

## Antioxidant activity of ginseng cultivated under mountainous forest with different growing years

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Ginseng cultivated and grown naturally under mountainous forest is formally called “Lin-Xia-Shan-Shen” (LXSS) and grown in manual condition is called garden ginseng (GG) according to *Chinese pharmacopoeia* (2010 edition). Usually the growing condition of LXSS is similar to wild ginseng and mostly used in Chinese folks in ancient times. The antioxidant properties of LXSS with different growing years were evaluated by their inhibitions of thiobarbituric acid-reactive substance (TBA-RS) formation in liver homogenate and 2, 2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging activity comparing with those of GG. The inhibitions of different polar extracts (*n*-butanol and water) of LXSS and GG on TBA-RS formation were also evaluated. The results showed that the antioxidant effects of LXSS were higher than those of GG and the TBA-RS formation inhibition of LXSS with longer growing years were stronger than those with shorter growing years, while the DPPH-radical scavenging activity of LXSS did not show significant difference with the change of the growing year. The results indicated that the inhibitory effect of TBA-RS formation and the DPPH-radical scavenging of LXSS were correlated with the contents of ginsenosides. In addition, the starch contents of LXSS and GG were determined by micro-amount method with spectrophotometer. It showed that the starch content in GG was higher than that of LXSS whose starch decreased gradually with the growing year.

**Keywords:** *Panax ginseng*, Lin-Xia-Shan-Shen, Antioxidant activity, Ginsenoside

### INTRODUCTION

Ginseng, the dried root and rhizome of *Panax ginseng*, has long been used in the belief that it is tonic and conducive to longevity in Chinese folks. Ginseng cultivated and grown naturally under mountainous forest is formally called “Lin-Xia-Shan-Shen” (LXSS) and grown in manual condition is called in China garden ginseng (GG) according to *Chinese pharmacopoeia* (2010 edition) [1]. Usually the growing condition of LXSS is similar to wild ginseng which was mostly used in Chinese folks in ancient times. Pharmacological effects of ginseng have been demonstrated to act in the central nervous system

and in cardiovascular, endocrine, and immune systems. Additionally, ginseng and its constituents possessed antineoplastic, antistress, and antioxidant activities [2]. GG now is still the main resource of ginseng materials, but its forest-ruined cultivation mode severely destroyed the ecological balance. In recent two decades, the cultivation of ginseng under mountain forest has been spread at large scale so as to preserve forest in China and imitate the growing conditions of wild ginseng. The cultivation of LXSS makes best use of forest to change the forest-ruined cultivation mode of GG. The natural growth of

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LXSS without pesticide and fertilizer reduces environmental contamination and provides green medicine materials. The similar appearance of LXSS with wild ginseng formed by longer growing years is also one of its attracting characteristics in the culture of traditional Chinese medicine. All these destine the planting of LXSS to become a prospective cultivation mode of ginseng.

Research indicated that the water, methanol, and ethanol extracts of leaves of wild ginseng were capable of scavenging free radicals, among which, the ethanol extract showed the highest 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical, hydroxyl radical scavenging, and ferrous ion chelating activities. On the other hand the highest superoxide radical scavenging activity was found in water extract. The differences in antioxidant activities seemed to be ascribed to the differences in concentrations of key antioxidants among various solvent extracts [3].

Previously the standard operating procedure for cultivation [4] and fingerprint of LXSS [5] were proposed. More recently, the accumulating profiles of ginsenosides in LXSS collected in different months [6] and different growing years [7] were explored. As a continuous study, anti-oxidative effects of LXSS and GG were compared. Meanwhile, the starch contents of LXSS and GG were determined. The results were attempted to explain the different activity of LXSS and GG, especially LXSS with different growing years.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats were purchased from Institute of Experimental Animals of Dalian Medical University (licence no. SCXK 2008-0002). The animals were maintained at a constant temperature of  $23\pm 2^{\circ}\text{C}$  and were fed standard laboratory chow (Qianmin food stuff factory in city of Shenyang) and tap water.

### Materials

Root and rhizome of LXSS and GG were purchased from Benxi, Liaoning province and were identified by Liu Feng-Yun (senior pharmacist of Benxi institute for drug control) and Wang Bing (professor of Liaoning University of traditional Chinese medicine). The growing years of LXSS were determined by the number of rhizome nodes together with cultivated time at the different collection areas. The voucher specimens were deposited in college of pharmacy, Liaoning University of Traditional Chinese Medicine.

### Preparation of water and methanol extracts of Lin-Xia-Shan-Shen and garden ginseng

The ginseng samples were dried in oven at  $50^{\circ}\text{C}$  to constant weight. Then they were crushed by high-speed pulverizer and weighed. 0.005, 0.01, and 0.02 g of the samples were set as low, moderate and high dosages and extracted with 50 times the volume of water at  $100^{\circ}\text{C}$  for two times, each time 0.5 h, and the solvents were evaporated at  $50^{\circ}\text{C}$  to give water extract of LXSS (LXSSW) and water extract of GG (GGW), respectively. These extracts were dissolved in 1 mL distilled water for the determination of inhibitory of thiobarbituric acid-reactive substance (TBA-RS) formation. Then LXSSW and GGW were extracted with chloroform and *n*-butanol successively to yield the chloroform, *n*-butanol, and water layers, respectively. Different polar layer extracts were dissolved in 1 mL 10% dimethyl sulfoxide for activity determination. While different dosages of ginseng powder were extracted with 50 times the volume of methanol for two times, each time 0.5 h, and the solvents were evaporated at  $50^{\circ}\text{C}$  to give methanol extract of LXSS (LXSSM) and methanol extract of GG (GGM). These extracts were dissolved in 1 mL methanol for the determination of DPPH radical-scavenging.

### HPLC analysis

The chemical analysis was achieved on an Agilent 1100 HPLC instrument with Agilent C18 column ( $150\times 4.6$  mm,  $5\ \mu\text{m}$ ), column temperature was set at  $30^{\circ}\text{C}$ , and flow rate at  $1.0\ \text{mL}\cdot\text{min}^{-1}$ . The ginsenosides were separated within 70 min by mobile phase consisted of acetonitrile (A) and 0.1% phosphoric acid (W) with the gradients as follows: 0 to 30 min, A :W from 19:81 to 29:71; 30 to 50 min, A:W from 29:71 to 32:68; 50 to 70 min, A:W from 32:68 to 51:49.

### Determination of ginsenosides

Contents of ginsenosides Rg1, Rb1, and Rd were determined by external standard method. Meanwhile ginsenosides Re, Rf, Rc, Rb2, and Rb3 were determined by multi-components quantization with one marker. Since the relative correction factors (RCFs) of usual ginsenosides to Rb1 were around 1 [8], so the RCFs of ginsenosides Re, Rf, Rc, Rb2, and Rb3 to Rb1 were determined approximately as 1. The amount of total ginsenoside was calculated by adding the contents of Rg1, Re, Rf, Rc, Rb1, Rb2, Rb3, and Rd together. The yields of total ginsenosides of water and methanol extracts of ginseng samples were listed in Tables 1 and 2 respectively.

**Table 1.** Effects of GGW and LXSSW on TBA-RS formation in the liver homogenate and amount of ginsenosides in the extracts

Extracts	Crude drug (mg/mL)	Yield of the extract (%) <sup>1)</sup>	Inhibition of TBA-RS formation (%)	Content of ginsenosides (mg/g) <sup>2)</sup>
Vitamin C			83.9±1.78	-
GGW	5	30.3	25.3±0.78	10.43±3.28
	10		37.7±0.71	
	20		42.6±1.88	
LXSSW (3-4 yr)	5	29.8	31.9±0.44**△△△	13.81±6.39
	10		38.7±0.85	
	20		46.9±1.05**△△△	
LXSSW (6-7 yr)	5	28.2	42.2±0.57***	15.44±3.02
	10		40.4±1.19*	
	20		58.5±1.23***	
LXSSW (12-15 yr)	5	37.4	49.4±0.46***△△△	15.05±7.35
	10		64.1±0.73***△△△	
	20		67.5±0.93***△△△	

Significant difference between GGW and LXSSW on the same dosage, \* $p<0.05$ , \*\* $p<0.01$ , and \*\*\* $p<0.001$ .

Significant difference between LXSSW with growing years 3 to 4 or 12 to 15 and 6 to 7 on the same dosage, △△△ $p<0.001$ .

GGW, water extract of garden ginseng; LXSSW, water extract of Lin-Xia-Shan-Shen; TBA-RS, thiobarbituric acid-reactive substance.

<sup>1)</sup> Yield=weight of the residue/raw material.

<sup>2)</sup> Calculated by adding the contents of Rg1, Re, Rf, Rc, Rb1, Rb2, Rb3, and Rd together.

**Table 2.** DPPH radical-scavenging activities of GGM and LXSSM

Extracts	Crude drug (mg/mL)	Yield of the extract (%)	DPPH radical-scavenging activity (%)	Content of ginsenosides (mg/g) <sup>1)</sup>
Vitamin C			96.0±2.03	-
GGM	5	4.1	44.7±0.22	7.67±1.16
	10		71.7±0.98	
	20		86.9±1.45	
LXSSM (3-4 yr)	5	3.8	49.2±0.76	10.24±0.05
	10		70.6±0.37	
	20		94.2±0.81**	
LXSSM (6-7 yr)	5	3.6	49.6±0.54*	11.65±1.14
	10		79.2±0.65**	
	20		95.3±1.07***	
LXSSM (12-15 yr)	5	4.8	51.1±0.39*	11.38±1.52
	10		81.4±0.81**	
	20		95.7±2.43**	

Significant difference between GGM and LXSSM on the same dosage, \* $p<0.05$ , \*\* $p<0.01$ , and \*\*\* $p<0.001$ .

DPPH, 2, 2-diphenyl-1-picrylhydrazyl; GGM, methanol extract of garden ginseng; LXSSM, methanol extract of Lin-Xia-Shan-Shen.

<sup>1)</sup> Calculated by adding the contents of Rg1, Re, Rf, Rc, Rb1, Rb2, Rb3, and Rd together.

### Determination of starch contents

The content of starch was determined by the micro-amount method with spectrophotometer [9]. Ginseng powder 10 mg was wetted by 100  $\mu$ L alcohol, then added 900  $\mu$ L 1 mol/L NaOH solvent. The mixture was dispersed in boiling water, then cooled rapidly, and centrifuged at 800 rpm for 5 min. The supernatant 300  $\mu$ L was added 1 mL 1 mol/L I<sub>2</sub>-KI/ethanol solvent and diluted

with water to 10 mL as test solution. Blank solution was prepared by the same procedure under the absence of the ginseng powder. Control solution was also prepared as above by adding soluble starch instead of ginseng powder. The solutions were placed 10 min before determination of absorbance at 580 nm. The content of starch was determined by standard curve method.

### **Inhibitory of thiobarbituric acid-reactive substance formation in rat liver homogenate**

Rats weighted 250 g were fasted for 12 h before being sacrificed by cervical dislocation. The liver was removed immediately and homogenized in a 9-fold weight of ice-cold 0.9% NaCl solution (pH 7.4) to give the 10% liver homogenate. Solutions of different concentrations of LXSSW and GGW were added to 1 mL 10% homogenate. The mixtures were placed in a shaking bath at 37°C for 90 min. Two milliliter of 10% trichloroacetic acid (TCA) was added to each centrifuging tube. Then after 5 min of reaction, 1 mL 0.67% 2-thiobarbituric acid (TBA) was added to the tubes, and then the tubes were scrolled to blend thoroughly. The mixtures were heated for 15 min in boiling water in the dark. After cooling under running water, the tubes were centrifuged (3,500 rpm, 5 min) and the absorbance was measured at 532 nm. The TBA value was expressed in terms of malondialdehyde.

Two milliliter of 10% TCA and 1 mL of 0.67% TBA aqueous solution were added to 1 mL of the homogenate together with 1 mL vitamin C in a 10-mL centrifuging tube. The mixture was centrifuged and the absorbance was measured at 532 nm as external standard.

### **2, 2-Diphenyl-1-picrylhydrazyl radical-scavenging activity**

Samples dissolved in 2 mL methanol were treated with 2 mL 65 µg/mL DPPH-methanol solution and the mixture were incubated at 30°C for 30 min (final concentration of DPPH, 32.5 µg/mL), and the absorbance change at 517 nm was measured. The reaction solution without DPPH was used as blank test.

### **Statistical analysis**

Values are expressed as mean±SEM. Student *t*-test was used for statistical analysis. A *p*-value of less than 0.05 was considered significant. The correlation analysis of data was achieved by SPSS (SPSS, Chicago, IL, USA).

## **RESULTS AND DISCUSSION**

Effects of LXSSW and GGW, together with the *n*-butanol and water layers of ginseng extracts on TBA-RS formation as a parameter of liver spontaneous lipid peroxidation were examined. These extracts inhibited TBA-RS formation in a dose and growing year-dependent manner (Table 1). The result suggested that LXSSW and GGW have antioxidant activity. Table 1 showed all the extracts of LXSSW and GGW reduced TBA-RS formation at a dose of 20 mg/mL. And the inhibition ef-

fect of LXSSW was stronger than that of GGW. On the other hand, ginsenosides have been reported to show antioxidant activity [10]. Therefore the total contents of ginsenosides in the extracts of GGW and LXSSW with different growing years were determined (Table 1). And a correlation ( $r=0.6731$ ,  $p<0.05$ ) was observed between the amount of total ginsenosides in the extract and their inhibitions of TBA-RS formation. The effects of different polar extracts of LXSSW and GGW on TBA-RS formation in liver homogenate were listed in Table 3, which showed the effects of *n*-butanol and water layers of LXSSW were stronger than those of GGW. While different from LXSSW, the inhibition of *n*-butanol and water layers of LXSSW did not significant increase as the growing year getting longer. This might be due to the different kind and content of antioxidants existed in different polar extracts, and the growing year-dependent inhibition of TBA-RS formation of LXSSW could be ascribed to the appropriate ratio of the *n*-butanol and water soluble components in LXSS. Water layers of all ginseng samples showed significant differences ( $p<0.01$ ) to the *n*-butanol layers on the same dosage, which indicated that the water-soluble components had stronger anti-lipid peroxidation than ginsenosides mainly existed in the *n*-butanol layer.

The DPPH radical, which is stable and shows absorption at 517 nm, has been used as a convenient tool for the radical-scavenging assay. When this compound accepts an electron or hydrogen radical to become a more stable compound, its absorption vanishes. DPPH radical-scavenging activities of the GGM and LXSSM were examined. As shown in Table 2, both LXSSM and GGM showed strong activity of scavenging DPPH radical, and the inhibition of LXSSM was stronger than that of GGM. These extracts scavenged DPPH radical in a dose-dependent manner (Table 2), but this activity did not increase as the growing years getting longer of LXSS. The activity of the high dosage group of LXSSM was equal to that of known antioxidant such as vitamin C [11]. A good correlation ( $r=0.9312$ ,  $p<0.001$ ) was observed between the amount of ginsenosides in the extracts and the DPPH radical-scavenging activity. The starch contents were 39.58%±0.40, 21.42%±0.23, 14.67%±0.14, and 12.79%±0.28 in GG, LXSS (3 to 4 yr), LXSS (6 to 7 yr), and LXSS (12 to 15 yr), respectively. The content of starch exhibited a trend of negative correlation with the anti-oxidative activity, indicating the starch in ginseng did not contribute to this effect, which could also explain the high potency of LXSS with longer growing years.

**Table 3.** Effects of different polar extracts of GGW and LXSSW on TBA-RS formation in the liver homogenate

Extracts		Yield (%) <sup>1)</sup>	Dosage (mg/mL)	Inhibition of TBA-RS formation (%)
GG	<i>n</i> -butanol layer	4.4	0.67	8.0±0.42
			1.34	36.4±0.81
			2.67	40.3±1.53
	Water layer	30.3	0.67	7.9±0.22
			1.34	40.2±1.69
			2.67	43.6±1.52
LXSS (3-4 yr)	<i>n</i> -butanol layer	5.0	0.67	11.4±0.12*
			1.34	47.3±1.09**
			2.67	63.2±0.45**
	Water layer	29.8	0.67	20.3±0.59**
			1.34	54.8±0.89***
			2.67	56.8±1.79***
LXSS (6-7 yr)	<i>n</i> -butanol layer	12.3	0.67	8.8±0.34
			1.34	41.4±0.52***
			2.67	51.8±0.37***
	Water layer	28.2	0.67	20.7±0.92***
			1.34	55.6±0.56***
			2.67	58.0±2.09***
LXSS (12-15 yr)	<i>n</i> -butanol layer	13.5	0.67	3.1±0.12
			1.34	45.4±0.69***
			2.67	65.4±0.66***
	Water layer	37.4	0.67	26.0±0.46
			1.34	62.6±1.32***
			2.67	62.0±1.82***

Significant difference between GGW and LXSSW on the same dosage of the same polar layer. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

GGW, water extract of garden ginseng; LXSSW, water extract of Lin-Xia-Shan-Shen; TBA-RS, thiobarbituric acid-reactive substance; GG, garden ginseng; LXSS, Lin-Xia-Shan-Shen.

<sup>1)</sup> Yield=weight of the residue/raw material.

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