

Full Length Research Paper

Free radical scavenging activity of extract from *Ilex rotunda* determined by electron spin resonance spectroscopy

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Antioxidant activity of the extract from the barks of *Ilex rotunda* was investigated using electron spin resonance (ESR) spectroscopy on 1,1-diphenyl-2-picrylhydrazyl (DPPH), alkyl, hydroxyl, and superoxide radicals. The extract exhibited strong free radical scavenging activity with scavenging percentages of DPPH radical ($82.2 \pm 1.8\%$), alkyl radical ($82.9 \pm 1.7\%$), hydroxyl radical ($89.9 \pm 2.1\%$), and superoxide radical ($92.5 \pm 2.6\%$) at concentration of 50 $\mu\text{g/ml}$, respectively. Activity increased with increasing extract concentrations. Therefore, these results presented the extract from the barks of *I. rotunda* with a potent antioxidant activity that could be useful in food, pharmaceutical, and cosmetic industries.

Key words: *Ilex rotunda*, antioxidant, free radical scavenging activity, electron spin resonance.

INTRODUCTION

Free radicals, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), alkyl, hydroxyl, and superoxide anion reactive groups, are generated during cellular metabolism and mitochondrial energy production (Li et al., 2009a). Under normal conditions, concentrations of free radicals are controlled by the balance between rates of their generation and rates of their scavenging by both enzymatic and nonenzymatic antioxidants. However, under pathological conditions, the balance between generation and elimination of free radicals is disrupted (Je et al., 2009). Uncontrolled generation of free radicals that attack a number of macro-molecules including lipids, proteins and DNA resulting in the cellular damage, is believed to be linked with many disease such as cardiovascular disease (Sugamura and Keaney, 2011), diabetes mellitus (Lappas et al., 2011), cancer (Lau et al., 2008), arthritis and inflammatory diseases (Gelderman et al., 2007; Moulton, 1996), Alzheimer's and Parkinson's disease (Dumont and Beal, 2011; Tabner et al., 2001). Antioxidants can act as free radical scavengers. Even though the synthetic antioxidants, such as butylated

hydroxyanisole, butylated hydroxytoluene, *tert*-butylhydroquinone, and propyl gallate are effective, their applications are restricted because of the potential risks related to health. Many pharmaceutical companies and researchers are growing interest in the search for antioxidants through the study of natural products that lack toxic and/or side-effects (Li et al., 2009b; Kim et al., 2010).

The Aquifoliaceae plant, *Ilex rotunda* Thunb. is widely cultivated as an ornamental or garden plant in China. The barks of *I. rotunda*, commonly known as "Jiu-Bi-Ying", have been used as traditional Chinese medicine for reducing fever and detoxification, removing damp, and analgesia. In the previous investigation, a large amount of triterpenes and triterpene glycosides were isolated from the fruits and leaves of *I. rotunda* (Nakatani et al., 1989; Amimoto et al., 1992, 1993a, b and c). With the aim of screening for antioxidants from medicinal plants, we found that the extract from the barks of *I. rotunda* exhibited significant antioxidant activities. Although some *Ilex* species have been reported as sources of antioxidants (Schinella et al., 2009; Thuong et al., 2009; Leonard et al., 2010), there is no report about the antioxidant activity of *I. rotunda*. In the present work, we describe the evaluation of its radical scavenging activity against DPPH, alkyl, hydroxyl, and superoxide radicals using an electron spin

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resonance (ESR) technique.

MATERIALS AND METHODS

General experimental procedures

The ESR spectra were recorded on a JES-TE100 ESR spectrometer (JEOL, Tokyo, Japan). Column chromatography was performed with macroporous resin D101 (Haiguang Chemical Ltd., Tianjin, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2-azobis-(2-amidinopropane) hydrochloride (AAPH), *R*-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitron (4-POBN), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), H_2O_2 , riboflavin, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant materials

The barks of *I. rotunda* were purchased in November 2009 from Chinese crude-drug market in Yulin, Guangxi Province, China, and authenticated by Prof. Baomin Feng, College of Bioengineering, Dalian University, China. A voucher specimen (IR20091101) is deposited at the Institute of Phytochemistry, Jilin Academy of Chinese Medicine Sciences, China.

Preparation of extract

The coarse powder of the barks of *I. rotunda* was obtained after comminution and filtration (20 to 40 meshes). The powder (1 kg) was extracted twice with 70% (v/v) ethanol aqueous solution under reflux for 2 h and the solvent was evaporated under reduced pressure to give a brown residue (113 g). The residue (100 g) was diluted with distilled water and subjected to D101 macroporous resin column chromatography and eluted with H_2O and 30 and 70% (v/v) ethanol aqueous solution, successively. From fraction eluted with 70% (v/v) ethanol aqueous solution, we obtained a yellowish residue (38 g), which was used for this study.

DPPH radical scavenging assay

The DPPH radical scavenging activity was measured using an ESR spectrometer according to the technique described by Nanjo et al. (1996). A 30 μl of the indicated concentration of tested extract in 10% DMSO was added to 30 μl of 60 μM DPPH in methanol. After mixing vigorously for 10 s, the solution was transferred into a Teflon capillary tube and fitted into the cavity of the ESR spectrometer. The spin adduct was measured on an ESR spectrometer exactly 2 min later. The measurement conditions were as follows: magnetic field, 336.5 ± 5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000 ; and sweep time, 30 s. All radical scavenging activities of the extract in the present study were calculated by scavenging rate = $[1 - (H_x/H_0)] \times 100\%$, in which H_0 and H_x are the ESR signal intensities of samples in the absence and presence of the extracts, respectively.

Alkyl radical scavenging assay

Alkyl radicals were generated by AAPH. The reaction mixture containing 20 μl of 40 mM AAPH, 20 μl of phosphate buffer solution (PBS, pH 7.4), 20 μl of 40 mM 4-POBN, and 20 μl of the indicated concentrations of tested extract, was incubated at 37°C for 30 min (Hiramoto et al., 1993). Subsequently, the reaction mixture was

transferred to a Teflon capillary tube and the spin adduct was recorded on an ESR spectrometer under the following measurement conditions: magnetic field, 336.5 ± 5 mT; microwave power, 10 mW; microwave frequency, 9441 MHz; modulation frequency, 100 kHz; and sweep time, 30 s. The alkyl radical scavenging activity (%) was presented as described above.

Hydroxyl radical scavenging assay

Hydroxyl radicals were generated by the iron-catalyzed Fenton Haber-Weiss reaction, and the generated hydroxyl radicals reacted rapidly with nitron spin trap DMPO. The resultant DMPO-OH adduct was detectable with an ESR spectrometer (Rosen and Rauckman, 1984). The ESR spectrum was recorded 2.5 min after mixing in a PBS (pH 7.4) with 20 μl of 0.3 mM DMPO, 20 μl of 10 mM FeSO_4 , 20 μl of 10 mM H_2O_2 , and 20 μl of the indicated concentration of tested extract using an ESR spectrometer set at the following conditions: magnetic field, 336.5 ± 10 mT; microwave power, 1 mW; modulation frequency, 100 KHz; amplitude, 1×200 ; and sweep time, 4 min. The hydroxyl radical scavenging activity (%) was presented as described above.

Superoxide radical scavenging assay

Superoxide radicals were generated by the UV-irradiated riboflavin-EDTA system (Zhao et al, 1989). The reaction mixture containing 60 μl of 0.3 mM riboflavin, 60 μl of 1.6 mM EDTA, 60 μl of 800 mM DMPO, and 60 μl of the indicated concentration of tested extract was irradiated for 1 min under UV lamp at 365 nm. The mixture was transferred to a Teflon capillary tube of the ESR spectrometer for measurement. The instrumental parameters were as following: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000 ; and sweep time, 1 min. The superoxide radical scavenging activity (%) was presented as described above.

Statistical analysis

The results were analyzed using the Statistical Package of Social Science Software (SPSS 12.0 for Windows, 2003, SPSS Inc, Chicago, IL). The data were expressed as the mean of three replicate determinations and standard deviation.

RESULTS AND DISCUSSION

ESR trapping technique is based on the measurement of transitions of unpaired electrons in a magnetic field, which provides a sensitive, direct, and accurate means of monitoring reactive species at room temperature, and the antioxidant activity can be effectively determined by ESR spectrometer (Antolovich et al., 2002). As it has been reported, different antioxidants exhibit differential scavenging activity on various reactive oxygen species (Wojcik et al., 2010). Reaction with hydroxyl radicals is non-specific, while reaction with other radicals is more specific (Singh et al., 2009). The antioxidant activities of the extract from the barks of *I. rotunda* were investigated using several free radicals generated from different *in vitro* systems by ESR spectrometer.

DPPH, a relative stable free radical, accepts electron or hydrogen radical to become a stable diamagnetic

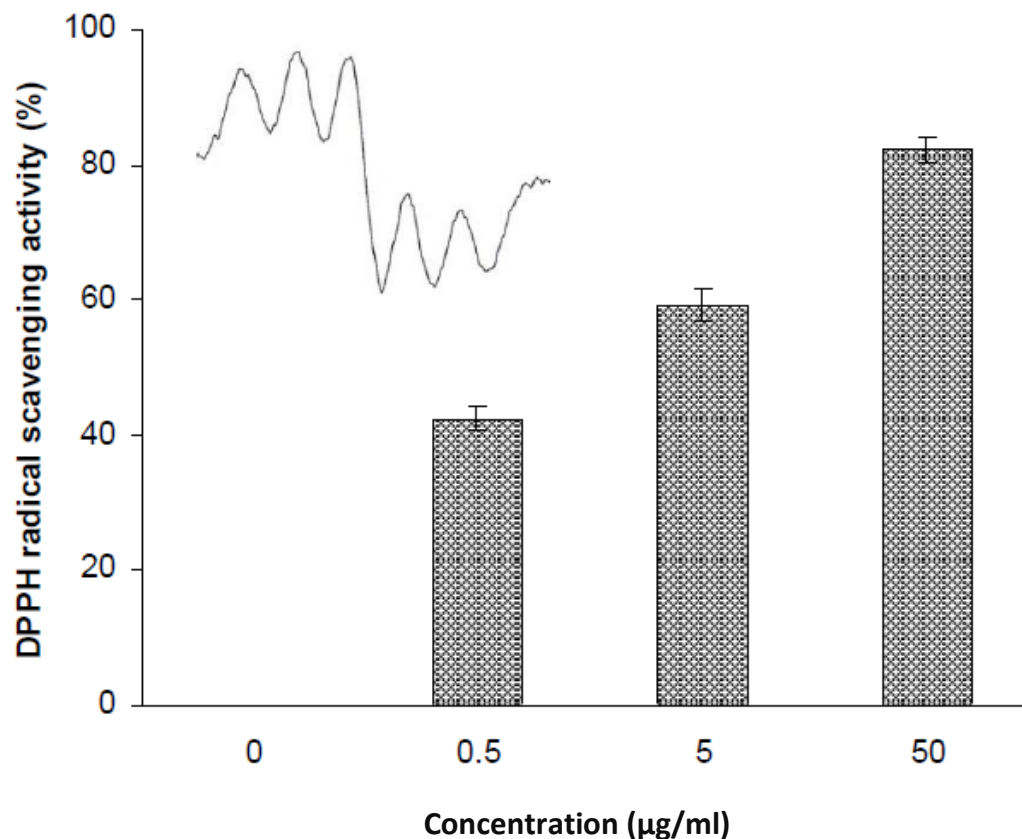


Figure 1. DPPH radical scavenging activity of the extract from the barks of *I. rotunda*. Values are the means \pm SD of three determinations.

molecule. Because it can accommodate sample in a short period and sensitive enough to detect sample at low concentration, it has been extensively used for screening antiradical activities of natural antioxidants functioning as proton radical scavengers or hydrogen donors (Sánchez-Moreno, 2002). The scavenging activity of the extract from the barks of *I. rotunda* towards DPPH radical was shown in Figure 1, which increased with increasing concentrations, with 42.3 ± 1.7 , 59.0 ± 2.4 and $82.2 \pm 1.8\%$ scavenging activities for 0.5, 5 and 50 µg/ml extracts respectively. The results indicated that the extract from the barks of *I. rotunda* possessed potential capacity against DPPH radical in a dose-dependent manner.

Alkyl radicals are a primary intermediate in many hydrocarbon reactions. They can be easily detected with ESR, a technique that has been found to be very useful in the characterization of solid surfaces elucidation of active surface sites and surface reactions (Adebajo and Gesser, 2001). The extract from the barks of *I. rotunda* exhibited significant potency to scavenge alkyl radicals at various concentrations with scavenging percentages at 30.5 ± 2.1 (0.5 µg/ml), 58.8 ± 3.5 (5 µg/ml) and $82.9 \pm 1.7\%$ (50 µg/ml) (Figure 2).

Hydroxyl radicals are extremely reactive free radicals

formed in biological systems. They have been implicated as a highly damaging species in free radical pathology capable of damaging almost every molecule found in living cells (Gulcin, 2006). In this study, the hydroxyl radicals generated in the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ system were trapped by DMPO forming a spin adduct which could be detected by an ESR spectrometer (Bindoli et al., 1992). The hydroxyl radical scavenging activity measured occurred in a dose-dependent manner for different concentrations of the extract from the barks of *I. rotunda* as shown in Figure 3. The extract from the barks of the plant showed hydroxyl radical scavenging activities of 57.5 ± 3.7 (0.5 µg/ml), 79.3 ± 2.9 (5 µg/ml) and $89.9 \pm 2.1\%$ (50 µg/ml).

Superoxide anions play important roles in the formation of ROS such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA (Pietta, 2000). A typical ESR spectrum of DMPO-OOH spin adduct was detected from irradiated riboflavin/EDTA system. As illustrated in Figure 4, the superoxide radical scavenging activity of the extract from the barks of *I. rotunda* increased with increasing concentrations indicating 62.4 ± 4.2 , 80.3 ± 3.9 and $92.5 \pm 2.6\%$ scavenging activities for 0.5, 5 and 50 µg/ml extracts, respectively. The results indicated that this

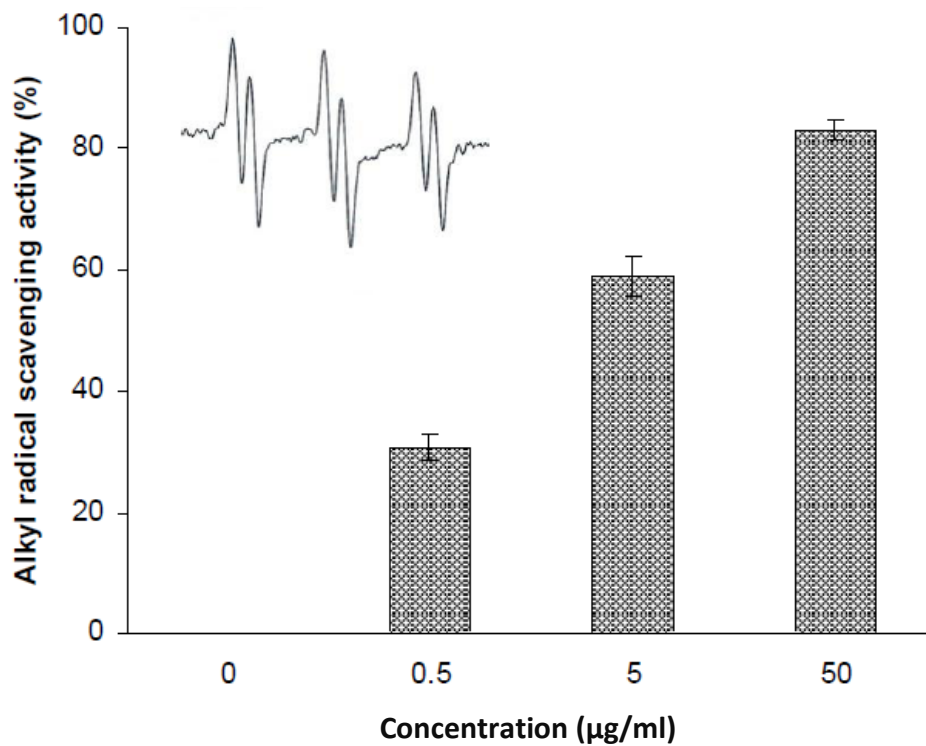


Figure 2. Alkyl radical scavenging activity of the extract from the barks of *I. rotunda*. Values are the means \pm SD of three determinations.

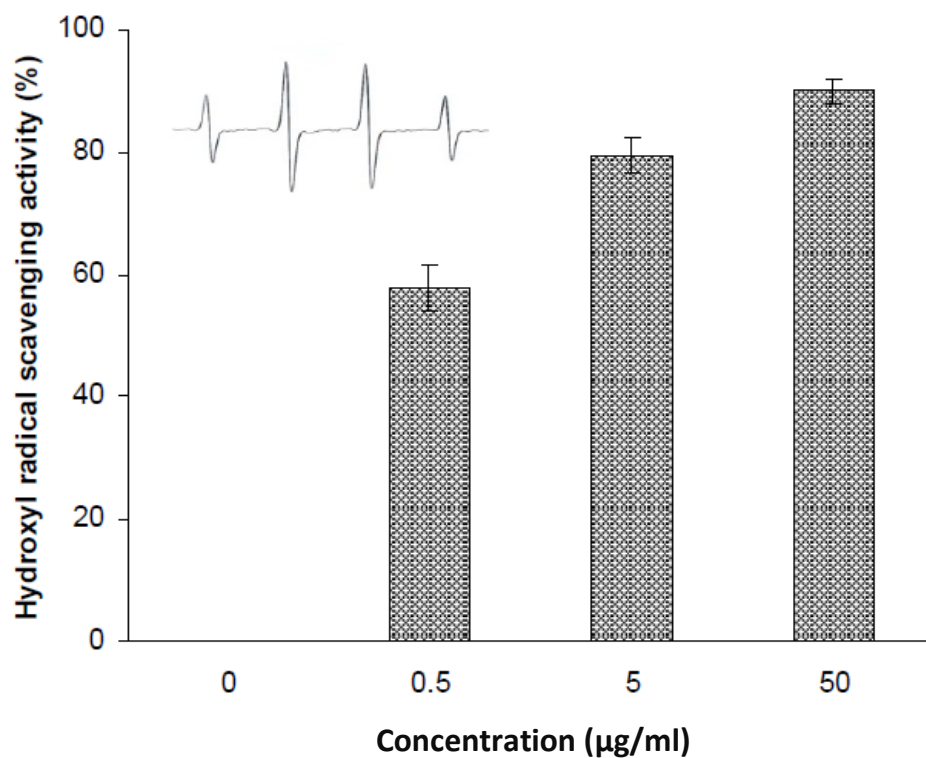


Figure 3. Hydroxyl radical scavenging activity of the extract from the barks of *I. rotunda*. Values are the means \pm SD of three determinations.

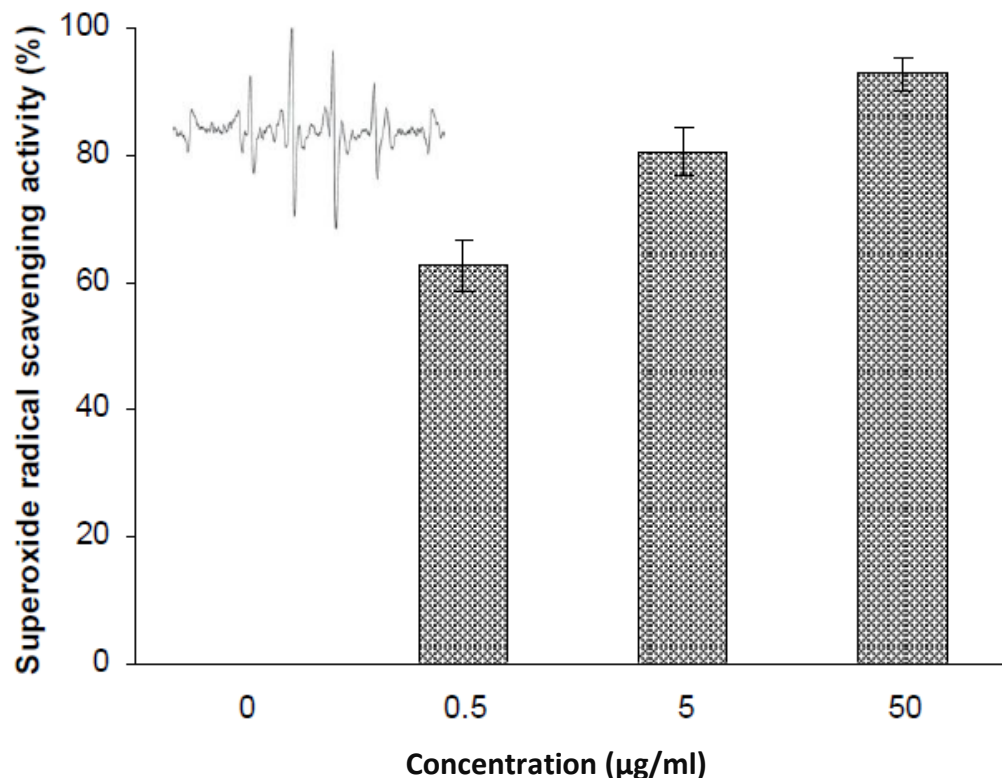


Figure 4. Superoxide radical scavenging activity of the extract from the barks of *I. rotunda*. Values are the means \pm SD of three determinations.

extract possessed potential capacity against superoxide radical in a dose-dependent manner.

Conclusion

Recently, many researches are interested in finding any natural antioxidants possessing safety and effectiveness source which can be substituted for current commercial synthetic antioxidants. The present study indicates that the extract from the barks of *I. rotunda* exhibited good antioxidant activity by effective scavenging various free radicals, such as DPPH, alkyl, hydroxyl, and superoxide radicals. The extract from the barks of *I. rotunda* may be a useful natural radical scavenger and a potential supplement for the food, pharmaceutical, and cosmetic industries. Further studies are needed to isolate and identify active constituents for a better understanding of the structure-functionality relationship.

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