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Short communication

Antitussive and expectorant properties of growing and fallen leaves of loquat (*Eriobotrya japonica*)

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ABSTRACT

Folium Eriobotryae, the dried leaves of loquat (*Eriobotrya japonica*, (Thunb.) Lindl., Rosaceae), is a traditional Chinese medicine used to treat cough with phlegm in China. Fallen and growing loquat leaves were tested for their effect on coughing and expectoration in mice. HPLC-ELSD and HPLC-MS analyses of aqueous and ethanol extracts of fallen or growing leaves were used to identify the chemical components responsible for this effect. Both the aqueous and ethanol extracts of growing and fallen leaves of loquat contained antitussive and expectorant activities. Moreover, an aqueous extract of growing loquat leaves with a higher flavonoid content displayed a stronger expectorant activity while the ethanol extract of fallen loquat leaves that contained a higher content of triterpenoid acids induced a stronger antitussive activity.

Introduction

Cough is the most common symptom in patients subjected to endogenous or exogenous irritants and it is an important symptom of various airway inflammatory diseases. It is also a major public health issue worldwide because it reduces comfort and causes sleep disturbances. Over recent decades, herbal medicines and active ingredients of natural products have garnered growing attention as potential therapeutic agents to prevent and treat coughing, due to their high efficacy and low risk of side effects (Zhou et al., 2013).

Loquat is a multipurpose plant cultivated as a kind of fruit tree and its dried leaves have been used as a traditional Chinese medicine to treat cough with phlegm for thousands of years. Recent pharmacological studies found that *Folium Eriobotryae* also possessed several other activities, such as anti-diabetic effects (Chen et al., 2008; Lü et al., 2009b), anti-tumor and anti-inflammatory effects (Huang et al., 2009), antioxidant effects (Huang et al., 2006), and a beneficial effect on non-alcoholic fatty liver disease (NAFLD) (Jian et al., 2017; Jian et al., 2018). Phytochemical studies indicated that flavonoids (Jung et al., 1999), triterpenoid acids (Chen et al., 2008) and sesquiterpene glycosides (Zhao et al., 2015) were the main chemical constituents in loquat leaves; however, its active compounds responsible for the antitussive activity have not be identified. In the present study, the aqueous and ethanol extract of growing leaves and fallen leaves of loquat were investigated for their content of secondary metabolites and their beneficial effects on coughing in mice. The results indicated an advantage in evaluation of the pharmacological effects and the potential effective substances of both growing and fallen leaves.

Materials and methods

Growing leaves (GL) and fallen leaves (FL) of loquat (*Eriobotrya japonica*, (Thunb.) Lindl., Rosaceae) were both manually collected in December 2015 in Suzhou, China (120.296235° S; 31.086002° W). The plant was identified by Professor Wei-Lin Li at the Institute of Botany, Jiangsu Province and the Chinese Academy of Sciences, Nanjing (China). A voucher specimen (No. 151228) was deposited in the herbarium of the Institute of Botany (Nanjing). The leaves were washed, dried at 25–30 °C before being cut into strips.

Pentoxyverine was purchased from Sinopharm Rongsheng Pharmaceutical Co. Ltd. (Henan, China). Acetonitrile and methanol (HPLC grade) were obtained from Tedia Co. Inc. (Fairfield, OH, USA). Ammonium hydroxide, sodium nitrite, sodium carboxyl methyl cellulose (CMC-Na), aluminum nitrate, sodium hydroxide, and Phenol Red were all purchased from Nanjing Chemical Reagent Co. Ltd. (Jiangsu, China), and other reagents of analytical grade were provided from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

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Chemical standards were purchased from the National Institutes for Food and Drug Control (Beijing, China).

Batches of air-dried GL (200 g) were extracted twice with 2000 ml of boiling water for 2 h, and the filtrate was concentrated in a vacuum to obtain the aqueous extract of GL (AGL). The filtered residue was subsequently extracted twice with 2000 ml of 80% ethanol at 80 °C for 2 h, and the filtrate was concentrated in a vacuum to obtain the ethanol extract of GL (EGL). The aqueous and ethanol extracts of FL (AFL and EFL) were obtained as for GL.

Total flavonoid content of AGL, EGL, AFL, and EFL was measured by the aluminum chloride colorimetric assay as reported previously from our laboratory (Lü et al., 2009b).

Aqueous solutions of AGL and AFL were prepared [AGL 17.35 mg (equal to 100 mg crude drug)/ml, AFL 11.85 mg (equal to 100 mg crude drug)/ml] and filtered through a 0.45-μm PVD filter for HPLC-MS analysis. Spectra were generated on an Agilent 6530 accurate-mass quadrupole time-of-flight system (Agilent, USA), with an ESI source operating in the positive ionization mode. Analyses were made using a MassHunter Qualitative Analysis software (B.05.00). Separation was carried out with an Agilent Zorbax SB-C_{18} column (1.8 μm, 4.6 × 100 mm; Waldbronn, Germany) using a capillary voltage of +4.0 kV. Methanol (A) and 0.1% formic acid (B) were used as the mobile phase under gradient conditions (0–5 min, 15% A, 85% B; 5–60 min, 15–55% A, 85–45% B; 60–75 min, 55–100% A, 45–0% B).

Methanol solutions of EGL and EFL and aqueous solutions of AGL and AFL were prepared [EGL 9.35 mg (equal to 100 mg crude drug)/ml, EFL 9.65 mg (equal to 100 mg crude drug)/ml] and filtered through a 0.45-μm PVD filter for HPLC-ELSD analysis. HPLC-ELSD analysis was performed with an Ultimate 3000 HPLC system (Thermo Fisher, USA) coupled with an Alltech 3300 ELSD detector. An Acclaim C_{18} column (4.6 × 250 mm, 5 μm) was used and the temperature was held at 30 °C. The mobile phases, acetonitrile (A) and 0.5% ammonium acetate (B) were used under gradient conditions as follows: 0–5 min, 50% A, 50% B; 5–23 min, 50–54% A, 50–46% B; 23–48 min, 54–90% A, 46–10% B; 48–50 min, 90–100% A, 10–0% B, with a flow rate of 1 ml/min. The temperature of the ELSD drift tube was 70 °C, with a flow rate of nitrogen of 1.5 l/min.

Six triterpenoid acids including euscaphic acid, tormentic acid, corosolic acid, maslinic acid, oleanolic acid, and ursolic acid were used as reference samples to quantify the contents in extracts.

The 6-week-old male ICR mice (18–22 g) were provided from the Comparative Medicine Center of Yangzhou University, China. All the experimental procedures and animal treatments were according to the Guide for the Care and Use of Laboratory Animals (No. 2008001680632).

In the evaluation of antitussive activity, the animals were acclimatized for one week before being randomly selected and placed in ten groups of ten biological replicates ($n = 10$). Then, they were treated orally for three days as follows: mice of control groups were fed with 0.5% CMC-Na/day, while low dose [2.5 g (crude drug)/kg (mouse body weight)/day] and high dose [5 g (crude drug)/kg (mouse body weight)/day] of AGL, AFL, EGL, and EFL were administrated to mice of the test groups. The positive group was treated with Pentoxyverine (17.5 mg/kg (mouse body weight)/day). Antitussive effects were examined by using a classical mouse cough model induced by ammonium hydroxide (Liu et al., 2015), the mice were each exposed to a 500 ml special glass chamber sprayed with 15% ammonium hydroxide (0.2 ml) as described in a previous study (Wang et al., 2012). The cough incubation period and the frequency of cough were recorded from each mouse during 3 min.

To evaluate expectorant activity, the mice animals were grouped and treated as described above, except that the positive group was treated with ammonium chloride (NH_4Cl, 250 mg/kg (mouse body weight)/day). After the third day of treatments, mice were administrated after a 12 h, overnight fast, then 30 min later, they were given Phenol Red (5%, w/v) by intraperitoneal injection. Fifteen min later, the mice were sacrificed by cervical dislocation without damaging the trachea, which was tied-off and then lavaged three times with 0.5 ml sodium bicarbonate (5%, w/v). The collected washing fluid was centrifuged and the absorbance at 546 nm measured at 37 °C. The concentration of Phenol Red was determined as described previously (Li et al., 2012).

The experimental results were expressed as mean ± standard deviation (mean ± SD). The statistical significance of differences between two groups was analyzed using One-way ANOVA analysis of variance followed by a Newman–Keuls post hoc test used to perform multiple comparisons (GraphPad Software, Inc., San Diego, CA, USA). Values of $p < 0.05$ were taken to indicate statistical significant pharmacological effects.

Results and discussion

The total flavonoid content of AGL, EGL, AFL, and EFL was 28.079 ± 0.606, 3.643 ± 0.253, 9.880 ± 0.522, and 2.143 ± 0.312 mg/g, respectively, indicated that the total flavonoid content in growing leaves was richer than that in fallen leaves, and that the total flavonoids content in aqueous extract was higher than that in the ethanol extract. To investigate further, both AGL and AFL were subjected to HPLC-MS analysis, and twelve compounds were assigned using a HPLC-MS method in both the AGL and AFL (Fig. 1) (Lü et al., 2009b). As shown in Fig. 1, the aqueous extracts of GL and FL presented qualitatively similar chromatograms, but with clear differences in peak heights. For twelve compounds were identified and assigned. Compounds 3–12 were flavonoids or their glycosides. Except for compounds 10 and 12, the peak areas of other compounds in AGL were higher than those of AFL, which was consistent with measurements of the total flavonoid content.

In the evaluation of expectorant activity, mice were injected intraperitoneally with Phenol Red, which was partially discharged within the trachea secretion. Expectorant drugs enhance secretion and dilute the phlegm in the respiratory tract, thus increasing the excretion of Phenol Red (Zhou et al., 2013). As shown in Fig. 2, relative to the control group, all treatments, with the exception of low dose of ethanol extracts of growing and fallen leaves (EGLL and EFLL) induced remarkable expectorant activity. However, aqueous extracts of growing and fallen leaves induced a higher effect than those of ethanol extracts, and the most effective was the high dose of aqueous extract of growing leaves (AGLH) ($p < 0.001$).

Airway mucus hypersecretion, the major cause of coughing and phlegm, is closely associated with the occurrence and development of chronic airway inflammation, and affects the lung function (Zhang and Zhou, 2014). Previous studies have demonstrated that loquat infusion has significant anti-inflammatory activities in both cell and animal models (Zar et al., 2014). Some of the identified flavonoids in the aqueous extract of loquat leaves are known to have anti-inflammatory activity through STAT-1 and NF-κB inhibition (Hamalainen et al., 2007; Hoensch and Oertel, 2012). NF-κB is known to play critical roles in the expression of proinflammatory cytokines. In our study, AGL with higher contents of flavonoids showed better expectorant activities; therefore, flavonoids may be the main constituents responsible for reducing phlegm.

Ammonia-induced cough is a commonly used model for assessing the antitussive effects of medical, bioactive components. In this model, the cough incubation period and cough incidence are often used as the assessing indices. Compared to the control group, the aqueous and ethanol extracts of both growing and fallen leaves of loquat delayed the cough incubation period for ammonia-induced cough and decreased the cough frequency (Fig. 3). The antitussive

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