Analysis of major triterpene acids and total polysaccharides in the leaves of 11 species of *Eriobotrya*

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Abstract. Loquat (*Eriobotrya japonica*) is a subtropical tree with commercially important fruit and with leaves that have medicinal uses against cough and asthma. Leaves contain significant amounts of phenols, flavonoids, triterpene acids, and polysaccharides. However, information of the triterpene acids and polysaccharides in leaves is limited. In this study, the contents of five major triterpene acids and total polysaccharides in the leaves of 11 species of Eriobotrya were determined using RP-HPLC and phenol-sulfuric acid method, respectively. Total concentration of triterpene acids varied from 8.38 to 22.35 mg g-1 DW. The concentration of triterpene acids was greater than 20 mg g-1 DW in *E. bengalensis*, *E. prinoides* and *E. fragrans*, and it was less than 10.0 mg g-1 DW in *E. elliptica*. Different drying temperature (40-80˚C) of leaves did not affect the content of polysaccharides with highest content (80.8 mg g-1 DW) from *E. prinoides*. The contents of triterpene acid in six wild species and polysaccharides in 5 wild species were higher than that in cultivated loquat.

1 Introduction

Dried leaves of loquat (*Eriobotrya japonica* (Thunb) Lindl.) have been used as a traditional Chinese medicine for the treatments of cough and asthma [1-2]. Most investigations on functional compounds in loquat leaves have been concentrated on only one species *Eriobotrya japonica* [3-9]There are a few studies on triterpene acids and polysaccharides [10].

Polysaccharides, which play an important role in the structural composition of living systems, are being identified via the processes of biological recognition, regulation, and information storage. In the last decades, some polysaccharides extracted from natural sources showed important biological activities, such as antitumor, immunomodulatory, hypoglycemic and anti-inflammatory and are attracting increasing attention in medicine. There are many investigations related to the viability of using ursolic acid as an effective medicine in anti-tumor treatment [11] and antifatigue agents [12].

2 Material and methods

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2.1 Materials

Mature leaves were obtained from 11 species of *Eriobotrya* trees grown in the orchard in South China Agriculture University. Leaves were dried for 3-4 days at about 40 °C, and then crushed into powder (80 mesh). The ground powder was stored at about -20 °C prior to analysis. The 12 samples of 11 species used in this study are listed in Table 1.

Table 1. The contents of triterpene acid (mg/g powder) and polysaccharide (amount to glucose mg/g DW) in various species of Eriobotrya.

Species	English name	A	B	C	D	E	The content of triterpene acid	The content of polysaccharide
E. japonica cv. Zaozhong No. 6	Common loquat cv. Zaozhong 6	3.13	1.47	1.02	1.90	7.35	14.88	42.83±2.05e
E. japonica Lindl.	Common loquat (wild tree)	2.50	0.64	0.50	1.69	5.57	10.90	44.97±1.66e
E. elliptica Lindl.	Tibet loquat	3.34	1.23	0.81	—	3.01	8.38	45.39±1.95e
E. prinoides var. *dadunensis*	Daduhe loquat	3.81	1.13	0.83	1.44	9.20	16.41	60.24±3.00c
E. prinoides Rehd & Wils	Oak leaf loquat	2.04	5.25	1.27	2.31	9.53	20.40	80.79±0.72a
E. deflexa Nakai	Taiwan loquat	2.11	1.52	0.61	1.30	5.86	11.40	45.23±0.33e
E. deflexa var. *koshunensis*	Hengchun loquat	1.51	1.79	0.86	1.36	9.73	15.25	42.88±0.55e
E. fragrans Champ	Fragrant loquat	1.53	5.76	0.98	2.81	9.13	20.21	72.68±3.23b
E. kwangsiensis Chun	Guangxi loquat	2.25	4.14	0.41	0.96	5.17	12.93	41.78±1.51ef
E. bengalensis Hook. f.	Bengal loquat	1.89	3.20	0.80	2.46	14.01	22.35	54.28±2.70d
E. obovata W. W. Smith	Obovata leaf loquat	2.91	5.64	0.51	—	3.57	12.63	41.94±1.95ef
E. cavaleriei Rehd	Big flower loquat	2.97	4.87	1.04	1.42	8.69	18.99	55.90±1.19d

Note: 1. A: tormentic acid; B: 2α,19α-dihydroxy-3-oxo-urs-12-en-28-oic acid;
C: 2α-hydroxyoleanolic acid; D: oleanolic acid; E: ursolic acid
2. Meaning with different letters within the same column are significantly different at the level of 5%.

2.2 Sample Preparation and HPLC Analysis of triterpene acid

Sample preparation and HPLC analysis were carried out according to Ju *et al* [4]. with some modifications: 0.8g dried powder was suspended in 20 mL chloroform and exposed to ultrasonic twice, each for 20 min, with an interval of 10 min. The suspension was kept over night (about 12 h, 25˚C), and then recovered to its original volume followed by filtration; 5 mL filtrate was dried and dissolved in 5 mL of methanol. The solution was filtered through a membrane with a pore size of 0.22 μm.

Filtrate (20μL) was injected into the HPLC apparatus, which is consist of an Aglien HP1100 quaternary HPLC pump system with VWD system, a SK5200H (200W) ultrasonic launder and a Laborata 4000 (Heidolph) evaporator. The column was 5μm hypersil ODS2 (250 mm * 4.6 mm I.D.) in 25 °C. The mobile phase was a solution composed of acetonitrile, methanol and 21 mg mL^{-1} aqueous solution of ammonium acetate (v:v:v=60: 14: 26), with a flow rate at 1.5 ml min^{-1}.The detector wavelength was 215 nm.

Ursolic acid and oleanolic acid were provided by the National Institute for the Control of Pharmacerticals and Biological Products (Beijing, China), and tormentic acid, 2α,19α-dihydroxy-3-oxo-urs-12-en-28-oic acid, 2α-hydroxyoleanolic acid (maslinic acid) were isolated from Fragrent loquat[8], all these were used as standard. Methanol and acetonitrile were of chromatographic purity and all the other chemicals used were of analytical purity. All the aqueous solutions were prepared with double distilled water and filtered through 0.45μm nylon filters before use. Data was acquired and analyzed using Aglien Chem Stations.

2.3 Sample preparation and determination of polysaccharides contents

2.3.1 Orthogonal assay of polysaccharides extraction

Three factors and three-level orthogonal test were carried out to determine the optimum extraction method of polysaccharides. The dried powder of 'Zaozhong 6' loquat were firstly defatted with ethanol solution and the resulting residues were suspended in deionized water of different volumn (Solid-liquid ratio were 1:10, 1:15, 1:20), and then treated for 3/6/9 hours by warm water bath (temperature were 60 °C, 80 °C, 100 °C), after that, the supernatant were collected for the analysis of polysaccharides by phenol - vitriolic colorimetry.

2.3.2 Dried temperature

Leaves of 'Zaozhong 6' loquat were dried in three temperatures (40°, 60° and 80°C) for 3-4 days.

2.3.3 Determination of polysaccharides contents

The dried powder (0.5 g) of leaves from each species was firstly defatted in 20 mL of 50 % ethanol solution by ultrasonic treatment, with 20 min, using an ultrasonic instrument (SK5200H Ultrasonic Launder, 200 W, Kedao Supersonic Equipment Co. Ltd., Shanghai, China). The residues were suspended in 10 mL of deionized water, and then treated for 3 hours by boiling water bath, after that, the suspension were centrifuged at 12000 g for 15 min.

The contents of polysaccharides of the supernatant were determined by phenol-vitriolic colorimetry. Firstly, 975 μL deionized water was added to 25 μL supernatant of each samples for diluting extraction, then 0.4 mL of 5% phenol was added to 0.2 mL of each diluted samples, after that, 2 mL of sulfuric acid was added immediately. The mixed solution was shaken vigorously under room temperature (about 25 ˚C) for 30 min. The absorption was measured at 490 nm, using distilled water as a blank. The amount of polysaccharides was measured and expressed as glucose equivalent (GE) on dry weight basis.

2.3.4 Data analysis

Each experiment was repeated by three times. The data were analyzed using the statistical package SPSS 10.0. Significant differences were calculated according to the Duncan's Multiple range test. Differences at the level of 5% were considered as statistically significant.

3 Results and discussion

3.1 Quantitative determination of triterpene acid

The samples of 'Zaozhong No.6' and ursolic acid were used for precision test, reproducibility test, recovery test and stability test. The results showed the method of HPLC was credible[6].

A series of solutions of tormentic acid, 2α,19α-dihydroxy-3-oxo-urs-12-en-28-oic acid, 2α-hydroxyoleanolic acid (maslinic acid), oleanolic acid and ursolic acid at gradient concentrations were prepared in methanol. The standard curve of concentration vs peak area was established. All the standard curves fitted perfect linear equation.

tormentic acid: Y=5.46827479X+10.15579 (r=0.99954);
2α,19α-dihydroxy-3-oxo-urs-12-en-28-oic acid: Y=3.99517844X+ 4.5830234 (r=0.99967);
2α-hydroxyoleanolic acid: Y=5.855837X+40.010592 (r=0.99954);
oleanolic acid: Y=5.3474258X+9.7835004 (r=0.99955);
ursolic acid: Y =4.33280277X＋19.362927 (r= 0.99969).

The concentration of triterpene acids in different species of *Eriobotrya* varied from 8.38 to 22.35 mg g^{-1} DW (table 1). Triterpene acids concentration was higher than 20.0 mg g^{-1} DW in Bengal loquat, Oak leaf loquat and Fragrant loquat, while it was lower than 10.0 mg g^{-1} DW in Tibet loquat. The content of triterpene acids in 6 species were higher than the normal loquat, it suggested that these wild species could be better candidate materials for utilizing triterpene acids.

3.2 Quantitative Determination of polysaccharides

3.2.1 Standard curve of glucose

A series of solutions of glucose which were dried to constant weight at gradient concentrations (40, 80, 120, 160 and 200 μg/mL) were prepared in deionized water. The OD490nm of those solutions were determined using phenol-vitriolic colorimetry. The standard curve of concentration vs OD490 nm was established. The standard curve fitted a perfect linear equation: Y=0.0051X-0.0047 (r= 0.999).

3.2.2 Orthogonal test of extraction

Polysaccharides were determined for 9 different treatments (Table 2). The analysis of variance results showed that the temperature of water bath and the Solid-liquid ratio influenced the yield of polysaccharides, the optimum condition was boiling water bath (100 °C) for 3 hours using a Solid: liquid ratio of 1:20.

Table 2. The result of orthogonal test of polysaccharid extraction.

No.	Temperature of water bath (°C)	Solid-liquid ratio	Time (hours)	Content of polysaccharide (mg GE /g DW)
1	60	1:10	3	10.448
2	60	1:15	6	17.176
3	60	1:20	9	21.776
4	80	1:10	6	18.089
5	80	1:15	9	23.343
6	80	1:20	3	26.759
7	100	1:10	9	23.670
8	100	1:15	3	34.410
9	100	1:20	6	36.895

3.2.3 Dried temperature and the contents of polysaccharides

The leaves of ‘Zaozhong 6’ loquat were dried in three temperatures (40°, 60° and 80°C) and the content of polyssachrids was 42.83±2.05 mg GE/g DW, 45.97±2.23 mg GE/g DW and 40.95±1.65 mg

GE/g DW respectively, the analysis of variance showed that the dried temperature had no apparent affect on the content of polysaccharides.

3.2.4 Determination of the contents of polysaccharides

Oak leaf loquat had the highest content of polysaccharaides (80.79mg GE/g DW, p=0.05), followed by Fragrant loquat, Daduhe loquat, Big flower loquat and Bengal loquat (Table 1). The lowest amount of polysaccharides (p=0.05) was observed in Guangxi loquat and Obovata leaf loquat. The contents of the polysaccharides of 5 species were higher than those of the normal loquat. This indicates that there is a significant difference in polysaccharides content among the 11 Eriobotrya species. Therefore, rather than the normal loquat, some wild species could be identified as better material for utilizing the polysccharides from the leaves.

4 Conclusion

The contents of triterpene acid and polysaccharides in the leaves of Oak leaf loquat, Fragrant loquat, Daduhe loquat, Big flower loquat and Bengal loquat were higher than those of the normal loquat while Oak leaf loquat, Daduhe loquat, Big flower loquat and Bengal loquat contained significantly higher level of total phenolics and flavonoids, and had stronger antioxidant activities compared with those of the cultivated species'Zaozhong 6' loquat [7]. It is reported that, the contents of polysaccharides and flavones in old leaves were quiet different between different loquat cultivars [13]. Our research also showed that the contents of polysaccharides, triterpene acid and flavones in leaves differed significantly among different species of *Eriobotyra*. Our study suggests that high level of functional components may be found in wild species of loquat and could be useful for medicinal use.

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