

## Simultaneous Determination of Benzoic Acid, Caffeic Acid and Chlorogenic Acid in Seeds of *Eriobotrya japonica* and their Antibacterial Effect

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**Abstract** We aim to develop a simple method for simultaneous and quantitative determination of benzoic acid, caffeic acid and chlorogenic acid in seeds of *Eriobotrya japonica*. In addition, antibacterial effect of these three phenolic acids was examined. A basic method is performed on the high performance liquid chromatography system coupled to an UV-detector (230 nm) and reverse phase C-18 column (4.6×150 mm, 5 µm). Each phenolic acid was confirmed via liquid chromatography-mass spectrometry (MS)/MS system under the multiple-reaction monitoring with negative-ion electrospray ionization (ESI(-)) mode. It is demonstrated that the method was could be applied to samples for an analytical study of the phenolic acids. On the other hand, three phenolic acids in seeds of *E. japonica* exhibited antibacterial effect against several pathogenic bacteria. Of these, benzoic acid was found to have stronger antibacterial effect.

**Keywords** antibacterial effect · benzoic acid · caffeic acid · chlorogenic acid · *Eriobotrya japonica* · high performance liquid chromatography

### Introduction

*Eriobotrya japonica*, known as loquat, is a fruit tree in the Rosaceae family. Its leaves have been used as traditional herbal medicine for the treatment of chronic bronchitis and coughs in Korea, Japan, China and other Asian countries. Moreover, some

seeds are used as a traditional herbal medicine to treat edema. However, most seeds of *E. japonica* are discarded and not used. For these reason, there is little report on the physiological activities of *E. japonica* seeds. According to the previous researches, *E. japonica* seeds contain amygdalin, unsaturated fatty acids, plant sterols, benzoic acid, caffeic acid, chlorogenic acid and benzaldehyde (Gray and Fowden, 1972; Yokota et al., 2006; Kim et al., 2009).

Phenolic acids such as benzoic acid, caffeic acid and chlorogenic acid are a group of phenolic compounds biosynthesized by the shikimic acid pathway (El-Basyouni et al., 1963). This class of phenolic compounds exhibits various physiological activities, including antibacterial, antioxidative, anti-inflammatory and anticarcinogenic (Nohynek et al., 2006; Russell and Duthie, 2011). Benzoic acid, caffeic acid and chlorogenic acid isolated from seeds of *E. japonica* have been reported as a tyrosinase inhibitor (Kim et al., 2009). However, there is no method for simultaneous analysis of these phenolic acids in seeds of *E. japonica*.

In this study, we aim to establish a simple method by high performance liquid chromatography (HPLC) to simultaneously estimate contents of the phenolic acids such as benzoic acid, caffeic acid and chlorogenic acid in seeds of *E. japonica*. In addition, we investigated the *in vitro* antibacterial effects of the phenolic acids against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus* and *Pseudomonas aeruginosa*.

### Materials and Methods

**Extraction and fractionation of plant material.** Seeds of *Eriobotrya japonica* were isolated from loquat fruits harvested in Goseong-gun, Gyoungsangnam-do, Korea in July 2011, and lyophilized. 2 kg of powdered plant material were extracted with 10 L of 99.6% methanol under reflux. The methanol crude extract was obtained by using a rotary vacuum evaporation system at 40°C (281 g). The crude extract (100 g) was diluted with distilled

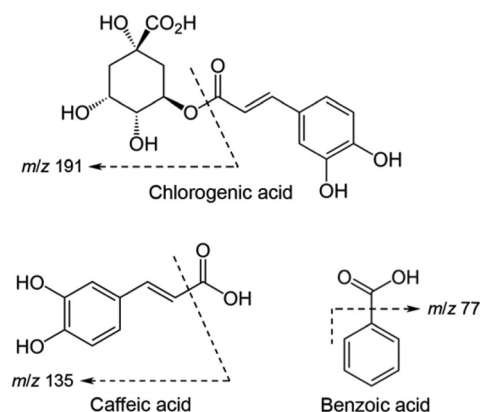
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**Table 1** Mass spectrometry parameters for analysis of phenolic acids

	Benzoic acid	Caffeic acid	Chlorogenic acid
Precursor/product ions ( $m/z$ ) <sup>a)</sup>	121/77	179/135	353/191
Fragment electric Voltage (V)	135	135	135
Collision energy (eV)	5	10	10

<sup>a)</sup>Derived from  $[M-H]^-$ .



**Fig. 1** Chemical structures and product ion of  $[M-H]$  for benzoic acid, caffeic acid and chlorogenic acid.

water and then fractionated by chloroform, ethyl acetate, *n*-butanol. After the solvents evaporated, 11.03% of chloroform fraction, 4.56% of ethyl acetate fraction, 16.62% of *n*-butanol fraction and 59.79% of aqueous fractions were obtained.

**Chemicals, bacterial strains, and culture conditions.** Benzoic acid, caffeic acid and chlorogenic acid (3-*O*-caffeoylquinic acid) was obtained from Sigma-Aldrich (USA). *Staphylococcus aureus* KCTC 3881 and *Staphylococcus epidermidis* KCTC 1917, *Bacillus cereus* KCTC 1012 and *Pseudomonas aeruginosa* KCTC 2004 were provided from the Korean Culture Type Collection (KCTC, Korea). Bacterial strains were cultured in Nutrient agar and broth medium at 37°C.

**Chromatographic methods.** The HPLC analysis was performed on a Shimadzu LC-10Avp series (Shimadzu, Japan) with an UV detector at 230 nm and the flow rate was maintained at 1 mL/min. The instrument was equipped with a TSK-gel ODS-80 column (4.6×150 mm, 5 μm, TOSHO, Japan). The analytes were eluted with a gradient mobile phase consisting of 0.1% formic acid (A) and acetonitrile (B). The gradient was programmed as follows: 0–35 min, 10–100% B; 35–40 min, 100–10% B; 40–45 min, 10% B.

**LC-MS/MS analysis.** Samples were analyzed using an Agilent LC-1200 series combined with an Agilent 6410 triple-quadrupole mass spectrometer (Agilent Technologies, USA) system. A XDB-C18 column (4.6×50 mm, 1.8 μm, Agilent, USA) was coupled and the flow rate was kept at 0.5 mL/min. The mobile phase was composed of 5 mM ammonium acetate in water with 0.1% formic acid (A) and 5 mM ammonium acetate in methanol with 0.1% formic acid (B). The gradient was programmed as follows: 0–

min, 75% A; 2–5 min, 75–60% A; 5–8 min, 60–0% A; 8–10 min, 0% A; 10–10.5 min, 0–75% A; 10.5–15, 75% A. The injection volume was 2 μL. Mass spectrometric analysis was performed in the multiple-reaction monitoring (MRM) with negative-ion electrospray ionization (ESI(-)) mode. The fragment electric voltage, collision energy and quantification of benzoic acid, caffeic acid and chlorogenic acid were achieved by monitoring the  $m/z$  of precursor/product ions (Table 1, Fig. 1).

**Disc diffusion assay.** The antibacterial activities of phenolic acids were performed by the disc diffusion assay (Bauer et al., 1966). Briefly, plates were prepared by spreading a pre-cultured strain broth (100 μL) on an agar medium using a sterilized cotton swab. The powdered samples were dissolved in the methanol to a final concentration of 50–100 mg/mL. Then they were sterilized by filtering through 0.2 μm syringe filters. Paper discs of 6 mm (ADVANTEC, Toyo Roshi Kaisha, Japan) saturated with samples was dried and placed on the surface of each inoculated plate. The plates were incubated for 24 h at 37°C. After this period, the diameter of growth inhibition area around the disc was measured by the measurement tool of Photoshop 6.0 (Adobe, USA) after scanning of cultured plates.

## Results and Discussion

**Analysis of phenolic acids by HPLC-UVD.** A simple HPLC method was developed for simultaneous and quantitative determination of benzoic acid, caffeic acid and chlorogenic acid in seeds of *Eriobotrya japonica*. Several phenolic compounds including these three phenolic acids isolated from seeds of *E. japonica* have been reported as a tyrosinase inhibitor (Kim et al., 2009). However, there is no report for simultaneous analysis of these three phenolic acids in seeds of *E. japonica*. In the test of wavelength for the detection of the phenolic acids, the maximum area and height of peaks of the phenolic acids could be obtained and the baseline of chromatogram was stable at 230 nm (Fig. 2). The method exhibited a good linear response for the three phenolic acids. Correlation coefficients ( $r^2$ ) were all >0.99. The equation of the calibration curve was:  $y=0.00004 \times 15.556$  ( $r^2=0.9988$ ) for benzoic acid,  $y=0.00004 \times 37.453$  ( $r^2=0.9922$ ) for caffeic acid and  $y=0.00005 \times 13.175$  ( $r^2=0.9991$ ) for chlorogenic acid.

The phenolic acid contents of the extract and fractions are shown in Table 2. The most abundant phenolic acid of the extract was chlorogenic acid. *n*-Butanol fraction had a significantly higher chlorogenic acid content than other samples. Chlorogenic acid is a phenolic acid which is commonly present in plant materials. Structurally, it is an ester of caffeic acid with the 3-hydroxyl group of a quinic acid (Fig. 1). It has been reported to possess many physiological activities including antioxidant, antimicrobial, chemopreventive, and so on (Chiang et al., 2004; Bouayed et al., 2007; Sung and Lee, 2010). On the other hand, chloroform and ethyl acetate fraction exhibited the highest content of benzoic acid and caffeic acid, respectively.

**Verification of phenolic acids by LC-MS/MS.** For the purpose

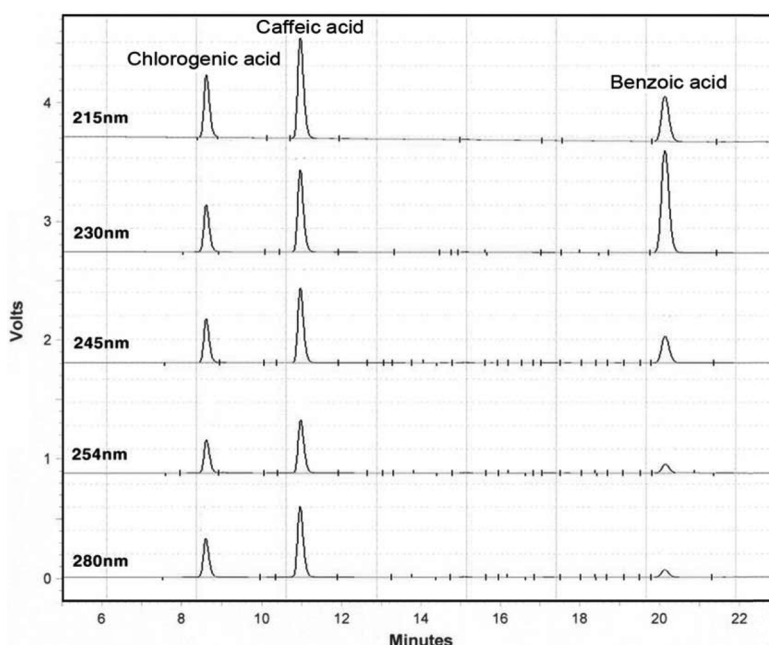


Fig. 2 LC-UVD chromatograms of phenolic acids at various wave length.

Table 2 Contents of phenolic acids in the extract and fractions

Samples	Phenolic acid contents (%)		
	Benzoic acid	Caffeic acid	Chlorogenic acid
Methanol extract	0.385±0.148 <sup>a)</sup>	0.071±0.025	0.957±0.098
Chloroform fraction	1.874±0.128	0.012±0.008	0.096±0.004
Ethyl acetate fraction	1.745±0.091	0.250±0.043	1.544±0.421
<i>n</i> -Butanol fraction	0.052±0.024	0.235±0.029	2.991±0.033
Aqueous fraction	0.005±0.005	0.023±0.008	0.265±0.003

<sup>a)</sup>Values are means of three replicate experiments ± SD.

Table 3 Antibacterial effects of phenolic acids against several pathogenic bacteria

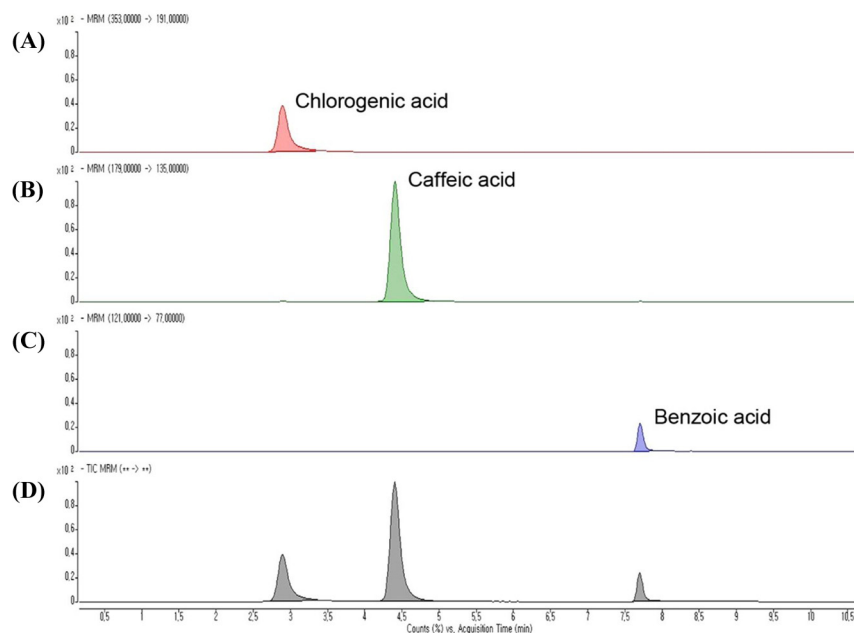
Samples	mg/disc	Zone of inhibition in diameter (mm)			
		<i>S. aureus</i>	<i>S. epidermidis</i>	<i>P. aeruginosa</i>	<i>B. cereus</i>
Benzoic acid	0.5	8.99±0.28 <sup>a)</sup>	12.83±0.81	11.47±1.16	11.58±1.05
	2.0	13.71±0.31	22.89±0.83	16.55±0.41	17.38±1.35
Caffeic acid	0.5	- <sup>b)</sup>	8.97±0.49	-	8.40±1.57
	2.0	9.70±0.84	11.10±0.85	8.57±0.94	10.82±0.61
Chlorogenic acid	0.5	9.40±0.25	-	-	6.54±0.93
	2.0	14.00±1.97	13.51±0.71	11.38±0.47	13.62±0.65

<sup>a)</sup>Values are means of three replicate experiments ± SD, <sup>b)</sup>No inhibition.

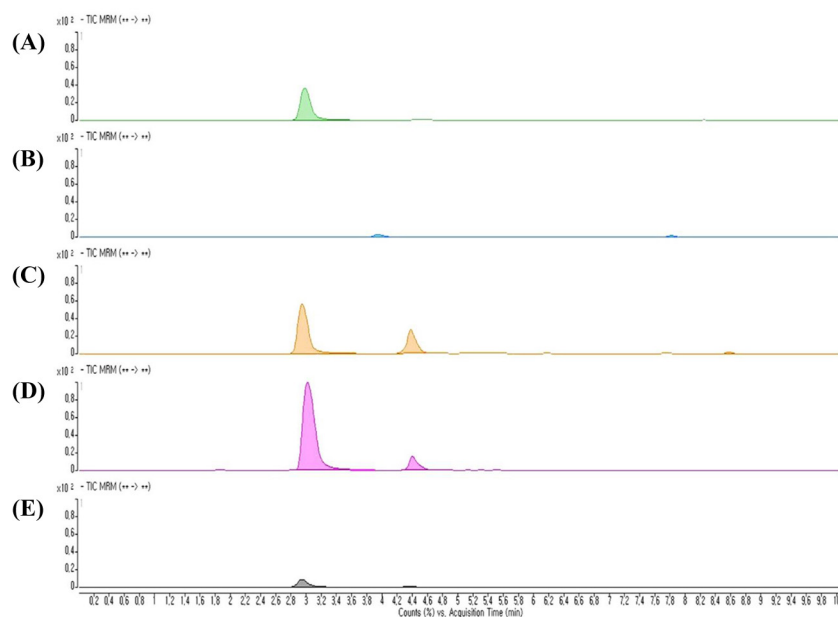
of correct identification, an LC-MS/MS analysis was performed on standard and samples under the MRM with ESI(-) mode. It is known that the negative-ion ESI mode is more suitable than positive-ion ESI mode for analyzing phenolic acids (Hu et al., 2005). As shown in Fig. 3, benzoic acid, caffeic acid and chlorogenic acid were separated and their retention times were at 7.78, 4.41 and 2.89, respectively. In addition, MRM chromatograms of the extract and its fractions of *E. japonica* are shown in Fig. 4. It seems to be similar pattern of results by HPLC-UVD analysis,

and a simple method by HPLC-UVD was could be applied to samples for accurate analysis of these three phenolic acids.

**Antibacterial effect of phenolic acids.** Antibacterial effects of the phenolic acids were evaluated against *S. aureus*, *S. epidermidis*, *B. cereus* and *P. aeruginosa*. In several previous researches, the antibacterial effects of phenolic acids such as caffeic acid and chlorogenic acid have been reported (Christina and Lisa, 2010; Karunanidhi et al., 2013). However, there is no study reported for simultaneous antibacterial test of benzoic acid, caffeic acid and



**Fig. 3** MRM chromatograms of phenolic acids by LC-MS/MS. (A) Extracted chromatogram of benzoic acid, (B) Extracted chromatogram of caffeic acid, (C) Extracted chromatogram of chlorogenic acid, (D) Total ion chromatogram of the mixture of phenolic acids.



**Fig. 4** MRM chromatograms of the extract and fractions from seeds of *E. japonica* by LC-MS/MS. (A) Methanol extract, (B) Chloroform fraction, (C) Ethyl acetate fraction, (D) *n*-Butanol fraction, (E) Aqueous fraction.

chlorogenic acid against same bacteria. The results obtained from disc diffusion assay are illustrated in Table 3. In the present study, benzoic acid exhibited the most effective antibacterial activity compared with chlorogenic and caffeic acid. The zone of inhibition ranged between 8.99–12.83 mm at 0.5 mg/disc and 13.71–22.89 mm at 2.0 mg/disc of benzoic acid against every tested pathogenic bacteria.

In conclusion, a simple method using HPLC-UVD verified

by LC-MS/MS has been proposed for simultaneous quantification of benzoic acid, caffeic acid and chlorogenic acid in the extract of seeds of *E. japonica* and its fractions. In addition, disc diffusion assay was performed for searching antibacterial activity of the phenolic acids against several pathogenic bacteria. Benzoic acid showed more effective antibacterial activity than caffeic acid and chlorogenic acid.

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