Antioxidant Activity of Ethyl Acetate Extract of *Amomum villosum* Lour.

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Abstract [**Objectives**] To study the antioxidant activity of the ethyl acetate extract of *Amomum villosum* Lour. [**Methods**] The removal rate of chelated iron ions, hydroxyl radicals, superoxide anion free radicals and DPPH free radicals by the ethyl acetate extract of *A. villosum* Lour. was determined by UV spectrophotometer. [**Results**] 0.500 0 μg/mL ethyl acetate extract of *A. villosum* Lour. had the strongest ability to chelate with ferrous ions and to remove hydroxyl radicals, superoxide anion free radicals and DPPH free radicals. The ability to chelate with ferrous ions was 95.14%, and the removal rate of the above free radicals was 86.217%, 81.44%, and 85.16%. [**Conclusions**] The ethyl acetate extract of *A. villosum* Lour. had a strong antioxidant effect, and its antioxidant capacity was related to the sample concentration, which provides a theoretical basis for its application in the development of antioxidant skin care products.

Key words *Amomum villosum* Lour., Extract, Antioxidant activity, Free radicals

1 Introduction

Amomum villosum Lour. is native to Yangchun, Guangdong Province, and is now distributed in Fujian, Guangdong, Guangxi and Yunnan of China. It is cultivated or wild in shade and wet mountain places. Its fruit can be used as medicine, and the quality of *A. villosum* Lour. from Guangdong is the best. In the *Kaibao Materia Medica*, *Compendium of Materia Medica* and related dictionaries, it is said that *A. villosum* Lour. belonging to Zingiberaceae is warm in nature and acrid in taste, has functions of promoting qi and flavoring, harmonize the stomach and refreshing one's mind, can is used to treat uncomfortable chest and abdomen, dyspepsia, dysphagia and vomiting, and other diseases. *A. villosum* Lour. contains many antioxidants, among which total flavonoids can inhibit apoptosis and protect myocardium injured by ischemia-reperfusion[1]. Besides, the ornamental value of *A. villosum* Lour. is high. The flowers can be appreciated in early summer, and the fruit can be observed in midsummer. Meanwhile, it is a good seasoning. In this study, it is hoped that the antioxidant activity of ethyl acetate extract of *A. villosum* Lour. was studied to fully understand the antioxidant activity of *A. villosum* Lour. and further develop the application of *A. villosum* Lour. in the market. In this experiment, the ability of 30%, 50%, 70% and 90% ethyl acetate extract of *A. villosum* Lour. to eliminate DPPH and superoxide anion free radicals, chelate metal ions (Fe^{2+}) and reduce Fe^{3+} was determined to study the antioxidant activity of *A. villosum* Lour., and the antioxidant activity and action mechanism of *A. villosum* Lour. were studied to provide reference for its further development.

2 Materials and methods

2.1 Materials

2.1.1 Experimental medicinal materials. The *A. villosum* Lour. brought from the market was ground into powder.

2.1.2 Experimental instruments. Main instruments included Uv-visible spectrophotometer (Yidian N4S, Shanghai Yidian Analytical Instrument Co., Ltd.), analytical balance (FA1204B, Shanghai Tianmei Balance Instrument Co., Ltd.), electric thermostatic water bath (HH-Z4, Jintan Chengdong Guangmang Instrument Factory), and chromatographic tubes (customized).

2.1.3 Experimental reagents. Main reagents were DPPH (Shanghai Jinsui Biotechnology Co., Led.), pyrotechnic (Tianjin Fuchen Chemical Reagent Factory), $FeSO_4 \cdot 7H_2O$ (Tianjin Damao Chemical Reagent Factory), phenazine (Shanghai Jinsui Biotechnology Co., Ltd.), BHT (Shanghai Jingchun Reagent Co., Ltd.), and AB-8 macroporous resin (Chengdu Chron Chemicals Co., Ltd.).

2.2 Experimental methods

2.2.1 Preparation of ethyl acetate extract of *A. villosum* Lour. and working solutions. A previous method[2] was adopted and adjusted appropriately. The *A. villosum* Lour. sample was ground into powder, and then 350 g of the powder was placed in a round-bottom flask, to which 1 750 mL of 70% (volume fraction) ethanol was added. It was treated by ultrasound twice at 78 ℃ and 200 W for 1 h each time. After being cooled down, the solution was filtered, and all of the filtrate was mixed. After being evaporated and concentrated, it was diluted with double distilled water until the concentration of recovered ethanol was 10%. Finally, the fluid-like extract was obtained, and was stored in a refrigerator at 4 ℃ for subsequent use. According to the pretreatment method

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of resin[3], the AB-8 macroporous resin soaked in 95% ethanol for 24 h was eluted until it had no alcohol flavor. At a certain flow rate, 30%, 50%, 70% and 90% ethanol were placed in chromatographic columns containing eluted resin, and then the same amount of fluid extract was placed in each chromatographic column. Finally, 30%, 50%, 70% and 90% *A. villosum* Lour. solution were collected, to which ethyl acetate was added. They were placed in a separating funnel respectively. After rotating, shaking, and standing, the upper solution was taken to obtain the extract of ethyl acetate layer, and then rotary evaporation was carried out to obtain the liquid to be tested. It was stored in a refrigerator at 4 ℃ for subsequent use.

2.2.2 Determination of the ability to remove DPPH free radical. 2 mL of the tested liquids with different concentrations of 30%, 50%, 70% and 90% were taken accurately to determine the ability to remove DPPH free radicals. At first, 10 mg of BHT was put in a volumetric flask, to which ethanol was added until the volume was up to 10 mL, and it was stored in a refrigerator. 0.100 0 mmol/LDPPH solution was prepared and stored away from light. Different concentrations of the tested solutions and 2 mL of 0.100 0 mmol/L DPPH solution were added to a 10 mL test tube and shaken well. It was away from light for 30 min at room temperature. Anhydrous ethanol was as reference to determine the absorbance of 2 mL of 0.100 0 mmol/L DPPH solution and 2 mL of anhydrous ethanol solution (A_0), 2 mL of 0.100 0 mmol/L DPPH solution and 2 mL of different concentrations of the tested liquids (A_1), and 2 mL anhydrous ethanol and 50% BHT solution (A_2) at 517 nm, respectively.

A_0: 2 mL of 0.100 0 mmol/L DPPH solution and 2 mL of anhydrous ethanol solution; A_1: 2 mL of 0.100 0 mmol/L DPPH solution and 2 mL of different concentrations of the tested liquids; A_2: 2 mL anhydrous ethanol and 50% BHT solution.

Each sample was tested three times to obtain the average. A_0, A_1 and A_2 were recorded, respectively, and the removal rate of DPPH free radicals (%) was calculated as follows: Removal rate $=[1-(A_1-A_2)/A_0]\times 100\%$.

2.2.3 Determination of the ability to remove superoxide anion radicals. A previous method[4] was adopted and adjusted appropriately. Firstly, 2 mL of different concentrations of liquids to be tested were mixed with 18 mL of 0.050 0 mmol/L phosphate buffer (pH =8.20) in a test tube with a plug, and then put in constant temperature water at 25 ℃ for 15 min. The absorbance of the mixture A_1 was determined. Afterwards, 6 mL of the mixture was taken, to which 0.4 mL of 45.000 0 mmol/L pyroquinol was added. After full reaction for 6 min, its absorbance A_2 was detected at 420 nm. 2.0 mL of anhydrous ethanol and 18.0 mL of 0.05 mmol/L phosphate buffer solution (pH =8.20) were mixed in a test tube with a plug. After the mixture was put in constant temperature water at 25 ℃ for 15 min, 6 mL of the mixture was taken, to which 0.4 mL of 45.000 0 mmol/L pyroquinol was added. After full reaction for 6 min, its absorbance A_0 was detected at 420 nm. The removal rate of superoxide anion radicals (O^{2-}) (%) was calculated as follows: Removal rate of O^{2-} $=[1-(A_1-A_2)/A_0]\times 100\%$.

2.2.4 Determination of the ability to remove hydroxyl radicals. The 50% liquid to be tested was taken to detect the ability to remove hydroxyl radicals. There were 3 colorimetric tubes in three groups, that is, each group had one colorimetric tube. In the blank group A_0, 2 mL of distilled water, 2 mL of 9.000 0 mmol/L salicylic acid-ethanol solution, and 2 mL of 9.000 0 mmol/L $FeSO_4$ solution were added to the colorimetric tube, and mixed evenly. In the experimental group A_1, 2 mL of 9.000 0 mmol/L of salicylic acid-ethanol solution and 2 mL of 8.800 0 mol/L H_2O_2 solution were added to the colorimetric tube, and mixed uniformly. Afterwards, 2 mL of 9.000 0 mmol/L $FeSO_4$ solution and 2 mL of the 50% solution to be tested were added, so that the solutions in the colorimetric tube could fully react. In the control group A_2, 2 mL of 9.000 0 mmol/L salicylic acid-ethanol solution and 2 mL of 8.800 0 mmol/L H_2O_2 solution were added to the colorimetric tube, and mixed evenly. Hereafter, 2 mL of distilled water and 2 mL of 9.000 0 mmol/L $FeSO_4$ solution were added to the colorimetric tube successively, shaken well and let stand.

A_0: 2 mL of distilled water +2 mL of 9.000 0 mmol/L salicylic acid-ethanol solution +2 mL of 9.000 0 mmol/L $FeSO_4$ solution; A_1: 2 mL of 9.000 0 mmol/L of salicylic acid-ethanol solution +2 mL of 8.800 0 mol/L H_2O_2 solution +2 mL of 9.000 0 mmol/L $FeSO_4$ solution and 2 mL of the 50% solution to be tested; A_2: 2 mL of distilled water +2 mL of 9.000 0 mmol/L salicylic acid-ethanol solution +2 mL of 8.800 0 mmol/L H_2O_2 solution +2 mL of 9.000 0 mmol/L $FeSO_4$ solution.

The three colorimetric tubes in groups A_0, A_1 and A_2 were concentrated with a rotary evaporator at 37 ℃ for 15 min. Distilled water was used for calibration, and the absorbance of the corresponding solution was detected 3 times by UV-visual spectrophotometer at 510 nm, and the average was obtained. The removal rate of hydroxyl radicals (%) was calculated as follows: Removal rate of hydroxyl radicals $=[A_2-(A_1-A_0)]/A_2\times 100\%$.

2.2.5 Determination of the ability to chelate with metal ions (Fe^{2+}). A previous method[5] was adopted and adjusted appropriately. At first, 0.04 g of $FeCl_2$ was dissolved in distilled water, and the volume was fixed to 100 mL to obtain 2.000 0 mmol/L $FeCl_2$ solution. Meanwhile, 0.125 5 g of phenazine was dissolved in distilled water, and the volume was fixed to 50 mL to obtain 5.000 0 mmol/L phenazine solution. Afterwards, 2 mL of different concentrations of samples were added to a test tube containing 0.1 mL of 2.000 0 mmol/L $FeCl_2$ solution. After incubation at room temperature, 0.2 mL of 5.000 0 mmol/L phiiazine reagent and 3 mL of distilled water were added to the tube in turn. After reaction for 10 min, the absorbance was detected at 562 nm.

A_0: 2 mL of distilled water +0.1 mL of $FeCl_2$ +0.2 mL of 5.000 0 mmol/L phenazine +3 mL of distilled water absorbance value; A_1: 2 mL of the sample +0.1 mL of $FeCl_2$ +0.2 mL of 5.000 0 mmol/L phenazine +3 mL of distilled water.

The ability to chelate with ferrous ions (%) was calculated

as follows: Ability to chelate with $Fe^{2+} = [A_0 - A_1/A_0] \times 100\%$.

3 Results and analysis

3.1 Ability to remove DPPH free radicals As can be seen from Table 1, with the increase of sample concentration, the ability to remove DPPH free radicals tended to increase firstly and then decrease. When the sample concentration was 0.500 0 μg/mL, the ethyl acetate extract of *A. villosum* Lour. had the strongest ability to remove DPPH free radicals, and the remove rate was 85.16%. It was more than 50% in the experimental concentration range, indicating that the ethyl acetate extract has good ability to remove DPPH free radicals.

Table 1 Removal rate of DPPH free radical by the ethyl acetate extract of *Amomum villosum* Lour.

Sample concentration μg/mL	A_0	A_1	A_2	Removal rate//%
0.300 0	0.256 0	0.058 0	0.005	79.30
0.500 0	0.256 0	0.043 0	0.005	85.16
0.700 0	0.256 0	0.065 0	0.005	76.57
0.900 0	0.256 0	0.081 0	0.005	70.31

3.2 Ability to remove superoxide anion free radicals Seen from Table 2, with the increase of sample concentration, the ability to remove superoxide anion free radicals increased firstly and then reduced. As the sample concentration was 0.500 0 μg/mL, the ethyl acetate extract of *A. villosum* Lour. had the strongest ability to remove superoxide anion free radicals, and the remove rate was 81.44%. It exceeded 50% in the experimental concentration range, showing that the ethyl acetate extract has good ability to remove superoxide anion free radicals.

Table 2 Removal rate of superoxide anion free radicals by the ethyl acetate extract of *Amomum villosum* Lour.

Sample concentration//μg/mL	A_0	A_1	A_2	Removal rate//%
0.300 0	0.431 0	0.986 0	0.836 0	65.20
0.500 0	0.431 0	0.929 0	0.849 0	81.44
0.700 0	0.431 0	0.935 0	0.840 0	77.96
0.900 0	0.431 0	0.958 0	0.838 0	72.16

3.3 Ability to remove hydroxyl radicals Table 3 shows that *A. villosum* Lour. had certain ability to remove hydroxyl radicals, and the ability was related to the sample concentration to a certain extent. With the increase of sample concentration, the ability to remove hydroxyl radicals changed accordingly. When the sample concentration was 0.500 0 μg/mL, the remove rate was up to 86.217%, and declined with the decrease of sample concentration. It shows that the ability to remove hydroxyl radicals was the strongest as the sample concentration was 0.500 0 μg/mL.

3.4 Ability to chelate with metal ions (Fe^{2+}) It can be seen from Table 4 that *A. villosum* Lour. had certain ability to chelate with metal ions (Fe^{2+}), and the ability was related to the sample concentration. When the sample concentration was 0.300 0, 0.500 0, 0.700 0 and 0.900 0 μg/mL, the ability of *A. villosum* Lour. to chelate with metal ions (Fe^{2+}) was 91.44%, 95.14%, 89.12% and 87.04%, respectively. When the sample concentration was 0.500 0 μg/mL, the ability of *A. villosum* Lour. to chelate with metal ions (Fe^{2+}) was the strongest. The results show that *A. villosum* Lour. had strong ability to chelate with metal ions (Fe^{2+}), which was closely related to the existence of reactive hydrogen.

Table 3 Removal rate of hydroxyl radicals by the ethyl acetate extract of *Amomum villosum* Lour.

Sample concentration// μg/mL	A_0	A_1	A_2	Removal rate//%
0.300 0	0.040 0	0.191 0	0.997 0	84.855
0.500 0	0.040 0	0.169 0	0.936 0	86.217
0.700 0	0.040 0	0.184 0	0.955 0	84.921
0.900 0	0.040 0	0.197 0	0.948 0	83.484

Table 4 Ability of the ethyl acetate extract of *Amomum villosum* Lour. to chelate with metal ions (Fe^{2+})

Sample concentration// μg/mL	A_0	A_1	Chelating ability//%
0.300 0	0.432 0	0.037 0	91.44
0.500 0	0.432 0	0.021 0	95.14
0.700 0	0.432 0	0.047 0	89.12
0.900 0	0.432 0	0.056 0	87.04

4 Conclusions

According to the above experiments, 0.500 0 μg/mL ethyl acetate extract of *A. villosum* Lour. had better antioxidant capacity, and the antioxidant capacity was related to the sample concentration. Among them, the ability to remove hydroxyl radicals was the strongest, and the ability to chelate with metal ions was also strong. The mechanism of antioxidant action is that antioxidant substances remove free radicals or complex with Fe^{2+} to form stable chelates to interrupt their own oxidation. The above results can provide a reference for the application of the ethyl acetate extract of *A. villosum* Lour. as an antioxidant in the field of beauty and skin care products.

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