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In vitro and *in vivo* antioxidant activity of aqueous extract from *Choerospondias axillaris* fruit

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Abstract

In the present study, an aqueous extract from *Choerospondias axillaries* fruit was evaluated for its *in vivo* antioxidant activity, using the D-galactose induced mouse aging model, and for its *in vitro* scavenging effects on the superoxide anions, DPPH, H_2O_2, $OH^{\cdot}$. The reducing power and Fe^{2+}-chelating ability, as well as the inhibition of lipid peroxidation were also evaluated. The flavonoid and phenolic contents of the extract were determined. Pertaining to the *in vivo* activity, the intragastric administration of the extract inhibited D-galactose induced oxidative damage. Furthermore, in the *in vitro* assays, the extract showed a high antioxidant effect, especially scavenging of DPPH anions and its reducing power. The total content of phenolic and flavonoid compounds was 568 mg of gallic acid equivalents/g dry material and 2.09 mg of quercetin equivalents/g dry material respectively. These results provide scientific support for the empirical use of *C. axillaries* fruit as a medicine for cardiovascular diseases.

Keywords: *Choerospondias axillaris*; Antioxidant activity; D-Galactose; Phenolic compounds; Flavonoid

1. Introduction

Choerospondias axillaris (Roxb.) B.L. Burtt and A.W. Hill (Anacardiaceae) is a medicinal plant used in Mongolia. Its fruit (abbreviated as CAF) is commonly used for the treatment of cardiovascular diseases. The constituents of CAF have been investigated chemically and shown to include phenolic compounds and flavonoid content (Lian, Zhang, Chong, & Zhou, 2003).

Phenolic compounds are widely found in the secondary products of medicinal plants, as well as in many edible plants (Hagerman et al., 1998). The ability of phenolic compounds to serve as antioxidants has been recognized, leading to speculation about the potential benefits of ingesting phenolic-rich foods. Several studies have described the antioxidant properties of medicinal plants, foods, and beverages which are rich in phenolic compounds (Brown & Rice-Evans, 1998; Krings & Berger, 2001).

In Mongolian medicine several properties, such as treatment of myocardial ischemia, calming nerves, ameliorating blood circulation and improving microcirculation, have been reported for CAF (Dai, Li, Chen, & Deng, 1992; Shi et al., 1985). The constituents responsible for the effects of CAF are always conceded to be the total flavonoids, because compounds of this kind are known to be antioxidants. The oxidative damage caused by reactive oxygen species (ROS), such as the superoxide radical ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$), on lipids, proteins and nucleic acids may trigger various diseases including cardiovascular disease. Epidemiological studies have shown that administration of antioxidants may decrease the probability of cardiovascular diseases (Abe & Berk, 1998; Madhavi, Deshpande, & Salunkhe, 1996).

In view of these findings, the aim of the present study was to evaluate the *in vivo* and *in vitro* antioxidant activity

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of a water extract from CAF. Because of the many ways in which an antioxidant can protect biological molecules against oxidative damage, we carried out different reactions to assess antioxidant activity, so as to determine the true antioxidant potential of the fruit (Aruoma, 2003). Furthermore, investigation into the presence of bioactive components, such as the flavonoid and phenolic content, was also pursued.

2. Materials and methods

2.1. Plant material

The plant material used was the dried fruit of *C. axillaris* which was obtained from the Inner Mongolia Mongolian medicine store and supply station (Huhhot, China), and authenticated by Dr. Minjian Qin (College of Traditional Chinese Medicine, China Pharmaceutical University, Nanjing, PR China) and a specimen was deposited in the Herbarium of College of Traditional Chinese Medicine, China Pharmaceutical University.

2.2. Chemical

All the chemicals and reagents were of analytical grade. D-Galactose was purchased from the Beijing Chemical-Regent Company (Beijing, China) and dissolved in 0.9% saline and distilled water at concentrations of 19 mg ml^{-1}. Commercial kits used for determination of MDA, GSH, SOD and GSH-px were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). α,α-Diphenyl-β-picrylhydrazyl (DPPH), Folin–Ciocalteu phenol reagent, gallic acid, 30% hydrogen peroxide, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) and ascorbic acid were purchased from Sigma–Aldrich (St. Louis, USA).

2.3. Animals

Male Kunming mice (20 ± 2 g) were purchased from the Experimental Animal Center of China Pharmaceutical University and they were group-housed (10 mice per cage) with free access to food and water, and kept in a regulated environment at 25 ± 1 °C under 12 h light/12 h dark conditions. After 1 week of acclimatization to the home cage, the mice were randomly divided into five groups and i.p. injected with D-galactose (150 mg kg^{-1}) once daily for 60 days. From day 15, the ageing control group (group AC) mice were p.o. administered with 0.3 ml of saline each; the CAF treatment group (group CAF) mice were p.o. administration CAF (250 mg kg^{-1}, 750 mg kg^{-1} and 2250 mg kg^{-1}). The normal control group (group NC) mice were i.p. injected with 0.3 ml of saline for 60 days and p.o. administration 0.3 ml saline.

2.4. Extract preparation

The fruit (500 g) was powdered with a blender and extracted in water at 100 °C for 1 h. The extract was filtered and extracted once again under the same conditions with new solvent. The filtered extracts were mixed and evaporated to 500 ml in a rotary evaporator under reduced pressure. This produced an aqueous extract containing 136 mg ml^{-1} of total dissolved solids (determined by freeze-drying). The extract was then frozen in 10 ml aliquots, stored at −20 °C and thawed as required. Upon thawing for assays, the extract was warmed to 40 °C to redissolve any solids that had formed during cooling.

2.5. Determination of total phenolic compounds and flavonoid content in extract

Total phenolic content was determined by the Folin–Ciocalteau method (Ordonez, Gomez, Vattuone, & lsla, 2006). CAF (0.5 ml, 0–20 mg ml^{-1}) was mixed with 2.5 ml of 0.2 N Folin–Ciocalteau reagent for 5 min and 2.0 ml of 75 g l^{-1} Na_2CO_3 were then added. The absorbance was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents.

Total flavonoids content was estimated according to a literature procedure (Ordonez et al., 2006). To 0.5 ml of each sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid content was calculated as quercetin from a calibration curve.

2.6. Measurement of super-oxide dismutase (SOD) activity and malondialdehyde (MDA) level in mouse brain

Mice were decapitated, and their brains were removed rapidly and homogenized in cold saline. The homogenate (10%) was centrifuged at 3000*g* at 4 °C for 20 min, and the supernatant was used for an assay. The assay for total SOD was based on its ability to inhibit the oxidation of oxymine by the xanthine–xanthine oxidase system (Oyanagui, 1984). The hydroxylamine nitrite produced by the oxidation of oxymine had an absorbance peak at 550 nm. SOD activities were expressed as units per microgram of brain protein. The thiobarbituric acid reaction (TBAR) method was used to determine the MDA (detected at 532 nm) (Ohkawa, Ohishi, & Yagi, 1979). MDA content was expressed as nanomoles per milligram of brain protein.

2.7. Determination of GSH content and glutathione peroxidase (GSH-px) activity

Mice were decapitated and the blood was collected, centrifuged at 4000*g*, at 4 °C for 10 min to collect serum for the assay. GSH was measured using dithiobisnitrobenzoic acid (DTNB) which reacts with the free thiol to give a

mixed disulfide plus 2-nitro-5-thiobenzoic acid, which can be quantified by its absorbance, at 412 nm (Ball, 1966). The activity of GSH-px was determined by quantifying the catalyzed reaction rate of H_2O_2 and GSH. One unit (U) of GSH-px was defined as the amount that reduced the level of GSH by 1 μmol.

2.8. Protein assay

Protein concentration was measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). Bovine serum albumin was used as the standard.

2.9. DPPH radical-scavenging activity assay

Each extract (0–20 mg ml^{-1}) dissolved in deionised water (2 ml), was mixed with 2 ml of methanolic solution containing DPPH radicals, resulting in a final concentration of 0.1 mM DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank (Hsu, Coupar, & Ng, 2006). Ascorbic acid was used as the control. The percentage scavenging effect was calculated as

$$\text{Scavenging rate} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract, A_2 was the absorbance without DPPH.

2.10. $OH^{\cdot}$-scavenging assay

$OH^{\cdot}$-scavenger ability was measured according to a literature procedure (Smirnoff & Cumbes, 1989) with a few modifications. OH radicals were generated from $FeSO_4$ and H_2O_2, and detected by their ability to hydroxylate salicylate. The reaction mixture (3 ml) contained 1 ml $FeSO_4$ (1.5 mM), 0.7 ml H_2O_2 (6 mM), 0.3 ml sodium salicylate (20 mM) and varying concentrations of CAF. After incubation for 1 h at 37 °C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as

$$\text{Scavenging rate} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract, A_2 was the absorbance without sodium salicylate.

2.11. Inhibition of lipid peroxidation in rat liver homogenate

Peroxidation of the liver homogenate was induced by $FeCl_2$–H_2O_2 (Yen & Hsieh, 1998). Briefly, 1% liver homogenate was incubated with 0.5 mM, each, of $FeCl_2$ and H_2O_2 with CAF (0–20 mg ml^{-1}). After incubation at 37 °C for 60 min, the formation of MDA in the incubation mixture was measured at 535 nm (Buege & Aust, 1978). BHT was used as the positive control. The percentage inhibitory effect was calculated as

$$\text{Inhibitory rate} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract, A_2 was the absorbance without liver homogenate.

2.12. $O_2^{\cdot-}$-scavenger activity assay

The enzyme xanthine oxidase catalyzes the oxidation of xanthine to uric acid. During this reaction, molecular oxygen acts as an electron acceptor, producing $O_2^{\cdot-}$. Xanthine oxidase activity was evaluated by spectrophotometric measurement of the production of uric acid from xanthine (Robak & Gryglewski, 1988). A 100 μM solution of xanthine in 0.1 M PBS at pH 7.8, with 0.04 U ml^{-1} of xanthine oxidase, was incubated at room temperature. The formation of uric acid was observed at 295 nm. After 10 min, the reaction was stopped by adding 0.1 M HCl. Different concentrations of CAF (0–0.5 mg ml^{-1}) were added to the test tubes before the addition of the enzyme. The inhibitory effect was calculated as

$$\text{Inhibitory rate} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract, A_2 was the absorbance without xanthine oxidase.

The non-enzymatic generation of superoxide anions was measured in samples which contained 10 μM PMS, 100 μM NADH and 600 μM NBT in 0.1 M PBS at pH7.8 (Robak & Gryglewski, 1988). After 2 min of incubation at room temperature, the reaction was stopped by adding 0.1 M HCl, and the absorbance was measured at 560 nm. Different concentrations of CAF (0–2 mg ml^{-1}) were added to the test tubes before the addition of PMS. The percentage scavenging effect was calculated as

$$\text{Scavenging rate} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract, A_2 was the absorbance without PMS.

2.13. H_2O_2 scavenging activity assay

H_2O_2 scavenging activity was measured according to a literature procedure (Zhao, Xiang, Ye, Yuan, & Guo, 2006) with a few modifications. H_2O_2 (1.0 ml, 0.1 mM) and 1.0 ml of various concentrations of the extract were mixed, followed by 100 μl 3% ammonium molybdate, 10 ml H_2SO_4 (2 M) and 7.0 ml KI (1.8 M). The mixed solution was titrated with $Na_2S_2O_3$ (5 mM) until the yellow color disappeared. The percentage scavenging effect was calculated as

$$\text{Scavenging rate} = (V_0 - V_1)/V_0 \times 100\%$$

where V_0 was volume of $Na_2S_2O_3$ solution used to titrate the control sample in the presence of hydrogen peroxide (without extract), V_1 was the volume of $Na_2S_2O_3$ solution used in the presence of the extract.

2.14. Reducing power assay

The reducing power was determined according to a literature procedure (Tsai, Huang, & Mau, 2006). Each extract (0–20 mg ml^{-1}) dissolved in deionised water (2.5 ml), was mixed with 2.5 ml of 200 mM PBS (pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$, and the mixture was incubated at 50 °C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 200*g* for 10 min. The upper layer (5 ml) was mixed with 5 ml of deionised water and 1 ml of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm against a blank. Ascorbic acid was used as the control. Increased absorbance of the reaction mixture indicates increased reducing power of the sample.

2.15. Fe^{2+}-chelating activity assay

Fe^{2+}-chelating activity was measured according to a literature procedure (Hsu et al., 2006) with a few modifications. The reaction mixture (2.15 ml) contained 500 μl CAF (0–20 mg ml^{-1}), 50 μl $FeCl_2$ (2 mM) and 1.6 ml deionised water. The mixture was shaken vigorously and left at room temperature for 5 min; 100 μl of ferrozine (5 mM in methanol) were then added, mixed and left for another 5 min to complex the residual Fe^{2+}. The absorbance of the Fe^{2+}–ferrozine complex was measured at 562 nm against a blank. EDTA-2Na was used as the control. The chelating activity of the extract for Fe^{2+} was calculated as

$$\text{Chelating rate} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract, A_2 was the absorbance without ferrozine.

2.16. Statistical analysis

All experiments were performed with each assay in triplicate. Data are presented as mean ± standard deviation of mean (SDM). The Duncan test and a one-way analysis of variance (ANOVA) were used for multiple comparisons (SPSS program, ver 12.0).

3. Results and discussion

3.1. Extract yields, total phenolic and flavonoid content

CAF is commonly used for the treatment of cardiovascular diseases in Mongolia. As usual, CAF and remedy adjuvants are pulverized and mixed with water to form a pill for oral use. However, water extraction of CAF and use of the extract, as raw materials of medicine, has been reported to retain the primary activities (Deng & Ji, 2002; Yang et al., 1999; Wu et al., 2000). We used water as our solvent and 13.6% (w/w) bulk materials were extracted from the fruit. Although most antioxidant activities from plant sources are derived from phenolic-type compounds (Bravo, 1998; Cai, Luo, Sun, & Corke, 2004), antioxidant activity does not always correlate with the presence of large quantities of these polyphenolic compounds, hence both data need to be examined together. For this, the extract was analyzed for total phenolic and flavonoid content. The total phenolic content in the fruit extract was 56.8% (w/w) of the extract material. However, it is surprising that the flavonoid content was low, yielding only 0.2% (w/w) of the extract material. This result is different to the 3.65% from dried CAF reported in literature (Fan, Sai, Aodeng, & Song, 2005), and it is speculated that the total flavonoid content may contain many kinds of flavonoid, most of which do not chelate with $AlCl_3$ and hence be spectrometrically detected, such as isoflavone and flavonone.

3.2. Antioxidant effects of CAF in D-galactose induced ageing mice

Rodents injected with D-galactose display symptoms which resemble accelerated ageing. The free radicals generated from oxidation of D-galactose overrun the capacity of cells to neutralize them. This causes the chain reaction of lipid peroxidation and the resultant end products, such as MDA, which combine with protein and phospholipid, lead to injury of the cellular membrane (Hayakawa, Hattori, Sugiyama, & Ozawa, 1992). The long-term administration of galactose induced changes in these redox-related biomarkers in mice, including decrease in SOD, GSH-px activities and GSH levels, as well as increase of the MDA level. In comparison to NC group mice, the SOD activity significantly declined by 35.9% in AC group mice and the MDA content remarkably increased by 33.5%. CAF could increase the activity of SOD and decrease the level of MDA (Table 1). The content of GSH and the activity of GSH-px in the AC group mice was significantly reduced by 20.8% and 19.1%, respectively. Treatment with CAF completely inhibited the reduction of GSH and GSH-px (Table 1). This is in accordance with the result obtained with adriamycin-induced rat heart and the liver peroxidation model (Bagenna, Tai, Wang, Xu, & Li, 2002).

3.3. DPPH radical-scavenging activity

The role of an antioxidant is to remove free radicals. One mechanism through which this is achieved involves donating hydrogen to a free radical and hence its reduction to an unreactive species. Addition of hydrogen removes the odd electron feature which is responsible for radical reactivity. Ascorbic acid showed an excellent scavenging activity ($IC_{50} = 0.1673$ mg ml^{-1}). It was observed that CAF also had strong scavenging activity, with an IC_{50} value of 0.5329 mg ml^{-1} (Fig. 1a).

3.4. OH-scavenging activity

Scavenging of OH is an important antioxidant activity because of very high reactivity of the OH radical which

Table 1
Effect of CAF on MDA, GSH content and SOD, GSH-px actificially senescent mouse brain

Group	MDA (nmol (mg protein)$^{-1}$)	SOD (U (mg protein)$^{-1}$)	GSH (mg l^{-1})	GSH-px (U l^{-1})
NC	0.468 ± 0.061	938 ± 75.9	365 ± 69.5	1102 ± 125.8
AC	0.625 ± 0.082$^{\#\#}$	601 ± 86.1$^{\#\#}$	289 ± 90.6$^{\#}$	891 ± 87.5$^{\#\#}$
CAF (250 mg kg^{-1})	0.600 ± 0.077	863 ± 77.4**	324 ± 84.5	949 ± 74.1
CAF (750 mg kg^{-1})	0.527 ± 0.074*	906 ± 64.0**	340 ± 58.8	1040 ± 83.2*
CAF (2250 mg kg^{-1})	0.488 ± 0.096**	929 ± 52.0**	353 ± 61.9	1097 ± 97.7**

$N = 10$.
* $P < 0.05$ vs. AC group.
** $P < 0.01$ vs. AC group.
$^{\#}$ $P < 0.05$ vs. NC group.
$^{\#\#}$ $P < 0.01$ vs. NC group.

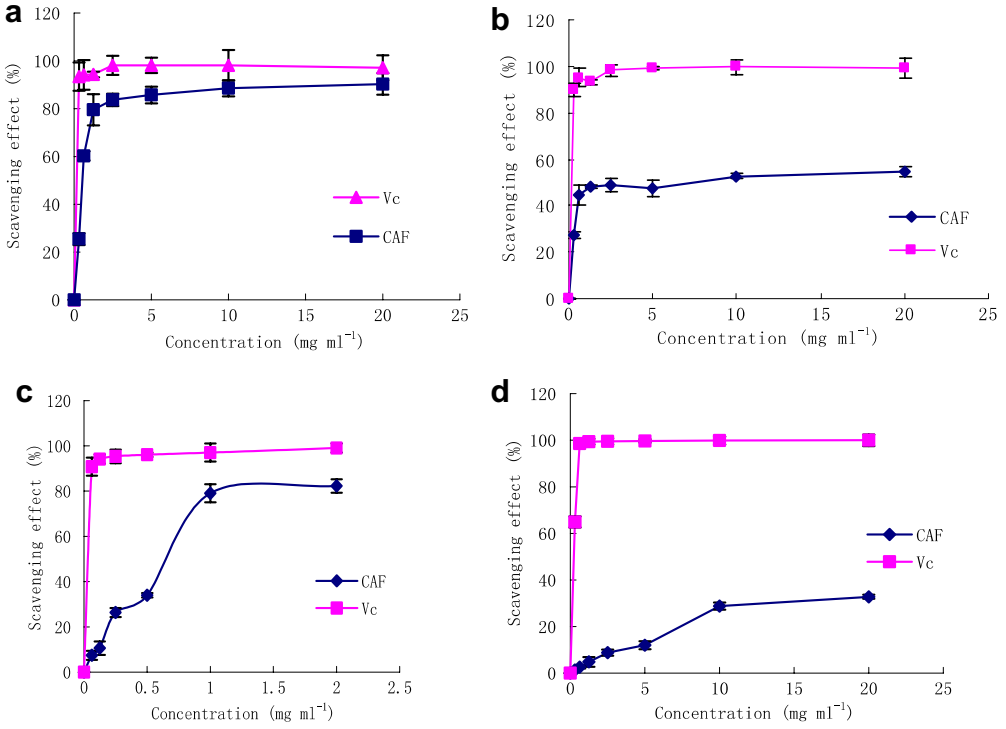

Fig. 1. (a) The DPPH radical-scavenging activity of CAF extract; (b) the OH·-scavenging activity of CAF extract; (c) the $O_2^{\cdot-}$-scavenging activity of CAF extract and (d) the H_2O_2-scavenging activity of CAF extract. The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effect (%) values ± 1 S.D. ($n = 3$) against extract concentration in mg extract per ml reaction volume.

enables it to react with a wide range of molecules found in living cells, such as sugars, amino acids, lipids and nucleotides. Although OH formation can occur in several ways, by far the most important mechanism *in vivo* is the Fenton reaction, where a transition metal is involved as a pro-oxidant in the catalyzed decomposition of superoxide and hydrogen peroxide (Stohs & Bagchi, 1995). With this assay, the IC_{50} value of CAF was 7.4462 mg ml^{-1}, while the value of ascorbic acid was 0.0164 mg ml^{-1} (Fig. 1b).

3.5. Inhibition of lipid peroxidation

In biological systems, lipid peroxidation generates a number of degradation products, such as MDA, and it is found to be an important cause of cell membrane destruction and cell damage (Yoshikawa, Naito, & Kondo, 1997). In the present study, we measured the potential of CAF to inhibit lipid peroxidation in rat liver homogenate, induced by the $FeCl_2$–H_2O_2 system. Obviously, CAF showed moderate activity at the concentrations tested. Even at 20 mg ml^{-1}, CAF only yielded 44.2% protection (Fig. 2). As the positive control, BHT showed a plateau of inhibitory ability ranging from 78.0–85.5% at 1.25–20 mg ml^{-1}. Decrease in lipid peroxidation by CAF may be a result of it scavenging OH produced by $FeCl_2$–H_2O_2 and H_2O_2 in the reaction system, and on the other hand, polyphenols in CAF may contain phenolic hydroxyl group, with the ability to accept electrons, which can combine with free radical competitively to decrease lipid peroxidation induced by free radicals.

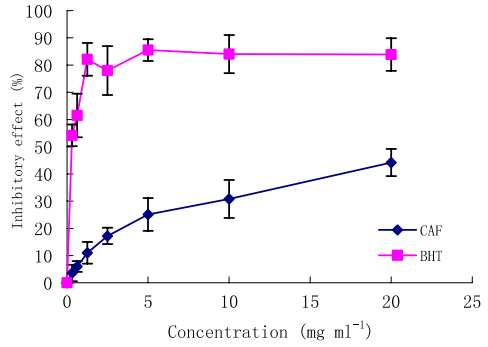

Fig. 2. Inhibition of lipid peroxidation by CAF extract. The absorbance values were converted to inhibitory effects (%) and the data plotted as the mean of replicate inhibitory effects (%) values ± 1 S.D. ($n = 3$) against extract concentration in mg extract per ml reaction volume.

3.6. $O_2^{\cdot-}$-scavenging activity

Xanthine oxidase catalyzes the oxidation of xanthine to uric acid. During oxidation of xanthine, $O_2^{\cdot-}$ and H_2O_2 are formed (Britigan, Pou, Rosen, Lilleg, & Buettner, 1990). This enzyme is consequently considered to be an important source of $O_2^{\cdot-}$. The superoxide anion is a radical which reacts with NO with a high rate constant, generating the oxidant peroxynitrite ($ONOO^-$) (Espey et al., 2002; Singh & Evans, 1997; White et al., 1994). At physiological pH, $ONOO^-$ and other reactive nitrogen species have been implicated in the pathophysiology of a variety of diseases including some cardiovascular diseases (Demiryurek, Cakici, & Kanzik, 1998; Persinger, Poynter, Ckless, & Janssen-Heininger, 2002). Ascorbic acid showed an excellent inhibitory activity ($IC_{50} = 0.0164$ mg ml^{-1}), while the IC_{50} value of CAF was 0.1012 mg ml^{-1} (Fig. 3).

In the non-enzymatic system, $O_2^{\cdot-}$ can be generated *in vitro* and assayed by the PMS/β-NADH/NTB system. Using this assay, the IC_{50} values of ascorbic acid and CAF were 0.0344 mg ml^{-1} and 0.6781 mg ml^{-1}, respectively (Fig. 1c).

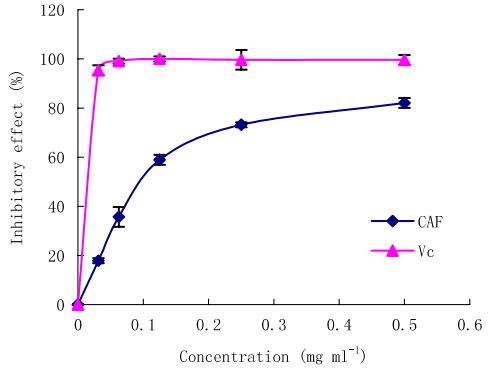

Fig. 3. Inhibition of xanthine oxidase by CAF extract. The absorbance values were converted to inhibitory effects (%) and the data plotted as the means of replicate inhibitory effects (%) values ± 1 S.D. ($n = 3$) against extract concentration in mg extract per ml reaction volume.

3.7. H_2O_2-scavenging activity

Although H_2O_2 is not very reactive, its high penetrability of cellular membrane leads to OH formation when it reacts with Fe^{2+} or the superoxide anion radical in the cell. As shown in Fig. 1d, CAF demonstrated H_2O_2 scavenging activity in a concentration dependent manner. Compared with CAF, ascorbic acid was more effective for scavenging H_2O_2. CAF only scavenged 32.8% of H_2O_2 even at the maximum concentration of 20 mg ml^{-1}, while ascorbic acid exhibited a scavenging rate of 100% at the same concentration.

3.8. Reducing power

An electron-donating reducing agent contributes to antioxidant activity by its capacity to donate an electron to free radicals, which results in neutralization of the radical, and the reduced species subsequently acquires a proton from solution. Another reaction pathway in electron donation is the reduction of an oxidized antioxidant molecule to regenerate the "active" reduced antioxidant. As showed in Fig. 4, the reducing power of CAF was 0.601 at 0.625 mg ml^{-1} and 0.828 at 2.5 mg ml^{-1}. However, ascorbic acid only showed slightly higher activity with a reducing power of 0.746 and 0.926 at 0.625 mg ml^{-1} and 2.5 mg ml^{-1}, respectively.

3.9. Fe^{2+}-chelating activity

Iron and copper are essential transition metal elements in the human body required for the activity of a large range of enzymes and for some proteins involved in cellular respiration, O_2 transport and redox reactions. Unfortunately, they contain unpaired electrons that enable them to participate in one-electron transfer reactions. Hence, they are powerful catalysts of autoxidation reactions (Lloyd, Hanna, & Mason, 1997). With this assay, EDTA-2Na showed strong Fe^{2+}-chelating activity. Even at the minimal concentration of 0.3125 mg ml^{-1}, its chelating rate was

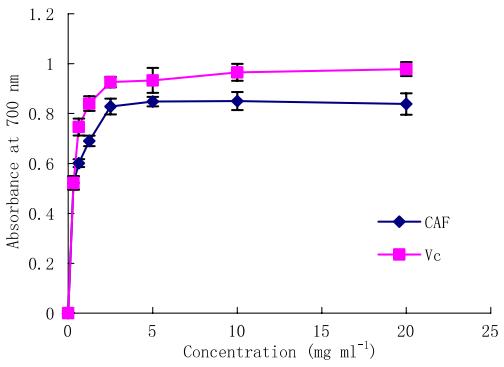

Fig. 4. Reducing power of CAF extract. The absorbance values were directly plotted as the mean of replicate absorbance values ± 1 S.D. ($n = 3$) against extract concentration in mg extract per ml reaction volume.

86.4%. However, CAF showed little Fe^{2+}-chelating activity. Even at 20 mg ml^{-1}, CAF did not reach a level of 6% (Fig. 5). It seems to be different from the literature reports stating that the main components in CAF are flavonoids, since flavonoid usually contains high Fe^{2+}-chelating activity. However, their scavenging potential and metal chelating ability are dependent upon their unique phenolic structure and the number and position of the hydroxyl groups (Pazos, Gallardo, Torres, & Medina, 2005). It may be that the flavonoids in the extract do not have the required structure, which attributes to this result. On the other hand, since the reaction is dependent on the affinity of an antioxidant towards Fe^{2+} in relation to ferrozine, the assay is affected by both the binding constant and the concentration of antioxidant and thus further investigations have to be carried out to assess whether the weak chelating ability for Fe^{2+} leads to this result.

CAF has been reported to be effective in treating cardiovascular diseases in humans. Many researches (Kirk, Sutllerland, Wang, Chait, & LeBoeuf, 1998; Youdim, Shukitt-Hale, MacKinnon, Kalt, & Joseph, 2000) indicated free radicals and lipid peroxidation metabolites as the main cause of cardiovascular diseases. In this study we demonstrated the antioxidant properties of an aqueous extract of CAF using various testing systems. The data suggested that CAF possesses direct and potent antioxidant activities through multiple mechanisms. Especially, the DPPH radical-scavenging activity and the reducing power of CAF were very potent and nearly as effective as ascorbic acid itself, which is an efficient organic electron and hydrogen donor (Fig. 1a and Fig. 4). Combined with the efficiency of *C. axillaris* in the treatment of cardiovascular diseases, the present study suggests that its biological functions are due, at least partially, to its protective effects against oxidation. The effect of inhibition of xanthine oxidase may be another important factor in this mechanism.

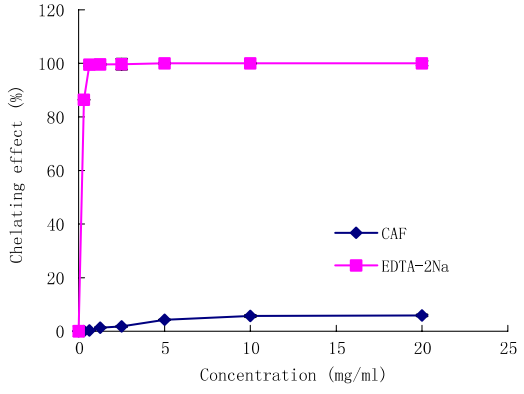

Fig. 5. Fe^{2+}-chelating activities of CAF extract. The absorbance values were converted to chelating effects (%) and data plotted as the mean of replicate chelating effects (%) ± 1 S.D. ($n = 3$) against extract concentration in mg extract per ml reaction volume.

Acknowledgments

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