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ORIGINAL ARTICLE

Analysis and identification of phenolic compounds with antiproliferative activity from Chinese olive (*Canarium album* L.) fruit extract by HPLC-DAD-SPE-TT-NMR

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Abstract

Chinese olives (*Canarium album* L.) are rich in phenolic compounds, exhibiting a broad spectrum of potential clinical applications. This study is the first report on the isolation and elucidation of bioactive compounds with high antiproliferative activity from the ethyl acetate fraction of a Chinese olive fruit methanolic extract (CO-EtOAc). We used the WST-1 assay to determine which subfractions of CO-EtOAc had significant antiproliferative activity using the murine colon cancer cell line CT26. Subsequently, the functional compounds were characterized by the hyphenated technique and high-performance liquid chromatography-diode array detector-solid phase extraction-transfer tube-nuclear magnetic resonance (HPLC-DAD-SPE-TT-NMR). Thirteen phenolic constituents were identified from the antiproliferation-enhancing subfractions of CO-EtOAc, including two new compounds, 2,4-didehydrochebulic acid 1,7-dimethyl ester (5) and 1-hydroxybrevifolin (7), which were further purified and found to exhibit marked antiproliferative activity. Chebulic acid dimethyl ester (2), which was isolated from *C. album* for the first time, also possessed antiproliferative activity.

Keywords: Antiproliferation, Chebulic acid 1,7-dimethyl ester, Chinese olive (*Canarium album* L.), HPLC-DAD-SPE-TT-NMR, 1-hydroxybrevifolin, 2,4-didehydrochebulic acid 1,7-dimethyl ester

1. Introduction

Chinese olive (*Canarium album* L.), a fruit tree belonging to the family Burseraceae, is cultivated mainly in southeast Asia. Compared to Mediterranean olive (*Olea europaea* L.) fruit, Chinese olive fruit has relatively low oil contents and is usually pickled before eating. Chinese olive fruit is a commonly-used folk herbal medicine in Asia and has been used as Materia medica to treat dysentery, faucitis, cough-hematemesis, snake bites, enteritis, and toxicosis from swellfish and alcohol [1–4]. Chinese olives were demonstrated to possess activity against CCl_4-induced hepatocyte damage and restrain hepatocyte lipid accumulation [5,6], lipopolysaccharide-induced inflammation [7], and metabolic dysfunction caused by the combination of a high-fat diet and streptozotocin administration [8]. These pharmacological characteristics might arise from the high content of polyphenolics in Chinese olives [9], including gallic acid [10], brevifolin, hyperin, ellagic acid, 3,3′-di-O-methylellagic acid [6], methyl gallate, ethyl gallate, brevifolin carboxylic acid, sinapic acid [11], corilagin, kaempferol-3-

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glucoside, and amentoflavone [12]. Our laboratory has reported that Chinese olive fruit extract inhibits colon cancer cell proliferation and tumor growth by promoting apoptosis through suppression of the NF-κB signaling pathway [13]. However, the antiproliferative compounds in Chinese olive fruit remain undetermined.

Colorectal cancer (CRC) is the third most prevalent cancer and the fourth leading cause of cancer-related deaths worldwide. This suffering is estimated to increase by 60% by 2030, and more than 2.2 million new cases and 1.1 million cancer deaths are predicted [14,15]. In Asia, the incidence and mortality of CRC are rapidly increasing at an alarming rate [16]. Given the impact of CRC, it is urgent to find effective drugs with minimal side effects for cancer therapy. Polyphenols are potential protective agents against CRC because they exhibit anti-inflammatory and antioxidant effects, induce cell cycle arrest and/or apoptosis, and inhibit proliferation, angiogenesis, and metastasis, as evidenced by both *in vitro* and *in vivo* studies. Chemopreventive phytochemicals, especially polyphenols, are considered the compounds that are the most essential against colon cancer [17,18]. Exploiting colon cancer proliferation pathways as potential targets is a central rationale for the treatment of CRC [19]. Thus, exploring new natural bioactive compounds with antiproliferative functions is important.

Identifying the chemical constituents of different materials requires instruments with a high capability for chemical analysis. Liquid chromatography-nuclear magnetic resonance (LC-NMR) is widely used for analytical separation and has the capability to more efficiently search for natural product structures [20,21]. Moreover, the HPLC-DAD-SPE-NMR hyphenated technique is a powerful tool for the thorough and comprehensive characterization of the natural products present in plants [22]. Thus, the present study aimed to explore the compounds with antiproliferative activity from Chinese olive fruit. With the assistance of HPLC-DAD-SPE-TT-NMR and comparison with existing compound information from other research groups, we identified 13 pure compounds from the antiproliferation-enhancing subfractions of the ethyl acetate soluble fraction of Chinese olive fruit extract (CO-EtOAc), two of which are new compounds.

2. Materials and methods

2.1. General experimental procedures

The following instruments and accompanying materials were used in this study. Off-line NMR spectra: A Bruker AV III-600 instrument equipped with an SEI $^{13}C-^{1}H$ probehead (methanol-d4, δ_H 3.30 and δ_C 49.0 ppm). HPLC-DAD-SPE-TT-NMR: An Agilent 1100 liquid chromatography system (Waldbronn, Germany) equipped with a photodiode array detector (Bruker DAD, Bruker, Rheinstetten, Germany) and a Knauer K120 HPLC pump (makeup pump) followed by a Prospekt 2 automated solid-phase extraction unit (Spark Holland, Emmen, Holland) containing 192 HySphere resin GP cartridges (10 × 2 mm, 10–12 μm), a Bruker nitrogen separator, and a MATCH tube transfer system (2 mm NMR MATCH tube, Bruker, Rheinstetten, Germany). HRESIMS: micrOTOF orthogonal ESI-TOF mass spectrometer (Bruker Daltonik).

2.2. Chemicals

Methanol (MeOH), n-hexane, ethyl acetate, n-butanol, trifluoroacetic acid, formic acid, sodium formate, Diaion HP-20, silica gel (63–200 μm), RPMI 1640 culture medium, fetal bovine serum (FBS), antibiotics, and trypsin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Water-soluble tetrazolium salt (WST-1) reagent was purchased from Abcam (Cambridge, MA, USA). HPLC-grade methanol was purchased from Mallinckrodt (KY, USA). MeOH-d4 was acquired from Cambridge Isotope Laboratories, Co. (MA, USA), and deionized water was prepared with a Barnstead water purification system (Dubuque, IA, USA).

2.3. Plant material

The Chinese olive fruits were obtained from Baoshan Township, Hsinchu County, Taiwan. After harvest, the fruits were dried at ambient temperature, blended into powders, and kept at 4 °C before use.

2.4. Extraction and isolation

The dry powder samples (18 kg) were macerated with water for 4 h at room temperature, and the resultant slurries were centrifuged. The residue (−10 kg) was dried and extracted with methanol (60 L) three times at room temperature. After complete extraction, the methanol solvent was removed by evaporation under reduced pressure at 45 °C to obtain the methanol extract (2.2 kg). The crude methanol extract (2 kg) was suspended in H_2O (3 L) and then successively partitioned with n-hexane (Hex), ethyl acetate (EtOAc), and n-butanol (BuOH, saturated with H_2O) (3 × 3 L each) to obtain fractions with different polarities. Each fraction (fr.) was

concentrated under reduced pressure, and the n-hexane fr. (CO-Hex), EtOAc fr. (CO-EtOAc), n-BuOH fr. (CO-BuOH), and H_2O fr. (CO–H_2O) were obtained. The fractionation protocol and yield of each fraction are displayed in Fig. 1.

CO-EtOAc was subjected to Diaion HP-20 column chromatography with gradient elution with MeOH–H_2O (1:9–1:0) to generate six subfractions (CO-EtOAc-frs. A–F). The yield of each subfraction is shown in Fig. 1. Active frs. C and D eluted with MeOH–H_2O (3:7–4:6) and (4:6–5:5), respectively, were mixed with n-hexane–EtOAc (7:3) to yield soluble (fr. C1 and fr. D1) and insoluble fractions (fr. C2 and fr. D2). Silica gel chromatography of the active frs. C2 and D2, eluted with n-hexane/EtOAc (70:30, 50:50 and 0:100), yielded frs. C2a-c and frs. D2a-c. Fr. C2b was then subjected to HPLC-SPE-TT-NMR analysis.

Semipreparative separation of each identified compound was further performed using a Lobar column (size C, 440 × 37 mm, LiChroprep RP-18, 40–63 μm, Merck) and a semipreparative HPLC column (Phenomenex Prodigy ODS3 column, 250 × 10 mm, 5 μm) (Torrance, CA, USA). The yield of each compound was as follows: 1 (0.03 g), 2 (0.08 g), 3 (0.10 g), 4 (0.08 g), 5 (0.06 g), 6 (0.04 g), 7 (0.07 g), 8 (0.05 g), 9 (0.05 g), 10 (0.10 g), 11 (0.07 g), and 12 (0.04 g). The compounds were further purified using a prepacked Lobar column (size C, 440 × 37 mm, LiChroprep RP-18, 40–63 μm, Merck) and semipreparative HPLC with a Phenomenex Prodigy ODS3 column (250 × 10 mm, 5 μm) (Torrance, CA, USA). Their structures are shown in Fig. 3. Each compound was identified by 1D-NMR and high-resolution electrospray ionization mass spectrometry (HRESIMS). The identities of the compounds were verified by referencing authentic samples with purities of 90–95% by TLC and HPLC.

2.5. HPLC conditions for HPLC-SPE-NMR and HPLC–MS analyses

HPLC separation for SPE-NMR was carried out on a YMC-packed, ODS-AM, 5 μm, 250 × 4.6 mm column with a linear gradient of methanol (solvent A) and 0.1% (v/v) TFA (aq) (solvent B) at a flow rate of 0.6 mL/min and detection at 280 nm. The linear gradient was 6%–62% A/B in 54 min followed by 85% A/B for 6 min. The delivery system for HPLC–MS was the same, except TFA was replaced with formic acid, and the eluate was introduced into the MS system via a splitter.

2.6. SPE-TT-NMR (600 MHz) and HRESIMS procedures

The SPE-TT-NMR strategy is as follows. After HPLC separation, postcolumn water was added to the eluate at a flow rate of 1.2 mL/min, and each compound peak was then passed through a HySphere resin GP cartridge to undergo solid-phase extraction. This process was repeated three times, injecting 250 μg of sample per run (5 μL, 50 mg/mL). The cartridges were then flushed with dry nitrogen supplied from a nitrogen separator to remove the residual solvents, and each compound was eluted into an NMR MATCH tube (2 mm) with CD_3OD (285 μL). The ^1H NMR spectrum of each separated compound was recorded using a multiple solvent suppression pulse program (LC1DCWPS) for residual proton and water signals in CD_3OD (δ 3.30, 4.80) and residual CH_3CN (δ 2.02). Shaped low-power rf pulse and CW decoupling on the F2 channel for decoupling of the ^{13}C satellites were utilized. All spectra were measured at 300 K, and the ^1H chemical shift was referenced to the residual signal of CD_2HOD at δ 3.30. A total of 24–1024 scans for each measurement were acquired to generate 16 K data points with a sweep width of 12000 Hz.

For HPLC-DAD-MS, an aliquot (10%) of each eluate separated by HPLC was introduced into the micrOTOF orthogonal ESI-TOF mass spectrometer via an Upchurch Scientific graduated microsplitter valve. MS data were acquired in a scan range between 100 and 1200 Da with an electrospray interface under positive ionization mode. The spray voltage was set at 4.0 kV with a dry nitrogen temperature of 180 °C. The nebulizer gas pressure was set at 0.4 bar with a dry gas flow of 4 L/min. The accurate masses were calibrated with sodium formate cluster ions as the internal standard.

2.7. Determination of total phenolic content

Total phenolics were determined using Folin–Ciocalteu reagent according to the method reported previously [23]. Briefly, 10 μL of each sample was incubated with 840 μL of deionized water and 50 μL of Folin–Ciocalteu reagent for 5 min, and then 100 μL of sodium carbonate solution (20% (w/v)) was added before mixing in the dark at room temperature for 30 min. The absorbance of the developed dark blue–purple color was measured by a spectrophotometer at 750 nm. The content of total phenolic compounds was determined using a

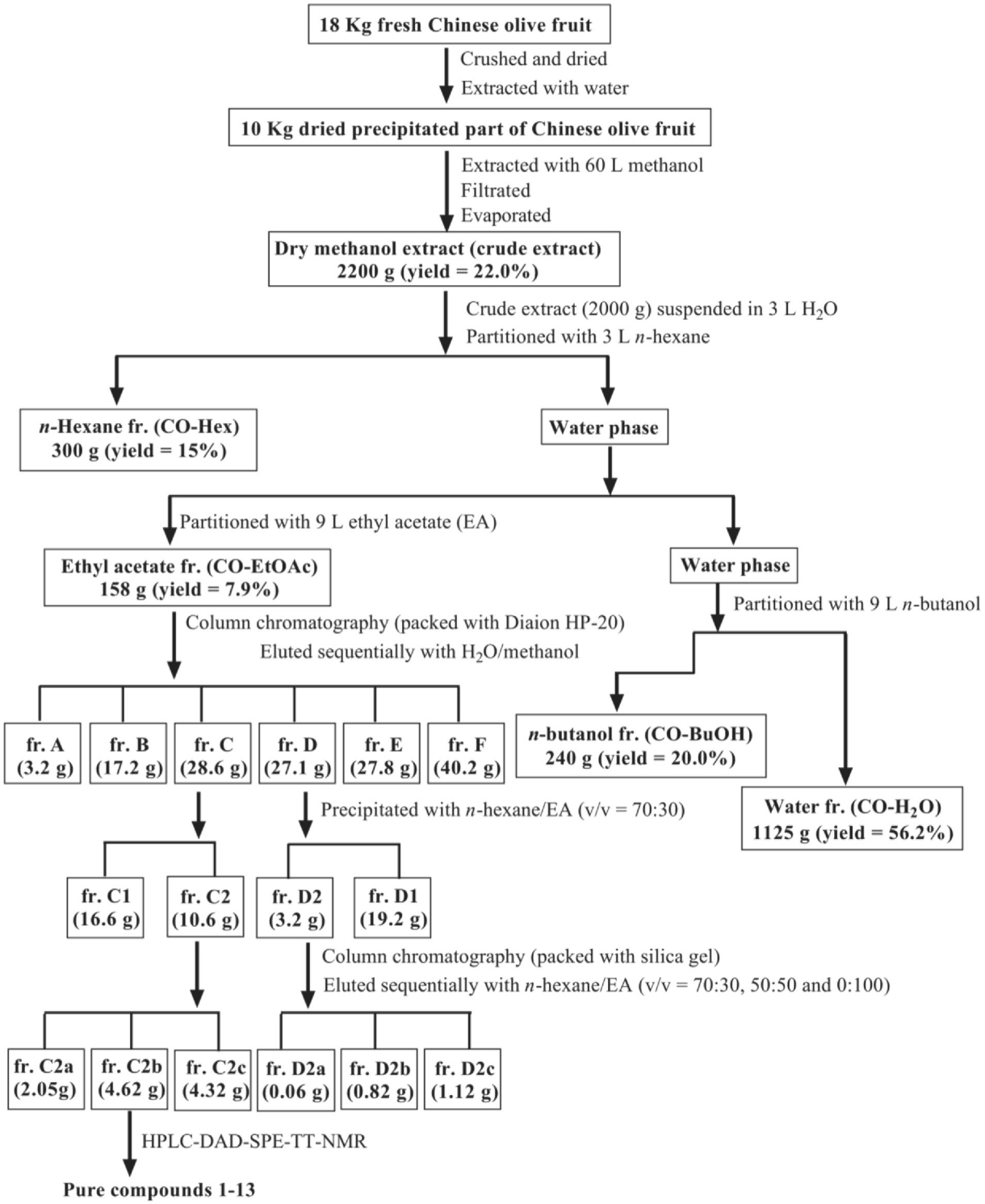

Fig. 1. Scheme for the preparation and isolation of the fractions and compounds from Chinese olive fruits with antiproliferative activity.

standard curve prepared from a gallic acid standard solution. The results are expressed as mg/g gallic acid equivalent (GAE) per gram of absolute dry weight (dw) (mg GAE/g dw).

2.8. Determination of total flavonoid content

The total flavonoid content was determined following the aluminum chloride colorimetric

method [24]. Briefly, 50 μL of each sample (1 mg/mL in ethanol) was diluted to 1 mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution. Next, 0.3 mL of 10% $AlCl_3$ solution was added after 5 min of incubation. Then, 2 mL of 1 mol/L NaOH solution was added, and the final volume of the mixture was brought to 10 mL with ddH_2O. The solution was mixed well, and the absorbance was measured against the reagent blank at 510 nm. The total flavonoid content was calculated using a standard curve prepared from a rutin standard solution, and the results are expressed as mg rutin equivalent (RE) per gram of absolute dry weight (dw) (mg RE/g dw).

2.9. Cell culture

The CT26 murine colon cancer cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). CT26 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U penicillin and 0.1 mg/mL streptomycin. The cells were maintained continuously in the exponential phase by frequent passage (every 2–3 days) at a cell concentration between 1×10^5 and 1×10^6 cells/mL in a humidified 37 °C incubator with 5% CO_2.

2.10. Cell proliferation assay

This method was based on the reduction of WST-1 to a yellow–orange soluble formazan product, by which the proliferation and survival of viable cells can be evaluated by measuring the optical density. Cells were plated at a density of 1×10^4 cells/well in 96-well plates. At the endpoint of each treatment, the optimal amount of WST-1 reagent was added to each well according to the manufacturer's recommendations. Cell viability was determined by measuring the absorbance of the samples at 450 nm with an ELISA plate reader, and the absorbance at 620 nm was also measured as a background reference.

2.11. Statistical analysis

All results were normally distributed and are expressed as the mean ± SEM. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey's multiple test. All statistical analyses were carried out using GraphPad 7.0 software (GraphPad Software Inc., La Jolla, CA, USA), and differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Determination of the antiproliferative activity and total phenolic and flavonoid contents in the solvent-extracted Chinese olive fruit fractions

Chinese olive fruits were collected, extracted and subjected to solvent–solvent partitioning as described in Materials and Methods. To identify the fractions with antiproliferative activities, we performed a WST-1 assay in a murine colon carcinoma cell line, CT26. CT26 cells were separately treated with the n-hexane (Co-Hex), ethyl acetate (CO-EtOAc), n-butanol (CO-BuOH), and H_2O (CO–H_2O) (Fig. 1) soluble fractions, and 0.2% DMSO was used as a control. As presented in Table S1 (https://www.jfda-online.com/journal/vol31/iss4/6/), CO-Hex, CO-EtOAc and CO-BuOH exhibited significant inhibitory effects on cell proliferation at concentrations higher than 100 μg/mL compared with the mock treatment (control). In particular, the antiproliferative activities of CO-EtOAc and CO-BuOH were greater than that of CO-Hex (Table S1 (https://www.jfda-online.com/journal/vol31/iss4/6/)).

Phenolic compounds are plant secondary metabolites with antioxidant activity that contribute to the prevention or treatment of certain diseases. Many studies have shown that flavonoids, phenolic compounds, and their synthetic analogs act as potential cytotoxic agents. Therefore, the total phenolic and flavonoid contents in different fractions were determined next (Table 1). The total flavonoid and phenolic contents in the Chinese olive methanol extract were 52.41 ± 3.13 mg rutin equivalents (RE)/g dw and 92.64 ± 6.82 mg gallic acid equivalents (GAE)/g dw, respectively. The total flavonoid contents in CO-Hex, CO-EtOAc, CO-BuOH, and CO–H_2O were 3.52 ± 0.69, 105.67 ± 12.5, 121.62 ± 23.5, and 22.76 ± 3.79 mg of RE/g dw, respectively. The total phenolic contents in CO-Hex, CO-EtOAc, CO-BuOH, and CO–H_2O were

Table 1. Total flavonoid and phenolic contents in the Chinese olive extract and its fractions.[a]

Material	Total Flavonoids Content (mg of RE/g dw[b])	Total Phenolic Content (mg of GAE/g dw[b])
100% Methanol Ext.	52.41 ± 3.13	92.64 ± 6.82
CO-Hex	3.52 ± 0.69	14.72 ± 2.75
CO-EtOAc	105.67 ± 12.5	280.21 ± 27.9
CO-BuOH	121.62 ± 23.5	186.82 ± 18.4
CO–H_2O	22.76 ± 3.79	48.21 ± 4.82

[a] Values are the mean ± standard deviation (n = 3).
[b] Dry weight (dw) basis of the sample from different fractions.

14.72 ± 2.75, 280.21 ± 27.9, 186.82 ± 18.4, and 48.21 ± 4.82 mg GAE/g dw, respectively. The total flavonoid and phenolic contents in CO-EtOAc were significantly higher than those in CO-Hex, CO-BuOH, and CO−H_2O (Table 1). Interestingly, our previous study demonstrated that CO-EtOAc possesses the most significant anti-inflammatory activity and suppresses cancer cell proliferation as well as tumor growth [13]. Therefore, CO-EtOAc was selected for further antiproliferation-based isolation of bioactive compounds.

3.2. Antiproliferative activity of the subfractions from CO-EtOAc

CO-EtOAc was then fractionated using a Diaion HP-20 column to obtain six subfractions (frs. A-F) (Fig. 1), and the antiproliferative activities of these subfractions were also measured (Table S2 (https://www.jfda-online.com/journal/vol31/iss4/6/)). Since subfractions C and D significantly reduced the viability of CT26 cells than their parental fraction, CO-EtOAc (Table S2 (https://www.jfda-online.com/journal/vol31/iss4/6/)), these two fractions were further separated with *n*-hexane-EtOAc (70:30) to give the respective soluble (frs. C1 and D1) and insoluble fractions (frs. C2 and D2) (Fig. 1). At a lower concentration (50 μg/mL), all subfractions from fr. C and fr. D showed an antiproliferative effect that was similar to their corresponding parent fraction (Table S2 (https://www.jfda-online.com/journal/vol31/iss4/6/)). However, frs. C1, C2, and D2 at 100 μg/mL exhibited a better antiproliferative effect than their corresponding parent fraction (Table S2 (https://www.jfda-online.com/journal/vol31/iss4/6/)), indicating that the antiproliferative activities were enriched after further fractionation.

3.3. Antiproliferative activity of the subfractions from Frs. C2 and D2

Frs. C2 and D2 were subsequently selected for further separation because their antiproliferative activity per gram was much higher than that of fr. C1. Frs. C2 and D2 were chromatographed on a silica gel column and eluted with hexane/ethyl acetate to obtain subfractions C2a, C2b, C2c, D2a, D2b and D2c. The WST-1 assay results showed that frs. C2b and C2c exhibited stronger antiproliferative effects than their parental fr. C2 at equal concentrations (Table S3 (https://www.jfda-online.com/journal/vol31/iss4/6/)), whereas frs. D2a, D2b and D2c showed no significant difference in antiproliferative activity compared with their parental fr. D2 (Table S3 (https://www.jfda-online.com/journal/vol31/iss4/6/)). These data indicate that separation enriched the bioactive components in frs. C2b and C2c.

3.4. The chemical structures of the compounds isolated from Fr. C2b

Through bioactivity-guided separation of the Chinese olive fractions, we dissected the active components into a narrow polarity spectrum. Fr. C2b was then selected for further analyses of the chemical structures contained within it because the antiproliferative activity per gram in fr. C2b was much higher than that in fr. C2c. Fr. C2b was subjected to HPLC-DAD-SPE-TT-NMR, and the resulting 12 peaks (Fig. 2) were identified as 13 phenolic compounds (Fig. 3) by ^{1}H and ^{13}C NMR together with high-resolution electrospray ionization mass spectrometry (Figs. S1 and S2 (https://www.jfda-online.com/journal/vol31/iss4/6/)).

The details of structure determination data of known compounds including 1, 2, 3, 4, 6, 8, 9a, 9b, 10, 11, and 12 are presented in supporting information. Based on the structure determination, compound 1 is gallic acid. A previous paper demonstrated that gallic acid had antiproliferative effects in the human colon cancer cell lines HCT116 and HT29 and tumor prevention abilities in a xenograft tumor model [25].

Compound **2**, *R*t 27.7 min (peak 2, Fig. 2), had a molecular formula of $C_{16}H_{16}O_{11}$ as deduced from (+) HRESIMS, showing $[M+H]^+$ at *m/z* 385.0766 (calcd for $C_{16}H_{17}O_{11}$, 385.0765). Its ^{1}H NMR spectrum showed signals at δ 7.03 (1H, s, H-3′), 5.27 (1H, d, $J = 1.2$ Hz, H-2), 3.84 (1H, dd, $J = 1.2$, 7.0 Hz, H-3), 3.66 (3H, s, OMe), 3.62 (3H, s, OMe), 2.86 (1H, dd, $J = 10.5$, 17.1 Hz, H-5a), and 2.45 (1H, dd, $J = 4.3$, 17.1 Hz, H-5b) (Fig. S4 (https://www.jfda-online.com/journal/vol31/iss4/6/)). These combined data identified **2** as chebulic acid 1,7-dimethyl ester [26]. To our best knowledge, chebulic acid 1,7-dimethyl ester was isolated from *C. album* for the first time.

Based on the structure determination, compound **3** is methyl gallate. Hung et al. found that treatment with methyl gallate markedly inhibited the proliferation of hepatocellular carcinoma both *in vitro* and *in vivo* [27].

Based on structure determination, compound 4 is aesculetin which has been shown to inhibit the proliferation of the colon cancer cell line SW480 by inhibiting the Wnt signaling pathway [28].

Compound **5**, *R*t 32.2 min (peak 5, Fig. 2), had a molecular formula of $C_{16}H_{12}O_{11}$ as deduced from

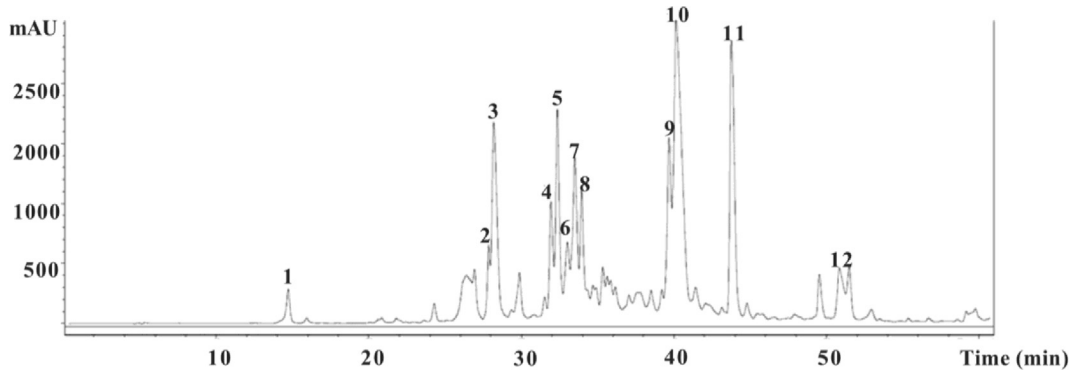

Fig. 2. HPLC chromatograms recorded at 280 nm showing the phenolic profile of the C2b subfractions. Peak: 1, gallic acid; 2, chebulic acid dimethyl ester; 3, methyl gallate; 4, aesculetin; 5, 2,4-didehydrochebulic acid 1,7-dimethyl ester; 6, vanillic acid or isovanillic acid; 7, 1-hydroxybrevifolin; 8, m-digallic acid 9, scopoletin (minor) and methyl brevifolin carboxylate (major); 10,3,4,8,9,10-pentahydroxydibenzo[b,d]pyran-6-one; 11, 5′-methoxyl-m-digallic acid or its isomer; and 12, ellagic acid.

(+)HRESIMS, showing $[M+H]^+$ at *m/z* 381.0457 (calcd for $C_{16}H_{13}O_{11}$, 381.0452). The molecular formula of **5** has four less hydrogens than that of **2**, corresponding to two ring and/or double bond equivalents. The ^1H NMR spectrum of **5** showed the following signals: δ 7.24 (1H, s, H-3′), 6.85 (1H, s, H-5), 3.78 (3H, s, OMe), and 3.69 (3H, s, OMe), thus lacking the aliphatic protons signals in **2** (Fig. S7 (https://www.jfda-online.com/journal/vol31/iss4/6/)) (Table 2). These combined data elucidated that **5** is likely 2,4-didehydrochebulic acid 1,7-dimethyl ester or its isomer at the ester moiety. Compound **5** is therefore a new natural product and is biogenetically related to **2**. To date, 2,4-didehydrochebulic acid or any of its derivatives have not been isolated.

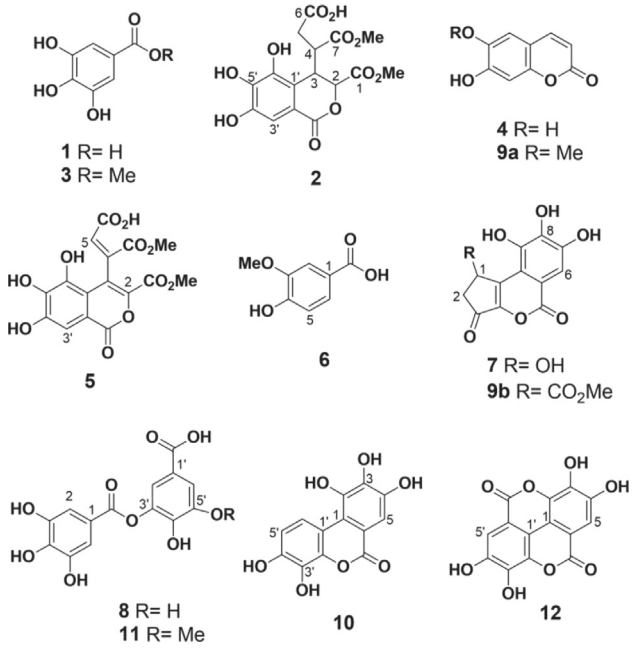

Fig. 3. Chemical structures of the phenolic compounds from Chinese olives. (1) Gallic acid; (2) chebulic acid dimethyl ester; (3) methyl gallate; (4) aescelutin; (5) 2,4-didehydrochebulic acid 1,7-dimethyl ester or its isomer; (6) vanillic acid or isovanillic acid; (7) 1-hydroxybrevifolin; (8) m-digallic acid; (9) scopoletin (a, minor) and methyl brevifolin carboxylate (b, major); (10) 3,4,8,9,10-pentahydroxydibenzo [b,d]pyran-6-one; (11) 5′-methoxyl-m-digallic acid or its isomer; and (12) ellagic acid.

Based on the structure determination, compound **6** is vanillic acid or its isomer isovanillic acid. Recently, vanillic acid has been shown to induce G1 phase arrest and inhibit the proliferation of human colon cancer HCT116 cells. Additionally, vanillic acid caused significant inhibition of tumor growth in a tumor xenograft model [29].

Compound **7**, *R*t 33.3 min (peak 7, Fig. 2), had a molecular formula of $C_{12}H_8O_7$ as deduced from (+) HRESIMS, showing $[M+H]^+$ at *m/z* 265.0342 (calcd for $C_{12}H_9O_7$, 265.0343). The ^1H NMR spectrum of **7** was similar to that of methyl brevifolin carboxylate (**9b**) (Table 2), showing signals at δ_H 7.30 (1H, s, H-6), 5.15 (1H, dd, J = 1.8, 6.2 Hz, H-1), 3.16 (1H, dd, J = 6.2, 18.5 Hz, H-2a), and 2.58 (1H, dd, J = 1.8, 18.5 Hz, H-2b), but lacked the ester methyl singlet (Fig. S9 (https://www.jfda-online.com/journal/vol31/iss4/6/)). We observed that the H-1 signal was shifted downfield relative to that in **9b**. In addition, the molecular formula of **7** corresponds to the loss of C_2H_2O from **9b**. We thus elucidated compound **7** as 1-hydroxybrevifolin, a new natural product. The C-1 configuration in **7**, however, remains to be clarified.

Based on the structure determination, compound **8** is *m*-digallic acid. Till now, the function of *m*-digallic acid on anti-tumor is still unknown.

*Table 2. ^{1}H NMR data of compounds **2**, **5**, **7**, and **9b** (δ m, J/Hz) (CD_3OD, 600 MHz).*[a]

No.	2	5	7	9b
1			5.15 dd (1.8, 6.2)	4.55 dd (2.2, 7.8)
2	5.27 d (1.2)		3.16 dd (6.2, 18.5)	3.00 dd (7.8, 18.8)
			2.58 dd (1.8, 18.5)	2.51 dd (2.2, 18.8)
3	3.84 dd (1.2, 7.0)			
4	—[b]			
5	2.86 dd (10.5, 17.1)	6.85 s		
	2.45 dd (4.3, 17.1)			
6			7.30 s	7.36 s
3′	7.03 s	7.24 s		
OMe	3.66 s	3.69 s		3.72 s
	3.62 s	3.78 s		

[a] Spectral data obtained from HPLC-SPE-NMR analysis.
[b] Overlapped with residual proton signal of D-solvent.

According to the analysis of ^{1}H NMR and (+)-HRESIMS data compounds **9a** and **9b** were elucidated as scopoletin and methyl brevifolin carboxylate, respectively. Notably, scopoletin has exhibited remarkable antitumorigenic activity in a tumor xenograft model [30].

According to the structure determination, compound **10** is 3,4,8,9,10-pentahydroxydibenzo-[*b*,*d*] pyran-6-one. To date, the function of 3,4,8,9,10-pentahydroxydibenzo-[*b*,*d*]pyran-6-one on anti-tumor is still unknown. According to the structure determination, compound **11** is methyl-*m*-digallate or its isomer. To date, the function of methyl-*m*-digallate on anti-tumor is still unknown.

According to the structure determination, compound **12** is ellagic acid. The antiproliferative activity of ellagic acid has been reported to modulates genes with proliferative, apoptotic, cell cycle-related, and angiogenic functions in HCT116 cells [31].

3.5. Antiproliferative activity of chebulic acid dimethyl ester (2), 2,4-didehydrochebulic acid 1,7-dimethyl ester (5) and 1-hydroxybrevifolin (7) identified from Fr. C2b

Thirteen phenolic constituents were identified from the antiproliferation-enhancing subfractions of CO-EtOAc. Chebulic acid dimethyl ester (**2**) is a known compound; however, it is isolated from *C. album* for the first time. Notably, 2,4-didehydrochebulic acid 1,7-dimethyl ester (**5**) and 1-hydroxybrevifolin (**7**) are newly discovered natural compounds. Therefore, we next assessed their antiproliferative activity. Treatment with compounds **2**, **5** and **7** significantly decreased the proliferation rate of CT26 cell compared to treatment of fr. C2b (Table 3). At a concentration of 10 μg/mL, treatment with fr. C2b exhibited no effect on cell viability. In contrast, compounds **2**, **5** and **7** markedly decreased cell viability by 32.1%, 50.0%, and 66.9%, respectively, versus the control group. These results indicate that we successfully isolated compounds from the parent fraction, fr. C2b, with antiproliferative effects (Table 3). This is the first report to investigate the biological effects of chebulic acid dimethyl ester (**2**), 2,4-didehydrochebulic acid 1,7-dimethyl ester (**5**), and 1-hydroxybrevifolin (**7**). To date, only chebulic acid, which has a structure similar to that of chebulic acid dimethyl ester (**2**), has been reported to participate in copper chelation and can thus affect some oxidation reactions that require copper as a cofactor. In addition, chebulic acid inhibited advanced glycation end product (AGE)-mediated protein linkage and the consequent inflammation and tumor progression [32–34]. Lee et al. also demonstrated that chebulic acid significantly reduced *tert*-butyl hydroperoxide (*t*-BHP)-induced cell cytotoxicity, decreasing intracellular reactive oxygen species

*Table 3. The effects of fr. C2b, chebulic acid dimethyl ester (**2**), 4-didehydrochebulic acid 1,7-dimethyl ester (**5**), and 1-hydroxybrevifolin (**7**) on the proliferation of CT26 cells.*

	Control	50 μg/mL	25 μg/mL	10 μg/mL
Fr. C2b	100.2 ± 4.21	63.1 ± 3.54[a]	76.4 ± 5.19[a]	93.1 ± 6.53
Compound (**2**)	100.7 ± 3.78	15.2 ± 5.11[a]	44.1 ± 0.69[a]	68.0 ± 7.12[a]
Compound (**5**)	100.5 ± 7.21	10.6 ± 2.10[a]	30.7 ± 6.89[a]	51.7 ± 10.1[a]
Compound (**7**)	100.5 ± 5.56	12.2 ± 3.52[a]	10.6 ± 4.19[a]	33.9 ± 2.36[a]

All experimental groups contained the same concentration of DMSO.
Cell proliferation (% of control) was calculated after sample treatment for 72 h.
Values are the mean ± standard deviation (n = 3).
[a] $p < 0.05$ versus the control group.

levels in rat isolated hepatocytes [35]. However, our results suggest that chebulic acid dimethyl ester (**2**) has an antiproliferative effect on colon cancer cells.

4. Conclusion

In this study, bioactivity-guided fractionation and chemical investigation of the ethyl acetate soluble fraction of *C. album* (Chinese olive) fruits resulted in the isolation of two novel compounds (**5** and **7**) in addition to 11 known compounds. Compounds **5** and **7** were identified as 2,4-didehydrochebulic acid 1,7-dimethyl ester and 1-hydroxybrevifolin, respectively, and we also isolated chebulic acid dimethyl ester (**2**) from *C. album* for the first time. Moreover, we confirmed the antiproliferative effects of compounds **2**, **5** and **7** in this study. Here, we provide the chemical ingredients responsible for the antiproliferative activity of the ethyl acetate soluble fraction of Chinese olive fruits and suggest that Chinese olive fruits could be a good source for the further development of natural nutraceuticals and functional foods for cancer treatment.

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Author contribution

Conceptualization, C.K.C. and S.S.L.; methodology, Y.T.Y.; software, C.K.C. and S.S.L.; validation, Y.C.L. and S.C.H.; formal analysis, Y.T.Y. and C.K.C.; investigation, Y.T.Y., C.K.C., Y.C.L. and S.S.L.; resources, Y.T.Y.; data curation, Y.C.L. and S.C.H.; writing—original draft preparation, Y.T.Y., C.K.C, Y.C.L., and S.C.H.; writing—review and editing, C.K.C., Y.C.L. and S.C.H.; visualization, Y.T.Y, C.K.C.; supervision, S.S.L, and S.C.H.; project administration, S.C.H.; funding acquisition, Y.C.L. and S.C.H. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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