Fitoterapia 104 (2015) 7–13

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Novel triterpenoid saponins from residual seed cake of *Camellia oleifera* Abel. show anti-proliferative activity against tumor cells

Jianfa Zong, Ruilong Wang, Guanhu Bao, Tiejun Ling, Liang Zhang, Xinfu Zhang, Ruyan Hou *

State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, Hefei 230036, PR China

ARTICLE INFO

Article history:
Received 17 February 2015
Accepted in revised form 28 April 2015
Accepted 3 May 2015
Available online 7 May 2015

Keywords:
Camellia oleifera
Saponins
Oleanane-type
Oleiferasaponin C_1–C_3
Anti-proliferative activity

ABSTRACT

Four oleanane-type triterpenoid saponins were isolated from the seed cake of *Camellia oleifera* Abel.: camelliasaponin B_1 and three new saponins, oleiferasaponin C_1–C_3 (**1**–**3**). Their structures were identified as 22-*O*-angeloyl-camelliagenin B 3-*O*-[β-D-galactopyranosyl-(1 → 2)]-[β-D-galactopyranosyl-(1 → 2)-α-L-arabinopyranosyl-(1 → 3)]-β-D-glucopyranosiduronic acid methyl ester (**1**); 22-*O*-angeloyl-camelliagenin A 3-*O*-[β-D-galactopyranosyl-(1 → 2)]-[β-D-glucopyranosyl-(1 → 2)-β-D-galactopyranosyl-(1 → 3)]-β-D-glucopyranosiduronic acid methyl ester (**2**); and 28-*O*-cinnamoyl-camelliagenin B 3-*O*-[β-D-galactopyranosylz-(1 → 2)] [β-D-galactopyranosyl(1 → 2)-α-L-arabinopyranosyl-(1 → 3)]-β-D-glucopyranosiduronic acid methyl ester (**3**) through 1D and 2D NMR, HR-ESI-MS, as well as GC–MS spectroscopic methods. The anti-proliferative activities of these four compounds were investigated on five human tumor cell lines (BEL-7402, BGC-823, MCF-7, HL-60 and KB). Compounds **1** and **2** and camelliasaponin B_1 showed significant cytotoxic activities.

1. Introduction

Triterpenoid saponins are an important class of natural products, which are distributed widely in plant kingdom. Their pharmacological effects, such as anticancer, antibacterial, anti-mutagenesis, anti-inflammatory and inhibition of cardio-cerebral vascular diseases, have been reviewed recently [1]. *Camellia oleifera* Abel., a green tea plant in the genus *Camellia*, is widely cultivated throughout southern China. Its seeds contain abundant amounts of unsaturated fatty acids, named as "eastern olive oil". In China, the annual production of tea seed is approximately 1 million tons, with 730,000 tons remaining as seed cake after oil extraction [2]. This remaining tea seed waste is usually discarded or used as organic fertilizer or fuel. However, it should be noted that the tea seed cake contains about 8% saponins [3]. These saponins could be used as active compounds in agricultural and pharmaceutical industry. Saponins exhibit anti-oxidant activity [4], anti-hyperlipidemic activity [5], anti-fungal activity [6,7], anti-tumor activity [8,9], among others [10–12].

LC–MS analysis has indicated that the seed cake of *C. oleifera* contains about 30 types of saponins [7]. Thus far, only three saponins have been isolated and structurally identified from *C. oleifera* seed cake, namely oleiferasaponin A_1 [3], oleiferasaponin B_1 and oleiferasaponin B_2 [13]. Furthermore, these three saponins were reported to exhibit significant cell protective or anticancer activity. Therefore, additional investigation into the saponins from tea oil seed cake is meaningful for both agricultural and pharmaceutical applications.

This study reports the isolation and structural elucidation of three new saponins, namely oleiferasaponin **C_1**, **C_2**, and **C_3**, and one known saponin, camelliasaponin B_1, from an *n*-butanol extract of *C. oleifera* seed cake. The structures were identified by extensive spectroscopic methods, including IR, UV, GC–MS, HR-ESI-MS, as well as 1D- and 2D-NMR (^{1}H and ^{13}C NMR, DEPT-90, DEPT-135, ^{1}H–^{1}H COSY, HSQC and HMBC). In addition, the isolated compounds were tested for cytotoxic activity against five human tumor cell lines (BEL-7402, BGC-823, MCF-7, HL-60 and KB).

2. Materials and methods

2.1. General

Several spectrometric instruments were used to obtain physical data on the four isolated saponins. IR was measured on a Nicolet 8700 FT-IR spectrophotometer (Thermo Scientific Instrument Co., USA). ^{1}H and ^{13}C NMR, DEPT-90, DEPT-135, ^{1}H–^{1}H COSY, HSQC and HMBC spectra were recorded in C_5D_5N using a Bruker AM-400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Coupling constants were expressed in Hz and chemical shifts were given on a δ (ppm) scale with tetramethylsilane (TMS) as an internal standard. HR-ESI-MS

Abbreviations: HPLC, high performance liquid chromatography; UV, ultraviolet; IR, infrared radiation; NMR, nuclear magnetic resonance; DEPT, distortionless enhancement of polarization transfer; COSY, correlated spectroscopy; HSQC, heteronuclear singular quantum coherence; HMBC, heteronuclear multiple bond coherence; HR, high resolution; ESI-MS, electro-spray ionization mass spectrometry; DMSO, dimethyl sulfoxide; GC–MS, gas chromatography mass spectrometry.

* Corresponding author. Tel./fax: +86 551 65786765.
E-mail address: hry@ahau.edu.cn (R. Hou).

http://dx.doi.org/10.1016/j.fitote.2015.05.001
0367-326X/

spectra were determined on an Electrostatic Field Orbital Trap Mass Spectrometer (Thermo Scientific) using an ESI source.

2.2. Chromatography

The HPLC purifications were performed with a YMC-Pack ODS—A semipreparative HPLC column (250 mm × 10 mm i.d., 5 μm, YMC Corp., Ltd., Japan) on a Varian Prostar HPLC instrument (Model 325) (Varian, Mulgrave, Australia) and a Waters 2695 separation module combined with a Waters 2489 UV detector with the detection wavelengths at 210 and 254 nm. The HPLC analyses were performed with a ZORBAX Eclipse Plus C_{18} HPLC column (250 mm × 4.6 mm i.d., 5 μm, Agilent Corp., Palo Alto, CA, USA) on an HPLC system composed of an LC-20AD pump with an SPD-M20A detector (Shimadzu Corp., Kyoto, Japan). AB-8 macroporous resin for column chromatography was purchased from Anhui San Xing resin technology Co. Ltd. (Anhui, China). GC–MS analyses were conducted on a GCMS-QP2010S (Shimadzu Corp.) with DB-5MS column (i.d. = 0.25 μm, length = 30 m, Agilent Technologies, USA).

2.3. Materials

Tea seed cakes of *C. oleifera* were obtained from a commercial tea oil producer in the city of Huangshan in Anhui Province, China. The samples had been defatted.

2.4. Extraction and isolation

The tea seed cake (4.0 kg) was crushed into powder and extracted three times with 70% EtOH at 60 °C under reflux. The extract was subjected to reduced pressure evaporation to obtain EtOH concentrated solution (990 g). The concentrated solution was extracted successively with petroleum ether, EtOAc and *n*-BuOH. The *n*-BuOH fraction (100 g) was dissolved in 1 L of distilled water, passed through a macroporous resin AB-8 column, and eluted with a gradient of aqueous EtOH (EtOH/H_2O 0, 30, 50, 70, 90%). The 70% EtOH eluate (11.8 g) was subjected to silica gel (100–200 mesh) column chromatography (70 mm × 800 mm) and then eluted with stepwise gradients of EtOAc and MeOH (10:0, 9:1, 7:1, 5:1, 4:1, 3:1, 2:1, 1:1, 0:10, each 12.0 L). Each fraction was collected and detected by TLC. Fractions with similar Rf values were combined, yielding five major fractions (A–E). Fraction D (2.4 g) was subjected to semipreparative HPLC [YMC-Pack ODS-A, CH_3CN-0.5% aqueous HCOOH (45:55, v/v), 2 mL/min] to yield five fractions [Fr. 1 (0.58 g), Fr .2 (0.17 g), Fr. 3 (0.15 g), Fr. 4 (0.12 g), and Fr. 5 (0.40 g)]. Fractions 1, 2, 3 and 4 were further purified by HPLC [Agilent C_{18}, 250 mm × 4.6 mm i.d., CH_3CN-0.5% aqueous HCOOH (45:55, v/v), 1 mL/min] to afford camelliasaponin B_1 (35 mg), compounds **1** (16.7 mg), **2** (14 mg), and **3** (10 mg).

2.4.1. Oleiferasaponin C_1 (**1**)

White amorphous powder; UV (MeOH) λ_{max} nm (log ε): 236 (4.18); IR (KBr) ν_{max} (cm^{-1}): 3416, 2950, 2927, 1720, 1644, 1442, 1385, 1241, 1159, 1078, 1046, 916, 895, 852, 782, 639, 588, and 533; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HR-ESI-MS (negative ion mode): *m/z* 1215.5770 $[M-H]^-$ (calcd for $C_{59}H_{91}O_{26}$, 1215.5799).

2.4.2. Oleiferasaponin C_2 (**2**)

White amorphous powder; UV (MeOH) λ_{max} nm (log ε): 210 (4.32); IR (KBr) ν_{max} (cm^{-1}): 3416, 2927, 1740, 1701, 1644, 1442, 1385, 1241, 1162, 1077, 1045, 914, 893, 855, 785, 640, and 606; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HR-ESI-MS (negative ion mode): *m/z* 1231.6084 $[M-H]^-$ (calcd for $C_{60}H_{95}O_{26}$, 1231.5748).

2.4.3. Oleiferasaponin C_3 (**3**)

White amorphous powder; UV (MeOH) λ_{max} nm (log ε): 280 (4.06); IR (KBr) ν_{max} (cm^{-1}): 3416, 2950, 2927, 1716, 1637, 1450, 1384, 1309, 1258, 1164, 1078, 1046, 917, 894, 769, 710, 684, 638, 588, and 535; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HR-ESI-MS (negative ion mode): *m/z* 1263.5768 $[M-H]^-$ (calcd for $C_{63}H_{91}O_{26}$, 1263.5799).

2.5. Acid hydrolysis and sugar analysis of compounds 1–3

Each saponin (1 mg) was dissolved in 1 M HCl (1 mL) for 3 h at 90 °C. The reaction mixture was extracted with chloroform, and the supernatant was evaporated to dryness under N_2 flow. The residue was dissolved in 0.2 mL of pyridine containing L-cysteine methyl ester hydrochloride (10 mg/mL) and reacted at 70 °C for 1 h. This reaction was evaporated under N_2 flow, after which 0.2 mL trimethylsilylimidazole (Adamas Reagent Co., Ltd) was added. The mixture was heated at 70 °C for another 1 h, and then partitioned between *n*-hexane and water. The organic phase was analyzed by GC–MS. Temperature conditions were as follows: injector temperature at 280 °C; the initial oven temperature was 160 °C for 1 min, then linearly increased to 200 °C at 6 °C/min. A further linear increase at 3 °C/min was performed to 280 °C and held for 5 min. The standard sugar samples were subjected to the same reaction and GC–MS conditions. The sugar units of compounds **1**–**3** were identified by comparison with authentic samples: L-arabinose (t_R 16.72 min), D-glucose (t_R 21.67 min), D-galactose (t_R 22.30 min), and D-glucuronic acid methyl ester (t_R 23.34 min). L-arabinose, D-galactose and D-glucuronic acid methyl ester were identified in a ratio of 1:2:1 for compounds **1** and **3**, while the sugar moieties of **2** were identified as D-glucose, D-galactose and D-glucuronic acid methyl ester in the ratio of 1:2:1.

2.6. HPLC analysis for the isolated saponins of seed cake

The four purified compounds were dissolved to 1 mg/mL with methyl alcohol separately. The solutions were filtered through a 0.22 μm membrane filter for HPLC analysis. HPLC column temperature was set 25 °C. The eluant was composed of mobile phase A (water containing 0.5% acetic acid) and mobile phase B (acetonitrile). The optimized gradient of mobile phase B was programmed as follows: 0–10 min, from 35% to 38%; 10–15 min, from 38% to 40%; 15–18 min, 40%; 18–28 min, from 40% to 43%; 28–45 min, from 43% to 55%; 45–52 min, from 55% to 80%; 52–58 min, and from 80% to 35%; then 8 min, 35%. Elution was performed at a solvent flow rate of 1.0 mL/min. The injection volume was 5 μL.

2.7. Anti-proliferative activity assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [14], was performed to evaluate the anti-proliferative activities of the isolated saponins against the human tumor cell lines BEL-7402, BGC-823, MCF-7, HL-60 and KB (Nanjing KeyGEN BioTECH Co., LTD, Jiangsu, China). The human tumor cell lines in culture medium (100 μL) were placed in a cell of 96-well plate at a concentration of 3–4 × 10^3 cells/mL and incubated at 37 °C in 5% CO_2 for 24 h. After 24 h, an additional 100 μL of complete medium with either: no additions (negative control), 0.1% DMSO (solvent control), 10 μg/mL Taxol (positive control), or different concentrations (0.781, 1.562, 3.125, 6.25, 12.5, 25, 50, and 100 μM) of the test saponins were added. The treated cells were incubated as above for 72 h. Then, 20 μL of MTT solution (5 mg/mL) was added to the culture medium, and the reaction mixture was incubated as above for 4 h. After 4 h, the medium was discarded and 150 μL DMSO were added. The optical density (OD) of each well was measured at 490 nm using a Tunable Microplate Reader (EL-x800, BioTek Instruments, USA). The inhibition rate of cell proliferation was calculated as:

$$\text{Inhibition rate}(\%) = \{[\text{OD(Negative control)} - \text{OD(Sample control)}] / \text{OD(Negative control)}\} \times 100\%.$$

The results were expressed as concentrations of compound producing 50% toxicity (IC_{50} value).