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Preparation of *Eucommia ulmoides* Leaves Antioxidant and Its Antioxidation Activity *In Vitro* and *In Vivo*

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Abstract: The adsorption and desorption efficiencies on flavonoids and polyphenols from *Eucommia ulmoides* leaves by different resins were investigated, and the most suitable resin for preparation of *Eucommia ulmoides* leaves antioxidant was chosen. Then the crude extract of *Eucommia ulmoides* leaves was adsorbed by resin, and the resin was eluted by 20%, 40%, 60% and 80% (in volume) ethanol elution solution, and then the antioxidants were gotten. The flavonoids and polyphenols in the antioxidants were tested for content. The antioxidation activity *in vitro* was studied by testing the scavenging capacities on HO·, O_2^{-}· and DPPH·. The antioxidation activity *in vivo* was studied by measuring the content of malondialchehyche (MDA), and superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) activity in mice orally administrated by 20% and 60% ethanol elution solution sample. The result showed: the 20%, 40% and 60% ethanol elution solution had good scavenging capacities on HO·, O_2^{-}· and DPPH·, and the antioxidation activities *in vitro* of the antioxidants was related to flavonoids and polyphenols, and polyphenols was the leading working material in antioxidation *in vitro*; 20% ethanol elution solution, of which there was more polyphenols in, showed more obvious antioxidation activities *in vivo*, which also meant that polyphenols was the leading working material in antioxidation *in vivo*.

Keywords: *Eucommia ulmoides* leaves, resin, antioxidant, antioxidation activity.

1. INTRODUCTION

Eucommia ulmoides is an endemic and economic useful tree in China, of which the extract has complex components and multiplex function [1-8]. In China, the bark of *Eucommia ulmoides* tree has been used as herb for two thousands years. The recent researches show that the leaves of *Eucommia ulmoides* tree have the similar components to bark, and the function is also similar, so leaves can be also used as herb instead of bark [9]. This provides a wide space for the comprehensive using of *Eucommia ulmoides*. There are more flavonoids and polyphenols in *Eucommia ulmoides* leaves [9], which can exhibit obvious antioxidation activity. Some researches show that *Eucommia ulmoides* has stronger capacity on scavenging free radicals [10], but the most researches objects are crude extract. In this study, we used resin to separate and purify *Eucommia ulmoides* leaves crude extract to prepare antioxidant, which was first researched as object to study *Eucommia ulmoides* leaves antioxidation activities *in vitro* and *in vivo*. At last, the antioxidation activities *in vitro* and *in vivo* of different antioxidants from *Eucommia ulmoides* leaves were evaluated.

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2. MATERIALS AND METHODS

2.1. Chemicals

LS-46 resin was supplied by Xi'an Lanshen Science and Technology Co., Ltd; HPD-600 resin was supplied by Hebei Cangzhou Baoen Chemical Co. Ltd; XDA-8 resin was supplied by Xi'an Lanxiao Science and Technology Co. Ltd. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was provided by Sigma-Aldric Company. All the kits were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China.

2.2. Animal

Male Clean Kunming mice weighing 20±2.0g (Experimental Animal Centre, Medical College of Xi'an Jiaotong University) were used in these experiments.

2.3. Preparation for Eucommia Ulmoides Leaves Crude Extract

After *Eucommia ulmoides* leaves were washed, they were dried at 50°C, and then crushed into powder. The powder was mixed with extraction solvent in 1:10, and the mixture was heated at 50°C in water bath for 1h. The extraction solution was filtered and the filtrate was collected and centrifuged. The supernatant liquor was crude extract liquor. The crude extract liquor was concentrated and dried in vacuum to become powder sample.

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2.4. Choosing Resin

LS-46、HPD-600and XDA-8 resins were prepared for testing. Static adsorption method was used to test adsorption efficiency of each type resin on flavonoids and ployphenols, and dynamic desorption method was used to test desorption efficiency of each type resin on flavonoids and ployphenols. Then based on adsorption and desorption efficiency, the suitable resin was chosen for separation and purification of *Eucommia ulmoides* leaves antioxidant.

2.5. Preparation of Eucommia ulmoides Leaves Antioxidant

The prepared resin was put into column, and 1BV crude extract liquor flowed through the resin in 1BV/h flow rate for adsorption, and each 4BV 20%, 40%, 60% and 80% (in volume) ethanol elution solution flowed through the resin in sequence with 1BV/h flow rate for desorption. Each ethanol elution solution flowing through the resin was collected, and then they were concentrated and dried in vacuum to become antioxidant samples in powder.

2.6. Flavonoids and Polyphenols Measurement

Each sample was measured for flavonoids with $Al(NO_3)_3$ method [11] using rutin as standard and ployphenols with Folin-Ciocalteu method [12] using gallic acid as standard.

2.7. Antioxidation Activity *In vitro*

Each sample, including crude extract and antioxidant samples, was prepared to 0.1mg/mL water solution. The scavenging efficiencies on different free radicals were measured, and Vc was taken as a reference sample. The scavenging capacities on HO·, O_2^{-}· and DPPH· were measured respectively as references [13] stated.

2.8. Animal Grouping and Administration

Mice weighed before experiment, were randomly divided into 3 groups at random (30 mice/group), including NC (negative control) group, EUI (20% ethanol elution) group and EUII (60% ethanol elution) group. All the mice were fed in laboratory animal room, where the temperature was kept in 25.0±2.0°C and relative humidity was kept in 40-60%. The mice were kept in separated cage and fed with standard feed. The mice freely drank water and ate feed. Before oral administration test, the mice were adapted in laboratory animal room for 3d. During the oral administration test, the mice were taken swimming exercise for 30 min in the same time everyday. EUI and EUII were dissolved with pure water to 0.2g/ml solution. The oral administration dose is 0.01 ml/10g everyday. The extracts were orally administered into mice in EUI and EUII group once per day for 15d using a feeding atraumatic needle.

2.9. Measurement of MDA, SOD, GSH-PX [14-16]

1 hour after the last oral administration, the mice (10 mice picked at random from per group) were forced to swim for 90 min without weight loading. The mice were anesthetized with ether and whole blood samples were collected in the tubes by the aorta puncture. The blood samples were anti-coagulated with heparin sodium in the tubes. Blood samples were centrifuged for 10 min at a speed of 4000 rpm. The serum was collected and the contents of MDA, the activity of SOD and GSH-PX were analyzed by spectrophotometric method with commercial kit.

2.10. Statistical Analysis

The data were expressed as mean ± standard error of the mean (mean±S.D.). Data were analyzed using Student's *t*-test. Differences at $p<0.05$ were considered to be significant.

3. RESULTS AND DISCUSSION

3.1 Adsorption Efficiency of Each Resin

The adsorption efficiencies of LS-46、HPD-600 and XDA-8 resin on flavonoids and ployphenols from *Eucommia ulmoides* leaves crude extract were shown in Table **1**. Table **1** showed that LS-46 had higher adsorption efficiency on flavonoids, and XDA-8 resin had higher adsorption efficiency on ployphenols

Table 1. Adsorption Efficiency of Resin on Flavonoids and Polyphenols

Resin	Adsorption Efficiency (%)	
	Flavonoids	Ployphenols
LS-46	82.66	64.63
HPD-600	63.5	58.13
XDA-8	81.2	68.75

3.2. Adsorption Efficiency of Each Resin

The desorption efficiency of LS-46、HPD-600 and XDA-8 resin on flavonoids and ployphenols from *Eucommia ulmoides* leaves crude extract was shown in Table **2**. XDA-8 resin had higher desorption efficiency on flavonoids and ployphenols. Following the results showed in Tables **1** and **2**, XDA-8 was chosen for preparation of *Eucommia ulmoides* leaves antioxidant.

Table 2. Desorption Efficiency of Resin on Flavonoids and Polyphenols

Ethanol/%	LS-46 Desorption Efficiency (%)		HPD-600 Desorption Efficiency (%)		XDA-8 Desorption Efficiency (%)	
	Flavonoids	Polyphenols	Flavonoids	Polyphenols	Flavonoids	Polyphenols
20	6.95	23.59	11.56	20.99	11.78	44.38
40	14.59	15.36	22.43	15.53	26.80	13.01
60	21.42	15.21	12.65	15.21	46.86	19.07
80	5.31	9.90	1.72	6.32	2.43	4.98

3.3. Flavonoids and Ployphenols Contents in Different Samples

Flavonoids and Ployphenols Contents in Different Samples were shown in Fig. (**1**).

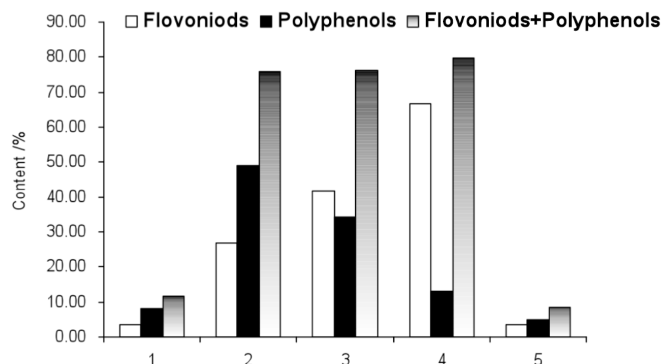

Fig. (1). Flavonoids and polyphenols contents in different samples. 1, crude extract; 2, 20% ethanol elution solution; 3, 40% ethanol elution solution; 4, 60% ethanol elution solution; 5, 80% ethanol elution solution.

It was shown in Fig. (**1**) that 20-60% ethanol elution solution enriched much more flavonoids and polyphenols, and the sums of contents of flavonoids and polyphenols in 20-60% ethanol elution solution were similar. However, the contents of flavonoids and polyphenols were changed with ethanol content. Polyphenols changed from high content to low content in 20%, 40% and 60% ethanol elution solution; Flavonoids changed from high content to low content in 60%, 40% and 20% ethanol elution solution. The result showed that polyphenols were separated first by low content ethanol elution solution (20%), but flavonoids were separated secondly by high content ethanol elution solution (60%). Therefore, when resin was used in separation and purification of flavonoids and polyphenols, each ethanol elution solution could be collected respectively to get products of different component content. In 80% ethanol elution solution, no matter flavonoids or polyphenols, the content was lowest and near to the content in crude extract.

3.4. The Antioxidation *In Vitro* of Each Antioxidant

The scavenging efficiencies on ·OH from different samples were shown in Fig. (**2**). Compared with crude extract, the scavenging efficiencies on ·OH from 20% ethanol elution solution sample was increased much significantly (p<0.01), and the scavenging efficiencies on ·OH from 40% and 60% ethanol elution solution sample was increased significantly (p<0.05), but for 80% ethanol elution solution sample and Vc, the differences were not obvious (p>0.05); compared with Vc, the scavenging efficiencies on ·OH from 20% ethanol elution solution sample was much significantly higher (p<0.01), and the scavenging efficiencies on ·OH from 40% and 60% ethanol elution solution sample were significantly higher (p<0.05), but for 80% ethanol elution solution sample, the difference was not obvious (p>0.05). This showed that after the *Eucommia ulmoides* leaves crude extract was separated and purified by resin, the antioxidative components were concentrated, and the fact that the scavenging efficiencies on ·OH from 20%, 40% and 60% ethanol elution solution sample were higher than crude extract proved this conclusion. Vc is a kind of strong and natural antioxidant. Fig. (**2**) showed that the scavenging capacities on ·OH from 20%, 40% and 60% ethanol elution solution sample were higher than Vc, especially for 20% ethanol elution solution sample, the scavenging capacities was much stronger Vc.

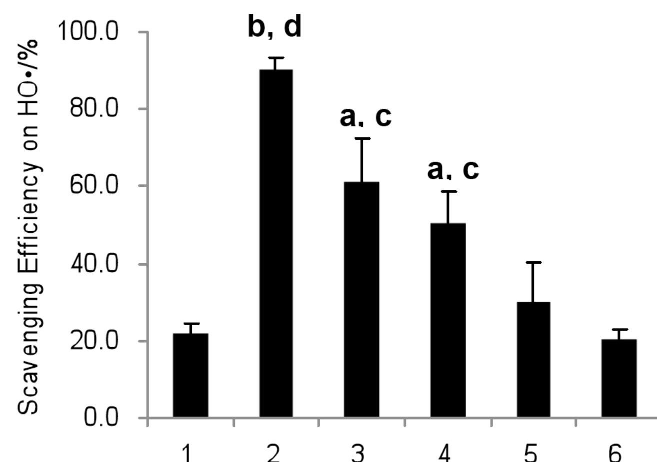

Fig. (2). Scavenging efficiency on •OH from different samples. 1, crude extract; 2, 20% ethanol elution solution; 3, 40% ethanol elution solution; 4, 60% ethanol elution solution; 5, 80% ethanol elution solution; 6, Vc. The data were expressed as mean±S.D. (n=3). The data were analyzed by t-test. Compared with crude extract, a means p<0.05, b means p<0.01; compared with Vc, c means p<0.05, d means p<0.01.

The scavenging efficiencies on O_2^{-}· from different samples were shown in Fig. (**3**). Compared with crude extract, the scavenging efficiencies on O_2^{-}· from 20% ethanol elution solution sample was increased much significantly (p<0.01), and the scavenging efficiencies on O_2^{-}· from 40% and 60% ethanol elution solution sample were increased significantly (p<0.05), but for 80% ethanol elution solution sample, the difference was not obvious (p>0.05)and for Vc, the difference was much significantly (p<0.01); compared with Vc, the difference of scavenging efficiencies on O_2^{-}· between 20% ethanol elution solution sample and Vc was not obvious (p>0.05), but for 40%, 60% and 80% ethanol elution solution sample, the differences were much significant (p<0.01). So, after the *Eucommia ulmoides* leaves crude extract was separated and purified by resin, except for 80% ethanol elution solution sample, the scavenging efficiencies on O_2^{-}·from each sample were enhanced obviously, especially for 20% ethanol elution solution sample, its scavenging capacities was much stronger than other samples. However, compared with Vc, which is a strong antioxidant, only 20% ethanol elution solution sample's scavenging capacity was near to it, and other samples were much weaker than it.

The scavenging efficiencies on DPPH· from different samples were shown in Fig. (**4**). Compared with crude extract, the scavenging efficiency on DPPH· from 20% ethanol elution solution sample was increased much significantly (p<0.01), and the scavenging efficiencies on DPPH· from 40% ethanol elution solution sample was increased significantly (p<0.05) and the difference between 60% solution sample and crude extract was not obvious, but

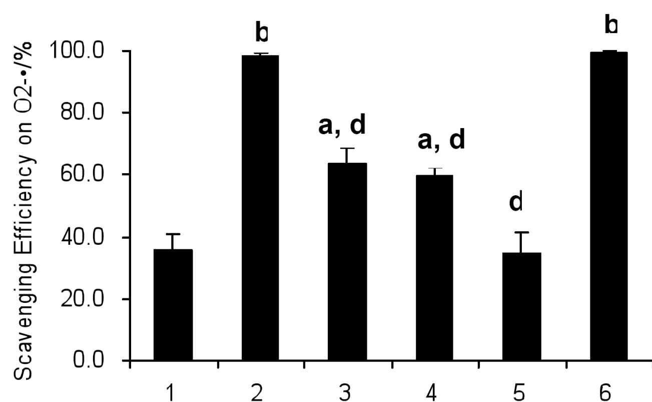

Fig. (3). Scavenging efficiency on $O_2^{-}\cdot$ from different samples. 1, crude extract; 2, 20% ethanol elution solution; 3, 40% ethanol elution solution; 4, 60% ethanol elution solution; 5, 80% ethanol elution solution; 6, Vc. The data were expressed as mean±S.D. (n=3). The data were analyzed by t-test. Compared with crude extract, a means p<0.05, b means p<0.01; compared with Vc, c means p<0.05, d means p<0.01.

for 80% ethanol elution solution sample, the scavenging efficiency was reduced much significantly (p<0.01) and for Vc, the difference was much significantly (p<0.01); compared with Vc, the difference of scavenging efficiencies on DPPH· between 20% ethanol elution solution sample and Vc was not obvious (p>0.05), but for 40%, 60% and 80% ethanol elution solution sample, the differences were much significant (p<0.01). DPPH· is a stable free radical in organic solvent. Except for 80% ethanol elution solution sample, the scavenging efficiencies on DPPH· from each sample were enhanced obviously, especially for 20% ethanol elution solution sample, its scavenging capacity was much stronger than other samples and near to Vc.

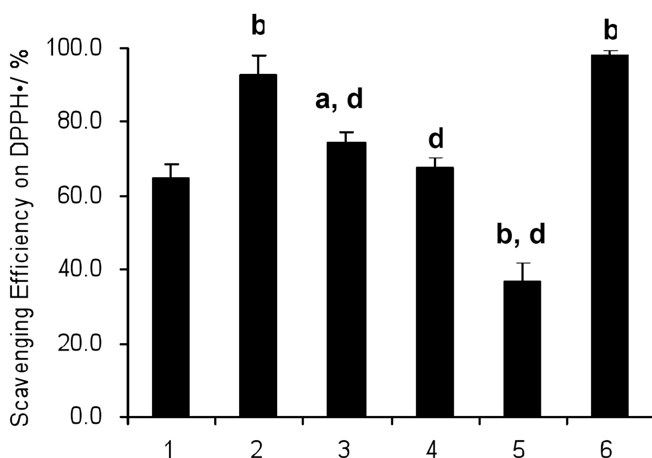

Fig. (4). Scavenging efficiency on DPPH· from different samples. 1, crude extract; 2, 20% ethanol elution solution; 3, 40% ethanol elution solution; 4, 60% ethanol elution solution; 5, 80% ethanol elution solution; 6, Vc. The data were expressed as mean±S.D. (n=3). The data were analyzed by t-test. Compared with crude extract, a means p<0.05, b means p<0.01; compared with Vc, c means p<0.05, d means p<0.01.

From Figs. (**2-4**), it could be seen that 20%, 40% and 60% ethanol elution solution had strong scavenging capacities on each free radicals, but for 80% ethanol elution solution sample, the difference of scavenging capacity between it and crude extract was not obvious, which was identical to that the contents of flavonoids and polyphenols in 20%, 40% and 60% ethanol elution solution sample were much higher than 80% ethanol elution solution sample and crude extract, but for 80% ethanol elution solution sample, the content was near to crude extract. So, flavonoids and polyphenols were antioxidative component in *Eucommia ulmoides* leaves. In addition, the sequence of free radicals scavenging capacities from high to low was 20%, 40%, 60% and 80% ethanol elution solution sample, and polyphenols content from high to low was also this sequence. Thus it could be seen that polyphenols played a leading role in antioxidation activity of *Eucommia ulmoides* leaves antioxidant.

3.5. The Antioxidation *In Vivo* of EUI and EUII

The result of effects on MDA, SOD, and GSH-PX of EUI and EUII in mice was shown in Figs. (**5-7**). Compared with NC group, the contents of MDA in blood serum of exercised mice in EUI and EUII group were reduced, and the difference between EUI and NC group was significant (p<0.05), but EUII and NC had no significant difference; the SOD activity of exercised mice in EUI and EUII group were increased, and the difference between EUI and NC group was very significant (p<0.01), but EUII and NC had no significant difference; the GSH-PX activity in both group were increased, and the difference between EUI and NC group was significant (p<0.05), EUII and NC had no significant difference. In body, SOD can deoxidize O^{2-} to H_2O_2 and O_2, and GSH-PX can deoxidize organic peroxide and H_2O_2 to H_2O and non-noxious substance. If there exists SOD and GSH-PX, the content of O^{2-}, organic peroxide and H_2O_2 will be reduced. So the peroxidative injury to cell and mitochondrial will be prevented, and MDA, which is the product of lipid perioxidation, will be reduced too. EUI could enhance body's SOD activity (p<0.01), reduced the content of MDA (p<0.05) and increase GSH-PX activity (p<0.05). The result showed that EUI could enhance the antioxidation ability in body.

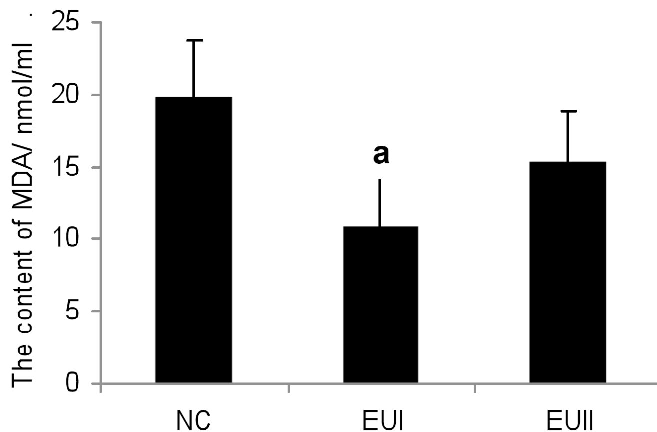

Fig. (5). MDA content in mice. NC, negative group; EUI, 20% ethanol elution solution group; EUII, 60% ethanol elution solution group. The data were expressed as mean±S.D. (n=10). The data were analyzed by t-test. Compared with NC, a means p<0.05, b means p<0.01.

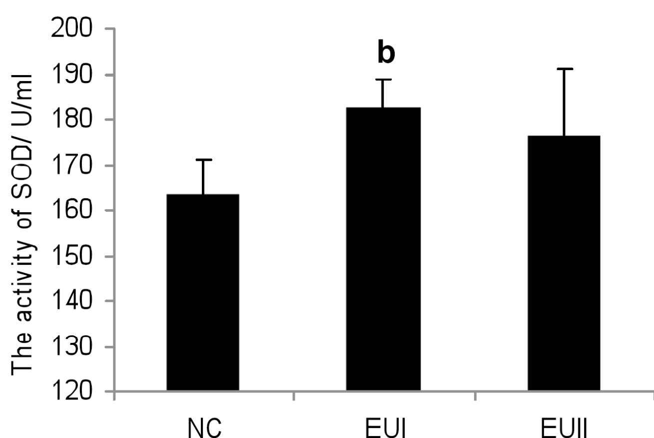

Fig. (6). SOD activity in mice. NC, negative group; EUI, 20% ethanol elution solution group; EUII, 60% ethanol elution solution group. The data were expressed as mean±S.D. (n=10). The data were analyzed by t-test. Compared with NC, a means p<0.05, b means p<0.01.

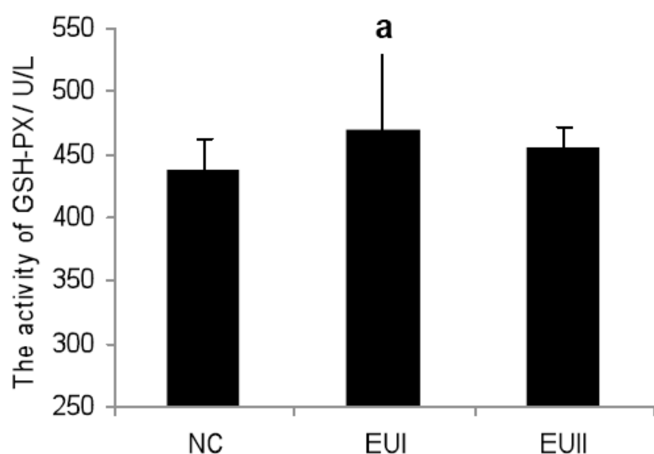

Fig. (7). GSH-PX activity in mice. NC, negative group; EUI, 20% ethanol elution solution group; EUII, 60% ethanol elution solution group. The data were expressed as mean±S.D. (n=10). The data were analyzed by t-test. Compared with NC, a means p<0.05, b means p<0.01.

4. CONCLUSIONS

- Through the investigation on adsorption efficiency and desorption efficiency on flavonoids and polyphenols from *Eucommia ulmoides* leaves crude extract by LS-46、HPD-600 and XDA-8 resin, the XDA-8 resin was chosen as a suitable resin for preparation of *Eucommia ulmoides* leaves antioxidant.
- Except 80% ethanol elution sample, each antioxidant had much more flavonoids and polyphenols, which were active component on scavenging free radicals, and polyphenols was the leading component. Different ethanol elution solution had different content of flavonoids and polyphenols. In 20% ethanol elution solution sample, polyphenols were enriched more; in 60% ethanol elution solution sample, flavonoids were enriched more.
- For antioxidation *in vitro*, the 20%, 40%, 60% and 80% ethanol elution solution sample had different scavenging capacities on HO·, O_2^{-}· and DPPH·, and 20%, 40% and 60% ethanol elution solution sample exhibited obviously better scavenging capacities than crude extract. However, 20% ethanol elution solution sample showed more obvious antioxidation *in vitro* than others, which showed polyphenols was the leading working material in antioxidation *in vitro*.
- For antioxidation *in vivo*, EUI (20% ethanol elution solution) showed more obvious antioxidation activities *in vivo*, which also meant that polyphenols was the leading working material in antioxidation *in vivo*.

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