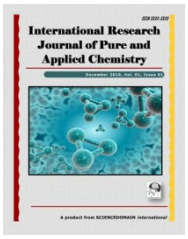

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Systematic Evaluation on Antioxidant of Magnolol *in vitro*

Xican Li[1*] and Chan Chen[2]

[1,2]School of Chinese Herbal Medicine, Guangzhou University of Chinese Medicine, Guangzhou Higher Education Mega Center, Guangzhou, China, 510006.

Research Article

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ABSTRACT

We systematically evaluated *in vitro* the antioxidant activity of magnolol, a natural phenolic phenylpropanoid, using DPPH (1, 1-Diphenyl-2-picrylhydrazyl radical), ABTS [2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)], Ferric ions (Fe^{3+}) reducing power, superoxide anion and hydroxyl radical-scavenging. In all assays, magnolol and positive controls exhibited effective antioxidant ability. The IC_{50} values of magnolol were calculated as 25.92, 0.85, 737.56, 29.97, 153.46 μg/mL, respectively, for DPPH, ABTS, Fe^{3+} reducing power, superoxide anion and hydroxyl radical-scavenging. In these five assays, the IC_{50} values of Trolox were respectively calculated as 5.08, 2.02, 58.92, 160.26, 62.69 μg/mL, while the IC_{50} values of BHT were respectively 4.17, 1.76, 75.83, 43.31, 202.64 μg/mL. Furthermore, as DPPH and ABTS assays were conducted in organic solutions, while the other assays were finished in aqueous solutions, magnolol is therefore regarded as an effective antioxidant *in vitro* in both lipid and aqueous media. Its various protective effects may be attributed to the antioxidant. The fact that magnolol could effectively scavenge both DPPH• and $ABTS^{+}$• suggested it may exert antioxidant action *in vitro* by donating hydrogen atom (H•) or electron (e).

Keywords: Systematic evaluation; magnolol; antioxidant; radical-scavenging; reducing power; in vitro.

**Corresponding author: Email: lixican@126.com;*

1. INTRODUCTION

Magnolol mainly derived from Houpu Magnolia (*Magnolia officinalis*) contains a peculiar chemical structure i.e. phenolic phenylpropanold which cannot easily be found in plants. As a typical phenolic phenylpropanold compound, magnolol was proved to possess protective effects against neuronal loss (Yasui and Akagi, 2004), against cell damage (Lin et al., 2006), against ischemia-reperfusion injury in hind limb (Chen et al., 2009), against DNA damage (Zhao and Liu, 2011), against liver damage in rats (Chen et al., 2009). These protective effects were assumed to be associated with its antioxidant activity.

On the other hand, the reports on antioxidant of magnolol were very limited. These studies mainly focused on its anti-lipid peroxidation (Ogata et al., 1997; Chiu et al., 1999), and on its structure-activity relationships between honokiol (Zhao and Liu, 2011; Ogata et al., 1997), or related phenolic compounds (Ogata et al., 1997).

Therefore, it is obviously necessary to systematically evaluate its antioxidant abilities and to further discuss the mechanism of its antioxidant.

2. MATERIALS AND METHODS

2.1 Chemicals

Magnolol [IUPAC name: 4-allyl-2-(5-allyl-2-hydroxy-phenyl) phenol, CAS number: 528-43-8, $C_{18}H_{18}O_2$] was obtained from NICPBP (National Institute for the Control of Pharmaceutical and Biological Products, China; lot number: 110729-200310); DPPH· (1,1-Diphenyl-2-Picrylhydrazyl radical), pyrogallol, linoleic acid, Trolox (± -6-hydroxyl-2,5,7,8-tetramethlychromane-2-carboxylic acid), BHA (Butylated hydroxyanisole), BHT (Butylatedhydroxy-toluene) were purchased from Sigma Co. (Sigma-Aldrich Shanghai Trading Co., China); ABTS diammonium salt [2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt)], and D-2-deoxyribose were obtained from Amresco Co.; All other chemicals were of analytic grade.

2.2 DPPH• Scavenging Activity

DPPH• radical-scavenging activity was determined as described by Wang (Wang et al., 2011). Briefly, 1 mL of DPPH• solution (0.1mmol/L) was mixed with 0.5 mL of various concentrations of samples dissolved in 95% ethanol. The mixture was kept at room temperature for 30 min, then the absorbance at 519 nm was measured on spectrophotometer (Unico 2100, Shanghai, China), using 95% ethanol as the blank. Trolox and BHT were used as the positive controls and the percentage DPPH• inhibition of the test samples was calculated:

$$\text{Inhibition \%} = (1 - A_S/A_0) \times 100\ \%$$

Where A_S is the absorbance in the presence of the sample magnolol or positive controls, while A_0 is the absorbance in the absence of the sample magnolol and positive controls.

2.3 ABTS$^{\cdot+}$ Scavenging Activity

The scavenging activity of ABTS$^{\bullet+}$ was measured by the method of Wang (Wang et al., 2011). The ABTS$^{\bullet+}$ was produced by mixing 0.35 mL of ABTS diammonium salt (7.4 mmol/L) with 0.35 mL of potassium persulfate (2.6 mmol/L). The mixture was kept in the dark at room temperature for 12 h to allow completion of radical generation, and then diluted with 95% ethanol (about 1:50) and its absorbance at 734 nm was measured on a spectrophotometer (Unico 2100, Shanghai, China). To determine the scavenging activity, 1.2 mL of ABTS$^{\bullet+}$ reagent was mixed with 0.3 mL of sample or negative control (95% ethanol), and the absorbance at 734 nm was measured 6 min after the initial mixing, using 95% ethanol as the blank. The percentage inhibition of the samples was calculated as:

Inhibition % = $(1 - A/A_0) \times 100$ %

Where A_0 is the absorbance at 734 nm of the negative control, A is the absorbance at 734 nm of the mixture with sample. Trolox and BHT, with a final concentration range of 0.67-2.67 μg/mL, were prepared as positive controls.

2.4 Ferric Ions (Fe^{3+}) Reducing Power

Ferric cyanide (Fe^{3+}) reducing power was determined by Oyaizu method (Oyaizu et al., 1986), as described by Wang (Wang et al., 2011). Samples (*x* μL) at various concentrations were mixed with Na_2HPO_4/KH_2PO_4 buffer (350-*x* μL, 0.2 mol/L, pH 6.6) and $K_3Fe(CN)_6$ (250 μL, 1g/100mL). The mixture was incubated at 50ºC for 20 min, 250μL of Trichloroacetic acid (10g/100mL) was added, and the mixture was centrifuged at 3,500×g for 10 min. The supernatant (400μL) was recovered, mixed with distilled water (400 μL) and $FeCl_3$ (400 μL, 0.1g/100mL) and placed immediately into the spectrophotometer (Unico 2100, Shanghai, China), and the timer was started. The absorbance at 700 nm was measured at 90 s. Samples were analyzed in groups of three, and when the analysis of one group has finished, the next group of three samples were mixed with $FeCl_3$ to avoid oxidization by air. Trolox and BHT were used as the positive controls, and an increased absorbance reading indicated increased reducing power. The relative percentage reducing power of the sample as compared to the maximum absorbance tested which appeared in magnolol at 10 μg/mL was calculated by using the formula: $(A-A_{min}) / (A_{max}-A_{min}) \times 100$. Here, A_{max} = absorbance of maximum absorbance tested, A_{min} = absorbance of minimum absorbance tested and A = absorbance of sample.

2.5 Superoxide Anion ($\bullet O_2^-$) Scavenging Activity

The scavenging ability at pH 8.2 of all test samples was determined by Marklund method (Marklund and Marklund, 1974), as described by Wang (Wang et al., 2011). Briefly, samples were dissolved in suitable solvents (absolute or 95% ethanol) at a concentration of 5mg/mL. The sample solution (*x* μL, where *x* = 0, 30, 60, 90, 120, 150, or 180 μL) was mixed with Tris-HCl buffer (2920 – *x* μL, 0.05 mol/L, pH 8.2) containing EDTA (1 mmol/L) and pyrogallol (80 μL, 6 mmol/L), then shook rapidly at room temperature. The absorbance at 325 nm of the mixture was measured (Unico 2100, Shanghai, China) against the Tris-HCl buffer every 30 s for 5 min. The slope of the correlation of absorbance with time was calculated. The reaction mixture without added sample was used as the control.

The $\bullet O_2^-$ scavenging ability was calculated as:

(1 – Slope of sample/Slope of control) × 100 %

2.6 Hydroxyl Radical (·OH) Scavenging Activity

The scavenging activity on the hydroxyl radical (•OH) was investigated by the deoxyribose degradation method with some modification (Wang et al., 2011). Our preliminary experiments demonstrated that almost organic solvents can greatly promote the inhibition percentage value. The inhibition of hydroxyl radical was evaluated as the following procedure: all test samples were firstly dissolved in ethanol (1 mg/mL), and 5-40 μL sample solution was transferred into mini tubes, the ethanol solvent was then removed at 80℃ to eliminate its interference. The reactions were performed in 0.2 mol/L phosphate buffer (pH 7.4), containing 2.8 mmol/L deoxyribose, 2.8 mmol/L H_2O_2, 25 μmol/L $FeCl_3$, 80 μmol/L Na_2EDTA, and the test sample (5-40 μg). The reaction was started by adding ascorbic acid to a final concentration of 100μmol/L and the reaction mixture (600 μL in total) was incubated for 20 min at 50℃ in a water bath. After incubation, the color was developed by addition of 0.5 mL 2-thiobarbituric acid (1 g/100 mL) followed by 0.5 mL trichloroacetic acid (5 g/100 mL) and heating in a boiling water bath for 15 min. The mixture was cooled and diluted two-fold with 95% ethanol, and the absorbance was measured at 532 nm against buffer (as blank). The reaction mixture without sample was used as control. The scavenging activity on hydroxyl radicals was expressed as:

Inhibition % = $(1 - A/A_0) \times 100$ %

Where A_0 is the absorbance at 532 nm of control (without sample), and A is the absorbance at 532 nm of the reaction mixture containing sample. Trolox and BHT were taken as positive controls.

2.7 Statistical Analysis

Data are given as the mean ± standard deviation (SD) of three measurements. The IC_{50} (concentration of the sample required to inhibit 50% of radical) were calculated by linear regression analysis. All linear regression in this paper was analyzed by Origin 6.0 professional software.

3. RESULTS AND DISCUSSION

3.1 DPPH· and ABTS·$^+$ Scavenging Activity

Both DPPH• and ABTS•$^+$ are stable free radicals and show characteristic absorptions at 519 nm or 734nm in methanol or ethanol solvent, respectively. When an antioxidant scavenges the free radicals by hydrogen (•H) donation, the colors in the DPPH and ABTS assay solutions become lighter. As presented in Fig.1, both the DPPH• and ABTS•$^+$ inhibition percentage values dose-dependently increased within the tested concentration, for magnolol and the positive controls Trolox and BHT. DPPH radical inhibition decreased in the order Trolox ≈ BHT > magnolol, while ABTS radical ion inhibition in the order magnolol > BHT > Trolox. The DPPH• and ABTS•$^+$ IC_{50} values of magnolol, Trolox and BHT were calculated and listed in Table 1.

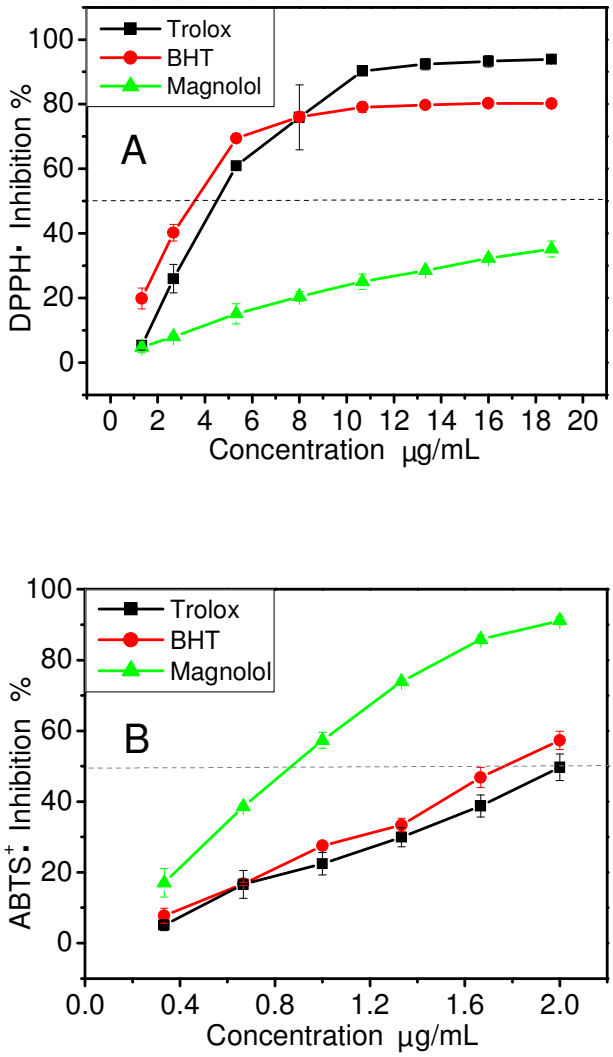


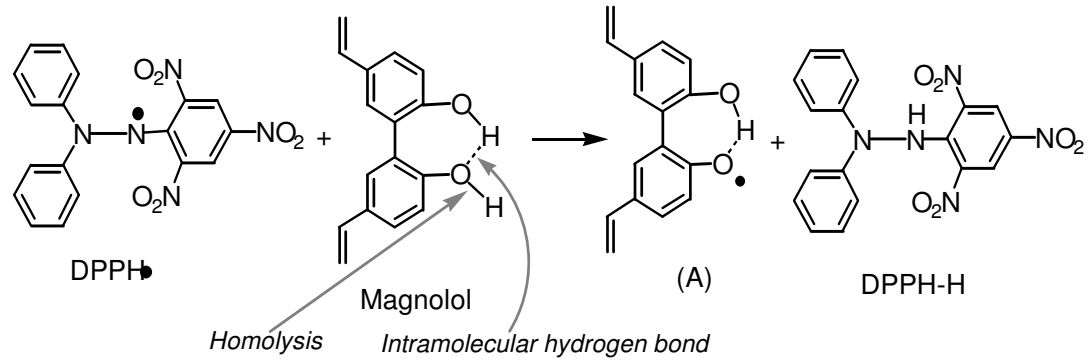
Fig. 1. Effect of different concentration of magnolol in free radical scavenging tests (A) DPPH assay, (B) ABTS assay

Each value is expressed as mean ± SD, n =3

Table 1. The values of IC_{50} of magnolol and positive controls (µg/mL)

	DPPH·	ABTS·+	Fe^{3+} reducing power	· O_2^-	· OH
Trolox	5.08	2.02	58.92	160.26	62.69
BHT	4.17	1.76	75.83	43.31	202.64
Magnolol	25.92	0.85	737.56	29.97	153.46

It is proposed that DPPH• may be scavenged by an antioxidant through donation of hydrogen atom (H·) to form a stable DPPH-H molecule which does not absorb at 519 nm. According to this hypothesis and previous study (Saito and Kawabata, 2005; Zhao and Liu, 2011), the reaction between DPPH• and magnolol could be explained by the following mechanism:



In the reaction, magnolol molecule donated H· via homolysis of one –OH group to produce radical (A) in which a conjugaitive system may lead to its stability. Of course, there was actually an intramolecular hydrogen bond between di-*ortho*-hydroxyl groups in magnolol molecule. The intramolecular hydrogen bond undoubtedly hindered the H· to be abstracted by DPPH•, the homolysis of the –OH became more difficult. Therefore, magnolol showed lower antioxidant ability than its isomer honokiol (Zhao & Liu, 2011).

However, the scavenging of $ABTS^{\bullet+}$ is assumed to be an electron transfer process:

$$ABTS^{+\bullet} + e \longrightarrow ABTS$$

$ABTS^{+\bullet}$ can be previously produced by the reaction between ABTS diammonium salt and potassium persulfate:

$$\underset{\text{yellow}}{2\,(NH_4)_2ABTS} + S_2O_8^{2-} \longrightarrow 2\,SO_4^{2-} + \underset{\text{green}}{2\,ABTS^{+\bullet}} + 2\,NH_4^+$$

3.2 Reducing Power Assay

Although a reductant is not necessarily an antioxidant, an antioxidant is commonly a reductant (Prior and Cao, 1999). The reducing power of a compound may therefore serve as a significant indicator of its potential antioxidant activity (Jung et al., 2008). It can be seen that the reducing power percentage values of magnolol and the positive controls were concentration related and increased with the increasing of sample concentration in the range of the tested concentration (Fig. 2). Their relative reducing powers on Fe^{3+} were as follows: Trolox > BHT > magnolol. The IC_{50} values of all tested samples were calculated and listed in Table 1.

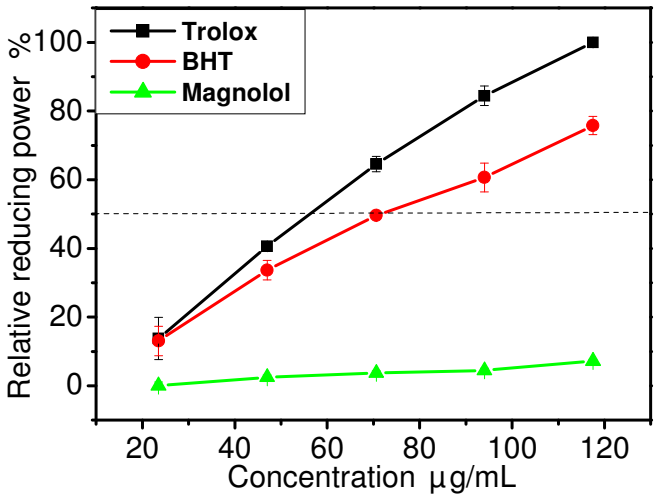


Fig. 2. The reducing power assay of magnolol and positive controls
Each value is expressed as mean ± SD, n =3

3.3 Superoxide Anion (·O_2^-) and Hydroxyl (·OH) Radical-scavenging Activity

ROS are various forms of activated oxygen including free radicals and non-free-radical species. Superoxide anion (•O_2^-) and hydroxyl radical (•OH) are two of the most important free radicals in living cells.

In our experiments, the superoxide radical was generated by the pyrogallol system at pH 8.2. Fig. 3A showed that magnolol and the positive controls Trolox and BHT demonstrated an ability to inhibit superoxide anion in a dose-dependent manner. Magnolol exhibited lower inhibition level than Trolox and BHT. Their IC_{50} values were calculated and listed in Table 1. The hydroxyl (•OH) radical-scavenging activity was evaluated by deoxyribose gradation assay. In this study, a mixture of Fe^{3+}-EDTA, hydrogen peroxide (H_2O_2), and ascorbic acid could generate hydroxyl radicals (•OH). Subsequently, •OH attacked deoxyribose and broke its furancycle to produce malondialdehyde (MDA). MDA combined with 2-thiobarbituric acid (TBA) to generate a chromogen with λ_{max} at 532 nm. Therefore, the value of A_{532nm} can reflect the amount of •OH radicals. The data in Fig. 3B showed that magnolol and the positive controls could effectively inhibit the formation of •OH in a concentration-dependent manner. According to the IC_{50} values (Table 1), magnolol possessed the higher scavenging activity than BHT, but lower than Trolox.

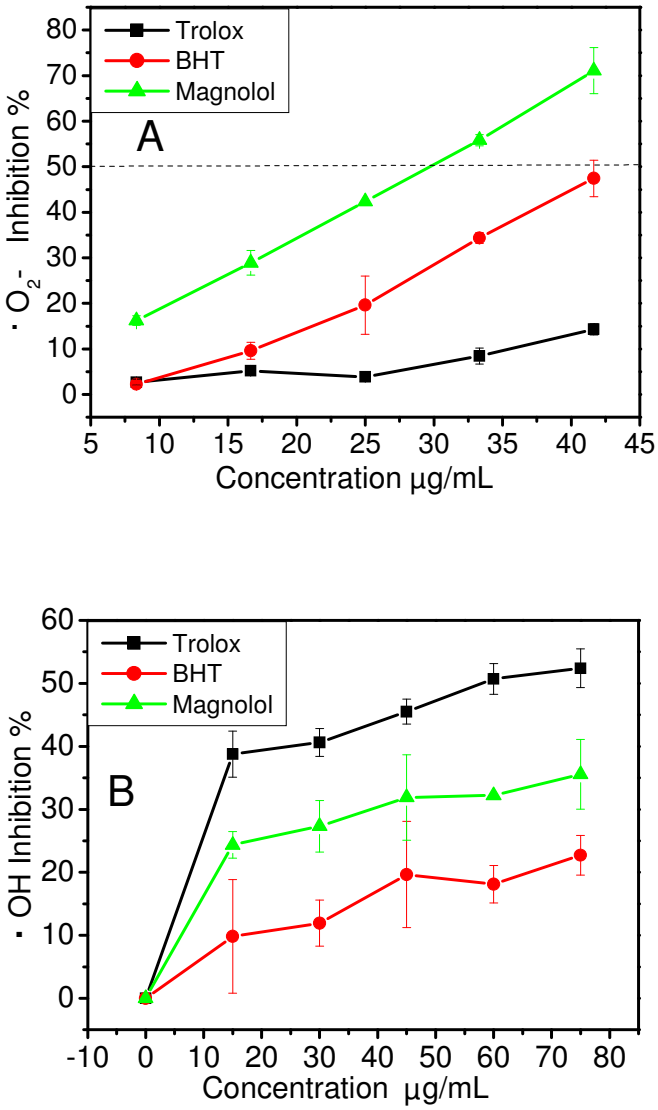


Fig. 3. Superoxide anion (A) and hydroxyl (B) radical scavenging activity of magnolol, Trolox and BHT

Each value is expressed as mean ± SD, n =3

4. CONCLUSIONS

Among the five assays, DPPH and ABTS assays were conducted in organic solutions, while Fe^{3+} reducing power, superoxide anion and hydroxyl radical-scavenging were finished in aqueous solutions. Magnolol exhibited a good concentration-dependent manner in both lipid and aqueous mediums. Thus, magnolol is regarded as an effective natural antioxidant in both media and it could be used in pharmacological or food industry as natural antioxidant.

The fact that magnolol could effectively scavenge both DPPH• and $ABTS^{+}$• suggested it may exert antioxidant action *in vitro* by donating hydrogen atom (H·) or electron (e).

The antioxidant of magnolol may be responsible for its various protective effects mentioned above.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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