文章编号:1001-6880(2014)3-0392-06

肾茶提取物抗氧化及保护线粒体作用研究

陈地灵[1],龙贺明[2],张鹤鸣[1*],李　宁[1],刘颂豪[1]

[1]华南师范大学药物研究院,广州 510631;[2]赣南医学院第一附属医院肿瘤科,赣州 341000

摘　要:肾茶水提液依次用石油醚、氯仿、乙酸乙酯萃取,与水层得到4个不同极性部位。以清除DPPH·、超氧阴离子和羟自由基的能力,络合Fe^{2+}能力,体外抗活性氧能力以及对$FeSO_4$和H_2O_2诱导小鼠肾脏组织匀浆脂质过氧化产物MDA和GSH-Px水平的影响,综合评价其抗氧化活性;以抑制Fe^{2+}-L-Cys诱导肾脏线粒体肿胀能力,初步评价其保护肾脏线粒体的作用。结果显示肾茶水提液4个不同极性部位均具有不同程度的清除DPPH·、$O_2^{\cdot-}$、·OH和络合Fe^{2+}能力;其中乙酸乙酯和水提部位具有显著体外抗ROS能力,降低MDA和升高GSH-Px水平,以及抑制肾脏线粒体肿胀的能力,且其作用效果均与剂量呈正相关。本实验结果可为肾茶用于治疗肾脏疾病的药效物质基础研究提供依据。

关键词:肾茶;抗氧化活性;金属络合能力;活性氧;线粒体肿胀

中图分类号:R285.5　　**文献标识码**:A

In vitro Antioxidant and Mitochondria Protective Activities of *Clerodendranthus spicatus* Extracts

CHEN Di-ling[1], LONG He-ming[2], ZHANG He-ming[1*], LI Ning[1], LIU Song-hao[1]

[1] *Southern Institute of Pharmaceutical Research, South China Normal University, Guangzhou Guangdong 510631, China*;

[2] *Department of Oncology, The First Affiliated Hospital of Gannan Medical University, Jiangxi Ganzhou 341000, China*

Abstract: Water extract of *Clerodendranthus spicatus* was successively fractionated using 3 different solvents to provide petroleum ether extract (PE), chloroform extract (CE), ethyl acetate extract (EAE) and water extract (WE). Different antioxidant assays including OH·, $O_2^{\cdot-}$, DPPH· scavenging activity, reducing power, metal chelating activity, anti-reactive oxygen species (anti-ROS) activity and effects on $FeSO_4$ and H_2O_2 induced MDA and GSH-Px production increase were employed to evaluate the antioxidant activities of four extracts. The inhibition rate of mitochondrial swelling induced by Fe^{2+}-L-Cys *in vitro* was employed to evaluate the protective effects on mitochondrion. The results showed that all the 4 different extracts had the ability of scavenging DPPH·, $O_2^{\cdot-}$, ·OH and chelating Fe^{2+}, anti-ROS *in vitro*, significantly lower the levels of MDA, rised the levels of GSH-Px in renal homogenate and inhibited the renal mitochondria swelling, in concentration dependent manner. The results provided a basis for *C. spicatus* to be used in the treatment of renal disease.

Key words: *Clerodendranthus spicatus*; antioxidant activity; metal chelating activity; reactive oxygen species; mitochondrial swelling

Introduction

Clerodendranthus spicatus Thunb., scattered in Fujian, Taiwan, Hainan, Guangdong and Yunnan provinces of China[1], is normally used as folk medicine to treat acute or chronic nephritis, urocystitis, urolithiasis, gallstone, rheumatic arthritis, *etc*[2]. More than 200 chemical constituents were isolated from *C. spicatus*, including flavonoids (5-hydroxy-6,7,4′-trimethoxyflavone and 5-hydroxy-6,7,3,4′-tetramethoxyflavone), phenolic acids (orthosiphol, siphonol, oleanolic acid, ursolic acid, rosmarinic acid and glucuronic acid), saponins and internal compensation inositol[3]. The pharmacological researches showed that the extracts of *C. spicatus* had antihypertensive effect, inhibitory effect on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*[4].

Received: March 22, 2013　Accepted: September 5, 2013

* Corresponding author Tel: 86-20-85217701; E-mail: d_zhm@163.com

Oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defense systems. Excessive ROS production is known to cause oxidative damage to major macromolecules in cells, including DNA, lipids and proteins, thereby disrupting cellular functions and integrity, which will cause cell death and tissue damage. The role of ROS has been implicated in several diseases, including cancer, diabetes and cardiovascular diseases, aging, *etc*. Antioxidants are vital substances which possess the ability to reduce oxidative damage caused by ROS. As the possible carcinogenic effects of synthetic antioxidants, there is a growing demand for replacing synthetic antioxidants by natural antioxidants from herbs. Hence, the development of natural antioxidant has gained more attentions.

To the best of the author's knowledge, no report about the antioxidant properties of *C. spicatus* has been published until now. The aim of this study is to evaluate the antioxidant properties of different extracts of *C. spicatus*, to investigate the relationship between concentration and antioxidant properties.

Materials and Methods

Chemicals

Trichloroacetic acid (TCA), 2-deoxy-D-ribose, ascorbic acid, linoleic acid, Tween-40, 3-(2-pyridyl)-5,6bis (4-phenylsulfonic acid)-1,2,4-triazine (ferrozine), ethylenediamine trtra acetic acid (EDTA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), phenazine methosulphate (PMS), potassium ferricyanide, thiobarbituric acids (TBA), nitroblue tetrazolium (NBT), β-nicotinamide adenine dinucleotide reduced (β-NADH) and ammonium thiocyanate were obtained from Sigma Aldrich Chemical Co. (USA). All other chemicals and solvents were of analytical or high performance liquid chromatography grade.

Animals and tissue homogenate preparation

Adult Kunming mice, 18-25 g, were obtained from Center of Laboratory Animal of Guangzhou University of Chinese Medicine, Lot No.: SCXK (Yue) 2008-0020, SYXK (Yue) 2008-0085. The mice were pair-housed in plastic cages in a temperature-controlled (25 ℃) colony room with a 12/12 h light/dark cycle. Food and water were available ad libitum. All experimental protocols were approved by the Center of Laboratory Animal of Guangzhou University of Chinese Medicine. All efforts were made to minimize the number of animals used. The mice were executed by cervical vertebra dislocation and the kidney were dissected, then grinded into 10% tissue homogenate using glass pestle with 4 ℃ physiological saline in ice-bath and stored at 4 ℃ before used.

Plant material

The whole plant was collected on August 20th, 2012 from Medicinal Plants Mountain in Guangzhou University of Chinese Medicine and was identified by Prof. LIN Li, a Chinese medicine resources specialist. The fresh plant was dried at room temperature, and then ground into fine powder. The voucher specimen (No. 20120820-SC) was deposited in botanic specimen center of college of Chinese Materia Medica, Guangzhou University of Chinese Medicine.

Preparation of extracts

10 g of the dried powder of *C. spicatus* was extracted with 3 × 200 mL water at 100 ℃ for 1.5 h each time. The water solutions were combined and evaporated to dryness under reduced pressure. The residue was dissolved in 200 mL of 30% aqueous ethanol (V/V) completely. The resulting solution was kept in a refrigerator at 4 ℃ overnight, and was centrifuged at 5000 r/min for 10 min to eliminate chlorophyll. The supernatant solution was collected and evaporated to dryness. The residue was added with 100 mL distilled water, and shaken vigorously to form a suspension, followed by fractionation using different solvents in order of: petroleum ether, chloroform and ethyl acetate. Three extracts and the water phase were evaporated to dryness under reduced pressure by rotary evaporator to provide petroleum ether extract (PE), chloroform extract (CE), ethyl acetate extract (EAE), and water extract (WE), respectively. All of these extracts were stored at 4 ℃ until used.

The stock solution of these extracts were weekly prepared at a concentration of crude drug 100 mg/mL in 50% aqueous methanol (V/V) and stored at 4 ℃. Di-

luted extracts were prepared daily by diluting the stock solutions with 50% aqueous methanol (V/V).

DPPH radical scavenging activity

The free-radical scavenging activity was measured by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH·) assay[5]. The testing of each sample was performed in triplicate. Percent inhibition was measured according to following formula and IC_{50} value was calculated by graph pad prism software. The radical-scavenging capacity (RSC) was calculated using the equation:

$RSC = [1-(A_1-A_0)/A_2] \times 100\%$

Where A_1 is the absorbance of probe, at a given sample concentration level (average of three probes); A_0 is the correction or the absorbance of the extract alone (without reagents), and A_2 is the absorbance of the DPPH radical reagent without extracts.

Superoxide radical scavenging activity

Superoxide radical scavenging activity (SRSA) was determined by the nitroblue tetrazolium reduction method described by Zhang *et al*[6]. All measurements were made in triplicate. The abilities to scavenge the superoxide radical were calculated using the following equation:

$SRSA = (1-A_1/A_0) \times 100\%$

Where A_1 is the absorbance of sample, A_0 is the absorbance of control.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging assay was carried out using a method described by Zhang *et al*[6] with slight modifications. The absorbance of supernatant was measured at 536 nm. The hydroxyl radical-scavenging activity was expressed as:

Scavenging percentage $= [(A_1-A_0)/(A-A_0)] \times 100\%$

Where A_1 is the absorbance of reaction mixture with sample, A_0 is the absorbance of reaction mixture with sample replaced by equivalent volume of deionized water, and A is the absorbance of the reaction mixture with sample and H_2O_2 replaced by equivalent volume of deionized water. Vitamin C was used as a positive control. The IC_{50} value was defined as the concentration of sample (mg/mL) require to scavenging 50% of hydroxyl radical and calculated by graph pad prism software.

Phosphomolybdenum (P-Mo) inhibition assay

The antioxidant activity was evaluated by phosphomolybdenum method according to the reported procedure[5]. Percent inhibition was calculated by the following formula.

Inhibition ratio = (1-absorbance of sample/absorbance of control) $\times 100\%$

Fe^{2+} chelating activity assay

1 mL of different concentrations of sample solution and 3.7 mL of 50% aqueous methanol (V/V) and 0.1 mL of 2 mmol/L $FeSO_4(7H_2O$ solution were mixed and reacted for 30 s, 0.1 mL of 5 mmol/L ferroustriazine solution, mixed and measured the absorbance A_1 at the wavelength of 562 nm after reaction at room temperature for 10 min. The sample's solvent was used as blank A_0. Percent chelating was measured according to following formula and IC_{50} value was calculated by graph pad prism software.

Chelating ability $= (A_1-A_0) / A_0 \times 100\%$

Effects on ROS in chemical modified systems

The levels of anti-reactive oxygen species (anti-ROS) were measured strictly following the instructions of determination kit purchased from Nanjing Jiancheng Bioengineering Institute. (China). The anti-ROS unit was calculated as 1 mL of sample solution lowering 1 mmol/(L·min) of H_2O_2 in the chemical modified systems at 37 °C.

Renal mitochondrial swelling degree assay

10 μL of different concentrations of sample solution and 40 μL of 0.01 mol/L L-Cys and 200 μL of 5 mmol/L $FeSO_4$ solution and 50 μL of 0.5 mg/mL prot renal mitochondria suspension liquid, then added to 3 mL with 0.1 mol/L PBS (pH7.4) mixed and measured the absorbance at the wavelength of 520 nm after reacting 0, 10, 15, 30 min. The absorbance without sample solution was used as control and the renal mitochondria suspension liquid was used as blank.

Effects on MDA in renal tissue homogenate

The levels of MDA were measured strictly following the instructions of determination kit purchased from Nanjing Jiancheng Bioengineering Institute (China). 0.2 mL of different concentrations of sample solution were

kept in 37℃ for 1h, then added 1 mL of 15% trichloroacetic acid solution and 1 mL of 0.67% TBA, vortex and developed in boiling water bath for 15 min, cooled and then centrifuged at 3000 rpm for 10 min, and measured the absorbance of supernatant fluid at 532 nm, and the normal, $FeSO_4$ and H_2O_2 induced renal tissue homogenate were measured, respectively. Percent inhibition was calculated by the following formula.

Inhibition ratio = $(A_0-A_1)/A_0) \times 100\%$

Where A_1 is the absorbance of reaction mixture with sample, A_0 is the absorbance of reaction mixture with sample replaced by equivalent volume of deionized water.

Effects on GSH-Px in renal tissue homogenate

280 μL of 10% renal tissue homogenate and 40 μL of different concentrations of sample solution were kept in the 37 ℃ for 1 h, ice-bath for 10 min and centrifuged at 3000 rpm for 10 min, measured the absorbance of supernatant fluid strictly following the instructions of determination kit purchased from Nanjing Jiancheng Bioengineering Institute (China). The control group tube was the absorbance of 5 mg/mL Vc solution and normal group was the absorbance of the PBS (pH7.4).

Statistical analysis

All assays were carried out in triplicates, and results were expressed as mean ± s. ANOVA was used to analyze the differences among IC_{50} of various samples for different antioxidant assays, with least significance difference (LSD) $P<0.01$ as a level of significance using SPSS 17.0 (SPSS, Abaus Concepts, Berkeley, CA) and graph pad prism (Graph Phad, San Diego, USA) software.

Results and Discussion

Antioxidant activity

The results of Duncan's multiple Range Tests showed that there were no significant differences ($P<0.05$) among IC_{50} (concentration of crude drug, similarly hereinafter) values of WE and EAE except on DPPH, while there were significant different from those of PE and CE, as shown in Table 1.

Table 1 The IC_{50} values of four extracts of *C. spicatus* (mean ± s, $n=3$)

Group	DPPH ·	$O_2^{-\cdot}$	· OH	P-Mo	Fe^{2+}
PE	460.22 ±21.48	330.54 ±12.46	38.55 ±2.61	1213.39 ±35.46	302.67 ±10.91
CE	721.42 ±15.17	314.72 ±8.42	29.75 ±2.45	1071.14 ±40.13	263.24 ±8.26
EAE	2.05 ±0.24	5.09 ±0.72	3.52 ±0.37	50.69 ±2.72	53.03 ±2.27
WE	1.22 ±0.07	3.84 ±0.46	2.89 ±0.21	50.21 ±3.84	47.17 ±1.84

Because the IC_{50} values of PE and CE on DPPH ·, $O_2^{-\cdot}$, · OH, P-Mo and Fe^{2+} were lower than that of EAE and WE (as shown in Table 1), only WE and EAE were used for the anti-ROS, inhibition MDA and GSH-Px and renal mitochondrial swelling assays.

As shown in Table 2, the WE and EAE showed significant activity on anti-ROS, the A_{520} of control tube was significant different from the sample tube ($P<0.01$). The anti-ROS activity assay showed significant dose-effect relationship at the concentrations 0.25-2.00 mg/mL. At the same concentration, the inhibition rate (IR) of WE was higher than that of EAE, but the difference was not significant ($P>0.05$).

Table 2 Effects of WE and EAE on ROS in chemical modified systems (mean ± s, $n=3$)

Sample	Dosage (mg/mL)	A_{520}	Inhibition rate (U/mg(prot.)
Control	–	0.827 ±0.012	–
EAE	0.25	0.517 ±0.007**	37.61
	1.00	0.207 ±0.001**	74.97
	2.00	0.089 ±0.003**	82.10
WE	0.25	0.429 ±0.016**	48.13
	1.00	0.187 ±0.009**	77.63
	2.00	0.024 ±0.002**	85.01

** $P<0.01$ vs control tube

The Fe^{2+} and H_2O_2 can induced the rising of levels of MDA in kidney tissue, as shown in Table 3, compared with the control tube, the levels of MDA of the Fe^{2+}, H_2O_2 induced tube and the spontaneous tube were significant different ($P<0.01$), respectively. It showed a

significant dose-effect relationship, and the effect of WE was better than that of EAE. The effect of extracts on H_2O_2-induced tube was the best, followed by the spontaneous tube and Fe^{2+}-induced tube. It suggested that the extracts of *C. spicatus* have a strong antioxidant activity. The same effects on levels of GAH-Px in renal homogenate were showed as in Table 4, the extracts of *C. spicatus* can significantly improve the antioxidant activity of kidney.

Table 3 Effects of EAE and WE on MDA in renal homogenate (mean ± s, $n=3$)

Sample	Dosage (mg/mL)	MDA content/(nmol/mg)					
		Normal	IR/%	$FeSO_4$-induced	IR/%	3% H_2O_2-induced	IR/%
Blank	–	2.515 ± 0.147	–	4.530 ± 0.461	–	4.048 ± 0.261	–
Control	–	13.911 ± 1.571△	–	21.460 ± 1.647△	–	19.529 ± 1.671△	–
EAE	0.25	8.507 ± 0.543**	38.85	17.829 ± 1.173*	16.92	12.338 ± 1.438**	36.82
	1.00	5.988 ± 0.416**	56.96	15.392 ± 1.249**	28.28	9.924 ± 1.084**	49.18
	2.00	4.745 ± 0.278**	65.89	12.283 ± 1.073**	42.76	5.695 ± 0.671**	70.84
WE	0.25	8.142 ± 0.671**	41.47	17.464 ± 1.548*	18.62	11.974 ± 1.004**	38.69
	1.00	5.623 ± 0.346**	59.58	15.027 ± 1.230**	29.98	9.560 ± 0.917**	51.05
	2.00	4.380 ± 0.315**	68.51	11.918 ± 0.973**	44.46	5.330 ± 0.536**	72.71

△$P<0.01$ vs blank tube; *$P<0.05$, **$P<0.01$ vs control tube.

Table 4 Effects of EAE and WE on GSH-Px and renal mitochondrial swelling (mean ± s, $n=3$)

Sample	Dosage (mg/mL)	GSH-Px content (nmol/mg)	Mitochondrial swelling A_{520}			
			0 min	5 min	15 min	30 min
Blank	–	48.162 ± 1.273	0.760 ± 0.021	0.751 ± 0.003	0.738 ± 0.005	0.728 ± 0.003
Control	–	84.511 ± 1.489△	0.749 ± 0.014	0.722 ± 0.005	0.687 ± 0.010	0.673 ± 0.004
EAE	0.25	69.614 ± 1.181	0.857 ± 0.018	0.839 ± 0.007	0.836 ± 0.009	0.833 ± 0.007
	1.00	95.894 ± 2.374*	0.876 ± 0.016	0.868 ± 0.006	0.866 ± 0.004	0.865 ± 0.009
	2.00	106.829 ± 2.813**	1.189 ± 0.024	1.184 ± 0.005	1.184 ± 0.012	1.183 ± 0.011
WE	0.25	81.278 ± 2.107	0.862 ± 0.017	0.859 ± 0.002	0.856 ± 0.005	0.854 ± 0.009
	1.00	101.453 ± 3.491**	0.891 ± 0.020	0.889 ± 0.001	0.887 ± 0.007	0.886 ± 0.007
	2.00	113.896 ± 3.617**	1.214 ± 0.024	1.213 ± 0.008	1.213 ± 0.013	1.212 ± 0.005

△$P<0.01$ vs blank tube; *$P<0.05$, **$P<0.01$ vs control tube.

Effects on renal mitochondrial swelling degree

The reaction of unsaturated fatty acid with free radicals induced by Fe^{2+}-L-Cys would produce much lipid peroxide and then the mitochondria would be swelling, and the absorbance at 520 nm would taper off with the extended response time. It meant that the mitochondria swelling will be more and more seriously. As shown in Table 4 and Fig. 1, the reduction rate of control tube was 12.69% and higher than the blank tube of 4.22%, the difference was significant ($P<0.01$), while the sample tube of WE and EAE were lower, which showed that the extracts of *C. spicatus* can significantly inhibit the mice renal mitochondria swelling and positively dependent on the concentration.

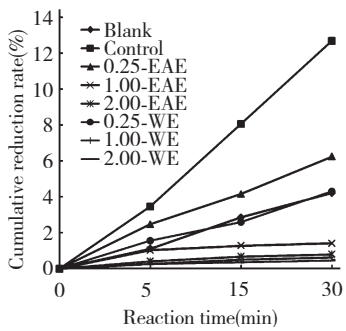

Fig. 1 The cumulative reduction rate of WE and EAE on absorbance of renal mitochondrial swelling

Conclusion

Recent studies showed that more than 30 flavonoids

were isolated from *C. spicatus*[7,8] which were tested to have strong antioxidant activities. More than 30 phenol and polyphenol acid were isolated which were tested to be the active ingredient of anti-inflammatory, antibiosis and diuresis[9]. Based on the results obtained in the present study, it was concluded that the *C. spicatus* had significant antioxidant activities and the extracts of *C. spicatus* can be used as an antioxidant for adjuvant therapy on renal diseases.

References

1 CAS Editorial Committee of Flora. Flora Republicae Popularis Sinicae, Volume 66. Beijing: Science Press, 1999. 574-577.

2 Cai HF, Shou Y, Wamg JJ, *et al*. Preliminary study on the pharmacological action spicatus. *J Chin Med Mater*, 1997, 20:38-40.

3 Tan JJ, Tan CH, Chen YL, *et al*. Chemical constituents of *Clerodendranthus spicatus*. *Nat Prod Res Dev*(天然产物研究与开发), 2009, 21:608-611.

4 Xu FC, Loubu ZD, Yang DJ. Study on antibacterial effects of the aqueous extract from *Clerodendranthus spicatus*(Thunb.) C. Y. Wu. *J Tibet Univ Nat Ed*, 2010, 25(2):82-85.

5 Aktumsek A, Zengin G, Guler GO, *et al*. Antioxidant potentials and anticholinesterase activities of methanolic and aqueous extracts of three endemic *Centaurea* L. species. *Food Chem Toxicol*, 2013, 55:290-296.

6 Zhang QA, Fan XH, Zhang ZQ, *et al*. Antioxidant for phenolic extracts from Semen Astragali Complanati. *Nat Prod Res Dev*(天然产物研究与开发), 2012, 24:955-958.

7 Chen YL, Tan CH, Tan JJ, *et al*. Progress of chemical and pharmacological studies on *Clerodendranthus spicatus*. *Nat Prod Res Dev*(天然产物研究与开发), 2009, 21:885-891.

8 Chen YL, Tan CH, Lu LL, *et al*. Water-soluble constituents *of Clerodendranthus spicatus*. *Chin Tradit Herb Drugs*, 2009, 40: 689-693.

9 Li YT, Huang RG, Zheng XZ. *Chin J Integr Tradit West Med*, 2002, 22:470-472.

(上接第 391 页)

6 Zhang ZP(张自萍), Guo R(郭荣), Liao GL(廖国玲), *et al*. Comparative study on methods for determination of betaine from *Lycium barbarum* L.. *Acta Agric Boreali-Occident Sin*(西北农业学报), 2007, 16:292-295.

7 Chen C(陈晨), Wen HX(文怀秀), Zhao XH(赵晓辉), *et al*. Determination of oligomeric proantho cyanidins in *Lycium Ruthenicum* Murr. Pigment. *Chin J Spectrosc Lab*(光谱实验室), 2011, 28:1767-1769.

8 Bao HM(鲍会梅). *Lycium* beta-carotene content analysis. *Food Ferment Tech*(食品与发酵科技), 2008, 44(2):63-66.

9 Zhang YJ(张宇金), Gao SY(高世勇), He LW(何立巍). Study on bioactivities of betaine. *J Harbin Univ Com, Nat Sci Ed*(哈尔滨商业大学学报, 自科版), 2006, 22:13-16.

10 Wang XJ(王晓菁), Wu Y(吴燕), Wang LM(王黎明). Determination of betaine in fruits of *Lycium barbarum* L. by solid phase extraction and high-performance liquid chromatography. *Food Sci*(食品科学), 2010, 31:199-201.

11 Zhu GH(朱桂花), Yang GW(杨高文), Chen XG(陈晓光). Spectrophotometry for determination of betaine in *Fructus Lycii* semi finished drinkables. *Chem Res App*(化学研究与应用), 1998, 10:433-435.