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Analysis of phenolic compounds in Chinese olive (*Canarium album* L.) fruit by RPHPLC–DAD–ESI–MS

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Abstract

Qualitative analysis of acetone extracts from Chinese olive (*Canarium album* L.) fruit pulp was performed by means of reversed phase high-performance liquid chromatography coupled to diode array detection and electrospray ionization mass spectrometry (RPHPLC–DAD–ESI–MS). Seven phenolic compounds were identified (gallic acid, methyl gallate, ethyl gallate, ellagic acid, brevifolin carboxylic acid, sinapic acid, and hyperin) by comparing their retention times, UV–Vis absorption spectra and mass spectra with authentic standards or literature data. In addition, six other phenolic compounds, including one kaempferol hexoside and five hexahydroxydiphenoyl (HHDP) derivatives, were partially identified. All of these phenolic compounds, except gallic acid, ellagic acid and hyperin, are reported in *Canarium album* L. for the first time. Quantification of phenolic compounds was performed by HPLC–DAD, which revealed that gallic acid (166 mg/100 g FW), ellagic acid (87.8 mg/100 g FW) and HHDP hexose (80.9 mg/100 g FW) were the major phenolic compounds in Chinese olive fruit.

Keywords: Chinese olive; *Canarium album* L.; Phenolic compounds; RPHPLC–DAD–ESI–MS

1. Introduction

Chinese olive (*Canarium album* L.) is a fruit tree belonging to the Burseraceae family. It is native to the southeast area of China and has been introduced to other Asian tropical and semi-tropical regions (Wei, Peng, & Mao, 1999). Like the Mediterranean olive (*Olea europaea* L.), Chinese olive fruit is a fusiform drupe; the fruit flesh has the organoleptic characteristics of strong bitter and astringent tastes (Yuan, Liu, & Tang, 2001). There are three kernels included in the hard stone, and our previous study has shown that fruit kernels of Chinese olive are rich in various nutritional components (He & Xia, 2007). Some fresh fruits are edible and, unlike their counterparts, Mediterranean olives, Chinese olive fruits have relatively low oil contents, and most of them are generally processed in the food industry to beverages, candy and confections (Ssonko & Xia, 2005).

The dried fruit of Chinese olive is also a traditional medicine material and has been widely used for the treatment of faucitis, stomatitis, hepatitis and toxicosis, in China (Ding, 1999). Recent studies have shown that pharmacological characteristics of *C. album* were intimately related to its phenolic compounds (Fu & Zhou, 1997; Kong et al., 1998; Yuan et al., 2001). However, to date, reports on analysis and identification of phenolic compounds from *C. album* are very scarce, and only five phenolics have been identified from *C. album*, namely gallic acid (Kong et al., 1998; Wei et al., 1999), brevifolin, hyperin, ellagic acid and 3,3′-di-*O*-methylellagic acid (Ito, Shimura, & Watanabe, 1990).

Reversed phase high-performance liquid chromatography, coupled to diode array detection and electrospray ionization mass spectrometry (RPHPLC–DAD–ESI–MS), has

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proved to be a powerful method for the identification of polyphenols with advantages of high sensitivity, short time and low consumption of samples (Amélia et al., 2005; Liu, Sun, Bi, & Guo, 2005; Pan & Cheng, 2006; Proestos, Sereli, & Komaitis, 2006; Zu, Li, Fu, & Zhao, 2006). This technique has been extensively and successfully applied to on-line structure elucidation of phenolic compounds in foodstuffs (Cristina, Gloria, Sonia, & Julián, 2004; Fang, Zhang, & Wang, 2007; Luigia & Giuseppe, 2006; Navindra, Lee, Scheuller, & David, 2006; Romina & Luis, 2007; Zoubida, Miloudi, Olga, Mohamed, & Dominique, 2007).

The objective of the present work, therefore, is to analyse the phenolic compounds in Chinese olive fruits, using the RPHPLC–DAD–ESI–MS technique, and to elucidate the phenolic profile of *C. album*.

2. Materials and methods

2.1. 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. The frozen fruits pulp was then kept at a temperature of −20 °C prior to analysis.

2.2. Chemicals and solvents

Pure phenolic standards of gallic acid, ellagic acid, ferulic acid, quercetin, syringic acid, protocatechic acid, chlorogenic acid, caffeic acid, sinapic acid, methyl gallate, ethyl gallate and hyperin were purchased from Sigma (St. Louis, MO, USA) and Fluka (Buchs, Switzerland). Standards were dissolved in methanol. Working solutions were prepared with methanol to make concentrations from 1.0 to 100.0 mg/l. Methanol (HPLC grade), acetone and acetic acid (analytical grade) were purchased from Shanghai Chemical Reagent Company, China. All solutions were prepared using distilled-deionized water.

2.3. Extraction of phenolic compounds

The frozen Chinese olive fruit pulp was ground into small particle, using a household flour mill (Tianjin, China). The mashed fruits (5 g) were placed into centrifugal tubes and extracted three times using 15 ml of 80% (v/v) acetone solution with intermittent mixing, centrifuged, and the supernatants obtained were pooled and evaporated to dryness with a rotary evaporator (SBW-1, Shanghai Shenbo Instrument Co., China) under reduced pressure in a water bath at 40 °C. The dry residue was redissolved in 5 ml of methanol and used for both the colorimetric and chromatographic analyses. For HPLC analysis, all samples were filtered through a 0.45 μm cellulose acetate filter (Millipore Corp., Bedford, MA, USA) before injections.

2.4. Colorimetric determination of total phenolics

Total phenolics were determined by using the Folin–Ciocalteau reagent according to the method reported by Fang et al. (2007). A calibration curve with equation: $y = 0.0054x - 0.0035$ ($R^2 = 0.9994$) was constructed using gallic acid solutions within the range 10–100 mg/l. Contents of total phenolics in Chinese olive fruits were expressed as gallic acid equivalents in milligrams per 100 g fresh weight (mg GAE/100 g FW). The results were averages of triplicate analyses.

2.5. RPHPLC–DAD–ESI–MS analysis

RPHPLC–DAD–ESI–MS analysis of phenolics in Chinese olive extracts 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 diversion of Waters Corp., Beverly, MA, USA). A reversed phase Lichrospher C-18 column (250 mm × 4 mm, i.d. and particle size 5 μm, Merck KGaA, Darmstadt, Germany) was used for separation. 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. UV–Vis absorption spectra were recorded on-line from 200 to 600 nm during HPLC analysis. Phenolics were detected at the wavelength of 280 nm.

Mass spectra were achieved by electrospray ionization in both positive and negative modes. The following ion optics were 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.

2.6. Identification and quantification of phenolic compounds

The phenolic compounds in Chinese olive extracts were identified by comparing their UV–Vis absorption spectra, ESI–MS spectra, and chromatographic characteristics with the literature data reported, or with reference standards available. The external standard method was used for the quantification of the individual phenolic compounds. Contents of gallic acid, ellagic acid, methyl gallate, ethyl gallate sinapic acid and hyperin were calculated with the regression equations from the standard curves. HHDP derivatives and brevifolin carboxylic acid were quantified as gallic acid because insufficient standards were available; quantification of kaempferol hexoside was performed by

using hyperin as standard. Concentrations were expressed as mg/100 g FW. Repeatability of the analysis was ±3%.

3. Results and discussion

3.1. Identification of the chromatographic peaks

Fig. 1 shows the HPLC chromatogram of acetone extracts from Chinese olive fruit. There were about 16 phenolic peaks separated in extracts using the reversed phase C-18 column. As shown in Table 1, peak identification was performed by comparing retention times (t_R), UV–Vis spectra and mass spectra with those of reference standards or literature data.

Among all the peaks in the chromatogram (Fig. 1), peak 3 was quite prominent, indicating that it was the predominant phenolic compound in Chinese olive fruit. This peak presented spectral characteristics of the hydroxybenzoic acids (Fang et al., 2007; Kong et al., 1998) with UV λ_{max} at 226 and 272 nm, and its t_R was at 7.86 min. The ESI–MS spectrum showed $[M-H]^-$ at m/z 169 and fragment ion $[M-COOH]^-$ at m/z 125. Compared with the standard, this compound was unambiguously identified as gallic acid. Peaks 9 and 11 had the same spectral characteristics as peak 3 (Table 1), with UV λ_{max} at 222 and 273 nm, 223 and 274 nm, respectively. Fragment ions at m/z 169, 125 were also observed in ESI–MS^- spectra of peaks 9 and 11, indicating that they were gallic acid derivatives. In reference to corresponding standards, peaks 9 and 11 were identified as methyl gallate and ethyl gallate, respectively, and these two gallates were first found in *C. album*.

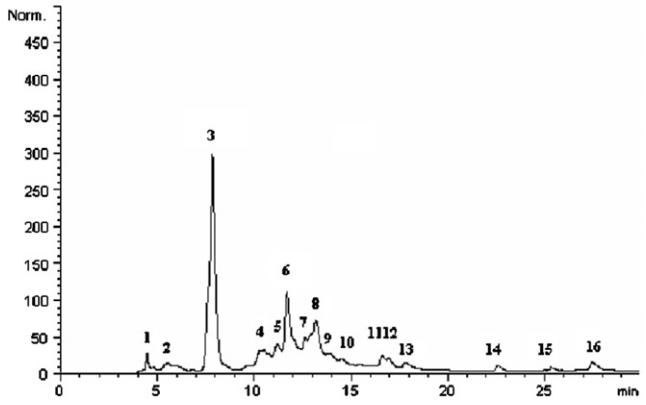

Fig. 1. HPLC chromatogram of the acetone extract from Chinese olive fruit detected at 280 nm. Peak numbers were consistent with those shown in Table 1.

Peaks 6, 10, 12, 13 and 15 showed UV absorptions in two regions, 200–280 nm and 300–380 nm, suggesting that multi-conjugate systems were present in their chemical construction (Alonso-Salces et al., 2004). Peak 6 was eluted at 11.7 min, with the pseudomolecular ions at m/z 301 $[M-H]^-$ and the fragment ions at m/z 257 $[M-H-COO]^-$, 229 $[M-H-COO-CO]^-$, and it was directly identified as ellagic acid by comparison with the authentic standard. Peak 12 exhibited a sodium pseudomolecular ion $[M+Na]^+$ at m/z 487, pseudomolecular ion $[M-H]^-$ at m/z 463 and fragment ions at m/z 303 $[M+H-162]^+$, 301 $[M-H-162]^-$, attributed to a hexosyl residue substituted to the quercetin aglycone, and was identified as hyperin (quercetin 3-galactoside) by comparison with the standard. Ito et al. (1990) reported that ellagic acid and hyperin were the important hepatoprotective compounds in Chinese olive. A fragment ion $[M-COOH]^-$ at m/z 247 attributed to brevifolin (Ito et al., 1990), was found in mass spectral peak 10, and spectra data were consistent with those reported in the literature (Sahar, Heba, Irmgard, & Mahmoud, 1997). Therefore, peak 10 was assigned as brevifolin carboxylic acid. Peak 13 had a pseudomolecular ion $[M-H]^-$ at m/z 447, and fragment ions at m/z 287 $[M+H-162]^+$, 285 $[M-H-162]^-$, which were the typical

Table 1
Identification of phenolic compounds in Chinese olive fruit by RPHPLC–DAD–ESI–MS

Peaks no.	t_R (min)	UV λ_{max} (nm)	MW	ESI–MS^+ (m/z)	ESI–MS^- (m/z)	Identification
1	4.47	225, 275	366	367, 345, 153	365, 343, 191	HHDP[c] alkyl derivative
2	5.53	224, 275	348	371, 349, 305	347, 303	Unknown
3	7.86	226, 272	170	–	169, 125	Gallic acid[a]
4	10.76	223, 276	380	381, 359, 153	357, 125	HHDP[c] alkyl derivative
5	11.19	232, 268	634	657, 465, 303	633, 463, 301	HHDP[c] hexosyl-gallate
6	11.70	254, 365	302	–	301, 257, 229	Ellagic acid[a]
7	12.64	224, 276	394	395, 373, 153	371, 169, 125	HHDP[c] alkyl derivative
8	13.18	230, 276	482	483, 465, 303	463, 301	HHDP[c] hexose
9	13.82	222, 273	184	185, 171	183, 169, 125	Methyl gallate[a]
10	14.49	277, 354	292	293	291, 247	Brevifolin carboxylic acid[b]
11	16.61	223, 274	198	199, 171, 127	197, 169, 125	Ethyl gallate[a]
12	16.95	257, 355	464	487, 303, 273	463, 301, 271	Hyperin[a]
13	17.83	266, 348	448	471, 287	447, 285	Kaempferol hexoside
14	22.57	270, 332	538	561, 539	537, 375	Unknown
15	25.36	236, 320	224	225, 100	–	Sinapic acid[a]
16	27.47	230, 276	400	423, 401, 383	399	Unknown

[a] Identified by comparison with reference standards.
[b] Identified by comparison with the literature data.
[c] Hexahydroxydiphenoyl.

mass of the kaempferol aglycone in the positive and negative modes, and fragmentation of the molecular ion showed that one hexose was linked to the kaempferol aglycone. However, the present data could not determine the hexose type or the location of the hexose at the kaempferol aglycone. Hence, peak 13 was tentatively identified as kaempferol hexoside. Peak 15 presented UV spectral characteristics of a cinnamoyl system with pseudomolecular ion $[M+H]^+$ at *m/z* 225, and was assigned to sinapic acid by comparison with the standard compound. Phenolic compounds of peaks 10, 13 and 15 have not been previously reported in *C. album*.

As observed in Table 1, peaks 1, 4, 5, 7 and 8 had UV spectral characteristics of the benzoyl system, and there was a typical ion fragmentation model of the hexahydroxydiphenoyl (HHDP) group (José, Hideyuki, & Takashi, 2004) in the mass spectra of these peaks, indicating the presence of a HHDP unit in the chemical structures of these compounds. HHDP is the main construction unit of ellagitannins in plant kingdom (Yasuyuki, 1996). Pseudomolecular ions $[M+H]^+$ of peaks 1, 4 and 7 were at *m/z* 367, 381 and 395, just equivalent to 2, 3 and 4 methyl radicals of mass difference compared with that of HHDP at *m/z* 339, which confirmed that peaks 1, 4 and 7 were HHDP alkyl derivatives. Peak 5 had a sodiated pseudomolecular ion $[M+Na]^+$ at *m/z* 657, a pseudomolecular ion $[M-H]^-$ at *m/z* 633, and fragment ions at *m/z* 463 $[M-H-170]^-$, 301 $[M-H-170-162]^-$ (see Fig. 2), coincident with a gallic acid group and a hexosyl residue attached to the HHDP, indicating that this was probably a HHDP hexosyl-gallate. Peak 8 had a pseudomolecular ion $[M+H]^+$ at *m/z* 483, fragment ions at *m/z* 465 $[M+H-18]^+$, and 303 $[M+H-18-162]^+$, corresponding to a hexosyl residue attached to HHDP. The chemical structures of peaks 5 and 8 were possibly similar to some ellagitannins, e.g. corilagin, strictinin, and 4,6-(*S*)-HHDP-glucopyranose (Bruno, Phila, Jean-Pierre, Saliou, & Edouard, 2005; Klaus & Herbert, 2000; Toshiyuki, Hideyuki, & Takashi, 2003; Yoshiaki et al., 1999). These HHDP derivatives (peaks 1, 4, 5, 7 and 8), though partially identified, are also first reported in *C. album*. Their exact structures need further confirmation and additional NMR data will be required. Some phenolics (peaks 2, 14 and 16) in the HPLC chromatogram were not identified.

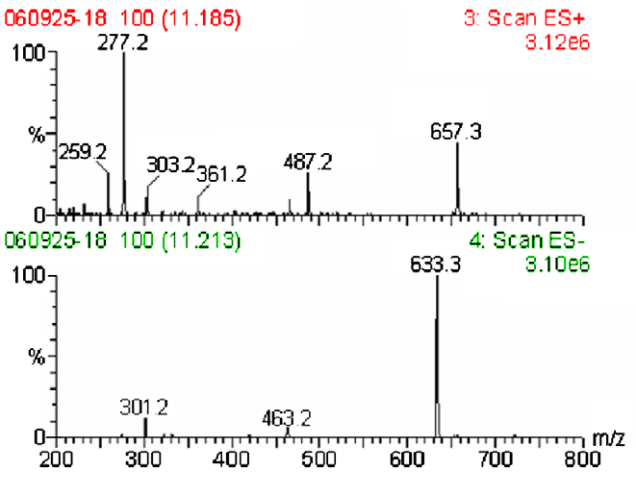

Fig. 2. ESI–MS$^{\pm}$ fragmentation pattern of HHDP hexosyl-gallate (peak 5) from Chinese olive fruits.

3.2. Quantification of phenolic compounds

Contents of phenolic compounds, excluding some unknown ones (peaks 2, 14 and 16), were determined by the HPLC–DAD method. The total contents of phenolics were calculated as the sum of the individual phenolic compounds and estimated by using the Folin–Ciocalteau method. The results obtained are presented in Table 2.

Gallic acid (166 mg/100 g FW), ellagic acid (87.8 mg/100 g FW) and HHDP hexose (80.9 mg/100 g FW) were the major phenolic compounds in Chinese olive fruits, and their amounts accounted for 32.9%, 17.4% and 16.0% of the total phenolics, respectively. Among all the quantified phenolics, phenolic acids and their two esters (methyl gallate and ethyl gallate) were in the highest amounts of 320 mg/100 g FW (more than 63% of the total phenolics), the content of HHDP derivatives in Chinese olive fruits reached 151 mg/100 g FW (around 30% of the total flavonoids compositions), including hyperin (14.5 mg/100 g FW), and kaemferol hexoside (18.2 mg/100 g FW) present in small amounts (<7% of the total). The phenolic profiles of Chinese olive fruits were absolutely different from their counterparts, Mediterranean olives (*Olea europaea* L.), in which the major phenolic compounds are oleuropein and demethyloleuropein (Danielle, Michael, Paul, Kevin, & Shimon, 2002).

As shown in Table 2, the content of total phenolics, as calculated by the sum of the individual phenolic compounds, was 504 mg/100 g FW, while the value (1291 mg

Table 2
Contents of phenolic compounds in Chinese olive fruit (values are the means of triplicate analyses)

Phenolic compounds	Content (mg/100 g fresh weight)
Gallic acid	166
Methyl gallate	24.7
Ethyl gallate	15.6
Ellagic acid	87.8
Brevifolin carboxylic acid[a]	21.7
Sinapic acid	4.1
Hyperin	14.5
Kaemferol hexoside[b]	18.2
HHDP alkyl derivative (peak 1)[a]	9.1
HHDP alkyl derivative (peak 4)[a]	10.3
HHDP alkyl derivative (peak 7)[a]	20.4
HHDP hexoyl-gallate[a]	30.6
HHDP hexose[a]	80.9
Total phenolics[c]	504
Total phenolics[d]	1291

[a] Quantified as gallic acid.
[b] Quantified as hyperin.
[c] Sum of the individual phenolic compounds.
[d] Determined following the Folin–Ciocalteau method (mg GAE/100 g fresh weight).

GAE/100 g FW) obtained by the Folin–Ciocalteau method was over 2.5 times that of the former. The substantial differences between the two analytical methods were due to the interference of other reducing substances in phenolic extracts, leading to overestimation of total phenolic contents in the Folin–Ciocalteau colorimetric analysis (Schieber, Keller, & Carle, 2001).

4. Conclusion

Reversed phase high-performance liquid chromatography, coupled to diode array detection and electrospray ionization mass spectrometry (RPHPLC–DAD–ESI–MS), was successfully employed in the qualitative analysis of phenolic compounds in Chinese olive fruit. Seven phenolic compounds were identified (gallic acid, methyl gallate, ethyl gallate, ellagic acid, brevifolin carboxylic acid, sinapic acid and hyperin) and six others (one kaempferol hexoside and five hexahydroxydiphenoyl (HHDP) derivatives) were tentatively identified. All of these phenolic compounds, except gallic acid, ellagic acid and hyperin, are reported in *Canarium album* L. for the first time. Quantitative analysis of phenolics indicated that gallic acid, ellagic acid and HHDP hexose were the three main phenolic compounds in Chinese olive fruits.

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