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In vitro antioxidant and antidiabetic activities of *Rehmannia glutinosa* tuberous root extracts

Hyun Ju Jeong[a], Ju-Sung Kim[b], Tae Kyung Hyun[c], Jinfeng Yang[a], Hak-Hee Kang[d], Jun-Cheol Cho[d], Hun-Myeong Yeom[d], Myong Jo Kim[a,*]

[a] Department of Applied Plant Sciences, Kangwon National University, 200-701, Chuncheon, Republic of Korea

[b] College of Applied Life Sciences, the Research Institute for Subtropical Agriculture and Biotechnology, Jeju National University, Jeju 690-756, Republic of Korea

[c] Department of Biochemistry, Gyeongsang National University, Jinju 660-701, Republic of Korea

[d] Skin Research Institute, AmorePacific Corporation R&D Centre, Yongin, Gyeounggi-do, 446-729, Republic of Korea

*Corresponding author, e-mail: kimmjo@kangwon.ac.kr

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ABSTRACT: To analyse the antioxidant and antidiabetic activities of *Rehmannia glutinosa* tuberous roots, extracts with nine different solvents were investigated. Antioxidant activity was measured with two methods, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and reducing power. Each extract exhibited a dose-dependent free radical scavenging action against DPPH and a reducing power, but diverse antioxidant activities. To determine the antidiabetic activity, we measured the α-glucosidase inhibitory capacity of each extract. We found that 10 μg/ml of 60% MeOH or 80% of MeOH extract inhibited more than 90% of α-glucosidase activity, and this inhibitory activity was not mediated by catalpol, a major iridoid constituent of *R. glutinosa* tuberous roots. Overall, the data suggested that the tuberous roots of *R. glutinosa* have potential as natural antioxidants and antidiabetics.

KEYWORDS: biological activity, α-glucosidase, solvent extract, HPLC, catalpol, 2,2-diphenyl-1-picrylhydrazyl

INTRODUCTION

Plant-based products have been known as both food and medicine since ancient times. Since acetylsalicylic acid, a synthetic derivative of salicin produced from willow bark, was first introduced in 1897[1], natural products have become major sources for chemical compounds used as starting materials[2]. In fact, approximately 40% of current mono-molecular medicinal drugs are derived directly or indirectly from plant species and their preparations[3]. In addition, many active substances such as alkaloids, phenolic compounds and mono-, di-, tri-, and sesquiterpens with different pharmaceutical functions have been introduced from a large number of plant species[3]. These indicate that plants are important sources of drugs as well as phytomedicines.

Rehmannia glutinosa Libosch., a species of the *Scrophulariaceae* family, is a perennial herb and one of the most important medicinal herbs in China and Korea. Its fresh and dried tuberous roots are used as a traditional Chinese medicinal ingredient for sedation, diabetes, haematological conditions, and insomnia[4–6]. The extract of its tuberous roots contains iridoids, monoterpenes, glycosides, fatty acids, amino acids, and microelements that have been reported to have various medical effects concerning reduction of blood pressure, blood circulation, immune capability, endocrine balance, and cardiovascular regulation[7–9]. Hence its economic importance is thought to result from the pharmaceutical activity present in the extracts of its tuberous roots[10]. Although these findings indicate that the extract of its tuberous roots has potential as a crude drug and a dietary health supplement, studies on the biological activity of tuberous roots have been limited.

Thus in this study, we analysed the biological activities of the crude extract from the tuberous roots of *R. glutinosa*. To better understand the biological activities of *R. glutinosa* tuberous roots, we determined the relationship between the amounts of phenols, flavonoid compounds and antioxidant activities. To determine the potential of *R. glutinosa* tuberous roots extracts as antidiabetic agents, we investigated the effect of extracts on the activity of α-glucosidase.

MATERIALS AND METHODS

Plant materials and extraction

The ground materials were extracted using different solvents: 60, 80, and 100% methanol, 60, 80, and 100% ethanol, ethyl acetate, acetone and water. Each solvent extract was evaporated to dryness using a rotary vacuum evaporator, and the solid residues were weighed.

DPPH free radical scavenging activity

The total free radical scavenging activity of various extracts was estimated by the DPPH method of Blois[11] with minor modifications. Initially, different concentrations of each extract in 4 ml MeOH were mixed with 1 ml DPPH (0.15 mM in MeOH). The reaction mixture was incubated for 30 min at 25 °C. The control contained all reagents without the extracts, while methanol was used as blank. The DPPH radical scavenging activity was determined by measuring the absorbance at 517 nm using a spectrophotometer. The DPPH radical scavenging activity of butylated hydroxytoluene (BHT) was analysed as a positive control. The DPPH activity was calculated as inhibition rate (%) according to

$$\left(1 - \frac{\text{absorbance value of sample}}{\text{absorbance value of control}}\right) \times 100\%.$$

The experiment was performed in triplicate.

Reducing power

The reducing power assay was determined according to the method described in Oyaizu[12]. The tested samples were mixed with 0.5 ml of 0.2 M Na_3PO_4 buffer (pH 6.6) and 1% potassium ferricyanide. After incubation at 50 °C for 20 min, the mixture was mixed with 2.5 ml of 10% trichloroacetic acid, and then incubated for 10 min. After centrifugation at 5000g for 10 min, the supernatant (0.5 ml) was mixed with distilled water (0.5 ml), and then ferric chloride (0.1 ml, 0.1%) was added. The absorbance was read spectrophotometrically at 700 nm. The reducing power of BHT, α-tocopherol was also determined for comparison.

Total phenolic compound analysis

Total phenol contents were determined using Folin-Ciocalteu reagent as described[13] with minor modifications. The extract (0.1 ml) was mixed with 50 µl of 2 N Folin-Ciocalteu reagent, and allowed to stand for 3–5 min at room temperature. Then, 0.3 ml of 20% Na_2CO_3 was added to the mixture. After 15 min at room temperature, 1 ml of distilled water was added. The absorbance was measured at 725 nm using a UV-spectrophotometer. Total phenolic were quantified by a calibration curve obtained from measuring the absorbance of gallic acid standard. The concentration was expressed as mg of gallic acid equivalents per gram of extract.

The analysis of total flavonoid contents

The total flavonoid content of the extract was determined according to the colorimetric method as described by Moreno et al[14]. To do so, 0.5 ml of each extract was added to test tubes containing 0.1 ml of 10% $Al(NO_3)_3$ (w/v), 0.1 ml of 1 M potassium acetate and 4.3 ml of 80% ethanol. After incubation for 40 min at room temperature, the absorbance was determined at 415 nm. The total flavonoid content was expressed in milligrams of quercetin equivalents (QE) per gram of extract.

α-Glucosidase inhibitory effect

B. stearothermophilus α-glucosidase inhibition assay was performed as described previously[15]. α-glucosidase (50 µl, 0.5 U/ml) and 0.2 M K_3PO_4 buffer (pH 6.8, 50 µl) were mixed with 50 µl of the test sample. After pre-incubation at 37 °C for 15 min, 3 mM pNPG (100 µl) was added. The enzymatic reaction was allowed to proceed at 37 °C for 10 min, and was stopped by the addition of 750 µl of 0.1 M Na_2CO_3. The 4-nitrophenol absorption was measured at 405 nm using a spectrophotometer. A solution without sample was used as a control, and a solution without substrate was used as a blank. The antidiabetic drug acarbose was also assayed as a standard reference. The percent inhibition of α-glucosidase was calculated as

$$\left(1 - \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{control}}}\right) \times 100\%$$

where $\text{Abs}_{\text{sample}}$ represents the absorbance of the experimental sample, $\text{Abs}_{\text{blank}}$ represents the absorbance of the blank, and $\text{Abs}_{\text{control}}$ represents the absorbance of the control.

Analysis of catalpol contents

To determine catalpol contents, the standard and samples were separately dissolved in methanol and filtered (0.45 µm) for HPLC analysis. For HPLC, a Younglin liquid chromatograph (Hogye-dong, Anyang, Korea), which consisted of a pump, a column chamber, a multi-wavelength detector, and a Midas for LC system, was used. Chromatographic separation was carried out at room temperature using a j'sphere ODS

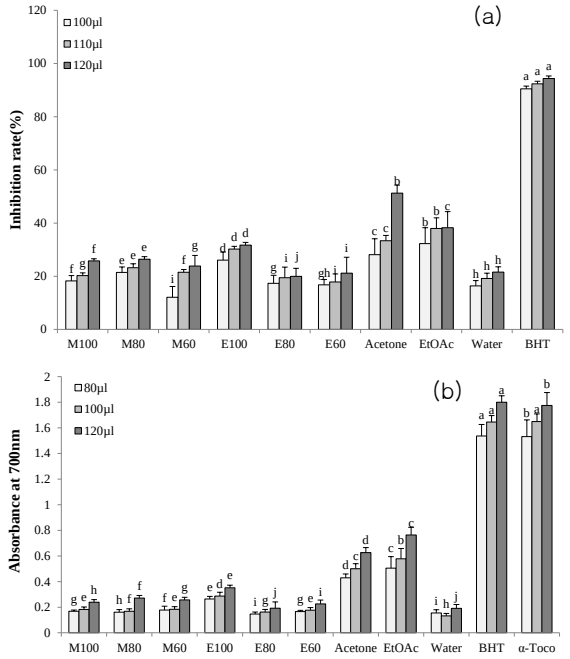

Fig. 1 The antioxidant activities of *R. glutinosa* tuberous root extracts. DPPH free radical scavenging activity (a) and reducing power (b) of extracts. Each value is mean ± SD of triplicate determinations. Mean separation within columns by Duncan's multiple range test at 0.1% level. M100, 100% MeOH; M80, 80% MeOH; M60, 60% MeOH; E100, 100% EtOH; E80, 80% EtOH; E60, 60% EtOH.

H80 analytical column (250 × 4.6 mm, 5 µl, YMC, Milford, USA). The mobile phase was a mixture of phosphatic acid/water (3:97 v/v) at a flow rate of 1.0 ml/min. The column temperature was 25 °C and the reading was taken at 204 nm wavelength.

Statistical analysis

The data are expressed as the means ± SD of the values. Statistical significance was determined by ANOVA. Duncan's multiple range tests were used to determine the significance of differences between the groups. A level of $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Antioxidant properties of *R. glutinosa* tuberous roots extract

As shown in Fig. 1a, DPPH free radical scavenging activity of all extracts increased in a dose-dependent manner. The highest activity was obtained from the acetone extract (51 ± 3%), whereas 80% EtOH (E80) extract exhibited the lowest DPPH free radical scavenging activity compared with other extracts. The percent scavenging activity of all fractions from *R. glutinosa* tuberous roots is presented in the following descending order: Acetone extract > ethyl ethanoate (EtOAc) extract > 100% EtOH (E100) extract > 80% MeOH (M80) extract > 100% MeOH (M100) extract > 60% MeOH (M60) extract > water extract > 60% EtOH (E60) extract > E80 extract. In the reducing power assay, more antioxidant compounds converted the Fe^{3+} in ferric chloride to Fe^{2+}. This assay is known as a robust and useful method for measuring a wide concentration range of antioxidant activities and capacities [16]. The results of this assay showed that the reducing power of the EtOAc and acetone extracts was higher than that of other extracts (Fig. 1b), suggesting that they possess stronger radical scavenging capacity than other extracts.

Table 1 Comparative analysis of extraction yield, total phenolic content and total flavonoid content in *R. glutinosa* tuberous root extracts.

Extraction solvent	TPC[a] (mg GAE/g)	TFC[b] (mg QE/g)
methanol	4.6 ± 1.1e	0.8 ± 0.1e
80% methanol	4.0 ± 0.4e	0.1 ± 0.2h
60% methanol	4.5 ± 0.6d	0.6 ± 0.1f
ethanol	8.8 ± 0.2c	1.9 ± 1.2b
80% ethanol	4.2 ± 0.7e	1.1 ± 0.5d
60% ethanol	4.1 ± 0.7e	1.4 ± 0.4c
acetone	26.6 ± 0.3a	5.7 ± 0.3a
ethyl acetate	11.2 ± 0.8b	5.7 ± 0.5a
water	5.00 ± 0.3d	0.2 ± 0.1g

[a] Total phenol content analysed as gallic acid equivalent mg/g of extract, values are averages of triplicates
[b] Total flavonoid content analysed as quercetin equivalent mg/g of extract, values are averages of triplicates
Each value is mean ± SD of triplicate determinations. Mean separation within columns by Duncan's multiple range test at 0.1% level.

To determine the relationship between the level of phenolic compounds and free radical scavenging activity, we analysed the total phenol content (TPC) and total flavonoid content (TFC) of different extracts of *R. glutinosa* tuberous roots. As shown in Table 1, the acetone extract contained the highest amount of total phenolic compounds. The total flavonoid levels of all extracts proceeded in the following order: Acetone extract > EtOAc extract > E100 extract > E60 extract > E80 extract > M100 extract > M60 extract > M80 extract > water extract. This indicates that phenolic acids and flavonoid compounds

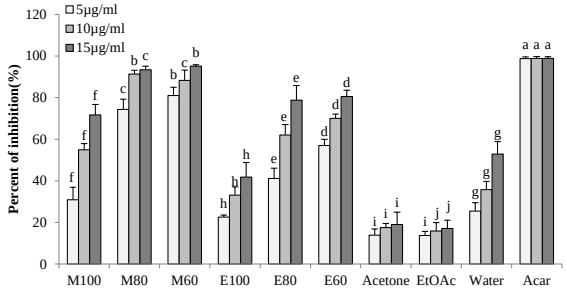

Fig. 2 Inhibitory effects of *R. glutinosa* tuberous root extracts on α-glucosidase activity. Each value is mean ± SD of triplicate determinations. Mean separation within columns by Duncan's multiple range test at 0.1% level.

Table 2 Content of catalpol in different extracts from *R. glutinosa* tuberous roots.

Extraction solvent	catalpol (μg/ml)
M100	55.7 ± 0.4d
M80	55.9 ± 0.8d
M60	59.9 ± 0.7b
E100	68.0 ± 0.4a
E80	55.0 ± 1.3e
E60	53.6 ± 2.0f
acetone	48.2 ± 4.1g
EtOAc	35.1 ± 2.1h
eater	57.3 ± 5.7c

Each value is mean ± SD of triplicate determinations. Mean separation within columns by Duncan's multiple range test at 0.1% level.

are the major antioxidant compounds in the tuberous roots of *R. glutinosa*. However, the EtOAc extract, particularly at low concentrations, exhibited a slightly higher level of antioxidant activity compared to the acetone extract (Fig. 1). It is remarkable that the antioxidant activity was higher, even though the acetone extract contained more than twice the amount of total phenolic compounds compared with the EtOAc extract (Table 1). It is not possible to determine a correlation between the total phenol and flavonoid contents and antioxidant capacities of the EtOAc and acetone extracts of *R. glutinosa* tuberous roots due to the presence of interfering phytochemicals like ascorbic acid, tocochromanols, and pigments. Alternatively, different types of phenolic compounds may have different free radical scavenging activities mediated by their structure[17,18]. Thus the EtOAc extracts of *R. glutinosa* tuberous roots may contain different types of phenolic compounds with higher antioxidant capacities compared with those in the acetone extracts.

α-Glucosidase inhibitory activity of *R. glutinosa* tuberous root extracts

We investigated the effects of different extracts from *R. glutinosa* tuberous roots on the activity of α-glucosidase, which is the target of drugs currently used to treat type 2 diabetes. As shown in Fig. 2, the M60 and M80 extracts significantly inhibited α-glucosidase activity, whereas the EtOAc and acetone extracts showed low inhibitory effects. In addition, 15 μg/ml of the E60 or E80 extract displayed about 80% of the inhibitory activity.

Catalpol is the major iridoid constituent of *R. glutinosa* tuberous roots[19], and has many pharmaceutical functions like protection of liver damage and reduction of elevated blood sugar[20]. Thus we hypothesized that the presence of high amount of catalpol in the M60 extract would result in a strong inhibition of α-glucosidase activity. To test this hypothesis, we measured the amount of catalpol in different extracts (Table 2). Most extracts contained similar amounts of catalpol (ranged from 48.2 μg/ml to 59.7 μg/ml), whereas the EtOAc and E100 extracts contained 35.1 μg/ml and 68.1 μg/ml of catalpol, respectively. This proves that catalpol is not an inhibitor compound of α-glucosidase. Similar levels of total phenolic and flavonoid contents were found in the M60, M80, and M100 extracts (Table 1). In addition, acetone and EtOAc extracts contained a notable amount of TPC and TFC (Table 1), whereas these extracts exhibited lower level of α-glucosidase inhibitory activity compared to other extracts (Fig. 2). Although phenolic compounds in medicinal plants do inhibit α-glucosidase[21], these findings indicate that the activity of α-glucosidase is probably independent on the levels of TPC and TFC in the extracts. This non-correlation between TPC (and TFC) and α-glucosidase inhibitory activity should be mediated by some of the polyphenols, which are existing in M60 and M80 and extremely active owing to their structural characteristics even if they are present in smaller quantities. Other possibility is that *R. glutinosa* tuberous roots contain other non-polyphenolic active compounds, such as polysaccharides, which possess strong α-glucosidase inhibitory activity[22–24]. Hence to clarify the anti-diabetic property of *R. glutinosa* tuberous roots, the purification and identification of active the compounds will be necessary.

Conclusions

To investigate the biological activities of *R. glutinosa* tuberous roots, we analysed the antioxidant and anti-diabetic activities of its extracts which were prepared

using different solvents. As a result, we found that the extracts of *R. glutinosa* tuberous roots have free radical scavenging activity and inhibitory activity against α-glucosidase. Although the effects of *R. glutinosa* tuberous root extracts have been established in vitro, these results indicate that *R. glutinosa* has potential as a crude drug and a dietary health supplement. Further studies on the isolation and characterization of active compounds from *R. glutinosa* extracts are needed using various techniques.

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