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Full Length Research Paper

Antioxidant activities of various fractions extracted from *Astragalus*

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The antioxidant activities in *vitro* and in *vivo* assays of the four extracts from the herb of *Astragalus* were investigated. 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) and reducing power assays were used to evaluate the antioxidant activities of the extracts *in vitro*. The effect of ethyl acetate extracts (EAE) in reducing oxidative stress in mice was evaluated. From the results of antioxidant assays *in vitro*, EAE was found to have the highest antioxidant activity. The antioxidant assay results showed that high doses of EAE significantly decrease the MDA level and increase the SOD activity of mice *in vivo*. These studies *in vitro* and *in vivo* demonstrated that the extract EAE from *Astragalus* has significant antioxidant and free radical scavenging activities. In summary, the results exhibited that the *Astragalus* could be used as a rich source of potentially natural antioxidants.

Key words: *Astragalus*, free radical, antioxidant activity.

INTRODUCTION

Reactive oxygen species are inevitably generated during normal and aberrant consumption of molecular oxygen. A vast amount of evidence implicates these free radicals are able to attack lipid membranes, proteins and DNA, and lead to some detrimental effects, such as lipid peroxidation of cell membranes, alteration of lipid-protein interactions, enzyme inactivation, DNA breakage (Halliwell and Gutteridge, 1998), and even result in cell death (Dean et al., 1993). So, the oxidative stress induced cell damage triggers both the physiological process of aging and many pathological progressions that eventually lead to serious health problems (Harman, 1993).

Antioxidants can reduce the cellular oxidative stress by inhibiting the formation of superoxide anions, and by detoxification of reactive oxygen species/reactive nitrogen species through upregulation of cellular defense mechanisms, such as superoxide dismutase, catalase, or glutathione peroxidase (Violi and Cangemi, 2005). Therefore, research on antioxidants, especially exploration of potent natural compounds with low cytotoxicity from plants, has become an important branch of biomedicine. Up to now, lots of plants such as *Jatropha curcas* (Diwani et al., 2009), *Strychnos henningsii* Gilg (Oyedemi et al., 2010), *Aster thomsonii* (Bibi et al., 2011), *Laurus nobilis* (Biljana et al., 2010], *Apocynum venetum* L. (Liang et al., 2010), *Kandelia candel* (Wei et al., 2010), *Gardenia jasminoides* Ellis (Fan et al., 2011) and so on, have been found to have exhibited strong antioxidant activity.

Astragalus is a precious herbal plant in Chinese traditional medicine as a therapeutant for intensifying phagocytosis of reticulo-endothelial systems, stimulate pituitary-adrenal cortical activity and restore depleted red blood cell formation in bone marrow (Cassileth et al., 2009).

Some studies have indicated that *Astragalus* contains flavonoids, phenolic acids polysaccharide and tannins. *Astragalus* is also one of the herbs known to stimulate the body natural production of interferon (Block and Mead, 2003; Zhao et al., 1990). The therapeutic potential offered is very exciting. However, the antioxidant activities of *Astragalus* have not been reported. Therefore, the purpose of the present investigation was to evaluate its antioxidant activities of various extracts from *Astragalus in vitro* and *in vivo*.

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EXPERIMENTAL

Materials and chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and Vitamin C were purchased from Sigma (St. Louis, MO, USA). 2,2-azinobis-6-(3-ethylbenzothiazoline sulfonic acid (ABTS) radical was purchased from Merck (Darmstadt, Germany). SOD Assay Kit001 and MDA Assay Kit A003 were purchased from the Institute of Biological Engineering of Nanjing Jianchen (Nanjing, China). Petroleum ether, ethanol, ethyl acetate, N-butanol and all other chemicals and reagents were of analytical grade.

Extraction and fractionation of antioxidants

Extraction of the antioxidants from *Astragalus* was done by using fractionated extraction according to the method described by Shi et al. (2010), with some modifications. The powder (100 g) of dried *Astragalus* was extracted with 95% ethanol (300 ml) at 80°C for 2 h and filtered; the process was repeated thrice. The filtrate was collected and combined, followed by concentrated using rotary evaporation at 40°C under vacuum to give a solid extract. The solid sample was further extracted with petroleum ether (300 ml) at 70°C for 2 h thrice and filtered; the solution was combined to yield the petroleum ether extraction (PEE, 0.7919 g). The residue was further fractionated with different solvents. Briefly, the residue was successively extracted with 300 ml of ethyl acetate, N-butanol and double-distilled water. Solvents from each fraction were removed with a rotary evaporator at 40°C under vacuum, and the residues were freeze-dried. After removal all of the solvents, three fractions were obtained, including the ethyl acetate (EAE), N-butanol (BE) and water (WE) fractions, with the solid residues being 0.2614, 0.2537 and 14.017 g, respectively.

Determination of total phenolic acids content

The total phenolic acids contents of these samples were determined by the Folin-Ciocalteu according to the method of Li et al. (2009) and Sim et al. (2010). Briefly, samples of the extract were taken into a test-tube and made to a volume of 0.5 ml with 95% ethanol, the Folin-Ciocalteu reagent (0.5 ml, 0.25 mol/L) and the Na_2CO_3 reagent (1.0 ml, 150 g/L) were added, and the mixture was incubated at room temperature for 30 min. The absorbance was measured at 760 nm and compared to gallic acid equivalents, using a gallic acid standard curve. The amount of total phenol was calculated as gallic acid equivalents from the calibration curve.

Assays for antioxidant activities

Determination of ABTS free radical-scavenging activity

The radicals scavenging activity of these samples (PEE, EAE, BE and WE) against radical cation ($ABTS^+$) were measured using the methods of Re et al. (1999) and Luo et al. (2010), with some modifications. $ABTS^+$ was produced by reacting 7 mmol/L of $ABTS^+$ solution with 2.45 mmol/L of potassium persulphate, and the mixture would be kept in the dark at room temperature for 16 h. In the moment of use, the $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Each sample (0.2 ml) with various concentrations (62.5 to 4000 µg/ml) were added to 2 ml of $ABTS^+$ solution and mixed vigorously. After reaction at room temperature for 6 min, the absorbance at 734 nm was measured. The $ABTS^+$ scavenging effect was calculated by the following formula:

ABTS scavenging effect (%) = [Ao-(A-Ab)]/Ao × 100

where Ao: A734 of ABTS without sample; A: A734 of sample and ABTS, and Ab: A734 of sample without ABTS.

Determination of DPPH free radical-scavenging activity

In the present test, DPPH scavenging activities of the different fractions were measured according to the method of Shimada et al (1992) and Fan et al. (2009), with some modifications. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1.0 ml of this solution was added with 3.0 ml of the samples of various concentrations (62.5 to 4000 µg/ml). The solution was kept at room temperature for 30 min, and the absorbance at 517 nm (A517) was measured. The DPPH scavenging effect was calculated as follows:

DPPH scavenging effect (%) = [Ao-(A-Ab)]/Ao × 100

Where Ao: A517 of DPPH without sample; A: A517 of sample and DPPH, and Ab: A517 of sample without DPPH.

Determination of reducing power assay

The reducing power of different test samples were quantified by the method described earlier by Luo et al. (2011, 2009), with some modifications. Briefly, the samples were used at differing concentrations (62.5 to 4000 µg/ml). 1.0 ml of sample was mixed with phosphate buffer (2.5 ml, 0.2 mol/l, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Then the reaction was terminated by 2.5 ml TCA solution (0.1%) and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 6 mmoll/L), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Antioxidant activity *in vivo*

Kunming mice (provided by Sichuan Academy of Medical Science, China), weighing in the range of 18 to 22 g, were kept in separated cages at a temperature of 21 ± 1°C and a 50 to 60% of relative humidity.

They underwent 12-h light-and-dark cycles with free access to food and water (Luo et al., 2011). All of the mice were evenly and randomly divided into five groups of ten mice each. Group I was given D-galactose and normal laboratory diet; Group II was given D-galactose, VC and normal laboratory diet; Group III was given D-galactose, the sample at a dose of 200 mg/kg per day and normal laboratory diet; Group IV was given D-galactose, the test sample at a dose of 100 mg/kg per day and normal laboratory diet; Group V was given D-galactose, the sample at a dose of 50 mg/kg per day and normal laboratory diet.

The dose of D-galactose of each group was 100 mg/kg/day body weight (Lv et al., 2007**)**. Twenty-four hours after the last drug administration, blood samples were obtained from the eyepit of the mice and processed for serum. Superoxide dismutase (SOD) activity (U/mL) was tested with the SOD Assay Kit. The concentrations of MDA in blood serum from the mice were determined with an MDA Assay Kit.

Statistical analysis

The data were presented as mean ± standard deviation. Statistical analysis was conducted with the SPSS 16.0 software package.

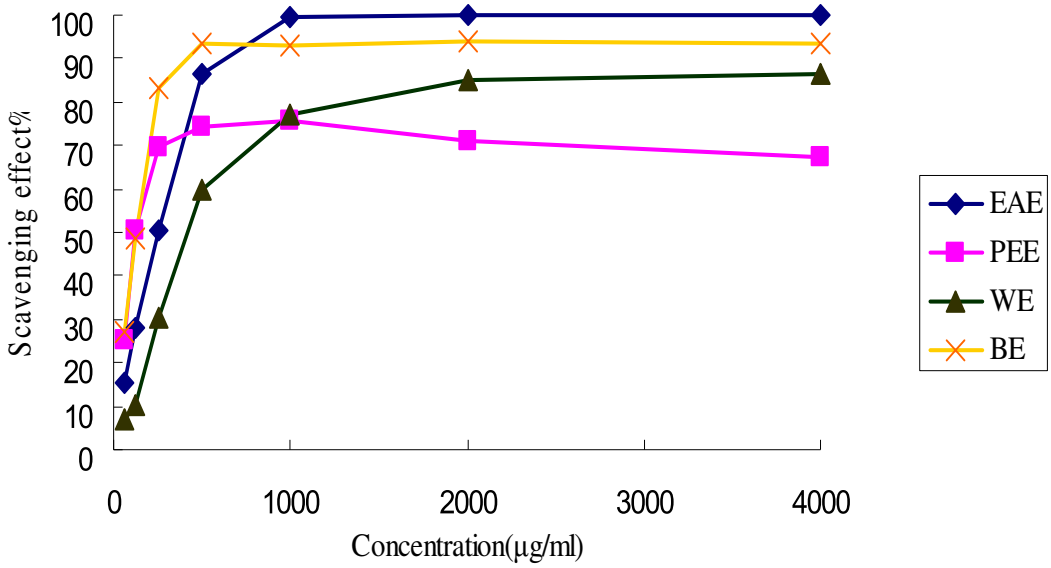

Figure 1. The ABTS scavenging activities of various extracts from *Astragalus*.

RESULTS AND DISCUSSION

Total phenolic acids content

The values of phenolic acid contents were measured by Folin-Ciocalteau method. The absorbance was measured at 760 nm and compared to gallic acid equivalents, using a gallic acid standard curve (y = 91.619x + 0.004, r = 0.9999). The amount of total phenol was calculated as gallic acid equivalents from the calibration curve. From the results, the amount of total phenolic acids differed significantly among the various extracts. The content of phenolic acid in the petroleum ether extracts was 2.89, 1.34 μg/ml in the butanol extracts and 0.73 μg/ml in the water extracts. However, the highest value of phenolic acid content was determined in the ethyl acetate extracts (3.94 μg/ml).

Effect of scavenging ABTS radicals

In the experiment, the scavenging abilities of various extracts from *Astragalus* on ABTS free radical were shown in Figure 1. As seen in the figure, the activities of all the samples increased in a concentration-dependent manner, and all the extracts showed valuable high radical scavenging activities in the higher doses (from 1000 to 4000 μg/ml). Especially for the ethyl acetate extracts (EAE) and butanol extracts (BE), at the dose of 1000 μg/ml, the scavenging abilities were 99.6 and 92.9%, respectively. Over the dose, the scavenging abilities of both samples increased slowly. On the contrary, the scavenging abilities on ABTS of petroleum ether extracts (PE) and water extracts(WE) were lower than that of ethyl acetate extracts. At the high dose of 4000 μg/ml, the scavenging abilities were 67.3 and 86.5% of both samples. Which were far lower than that of the ethyl acetate extracts (99.9%). Therefore, the ethyl acetate extracts fraction has significant effect on ABTS radical scavenging.

Effect of scavenging DPPH radicals

DPPH is a stable free radical that shows maximum absorption at 517 nm in methanol. In the test, the antioxidants were able to reduce the stable DPPH radical and the absorbance at 517 nm. The effect of antioxidants on DPPH radical scavenging was conceived to be due to their proton-donating ability. Therefore, the antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH free radical. In this experiment, the scavenging abilities of various extracts from *Astragalus* on DPPH free radical were shown in Figure 2. The results indicated that four samples showed in all concentrations dose-dependent DPPH radical scavenging activities. Furthermore, the scavenging activities of ethyl acetate extracts increased very significantly with increasing concentrations and stronger than butanol extracts, petroleum ether extracts and water extracts at every concentration points. Especially in the higher doses (from 1000 to 4000 μg/ml), ethyl acetate extracts exhibited very high radical scavenging activity (from 66.2 to 82.0%), which was far higher than that of the others. Therefore, it was obvious that the ethyl acetate extracts has strong antioxidant activity in the higher doses, followed by butanol extracts. Petroleum ether extracts has no significant effects on DPPH radical scavenging.

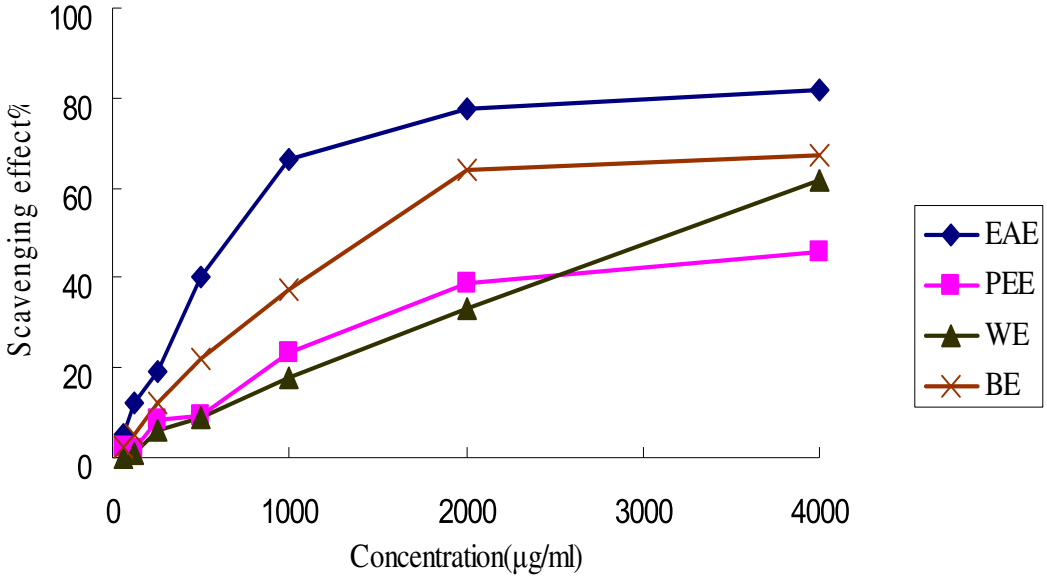

Figure 2. The DPPH scavenging activities of various extracts from *Astragalus.*

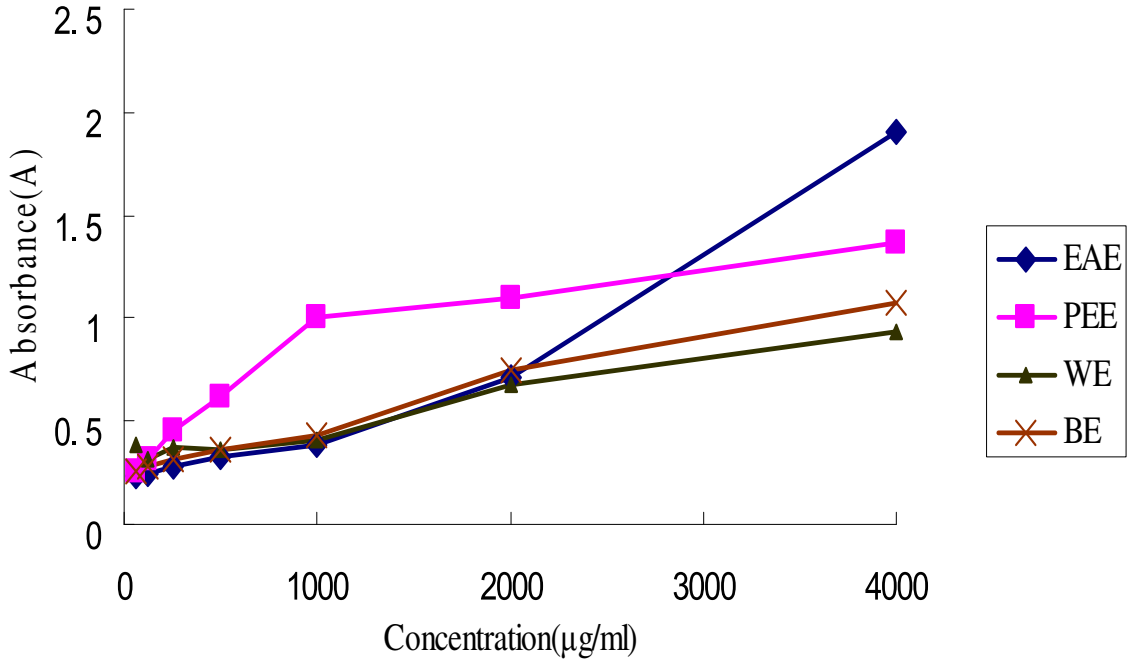

Figure 3. Reductive abilities of various extracts from *Astragalus.*

Reducing power assay

Research has revealed that there is a direct correlation between antioxidant activities and reducing power (Yildirim et al., 2001). To measure reductive powers of various extracts from Astragalus, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of different concentrations samples. The results of reductive capabilities of various extracts from *Astragalus* were exhibited as Figure 3. From the result, reducing power of all samples were in a concentration-dependent manner, and all extracts showed low reducing power in the low doses (from 62 to 2000 μg/ml). At a high concentration of 4000 μg/ml, butanol extracts, petroleum ether extracts and water extracts also exhibited low reducing powers, however, ethyl acetate extracts has significant reducing effect.

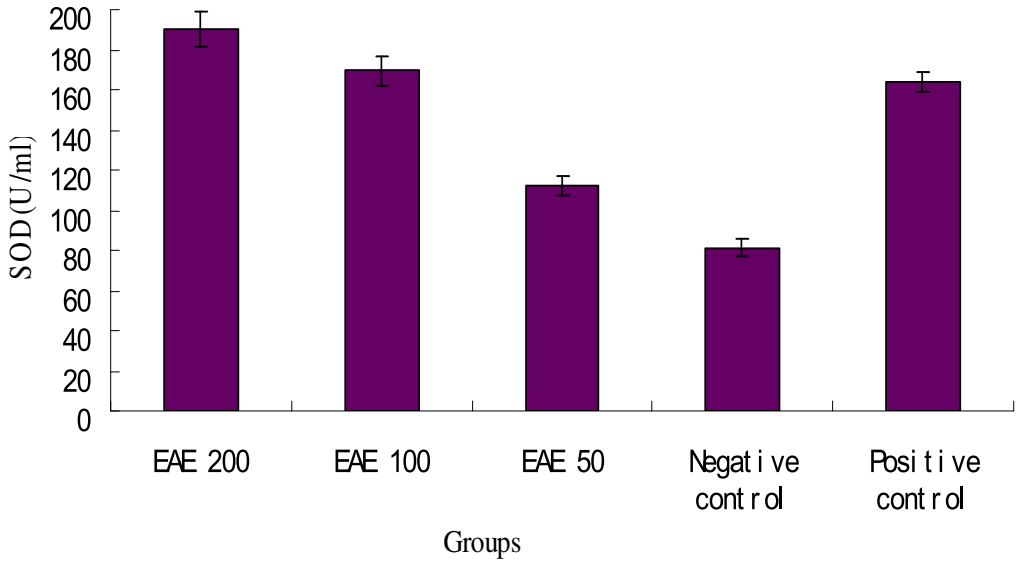

Figure 4. SOD activity of various samples *in vivo*. Results are presented as means ± standard deviations.

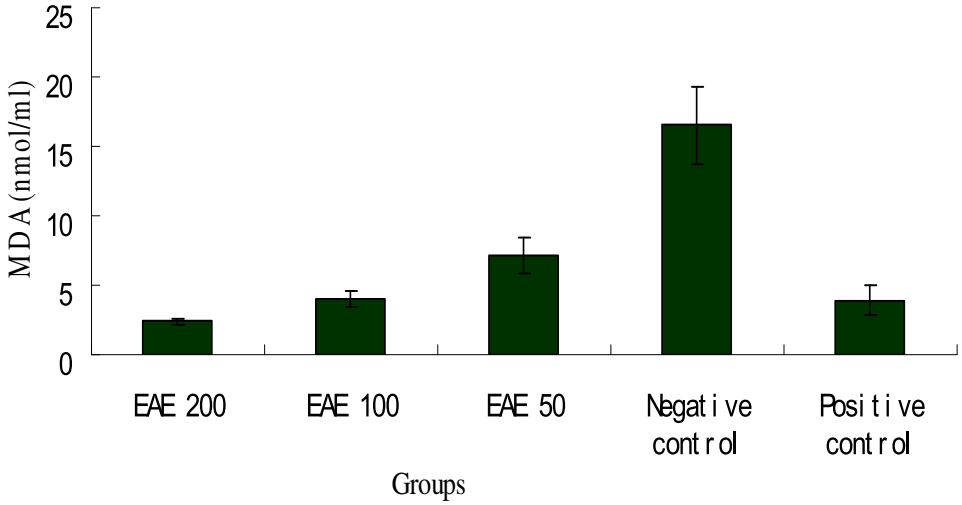

Figure 5. Determination of MDA contents in blood serum from various sample groups. Results are presented as means ± standard deviations.

Antioxidant activity in vivo

Superoxide dismutase (SOD) activity (U/ml) was tested with the SOD Assay Kit. Superoxide was generated in xanthine oxidase and hypoxanthine, and the superoxide scavenging effect of serum was determined according to the method of Oyanagui (1984). SOD activity of the serum was expressed in U/ml of the sample. As shown in Figure 4, SOD activities of different dose of all samples exhibited dose-dependent manner. At 200 mg/kg, particularly, SOD activity of ethyl acetate extracts was 190.27 U/ml, which was higher than that of vitamin C. However, SOD activity at low concentrations was much less evident, which is closed to that of the negative control. The results were therefore an indication of enhancement SOD activity of ethyl acetate extracts for high concentrations.

The concentrations of MDA in blood serum from the mice were determined with an MDA Assay Kit. The MDA value was estimated according to the thiobarbituric acid (TBA) method (Asakawa and Matsuhita, 1980). The samples added with TBA were heated in an acidic environment. The absorbance of the resulting solution was measured at 532 nm. The results in Figure 5 exhibited

a significant pattern of a decreasing MDA concentration in blood serum with increasing ethyl acetate extracts concentration. At 200 mg/kg, the concentration of MDA was 2.39 nmol/ml, which is stronger than that of the positive control (Vc). This can be interpreted as a significant effect of ethyl acetate extracts at high concentrations on MDA scavenging *in vivo*.

Conclusions

In the present study, the four extracts (ethyl acetate extracts, petroleum ether extracts, water extracts and butanol extracts) were first isolated from the Astragalus. Free radicals scavenging activities *in vitro* indicated that ethyl acetate extracts has significant radicals scavenging abilities on ABTS and DPPH radicals, and the scavenging effects were powerful, which is far higher than that of the other extracts. The ethyl acetate extracts also exhibited significant reducing power at high dose. Therefore, the ethyl acetate extracts (EAE) should be explored as novel potential antioxidants, and the strong antioxidant abilities may be relevant to the high phenolic acid contents. For *in vivo* assays, the ethyl acetate extracts was found to increase the levels of antioxidant enzymes (SOD) and to decrease the MDA content in blood serum. It was confirmed that ethyl acetate extracts could protect tissues against oxidative damages. Enhanced SOD activity in mice blood serum also can be related to the *in vivo* antioxidant activity of ethyl acetate extracts. With such strong antioxidant ability, ethyl acetate extracts was identified as a potential antioxidant.

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