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Full Length Research Paper

Bioactivity-guided fractionation for antioxidant property of *Athyrium multidentatum* (Doll) Ching. rhizome

Ji-wen Sheng[1]*, Dong-mei Liu[1], Zhi-jian Li[1], Li Qi[2] and Wei-fen Zhang[1]

[1]Department of Pharmacy and Biological Sciences, Weifang Medical College, Weifang 261053, China.
[2]Clinical College of Medicine, Weifang Medical College, Weifang 261031, China.

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In this paper, methanolic extract of *Athyrium multidentatum* (Doll) Ching. (AMC) rhizome was fractionated into three fractions (petroleum ether fraction (PEF), ethyl acetate fraction (EAF) and n-butanol fraction (BF)) on the basis of polarity. Each fraction had their antioxidant activities estimated employing various established *in vitro* systems, including superoxide/hydroxyl radical scavenging activity and reducing power. BF showed the strongest antioxidant activity and was chromatographed on a silica gel column followed by gradient elution. Fractions with CH_2Cl_2 – EtOAc 10:1, CH_2Cl_2 – EtOAc 5:1~1:1 and EtOAc – CH_3OH 10:1~1:1 as eluent showed significant antioxidant activity against superoxide anion radical and reducing power. All the results showed that AMC could be potential rich sources of natural antioxidants.

Key words: *Athyrium multidentatum* (Doll.) Ching, antioxidant activity, radical scavenging activity, reducing power, active fraction.

INTRODUCTION

Free radicals and other reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide are constantly generated through many biological processes and may be considered as a measure of biological inefficiency (Al-Omar et al., 2005). A vast amount of circumstantial evidence implicates oxygen-derived free radicals (especially superoxide and hydroxyl radical) as mediators of inflammation, shock and ischemia/reperfusion injury. Furthermore the radicals also play a role in the process of aging and carcinogenesis (Cuzzocrea et al., 2001). Antioxidants are able to prevent the radical chain reactions of oxidation by interrupting the free-radical chain of oxidation and donating hydrogen, thereby, forming stable free radicals, which do not initiate or propagate further oxidation of lipids (Wade et al., 1987). The most commonly used antioxidants at the present time are butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ). However, BHA and BHT are restricted by legislative rules because they are suspected to have some toxic effects, such as liver damage and carcinogens (Soubra et al., 2007). Therefore, with safety concerns identified for these synthetic antioxidants, considerable interest has arisen in finding alternative natural antioxidants. AMC is one of the most common ferns in Northeast of China and possesses extensive pharmacological effects, such as tranquilization, lowering blood pressure and diuresis.

The utilization of AMC as a drug has been documented in traditional Chinese medicine for over a thousand years. Mentionablely, AMC is also a delicious and nutritious potherb in Changbai Mountain area of China since ancient times and is very popular in South Korea, Japan and other countries. However, there are very few studies so far in the literature on antioxidant activity associated to AMC. Our previous studies have demonstrated that AMC polysaccharides had pronounced antioxidant activity against superoxide radical (Liu et al., 2011). The methanolic extract of AMC displayed stronger antioxidant

*Corresponding author. E-mail: sjwchy@wfmc.edu.cn.

Abbreviations: AMC, *Athyrium multidentatum* (Doll) Ching; **PEF,** petroleum ether fraction; **EAF,** ethyl acetate fraction; **BF,** n-butanol fraction; **ROS,** reactive oxygen species; **BHA,** butylated hydroxyanisol; **BHT,** butylated hydroxytoluene; **PG,** propyl gallate; **TBHQ,** tert-butylhydroquinone; **TCA,** trichloroacetic acid; **SOD,** superoxide dismutase; **CAT,** catalase; **GPx,** glutathione peroxidase.

activity than polysaccharides. In this paper, the methanolic extract of AMC was fractionated into three fractions: PEF, EAF and BF. Each fraction had their antioxidant activities investigated and the fraction showed the strongest antioxidant activity was chromatographed on a silica gel column followed by gradient elution. Concentrated elution fractions were screened by superoxide anion scavenging and reducing power-guided activity assay. The aim of the present study was to investigate the antioxidant activity of fractions of AMC.

MATERIALS AND METHODS

Materials and chemicals

Rhizome of AMC was harvested in Changbai Mountain area of China (September, 2009), air-dried and kept in plastic bags at room temperature before using. Pyrogallol, trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), and Vitamin C (Vc) were purchased from Sigma (St. Louis, MO, USA). Other reagents were of analytical grade and purchased from China National Pharmaceutical Group Corporation.

Preparation of the samples

A total of 1 kg of AMC rhizome was crushed and refluxed in methanol for 3 h. 22 g of crude extract (yield, 2.2%) was obtained from AMC rhizome after the hot extraction solution was separated from the residues and evaporated under reduced pressure. This extract was suspended in distilled water and partitioned successively with petroleum ether, ethyl acetate and aqua-saturated n-butanol. 1.5 g of petroleum ether, 3 g of ethyl acetate and 4.2 g of n-butanol extract were prepared after each fraction was concentrated under reduced pressure and the yield was 6.8, 3.6 and 19.1%, respectively. 0.1 g of petroleum ether extract was dissolved in 95% ethanol at a concentration of 10 mg/ml, then diluted to a final concentration of 666.67 µg/ml and stored at 4°C until further use. Sample of PEF, EAF and BF was obtained and were prepared in the same way.

Reducing power assay

The reducing power of all samples was determined by the method described by Yamaguchi et al. (1998), modified slightly. All solutions were used on the day of preparation. Briefly, 9.0 ml of reaction mixture, containing different concentrations of samples (133.34 to 666.67 µg/ml) in phosphate buffer (0.2 M, pH 6.8), was incubated with potassium ferricyanide (1%, w/v) at 50°C for 20 min. The reaction was terminated by TCA (10%, w/v) solution. Then the solution was mixed with distilled water and $FeCl_3$ (0.1%, w/v) solution. The reaction mixture was shaked vigorously and left stillness for 1 h. Increased absorbance at 700 nm indicated increased reducing power. Vc was used as positive control.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging ability of all samples was assessed by the method of Cao et al. (2008) with minor modification. The reaction mixture, containing H_2O_2 (1.0 mM) and $FeSO_4$ (2.0 mM), was incubated with salicylate (6.0 mM) at 37°C for 15 min. The absorbance A_1 of the mixture was determined at 510 nm against the blank. The absorbance A_2 was determined after 1.0 ml of sample was added. In order to offset the reduced absorbance caused by the adding of sample, the absorbance A_3 and A_4 were determined. The absorbance A_3 was determined after the mixture was incubated at 37°C for another 10 min. The absorbance A_4 was determined after distilled water (equal volume with sample) was added into the mixture. The hydroxyl radical inhibition percentage was calculated according to the given formula: Scavenging effect (%) = $[A_1 – A_2 – (A_3 – A_4)] \times 100 / A_1$

Superoxide radical scavenging assay

Measurement of superoxide radical scavenging activity was based on the method of Cao et al. (2008) with slight modification. Superoxide radicals were generated in the pyrogallol autoxidantion system containing 4.5 ml Tris-HCl buffer (50 mM, pH 8.2), 0.3 ml pyrogallol (3 mM), and varying concentrations of samples (31.75 to 158.73 µg/ml), the mixture prepared earlier was incubated at room temperature for 20 min. The absorbance A_1 at 319 nm was read every 30 s for 5 min against the blank. The speed of pyrogallol autoxidantion V_1 within 5 min can be calculated. In the control, sample was substituted with Tris-HCl buffer, the absorbance A_0 was measured and the speed of pyrogallol autoxidantion V_0 within 5 min can be calculated following the same method. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The capability of scavenging to superoxide radical was calculated as follows: Scavenging effect (%) = $(V_0 – V_1) \times 100 / V_0$

Screening of active fractions

The screening of active fraction was investigated by reducing power and superoxide anion scavenging-guided activity assay using the same method described by Yamaguchi et al. (1998) and Cao et al. (2008). Among PEF, EAF and BF, the most powerful antioxidant activity of fraction was chromatographed on a silica gel column and eluted successively with CH_2Cl_2–EtOAc, EtOAc–CH_3OH and CH_3OH–H_2O gradient elution system. Elution fractions were collected and combined according to TLC analysis. Each condensed elution fraction was dissolved in 95% ethanol at a concentration of 1 mg/ml and investigated the scavenging activity against superoxide radical and reducing power. The fractions showed strong scavenging capacity and reducing power compared with Vc were considered as active fractions.

Statistical analysis

The data presented are means ± SD of three determinations and followed by Student's *t* test. Differences were considered to be statistically significant if $P < 0.05$.

RESULTS AND DISCUSSION

Reducing power assay

The reducing capacity of plant extract components may serve as a significant indicator of its potential antioxidant activity. A higher absorbance indicates a higher ferric reducing power. Figure 1 depicted the reducing power of all tested samples. As shown in Figure 1, all fractions exhibited stronger reducing power than Vc. The reducing power of BF was stronger than that of PEF and EAF. However, the reducing power of BF was decreased at a

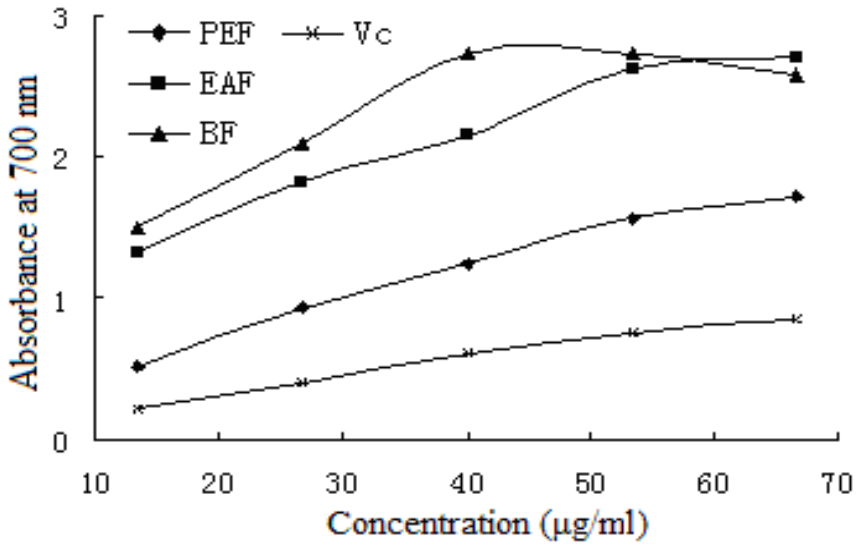

Figure 1. Reducing power of petroleum ether fraction (PEF), ethyl acetate fraction (EAF), n-butanol fraction (BF), and Vitamin C (Vc). Values are means ± S.D. (*n* = 3).

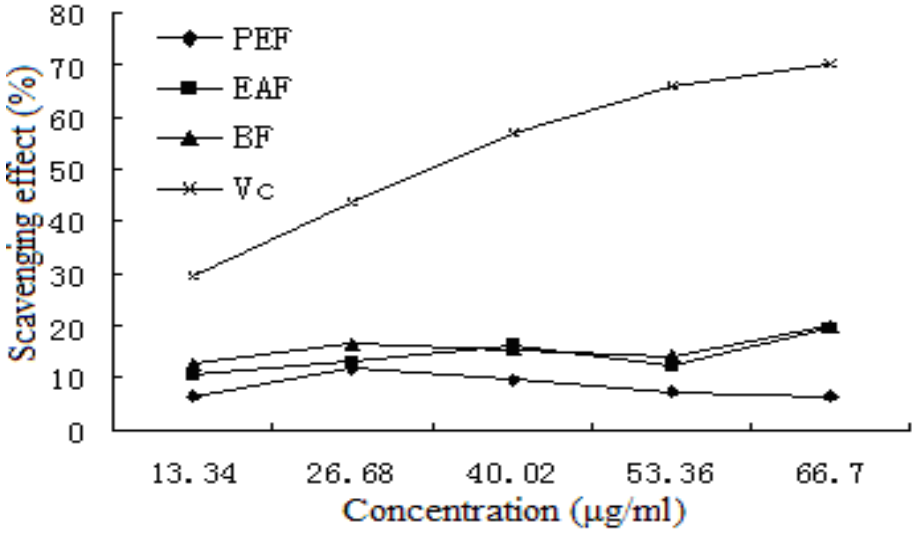

Figure 2. Hydroxyl radical scavenging activity of petroleum ether fraction (PEF), ethyl acetate fraction (EAF), n-butanol fraction (BF), and Vitamin C (Vc). Values are means ± S.D. (*n* = 3).

higher concentration of 40.02 μg/ml. PEF and EAF exhibited obvious reducing power in a concentration dependent manner. Among the tested samples, the reducing power was found to be increased in the order of BF > EAF > PEF at concentrations from 13.34 to 53.36 μg/ml. The reducing properties are generally associated with the presence of reductones. Reductones have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). It was reported that in most cases, irrespective of the stage in the oxidative chain in which the antioxidant action is assessed, most non-enzymatic antioxidative activity, such as scavenging of free radicals or inhibition of peroxidation, is mediated by redox reaction (Zhu et al., 2002). Our data on the reducing power of all fractions, especially BF and EAF, suggested that it was likely to contribute significantly toward the observed antioxidant effect.

Hydroxyl radical scavenging assay

The hydroxyl radical is the most active among the oxygen

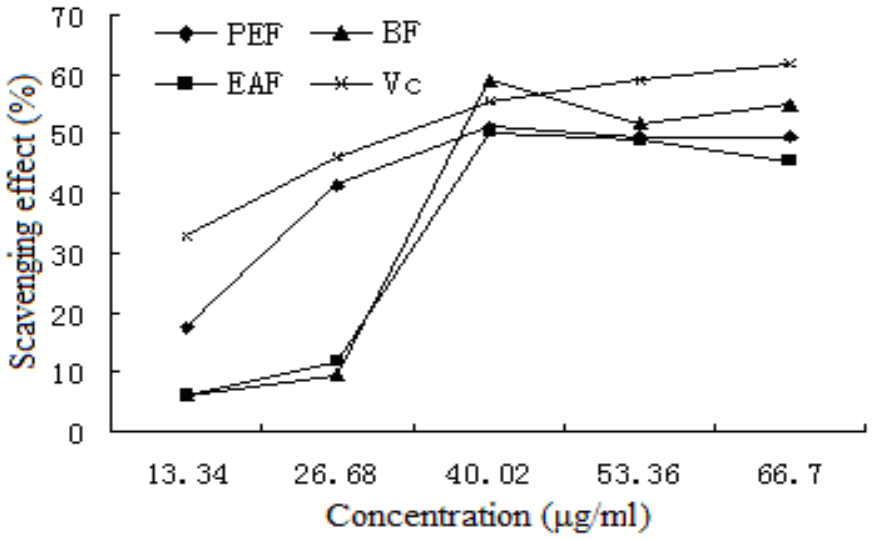

Figure 3. Superoxide radical scavenging activity of petroleum ether fraction (PEF), ethyl acetate fraction (EAF), n-butanol fraction (BF), and Vitamin C (Vc). Values are means ± S.D. (*n* = 3).

radicals and induces severe damage to the adjacent biomolecular. Thus, to evaluate the *in vitro* antioxidant activity of PEF, EAF and BF, the hydroxyl radical scavenging ability was measured by using Fenton reaction system in the present study. As shown in Figure 2, the scavenging activities of PEF, EAF and BF were weaker than that of Vc. The scavenging activities of BF and EAF were slightly stronger than PEF at concentrations from 13.34 to 66.70 μg/ml. The greatest inhibition percentage of PEF, EAF and BF on hydroxyl radical was 11.88, 19.30 and 19.80%, respectively. Previous studies had reported two types of antioxidant mechanism: suppression against hydroxyl radical generation and cleaning hydroxyl radical generated (Shon et al., 2003). In the former, the antioxidant activity may ligate to the metal ions which H_2O_2 to give the metal complexes. The metal complexes thus formed cannot further react with H_2O_2 to give a hydroxyl radical. The molecules that could chelate iron and render them inactive of poorly active Fenton reaction might have scavenging ability on hydroxyl radical (Macdonald et al., 2003). In another assay system in this study, we investigated the iron chelating ability of PEF, EAF and BF. All samples exhibited poor iron chelating ability. The mechanism would provide a good explanation about the weak scavenging effect of the tested samples on hydroxyl radical.

Superoxide radical scavenging assay

In this study, superoxide radicals were generated by pyrogallol autoxidation system (Jiao et al., 2005). Pyrogallol can autoxidate fast in alkali conditions and release superoxide anions and, in return, the superoxide anions can accelerate the autoxidation. However, the superoxide anions can be scavenged by adding some scavenger or antioxidant, the autoxidation will thus be depressed. As shown in Figure 3, the inhibition effects of all tested samples were relatively feeble at lower concentrations and significantly increased at a concentration of 40.02 μg/ml. The maximum scavenging percentage of PEF, EAF and BF was 51.04, 50.2 and 58.89%, respectively. BF exhibited stronger scavenging capacity on superoxide radicals than Vc at a concentration of 40.02 μg/ml. A further increasing of scavenging percentage was not observed at higher concentrations. Superoxide radical is believed to be the cause of other ROS formations such as hydrogen peroxide, peroxynitrite and hydroxyl radicals. Apart from that, the presence of superoxide anion can magnify the cellular damage because it produces other kinds of free-radicals and oxidizing agents, further induced pathological events such as arthritis and Alzheimer's disease (Zhu et al., 2004). Therefore, Superoxide radical scavenging capacity in the human body is the first line of defense against oxidative stress (Schauss et al., 2006).These results clearly showed that the antioxidant activities of all samples were related to the abilities of scavenging superoxide radical.

Screening of active fractions

According to the results of hydroxyl/superoxide radical scavenging activity and reducing power, BF showed the strongest antioxidant activity and was chromatographed on a silica gel column. Five fractions were obtained by

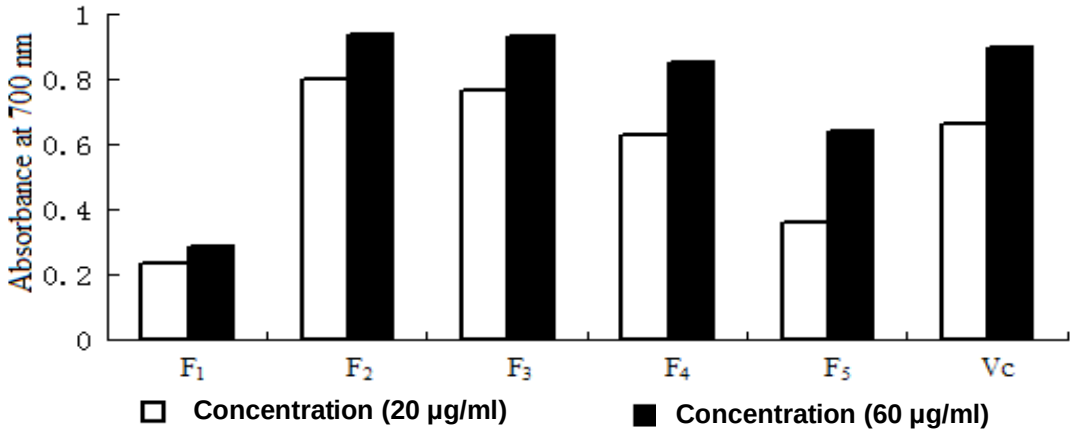

Figure 4. Reducing power of F_1, F_2, F_3, F_4, F_5, and Vitamin C (Vc). Values are means ± S.D. (*n* = 3).

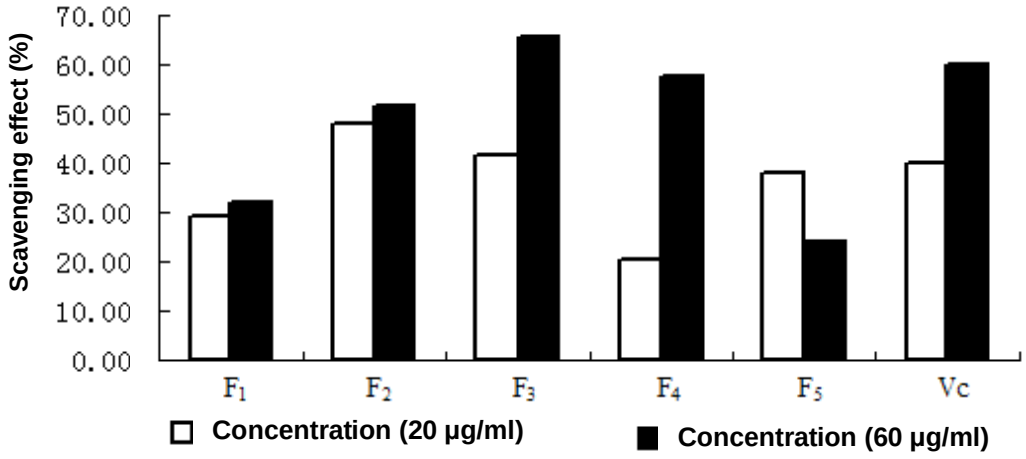

Figure 5. Superoxide radical scavenging activity of F_1, F_2, F_3, F_4, F_5, and Vitamin C (Vc). Values are means ± S.D. (*n* = 3).

elution with CH_2Cl_2 (F_1), CH_2Cl_2–EtOAc 10:1 (F_2), CH_2Cl_2–EtOAc 5:1~1:1 (F_3), EtOAc–CH_3OH 10:1~1:1 (F_4), and CH_3OH–H_2O 10:1 (F_5) gradient system. Obvious differences in chemical constituents between fractions were observed by TLC analysis. Since BF showed poor hydroxyl radical scavenging activity, screening of active fraction was preceded by superoxide radical scavenging activity and reducing power assay. Figures 4 and 5 depicted the screening results of active fractions. As shown in Figure 4, F_2, F_3 and F_4 exhibited outstanding reducing power at 20 and 60 μg/ml. The reducing power of F_2 and F_3 were stronger than that of Vc and F_4. All samples were observed in a concentration dependent manner at test concentration range. Superoxide radical scavenging effects were exhibited in Figure 5. As shown in Figure 5, all samples showed superoxide radical scavenging capacities. Scavenging activities of F_2 and F_3 were stronger than that of Vc at 20 μg/ml. F_3 exhibited stronger scavenging activity than Vc at 60 μg/ml. Except for F_5, all tested samples were observed in a concentration dependent manner at test concentration range. Our data showed that F_2, F_3 and F_4 were prominent in superoxide anion scavenging assay. According to the results of superoxide radical scavenging activity and reducing power, F_2, F_3 and F_4 were screened as the potential active fractions. Several chemical constituents existed in F_2, F_3 and F_4, which was confirmed by TLC analysis.

In our experiment, the inhibition effects of F_2, F_3 and F_4 on superoxide anion radical were superior to that of BF, however their reducing power were weaker than BF at the same concentration. That probably contributed to the different chemical constituents between BF and each active fraction.

Antioxidant is a chemical that in low concentration prevents oxidation and production of oxygen free radical

species. There are some antioxidants that change the oxygen free radicals to some compounds that are less harmful. These include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes (Masella et al., 2005). For example, SOD and CAT act in concert to inactivate superoxide radical and H_2O_2. This prevents the formation of the most reactive form of ROS, the hydroxyl radical and subsequent cellular damage (Fridovich, 1986). Obviously, the direct scavenging capabilities of F_2, F_3 and F_4 on superoxide radical have been observed in our studies. The relationship between the three active fractions and enzyme activities (SOD, CAT and GPx) will be further investigated by plural experimental methods in future. The results obtained in the present study clearly demonstrate that PEF, EAF and BF possessed marked antioxidant activity. BF exhibited the strongest antioxidant activity among the three fractions. The effective antioxidant fractions (F_2, F_3 and F_4) were obtained from BF by superoxide anion scavenging and reducing power-guided activity assay. Isolation and separation of active constituents will be the focus of our future studies.

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