

# Genetic Stability, Active Constituent, and Pharmacoactivity of *Salvia miltiorrhiza* Hairy Roots and Wild Plant

Yuan Yuan<sup>a,§</sup>, Yunjun Liu<sup>b,§</sup>, Dongmei Lu<sup>a</sup>, Luqi Huang<sup>a,\*</sup>, Rixin Liang<sup>a</sup>, Zhaochun Yang<sup>a</sup>, and Shunqin Chen<sup>a</sup>

<sup>a</sup> Institute of Chinese Materia Medica, Academy of Chinese Medical Sciences, Beijing 100700, China. E-mail: huangluqi@263.net

<sup>b</sup> Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China

\* Author for correspondence and reprint requests

Z. Naturforsch. **64c**, 557–563 (2009); received April 3/May 17, 2009

*Salvia miltiorrhiza* is an annual plant growing in China, Mongolia, Korea and some other Asian countries. The extract from *S. miltiorrhiza* roots has been used for supporting healthy cardiovascular and circulatory systems during the last decade. The active constituents of *S. miltiorrhiza* from different areas vary significantly, and the wild resources are overexploited. To adapt the demand for active constituents of *S. miltiorrhiza* against cardiovascular-related diseases, alternative materials need to be developed. The aim of the present work was to investigate the possibility of *S. miltiorrhiza* hairy roots as the alternative materials. The results showed that *S. miltiorrhiza* hairy roots are genetically stable. The contents of salvianolic acid B and tanshinone IIA, two main active constituents in hairy roots, determined by the assessment of combining flow cytometry and phytochemical analysis, are comparable to or significantly lower than in wild plant roots. The extract from *S. miltiorrhiza* hairy roots also had similar protection activity for hypoxia and reoxygenation injury in rat cardiac myocytes like that from wild plant roots. *S. miltiorrhiza* hairy roots may be alternative materials to obtain the drug or healthy food for cardiovascular-related diseases.

**Key words:** *Salvia miltiorrhiza*, Hairy Roots, Genetic Stability, Pharmacoactivity

## Introduction

*Salvia miltiorrhiza* is an annual plant growing in China, Mongolia, Korea and some other Asian countries. *S. miltiorrhiza* root is an important herbal medicine and has been used for the treatment of cardiovascular-related diseases because it improves the myocardial energy metabolism level during prolonged hypoxia and subsequent reoxygenation, protects the myocardium against hypoxia/reoxygenation injury, significantly attenuates acidosis during hypoxia, and prevents the appearance of overacidic areas of the myocardium after reoxygenation (Lin *et al.*, 2000). Because the active constituents of *S. miltiorrhiza* from different areas vary significantly and the wild resources

are overexploited, alternative materials need to be developed.

Hairy roots, obtained by infection with *Agrobacterium rhizogenes*, have a high growth rate and can grow in hormone-free media. Hairy root culture is an effective way to obtain active compounds of some medicinal plants, especially the interesting secondary metabolites that are accumulated in roots (Peebles *et al.*, 2007). However, genetic instability would be induced during continuous *in vitro* maintenance. The percentage of *Onobrychis viciaefolia* hairy root cells with a normal chromosome number was reduced from 85.0% to 23.5% after four months of subculture, and only 4.1% left eight months later. A *S. miltiorrhiza* hairy root culture was established by infecting leaves with *Agrobacterium rhizogenes* strain 15834 (Zhang *et al.*, 1995), and its mass culture was done in a 10-l ball-type airlift bioreactor, obtaining 889.5 g fresh weight from 3.68 g inoculation mass and 1.1 mg/g dry weight tanshinone after 50 days. In a previous work in our laboratory, we studied the mechanism of active components

<sup>§</sup> These authors contributed equally to this work.

**Abbreviations:** DI, DNA fluorescence index; FCM, flow cytometry; MS, Murashige and Skoog; LDH, lactate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase; H/R, hypoxia and reoxygenation.

accumulation in *S. miltiorrhiza* hairy roots. However, it is not clear whether the genetic change would occur during culture and how it affects the contents of active components.

In the present study, we assessed the genetic change and capacity to produce active constituents in *S. miltiorrhiza* hairy roots and roots of the wild plant, using an approach combining flow cytometry (FCM) and phytochemical analysis. We also evaluated the effects of the secondary metabolites from *S. miltiorrhiza* hairy roots on hypoxia and reoxygenation injury in rat cardiac myocytes. The results indicate that hairy roots may be a new source to obtain the drug against cardiovascular diseases, despite of the difference of nuclear DNA and active constituent contents to the wild plant.

## Material and Methods

### *In vitro* propagated hairy roots and phenotypic evaluation

*S. miltiorrhiza* is a diploid ( $2n = 2x = 16$ ) species. A *S. miltiorrhiza* hairy root culture was established by Zhang *et al.* (1995) and has been *in vitro* cultured on 67-V medium till now. 1 g hairy roots was transferred to new solid 67-V medium (100 ml) and the experiment material was sampled every 6 weeks. 10 samples were taken and named as S1 to S10. One-year-old wild plants of *S. miltiorrhiza* were obtained from Xingtang planting base, Hebei, China and identified by Prof. Luqi Huang. Young shoots were sterilized with 0.1%  $\text{HgCl}_2$  and grown to seedlings on Murashige and Skoog (MS) medium. Root samples were used for FCM analysis 3 months later.

### Genomic PCR

Genomic DNA was extracted from hairy root cultures and the wild plant as previously described. The *rolC* gene, an important gene in the T-DNA region of the Ri plasmid, was amplified using the PCR method to confirm the insertion of Ri T-DNA into the genome with the primers 5'-GATATATGCCAAATTTACTAG-3' and 5'-GTTAACAAAGTAGGAAACAGG-3'. The expected PCR band size was 577 bp. The PCR conditions were as follows: 95 °C for 1 min, followed by 30 cycles at 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 1 min, with a final extension time of 10 min at 72 °C.

### Quantitative analysis

The contents of tanshinone IIA and salvianolic acid B in *S. miltiorrhiza* hairy roots and roots of the wild plant were analyzed according to the methods of Chinese Pharmacopoeia. Powdered material (150 mg) obtained from samples was extracted for 1 h in a refluxing bath with 25 ml methanol in the case of tanshinone IIA and 70% methanol in the case of salvianolic acid B, respectively. The solutions were filtered through a membrane filter (0.45  $\mu\text{m}$ ). Their concentrations were determined by a HPLC system with a flow rate of 1 ml/min. HPLC conditions for the quantification of tanshinone IIA were as follows: Waters SymmetryShield™ (Milford, USA) RP<sub>18</sub> column (3.9 mm  $\times$  150 mm, 5  $\mu\text{m}$ ), the elution solvent was 75% methanol and the detection wavelength was 270 nm. HPLC conditions for the quantification of salvianolic acid B were: Waters SymmetryShield™ RP<sub>18</sub> column (3.9 mm  $\times$  150 mm, 5  $\mu\text{m}$ ), the elution solvent was  $\text{MeOH}/\text{C}_2\text{H}_5\text{N}/\text{HCOOH}/\text{H}_2\text{O}$  (30:10:1:59) and the detection wavelength was 286 nm. Peaks were identified by comparison with the retention time of standards, which were purchased from National Institute for the Control of Pharmaceutical and Biological Products (China). Two standard solutions contained 8.56  $\mu\text{g}/\text{ml}$  tanshinone IIA and 0.142 mg/ml salvianolic acid B, respectively. The injection volume was 10  $\mu\text{l}$  for both standard solutions and sample solutions. The analyses were repeated 6 times.

### Flow cytometry analysis

FCM analyses were carried out with *S. miltiorrhiza* hairy roots cultured on new medium for 6 weeks and 3-month-old wild plants cultured on MS medium. *Glycine max* (L.) Merrill was used as internal reference standard, the genome size of which has been captured recently as approx. 975 Mb in 20 chromosomes (<http://www.phytozome.net/soybean>) and nuclear DNA (pg) was obtained as 1.99 pg/2C by converting Mbp to pg according to the factor 978 Mbp = 1 pg (Dolezel *et al.*, 2003). Nuclei samples were prepared according to a protocol adopted from Alan *et al.* (2007) with some modifications (Yuan *et al.*, 2008). Briefly, 50 mg of *S. miltiorrhiza* and the same amount of *G. max* leaf tissues were simultaneously chopped with a razor blade with 1 ml of ice-cold nuclei isolation buffer [15 mM HEPES, 1 mM  $\text{Na}_2\text{EDTA}$ , 80 mM KCl, 20 mM NaCl, 300 mM sucrose, 0.2%

(v/v) Triton X-100, 0.5 mM spermine, 1% (w/v) polyvinylpyrrolidone-40, pH 7.5]. The homogenate was filtered into an 1.5-ml Eppendorf tube through a 37- $\mu$ m nylon filter and centrifuged at  $8,000 \times g$  for 5 min. The supernatant was poured out and the tube was placed upside down on a paper towel for several minutes to drain the remaining supernatant. The nuclei pellet was rinsed in 70% ethanol for 12 h and washed with isolation buffer for three times. The nuclei pellet was resuspended in 300  $\mu$ l isolation buffer with 25  $\mu$ g/ml RNase A. The nuclei were stained with 10  $\mu$ l of propidium iodide (1 mg/ml) per sample before analysis. The nuclei samples were also prepared without internal standard to determine the potential effect of *G. max* cell components on the DNA analysis. Samples were analyzed with a Coulter EPICS XL flow cytometer (Beckman-Coulter, Inc., Fullerton, CA, USA) equipped with an argon laser emitting at 488 nm using a 610-nm band pass filter. The instrument was checked for linearity with fluorescent Flow Check beads (Beckman-Coulter) and the amplification was adjusted to position the 2C peak of hairy roots and wild plants of *S. miltiorrhiza* nuclei approx. at channel 120 and 360 separately. Ten nuclei samples were prepared and 3000–5000 nuclei were analyzed from each sample. The means and standard errors (SEs) of the nuclear DNA fluorescence index ( $DI = 2C_{\text{Sample}} / 2C_{G.\text{max}}$ ) and the nuclear DNA content (pg/2C) were calculated according to the formula adopted from Dolezel and Bartos (2005). The mean coefficient variations [ $CV = (SD \text{ of } 2C \text{ peak} / 2C \text{ peak mean}) \cdot 100$ ] of 2C nuclei were calculated for all samples. Percentages of 2C, 4C, and 8C nuclei were measured to detect the possible occurrence of mixoploidy and polyploidy. This was repeated three times.

#### *Evaluating the effects of secondary metabolites from S. miltiorrhiza hairy roots in hypoxia and reoxygenation injury on rat myocardial cells*

Rat myocardial cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Beijing Chemical Co, China) containing 20% fetal bovine serum (Hyclone Laboratories Inc., USA) for 3 d. After 3 h of hypoxia and 1 h of reoxygenation, cells were incubated in different doses of 70% ethanolic extract from hairy roots (HP-1, HP-2, HP-3) and roots of the wild plant (WP-1, WP-2, WP-3). The beat frequency and cell survival were

measured using an inverted microscope in hypoxia and reoxygenation. Then the cells were treated with 100  $\mu$ l 5'-diphenyl tetrazolium bromide solution (Sigma Chemical Co., USA, 0.5 mg/ml, dissolved in DMEM without serum) at 37 °C for 4 h. After removing the solution, the cells were treated with 100  $\mu$ l dimethyl sulfoxide (DMSO) and oscillated for 10 min to dissolve crystals sufficiently. The leakage of lactate dehydrogenase (LDH), content of malondialdehyde (MDA) and activity of superoxide dismutase (SOD) of upper solution were measured using ELISA kits purchased from Nanjing Jiancheng Bioengineering Institute, China. Data are analyzed by SPSS13.0 and presented as mean  $\pm$  SE.

## Results and Discussion

### *Difference of nuclear DNA content between hairy roots and wild plants of S. miltiorrhiza*

Our previous results have shown that the *S. miltiorrhiza* hairy root growth increased twelve-fold after transferred to 67-V medium for 6 weeks. After 6 weeks, hairy roots did not grow further and the contents of the active constituents tanshinone IIA and salvianolic acid B decreased. So, in the present study 6-week-old hairy roots were chosen as experiment material. To confirm the presence of the T-DNA fragment of *Agrobacterium rhizogenes* in hairy roots, PCR was performed with specific primers for the *rolC* gene, which should be inserted into genomic DNA to maintain the hairy root growth (Bonhomme *et al.*, 2000). PCR results showed that the 577-bp *rolC* fragments were detected in all hairy root samples (Fig. 1), suggesting the stability of *rolC* and the T-DNA fragment of *A. rhizogenes* in long-term maintained *S. miltiorrhiza* hairy roots.

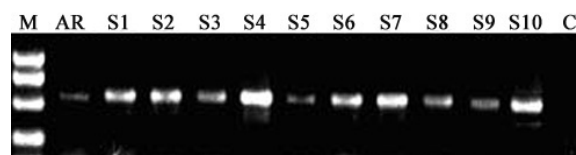


Fig. 1. PCR analysis of *rolC* gene in *S. miltiorrhiza* hairy roots. M, 2000-bp DNA marker; AR, *Agrobacterium rhizogenes*; S1–S10, different *S. miltiorrhiza* hairy root samples; C, wild plant of *S. miltiorrhiza* as control. Primers were designed specifically to obtain a 577-bp *rolC* gene fragment.

Ploidy variation is easily induced in tissue cultures and can cause genetic instability. The nuclear DNA content can be well estimated by FCM in plants. Wu *et al.* (2006) reported the genetic variation of Chinese *Cynodon* using FCM analysis. In the present study, the possible variations in the nuclear DNA content and ploidy level of samples were detected by FCM analysis. First, we tried the nuclei sample preparation protocol from Alan *et al.* (2007); however, it was not suitable for the hairy roots because of the big noise in FCM analysis. So we modified the method using root tissue as sample. The modified method was successfully used in FCM analyses of the *Scutellaria baicalensis* genome size using root samples (data not shown), which is similar to the results using leaf samples (Alan *et al.*, 2007). For FCM analyses of *S. miltiorrhiza* hairy roots and wild plants, only those samples with 3000–5000 nuclei were used. The nuclei samples of *S. miltiorrhiza* hairy roots and wild plants, separately, showed sharp 2C peaks with CVs averaging 6.7% and 6.9%, and 4C peaks were barely detectable above the debris. The ranges of CVs in the present study were similar to the analysis results from *Scutellaria baicalensis* (Alan *et al.*, 2007). The results of cytogenetic analysis showed that the frequency of chromosomal alterations was very low in hairy roots of *Datura stramonium*, which is not the case with normal root cultures (Baiza *et al.*, 1999). *G. max* nuclei were used as internal standard and did not change the positions of *S. miltiorrhiza* hairy root and wild plant peaks. FCM analysis of *S. miltiorrhiza* hairy roots provided mean nuclear DIs of 0.197, ranging from 0.193 to 0.225, and nuclear DNA contents ranging from 0.384 to 0.448 pg/2C. Our results showed that the diploid level was well preserved among *in vitro* maintained hairy roots, with nuclei rate in 2C peaks. Nuclear DNA contents were similar among the 10 samples of hairy roots, confirming the genetic stability of *in vitro* long-term cultured hairy roots. While Walker *et al.* (2006) reported that environmental factor effects on DNA determination of Fabaceae populations and the mean nuclear DNA content correlated significantly with the mean temperature and longitude, in our study, wild plants provided mean nuclear DIs of 0.599 and nuclear DNA contents of 1.192 pg/2C. The 1C genome size of hairy roots decreased compared with the wild plant. A genetic change was found between hairy roots and the wild plant of *S. miltiorrhiza*, suggesting

that plants may discard their unnecessary nuclear DNA when they only have a root organ.

#### *Comparison of active constituents in hairy roots and wild plants of S. miltiorrhiza*

Contents of tanshinone IIA and salvianolic acid B, the important indices for the quality of *S. miltiorrhiza* medicine, were determined in methanolic extracts of hairy roots. In a typical HPLC chromatogram (Fig. 2), peaks 1 and 2 were readily identified as tanshinone IIA and salvianolic acid

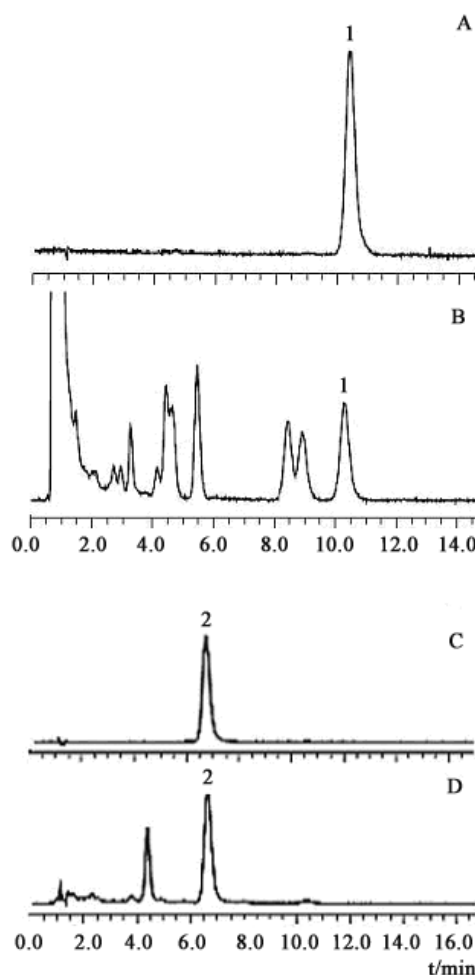


Fig. 2. HPLC analysis of *S. miltiorrhiza* hairy roots and wild plants. (A) Standard sample of tanshinone IIA; (B) mean chromatogram of *S. miltiorrhiza* hairy root samples for detecting tanshinone IIA; (C) standard sample of salvianolic acid B; (D) mean chromatogram of *S. miltiorrhiza* hairy root samples for detecting salvianolic acid B; 1, tanshinone IIA; 2, salvianolic acid B.

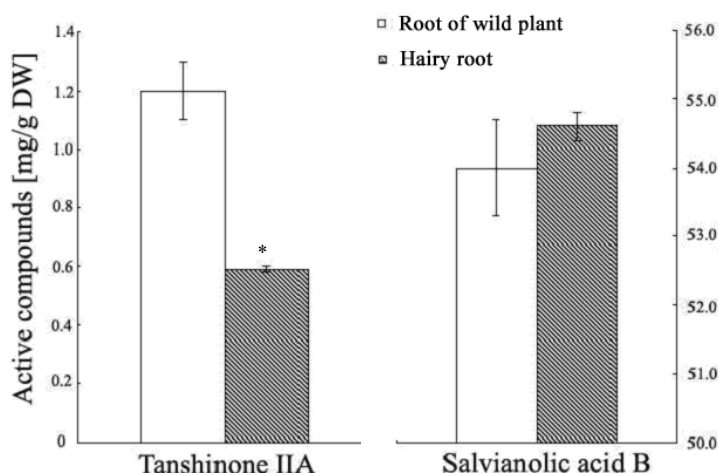


Fig. 3. Contents of tanshinone IIA and salvianolic acid B in *S. miltiorrhiza* hairy roots and roots of wild plant samples. Data are shown as means  $\pm$  SE for 6 independent experiments; \* denotes the significant difference between hairy roots and roots of wild plants at  $P < 0.05$ .

B, respectively, by comparing their UV spectra and retention times with those of corresponding authentic standards. The results showed that all *S. miltiorrhiza* hairy root samples maintained the capacity to produce characteristic active constituents under *in vitro* conditions. On average, the hairy root samples contained 0.59 mg/g tanshinone IIA and 54.6 mg/g salvianolic acid B (Fig. 3). But significant differences were demonstrated in these two substances amounts in *in vitro* maintained hairy roots. The CVs of tanshinone IIA and salvianolic acid B content in all hairy root samples was 18.9% and 21.1%, respectively, which was lower than the 50.0% and 25.3% of five cultivated populations (Zeng *et al.*, 2006), suggesting the quality stability of hairy roots as medicine. Fu *et*

*al.* (2006) reported that the genetic modification of roots leads to differential regulation mechanisms of gene expression for even closely related compounds, causing different contents of active constituents between hairy roots and wild plants. Hairy root cultures in this study did not, however, exhibit superior potentials for active compounds. The contents of tanshinone IIA and salvianolic acid B in hairy root cultures were about half of or similar to those in the roots of wild plants. The contents of active constituents in hairy roots could be regulated easier than in wild plant roots, so *S. miltiorrhiza* hairy roots might be a better material to obtain active constituents, *e. g.* tanshinone IIA and salvianolic acid B, than plant roots.

Table I. Effect in beat frequency of rat myocardial cells in hypoxia/reoxygenation injury.

Parameter/group		Tanshinone IIA [ $\mu\text{g/ml}$ ]	Salvianolic acid B [ $\mu\text{g/ml}$ ]	Hypoxia [ $\text{min}^{-1}$ ]	Reoxygenation [ $\text{min}^{-1}$ ]
Control		–	–	69 $\pm$ 10	67 $\pm$ 6*
H/R		–	–	69 $\pm$ 16	38 $\pm$ 7
Roots of wild plants	WP-1	0.35	15.66	70 $\pm$ 14	54 $\pm$ 7**
	WP-2	0.09	3.94	66 $\pm$ 14	46 $\pm$ 3*
	WP-3	0.02	0.97	70 $\pm$ 19	38 $\pm$ 5
Hairy roots	HR-1	0.35	15.66	71 $\pm$ 8	58 $\pm$ 11**
	HR-2	0.09	3.94	70 $\pm$ 14	50 $\pm$ 7*
	HR-3	0.02	0.97	69 $\pm$ 11	48 $\pm$ 11

\* and \*\* denote the significant difference to H/R group at  $P < 0.05$  and  $P < 0.01$  level.

Table II. Cell viability, LDH, MDA, and SOD of rat myocardial cells in hypoxia/reoxygenation injury.

Parameter/group		Tanshinone IIA [ $\mu\text{g/ml}$ ]	Salvianolic acid B [ $\mu\text{g/ml}$ ]	Cell viability (%)	LDH [U/l]	MDA [nmol/l]	SOD [U/l]
Control		—	—	$79.9 \pm 2.4^{***}$	$229.6 \pm 101.1^{***}$	$1.4 \pm 0.1^*$	$3.0 \pm 0.7^{**}$
H/R		—	—	$67.5 \pm 1.7$	$1179.0 \pm 52.0$	$1.7 \pm 0.2$	$1.8 \pm 0.8$
Roots of wild plants	WP-1	0.35	15.66	$72.7 \pm 3.8^*$	$946.7 \pm 67.7^{**}$	$0.9 \pm 0.2^{***}$	$3.8 \pm 0.2^{***}$
	WP-2	0.09	3.94	$71.1 \pm 1.1$	$1042.8 \pm 100.0^*$	$1.1 \pm 0.2^{***}$	$4.1 \pm 0.5^{***}$
	WP-3	0.02	0.97	$71.3 \pm 1.5$	$1068.5 \pm 142.3$	$1.2 \pm 0.2^{**}$	$4.3 \pm 0.5^{***}$
Hairy roots	HR-1	0.35	15.66	$70.1 \pm 1.7^{**}$	$1142.9 \pm 85.2$	$1.1 \pm 0.1^{***}$	$4.3 \pm 0.1^{***}$
	HR-2	0.09	3.94	$69.5 \pm 1.8^{**}$	$1169.2 \pm 91.8$	$1.2 \pm 0.2^{**}$	$3.9 \pm 0.4^{***}$
	HR-3	0.02	0.97	$67.7 \pm 0.1^{**}$	$1177.7 \pm 61.0$	$1.5 \pm 0.6$	$3.6 \pm 1.1^{***}$

\*, \*\* and \*\*\* denote the significant difference to H/R group at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$  level.

#### Protective effects of *S. miltiorrhiza* hairy roots against myocardial hypoxia/reoxygenation injury

It has been reported that the medicinal function of *S. miltiorrhiza* is to cure cardiovascular disease (Wei *et al.*, 2007). Our results showed that the content of the active compound tanshinone IIA in hairy roots is lower than that in plant roots, causing the question whether *S. miltiorrhiza* hairy roots could maintain the pharmacological activity. Thus pharmacological experiments using the extract from *S. miltiorrhiza* hairy roots against myocardial hypoxia/reoxygenation (H/R) injury in rat myocardial cells were performed.

Our results showed that the rat myocardial cells were injured significantly in the H/R group. The beat frequency of injured cells increased significantly after rat myocardial cells were treated with different doses of the extract from hairy roots and roots of wild plants (Table I). Compared with the control group, the absorbency decreased in the methylthiazolyldiphenyl tetrazolium experiment. The leakage of LDH and content of MDA in culture solution increased, but the activity of SOD decreased. Compared with the H/R group, the number of live cells increased after being treated with the extract from hairy roots (HR-1, HR-2,

HR-3) and in the WP-1 group. There was no significant difference in the LDH release between the wild plant groups and hairy root groups. The MDA level in culture solution decreased in the three wild plant groups, HR-1 group, and HR-2 group, and the activity of SOD increased in the three wild plant groups and the three hairy root groups. The LDH and MDA level decreased following the increase of drug dosage (Table II). These results suggest that *S. miltiorrhiza* hairy roots could resist injury in rat cardiac myocytes and prevent rat cardiac myocytes from H/R injury, the mechanism of which is likely related to antioxidation and scavenging of free radicals from cardiomyocytes. The results show that the extraction from *S. miltiorrhiza* hairy roots has similar protection activity to that from wild plant roots, suggesting that *S. miltiorrhiza* hairy roots might be used as alternative to *S. miltiorrhiza* roots.

#### Acknowledgements

This work was supported by the China National Basic Research Program (grant No. 2006CB504700) and by Chinese National Programs for High Technology Research and Development (grant No. 2007AA02Z104).

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