Activities constituents from Yaowang Tea (*Potentilla glabra* Lodd.)

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**Yaowang tea**, the leaves and flowers of *Potentilla glabra* Lodd. (family Rosaceae), has been used for a long time to prevent and mitigate hyperlipidemia, hypertension, diabetes and so on. According to this research, twelve flavonoids were isolated and all the flavonoids were obtained for the first time in *P. glabra*. The different polar portions (petroleum ether portion, ethyl acetate portion, *n*-butanol portion and water portion) and six flavonoids from *P. glabra* were screened, and their antioxidant abilities were measured by DPPH and FRAP assays. Six tested flavonoids showed significant free radical scavenging capacity in the DPPH assay and total antioxidant activity in the FRAP assay compared with Vitamin C (Vc). This work provides an understanding for the flavonoids of *P. glabra* and lays the foundation for the future research.

Keywords: Yaowang tea, *Potentilla glabra* Lodd., flavonoids, antioxidant

**Introduction**

*Potentilla glabra* Lodd., belonging to genus *Potentilla* (family Rosaceae), is mainly distributed in China, Mongolia, Soviet Union, and North Korea (Chinese academy of sciences, 1985). The leaves and flowers of *P. glabra* have been used as a tea named *Yaowang tea* (Chinese: 药王茶) or *Guanyin tea* (Chinese: 观音茶) in folk. As a herb tea, *P. glabra* has been widely drunk for a long time. According to legend, in AD 649, king medicine Sun Simiao often picked the leaves of *chapazi* (the nickname of *P. glabra*) and drank it as tea, then this method was introduced to the local people and folk doctors to fortify their health (Bai et al., 2012). So far, the habit of drinking *Yaowang tea* was preserved and *Yaowang tea* had become the representative drink in Taebak Mountains areas (Kang & Li, 2005). The *Yaowang tea* is rich in phenolic and other antioxidant properties. Modern pharmacological research suggested that *P. glabra* showed obvious antioxidant and antibiosis activities (Wang et al., 2013). For this reason, *P. glabra* also had been developed into different health-related products, such as *Qingre Jianwei Yinlumei Liangcha* (Patent CN102406024B), to prevent and mitigate hyperlipidemia, hypertension, diabetes and so on. Although phytochemistry and pharmacologic actions of *P. glabra* had been reported, its specific pharmaceutically active ingredient is still unclear.

In order to find the active components of *P. glabra*, we have isolated these compounds through the antioxidant activity-guided fractionation, and measured the antioxidant capacity of the different polar portions and compounds. Our study will provide comprehensive insight into these excellent natural antioxidants from *P. glabra*. It will construct a new foundation for further study on mechanism and development of healthy product from *P. glabra*.

**Materials and Methods**

**Plant materials** The aerial parts of *P. glabra* were collected from Inner Mongolia Jiufeng mountain natural reserve in August 2013. A voucher specimen (Bt2013091605) has been deposited in Baotou Medical College, China.

**Chemicals, reagents and apparatus** Methanol (MeOH), petroleum ether (PE), ethyl acetate (EtOAc), and *n*-butanol
(n-BuOH) were obtained from Tianjin Fengchuan Chemical Reagent Science And Technology Co., Ltd. (Tianjin, China). Ferric chloride, ferrous sulphate, 1,1-diphenyl-2-picrylhydrazyl (DPPH, 98%), 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ) solution and Vitamin C (Ve, L-Ascorbic Acid, 99%) were purchased from Sigma Chemical Co. (St Louis, MO). All reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All chemicals and reagents used were analytical grade. Micromass ZabSpec high resolution mass spectrometer (resolution: 1000). (Waters, America), Varian INOVA-400 instrument (Varian, America).

**Extraction** The air-dried aerial parts of *P. glabra* (4.70 kg) were extracted with MeOH (70%) for 3 times at 70°C. A sample (480 g), through vacuum concentration, the residue of the extract was suspended in water (4 L) and fractionated by successive partitioning with PE, EtOAc and n-BuOH, respectively. And the different polar portions were produced: PE portion (I), EtOAc portion (II), n-BuOH portion (III) and water portion (IV).

The EtOAc portion (II) (80 g) was subjected to dry column on silica gel (200 – 300 mesh, 1000 g) and gradient eluting with PE: EtOAc (10:1 to 2:1, v/v) to get 6 main fractions (Frs. A1-6). Then fraction A3 (12.5 g) was further chromatographed on silica gel (300 – 400 mesh, 300 g) eluting with CHCl3, and increasing percentage of MeOH (20 – 80%, v/v) to give 70 fractions (Frs. B1-70). The combined fraction B35-50 was further purified by repeated Sephadex LH-20 eluting with MeOH: H2O (80:20, v/v) to obtain compounds 1 (22 mg), 2 (9 mg) and 3 (25 mg). The n-BuOH portion (III) (100 g) was subjected to dry column on silica gel (200 – 300 mesh, 1.0 kg) and gradient eluting with CHCl3: MeOH (5:1 to 1:1, v/v) to get 6 main fractions (Frs. C1-6). Then fraction C2 (17.5 g) was then further chromatographed on silica gel (300 – 400 mesh, 600 g) eluting with CHCl3: MeOH: H2O (7:3:0.5, v/v) to give 48 fractions (Frs. D1-48). D10-20 was further chromatographed by repeated Sephadex LH-20 eluting with MeOH: H2O (70:30, v/v) to obtain compound 4 (6 mg). D25-40 was purified by repeated ODS with the percentage of MeOH and H2O (40%) to obtain 5 (9 mg), 6 (8 mg) and 7 (22 mg). Fraction C4 (21.3 g) was further separated on Sephadex LH-20 column eluted repeatedly with 70% MeOH: H2O to give 60 fractions (Frs. E1-60). E5-15 was further chromatographed by repeated Sephadex LH-20 eluting with MeOH: H2O (60:40, v/v) to obtain compound 8 (24 mg). The combined fraction E20-35 was purified by preparative HPLC with 40% MeOH: H2O to give compound 9 (27 mg), 10 (9 mg) and 11 (7 mg). Frs. E40-55 was further chromatographed by repeated Sephadex LH-20 and purified by preparative HPLC to obtain compound 12 (22 mg).

The structures of twelve compounds were elucidated. Mass spectra were measured by Micromass ZabSpec high resolution mass spectrometer (resolution: 1000). (Waters, America). NMR spectra were recorded at 400 MHz (1H) and 100 MHz (13C) on a Varian INOVA-400 instrument (Varian, America). The NMR spectra were recorded in DMSO-d6, or CD3OD, and chemical shifts were given in δ (ppm) relative to Tetramethylsilane (TMS) as internal standard.

**Antioxidant activity assays**

a) DPPH assay

The DPPH assay was conducted according to the method reported by Li et al. (2012). 0.1 mM solution of DPPH in ethanol was prepared before measure and 0.1 mL various concentrations of the sample solutions were thoroughly mixed with 0.1 mL of freshly prepared DPPH in microplate. Vc (5.68 μmol/L - 45.45 μmol/L) was used as the positive control and MeOH was used as a blank. After 30 min of reaction at room temperature in the dark, the absorbances were measured at 517 nm using a 721-visible spectrophotometer (S22PC, Shanghai Lengguang Technology Co., Ltd., China). The tests were carried out in triplicate.

The capability to scavenge the free radical DPPH in percentage of sample (X%) was calculated according to the following equation:

\[ X\% = (A_2 - A_1) / A_2 \]

Where \( A_2 \) is the absorbance of the incubation mixture containing both DPPH solution and ethanol, \( A_1 \) is the absorbance of the incubation mixture containing both the test sample and ethanol. \( A_1 \) is the absorbance of the incubation mixture containing both the test sample and MeOH solution. Extract concentration providing 50% inhibition (IC50) was calculated using the graph by plotting inhibition percentage against extract concentration.

b) FRAP assay

The total antioxidant activity was determined by FRAP assay (Chen et al., 2010). Fresh working FRAP reagent was prepared daily by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL TPTZ solution (10 mM) and 2.5 mL of FeCl3 solution (20 mM). Preparation of FeSO4 series solution: weigh accurately FeSO4 15.200 mg. Mixed with ultra pure water, the FeSO4 solution is configured with a series of concentration of 50, 100, 150, 200, 300, 400, 800, 1600 μmol·L⁻¹. The sample solution preparation: according to the results of pre experiment, the different polar portions were equipped with 0.10 mg/mL. The test compounds and Vc with concentration (100 μmol/L, 5 μL) were added to FRAP reagent (180 μL) and the absorbances of the samples were measured at 593 nm by using the spectrophotometer. The FRAP reagent with distilled water was used as a blank. The reaction mixture was incubated for 8 min at 37°C in water. 1.00 mmol/L FeSO4 was chosen as reference and total antioxidant activity was expressed in micromolar FeSO4 equivalents. All tests were run in triplicate and the results were expressed as means ± standard deviation (SD).

The radical-scavenging activities of samples were determined as the percentage decrease in the absorbance compared to a blank test. The inhibition percentage of the radicals by the samples was calculated using the following equation:
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is absorbance of the control and $A_0$ absorbance of sample after reacting for a specified time period.

Statistical analysis Significant differences were determined using Student’s t test, where differences were considered significant if $p < 0.05$.

Results and Discussion

Structure determination The antioxidant activity assays of the different polar portions have been detected in our study. We have carried out the separation and purification of EtOAc and $n$-BuOH portion that possessed great scavenging free radical capacity and total antioxidant activity. Twelve flavonoids were isolated and the structures of these compounds were elucidated by spectral data ($^1$H-NMR, $^{13}$C-NMR and ESI-MS) and compared with the references as Kaempferol (1) (Lin et al., 2000; Ni et al., 2013), Rhamnetin (2) (Wang et al., 2000; Bankova et al., 1983; Chen et al., 2012), Quercetin (3) (El-Sawi & Sleem, 2010; Koolen et al., 2012), Rhamnetin-3-O-rhamnoside (4) (Kim et al., 2004), Rhamnetin-3-O-glucoside (5) (Li et al., 1996; Zhao et al., 2013), Quercetin-3-O-galactoside (6) (Olszewska 2005; Liu et al., 2009), Quercetin-3-O-xylloside (7) (Yan et al., 2002; Yuan et al., 2010), Quercetin-3-O-rhamnoside (8) (Kim et al., 2004; Lei et al., 2012), Quercetin-3-O-glucoside (9) (Lei et al., 2012), Quercetin-3-O-glucuronide (10) (Moon et al., 2001; Li et al., 2011), Rhamnetin-3-O-rutinoside (11) (El-Alfy et al., 2011; Pan et al., 2005) and Rutin (12) (El-Sawi & Sleem, 2010; Koolen et al., 2012). The structures of compounds isolated from P. glabra were shown in Fig. 1 and the spectral data ($^1$H-NMR, $^{13}$C-NMR and ESI-MS) were shown in Table 1. All the flavonoids were obtained from this plant for the first time.

Antioxidant activity assays We have selected the different polar portions (I-IV) and six sufficient amounts of compounds (1, 3, 7, 8, 9 and 12) to do the antioxidant experiment. The results exhibited antioxidant effect by DPPH and FRAP assay (Fig. 2).

Free radicals produced by the human body and induced oxidation reactions have closely relationship with a variety of diseases, which can accelerate human aging, antioxidants effectively remove free radicals, delaying human aging and disease prevention. As an artificial free radical, DPPH is usually to measure the free radical scavenging capacity in vitro. At present, synthetic antioxidants are widely used in the field of medical and food. Meanwhile, more and more people are increasingly in pursuit of environmental protection. Therefore, looking a security and natural biological antioxidants has been gradually a research hotspot. (Chen et al., 2011).

DPPH assay is based on the measurement of the scavenging ability of the stable radical DPPH$^\cdot$. It is considered a valid and easy assay to evaluate radical-scavenging activity of antioxidants and the ability of captured DPPH free radical is acting as an index (Sun et al., 2011). In this experiment, the radical scavenging activities of the different polar portions (I-IV) and six isolated compounds from P. glabra were measured by DPPH assay. Vc was used as positive control. The data in Fig. 2A has shown that the IC$_{50}$ values of Vc, PE portion (I), EtOAc portion (II), $n$-BuOH portion (III) and water portion (IV) were 4.22 μg/mL, 23.35 μg/mL, 4.57 μg/mL, 5.43 μg/mL and 16.84 μg/mL, respectively. The EtOAc portion (II) and the $n$-BuOH portion (III) displayed slightly lower antioxidant activity than Vc. The IC$_{50}$ value of Vc, Kaempferol (1), Quercetin (3),

\[
\text{Inhibition (\%)} = \frac{[A_0 - A_t]}{A_0} \times 100\% \quad \text{--- Eq. 2}
\]

Where $A_0$ is absorbance of the control and $A_t$ is absorbance of sample after reacting for a specified time period.

Fig. 1. Structures of compounds isolated from P. glabra.

Fig. 2. The DPPH free radical scavenging potential and the antioxidant power by FRAP assay group of the compounds. (A) DPPH scavenging activity of different polar portions. (I) PE portion; (II) EtOAc portion; (III) $n$-BuOH portion and (IV) water portion. (B) DPPH scavenging activity of compounds. (1) Kaempferol; (3) Quercetin; (7) Quercetin-3-O-xylloside; (8) Quercitrin; (9) Quercetin-3-O-glucoside and (12) Rutin. Each value represents means ± SD (n = 3), ($p < 0.05$). (C) FRAP scavenging activity of different polar portions at 0.1 mg/mL. (I) PE portion; (II) EtOAc portion; (III) $n$-BuOH portion and (IV) water portion. (D) FRAP scavenging activity of compounds at 100 μmol/L. (1) Kaempferol; (3) Quercetin; (7) Quercetin-3-O-xylloside; (8) Quercitrin; (9) Quercetin-3-O-glucoside and (12) Rutin. Each value represents means ± SD (n = 3), ($p < 0.05$).

\[
\frac{t - BuOH}{A_0} \cdot 100\% \quad \text{--- Eq. 3}
\]
Table 1. ESI-MS, $^1$H and $^{13}$C NMR data

<table>
<thead>
<tr>
<th>Name</th>
<th>$^1$H NMR (400 MHz)</th>
<th>$^{13}$C NMR (100 MHz)</th>
<th>ESI-MS</th>
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<tr>
<td>Kaempferol (1)</td>
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<tr>
<td>(DMSO-d$_6$) δ: 12.48 (1H, s, 5-OH), 10.77 (1H, s, 7-OH), 10.09 (1H, s, 3-OH), 9.38 (1H, s, 4'-OH), 8.04 (2H, d, J=8.8 Hz, H-2´), 6.92 (1H, d, J=8.8 Hz, H-3´), 6.43 (1H, d, J=2.0 Hz, H-8), 6.19 (1H, d, J=8.4 Hz, H-5´), 5.0, 156.1 (C-5), 146.7 (C-2), 135.5 (C-3), 129.4 (C-2´), 129.4 (C-6), 121.5 (C-1´), 115.3 (C-3´), 115.3 (C-5´), 103.0 (C-10), 98.1 (C-6), 93.4 (C-8)</td>
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<tr>
<td>Rhamnetin (2)</td>
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<td>(DMSO-d$_6$) δ: 12.47 (1H, s, 5-OH), 9.60 (1H, s, 3-OH), 9.44 (1H, s, 4'-OH), 7.95 (1H, d, J=2.1 Hz, H-2´), 7.56 (1H, d, J=8.4 Hz, H-5´), 6.88 (1H, d, J=8.5 Hz, H-5´), 6.69 (1H, d, J=2.0 Hz, H-8), 6.34 (1H, d, J=2.1 Hz, H-6), 3.86 (3H, s, 7-OCH$_3$)</td>
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<td>Quercetin (3)</td>
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<td>(DMSO-d$_6$) δ: 12.49 (1H, s, 5-OH), 10.78 (1H, 7-OH), 9.60 (1H, s, 3-OH), 9.37 (1H, 3-OH), 9.31 (1H, s, 4'-OH), 7.63 (1H, d, J=2.0 Hz, H-2´), 7.53 (1H, dd, J=2.0, 8.5 Hz, H-6), 6.87 (1H, d, J=2.0 Hz, H-8), 6.36 (1H, d, J=2.0 Hz, H-5´), 6.39 (1H, d, J=2.0 Hz, H-6), 6.17 (1H, d, J=2.0 Hz, H-6), 9.2 (C-8)</td>
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<tr>
<td>Rutin (12)</td>
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<tr>
<td>(DMSO-d$_6$) δ: 12.51 (1H, s, 5-OH), 10.77 (1H, s, 7-OH), 10.09 (1H, s, 3-OH), 9.38 (1H, s, 4'-OH), 8.04 (2H, d, J=8.8 Hz, H-2´), 6.92 (1H, d, J=8.8 Hz, H-3´), 6.43 (1H, d, J=2.0 Hz, H-8), 6.19 (1H, d, J=8.4 Hz, H-5´), 5.0, 156.1 (C-5), 146.7 (C-2), 135.5 (C-3), 129.4 (C-2´), 129.4 (C-6), 121.5 (C-1´), 115.3 (C-3´), 115.3 (C-5´), 103.0 (C-10), 98.1 (C-6), 93.4 (C-8)</td>
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Notes: coupling constant J = Hz in parentheses.
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Quercetin-3-O-xylloside (7), Quercitrin (8), Quercetin-3-O-glucoside (9) and Rutin (12) were found to be 23.85 μmol/L, 20.52 μmol/L, 9.31 μmol/L, 11.10 μmol/L, 14.92 μmol/L, 11.92 μmol/L and 17.29 μmol/L (Fig. 2B). The IC₅₀ value of Quercetin (3) was lower than the others. The exhibited radical scavenging activities were in the order 3 > 7 > 9 > 8 > 12 > 1 > Vc.

The FRAP assay is often used to measure the antioxidant capacity of foods, beverages and nutritional supplements containing polyphenols (Guo et al., 2003). It is used to measure the ferric reducing ability of plasma. Fe²⁺-TPTZ (blue) was synthesized from the test compounds and Fe²⁺-TPTZ and evaluation standard is concentration of equivalent Fe²⁺. In FRAP assay, the data in Fig. 2C has shown that the equivalent of Fe²⁺ (mmol/L) concentration of Vc, PE portion (I), EtOAc portion (II), n-BuOH portion (III) and water portion (IV) were 0.43 mmol/L, 0.20 mmol/L, 1.70 mmol/L, 1.60 mmol/L and 1.13 mmol/L at 0.10 mg/mL, respectively. Among the FRAP assay, the antioxidant capacity of 0.10mg/mL EtOAc portion (II) was highest followed by n-BuOH portion (III). PE portion (I) and water portion (IV) were relatively low. The data in Fig. 2D has shown that the equivalent of Fe²⁺ (mmol/L) concentration of Vc, Kaempferol (1), Quercitin (3), Quercetin-3-O-xylloside (7), Quercitrin (8), Quercetin-3-O-glucoside (9) and Rutin (12) were found to be 0.38 mmol/L, 0.76 mmol/L, 1.27 mmol/L, 0.60 mmol/L, 0.57 mmol/L, 0.61 mmol/L and 0.49 mmol/L at 100 μmol/L. The exhibited radical scavenging activities were in the order 3 > 1 > 9 > 7 > 8 > 12 > Vc. All the tested compounds were at concentration of 100 μmol/L.

During the past decades, concerns over the possible adverse health effects caused by the use of synthetic antioxidants, the use of natural antioxidants and plant-derived extracts have attracted more and more attention due to their low side effects. It has been reported that the genus Potentilla is a natural source of antioxidants, and the antioxidant activity is stronger than the synthetic antioxidants (Tori et al., 2011; Miliauskas et al., 2004).

The genus Potentilla species are rich in polyphenolic compounds, and they have obvious therapeutic effect on some chronic diseases (Michal & Klaus, 2009; Dai & Mumper, 2010). Total phenolics and total flavonoids were the main antioxidant active constituents. Our study indicated flavonoids in P. glabra showed the similar antioxidant activity, and were suspected as the responsible compounds. The position and number of -OH could affect the antioxidant capacity of the compound. The flavonoids we yielded have a degree of regularity in structure, flavonoids with many hydroxyl groups have stronger ability in scavenging free radicals, and phenolic hydroxyl group located in C-5, C-7 is essential to maintain the activity and it has a relationship with the transition metal complex (Cao et al., 2003). Phenolic hydroxyl number and hydrogen bonds number is related to the antioxidant activity of molecular. This is an important factor for flavonoids with strong antioxidant properties (Zhao et al., 2001). The ability to scavenge free radicals is related to the phenolic hydroxyl group of the compounds. By comparison of six flavonoids, the ability of scavenging free radicals is increasing with the augment of phenolic hydroxyl groups. The number of phenolic hydroxyl group in quercetin is 5, and the number of phenolic hydroxyl group in other compounds is relative less. Therefore, the antioxidant activity of quercetin is relative higher. The different aglycone with the same number of phenolic hydroxyl group (such as compounds 1, 7, 8, 9 and 12), the antioxidant activity of compound I is lower than compounds 7, 8, 9 and 12, which could speculate that the impact of hydroxy located in C-3 on the ability to scavenging free radicals and antioxidant capacity of compounds is bigger than hydroxy located in C-3. In the case of the same aglycone (such as compounds 7, 8, 9 and 12), the antioxidant activity of compounds decreased after connecting with glycosides and was directly proportional to the number of glycosides in the same position. It may be related to the structure of glycosides that hindered the ability of scavenging free radicals and intramolecular hydrogen bond.

Conclusions

In this research, the different polar portions (crude extracts I-IV) were selected for antioxidant experiment. EtOAc portion (II) and n-BuOH (III) portion have showed relatively high free radical scavenging activity, PE portion (I) and water portion (IV) were relatively low. We make a further separation of two active portions. Twelve flavonoids were identified by spectral data (¹H-NMR, ¹³C-NMR and ESI-MS) analysis, and all flavonoids were isolated for the first time in P. glabra. The six large amount flavonoids (compounds 1, 3, 7, 8, 9 and 12) had been analyzed by DPPH and FRAP, and the other parts of compounds were not enough to detected. All the tested compounds showed higher activity in the DPPH and FRAP assay compared to Vc at 100 μmol/L. Above all, the remarkable antioxidant properties survey demonstrated that P. glabra could be regarded as a complement to rich bioactive nutraceuticals that may help and enhance endogenous antioxidant protection.

Acknowledgements

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Reference


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