Anticancer effects in vitro and chemical compositions of extracts of Acanthopanax henryi

Zhi Li¹, Jonghwan Kim², Xiang-Qian LIU*, Kyungtae Lee³, Changsoo Yook³

a. Hunan Key Laboratory of Traditional Chinese Medicine modernization, School of Pharmacy, Hunan University of Chinese Medicine, Hunan Changsha 410208, China
b. Herbal Medicine Research Division, Korea Food & Drug Administration, Seoul 363-700, Korea
c. College of Pharmacy, Kyung-Hee University, Seoul 130-701, Korea

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Abstract

Objective: To study on anticancer effect in vitro and chemical compositions of root bark extracts of Acanthopanax henryi.

Methods: The hot methanol extract of the root bark of A. henryi was through XAD-4 column chromatography eluting with a gradient of methanol in water. The anticancer effects of MeOH fractions were evaluated by MTT method. Then compounds were isolated by a combination of chromatographic materials, silica gel, ODS, and Sephadex LH-20, and finally methods of recrystallization were used to purify products. Their structures were elucidated on the basis of spectroscopic methods.

Results: The 100% MeOH fraction showed better cytotoxicity on HL-60 cells, HT-29 cells and A549 cells as judged by IC₅₀ than other MeOH fractions. Six compounds were identified as stigmasterol (I), β-sitosterol (II), eleutheroside E (III), acanthoic acid (IV), kaurenoic acid (V), and daucosterol (VI), respectively.

Conclusion: 100% MeOH fraction of the root bark of A. henryi has significant inhibition on HL-60 human leukemia cells, HT-29 human colon cancer cells, A549 human lung cancer cells. Compounds IV and V were obtained from this plant for the first time. These results provide a pharmacological basis for the treatment of cancer in the clinical application of this herb.

Keywords: Acanthopanax henryi (Oliv.)Harms, root bark extracts, anticancer effect, chemical compositions

1 Introduction

Acanthopanax henryi (Oliv.)Harms (Araliaceae) has been used as a traditional medicine for the treatment of rheumatism, inflammation, fatigue, paralysis and so on1-2. The root barks of A. henryi (Oliv.)Harms as “Wujiaipi” was included at Hunan local medicinal materials standard3. Phytochemical researches revealed that some lignans, diterpenoids, flavonoids, etc. were isolated from Acanthopanax spp.4-9. Pharmacological studies indicated that 80% MeOH fraction of root bark of A. henryi showed significant anti-inflammatory activity. However, the anti-cancer effects of A. henryi were not reported until now. As known, cancer caused by disorders of controlling cell proliferation, is the most terrible cause of death. More and more researchers are committed to discover more lead compounds from plants to develop new antitumor agents for treatment of neoplasms10-11. Researches on diterpenoids from Acanthopanax spp. have proved the diterpenoids are one of active
compounds of anticancer 12-13. Therefore, the aim of this study was to test the anti-cancer effect in vitro and isolate and identify their chemical compositions from the MeOH fraction of the root bark of A. henryi.

2 Materials and methods

2.1 Plant Material
The root barks of A. henryi material was collected in August 2008 in Mt. DAXIONG, Hunan province, China, and dried in the shade. The plant was identified by Emeritus Professor Chang-Soo Yook, Department of Pharmacy, Kyung Hee University. The voucher specimens have been deposited in the Pharmacognosy Laboratory, Department of Pharmacy, Kyung Hee University (KHUP-0714 / A. henryi root barks / 2008).

2.2 Chemicals and instruments
Melting points (uncorrected) were measured using a Boetius micromelting point apparatus. Optical rotations were determined on a JASCO DIP-1000KUY polarimeter (l=0.5). IR spectra were obtained with a Hitachi 270-30 type spectrophotometer. FAB-MS were obtained in a glycerol matrix in the positive ion mode using JEOL JMS-DX300 and JMS-DX303HF instruments, and NMR spectra were measured in pyridine-d$_5$ and d$_2$-methanol on a JEOL-$\alpha$-500 spectrometer and chemical shifts were relative to tetramethylsilane (TMS). Column chromatography (CC) was carried out on silica gel 230–400 mesh (Merk), Diaion HP-20P (Mitsubishi Chem. Ind. Co., Ltd., Japan), Chromatorex ODS (30-50μm, Fuji Silysia Chem. Ind. Co., Ltd., Japan) and Sephadex LH-20 (Pharmacia Biotech, Sweden). TLC was performed on precoated silica gel plates (Merck) and RP-18F$_{254}$ (Merck) plates. Spots were visualized by heating silica gel plates sprayed with 10% H$_2$SO$_4$ in EtOH. Chloroform, methanol, water and other reagents were analytical grade.

Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin were purchased from Life Technologies Inc. (Grand Island, NY, USA).

2.3 Cell Culture

2.3.1 Cell culture and preincubation of drugs
The HL-60 cells, HT-29 human colon cancer cells and A549 human lung cancer cells were grown at 37 ºC in DMEM supplemented with 10% FBS, penicillin (100 units·mL$^{-1}$), and streptomycin sulfate (100 μg·mL$^{-1}$) in a humidified 5% CO$_2$ atmosphere. Cells were incubated with every MeOH fraction at the indicated concentrations.

2.3.2 MTT assay for cell viability
The HL-60 cells, HT-29 human colon cancer cells, A549 human lung cancer cells after 24 h of continuous exposure to the tested compounds was measured with a colorimetric assay based on the ability of the mitochondria in viable cells to reduce MTT. Percentage of cell viability was calculated as the absorbance of treated cells/control cells × 100.

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HL-60 cells, HT-29 human colon cancer cells and A549 human lung cancer cells were cultured in 96-well plates for 24 h, followed by treatment with MeOH fraction of the root bark extract of A. henryi. After 24 h incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution (50 μL; 2 mg·mL$^{-1}$ in PBS) was added to the medium, and the medium was incubated for 4 h. Then, the supernatant was removed, and the obtained formazan crystals were dissolved in 200 μL of dimethylsulfoxide (DMSO). Absorbance was measured at 540 nm. Percent of cells showing cytotoxicity was determined relative to the control group.

2.3.3 Proliferation assay of HL-60 cells, HT-29 human colon cancer cells, A549 human lung cancer cells
Nitroblue tetrazolium (NBT) reduction test: the percentage of HL-60 cells, HT-29 human colon cancer cells and A549 human lung cancer cells capable of reducing NBT was measured by counting the number of cells containing the precipitated formazan particles after the cells had been incubated with the NBT (1.0 mg/mL) at 37 ºC for 30 min. 12-O-Tetradecanoylphorbol-13-acetate was used to stimulate the formation of formazan. Phagocytosis
test: the HL-60 cells, HT-29 human colon cancer cells and A549 human lung cancer cells (1 x 10^6 cells/mL) were suspended in serum-free RPMI 1640 medium containing 0.2% of the latex particles (average diameter, 0.81 μM) and incubated at 37°C for 4 h. After incubation, the cells were washed once with PBS. The cells containing > 10 latex particles were scored as being phagocytic cells.

Esterase activity test: a smear preparation was chemically stained for α-naphthyl acetate esterase and naphthol AS-D chloroacetate esterase using the standard techniques.

Flow cytometry: the HL-60 cells, (2 x 10^5 cells/mL) exposed to each MeOH Ex. & Fr. were collected and washed twice with ice-cold PBS. The cells were then incubated with the direct fluorescein isothiocyanate-labeled anti-CD 14 or anti-CD 66b antibodies (Pharmingen, San Diego, CA, USA) on ice for 30 min, washed twice with PBS, and the level of antibody binding to the cells was quantified using fluorescence-activated cell sorting flow cytometry (Becton Dickinson Co.).

2.3.4 Statistical analysis
The results were expressed as the means ± S.D. of triplicate experiments. Statistically significant values were compared using ANOVA and Dunnett’s post-hoc test, and p-values of less than 0.05 were considered statistically significant.

2.4 Extraction and isolation
The dried root bark of *A. henryi* (3 kg) were extracted with hot MeOH (65 °C) 3 times and concentrated under reduced pressure to obtain extracts (129 g), and then was chromatographed on XAD-4 by elution with H2O, 30%, 50%, 80%, 100% MeOH and acetone (Fr.1, 2, 3, 4, 5, 6), successively. 100% MeOH fraction was mixed with acetone fraction have the almost same spots by RP-TLC analysis, and evaporated to dryness in vacuo to give a residue (29 g), 14 g of which was subsequently chromatographed on silica gel by elution with n-hexane:acetone (100 : 1 → 1 : 5) to give several fractions (Fr. 5/6-A, Fr. 5/6-B, Fr. 5/6-C, Fr. 5/6-D, Fr. 5/6-E etc). These fractions were chromatographed on ODS by elution with 50% MeOH to MeOH to give several fractions including Fr. C. Compounds I–VI were isolated according to XAD-4, silica gel, ODS, sephadex LH-20 column chromatography.

3 Results

3.1 Cytotoxicity of the MeOH fractions of the MeOH extract of root bark of *A. henryi* on cancer cell lines
The MeOH extract showed some degrees of cytotoxicity on HL-60 cells, HT-29 cells and A549 cells as judged by IC_{50} and its value is 120.25, 260.94, and 366.87 μg/mL, respectively. 50% MeOH fraction showed some degrees of cytotoxicity on HL-60 cells, HT-29 cells and A549 cells as judged by IC_{50} and its value is 164.39, > 400, > 400 μg/mL, respectively. 80% MeOH fraction showed some degrees of cytotoxicity on HL-60 cells, HT-29 cells and A549 cells as judged by IC_{50} and its value is 79.12, 376.97, > 400 μg/mL, respectively. 100% MeOH fraction showed better cytotoxicity on HL-60 cells, HT-29 cells and A549 cells as judged by IC_{50} and its value is 50.41, 102.05, 147.57 μg/mL, respectively (Table).

<table>
<thead>
<tr>
<th>Samples</th>
<th>HL-60 human leukemia cells (μg/mL)</th>
<th>HT-29 human colon cancer cells (μg/mL)</th>
<th>A549 human lung cancer cells (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH Ex.</td>
<td>120.25</td>
<td>260.94</td>
<td>366.87</td>
</tr>
<tr>
<td>50% MeOH Fr.</td>
<td>164.39</td>
<td>&gt; 400</td>
<td>&gt; 400</td>
</tr>
<tr>
<td>80% MeOH Fr.</td>
<td>79.12</td>
<td>376.97</td>
<td>&gt; 400</td>
</tr>
<tr>
<td>100% MeOH Fr.</td>
<td>50.41</td>
<td>102.05</td>
<td>147.57</td>
</tr>
</tbody>
</table>

3.2 Identification of the compounds from the MeOH extract of *A. henryi* root barks
The compounds I - VI were identified as
stigmasteryl, β-sitosterol, eleutheroside E, acahanthic acid, kaurenoic acid, and daucosterol by comparison of the NMR and MS data with those reported.

Compound I: C29H40O6, white powder, mp 170~172̊C; gave a positive reaction in the Liebermann-Burchard and Salkowski tests; 1H-NMR(400 MHz, CD3OD) δ: 5.40(1H, m, H-6), 5.14(1H, dd, J=14.6, 8.0 Hz, H-22), 4.98(1H, dd, J=14.5, 7.8 Hz, H-23), 3.30(1H, m, H-3a), 0.97(3H, s, H-19), 0.91(3H, d, J=6.5 Hz, H-21), 0.82(3H, d, J=6.2 Hz, H-26), 0.82(3H, t, J=7.5 Hz, H-29), 0.77(3H, d, J=6.0 Hz, H-27), 0.65(3H, s, H-18). 13C-NMR(100 MHz, CD3OD) δ: 142.2(C-5), 140.0(C-22), 130.6(C-23), 122.4(C-6), 72.4(C-3), 58.2(C-14), 57.4(C-17), 52.2(C-24), 49.4(C-9), 43.0(C-4), 41.9(C-13), 41.1(C-20), 41.0(C-12), 38.5(C-1), 37.4(C-10), 35.1(C-25), 33.2(C-2), 33.2(C-7), 32.3(C-8), 29.3(C-16), 27.1(C-28), 23.1(C-15), 21.7(C-21), 21.6(C-26), 21.1(C-11), 19.8(C-19), 19.3(C-27), 12.6(C-29), 12.3(C-18). All of the data above are in agreement with those of stigmasteryl.

Compound II: C29H40O6, white powder, mp 139~141̊C; gave a positive reaction in the Liebermann-Burchard and Salkowski tests; EI-MS m/z: 414 [M]+, 1H-NMR(400 MHz, CD3OD) δ: 5.37(1H, d, J=4.97 Hz, H-6), 3.51(1H, m, H-3), 1.00(3H, s, H-19), 0.92(3H, d, J=6.5 Hz, H-21), 0.88(3H, d, J=1.86 Hz, H-29), 0.85(3H, d, J=3.71 Hz, H-26), 0.82(3H, s, H-27), 0.70(3H, s, H-18). All of the data above are in agreement with those of β-sitosterol.

Compound III: C29H40O15, white needle crystal, mp 257~259̊C; IR(KBr) cm−1: 3400, 3030, 2960, 1600, 1530, 1150, 1020, 600. 1H-NMR(400 MHz, CD3OD) δ: 6.80(4H, s, H-2, 6, 2′, 6′), 4.86(2H, d, J=5.0 Hz, gluc-H-1, gluc-H-1′), 4.60(2H, d, J=4.0 Hz, H-7,7′), 3.70(12H, s, 4×OCH3), 3.10(2H, m, H-8,8′), 13C-NMR(100 MHz, CD3OD) δ:152.9(C-3, 3′, 5′), 137.1(C-4, 4′), 134.3(C-1, 1′), 104.5(C-2, 2′), 104.5(C-6, 6′), 103.3(gluc-1, 1′), 85.4(C-7, 7′), 79.3(gluc-3, 3′), 77.5(gluc-5, 5′), 76.8(gluc-2, 2′), 74.6(C-9, 9′), 71.8 (gluc-4, 4′), 70.3(gluc-6, 6′), 56.9(OCH3), 56.8(OCH3), 54.1(C-8, 8′). All of the data above are in agreement with those of eleutheroside E16.

Compound IV: C29H38O2, amorphous powder, mp 135~136̊C; IR(KBr) cm−1: 3290(OH), 1690(COOH), 1638(C=C); 1460; 1075; 965. 1H-NMR(500MHz, CD3OD) δ: 5.84(1H, dd, J=17.5Hz, 10.8Hz, H-15), 5.41(1H, m, H-11), 4.98(1H, dd, J=17.5Hz, 1.3Hz, H-16), 4.86(1H, dd, J=10.3Hz, 1.4Hz, H-16′), 1.18(3H, s,H-18), 1.07(3H, s, H-17), 1.03(3H, s, H-20); 13C-NMR(125MHz, CD3OD) δ: 181.68(C-19), 151.51(C-15), 151.38(C-9), 117.72(C-11), 109.73(C-16), 49.09(C-5), 45.16(C-4), 43.30(C-14), 43.24(C-1), 39.65(C-10), 39.57(C-12), 38.70(C-3), 35.91(C-13), 29.96(C-8), 29.24(C-18), 29.19(C-7), 23.10(C-20), 22.76(C-17), 21.62(C-6), 20.28(C-2). All of the data above are in agreement with those of acahanthic acid15,16.

Compound V: C29H39O6, needle crystals, mp 179~180̊C; IR(KBr) cm−1: 3420(OH), 1690(COOH), 1655, 875(C=C); 1H-NMR(500MHz, CD3OD) δ: 4.78(1H, brs, H-17), 4.72(1H, brs, H-17′), 2.61(1H, m, H-13), 1.18(3H, s, H-18), 0.97(3H, s, H-20); 13C-NMR(125MHz, CD3OD) δ: 181.68(C-19), 156.77(C-16), 103.75(C-17), 58.29(C-5), 56.56(C-9), 50.18(C-15), 45.45(C-8), 45.26(C-13), 44.68(C-4), 42.59(C-7), 42.10(C-1), 40.84(C-14), 40.81(C-10), 39.24(C-3), 34.26(C-12), 29.56(C-18), 23.13(C-6), 20.37(C-2), 19.50(C-11), 16.39(C-20). All of the data above are in agreement with those of kaurenoic acid19.

Compound VI: C39H44O14, white powder, mp 291~292.5̊C; gave a positive reaction in the Liebermann-Burchard and Molish tests, IR (KBr) cm−1: 3414 (OH), 2936, 2875, 1639 (C=C), 1426, 1379, 1076, 1026, 894. EI-MS m/z (%): 414 (M-Glc)1+, 396 (M-H2O)+, 255, 213, 147. 1H-NMR (DMSO) δ: 5.32 (1H, 6-H), 4.42 (1H, t, 3-H), 4.21 (1H, d, 1′-H); 13C-NMR (CDCl3, 100 MHz) δ: 37.76 (C-1), 30.44 (C-2), 78.67 (C-3), 40.30 (C-4), 141.37 (C-5), 121.85 (C-6), 32.42 (C-7), 32.42 (C-8), 50.30 (C-9), 37.20 (C-10), 21.52 (C-11), 39.62 (C-12), 42.82 (C-13), 57.20 (C-14), 24.66 (C-15), 28.55 (C-16), 56.75 (C-17), 12.11 (C-18), 19.51 (C-19), 36.48 (C-20), 19.21 (C-21), 34.64 (C-22), 27.21 (C-23), 46.54 (C-24), 30.06 (C-25), 19.51 (C-26), 20.00 (C-27), 23.79 (C-28), 12.28 (C-29), 102.79 (C-1′), 75.35
(C'-2), 78.60 (C'-3), 72.04 (C'-4), 78.05 (C'-5), 63.00 (C'-6). All of the data above are in agreement with those of daucosterol\textsuperscript{20}.

4 Discussion and Conclusions

In this manuscript, the anti-cancer effect the root bark of \textit{A. henryi} was determined by MTT methods and showed that 100% MeOH fraction has better cytotoxicity on HL-60 cells, HT-29 cells and A549 cells as judged by IC\textsubscript{50} and six compounds were isolated and identified by spectroscopic methods, they are stigmasterol (I), \beta-sitosterol (II), eleutheroside E (III), acanthoic acid (IV), kaurenoic acid (\textit{V}), and daucosterol (VI), compounds IV and V were obtained from this plant for the first time. Some studies have proved that acanthoic acid, kaurenoic acid have better activities of inhibition on TNF-\textalpha and interleukin-1, and some of their derivatives are considered as important compounds in the development of effective antitumor chemotherapeutic agents owing to their cytotoxic properties in the induction of apoptosis\textsuperscript{21-23}.

However, further studies on the action mechanism of the root bark of \textit{A. henryi} are very necessary. The present results will provide a pharmacological basis for the treatment of cancer in the clinical application of this herb and also reveal the herb is worth resource to develop.

Competing interests

The authors declare that they have no competing interests.

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