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Full Length Research Paper

In vitro and *in vivo* studies of antioxidant activities of flavonoids from *Adiantum capillus-veneris* L.

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In this study, ultrasonic-assisted extraction was used to extract flavonoids from *Adiantum capillus-veneris* L. The crude flavonoids of Adiantum (FA) were then purified with the AB-8 macroporous adsorption resin. The antioxidant activities of the purified FA were evaluated and compared with synthetic antioxidants in scavenging capability of 1, 1-diphenyl 1-2-picryl-hydrazyl (DPPH) free radical, scavenging capacity of superoxide anion, chelating capability of ferrousion and reducing power *in vitro*. The antioxidant activities of FA *in vivo* also were investigated through acute mice liver injury experiment. The results showed that antioxidant activity of FA was relevant to concentration; the antioxidant activities of FA *in vitro* were approximate or more significant than that of some synthetic antioxidants (butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA) and ascorbic acid). The liver intoxicated by CCl_4 in mice showed a significant decrease in superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) levels, simultaneously significant increase in the level of malondialdehyde (MDA) ($P < 0.01$). Compared with the toxic control group that received only CCl4, the group that received vitamin E and higher dose of FA showed significant increase in activity of SOD, CAT and GSH, together with notable decrease in activity of MDA. These changes were in a dose dependent manner. The results showed that the FA possesses potent antioxidant properties, and might be a potential natural source of antioxidants that could be applicable to the medical and food fields.

Key words: Flavonoids, antioxidant, *in vitro, in vivo*, *Adiantum capillus-veneris* L.

INTRODUCTION

Free radicals, chemical reactions and several redox reactions of various compounds may cause protein oxidation, DNA damage and lipid peroxidation in living cells (Sahreen et al., 2010). Therefore, oxidation have been claimed to play an important role in human health and causing several diseases, including cancer, hypertension, heart attack and diabetes (Halliwell, 2007). However, living organisms have developed antioxidant systems to counteract reactive species and to reduce their damage. These complex antioxidant systems include enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) macromolecules and an array of small molecules, including ascorbic acid, α-tocopherol, carotenoids, polyphenols, uric acid and bilirubin (Yu, 1994). Oxidative damage occurs when this system is overwhelmed. Antioxidants could increase these complex antioxidant systems and protect the human body against free radicals that may cause pathological conditions, such as anaemia, arthrists, inflammation, neurodegeneration, ageing process and perhaps dementias (Polterait, 1997). Free radicals can be scavenged through utilizing natural antioxidant compounds present in medicinal plants. Some medicinal plants have been shown to have both chemopreventive and/or therapeutic effects on human diseases (Sabu and Kuttan, 2002).

Adiantum capillus-veneris L. (Adiantum) is a kind of medicinal and ornamental ferns belonging to Adiantaceae. It is one of the common and widely

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distributed Chinese medicinal herb (Tang et al., 2007). As described in traditional Chinese medical literature, it have been utilized as diuretic and was used to treat cold, fever, cough, bronchial disorders, stimulant, emollient, purgative, demulcent, skin diseases and tumors of liver and other viscera (Singh et al., 2008). It is a perennial fern and is proved rich in bioactive compounds, such as phenolic, hydrolysable tannins and polysaccharides (Tang et al., 2007). Flavonoids are a large group of phenolic plant constituents. The antioxidant activity of phenolic compounds in plants is mainly due to their redox properties and chemical structure, which can play an important role in neutralizing free radicals, chelating transitional metals and quenching triplet oxygen, by delocalization or decomposing peroxides (Zheng and Wang, 2001). Moreover, some findings indicated that flavonoids possess various clinical properties, such as antiatheros- clerotic, antiinflammatory, antitumour, antiosteoporotic and antiviral effects (Zheng and Wang, 2001). There are, however, few reports about antioxidant activity of FA *in vitro* and *in vivo*. Antioxidant activity of FA *in vitro* and *in vivo* was determined in this study.

MATERIALS AND METHODS

Apparatus

Adiantum were purchased from Bozhou Chinese herbal market (Anhui, China). They were air dried, smashed and passed through a 60-mesh sieve.

Superoxide dismutase (SOD), catalase (CAT), malondial dehyde (MDA) and glutathione (GSH) assay kits were purchased from Nanjing Jianchen Bioengineering Institute (Nanjing, China). All other chemical reagents used in the experiments were of analytical grade as commercially available.

Test animals are Kunming mice (20 ± 2 g, equal male and female), which were obtained from the Laboratory Animal Center of Jiangsu University (Zhenjiang, China). Mice were housed in polypropylene cages after acclimatization for a period of one week in a new environment with free access to standard food and water ad libitum. Animals were handled according to the rules and regulations of Institutional Animal Ethics Committee (IAEC), Jiangsu University, China.

Spectrophotometer (UV-9100 Ruili Corporation, Beijing, China) was used for total flavonoids analysis, heating and drying oven (DHG-9240A Shanghai Yiheng Science and Technology Co, Ltd., Shanghai, China) was used for dryness of sample, FD-1 freeze drier (Beijing Boyikang, China) was used for freeze-dried.

Ultrasonic-assisted extraction of flavonoids

Adiantum powder (15 g) was accurately weighed and put into Soxhlet extractor with adding petroleum ether to extract lipid until it became colorless, then it was cooled and dried at 45°C in heating and drying oven.

The dried and defatted Adiantum powder (10 g) was placed into a volumetric flask (400 ml), soaked with ethanol solvent (ethanol concentration is 80%, v/v) and then was extracted by ultrasound at 120W for 90 min. The extract was filtered, and the filtrate was collected and stored at 4°C (Wang et al., 2008).

Purification of flavonoids by macroporous resin adsorption

The crude flavonoids extract was purified by AB-8 macroporous adsorption resin in a column (1.5 × 1.5 × 25 cm^3). The conditions of purification were: injecting concentration 1.21 mg/ml, pH 5, 50% (v/v) ethanol as desorption solvent, the purified extract of flavonoids was collected and then freeze-dried for determination of bioactivity (Huang et al., 2009).

Determination of total flavonoids content

The total flavonoids content was determined with a colorimetric method described by Jia et al. (1999) with some modification. Briefly, 0.5 ml diluted sample was transferred to 10 ml colorimetric tube, then 0.5 ml of 5% (w/v) sodium nitrite, 0.5 ml of 10% (w/v) aluminum nitrate and 4 ml of 4% (w/v) sodium hydroxide were added in the order stated. The final volume was adjusted to 10 ml with 95% (v/v) ethanol. The mixture was allowed to react for 15 min; the absorbance was then measured at 510 nm against a blank. The amount of the total flavonoids was expressed as rutin equivalents (μg rutin/g sample) through the calibration curve of rutin. The calibration curve was A = 0.5717C + 0.0041, where A was absorbance of sample, C was sample concentration (R2 = 0.9996).

In vitro antioxidant activities

Scavenging activity on DPPH radical

The scavenging activity on DPPH radical of the purified FA and BHT was measured according to the method of Liu et al. (2009) with some modifications. The 0.04 g/L solution of DPPH in 100% ethanol was prepared before UV measurements. 1 ml of the samples of purified FA and BHT from 0.1 to 1.6 mg/ml were thoroughly mixed with 4 ml of prepared DPPH and 4 ml of 100% ethanol. The mixture was shaken vigorously and left to react 20 min in the dark, and the absorbance was then measured at 517 nm against a blank. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. BHT was used as positive control. The capability to scavenge the DPPH radical was calculated using the formula given as:

$$\text{Scavenging activity (\%)} = [1-(Ai-Aj)/A0] \times 100$$

where A0 was the absorbance of DPPH solution without sample (4 ml DPPH + 4 ml of 100% ethanol); Ai was the absorbance of the test sample mixed with DPPH solution (4 ml sample + 4 ml DPPH) and Aj was the absorbance of the sample without DPPH solution (4 ml sample + 4 ml of 100% ethanol).

Scavenging activity on superoxide anion radical

Superoxide anion radical scavenging activity was determined as described by Nagendra et al. (2009) with some modifications. All solutions were prepared in 0.2 M phosphate buffer (pH 7.4). The samples of purified FA and BHT from 0.05 to 0.8 mg/ml were mixed with 3 ml of reaction buffer solution (pH 7.4) containing 1.3 μM riboflavin, 0.02 M methionine and 5.1 μM nitro blue tetrazolium. The reaction solution was exposed to two 30W fluorescent lamps for 20 min and the absorbance was then measured at 560 nm. BHT was used as a comparison. The superoxide anion radical scavenging activity (%) was calculated using the formula given as:

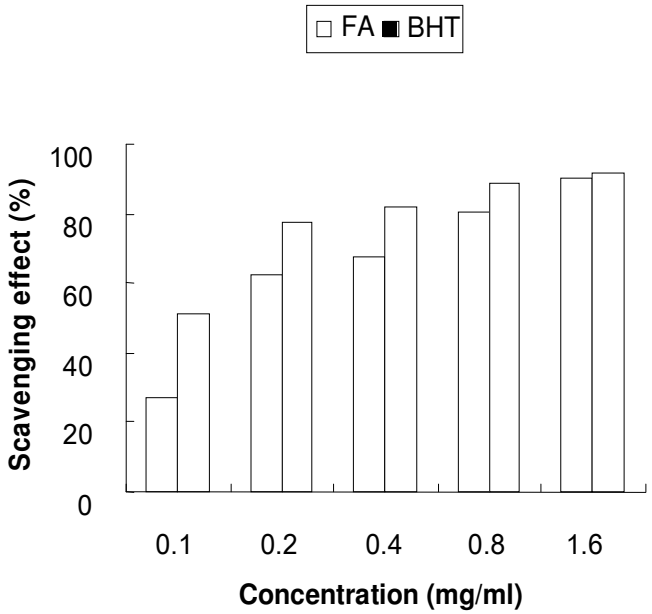

Figure 1. DPPH free radical scavenging effects of FA and BHT.

Scavenging activity (%) = (1–Asample/A control) × 100.

The ability of chelating ferrous ions determination

Chelating ability was measured according to the method of Tumay et al. (2009). Sample (0.1 to 1.6 mg/ml) in ethanol (1 ml) was mixed with 4.7 ml of methanol and 0.1 ml of 2 mmol/L ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mmol/l ferrozine. After 10 min at room temperature, the absorbance of the mixture was measured at 562 nm against blank. The results were expressed as percentage of inhibition of the ferrozine-Fe^{2+} complex formation. The percentage of inhibition of the ferrozine-Fe^{2+} complex formation was calculated using the formula given as:

Chelating ability (%) = [(Acontrol - Asample)/Acontrol] × 100;

where Acontrol is the absorbance of the ferrozine-Fe^{2+} complex and Asample is the absorbance in the presence of purified FA. EDTA was used as positive control.

Reducing power

The determination of reducing power was carried out as described by Vaquero et al. (2010). 1 ml sample (0.05 to 0.8 µg/ml) was mixed with 2.5 ml of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide. After the mixture was incubated at 50°C for 20 min, 2.5 ml of 10% (w/v) trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer of 5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% (w/v) ferric chloride for 10 min, and then the absorbance was measured at 700 nm against a blank. The reducing power of ascorbic acid was also determined for a comparison. High absorbance of the reaction mixture indicates strong reducing power.

The four experiments mentioned previously were all performed three times and averaged.

Antioxidant activities *in vivo*

Experimental design and sample collection

Mice were randomly divided into six groups of six in each. Group I received distilled water containing 0.3% sodium carboxymethylcellulose (CMC-Na) (1 µl/g body weight, p.o.) for 5 days, and olive oil (1 µl/g body weight, s.c.) on days 2 and 3. Besides 0.3% CMC-Na (1 µl/g body weight, p.o.) for 5 days, Group II, III, IV, V and VI also given a mixture of CCl_4 and olive oil at rate of 1:1 (2 µl/g body weight, s.c.) on day 2 and 3. Additionally, Group III received vitamin E (50 µl/g body weight, p.o.) daily for 5 days. Groups IV, V and VI were treated by FA in a dose of 50, 100 and 200 µl/g body weight, respectively for 5 days. Mice were then sacrificed by bleeding on day 6, the liver was removed and homogenized for the measurement of antioxidant activity (huang et al., 2011).

Biochemical detection

Liver homogenates (10% w/v) were prepared in potassium phosphate buffer (50 mM, pH 7.4), and the suspension was centrifuged at 4°C, 1000 rpm for 12 min. The supernatant was used for the determination of SOD, CAT, MDA and GSH. They were assayed based on the method provided by KITs.

Statistical analysis

Data was presented as mean (x) ± standard deviation (SD). The differences among different groups were analyzed using one-way analysis of variance (ANOVA), differences were considered to be statically significant at $P < 0.05$.

RESULTS AND DISCUSSION

In vitro the antioxidant activities of the FA

DPPH is a commercial oxidizing radical, which can be reduced by antioxidants. The disappearance of the DPPH radical based on the absorbance at 517 nm wavelength can be monitored by decreased optical density (Blois, 1958). The result of DPPH free radical-scavenging ability of purified FA was depicted in Figure 1, in which BHT was as synthetic antioxidants control. It can be seen from Figure 1 that the DPPH radical scavenging increased from 27.44 to 90.27% when the concentration increased from 0.1 to 1.6 mg/ml. The activities of BHT were from 51.23% at 0.1 mg/ml to 91.78% at 1.6 mg/ml. BHT and purified FA had the approximate radical-scavenging abilities, where sample had the highest concentration. At the concentration of 1.6 mg/ml, the activities of purified FA was 90.27%, only little lower than those of BHT. The results indicated that the purified FA have significant DPPH radical scavenging activity at higher concentration.

The superoxide anion can be generated by illuminating a solution containing riboflavin. In this study, the superoxide anion scavenging effects of purified FA were depicted in Figure 2. Purified FA exhibited excellent superoxide anion scavenging activity, and was higher

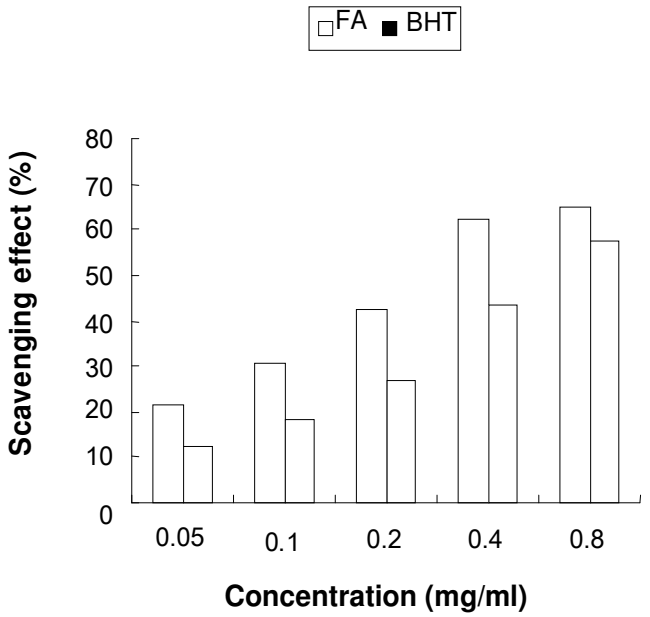

Figure 2. Superoxide radical scavenging effects of FA and BHT.

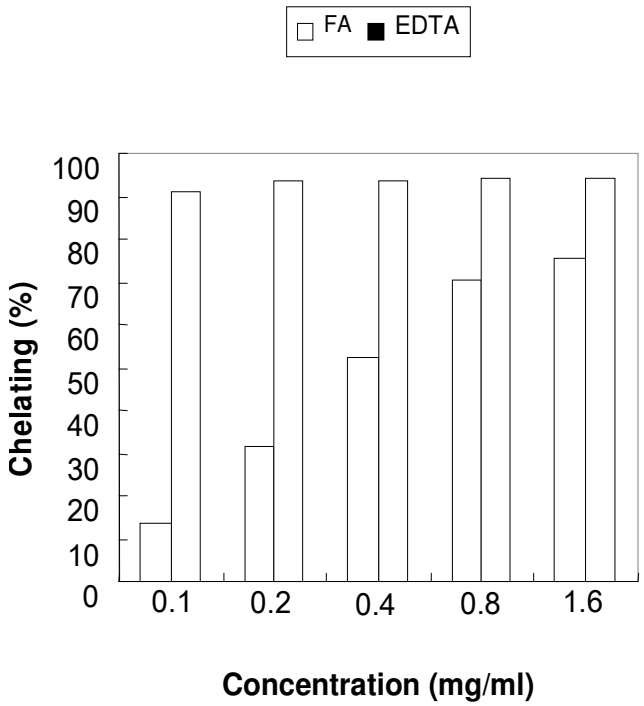

Figure 3. Ability of chelating ferrousion of FA and EDTA.

than that of BHT. At 0.8 mg/ml, the superoxide scavenging activities of purified FA was 64.74% and BHT was 57.62%. These findings are noteworthy because purified FA may be useful in the treatment of many kinds of diseases related to free radical oxidations. Notably, purified FA would be well adapted to the pathogenesis of ischemic disease, which is characterized by an overproduction of the superoxide anion due to a leak of electrons in the mitochondrial respiratory chain and to the conversion of xanthine dehydrogenase to XOD, which produces O^{2-} when converting hypoxanthine successsively to xanthine, then uric acid. Thus, FA being able to inhibit both XOD and to scavenge O^{2-} may be useful as protecting agents against cellular injury during reperfusion of ischemic tissues (Werns and Lucchesi, 1990).

The chelating ability of purified FA was depicted in Figure 3. It had been recognized that the FA interfering with the catalytic activity of metal ions could affect the peroxidative process. Fe^{2+} ions are the most powerful prooxidant among various species of metal ions. Chelating ability of the purified FA was determined by the ferrozine assay. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating, the complex formation was disrupted with the result that the red color of the complex is decreased. Measurement of the rate of color reduction therefore allows estimation of the chelating activity of the coexisting chelator (Yamaguchi et al., 2000). In this assay, EDTA exhibited an excellent ferrous ion-chelating ability of 94.51% at a concentration of 1.6 mg/ml, the ability of purified FA was 75.38% at same concentration. It indicated that FA had higher chelating ability of ferrousion in higher concentration.

The reducing powers of the purified FA and ascorbic acid were shown in Figure 4. Higher absorbance value indicated higher antioxidant activity. Reducing power is generally associated with the presence of reductones, which exert antioxidant action by breaking the free radical chain through donating a hydrogen atom (Duan et al., 2007). Flavonoids appear to function as good electron and hydrogen-atom donors, and therefore should be able to terminate radical chain reactions by converting free radicals to more stable products. In this assay, the presence of purified FA in the tested samples would result in reducing Fe3+/ferricyanide complex to the ferrous form (Fe^{2+}). The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The FeCl3/K3Fe(CN)6 system offers a sensitive method for the "semi-quantitative" determination of dilute concentrations of flavonoids, which participate in the redox reaction. In the present study, purified FA exhibited a stronger reducing power, which is approximately seen in ascorbic acid. Furthermore, the reducing power of the purified FA increased with concentration. At a 0.8 mg/ml concentration, the reducing power of purified FA was 3 times superior to that of the 0.05 mg/ml concentration. At the concentration of 0.8 mg/ml, the reducing power of purified FA was 2.24, only little lower than those of ascorbic acid. Furthermore, this reducing power of purified FA approximate that of a green tea extract. Yen and Chen (1995) reported that the reducing powers of green, pouchong, oolong and black tea extracts were 2.47, 2.38, 2.75 and 1.32 at a 1.0 mg dose, respectively.

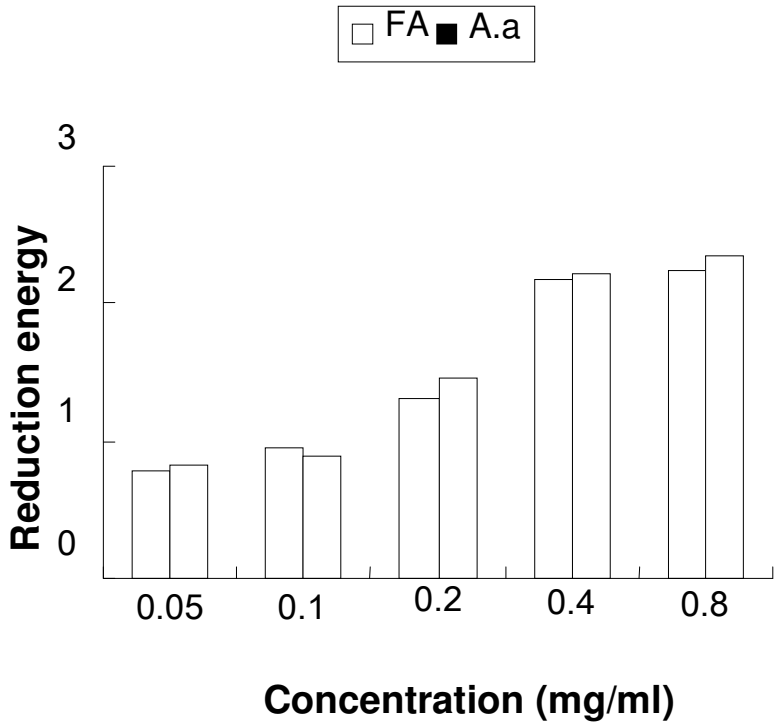

Figure 4. Reducing power of purified FA and ascorbic acid (A.a).

Table 1. Effects of FA on liver SOD, MDA, CAT and GSH in CCl_4-intoxicated mice.

Group	SOD (U/mg protein)	MDA (nmol/mg protein)	CAT (U/mg protein)	GSH (mg/g protein)
I	331.67 ± 30.48	2.74 ± 0.33	62.58 ± 10.07	6.07 ± 0.87
II	137.50 ± 34.56$^{\#\#}$	5.85 ± 0.44$^{\#\#}$	32.41 ± 9.58$^{\#\#}$	2.23 ± 0.52$^{\#\#}$
III	251.82 ± 28.53**	4.62 ± 0.59**	48.43 ± 5.83*	3.37 ± 0.78*
IV	161.13 ± 38.21	4.84 ± 0.90*	31.83 ± 6.62	2.89 ± 0.25*
V	196.37 ± 48.48*	3.08 ± 0.58**	44.60 ± 7.39*	4.23 ± 0.47**
VI	262.23 ± 35.87**	4.94 ± 0.66*	46.47 ± 4.02**	4.21 ± 0.44**

The data were expressed in mean ± SD and n = 6 in each group. Compared with Group I, $^{\#}$, $P < 0.05$ and $^{\#\#}$, $P < 0.01$. Compared with Group II, *, $P < 0.05$, and **, $P < 0.01$.

According to these results, purified FA was found as the better radical reducer for this system.

In vivo the antioxidant activities of the FA

Table 1 showed the antioxidant activities of FA in liver of mice. Group I was control group, Group II was the toxic control group that received CCl4, Group III received CCl_4 + vitamin E 50 (μg/g), group IV received CCl_4 + FA 50 (μg/g), group V received CCl_4 + FA 100 (μg/g), Group VI received CCl_4 + FA 200 (μg/g). Compared with the Group I, Group II exhibited a significant decrease in SOD, CAT and GSH levels, together with significant increase in the level of MDA ($P < 0.01$). Compared with the Group II, Vitamin E and higher dose of FA showed significant increase in activity of SOD, CAT and GSH ($P < 0.01$), and notable decrease in activity of MDA ($P < 0.05$). These changes were in a dose dependent relationship.

The pathological disturbances caused by CCl4- are extensively used for the evaluation of antioxidant effects of drugs and extracts of plant (Avijeet et al., 2008). The hepatic damage induced by CCl_4- is well known to be evidenced by free radical metabolites, such as trichloromethyl-free radicals ($\bullet CCl_3$) and trichloromethyl peroxy radicals ($\bullet OOCCl_3$), which could readily interact with unsaturated membrane lipid to produce lipid peroxidation, and lead to cell damage (Snyder and Andrews, 1996). CCl_4 liver toxicity depends on cytochrome P450 in endoplasmic reticulum in which there is catalytic reduction from dehalogenation of CCl_4 to radical instability $\bullet CCl_3$ (Yan et al., 2011). Covalent binding reaction with macromolecular material in the cells was

followed with cell structure damage and enzyme activity decline. •CCl_3 and oxygen can also form a high activity of •$OOCCl_3$; although, lipid peroxidation reaction occurs, and it results in the destruction of poly-unsaturated fatty acids (Yan et al., 2011). It has been reported that one of the principal causes of CCl_4- induced damage is lipid peroxidation that is induced and accelerated by free radical derivatives of CCl_4 (Avijeetet et al., 2008). Therefore, free radical scavenging shows important means to protect against CCl_4-induced oxidative damage.

SOD represents one protection against oxidative tissue-damage. SOD metabolizes O^{2-} to H_2O_2. CAT converted H_2O_2 into non-toxic products. The decrease of SOD and CAT may result in many deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide (Srinivasan et al., 2007). GSH was another important parameter that evidenced oxidative damage. Decrease in liver GSH activity in CCl_4- treated mice indicates the damage to the cells and peroxidation (Halliwell, 1994). On the contrary, MDA is a cytotoxic product of lipid peroxidation. In this study, higher dose of FA were shown to have significant increase in the activity of SOD, CAT and GSH, and notable decrease in activity of MDA. The fact of FA treatment indicated that FA might prevent the peroxidation by CCl_4.

Our investigation showed that FA has strong radical scavenging activity and antioxidant activity *in vitro* in this study. Reducing powers of flavonoids are generally related to the presence of their reductones. The antioxidant mechanism used by reductones breaks free radical chain reactions by donating a hydrogen atom, or by reacting with certain precursors to prevent peroxide formation (Gordon, 1990). Therefore, radical scavenging is associated with the inhibition of peroxidation (Rekka and Kourounakis, 1991). The data from this study indicated that the radical scavenging activity of FA seem to result in its antioxidant activity *in vivo*. Further study should be carried on revealing the concrete molecular mechanism of FA reversing CCl_4- treated hepatocytes damage.

Conclusions

In this study, the antioxidant activities of the FA were evaluated *in vitro* and *in vivo*. The results indicated that the FA had significant antioxidant activities, which can be used as a source of potential antioxidant and functional medicinal material. The use of Adiantum as a natural antioxidant source appears to be an alternative to synthetic antioxidants. The knowledge gained from this study should be useful for further exploitation and application of the Adiantum resource.

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