Key words: Anwulignan, apoptosis, human cancer cells, macelignan, *Schisandra chinensis*

SUMMARY

- Our research achieved the isolation of an active component from *Schisandra chinensis* fruits and identification as anwulignan. Furthermore, it was evaluated for the cytotoxicity against various human cancer cell lines. As a result, anwulignan was revealed to be promising anti-cervical and anti-gastric cancer agents.



Abbreviations used: Fr: Fraction; CC.: Column chromatography; PI: Propidium iodide; AGS: Human stomach adenocarcinoma; HT29: Human colon cancer; HeLa: Human cervical cancer; Ext: Extracts; SCE: *Schisandra chinensis* EtOAc fraction; SiO_2: Silica gel; ODS: octadecyl SiO_2; Ev/Tv: elution volume/total volume; NMR: Nuclear magnetic resonance spectroscopy; IR: Infrared spectroscopy; EIMS: Electron Ionization Mass Spectrometer.

Correspondence:

Prof. Nam-In Baek,
Department of Oriental Medicine Biotechnology, Kyung Hee University, Yongin-Si 17104, Korea.
E-mail: nibaek@khu.ac.kr

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INTRODUCTION

Schisandra chinensis (*Schisandraceae*) is a deciduous fruit-bearing vine distributed in the far eastern parts of Asia. The fruits of the plant, called "Omija" in Korean meaning "five-flavor-fruits," are reddish berries in hanging clusters with 10 cm long. They are used as an "adaptogen" to enhance resistance to disease and stress, increasing one's energy as well as physical performance and endurance.[1] In the traditional Chinese medicine, the berries are considered beneficial to the human body as well as the life force or energy inherent in all living organisms[2] and to have favorable effects on Alzheimer's disease and fatty liver diseases.[3] The berries of *S. chinensis* contain a significant number of lignans, which are known as phytoestrogens and possess antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial, antiallergic, antitumor, antiangiogenic, antiseptic, and protective effects on cytotoxic effect and oxidative stress.[4-6] The principal constituents of the fruits have been reported to be lignans, especially dibenzocyclooctadiene-type lignans.[7,8] However, other types of lignans such as sesquiterpenes, 3,4-seco-21,26-olide-artane type triterpeonoids, and bisnortriterpenoids with unusual skeletons also have been isolated and assessed for pharmacological activities.[9-11] Recently, the authors found the alcohol extracts from *S. chinensis* fruits to show cytotoxicity on human cancer cell lines. This study represents the process of isolation, identification, as well as the evaluation of the cytotoxicity to human cancer, human stomach adenocarcinoma (AGS), human colon (HT29), and human cervical cancer (HeLa) cancer cells.

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MATERIALS AND METHODS

General experimental procedures

The materials for the isolation of lignans, instruments for structure determination, and drugs for the evaluation of cytotoxicity were referred to the previous authors' literature.[12-14]

Plant materials

S. chinensis fruits were provided by RDA (Eumseong, Korea) in 2019, which was confirmed by Prof. D. G. Kim of Woosuk University (Jeonju, Korea). A guarantee sample (KHU-NPCL-201904) was stored at NPCL, Kyung Hee University.

Extraction and isolation

The *S. chinensis* fruits (dry weight, 5.4 kg) were soaked in 80% MeOH (54 L × 2) at 25°C for 24 h, poured on filter paper and concentrated in a vacuum to provide black–brown sticky extracts (Ext, 1.26 Kg). The residue was poured in H_2O (4.2 L) and extracted using EtOAc (4.2 L × 2). The organic components were dehydrated by the addition of anhydrous $MgSO_4$, filtered on filter paper and concentrated to yield the EtOAc fraction (Fr, SCE; 330 g). Column chromatography (CC) for SCE (329 g) was conducted on a SiO_2 column (11 × 16 cm) using *n*-hexane-EtOAc (15:1 → 10:1 → 5:1 → 1:1, 900 mL of each) as eluting solvent. The eluates were assessed by the TLC experiment and gathered according to TLC pattern to finally yield 12 Fr (SCE-1 to SCE-12). Fraction SCE-5 (8.7 g, elution volume/total volume [Ev/Tv] 0.108–0.276) was applied to an ODS column (7 × 6 cm) using acetone-H_2O (2:1, 3.0 L) to afford 10 Fr (SCE-5-1 to SCE-5-10). Fr SCE-5-3 (3.6 g, Ev/Tv 0.167-0.267) was SiO_2 CC (4.5 cm × 20 cm) using $CHCl_3$-EtOAc = 35:1, 2.9 L) to yield 11 Fr (SCE-5-3-1 to SCE-5-3-11) along with a purified lignan (SCE-5-3-3, Anwulignan, 42 mg, Ev/Tv 0.200-0.390).

Anwulignan (1): TLC, R_f 0.66 (SiO_2, EtOAc-$CHCl_3$ = 1:12), R_f 0.57 (ODS, H_2O-acetone = 1:4); colorless solid; $[\alpha]_D^{25}$ +6.2° (*c* 0.79, $CHCl_3$); UV (MeOH): 285 nm; IR (LiF plate, *ν*) 3502 (OH), 2956 (CH), 1608 (phenyl), 1514 (phenyl) cm^{-1}; EIMS *m/z* 328 [M] +, 265, 237, 135; NMR (600 MHz, $CDCl_3$) [Table 1].

Cell culture and cell viability assay

The condition for the culture of HeLa, AGS, and HT29 cells was same as the previously reported literature.[15-17] Cell viability was calculated using MTT assay as previously reported.[18] The treated concentrations of Anwulignan were 10, 25, and 50 μg/mL.

Microscopic analysis of the cells stained with Hoechst 33,258 and propidium iodide

AGS and HeLa cells were seeded onto the coverslip in the cell culture dish, incubated for 24 h and treated by Anwulignan (8 μg/mL) with additional incubation for 24 h. The dishes were washed twice using 100 μL PBS. Next, 5 μL of Hoechst 33,258 (10 mg) or propidium iodide (PI; 5 mg) dye solution was treated on the dishes for staining. The concoction was incubated at 25°C for 30 min in total darkness. Subsequently, the stained suspension (10 μL) was placed between a slide and coverslip. The cells were envisaged using a fluorescence microscope, a Leica DMIL LED (Leica Camera, Wetzlar, Germany). Not <300 cells were calculated on each slide with the performance of triplicate.

RESULTS AND DISCUSSION

Structural identification

Dried *S. chinensis* fruits were extracted in MeOH and partitioned into EtOAc, *n*-BuOH, and H_2O Fr. Repeated CC of the EtOAc fraction led to the isolation of a lignan (1), which was identified to be anwulignan on the basis of intensive analyses of NMR, IR, EIMS, as well specific rotation data [Figure 1].

Table 1: ^{1}H- (600 MHz) and ^{13}C-NMR (125 MHz) data of Anwulignan ($CDCl_3$)

Number of C	δ_H	δ_C
1	-	133.78
2	6.65, 1H, d, *J*=1.6 Hz	111.47
3	-	146.32
4	-	143.58
5	6.86, 1H, d, *J*=7.8 Hz	114.05
6	6.68, 1H, dd, *J*=7.8, 1.6 Hz	121.72
7	2.750, 1H, dd, *J*=13.2, 5.4 Hz 2.28, 1H, dd, *J*=13.2, 9.6 Hz	38.87
8	1.77, 1H, m	39.30
9	0.87, 3H, d, *J*=6.6 Hz	16.25
1′	-	135.73
2’	6.69, 1H, d, *J*=1.2 Hz	109.38
3′	-	147.49
4′	-	145.49
5′	6.76, 1H, d, *J*=7.8 Hz	107.96
6′	6.64, 1H, dd, *J*=7.8, 1.2 Hz	121.85
7′	2.753, 1H, dd, *J*=13.2, 6.0 Hz 2.32, 1H, dd, *J*=13.2, 9.6 Hz	39.10
8′	1.77, 1H, m	39.38
9′	0.86, 3H, d, *J*=6.6 Hz	16.14
OMe	3.89, 3H, s	55.85
-O-CH_2-O-	5.95, 1H, d, *J*=1.8 Hz 5.94, 1H, d, *J*=1.8 Hz	100.74
4-OH	5.55, 1H, s	-

Compound 1, a colorless solid, showed ultraviolet absorption characteristics at 285 nm and displayed a violet color on TLC with 10% H_2SO_4 spray and baking. The IR spectrum revealed the presence of hydroxyl (3502) and aromatic (1608, 1514 cm^{-1}) groups. The molecular weight was conclude as 328 Da (Molecular ion peak *m/z* 328 [M] +· EIMS). ^{1}H-NMR data [Table 1] included typical signals due to two 3,4-disubstiltuted phenyl moieties (chemical shift, coupling pattern, *J* in Hz; 6.65, d, 1.6, H-2; 6.86, d, 7.8, H-5; 6.68, dd, 7.8, 1.6, H-6; 6.69, d, 1.2, H-2’; 6.76, d, 7.8, H-5’; 6.64, dd, 7.8, 1.2, H-6’),[13] two methylenes with germinal coupling (2.75, dd, 13.2, 5.4, H-7a; 2.28, dd, 13.2, 9.6, H-7b; 2.753, dd, 13.2, 6.0, H-7’a; 2.32, dd, 13.2, 9.6, H-7’b), two methines (1.77, m, H-8; 1.77, m, H-8’), two doublet methyls (δ_H 0.87, d, 6.6, H-9; δ_H 0.86, d, 6.6, H-9’), a dioxymethylene with germinal coupling (5.95, d, 1.8; δ_H 5.94, d, 1.8) and a methoxy (3.89, s, H-OMe), suggesting that compound 1 was a dibenzylbutane type lignan.[14] In addition, a hydroxyl (5.55, s, H-4-OH) proton signal was observed, which indicated a hydrogen bond in the molecule. ^{13}C-NMR Data [Table 1] were composed of 18 carbon signals including methoxy (δ_C 55.85, C-OMe) and dioxymethylene (δ_C 100.74, C-O-CH_2-O) moieties. The carbon signals derived from a lignan moiety were four oxygenated olefin quaternaries (δ_C 146.32, C-3; 143.58, C-4; 147.49, C-3’; 145.49, C-4’), two olefin quaternaries (δ_C 133.78, C-1; 135.73, C-1’), six olefin methines (δ_C 111.47, C-2; 114.05, C-5; 121.72, C-6; 109.38, C-2’; 107.96, C-5’; 121.85, C-6’), two methines (δ_C 39.30, C-8;

39.38, C-8'), two methylenes (δ_C 38.37, C-7; 39.10, C-7'), and two methyls (δ_C 16.25, C-9; 16.14, C-9'). As shown in Figure 2, the proton signal of a dioxymethylene proton (δ_H 5.95 and 5.94) showed HMBC correlations with two oxygenated olefin carbon signals (δ_C 147.49, C-3'; 145.49, C-4'). Moreover, a cross peak was detected between a methoxy proton (δ_H 3.89) and an oxygenated olefin quaternary carbon (δ_C 146.32, C-3) signals. Further, a proton signal due to a hydroxyl group (δ_H 3.89) exhibited cross-peaks with three oxygenated olefin quaternary carbon signals through one J^2 coupling (δ_C 143.58, C-4) and two J^3 couplings (δ_C 146.32, C-3; 114.05, C-5). The proton of OH-4 was revealed to form a hydrogen bond with the oxygen of OCH_3-3. Collectively, taken together, compound 1 was identified to be a dibenzylbutane type lignan, 4-[4-(1,3-benzodioxol- 5-yl)-2,3-dimethylbutyl]-2-methoxyphenol, which has two chiral centers, C-8 and C-8′. Comparison of the specific rotation value measured in this study (+6.2°) with those reported in previous research (+5.28°, +5.5° and + 4.0°),[19-21] respectively, confirmed the configuration of C-8 and C-8′ to be *S* and *R*. Finally, the chemical structure of compound 1 was identified to be 4-[2*S*,3*R*-4-(1,3-benzodioxol-5-yl)-2,3-dimethylbutyl]-2-metho xyphenol, which has been previously isolated from *S. spenanthera*, *Myristica fragrans,* and *Virola calophylla* and labeled as anwulignan,[22] macelignan,[23] and calophyn,[24] respectively.

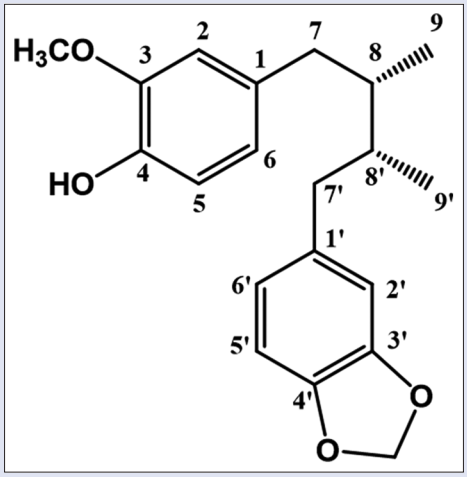

Figure 1: Chemical structure of anwulignan from the fruits of *Schisandra chinensis*

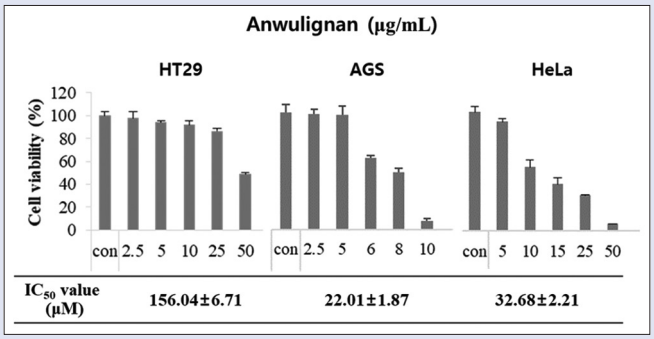

Figure 3: Cytotoxicity of anwulignan from the fruits of *Schisandra chinensis* on human cancer cell lines. HT29, colon; AGS, gastric; HeLa, cervical

Cytotoxicity of anwulignan on human cancer cell lines

The extract from *S. chinensis* and lignans is reported to have an anticancer effect including cytotoxicity on cancer cell lines.[19] Anwulignan has been shown to have an inhibitory effect against chemical-induced toxicity and protective effects against D galactose-induced hepatic injury.[2,3] Therefore, the cytotoxicity of anwulignan against and colon (HT-29), gastric (AGS) and cervical (HeLa) cancer cells was estimated by the use of MTT test and expressed as IC_{50} value [Figure 3]. The data indicated that Anwulignan concentration-dependently suppressed the proliferation and viability of all three cancer cells. Even though IC_{50} in AGS (22.01 ± 1.87 μM), HeLa (32.68 ± 2.21 μM), and HT29 (156.04 ± 6.71 μM) cells were low relative to those of the well-known and clinically used anticancer compound doxorubicin (IC_{50} value: 0.25 μM, AGS; 1.45 ± 0.15 or 3.7 ± 0.3 μM, HeLa; 11.39 or 0.75 μM, HT29),[25-29] the values are very high in comparison to naturally occurring compounds (IC_{50} value: baicalein on AGS, 85 μM; galactosyl diglyceride on AGS, 49.1-83.4 μM; clausenidin on HT29, 42.07 μM; quercetin on HT29, 75 μM).[28-31]

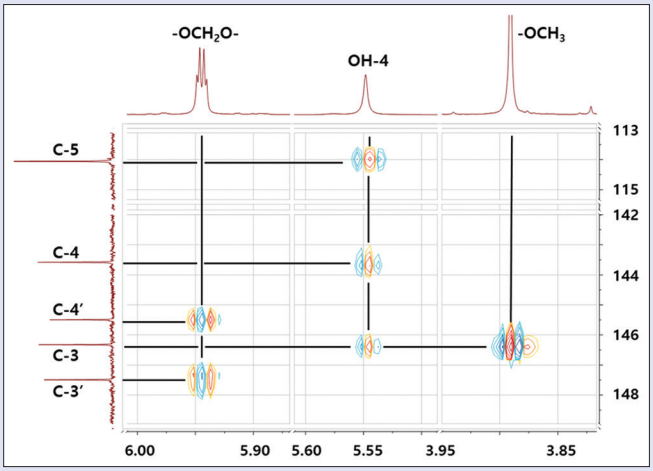

Figure 2: HMBC spectrum of anwulignan (600 MHz, $CDCl_3$)

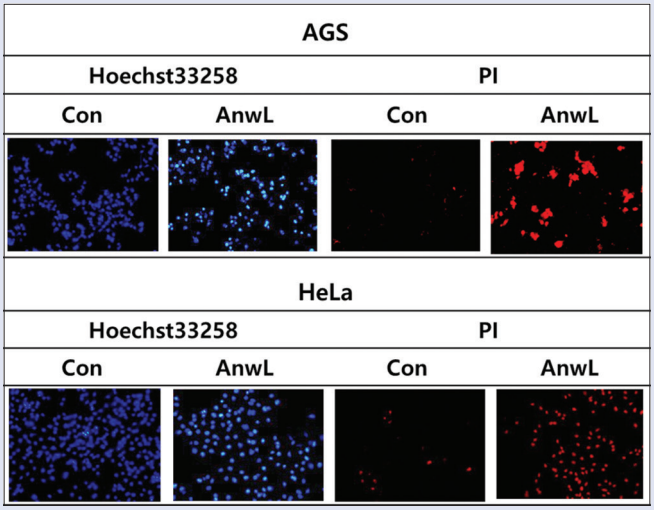

Figure 4: Fluorescent microscopy images for Hoechst 33258 and PI staining in cultured AGS and HeLa cell lines. Con, control; Anwl, anwulignan

Cell staining study

Cell death is commonly described in dichotomy as either necrosis or apoptosis. Apoptosis is characterized as an active, scheduled proceeding of spontaneous cellular collapse that challenges inflammation, while necrosis is described as passive, incidental cellular break down because of external disruptions on a par with toxins, infection, or trauma that culminates in uncontrolled extinction with unregulated release of inflammatory cellular contents. Apoptosis leads to cellular morphological changes through chromatin condensation and nuclear fragmentation into membrane-bound fragments and formation of apoptotic bodies. Necrosis leads to the destruction of cell membrane function resulting in ungoverned liberation of cell death products into the extracellular domain. DNA fragments are in current one of the most reliable proof of cell death. Hoechst 33,258 fluorochrome is a fluorescent stain for labeling DNA in fluorescence microscopy and may be used on live or fixed cells. The cells in progress of apoptois showed morphological variation in the chromatin of nuclear. PI is a fluorescent dye that is unable to penetrate undamaged lipid membranes resulting in ejection by living cells. PI is an easy-to-use nucleic acid stain for distinguishing dead cells from live ones. Hoechst 33,258, blue fluorescent is capable of penetration into undamaged membrane of intact cell membranes and tinctures the concentrated chromatin of apoptotic cells with high bright comparing to the unbound one of common cells, leading to tracking the variance in nuclear resulting from apoptosis. PI, a red-fluorescent, is kept out by live cells or apoptotic cell in the early stage. Therefore, the penetration of PI into cell infers a deprivation of membrane function, which is a typical property of apoptotic cells in the late stage or necrotic cells. Necrotic, late apoptotic, early apoptotic, and viable cells are observed as intact pink nuclei, condensed/fragmented pink nuclei, condensed/fragmented blue nuclei, and intact blue nuclei in microscopic images, respectively [Figure 4]. The present research confirmed Anwulignan to bring about necrosis or apoptosis in AGS and HeLa cells was evaluated by treating cells for 24 h with the compound. Numerous alterations in the shape of the nucleus, cell structure, and size as characteristics of apoptosis and necrosis were detected, indicating the drug brought about apoptosis and necrosis to result of cell death.

CONCLUSION

In summary, our study led to the isolation of a lignan from *S. chinensis* fruits, identified as anwulignan, a dibenzylbutane-type lignan, based on intensive spectroscopic study. Anwulignan significantly induced the death of the human cancer cell lines AGS and HeLa. A staining study using Hoechst 33,258 and PI elucidated that the cell death was induced by mainly apoptosis but also partly necrosis.

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Conflicts of interest

There are no conflicts of interest.

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