The Role of C-8 OH on the Antioxidant Activity of Norwogonin and Isowogonin

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Abstract

In the present study, the antioxidant property of 4 flavones (moslosooflavone, wogonin, isowogonin, and norwogonin) was evaluated using 6 different assays: 1,1-diphenyl-2-picrylhydrazyl (DPPH·), superoxide (O2•−), and nitric oxide (NO) radical scavenging assays, ferrous iron chelation, reducing power, and total antioxidant capacity. The 4 flavones exhibited antioxidant activities with decreasing order as norwogonin > isowogonin >> wogonin > moslosooflavone. The present results demonstrated that norwogonin and isowogonin exhibited excellent antioxidant activity, which was mainly based on the presence of C-8 hydroxyl group.

Keywords

flavones, norwogonin, isowogonin, wogonin, moslosooflavone, C-8 hydroxyl group, antioxidant activity

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Flavonoids are a large group of plant secondary metabolites with significant antioxidant properties and widespread in fruits, vegetables, and beverages. Recently, they have received much more attention due to their positive impact on human health. Flavones are one of the important subgroups of flavonoids and own a wide range of biological activities, such as antimicrobial, antiviral, antiproliferative, anti-diabetic, anti-ischemic, anti-inflammatory, and cardioprotective effects. It is believed that these biological effects come from the antioxidant activity of flavones, which is due to their ability to directly scavenge free radicals and to reduce free radical formation by binding metal ions and inhibiting enzymatic systems.

According to the results of the structure-activity relationships, the antioxidant activity of flavones increases with an increase in the number of hydroxyl groups in molecule. Besides that, the presence of C2-C3 double bond, C4 keto group, hydroxyl groups in positions 3 and 5, and ortho-dihydroxy group in molecule is also considered as important structural characteristics for antioxidant potency. However, very little is known about the effects of C-8 OH on the antioxidant activity of flavones.

In the present study, 4 bioactive polyhydroxy flavones isolated from Scutellaria baicalensis Georgi (Huang Qin in Chinese): wogonin (5,7-dihydroxy-8-methoxyflavone), isowogonin (5,8-dihydroxy-7-methoxyflavone), norwogonin (5,7,8-trihydroxyflavone), and moslosooflavone (5-hydroxy-7,8-dimethoxyflavone), were selected to explore the role of C-8 OH on the antioxidant activity of flavones. Structurally, the B-ring in the 4 flavones does not contain any hydroxy, methoxy, or other substituent. The only difference among them is the presence of hydroxy or methoxy groups at the positions 5, 7, and 8 of the A-ring (Figure 1).

Results and Discussion

Due to the fact that flavones exert their antioxidant activity through various mechanisms, including chelating ferrous iron, degrading peroxide, and scavenging free radicals, more than one antioxidant assay should be used in order to fully determine and compare the antioxidant effects of the flavones. Therefore, the antioxidant activity of the 4 flavones was investigated by assessing their effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH·), superoxide anion (O2•−), nitric oxide (NO) radical scavenging activity, metal chelating ability, reducing power, and total antioxidant capacity (TAC).
The DPPH· radical scavenging assay is an easy, rapid, and sensitive method and has widely been used for evaluating the free radical-scavenging capacities of flavones. This methodology is based on the theory that DPPH· is scavenged by an antioxidant through a donation of hydrogen to form a stable DPPH-H molecule. The 5,7,8-trihydroxyflavone norwogonin has 2 hydroxyl groups in ortho position, which is considered as one of the best features for an antioxidant compound. As expected, norwogonin exhibited the strongest scavenging efficiency on DPPH·, and its EC_{50} value was 0.26 ± 0.01 mmol·mL^{-1} (Figure 2a and Table 1). Generally speaking, the more hydroxyl means higher electron transfer/hydrogen donating ability, while the introduction of methoxyl group increased the space steric which is adverse to electron transfer process and then decreased the radical scavenging activity. Replacement of C-7 hydroxyl group of norwogonin by a methoxyl group (isowogonin) only slightly decreased the DPPH· scavenging activity (EC_{50} = 0.28 ± 0.01 mmol·mL^{-1}), whereas replacement of C-8 hydroxyl group of norwogonin by a methoxyl group (wogonin and moslosooflavone) exhibited little or no DPPH· radical scavenging activity at the concentrations tested. The weak free radical scavenging activity on DPPH· of wogonin has also been supported by the study of Woźniak et al.\textsuperscript{11} and Liau et al.\textsuperscript{11} One hypothesis for the observed difference might be attributed to the fact that C-8 hydroxyl group owned stronger hydrogen donating ability than C-7 hydroxyl group. From the above results, it was concluded that the hydroxyl group in position 8 on the A-ring played a vital role in DPPH· free radical scavenging activity of flavones and ortho-dihydroxy group could improve the scavenging activity.

Superoxide radicals, generated via the leakage of an electron from the electron transport chain to molecular oxygen during oxidative phosphorylation in mitochondria, are the primary ROS in vivo and involved in many pathological processes.\textsuperscript{12} Although they are not highly reactive, they can form stronger ROS, including hydrogen peroxide and hydroxyl radicals, and initiate damage to DNA.\textsuperscript{13} Many diseases, such as cancer, cardiovascular disease, and neurodegenerative disorders, and aging can contribute to the excessive production of O_{2}^{•−}.\textsuperscript{14} Flavones are superoxide scavengers and would be promising agents for the treatment of related diseases.\textsuperscript{18} In the present study, the scavenging capacity of the flavones toward superoxide anion radicals was evaluated by using PMS-NADH-NBT system. As shown in Figure 2(b) and Table 1, the O_{2}^{•−} radical scavenging activity of flavones was in the order norwogonin > isowogonin >> wogonin > moslosooflavone. Norwogonin and isowogonin could significantly inhibit O_{2}^{•−} radical with EC_{50} value of 0.11 ± 0.01 mmol·mL^{-1} and 0.96 ± 0.10 mmol·mL^{-1}. The O_{2}^{•−} radical scavenging activity of norwogonin is greater than that of rutin (EC_{50} = 0.40 ± 0.03 mmol·mL^{-1}) due to the presence of ortho-dihydroxy group.\textsuperscript{16} Isowogonin and wogonin have the same number of hydroxyl group, but wogonin showed little scavenging activity on O_{2}^{•−} radical with EC_{50} values over 2 mmol·mL^{-1}, which is in agreement with previous report.\textsuperscript{11} Moslosooflavone almost did not have scavenging activity on O_{2}^{•−} radical. The presence of C-8 hydroxyl group maintains the O_{2}^{•−} radical scavenging activity, while the methoxyl group in C-8 prevents wogonin and moslosooflavone from reacting efficiently with the O_{2}^{•−} radicals. This suggests that the hydroxyl group at position 8 should be responsible for the difference in the observed activity.

NO, produced by endothelial cells and macrophages, plays an important role in maintaining the dilation of blood vessels under physiological conditions.\textsuperscript{15} Same as O_{2}^{•−}, NO is not a very reactive free radical, but it is able to react with O_{2}^{•−} to form more reactive peroxynitrite, which can trigger nitrosative damage on biomolecules. Furthermore, overgeneration of NO can result in oxidative damage and is involved in ischemia reperfusion and neurodegenerative and chronic inflammatory diseases.\textsuperscript{18,20} It was reported that flavones could directly scavenge NO.\textsuperscript{21} Previous study showed that wogonin displayed potent inhibition of NO production in LPS-elicited RAW 264.7 macrophages with IC_{50} = 45.3 ± 0.2 µM.\textsuperscript{22} In the present study, we also got similar results. As shown in Figure 2(c) and Table 1, at 2 mmol·mL^{-1} concentration, norwogonin, isowogonin, wogonin, and rutin could scavenge NO radical by 54.27%, 49.49%, 30.81%, and 54.36%, respectively, while moslosooflavone, in which both C-7 and C-8 hydroxyl groups were methylated, exhibited very low scavenging activity on NO radical (only 2.23%) at this concentration. In addition, the NO scavenging activity also decreased when the hydroxyl group at the C-7 or C-8 position of the A-ring was replaced by methoxyl group, but isowogonin was more potent than wogonin in scavenging NO. These results indicated that both C-7 and C-8
hydroxyl groups contribute to the NO scavenging activity of flavones, while C-8 hydroxyl group is more efficient than C-7 hydroxyl group.

Transition metal ions are essential for many physiological functions, but excessive concentration of metal ions may be toxic, leading to formation of free hydroxyl radicals by Fenton reaction. Transition metal ions are essential for many physiological functions, but excessive concentration of metal ions may be toxic, leading to formation of free hydroxyl radicals by Fenton reaction.23 In addition to directly scavenging free radicals, flavones can exhibit antioxidant activity as a metal ion chelator. Since iron is the most abundant metal in the body, the ferrous iron chelating ability of flavones was investigated in the present study. As shown in Figure 2(d) and Table 1, EDTA shows very strong \( \text{Fe}^{2+} \) chelating capacity with an IC\(_{50} = 0.26 \pm 0.01 \) mmol mL\(^{-1}\), while the 4 flavones only exhibited weak chelating activity on \( \text{Fe}^{2+} \) with EC\(_{50} > 2 \) mmol mL\(^{-1}\). At concentration of 2 mmol mL\(^{-1}\), the \( \text{Fe}^{2+} \) chelating activity of norwogonin, isowogonin, wogonin, and moslossoflavone was 21.52\%, 10.03\%, 8.98\%, and 8.23\%, respectively. Results showed that norwogonin containing the 7,8-dihydroxyl substitution had a higher chelating ability than other flavones, blockade of the hydroxyl at C-7 or C-8 by methylation dramatically

Figure 2. Antioxidant effect of moslossoflavone, wogonin, isowogonin, and norwogonin. (a) DPPH• radical scavenging assay; (b) superoxide radical scavenging assay; (c) NO radical scavenging assay; (d) Fe(2+) chelating ability; (e) reducing capacity; (f) total antioxidant capacity (means ± SD, \( n = 3 \)). DPPH, 1,1-diphenyl-2-picrylhydrazyl.
reduced the activity. Our study suggested that the 7,8-dihydroxyl groups played an important role in the ferrous iron chelating ability of norwogonin, while C-7 or C-8 hydroxyl group alone had little contribution to the activity. Interestingly, the ferrous iron chelating ability of isowogonin, wogonin, and moslosooflavone is nearly equivalent. Moslosooflavone, in which both C-7 and C-8 hydroxyl groups were replaced by methoxyl group, still exhibited weak ferrous iron chelating ability, which may be attributed to the C-4 keto group and C-5 hydroxyl group, still exhibited weak ferrous iron chelating ability. It is reported that reducing power is concomitant with antioxidant activity and related to the electron transfer ability. It is reported that reducing power is concomitant with antioxidant activity and related to the electron transfer ability. Flavones are considered as good electron and hydrogen atom donors and may serve as excellent reducing agents. As evidenced in Figure 2e and Table 1, norwogonin and isowogonin presented linearly dose-dependent increases in absorbance, while wogonin and moslosooflavone showed very weak reducing power. Their reducing power followed the order norwogonin > isowogonin >> wogonin ≈ moslosooflavone. This order was same as the aforementioned results in DPPH· and O2•− scavenging capacity and reducing power, suggesting flavones that possess C-8 hydroxyl group appear to have excellent TAC. In contrast the C-5 and C-7 hydroxyl groups in flavones did not significantly contribute to TAC.

### Conclusions

Collectively, our current study suggested a fundamental structure-activity relationship concerning antioxidant activity of the 4 natural flavones. We could draw a conclusion that both norwogonin and isowogonin had excellent antioxidant activity, suggesting that C8-OH was the prominent antioxidant active groups for them. We hope our data will provide enlightenment for the coming studies of designing B-ring unsubstituted flavone derivatives with excellent antioxidant activity. More importantly, the findings indicated that norwogonin and isowogonin afforded outstanding antioxidant capacity and might be promising candidates as dietary natural products in the treatment of oxidative related diseases.

### Experimental

#### Reagents and Materials

Wogonin (batch no: 20150812) was purchased from Ci Yuan Biotechnology Co., Ltd (Xian, Shannxi, China) whereas moslosooflavone, isowogonin, and norwogonin were synthesized according to our previous reported method. Wogonin, isowogonin, norwogonin, and moslosooflavone were dissolved in dimethyl sulfoxide (DMSO), stored at −20°C, and diluted in medium immediately before using. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Nicotinamide adenine dinucleotide (NADH), phenazinemethosulfate (PMS), nitroblue tetrazolium chloride (NBT), 2-thiobarbituric acid (TBA), sodium nitroprusside, N-(1-naphthyl)ethylenediamine

#### Table 1. Determination of Antioxidant Activity of Norwogonin, Isowogonin, Wogonin, and Moslosooflavone.

| Compound            | EC50 (mmol·mL⁻¹) | EC50 (mmol·mL⁻¹) | EC50 (mmol·mL⁻¹) | EC50 (mmol·mL⁻¹) | Reducing power | TAC  |
|---------------------|------------------|------------------|------------------|------------------|---------------|------|
| Norwogonin          | 0.28 ± 0.01⁷     | 0.11 ± 0.01⁷     | 54.27 ± 0.58⁴    | 21.52 ± 0.48⁴    | 0.32 ± 0.01⁷  | 0.45 ± 0.01⁷ |
| Isowogonin          | 0.26 ± 0.01⁸     | 0.96 ± 0.09⁹     | 49.49 ± 1.61⁴    | 10.03 ± 0.15⁷    | 0.34 ± 0.01⁹  | 0.54 ± 0.01⁸ |
| Wogonin             | >1               | >1               | 30.81 ± 1.04⁶    | 8.89 ± 0.39⁷     | >0.5          | >1   |
| Moslosooflavone     | >1               | >1               | 2.23 ± 0.38⁸     | 8.24 ± 0.14⁷     | >0.5          | >1   |
| Rutin               | 0.15 ± 0.01⁸     | 0.40 ± 0.03⁹     | 54.36 ± 0.53⁴    | ND               | 0.15 ± 0.01⁸  | 0.67 ± 0.01⁷ |
| EDTA                | ND               | ND               | ND               | 100 ± 0.13       | ND            | ND   |

Note: The data in the table were represented as means ± SD (n = 3). The statistically different results were represented by different superscript letters in each column, P < 0.05.
DPPH· radical scavenging assay. The ability of flavones to scavenge the DPPH· radical was determined according to Girgih et al’s method with slight modification. In brief, 125 µL of various concentrations of flavones dissolved in DMSO was mixed with 125 µL of the DPPH· solution (100 µM∙mL⁻¹ in methanol) in a 96-well plate. After an incubation period of 30 minutes in dark at room temperature, the absorbance of the samples was read at 517 nm. The blank reaction consisted of 100 µL DMSO mixed with 100 µL of the DPPH· solution. Rutin was used as the positive control. Lower absorbance indicated higher DPPH· scavenging activity. Inhibition of DPPH radical was calculated using the equation below:

\[
\text{DPPH· scavenging effect (\%)} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \quad (1)
\]

where \(A_0\) is the absorbance of blank and \(A_1\) is the absorbance of samples at different concentrations. The EC \(_{50}\) value is calculated as the concentration of samples that causes 50% inhibition of DPPH· radical formation. A lower EC \(_{50}\) value corresponds to a higher antioxidant activity.

Superoxide anion scavenging assay. Superoxide anion scavenging activity was determined according to the method of Shukla with some modifications. About 100 µL of various concentrations of flavones, 50 µL of NADH (0.5 mmol∙L⁻¹ in 0.1M Tris-Hcl, pH 8.0) solution, and 50 µL of NBT (0.2 mmol∙L⁻¹ in distilled water) were mixed well. Then 50 µL of PMS (25 µmol∙L⁻¹ in distilled water) was added. The reaction mixture was incubated at room temperature for 15 minutes. The absorbance was measured at 570 nm. Rutin was used as a positive control. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage of scavenging was calculated by equation (1).

Nitric oxide (NO) scavenging assay. The NO radical scavenging activity was measured by the inhibition of spontaneous NO formation from sodium nitroprusside in solution. In brief, 50 µL of various concentrations of flavones was added to 50 µL of sodium nitroprusside (10 mmol∙L⁻¹ in phosphate buffer, pH 7.4). The resulting solution was incubated under light at room temperature for 150 minutes. Then 50 µL of 0.33% (w/v) sulfanilamide in 28 mM sodium phosphate and 4 mM ammonium molybdate in 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride was added. The absorbance at 540 nm was recorded in a microplate reader after 30 minutes. Rutin was used as reference standard. The NO radical scavenging activity was calculated according to equation (1).

Ferrous iron chelation. The ability of the sample to chelate ferrous ions was determined according to the reported method. Briefly, 50 µL of FeCl₂·4H₂O solution (2 mmol∙L⁻¹) was added to 1 mL of various concentrations of flavones. Then 200 µL of ferrozine solution (5 mmol∙L⁻¹) was added to the mixture to initiate the reaction. The resulting mixture was shaken vigorously and left at room temperature for 10 minutes. The absorbance of the solution was read at 562 nm. EDTA was used as the positive control. The percentage inhibition of ferrozine-Fe²⁺ complex formation was calculated using equation (1).

Reducing power. The reducing power assay was performed according to the method of Oyaizu. Accordingly, 100 µL of various concentrations of flavones was mixed with 2.5 mL of sodium phosphate buffer (0.2 mol∙L⁻¹, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated for 30 minutes at 50°C in a water bath follow by the addition of 2.5 mL of 10% TCA. The mixture was centrifuged at 3000 rpm for 10 minutes. The upper layer fraction (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. The absorbance was read at 700 nm after 10 minutes. Rutin was used as a positive control. A higher absorbance indicated a higher reducing power. The EC \(_{50}\) value is calculated as the concentration of samples at which the absorbance is 0.5.

Phosphomolybdate assay (total antioxidant capacity). Total antioxidant activity of the flavones was determined by the phosphomolybdate method. About 100 µL of various concentrations of flavones was mixed with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The resulting solution was incubated at 95°C for 90 minutes. After the resulting solution was cooled to room temperature, the absorbance of the solution was measured at 695 nm. Rutin was used as a positive control. A higher absorbance indicated a higher TAC. The EC \(_{50}\) value is calculated as the concentration of samples at which the absorbance is 0.5.

Statistical Analyses

All experiments were performed in triplicate, and the results were expressed as means ± standard deviation (SD). The statistical analysis was conducted with one-way ANOVA, followed by the Tukey’s test. Statistical significance was defined as \(P < 0.05\).

Declaration of Conflicting Interests

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