

Isolation and structure elucidation of phenolic compounds in Chinese olive (*Canarium album* L.) fruit

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Abstract Chinese olive (*Canarium album* L.), one native and well-known tropical fruit tree in the southeast of China, contain a large amount of phenolics and possess great pharmacological activities. In this study, phenolics were extracted from Chinese olive fruit using 80% (v/v) aqueous acetone, and seven phenolic compounds were isolated and purified by Polyamide column and Toyopearl HW-40 column chromatography from crude extracts. Their structures were elucidated by high performance liquid chromatography coupled to diode array detection and electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS), and where possible by ^1H NMR and ^{13}C NMR spectrometry. Except gallic acid and hyperin, five phenolic compounds including methyl gallate, ethyl gallate, corilagin, kaempferol-3-glucoside and amentoflavone were first identified in Chinese olive.

Keywords Chinese olive · *Canarium album* L. · Phenolic compounds · Isolation · Structure elucidation

Introduction

Phenolic compounds are broadly distributed in the plant kingdom and are the most abundant secondary metabolites found in plants [1], which are a very heterogeneous group

including simple phenolic acids, cinnamic acid and its derivatives, flavonoids, lignans, and tannins [2]. In the recent decades, phenolics from plants have gained much attention, due to their antioxidant activities and free radical scavenging abilities, which potentially have beneficial implications in human health [3–6]. Some epidemiological studies have indicated that increased consumption of foods rich in phenolic compounds (fruits, vegetables, whole grain cereals, red wine, tea) is correlated with reduced risk of cardiovascular disease, neuro-degenerative disease, and certain types of cancer [7, 8].

Chinese olive (*Canarium album* L.) is a fruit tree belonging to the Burseraceae family. It is indigenous to the southeast area of China and now has been widely cultivated in other Asian tropical and semi-tropical regions [9]. Similar to the the Mediterranean olive (*Olea europaea* L.), Chinese olive fruit is a fusiform drupe, and also has the organoleptic characteristics of strong bitter and astringent tastes [10]. Some fresh fruits are edible and, unlike their Mediterranean counterparts of olives, Chinese olive fruits have relatively low oil contents, and most of them are generally processed in food industry to beverage, candy and confections [11].

Dried fruit of Chinese olive is also a traditional medicine material in China and possesses some pharmacological functions, such as anti-bacterium, anti-virus, anti-inflammation and detoxification [10, 12–14]. Previous study has showed Chinese olive fruit was rich in phenolic compounds, which closely related to its organoleptic and pharmacological characteristics [15]. However, up to date, reports on isolation and identification of phenolic compounds from Chinese olive were very scarce, and only five phenolics have been identified from Chinese olive fruits and leaves as gallic acid [9, 14], brevifolin, hyperin, ellagic acid and 3,3'-Di-O-methyllellagic acid [16].

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Therefore, further separation and identification of phenolic compounds was necessary for complete characterization of phenolic profiles of Chinese olive. The purpose of the present work is to isolate and purify the phenolic compounds from Chinese olive fruits by Polyamide column and Toyopearl HW-40 column chromatography, and then elucidate their structures by chromatographic (HPLC, TLC) and spectrometric (UV, ESI-MS, ^1H NMR, ^{13}C NMR) techniques.

Materials and methods

Plant materials

Mature Chinese olive fruits were obtained from plants that grow widely in Fujian province of Southeastern China. The fruits were washed, depitted and quickly frozen with liquid nitrogen. The frozen fruits pulp were then kept at a temperature of -20°C ready for use.

Chemicals

Pure phenolic and monosaccharide standards of gallic acid, ferulic acid, kaempferol, quercetin, chlorogenic acid, caffeic acid, methyl gallate, ethyl gallate, hyperin, as well as glucose, fructose, galactose, arabinose, xylose, and mannose were purchased from Sigma (St. Louis, MO, USA) and Fluka (Buchs, Switzerland). Methanol, tetramethylsilane (TMS) and deuterated methanol (CD_3OD) were of HPLC grade (Sigma), ethanol, butanol, acetone, acetic acid, hydrochloric acid, pyridine, anilinium hydrogen phthalate and ferric chloride (analytical grade) were purchased from Shanghai Chemical Reagent Company, China. All solutions were prepared using distilled-deionized water.

Extraction of phenolic compounds

The frozen Chinese olive fruits pulp was ground into small particles using a household flour mill (Tianjin, China). The mashed fruits (500 g) were extracted three times at room temperature using 1,500 ml of 80% (v/v) aqueous acetone with intermittent mixing and 3 h each time, the mixture were then centrifuged, and the supernatants were pooled and evaporated to remove acetone with a rotary evaporator (SBW-1, Shanghai Shenbo Instrument Co., China) under reduced pressure at 40°C . The concentrated extracts were further partitioned with butanol for three times.

Isolation and purification of phenolic compounds

The butanol fractions were applied on a polyamide column (600 mm \times 40 mm i.d., Chemical plant of Nankai University,

Tianjin, China) eluted with aqueous ethanol to give six fractions (F1–F6), the ethanol concentration being increased from 0 to 100% in increments of 20%. Fractions (F2–F5) obtained from the 20–80% aqueous ethanol containing the main bulk of the phenolics were separately subjected to further chromatographic separation on Toyopearl HW-40 column (300 mm \times 25 mm i.d., Tosoh, Japan). Fractions were collected using a fraction auto-collector (SBS-100, Shanghai Qingpu Huxi Instrument Co., Shanghai, China) and monitored by thin layer chromatography (TLC). Fractions showing similar TLC patterns were combined and rechromatographed until pure compounds were isolated. Repeated column chromatography on Toyopearl HW-40, eluted with aqueous ethanol (0–100%), led to the isolation of compound **1** from fraction F2, **2**, **3** and **4** from fraction F3, **5**, **6** from fraction F4, and **7** from fraction F5.

Thin layer chromatography analysis

Thin layer chromatography (TLC) analysis of phenolic fractions was carried on silica gel G-precoated plates (200 mm \times 100 mm, Qingdao Haiyang Chemical Co., Qingdao, China), which were developed with butanol–acetic acid–water (4:1:1, v/v). The plates were visualized by spraying with 2% ferric chloride (FeCl_3) ethanol solution, followed by heating the plates with a hot air blower. The phenolic acids and flavonoids were revealed as dark blue spots.

Hydrolysis of phenolic glycosides

The hydrolysis of phenolic glycosides was performed according to the method described by Lu and Foo [17]. A small sample of purified phenolic glycosides (1–2 mg) was hydrolyzed by dissolving it in 1 ml of 2 mol/l HCl and incubated at 100°C for 1 h in a shaking water bath. The hydrolysates were compared with standard sugar samples on TLC developed with butanol–pyridine–water (6:4:3, v/v). The sugar spots were visualized by spraying with a methanol solution of anilinium hydrogen phthalate and heating in an oven at 110°C for 10 min.

Zirconium oxychloride–citric acid color test

The zirconium oxychloride–citric acid color test was used to identify 3-hydroxy flavonoids [18]. The 2% zirconium oxychloride methanol solution was added into the test sample, the flavonoid sample with free 3-hydroxy or 5-hydroxy would present yellow color. Then, the 2% citric acid methanol solution was also added into the sample, if the yellow color of sample remained, it indicated that there was free 3-hydroxy existing in the flavonoid. If the color faded after addition of citric acid, there was no free 3-hydroxy or the flavonoid was glycosided at 3 positions.

HPLC-DAD-ESI-MS analysis

HPLC-DAD-ESI-MS analysis of purified phenolics from Chinese olive was performed using a Waters platform ZMD 4000 system, composed of a Micromass ZMD mass spectrometer and a Waters 2690 HPLC equipped with a Waters 996 diode array detector (Waters Corp., Milford, MA, USA). Data were collected and processed via a personal computer running MassLynx software version 3.1 (Micromass, a division of Waters Corp., Beverly, MA, USA). A reversed-phase Purospher STAR C-18 column (250 mm × 4.6 mm, i.d. and particle size 5 μm, Merck KGaA, Darmstadt, Germany) was used for separation with column temperature set at 30 °C. The sample injection volume was 10 μl. Gradient elution was performed with 0.5% (v/v) acetic acid (solvent A) and methanol (solvent B) at a constant flow rate of 0.6 ml/min. The linear gradient profile was as follows: 100% A and 0% B at the start, then to 10% A and 90% B at 20 min, remaining at 10% A and 90% B from 20 to 25 min, and falling back to 100% A and 0% B at 30 min. The purity of individual phenolics was checked by HPLC-DAD. UV-Vis spectra were recorded on-line from 200 to 600 nm during HPLC analysis. Phenolics were detected at 280 nm.

Mass spectra were achieved by electrospray ionization in both positive and negative modes. The following ion optics was used: capillary 3.88 kV (negative) and 3.87 kV (positive), cone 30 V (negative) and 24 V (positive), and extractor 5 V. The source block temperature was 120 °C and the desolvation temperature was 300 °C. The electrospray probe-flow was adjusted to 70 ml/min. Continuous mass spectra were obtained by scanning from 100 to 800 *m/z*.

Nuclear magnetic resonance spectroscopy (NMR)

¹H NMR and ¹³C NMR spectra for phenolic compounds **4** and **7** were recorded on a Bruker AC-400 (Bruker Analytik, Rheinstetten, Germany) spectrometer working at 400 MHz

for ¹H and 100 MHz for ¹³C NMR. Purified samples (10–20 mg) were dissolved in 0.4 ml of deuterated methanol (CD₃OD) and Tetramethylsilane (TMS) was used as internal standard.

Results and discussion

The 80% aqueous acetone extract of Chinese olive fruit was partitioned with butanol, and preliminary analysis of butanol fraction showed its fairly complex composition. After repeated chromatographic separation and purification on polyamide and Toyopearl HW-40 column, seven individual phenolic compounds were successfully isolated from butanol fraction. HPLC-DAD analysis of isolated phenolics **1–7** demonstrated their high-purity, and they were identified by comparison of their UV spectra and HPLC retention times (*t_R*) with those of reference standards and by mass spectrometry (Table 1), TLC, chemical methods, as well as NMR spectrometry (Tables 2, 3) where possible. Their chemical structures were shown in Fig. 1.

Compounds **1**, **2** and **3** were white amorphous powder, presented spectral characteristics of the hydroxybenzoic acids [14, 19] with UV λ_{\max} at 226 and 272 nm, 222 and 273 nm, 223 and 274 nm, respectively (Table 1). They were directly identified as gallic acid (**1**), methyl gallate (**2**) and ethyl gallate (**3**), by comparison of *t_R* and UV spectra with those of standards and confirmed by ESI-MS spectra. The characteristic ions at *m/z* 169 and 125 were observed in mass spectra of **1–3**.

Compound **4** was a white amorphous powder. As shown in Table 1, it had UV spectral characteristics of benzoyl system, and there was a typical ion fragmentation model of hexahydroxydiphenoyl (HHDP) group [20] in ESI-MS spectra, indicating the presence of HHDP unit in compound **4**, which was further evidenced by the presence of two isolated one-proton singlets at δ_{H} 6.74 and 6.71 in ¹H NMR spectrum (Table 2). The mass spectra revealed compound **4**

Table 1 Analysis of phenolic compounds **1–7** by HPLC-DAD-ESI-MS

Compound	<i>t_R</i> (min)	UV λ_{\max} (nm)	MW	ESI-MS ⁺ (<i>m/z</i>)	ESI-MS ⁻ (<i>m/z</i>)	Identification
1	5.47	226, 272	170	–	169, 125	Gallic acid ^a
2	10.88	222, 273	184	185, 171	183, 169, 125	Methyl gallate ^a
3	10.91	223, 274	198	199, 171, 127	197, 169, 125	Ethyl gallate ^a
4	11.07	232, 268	634	657, 465, 303, 277	633, 463, 301, 275	Corilagin ^c
5	13.77	257, 355	464	487, 303, 273	463, 301, 271	Hyperin ^{ab}
6	14.88	266, 348	448	471, 287	447, 285	Kaempferol-3-glucoside ^b
7	21.25	270, 332	538	561, 539	537	Amentoflavone ^c

^a Identified by comparison with reference standards

^b Identified by MS, chemical method and TLC

^c Identified by MS and NMR spectra

Table 2 ^1H NMR and ^{13}C NMR spectra data of phenolic compound **4** in CD_3OD

^1H NMR (δ_{ppm})		^{13}C NMR (δ_{ppm})					
Glc H-1	6.35	Glc C-1	95.37	Galloyl C-4	140.4	HHDP C-6	145.52
Glc H-2	4.03	Glc C-2	69.07	Galloyl C-5	146.52	HHDP C-7	168.74
Glc H-3	4.83	Glc C-3	65.18	Galloyl C-6	111.52	HHDP C-1'	125.96
Glc H-4	4.47	Glc C-4	71.56	Galloyl C-7	166.94	HHDP C-2'	117.47
Glc H-5	4.50	Glc C-5	76.38	HHDP C-1	125.85	HHDP C-3'	108.80
Glc H-6	4.12, 4.93	Glc C-6	62.25	HHDP C-2	116.85	HHDP C-4'	138.37
Galloyl H-2,-6	7.08	Galloyl C-1	121.00	HHDP C-3	108.87	HHDP C-5'	146.00
HHDP H-3	6.74	Galloyl C-2	110.89	HHDP C-4	137.83	HHDP C-6'	145.50
HHDP H-3'	6.71	Galloyl C-3	146.42	HHDP C-5	145.36	HHDP C-7'	169.51

Table 3 ^1H NMR and ^{13}C NMR spectra data of phenolic compound **7** in CD_3OD

^1H NMR (δ_{ppm})		^{13}C NMR (δ_{ppm})					
H-3	6.57	C-2	166.17	C-2'	129.56	C-7''	166.53
H-6	6.35	C-3	103.65	C-3'	116.29	C-8''	95.53
H-2',6'	7.53	C-4	184.41	C-4'	162.79	C-9''	159.63
H-3',5'	6.69	C-5	162.68	C-5'	117.15	C-10''	105.46
H-3''	6.56	C-6	100.57	C-6'	129.56	C-1'''	123.16
H-6''	6.17	C-7	163.36	C-2''	166.32	C-2'''	133.08
H-8''	6.38	C-8	106.27	C-3''	104.24	C-3'''	122.44
H-2'''	7.97	C-9	156.78	C-4''	184.03	C-4'''	161.78
H-5'''	7.10	C-10	105.58	C-5''	165.38	C-5'''	118.33
H-6'''	7.86	C-1'	123.49	C-6''	101.06	C-6'''	128.96

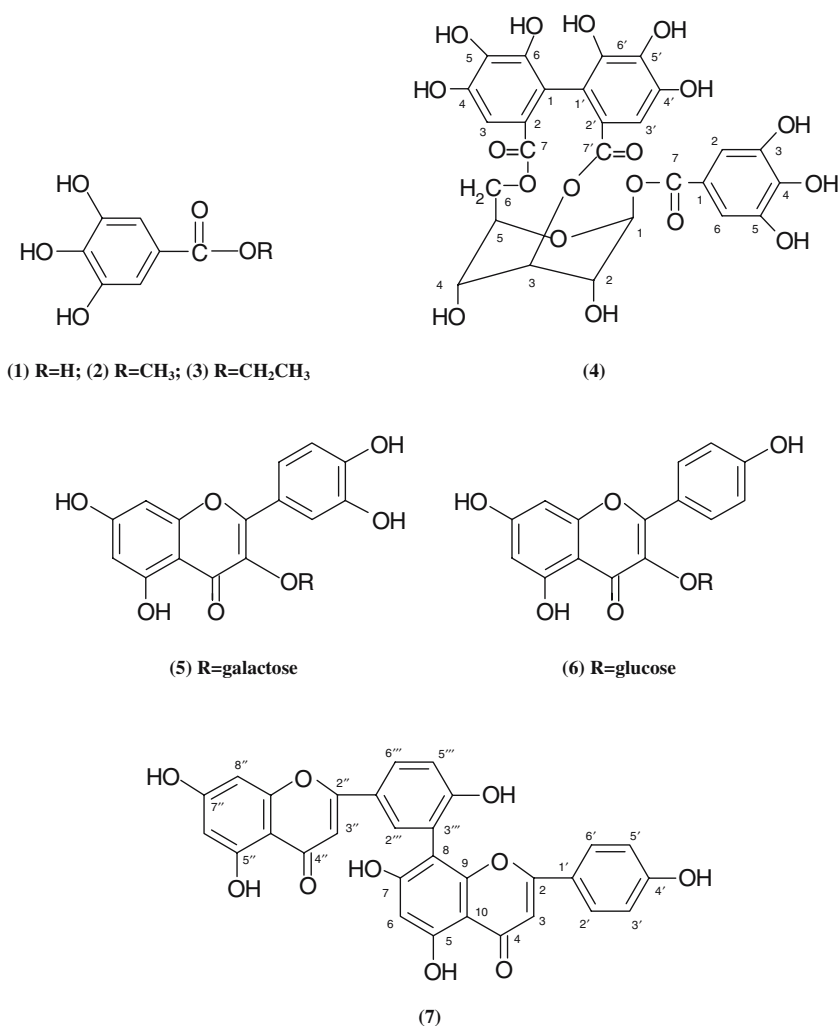
had a sodiated pseudomolecular ion $[\text{M} + \text{Na}]^+$ at m/z 657, a pseudomolecular ion $[\text{M} - \text{H}]^-$ at m/z 633, and fragment ions at m/z 463 $[\text{M} - \text{H}-170]^-$, 301 $[\text{M} - \text{H}-170-162]^-$, coincident with a gallic acid group and a HHDP moiety attached to one hexose. The ^1H NMR spectrum showed chemical shifts between δ_{H} 4.03 and δ_{H} 4.93 due to protons of glucose at 2–6 positions. The signals of the anomeric proton, H-3 and H-6 appeared at significantly lower field than those of remaining glucose protons, indicating the acylation of hydroxyl groups at C-1, C-3 and C-6 of the glucose core. The ^{13}C NMR spectrum of **4** indicated the signals of three-carbonyl carbon (HHDP δ_{C} 168.74, 169.51; galloyl δ_{C} 166.94), nine oxygenated aromatic carbons (six for HHDP and three for galloyl), and six carbons of glucose (see Table 2). The data of ^1H and ^{13}C NMR corresponded to those in the literature [21], thus, compound **4** was identified as 1-*O*-galloyl-3,6-*O*-hexahydroxy diphenyl- β -D-glucopyranose (corilagin). Corilagin was a bioactive ellagitannin also found in *Acalypha hispida* [22], *Cunonia macrophylla* [23] and *Phyllanthus amarus* [24].

Compounds **5**, **6** were yellow powder, exhibited two major absorption peaks in two regions of 200–280 and 300–380 nm (Table 1), suggesting the identities of flavonoids [25]. The mass spectra of compound **5** showed pseudomolecular ion $[\text{M} - \text{H}]^-$ was at m/z 463, with fragment ions at m/z 303 $[\text{M} + \text{H}-162]^+$, 301 $[\text{M} - \text{H}-162]^-$,

referring to a hexosyl residue was substituted to the flavonol aglycone. TLC analysis of acid hydrolysates of **5** revealed the presence of quercetin and galactose. Therefore, **5** was assigned to hyperin (quercetin 3-galactoside) in comparison with the standard. Similar to **5**, compound **6** was also one flavonol glycoside with pseudomolecular ion $[\text{M} - \text{H}]^-$ at m/z 447 and fragment ion at m/z 285 $[\text{M} - \text{H}-162]^-$. Kaempferol aglycone and glucose were detected from acid hydrolysates of **6** by TLC. To determine the location of glucose at kaempferol, zirconium oxychloride–citric acid color test was performed here, and result showed kaempferol was substituted by glucose at the 3rd position. Then, the structure of compound **6** was confirmed as kaempferol-3-glucoside.

Compound **7** was pale yellow powder, presented spectral characteristics of flavonoids with UV λ_{max} at 270 and 332 nm. ESI-MS spectra showed a sodiated pseudomolecular ion $[\text{M} + \text{Na}]^+$ at m/z 561 and pseudomolecular ions at m/z 539 $[\text{M} + \text{H}]^+$, 537 $[\text{M} - \text{H}]^-$ (Table 1), indicating molecular weight (MW) of **7** was 538. The ^1H NMR spectrum of **7** (Table 3) showed two olefinic signals at δ_{H} 6.56, 6.57 (1H, *s*, H-3, H-3''), characteristic of two flavones structure, and presented only two meta-coupled proton signals at δ_{H} 6.17 and 6.38 (*d*, $J = 2.1$ Hz, H-6'', H-8''). Since no similar doublet was observed for H-6 and H-8, the linkage for the biflavones should involve either C-6 or C-8. The

Fig. 1 Chemical structures of phenolic compounds 1–7 from Chinese olive, gallic acid (1), methyl gallate (2), ethyl gallate (3), corilagin (4), hyperin (5), kaempferol-3-glucoside (6), amentoflavone (7)



coupled system H-2'/H-6'–H-3'/H-5' of aromatic protons appeared as two distinct doublets at δ_{H} 7.53 and 6.69 (d , $J = 8.8$ Hz), respectively, excluded the possibility of any linkage between the flavone moieties at C-3, C-2', C-3', C-5' or C-6' with C-3'''. In ^{13}C NMR spectrum (Table 3), chemical shifts of C-3''' (δ_{C} 122.44) and C-8 (δ_{C} 106.27) were at significantly lower field compared with corresponding C-3' (δ_{C} 116.29) and C-8'' (δ_{C} 95.53), indicating a C-3'''–C-8 interflavone linkage. All these data were in accordance with those reported previously [26, 27], the structure of compound 7 was established as amentoflavone. Amentoflavone was a dimer of apigenin, naturally occurring in some plants with medicinal properties, including *Ginkgo biloba* and *Hypericum perforatum* [28], and exhibited far stronger biological activity than monomer [29].

Conclusion

In this study, seven phenolic compounds were isolated and purified by Polyamide column and Toyopearl HW-40

column chromatography from acetone extract of Chinese olive fruit, and identified as gallic acid, methyl gallate, ethyl gallate, corilagin, hyperin, kaempferol-3-glucoside, amentoflavone by HPLC-DAD-ESI-MS analysis, where possible by NMR spectrometry. To our knowledge, except gallic acid and hyperin, other five phenolic compounds were first found in Chinese olive.

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