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The radical scavenging activities of *radix puerariae* isoflavonoids: A chemiluminescence study

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Abstract

The purpose of this paper was to determine the radical scavenging activities of *radix puerariae* isoflavonoids (RPI) toward different reactive oxygen species (ROS), including superoxide anion($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), lipid-derived radicals ($R^{\cdot}$) and singlet oxygen ($^1O_2$), using a chemiluminescence technique. RPI used here contained puerarin (PU), daidzin, daidzein and others as tested by HPLC. $O_2^{\cdot-}$, $OH^{\cdot}$, $R^{\cdot}$, and $^1O_2$ were generated from a pyrogallol autoxidation system, Fenton reaction system, H_2O_2-induced oxidation of unsaturated fatty acid system and NaClO–H_2O_2 system, respectively. RPI could effectively scavenge $O_2^{\cdot-}$, $OH^{\cdot}$, $R^{\cdot}$, and $^1O_2$ at EC_{50} of 0.08, 0.005, 0.03, and 0.004 mg/ml, respectively. In addition, the radical scavenging activities of RPI were much higher than that of its main component PU, and similar to that of tea polyphenols, indicating that RPI, not PU, was a powerful antioxidant.

Keywords: Isoflavonoids; *Radix puerariae*; Reactive oxygen species; Chemiluminescence

1. Introduction

Modern theory of free radical biology and medicine (Freeman & Crapo, 1982; Halliwell, Antonia, Susanna, & Okezie, 1995) suggests that reactive oxygen species (ROS), such as $O_2^{\cdot-}$, $OH^{\cdot}$, $R^{\cdot}$ and $^1O_2$, are involved in the course of aging, cancers and cardiovascular diseases, and supplementation of plant-derived antioxidants is believed to contribute a good protection against such disorders. Therefore, plant-derived antioxidants are now receiving a special attention.

Isoflavonoids are plant polyphenolic antioxidants (Qiong, Gerald, Hadi, Moini, & Lester, 2002) that are primarily found in legumes, especially in soybean and various soybean-based food products (Hodgson, Croft, Puddey, Mori, & Beilin, 1996). Recent studies show that isoflavonoids also occur abundantly in *radix puerariae*, the root of a wild leguminous creeper, *Pueraria lobata* (Wild) Ohwi, which has long been used as herbal medicine (Fang, 1980; Keung & Vallee, 1993), as well as the raw material of functional food in China. The antioxidant activities of *radix puerariae* isoflavonoids have been roughly evaluated in rats (Speroni et al., 1996) as well as in peroxidase/H_2O_2/luminol/enhancer systems (Guerra et al., 2000). However, chemically, its radical scavenging activities toward reactive oxygen species (ROS) have not, to our knowledge, been well investigated to date.

Chemiluminescence (Archer, Nelson, & Weir, 1989) is a simple, direct and effective method for free radical and antioxidant study. Previous work (Yu, Zhao, Xue, Jin, & Wang, 2001) has established a series of ROS chemiluminescene systems.

In the present work, the radical scavenging activities of RPI towards ROS were evaluated in different chemical systems and were compared with those of its main component, puerarin, as well as those of tea polyphenols.

2. Materials and methods

2.1. Materials

Radix Puerariae was from Huangshan Crude Drug Co. (Huangshan, China). Luminol and 1,10-phenanthroline

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were purchased from Sigma Co. (St. Louis, MO, USA). Daidzein and daidzin were from Xiehe Med. Factory of Science Institute of China (Beijing, China). Tea polyphenols were from Qunli Natural product Co. (Hai Nan, China). Pyrogallol was from Beijing Chemical Co. Ltd. (Beijing, China). Buffered saline solution (BSS, pH 10.2, 7.38) and distilled water were prepared in our laboratory.

2.2. Extraction and analysis of RPI and purification of PU

One kilogramme of *radix puerariae* was first gently pulverized and was then refluxed with 3000 ml 95% ethanol for 3 h at 80 °C under the protection of N_2. After that, the mixture was filtered through a paper filter and the filtered solution was evaporated to dryness under a stream of nitrogen with a vacuum evaporator (Shanghai No.6 instrument Factory, Shanghai, China) to obtain 164.2 g RPI which was assessed on a Waters 2690 HPLC using a 5 μm particle size Hypersil C18 column (4.6 mm × 200 mm) (Chrompack, USA). The flow phase was a mixture of methanol, chloroform and water (40/2/58, v/v/v) and the flow rate was 1 ml/min. The elution peaks were detected at 280 nm.

500 ml HCl (6%) was added into 164.2 g RPI to form a hydrolysis solution at 35 °C after 2.5 h. The hydrolysis solution was then extracted twice by 300 ml chloroform and the chloroform solution was diluted twofold and kept below 0 °C for 3 days; then a crude PU crystal substance was obtained, and finally the crystals were recrystallized twice move from acetic acid and methanol solution (1:1, v/v) to obtain 1.4 g PU (purity of 97.0% as measured by HPLC).

2.3. Evaluation of the radical scavenging activity of RPI, PU, tea polyphenols

Radical scavenging activities of RPI were assessed on a SH-G Biochemistry Chemiluminescence Meter (BCM). (Shanghai Measurement Equipment Factory, Shanghai, China). The BCM is composed of three parts: an automatically rotating sample support in which 12 sample cells (glass tube, diameter = 10 mm, height = 20 mm) can be placed, a chemiluminescence monitor and a data processor. Each sample cell can rotate and cross the monitor at a set time interval according to a self-made programme. When testing, the chemiluminescence intensity (CL) of a reaction system can be recorded in the data processor at the set time interval.

A definite weight of RPI sample was first dissolved in distilled water to form a solution, which was then diluted into a series of different concentrations of solutions for the following test. PU and tea polyphenol samples were treated in a similar way. Superoxide anion assay (Yu et al., 2001): superoxide anion was generated by pyrogallol autoxidation. The reaction mixture contained 50 μl pyrogallol (1×10^{-3}M), 700 μl BSS (pH 10.2), 20 μl luminol (1×10^{-3} M). A sample cell loaded with the mixture was first placed in the BCM. When the cell crossed the monitor, a known concentration of sample was injected into the cell in situ. The CL was simultaneously recorded in the processor and then was recorded once every 6 s (CBSS instead of specimen was present in control). The scavenging rate was obtained according to the formula: scavenging rate (%) = (CL(control) − CL(sample)) × 100/CL(control).

Hydroxyl radical was assigned (Yu et al., 2001) after generation by a Fenton-type reaction. The reaction mixture included 20 μl $FeCl_2$ (1×10^{-3} M), 30 μl 1,10-phenanthroline (1×10^{-3} M), 800 μl BSS (pH 7.38) and 50 μl H_2O_2 (0.6%). The testing procedure and scavenging rate formula were similar to those for the superoxide anion assay.

Lipid-derived radicals were assigned after lipid peroxidation, generated by the oxidation of primrose oil (unsaturated fatty acid content >80%), induced by H_2O_2. The reaction mixture contained 100 μl evening primrose oil, 800 μl BSS (pH 7.38), 100 μl H_2O_2 (0.6%) in sample cells. The testing procedure and scavenging rate formula were similar to those for the superoxide anion assay.

Singlet oxygen was asigned (Yu et al., 2001) after singlet oxygen was chemically generated by the reaction between NaOCl and H_2O_2 in a solution of pH 8 at 37 °C. The reaction mixture included 200 μl NaOCl (1.4 mmol/l), and 800 μl BSS (pH 8) and 50 μl H_2O_2 (0.6%). The testing procedure and scavenging rate formula were similar to those for the superoxide anion assay.

2.4. Statistical analysis

Chemiluminescence data were processed using an origin 6.0 software (Microcal Software, Inc., Northampton, MA, USA). The experiments were repeated three times.

3. Results and discussion

3.1. HPLC spectrum of RPI

Fig. 1 shows the HPLC spectrum of RPI, where three peaks, at retention times of 4.227, 7.498 and 11.532 min are identified as daidzein, PU, daidzin, respectively, and where PU is the main component. This result is consistent with those obtained by Chen, Zhang, and Ye (2001) and Wing and Bert (1998). However, they also identified other isoflavonoid compounds, such as rutin, genistein and formononetin in *radix puerariae*, which may appear as "the unidentified peaks" in our HPLC spectrum.

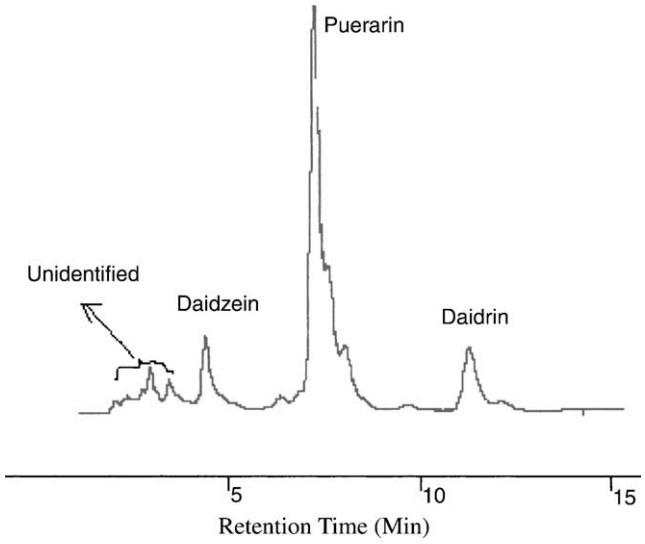

Fig. 1. HLPC spectrum of *radix puerariae* isoflavonoids.

3.2. Radical scavenging activities of RPI

Fig. 2 shows the time-dependent scavenging effect of RPI on $O_2^{\cdot-}$. In the present system, $O_2^{\cdot-}$, generated from pyrogallol autoxidation, could oxidize luminol to produce a strong chemiluminescence signal that was proportional to the concentration of $O_2^{\cdot-}$. Therefore, the scavenging effect of RPI on $O_2^{\cdot-}$ could be obtained by observing its inhibition of the CL. In Fig. 2, the CL of the control increased quickly and then remained relative stable. After adding RPI, the CL decreased slowly and dose-dependently, and tended to be flat after 50 s. Efficient concentration (EC_{50}) which was defined as the concentration to inhibit 50% chemiluminescence intensity, was usually used to express the radical scavenging activities. EC_{50} of RPI towards $O_2^{\cdot-}$ was 0.10 mg/ml.

Fig. 3 shows that the time-dependent scavenging effect of RPI on OH·. OH·, generated in a Fenton-type reaction, could oxidize luminol to produce a strong chemiluminescence signal, proportional to the concentration of OH· (Gülüzar & Demiryürek, 1998). The CL of the control was quickly increased with time and reached a plateau after 6 s. After addition of RPI, the CL intensity was decreased in a dose-dependent fashion. EC_{50} toward OH· was 0.005 mg/ml.

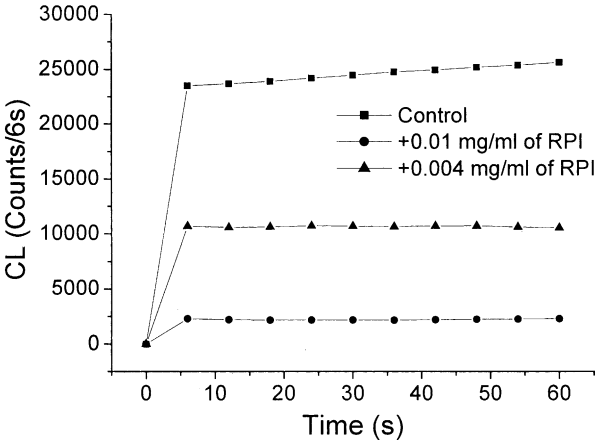

Fig. 3. The time-dependent scavenging effect of RPI on OH·.

Fig. 4 shows the time-dependent scavenging effect of RPI on lipid-derived radicals (R·). R·, generated in an H_2O_2/evening primrose oil system, could produce relatively strong CL, not depending on luminol. The CL of the control increased with time and reach a plateau after 30 s. The CL intensity was dose-dependently decreased after the addition of RPI. EC_{50} towards lipid-derived radicals was 0.03 mg/ml.

Fig. 5 shows the time-dependent scavenging effect of RPI on $^1O_2$. $^1O_2$ was generated in the reaction between NaOCl and H_2O_2 in a solution of pH 8 and detected by the CL technique. The CL of the control produced a peak at 16 s and then decayed quickly with time. The CL dose-dependently decreased with the addition of RPI. EC_{50} towards $^1O_2$ was 0.004 mg/ml.

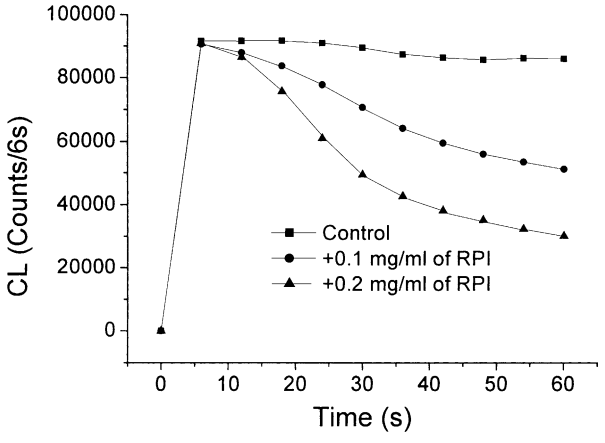

Fig. 2. The time-dependent scavenging effect of RPI on $O_2^{\cdot-}$.

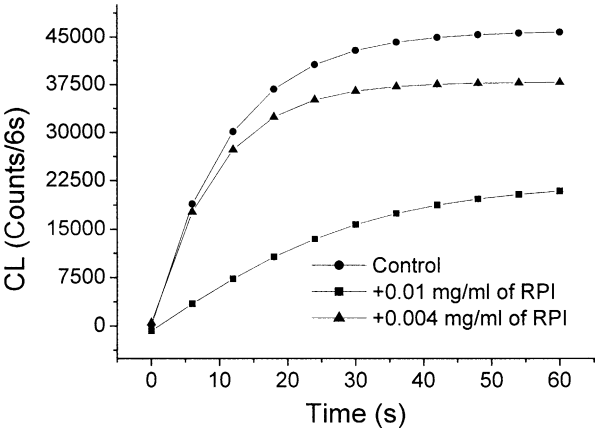

Fig. 4. The time-dependent scavenging effect of RPI on lipid-derived radicals.

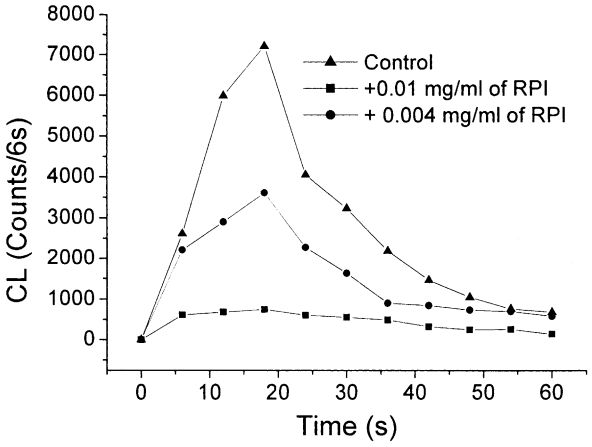

Fig. 5. The time-dependent scavenging effect of RPI on $^1O_2$.

The higher the EC_{50} value, the lower were the radical scavenging activities. Referring to EC_{50}, it could be observed that the radical scavenging activities of RPI towards ROS decreased in the following sequence: $^1O_2 \approx OH^{\cdot} > R^{\cdot} > O_2^{\cdot-}$, which was consistent with the decreasing sequence of ROS activities.

3.3. Comparison of RPI with PU, tea polyphenols

Table 1 compares the EC_{50} of RPI, PU and tea polyphenols and indicates that the radical scavenging activities of RPI were much higher than that of PU and similar to that of tea polyphenols, referring to their EC_{50} values.

This result suggests that RPI could scavenge all the ROS studied here. RPI is a term for a group of isoflavonoid compounds, such as PU, daidzein, PU and daidzin. Eric, Ren, and Huynh (1999) suggested that the free radical scavenging activities of isoflavonoid compounds were attributable to the hydroxyl groups on the backbone structure. Thus, the free radical scavenging activities of RPI toward ROS, observed above, could be attributed to hydroxyl groups on the backbone structure of isoflavonoid compounds contained therein. This result also shows that the free radical scavenging activities of RPI were higher than that of PU in any one of the ROS systems studied here, since PU was the main isoflavonoid compound, accounting for about 70% of the total isoflavonoids. This result also indicates that the isoflavonoid compounds in RPI would act synergistically in the present state or there were other components that had much stronger radical scavenging activities. In fact, Guerra et al. (2000) used peroxidase/H_2O_2/luminol/enhancer systems to compare the antioxidant activity of RPI and PU and the results they obtained were similar to ours. In addition, the free radical scavenging activities of RPI were also comparable with those of tea polyphenols, well-known antioxidants, showing that RPI was a powerful ROS scavenger.

Table 1
EC_{50} comparison between RPI, PU and tea polyphenols (mg/ml)

ROS	RPI	PU	Tea polyphenols
$O_2^{\cdot-}$	0.10	0.5	0.08
$OH^{\cdot}$	0.005	0.02	0.004
$R^{\cdot}$	0.03	0.14	0.02
$^1O_2$	0.004	0.05	0.005

Radix puerariae, as a raw material, has been applied in the fields of medicine and functional food in China and other countries for a some time. Our result, that RPI extracted from *radix puerariae* possessed a powerful ROS scavenging activities, may supply the scientific evidence for its application. However, there are some questions as to the exact mechanism by which RPI was more efficient that PU in scavenging ROS, which should be resolved in the future.

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